EFFECTS OF ELECTRONIC CIGARETTES ON RESPIRATORY IMMUNE HOMEOSTASIS USING TRANSLATIONAL *IN VITRO* AND *IN VIVO* APPROACHES

Elise Hickman

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Toxicology and Environmental Medicine.

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Approved by:

Neil Alexis

Michelle Hernandez

Ilona Jaspers

Kun Lu

James Samet

ABSTRACT

Elise Hickman: Effects of Electronic Cigarettes on Respiratory Immune Homeostasis using Translational *In Vitro* and *In Vivo* Approaches
(Under the direction of Ilona Jaspers)

Millions of people are current e-cigarette users. E-cigarettes are commonly perceived to be safer than cigarettes, but their inhalation toxicity has not been fully described. Previous work in cells, rodents, and humans has demonstrated that e-liquids, e-liquid chemical components, and e-cigarette aerosols can be cytotoxic and modulate cellular and respiratory host defense function. However, with only a decade of research on e-cigarettes, many knowledge gaps remain, and the variety of e-cigarette devices and e-liquid formulations constantly being introduced to the market presents additional challenges when investigating the effects of e-cigarettes on respiratory health. The goal of this dissertation was to address specific knowledge gaps pertaining to the effects of e-cigarettes on respiratory host defense, including whether e-cigarette flavoring chemicals affect neutrophil function, whether e-cigarette use is associated with respiratory microbiome dysbiosis, and whether different e-cigarette device types are associated with different central airway immune phenotypes using cells and clinical samples from human subjects. Our data demonstrate that aromatic aldehyde e-cigarette flavoring chemicals can impair neutrophil phagocytosis and oxidative burst. We also observed unique nasal microbiome dysbiosis in e-cigarette users relative to smokers and non-smokers, and this signature was associated with changes in proteins that are associated with the host-microbiome interaction. Furthermore, we found that users of newer generation e-cigarettes such as JUUL had significantly lower expression of soluble immune mediators in cell-free induced sputum in comparison with smokers, non-smokers, and users of older generation devices. Taken together, these findings demonstrate dysregulated immune homeostasis in association with e-cigarette use, with a trend toward impaired immune responses. Additional contributions of this dissertation include development and characterization of a human monocyte-derived macrophage cell

culture model for use in air-liquid interface exposures, assessment of sex differences in neutrophil function, and creation of tools (high school biology lessons, clinical vaping questionnaire) to disseminate e-cigarette science to youth and facilitate conversations about vaping. Overall, these findings highlight the need for continued investigation of the mechanisms underlying the effects of e-cigarettes and have direct implications for e-cigarette regulation, including the importance of device type in e-cigarette toxicity.

“You've got to jump off cliffs all the time and build your wings on the way down." – Ray Bradbury

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I would also like to thank my committee members – Dr. Neil Alexis, Dr. Jim Samet, Dr. Michelle Hernandez, and Dr. Kun Lu – for their feedback on my projects over the years and our additional collaborators – Dr. Andrew Hinton, Dr. Peter Mucha, Alexis Payton, Dr. Julia Rager, Heather Wells, Dr. Robert Immormino, and Dr. Timothy Moran – for their contributions to my training and the research presented in this dissertation. I feel so lucky to have been surrounded by so many amazing scientists, and it was a pleasure to work with and learn from you all. I am also incredibly grateful to Dana Haine for being a wonderful science outreach collaborator and for mentoring me in lesson development and science communication. Working with you has added another layer of enjoyment and meaning to my graduate school experience.

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To my parents, Beth and Mike Hickman, my first and most important teachers, thank you for your constant encouragement. Thank you for instilling in me a love of reading and writing, both of which have been essential to my success as a scientist. You have always inspired me to be curious about this world we live in, to follow my passions, and to keep a healthy perspective on life. I am incredibly grateful that you are always on the other end of the phone. I would not be where I am today without your nurturing and support.

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PREFACE

Research is becoming increasingly interdisciplinary. The work presented in this dissertation was produced in collaboration with many other accomplished and talented scientists, and it is important to recognize their contributions. For each of the studies presented here, Dr. Ilona Jaspers was the principal investigator and provided guidance and feedback on project direction, experimental design, and manuscript preparation.

Chapter 1 was written entirely by me with edits from Dr. Jaspers. Parts of Chapter 1 are adapted from previously published work with the following citation:

> Hickman, E. & Jaspers, I. (2020) Current E-Cigarette Research in the Context of Asthma. *Current Allergy and Asthma Reports*. 20(10): 62. DOI: 10.1007/s11882-020-00952-2

For Chapter 2, I am grateful to Carolina Herrera for optimizing the neutrophil isolation and phagocytosis protocols and for performing phagocytosis assays with isoamyl acetate. All other data presented in Chapter 2 were collected by me. This chapter was written entirely by me with feedback from Dr. Jaspers and Ms. Herrera. This chapter was previously published as:

> Hickman, E., Herrera, C.A., & Jaspers, I. (2019) Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chemical Research in Toxicology*. 10.1021/acs.chemrestox.9b00171

Chapter 3 was a highly collaborative project involving many contributors. Clinical sample collection for this study spanned many years and required assistance from a clinical study team to collect and process all samples. With oversight from Dr. Jaspers, I conceived and led the research described in this chapter. Sequencing of the 16S bacterial gene and sequence alignment to operational taxonomic units were provided by Bryan Zorn and Dr. Matthew Wolfgang. I measured the proteins in matching nasal lavage fluid and carried out preliminary analyses of the microbiome and protein data. Andrew Hinton and Dr. Peter Mucha provided additional bioinformatics expertise for integration of 16S sequencing data and

soluble mediator data and for application of the SelEnergyPerm method. Mr. Hinton and myself, under the mentorship of Dr. Jaspers and Dr. Mucha, coauthored a manuscript, which is currently under peer review

Chapter 4 describes unpublished primary research investigating differences in central airway phenotypes in users of different types of e-cigarettes. For Chapter 4, induced sputum was collected by the clinical study team in the Center for Environmental Medicine, Asthma, and Lung Biology and processed by the lab of Dr. Neil Alexis, under the supervision of Heather Wells. I measured soluble mediators with assistance from Stephanie Brocke, Aleah Bailey, and Dr. Meghan Rebuli. Rachel Church analyzed biomarkers of liver injury in serum samples. Alexis Payton and Dr. Julia Rager provided expertise and training on data selection and machine learning approaches.

The data in Chapter 5 were collected primarily by me. Catalina Cobos-Uribe and Dr. Meghan Rebuli performed the Mesoscale Discovery assay, and Dr. Robert Immormino and Dr. Timothy Moran performed the flow cytometry experiments. I led the analysis of the data and preparation of the manuscript with guidance from Dr. Neil Alexis and Dr. Ilona Jaspers. This study is currently under peer review.

Chapter 6 describes unpublished primary research assessing demographic associations with neutrophil function and the effects of newer generation e-cigarettes on sputum cell function. I am grateful to Carolina Herrera for her original observation of sex differences in neutrophil phagocytosis, which prompted continued investigation of these phenomena. As in Chapter 4, induced sputum was collected by the clinical study team in the Center for Environmental Medicine, Asthma, and Lung Biology and processed by the lab of Dr. Neil Alexis, under the supervision of Heather Wells. Dr. Parker Duffney assisted with optimization of sputum cell functional assays and shared the experimental load with me throughout the study. I analyzed the data and wrote the chapter with feedback from Dr. Ilona Jaspers.

Chapter 7 is unpublished and describes the outreach work I have completed in collaboration with the UNC Institute for the Environment and the Mountain Area Health Education Center (MAHEC). I co-wrote the section of this chapter describing collaborative biology lesson development and teacher

professional development with Dana Haine, and feedback was provided by Dr. Ilona Jaspers. Noah Rice generated the nicotine equivalency data that was included on the back of the clinical vaping questionnaire, and the questionnaire was iteratively refined through feedback from MAHEC collaborators and focus groups. The section describing development of the clinical vaping questionnaire was written entirely by me with feedback from Dr. Ilona Jaspers.

Chapter 8 represents a summary of the research presented in previous chapters, integration of these findings with the literature, limitations of this dissertation, and future directions. I wrote this chapter, and edits were provided by Dr. Ilona Jaspers. Parts of Chapter 8 were adapted from previously published work with the following citation:

> Hickman, E. & Jaspers, I. (2021) Evolving chemical landscape of e-cigarettes, 2021. *Tobacco Control*. DOI: 10.1136/tobaccocontrol-2021-056808

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ACE – Abundance-based coverage estimator

ACE2 – Angiotensin-converting enzyme 2

ACTB – Beta actin

ANCOM – Analysis of composition of microbiomes

ANCOVA – Analysis of covariance

ANOSIM – Analysis of similarities

AP – Advanced placement

ARG1 – Arginase 1

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

AUC – Area under the curve

AUROC – Area under the receiver operator characteristic

BAL – Bronchoalveolar lavage

BH – Benjamini and Hochberg

BMI – Body mass index

BRFSS – Behavioral Risk Factor Surveillance System

BZ – Benzaldehyde

BZPGA – Benzaldehyde propylene glycol acetal

CA – Cinnamaldehyde

CBD – Cannabidiol

CCCP – Carbonyl cyanide m-chlorophenyl hydrazone

CD – Cluster of differentiation

cDNA – Complementary deoxyribonucleic acid

CCL17 – C-C motif chemokine ligand 17 (also known as TARC)

CCL18 – C-C motif chemokine ligand 18

CLR – Centered log ratio

CO_2 – Carbon dioxide

CoDA – Compositional data analysis

COPD – Chronic obstructive pulmonary disease

COVID-19 – Coronavirus disease 2019

CRP – C-reactive protein

CYP2A6 – Cytochrome P450 family 2 subfamily A member 6

DCV – Differential compositional variation

DEFB4A-2 – Defensin, Beta 4A

DMSO – Dimethyl sulfoxide

DPBS – Dulbecco's phosphate-buffered saline

dsDNA – Double-stranded deoxyribonucleic acid

DTT – Dithiothreitol

EC – E-cigarette

ECAR – Extracellular acidification rate

E-cig – Electronic cigarette/e-cigarette

EDTA – Ethylenediamine tetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

EV – Ethyl vanillin

EVALI – E-cigarette or vaping product use associated lung injury

FBS – Fetal bovine serum

FCCP – Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

FDA – Food and Drug Administration

FDR – False discovery rate

FeNO – Fractional exhaled nitric oxide

FEV1 – Forced expiratory volume in one second

Flt1 – Fms related receptor tyrosine kinase 1 (also known as the VEGF receptor 1)

Gen – Generation

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GRAS – Generally recognized as safe

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA-DR – Human leukocyte antigen – DR isotype

hMDM – Human monocyte-derived macrophage

HMOX-1 – Heme oxygenase 1

HSD – Honestly significant difference test

IA – Isoamyl acetate

IC_{50} – Half-maximal inhibitory concentration

IFN-γ – Interferon gamma

IgA – Immunoglobulin A

IgE – Immunoglobulin E

IL – Interleukin

IRB – Institutional review board

K-12 – Kindergarten through 12^{th} grade

LAIV – Live-attenuated influenza virus

LDH – Lactate dehydrogenase

LLD – Lower limit of detection

LPS – Lipopolysaccharide

mBMDM – Mouse bone-marrow-derived macrophage

MCP-1 – Monocyte chemoattractant protein 1

MCP-4 – Monocyte chemoattractant protein 4

M-CSF – Macrophage colony-stimulating factor

MDC – Macrophage-derived chemokine

MDM – Monocyte-derived macrophage

MFI – Mean fluorescence intensity

MIP-1α – Macrophage inflammatory protein 1 alpha

MIP-1β – Macrophage inflammatory protein 1 beta

miRNA – Micro ribonucleic acid

MLR – Multinomial logistic regression

MMP2 – Matrix metalloproteinase 2

MMP9 – Matrix metalloproteinase 9

MPO – Myeloperoxidase

MRC1 – Mannose receptor C-type 1

MRSA – Methicillin-resistant Staphylococcus aureus

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUC5AC – Mucin 5AC

NADPH – Nicotinamide adenine dinucleotide phosphate hydrogen

NGSS – Next generation science standards

NHIS – National Health Interview Survey

NIEHS – National Institute for Environmental Health Sciences

NLF – Nasal lavage fluid

NO – Nitric oxide

NOS2 – Nitric oxide synthase 2

NQO1 – Nicotinamide adenine dinucleotide plus hydrogen quinone oxidoreductase 1

NRT – Nicotine replacement therapy

NS – Non-smoker

NS/NV – Non-smoker/non-vaper

NYTS – National Youth Tobacco Survey

OCR – Oxygen consumption rate

OTU – Operational taxonomic unit

OXPHOS – Oxidative phosphorylation

PATH – Population Assessment of Tobacco and Health

PBS – Phosphate-buffered saline

PCA – Principal components analysis

PCC – Pearson's correlation coefficients

PCoA – Principal coordinates analysis

PCR – Polymerase chain reaction

PD – Professional development

PERMANOVA – Permutational analysis of variance

PFA – Paraformaldehyde

PG – Propylene glycol

PKC – Protein kinase C

PLS-DA – Partial least squares discriminant analysis

PMA – Phorbol 12-myristate 13-acetate

PMN – Polymorphonuclear cell (neutrophil)

Poly I:C – Polyinosinic:polycytidylic acid

pRDA – Partial redundancy analysis

PTGS2 – Prostaglandin-endoperoxide synthase 2

QDA – Quadratic discriminant analysis

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

ROC – Receiver operating characteristic

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute

SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2

SD – Standard deviation

SEM – Standard error of the mean

sICAM1 – Soluble intercellular adhesion molecule 1

STEM – Science, technology, engineering, and math

sVCAM1 – Soluble vascular adhesion molecule 1

TARC – Thymus- and activation-regulated chemokine (also known as CCL17)

THC – Tetrahydrocannabinol

Tie2 – TEK tyrosine kinase (also known as angiopoietin-1 receptor)

TNF-α – Tumor necrosis factor alpha'

TNF-β – Tumor necrosis factor beta

TRPA1 – Transient receptor potential cation channel subfamily A member 1

UNC – University of North Carolina at Chapel Hill

UNG – Uracil N-glycosylase

U.S. – United States

VEGF – Vascular endothelial growth factor (A)

VEGFD – Vascular endothelial growth factor D

VG – Vegetable glycerin

XF – Extracellular flux

CHAPTER 1: HISTORY, EVOLUTION, AND BIOLOGICAL EFFECTS OF E-CIGARETTES[1]

E-cigarette Invention and Use

Cigarette smoking and impetus for e-cigarettes

Tobacco has a deep history around the world, and it has been used for centuries by many cultures (1). Cigarette smoking, one of the most popular forms of tobacco consumption in the modern era, peaked in the early 1950s in the United States, with approximately 47% of adults smoking cigarettes (2). It was not until 1964, when the report from the Surgeon General's Advisory Committee describing the health effects associated with cigarette use was released (3), that public opinion surrounding cigarette use began to shift significantly. Over the past 60 years, cigarette smoking has declined substantially, reaching a low of 13.7% of U.S. adults in 2018 (4), a notable public health achievement. However, cigarette smoking is still responsible for over 7.5 million deaths per year worldwide and 480,000 deaths per year in the United States (5). Alternatives to cigarette smoking that reduce exposure to harmful chemicals and facilitate smoking cessation have been, and continue to be, critical to fighting this tobacco-associated morbidity and mortality. Electronic cigarettes, or e-cigarettes, were invented by Hon Lik (6), a Chinese pharmacist, who thought that these devices would aid in smoking cessation. Thus, when e-cigarettes were first introduced in the U.S. in 2007, they were touted as a safer alternative to smoking and as a product that could aid in smoking cessation. However, e-cigarettes entered the market without any regulatory oversight, data addressing potential health effects, or evidence supporting their efficacy for smoking cessation. Additionally, e-cigarettes were marketed to young never-smokers using tactics previously

[1] Parts of this chapter previously appeared as an article in *Current Allergy and Asthma Reports* with the citation: Hickman, E. and Jaspers, I. Current e-cigarette research in the context of asthma. *Current Allergy and Asthma Reports* 20(10):62. 2020. Content was adapted/updated to reflect additional research since publication. Reproduced with permission from Springer Nature.

employed by the tobacco industry to market cigarettes (7, 8). These factors have created complex regulatory, biomedical, and public health challenges over the past 15 years.

Prevalence and patterns of e-cigarette use within the United States

Prevalence of e-cigarette use in the United States is assessed separately in adults (aged 18 years and older) and youth (under 18 years of age). Two mechanisms used to collect information about adult e-cigarette use in the United States are the National Health Interview Survey (NHIS) and the Behavioral Risk Factor Surveillance System (BRFSS), both administered by the U.S. Centers for Disease Control and Prevention. These mechanisms both aim to collect national estimates for health outcomes and risks but accomplish this using different approaches – data for the NHIS is collected via in-person household interviews, while data for the BRFSS is collected via telephone surveys (9). In 2018, The NHIS indicated that 3.2% of adults in the U.S. (8.1 million) were current e-cigarette users (10), and the BRFSS indicated that 5.4% of adults in the U.S. (13.7 million) were current e-cigarette users (11).

For youth, the primary mechanism for assessing current e-cigarette use is the National Youth Tobacco Survey (NYTS), administered yearly by the CDC in schools. The 2021 NYTS data indicate that 13.1% of middle and high school students (1.7 million) were current e-cigarette users, including 11.3% of high school and 2.8% of middle school students (12), representing a sharp decline from the 2020 use prevalence of 19.6% for high school students and 4.7% for middle school students (13). Importantly, the 2021 NYTS was conducted in a different setting than previous years because of the COVID-19 pandemic. Some participants took the survey at home, while some took the survey at school, and youths who took the survey at school reported a higher prevalence of current e-cigarette use (15%) than youths who took the survey at home (8.1%)(12). Because of this observation, the authors caution against comparing 2021 NYTS data to data collected in previous years (12). Altogether, these data suggest that there are approximately 9.8-17 million current e-cigarette users in the United States.

E-Cigarette Technology

E-cigarette regulations and device evolution

E-cigarettes are battery-powered devices that heat and aerosolize e-liquids containing nicotine and flavoring chemicals dissolved in a base liquid made up of propylene glycol and glycerin. The user then inhales the resulting aerosol, enabling nicotine and other chemicals to enter lungs and blood stream. The first generation of e-cigarettes were called "cig-a-likes" because they resembled cigarettes. These devices were disposable, low-powered, and typically tobacco or menthol flavored, similar to traditional combustible cigarettes (14, 15). When e-cigarettes entered the market, tobacco products were not regulated by the government. In 2009, President Obama signed into law the Family Smoking Prevention and Tobacco Control Act, which gave the U.S. Food and Drug Administration (FDA) the power to regulate tobacco products (16). However, e-cigarettes were not covered under this law and continued to gain uncontrolled market strength (17) . Around this time, the first peer-reviewed publications on e-cigarettes were published (18-20), and device types such as vape pens and box mods, also referred to as second and/or third generation devices, gained popularity (17). These devices allowed for increased customization of the vaping experience, including thousands of uniquely flavored e-liquids, control over aerosolization settings such as temperature and power, and in some cases, user choice over components such as the wick and coil (21-23). The e-cigarette devices themselves and the e-liquids used to fill them were also increasingly appealing, with bright colors, modern designs, and creative candy, pastry, and fruity flavors (21). As a result of this appeal and of successful marketing by tobacco companies (7, 17), e-cigarette use surpassed cigarette use in high schoolers in 2014 (24).

JUUL, a sleek, discreet, pod-based e-cigarette, was introduced in the U.S. 2015. JUUL was different than other e-cigarettes in its design, which looks like a USB computer accessory, and its nicotine formulation (25, 26), both of which likely contributed to the vaping epidemic in youth during the subsequent years (27, 28). Following JUUL's skyrocketing popularity (29), especially among teenagers, a new wave of similar devices entered the market, and in 2020, pre-filled pods or cartridges (also known as fourth generation e-cigarettes) were still the most popular e-cigarette device type among U.S. high school

students (13, 30). Although in 2016 the "Deeming Rule" was signed into law, giving the FDA authority to regulate other products deemed tobacco products (31), including e-cigarettes, the FDA and state governments are still engaged in lawsuits with the tobacco industry over the regulation of e-cigarettes products and marketing (32). Meanwhile, e-cigarettes have largely gone unregulated, and e-cigarettes are not required to be tested for toxicity before they are sold to the public. Only recently has the FDA made strides to curb e-cigarette use in young adults by restricting the sale of flavored disposable pods (33), but these efforts have largely been circumvented by the creation of new e-cigarette device types (34). In response to these restrictions, disposable e-cigarettes gained popularity, with the percentage of youth vapers using disposables increasing from 2.5% in 2019 to 26.5% in 2020 (13, 35, 36). Thus, e-cigarette devices are constantly evolving in response to regulatory actions, which in turn means that e-cigarette researchers are typically behind the curve in investigating the health effects of these emerging devices (37). Notably, the FDA recently granted its first e-cigarette marking authorization to Vuse Solo (38), a nicotine-salt-containing fourth generation e-cigarette, with similar devices under review. A timeline representing the evolution of e-cigarette devices and pertinent regulatory events related to e-cigarettes is depicted in Figure 1-1.

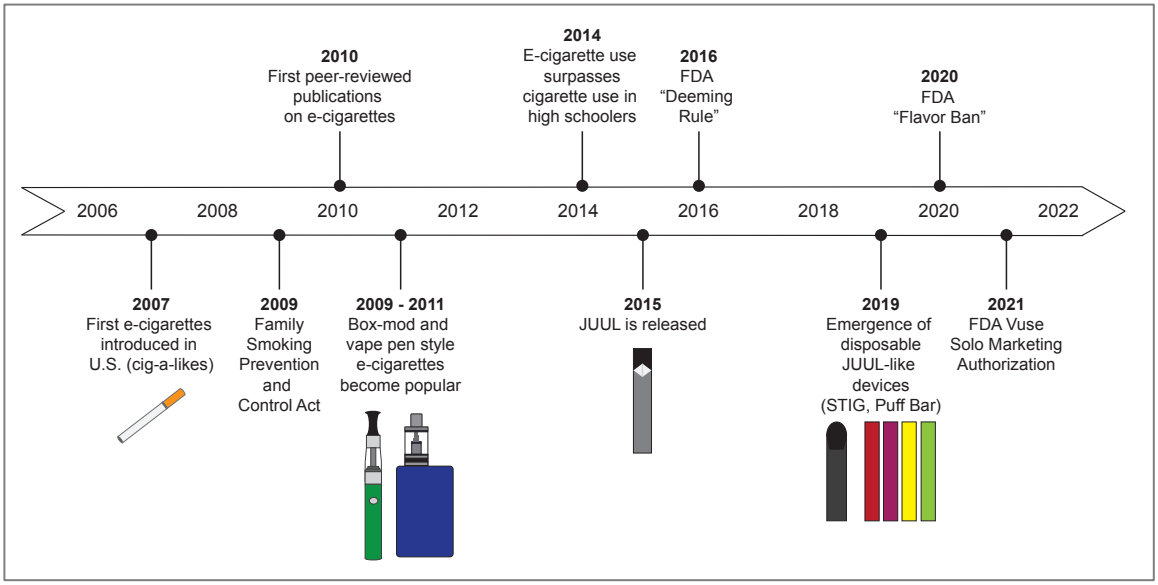


Figure 1-1. Timeline representing the evolution of e-cigarette devices and pertinent regulatory events related to e-cigarettes.

E-cigarette chemical components

Although e-cigarette devices can vary widely in appearance and aerosolization settings, most e-liquids used in these devices share common chemical components, including nicotine, flavoring chemicals, propylene glycol, and vegetable glycerin.

Nicotine

Nicotine is a stimulant and is the main psychoactive ingredient in e-liquids. Although nicotine-free e-liquids exist, most e-cigarettes contain nicotine. Nicotine activates nicotinic acetylcholine receptors in the brain, resulting in the release of neurotransmitters such as dopamine and, over time, is highly addictive (39). Nicotine exists in three forms: freebase, monoprotonated, and diprotonated, and nicotine can transition between forms depending on the pH in the nicotine solution (40). Freebase nicotine is most commonly found in e-liquids used with earlier generations of e-cigarettes, such as vape pens and box mods, making it relatively harsh when inhaled (41, 42). Nicotine salts, which were first used in JUUL e-cigarettes, are formulated by combining freebase nicotine with an organic acid, such as lactic acid, benzoic acid, or levulinic acid (43, 44). This combination results in the formation of monoprotonated nicotine and lowers the pH of the mixture and the inhaled aerosol in comparison with freebase-nicotine-containing e-cigarettes (25, 26, 42). The proportion of nicotine in the aerosol in protonated and freebase forms impacts the user sensory experience, with nicotine-salt-containing e-cigarettes providing a "smoother" feeling and allowing for inhalation of higher concentrations of nicotine (45). Because of the relationship between nicotine form and sensory experience, e-liquids containing freebase nicotine typically contain 1-3% nicotine, while nicotine-salt containing e-liquids typically have a higher nicotine concentration of 5-7% (41, 46).

Flavoring Chemicals

Flavoring chemicals in e-cigarettes provide the aerosol with an appealing aroma and taste. In 2014, Zhu and colleagues reported that there were over 7,000 unique flavors of e-liquids (21). Each e-liquid flavor is a proprietary blend of multiple flavoring chemicals (47-50). Previous studies suggest that there are approximately 100-200 unique flavoring chemicals used to create the flavors found in e-liquids and that these flavoring chemicals are present in a wide range of concentrations, many greater than 1 mg/mL (47-49, 51). Most of these flavoring chemicals are Generally Recognized as Safe (GRAS) for consumption in food by the FDA but have not been tested for inhalational toxicity (52). These flavoring chemicals belong to many chemical classes, including esters, terpenes, ketones, alcohols, and aldehydes (48). Some of the most common flavoring chemicals in e-liquids include cinnamaldehyde (cinnamon), benzaldehyde (almond or cherry), vanillin (vanilla), ethyl vanillin (vanilla), menthol (mint or cooling), ethyl maltol (sweet), and maltol (sweet) (47-51, 53). Flavoring chemicals can also react with PG in the e-liquid to generate flavorant PG acetals, which may have enhanced biological reactivity (54-56).

Propylene glycol and vegetable glycerin

Propylene glycol (PG) and vegetable glycerin (VG) comprise the "base liquid" of an e-liquid, in which the nicotine and flavoring chemicals are dissolved. Each contributes a unique quality to the resulting e-cigarette aerosol – PG provides the feeling of a "throat hit", while VG contributes sweetness and a larger cloud (57). PG and VG are mixed at different ratios in e-liquids depending on the device being used and desired cloud. Popular ratios of PG/VG include 55/45 in higher-powered, freebase-nicotine-containing devices and 30/60 in lower powered, nicotine-salt-containing devices such as JUUL.

Effects of aerosolization on e-liquid chemical components

The composition of e-cigarette aerosol is highly dependent on e-liquid composition, device type, wattage, puff topography, coil type, and coil resistance. In general, higher power aerosolization generates

more aerosol and results in more thermal decomposition of the e-liquid (26, 58-62). Some of the chemicals that have been detected as decomposition products of e-liquids in the aerosol include formaldehyde, acetaldehyde, propionaldehyde, glyoxal, methylglyoxal, diacetyl, and acrolein, though the concentration of these chemicals detected is highly variable (26, 59, 60, 62-67). While most of these decomposition products are attributed to decomposition of PG and VG in the e-liquid, it is also possible that flavoring chemicals could contribute to formation of aldehydes in the e-cigarette aerosol (68). Flavoring chemicals and their PG acetal derivatives do transfer from the e-liquid to the aerosol, with efficiency estimated to be 40-80% (46, 55, 67); however, the extent to which they break down upon aerosolization is still debated (69, 70). Metals, such as zinc, aluminum, copper, iron, tin, nickel, and lead, have also been detected in e-cigarette aerosol and are thought to originate from e-cigarette atomizers (14, 71-74). Taken together, these studies demonstrate that inhaling aerosolized e-liquid exposes e-cigarette users to a complex chemical mixture that is highly variable depending on the vaping parameters.

Other chemicals found in e-cigarettes

Because e-liquid and e-cigarette manufacturers are not required to quantify and list all ingredients in their products, and there is little regulatory oversight of the manufacturing process, many e-liquids contain chemicals beyond nicotine, flavorings, propylene glycol, vegetable glycerin, and organic acids. For example, Holt and colleagues performed untargeted gas chromatography-mass spectrometry and detected additional additives such as caffeine and theobromine in JUUL pods (75), and contaminants such as pharmaceutical and industrial chemicals have also been detected in e-liquids (76, 77).

E-cigarettes were first popularized for nicotine delivery, and nicotine-containing e-cigarettes are the focus of this dissertation, but it is important to recognize that e-cigarettes containing other biologically active compounds are now also available. For example, cannabis vaping is increasingly popular, and cannabis e-cigarettes can contain psychoactive chemicals such as Δ-9-tetrahydrocannabinol (Δ-9-THC) and cannabidiol (Δ-8-CBD or Δ-9-CBD) (78, 79). THC and CBD are typically dissolved in either medium-chain triglycerides or in a PG/VG mixture. Other vaping products feature compounds such as

melatonin, caffeine, and vitamins. Very little research has addressed potential benefits and risks of these types of e-cigarettes.

Biological effects of e-cigarettes on the respiratory system

Aerosol from e-cigarettes is inhaled, so the respiratory system is the most highly exposed organ system to the chemicals in e-cigarette aerosol. The respiratory system is responsible not only for gas exchange to oxygenate the blood but also for protecting the body from inhaled pathogens and pollutants. This innate respiratory host defense system is complex, consisting of an epithelial cell barrier with varied cellular composition along the respiratory tract, and immune cells, such as neutrophils and macrophages. The following sections detail current understanding of the effects of e-cigarettes on different components of the respiratory system. Reviewed studies for specific cell types are summarized in Table 1-1.

Association of e-cigarette use with respiratory disease in population-based human studies

The Population Assessment of Tobacco and Health (PATH) study is a longitudinal study of approximately 49,000 people ages 12 and over that evaluates how tobacco use affects health. Bhatta *et al*. found a significant association between former or current e-cigarette use at Wave 1 of the PATH study and having any respiratory disease (COPD, chronic bronchitis, emphysema, asthma) at Waves 2 or 3, controlling for factors such as smoking and other demographic and clinical variables (80). For asthma specifically, there was a significant association between current e-cigarette use at Wave 1 and newly reported asthma at Wave 2 (80). Dai and Khan also found an association between e-cigarette use and respiratory symptoms and linked these symptoms to biomarkers of tobacco exposure (81).

Furthermore, e-cigarette use has been associated with increased odds ratio of self-reported asthma (82, 83), which was higher in daily as compared to occasional e-cigarette users (82). In a small pilot study, e-cigarette users had an increase in plasma IgE in comparison with nonsmokers (84), suggesting increased markers of atopy. In adolescents, e-cigarette use has been associated with having asthma, bronchitic symptoms, and days absent for asthmatic high schoolers due to severe asthma, controlling for

other substance use and demographic variables (85-88). Bayly *et al* demonstrated increased risk for asthma attacks following secondhand e-cigarette exposure in adolescents (89), but Chaffee *et al.* did not find an association with e-cigarette in study that examined four separate populations of adolescents and young adults (88). Another study did not find any association between bronchitic symptoms, wheeze, or shortness of breath in young adults when adjusting for cannabis vaping (90), suggesting that cannabis vaping may be a confounder in previous studies associating e-cigarette use with respiratory symptoms, and another study found no increase in odds of asthma with e-cigarette use (91). Cross-sectional studies have also found associations between e-cigarette use and COPD in never cigarette smokers (83, 92).

Overall, this body of epidemiological research suggests that there is a link between e-cigarettes and respiratory diseases such as asthma and COPD, but additional longitudinal studies are needed to validate these findings, assess the strength of this association, and address additional factors such as cannabis vaping.

Effects of e-cigarettes on inflammation, respiratory function, and airway hyperresponsiveness

Studies assessing the overall inflammatory effects of e-cigarettes on the lungs have been conducted primarily in mouse models. Some studies have found that e-cigarettes induce pro-inflammatory changes in the lungs (93, 94), while other studies have found no induction of inflammation (95) or a mixture of pro- and anti-inflammatory changes (96, 97). Two studies have reported dysregulation in lung lipid homeostasis pathways following e-cigarette exposure in mice (95, 97). Divergent findings from mouse studies are likely the result of differing exposure lengths (ranging from days to months), e-cigarette devices, sex of animals, e-cigarette device, and composition of e-liquid (presence or absence of nicotine and flavors, concentration of nicotine, flavor); however, overall, these studies suggest that e-cigarettes disrupt immune homeostasis in the lungs.

Additional evidence in human controlled exposure studies and case reports suggests that e-cigarettes can alter airway function and inflammation and that asthmatics are more susceptible to the effects of e-cigarettes than healthy vapers. Lappas *et al.* found that both healthy smokers and those with

mild asthma experienced bronchoconstriction coupled with a decrease in FeNO following a session of vaping and that mild asthmatics took longer to recover from these effects (98). However, Boulay *et al.* found that vaping PG/VG alone does not have acute effects on respiratory function in asthmatics or non-asthmatics (99). Two case reports of adolescent asthmatic vapers who had recently vaped and presented to the emergency room with life-threatening status asthmaticus, necessitating veno-venous extracorporeal membrane oxygenation have also been described (100). Interestingly, the prevalence of underlying asthma in patients who had EVALI was higher than the prevalence of asthma in the general population (101).

Only one study has assessed airway hyperresponsiveness following e-cigarette exposure. Chapman *et al.* challenged BALB/c mice with either phosphate buffered saline or house dust mite and then exposed them to aerosols from different flavored e-cigarettes. These mice had varied responses depending on the flavor, with cinnamon-flavored aerosol increasing airway hyperresponsiveness and banana-flavored aerosol increasing lung collagen deposition, indicating that e-cigarettes can induce abnormal lung function in an animal model of allergic airway disease (102). Collectively, these pieces of evidence from human and animal studies, both controlled exposure and observational clinical studies, indicate that asthmatics may be a susceptible subpopulation for the effects of e-cigarettes, yet the mechanisms mediating these effects are unknown.

Effects of e-cigarettes on respiratory epithelial cells

The airway epithelial cell barrier is critical to maintaining respiratory health and immune homeostasis. Airway epithelial cells secrete mucus and cytokines and are part of the mucociliary escalator, which transports mucus and debris up and out of the airways. Dysregulation of epithelial cell function is associated with many airway diseases, including asthma, COPD, and fibrosis (103-108). E-cigarettes and e-liquid components have been shown to alter mucin composition and mucociliary escalator function. E-cigarette users had increased levels of MUC5AC in induced sputum and bronchoalveolar lavage fluid (109, 110), and aerosolized PG/VG alone increased MUC5AC in primary

human bronchial epithelial cells exposed *ex vivo* and in mouse nasal epithelial cells from mice exposed *in vivo* (110). E-cigarette aerosols and e-liquid components can also impair mucociliary function in primary human bronchial epithelial cells and in sheep (111, 112). Transient Receptor Potential Cation Channel Subfamily A Member 1 (TRPA1), a calcium ion channel, was found to be responsible for initiating these effects in the study by Chung *et al.*, and interestingly, flavoring chemicals and their propylene glycol acetals can also activate TRPA1 (55, 112). These findings are particularly notable given that MUC5AC is often increased in asthmatics and TRPA1 is hypothesized to play a role in asthmatic airway hyperresponsiveness (103, 113, 114).

Expression of certain cytokines, chemokines, and signaling molecules by epithelial cells mediates the recruitment and activation of immune cells involved in the pathogenesis of airway diseases (115, 116). E-cigarettes and e-liquid flavoring chemicals can alter cytokine expression in airway epithelial cells (58, 93, 117, 118). Gene expression studies have demonstrated that e-cigarette users have suppressed immune gene expression in the nasal epithelia (119), that flavoring chemicals alone can induce changes in cytoskeletal- and cilia-related gene expression in airway epithelial cells (120), that e-cigarette condensates can dysregulated ribosomal and protein synthesis pathways (67), and that flavored e-liquids can alter epithelial cell metabolism (121). Additionally, both unflavored e-cigarette aerosol, e-cigarette condensate, and e-cigarette flavoring chemicals have been shown to impair airway epithelial cell barrier function (117, 118, 122, 123). Another important consideration is that almost all studies assessing the effects of e-cigarettes on cellular function use cells from healthy, non-smoking donors. A recent study published by Escobar *et al* demonstrates that nasal epithelial cells from smokers and nonsmokers have differential responses to e-cigarette aerosol (124), highlighting the importance of assessing the effects of e-cigarettes in populations other than healthy non-smokers. Overall, these data convincingly show that epithelial cell function is altered by e-cigarettes; however, the mechanisms underlying these effects, the contributions of each e-cigarette chemical, and differential responses in individuals with increased susceptibility due to disease state or smoking status are active areas of research.

Effects of e-cigarettes on macrophages

Macrophages are the most highly abundant immune cells in the airways, and they are responsible for a wide range of pro- and anti-inflammatory functions to maintain host defense and respiratory immune homeostasis. Studies on the effects of e-cigarettes and e-liquid components suggest that e-cigarettes can alter macrophage function. Multiple studies have shown that *in vitro* exposure of macrophages to e-cigarette condensate, flavoring chemicals, and nicotine can modulate cytokine release. For example, IL-8 release from macrophages is increased following exposure to flavored and unflavored condensate, as well as many e-cigarette flavoring chemicals alone, and one study found that this response was nicotine dependent (125-127). Other cytokines that have been shown to be modulated by e-cigarette exposure include IL-6 and TNF-α, though whether their release is increased or decreased in response to e-cigarette exposure is not consistent between studies (125, 127). Phagocytosis, another key macrophage function, is decreased following both *in vitro* and *in vivo* exposure to e-cigarettes and e-liquid components (127-129), and this impairment can be reversed or partially reversed by treatment with dithiothreitol or N-acetylcysteine, suggesting that oxidant imbalance contributes to impaired phagocytic function (127, 129). E-cigarettes have also been shown to increase reactive oxygen species production, increase nitric oxide production, increase protease release, alter surface marker expression, and dysregulate lipid homeostasis and gene expression in macrophages (95, 118, 125, 127, 130). As macrophages are such central regulators of respiratory immunity, alterations of their function by e-cigarette use will likely impact overall respiratory health and potential pathologies associated with e-cigarette use.

Effects of e-cigarettes on neutrophils

Neutrophils are one of the respiratory system's first lines of defense, and neutrophilic inflammation is associated with diseases such as asthma and COPD (131-135). E-cigarette users have significantly elevated markers of neutrophil activation in induced sputum and bronchoalveolar lavage fluid in comparison with nonsmokers, and peripheral blood neutrophils from e-cigarette users are more susceptible to PMA-induced NETosis (109, 130), indicating that chronic e-cigarettes use may cause

neutrophils to become even more reactive. However, exposure of human peripheral blood neutrophils from non-vaping subjects to e-cigarette aerosol condensate *ex vivo* decreases chemotaxis, reactive oxygen species production, NETosis, and phagocytosis (136). We have shown that exposure of human peripheral blood neutrophils to flavoring chemicals alone can suppress phagocytosis and oxidative burst, and that flavored e-liquids can modulate NETosis (56, 129) (Chapter 2). Though these *in vivo* and *in vitro* findings may seem to be contradictory, they are likely the result of different routes, lengths, and chemical compositions of exposure. Together, they indicate that e-cigarettes can dysregulate neutrophil function and that acute exposure to e-cigarette aerosol or specific flavorings could impair key neutrophil functions, resulting in decreased host defense. Interestingly, nicotine alone can elicit a dose-dependent increase in protease release (130), which could have implications for the development or exacerbation of airway diseases due to the connection between proteases and airway remodeling in airway diseases.

Table 1-1. Summary of reviewed studies evaluating the effects of e-cigarettes on the function of epithelial cells, macrophages, and neutrophils.

Cell Type	Study	Experimental System	Results
Epithelial Cells	Chung *et al.* 2019 (112)	Primary human bronchial epithelial cells exposed *in vitro* to e-cigarette aerosol and nebulized nicotine	↑ mucus viscosity
		Sheep exposed *in vivo* to e-cigarette aerosol and nebulized nicotine	↓ tracheal mucus velocity
	Clapp *et al.* 2019 (111)	Primary human bronchial epithelial cells and BEAS2Bs exposed *in vitro* to cinnamon-flavored e-liquids and aerosols and to cinnamaldehyde alone	↓ cilia beat frequency ↓ mitochondrial respiration
	Crotty Alexander *et al.* 2018 (122)	Primary human bronchial epithelial cells exposed *in vitro* to e-cigarette aerosol	↓ barrier function
	Escobar *et al.* 2020 (58)	Primary human bronchial cells and 16HBEs exposed *in vitro* to e-cigarette aerosol	↑ IL-6, IL-8, HMOX-1, NQO1
	Escobar *et al.* 2021 (124)	Primary human nasal epithelial cells from smokers and non-smokers exposed *in vitro* to e-cigarette aerosol	↑ mucins, pro-inflammatory cytokines; dependent on smoking status and nicotine type
	Gerloff *et al.* 2017 (117)	BEAS-2Bs and 16HBEs exposed *in vitro* to flavoring chemicals	↑ IL-8 release ↓ barrier function
	Ghosh *et al.* 2018 (110)	Bronchial brush biopsies from nonsmokers, e-cigarette users, and smokers	↑ MUC5AC expression
		Primary human bronchial epithelial cells exposed *in vitro* to e-cigarette aerosol with PG/VG only	↑ MUC5AC expression
		Mice exposed *in vivo* to e-cigarette aerosol with PG/VG only	↑ MUC5AC expression
	Ghosh *et al.* 2020 (123)	Human bronchial epithelial cells exposed to e-cigarette aerosol	↓ barrier function
	Lerner *et al.* 2015 (93)	H292 cells exposed *in vitro* to e-cigarette aerosol	↑ IL-8 and IL-8 release

		C57BL/6J mice exposed *in vivo* to e-cigarette aerosol	↑ IL-6 and MCP-1 in bronchoalveolar lavage fluid
	Martin *et al.* 2016 (119)	Human nasal epithelial cells from nonsmokers, e-cigarette users, and smokers	↓ expression of immune-related genes
	Park *et al.* 2019 (120)	Human bronchial epithelial cells exposed to flavoring chemicals diacetyl and 2,3-pentanedione	↓ expression of cytoskeletal- and cilia-related genes
	Park *et al.* 2021	Human bronchial epithelial cells exposed *in vitro* to vanilla e-cigarette condensate	↓ expression of ribosomal and protein biogenesis genes
	Pinkston *et al.* 2020 (118)	H292 cells exposed *in vitro* to e-cigarette aerosol	↑ ROS production Dysregulated gene expression
		BEAS-2Bs exposed *in vitro* to e-cigarette aerosol	↑ ROS and NO production ↓ barrier function Dysregulated gene expression
	Reidel *et al.* 2017 (109)	Induced sputum from nonsmokers, e-cigarette users, and smokers	↑ MUC5AC expression
	Smith *et al.* 2021 (121)	BEAS-2Bs exposed *in vitro* to PG/VG or PG/VG with vanilla flavoring	Dysregulation in metabolites associated with amino acid metabolism, antioxidant responses, and bioenergetic pathways
Macrophages	Clapp *et al.* 2017 (129)	Alveolar macrophages from healthy subjects exposed i*n vitro* to flavored e-liquids and cinnamaldehyde	↓ phagocytosis ↑ ↓ IL-6, ↓ IL-8
	Ghosh *et al.* 2019 (130)	Alveolar macrophages from healthy subjects exposed i*n vitro* to nicotine	↑ protease release
	Madison *et al.* 2019 (95)	C57BL/6J mice exposed *in vivo* to e-cigarette aerosol	↑ phospholipid ↓ M1 polarization ↓ interferon response to poly I:C
	Muthumalage *et al.* 2017 (126)	MM6 and U937 (monocytic) cells exposed *in vitro* to flavoring chemicals	↑ IL-8 release
	Pinkston *et al.* 2020 (118)	RAW246.7 cells exposed *in vitro* to e-cigarette aerosols	Altered cellular morphology ↑ ROS and NO production Dysregulated gene expression
	Scott *et al.* 2018 (127)	Alveolar macrophages and THP-1 cells exposed *in vitro* to e-cigarette condensate	↑ reactive oxygen species ↑ proinflammatory cytokine release ↓ phagocytosis
	Sussan *et al.* 2015 (128)	C57BL/6J mice were exposed *in vivo* to e-cigarette aerosol	↓ phagocytosis
	Ween *et al.* 2017 (125)	THP-1 cells exposed *in vitro* to e-cigarette condensate	↑ IL-8 release ↓ other proinflammatory cytokine release ↓ phagocytosis
Neutrophils	Clapp *et al.* 2017 (129)	Peripheral blood neutrophils from healthy subjects exposed i*n vitro* to flavored e-liquids and cinnamaldehyde	↓ phagocytosis, ↑ ↓ NETosis, ↑ IL-8 release
	Ghosh *et al.* 2019 (130)	Peripheral blood neutrophils from healthy subjects exposed i*n vitro* to nicotine	↑ protease release
	Hickman *et al.* 2019 (56)	Peripheral blood neutrophils from healthy subjects exposed *in vitro* to flavoring chemicals	↓ phagocytosis ↓ oxidative burst
	Hwang *et al.* 2016 (136)	Peripheral blood neutrophils from healthy subjects exposed i*n vitro* to e-cigarette condensate	↓ phagocytosis ↓ oxidative burst ↓ chemotaxis, ↓ NETosis
	Reidel *et al.* 2017 (109)	Peripheral blood neutrophils from nonsmokers, e-cigarette users, and smokers	↑ NETosis in cells from e-cigarette users
		Induced sputum from nonsmokers, e-cigarette users, and smokers	↑ markers of neutrophil activation in e-cigarette users

Effects of e-cigarettes on responses to infection

Two studies in mice have shown that exposure to e-cigarette aerosol impairs antiviral responses to influenza (95, 128), and as compared to nonsmokers, bronchial expression of toll-like receptor 3, which recognizes double-stranded RNA and is important for antiviral responses, was decreased in vapers (110). Additionally, e-cigarette users who were administered the live-attenuated influenza virus (nasal flu vaccine) had lower production of nasal LAIV-specific IgA and downregulated nasal immune gene and mediator expression in comparison with non-smokers following vaccination (137). These studies are all in agreement that e-cigarettes can suppress antiviral responses; therefore, e-cigarettes users may be at increased risk for viral infections.

Consequently, whether e-cigarette users are at increased risk of infection by SARS-CoV-2 has been of great interest given the ongoing COVID-19 pandemic (138). Although vaping has been shown to increase coronavirus disease severity (139) and upregulate expression of ACE2 (96, 140), the receptor through which SARS-CoV-2 enters cells, in mouse models, epidemiological studies have yet to find an association between e-cigarette use and COVID-19 diagnosis or severity (141, 142).

The respiratory microbiome is another important component in respiratory mucosal homeostasis, and respiratory microbiome dysbiosis has been associated with respiratory diseases such as cystic fibrosis, chronic obstructive pulmonary disease, asthma, and chronic rhinosinusitis (143-149). Although the respiratory microbiome in e-cigarette users has not yet been compared to that of nonsmokers, smoking has been shown to be associated with changes in the nasal microbiome (150), and e-cigarette use has been associated with changes in the oral microbiome (151-153), indicating that it is likely that the respiratory microbiome is also altered by e-cigarette use. Studies have shown that exposure to e-cigarettes can increase bacterial burden of *Streptococcus pneumoniae* in mice (128, 154) and decrease survival of mice infected with methicillin-resistant *Staphylococcus aureus* (MRSA) (136). Additionally, e-cigarette condensate can have direct effects on MRSA, including increasing cellular invasion, virulence gene expression, and resistance to killing by antimicrobial peptide LL-37 (136). These pieces of evidence suggest that e-cigarette use can both directly and indirectly modulate bacterial colonization in the

respiratory tract, and future studies seeking to understand how this affects respiratory microbial communities are needed.

Ongoing Public Health Challenges and Needs

E-cigarette research is still an emerging field, with many unknowns regarding e-cigarette toxicity, and hundreds of papers published each year (Figure 1-2), which makes communicating risks about e-cigarettes to the public challenging. Notably, PubMed includes over 6,000 entries with "electronic cigarettes", "electronic cigarette", "e-cigarettes," "e-cigarette," "vaping," "vape," "vapes", "electronic nicotine delivery system", or "electronic nicotine delivery systems" in the title, with almost half of those published in the past two years (Figure 1-2). These publications include a wide range of topics, including health effects, smoking cessation, and risk perception (Table 1-2). However, as the number of e-cigarette publications has increased, the prevalence of youth vaping has also increased (Figure 1-2), demonstrating that these publications do not have immediate impact and that research findings are not reaching youth. Tobacco use often begins at an early age (17, 155), and vaping is associated with subsequent smoking initiation (156-158), so educating youth about e-cigarettes and engaging youth in e-cigarette science to prevent future tobacco-related morbidity and mortality is critically needed.

Unfortunately, the emergent nature of e-cigarette research coupled with often unclear or little messaging to youth about e-cigarettes has contributed to the vaping epidemic due to misperceptions youth have about the safety and addictiveness of e-cigarettes (159, 160). Early studies demonstrated that e-cigarettes are often perceived by youth as less addictive and less harmful than cigarettes (159, 161). Recent studies show that although youths' awareness of the potential for addiction and harm associated with e-cigarettes is increasing (162, 163), approximately 30% of youth still believe that it is very unlikely, somewhat unlikely, or neither likely nor unlikely that JUUL is addictive, and less than half of past 30-day JUUL users thought that JUUL always contains nicotine (163, 164). Other studies have demonstrated additional misperceptions, including that fruity flavors are less harmful than other flavors of e-cigarettes, that e-cigarette aerosol is water vapor, and that e-cigarettes are not a tobacco product (165-167).

Importantly, decreased perception of e-cigarette addictiveness and risk for adverse health effects is associated with an increased likelihood of trying e-cigarettes and being a current e-cigarette user (159, 161, 163, 168), further underscoring the urgency of communicating about e-cigarettes to youth. Collectively, these studies highlight how findings about e-cigarettes published in the literature are not reaching youth. To address this challenge, it is necessary that biomedical scientists investigating the health effects of e-cigarettes engage with other sectors, such as medicine, government, education, and the public. Translating research findings is needed so that these findings can reach the true stakeholders in this research – those who use e-cigarettes or are likely to begin using e-cigarettes – and so that biomedical research remains relevant to needs in these other sectors (Figure 1-3). This approach has been termed convergence science – problem solving that crosses disciplinary boundaries to solve complex societal problems (169) – and it will be critical in addressing the complex challenges presented by the vaping epidemic.

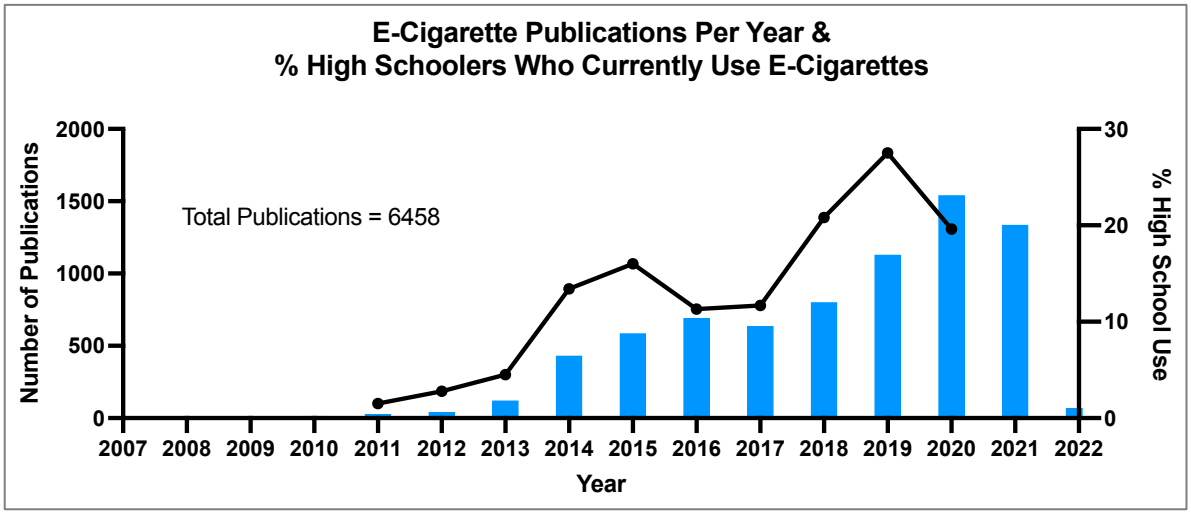


Figure 1-2. Number of e-cigarette publications and percentage of high school students who are current e-cigarette users overlaid. Number of publications was determined using the PubMed database (pubmed.ncbi.nlm.nih.gov) as of January 12, 2022, and percentage of high school students who are current e-cigarette users was based on NYTS data. Publications were included that contained any of the following words in the title: "electronic cigarette", "electronic cigarettes", "e-cigarettes," "e-cigarette," "vaping," "vape," "vapes," "electronic nicotine delivery system", or "electronic nicotine delivery systems" and were published after 2006. Data from the 2021 NYTS (11.3%) were not included because the authors caution against comparing these results with previous years due to differences in the administration of the survey (12).

Table 1-2. Number of PubMed database entries that contain "e-cigarette" or related phrase in the title and match additional search criteria based on topic or article type.

Text Contains	Count
Health effects	393
Respiratory	487
Smoking cessation	1771
Risk perception	46
Harm perception	32
Title Contains	**Count**
Case report	38
Case series	28
Meta-analysis	44
Article Type	**Count**
Editorial	248
Review or systematic review	567

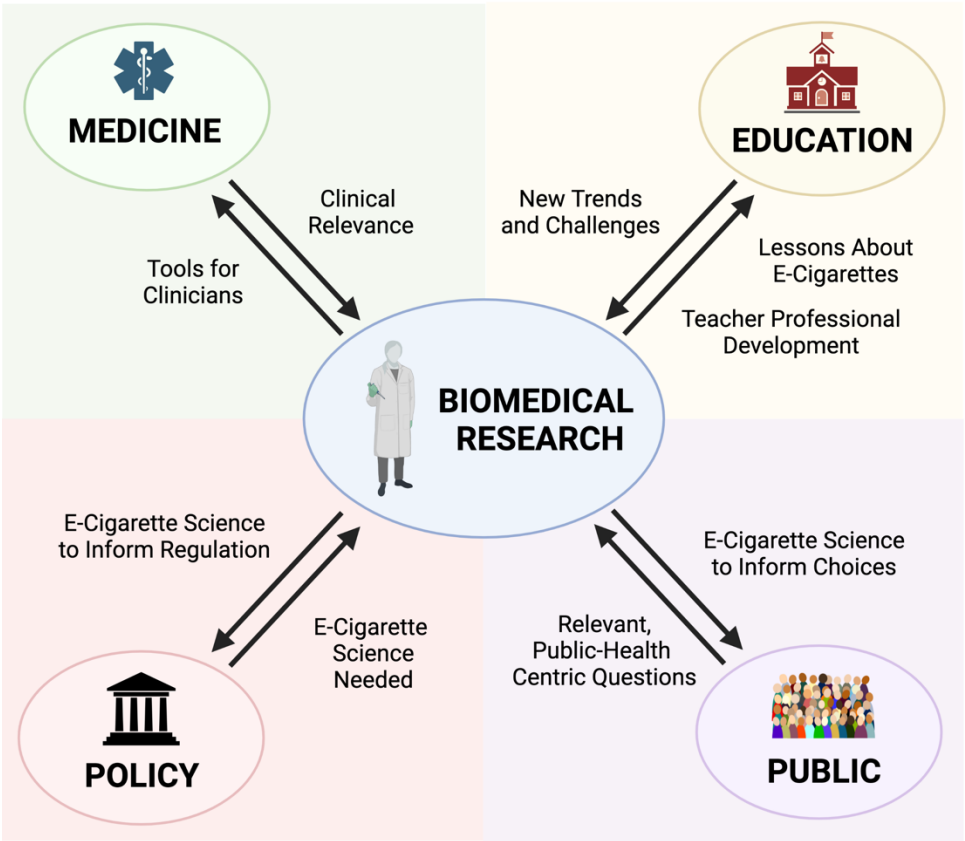


Figure 1-3. Convergence science approach to e-cigarette research.
Figure created with biorender.com.

Purpose of this study and overall impact

Previous work from our laboratory and from others has demonstrated the potential for e-cigarette toxicity and impairment of respiratory immune defense using *in vitro* and *in vivo* models as well as in samples from human subjects (95, 109, 111, 119, 129, 130). However, the effect of e-cigarettes on the respiratory microbiome, which interfaces with the host immune system and is associated with many respiratory diseases, (147, 149) has not been evaluated. Furthermore, pod-based e-cigarettes, such as JUUL, and disposable e-cigarettes, now represent the majority of the e-cigarette market (29, 36), but most published research has been conducted with model systems or subjects exposed to previous generation e-cigs (vape pens & box mods) (95, 109, 111, 119, 129, 130), which differ from pod e-cigs in their aerosolization parameters, puff topography, and nicotine formulation (25, 46). Hence, whether and how exposure to pod-based devices, such as JUUL, differently affects respiratory mucosal immune responses also represents a critical knowledge gap.

The overarching goal of this dissertation to is integrate data obtained from clinical human *in vivo* and macrophage and neutrophil *in vitro* studies to determine the effects of exposure to e-cigs on respiratory immune responses and fill critical knowledge gaps described above. My general hypotheses are that e-cigarette use is associated with nasal microbiome dysbiosis and altered nasal host-microbiota interactions; users of newer generation e-cigarettes have unique innate immune responses in the central airways in comparison with nonsmokers, smokers, and previous generation e-cig users; and that newer generation e-cigarette aerosols impair macrophage function. Chapter 2 represents a study conducted during my first-year rotation in the lab that demonstrates that e-liquid flavoring chemicals can impair neutrophil function. The findings of this study, in combination with studies from other lab, formed the basis for my investigation into the effects of e-cigarettes on the nasal microbiome, detailed in Chapter 3. The data presented in Chapter 3 demonstrate that e-cigarette use is associated with dysbiosis of the nasal microbiome and that these changes are unique from nasal microbiome dysbiosis observed in smokers. Importantly, these changes were linked with proteins that mediate the interaction between the nasal microbiome and host in matched samples. Chapter 4 is a clinical study that evaluates biomarkers of

respiratory immune homeostasis in users of different e-cigarette devices. The data presented in this chapter demonstrate that users of newer, pod-based or disposable e-cigarettes that contain nicotine salts have a unique immune mediator profile from previous generation device users, nonsmokers, and smokers. In this study, we also applied novel computational tools to understand differences in overall immune mediator profiles to complement a more traditional variable-by-variable analysis approach. To follow up on this human *in vivo* study, I next planned to assess the effects of newer generation e-cigarette aerosol on macrophage function *in vitro*. However, the COVID-19 pandemic limited my ability to access bronchoalveolar lavage macrophages, the intended cell type for these studies, so I developed a macrophage model using human monocyte-derived macrophages (hMDMs). Chapter 5 details the phenotypic characterization of my hMDMs and expands upon previous literature to include assessment of additional mediators secreted by M0, M1, and M2 hMDMs than are traditionally used to assess macrophage differentiation and polarization. Additionally, we assessed the bioenergetic profiles of the polarized hMDMs and found that they were similar to bioenergetic profiles of induced sputum and bronchoalveolar lavage macrophages. Chapter 6 contains unpublished preliminary data, including evaluation of sex differences in human neutrophil phagocytosis and oxidative burst and analysis of sputum macrophage function in newer generation e-cigarette users in comparison to nonsmokers/non-vapers. Chapter 7 describes products I have helped develop to disseminate and translate e-cigarette science and tools to youth, through biology lessons, and clinicians, through a vaping questionnaire for primary care clinics.

Taken together, research in this dissertation addresses critical knowledge gaps in the field of e-cigarette toxicology, providing novel insights that will serve as the basis for future translational and mechanistic studies to improve understanding of the effects of e-cigarettes on the respiratory immune system.

REFERENCES

1. **Doll R**. Uncovering the effects of smoking: historical perspective. *Stat Methods Med Res* 7: 87-117, 1998.

2. **Roper E**. A Study of People's Cigarette Smoking Habits and Attitudes 1953.

3. Smoking and Health: Report of the Advisory Committe to the Surgeon General of the Public Health Service 1964.

4. Cigarette Smoking Among U.S. Adults Hits All-Time Low. United State Centers for Disease Control 2019.

5. **Reitsma MB, Flor LS, Mullany EC, Gupta V, Hay SI, and Gakidou E**. Spatial, temporal, and demographic patterns in prevalence of smoking tobacco use and initiation among young people in 204 countries and territories, 1990–2019. *The Lancet Public Health* 6: e472-e481, 2021.

6. **Lik H**. Electronic Atomization Cigarette, Patent Number US20070267031A1. In: *Google Patents*. The United States: Best Partners Worldwide Ltd Fontem Holdings 1 BV.

7. **Collins L, Glasser AM, Abudayyeh H, Pearson JL, and Villanti AC**. E-Cigarette Marketing and Communication: How E-Cigarette Companies Market E-Cigarettes and the Public Engages with E-cigarette Information. *Nicotine Tob Res* 21: 14-24, 2019.

8. **Jackler RK, Chau C, Getachew BD, Whitcomb MM, Lee-Heidenreich J, Bhatt AM, Kim-O'Sullivan SHS, Hoffman ZA, Jackler LM, and Ramamurthi D**. JUUL Advertising Over its First Three Years on the Market 2019.

9. **Nelson DE, Powell-Griner E, Town M, and Kovar MG**. A comparison of national estimates from the National Health Interview Survey and the Behavioral Risk Factor Surveillance System. *Am J Public Health* 93: 1335-1341, 2003.

10. **Creamer MR, Wang TW, Babb S, Cullen KA, Day H, Willis G, Jamal A, and Neff L**. Tobacco Product Use and Cessation Indicators Among Adults - United States, 2018. *MMWR Morb Mortal Wkly Rep* 68: 1013-1019, 2019.

11. **Obisesan OH, Osei AD, Uddin SMI, Dzaye O, Mirbolouk M, Stokes A, and Blaha MJ**. Trends in e-Cigarette Use in Adults in the United States, 2016-2018. *JAMA Intern Med* 180: 1394-1398, 2020.

12. **Park-Lee E, Ren C, Sawdey MD, Gentzke AS, Cornelius M, Jamal A, and Cullen KA**. Notes from the Field: E-Cigarette Use Among Middle and High School Students - National Youth Tobacco Survey, United States, 2021. *MMWR Morb Mortal Wkly Rep* 70: 1387-1389, 2021.

13. **Wang TW, Neff LJ, Park-Lee E, Ren C, Cullen KA, and King BA**. E-cigarette Use Among Middle and High School Students - United States, 2020. *MMWR Morb Mortal Wkly Rep* 69: 1310-1312, 2020.

14. **Goniewicz ML, Knysak J, Gawron M, Kosmider L, Sobczak A, Kurek J, Prokopowicz A, Jablonska-Czapla M, Rosik-Dulewska C, Havel C, Jacob P, 3rd, and Benowitz N**. Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. *Tob Control* 23: 133-139, 2014.

15. **Hess CA, Olmedo P, Navas-Acien A, Goessler W, Cohen JE, and Rule AM**. E-cigarettes as a source of toxic and potentially carcinogenic metals. *Environmental research* 152: 221-225, 2017.

16. Family Smoking Prevention and Tobacco Control Act of 2009 (Public Law 111–13), 111th United States Congress. https://www.govinfo.gov/content/pkg/PLAW-111publ31/pdf/PLAW-111publ31.pdf. 2009.

17. **U.S. Department of Health and Human Services**. E-Cigarette Use Among Youth and Young Adults. A Report of the Surgeon General Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2016.

18. **Flouris AD, and Oikonomou DN**. Electronic cigarettes: miracle or menace? *Bmj* 340: c311, 2010.

19. **Etter JF**. Electronic cigarettes: a survey of users. *BMC Public Health* 10: 231, 2010.

20. **Vardavas CI, Anagnostopoulos N, Kougias M, Evangelopoulou V, Connolly GN, and Behrakis PK**. Short-term pulmonary effects of using an electronic cigarette: impact on respiratory flow resistance, impedance, and exhaled nitric oxide. *Chest* 141: 1400-1406, 2012.

21. **Zhu SH, Sun JY, Bonnevie E, Cummins SE, Gamst A, Yin L, and Lee M**. Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tob Control* 23 Suppl 3: iii3-9, 2014.

22. **DeVito EE, and Krishnan-Sarin S**. E-cigarettes: Impact of E-Liquid Components and Device Characteristics on Nicotine Exposure. *Curr Neuropharmacol* 16: 438-459, 2018.

23. **Brown CJ, and Cheng JM**. Electronic cigarettes: product characterisation and design considerations. *Tob Control* 23 Suppl 2: ii4-10, 2014.

24. **Arrazola RA, Singh T, Corey CG, Husten CG, Neff LJ, Apelberg BJ, Bunnell RE, Choiniere CJ, King BA, Cox S, McAfee T, and Caraballo RS**. Tobacco use among middle and high school students - United States, 2011-2014. *MMWR Morb Mortal Wkly Rep* 64: 381-385, 2015.

25. **Talih S, Salman R, El-Hage R, Karam E, Karaoghlanian N, El-Hellani A, Saliba N, and Shihadeh A**. Characteristics and toxicant emissions of JUUL electronic cigarettes. *Tob Control* 2019.

26. **Reilly SM, Bitzer ZT, Goel R, Trushin N, and Richie JP**. Free Radical, Carbonyl, and Nicotine Levels Produced by Juul Electronic Cigarettes. *Nicotine Tob Res* 21: 1274-1278, 2019.

27. **Kaplan B, Cherukupalli R, Welding K, Kennedy RD, and Cohen JE**. The youth e-cigarette epidemic: New estimates of JUUL Labs' revenue from youth users in the US. *Tob Induc Dis* 19: 33, 2021.

28. **Mantey DS, Case KR, Omega-Njemnobi O, Springer AE, and Kelder SH**. Use frequency and symptoms of nicotine dependence among adolescent E-cigarette users: Comparison of JUUL and Non-JUUL users. *Drug Alcohol Depend* 228: 109078, 2021.

29. **Huang J, Duan Z, Kwok J, Binns S, Vera LE, Kim Y, Szczypka G, and Emery SL**. Vaping versus JUULing: how the extraordinary growth and marketing of JUUL transformed the US retail e-cigarette market. *Tob Control* 28: 146-151, 2019.

30. **Goniewicz ML, Boykan R, Messina CR, Eliscu A, and Tolentino J**. High exposure to nicotine among adolescents who use Juul and other vape pod systems ('pods'). *Tobacco Control* 28: 676, 2019.

31. **The United States Food and Drug Administration**. Deeming Tobacco Products To Be Subject to the Federal Food, Drug, and Cosmetic Act, as Amended by the Family Smoking Prevention and Tobacco Control Act; Restrictions on the Sale and Distribution of Tobacco Products and Required Warning Statements for Tobacco Products. 2016.

32. **Dyer O**. E-cigarette maker Juul pays $40m to North Carolina in landmark settlement. *Bmj* 374: n1669, 2021.

33. FDA finalizes enforcement policy on unauthorized flavored cartridge-based e-cigarettes that appeal to children, including fruit and mint U.S. Food and Drug Administration. https://www.fda.gov/news-events/press-announcements/fda-finalizes-enforcement-policy-unauthorized-flavored-cartridge-based-e-cigarettes-appeal-children.

34. **Wang TW, Gentzke AS, Neff LJ, Glidden EV, Jamal A, Park-Lee E, Ren C, Cullen KA, King BA, and Hacker KA**. Disposable E-Cigarette Use among U.S. Youth - An Emerging Public Health Challenge. *The New England journal of medicine* 384: 1573-1576, 2021.

35. **Hahn SM**. National Survey Shows Encouraging Decline in Overall Youth E-Cigarette Use, Concerning Uptick in Use of Disposable Products US Food and Drug Administration. https://www.fda.gov/news-events/press-announcements/national-survey-shows-encouraging-decline-overall-youth-e-cigarette-use-concerning-uptick-use.

36. **Anic G, Cullen KA, Gardner LD, and Liu ST**. E-Cigarette Device Types Used by Middle School Students and High School Students-The U.S., 2019. *J Adolesc Health* 69: 515-518, 2021.

37. **Hickman E, and Jaspers I**. Evolving chemical landscape of e-cigarettes, 2021. *Tobacco Control* tobaccocontrol-2021-056808, 2021.

38. FDA Permits Marketing of E-Cigarette Products, Marking First Authorization of Its Kind by the Agency https://www.fda.gov/news-events/press-announcements/fda-permits-marketing-e-cigarette-products-marking-first-authorization-its-kind-agency.

39. **Benowitz NL**. Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. *Annu Rev Pharmacol Toxicol* 49: 57-71, 2009.

40. **El-Hellani A, El-Hage R, Baalbaki R, Salman R, Talih S, Shihadeh A, and Saliba NA**. Free-Base and Protonated Nicotine in Electronic Cigarette Liquids and Aerosols. *Chemical research in toxicology* 28: 1532-1537, 2015.

41. **El-Hellani A, Salman R, El-Hage R, Talih S, Malek N, Baalbaki R, Karaoghlanian N, Nakkash R, Shihadeh A, and Saliba NA**. Nicotine and Carbonyl Emissions From Popular Electronic Cigarette Products: Correlation to Liquid Composition and Design Characteristics. *Nicotine Tob Res* 20: 215-223, 2018.

42. **Duell AK, Pankow JF, and Peyton DH**. Free-Base Nicotine Determination in Electronic Cigarette Liquids by (1)H NMR Spectroscopy. *Chemical research in toxicology* 31: 431-434, 2018.

43. **Harvanko AM, Havel CM, Jacob P, and Benowitz NL**. Characterization of Nicotine Salts in 23 Electronic Cigarette Refill Liquids. *Nicotine Tob Res* 2019.

44. **Bowen A, and Xing C**. Nicotine salt formulations for aerosol devices and methods thereof, United States Patent US9215895B2. In: *Google Patents*. The United States: Juul Labs Inc.

45. **Leventhal AM, Madden DR, Peraza N, Schiff SJ, Lebovitz L, Whitted L, Barrington-Trimis J, Mason TB, Anderson MK, and Tackett AP**. Effect of Exposure to e-Cigarettes With Salt vs Free-Base Nicotine on the Appeal and Sensory Experience of Vaping: A Randomized Clinical Trial. *JAMA Network Open* 4: e2032757-e2032757, 2021.

46. **Omaiye EE, McWhirter KJ, Luo W, Pankow JF, and Talbot P**. High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations. *Chemical research in toxicology* 32: 1058-1069, 2019.

47. **Tierney PA, Karpinski CD, Brown JE, Luo W, and Pankow JF**. Flavour chemicals in electronic cigarette fluids. *Tob Control* 25: e10-15, 2016.

48. **Omaiye EE, McWhirter KJ, Luo W, Tierney PA, Pankow JF, and Talbot P**. High concentrations of flavor chemicals are present in electronic cigarette refill fluids. *Sci Rep* 9: 2468, 2019.

49. **Behar RZ, Luo W, McWhirter KJ, Pankow JF, and Talbot P**. Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Sci Rep* 8: 8288, 2018.

50. **Krüsemann EJZ, Pennings JLA, Cremers J, Bakker F, Boesveldt S, and Talhout R**. GC-MS analysis of e-cigarette refill solutions: A comparison of flavoring composition between flavor categories. *J Pharm Biomed Anal* 188: 113364, 2020.

51. **Behar RZ, Luo W, Lin SC, Wang Y, Valle J, Pankow JF, and Talbot P**. Distribution, quantification and toxicity of cinnamaldehyde in electronic cigarette refill fluids and aerosols. *Tob Control* 25: ii94-ii102, 2016.

52. **FEMA**. Safety Assessment and Regulatory Authority to Use Flavors—Focus on Electronic Nicotine Delivery Systems and Flavored Tobacco Products. 2016.

53. **Omaiye EE, Luo W, McWhirter KJ, Pankow JF, and Talbot P**. Flavour chemicals, synthetic coolants and pulegone in popular mint-flavoured and menthol-flavoured e-cigarettes. *Tobacco Control* tobaccocontrol-2021-056582, 2021.

54. **Erythropel HC, Davis LM, de Winter TM, Jordt SE, Anastas PT, O'Malley SS, Krishnan-Sarin S, and Zimmerman JB**. Flavorant-Solvent Reaction Products and Menthol in JUUL E-Cigarettes and Aerosol. *Am J Prev Med* 57: 425-427, 2019.

55. **Erythropel HC, Jabba SV, DeWinter TM, Mendizabal M, Anastas PT, Jordt SE, and Zimmerman JB**. Formation of flavorant-propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob Res* 2018.

56. **Hickman E, Herrera CA, and Jaspers I**. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chemical research in toxicology* 32: 982-985, 2019.

57. **Li Q, Zhan Y, Wang L, Leischow SJ, and Zeng DD**. Analysis of symptoms and their potential associations with e-liquids' components: a social media study. *BMC Public Health* 16: 674, 2016.

58. **Escobar YH, Nipp G, Cui T, Petters SS, Surratt JD, and Jaspers I**. In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System. *Chemical research in toxicology* 2020.

59. **Uchiyama S, Noguchi M, Sato A, Ishitsuka M, Inaba Y, and Kunugita N**. Determination of Thermal Decomposition Products Generated from E-Cigarettes. *Chemical research in toxicology* 33: 576-583, 2020.

60. **Sleiman M, Logue JM, Montesinos VN, Russell ML, Litter MI, Gundel LA, and Destaillats H**. Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ Sci Technol* 50: 9644-9651, 2016.

61. **Son Y, Weisel C, Wackowski O, Schwander S, Delnevo C, and Meng Q**. The Impact of Device Settings, Use Patterns, and Flavorings on Carbonyl Emissions from Electronic Cigarettes. *Int J Environ Res Public Health* 17: 2020.

62. **Li Y, Burns AE, Tran LN, Abellar KA, Poindexter M, Li X, Madl AK, Pinkerton KE, and Nguyen TB**. Impact of e-Liquid Composition, Coil Temperature, and Puff Topography on the Aerosol Chemistry of Electronic Cigarettes. *Chemical research in toxicology* 34: 1640-1654, 2021.

63. **Son Y, Mainelis G, Delnevo C, Wackowski OA, Schwander S, and Meng Q**. Investigating E-Cigarette Particle Emissions and Human Airway Depositions under Various E-Cigarette-Use Conditions. *Chemical research in toxicology* 33: 343-352, 2020.

64. **Cirillo S, Urena JF, Lambert JD, Vivarelli F, Canistro D, Paolini M, Cardenia V, Rodriguez-Estrada MT, Richie JP, Jr., and Elias RJ**. Impact of electronic cigarette heating coil resistance on the production of reactive carbonyls, reactive oxygen species and induction of cytotoxicity in human lung cancer cells in vitro. *Regulatory toxicology and pharmacology : RTP* 109: 104500, 2019.

65. **Klager S, Vallarino J, MacNaughton P, Christiani DC, Lu Q, and Allen JG**. Flavoring Chemicals and Aldehydes in E-Cigarette Emissions. *Environmental Science & Technology* 51: 10806-10813, 2017.

66. **Azimi P, Keshavarz Z, Lahaie Luna M, Cedeno Laurent JG, Vallarino J, Christiani DC, and Allen JG**. An Unrecognized Hazard in E-Cigarette Vapor: Preliminary Quantification of Methylglyoxal Formation from Propylene Glycol in E-Cigarettes. *Int J Environ Res Public Health* 18: 2021.

67. **Park HR, Vallarino J, O'Sullivan M, Wirth C, Panganiban RA, Webb G, Shumyatcher M, Himes BE, Park JA, Christiani DC, Allen J, and Lu Q**. Electronic cigarette smoke reduces ribosomal protein gene expression to impair protein synthesis in primary human airway epithelial cells. *Sci Rep* 11: 17517, 2021.

68. **Khlystov A, and Samburova V**. Flavoring Compounds Dominate Toxic Aldehyde Production during E-Cigarette Vaping. *Environ Sci Technol* 50: 13080-13085, 2016.

69. **Farsalinos K, Gillman G, Kistler K, and Yannovits N**. Comment on "Flavoring Compounds Dominate Toxic Aldehyde Production during E Cigarette Vaping". *Environ Sci Technol* 51: 2491-2492, 2017.

70. **Khlystov A, and Samburova V**. Response to Comment on "Flavoring Compounds Dominate Toxic Aldehyde Production during E Cigarette Vaping". *Environ Sci Technol* 51: 2493-2494, 2017.

71. **Williams M, Li J, and Talbot P**. Effects of Model, Method of Collection, and Topography on Chemical Elements and Metals in the Aerosol of Tank-Style Electronic Cigarettes. *Sci Rep* 9: 13969, 2019.

72. **Williams M, Villarreal A, Bozhilov K, Lin S, and Talbot P**. Metal and silicate particles including nanoparticles are present in electronic cigarette cartomizer fluid and aerosol. *PLoS One* 8: e57987, 2013.

73. **Williams M, Bozhilov K, Ghai S, and Talbot P**. Elements including metals in the atomizer and aerosol of disposable electronic cigarettes and electronic hookahs. *PLoS One* 12: e0175430, 2017.

74. **Pappas RS, Gray N, Halstead M, Valentin-Blasini L, and Watson C**. Toxic Metal-Containing Particles in Aerosols from Pod-Type Electronic Cigarettes. *J Anal Toxicol* 45: 337-347, 2021.

75. **Holt AK, Poklis JL, Cobb CO, and Peace MR**. Identification of Gamma-Butyrolactone in JUUL Liquids. *J Anal Toxicol* 45: 892-900, 2021.

76. **Hadwiger ME, Trehy ML, Ye W, Moore T, Allgire J, and Westenberger B**. Identification of amino-tadalafil and rimonabant in electronic cigarette products using high pressure liquid chromatography with diode array and tandem mass spectrometric detection. *J Chromatogr A* 1217: 7547-7555, 2010.

77. **Tehrani MW, Newmeyer MN, Rule AM, and Prasse C**. Characterizing the Chemical Landscape in Commercial E-Cigarette Liquids and Aerosols by Liquid Chromatography-High-Resolution Mass Spectrometry. *Chemical research in toxicology* 34: 2216-2226, 2021.

78. **Chadi N, Minato C, and Stanwick R**. Cannabis vaping: Understanding the health risks of a rapidly emerging trend. *Paediatr Child Health* 25: S16-s20, 2020.

79. **Trivers KF, Phillips E, Gentzke AS, Tynan MA, and Neff LJ**. Prevalence of Cannabis Use in Electronic Cigarettes Among US Youth. *JAMA Pediatr* 172: 1097-1099, 2018.

80. **Bhatta DN, and Glantz SA**. Association of E-Cigarette Use With Respiratory Disease Among Adults: A Longitudinal Analysis. *Am J Prev Med* 58: 182-190, 2020.

81. **Dai H, and Khan AS**. A Longitudinal Study of Exposure to Tobacco-Related Toxicants and Subsequent Respiratory Symptoms Among U.S. Adults with Varying E-cigarette Use Status. *Nicotine Tob Res* 22: S61-s69, 2020.

82. **Osei AD, Mirbolouk M, Orimoloye OA, Dzaye O, Uddin SMI, Dardari ZA, DeFilippis AP, Bhatnagar A, and Blaha MJ**. The association between e-cigarette use and asthma among never combustible cigarette smokers: behavioral risk factor surveillance system (BRFSS) 2016 & 2017. *BMC Pulm Med* 19: 180, 2019.

83. **Bircan E, Bezirhan U, Porter A, Fagan P, and Orloff MS**. Electronic cigarette use and its association with asthma, chronic obstructive pulmonary disease (COPD) and asthma-COPD overlap syndrome among never cigarette smokers. *Tob Induc Dis* 19: 23, 2021.

84. **Jackson M, Singh KP, Lamb T, McIntosh S, and Rahman I**. Flavor Preference and Systemic Immunoglobulin Responses in E-Cigarette Users and Waterpipe and Tobacco Smokers: A Pilot Study. *Int J Environ Res Public Health* 17: 2020.

85. **Schweitzer RJ, Wills TA, Tam E, Pagano I, and Choi K**. E-cigarette use and asthma in a multiethnic sample of adolescents. *Prev Med* 105: 226-231, 2017.

86. **McConnell R, Barrington-Trimis JL, Wang K, Urman R, Hong H, Unger J, Samet J, Leventhal A, and Berhane K**. Electronic Cigarette Use and Respiratory Symptoms in Adolescents. *Am J Respir Crit Care Med* 195: 1043-1049, 2017.

87. **Cho JH, and Paik SY**. Association between Electronic Cigarette Use and Asthma among High School Students in South Korea. *PLoS One* 11: e0151022, 2016.

88. **Chaffee BW, Barrington-Trimis J, Liu F, Wu R, McConnell R, Krishnan-Sarin S, Leventhal AM, and Kong G**. E-cigarette use and adverse respiratory symptoms among adolescents and Young adults in the United States. *Preventive Medicine* 153: 106766, 2021.

89. **Bayly JE, Bernat D, Porter L, and Choi K**. Secondhand Exposure to Aerosols From Electronic Nicotine Delivery Systems and Asthma Exacerbations Among Youth With Asthma. *Chest* 155: 88-93, 2019.

90. **Braymiller JL, Barrington-Trimis JL, Leventhal AM, Islam T, Kechter A, Krueger EA, Cho J, Lanza I, Unger JB, and McConnell R**. Assessment of Nicotine and Cannabis Vaping and Respiratory Symptoms in Young Adults. *JAMA Netw Open* 3: e2030189, 2020.

91. **Walker CJ, and Christian WJ**. Estimating the Population Attributable Fraction of Asthma Due to Electronic Cigarette Use and Other Risk Factors Using Kentucky Behavioral Risk Factor Survey Data, 2016-2017. *Subst Use Misuse* 56: 353-358, 2021.

92. **Antwi GO, and Rhodes DL**. Association between E-cigarette use and chronic obstructive pulmonary disease in non-asthmatic adults in the USA. *Journal of Public Health* fdaa229, 2020.

93. **Lerner CA, Sundar IK, Yao H, Gerloff J, Ossip DJ, McIntosh S, Robinson R, and Rahman I**. Vapors produced by electronic cigarettes and e-juices with flavorings induce toxicity, oxidative stress, and inflammatory response in lung epithelial cells and in mouse lung. *PLoS One* 10: e0116732, 2015.

94. **Wang Q, Sundar IK, Li D, Lucas JH, Muthumalage T, McDonough SR, and Rahman I**. E-cigarette-induced pulmonary inflammation and dysregulated repair are mediated by nAChR α7 receptor: role of nAChR α7 in SARS-CoV-2 Covid-19 ACE2 receptor regulation. *Respir Res* 21: 154, 2020.

95. **Madison MC, Landers CT, Gu BH, Chang CY, Tung HY, You R, Hong MJ, Baghaei N, Song LZ, Porter P, Putluri N, Salas R, Gilbert BE, Levental I, Campen MJ, Corry DB, and Kheradmand F**. Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine. *J Clin Invest* 129: 4290-4304, 2019.

96. **Masso-Silva JA, Moshensky A, Shin J, Olay J, Nilaad S, Advani I, Bojanowski CM, Crotty S, Li WT, Ongkeko WM, Singla S, and Crotty Alexander LE**. Chronic E-Cigarette Aerosol Inhalation Alters the Immune State of the Lungs and Increases ACE2 Expression, Raising Concern for Altered Response and Susceptibility to SARS-CoV-2. *Front Physiol* 12: 649604, 2021.

97. **Szafran BN, Pinkston R, Perveen Z, Ross MK, Morgan T, Paulsen DB, Penn AL, Kaplan BLF, and Noël A**. Electronic-Cigarette Vehicles and Flavoring Affect Lung Function and Immune Responses in a Murine Model. *Int J Mol Sci* 21: 2020.

98. **Lappas AS, Tzortzi AS, Konstantinidi EM, Teloniatis SI, Tzavara CK, Gennimata SA, Koulouris NG, and Behrakis PK**. Short-term respiratory effects of e-cigarettes in healthy individuals and smokers with asthma. *Respirology* 23: 291-297, 2018.

99. **Boulay M, Henry C, Bossé Y, Boulet LP, and Morissette MC**. Acute effects of nicotine-free and flavour-free electronic cigarette use on lung functions in healthy and asthmatic individuals. *Respir Res* 18: 33, 2017.

100. **Bradford LE, Rebuli ME, Ring BJ, Jaspers I, Clement KC, and Loughlin CE**. Danger in the vapor? ECMO for adolescents with status asthmaticus after vaping. *J Asthma* 1-5, 2019.

101. **Clapp PW, Peden DB, and Jaspers I**. E-cigarettes, vaping-related pulmonary illnesses, and asthma: A perspective from inhalation toxicologists. *J Allergy Clin Immunol* 145: 97-99, 2020.

102. **Chapman DG, Casey DT, Ather JL, Aliyeva M, Daphtary N, Lahue KG, van der Velden JL, Janssen-Heininger YMW, and Irvin CG**. The Effect of Flavored E-cigarettes on Murine Allergic Airways Disease. *Sci Rep* 9: 13671, 2019.

103. **Bonser LR, and Erle DJ**. Airway Mucus and Asthma: The Role of MUC5AC and MUC5B. *J Clin Med* 6: 112, 2017.

104. **Lachowicz-Scroggins ME, Yuan S, Kerr SC, Dunican EM, Yu M, Carrington SD, and Fahy JV**. Abnormalities in MUC5AC and MUC5B Protein in Airway Mucus in Asthma. *Am J Respir Crit Care Med* 194: 1296-1299, 2016.

105. **Mims JW**. Asthma: definitions and pathophysiology. *Int Forum Allergy Rhinol* 5 Suppl 1: S2-6, 2015.

106. **Kesimer M, Ford AA, Ceppe A, Radicioni G, Cao R, Davis CW, Doerschuk CM, Alexis NE, Anderson WH, Henderson AG, Barr RG, Bleecker ER, Christenson SA, Cooper CB, Han MK, Hansel NN, Hastie AT, Hoffman EA, Kanner RE, Martinez F, Paine R, 3rd, Woodruff PG, O'Neal WK, and Boucher RC**. Airway Mucin Concentration as a Marker of Chronic Bronchitis. *The New England journal of medicine* 377: 911-922, 2017.

107. **Zoz DF, Lawson WE, and Blackwell TS**. Idiopathic pulmonary fibrosis: a disorder of epithelial cell dysfunction. *Am J Med Sci* 341: 435-438, 2011.

108. **Stancil IT, Michalski JE, Davis-Hall D, Chu HW, Park J-A, Magin CM, Yang IV, Smith BJ, Dobrinskikh E, and Schwartz DA**. Pulmonary fibrosis distal airway epithelia are dynamically and structurally dysfunctional. *Nature Communications* 12: 4566, 2021.

109. **Reidel B, Radicioni G, Clapp PW, Ford AA, Abdelwahab S, Rebuli ME, Haridass P, Alexis NE, Jaspers I, and Kesimer M**. E-Cigarette Use Causes a Unique Innate Immune Response in the Lung, Involving Increased Neutrophilic Activation and Altered Mucin Secretion. *Am J Respir Crit Care Med* 197: 492-501, 2018.

110. **Ghosh A, Coakley RC, Mascenik T, Rowell TR, Davis ES, Rogers K, Webster MJ, Dang H, Herring LE, Sassano MF, Livraghi-Butrico A, Van Buren SK, Graves LM, Herman MA, Randell SH, Alexis NE, and Tarran R**. Chronic E-Cigarette Exposure Alters the Human Bronchial Epithelial Proteome. *Am J Respir Crit Care Med* 198: 67-76, 2018.

111. **Clapp PW, Lavrich KS, van Heusden CA, Lazarowski ER, Carson JL, and Jaspers I**. Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *Am J Physiol Lung Cell Mol Physiol* 316: L470-l486, 2019.

112. **Chung S, Baumlin N, Dennis JS, Moore R, Salathe SF, Whitney PL, Sabater J, Abraham WM, Kim MD, and Salathe M**. Electronic Cigarette Vapor with Nicotine Causes Airway Mucociliary Dysfunction Preferentially via TRPA1 Receptors. *Am J Respir Crit Care Med* 200: 1134-1145, 2019.

113. **Yang H, and Li S**. Transient Receptor Potential Ankyrin 1 (TRPA1) Channel and Neurogenic Inflammation in Pathogenesis of Asthma. *Med Sci Monit* 22: 2917-2923, 2016.

114. **Bessac BF, and Jordt SE**. Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology (Bethesda)* 23: 360-370, 2008.

115. **Bartemes KR, and Kita H**. Dynamic role of epithelium-derived cytokines in asthma. *Clin Immunol* 143: 222-235, 2012.

116. **Barnes PJ**. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 118: 3546-3556, 2008.

117. **Gerloff J, Sundar IK, Freter R, Sekera ER, Friedman AE, Robinson R, Pagano T, and Rahman I**. Inflammatory Response and Barrier Dysfunction by Different e-Cigarette Flavoring Chemicals Identified by Gas Chromatography-Mass Spectrometry in e-Liquids and e-Vapors on Human Lung Epithelial Cells and Fibroblasts. *Appl In Vitro Toxicol* 3: 28-40, 2017.

118. **Pinkston R, Zaman H, Hossain E, Penn AL, and Noël A**. Cell-specific toxicity of short-term JUUL aerosol exposure to human bronchial epithelial cells and murine macrophages exposed at the air–liquid interface. *Respiratory Research* 21: 269, 2020.

119. **Martin EM, Clapp PW, Rebuli ME, Pawlak EA, Glista-Baker E, Benowitz NL, Fry RC, and Jaspers I**. E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 311: L135-144, 2016.

120. **Park HR, O'Sullivan M, Vallarino J, Shumyatcher M, Himes BE, Park JA, Christiani DC, Allen J, and Lu Q**. Transcriptomic response of primary human airway epithelial cells to flavoring chemicals in electronic cigarettes. *Sci Rep* 9: 1400, 2019.

121. **Smith MR, Jarrell ZR, Orr M, Liu KH, Go YM, and Jones DP**. Metabolome-wide association study of flavorant vanillin exposure in bronchial epithelial cells reveals disease-related perturbations in metabolism. *Environ Int* 147: 106323, 2021.

122. **Crotty Alexander LE, Drummond CA, Hepokoski M, Mathew D, Moshensky A, Willeford A, Das S, Singh P, Yong Z, Lee JH, Vega K, Du A, Shin J, Javier C, Tian J, Brown JH, and Breen EC**. Chronic inhalation of e-cigarette vapor containing nicotine disrupts airway barrier function and induces systemic inflammation and multiorgan fibrosis in mice. *Am J Physiol Regul Integr Comp Physiol* 314: R834-r847, 2018.

123. **Ghosh B, Reyes-Caballero H, Akgün-Ölmez SG, Nishida K, Chandrala L, Smirnova L, Biswal S, and Sidhaye VK**. Effect of sub-chronic exposure to cigarette smoke, electronic cigarette and waterpipe on human lung epithelial barrier function. *BMC Pulm Med* 20: 216, 2020.

124. **Escobar YH, Morrison CB, Chen Y, Hickman E, Love CA, Rebuli ME, Surratt JD, Ehre C, and Jaspers I**. Differential responses to e-cig generated aerosols from humectants and different forms of nicotine in epithelial cells from nonsmokers and smokers. *Am J Physiol Lung Cell Mol Physiol* 320: L1064-l1073, 2021.

125. **Ween MP, Whittall JJ, Hamon R, Reynolds PN, and Hodge SJ**. Phagocytosis and Inflammation: Exploring the effects of the components of E-cigarette vapor on macrophages. *Physiol Rep* 5: e13370, 2017.

126. **Muthumalage T, Prinz M, Ansah KO, Gerloff J, Sundar IK, and Rahman I**. Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine. *Front Physiol* 8: 1130, 2017.

127. **Scott A, Lugg ST, Aldridge K, Lewis KE, Bowden A, Mahida RY, Grudzinska FS, Dosanjh D, Parekh D, Foronjy R, Sapey E, Naidu B, and Thickett DR**. Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages. *Thorax* 73: 1161-1169, 2018.

128. **Sussan TE, Gajghate S, Thimmulappa RK, Ma J, Kim JH, Sudini K, Consolini N, Cormier SA, Lomnicki S, Hasan F, Pekosz A, and Biswal S**. Exposure to electronic cigarettes impairs pulmonary anti-bacterial and anti-viral defenses in a mouse model. *PLoS One* 10: e0116861, 2015.

129. **Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I**. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278-L292, 2017.

130. **Ghosh A, Coakley RD, Ghio AJ, Muhlebach MS, Esther CR, Jr., Alexis NE, and Tarran R**. Chronic E-Cigarette Use Increases Neutrophil Elastase and Matrix Metalloprotease Levels in the Lung. *Am J Respir Crit Care Med* 2019.

131. **Grunwell JR, Stephenson ST, Tirouvanziam R, Brown LAS, Brown MR, and Fitzpatrick AM**. Children with Neutrophil-Predominant Severe Asthma Have Proinflammatory Neutrophils With Enhanced Survival and Impaired Clearance. *J Allergy Clin Immunol Pract* 7: 516-525.e516, 2019.

132. **Mosca T, Menezes MC, Silva AV, Stirbulov R, and Forte WC**. Chemotactic and Phagocytic Activity of Blood Neutrophils in Allergic Asthma. *Immunol Invest* 44: 509-520, 2015.

133. **Radermecker C, Louis R, Bureau F, and Marichal T**. Role of neutrophils in allergic asthma. *Curr Opin Immunol* 54: 28-34, 2018.

134. **Hoenderdos K, and Condliffe A**. The neutrophil in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 48: 531-539, 2013.

135. **Stănescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM, and Maestrelli P**. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 51: 267-271, 1996.

136. **Hwang JH, Lyes M, Sladewski K, Enany S, McEachern E, Mathew DP, Das S, Moshensky A, Bapat S, Pride DT, Ongkeko WM, and Crotty Alexander LE**. Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria. *J Mol Med (Berl)* 94: 667-679, 2016.

137. **Rebuli ME, Glista-Baker E, Hoffman JR, Duffney PF, Robinette C, Speen AM, Pawlak EA, Dhingra R, Noah TL, and Jaspers I**. Electronic-Cigarette Use Alters Nasal Mucosal Immune Response to Live-attenuated Influenza Virus. A Clinical Trial. *Am J Respir Cell Mol Biol* 64: 126-137, 2021.

138. **McAlinden KD, Eapen MS, Lu W, Chia C, Haug G, and Sohal SS**. COVID-19 and vaping: risk for increased susceptibility to SARS-CoV-2 infection? *Eur Respir J* 56: 2020.

139. **Sivaraman V, Parker D, Zhang R, Jones MM, and Onyenwoke RU**. Vaping Exacerbates Coronavirus-Related Pulmonary Infection in a Murine Model. *Front Physiol* 12: 634839, 2021.

140. **Lallai V, Manca L, and Fowler CD**. E-cigarette vape and lung ACE2 expression: Implications for coronavirus vulnerability. *Environ Toxicol Pharmacol* 86: 103656, 2021.

141. **Kale D, Herbec A, Perski O, Jackson SE, Brown J, and Shahab L**. Associations between vaping and Covid-19: Cross-sectional findings from the HEBECO study. *Drug Alcohol Depend* 221: 108590, 2021.

142. **Jose T, Croghan IT, Hays JT, Schroeder DR, and Warner DO**. Electronic Cigarette Use Is Not Associated with COVID-19 Diagnosis. *J Prim Care Community Health* 12: 21501327211024391, 2021.

143. **Noval Rivas M, Crother TR, and Arditi M**. The microbiome in asthma. *Curr Opin Pediatr* 28: 764-771, 2016.

144. **Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L, Pachter L, Moffatt MF, and Cookson WO**. Disordered microbial communities in asthmatic airways. *PLoS One* 5: e8578, 2010.

145. **McCauley K, Durack J, Valladares R, Fadrosh DW, Lin DL, Calatroni A, LeBeau PK, Tran HT, Fujimura KE, LaMere B, Merana G, Lynch K, Cohen RT, Pongracic J, Khurana Hershey GK, Kercsmar CM, Gill M, Liu AH, Kim H, Kattan M, Teach SJ, Togias A, Boushey HA, Gern JE, Jackson DJ, and Lynch SV**. Distinct nasal airway bacterial microbiotas differentially relate to exacerbation in pediatric patients with asthma. *J Allergy Clin Immunol* 144: 1187-1197, 2019.

146. **Loverdos K, Bellos G, Kokolatou L, Vasileiadis I, Giamarellos E, Pecchiari M, Koulouris N, Koutsoukou A, and Rovina N**. Lung Microbiome in Asthma: Current Perspectives. *J Clin Med* 8: 2019.

147. **Man WH, de Steenhuijsen Piters WA, and Bogaert D**. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* 15: 259-270, 2017.

148. **Ubags NDJ, and Marsland BJ**. Mechanistic insight into the function of the microbiome in lung diseases. *Eur Respir J* 50: 2017.

149. **Ramakrishnan VR, Hauser LJ, and Frank DN**. The sinonasal bacterial microbiome in health and disease. *Curr Opin Otolaryngol Head Neck Surg* 24: 20-25, 2016.

150. **Charlson ES, Chen J, Custers-Allen R, Bittinger K, Li H, Sinha R, Hwang J, Bushman FD, and Collman RG**. Disordered microbial communities in the upper respiratory tract of cigarette smokers. *PLoS One* 5: e15216, 2010.

151. **Pushalkar S, Paul B, Li Q, Yang J, Vasconcelos R, Makwana S, Gonzalez JM, Shah S, Xie C, Janal MN, Queiroz E, Bederoff M, Leinwand J, Solarewicz J, Xu F, Aboseria E, Guo Y, Aguallo D, Gomez C, Kamer A, Shelley D, Aphinyanaphongs Y, Barber C, Gordon T, Corby P, Li X, and Saxena D**. Electronic Cigarette Aerosol Modulates the Oral Microbiome and Increases Risk of Infection. *iScience* 23: 100884, 2020.

152. **Ganesan SM, Dabdoub SM, Nagaraja HN, Scott ML, Pamulapati S, Berman ML, Shields PG, Wewers ME, and Kumar PS**. Adverse effects of electronic cigarettes on the disease-naive oral microbiome. *Sci Adv* 6: eaaz0108, 2020.

153. **Chopyk J, Bojanowski CM, Shin J, Moshensky A, Fuentes AL, Bonde SS, Chuki D, Pride DT, and Crotty Alexander LE**. Compositional Differences in the Oral Microbiome of E-cigarette Users. *Front Microbiol* 12: 599664, 2021.

154. **Miyashita L, Suri R, Dearing E, Mudway I, Dove RE, Neill DR, Van Zyl-Smit R, Kadioglu A, and Grigg J**. E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells. *Eur Respir J* 51: 2018.

155. **U.S. Department of Health and Human Services**. Preventing tobacco use among youth and young adults. Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention, 2012.

156. **Loukas A, Marti CN, and Harrell MB**. Electronic nicotine delivery systems use predicts transitions in cigarette smoking among young adults. *Drug Alcohol Depend* 231: 109251, 2021.

157. **Hammond D, Reid JL, Cole AG, and Leatherdale ST**. Electronic cigarette use and smoking initiation among youth: a longitudinal cohort study. *Cmaj* 189: E1328-e1336, 2017.

158. **O'Brien D, Long J, Quigley J, Lee C, McCarthy A, and Kavanagh P**. Association between electronic cigarette use and tobacco cigarette smoking initiation in adolescents: a systematic review and meta-analysis. *BMC Public Health* 21: 954, 2021.

159. **Amrock SM, Lee L, and Weitzman M**. Perceptions of e-Cigarettes and Noncigarette Tobacco Products Among US Youth. *Pediatrics* 138: 2016.

160. **Amrock SM, Zakhar J, Zhou S, and Weitzman M**. Perception of e-cigarette harm and its correlation with use among U.S. adolescents. *Nicotine Tob Res* 17: 330-336, 2015.

161. **Parker MA, Villanti AC, Quisenberry AJ, Stanton CA, Doogan NJ, Redner R, Gaalema DE, Kurti AN, Nighbor T, Roberts ME, Cepeda-Benito A, and Higgins ST**. Tobacco Product Harm Perceptions and New Use. *Pediatrics* 142: 2018.

162. **Miech R, Leventhal A, Johnston L, O'Malley PM, Patrick ME, and Barrington-Trimis J**. Trends in Use and Perceptions of Nicotine Vaping Among US Youth From 2017 to 2020. *JAMA Pediatr* 175: 185-190, 2021.

163. **Russell C, Katsampouris E, and McKeganey N**. Harm and Addiction Perceptions of the JUUL E-Cigarette Among Adolescents. *Nicotine & Tobacco Research* 22: 713-721, 2020.

164. **Willett JG, Bennett M, Hair EC, Xiao H, Greenberg MS, Harvey E, Cantrell J, and Vallone D**. Recognition, use and perceptions of JUUL among youth and young adults. *Tobacco Control* 28: 115, 2019.

165. **Pepper JK, Ribisl KM, and Brewer NT**. Adolescents' interest in trying flavoured e-cigarettes. *Tobacco Control* 25: ii62, 2016.

166. **Meernik C, Baker HM, Kowitt SD, Ranney LM, and Goldstein AO**. Impact of non-menthol flavours in e-cigarettes on perceptions and use: an updated systematic review. *BMJ open* 9: e031598, 2019.

167. **Gorukanti A, Delucchi K, Ling P, Fisher-Travis R, and Halpern-Felsher B**. Adolescents' attitudes towards e-cigarette ingredients, safety, addictive properties, social norms, and regulation. *Prev Med* 94: 65-71, 2017.

168. **Strong DR, Messer K, White M, Shi Y, Noble M, Portnoy DB, Persoskie A, Kaufman AR, Choi K, Carusi C, Bansal-Travers M, Hyland A, and Pierce J**. Youth perception of harm and addictiveness of tobacco products: Findings from the Population Assessment of Tobacco and Health Study (Wave 1). *Addict Behav* 92: 128-135, 2019.

169. **Committee on Key Challenge Areas for Convergence and Health; Board on Life Sciences; Division on Earth and Life Studies; National Research Council**. Convergence: Facilitating Transdisciplinary Integration of Life Sciences, Physical Sciences, Engineering, and Beyond. Washington (DC): National Academies Press 2014.

CHAPTER 2: COMMON E-CIGARETTE FLAVORING CHEMICALS IMPAIR NEUTROPHIL PHAGOCYTOSIS AND OXIDATIVE BURST[2]

Introduction

The growing popularity of e-cigarettes has been linked to the public opinion and promotion of them as a safer alternative to tobacco cigarettes (1) and some studies suggesting e-cigarettes help in smoking cessation (2). Of particular concern is adolescent use of e-cigarettes. Currently, 20.8% of high school students in the US are e-cigarette users (3). E-cigarettes heat and aerosolize e-liquids, which are typically composed of humectants (propylene glycol (PG) and vegetable glycerin), nicotine, and flavoring chemicals. The wide variety of e-liquid flavors is one of the primary reasons that using e-cigarettes is so appealing to adolescents (4, 5), yet many of these flavoring chemicals have not been evaluated for inhalational toxicity (6). Hence, understanding the potential toxicity of e-cigarette flavorings is necessary to inform regulation of e-liquid manufacturing and to educate the public about e-cigarette safety.

Neutrophils, along with airway macrophages, are one of the body's first lines of defense against inhaled pathogens (7), making them an important target for inhaled toxicants. There is emerging appreciation for the complex interplay between cellular metabolism and innate immune response as well as the concept that bioenergetic changes are at the center of innate immune dysfunction and immune cell phenotypes (8). However, bioenergetic characterization of the innate immune system has been limited primarily to macrophages while neutrophils have been largely ignored (9, 10). Furthermore, the ability of toxicants to alter neutrophil bioenergetics and downstream functions remains unclear. Our lab has demonstrated that the flavoring chemical cinnamaldehyde significantly affects cellular bioenergetics, which in turn causes impairment of key innate immune functions of respiratory epithelial cells (11).

[2] This chapter previously appeared as an article in Chemical Research in Toxicology. The original citation is as follows: Hickman E, Herrera CA, and Jaspers I. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chemical research in toxicology* 32: 982-985, 2019. Reproduced with permission from Chemical Research in Toxicology. Copyright 2020 American Chemical Society.

Hence, disruption of cellular bioenergetics could be a central mechanism mediating adverse effects of flavoring chemicals.

In this study, we investigated the effects of four common flavoring chemicals – cinnamaldehyde (cinnamon), ethyl vanillin (vanilla), benzaldehyde (almond or cherry), and isoamyl acetate (banana) – on neutrophil oxidative burst and phagocytosis. Because we had previously observed impairment of airway and immune cell function by cinnamaldehyde (11, 12), we were interested in whether other common aromatic aldehydes found in e-cigarettes also impaired cellular function (13-15). We chose isoamyl acetate, an ester that is commonly detected in e-cigarettes (15), for comparison. We also assessed the effects of benzaldehyde PG acetal, which can form via the reaction of benzaldehyde and PG in e-liquids (16) and has been identified by multiple research groups in e-liquids and e-cigarette aerosols (13-15).

Methods

Blood Collection and Neutrophil Isolation

We isolated neutrophils from venous blood of healthy human subjects as described previously (12). Venus blood was obtained from subjects (males and females between 21-56 years of age) for the isolation of peripheral blood neutrophils (Table 2-1). Subjects were self-reported healthy volunteers with no acute illness or allergy symptoms. Other exclusion criteria were current nicotine use, asthma, and/or pregnant and nursing women. Informed consent was obtained from all subjects and all studies were approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board (IRB #11-1363 and #97-0845). All studies were performed in accordance with The Code of Ethics of the World Medical Association. Venous blood was collected in EDTA-coated Vacutainer tubes (BD, Thermo Fisher Scientific, Waltham, MA) and neutrophils were isolated and resuspended as described previously (12). Cytotoxicty was determined by measuring lactate dehydrogenase (LDH) in supernatants, as described by us before (data not shown) (12).

Table 2-1. Overall study demographics. Data represents mean ± SEM

	Male	Female
n	15	17
Age	30.2 ± 2.1	28.4 ± 2.0
BMI	25.9 ± 1.0	24.9 ± 1.2
Race (White/African American/Asian)	11/3/1	15/1/1

Flavoring Chemicals

Cinnamaldehyde (99%, 7.79 M), benzaldehyde (≥ 98%, 9.64 M), benzaldehyde propylene glycol acetal (≥ 95%, 6.16 M), isoamyl acetate (≥ 97%, 6.73 M), and ethyl vanillin (≥ 98%, powdered) were acquired from Sigma-Aldrich (St. Louis, MO) and stored as indicated by the manufacturer. Ethyl vanillin was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich) at a concentration of 5 M. All other flavoring chemicals were diluted directly into cell culture media from the manufacturer stock solutions immediately prior to the experiment. The highest concentration of DMSO for the ethyl vanillin experiments was 0.1%.

Seahorse Extracellular Flux Analysis

We developed and optimized a Seahorse extracellular flux assay based on existing literature and manufacturer guidelines (17). Briefly, 24-well XF assay plates (Agilent Technologies, Santa Clara, CA) were coated with 0.672 μg Cell-Tak (Corning, Thermo Fisher Scientific, Waltham, MA) per well and stored at 4°C for no more than one week before use. Coated plates were allowed to warm to room temperature (approximately 21°C) before plating 1.5 x 10^5 isolated neutrophils per well in 100 μL of Seahorse media (Seahorse XF RPMI media without phenol red, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM HEPES, and 10 mM glucose, pH 7.4). The plate was centrifuged at 300 x g for 1 minute, rotated 180 degrees, and centrifugation was repeated for 1 minute. Cells were allowed to rest in a non-CO_2 incubator at 37°C for approximately 20 minutes, followed by the addition of 400 μL Seahorse media and an additional 20 minutes of incubation before the start of the assay. The effect of flavoring agents on neutrophil oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was measured via

sequential injection of a flavoring compound (0 – 5 mM final concentration) followed by injection of PKC agonist phorbol 12-myristate 13-acetate (PMA, 100 ng/mL final concentration) (Sigma Aldrich) on a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) at 37°C.). 0 – 5 mM was used because this is approximately 2 orders of magnitude below maximum concentrations of these flavorings in e-liquids, and we have observed cellular effects on other cell types in this concentration range (12-15). Seahorse Extracellular Flux assays simultaneously measure oxygen consumption and extracellular acidification. Extracellular acidification can be used as an indicator of glycolytic activity because the protons produced by lactate dehydrogenase during glycolysis acidify the extracellular space. Neutrophils generate the energy and NADPH needed to perform oxidative burst almost exclusively through glycolysis and the pentose phosphate pathway. Three baseline readings were taken before injection of the flavoring compound, three readings were taken after injection of the flavoring compound, and 30 readings were taken after injection of PMA. The assay was run with mix/wait/measure cycles of 3 minutes/0 minutes/3 minutes. The total oxygen consumption during oxidative burst represents the amount of oxygen converted by the neutrophils to superoxide. This was quantified by calculating the area under the curve after PMA stimulation using OCR data (Figure 2-1A, Figure S2-1).

Phagocytosis

$1x10^5$ isolated neutrophils were plated in 100 μL neutrophil media in a black clear-bottom 96-well plate. Cells were incubated (37°C, 5% CO_2) for 30 minutes. Neutrophils were then challenged in triplicate with 0-5 mM of flavoring chemical for 1 hour at 37°C and phagocytosis was measured using pHrodo Red *Staphylococcus aureus* BioParticles (Thermo Fischer Scientific, Waltham, MA) as described by us before (12). Cinnamaldehyde (CA, 1 mM) was used as a positive control for inhibition of phagocytosis (12).

Statistics

Each assay was performed with isolated neutrophils from at least n=3 male and n=3 female subjects. For the Seahorse experiments, data were analyzed using area under the curve analysis. Oxidative burst and phagocytosis data were normalized by calculating the percentage of response relative to the media or DMSO control for each subject. Normality of each data set was assessed using the Shapiro-Wilk test. Significance of normal dose-response data sets was assessed with a repeated-measures one-way ANOVA followed by Tukey's HSD test. Significance of non-normal dose-response data sets was assessed with a Friedman test followed by Dunn's post-test. We tested for but did not find significant sex differences in the effects of the flavoring chemicals on neutrophil oxidative burst or phagocytosis (data not shown).

Results

Cinnamaldehyde and ethyl vanillin decreased total oxygen consumption in a dose-dependent manner (Figures 2-1B, 2-1C, 2-2A, 2-2B), with a calculated IC_{50} of 0.39 +/- 0.04 mM and 0.42 +/- 0.1 mM respectively. Benzaldehyde and benzaldehyde PG acetal significantly decreased oxygen consumption only at the highest dose, 5 mM (Figures 2-1D, 2-1E, 2-2C, 2-2D). Isoamyl acetate did not affect oxygen consumption during oxidative burst (Figures 2-1F).

Using the area under the curve analysis, we also obtained the maximum OCR, time to the maximum OCR, and time to baseline OCR. The data for the maximum OCR followed similar trends to the total oxygen consumption data (Table S2-1). The highest doses of ethyl vanillin and benzaldehyde (5mM) significantly decreased the time to maximum OCR ($p < 0.05$, Table S2-2). There was no significant difference in the time it took to return to baseline for any of the doses of the flavoring chemicals (data not shown). The ECAR data mirror the OCR data, as expected (Figure S2-2, Table S2-3, Table S2-4).

We have previously shown that cinnamaldehyde impairs neutrophil phagocytosis (12). Here, we show that other aromatic aldehydes are also capable of impairing neutrophil phagocytosis. Ethyl vanillin,

benzaldehyde, and benzaldehyde PG acetal significantly decreased neutrophil phagocytosis (Figure 2-2A, 2-2B, 2-2C), while isoamyl acetate (Figure 2-2D) had no effect. The inhibition caused by benzaldehyde PG acetal was more potent than that observed with benzaldehyde, with IC_{50} values of 4.72 +/- 2.05 and 1.89 +/- 0.66 mM for benzaldehyde and benzaldehyde PG acetal, respectively, suggesting that chemicals formed via the reaction of flavoring chemicals and e-liquid constituents under ambient conditions may exert stronger toxicological effects than those of the base favoring chemical.

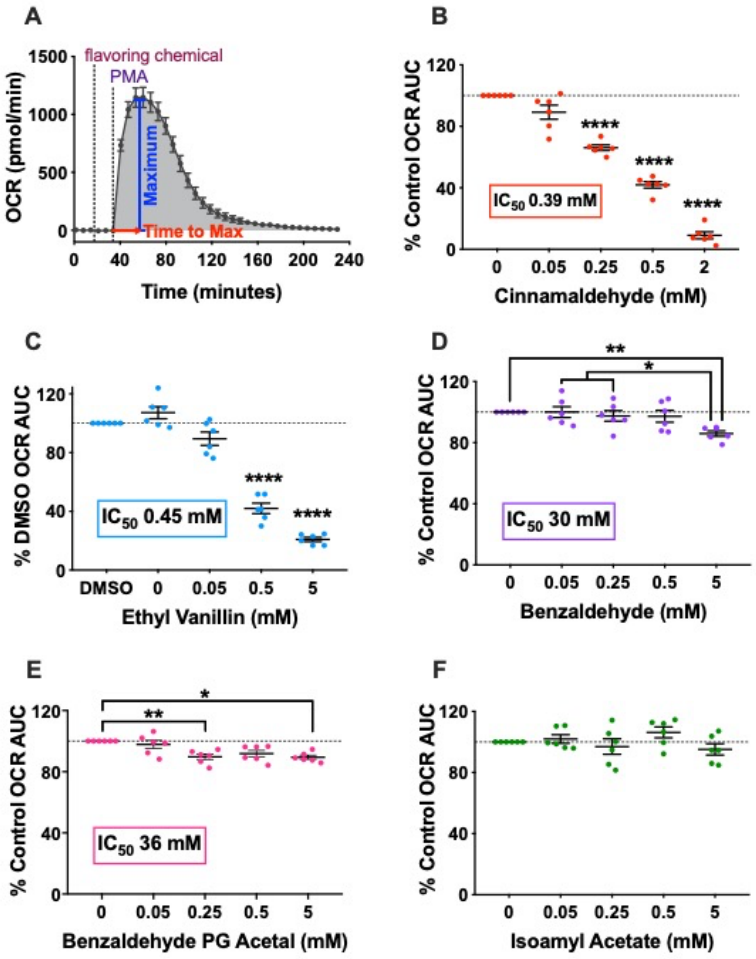


Figure 2-1. Neutrophil oxidative burst following exposure to e-cigarette flavoring chemicals.A Seahorse Extracellular Flux assay (A) was used to measure the effects of flavoring chemicals cinnamaldehyde (B), ethyl vanillin (C), benzaldehyde (D), benzaldehyde PG acetal (E), and isoamyl acetate (F) on PMA-stimulated neutrophil oxidative burst. Oxygen consumption rate (OCR, pmol/min) was used to determine total oxygen consumption by integrating the oxygen consumption rate (OCR, pmol/min) of PMA-stimulated neutrophils over time (area under the curve, AUC). Data represents mean +/- SEM with n = 6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

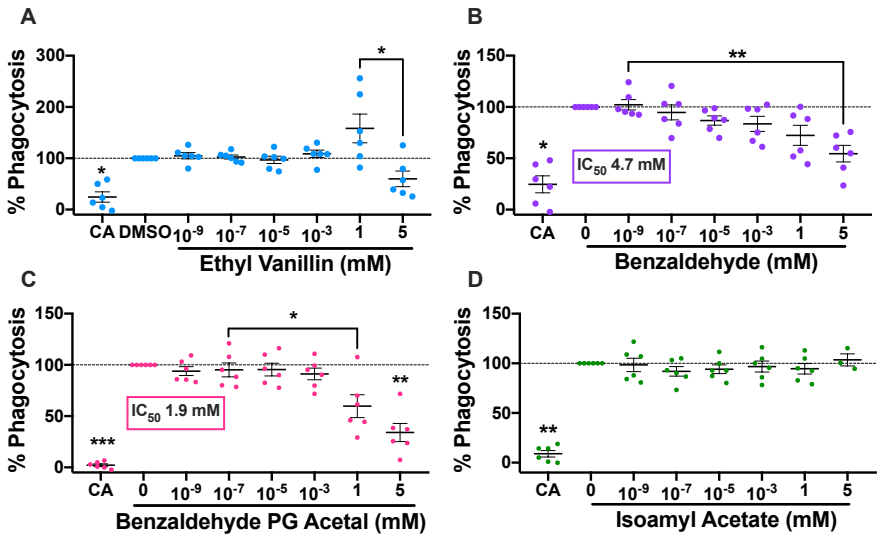


Figure 2-2. Neutrophil phagocytosis following exposure to e-cigarette flavoring chemicals. Effects of flavoring chemicals ethyl vanillin (A), benzaldehyde (B), benzaldehyde PG acetal (C), and isoamyl acetate (D) on neutrophil phagocytosis as assessed with pHrodo red S. aureus BioParticles. Data represents mean +/- SEM with n = 6, with the exception of n = 3 for 5 mM iso-amyl acetate. * $p < 0.05$, ** $p < 0.01$.

Discussion

While there are thousands of different commercially available flavored e-liquids (18), they are created using a much smaller set of flavoring chemicals that are combined to create unique flavors (13-15). Chemical analyses of flavored e-liquids and e-cigarette aerosols from separate studies reveal that many different flavored e-liquids share common flavoring chemicals and that these flavoring chemicals are present in e-liquids at up to molar concentrations (13-15). Interestingly, the flavoring chemicals we found to impair neutrophil function share a common chemical class – they are all aromatic aldehydes or their derivatives.

Though they share a common chemical class, these flavorings have unique functional groups which may explain their differential activities. For example, Cinnamaldehyde is an α,β-unsaturated aldehyde that can covalently bind and modify thiols, including interactions with cysteinyl groups on proteins (19), whereas vanillin (the sister compound to ethyl vanillin) has been shown to interact with proteins via formation of Schiff bases (20). Benzaldehyde shares structural similarity to cinnamaldehyde but lacks the reactive α,β-unsaturated moiety, which may explain why it inhibited neutrophil function to a

lesser extent than cinnamaldehyde. Potential mechanisms underlying decreased oxidative burst observed here could include direct inhibition of key proteins in glycolysis or the pentose phosphate pathway via thiol modification, alteration of glucose uptake, or interference with NADPH oxidase complex formation and activation. The bioenergetic effects of these chemicals could also have implications for assays such as the MTT assay, which relies on mitochondrial metabolism as an indicator of cytotoxicity.

Erythropel et al. recently demonstrated that aldehydic flavoring chemicals such as cinnamaldehyde and benzaldehyde can react with PG under ambient conditions to form flavorant PG acetals (16), which have been reported in e-liquids and e-cigarette aerosols (13-15). Here, we demonstrated that benzaldehyde PG acetal can impair phagocytosis more potently than benzaldehyde (Figure 2-2B/C). However, benzaldehyde impaired oxidative burst slightly more potently than benzaldehyde PG acetal (Figure 2-1D, 2-1E). These results further support the previously published notion that flavoring chemicals present in e-liquids can also form secondary or tertiary reaction products through interactions with various components of the e-liquid, which alters their biological activities and toxicities.

Among the most challenging components of *in vitro* experimental research models is estimating physiologically relevant doses of inhaled flavoring chemicals. The flavoring chemicals we studied have been reported in e-liquids in hundreds of millimolar to molar concentrations (12, 14, 15, 21), and these flavoring chemicals carry over to aerosol with high efficiency (16, 21). Our highest doses are two orders of magnitude below these ranges; however, because human *in vivo* exposure has not been precisely quantified, it is difficult to compare the doses at which we found effects to the level of exposure in e-cigarette users.

In addition, our results show differing potencies and effects of the flavoring chemicals on oxidative burst and phagocytosis (Table 2-2). This discrepancy may be due to the method of neutrophil activation used. Uptake of *S. aureus* BioParticles more accurately recapitulates neutrophil phagocytosis *in vivo,* while stimulation of neutrophils with PMA directly activates protein kinase C, in the absence of a relevant pathogen.

Table 2-2. IC_{50} values for neutrophil oxidative burst AUC and phagocytosis. Data represents mean ± SEM. No data = n.d., Not calculable = n.c. (confidence interval for IC_{50} included infinity).

Flavoring	Oxidative Burst IC_{50} (mM)	Phagocytosis IC_{50} (mM)
Cinnamaldehyde	0.3860 ± 0.0365	n.d.
Ethyl Vanillin	0.4230 ± 0.0962	n.c.
Benzaldehyde	29.92 ± 13.93	4.715 ± 2.051
Benzaldehyde PG Acetal	36.39 ± 24.45	1.888 ± 0.6615
Isoamyl Acetate	n.c.	n.c.

Overall, our data demonstrate that flavoring chemicals present in e-liquids can impair neutrophil function at different levels (Figure 2-3). Impaired neutrophil function has been shown to play a role in decreased bacterial clearance, especially in vulnerable patients, such as those with chronic obstructive pulmonary disease (22). Thus, inhalation of e-cigarette flavorings, especially aldehydes, could significantly impair neutrophil function and consequently increase susceptibility to infection and respiratory disease. Since we found that chemicals from the same class (e.g. aromatic aldehydes) may affect immune cell function in a similar manner, assessing the toxicity of flavoring chemicals in e-cigarettes could be done by chemical class rather than by individual compound.

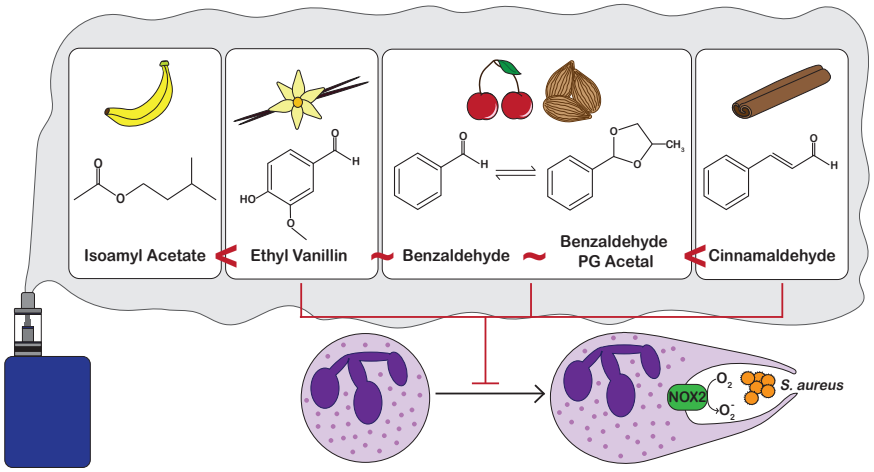


Figure 2-3. Summary of findings.

REFERENCES

1. **Amrock SM, Zakhar J, Zhou S, and Weitzman M**. Perception of E-Cigarette Harm and Its Correlation With Use Among U.S. Adolescents. *Nicotine & Tobacco Research* 17: 330-336, 2015.

2. **Hajek P, Phillips-Waller A, Przulj D, Pesola F, Myers Smith K, Bisal N, Li J, Parrott S, Sasieni P, Dawkins L, Ross L, Goniewicz M, Wu Q, and McRobbie HJ**. A Randomized Trial of E-Cigarettes versus Nicotine-Replacement Therapy. *The New England journal of medicine* 380: 629-637, 2019.

3. **Teresa W. Wang AG, Saida Sharapova, Karen A. Cullen, Bridget K. Ambrose, Ahmed Jamal**. Tobacco Product Use Among Middle and High School Students — United States, 2011–2017. *MMWR Morb Mortal Wkly Rep* 67: 629-633, 2018

4. **Jongenelis MI, Kameron C, Brennan E, Rudaizky D, Slevin T, and Pettigrew S**. E-cigarette product preferences among Australian young adult e-cigarette users. *Aust N Z J Public Health* 42: 572-574, 2018.

5. **Audrain-McGovern J, Strasser AA, and Wileyto EP**. The impact of flavoring on the rewarding and reinforcing value of e-cigarettes with nicotine among young adult smokers. *Drug Alcohol Depend* 166: 263-267, 2016.

6. **FEMA**. Safety Assessment and Regulatory Authority to Use Flavors—Focus on Electronic Nicotine Delivery Systems and Flavored Tobacco Products. 2016.

7. **Mócsai A**. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *The Journal of experimental medicine* 210: 1283-1299, 2013.

8. **Weinberg SE, Sena LA, and Chandel NS**. Mitochondria in the regulation of innate and adaptive immunity. *Immunity* 42: 406-417, 2015.

9. **O'Neill LA, Kishton RJ, and Rathmell J**. A guide to immunometabolism for immunologists. *Nat Rev Immunol* 16: 553-565, 2016.

10. **Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, and Alexis NE**. Macrophages from The Upper and Lower Human Respiratory Tract Are Metabolically Distinct. *Am J Physiol Lung Cell Mol Physiol* 2018.

11. **Clapp PW, Lavrich KS, van Heusden C, Lazarowski ER, Carson JL, and Jaspers I**. Cinnamaldehyde in Flavored E-Cigarette Liquids Temporarily Suppresses Bronchial Epithelial Cell Ciliary Motility by Dysregulation of Mitochondrial Function. *Am J Physiol Lung Cell Mol Physiol* 2019.

12. **Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I**. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278-L292, 2017.

13. **Peace MR, Mulder HA, Baird TR, Butler KE, Friedrich AK, Stone JW, Turner JBM, Poklis A, and Poklis JL**. Evaluation of Nicotine and the Components of e-Liquids Generated from e-Cigarette Aerosols. *J Anal Toxicol* 42: 537-543, 2018.

14. **Tierney PA, Karpinski CD, Brown JE, Luo W, and Pankow JF**. Flavour chemicals in electronic cigarette fluids. *Tob Control* 25: e10-15, 2016.

15. **Omaiye EE, McWhirter KJ, Luo W, Tierney PA, Pankow JF, and Talbot P**. High concentrations of flavor chemicals are present in electronic cigarette refill fluids. *Sci Rep* 9: 2468, 2019.

16. **Erythropel HC, Jabba SV, DeWinter TM, Mendizabal M, Anastas PT, Jordt SE, and Zimmerman JB**. Formation of flavorant-propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids. *Nicotine Tob Res* 2018.

17. **Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballinger SW, and Darley-Usmar VM**. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. *Lab Invest* 93: 690-700, 2013.

18. **Zhu SH, Sun JY, Bonnevie E, Cummins SE, Gamst A, Yin L, and Lee M**. Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tob Control* 23 Suppl 3: iii3-9, 2014.

19. **Weibel H, and Hansen J**. Interaction of cinnamaldehyde (a sensitizer in fragrance) with protein. *Contact dermatitis* 20: 161-166, 1989.

20. **Chobpattana W, Jeon IJ, and Smith JS**. Kinetics of interaction of vanillin with amino acids and peptides in model systems. *J Agric Food Chem* 48: 3885-3889, 2000.

21. **Behar RZ, Luo W, McWhirter KJ, Pankow JF, and Talbot P**. Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Sci Rep* 8: 8288, 2018.

22. **Stockley JA, Walton GM, Lord JM, and Sapey E**. Aberrant neutrophil functions in stable chronic obstructive pulmonary disease: the neutrophil as an immunotherapeutic target. *International immunopharmacology* 17: 1211-1217, 2013.

CHAPTER 3: E-CIGARETTE USE, CIGARETTE USE, AND SEX ARE ASSOCIATED WITH A MODIFIED NASAL MICROBIOME AND MARKERS OF NASAL HOST-MICROBIOTA INTERACTIONS

Introduction

Approximately 7 million adults and more than 3.5 million youth are current electronic cigarette (e-cigarette) users (1-3). E-cigarettes heat and aerosolize e-liquids containing nicotine and flavorings dissolved in humectants propylene glycol and glycerin. E-cigarette use has been steadily increasing over the past decade, especially among teenagers and young adults, reversing the previous decline in youth tobacco use (3, 4). Public health crises, such as the outbreak of e-cigarette and vaping-associated lung injury in 2019-2020 and the ongoing SARS-CoV-2 global pandemic, highlight the importance of research examining the effects of e-cigarettes on respiratory immune function (5, 6).

There is emerging evidence that e-cigarettes disrupt respiratory innate immunity. Previous work has demonstrated the potential for e-cigarette toxicity and impairment of respiratory immune defense using *in vitro* and *in vivo* models as well as in samples from human subjects (7-12). For example, e-cigarette users have altered markers of innate immune responses in induced sputum and bronchoalveolar lavage fluid in comparison with smokers and nonsmokers (8, 12) and chronic e-cigarette exposure in mice can dysregulate endogenous lung lipid homeostasis and innate immunity (11, 13). *In vitro* studies have demonstrated that e-liquids, e-cigarette aerosols, and their components can impair the function of ciliated airway cells and respiratory immune cells (9, 14-18). Furthermore, e-cigarette exposure has been shown to enhance bacterial virulence and adhesion to airway cells (19, 20), suggesting that e-cigarette exposure may impact the respiratory microbiome. However, the effects of e-cigarette use on the respiratory microbiome in humans have not been evaluated.

The respiratory microbiome includes distinct communities of microbiota along the length of the respiratory tract (21). Similar to microbial communities at other body sites, respiratory microbiota

interface with the host immune system, and dysbiosis of the respiratory tract microbiome has been associated with diseases, including cystic fibrosis, chronic obstructive pulmonary disease, asthma, and chronic rhinosinusitis, as well as with disease exacerbations and smoking cigarettes (21-24). Sampling the nasal microbiome is straightforward in contrast to the lower airway microbiome, which is easily contaminated with oral microbiota during specimen collection (25). In addition, the nose is an important gatekeeper in the respiratory tract, as potential pathogens must often colonize this region before progressing to the lower respiratory tract (21). This role has become even more clear and relevant with the emergence of SARS-CoV-2, with recent studies showing associations between the nasal microbiome and SARS-CoV-2 infection (26, 27). Of note is that dysbiosis of the nasal microbiome specifically has been associated with smoking cigarettes (23), and gene expression and histopathological changes due to smoking are similar in the nasal and lower airway epithelium (7), supporting the use of the nasal microbiome for studying the effects of environmental exposures on the respiratory microbiome.

Mechanistic study of the human microbiota is an important focus when studying the human microbiome, where identifying microbes associated with disease is paramount (28). To uncover complex interactions in microbiome association studies changes to classical statistical methods are required (29). In addition, computational methods that robustly integrate disparate data types with 16S microbiome data for association testing have been limited (30). In particular, microbiome datasets have interspecies interactions, small sample sizes, high dimensionality (where the number of features greatly exceed the number of samples), are sparse (where the data matrix contains many zeroes), and when converted to relative abundance are compositional, meaning the total number of reads is not informative (31). Combined, these challenges significantly confound the multivariate integrative analysis required to improve our understanding of host-microbiome interactions. Thus, novel analytical tools are necessary to uncover true signals hidden within small sample size microbiome data.

In this study, we sampled the nasal microbiomes of smokers, nonsmokers, and e-cigarette users using a non-invasive absorptive strip to collect nasal epithelial lining fluid. We then used high-throughput sequencing of the bacterial 16S rRNA gene from the strips to identify bacteria present and analyze the

bacterial composition of the nasal microbiome in our subjects. Because these microbial communities are composed of highly interdependent taxa that have complex interaction patterns, multivariate data analysis is critical to extract biologically relevant information.

Here, we leverage Selection Energy Permutation (32)[pre-print], a novel multivariate association test that simultaneously tests associations while identifying robust subsets of pairwise log ratios in the setting of high-dimensional, low sample size data. These reduced subsets are then used to integratively analyze nasal microbiome and matched cell-free nasal lavage fluid mediator data to determine: 1) whether there were significant compositional differences in the nasal microbiomes of E-cigarette users, smokers, and nonsmokers, 2) whether levels of nasal lavage fluid (NLF) mediators are significantly different in e-cigarette users and smokers in comparison with nonsmokers, and 3) whether changes in levels of these mediators correlate with nasal microbiome dysbiosis. Our data demonstrate nasal microbiome dysbiosis and unique networks of host-microbiota mediators in e-cigarette users and smokers in comparison with nonsmokers. This is indicative of disrupted respiratory mucosal immune responses in these groups and potentially increased susceptibility to infection by specific bacterial taxa. We also observed significant sex differences in the nasal microbiome, highlighting the importance of including sex as a biological variable in nasal microbiome studies.

Methods

Subject recruitment

Nasal epithelial lining fluid (NELF) strips, nasal lavage fluid (NLF), and venous blood were obtained from healthy adult human e-cigarette users, smokers, and nonsmokers as described previously (Table 3-1) (33), forming our exposure groups. Inclusion criteria were healthy adults age 18-50 years who are either nonsmokers not routinely exposed to environmental tobacco smoke, active regular cigarette smokers, or active e-cigarette users. Active cigarette smoking and e-cigarette use were determined as described previously (7). Exclusion criteria were current symptoms of allergic rhinitis (deferred until symptoms resolve), asthma, fractional expiratory volume in one second (FEV_1) less than 75% predicted at

screen, bleeding disorders, recent nasal surgery, immunodeficiency, current pregnancy, chronic obstructive pulmonary disease, cardiac disease, or any chronic cardiorespiratory condition. After the consent process was completed, a medical history and substance use questionnaire was obtained, and subjects were issued a diary to document smoking/vaping for up to 4 weeks, after which they returned for sample collection. E-cigarette users averaged less than 1.5 cigarettes/day in their smoking/vaping diaries, while cigarette users ranged from 4.93-20 cigarettes per day in their diaries. To compare demographic characteristics between subjects in the different exposure groups, age, BMI, and serum cotinine levels were tested for normality using the Shapiro-Wilk test, and groups were compared using the Kruskal-Wallis test followed by the Steel-Dwass method for non-parametric multiple comparisons (analogous to a one-way ANOVA with Tukey's Honestly Significantly Different Test (HSD) for parametric data). All samples were collected between 2014 – 2018 (prior to the COVID-19 pandemic).

Table 3-1. Subject demographics. Reported values are mean ± standard error. Groups were compared using the Steel Dwass method for non-parametric multiple comparisons. AA = African American. # $p < 0.05$ in comparison with nonsmokers and smokers. **** $p < 0.0001$ in comparison with nonsmokers.

	Nonsmokers	E-Cigarette Users	Smokers
n	20	28	19
Sex (Male/Female)	8/12	19/9	10/9
Race (White/AA/Asian/Other)	16/1/2/1	18/4/5/1	10/8/0/1
Age	30.75 ± 1.32	26.39 ± 1.44#	31.89 ± 1.91
BMI	27.11 ± 1.31	30.07 ± 1.51	27.65 ± 1.43
Cigarettes/Day	0 ± 0	0.14 ± 0.07	12.68 ± 0.96
mL E-Liquid/Day	0 ± 0	3.60 ± 0.70	0.015 ± 0.015
E-Cigarette Puffs/Day	0 ± 0	53.90 ± 16.54	0.466 ± 0.414
E-Liquid Nicotine (mg/mL)	0 ± 0	19.43 ± 4.92	0.158 ± 0.158
Former Cigarette Smoker (Yes/No)	0/20	22/6	19/0
Marijuana Use (Yes/No)	0/20	4/24	4/15
Serum Cotinine (ng/mL)	0 ± 0	127.99 ± 15.42****	170.16 ± 21.41****

Serum Cotinine Measurement

Venous blood was collected in BD Vacutainer serum-separating tubes (Fisher Scientific, Waltham, MA) and allowed to clot for a minimum of 15 minutes at room temperature. The blood was then centrifuged at 1200 x g for 10 minutes, and the serum layer was transferred to a fresh tube and stored at -80°C until samples were collected from all subjects. Serum was assayed for cotinine, a metabolite of nicotine that can be measured as a biomarker of nicotine consumption, using a commercially available ELISA kit (Calbiotech, Mannheim, Germany) per manufacturer's instructions. Absorbance was read on a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany). The limit of quantification for serum cotinine was 5 ng/mL. For samples below the limit of detection, a value of zero was assigned. Serum was not available for one subject in the cohort.

NELF Strip 16S rRNA Gene Sequencing

DNA was extracted from whole NELF strips using Powersoil DNA Isolation Kit (MoBio Laboratories). Sequencing libraries were prepared as previously described.(34) Samples were sequenced on an Illumina MiSeq kit version V3 2x300 paired end over the V3-V4 bacterial 16S rRNA gene. Raw sequencing data were demultiplexed and processed to generate a table of operational taxonomic units (OTUs). Specific primer schema, qPCR data, and the OTU table (having at least 10 sequences per OTU across all samples) are provided in the supplement. Raw sequence data have been uploaded under the BioProject accession number PRJNA746950 within the Sequence Read Archive.

NLF Processing and Soluble Mediator Measurement

Cell-free nasal lavage fluid was obtained via processing of raw nasal lavage fluid as described previously.(35) Briefly, raw nasal lavage fluid from each nostril was pooled and centrifuged at 500x g through a 40 μm strainer for 10 minutes. Supernatant (cell-free NLF) was collected and stored at -80°C until samples were collected from all subjects. Due to limitations in sample volume, a targeted list of

soluble mediators was chosen for measurement in cell-free NLF. Cell-free NLF was assayed for mediators of host-microbiota interaction (neutrophil elastase, immunoglobulin A (IgA), lactoferrin, lysozyme, interleukin 8 (IL-8), alpha-defensin 1, beta-defensin 1, and beta-defensin 2 using commercially available ELISA kits per manufacturer's instructions as described in Table S3-1. These mediators were chosen based on their previously described roles in respiratory mucosal host-microbiome interactions, association with respiratory disease and tobacco product exposure, and detectable concentrations in nasal lavage fluid, which is a relatively dilute sample. (21, 36-39) Absorbance was read on a CLARIOstar plate reader and concentrations (pg/mL) were interpolated using GraphPad Prism 9. For samples below the limit of detection, a value of ½ the lowest standard was assigned. Cell-free nasal lavage fluid was not available for one subject in the cohort (Figure S3-1).

Sequencing Data Processing and Filtering

Five samples were removed from the dataset due to a low number of reads (Figure S3-1). A spiked pseudomonas positive control was identified correctly as pseudomonas. To control for potential contamination on the NELF strips, the decontam R package was used to remove contaminants.(40) This package uses an algorithm that takes into account the relative abundance of operational taxonomic units (OTUs) in samples and controls to remove the most likely contaminants and has been shown useful for respiratory samples.(41) This reduced the number of OTUs from 5346 to 4677. Alpha diversity measures (Observed, Chao1, ACE, Shannon, Simpson, Fisher) were calculated using the phyloseq R library before trimming OTU counts less than 5 for downstream analysis. This brought the number of OTUs to 3059 for downstream analysis.

Alpha diversity

Shannon and Simpson diversity indices were computed for each sample. Diversity indices were tested for normality using the Shapiro-Wilk test and further statistical tests to compare groups were

carried out using the appropriate parametric (two-tailed t-test, ANOVA) or non-parametric (Kruskal-Wallis, Steel Dwass) tests. These analyses were performed using JMP Pro 14 and GraphPad Prism 8.

Nasal Microbiome Compositional Data Analysis

To limit spurious findings and because absolute sequencing counts are uninformative (31, 42, 43), compositional data analysis (CoDA)(44) was carried out on the OTU count table after aggregating OTUs (O = 3059) by family (min. level assigned) and genera (max level assigned) and removing taxa not present in at least 20% of samples. The 20% sparsity threshold was selected to maximize class-specific information (Sex, Exposure group) while ensuring the microbial signatures were robust and contained minimal noise due to excessive sparsity. After aggregating OTUs, we define the taxa count matrix, $\mathbf{X} \in \mathbb{R}^{nxp}$, with n = 62 samples and p = 143 taxa. The closure operator, $C[\cdot]$, was then used to map the count data of each element x_{ij} of $\mathbf{X}$ onto its corresponding coordinate on the unit-sum simplex, defining $\mathbf{X}' = C[\mathbf{X}]$ in terms of matrix elements as

$$x'_{ij} = (C[\boldsymbol{X}])_{ij} = \frac{x_{ij}}{\sum_{k=1}^{P} x_{ik}}$$

Because the presence of zeros is a major limitation of the log ratio transformation essential to CoDA, all zeroes must be robustly imputed to non-zero values. To overcome this we use the ratio-preserving multiplicative replacement strategy which has been shown to have several theoretical advantages over simple additive replacement (45): we set the δ imputed values to a single constant equal to the smallest nonzero value encountered in $\mathbf{X}'$. From this, we impute zeros and replace $\mathbf{X}'$ with $\mathbf{Z}$ defined in matrix elements as:

$$z_{ij} = \begin{cases} \delta & , \quad x'_{ij} = 0 \\ \left(1 - \sum_{k|x_{ik}=0} \delta\right) x'_{ij} & , \quad x'_{ij} > 0 \end{cases}$$

Partial redundancy analysis to remove variation due to Sex

To remove the significant effect of Sex (which otherwise obscures the exposure group effect) on **Z**, partial Redundancy Analysis (pRDA)(46) was used. Here we encode the Sex variable into the design matrix **S.** Additionally, to ensure multiple regression computations used in pRDA are performed on symmetric vectors in real space that preserves the inter-sample Euclidean distances, a center log ratio (clr) transformation was applied(44) to **Z**, defining the clr values **C** for each sample as $\boldsymbol{c}_i = [c_1, \ldots, c_p]$ such that:

$$\mathrm{c_{ij}} = \log\left(\frac{\mathrm{z_j^i}}{\mathrm{G_i}}\right) \text{ where } G_i = \left(\prod_j z_{ij}\right)^{\frac{1}{p}}$$

With **C** defined, pRDA was carried out in the vegan R package (47). Multivariate linear regression of **C** on **S** (i.e. computed as a series of multiple linear regression on individual features) was used to produce the fitted values $\widehat{\mathbf{C}}$. To remove the Sex effect as in pRDA, the adjusted values of **C** were computed by $\mathbf{P} = \mathbf{C} - \widehat{\mathbf{C}}$ where $\widehat{\mathbf{C}}$ contains all variation attributable to Sex. With **P** defined in Euclidean coordinates which are not suitable for downstream pairwise log ratio transformations, an inverse clr transformation was applied to map the adjusted coordinates back to the unit-sum simplex. The Sex adjusted relative abundance matrix **M** with elements m_{ij} is computed as:

$$m_{ij} = \frac{\exp(p_{ij})}{\sum_{k=1}^{p} \exp(p_{ik})}$$

Nasal Microbial Signature identification using Selection Energy Permutation

To identify microbial log ratio signatures in the setting of high-dimensional low sample size data we utilized the recently developed Selection Energy Permutation (SelEnergyPerm) method, which has been shown to have increased statistical power over several existing multivariate hypothesis testing methods under hypothesis testing settings like this (32). The SelEnergyPerm method simultaneously selects a reduced subset of log ratios while maximizing the association between groups. Let the group

distributions be defined as $X \in \mathbb{R}^{n\,x\,f}$ and $Y \in \mathbb{R}^{m\,x\,f}$. In this work, we use SelEnergyPerm with the energy statistic (E-statistic)(48) defined by

$$\mathcal{E}_{n,m}(X,Y) = 2A - B - C,$$

where A, B, and C are specified, in terms of the vectors of $\mathbb{R}^f$ indexed by sample, by

$$A = \frac{1}{nm}\sum_{i=1}^{n}\sum_{j=1}^{m}\left\|\boldsymbol{x}_i - \boldsymbol{y}_j\right\|, \qquad B = \frac{1}{n^2}\sum_{i=1}^{n}\sum_{j=1}^{n}\left\|\boldsymbol{x}_i - \boldsymbol{x}_j\right\|, \qquad C = \frac{1}{m^2}\sum_{i=1}^{m}\sum_{j=1}^{m}\left\|\boldsymbol{y}_i - \boldsymbol{y}_j\right\|$$

From this, the pooled multi-class (#classes (c) $\geq$ 2) E-statistic becomes

$$S = \sum\nolimits_{1\leq j<k\leq k}\left(\frac{n_j + n_k}{2N}\right)\left[\frac{n_j n_k}{n_j + n_k}\,\mathcal{E}_{\mathrm{n_j},n_k}(X_j, X_k)\right]$$

The pooled E-statistic is then maximized using forward selection on a subset selected from the full set of pairwise log ratios to explain maximal variation when compared to the full set of pairwise log ratios. Similar to the approach in Greenacre et al. (49), the reduced subset of log ratios are selected from the $\frac{p(p-1)}{}$ 2-dimensional feature space (all pairs). However, there are p^{p-2} possible ways to select a subset of log ratios that explain the total log ratio variance. To overcome this, SelEnergyPerm scores each log ratio using the differential compositional variation scoring method and then iteratively computes acyclic subsets of log ratios (32), with permutation testing via Monte Carlo sampling(50) to assess the significance and prevent overfitting of the log ratio signature. Specifically, given a log ratio signature discovered with true labels, SelEnergyPerm tests if the observed pooled E-statistic (S^*) is more extreme than E-statistics sampled from the permutation distribution of log ratio signatures selected under random labels (S_i, indexing different random-label samples). With γ such E-statistics randomly sampled from the permutation distribution the one-sided p-value becomes

$$\hat{p} = \frac{1 + \sum_{i=1}^{\gamma} I(S_i > S^*)}{\gamma + 1}$$

As expected, we find that removing large numbers of uninformative features increases statistical power in the high-dimensional low-sample-size setting. To identify the Sex nasal microbial signatures in this study we utilized **Z** with labels $= Sex$ and for the Exposure group microbial signature we utilized **M** with

labels $= Subject\ Group$. Using these data, we applied the SelEnergyPerm method with default settings using 200 permutations. Additionally, to reduce noise from sparse features, we further reduced taxa included in the analysis by first identifying the number of taxa to include in the microbial signature. We tested the following subset sizes: $[5,10,20,40,60,80,100]$. Applying the SelEnergyPerm method on each subset and normalizing the energy statistic(48) we selected the subset that maximized the normalized pooled energy statistic (**Figure S3-2**) and then tested if the observed S^* was more extreme than random. In this way, we increase the statistical power of our analysis and reduce the chance of overfitting. While this is good for identifying associations, it can come at the expense of reduced overall discriminatory potential.

Network Visualization of Microbial signature

To visualize the microbial log ratio signatures, we constructed undirected graphs connecting the key taxa (vertices/nodes) by edges representing the formation of a ratio between two taxa with edge weight corresponding to the between-group Kruskal-Wallis H-statistic. While the full log ratio structure is directed in distinguishing numerators from denominators, directedness in the visualizations used here does not fundamentally change our interpretation. Graphs were visualized using Gephi (51) and R-igraph (52).

Multivariate statistical test for microbial signals

To confirm associations between microbial log ratio signatures and Sex/Exposure group multivariate hypothesis testing was done using permutational multivariate analysis of variation(53) and implemented using the R vegan package (47). Unsupervised lower-dimensional projections of samples and group centroids were done using principal coordinate analysis (PCoA) and were implemented using the R stats package.

Partial Least Squares Discriminate Analysis

We utilized partial least squares discriminate analysis (PLS-DA)(54, 55), a versatile multivariate statistical regression technique, to model and understand the relationship between Sex/Exposure group to their microbial signatures. Shown to have reliable performance on compositional and genomic datasets (56, 57), PLS-DA models perform classification, inference, and are inherently linear thus offering improved model interpretability. We specified a priori the number of PLS-DA components (ncomp) as follows: for the between Sex nasal microbial signature, ncomp = 1; for the between Exposure group nasal microbial signature, ncomp = 2. Model fitting was done using the R caret (58) *plsda* function, with latent space projections and loadings extracted from the final models fit using all samples using R caret (58). PLS-DA biplots were created by scaling and superimposing the loading vectors onto the score coordinates extracted from the final fitted model. PLS-DA biplots were visualized using the R ggplot2 package (59).

Receiver operating characteristic curve analysis and PLS-DA performance metric

To understand how well the binary PLS-DA models discriminate between Sex using the nasal microbiome signature, we utilized the area under the receiver operating characteristic metric, AUC, which represents the probability that a randomly selected instance of class 1 will be ranked higher than a randomly selected instance of class 2 (60). Additionally, to understand the discriminatory potential of the ternary PLS-DA Exposure group models, the multi-class AUC metric was used. The multi-class AUC generalizes binary AUC through pairwise class AUC averaging and has the useful property of being independent of cost and priors as in AUC while having a similar interpretation to misclassification rate (61). AUC metrics were estimated using repeated k-fold cross-validation (62). The R pROC package (63) was used to compute all AUC metrics. ROC curves, which graph the false positive and true positive rate of a classifier over a range of thresholds, were computed using the R pROC package (63) and visualized using the R ggplot2 package (59).

NLF mediator and microbiome data integration

We define the nasal lavage data matrix, $\mathbf{L} \in \mathbb{R}^{n \times f}$, where n = 66 samples and f = 7 mediators. Treating the data as relative such that sample-wise absolute concentrations in our study are considered unimportant (Figure S3-3A), zeroes were imputed after applying the closure operator to **L** as described in our compositional data analysis methods. From this, we define $\mathbf{L}' \in \mathbb{R}^{n \times k}$, with k = 21, to include all pairwise log ratios from **L**. To remove uninformative NLF mediators, we computed the differential compositional variation (DCV) score (32) and assigned each NLF mediator log ratio a score by averaging the within-fold DCV score using 20 repeats of 10-fold cross-validation. NLF log ratios with a DCV score < 0 were considered uninformative and were removed (Figure S3-3B). From this ' was reduced to $\hat{\mathbf{L}} \in \mathbb{R}^{n \times k}$ where k=4 (Figure S3-3C) log ratios. To test for univariate associations between NLF mediator log ratios and Exposure group the Kruskal-Wallis test was applied followed by pairwise Wilcoxon rank-sum testing if $\alpha < 0.05$. The nasal microbiome signal was obtained by applying the SelEnergyPerm method to **M** to get $\hat{\mathbf{M}} \in \mathbb{R}^{n \times r}$ where n = 62 and r = 9 log ratios.

Concatenating these data, we define the integrated NLF mediator and nasal microbiome matrix as $\mathbf{D} \in \mathbb{R}^{n \times f}$ where n = 61 (6 samples were removed due to either missing nasal microbiome or NLF data) and f = 13 (4-nasal lavage and 9 microbiome log-ratio features). Exposure group discrimination was estimated separately for each of $\hat{\mathbf{L}}$, $\hat{\mathbf{M}}$, and **D** using multi-class AUC from 50 repeats of 10-fold cross-validation using 2-component PLS-DA models. Multi-class AUC estimates using $\hat{\mathbf{L}}$, $\hat{\mathbf{M}}$, and **D** were compared between groups using the non-parametric Wilcoxon rank-sum test.

Nasal NLF mediator and microbiome association analysis

A final 2-component PLS-DA model to discriminate between exposure groups was fit to $\hat{\mathbf{M}}$. Using dimensionality reduction inherent to PLS-DA, the first PLS-DA component (explaining the most variation) was extracted as a latent variable for further analysis. Pearson's correlation coefficients (PCC) and subsequent p-values were computed between the first PLS-DA component and **L''** represent the

reduced nasal microbiome exposure group signature. PCC p-values, adjusted for multiple comparisons (q-value) using the Benjamini-Hochberg (BH) correction,(64) were considered significant if $q \leq 0.10$. These analyses were carried out using the R stats and caret packages.

Between Exposure group correlation analysis

Partitioning the samples of **D** into 3 matrices based on exposure group (nonsmokers, e-cig users, or smokers), we calculate all pairwise PCC and p-values between features for each group. We also report q-values after adjusting for multiple comparisons within each group using the BH method. Correlations were considered significant if $q \leq 0.10$. Significant PCC within each subject were then aggregated across all exposure groups and visualized as a graph using the R igraph package (52).

Confidence Intervals and univariate statistical test for log ratio

Log ratio 95% confidence interval estimates were calculated by

$$CI_i = \bar{x_i} \pm 1.96 \frac{s_i}{\sqrt{n}}$$

where for the *ith* log ratio, $\bar{x_i}$ = sample mean, s_i = sample standard deviation and n =number samples. Log ratios with confidence intervals bounds that do not include 0 are interpreted as enriched on average for the numerator if $\bar{x} > 0$ or denominator if $\bar{x} < 0$. The Kruskal-Wallis and Wilcoxon rank-sum test were used for univariate comparisons of log ratios between Sex or Exposure groups. Moreover, p-values were adjusted for multiple comparisons using the BH correction using the R stats library and are reported as q-values.

Data Availability

Raw sequencing data is available under the SRA BioProject accession number PRJNA746950. Processed OTU and NLF tables by exposure group and sex have been deposited in the github repository: https://github.com/andrew84830813/nasalMicrobiome_EcigSmoking/Data/

Code accessibility

All nasal microbiome analyses were done using version 4.0.0 of the R statistical programming language. All input data, R script, and functions used in the analysis presented here can be retrieved from the github repository: https://github.com/andrew84830813/nasalMicrobiome_EcigSmoking.git

Results

Subject Demographics

Demographic, questionnaire, and smoking/vaping diary data are summarized in Table 3-1. The study cohort was comprised of 30% nonsmokers (n = 20), 42% e-cigarette users (n = 28), and 28% smokers (n = 19) with at least n = 8 per sex within each exposure group. E-cigarette users were significantly younger (26.39 ± 1.44) than nonsmokers (30.75 ± 1.32) and smokers (31.89 ± 1.91) ($p < 0.05$). BMI did not differ significantly between the exposure groups. Questionnaires and smoking/vaping diaries were completed for 95% (19/20) of nonsmokers and 100% of e-cigarette users and smokers. However, there was variability in the completeness of diaries filled out by e-cigarette users, particularly for the e-cigarette use parameters (mL/day, puffs/day, nicotine concentration, flavor, device). Cigarette users smoked an average of 12.68 ± 0.96 cigarettes per day, whereas 25% (7/28) of e-cigarette users smoked a cigarette during the diary period with an average of 0.14 ± 0.07 cigarettes per day, while 13 e-cigarette users reported puffs per day and 16 reported mL e-liquid/day and e-liquid nicotine concentration in mg/mL. These e-cigarette users averaged 53.90 ± 16.54 puffs/day, 3.60 ± 0.70 mL of e-liquid, and 19.43 ± 4.92 mg/mL nicotine in e-liquids. One smoker reported vaping on one day of the diary, which is the reason for the non-zero values for e-cigarette use parameters in the smoker category. Nonsmokers did

not report previous cigarette smoking or marijuana use, whereas 79% (22/28) of e-cigarette users were former cigarette smokers, while 14% (4/28) of e-cigarette users and 21% (4/19) of smokers reported marijuana use in their diaries. Cotinine, a metabolite of nicotine, was not detectable in the serum of nonsmokers and was significantly elevated in the serum of e-cigarette users (127.99 ± 15.42) and smokers (170.16 ± 21.41) in comparison with nonsmokers ($p < 0.0001$), as expected.

Nasal Microbiome Characteristics

The 4677 OTUs included in the dataset represented OTUs from 19 unique phyla and 225 unique genera. The top four most abundant phyla by average relative abundance across all samples were *Actinobacteria* (50.2%), *Firmicutes* (36%), *Proteobacteria* (12.0%), and *Bacteroidetes* (1.6%). The top six most abundant genera by average relative abundance across all samples were *Corynebacterium* (40.7%), *Staphylococcus* (19.9%), *Propionibacterium* (11.8%), *Alliococcus* (8.5%), *Moraxella* (5.3%), and *Streptococcus* (4.2%). This microbial composition is similar to previously reported studies of the nasal microbiome.(65, 66) These data are summarized in Figure 3-1, where relative abundances by exposure group and sex are plotted for the most highly abundant phyla and genera.

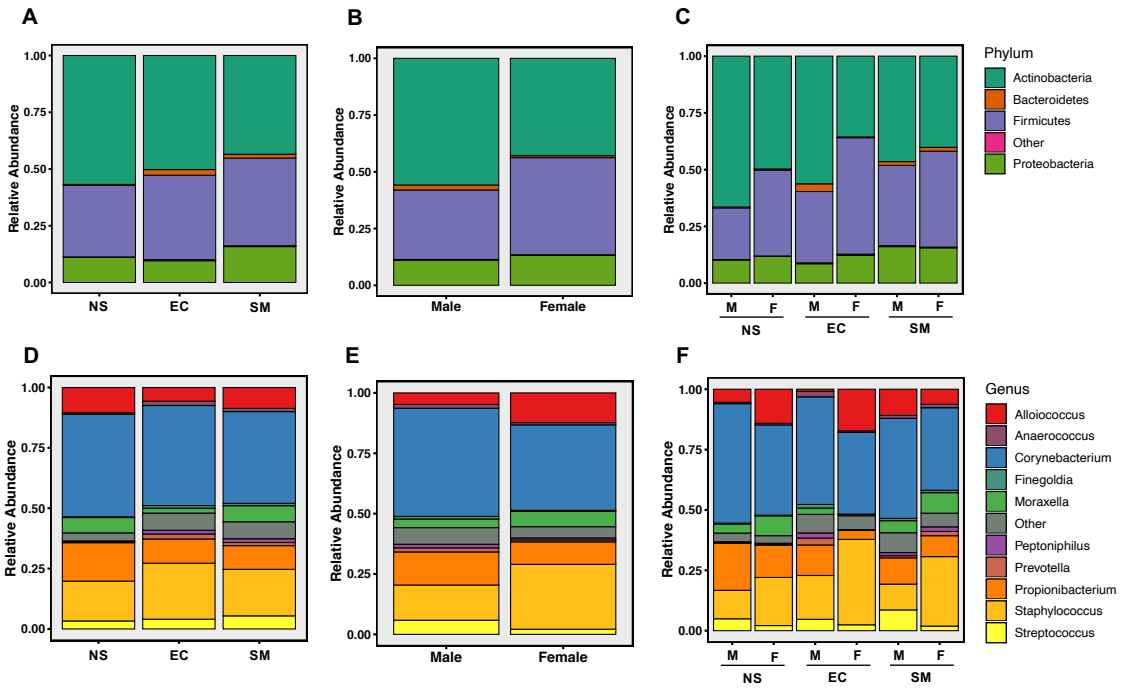


Figure 3-1. Average relative abundances of the top 4 phyla and top 10 genera plotted by exposure group, sex, and sex within exposure groups. NS = nonsmoker, EC = e-cigarette user, SM = smoker, M = male, F = female.

Alpha Diversity

To determine whether there are differences in alpha diversity between the nasal microbiomes of smokers, nonsmokers, and e-cigarette users, we calculated alpha diversity indices (Observed, Chao1, ACE, Shannon, Simpson, Fisher) using phyloseq (67). We did not find any statistically significant differences between the exposure groups for any measure of alpha diversity; however, we did observe a non-significant trend of increased alpha diversity in smokers (Figures 3-2A and 3-2B). Because our group and others have previously observed sex differences in respiratory mucosal immune responses(68, 69) we also tested whether alpha diversity was significantly different between male and female subjects. We found that both the Shannon and Simpson indices were significantly higher in males than females ($p = 0.021$ and $p = 0.0078$, respectively) (Figures 3-2C and 3-2D). We then tested for the interaction between sex and exposure group and found that sex was a significant source of observed variation ($p = 0.0286$ for Shannon; $p = 0.0102$ for Simpson), while exposure group was not. When the data were stratified by exposure group, the only male-female comparison that remained significant was in the e-cigarette user group ($p = 0.0361$ for Shannon; $p = 0.0124$ for Simpson) (Figures 3-2E and 3-2F). These results suggest that sex is an important biological variable to consider in studies of the nasal microbiome.

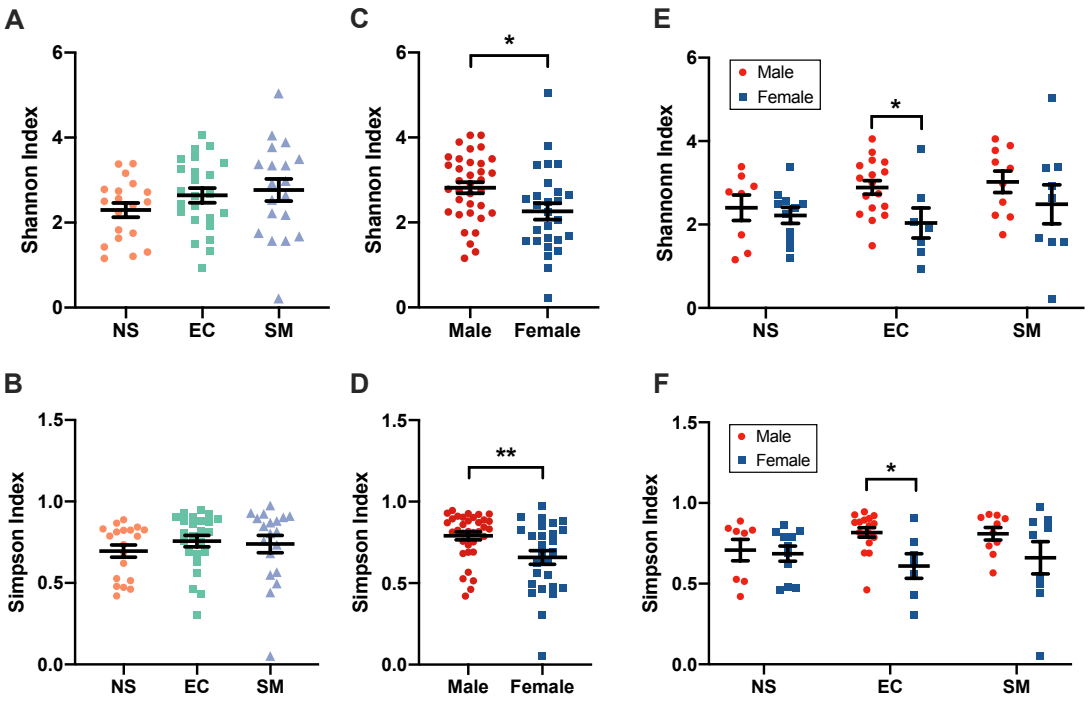


Figure 3-2. Shannon and Simpson indices of alpha diversity are significantly different between sexes, and this difference is most pronounced in e-cigarette users. The Shannon and Simpson indices for alpha diversity were calculated and plotted by exposure group (A, B), sex (C, D), and sex within exposure groups (E, F). NS = nonsmoker, EC = e-cigarette user, SM = smoker. Data are presented as mean ± standard error. * $p < 0.05$, ** $p < 0.01$ by t-test (C), Kruskal-Wallis test (D), or two-way ANOVA with Fisher's LSD (E, F).

Compositional Difference of the Nasal Microbiome by Sex

Because we observed distinctions in alpha diversity between sexes, we next tested whether there were significant compositional differences between the sexes and to identify specific genera capable of explaining these dissimilarities. Given challenges presented by sparse, compositional 16S rRNA gene sequencing data combined with high-dimensionality (genera = 255) and small sample size (n=62), we leveraged the SelEnergyPerm (32) method to identify a robust signature of nasal microbiome taxa (among sparse noisy data) capable of explaining compositional differences between sexes.

By applying this method, we discovered (beyond random noise) a subset of genera (g = 6) capable of maximizing the energy distance between male and female samples ($p = 0.0123$, Figure S3-2A). This microbial signature was comprised of four log ratios between *Rhodococcus*, *Finegoldia*, *Sneathia*, *Abiotrophia*, *Tannerella*, and *Yaniella* genera (Figure 3-3A). Using the identified log ratio signature,

PERMANOVA analysis (pseudo-F = 16.586, p =0.0002, Figure 3-3B) also confirmed the existence of differences in the nasal microbiome composition between sex. Analysis of individual taxa log ratios between sexes demonstrated important nasal microbiome compositional differences (Figure 3-3C). In female samples, *Yaniella* was more abundant on average than *Rhodoccous* and *Tannerella,* while the reverse was true for males. In male samples, *Abiotrophia* was more abundant on average than *Sneathia,* while the opposite was true for females. Finally, in both males and females, *Finegoldia* was observed to be more abundant than *Yaniella,* however, *Finegoldia* was significantly more enriched relative to *Yaniella* in males compared to females.

Next, we analyzed the microbial signature as a whole using Partial Least Squares Discriminate Analysis (PLS-DA) with a single component to predict sex. Using 20 repeats of 10-fold cross-validation, the average area under the receiver operating characteristic curve (AUC) for predicting sex given the reduced microbial signature was 0.862 (95% CI 0.842 – 0.883, Figure 3-3D). With strong cross-validated predictive performance, a final PLS-DA model was trained on all samples (n=62). Scores from the single PLS-DA component indicated strong separation between sexes (Figure 3-3E). The PLS-DA loading plot (Figure 3-3F), which shows how each log ratio contributes to the final score, demonstrates key relationships between taxa log ratios. Increased abundance of *Abiotrophia and Finegoldia* (in log ratios where they appear) were characteristic of males, and increased abundance of *Yaniella* was associated with females. Overall, these findings indicate there exists a compositionally distinct taxa subset that differs strongly in the nasal microbiomes of males and females. Therefore, controlling for sex differences present in the nasal microbiome is important in further analysis.

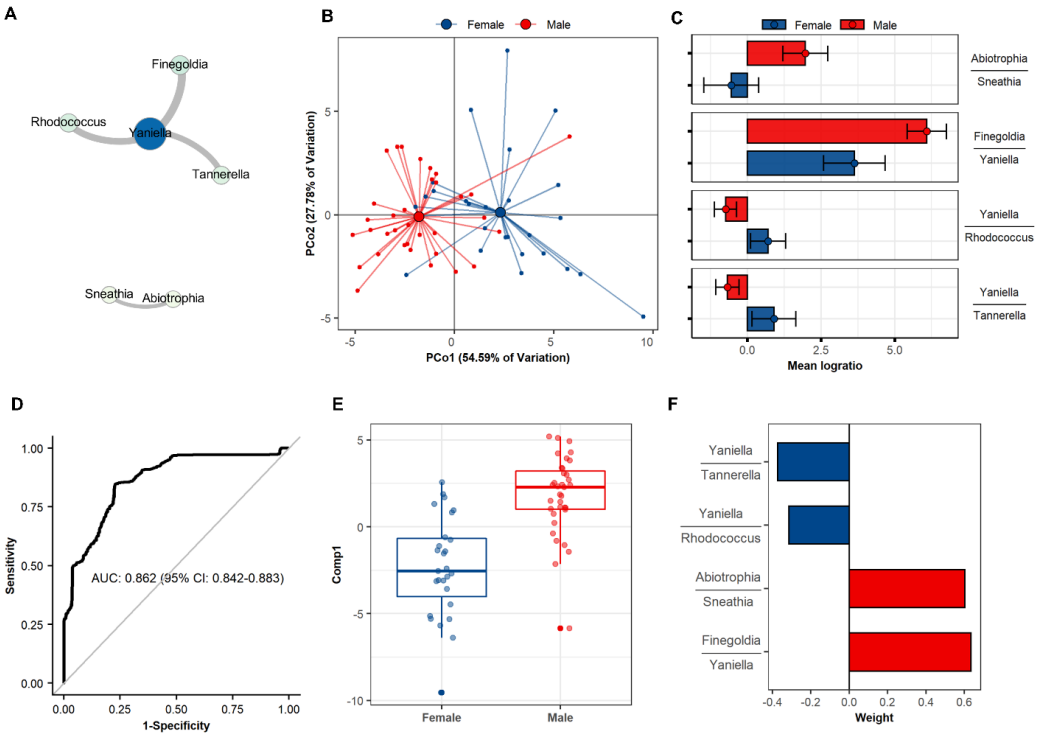


Figure 3-3. Nasal microbiome differences between sexes. (Males: n=35; Females: n=27). (A) Network representation of SelEnergyPerm (p=0.0123) derived genus aggregated taxa log ratio signature of nasal microbiome differences between sexes (Node = genera; edge = log ratio between taxa, Edge-weight = Kruskal Wallis H-statistic between sexes, Size/Color = node strength). (B) Principal coordinate analysis plot of nasal microbiome log ratio signature between sex explaining 82.37% of the total variation. (C) Univariate analysis of log ratio signature showing average depletion or enrichment of specific taxa log ratios between sexes. Error bars reflect 95% confidence intervals of the mean log-ratio value for males and females. (D) Receiver operating characteristics (ROC) curve displaying the area under the curve (AUC) predictive performance (20x10-fold cross-validation) of 1-component partial least squares discriminant analysis (PLS-DA) models trained on nasal microbiome signature between sexes. (E) PLS-DA scores plot of single discriminating component between sexes. Final PLS-DA model fit using all samples (n=62). (F) PLS-DA loadings plot showing contributions of each log ratio to final scores.

Compositional Difference of the Nasal Microbiome by Exposure group

We next examined whether there were distinct nasal microbiome compositions between exposure groups (e-cigarette users: n = 24; smokers: n=19; nonsmokers: n=19; See Methods and Table 3-1). Taking into account nasal microbiome sex differences and applying SelEnergyPerm, we identified a subset of genera (g = 12) important for explaining key nasal microbiome alterations between exposure groups (p = 0.032). This microbial signature comprised nine log ratios (edges) between 12 key genera (nodes) (Figure 3-4A). PERMANOVA analysis (pseudo-F = 8.4889, p =0.0002, Figure 3-4B) confirmed differences in nasal microbiome composition between exposure groups given the microbial signature of 9 log ratios.

Individual analyses of log ratios elucidated specific compositional differences between exposure groups (Figure 3-4C). In e-cigarette users, *Lactobacillus* taxa were significantly more abundant relative to *Bacillus* taxa, while in smokers and nonsmokers, these taxa presented in similar proportions, suggesting an enrichment of *Lactobacillus* among e-cigarette users. E-cigarette users' nasal microbiomes also contained significantly more *Staphylococcus* relative to *Bacillus* than what was observed in nasal microbiomes of both smokers ($q = 0.0097$) and nonsmokers ($q = 0.0031$). In smokers, *Maccrocus* genera were significantly more abundant on average relative to *Hymenobacter*, *Mycobacterium*, *Varibaculum*, and *Rhodococcus,* suggesting that smoking may enrich *Macrococcus* taxa populations in the nasal passage. Additionally, smoker nasal microbiomes contained more *Hymenobacter* relative to *Moryella,* whereas the opposite was true for nonsmokers, both in contrast to e-cigarette users, which maintained on average equal amounts of both genera. In nonsmokers, *Lautropia* taxa were significantly more abundant relative to *Bulleidia,* but this was not observed in smokers and e-cigarette users.

To understand how taxa log ratios work together to discriminate between exposure groups, PLS-DA was used with 20 repeats of 10-fold cross-validation (Figure 3-4D). The estimated multi-classification AUC was 0.851 (95% CI 0.835 – 0.866) suggesting excellent exposure group discrimination. Pairwise examination of exposure group classifications shows strong differences between the nasal microbiomes of nonsmokers/e-cigarette users (AUC = 0.895: 95% CI 0.874 – 0.915) and smokers/e-cigarette users (AUC = 0.893: 95% CI 0.873 – 0.913), with weaker yet distinct differences between smokers/nonsmokers (AUC = 0.803: 95% CI 0.773 – 0.833) (Figure 3-4D). The relative importance of taxa log ratios for discriminating between exposure groups was computed using a final PLS-DA model fit using all samples (n=62). The log ratio between *Macrococcus* relative to *Hymenobacter* was found to be most important for classifying samples as smoker (least important for e-cigarette user classification), and the log ratio between *Bacillus* taxa relative to taxa from the *Micrococcaceae* family was most important for samples to be classified as e-cigarette users (least important to be classified as smokers) (Figure 3-4E). Interestingly, inspection of relative log ratio importance data failed to uncover log ratios disproportionately important for nonsmokers. This observation suggests smoking and e-cigarette use recognizably alter the nasal

microbiome in otherwise healthy adults. Overall, analysis of the taxa log ratios signature suggests alterations in *Macrococcus* and *Bacillus* genera are important for distinguishing between these exposure groups.

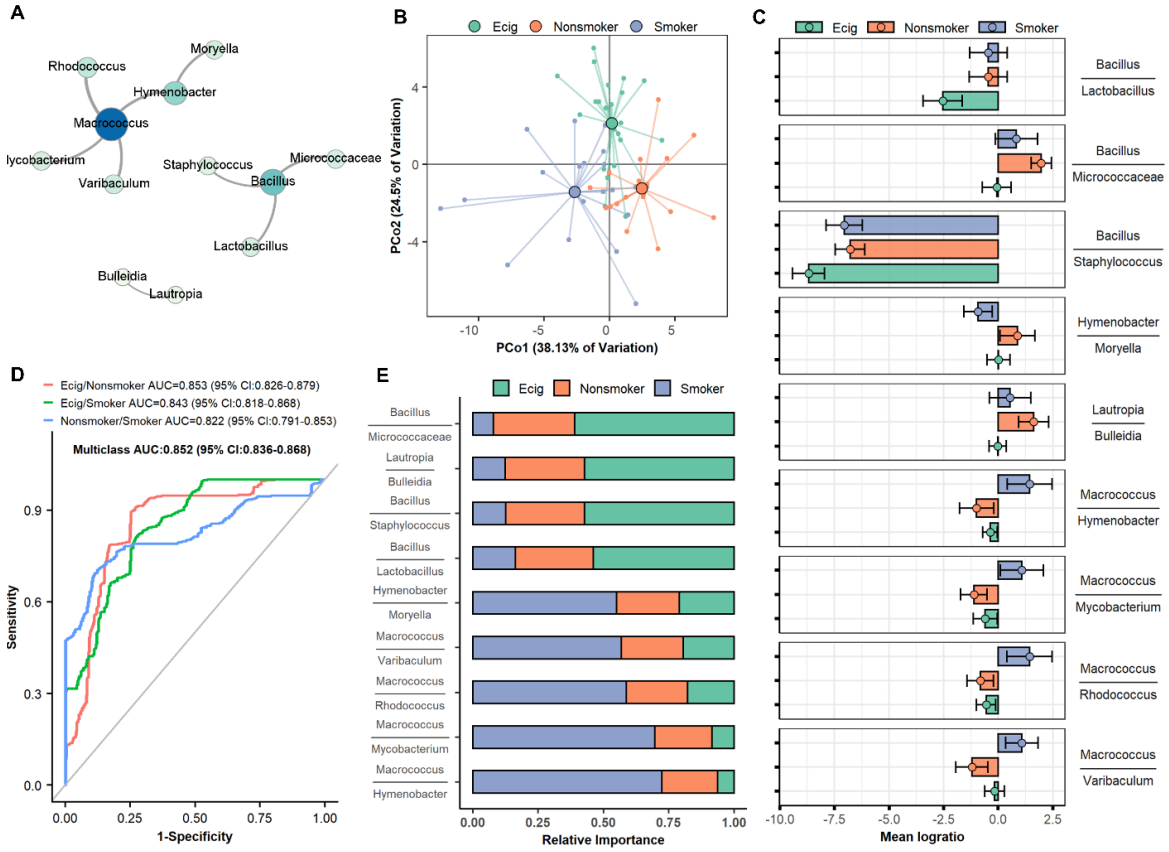


Figure 3-4. Nasal microbiome differences between exposure groups. (Ecig-users: n=24; Nonsmokers: n=19; and Smokers: n=19) adjusted for sex. (A) Network representation of SelEnergyPerm (p=0.032) derived genus aggregated taxa log ratio signature of nasal microbiome differences between exposure groups (Node = genera; edge = log ratio between taxa, Edge-weight = Kruskal Wallis H-statistic between sex, Size/Color = node strength). (B) Principal coordinate analysis plot of nasal microbiome log ratio signature between exposure groups explaining 62.63% of the total variation. (C) Univariate analysis of log ratio signature showing average depletion or enrichment of specific taxa log ratios between exposure groups. Error bars reflect 95% confidence intervals of the mean log-ratio value for each exposure group. (D) ROC curve displaying the multi-classification AUC for predicting exposure group (20x10-fold cross-validation) of 2-component PLS-DA models trained on nasal microbiome signature between exposure groups. (E) Relative importance of log ratios for distinguishing between exposure groups in PLS-DA model trained on all samples (n=62).

Differences in NLF mediator Expression Patterns Between Exposure groups

Because smoking and e-cigarette use were associated with distinct changes in the nasal microbiome, we next explored if there was altered expression of innate immune response mediators in the exposure groups. Accounting for differences in absolute concentration (Figure S3-3A) and subsequently applying differential compositional variation scoring (32) (See Methods, Figure S3-3B), we identified four log ratios among NLF mediators that showed strong intergroup variability (Figure S3-3C). These ratios comprised the following NLF mediators: IL-8, DEFB4A-2, neutrophil elastase, IgA, and lactoferrin. Kruskal-Wallis one-way testing (Figure S3-3D) of each log ratio suggest there exist intergroup differences in NLF mediator expression formed between the concentrations of neutrophil elastase relative to IL-8 ($H = 6.4417$; $p = 0.0399$; $q = 0.0798$) and lactoferrin relative to IL-8 ($H = 8.2080$; $p = 0.0165$; FDR = 0.0660). There were no significant differences between exposure groups among log ratios formed by IgA relative to IL-8 or DEFB4A-2 relative to neutrophil elastase. However, multivariate analysis with PERMANOVA (pseudo-$F = 3.7678$, $p = 0.0030$) using the four key log ratios confirmed there were differences in NLF mediator expression patterns between exposure groups when considered together. To better understand which groups were different, we applied PLS-DA. Training a PLS-DA model with the NLF mediator expression patterns revealed the strongest between-subject-group discrimination to be among Smokers and Nonsmokers (AUROC = 0.8230, 95%CI 0.7920-0.8530, Figure S3-3E). Notably, e-cigarette users' NLF mediators were weakly distinguishable from nonsmokers (AUROC = 0.6720, 95%CI 0.6350-0.7100, Figure S3-3E) but more discernible from smokers (AUROC = 0.7480, 95%CI 0.7130-0.7820, Figure S3-3E). Together, these results suggest that the expression of NLF mediators in smokers was distinct from that of e-cigarette users and healthy adults.

Integration of NLF mediators and nasal microbiome composition improves exposure group discrimination

Finally, we aimed to understand if alterations in NLF mediator expression are associated with nasal microbiome dysbiosis resulting from smoking or e-cigarette use. To this end, we first estimated the discriminatory AUROC of a 2-component PLS-DA model fit on log ratios from NLF mediators (Figures S3-2C and S3-3), nasal microbiome (Figures 3-4A and S3-4A), or both nasal microbiome and NLF mediators (Figure 3-5A). When compared to individual signatures, improved discriminatory AUROC (Figure 3-5B) was observed when PLS-DA models were fit using the combined nasal microbiome and NLF mediator signatures. Therefore, with established synergy between mediator expression and nasal microbiome composition in discriminating between exposure groups, we next examined if correlations were present between the two.

Association between altered NLF mediator expression and nasal microbiome dysbiosis

Using the first PLS-DA component of the nasal microbiome signature, we found significant correlations with NLF mediator expression, showing an association between the nasal microbiome composition and NLF mediator expression (Figure 3-5C). Examination of the location of samples by exposure group projected along the first PLS-DA component show important projective distinctions between smokers (on average negative projections) and both e-cigarette users and nonsmokers (on average positive projections) (Figure S3-4B). Given this, these correlations suggest nasal microbiome dysbiosis caused by cigarette smoke exposure is associated with increased expression of IL-8 relative to neutrophil elastase, Total IgA, and lactoferrin (Figure 3-5C). Moreover, the loadings along the first PLS-DA component (Figure S3-4C) show log ratios with higher abundance of *Maccroccous* as being the most important contributor to negative projections. Combined, these data propound an important link between dysbiosis in *Macrococcus* communities within the nasal microbiome and NLF IL-8 expression.

Microbial functional and mediator expression differences between exposure groups

Correlation analysis of the combined NLF mediator expression and nasal microbiome signature reveal distinct correlation patterns within exposure groups suggesting distinct functional differences (Figures 3-5D). Most notably, a significant negative correlation between log ratios formed by *Hymenobacter/Moryella* and *Macrococcus/Hymenobacter* was observed only in the nonsmoker group. This negative correlation highlights a possible role of *Hymenobacter*, in that it appears to be important for maintaining a healthy balance of *Maccroocous* and *Moryella.* In the e-cigarette and smoking groups, we observed a significant positive correlation between the log ratios formed by IgA/IL-8 *and* Lactoferrin/IL-8. Analysis of this correlation pattern reveals that increased expression of IL-8 in these groups may come at the expense of decreased expression of IgA and lactoferrin or vice versa. We also observed a significant negative correlation between the log ratios formed by Neutrophil Elastase/IL-8 and DEFB4A-2/Neutrophil Elastase in the e-cigarette and smoking groups. These strong negative correlations show that increased expression of IL-8 and DEFB4A-2 is strongly associated with decreased expression of neutrophil elastase. The final significant correlation pattern observed was in smokers only and consisted of four positively correlated log ratios formed by *Macrococcus* relative to *Hymenobacter*, *Mycobacterium*, *Varibaculum*, and *Rhodococcus* (Figure 3-5D). Relatively interpreting these correlations between log ratios suggests that as *Macrococcus* becomes more abundant (among these ratios) the abundance of *Hymenobacter*, *Mycobacterium*, *Varibaculum*, and *Rhodococcus* decreases. This suggests that cigarette smoke exposure may produce favorable colonization conditions for *Maccroccous* genera which subsequently reduces the abundance of Hymenobacter, *Mycobacterium*, *Varibaculum*, and *Rhodococcus*.

From these analyses, our results demonstrate there exists a strong association between altered NLF mediator expression and nasal microbiome dysbiosis. Our findings indicate nasal microbiome dysbiosis from smoking is associated with simultaneous increase in IL-8 expression and *Maccroccous* abundance. Additionally, variations in the correlation networks among e-cigarette users and smokers,

while similar, were distinct from nonsmokers, suggesting functional differences at the microbial and mediator levels between exposure groups.

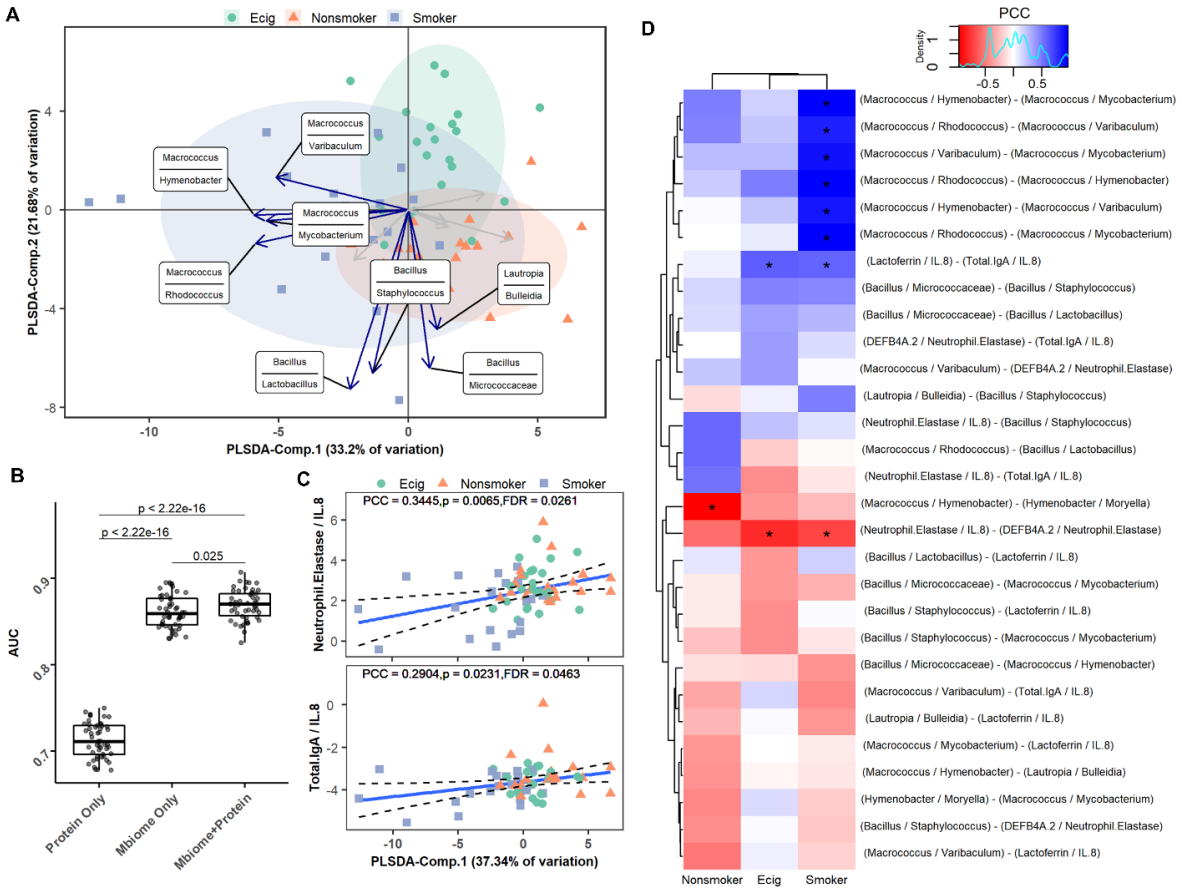


Figure 3-5. Integrating data uncovers association between NLF mediators and nasal microbiome along with identifying distinct correlation patterns between exposure groups.(Ecig-users: n=23; Nonsmokers: n=19; and Smokers: n=19). (A) PLS-DA biplot of integrated NLF mediators and nasal microbiome (B) Box and whisker's plot comparing area under the receiver operating characteristic curve performance of 2-component PLS-DA model (50x10-fold cross-validation) using each data type alone or integrated. (C) Scatter plot showing correlations between log ratios formed between concentrations$(\mu g/mL)$ of Lactoferrin, Neutrophil Elastase relative to IL-8 and the first PLS-DA component of the nasal microbiome. (D) Correlation heatmap showing Pearson's correlation coefficients (PCC) between and within the microbiome and protein log ratio signatures. ($*$ indicates within group $q \leq 0.10$)

Discussion

Despite the growing body of research showing that e-cigarette use can disrupt the respiratory immune system, no studies to date have assessed the effects of e-cigarettes on the respiratory microbiome and host-microbiota interactions. In this study, after adjusting for sex differences, we found that e-cigarette users, smokers, and nonsmokers have unique nasal microbiomes, with differences driven by the relationships between a subset of key taxa. We also found a subset of immune mediators that had distinct relationships between each other in the different exposure groups. Importantly, we found a link between nasal microbiome dysbiosis and soluble immune mediator networks.

A fundamental feature of our study is that we detected microbial signatures from the nasal microbiome that explained differences between sex and exposure groups using the novel SelEnergyPerm computational method. This method directly accounts for the sparse, high-dimensional and compositional nature of the 16S amplicon relative abundance data. Additionally, SelEnergyPerm identifies subsets of robust log ratios between taxa, as opposed to analyzing taxa relative abundance alone, yielding higher statistical power in the sparse association setting with low-sample-size compositional data (32). Most importantly, traditional statistical techniques such as PERMANOVA, ANOSIM, and ANCOM alone were unable to detect these sparse associations within the high-dimensional nasal microbiome feature space. Further, our parsimonious yet statistically significant signatures were then integrated with NLF mediators where we were then able to uncover novel interactions between a taxa subset within the nasal microbiome and the NLF mediators in response to exposure to cigarette or e-cigarette aerosol.

We observed that there were relationships between a subset of taxa that were important in separating the microbial communities of smokers, nonsmokers, and e-cigarette users (Figure 3-4). Only a few studies have previously compared the nasal microbiome of smokers and nonsmokers.(23, 70) Charlson et al. found specific bacteria genera that were differentially abundant in smokers and that some genera belonging to the phylum *Firmicutes* were important in distinguishing smokers from nonsmokers (23). Other studies did not find any significant differences in diversity measures or relative taxa abundance between smokers and nonsmokers (70). In our study, which focused on the composition of the

nasal microbiome and ratios between taxa rather than relative abundance of individual taxa, we found that alterations in *Macrococcus* and *Bacillus* genera are important for distinguishing between exposure groups. Our data also suggest an enrichment of *Lactobacillus* and *Staphylococcus* relative to *Bacillus* in e-cigarette users and enrichment of *Macrococcus* relative to *Hymenobacter*, *Mycobacterium*, *Varibaculum*, and *Rhodococcus* in smokers. A shift from *Lactobacillus* to *Bacillus* in the lung microbiome has been previously demonstrated in response to influenza A infection and increases in anaerobic bacteria, such as *Lactobacillus,* have been associated with chronic rhinosinusitis (65). Furthermore, *Bacillus* have been shown to produce antimicrobials against *S. aureus* (71), indicating that the patterns we have observed may be directly linked to specific interactions between taxa. An increase in *Staphylococcus* relative to *Bacillus* in e-cigarette users is also notable due to the role of species such as *Staphylococcus aureus*, which is carried normally by about 30% of people and is also considered to be a potential pathogen of the skin and mucosal surfaces (72, 73). Our data provide evidence that e-cigarette and smoker nasal microbiomes are distinctly shifted from nonsmokers. Importantly, we also observed that different subsets of taxa were important in separating e-cigarette users and smokers, rather than effects on a continuum from nonsmokers to e-cigarette users to smokers, highlighting the concept that the effects of e-cigarettes are likely unique from those of smokers, even though they are commonly directly compared.

We also measured concentrations of mediators of host-microbiota interactions in nasal lavage fluid to determine whether the changes in the nasal microbiome in different exposure groups are potentially caused by direct effects on the microbiome, mediated by changes in the host immune system, or both. Our data indicate that the expression of immune mediators in nasal lavage fluid samples differed among exposure groups and was driven by shifts in neutrophil elastase and lactoferrin relative to IL-8. Neutrophil elastase and IL-8 are associated with inflammation and neutrophil recruitment, while lactoferrin is an antimicrobial protein primarily produced by epithelial cells and has a wide array of functions, including antioxidant and immune-modulating properties (74). Our results suggest that e-cigarette users and smokers may have altered immune mediator milieu, indicating a shift away from

immune homeostasis and towards increased inflammation and neutrophil recruitment. This shift could be partially driving observed differences in the nasal microbiome.

Our data indicate that both e-cigarette users and smokers have altered nasal microbial communities and relationships between markers of innate immune response, which could imply that they are at increased susceptibility to respiratory infections and/or that they exist in a state of inflammation and altered immune response. We also uncovered interactions of key immune mediators with the host and microbiota, such as IL-8, neutrophil elastase, and lactoferrin, that are also disrupted by e-cigarette and cigarette use. The microbial shifts we observed in association with e-cigarette and cigarette use could be driven by changes in the microenvironment, such as temperature, pH, free radical formation, and availability of metabolic substrates (e.g. sugars) that could then alter the fitness of different bacteria in the nasal microbial community. The shifts we observed could also be mediated through direct effects on respiratory host defense function, inflammation, and/or specific microbes. Multiple processes are likely at play, but our novel findings on the effects of e-cigarettes on the nasal microbiome add to the growing body of literature demonstrating that e-cigarettes are not without health effects and that they should be more thoroughly investigated for inhalational toxicity.

Because sex differences in the human immune system and its response to respiratory disease and toxicant exposure have been observed previously (68, 75), we also investigated whether there were sex differences in the nasal microbiomes of our subjects. We observed that the relationships between six genera were important in separating the nasal microbiomes of males and females (Figure 3-4A). Increased abundance of *Abiotrophia* and *Finegoldia* (in log ratios where they appear) were characteristic of males, and increased abundance of *Yaniella* was associated with Females. Many of these genera have been detected in previous studies of skin, oral, and/or respiratory microbiomes (23, 65, 76-80), but detailed information on the functions of these bacteria as part of the microbial community, as well as their impact on host health, are not available for all taxa. Although some of these genera, such as *Abiotrophia* and *Finegoldia* have been associated with disease- and exposure-driven alterations in the respiratory microbiome (23, 65, 76, 77), we hypothesize that the observed sex difference is neither good nor bad;

rather, it is reflective of a different baseline composition in males and females or altered microenvironments in males and females due to differences in toxicant metabolism rates or mechanisms of immune regulation (81, 82). In other body sites, such as the gut, sex differences have been detected and have been attributed to a variety of factors, including sex hormone levels, pharmaceutical use, and diet (83, 84). In mice, sex-related differences in gut microbiota were shown to impact pulmonary responses to ozone (69). However, few studies have explored sex differences in the respiratory microbiome (85). In the studies that have analyzed data by sex, detection of sex differences is not consistent between studies and is typically not explored in-depth (66, 72, 86). Importantly for the data presented here, compositional differences in the nasal microbiomes of e-cigarette users, smokers, and nonsmokers were not apparent until sex was properly adjusted for, further underscoring the importance of considering sex as a biological variable which significantly modifies exposure effects and can substantially affect data interpretation.

Though our study reveals important community shifts in nasal microbiota and immune mediators associated with e-cigarette and cigarette use as well as with sex, there are limitations to our study. Our novel analysis approach, while properly accounting for the compositional nature of the data, limits us in comparing our work to previous studies, which have been more focused on specific taxa rather than ratios across the microbial community as a whole. As with any study of human subjects, there is also inherent inter-subject variability that can interfere with detection of differences between groups. In our e-cigarette user group, there was considerable variability in factors that could impact the exposure subjects are receiving, including e-liquid flavor, device, nicotine content, and frequency of use. The e-cigarette user group also includes previous smokers and some marijuana use was reported in both smoker and e-cigarette user questionnaires. These factors were included in our analysis and did not show a significant impact on our overall findings due to the nature of the computational models we used. Although our study had a similar number of subjects per cohort to previous comparable studies (23, 87, 88), future studies with larger cohort sizes coupled with more extensive questionnaires could improve the ability to detect which, if any, of these factors may be driving changes in microbiota composition. Larger cohort sizes would also increase power to detect overall changes and shifts in the nasal microbiomes of such subjects

given the compositional and sparse nature of 16S sequencing data. Finally, although 16S amplicon sequencing is a common and accessible method to study the composition of the human microbiome, 16S amplicon sequencing is not typically able to resolve taxa at the species or strain level, which, given that species and strains within the same genus can have different implications for health and disease, is a key methodological limitation (89). Future studies using sequencing approaches with higher biological resolution, such as shotgun metagenomic sequencing, will allow for improved understanding of the health implications associated with shifts in microbial communities associated with tobacco product use.

As a whole, our results support and expand on the previously published notion that exposure to inhaled toxicants, including tobacco products, can influence the respiratory microbiome (23, 90, 91). The novel, robust computational approach in terms of pairwise log ratios that we applied allowed us to uncover both exposure- and sex-dependent effects on nasal mucosal host defense responses using straightforward, non-invasive sampling of the upper respiratory tract of human subjects. Importantly, we were able to integrate 16S amplicon sequencing data with expression of soluble immune mediators to understand interactions between the nasal microbiome and host milieu by appropriately handling the sparse, compositional data generated by 16S amplicon sequencing, accounting for inter-individual variability between subjects' mediator levels, and selecting for features that were most important for separating classes, resulting in interpretable, biologically meaningful results. Conventional analysis pipelines would have limited our ability to integrate these two types of data and detect the exposure and sex-dependent effects we observed, highlighting the importance of applying innovative computational methods to address specific research questions and integrating multiple factors in understanding biological outcomes of exposure and disease.

REFERENCES

1. **Wang T, Asman K, Gentzke A, and al. e**. Tobacco Product Use Among Adults — United States, 2017. *MMWR Morb Mortal Wkly Rep* 67: 1225–1232, 2017.

2. **Leventhal AM, Miech R, Barrington-Trimis J, Johnston LD, O'Malley PM, and Patrick ME**. Flavors of e-Cigarettes Used by Youths in the United States. *Jama* 2019.

3. **Wang TW, Neff LJ, Park-Lee E, Ren C, Cullen KA, and King BA**. E-cigarette Use Among Middle and High School Students - United States, 2020. *MMWR Morb Mortal Wkly Rep* 69: 1310-1312, 2020.

4. **Cullen KA, Gentzke AS, Sawdey MD, Chang JT, Anic GM, Wang TW, Creamer MR, Jamal A, Ambrose BK, and King BA**. e-Cigarette Use Among Youth in the United States, 2019. *Jama* 2019.

5. **Kiernan E, Click ES, Melstrom P, Evans ME, Layer MR, Weissman DN, Reagan-Steiner S, Wiltz JL, Hocevar S, Goodman AB, and Twentyman E**. A Brief Overview of the National Outbreak of e-Cigarette, or Vaping, Product Use-Associated Lung Injury and the Primary Causes. *Chest* 159: 426-431, 2021.

6. **McAlinden KD, Eapen MS, Lu W, Chia C, Haug G, and Sohal SS**. COVID-19 and vaping: risk for increased susceptibility to SARS-CoV-2 infection? *Eur Respir J* 56: 2020.

7. **Martin EM, Clapp PW, Rebuli ME, Pawlak EA, Glista-Baker E, Benowitz NL, Fry RC, and Jaspers I**. E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 311: L135-144, 2016.

8. **Reidel B, Radicioni G, Clapp PW, Ford AA, Abdelwahab S, Rebuli ME, Haridass P, Alexis NE, Jaspers I, and Kesimer M**. E-Cigarette Use Causes a Unique Innate Immune Response in the Lung, Involving Increased Neutrophilic Activation and Altered Mucin Secretion. *Am J Respir Crit Care Med* 197: 492-501, 2018.

9. **Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I**. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278-L292, 2017.

10. **Clapp PW, Lavrich KS, van Heusden CA, Lazarowski ER, Carson JL, and Jaspers I**. Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *Am J Physiol Lung Cell Mol Physiol* 316: L470-l486, 2019.

11. **Madison MC, Landers CT, Gu BH, Chang CY, Tung HY, You R, Hong MJ, Baghaei N, Song LZ, Porter P, Putluri N, Salas R, Gilbert BE, Levental I, Campen MJ, Corry DB, and Kheradmand F**. Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine. *J Clin Invest* 129: 4290-4304, 2019.

12. **Ghosh A, Coakley RD, Ghio AJ, Muhlebach MS, Esther CR, Jr., Alexis NE, and Tarran R**. Chronic E-Cigarette Use Increases Neutrophil Elastase and Matrix Metalloprotease Levels in the Lung. *Am J Respir Crit Care Med* 2019.

13. **Sussan TE, Gajghate S, Thimmulappa RK, Ma J, Kim JH, Sudini K, Consolini N, Cormier SA, Lomnicki S, Hasan F, Pekosz A, and Biswal S**. Exposure to electronic cigarettes impairs pulmonary anti-bacterial and anti-viral defenses in a mouse model. *PLoS One* 10: e0116861, 2015.

14. **Gerloff J, Sundar IK, Freter R, Sekera ER, Friedman AE, Robinson R, Pagano T, and Rahman I**. Inflammatory Response and Barrier Dysfunction by Different e-Cigarette Flavoring Chemicals Identified by Gas Chromatography-Mass Spectrometry in e-Liquids and e-Vapors on Human Lung Epithelial Cells and Fibroblasts. *Appl In Vitro Toxicol* 3: 28-40, 2017.

15. **Muthumalage T, Prinz M, Ansah KO, Gerloff J, Sundar IK, and Rahman I**. Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine. *Front Physiol* 8: 1130, 2017.

16. **Behar RZ, Luo W, McWhirter KJ, Pankow JF, and Talbot P**. Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Sci Rep* 8: 8288, 2018.

17. **Clapp PW, Lavrich KS, van Heusden C, Lazarowski ER, Carson JL, and Jaspers I**. Cinnamaldehyde in Flavored E-Cigarette Liquids Temporarily Suppresses Bronchial Epithelial Cell Ciliary Motility by Dysregulation of Mitochondrial Function. *Am J Physiol Lung Cell Mol Physiol* 2019.

18. **Hickman E, Herrera CA, and Jaspers I**. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chemical research in toxicology* 32: 982-985, 2019.

19. **Hwang JH, Lyes M, Sladewski K, Enany S, McEachern E, Mathew DP, Das S, Moshensky A, Bapat S, Pride DT, Ongkeko WM, and Crotty Alexander LE**. Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria. *J Mol Med (Berl)* 94: 667-679, 2016.

20. **Miyashita L, Suri R, Dearing E, Mudway I, Dove RE, Neill DR, Van Zyl-Smit R, Kadioglu A, and Grigg J**. E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells. *Eur Respir J* 51: 2018.

21. **Man WH, de Steenhuijsen Piters WA, and Bogaert D**. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* 15: 259-270, 2017.

22. **Ubags NDJ, and Marsland BJ**. Mechanistic insight into the function of the microbiome in lung diseases. *Eur Respir J* 50: 2017.

23. **Charlson ES, Chen J, Custers-Allen R, Bittinger K, Li H, Sinha R, Hwang J, Bushman FD, and Collman RG**. Disordered microbial communities in the upper respiratory tract of cigarette smokers. *PLoS One* 5: e15216, 2010.

24. **Ramakrishnan VR, Hauser LJ, and Frank DN**. The sinonasal bacterial microbiome in health and disease. *Curr Opin Otolaryngol Head Neck Surg* 24: 20-25, 2016.

25. **Grønseth R, Drengenes C, Wiker HG, Tangedal S, Xue Y, Husebø GR, Svanes Ø, Lehmann S, Aardal M, Hoang T, Kalananthan T, Hjellestad Martinsen EM, Orvedal Leiten E, Aanerud M, Nordeide E, Haaland I, Jonassen I, Bakke P, and Eagan T**. Protected sampling is preferable in bronchoscopic studies of the airway microbiome. *ERJ Open Research* 3: 00019-02017, 2017.

26. **Di Stadio A, Costantini C, Renga G, Pariano M, Ricci G, and Romani L**. The Microbiota/Host Immune System Interaction in the Nose to Protect from COVID-19. *Life (Basel)* 10: 345, 2020.

27. **Rosas-Salazar C, Kimura KS, Shilts MH, Strickland BA, Freeman MH, Wessinger BC, Gupta V, Brown HM, Rajagopala SV, Turner JH, and Das SR**. SARS-CoV-2 Infection and Viral Load are Associated with the Upper Respiratory Tract Microbiome. *J Allergy Clin Immunol* 2021.

28. **Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, and Knight R**. Current understanding of the human microbiome. *Nature Medicine* 24: 392-400, 2018.

29. **Li H**. Statistical and Computational Methods in Microbiome and Metagenomics. In: *Handbook of Statistical Genomics*2019, p. 977-550.

30. **Jiang D, Armour CR, Hu C, Mei M, Tian C, Sharpton TJ, and Jiang Y**. Microbiome Multi-Omics Network Analysis: Statistical Considerations, Limitations, and Opportunities. *Frontiers in Genetics* 10: 2019.

31. **Gloor GB, Macklaim JM, Pawlowsky-Glahn V, and Egozcue JJ**. Microbiome Datasets Are Compositional: And This Is Not Optional. *Frontiers in Microbiology* 8: 2017.

32. **Hinton AL, and Mucha PJ**. A simultaneous feature selection and compositional association test for detecting sparse associations in high-dimensional metagenomic data (PREPRINT - Version 1). *PREPRINT Version 1 available at Research Square* 2021.

33. **Rebuli ME, Speen AM, Clapp PW, and Jaspers I**. Novel applications for a noninvasive sampling method of the nasal mucosa. *Am J Physiol Lung Cell Mol Physiol* 312: L288-l296, 2017.

34. **Muhlebach MS, Zorn BT, Esther CR, Hatch JE, Murray CP, Turkovic L, Ranganathan SC, Boucher RC, Stick SM, and Wolfgang MC**. Initial acquisition and succession of the cystic fibrosis lung microbiome is associated with disease progression in infants and preschool children. *PLoS Pathog* 14: e1006798, 2018.

35. **Horvath KM, Herbst M, Zhou H, Zhang H, Noah TL, and Jaspers I**. Nasal lavage natural killer cell function is suppressed in smokers after live attenuated influenza virus. *Respir Res* 12: 102, 2011.

36. **Baines KJ, Wright TK, Simpson JL, McDonald VM, Wood LG, Parsons KS, Wark PA, and Gibson PG**. Airway β-Defensin-1 Protein Is Elevated in COPD and Severe Asthma. *Mediators Inflamm* 2015: 407271, 2015.

37. **Hiemstra PS, Amatngalim GD, van der Does AM, and Taube C**. Antimicrobial Peptides and Innate Lung Defenses: Role in Infectious and Noninfectious Lung Diseases and Therapeutic Applications. *Chest* 149: 545-551, 2016.

38. **Hariri BM, and Cohen NA**. New insights into upper airway innate immunity. *Am J Rhinol Allergy* 30: 319-323, 2016.

39. **Salzano FA, Marino L, Salzano G, Botta RM, Cascone G, D'Agostino Fiorenza U, Selleri C, and Casolaro V**. Microbiota Composition and the Integration of Exogenous and Endogenous Signals in Reactive Nasal Inflammation. *J Immunol Res* 2018: 2724951, 2018.

40. **Davis NM, Proctor DM, Holmes SP, Relman DA, and Callahan BJ**. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6: 226, 2018.

41. **Drengenes C, Wiker HG, Kalananthan T, Nordeide E, Eagan TML, and Nielsen R**. Laboratory contamination in airway microbiome studies. *BMC Microbiol* 19: 187, 2019.

42. **Quinn TP, Erb I, Gloor G, Notredame C, Richardson MF, and Crowley TM**. A field guide for the compositional analysis of any-omics data. *GigaScience* 8: 2019.

43. **Tsilimigras MCB, and Fodor AA**. Compositional data analysis of the microbiome: fundamentals, tools, and challenges. *Annals of Epidemiology* 26: 330-335, 2016.

44. **Aitchison J**. The Statistical Analysis of Compositional Data. *Journal of the Royal Statistical Society Series B (Methodological)* 44: 139-177, 1982.

45. **Martın-Fernandez JA, Barcelo-Vidal C, and Pawlowsky-Glahn V**. Dealing with Zeros and Missing Values in Compositional Data Sets Using Nonparametric Imputation. *Mathematical Geology* 26, 2003.

46. **Legendre P, and Legendre L**. Canonical analysis. In: *Developments in Environmental Modelling*Elsevier, 2012, p. 625-710.

47. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O'Hara B, Simpson G, Solymos P, Stevens H, and Wagner H**. Vegan: Community Ecology Package. *R Package Version 22-1* 2: 1-2, 2015.

48. **Rizzo ML, and Székely GJ**. Energy distance. *Wiley Interdisciplinary Reviews: Computational Statistics* 8: 27-38, 2016.

49. **Greenacre M**. Variable Selection in Compositional Data Analysis Using Pairwise Logratios. *Mathematical Geosciences* 51: 649-682, 2019.

50. **Ernst MD**. Permutation Methods: A Basis for Exact Inference. *Statistical Science* 19: 676-685, 2004.

51. **Bastian M, Heymann S, and Jacomy M**. *Gephi: An Open Source Software for Exploring and Manipulating Networks*. 2009.

52. **Csardi G, and Nepusz T**. The Igraph Software Package for Complex Network Research. *InterJournal* Complex Systems: 1695, 2005.

53. **Anderson MJ**. Permutational Multivariate Analysis of Variance (PERMANOVA). In: *Wiley StatsRef: Statistics Reference Online*American Cancer Society, 2017, p. 1-15.

54. **Barker M, and Rayens W**. Partial least squares for discrimination. *Journal of Chemometrics* 17: 166-173, 2003.

55. **Brereton RG, and Lloyd GR**. Partial least squares discriminant analysis: taking the magic away. *Journal of Chemometrics* 28: 213-225, 2014.

56. **Kalivodová A, Hron K, Filzmoser P, Najdekr L, Janečková H, and Adam T**. PLS-DA for compositional data with application to metabolomics. *Journal of Chemometrics* 29: 21-28, 2015.

57. **Boulesteix A-L, and Strimmer K**. Partial least squares: a versatile tool for the analysis of high-dimensional genomic data. *Briefings in Bioinformatics* 8: 32-44, 2006.

58. **Kuhn M**. Building Predictive Models in *R* Using the **caret** Package. *Journal of Statistical Software* 28: 2008.

59. **Wickham H**. *ggplot2: Elegant Graphics for Data Analysis*. Springer International Publishing, 2016.

60. **Fawcett T**. An introduction to ROC analysis. *Pattern Recognition Letters* 27: 861-874, 2006.

61. **Hand DJ, and Till RJ**. A Simple Generalisation of the Area Under the ROC Curve for Multiple Class Classification Problems. *Machine Learning* 45: 171-186, 2001.

62. **Stone M**. Cross-Validatory Choice and Assessment of Statistical Predictions. *Journal of the Royal Statistical Society: Series B (Methodological)* 36: 111-133, 1974.

63. **Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, and Müller M**. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 12: 77, 2011.

64. **Benjamini Y, and Hochberg Y**. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)* 57: 289-300, 1995.

65. **Kumpitsch C, Koskinen K, Schöpf V, and Moissl-Eichinger C**. The microbiome of the upper respiratory tract in health and disease. *BMC Biol* 17: 87, 2019.

66. **De Boeck I, Wittouck S, Wuyts S, Oerlemans EFM, van den Broek MFL, Vandenheuvel D, Vanderveken O, and Lebeer S**. Comparing the Healthy Nose and Nasopharynx Microbiota Reveals Continuity As Well As Niche-Specificity. *Frontiers in microbiology* 8: 2372-2372, 2017.

67. **McMurdie PJ, and Holmes S**. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8: e61217, 2013.

68. **Rebuli ME, Speen AM, Martin EM, Addo KA, Pawlak EA, Glista-Baker E, Robinette C, Zhou H, Noah TL, and Jaspers I**. Wood Smoke Exposure Alters Human Inflammatory Responses to Viral Infection in a Sex-Specific Manner: A Randomized, Placebo-Controlled Study. *Am J Respir Crit Care Med* 2018.

69. **Cho Y, Abu-Ali G, Tashiro H, Brown TA, Osgood RS, Kasahara DI, Huttenhower C, and Shore SA**. Sex Differences in Pulmonary Responses to Ozone in Mice. Role of the Microbiome. *Am J Respir Cell Mol Biol* 60: 198-208, 2019.

70. **Yu G, Phillips S, Gail MH, Goedert JJ, Humphrys MS, Ravel J, Ren Y, and Caporaso NE**. The effect of cigarette smoking on the oral and nasal microbiota. *Microbiome* 5: 3, 2017.

71. **Piewngam P, Zheng Y, Nguyen TH, Dickey SW, Joo H-S, Villaruz AE, Glose KA, Fisher EL, Hunt RL, Li B, Chiou J, Pharkjaksu S, Khongthong S, Cheung GYC, Kiratisin P, and Otto M**. Pathogen elimination by probiotic Bacillus via signalling interference. *Nature* 562: 532-537, 2018.

72. **Liu CM, Price LB, Hungate BA, Abraham AG, Larsen LA, Christensen K, Stegger M, Skov R, and Andersen PS**. Staphylococcus aureus and the ecology of the nasal microbiome. *Science Advances* 1: e1400216, 2015.

73. **Sakr A, Brégeon F, Mège J-L, Rolain J-M, and Blin O**. Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. *Frontiers in microbiology* 9: 2419-2419, 2018.

74. **Actor JK, Hwang SA, and Kruzel ML**. Lactoferrin as a natural immune modulator. *Curr Pharm Des* 15: 1956-1973, 2009.

75. **Casimir GJ, Lefevre N, Corazza F, and Duchateau J**. Sex and inflammation in respiratory diseases: a clinical viewpoint. *Biology of sex differences* 4: 16, 2013.

76. **Man WH, van Houten MA, Mérelle ME, Vlieger AM, Chu M, Jansen NJG, Sanders EAM, and Bogaert D**. Bacterial and viral respiratory tract microbiota and host characteristics in children with lower respiratory tract infections: a matched case-control study. *Lancet Respir Med* 7: 417-426, 2019.

77. **Neumann A, Björck L, and Frick I-M**. Finegoldia magna, an Anaerobic Gram-Positive Bacterium of the Normal Human Microbiota, Induces Inflammation by Activating Neutrophils. *Frontiers in Microbiology* 11: 2020.

78. **Hoggard M, Wagner Mackenzie B, Jain R, Taylor MW, Biswas K, and Douglas RG**. Chronic Rhinosinusitis and the Evolving Understanding of Microbial Ecology in Chronic Inflammatory Mucosal Disease. *Clinical Microbiology Reviews* 30: 321, 2017.

79. **Chiu C-Y, Chan Y-L, Tsai M-H, Wang C-J, Chiang M-H, Chiu C-C, and Su S-C**. Cross-talk between airway and gut microbiome links to IgE responses to house dust mites in childhood airway allergies. *Scientific Reports* 10: 13449, 2020.

80. **Bacci G, Paganin P, Lopez L, Vanni C, Dalmastri C, Cantale C, Daddiego L, Perrotta G, Dolce D, Morelli P, Tuccio V, De Alessandri A, Fiscarelli EV, Taccetti G, Lucidi V, Bevivino A, and Mengoni A**. Pyrosequencing Unveils Cystic Fibrosis Lung Microbiome Differences Associated with a Severe Lung Function Decline. *PLoS One* 11: e0156807, 2016.

81. **Zanger UM, and Schwab M**. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & Therapeutics* 138: 103-141, 2013.

82. **Vemuri R, Sylvia KE, Klein SL, Forster SC, Plebanski M, Eri R, and Flanagan KL**. The microgenderome revealed: sex differences in bidirectional interactions between the microbiota, hormones, immunity and disease susceptibility. *Semin Immunopathol* 41: 265-275, 2019.

83. **Kim YS, Unno T, Kim BY, and Park MS**. Sex Differences in Gut Microbiota. *World J Mens Health* 38: 48-60, 2020.

84. **Shin JH, Park YH, Sim M, Kim SA, Joung H, and Shin DM**. Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome. *Res Microbiol* 170: 192-201, 2019.

85. **Han MK, Arteaga-Solis E, Blenis J, Bourjeily G, Clegg DJ, DeMeo D, Duffy J, Gaston B, Heller NM, Hemnes A, Henske EP, Jain R, Lahm T, Lancaster LH, Lee J, Legato MJ, McKee S, Mehra R, Morris A, Prakash YS, Stampfli MR, Gopal-Srivastava R, Laposky AD, Punturieri A, Reineck L, Tigno X, and Clayton J**. Female Sex and Gender in Lung/Sleep Health and Disease. Increased Understanding of Basic Biological, Pathophysiological, and Behavioral Mechanisms Leading to Better Health for Female Patients with Lung Disease. *Am J Respir Crit Care Med* 198: 850-858, 2018.

86. **De Boeck I, Wittouck S, Martens K, Claes J, Jorissen M, Steelant B, van den Broek MFL, Seys SF, Hellings PW, Vanderveken OM, and Lebeer S**. Anterior Nares Diversity and Pathobionts Represent Sinus Microbiome in Chronic Rhinosinusitis. *mSphere* 4: e00532-00519, 2019.

87. **Chopyk J, Bojanowski CM, Shin J, Moshensky A, Fuentes AL, Bonde SS, Chuki D, Pride DT, and Crotty Alexander LE**. Compositional Differences in the Oral Microbiome of E-cigarette Users. *Front Microbiol* 12: 599664, 2021.

88. **Ganesan SM, Dabdoub SM, Nagaraja HN, Scott ML, Pamulapati S, Berman ML, Shields PG, Wewers ME, and Kumar PS**. Adverse effects of electronic cigarettes on the disease-naive oral microbiome. *Sci Adv* 6: eaaz0108, 2020.

89. **Janda JM, and Abbott SL**. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* 45: 2761-2764, 2007.

90. **Chen YW, Li SW, Lin CD, Huang MZ, Lin HJ, Chin CY, Lai YR, Chiu CH, Yang CY, and Lai CH**. Fine Particulate Matter Exposure Alters Pulmonary Microbiota Composition and Aggravates Pneumococcus-Induced Lung Pathogenesis. *Front Cell Dev Biol* 8: 570484, 2020.

91. **Mariani J, Favero C, Spinazzè A, Cavallo DM, Carugno M, Motta V, Bonzini M, Cattaneo A, Pesatori AC, and Bollati V**. Short-term particulate matter exposure influences nasal microbiota in a population of healthy subjects. *Environmental research* 162: 119-126, 2018.

CHAPTER 4: INDUCED SPUTUM BIOMARKERS OF AIRWAY IMMUNE HOMEOSTASIS: A COMPARATIVE ANALYSIS IN 3RD VERSUS 4TH GENERATION E-CIGARETTE USERS

Introduction

E-cigarettes continue to be of public health concern. In 2020, more than 3.5 million youth in the United States were e-cigarette users, including 19.6% of high schoolers (1). Additionally, an estimated 5-8 million U.S. adults also use e-cigarettes (2, 3). (Note: the 2021 NYTS data collection was hampered by the COVID 19 pandemic (4)). Over the past decade, an increasing number of studies have demonstrated that e-cigarettes and their chemical constituents can affect the respiratory system, including respiratory immune homeostasis [reviewed in (5, 6)], but the extent of these effects, including their magnitudes, affected cell types, and biological implications for human health, is an active area of investigation.

A major challenge in the field of e-cigarette toxicology is the constant evolution of e-cigarette devices and e-liquid formulations. The most notable example of this is the shift in the e-cigarette industry that started when JUUL, a discreet, pod-based e-cigarette formulated with nicotine salts, was introduced in the U.S. 2015. Following JUUL's skyrocketing popularity (7), a new wave of similar devices entered the market, and in 2020, pre-filled pods or cartridges were still the most popular e-cigarette device type among U.S. high school students (1, 8). In response to the FDA's restrictions on flavored, cartridge-based e-cigarettes (9), disposable e-cigarettes have also gained popularity, with the percentage of youth vapers using disposables increasing from 2.5% in 2019 to 26.5% in 2020 (1, 10).

This newer generation of e-cigarettes (sleek, low-power pod, cartridge, and disposable e-cigarettes, also referred to as 4th generation e-cigarettes) is unique from previous generation e-cigarettes (vape pens and box mods, also referred to as 3rd generation e-cigarettes) in their aerosolization parameters and nicotine formulation (11-14). E-liquid found in 4th generation e-cigarettes typically contains nicotine salts, which are generated by combining freebase nicotine with an organic acid (15). This results in the

formation of monoprotonated nicotine and lowers the pH of the mixture and therefore also the resulting aerosol (12, 15, 16). The proportion of nicotine in the aerosol in protonated and freebase forms impacts the user sensory experience, with nicotine-salt-containing e-cigarettes providing a "smoother" feeling and allowing for inhalation of higher concentrations of nicotine (8, 17). Importantly, the inhalation toxicity of organic acids commonly used in the formulation of nicotine-salt-containing e-liquids is poorly understood.

Previous studies have shown increased proteinase levels, enhanced neutrophil activation, and altered mucin composition in airway samples from e-cig users (18, 19). However, the samples used in these studies were collected from subjects who used 3rd generation e-cigs, as 4th generation e-cigs such as JUUL had not yet gained popularity at the time of these studies. Because 4th generation e-cigarettes are now the most popular type of e-cigarette, particularly among young never-smokers, there is a need to assess the respiratory effects of these types of e-cigs in human subjects and determine whether these effects are unique from those observed in previous generation e-cig users. This research is also critical given the recent marketing authorization of Vuse Solo, a nicotine-salt-containing 4th generation e-cigarette, by the FDA, with other similar devices still under review (20).

In this study, we collected induced sputum samples from a cohort of non-smokers/non-vapers, smokers, 3rd generation and 4th generation e-cigarette users to determine whether 4th generation e-cigarette users exhibited unique central airways immune profiles. We evaluated cellular composition and soluble mediators associated with inflammation, host defense, and lung injury in sputum samples. We then applied both a standard variable-by-variable analysis and a multivariate predictive modeling analysis to enhance our resolution to distinguish inflammatory expression profiles between subject cohorts. Our results demonstrate that there are significant differences between these markers of respiratory immune homeostasis between 4th generation e-cigarette users and other groups, underscoring the importance of considering device type when assessing the inhalation toxicity of e-cigarettes.

Methods

Study cohort and sample collection

Subject recruitment

Healthy adult human nonsmokers, smokers, and e-cigarette users between 18-50 years old were recruited to participate in this study. Active cigarette smoking and vaping were determined as described previously (21). E-cigarette users were classified as 3rd generation e-cigarette users if they reported using primarily vape pens, box mods, or similar devices that contain freebase nicotine. E-cigarette users were classified as 4th generation e-cigarette users if they reported using primarily JUUL or other sleek, discreet, low-powered e-cigarettes that contain nicotine salts. Two subjects used both 3rd and 4th generation e-cigarettes regularly and were excluded from the study. A flow chart showing inclusion and exclusion of clinical study subjects by device type is available in Figure S4-1. Exclusion criteria included current symptoms of allergic rhinitis, chronic cardiorespiratory disease, immunodeficiency, bleeding disorders, current pregnancy, and FEV_1 less than 75% predicted during the screen visit. All studies were approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board (IRB #13-3454 and #17-2275).

Sample collection and processing

Induced sputum collection, processing, acquisition of fluid phase samples, and differential cell counts were performed as described previously (22, 23). To obtain serum, venous blood was collected in BD Vacutainer serum-separating tubes (Fisher Scientific, Waltham, MA), allowed to clot for a minimum of 15 minutes, and centrifuged at 1200 x g for 10 minutes. The serum layer was collected and stored at -80°C until samples were collected from all subjects.

Experimental procedures

Serum cotinine measurement

Serum cotinine, a metabolite of nicotine, was measured using a commercially available ELISA kit (Calbiotech) to confirm smoking status. For samples below the limit of detection (5 ng/mL), a value of zero was assigned. Serum was not available for some subjects in each group. The number of subjects for which serum was available in each group is denoted in Table 4-1.

Induced sputum soluble mediator measurement

Soluble mediator concentrations in cell-free induced sputum supernatants were determined using commercially available single-plex ELISAs (R&D Systems) and Mesoscale Discovery multi-plex ELISAs. dsDNA concentrations were measured using the Quant-iT Picogreen assay (Thermo Fisher). To account for the presence of dithiothreitol (DTT, 3.25 mM) in sputum supernatant samples, ELISA standards were diluted in assay diluent with an equivalent concentration of DTT as was in the sputum supernatants for that assay, thus generating a DTT and non-DTT standard curve. During dilution optimization assays for samples, we compared both standard curves and found no significant interference of DTT at the concentrations equivalent to those in our diluted samples (data not shown).

Liver Injury Analysis

To determine whether there were any effects of cigarette and/or e-cigarette exposure on liver health, serum samples were analyzed for markers of liver injury. Although we observed biomarker levels above or below the normal limits in some individual subjects, overall, our data do not indicate clinically significant changes between groups (Table S4-1).

Data Analysis

Data availability

Data input files and code used for the analysis can be found at: https://github.com/ehickman0817/dissertation-ch4-devicecomparison . All analyses were conducted using R v4.1.1 using baseline statistical packages unless otherwise noted below (24). An overview of the analysis structure for soluble mediators is provided in Figure 4-1.

Normality testing

For all continuous variables, normality was tested prior to between-group comparative analyses using the Shapiro-Wilk test. Normally distributed data were analyzed using parametric tests, and non-normally distributed data were analyzed using non-parametric tests, when possible. Normality was also assessed through examination of histograms and quantile-quantile plots through the ggplot2 package.

Demographics

To determine which covariates were significantly different between exposure groups, we performed either a Fisher's exact test (categorical variables) or a Kruskal-Wallis test with Dunn's test (continuous variables) for multiple comparisons. Covariates that were significantly different between groups were included in analyses as described below. Due to the small number of Black, Asian and Pacific Islander, and Mixed/Other subjects in each group, these groups were collapsed into "Non-White" for further analyses.

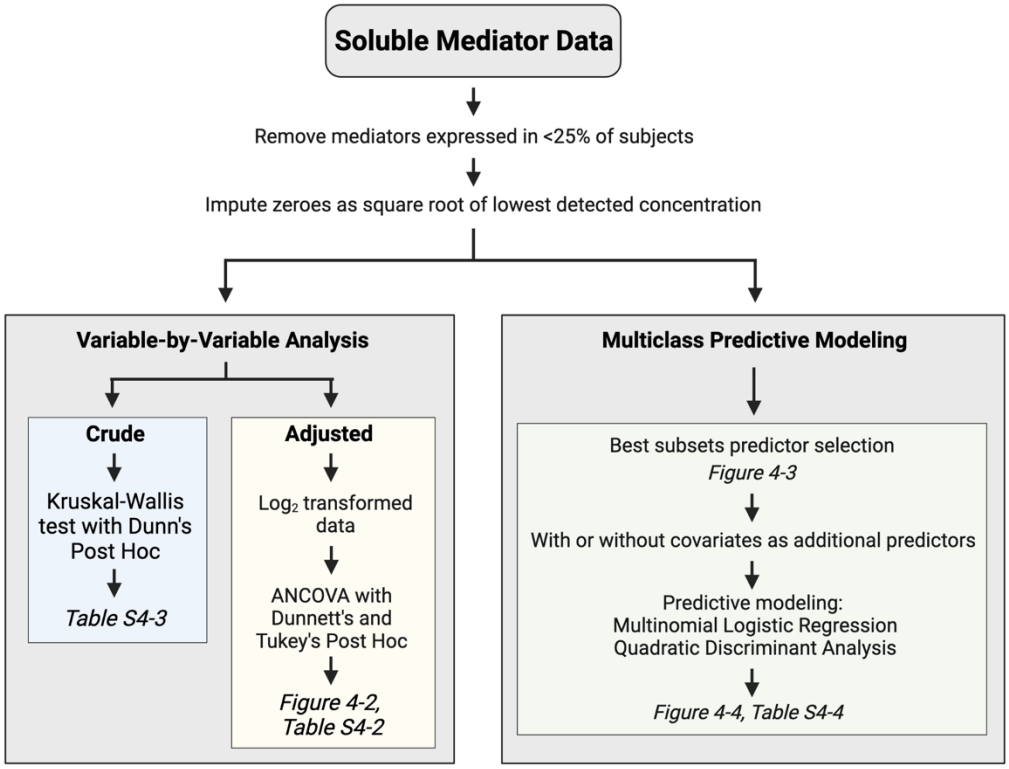


Figure 4-1. Flow chart depicting analysis workflow for investigating differences in soluble mediators between exposure groups. Figure created using biorender.com.

Differences in Individual Sputum Differential Metrics and Soluble Mediators Between Exposure Groups

We employed two different approaches to determine whether there were significant differences in individual sputum cell differential metrics or the expression soluble mediators between exposure groups. First, we carried out a Kruskal-Wallis test followed by a Dunn's test for non-parametric multiple comparisons on the raw data for each cell metric and mediator value. Next, because there were significant differences between demographic variables across exposure groups, we also ran analysis of covariance (ANCOVA) tests, which allowed us to control for the differences in age, sex, and race in our exposure groups. Because post-hoc tests used by ANCOVA assume normality, prior to running ANCOVA, the data were pseudo $\log_2$ transformed by adding one and then taking the log of the metric or mediator concentration. This approach ensured that all values in the dataset were positive and moved the data closer to a normal distribution. For variables with significant ($p < 0.05$) overall associations with exposure

group status using ANCOVA, Dunnett's post-hoc test was performed on the ANCOVA output to evaluate significant differences between controls and exposure groups. We were also interested in whether there were significant differences in these cell and mediator values specifically between 3rd and 4th generation e-cigarette users. For this, we employed Tukey's post hoc test, which compares means between each pair of groups, and examined the unadjusted p-values for the specific comparison between 3rd and 4th generation e-cigarette users.

Variable Selection and Predictive Modeling to Enhance Resolution of Between Group Differences in Sputum Soluble Mediators

To determine whether these overall differences in soluble mediators were significant enough to separate subjects in each exposure group from the others, we employed machine learning models. Prior to applying machine learning models, data were pre-processed by removing mediators that were undetected in more than 75% of subjects. For remaining mediators, zeroes (undetected mediators below the LLD) were imputed using the square root of the lowest detected concentration for each mediator. When designing the machine learning models for soluble mediators, we considered mediator data as primary predictor data. Next, we considered covariates identified as significantly different between exposure groups, including age, sex, and race, for inclusion as predictor variables. We also considered induced sputum cell metrics for inclusion as predictors; however, given that these data were only available for a subset of subjects in the cohort (Figure S4-1) and that the scale and resolution of these data differs from the soluble mediator data, we decided to build models only with mediator data alone or mediator data in combination with covariates. The evaluated outcome variables were exposure group classifications: non-smoker/non-vaper, smoker, 3rd generation e-cig user, 4th generation e-cig user. We also applied the variable selection and data reduction method of Best Subsets Regression (through the leaps package, v3.1 (25)) to address our study questions and optimize performance of the machine learning models. We tested many methods for data reduction (e.g., PCA) and variable selection (e.g., Spearman's correlation, Lasso regression, Best Subsets regression), and we found that Best Subsets regression, which tested all

linear combinations of variables and prioritized which variables best explained separation between exposure group classifications (26), was the best choice to address our study's questions and optimize performance of machine learning models. Predictive models were built using both quadratic discriminant analysis (QDA) and multinomial logistic regression (MLR), as these machine learning methods allow for prediction of multiple categorical classes. Using the *caret* package (v6.0-90) (27), data were randomly split into training and test datasets (5-fold cross-validation) before application of machine learning models to the datasets. Similar to previously published work (28, 29), model performance parameters were calculated based on the resulting confusion matrix, which summarizes classification of subjects based on correct and incorrect identification of subjects as belonging to or not belonging to a specific exposure group.

Results

Subject Demographics

Demographic data are summarized in Table 4-1. The study cohort was comprised of 27% non-smokers/non-vapers (NS/NV; n = 28), 20% smokers (n = 21), 26% 3rd generation e-cigarette users (n = 27), and 26% 4th generation e-cigarette users (n = 27). Each exposure group contained both males and females, with a minimum of n = 8 per sex per group. 4th generation e-cigarette users were significantly younger on average than all other groups, while smokers were significantly older. These age differences were expected given the rise in popularity of 4th generation e-cigarettes and decline in popularity of cigarettes among youth (30, 31). The distribution of subjects' races was significantly different across exposure groups ($p = 0.0095$), which mirrors previous studies showing that e-cigarette users are more likely to be white (3, 32). BMI was not significantly different between exposure groups. As expected, smokers, 3rd generation e-cig users, and 4th generation e-cigarette users had significantly elevated levels of serum cotinine, a metabolite of nicotine, in comparison with NS/NV. There were no significant differences in serum cotinine between the three tobacco user groups, indicating similar nicotine exposure at the time of sample collection.

Table 4-1. Study demographics. NS/NV = non-smokers/non-vapers. For continuous variables, groups were compared using the Kruskal-Wallis test with Dunn's test for non-parametric multiple comparisons. For categorical variables, groups were compared using Fisher's Exact Test. *** at least $p < 0.001$ in comparison with all other groups. ^ $p < 0.05$ in comparison with smokers.

	NS/NV (N=28)	Smoker (N=21)	3rd Gen (N=27)	4th Gen (N=27)	P-value
Sex					0.004
Male	9.00 (32.1%)	8.00 (38.1%)	19.0 (70.4%)	19.0 (70.4%)	
Female	19.0 (67.9%)	13.0 (61.9%)	8.00 (29.6%)	8.00 (29.6%)	
Race					0.0095
White	21.0 (75.0%)	10.0 (47.6%)	18.0 (66.7%)	23.0 (85.2%)	
Black	5.00 (17.9%)	10.0 (47.6%)	3.00 (11.1%)	1.00 (3.7%)	
Asian/Pacific Islander	1.00 (3.6%)	0 (0%)	4.00 (14.8%)	2.00 (7.4%)	
Mixed/Other	1.00 (3.6%)	1.00 (4.8%)	2.00 (7.4%)	1.00 (3.7%)	
Hispanic					0.418
No	24.0 (85.7%)	20.0 (95.2%)	26.0 (96.3%)	23.0 (85.2%)	
Yes	4.00 (14.3%)	1.00 (4.8%)	1.00 (3.7%)	4.00 (14.8%)	
Age					<0.001
Mean (SD)	26.5 (5.29) ^	32.1 (7.42)	27.2 (7.42) ^	21.6 (3.17) ***	
BMI					0.133
Mean (SD)	26.2 (5.61)	27.3 (5.98)	27.2 (5.94)	24.0 (4.04)	
Serum Cotinine (ng/mL)					<0.001
Mean (SD)	0 (0) ***	188 (87.5)	143 (82.2)	110 (90.5)	
N measured	21	20	25	12	

Induced sputum differential cell counts

Induced sputum differential cell counts are summarized in Table 4-2. Because ANCOVA performed on sputum differential metrics indicated no significant associations with sex and race and only one significant association with age (bronchial cells per mg, $p = 0.011$), Kruskal-Wallis p-values with Dunn's test for multiple comparisons between exposure groups are reported for induced sputum cell differential data in Table 4-2. We observed that smokers' induced sputum contained a significantly higher percentage of neutrophils in comparison with all other exposure groups, which is consistent previous reports of smoking-associated increases in inflammatory cell recruitment to the airways (33-35). We found that 3rd generation e-cigarette users had significantly more macrophages per mg of sputum, fewer lymphocytes per mg of sputum and a lower percentage of lymphocytes than smokers. Interestingly, 4th generation e-cigarette users had significantly greater absolute cells per mg and percent bronchial epithelial cells than both non-smokers/non-vapers and 3rd generation e-cigarette users.

Table 4-2. Sputum differential data. Data are presented as mean (standard error). Groups were compared using the Kruskal-Wallis test with Dunn's test for non-parametric multiple comparisons. * at least $p < 0.05$ in comparison with all other groups, ^^ $p < 0.01$ in comparison with smokers, # $p < 0.05$ and ## $p < 0.01$ in comparison with 3rd gen, & $p < 0.05$ in comparison with NS/NV.

	NS/NV (N=20)	Smoker (N=19)	3rd Gen (N=25)	4th Gen (N=25)	Overall P-value
Total Cells/mg	766 (190)	727 (225)	976 (135)	780 (151)	0.0956
Macrophages/mg	469 (122)	204 (54.5)	**544 (89.4) ^^**	505 (132)	**0.0119**
% Macrophage	67.2 (6.04)	**36.5 (5.59) ***	59.1 (5.09)	57.7 (5.12)	**0.0032**
PMN/mg	259 (97.0)	505 (174)	421 (99.8)	256 (40.2)	0.102
% PMN	26.5 (5.07)	**61.3 (5.39) ***	39.8 (5.12)	38.5 (5.29)	**<0.001**
Eosinophils/mg	1.15 (0.678)	6.26 (4.01)	5.92 (3.70)	2.48 (1.16)	0.394
% Eosinophil	0.230 (0.135)	0.775 (0.278)	0.456 (0.224)	0.704 (0.505)	0.564
Lymphocytes/mg	2.75 (1.73)	1.95 (1.42)	**0.04 (0.04) &**	1.12 (0.401)	**0.0175**
% Lymphocyte	0.233 (0.092)	0.135 (0.084)	**0.008 (0.008) &**	**0.094 (0.028) #**	**0.0133**
Bronchial Cells/mg	2.30 (0.927)	10.5 (7.69)	4.40 (1.74)	**15.5 (5.06) &, ##**	**0.0041**
% Bronchial Cells	0.870 (0.355)	1.33 (0.460)	0.562 (0.203)	**3.03 (1.24) ##**	**0.0138**

Soluble mediators that were significantly different between exposure groups

ANCOVA performed on soluble mediator data indicated that there were mediators that had significant associations with exposure group, age, sex, and race (Table S4-2). 12 soluble mediators were significantly affected by exposure group: CRP, Flt1, IFN-γ, IL-6, MCP-1, MIP-1β, MMP-2, sICAM-1, sVCAM-1, Tie-2, uteroglobin, and VEGF (Figure 4-2). Of these, a majority included significant differences between 4th generation and 3rd generation e-cigarette users, and a majority were significantly decreased in 4th generation e-cigarette users in comparison with the other exposure groups. Specifically, levels of CRP, IFN-γ, MCP-1, uteroglobin, and VEGF were significantly lower in 4th vs 3rd generation e-cigarette users. And, although these comparisons did not reach statistical significance, we observed reduced expression of CRP, IFN-γ, MCP-1, and uteroglobin in 4th generation e-cigarette users relative to non-smokers/non-vapers. The concentrations of sICAM1 and sVCAM1 were significantly lower in 4th vs 3rd generation e-cigarette users and non-smokers/non-vapers. Additionally, IL-6 concentration was significantly increased in smokers in comparison with non-smokers/non-vapers, as expected (33, 36). Average concentrations stratified by exposure group with Kruskal-Wallis p-values (crude/unadjusted) for all mediators are summarized in Table S4-3. Overall, these results indicate suppression of soluble mediator levels in the airways in 4th generation e-cigarette users, which suggests dysregulated immune homeostasis in the form of overall immune suppression in these subjects.

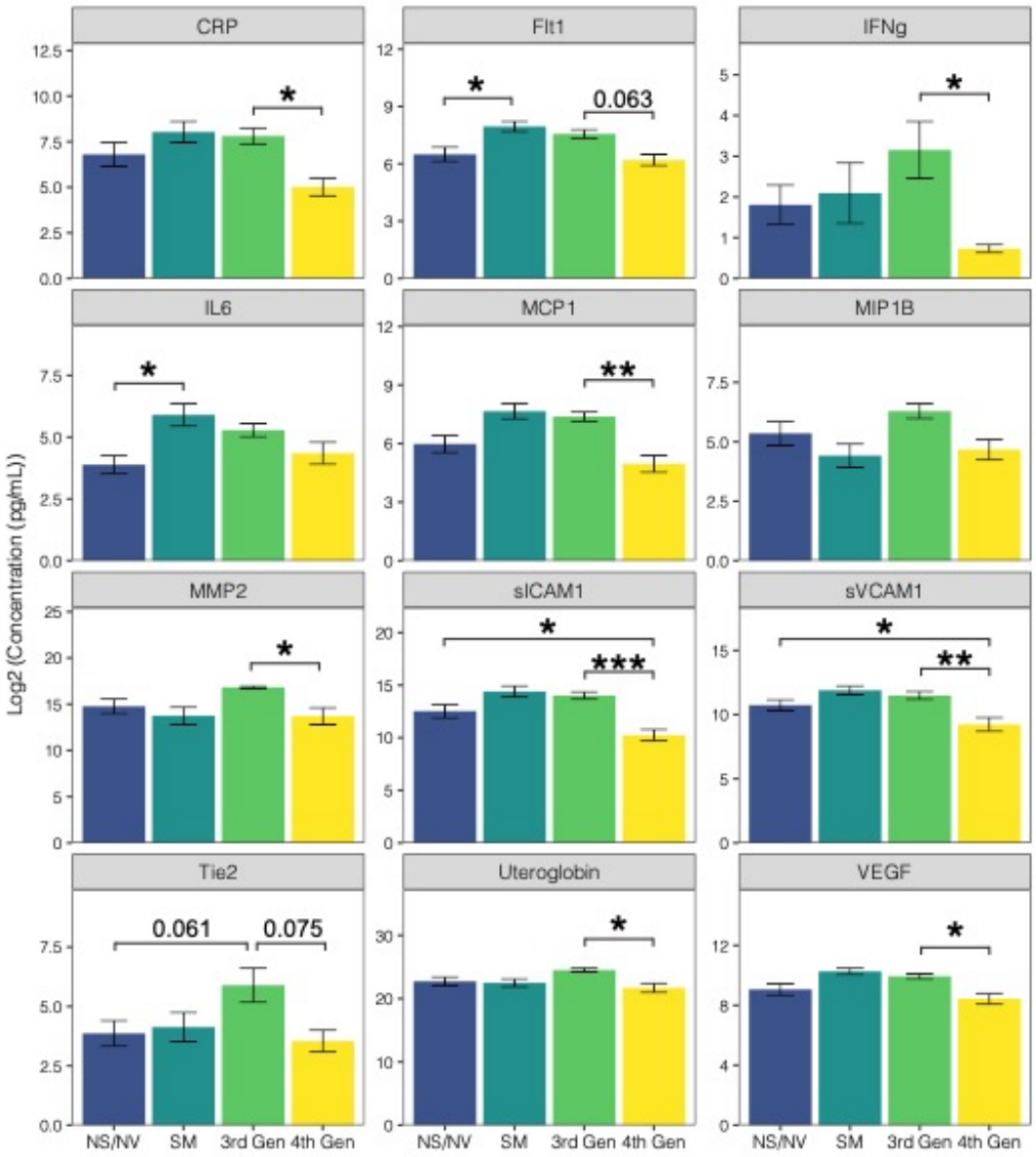


Figure 4-2. Soluble mediators that were significantly different between exposure groups after adjusting for age, sex, and race differences between exposure groups.

Results are presented as mean ± standard error of log_2 transformed mediator concentrations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using ANCOVA followed by Dunnett's (comparisons with NS/NV) and Tukey's (3^{rd} v. 4^{th} Gen) post-hoc tests. NS/NV = non-smoker/non-vaper, SM = smoker. N = 28 non-smokers/non-vapers, 21 smokers, 27 3^{rd} generation e-cig users, and 27 4^{th} generation e-cig users.

Variable selection and predictive modeling to evaluate the separation between all four exposure groups.

Although we found individual significant differences in induced sputum cell differential metrics and soluble mediators between exposure groups, we also wanted to understand whether there were significant overall differences in soluble mediator signatures between groups using predictive modeling. Given our cohort size and number of subjects per group, we determined that the maximum number of predictors our models would accept was nine. We applied best subsets regression to our soluble mediator data to determine the nine variables that best separated the exposure groups. The mediators that best separated the exposure groups were: eotaxin-3, IL-6, IL-10, MIP-1α, MMP-9, MPO, TARC, Tie2, and VEGFD (Figure 4-3). These mediators are associated with a number of different cell functions, including immune cell recruitment, type 2 inflammation, angiogenesis, and tissue remodeling. These mediators were then used as predictors to build models using either multinomial logistic regression or quadratic discriminant analysis, with or without covariates additionally included as predictors. Most of these variables were different from the variables determined to be significantly different between exposure groups by ANCOVA and ANOVA, indicating novel trends that are not captured by traditional group comparison approaches. Notably, trends that we observed in these mediators were: decreased expression of TARC and VEGF and increased expression of IL-10, MMP9, and eotaxin-3 in 4th generation e-cigarette users, increased expression of Tie2 and MPO in 3rd generation e-cigarette users, and increased expression of IL-6 in smokers (Figure 4-3). Unsupervised hierarchical clustering of mean mediator expression by exposure group showed that the expression profile of 4th generation e-cigarette users was the most different from the other three exposure groups (Figure 4-3).

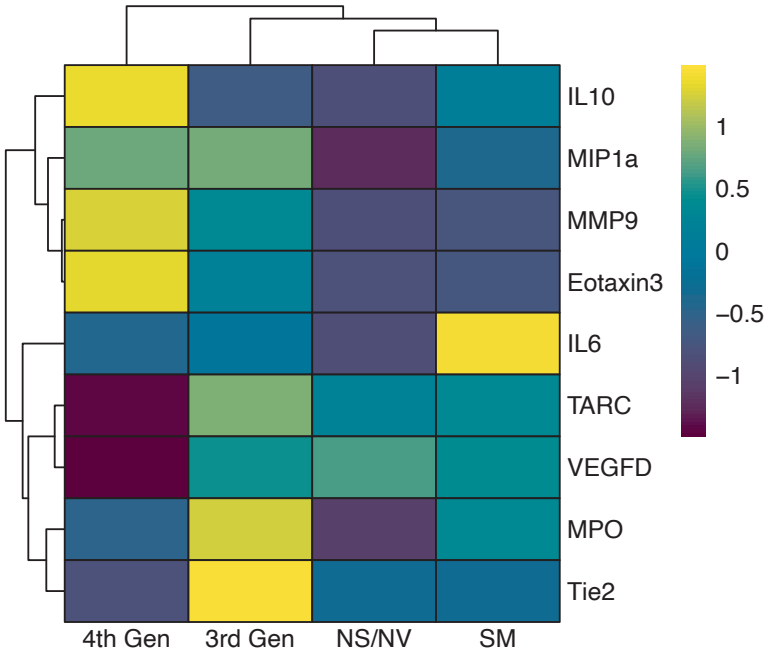


Figure 4-3. Heatmap showing mean relative expression (row-scaled) of soluble mediators selected by best subsets regression by exposure group.

Overall and by class performance metrics for each model are summarized in Figure 4-4 and Table S4-4. Performance for all models followed similar trends, with specificity and negative predictive value relatively high, and accuracy, sensitivity, and positive predictive value lower, indicating that the models could better predict which subjects were not members of a specific group than predict which subjects were members of a specific group. Notably, for most of the models, performance metrics for 4th generation e-cigarette users were higher than for other groups, indicating higher predictivity for classification of 4th generation e-cigarette users based on soluble mediator concentrations.

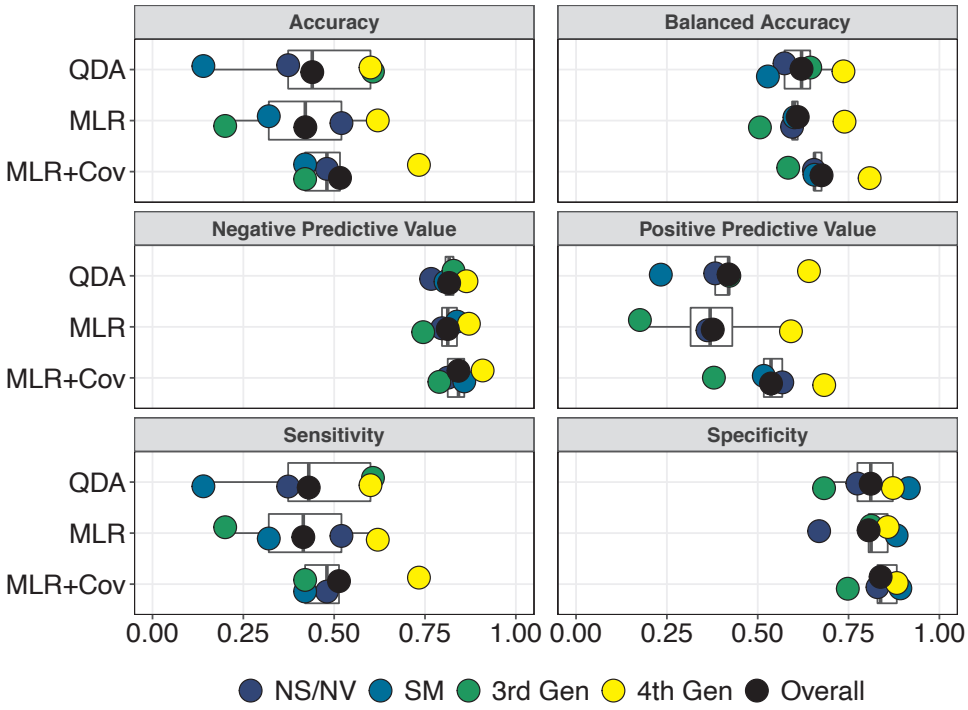


Figure 4-4. Performance parameters for predictive models built using soluble mediators as predictor variables. QDA = Quadratic Discriminant Analysis; MLR = multinomial logistic regression; MLR + Cov = multinomial logistic regression with covariates included as additional predictors.

Discussion

In this study, we collected induced sputum from healthy human non-smokers/non-vapers, smokers, 3rd generation e-cigarette users, and 4th generation e-cigarette users and employed a multifaceted approach to understand differences in biomarkers of respiratory immune homeostasis between groups. Using both variable-by-variable analyses and predictive modeling, we demonstrated significant differences in both induced sputum cell differentials and soluble mediator milieu between exposure groups. Most notably, we observed significantly decreased expression of soluble mediators in 4th generation e-cigarette users, which could indicate airway immune dysfunction in these subjects. To our knowledge, this is the first study directly comparing respiratory immune biomarkers in e-cigarette users who use different device types.

Importantly, we observed significantly different expression profiles of soluble mediators in 4th generation e-cigarette users in comparison with non-smokers/non-vapers and 3rd generation e-cigarette

users. After adjusting for sex, age, and race, we found that expression of sICAM-1 and sVCAM-1 was significantly lower in 4th generation e-cigarette users in comparison with other groups. Increased levels of both sICAM-1 and sVCAM-1 have been associated with acute lung injury (37, 38), but decreased levels of these molecules in response to a disease state or toxicant exposure, particularly in airway fluid, have not been documented. Previous studies have demonstrated that sICAM-1 can be protective during rhinovirus infection, as sICAM-1 can bind the virus, thereby reducing the amount of virus binding mICAM-1 and entering epithelial cells (39-41). Therefore, the significantly reduced expression of sICAM-1 we observed in 4th generation e-cigarette users may indicate increased susceptibility to rhinovirus infection in 4th generation e-cigarette users.

We also observed significantly decreased expression of CRP, IFN-γ, MCP-1, and uteroglobin in 4th generation e-cigarette users compared with 3rd generation e-cigarette users, although not all these comparisons reached statistical significance with non-smokers/non-vapers. Each of these mediators are known to play important roles in respiratory host defense. On mucosal surfaces, such as in the respiratory tract, CRP acts as an antimicrobial and binds to specific residues on cell walls of bacteria that are prevalent in the respiratory tract (42, 43). As it is constitutively expressed, it is hypothesized to play a role in mediating host-microbe interactions in the respiratory tract, though more work is needed to characterize CRP in the respiratory mucosa in association with disrupted respiratory immune homeostasis. IFN-γ and MCP-1 are pleiotropic molecules that mediate activity of both the innate and adaptive immune systems, including priming of macrophages to respond to pro-inflammatory stimuli, chemotaxis, and maintenance of immune homeostasis (44-47). Uteroglobin, also known as club cell secretory protein, is regarded as anti-inflammatory, and decreases in uteroglobin have been observed previously in smokers and subjects with COPD (48). Therefore, decreased expression of these mediators demonstrates suppressed host defense in association with 4th generation e-cigarette use.

Here, we also wanted to determine whether soluble mediator concentrations measured in induced sputum could be used to predict whether subjects were non-smokers/non-vapers, smokers, 3rd generation e-cigarette users, or 4th generation e-cigarette. Before applying predictive models, we used best subsets

regression to reduce the number of input variables, which allowed us to identify soluble mediators that were important in separating exposure groups. Unsupervised hierarchical clustering of the mean expression of the nine mediators identified by best subsets regression showed that expression patterns of these mediators in 4th generation e-cigarette users separated the most from the other three exposure groups. These soluble mediators spanned a wide range of general functions, including cytokine and chemokine signaling, host defense, and vasculature maintenance. We then applied multiclass machine learning models (multinomial logistic regression and quadratic discriminant analysis) and found that 4th generation e-cigarette users showed the highest predictivity in a majority of our models, suggesting that the soluble mediator milieu associated with 4th generation e-cigarette use is unique from the milieu observed in the other three exposure groups. This approach allowed us to detect shifts in the composition of soluble mediator expression that were not evident when examining mediators one-by-one and to consider the complexity underlying soluble mediator signaling that maintains immune homeostasis, which could provide the basis for more targeted studies assessing the respiratory effects of e-cigarette use. Future studies are needed to directly assess the relationship between e-cigarette use and the cellular processes highlighted in this analysis, particularly those beyond standard inflammatory pathways commonly interrogated in studies of e-cigarette effects.

Furthermore, we observed that there were significant differences in induced sputum cell differentials between exposure groups, indicative of disrupted immune homeostasis from the baseline sputum cell composition observed in non-smokers/non-vapers. We found that 4th generation e-cigarette users had a significantly higher percentage of bronchial epithelial cells in their induced sputum than non-smokers/non-vapers, which could indicate airway injury; however, few studies have established normal ranges for bronchial epithelial cells in induced sputum, making it difficult to assess if the magnitude of effect observed is biologically significant. This finding is also particularly interesting in the absence of significant increases in markers of airway injury in 4th generation e-cigarette users.

We hypothesize that the differences we observed between 3rd and 4th generation e-cigarette users could be driven by the following mechanisms. First, 4th generation e-cigarettes contain nicotine salts,

which are formulated using organic acids such as benzoic acid, lactic acid, and levulinic acid (15). The effects of inhaling these organic acids are currently unknown and therefore could be driving some of the effects we observed. Second, 4th generation e-cigarettes contain a higher concentration of nicotine than 3rd generation e-cigarettes (11), and nicotine is known to be immunosuppressive (49, 50) . Although we observed similar serum cotinine levels across tobacco use groups, it is possible that serum cotinine is not an accurate reflection of nicotine concentrations in the respiratory mucosal microenvironment and that the high level of nicotine present in the aerosol of 4th generation e-cigarette users exerts immunosuppressive effects. Third, 4th generation e-cigarettes aerosolize the e-liquid at lower temperatures than 3rd generation e-cigarettes, thereby producing fewer free radicals and carbonyls (13, 14). This phenomenon likely explains the absence of overt acute inflammation observed in 4th generation e-cigarette users and our observation that increased expression of inflammatory biomarkers 3rd generation e-cigarette users was more frequent than in 4th generation e-cigarette users. Lastly, 4th generation e-cigarette users are significantly younger, less likely to be former smokers, and more likely to be dual users with marijuana. We did not assess inhalation of cannabinoids, which may be particularly relevant given that dual use of nicotine-containing e-cigarettes and marijuana (either smoked or vaped) is prevalent in young adult users (51-54) and that cannabinoids can modulate the immune system (55). These factors may also contribute to the different pattern of soluble mediator expression we observed in 4th versus 3rd generation e-cigarette users.

Although our study provides novel insight into differences in airway biomarkers of immune homeostasis between exposure groups, there are limitations to our study that are important to consider and that warrant exploration in future studies. Beyond categorizing subjects as current e-cigarette users of a specific device type, we did not collect additional information about e-cigarette use parameters, such as flavor, number of puffs per day, previous smoking history, or length of e-cigarette use. These variations in e-cigarette use patterns may have contributed to the high variability we observed within each of our e-cigarette groups. Future studies with larger cohort sizes and more detailed e-cigarette use questionnaires will be required to control for this variation and to more fully elucidate factors driving respiratory effects

observed in e-cigarette users. Furthermore, as a cross-sectional observational study, our results are primarily associative and hypothesis-generating. Prospective studies that collect and analyze samples from e-cigarette users over time will be required to more fully understand respiratory effects associated with e-cigarette use. And, because of the short length of time e-cigarettes have been on the market, and therefore the relatively short vaping histories of e-cigarette users, it is not yet possible to evaluate the long-term effects of vaping.

Taken together, our data demonstrate that there are significant differences in biomarkers of respiratory immune homeostasis in 4th generation e-cigarette users in comparison with non-smokers/non-vapers and with 3rd generation e-cigarette users (Figure 4-5). Our findings highlight the importance of considering device type in studies of e-cigarette inhalation toxicity, the utility of leveraging multiple analysis approaches to understand differences between exposure groups, and the need for continued investigation of the mechanisms underlying the effects of popular e-cigarette devices, including those that have recently been authorized for use by the FDA.

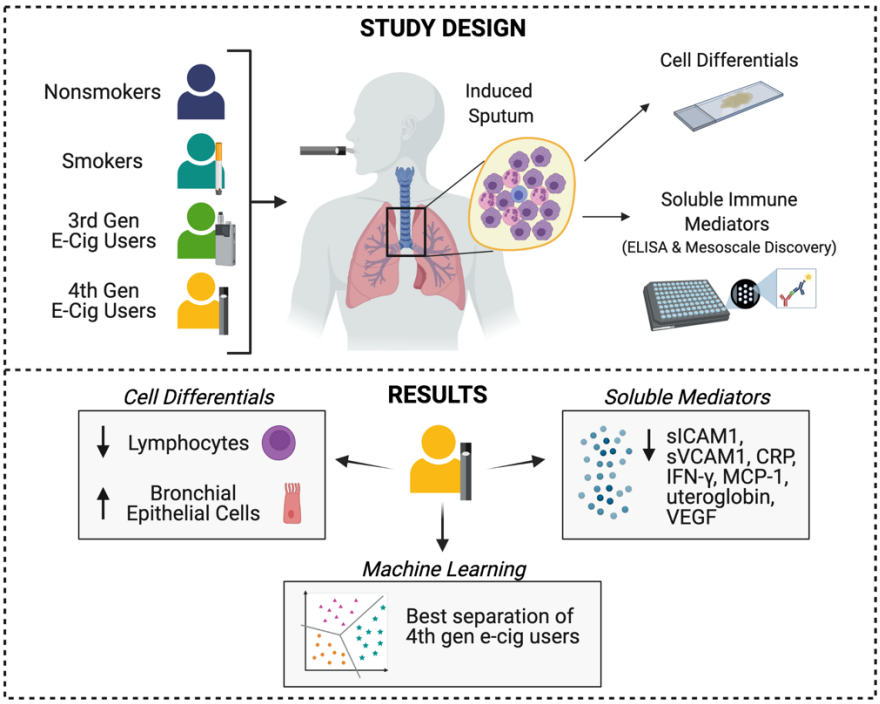


Figure 4-5. Summary of findings.

REFERENCES

1. **Wang TW, Neff LJ, Park-Lee E, Ren C, Cullen KA, and King BA**. E-cigarette Use Among Middle and High School Students - United States, 2020. *MMWR Morb Mortal Wkly Rep* 69: 1310-1312, 2020.

2. **Creamer MR, Wang TW, Babb S, Cullen KA, Day H, Willis G, Jamal A, and Neff L**. Tobacco Product Use and Cessation Indicators Among Adults - United States, 2018. *MMWR Morb Mortal Wkly Rep* 68: 1013-1019, 2019.

3. **Mayer M, Reyes-Guzman C, Grana R, Choi K, and Freedman ND**. Demographic Characteristics, Cigarette Smoking, and e-Cigarette Use Among US Adults. *JAMA Network Open* 3: e2020694-e2020694, 2020.

4. **Park-Lee E, Ren C, Sawdey MD, Gentzke AS, Cornelius M, Jamal A, and Cullen KA**. Notes from the Field: E-Cigarette Use Among Middle and High School Students - National Youth Tobacco Survey, United States, 2021. *MMWR Morb Mortal Wkly Rep* 70: 1387-1389, 2021.

5. **Gotts JE, Jordt SE, McConnell R, and Tarran R**. What are the respiratory effects of e-cigarettes? *Bmj* 366: l5275, 2019.

6. **Hickman E, and Jaspers I**. Current E-Cigarette Research in the Context of Asthma. *Curr Allergy Asthma Rep* 20: 62, 2020.

7. **Huang J, Duan Z, Kwok J, Binns S, Vera LE, Kim Y, Szczypka G, and Emery SL**. Vaping versus JUULing: how the extraordinary growth and marketing of JUUL transformed the US retail e-cigarette market. *Tob Control* 28: 146-151, 2019.

8. **Goniewicz ML, Boykan R, Messina CR, Eliscu A, and Tolentino J**. High exposure to nicotine among adolescents who use Juul and other vape pod systems ('pods'). *Tobacco Control* 28: 676, 2019.

9. FDA finalizes enforcement policy on unauthorized flavored cartridge-based e-cigarettes that appeal to children, including fruit and mint U.S. Food and Drug Administration. https://www.fda.gov/news-events/press-announcements/fda-finalizes-enforcement-policy-unauthorized-flavored-cartridge-based-e-cigarettes-appeal-children.

10. **Hahn SM**. National Survey Shows Encouraging Decline in Overall Youth E-Cigarette Use, Concerning Uptick in Use of Disposable Products US Food and Drug Administration. https://www.fda.gov/news-events/press-announcements/national-survey-shows-encouraging-decline-overall-youth-e-cigarette-use-concerning-uptick-use.

11. **Omaiye EE, McWhirter KJ, Luo W, Pankow JF, and Talbot P**. High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations. *Chemical research in toxicology* 32: 1058-1069, 2019.

12. **Talih S, Salman R, El-Hage R, Karam E, Karaoghlanian N, El-Hellani A, Saliba N, and Shihadeh A**. Characteristics and toxicant emissions of JUUL electronic cigarettes. *Tob Control* 2019.

13. **Reilly SM, Bitzer ZT, Goel R, Trushin N, and Richie JP**. Free Radical, Carbonyl, and Nicotine Levels Produced by Juul Electronic Cigarettes. *Nicotine Tob Res* 21: 1274-1278, 2019.

14. **El-Hellani A, Salman R, El-Hage R, Talih S, Malek N, Baalbaki R, Karaoghlanian N, Nakkash R, Shihadeh A, and Saliba NA**. Nicotine and Carbonyl Emissions From Popular Electronic Cigarette Products: Correlation to Liquid Composition and Design Characteristics. *Nicotine Tob Res* 20: 215-223, 2018.

15. **Harvanko AM, Havel CM, Jacob P, and Benowitz NL**. Characterization of Nicotine Salts in 23 Electronic Cigarette Refill Liquids. *Nicotine Tob Res* 2019.

16. **Duell AK, Pankow JF, and Peyton DH**. Free-Base Nicotine Determination in Electronic Cigarette Liquids by (1)H NMR Spectroscopy. *Chemical research in toxicology* 31: 431-434, 2018.

17. **Leventhal AM, Madden DR, Peraza N, Schiff SJ, Lebovitz L, Whitted L, Barrington-Trimis J, Mason TB, Anderson MK, and Tackett AP**. Effect of Exposure to e-Cigarettes With Salt vs Free-Base Nicotine on the Appeal and Sensory Experience of Vaping: A Randomized Clinical Trial. *JAMA Network Open* 4: e2032757-e2032757, 2021.

18. **Reidel B, Radicioni G, Clapp PW, Ford AA, Abdelwahab S, Rebuli ME, Haridass P, Alexis NE, Jaspers I, and Kesimer M**. E-Cigarette Use Causes a Unique Innate Immune Response in the Lung, Involving Increased Neutrophilic Activation and Altered Mucin Secretion. *Am J Respir Crit Care Med* 197: 492-501, 2018.

19. **Ghosh A, Coakley RD, Ghio AJ, Muhlebach MS, Esther CR, Jr., Alexis NE, and Tarran R**. Chronic E-Cigarette Use Increases Neutrophil Elastase and Matrix Metalloprotease Levels in the Lung. *Am J Respir Crit Care Med* 2019.

20. FDA Permits Marketing of E-Cigarette Products, Marking First Authorization of Its Kind by the Agency https://www.fda.gov/news-events/press-announcements/fda-permits-marketing-e-cigarette-products-marking-first-authorization-its-kind-agency.

21. **Martin EM, Clapp PW, Rebuli ME, Pawlak EA, Glista-Baker E, Benowitz NL, Fry RC, and Jaspers I**. E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 311: L135-144, 2016.

22. **Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, and Alexis NE**. Macrophages from The Upper and Lower Human Respiratory Tract Are Metabolically Distinct. *Am J Physiol Lung Cell Mol Physiol* 2018.

23. **Alexis NE**. Biomarker sampling of the airways in asthma. *Curr Opin Pulm Med* 20: 46-52, 2014.

24. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R Foundation for Statistical Computing, Vienna, Austria. . https://www.R-project.org/.

25. Thomas Lumley based on Fortran code by Alan Miller (2020). leaps: Regression Subset Selection. R package version 3.1. https://CRAN.R-project.org/package=leaps.

26. **Heinze G, Wallisch C, and Dunkler D**. Variable selection - A review and recommendations for the practicing statistician. *Biom J* 60: 431-449, 2018.

27. Max Kuhn (2021). caret: Classification and Regression Training. R package version 6.0-90. https://CRAN.R-project.org/package=caret.

28. **Clark J, Avula V, Ring C, Eaves LA, Howard T, Santos HP, Smeester L, Bangma JT, O'Shea TM, Fry RC, and Rager JE**. Comparing the Predictivity of Human Placental Gene, microRNA, and CpG Methylation Signatures in Relation to Perinatal Outcomes. *Toxicological sciences : an official journal of the Society of Toxicology* 183: 269-284, 2021.

29. **Ring C, Sipes NS, Hsieh J-H, Carberry C, Koval LE, Klaren WD, Harris MA, Auerbach SS, and Rager JE**. Predictive modeling of biological responses in the rat liver using in vitro Tox21 bioactivity: Benefits from high-throughput toxicokinetics. *Computational Toxicology* 18: 100166, 2021.

30. **Gentzke AS, Creamer M, Cullen KA, Ambrose BK, Willis G, Jamal A, and King BA**. Vital Signs: Tobacco Product Use Among Middle and High School Students - United States, 2011-2018. *MMWR Morb Mortal Wkly Rep* 68: 157-164, 2019.

31. **Wang TW, Gentzke A, Sharapova S, Cullen KA, Ambrose BK, and Jamal A**. Tobacco Product Use Among Middle and High School Students - United States, 2011-2017. *MMWR Morb Mortal Wkly Rep* 67: 629-633, 2018.

32. **Perikleous EP, Steiropoulos P, Paraskakis E, Constantinidis TC, and Nena E**. E-Cigarette Use Among Adolescents: An Overview of the Literature and Future Perspectives. *Front Public Health* 6: 86, 2018.

33. **Strzelak A, Ratajczak A, Adamiec A, and Feleszko W**. Tobacco Smoke Induces and Alters Immune Responses in the Lung Triggering Inflammation, Allergy, Asthma and Other Lung Diseases: A Mechanistic Review. *Int J Environ Res Public Health* 15: 2018.

34. **van der Vaart H, Postma DS, Timens W, Hylkema MN, Willemse BW, Boezen HM, Vonk JM, de Reus DM, Kauffman HF, and ten Hacken NH**. Acute effects of cigarette smoking on inflammation in healthy intermittent smokers. *Respir Res* 6: 22, 2005.

35. **Stănescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM, and Maestrelli P**. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 51: 267-271, 1996.

36. **Carpagnano GE, Kharitonov SA, Foschino-Barbaro MP, Resta O, Gramiccioni E, and Barnes PJ**. Increased inflammatory markers in the exhaled breath condensate of cigarette smokers. *European Respiratory Journal* 21: 589, 2003.

37. **Attia EF, Jolley SE, Crothers K, Schnapp LM, and Liles WC**. Soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1) Is Elevated in Bronchoalveolar Lavage Fluid of Patients with Acute Respiratory Distress Syndrome. *PLoS One* 11: e0149687, 2016.

38. **Mendez MP, Morris SB, Wilcoxen S, Greeson E, Moore B, and Paine R**. Shedding of soluble ICAM-1 into the alveolar space in murine models of acute lung injury. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 290: L962-L970, 2006.

39. **Crump CE, Arruda E, and Hayden FG**. In vitro Inhibitory Activity of Soluble ICAM-1 for the Numbered Serotypes of Human Rhinovirus. *Antiviral Chemistry and Chemotherapy* 4: 323-327, 1993.

40. **Arruda E, Crump CE, Marlin SD, Merluzzi VJ, and Hayden FG**. In vitro studies of the antirhinovirus activity of soluble intercellular adhesion molecule-1. *Antimicrob Agents Chemother* 36: 1186-1191, 1992.

41. **Whiteman SC, and Spiteri MA**. IFN-gamma regulation of ICAM-1 receptors in bronchial epithelial cells: soluble ICAM–1 release inhibits human rhinovirus infection. *Journal of Inflammation* 5: 8, 2008.

42. **Gould JM, and Weiser JN**. Expression of C-reactive protein in the human respiratory tract. *Infect Immun* 69: 1747-1754, 2001.

43. **Weiser JN, Pan N, McGowan KL, Musher D, Martin A, and Richards J**. Phosphorylcholine on the lipopolysaccharide of Haemophilus influenzae contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J Exp Med* 187: 631-640, 1998.

44. **Ivashkiv LB**. IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nat Rev Immunol* 18: 545-558, 2018.

45. **Zhang J**. Yin and yang interplay of IFN-gamma in inflammation and autoimmune disease. *J Clin Invest* 117: 871-873, 2007.

46. **Mitchell C, Provost K, Niu N, Homer R, and Cohn L**. IFN-γ acts on the airway epithelium to inhibit local and systemic pathology in allergic airway disease. *J Immunol* 187: 3815-3820, 2011.

47. **Gschwandtner M, Derler R, and Midwood KS**. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front Immunol* 10: 2019.

48. **Laucho-Contreras ME, Polverino F, Gupta K, Taylor KL, Kelly E, Pinto-Plata V, Divo M, Ashfaq N, Petersen H, Stripp B, Pilon AL, Tesfaigzi Y, Celli BR, and Owen CA**. Protective role for club cell secretory protein-16 (CC16) in the development of COPD. *Eur Respir J* 45: 1544-1556, 2015.

49. **Cui WY, and Li MD**. Nicotinic modulation of innate immune pathways via α7 nicotinic acetylcholine receptor. *J Neuroimmune Pharmacol* 5: 479-488, 2010.

50. **Sopori M**. Effects of cigarette smoke on the immune system. *Nat Rev Immunol* 2: 372-377, 2002.

51. **Westling E, Rusby JC, Crowley R, and Light JM**. Electronic Cigarette Use by Youth: Prevalence, Correlates, and Use Trajectories From Middle to High School. *J Adolesc Health* 60: 660-666, 2017.

52. **Wills TA, Leventhal AM, Sargent JD, and Pagano I**. Concurrent Use of E-cigarettes, Combustible Cigarettes, and Marijuana. *Pediatrics* 148: 2021.

53. **Boykan R, Messina CR, Chateau G, Eliscu A, Tolentino J, and Goniewicz ML**. Self-Reported Use of Tobacco, E-cigarettes, and Marijuana Versus Urinary Biomarkers. *Pediatrics* 143: 2019.

54. **Trivers KF, Phillips E, Gentzke AS, Tynan MA, and Neff LJ**. Prevalence of Cannabis Use in Electronic Cigarettes Among US Youth. *JAMA Pediatr* 172: 1097-1099, 2018.

55. **Turcotte C, Blanchet MR, Laviolette M, and Flamand N**. Impact of Cannabis, Cannabinoids, and Endocannabinoids in the Lungs. *Front Pharmacol* 7: 317, 2016.

CHAPTER 5: EXPANDED FUNCTIONAL CHARACTERIZATION OF M0, M1, AND M2 HUMAN MONOCYTE-DERIVED MACROPHAGES SUPPORTS THEIR UTILITY AS A MODEL FOR RESPIRATORY MACROPHAGES

Introduction

Macrophages play vital roles in maintaining immune homeostasis in the large and distal airways. Key macrophage functions include phagocytosis of microbial pathogens, release of cell signaling molecules, and participation in tissue remodeling (1-3). It is well-established that macrophages exhibit functional and cell surface phenotype plasticity that can be altered depending on their location within the respiratory tract and in response to stimuli present in their microenvironment (3-5). As a result, it can be challenging to accurately recapitulate *in vitro* the macrophage characteristics observed *in vivo* including the distinct macrophage subpopulations found on the surfaces of the central airways and in the distal alveolar region.

Many factors limit the routine experimental use of airway macrophages collected from human volunteers. Whether it is by induced sputum for central airways cells, or from bronchoalveolar lavage (BAL) for distal airway cells, both sampling techniques require specific expertise, are time consuming, involve a degree of subject risk, and, for BAL in particular, is costly (6-8). Accessing primary human airway macrophages for research purposes has become even more challenging, and at many institutions, impossible, during the COVID-19 pandemic due to the risk of aerosol transmission during sample collection (9-11). Therefore, *in vitro* models are necessary for macrophage experimentation and have been used extensively to model *in vivo* macrophage function and phenotype. Due to recent studies that have characterized the bioenergetic profile of sputum and BAL macrophages (12), more is known about the phenotype of these cells *in vivo*. Consequently, it is crucial to determine if these routinely used *in vitro* models of macrophages accurately reflect *in vivo* macrophage phenotypes, particularly from a bioenergetic cell metabolism perspective.

One such model uses human monocyte-derived macrophages (hMDMs), which are isolated from the peripheral blood of human subjects and then differentiated into various macrophage subsets. This model is accessible, biologically relevant, and relatively easy to use. Across studies, there is considerable variability in the way that hMDMs are differentiated and polarized into subsets, including length of differentiation, differentiation stimulus, and polarization stimuli. Most commonly, hMDMs are differentiated over about one week with M-CSF or GM-CSF (M0) and then polarized into M1 and/or M2 macrophages using stimuli such as IFN-γ and lipopolysaccharide (LPS) (M1) or IL-4 and IL-13 (M2), and others, depending on the desired phenotype (4, 13-15).

Most studies utilizing hMDMs as models rely on a narrow set of parameters to confirm successful differentiation and polarization. Previous studies have characterized the phenotypes of hMDMs differentiated into M1 and M2 macrophages, with a significant focus on transcriptional changes and to a lesser extent, on cell surface receptor expression and cell functions such as phagocytosis. (4, 14, 15). There is also emerging appreciation for the important role that cellular metabolism (bioenergetics) plays in mediating macrophage activation and polarization that drive subsequent innate immune responses (16, 17). For example, when macrophages encounter pro-inflammatory stimuli, signal transduction cascades are initiated that shift cellular metabolism from mitochondrial-based oxidative phosphorylation pathways to glycolysis, thereby allowing the cell to more rapidly respond to increased energy demands during active infection or inflammation (18-20). It has also been recently reported that the baseline bioenergetic profiles of airway macrophages recovered from healthy volunteers differs depending on their location within the respiratory tract (12). Our group observed that sputum macrophages from the surfaces of the central airways reflect an M1 glycolysis-dependent phenotype, whereas BAL macrophages recovered from the distal airways reflect an M2 oxidative phosphorylation-dependent phenotype. Animal studies using MDMs have also demonstrated bioenergetic differences between subsets of polarized macrophages (21-23), but no studies to date have established complete bioenergetic profiles of polarized hMDMs to evaluate their congruence with human macrophages in vivo. Thus, further understanding of polarized hMDM phenotype and function is critically needed. In this study, our objective was to generate human

monocyte-derived macrophages polarized into M1- and M2-like states and to thoroughly integrate their phenotype profiles by measuring cell surface marker and gene expression, cytokine secretion, phagocytic capacity, and cellular bioenergetics.

Methods

Subjects

Healthy non-smoking adult human subjects participated in a venous blood draw. Study exclusion criteria included current nicotine use, acute illness, allergy symptoms, asthma, and/or pregnant and nursing women. The sex of subjects in each experiment in reported in the figure legend. Informed consent was obtained from all subjects, and all studies were approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board (IRB #11-1363).

Monocyte isolation

Venous blood was collected in BD Vacutainer tubes with EDTA. Peripheral blood mononuclear cells were isolated using Ficoll-Paque Plus (Cytivia) density centrifugation and washed 3 times with DPBS. CD14+ monocytes were isolated using magnetic bead negative selection per the manufacturer's protocol (EasySep Human Monocyte Isolation Kit, Stemcell Technologies). After negative selection, the purity of the resulting cell population was verified using flow cytometry, with an average of ~90% CD14+ monocytes (data not shown).

Monocyte differentiation

Immediately following isolation, CD14+ monocytes were seeded at a density of 187,500 cells/cm^2 in various sizes of tissue-culture treated multi-well plates or Transwell inserts appropriate for assays performed. Monocytes base media was RPMI-1640 media (Gibco) with 10% FBS (Millipore Sigma) and 1% penicillin/streptomycin (100 U/mL, Gibco). L-glutamine (Gibco) was added to the base media immediately before cell seeding and/or feeding (2 mM final concentration). Monocytes were

differentiated into naïve (M0) macrophages with base media + 40 ng/mL M-CSF. Four days after the isolation, the media was replaced. Six days after the isolation, differentiation media was removed, and cells were polarized into M1 hMDMs with 20 ng/mL IFN-γ + 20 ng/mL LPS, M2 hMDMs with 20 ng/mL IL-4, or M0 hMDMs with no stimulants added to the base media. Samples were collected and phenotype assays were performed approximately 24 hours after polarization.

Gene expression

24 hours after polarization, hMDMs were washed with DBPS and lysed in Ambion lysis buffer with 1% β-mercaptoethanol. Lysate was stored at -80°C until samples were collected from all subjects. Total RNA was isolated using the Ambion Pure Link RNA Mini Kit (Life Technologies). RNA was reverse transcribed into cDNA as described previously (24). Real-time quantitative PCR was performed with cDNA using Applied Biosystems TaqMan Universal Master Mix II with UNG (Thermo Fisher Scientific), TaqMan assays, and the QuantStudio3 Real-Time PCR System (Thermo Fisher Scientific). TaqMan assays were as follows: Hs00968979_m1 (*ARG1*), Hs00267207_m1 (*MRC1*), Hs01075529_m1 (*NOS2*), Hs00153133_m1 (*PTGS2*). Gene expression differences were calculated using the $2^{-\Delta\Delta Ct}$ method with *ACTB* as the endogenous control and M0-like hMDMs as the reference phenotype.

Cytokine secretion

24 hours after polarization, media was collected and centrifuged at 1000 x g for 10 minutes to remove any cellular debris. Supernatant was transferred to a new tube and stored at -80°C until all samples were collected. Protein concentrations were measured using commercially available single- and multi-plex ELISAs (IL-6 and IL-8: BD Bioscience; TNF-α, CCL17, CCL18, MMP-2, MMP-9: R&D Systems; V-PLEX Human Cytokine 30-plex: Mesoscale Discovery). For single-plex ELISAs, absorbance was quantified using a CLARIOstar plate reader (BMG Labtech) per assay instructions. The V-PLEX Human Cytokine 30-plex was read on the MESO QuickPlex SQ 120 (Mesoscale Discovery).

Phagocytosis

24 hours after polarization, hMDM phagocytosis of *S. aureus* and Zymosan A pHrodo Red Bioparticles (Thermo Fisher Scientific) was measured as described previously (25), with a Bioparticle incubation time of 2 hours. Fluorescence in each well was quantified using a CLARIOstar plate reader (BMG Labtech).

Cellular bioenergetics

24 hours following polarization, hMDMs were assayed for bioenergetic parameters using the Seahorse Extracellular Flux Modified Cell Mito Stress Test (Agilent) as described previously (12). Briefly, polarization media was replaced with Seahorse XF RPMI, pH 7.4, supplemented with 2 mM L-glutamine. Then, hMDMs were incubated in a non-CO_2 incubator for 30-40 minutes before the start of the assay. Injection order and final concentrations of treatments were as follows: Port A – 10 mM glucose; Port B – 1 μM oligomycin; Port C – 1.25 μM FCCP; Port D – 0.5 μM rotenone and 0.5 μM antimycin A. Mix-wait-measure times were 3 min – 2 min – 3 min, per manufacturer's instructions. Mitochondrial and glycolytic parameters were calculated as described previously and as recommended by the manufacturer (12). Immediately following the assay, nuclei were stained using Hoechst 33342 (Thermo Fisher Scientific), and fluorescence in each well was quantified using a CLARIOstar plate reader (BMG Labtech). Data were normalized by dividing bioenergetic parameters by mean Hoechst 33342 fluorescence intensity in each well.

Flow cytometry

24 hours after polarization, hMDMs were washed three times with DPBS and dissociated via incubation (37°C, 5% CO_2) with Cellstripper (Corning) for 30 minutes, followed by thorough washing over the well with a micropipette to aid in cell detachment. Cells were pelleted (400 x g for 5 minutes) and counted with a hemocytometer. Prior to staining, 2-5x10^5 hMDMs were incubated with

human TruStain FcX (BioLegend) for 5 min to block Fc receptors. Live/dead cell discrimination was achieved using Zombie Aqua (BioLegend). Direct fluorochrome-conjugated antibodies against CD64 (clone 10.1; PerCP-Cy5.5), CD206 (15-2; FITC), HLA-DR (L243; PE-Cy7), CD86 (IT2.2; PE), CD14 (HCD14; APC-Cy7) and CD163 (GHI/61; AF-647) were used for detection of hMDM surface markers. Following staining cells were washed and then fixed with 4% PFA in PBS. Flow cytometry data were acquired with a four-laser LSRII (BD Biosciences) and analyzed using FlowJo software. Only single cells were analyzed. All antibodies were purchased from BioLegend.

Statistical analyses

All statistical analyses were performed in GraphPad Prism 9. For each set of data, normality was assessed using the D'Agostino & Pearson test. Normally distributed data were analyzed using matched one-way ANOVA with Tukey's multiple comparisons test. Non-normally distributed data were analyzed using the Friedman test with Dunn's multiple comparisons test (if no values were missing) or the Friedman test with Holm-Sidak multiple comparisons test (which allows for missing values). When possible, we investigated sex differences in hMDM function. In the cohort of samples used for single-plex ELISAs, we tested for sex differences in the expression of proteins in each treatment group using a two-way ANOVA with sex and polarization as factors. Except for MMP-9 ($p = 0.0136$ overall; $p = 0.0639$ between males and females in the M1 group, with cells from females having higher expression than cells from males), sex was not a significant source of variation in our data. We also tested for but did not detect sex differences in phagocytosis or cellular bioenergetics. Multi-plex ELISA data are displayed using a row scaled heatmap, which was generated in R version 4.1.1 (26) using the *pheatmap* (27) and *viridis* (28) packages. Raw concentrations and additional statistical comparisons for multi-plex ELISA data are available in Table S5-1. To reduce dimensionality in our data and further explore differences in soluble mediator expression between subsets of polarized hMDMs, we performed Principal Component Analysis (PCA) using the R packages *ggfortify* (29, 30) and *factoextra* (31). Input data and R code used for these analyses are publicly available at https://github.com/ehickman0817/hMDM-phenotypes.

Results

M0-, M1-, and M2-like hMDMs display significantly different cell surface markers.

We measured the expression of cell surface markers that have been assessed previously in the context of macrophage phenotyping. Using flow cytometry, we found that M1 hMDMs had significantly higher expression of CD64 than M0 and M2 hMDMs and CD86 than M0 hMDMs (Figures 5-1A and 5-1B). There was no significant difference in HLA-DR expression between the polarization states (Figure 5-1C). As expected, M2 hMDMs expressed significantly more CD206 than M0 hMDMs (Figure 5-1D), and M1 hMDMs expressed less CD163 than M0 and M2 hMDMs, though this difference did not reach statistical significance (Figure 5-1E). Overall, our results are similar to previously published studies assessing cell surface marker expression following M1 and M2 hMDM polarization (13, 21, 32).

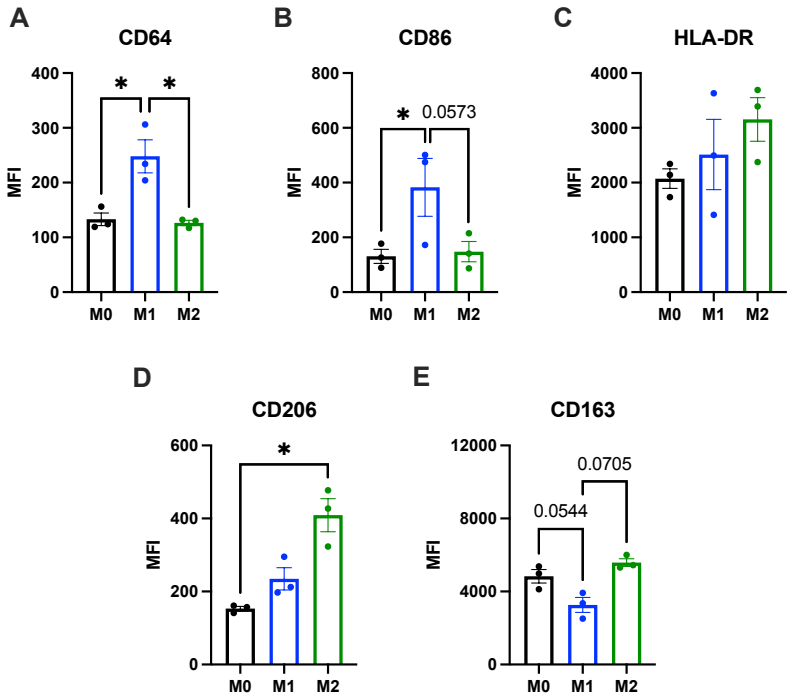


Figure 5-1. Polarization of hMDMs induces changes in cell surface markers. M1 hMDMs express higher levels of CD64 (A) and CD86 (B) than M0 and M2 hMDMs. There were no significant differences in HLA-DR (C) across polarization states. CD206 expression (D) is significantly increased in M2 hMDMs, and CD163 expression (E) is decreased in M1 hMDMs in comparison to M0- and M2 hMDMs. n = 3 (all males). Data are presented as mean ± SEM. * $p < 0.05$ by repeated measures one-way ANOVA with Tukey's test for multiple comparisons.

Polarization of macrophages to M0-, M1-, and M2-like phenotypes significantly changes gene expression.

We measured the expression of four genes that have previously been shown to be modulated in response to M1 and M2 polarization (Figure 5-2) and found that M1 hMDMs had significantly higher expression of *NOS2* in comparison with M0 and M2 hMDMs and significantly higher *PTGS2* expression in comparison with M2 hMDMs (Figures 5-2A and 5-2B). We did not detect any significant differences in *ARG1* between polarization conditions. Expression of *MRC1*, the gene that encodes CD206, was significantly increased in M2 hMDMs and significantly decreased in M1 hMDMs (Figure 5-2D). This increase in *MRC1* gene expression in M2 hMDMs mirrors the increase in CD206 expression measured with flow cytometry. However, *MRC1* expression in M0 and M1 like hMDMs did not follow the same pattern observed in CD206 surface expression. Overall, these findings agree with previous studies that evaluated gene expression changes in M1 and M2 polarized macrophages *in vitro* (13, 21).

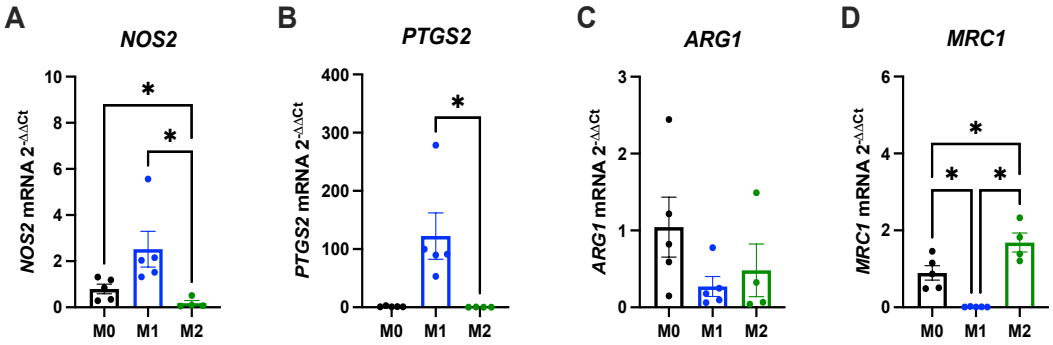


Figure 5-2. hMDM polarization-induced changes in gene expression.(A) *NOS2*, (B) *PTGS2*, (C) *ARG1*, and (D) *MRC1*. N = 5 (3 males, 2 females for M0 and M1; RNA extraction failed on one M2 sample, resulting in 3 males and 1 female for the M2 group). Data are presented as mean ± SEM. * $p < 0.05$ by Friedman test with Holm-Sidak multiple comparisons test (which allows for missing values).

Polarization of macrophages to M0-, M1-, and M2-like phenotypes significantly changes cytokine secretion.

We measured the concentrations of secreted cytokines and immune mediators in our different macrophage subsets that have previously been shown to differentiate macrophage subtypes (13) (Figure 5-3). We found that M1 hMDMs secreted significantly more IL-6, IL-8, and TNF-α than M0 and M2 hMDMs (Figures 5-3A, 5-3B, and 5-3C) and that M2 hMDMs secreted significantly more CCL17 than M0 and M1 hMDMs and more CCL18 than M0 hMDMs (Figures 5-3D and 5-3-E), as expected. We were

also interested in whether these cells secreted matrix metalloproteinases, such as MMP2 and MMP9, due to their important roles in tissue remodeling in the lungs. All hMDMs secreted MMP-9, with M1 hMDMs secreting significantly less than M0 hMDMs (Figure 5-3F). MMP-2 was not secreted by the hMDMs. We also found that secretion patterns for these mediators across polarization states were similar when hMDMs were grown on Transwell inserts (Figure 5-4), indicating that these cells maintain their phenotype and can be used for brief exposures at air-liquid interface.

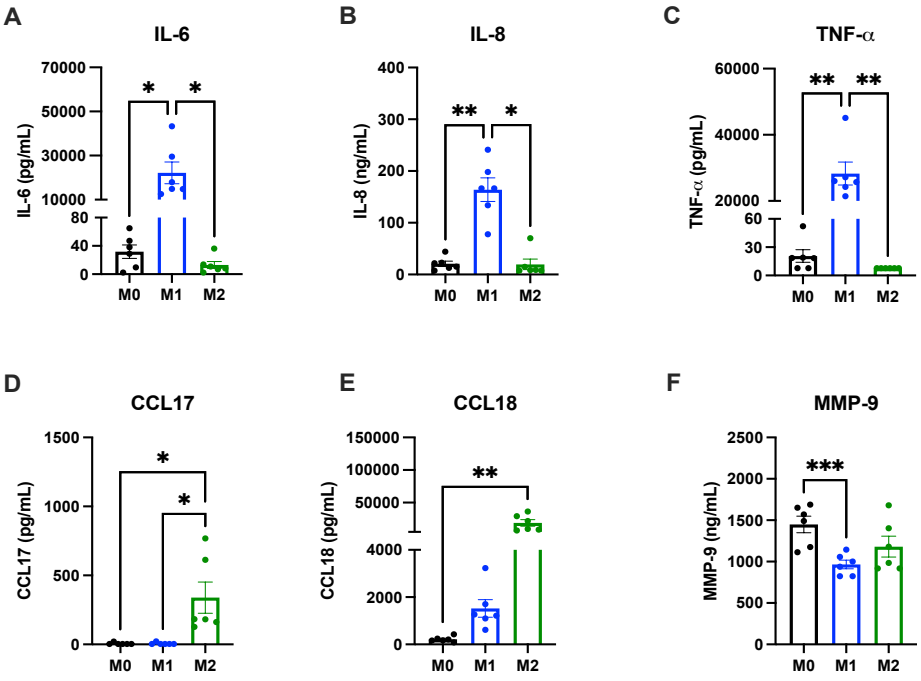


Figure 5-3. hMDM polarization-induced changes in cytokine secretion. M1 hMDMs secrete significantly higher levels of (A) IL-6, (B) IL-8, and (C) TNF-α than M0 or M2 hMDMs, while M2 hMDMs secrete significantly higher levels of (D) CCL17 than M0 or M1 hMDMs and (E) CCL18 than M0 hMDMs. All hMDMs secreted (F) MMP-9, with M1 hMDMs secreting significantly less than M0 hMDMs. n =6 (3 males, 3 females). Data are presented as mean ± SEM. Normally distributed data were analyzed using matched one-way ANOVA with Tukey's multiple comparisons test (B, C, F). Non-normally distributed data were analyzed using the Friedman test with Dunn's multiple comparisons test (A, D, E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

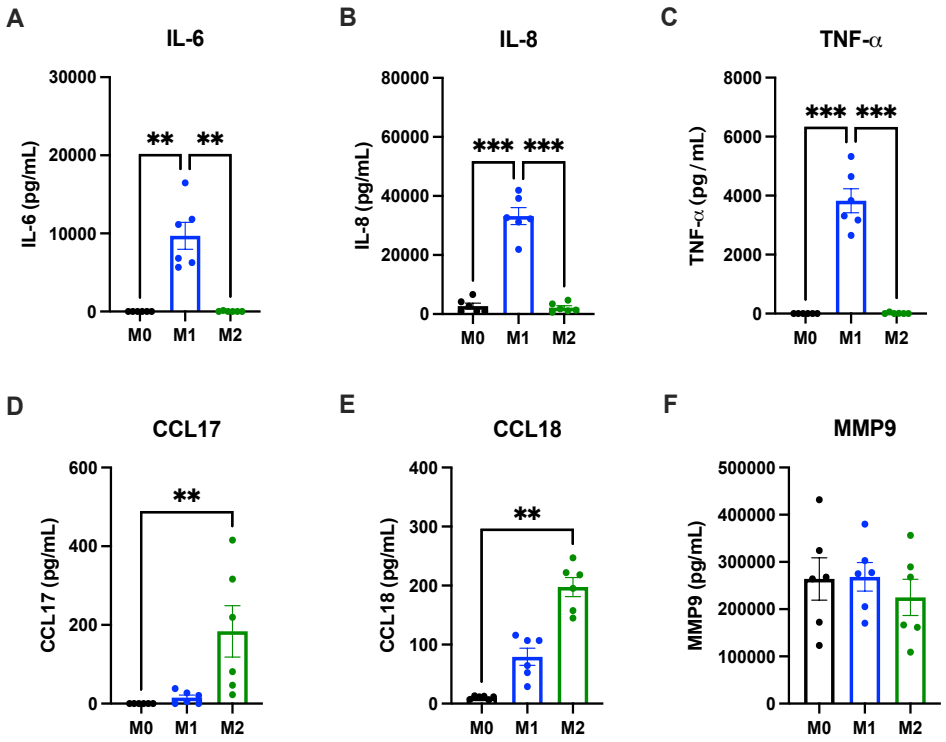


Figure 5-4. hMDM polarization-induced changes in cytokine secretion for hMDMs grown on Transwell supports. M1 hMDMs secrete significantly higher levels of (A) IL-6, (B) IL-8, and (C) TNF-α than M0 or M2 hMDMs, while M2 hMDMs secrete significantly higher levels of (D) CCL17 and (E) CCL18 than M0 hMDMs. All hMDMs secreted (F) MMP-9, with no significant differences between groups. n = 6 (4 males, 2 females). Data are presented as mean ± SEM. Normally distributed data were analyzed using matched one-way ANOVA with Tukey's multiple comparisons test (A, B, C, F). Non-normally distributed data were analyzed using the Friedman test with Dunn's multiple comparisons test (D, E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

We expanded our cytokine analysis to better understand the secretome of polarized hMDMs. To that end, we measured a panel of 27 cytokines, chemokines, and other secreted mediators using multi-plex ELISA on a separate set of samples (Figure 5-5, Table S5-1). We found that there were secreted mediators unique to each polarization state; for example, M2 hMDMs secreted high levels of MCP4, MDC, and TARC, while M1 hMDMs secreted high levels of many pro-inflammatory cytokines and chemokines (Figure 5-5A). In comparison with M0 hMDMs, M2 hMDMs secreted significantly more MCP4 and TARC, and M1 hMDMs secreted significantly more MCP4, eotaxin, eotaxin-3, IL12p70, IL-1α, IL15, TNF-β, IL-6, TNF-α, IL12p40, IL-13, and IL-2. Principal component analysis demonstrated clear separation between polarized hMDM subsets (Figure 5-5B) that are driven by differences in soluble

mediators similar to those that were significantly different between groups using variable-by-variable analysis (Figure 5-5C). These data support the previously published paradigm that M1 hMDMs are more pro-secretory and provide additional data to establish baseline secretory states of polarized hMDMs (13, 32).

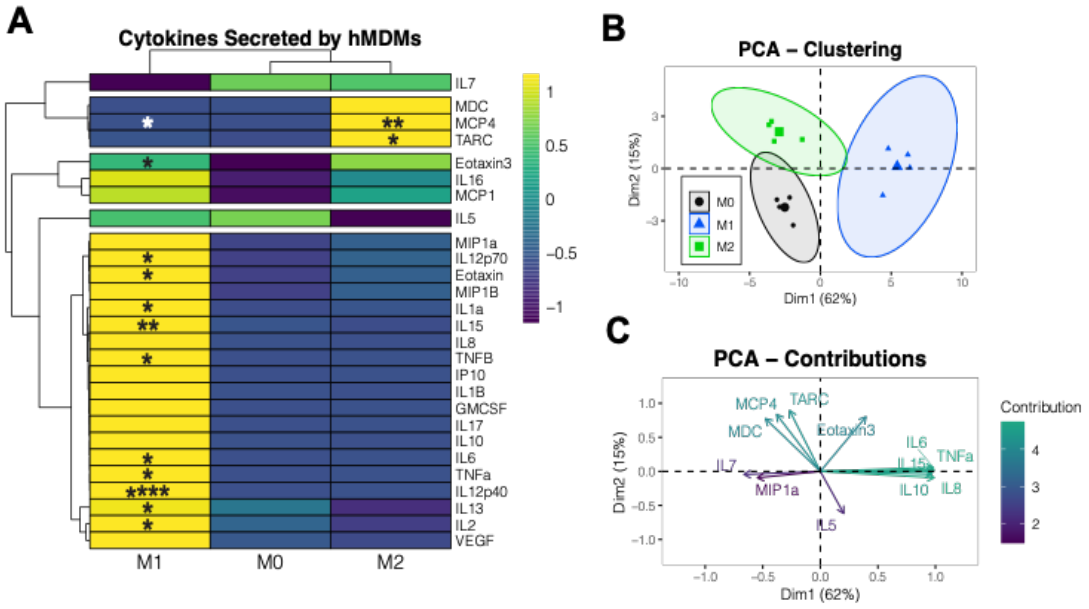


Figure 5-5. Expanded characterization of cytokines, chemokines, and soluble mediators expressed by hMDMs.(A) Row-scaled heatmap showing protein expression. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ in comparison with M0 hMDMs by either matched one-way ANOVA with Tukey's multiple comparisons test or Friedman test with Dunn's multiple comparisons test. Raw concentrations and significant comparisons between M1 and M2 hMDMs are reported in Table S1. (B) PCA plot showing clustering of cytokine secretion by polarized hMDMs. (C) Percentage contributions of each variable to the variation observed in the first two PCA dimensions. For all plots, n = 4 (1 male, 3 females).

All hMDM polarization subsets are phagocytic.

We wanted to confirm that the polarized hMDMs we generated were phagocytic and determine if there were baseline differences in phagocytosis between polarization states. To determine phagocytic capacity of the polarized hMDMs we used pHrodo Red *S. aureus* and zymosan A BioParticles to test bacterial and fungal phagocytosis, respectively. We found that all polarization states had a similar phagocytic capacity for *S. aureus* (bacterial) BioParticles (Figure 5-6A). M1 hMDMs had significantly lower phagocytic capacity for zymosan A (fungal) BioParticles than M0- and M2 hMDMs (Figure 5-6B).

These data agree with previous studies demonstrating increased phagocytosis of zymosan and acidification of the phagolysosome in M2 macrophages (33, 34).

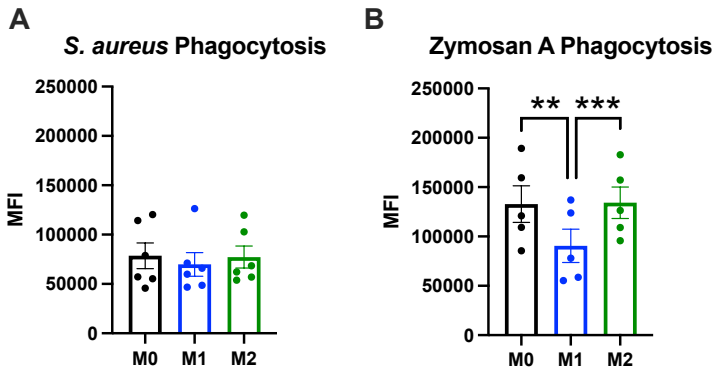


Figure 5-6. Polarized hMDMs are phagocytic, and M1 hMDMs are significantly less phagocytic of Zymosan A pHrodo Red BioParticles than M0 or M2 hMDMs.hMDMs were assayed for phagocytosis of (A) *S. aureus* and (B) Zymosan A pHrodo Red BioParticles over two hours. n = 6 (3 males, 3 females) for Figure 5A and n = 5 (2 males, 3 females) for Figure 5B. Data are presented as mean ± SEM.

M0, M1, and M2 hMDMs have unique bioenergetic profiles that are similar to airway macrophages.

To determine bioenergetic differences between our polarized macrophages subsets, we performed Seahorse Extracellular Flux assays, which simultaneously measure the oxygen consumption rate (OCAR) and extracellular acidification rate (ECAR) in cell media following exposure of cells to mitochondrial and glycolytic inhibitors (Figures 5-7A and 5-7B). We found that M1 hMDMs had significantly higher proton leak and lower mitochondrial respiration, spare respiratory capacity, and coupling efficiency than M0 and M2 hMDMs (Figures 5-7C-F), indicating less efficient generation of energy/ATP via oxidative phosphorylation (OXPHOS) in the mitochondria. We also found that M1 hMDMs were significantly more glycolytic than M0 and M2 hMDMs (Figure 5-7G). Interestingly, both M1 and M2 hMDMs had significantly higher glycolytic capacity than M0 hMDMs (Figure 5-7H), and M2 hMDMs had significantly higher glycolytic reserve than M0 and M1 hMDMs. Overall, these results support the notion that M1 hMDMs rely more on glycolysis and exist in a more high-energy, activated state at baseline, while M2 hMDMs rely more on oxidative phosphorylation for ATP generation and are better able to respond to increased demand for energy via OXPHOS and glycolysis.

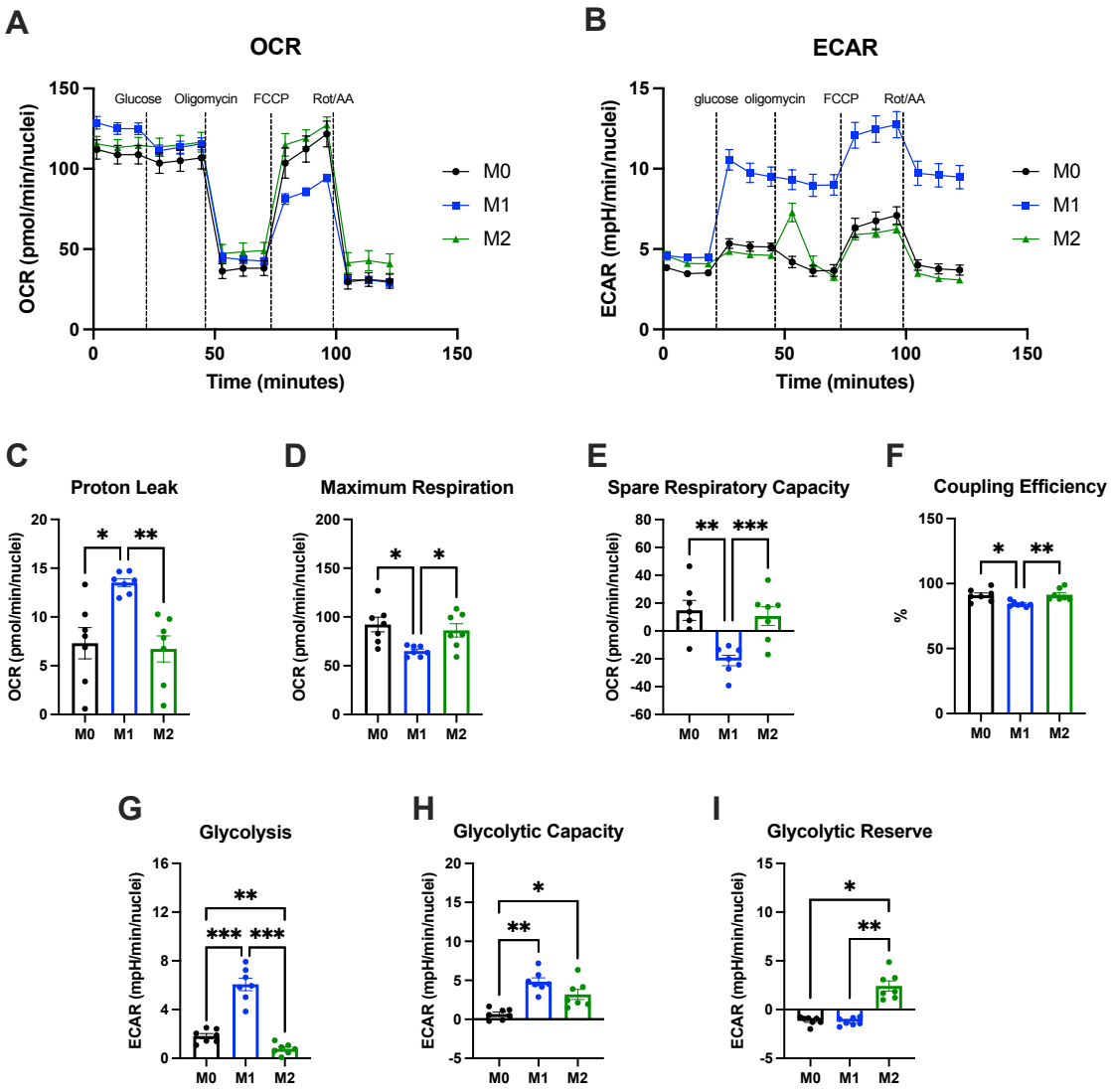


Figure 5-7. hMDM polarization induces bioenergetic changes.hMDM OCR (A) and ECAR (B) were measured as proxies for mitochondrial and glycolytic function using Seahorse Extracellular Flux. M1 MDMs had significantly higher proton leak (C) and significantly lower maximum respiration (D), spare respiratory capacity (E), and coupling efficiency (F) than M0 and M2 hMDMs. M1 MDMs were significantly more glycolytic (G) than other polarization states, and both M1 and M2 hMDMs had significantly higher glycolytic capacity (H) than M0 like hMDMs. M2 hMDMs had significantly more glycolytic reserve than M0 or M1 hMDMs (I). n = 7 (3 males, 4 females). Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by matched one-way ANOVA with Tukey's multiple comparisons test (C-H) or Friedman test with Dunn's multiple comparisons test (I).

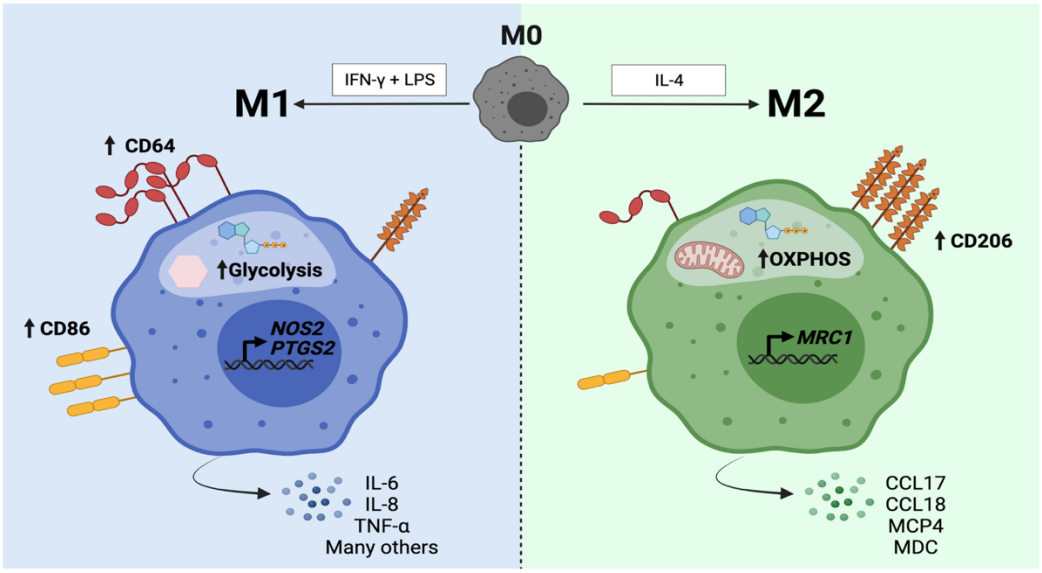


Figure 5-8. Summary of M1 and M2 hMDM phenotypes. Created with biorender.com.

Discussion

In this study, we thoroughly characterized cellular phenotypes and functions of human monocyte-derived macrophages polarized into M1- and M2-like states (Figure 5-8). We observed significant changes in cell surface marker expression, gene expression, and cytokine secretion that were consistent with previous studies of hMDMs (13, 21, 32). To evaluate the potential for these cells to retain their phenotype when grown on Transwell inserts, allowing for short exposures at air-liquid interface, we also evaluated cytokine secretion of hMDMs differentiated and polarized on Transwells and found that cytokine secretion profiles could be recapitulated in the cells grown on Transwells. We further expanded characterization of polarized hMDMs from previous studies by demonstrating additional significant differences in soluble mediator expression and in cellular bioenergetics between the different polarization states. Importantly, the bioenergetic profiles we observed *in vitro* following polarization were similar to bioenergetic profiles previously observed *in vivo* from BAL-derived alveolar and sputum-derived central airway macrophages (12).

Because cellular bioenergetics is thought to be a central regulator of macrophage function and downstream engagement of innate and acquired immune cells, and because most previous bioenergetic work has been performed using mouse bone-marrow-derived macrophages (mBMDMs) (21-23, 32), we

investigated the important question of how polarization shifts hMDM bioenergetic profiles using Seahorse Extracellular Flux assays. We found that our polarization protocol significantly shifted cellular bioenergetics of hMDMs, with M1-like hMDMs significantly more glycolytic than M0- and M2-like hMDMs. We also found that M1-like hMDMs were less able to respond to increased energy demand via mitochondrial respiration or glycolysis. These findings agree in part with data presented in studies using mBMDMs in that the M1-like hMDMs were more glycolytic than M0 or M2 (21, 23). However, unlike mBMDMs, our M1-like hMDMs were much more responsive to mitochondrial inhibitors (21-23, 35), and our M0- and M2-like hMDMs exhibited more significantly reduced glycolytic parameters than previously reported in mBMDMs (21). These lines of evidence support the previously proposed concept that mechanisms regulating macrophage polarization and bioenergetics are divergent between species and that, while of immense utility, polarized hMDMs display unique features not observed in mBMDMs (32). Importantly, the bioenergetic profiles we observed in our polarized hMDMs were similar to the bioenergetic profiles of sputum and BAL-derived macrophages (12). M1-like hMDMs shared a similar bioenergetic profile to induced sputum macrophages, with significantly lower maximal respiration and higher glycolytic capacity than M2-like hMDMs, which had a bioenergetic profile more similar to BAL macrophages (12). These divergent bioenergetic profiles *in vivo* suggest distinct functions of macrophage subsets in different anatomic locations in the respiratory tract, with M1-like, highly glycolytic macrophages on the surfaces of the large airways ready to quickly respond to the constant presence of inhaled pathogens, while M2-like macrophages remain more bioenergetically quiescent in the pathogen-protected distal regions of the airways mediating homeostasis.

In addition to expanding the phenotype characterization of hMDMs with bioenergetic analysis, we included measurement of soluble mediators to a larger panel than is typically used to assess macrophage differentiation and polarization. We found clear differences in mediator secretion in comparison with M0 hMDMs and revealed specific clustering of hMDM phenotypes via principal component analysis performed on mediator data (Figure 5-5). Our principal component analysis showed several novel findings that included expression levels of MDC, MCP4, and TARC/CCL17 contributed to

distinguishing the M2 phenotype, expression levels of IL-7, MIP-1α, and IL-5 together contributed to distinguishing the M0 phenotype, and expression levels of many pro-inflammatory mediators contributed to distinguishing the M1 phenotype. Understanding clustering of these mediators is a useful tool that could be applied to understanding shifts in macrophage populations associated with disease states or toxicant exposure.

For other endpoints, comparisons between our hMDMs raised in culture and lung macrophages recovered from human volunteers are difficult given that there are few studies that have directly compared these specific endpoints in different subpopulations of human respiratory macrophages. Studies evaluating surface marker expression and gene expression of different human lung macrophage populations have revealed a high degree of complexity in expression patterns and have demonstrated that even within regions of the airways, distinct subpopulations exist (5, 36, 37). For example, although the mannose receptor CD206, which mediates endocytosis and phagocytosis of mannoglycoprotein-expressing microorganisms and debris, has generally been considered a marker of M2 macrophages, CD206 is expressed on the surface of both alveolar and tissue resident macrophages and is co-expressed with markers associated with M1 polarization, such as HLA-DR (5, 38, 39).

Overall, our findings represent a thorough characterization of differentiated and polarized hMDMs and suggest that polarized hMDMs may provide an acceptable *in vitro* model to study and extrapolate findings to human respiratory macrophages. Importantly, we confirmed cytokine secretion profiles in M0, M1, and M2 hMDMs differentiated on Transwell membranes to support the use of this macrophage model for inhalational exposure studies. However, it is critical to acknowledge that these *in vitro* models do not fully recapitulate the phenotypic diversity and plasticity of macrophages *in vivo.* Recent studies have demonstrated the generation of alveolar-macrophage-like cells in mice using novel differentiation techniques (40, 41), and there is a pressing need to build on the observations presented here and to develop more robust models of human lung macrophages to investigate how disease states and inhalational perturbations (e.g. microbes, toxicant exposures) alter human macrophage function.

REFERENCES

1. **Martin TR, and Frevert CW**. Innate immunity in the lungs. *Proceedings of the American Thoracic Society* 2: 403-411, 2005.

2. **Iwasaki A, Foxman EF, and Molony RD**. Early local immune defences in the respiratory tract. *Nat Rev Immunol* 17: 7-20, 2017.

3. **Byrne AJ, Mathie SA, Gregory LG, and Lloyd CM**. Pulmonary macrophages: key players in the innate defence of the airways. *Thorax* 70: 1189, 2015.

4. **Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, De Nardo D, Gohel TD, Emde M, Schmidleithner L, Ganesan H, Nino-Castro A, Mallmann MR, Labzin L, Theis H, Kraut M, Beyer M, Latz E, Freeman TC, Ulas T, and Schultze JL**. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* 40: 274-288, 2014.

5. **Yu YR, Hotten DF, Malakhau Y, Volker E, Ghio AJ, Noble PW, Kraft M, Hollingsworth JW, Gunn MD, and Tighe RM**. Flow Cytometric Analysis of Myeloid Cells in Human Blood, Bronchoalveolar Lavage, and Lung Tissues. *Am J Respir Cell Mol Biol* 54: 13-24, 2016.

6. **Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanović R, Maestrelli P, and Sterk PJ**. Sputum induction. *European Respiratory Journal* 20: 3s, 2002.

7. **Weiszhar Z, and Horvath I**. Induced sputum analysis: step by step. *Breathe* 9: 300, 2013.

8. **Collins AM, Rylance J, Wootton DG, Wright AD, Wright AK, Fullerton DG, and Gordon SB**. Bronchoalveolar lavage (BAL) for research; obtaining adequate sample yield. *Journal of visualized experiments : JoVE* 2014.

9. **Lentz RJ, and Colt H**. Summarizing societal guidelines regarding bronchoscopy during the COVID-19 pandemic. *Respirology* 25: 574-577, 2020.

10. **Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, and Tan W**. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *JAMA* 323: 1843-1844, 2020.

11. **van Doremalen N, Bushmaker T, Morris DH, Holbrook MG, Gamble A, Williamson BN, Tamin A, Harcourt JL, Thornburg NJ, Gerber SI, Lloyd-Smith JO, de Wit E, and Munster VJ**. Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1. *New England Journal of Medicine* 382: 1564-1567, 2020.

12. **Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, and Alexis NE**. Macrophages from The Upper and Lower Human Respiratory Tract Are Metabolically Distinct. *Am J Physiol Lung Cell Mol Physiol* 2018.

13. **Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, and Fantino E**. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am J Respir Cell Mol Biol* 53: 676-688, 2015.

14. **Beyer M, Mallmann MR, Xue J, Staratschek-Jox A, Vorholt D, Krebs W, Sommer D, Sander J, Mertens C, Nino-Castro A, Schmidt SV, and Schultze JL**. High-resolution transcriptome of human macrophages. *PLoS One* 7: e45466, 2012.

15. **Martinez FO, Gordon S, Locati M, and Mantovani A**. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 177: 7303-7311, 2006.

16. **Weinberg SE, Sena LA, and Chandel NS**. Mitochondria in the regulation of innate and adaptive immunity. *Immunity* 42: 406-417, 2015.

17. **Sandhir R, Halder A, and Sunkaria A**. Mitochondria as a centrally positioned hub in the innate immune response. *Biochim Biophys Acta Mol Basis Dis* 1863: 1090-1097, 2017.

18. **Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, Knapp B, Haas R, Schmid JA, Jandl C, Amir S, Lubec G, Park J, Esterbauer H, Bilban M, Brizuela L, Pospisilik JA, Otterbein LE, and Wagner O**. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab* 15: 813-826, 2012.

19. **O'Neill LA, Kishton RJ, and Rathmell J**. A guide to immunometabolism for immunologists. *Nat Rev Immunol* 16: 553-565, 2016.

20. **Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragones J, Martin-Sanz P, Cascante M, and Bosca L**. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *J Immunol* 185: 605-614, 2010.

21. **Van den Bossche J, Baardman J, Otto NA, van der Velden S, Neele AE, van den Berg SM, Luque-Martin R, Chen HJ, Boshuizen MC, Ahmed M, Hoeksema MA, de Vos AF, and de Winther MP**. Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. *Cell Rep* 17: 684-696, 2016.

22. **Huang SC-C, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, Beatty W, Love-Gregory L, Lam WY, O'Neill CM, Yan C, Du H, Abumrad NA, Urban JF, Artyomov MN, Pearce EL, and Pearce EJ**. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nature Immunology* 15: 846-855, 2014.

23. **Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, Chmielewski K, Stewart KM, Ashall J, Everts B, Pearce EJ, Driggers EM, and Artyomov MN**. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity* 42: 419-430, 2015.

24. **Escobar YH, Nipp G, Cui T, Petters SS, Surratt JD, and Jaspers I**. In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System. *Chemical research in toxicology* 2020.

25. **Hickman E, Herrera CA, and Jaspers I**. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chemical research in toxicology* 32: 982-985, 2019.

26. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R Foundation for Statistical Computing, Vienna, Austria. . https://www.R-project.org/.

27. **Kolde R**. (2019) pheatmap: Pretty Heatmaps. R package version 1.0.12. https://CRAN.R-project.org/package=pheatmap.

28. **Garnier S, Ross N, Rudis R, Camargo AP, Sciaini M, and Scherer S**. (2021) Rvision - Colorblind-Friendly Color Maps for R. R package version 0.6.2.

29. **Tang Y, Horikoshi M, and Li W**. ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages. *The R Journal* 8.2: 478-489, 2016.

30. **Horikoshi M, and Tang Y**. (2016) ggfortify: Data Visualization Tools for Statistical Analysis Results. https://CRAN.R-project.org/package=ggfortify.

31. **Kassambara A, and Mundt F**. factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.7. https://CRAN.R-project.org/package=factoextra. 2020.

32. **Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, Foresti R, and Immenschuh S**. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis. *Redox Biology* 22: 101147, 2019.

33. **Mendoza-Coronel E, and Ortega E**. Macrophage Polarization Modulates FcγR- and CD13-Mediated Phagocytosis and Reactive Oxygen Species Production, Independently of Receptor Membrane Expression. *Front Immunol* 8: 303, 2017.

34. **Canton J, Khezri R, Glogauer M, and Grinstein S**. Contrasting phagosome pH regulation and maturation in human M1 and M2 macrophages. *Mol Biol Cell* 25: 3330-3341, 2014.

35. **Huang J, Duan Z, Kwok J, Binns S, Vera LE, Kim Y, Szczypka G, and Emery SL**. Vaping versus JUULing: how the extraordinary growth and marketing of JUUL transformed the US retail e-cigarette market. *Tob Control* 28: 146-151, 2019.

36. **Takiguchi H, Yang CX, Yang CWT, Sahin B, Whalen BA, Milne S, Akata K, Yamasaki K, Yang JSW, Cheung CY, Vander Werff R, McNagny KM, Leitao Filho FS, Shaipanich T, van Eeden SF, Obeidat Me, Leung JM, and Sin DD**. Macrophages with reduced expressions of classical M1 and M2 surface markers in human bronchoalveolar lavage fluid exhibit pro-inflammatory gene signatures. *Scientific Reports* 11: 8282, 2021.

37. **Dewhurst JA, Lea S, Hardaker E, Dungwa JV, Ravi AK, and Singh D**. Characterisation of lung macrophage subpopulations in COPD patients and controls. *Scientific Reports* 7: 7143, 2017.

38. **Linehan SA, Martínez-Pomares L, Stahl PD, and Gordon S**. Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: In situ expression of mannose receptor by selected macrophages, endothelial cells, perivascular microglia, and mesangial cells, but not dendritic cells. *J Exp Med* 189: 1961-1972, 1999.

39. **Bharat A, Bhorade SM, Morales-Nebreda L, McQuattie-Pimentel AC, Soberanes S, Ridge K, DeCamp MM, Mestan KK, Perlman H, Budinger GR, and Misharin AV**. Flow Cytometry Reveals Similarities Between Lung Macrophages in Humans and Mice. *Am J Respir Cell Mol Biol* 54: 147-149, 2016.

40. **Gorki AD, Symmank D, Zahalka S, Lakovits K, Hladik A, Langer B, Maurer B, Sexl V, Kain R, and Knapp S**. Murine ex vivo Cultured Alveolar Macrophages Provide a Novel Tool to Study Tissue-Resident Macrophage Behavior and Function. *Am J Respir Cell Mol Biol* 2021.

41. **Luo M, Lai W, He Z, and Wu L**. Development of an Optimized Culture System for Generating Mouse Alveolar Macrophage-like Cells. *J Immunol* 207: 1683-1693, 2021.

CHAPTER 6: ADDITIONAL EXPERIMENTS

Understanding the Relationship Between Neutrophil Function and Demographic Variables

Introduction

Neutrophils are the most abundant white blood cell in circulation, and the largest pool of systemic neutrophils resides in the vasculature surrounding the lungs (1). Neutrophils, along with airway macrophages, are one of the body's first lines defense against inhaled pathogens (1-3). Additionally, neutrophilic inflammation and neutrophil dysfunction are associated with chronic airway diseases, such as cystic fibrosis, COPD, and asthma (4). These factors demonstrate the importance of understanding neutrophil biology in respiratory toxicology and disease, but neutrophils remain much less studied *in vitro* relative to other cells of innate respiratory host defense such as macrophages and airway epithelial cells. This is largely because primary neutrophils are difficult to maintain in culture, and few functionally and phenotypically accurate neutrophil cell lines exist (5). Primary neutrophils isolated from the peripheral blood of human subjects are considered the most translationally relevant way to study neutrophil function *in vitro*, but using cells from human subjects introduces the potential for functional variability between donors. Although some studies in mice have demonstrated sex- and age- dependent differences in neutrophil function (6, 7), and one study has demonstrated sex differences in baseline neutrophil transcriptional profiles, type 1 interferon responses, and bioenergetics (8), no studies have addressed demographic associations with phagocytosis and oxidative burst in human neutrophils. The objective of this study was to determine whether neutrophil oxidative burst and phagocytosis were significantly associated with donor age, BMI, or sex to inform clinical study design and provide the basis for future studies elucidating the role of demographic variables in neutrophil function.

Methods

Subjects

Venous blood was obtained from subjects (males and females between 19-62 years of age) for the isolation of peripheral blood neutrophils (Table 6-1). Subjects were self-reported healthy volunteers with no acute illness or allergy symptoms. Other exclusion criteria were current nicotine use, asthma, and/or pregnant and nursing women. Informed consent was obtained from all subjects and all studies were approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board (IRB #11-1363 and #97-0845). All studies were performed in accordance with The Code of Ethics of the World Medical Association.

Neutrophil Isolation

Venous blood was collected in EDTA-coated Vacutainer tubes (BD, Thermo Fisher Scientific, Waltham, MA). Neutrophils were isolated by density centrifugation of venous blood through Histopaque 1119 (Sigma Aldrich) and a discontinuous Percoll (GE Healthcare Life Sciences, Marlborough, MA) gradient as described previously (9). Isolated neutrophils were resuspended in either Seahorse media or neutrophil media. Cell count and viability were determined using a hemocytometer and trypan blue (Sigma Aldrich) exclusion.

Seahorse Extracellular Flux Analysis

We developed and optimized a Seahorse extracellular flux assay based on existing literature and manufacturer guidelines (10, 11). To our knowledge, this is the first publication reporting neutrophil oxidative burst using this assay and analysis paradigm. 24-well XF assay plates (Agilent Technologies, Santa Clara, CA) were coated with 0.672 μg Cell-Tak (Corning, Thermo Fisher Scientific, Waltham, MA) per well, according to manufacturer instructions, and stored at 4°C for no more than one week before assay. Coated plates were allowed to warm to room temperature (approximately 21°C) before cell plating. 1.5×10^5 isolated neutrophils were plated in 100 μL of Seahorse media. The plate was centrifuged at 300

x g for 1 minute, rotated 180 degrees, and centrifugation was repeated for 1 minute. Cells were allowed to rest in a non-CO_2 incubator at 37°C for approximately 20 minutes, followed by the addition of 400 μL Seahorse media and an additional 20 minutes of incubation before the start of the assay. Neutrophil oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured following injection of PKC agonist phorbol 12-myristate 13-acetate (PMA, 100 ng/mL final concentration) (Sigma Aldrich) on a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) at 37°C. Seahorse Extracellular Flux assays simultaneously measure oxygen consumption and extracellular acidification. Extracellular acidification can be used as an indicator of glycolytic activity because the protons produced by lactate dehydrogenase during glycolysis acidify the extracellular space. Neutrophils generate the energy and NADPH needed to perform oxidative burst almost exclusively through glycolysis and the pentose phosphate pathway. Three baseline readings were taken before injection of the flavoring compound, three readings were taken after injection of the flavoring compound, and 30 readings were taken after injection of PMA. The assay was run with mix/wait/measure cycles of 3 minutes/0 minutes/3 minutes. Data was analyzed using GraphPad Prism 8 area under the curve analysis. The area under the curve analysis generates: area under the curve, maximum OCR and ECAR, time to maximum OCR and ECAR, and time to baseline OCR and ECAR.

Phagocytosis

$1x10^5$ isolated neutrophils were plated in 100 μL neutrophil media in a black clear-bottom 96-well plate. Cells were incubated (37°C, 5% CO_2) for 30 minutes. Neutrophils were then challenged in triplicate with 0-5 mM of flavoring chemical for 1 hour at 37°C. Cinnamaldehyde (CA, 1 mM) was used as a positive control for inhibition of phagocytosis (9). pHrodo Red *Staphylococcus aureus* BioParticles (Thermo Fischer Scientific, Waltham, MA) were then opsonized and prepared according to manufacturer instructions and added to each well after a 1-hour incubation with the flavoring compounds. Neutrophils and BioParticles were incubated together at 37°C for 3 hours, and BioParticle phagocytosis was assessed

via quantification of mean fluorescence intensity (MFI) at a gain of 2500 using a CLARIOstar fluorescent microplate reader (BMG LABTECH, Ortenberg, Germany).

Statistics

To determine the influence of demographic variables on baseline phagocytic and oxidative burst capacity, analyses were conducted in R v4.1.1 using baseline statistical packages unless otherwise noted (12). For all continuous variables, normality was tested prior to further analyses using the Shapiro-Wilk test. Significant differences between males and females were tested with a two tailed t-test for parametric data or a Wilcoxon signed-rank test for nonparametric data. Correlations were determined using Spearman's rank correlation. Correlations were plotted using the *corrplot* package (13). Input data and R code used for these analyses are publicly available at https://github.com/ehickman0817/dissertation-extradatachapter.

Results

Subject characteristics.

Demographic composition of each data set is summarized in table 6-1. Each data set contained roughly equal proportions of males and females, with a majority of subjects being non-Hispanic and white. The mean age for each data set was between 26-29 years old, with a range of 19-62 years old. The mean BMI was 24-25, with a range of 18.3-38.4.

Neutrophils from male subjects are more phagocytic of S. aureus *BioParticles than neutrophils from female subjects.*

There were no significant associations between neutrophil bioenergetic parameters and BMI or age, and neutrophil bioenergetic parameters were not significantly different between males and females (Table 6-2). There were also no significant associations between *S. aureus* phagocytic capacity and age or

BMI (Table 6-2). However, the magnitude of neutrophil phagocytosis of *S. aureus* BioParticles in males was significantly higher than the magnitude in females (Table 6-2, Figure 6-1).

Table 6-1. Subject demographics for the three sets of data analyzed.

	Bioenergetics (Seahorse XF) (N=29)	***S. aureus* Phagocytosis (N=20)**	**Bioenergetics & Phagocytosis (N=10)**
Sex			
Female	14 (48.3%)	10 (50.0%)	4 (40.0%)
Male	15 (51.7%)	10 (50.0%)	6 (60.0%)
Race			
White	21 (72.4%)	17 (85.0%)	8 (80.0%)
Black	3 (10.3%)	1 (5.0%)	1 (10.0%)
Asian	4 (13.8%)	1 (5.0%)	0 (0%)
Mixed/Other	1 (3.4%)	1 (5.0%)	1 (10.0%)
Hispanic			
No	25 (86.2%)	17 (85.0%)	8 (80.0%)
Yes	4 (13.8%)	3 (15.0%)	2 (20.0%)
Age			
Mean (SD)	28.2 (7.75)	29.0 (9.96)	26.6 (6.33)
Median [Min, Max]	25.0 [19.0, 46.0]	24.5 [20.0, 62.0]	25.0 [22.0, 44.0]
BMI			
Mean (SD)	24.9 (4.31)	24.2 (4.55)	24.0 (3.48)
Median [Min, Max]	24.4 [18.3, 34.4]	24.0 [18.7, 38.4]	24.1 [18.7, 31.0]

Table 6-2. P-values and Spearman correlation coefficients to assess whether sex, age, or BMI are associated with neutrophil bioenergetics or *S. aureus* phagocytosis. Significant differences between males and females were tested with a two tailed t-test for parametric data and a Wilcoxon signed-rank test for nonparametric data. Significant p-values are bolded.

Metric	**Sex**	**Age**		**BMI**	
	P-value	***Correlation***	***P-value***	***Correlation***	***P-value***
OCR Maximum (pmol/min)	0.9962	-0.13	0.4857	0.10	0.6144
OCR AUC (total pmol)	0.6579	-0.15	0.4348	-0.07	0.7124
OCR Time to Maximum (minutes)	0.8613	-0.10	0.5925	-0.02	0.9059
OCR Time to Baseline (minutes)	0.2196	-0.14	0.4610	0.01	0.9756
ECAR Maximum (mpH/min)	0.6517	-0.11	0.5661	-0.05	0.7799
ECAR AUC (total mpH)	0.4797	-0.20	0.2887	-0.22	0.2436
ECAR Time to Maximum (minutes)	0.6783	-0.03	0.8862	-0.11	0.5761
ECAR Time to Baseline (minutes)	0.4559	-0.20	0.2968	-0.06	0.7573
Phagocytosis (MFI)	**0.0374**	-0.12	0.6217	0.20	0.3995

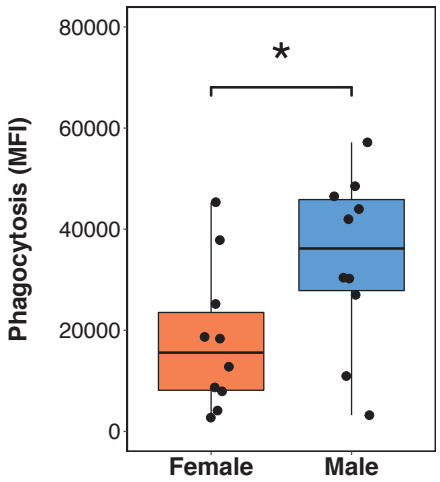


Figure 6-1. Sex differences in baseline neutrophil phagocytosis. Neutrophil phagocytosis of pHrodo red *S. aureus* BioParticles (n = 10 males, n = 10 females) was significantly different in cells collected from female and male subjects. * $p < 0.05$ by t-test.

Correlations between neutrophil bioenergetic parameters and phagocytosis are sex-dependent

To determine the relationship between phagocytic capacity of *S. aureus* BioParticles and bioenergetic parameters in neutrophils, we analyzed correlations between these measurements in subjects whose cells had been used for both types of experiments (Table 6-1). With data from all subjects combined, there were significant correlations between maximum OCR, OCR area under the curve, maximum ECAR, and ECAR area under the curve, demonstrating concurrent oxygen consumption and extracellular acidification following PMA stimulation (Figure 6-2A). This is logical given that the area under the curve is dependent on the peak pmol/min and mpH/min values, and the correlation between OCR and ECAR agrees with previously published literature demonstrating that PMA stimulation induces both oxidative burst and glycolysis in neutrophils (10, 14). However, there were no significant correlations between phagocytosis and bioenergetic parameters, indicating differences in the cellular pathways activated in each assay (Figure 6-2A).

Because we observed significant sex differences in baseline neutrophil phagocytosis (Figure 6-1), we next wanted to determine whether the correlations were similar when the data were sex disaggregated. We found that phagocytic capacity of neutrophil collected from male subjects was significantly positively correlated with time to maximum OCR and ECAR, while this pattern was not observed in neutrophils

from female subjects (Figure 6-2B, 6-2C, 6-3A, 6-3B). Significant correlations between Seahorse parameters were also different in cells collected from male and female subjects (Figure 6-2B, 6-2C), potentially indicating sex differences in regulation of neutrophil bioenergetics following PMA stimulation.

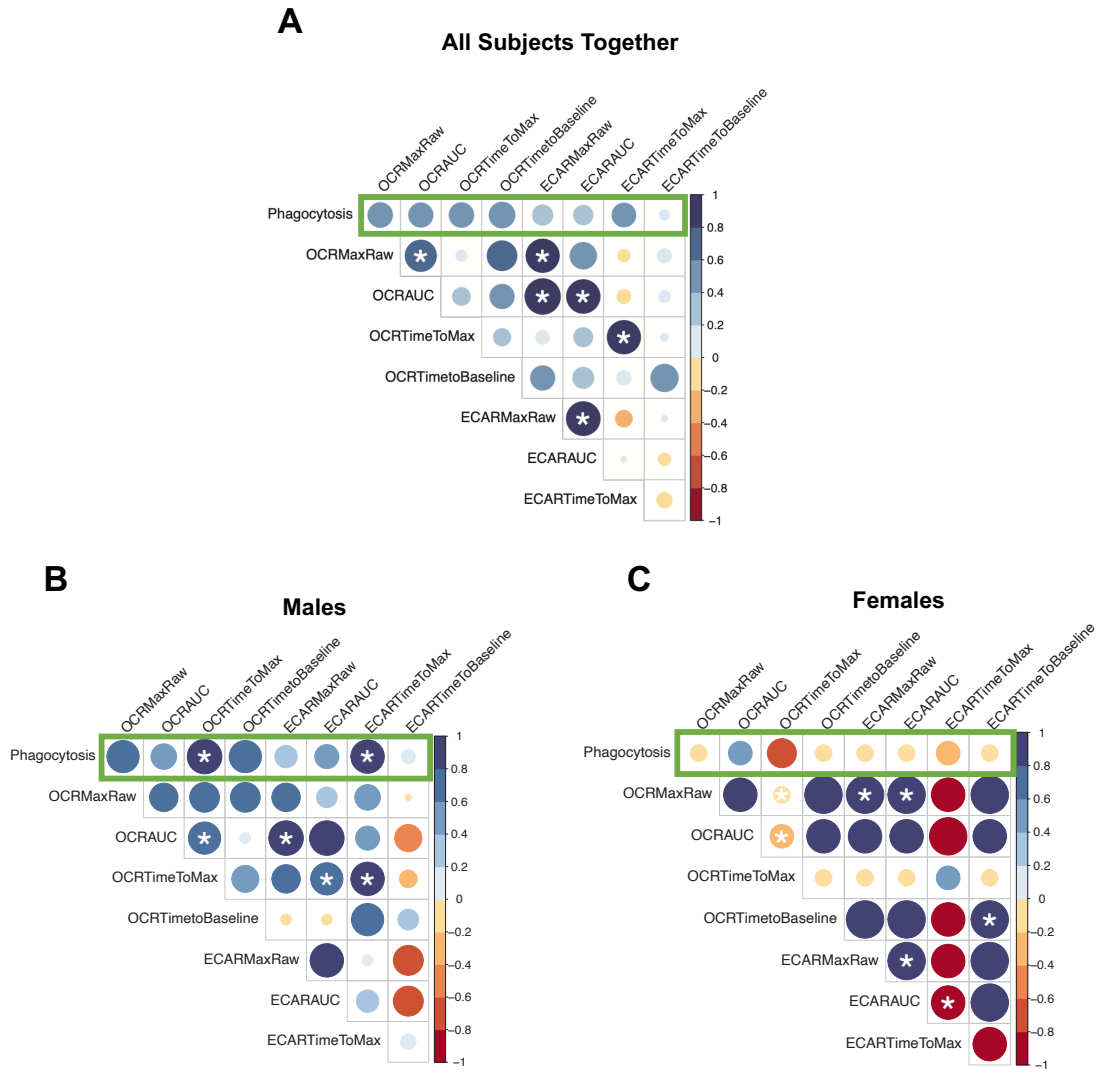


Figure 6-2. Correlation plot showing Spearman's rank correlation coefficients between neutrophil bioenergetic parameters and phagocytic capacity. The size of the corresponding circle and the color of the circle indicate the magnitude of the correlation coefficient. Asterisks indicate correlations that were significant with $p < 0.05$. Correlations of particular interest (between phagocytosis and bioenergetic parameters) are outlined in green. (A) All subjects together (n = 10); (B) Males only (n = 6); (C) Females only (n = 4).

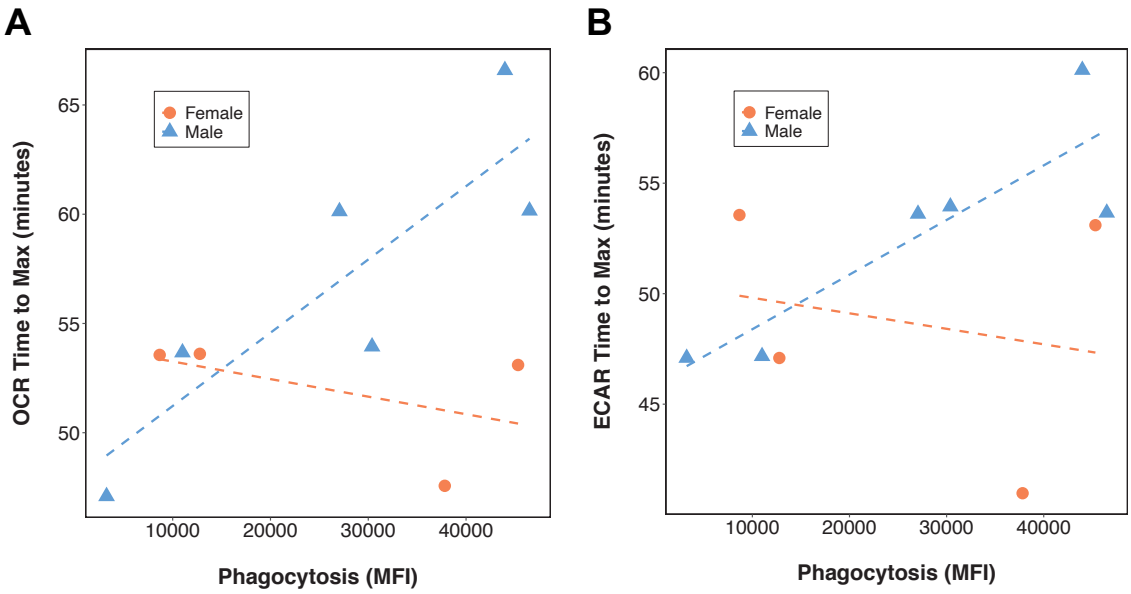


Figure 6-3. Significant correlations between phagocytosis capacity and time to maximum OCR and ECAR. Phagocytosis was significantly correlated with OCR and ECAR time to maximum in males but not in females. (A) Time to maximum OCR correlated with phagocytosis. R = 0.89 and p = 0.03 in males; R = -0.6 and p = 0.50 in females. (B) Time to maximum ECAR correlated with phagocytosis. R = 0.83 and p = 0.023 in males; R = -0.4 and p = 0.79 in females. n = 6 males, n = 4 females. Correlations calculated using Spearman's rank correlation.

Discussion

The results of this study introduce a novel finding of sexual dimorphism in human neutrophil phagocytic capacity. We found that neutrophils from male subjects were more phagocytic than neutrophils from female subjects (Table 6-2, Figure 6-1). However, we did not find sexual dimorphism in PMA-stimulated oxidative burst bioenergetic parameters (Table 6-2). This discrepancy may be due to the method of neutrophil activation used. Phagocytosis of *S. aureus* BioParticles more accurately recapitulates neutrophil phagocytosis *in vivo,* while stimulation of neutrophils with PMA directly activates protein kinase C (14), which does not occur *in vivo.* Though studies in animal models report higher phagocytosis in females than in males (7), to our knowledge, this is the first study reporting sex difference in human neutrophil phagocytosis. Some mechanisms hypothesized to underlie sex differences in neutrophil number and function include hormonal regulation via the G-protein coupled estrogen receptor and nuclear estrogen receptors (7, 15-18), as well as X-chromosome mosaicism for immune genes (7, 19) and miRNAs (20, 21). Given these hypothesized mechanisms, another important

consideration is that culturing and assaying neutrophils *ex vivo*, in the absence of endogenous hormones, may result in discrepancies between sexual dimorphisms observed *ex vivo* and *in vivo*.

Importantly, our observation of sexual dimorphism in phagocytosis is limited to phagocytosis of *S. aureus* BioParticles. Phagocytosis is mediated by complex cellular signaling pathways stimulated by binding of microbial ligands to cell surface receptors (22, 23); therefore, the cell signaling pathway activated by *S. aureus* BioParticles may be different from phagocytosis of other bacteria, yeast, or cellular debris and may be different from phagocytosis *in vivo*. Another limitation of this study is that phagocytosis assays and Seahorse assays were conducted on two separate days, so within-subject correlations between phagocytic capacity and bioenergetic parameters as determined in this study may be weaker than if assays were performed on the same day. Additionally, although most subjects contributed samples to this study on their first visit, BMI was only collected at each subject's first visit as part of a general sample collection protocol, so it is possible that recorded BMI differed from the BMI of the subject on the day that assays were performed if subjects returned for additional sample collection.

We also observed that phagocytosis was significantly correlated with the amount of time that it took cells to achieve maximum OCR and ECAR following PMA stimulation in males but not in females (Figures 6-2 and 6-3). Although our sample size per sex is relatively small, and there is a lack of prior literature with which to compare these findings, this is a notable observation that supports sexual dimorphism in neutrophil activation and warrants future investigation. While addressing the mechanism underlying these observed sex differences is outside the scope of this study, our data provide cellular evidence to support clinical and animal model data suggesting sexual dimorphism in the immune system and underscore the importance of considering and reporting biological sex when colleting samples from human subjects. Future studies that investigate sex differences in additional neutrophil functional endpoints and the mechanisms underlying these differences are needed and will be highly applicable to translational research.

Effects of 4th Generation E-Cigarettes on Sputum Macrophage Function

Introduction

Macrophages are a critical cell type in maintenance of respiratory host defense. Macrophage dysfunction is associated with a wide array of respiratory diseases and pathologies (24), but the effects of e-cigarettes and e-liquid components on respiratory macrophages remain unclear. Previous literature on the effects of e-cigarettes on macrophages suggest that e-cigs can be cytotoxic, reduce phagocytosis, and modulate cytokine release in macrophages (25-28). However, the roles of nicotine and flavoring chemicals in mediating these effects are not consistent between studies, and data regarding whether e-cigarettes induce or suppress cytokine release are also inconsistent. Furthermore, there are no studies that assess the effects of nicotine-salt-containing pod and disposable e-cigarettes, which are currently the current most popular e-cigarette type, on macrophages.

It can be challenging to determine concentrations of e-liquids or condensates most appropriate to expose cell *in vitro* because the deposition of e-cigarette aerosol in the airways and therefore the concentrations of aerosol components that cells are exposed to are currently unknown. Thus, taking cells from e-cigarette users, which have already been exposed e-cigarette aerosol *in vivo*, and measuring their function represents a viable alternative to *in vitro* exposures and can be used to complement *in vitro* studies. In this study, we collected induced sputum cells from 4th generation e-cigarette users and non-vapers, enriched the cells for macrophages, and measured mitochondrial membrane potential (as a proxy for cellular bioenergetics) and phagocytic capacity to determine whether e-cigarettes alter these key cellular functions.

Methods

Subject recruitment

Healthy adult human nonsmokers and e-cigarette users between 18-50 years old were recruited to participate in this study. Active vaping was determined as described previously (20). E-cigarette users were classified as 4th generation e-cigarette users if they reported using primarily JUUL or other sleek, discreet, low-powered e-cigarettes that contain nicotine salts. Exclusion criteria included current symptoms of allergic rhinitis, chronic cardiorespiratory disease, immunodeficiency, bleeding disorders, current pregnancy, and FEV1 less than 75% predicted during the screen visit. All studies were approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board (IRB # #17-2275).

Sample collection and processing

Induced sputum collection, processing, acquisition of fluid phase samples, and differential cell counts were performed as described previously (29, 30). To obtain serum, venous blood was collected in BD Vacutainer serum-separating tubes (Fisher Scientific, Waltham, MA), allowed to clot for a minimum of 15 minutes, and centrifuged at 1200 x g for 10 minutes. The serum layer was collected and stored at -80°C until samples were collected from all subjects.

Sputum cell mitochondrial membrane potential assay

3 x 10^5 sputum cells per well were seeded into a 96-well black-walled, clear bottomed plate. The plate was centrifuged at 300 x g for 2 minutes with low acceleration and braking. Cells were incubated for one hour (37 °C, 5% CO_2) to allow settling. After one hour, a solution of JC-1 dye (Thermo Fisher) with or without carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a positive control for electron transport chain uncoupling, was added to the wells for final concentrations of 2 mM JC-1 and 50 mM CCCP. After a 30-minute incubation (37 °C, 5% CO_2) with JC-1 or JC-1 + CCCP (Figure 6-4), staining solution and nonadherent cells were removed (to enrich for macrophages) and replaced with fresh media and cells

were incubated for ten minutes. The ratio of J-aggregate (red) to J-monomer (green) was obtained by measuring the fluorescent signal of each well using well scan mode on a CLARIOstar plate reader with excitation wavelength set at 488 nm and detection wavelengths set at 535 nm (green) and 595 nm (red).

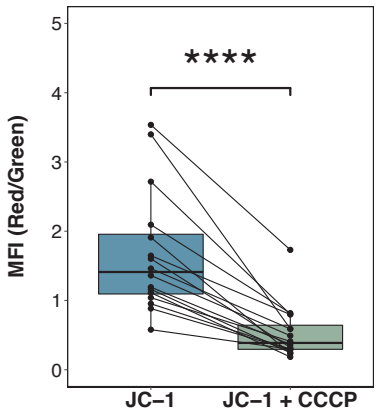


Figure 6-4. JC-1 red/green ratio of mean fluorescence intensity (MFI) is decreased by CCCP treatment. Induced sputum macrophages were stained using JC-1 following treatment with or without CCCP. Matched samples are connected with a line. **** $p < 0.0001$ by paired Wilcoxon test. n = 16

Sputum cell S. aureus phagocytosis assay

2 x 10^5 sputum cells per well were seeded into a 96-well black-walled, clear bottomed plate. Cells were allowed to incubate (37 °C, 5% CO_2) for one hour before media was replaced to remove all non-adherent cells and enrich for macrophages. Then, phagocytosis was measured using pHrodo Red Staphylococcus aureus BioParticles (Thermo Fischer Scientific) as described previously (22), with an incubation time of three hours.

Analysis

All statistical analyses were conducted in R v4.1.1 using baseline statistical packages unless otherwise noted (12). For all continuous variables, normality was tested using the Shapiro-Wilk test. Differences between groups were tested with a two tailed t-test for parametric data or a Wilcoxon signed-rank test for nonparametric data. The JC-1 normality test was on the borderline between significant and non-significant ($p = 0.02$), so both a t-test and Wilcoxon signed-rank test were carried out. Correlations

were determined using Spearman's rank correlation. Input data and R code used for these analyses are publicly available at https://github.com/ehickman0817/dissertation-extradatachapter.

Results

Subject characteristics

The demographic characteristics of the subjects from each group are summarized in Table 6-3. Each group contained both males and females and was majority non-Hispanic white. The mean age was slightly higher in non-vapers (26.7 years) than 4th generation e-cigarette users (21.4 years old) ($p = 0.0514$). In the 4th generation e-cigarette user group, 8 subjects used only JUUL, 1 subject used a mixture of JUUL and disposables, and 2 subjects used only disposables.

Table 6-3. Subject demographics by group. Groups were compared using a Wilcoxon Test (for continuous variables) or Fisher's Exact Test (categorical variables).

	Non-vapers (N=6)	4th Gen E-Cig Users (N=11)	P-value
Sex			
Female	4 (66.7%)	2 (18.2%)	0.109
Male	2 (33.3%)	9 (81.8%)	
Race			
White	5 (83.3%)	9 (81.8%)	0.432
Black	1 (16.7%)	0 (0%)	
Asian	0 (0%)	2 (18.2%)	
Hispanic			
No	6 (100%)	9 (81.8%)	0.515
Yes	0 (0%)	2 (18.2%)	
Age			
Mean (SD)	26.7 (5.89)	21.4 (1.91)	0.0514
Median [Min, Max]	26.5 [21.0, 37.0]	21.0 [19.0, 26.0]	
BMI			
Mean (SD)	24.5 (5.48)	23.6 (2.87)	0.961
Median [Min, Max]	24.1 [18.8, 34.5]	22.9 [20.0, 29.3]	
Device			
JUUL	0 (0%)	8 (72.7%)	<0.001
JUUL/Disposable	0 (0%)	1 (9.1%)	
Disposable	0 (0%)	2 (18.2%)	
None	6 (100%)	0 (0%)	

Sputum cell differentials were not significantly different between non-vapers and 4th generation e-cigarette users

Cell differential data pertinent to cellular functional assays are reported in Table 6-4. There were no significant differences between select sample weight, total cell count, cells per milligram of sputum, percent neutrophils, percent macrophages, or percent viability between non-vapers and 4th generation e-cigarette users.

Table 6-4. Sputum sample cell characteristics. Values are reported as mean (SEM). Groups were compared using a Wilcoxon Test.

	Non-vapers (N=6)	4th Gen E-Cig Users (N=11)	P-value
Select Sample Weight	2140 (704)	1670 (220)	0.884
Total Cell Count	1640000 (733000)	5170000 (2420000)	0.191
Cells/mg	680 (89.7)	2600 (923)	0.216
% PMN	42.7 (12.6)	45.2 (8.82)	0.733
% Macrophage	55.9 (12.9)	53.2 (8.61)	0.591
% Viable	0.755 (0.0622)	0.832 (0.0306)	0.268

4th generation e-cigarette users have high sputum macrophage mitochondrial membrane potential than non-vapers.

To understand whether 4th generation e-cigarette use was associated with alterations in sputum macrophage cellular function, we measured mitochondrial membrane potential and *S. aureus* phagocytosis in sputum macrophages from non-smokers/non-vapers and 4th generation e-cigarette users. We found that mitochondrial membrane potential was significantly higher in macrophages from 4th generation e-cigarette users in comparison with non-smokers/non-vapers (Figure 6-5A) potentially indicative of a more pro-inflammatory phenotype. We did not observe significant differences in phagocytosis between 4th generation e-cigarette users and non-smokers/non-vapers (Figure 6-5B).

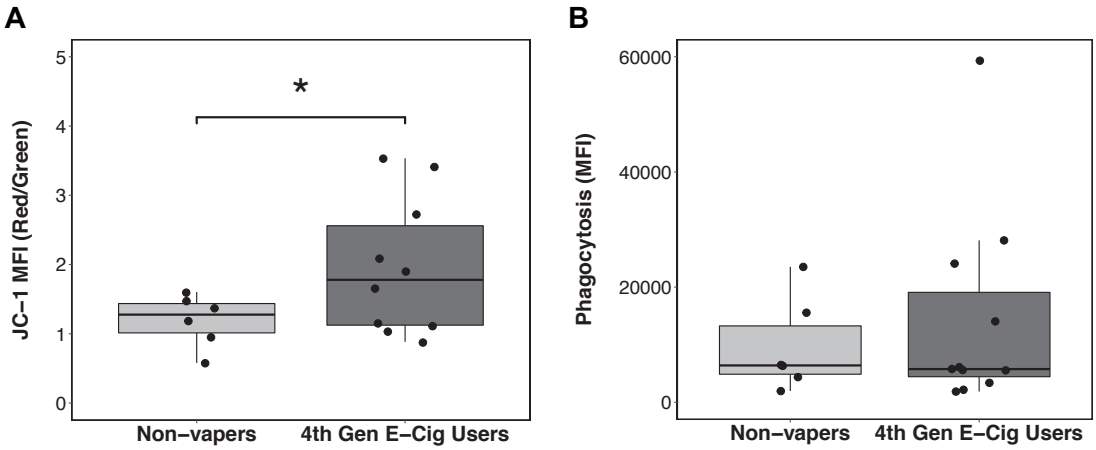


Figure 6-5. 4th generation e-cigarette users have higher sputum macrophage mitochondrial membrane potential than non-vapers. Sputum macrophages were assayed for (A) mitochondrial membrane potential using JC-1 dye (n = 6 non-vapers; n = 10 4th generation e-cigarette users), * $p < 0.05$ by unpaired t-test and $p = 0.18$ by Wilcox test; (B) phagocytic potential using pHrodo Red S. aureus BioParticles dye (n = 6 non-vapers; n = 11 4th generation e-cigarette users).

Discussion

In this study, we found that sputum macrophages from 4th generation e-cigarette users had significantly higher mitochondrial membrane potential than sputum macrophages from non-vapers (Figure 6-5A). This is notable because cellular bioenergetics is recognized to be a central regulator of macrophage function (31, 32). Macrophages polarized toward an inflammatory phenotype require large amounts of ATP generated through glycolysis to perform phagocytosis, and additionally, the mitochondria generate reactive oxygen species (31, 33). Previous studies have observed increased mitochondrial membrane potential as part of metabolic reprogramming that occurs in response to inflammatory stimuli (33). Therefore, the higher mitochondrial membrane potential in macrophages from 4th generation e-cigarette users in comparison with those from non-vapers may be reflective of a more pro-inflammatory phenotype. However, we did not observe a significant difference in phagocytic capacity in 4th generation e-cigarette users (Figure 6-5B), suggesting that these subjects' macrophages have equal antimicrobial capacity towards *S. aureus.*

The most significant limitation in this study was the unavoidable variability in sputum cell composition and cellular viability. Induced sputum contains a mixed cell population primarily consisting of macrophages and neutrophils, and although there were not significant differences in percentage of

macrophages and neutrophils between groups, and macrophages were selected for via centrifugation and adherence, the proportions of each cell type were different across subjects. Generally, this is difficult to address experimentally because cell differentials are not determined until after assays are performed with live cells. The JC-1 assay readout is ratiometric and thus controls well for differences in cell number and composition across assays. The phagocytosis assay is more susceptible to differences in cell populations because there is no normalizing component in the assay and because macrophages are generally more highly phagocytic of *S. aureus* BioParticles than neutrophils. And, although cells were seeded based on the number of viable cells, it is possible that low cell viability after sample processing could influence behavior of live cells remaining in the sample. Therefore, it is possible that the effects of e-cigarettes on cellular function were masked by these factors, particularly for the phagocytosis assay. Another significant limitation in this study was the number of subjects in each cohort, which was limited by the COVID-19 pandemic. Future studies including larger cohort sizes and additional experimental steps to ensure selection of only macrophages and an equal number of viable macrophages for each subject would provide more robust data and impactful conclusions about the effects of e-cigarettes on sputum macrophage function.

REFERENCES

1. **Hartl D, Tirouvanziam R, Laval J, Greene CM, Habiel D, Sharma L, Yildirim A, Dela Cruz CS, and Hogaboam CM**. Innate Immunity of the Lung: From Basic Mechanisms to Translational Medicine. *Journal of innate immunity* 10: 487-501, 2018.

2. **Mócsai A**. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *The Journal of experimental medicine* 210: 1283-1299, 2013.

3. **Zhang P, Summer WR, Bagby GJ, and Nelson S**. Innate immunity and pulmonary host defense. *Immunological reviews* 173: 39-51, 2000.

4. **Jasper AE, McIver WJ, Sapey E, and Walton GM**. Understanding the role of neutrophils in chronic inflammatory airway disease. *F1000Research* 8: 2019.

5. **Blanter M, Gouwy M, and Struyf S**. Studying Neutrophil Function in vitro: Cell Models and Environmental Factors. *J Inflamm Res* 14: 141-162, 2021.

6. **Lu RJ, Taylor S, Contrepois K, Kim M, Bravo JI, Ellenberger M, Sampathkumar NK, and Benayoun BA**. Multi-omic profiling of primary mouse neutrophils predicts a pattern of sex- and age-related functional regulation. *Nature Aging* 1: 715-733, 2021.

7. **Casimir GJ, Lefevre N, Corazza F, and Duchateau J**. Sex and inflammation in respiratory diseases: a clinical viewpoint. *Biology of sex differences* 4: 16, 2013.

8. **Gupta S, Nakabo S, Blanco LP, O'Neil LJ, Wigerblad G, Goel RR, Mistry P, Jiang K, Carmona-Rivera C, Chan DW, Wang X, Pedersen HL, Gadkari M, Howe KN, Naz F, Dell'Orso S, Hasni SA, Dempsey C, Buscetta A, Frischmeyer-Guerrerio PA, Kruszka P, Muenke M, Franco LM, Sun H-W, and Kaplan MJ**. Sex differences in neutrophil biology modulate response to type I interferons and immunometabolism. *Proceedings of the National Academy of Sciences* 117: 16481, 2020.

9. **Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I**. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278-L292, 2017.

10. **Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballinger SW, and Darley-Usmar VM**. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. *Lab Invest* 93: 690-700, 2013.

11. **Swain PM, Romero N, Jastromb N, and Dranka BP**. Quantitative Analysis of Neutrophil Activation Using Agilent Seahorse XF Technology. 2018.

12. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R Foundation for Statistical Computing, Vienna, Austria. . https://www.R-project.org/.

13. **Wei T, and Simko V**. R package 'corrplot': Visualization of a Correlation Matrix (Version 0.92). Available from https://github.com/taiyun/corrplot. 2020.

14. **Tauber AI**. Protein kinase C and the activation of the human neutrophil NADPH-oxidase. *Blood* 69: 711-720, 1987.

15. **Rodenas MC, Tamassia N, Cabas I, Calzetti F, Meseguer J, Cassatella MA, García-Ayala A, and Mulero V**. G Protein-Coupled Estrogen Receptor 1 Regulates Human Neutrophil Functions. *Biomedicine Hub* 2: 2-2, 2017.

16. **Dougherty SM, Mazhawidza W, Bohn AR, Robinson KA, Mattingly KA, Blankenship KA, Huff MO, McGregor WG, and Klinge CM**. Gender difference in the activity but not expression of estrogen receptors alpha and beta in human lung adenocarcinoma cells. *Endocrine-related cancer* 13: 113-134, 2006.

17. **Bekesi G, Kakucs R, Varbiro S, Racz K, Sprintz D, Feher J, and Szekacs B**. In vitro effects of different steroid hormones on superoxide anion production of human neutrophil granulocytes. *Steroids* 65: 889-894, 2000.

18. **Bekesi G, Tulassay Z, Racz K, Feher J, Szekacs B, Kakucs R, Dinya E, Riss E, Magyar Z, and Rigo J, Jr.** The effect of estrogens on superoxide anion generation by human neutrophil granulocytes: possible consequences of the antioxidant defense. *Gynecol Endocrinol* 23: 451-454, 2007.

19. **Chandra R, Federici S, Nemeth ZH, Horvath B, Pacher P, Hasko G, Deitch EA, and Spolarics Z**. Female X-chromosome mosaicism for NOX2 deficiency presents unique inflammatory phenotype and improves outcome in polymicrobial sepsis. *J Immunol* 186: 6465-6473, 2011.

20. **Klein SL, and Flanagan KL**. Sex differences in immune responses. *Nat Rev Immunol* 16: 626-638, 2016.

21. **Pinheiro I, Dejager L, and Libert C**. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. *Bioessays* 33: 791-802, 2011.

22. **van Kessel KPM, Bestebroer J, and van Strijp JAG**. Neutrophil-Mediated Phagocytosis of Staphylococcus aureus. *Front Immunol* 5: 2014.

23. **Garcia-Garcia E, and Rosales C**. Adding Complexity to Phagocytic Signaling: Phagocytosis-Associated Cell Responses and Phagocytic Efficiency. In: *Madame Curie Bioscience Database [Internet]*. Austin, TX: Landes Bioscience, 2000-2013.

24. **Belchamber KBR, and Donnelly LE**. Macrophage Dysfunction in Respiratory Disease. *Results Probl Cell Differ* 62: 299-313, 2017.

25. **Muthumalage T, Prinz M, Ansah KO, Gerloff J, Sundar IK, and Rahman I**. Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine. *Front Physiol* 8: 1130, 2017.

26. **Ween MP, Whittall JJ, Hamon R, Reynolds PN, and Hodge SJ**. Phagocytosis and Inflammation: Exploring the effects of the components of E-cigarette vapor on macrophages. *Physiol Rep* 5: e13370, 2017.

27. **Leslie LJ, Vasanthi Bathrinarayanan P, Jackson P, Mabiala Ma Muanda JA, Pallett R, Stillman CJP, and Marshall LJ**. A comparative study of electronic cigarette vapor extracts on airway-related cell lines in vitro. *Inhal Toxicol* 29: 126-136, 2017.

28. **Scott A, Lugg ST, Aldridge K, Lewis KE, Bowden A, Mahida RY, Grudzinska FS, Dosanjh D, Parekh D, Foronjy R, Sapey E, Naidu B, and Thickett DR**. Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages. *Thorax* 73: 1161-1169, 2018.

29. **Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, and Alexis NE**. Macrophages from The Upper and Lower Human Respiratory Tract Are Metabolically Distinct. *Am J Physiol Lung Cell Mol Physiol* 2018.

30. **Alexis NE**. Biomarker sampling of the airways in asthma. *Curr Opin Pulm Med* 20: 46-52, 2014.

31. **O'Neill LA, Kishton RJ, and Rathmell J**. A guide to immunometabolism for immunologists. *Nat Rev Immunol* 16: 553-565, 2016.

32. **Wang Y, Li N, Zhang X, and Horng T**. Mitochondrial metabolism regulates macrophage biology. *J Biol Chem* 297: 100904, 2021.

33. **Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, Tourlomousis P, Däbritz JHM, Gottlieb E, Latorre I, Corr SC, McManus G, Ryan D, Jacobs HT, Szibor M, Xavier RJ, Braun T, Frezza C, Murphy MP, and O'Neill LA**. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* 167: 457-470.e413, 2016.

CHAPTER 7: OUTREACH

Bridging The Gap Between E-Cigarette Research And High School Students Through Collaborative STEM Lesson Development

Introduction

Especially among adolescents, e-cigarette use represents a significant public health concern, with 3.6 million middle and high school students reporting use in 2020 (1-4) (Note: 2021 National Youth Tobacco Survey data cannot be compared to previous years due to differences in the administration of the survey as a result of COVID-19 (2)). E-cigarettes are battery-powered devices that heat and aerosolize an e-liquid containing nicotine, flavoring chemicals, and base liquids (propylene glycol and vegetable glycerin), allowing the user to inhale the resulting cloud. E-cigarettes have been proposed as a tool that could aid in smoking cessation and reduce exposure to harmful chemicals in cigarettes, but most youth users of e-cigarettes have never smoked, and studies reveal that these youth are also more likely to subsequently start smoking (5-8). The youth vaping epidemic, which was recognized by the United States Surgeon General in 2016 (9), has raised concern in public health circles about youth addiction to nicotine. And, with 85% of youth reporting they use flavored e-cigarettes (2), the unknown respiratory effects of e-liquid constituents such as flavorings also elicit concern. The prevalence of e-cigarette use among youth highlights the need for education about the potential health effects of e-cigarettes.

Preparing youth to make informed, health-protective decisions about e-cigarette use represents an opportunity to engage them in science, technology, engineering, and math (STEM) activities while also promoting biomedical research careers, like those in the field of toxicology. Well-informed STEM teachers are critical to the success of these endeavors. Building the capacity of STEM teachers to incorporate the evolving science of e-cigarette research into instruction is one avenue to communicate risk

to a vulnerable population while addressing curriculum standards. Table 7-1 shows the numerous curriculum connections that can be made when exploring the science of e-cigarettes.

Table 7-1. Numerous curriculum connections can be made when exploring the science of e-cigarettes.

Science Discipline	Curriculum connections
Life sciences	Anatomy & Physiology (Respiratory system; Immune system) Cell structure & function (Ciliated epithelial cells; cellular respiration) Gene expression Immunology Nicotine addiction Toxicology
Physical sciences	Functional groups Solutions (solvent, solutes) Chemical reactions pH Temperature (heat) Aerosols
Environmental sciences	Single-use plastics; not recyclable Litter Leak heavy metals and residual nicotine into environment Secondhand aerosol

E-cigarettes provide a timely and relevant context in which students can explore and refine their knowledge of fundamental anatomy and physiology, biology, and chemistry concepts. However, teachers may not have the relevant content knowledge to identify curriculum connections and successfully incorporate this topic into their instruction. A rapidly evolving biomedical research landscape coupled with curriculum constraints are additional challenges teachers face when trying to incorporate current scientific research into their teaching, and this challenge exists for both new and veteran teachers (10, 11). Furthermore, this disconnect between science curriculum standards and the cutting-edge biomedical research taking place in today's laboratories has real implications for the future biomedical workforce, which is dependent upon K-12 teacher and student knowledge of current science and science careers. Therefore, providing teachers with curriculum-relevant content and STEM-based activities that showcase current science, technologies, and careers can promote science literacy while also cultivating the biomedical workforce.

In response to these challenges, and to the goal of bringing e-cigarette research findings from the laboratory to youth, researchers investigating the effects of e-cigarette solvents and flavorings on respiratory health at the University of North Carolina (UNC) at Chapel Hill partnered with science education experts at the UNC Center for Environmental Health and Susceptibility to bring e-cigarette research to high school life science classrooms. To address this goal, we 1) developed three unique data interpretation activities or "lessons" centered around e-cigarette research, and 2) provided content-rich teacher professional development, including modeling lesson implementation, to increase teacher knowledge of e-cigarettes and to increase teacher preparedness introducing this content in their classrooms.

In this article, we describe our collaborative process for involving scientists in curriculum development and teacher professional development activities to convey the potential health effects of vaping that extend beyond those of nicotine, while simultaneously cultivating students' data literacy and demonstrating the process by which emerging science unfolds in the laboratory.

Methods

Co-Design Team

Dana Haine, MS and Elise Hickman, BS worked together to collaboratively develop lessons and execute teacher professional development activities. Ms. Haine is the K-12 Science Education Manager with the UNC Institute for the Environment's Center for Public Engagement with Science and also conducts outreach for the UNC Center for Environmental Health and Susceptibility in the Gillings School of Global Public Health. Ms. Hickman is a PhD candidate in the Curriculum in Toxicology and Environmental Medicine at UNC Chapel Hill. Ms. Haine and Ms. Hickman were connected in 2018 by, Dr. Ilona Jaspers, and have been working together over the past four years to design curricula as well as develop and implement corresponding teacher professional development activities. As a biomedical scientist, Ms. Hickman's contributions included explaining the context and relevance of current e-cigarette research, providing access to data for use in the lesson, ensuring accurate representation of e-

cigarette science, and modeling science practices. As a STEM education expert with a biomedical background, Ms. Haine's contributions included aligning content to state and national science education standards, ensuring lessons were feasible given the realities of the science classroom, providing background information and classroom ready resources, and making sure data was presented in an accessible way for high school classrooms. Through a collaborative and iterative design process, Ms. Haine and Ms. Hickman developed three unique data interpretation activities and modeled these lessons during teacher professional development sessions at state and national practitioner conferences and during a four-part workshop series described in this article.

Lesson Development Process

Recognizing the relevance of e-cigarettes to students' lives and health outcomes and the opportunity to use this content to reinforce curriculum standards, we sought to develop educational materials that would bring the e-cigarette research taking place in biomedical laboratories to secondary life science classrooms. We proceeded through the following steps to collaboratively develop research-based lessons that would educate students about the potential harms of e-cigarettes while engaging them in the practices of science as described in the Framework for K-12 Science Education (12).

Identify key concepts

Our collaboration began with dialogue to identify key concepts that could be conveyed through learning about e-cigarettes and the corresponding research to understand the respiratory health effects of e-liquids and aerosols. Examples of key concepts included: flavorings are chemicals, e-liquids are complex chemical mixtures, e-cigarettes produce an aerosol (not a vapor) when heated, and chemicals in e-cigarette liquids and aerosols can affect cellular function. We noted that e-cigarette research is particularly relevant to courses such as biology, anatomy and physiology, and biomedical science because understanding this research requires students to explore and refine what they know about cell structure and function, the immune system, and cellular respiration.

Identify relevant science and engineering practices

As we discussed the research methods and the research findings, we identified relevant science and engineering practices that could be highlighted to create authentic learning experiences for students. By focusing on current biomedical science content, many of the Science and Engineering Practices outlined in the *Next Generation Science Standards* (13) and in the AP Biology Curriculum (14) are easily integrated. For example, in each lesson students observe and analyze data and evaluate evidence (NGSS Practice 4 and AP Biology Science Practice 5).

Align to standards

Next, we aligned content to state and national curriculum standards as shown in Tables 7-2 and 7-3. Lesson content is relevant to NGSS Disciplinary Core Ideas in the Life Sciences (LS1 and LS3) and to AP Biology Big Ideas 2, 3, 4. While e-cigarettes and vaping are not explicitly mentioned in today's curriculum standards, this content can be used to reinforce standards by providing up to date examples of biological phenomena (e.g., inflammation triggered by chemical exposure) or by providing opportunities to apply learning about a fundamental biological process like cellular respiration. For example, data featured in Lesson 2 demonstrates how a specific e-cigarette flavoring chemical, cinnamaldehyde, can impair mitochondrial function, resulting in decreased ATP production and decreased cilia beating in airway epithelial cells. Thus, this specific example could be used to reinforce what students learned about the role of mitochondria and ATP in cells.

Table 7-2. Curriculum alignment with Advanced Placement Biology.

Advanced Placement Biology	
Science Practices	**Big Ideas**
This activity invites students to use all six science practices: • Concept Explanation • Visual Representations • Questions and Methods • Representing and Describing Data • Statistical Tests and Data Analysis • Argumentation	BIG IDEA 2: ENERGETICS (ENE) • **Enduring understanding 2.D, 2.E** BIG IDEA 3: INFORMATION STORAGE AND TRANSMISSION (IST) • **Enduring understanding 3.B** BIG IDEA 4: SYSTEMS INTERACTIONS (SYI) • **Enduring understanding 4.C**
Relevant Units: • Unit 1: Chemistry of Life (Biological Macromolecules (Proteins)) • Unit 2: Cell Structure and Function • Unit 3: Cellular Energetics (Enzymes)	

Table 7-3 Curriculum alignment with Next Generation Science Standards.

Next Generation Science Standards		
Disciplinary Core Ideas	**Relevant Science & Engineering Practices**	**Relevant Cross Cutting Concepts**
LS1: From Molecules to Organisms: Structures and Processes **LS3:** Heredity: Inheritance and Variation of Traits	• Asking questions and defining problems • Analyzing and interpreting data • Developing and using models • Constructing explanations • Obtaining, evaluating, and communicating information	• Patterns • Cause and effect: mechanism and explanation • Scale, proportion, and quantity • Systems and system models • Structure and Function • Stability and change
Relevant Performance Expectation: HS-LS1-2. Develop and use a model to illustrate the hierarchical organization of interacting systems that provide specific functions within multicellular organisms.		

Promote data literacy

Because of the emerging nature of research taking place to understand the health effects of e-cigarettes, we felt it was important to ground our lessons in the literature. We proceeded to develop lessons that would enable students to interact directly with primary research data pertaining to the toxicological effects of e-cigarettes and draw their own conclusions about the health effects of vaping. Notably, this approach aligns with two evidence-based recommendations for effective vaping prevention

strategies outlined on the Vaping Prevention Resource website: 1) demonstrate to youth how chemicals they inhale when vaping can affect their health, and 2) emphasize how chemicals can have health effects on the respiratory and cardiovascular systems (15).

Furthermore, research shows that data-enhanced learning experiences, especially those that include data relevant to students' lives, are important for developing essential science skills (16, 17). Thus, by centering our lessons around student-led analysis of primary data, we sought to cultivate data literacy skills and promote student awareness about the process by which new scientific knowledge is obtained and communicated. For all lessons, we used recently published peer-reviewed publications from research groups at UNC-Chapel Hill due to the design team's familiarity with this research and to maximize the ability for the design team to interact with the authors. From these papers, we selected data that would both support student learning about the key concepts we identified and be easily adapted for use in the classroom.

In addition to offering data-enhanced learning experiences, another intention behind the development of these lessons was to offer teachers a critical thinking or "minds-on" activity that did not require the purchase of lab materials, use of fume hoods, or extensive preparation in an effort to make the lessons both accessible and adaptable for in person or virtual instruction. Our approach to incorporating scientific data into each lesson was to provide guiding questions and one or two corresponding data sets along with background information and questions to prompt student interaction with the data and to prepare them to communicate their findings to others. To verify that teachers would be responsive to this approach, we acquired teacher feedback about our conceptualizations from the beginning and throughout the lesson development process. For example, in hearing that AP Biology teachers cover standard error, we made sure to keep error bars in featured graphs and we offered guidance to students on interpreting error bars and evaluating statistical significance. These elements can easily be hidden on graphs to engage students not yet prepared to address these concepts.

Lessons 1 and 2 utilize the 5E instructional model, in which students explore a phenomenon by progressing through the following stages: engagement, exploration, explanation, elaboration, and evaluation (18). We designed these lessons to enable teachers to customize their approach to introducing students to one or more data sets. For each lesson we offer six guiding questions, with 1-2 graphs per question, and while we envisioned that teachers could conduct this as a jigsaw activity, the guiding questions and corresponding graphs could be adapted into worksheets, lecture based discussion, or placed at stations. Although these lessons were designed for in-person implementation, the materials are well suited for use in a virtual learning environment.

For our third lesson, which we developed in response to the pandemic and the shift to remote learning, we incorporated a data analysis section into a digital interactive notebook (Google slide set). In this lesson, we provided one guiding question that was accompanied by three sets of data and teachers could conduct this as a jigsaw activity or have students examine all three data sets.

A key feature of these lessons is that students summarize their answers to the questions paired with the graphs they were given and then communicate their findings to the class. To keep the class on task during presentations, students complete a summary table while they hear from other groups. After the data interpretation activity is complete, teachers then lead a discussion with the entire class to synthesize the findings presented and consider their broad impact as well as discuss future directions for research.

Make data accessible

With permission from the authors of the featured research papers, we worked to make the data presented in graphs more accessible to students by re-labeling axes and providing additional context for the experiments performed to collect the data (Figures 7-1 and 7-2). To encourage student interaction with the data, we also provided question prompts for each set of graphs that start with a review of key concepts, followed by specific questions about the data (Figure 7-3).

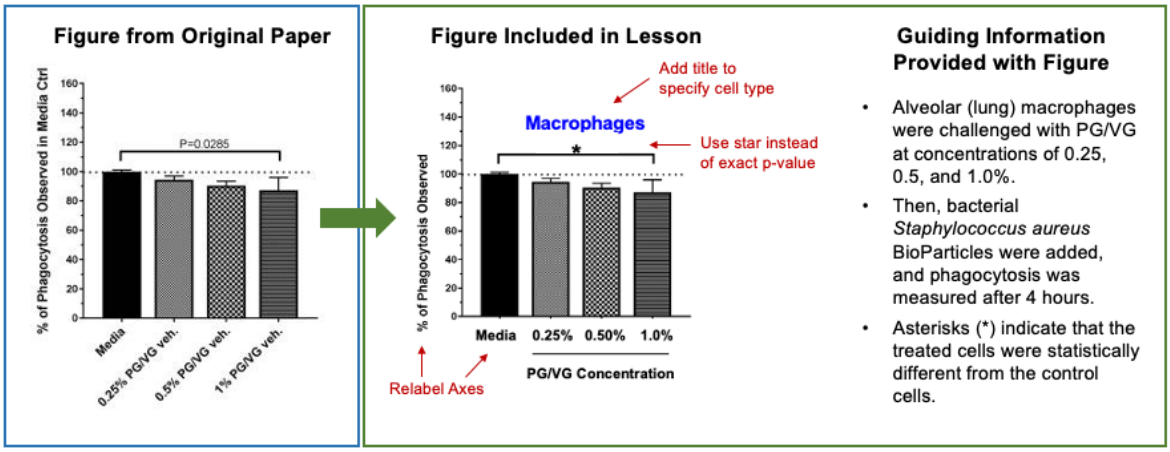


Figure 7-1. Example demonstrating modification of a graph from the primary literature to increase accessibility to students. Figure adapted from Clapp et al 2017 "Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function" in the *American Journal of Physiology Lung Cellular and Molecular Physiology* (19).

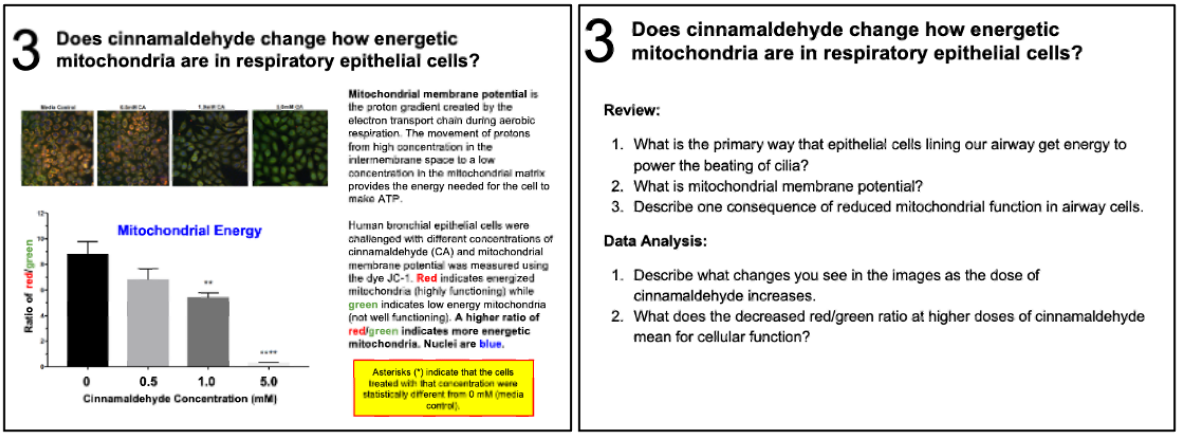


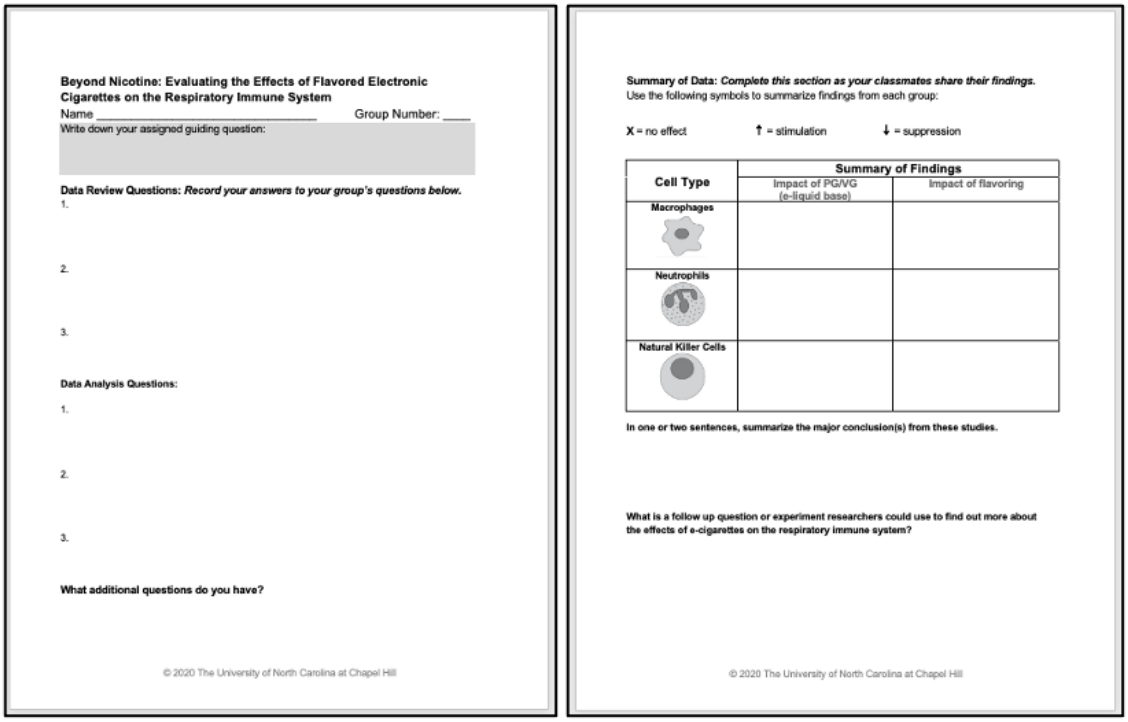
Figure 7-2. Slides from Lesson 2 demonstrate an additional example of context provided with graphs to aid in data interpretation and questions used to guide students through data analysis. Figures adapted from Clapp et al 2019 "Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function" in the American Journal of Physiology Lung Cellular and Molecular Physiology (17).

Develop supporting materials

To support teachers in implementing each lesson, we created a facilitator guide to convey learning objectives, provide background information, describe the activity procedure and opportunities to extend learning, and offer an answer key. In addition, we created a companion slide set which includes slides that can be used to introduce pertinent background on e-cigarettes, including how e-cigarettes work, what chemicals are found in e-cigarette liquids, and why scientists are interested in studying e-cigarette

flavoring chemicals. The slides also cover the source of the cells used in the experiments and describe how the experiments were designed (e.g., control and experimental groups). Lastly, the slides cover how to read bar graphs, including interpretation of error bars and significance markers. In addition, we created a worksheet/data summary table to guide students in synthesizing information and drawing their own conclusions (Figure 7-3).

A central component of developing these supporting materials was the creation and use of visual aids to depict experimental and cellular processes, which were developed using tools such as Adobe Illustrator and BioRender (biorender.com). Figure 7-4 shows a slide we developed using images from Biorender to enable students to build a model of a healthy respiratory epithelium in preparation for the data analysis activity.



Beyond Nicotine: Evaluating the Effects of Flavored Electronic Cigarettes on the Respiratory Immune System

Name _____ Group Number: ____

Write down your assigned guiding question:

Data Review Questions: ***Record your answers to your group's questions below.***

1.

2.

3.

Data Analysis Questions:

1.

2.

3.

What additional questions do you have?

Summary of Data: ***Complete this section as your classmates share their findings.***
Use the following symbols to summarize findings from each group:

X = no effect ↑ = stimulation ↓ = suppression

Cell Type	Summary of Findings	
	Impact of PG/VG (e-liquid base)	Impact of flavoring
Macrophages		
Neutrophils		
Natural Killer Cells		

In one or two sentences, summarize the major conclusion(s) from these studies.

What is a follow up question or experiment researchers could use to find out more about the effects of e-cigarettes on the respiratory immune system?

Figure 7-3. Student worksheet for facilitating the synthesis of data presented in Lesson 1. Cell images were generated in BioRender.

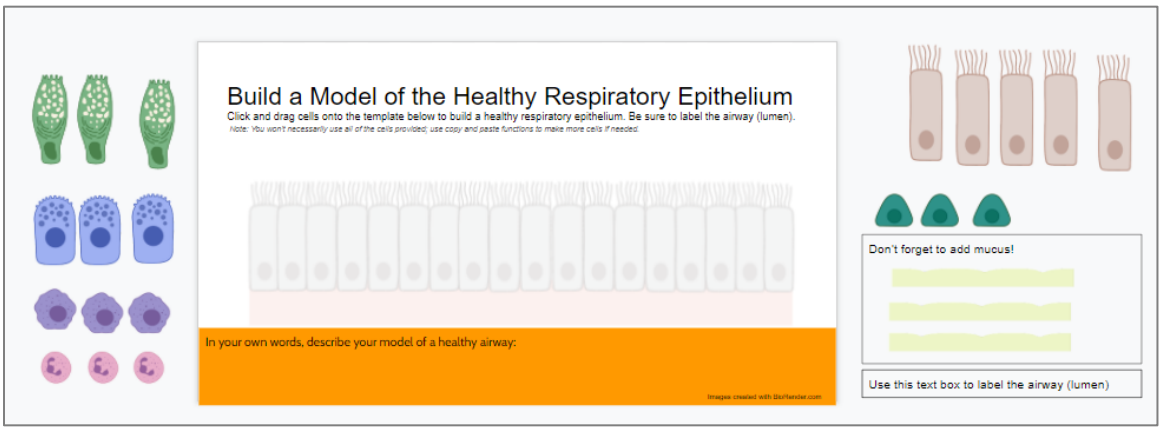


Figure 7-4. Interactive Google slide from Lesson 3 in which students click and drag cells to build a model of a healthy respiratory epithelium. Cell images were generated using biorender.com.

Acquire expert review

Once we had developed our lessons and ancillary materials, we solicited feedback from biomedical scientists, STEM outreach experts, and classroom teachers. The value of having a scientist involved from the beginning makes the scientific review process easier, and results in what we think is a more authentic and more robust educational activity. The value of having teachers involved in the expert review process as well is to ensure that the final lesson will be most useful to teachers, which will increase the likelihood of classroom adoption.

Pilot and refine lessons

In addition to acquiring expert review, we invited teachers to pilot the lessons and we piloted the lessons with students and teachers as well. Each time we received constructive feedback from teachers or students, we refined the lessons accordingly. Much of our refinements were related to guiding students through interpreting graphs.

Disseminate lessons

The lessons developed as a result of this collaboration have been disseminated at practitioner conferences such as the National Science Teaching Association, the National Association of Biology Teachers and the North Carolina Science Teachers Association. It is through these presentations that we expanded our teacher network and acquired additional feedback to improve subsequent revisions to lessons. Furthermore, given that this project was partially funded by the National Institute for Environmental Health Sciences (NIEHS), these lessons have also been shared with the larger NIEHS community via the Partnerships for Environmental Public Health Resource Center. The lessons materials are publicly available at: https://ie.unc.edu/cpes/environmental-health-resources/

Update lessons

A notable challenge in developing lessons around the emerging field of e-cigarette toxicology is that there is new research being published consistently and an ever-changing landscape of e-cigarette regulations, both of which could impact the background information provided with the lessons and the relevancy of the lessons themselves. In consideration of this challenge, we revisit the lessons each year to update the background information, and lesson 3, as a Google Slides deck, is a "living document" that we can continuously update, ensuring that teachers have access to the most up to date information.

The Lessons

This work resulted in three data interpretation activities, which are summarized in Figure 7-5. All three lessons share a common theme in that students learn about the experimental models and technologies being used to investigate the health effects of e-cigarettes by interacting with published scientific data showing how e-liquids and e-cigarette aerosols influence biological responses such as phagocytosis, cellular respiration, and inflammation. Teachers can assess student learning through a variety of mechanisms, including but not limited to collecting completed worksheets/data summary

tables, asking students to summarize their responses to their data analysis in writing or through a presentation, or by asking students to propose a new research question based on what they learned.

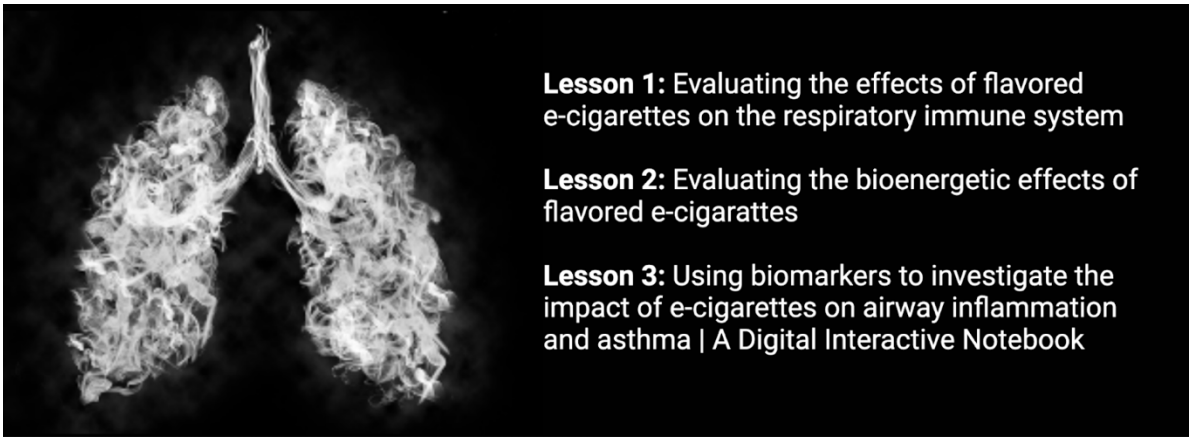


Figure 7-5. Three data interpretation lessons were developed as a result of this collaboration.

In Lesson 1, *"Evaluating the Effects of Flavored Electronic Cigarettes on the Respiratory Immune System"*, students learn about the experimental models and technologies being used to investigate the health effects of flavored e-cigarettes by interacting with published scientific data showing how flavored e-liquids and inhaled aerosols derived from these liquids influence biological function (e.g., phagocytosis) of respiratory immune cells.

In Lesson 2, "*Evaluating the Bioenergetic Effects of Flavored Electronic Cigarettes on Respiratory Immune Cells*", students interact with data showing the effects of cinnamon flavored e-liquid (containing the chemical cinnamaldehyde) on cilia beating and cellular metabolism in airway epithelial cells and consider implications for human health. For example, after evaluating evidence that cinnamaldehyde impairs cilia beating, students consider what these experimental findings might mean for the lungs of vapers, particularly their ability to clear debris and pathogens like SARS-CoV-2.

Lesson 3, *"Biomedical research on e-cigarettes & vaping | Investigating the impact of e-cigarettes on airway inflammation and asthma"*, was designed with a virtual classroom in mind and features a three-part digital interactive notebook that students can work through either independently or in pairs/small groups. In Part 1, students learn about the respiratory tract and the cells that make up the respiratory epithelium and how these cells behave during inflammation through building models of

healthy and inflamed epithelia. Students are also introduced to biomarkers, which are evaluated in Part 2. In Part 2, students interpret data from a study in which biomarkers of neutrophil activation, mucus production, and chemical detoxification were measured in the airways of smokers, non-smokers, and e-cigarette users to determine if e-cigarette use causes inflammation of the airway. In Part 3, students are introduced to an emerging research question that is relevant to many youth: Are asthmatics more susceptible to the respiratory health effects of e-cigarettes? Students learn about the features of asthma and are asked to design an experiment to determine whether e-cigarettes differently affect asthmatics. Similar to Lessons 1 and 2, teachers can customize which slides and/or parts of Lesson 3 they want to use based on their instructional goals.

In response to hearing teachers' requests for a wet-lab activity about vaping, we developed a data-based, qualitative wet-lab simulation in collaboration with the University of Rochester where students test simulated "sputum" samples from smokers, vapers and non-smokers/non-vapers to detect levels of select protein biomarkers. This activity uses inexpensive non-toxic materials (e.g., buffers and pH strips) and requires minimal preparation and clean-up. By investigating how e-cigarette use influences levels of biomarkers associated with inflammation among nonsmokers, cigarette smokers and e-cigarette users, students get a glimpse into this emerging area of research as they learn how scientists are working to understand how e-cigarettes and the chemicals they contain influence respiratory health.

Teacher Professional Development Series

To disseminate these lessons and promote adoption by teachers, we collaborated to develop a teacher professional development experience. Due to the pandemic, we had to pivot from our original plan to offer an in-person workshop with lab tours to instead offering a four part (6 hours total) virtual teacher professional development (PD) series, titled *Introducing students to the health effects of vaping on the respiratory system and to the multidisciplinary field of toxicology* that ran in February 2021. By providing content-rich teacher professional development that included presentations by diverse researchers and modeling of lesson implementation, we sought to increase teacher knowledge of e-

cigarettes and the research taking place to understand their health effects and to increase teacher preparedness to introduce this content in their classrooms. In addition, participating teachers were invited to pilot one or more lessons in spring 2021 and provide feedback to improve lessons for the 2021-2022 academic year. Recordings of presentations that were part of the teacher professional development series are publicly available on YouTube. Table 7-4 shows the outline for each PD session.

Table 7-4. Outline of virtual PD series.

Session title	Title of featured lesson	Titles of scientist "microtalks"
Session 1: Vaping and adolescents: How did we get here?	None	"Introduction to E-Cigarettes" "The Flavor Story" "Effects of E-Cigarette Salts" "Marketing Electronic Cigarettes to Adolescents"
Session 2: Cell, rodents, and humans: Experimental models for studying flavored e-cigarettes	Lesson 1: Evaluating the effects of flavored e-cigarettes on the respiratory immune system	"Cell Culture Models in Respiratory Toxicology" "Rodent Models in Respiratory Toxicology" "Human Subjects Research in Respiratory Toxicology"
Session 3: Flavored e-cigarettes & cell bioenergetics	Lesson 2: Evaluating the bioenergetic effects of flavored e-cigarettes	"Vaping: Is it the flavor of the decade or the decade of flavors?"
Session 4: E-cigarettes & lung health	Lesson 3: Using biomarkers to investigate the impact of e-cigarettes on airway inflammation and asthma | A digital interactive notebook and wet-lab simulation activity (extension)	"Vaping and Health Effects" "E-Cigarettes and Respiratory Infections"

We want to call out a number of features that we think contributed to the series' success for those wanting to integrate scientists and research findings into teacher PD activities:

1. *Provide teachers with direct access to researchers.* Involving researchers in professional development activities is not only an avenue for delivering up to date science content to teachers but also fosters a sense of professionalism among teachers. Teachers appreciate learning science content from researchers and are especially receptive when they are viewed by researchers as being capable of learning the content being presented and provided with opportunities to interpret findings versus being told experimental results. Anecdotally, we know that these authentic learning experiences provide teachers with real stories they can then share with their students, with some teachers reporting that access to the scientific data and the resulting stories they bring to class results in their being seen as credible sources of information by students.
2. *Offer 12–15-minute researcher "microtalks."* We encouraged researchers to keep their presentations short and to tailor their presentations to a teacher audience where there would be a range of background knowledge. We asked researchers to avoid use of unnecessary jargon and unexplained acronyms, and to consider using highly visual slides and easy to read/interpret graphs that could be used with students. We also invited each researcher to consider incorporating opportunities to make their presentation interactive, through the use of question prompts, poll questions, or short videos describing research activities or experimental set-ups.
3. *Feature diverse biomedical researchers.* Using a "microtalk" approach enabled us to offer more presentations in an effort to showcase the diverse biomedical research taking place to understand the health effects of vaping. By including toxicologists, geneticists, and air quality researchers in programming, we were able to emphasize not only the interdisciplinary research taking place to study the health effects of e-cigarettes but also the varying scopes of

research (cells, tissues, human subjects) to reinforce learning about research methods and experimental design.

4. *Model classroom activities.* In sessions 2, 3, and 4 of the PS series, we modeled the lessons for teachers as a means of encouraging classroom adoption and prompting subsequent discussion of classroom integration strategies. We adapted the data interpretation activities in Lessons 1 and 2 to the virtual environment by moving teachers into Zoom breakout rooms to discuss one of six guiding questions. For Lesson 3, teachers worked in small groups to complete part I of the digital interactive notebook (building a model of a healthy respiratory epithelium) and then we walked through Parts 2 and 3 together since we had modeled an approach to data analysis in previous sessions. For all breakout sessions, we circulated among breakout rooms to answer questions and monitor each group's progress.

Program Evaluation

Program evaluation was conducted to not only evaluate teacher satisfaction with each PD session and provide teachers with the opportunity to offer suggestions for improvement for each lesson but also to capture the extent to which participation in the series resulted in changes in teacher knowledge of e-cigarette research and preparedness to incorporate this research into instruction.

Teacher self-reported knowledge and preparedness regarding e-cigarettes prior to professional development series

Teachers (n=83) applying to participate in the February 2021 PD series were asked to self-report their knowledge of the following: the chemical composition of e-cigarette liquids and inhaled aerosols; the experimental models and technologies being used to study the health effects of flavored e-cigarettes; and emerging findings on the health effects of e-cigarette use or vaping. Figure 7-6 reveals a knowledge gap that we set out to address through this PD series by connecting teachers with scientists studying the health effects of e-cigarettes and with the data arising from this research.

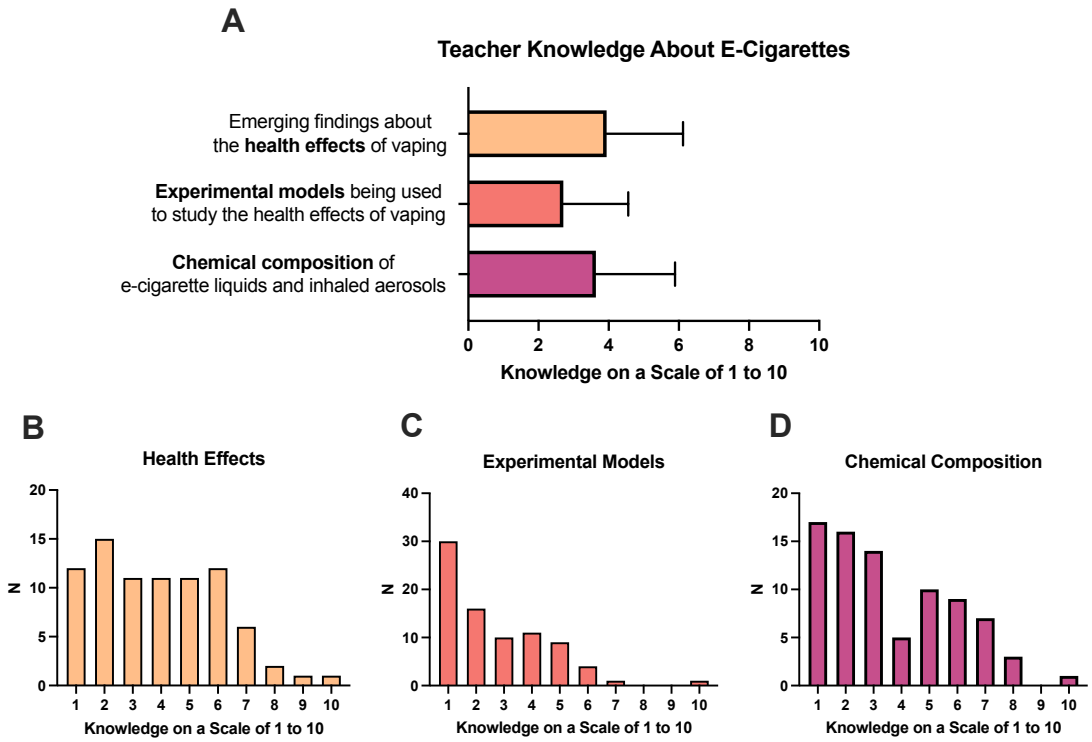


Figure 7-6. Teacher self-reported knowledge prior to participating in a 4-part professional development series. Teachers (n=83) applying to participate in the series were asked to rate their knowledge of the following items using a scale of 1 ("not knowledgeable") to 10 ("extremely knowledgeable"). Values for the subset of teachers who participated in the series were similar and are reported in Figure 8. A) Mean and standard deviation reported by topic. B-D) Distribution of responses (n= 82, one teacher did not provide responses to these questions).

80% of teachers reported they had incorporated e-cigarettes/vaping into life science instruction "to a little extent" or "not at all" (data not shown). 58% (n=83) reported that they were either "not at all" prepared or prepared "to a little extent" to facilitate classroom discussions about the health effects of vaping, and 81% indicated they were either "not at all" prepared or prepared "to a little extent" to speak authoritatively about the biomedical research taking place to study the health effects of vaping (Figure 7-7).

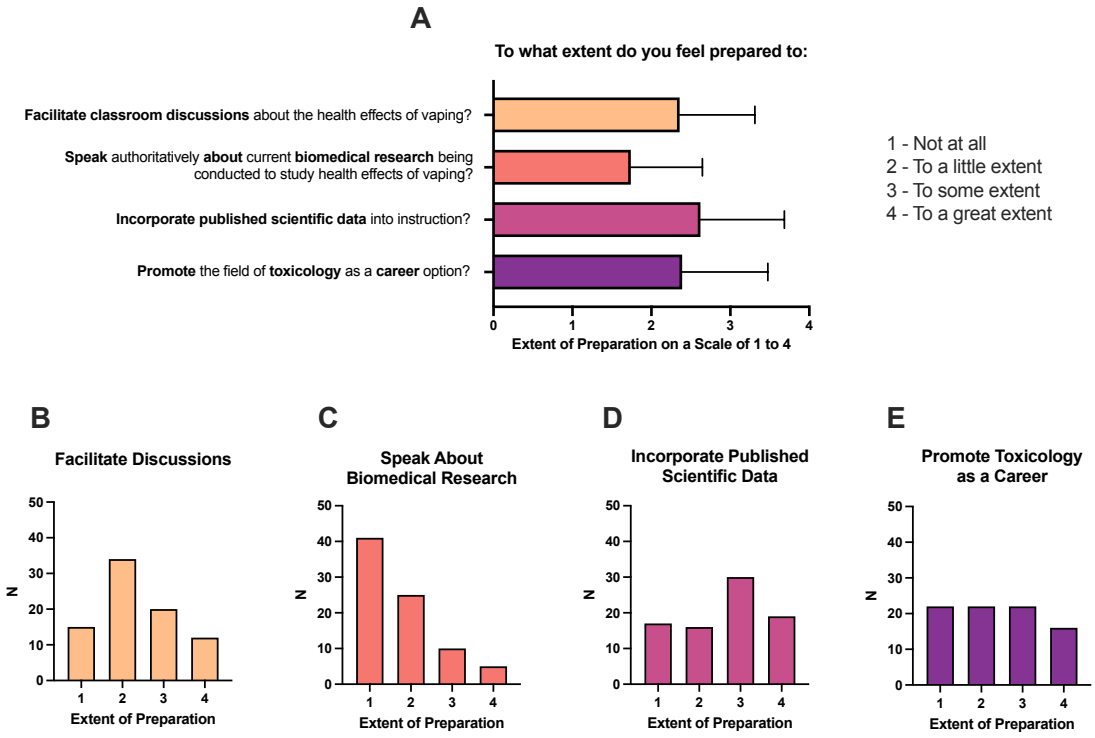


Figure 7-7. Teacher preparedness. Applicants were asked to rate their preparedness to promote toxicology as a career, incorporate published data into instruction, speak about biomedical research, and facilitate discussions. Answers were collected using a Likert scale and converted to numeric values for analysis (1 - "not at all", 2 - "to a little extent", 3 - "to some extent", 4 - "to a great extent"). A) Mean and standard deviation reported by topic. B-E) Distribution of responses (n = 82 except for "facilitate discussions" where n = 81).

These data suggest that the majority of teachers applying to participate in this PD series had not had the opportunity to acquire relevant content knowledge that would enable them to identify curriculum connections and successfully incorporate this topic into their instruction. Thirty-two of these teachers were invited to participate in this four-part PD series and 26 opted to participate in the series, with 20 (77%) attending all four sessions. With the exception of a high school chemistry teacher, all participants taught one or more life science courses ranging from biology to anatomy and physiology to biomedical science (e.g., Project Lead the Way). Twenty-three (88%) of teachers were high school teachers and the remaining three teachers taught biology courses at the college level. At the conclusion of each session, participants were asked to complete an online evaluation form. For each session, evaluation data revealed that the majority (95% or greater) of teachers reported the session increased their knowledge of featured

content, that the session was relevant to the content they teach, and that it gave them ideas for incorporating e-cigarette research into teaching (data not shown).

Changes in teacher knowledge and preparedness

On the session 4 evaluation, teachers were once again asked to rate their knowledge of e-cigarettes and preparedness to discuss biomedical research in their classrooms. Figure 8 depicts self-reported changes in knowledge and preparedness as a result of participating in this PD series. These results reveal that as a result of participating in this PD series teachers felt more knowledgeable about the chemical composition of e-cigarettes, experimental models being used to study e-cigarettes, and emerging findings in the field of e-cigarette toxicology (Figure 7-8A). Data also revealed that teachers felt more prepared to facilitate discussions about e-cigarettes and speak about current biomedical research as a result of participating in this PD series (Figure 7-8B). However, the series did not significantly increase the extent to which teachers' felt prepared to incorporate published data into instruction or to promote toxicology as a career (Figure 7-8B). We hypothesize that these findings could be because teachers who registered for the series already had some level of comfort with and/or interest in published scientific data and toxicology, and a desire to learn more about e-cigarettes and current biomedical research attracted them to the professional development series.

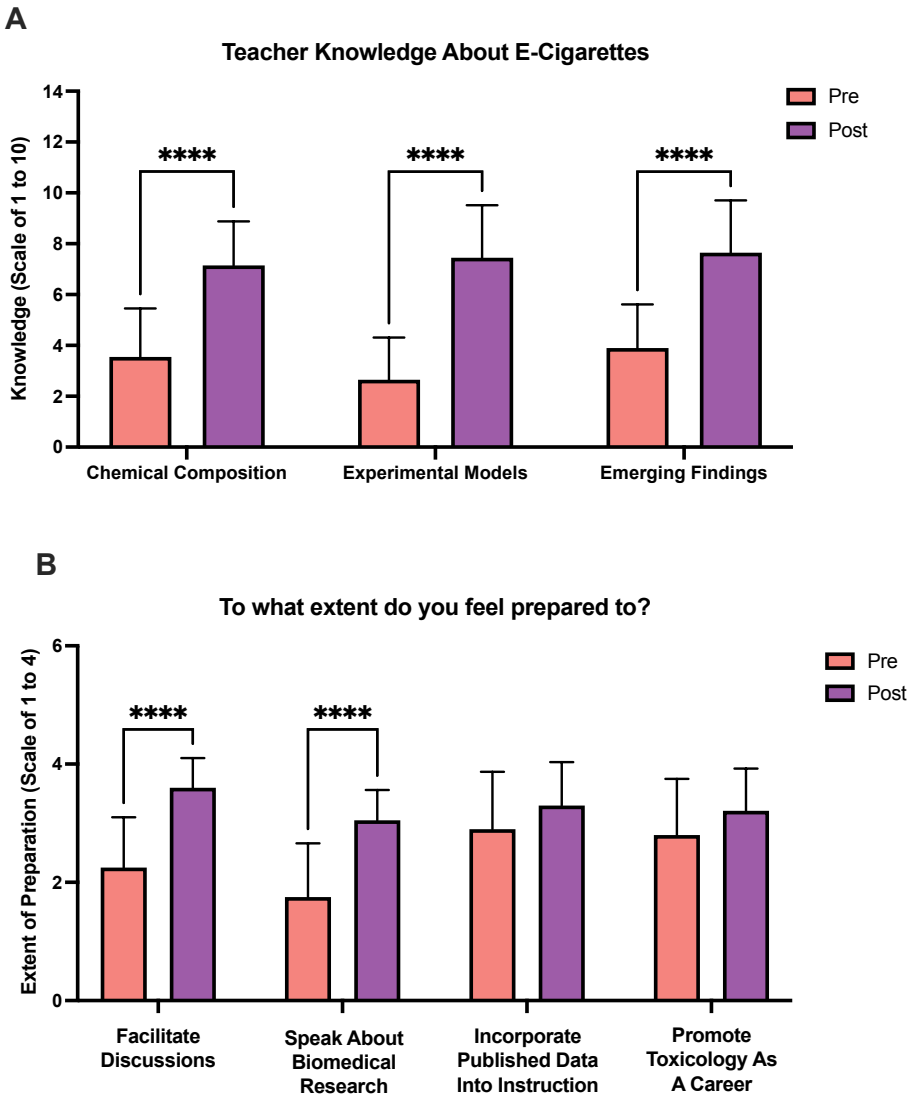


Figure 7-8. Teacher self-reported changes in content knowledge and preparedness as a result of participating in the PD series. A) Teachers were asked to rate their knowledge on a scale from 1 ("not at all knowledgeable") to 10 ("extremely knowledgeable") and their B) preparedness on a scale from 1 ("not at all") to 4 ("to a great extent") as part of their program application (January 2021) and on the final program evaluation (session 4, February 2021). Mean and standard deviation are reported for matched pre/post responses (n=20). Pre-post data were compared using a matched two-way analysis of variance with Sidak's multiple comparisons test (**** $p < 0.0001$).

Plans to implement activities in classrooms

Following each session that featured a lesson, teachers were asked to indicate on the session evaluation whether they planned to incorporate the featured lesson. Interestingly, a much higher percentage of teachers planned to incorporate Lesson 2 (flavorings & bioenergetics) and Lesson 3 (biomarkers & asthma) than Lesson 1 (flavorings & immune cell function) (Figure 7-9). This finding suggests that the material presented in lessons 2 and 3 may have been more directly aligned with teachers' curricula. Alternatively, teachers may have become more comfortable with the content as the professional development series progressed, with this increased comfort contributing to an increased percentage who planned to incorporate the lessons into their classrooms.

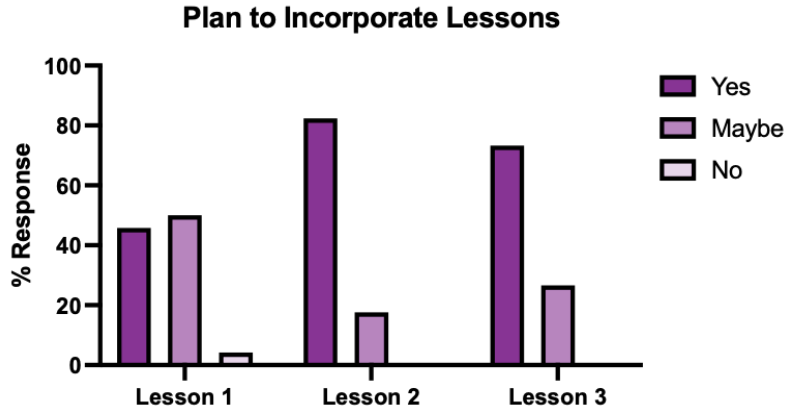


Figure 7-9. Teacher self-reported plans to incorporate featured lessons into instruction. At the conclusion of each session featuring a lesson (sessions 2-4), teachers were asked to indicate on their program evaluation if they planned to incorporate the featured lesson (Lesson 1, n=24; Lesson 2, n = 17; Lesson 3, n = 15).

Additional feedback from professional development series attendees

Additional comments submitted by teachers as part of the evaluation support the quantitative impact on knowledge and preparedness described above. These comments reflect that teachers found the series valuable, appreciated the way the series was organized, and thought the presentations were understandable and accessible:

1. “The whole series was fantastic. The lessons are relevant and accessible and the speakers were very interesting. I'm excited to use some or all of what I've learned and the lessons in my teaching!”
2. “This has been one of the best professional development experiences. The information is relevant & up to date. The speakers were well-prepared and excited about sharing their research. They neither over-simplified their presentations, nor over-complicated them with specific technical language that only someone in that field would understand. There was a good mix of background knowledge & activities for the classroom which could be tailored to meet the needs of different courses at different levels of instruction.”
3. “I greatly appreciate the level of expertise and professional content and delivery of this workshop. It's great to be challenged and learn new and relevant information to share with my students.”
4. “Incredible series… Every talk and resource is incredibly meaningful and I can see it fitting nicely in many different versions of my curriculum. [It is] something students will find interesting and meaningful in their day to day which really increases engagement.”

Despite overwhelmingly positive comments and evaluation results for the PD series, teachers also offered constructive criticism, including a desire for more hands-on lab activities and for data to be accessible to students in less-advanced courses. This feedback is incredibly valuable in considering how to build upon the team’s work thus far:

1. “Would love to see more labs/activities to use with the students.”

2. “I found the research very interesting, and it was great to hear the talks… but most of the lessons provided are more appropriate for an advanced biology or AP bio course at the high school level and really wouldn't be appropriate… for general bio or middle school life science (the two courses I teach). Many of the graphs and information really need to be adjusted to different levels (differentiated for student reading and graphing analysis).”

Discussion

Taken together, this program description highlights the effectiveness of collaborative efforts to develop high school science lessons and accompanying teacher professional development focused on e-cigarette research. Our work demonstrates how scientists can help to bridge the gap between current research and curriculum standards by partnering with science education experts and teachers to bring current science into the classroom. Feedback on the lessons from teachers and classrooms shows that these lessons are engaging for students and provide a mechanism for reinforcing key concepts and science practices through the lens of a topic that is highly relevant to their lives. Evaluation data demonstrate the effectiveness of combining delivery of science content with data interpretation activities to increase teacher knowledge about e-cigarettes and preparedness to incorporate e-cigarette science in their classrooms.

However, these lessons were developed with data analysis and data literacy in mind, so they are very data-rich and are most appropriate for advanced learners, including honors, AP, and college-level courses. There is a critical need to develop similar lessons that are accessible to students at all levels through simplification of data presented and the messages conveyed in the lessons. In our lessons, we provide examples for how teachers can pare down the lessons themselves, but lessons that are “classroom ready” and do not require additional work to reframe at a lower level will increase adoption by teachers. Developing such lessons is incredibly challenging to do in a scientifically accurate way, particularly for a relatively new field such as e-cigarette toxicology, where health effects are not completely understood and are not as black-and-white as they are with an exposure like smoking. Careful consideration will be

needed when developing these more-accessible lessons to ensure they are understandable and that they accurately reflect the scientific literature.

Though these lessons and professional development activities are specific to e-cigarette research, they serve as examples for how biomedical scientists in all fields can partner with science education experts to develop standards-aligned curricula. Partnerships with scientists and teachers during lesson development process are critical to the success of such curricula to provide educational content that is real, relevant, and robust. For science education experts, partnering with scientists results in educational materials and learning experiences that provide deeper engagement among science learners by providing authentic and relevant content. By interacting with published data and having the opportunity to use science and engineering practices, learners get a glimpse of the exciting research taking place in today's biomedical laboratories.

For scientists, partnering with science education experts, including teachers themselves, can enhance research endeavors and science communication skills. Scientists are accustomed to sharing their research with those in their specialized field, often through highly technical presentations and papers, but can find it challenging to explain their research to a non-expert audience and to communicate research impact clearly. Because of this, scientific research often does not reach the stakeholders impacted directly by the research findings. Engaging in outreach, including the collaborative lesson design process and teacher professional development activities described here, provides scientists with the opportunity to practice communicating their science and provides an avenue through which relevant stakeholders can be engaged and even contribute to the education of the scientist. For e-cigarettes in particular, visiting classrooms and interacting with students was useful for informing researchers about e-cigarette devices being used by adolescents, current vaping trends among adolescents, and questions that students and teachers have about vaping, which in turn can directly influence e-cigarette research. This was the experience of Dr. Ilona Jaspers, who accompanied the authors to a number of outreach events that entailed dialogue with students, teachers, and parents. "I gained a lot of insights into the actual public health problem through real life stories…….getting feedback from the community has informed our

research and made it truly translational," says Jaspers when reflecting on the value of outreach to her research endeavors. Engaging in outreach can also cultivate science communication skills and positively affect scientists' presentation skills and grant writing skills within their specialized fields.

For co-author and design team member Ms. Hickman, this collaboration serves as an example for how others in biomedical science graduate programs could approach their research training not only from a scientific perspective but also with the goal of ensuring their research findings reaches populations that can benefit from this knowledge. The motivation driving this collaboration between a scientist and a science education expert is to cultivate a network of life science teachers better prepared to integrate e-cigarette research into instruction in an effort to respond to the youth vaping epidemic. Through the development of engaging, science-based, and health-centered curricula and teacher professional development, this work brings a relevant topic and authentic learning opportunities to classrooms filled with members of a population highly vulnerable to the impacts of vaping: adolescents.

Development of a Vaping Questionnaire for Use in Clinical Settings

Clinical assessment of e-cigarette use can be challenging due to the wide variety of e-cigarette devices and ways in which they are used, including frequency, nicotine strength, flavor, and potential for dual use with marijuana or cannabidiol vaping, as discussed in Chapters 3 and 4. Currently, few tools exist to facilitate discussions between clinicians and their patients about e-cigarette use (20), and inclusion of vaping history in patient screening and electronic health records is rare (21, 22). Importantly, language used by clinicians and patients to discuss e-cigarettes is often different – for example, clinicians may only ask patients about tobacco use, and patients may not consider vaping to be equivalent to tobacco use (21, 23). These discussions are particularly important for interactions with adolescents given that using e-cigarettes increases risk for subsequent smoking initiation (6-8).

To address this need, I developed a visual questionnaire, which includes photos of different device types, sections to indicate vaping frequency, strength, flavor, and chemical components, and a nicotine equivalency chart so that e-cigarette and cigarette nicotine content can be compared (Figures 7-10 and 7-11). The questionnaire was recently reviewed by a focus group of young adults, who overwhelmingly supported its use and reported that it was an engaging, effective way to prevent vaping. A co-author publication describing implementation of this vaping questionnaire is currently under peer review, and the questionnaire is publicly available for any clinician to use in their practice. Aside from utility in primary care clinics, this questionnaire also has potential for use in collection of more accurate vaping histories in clinical studies of e-cigarette users and as a model for fields to include as part of patients' vaping histories in electronic health records.

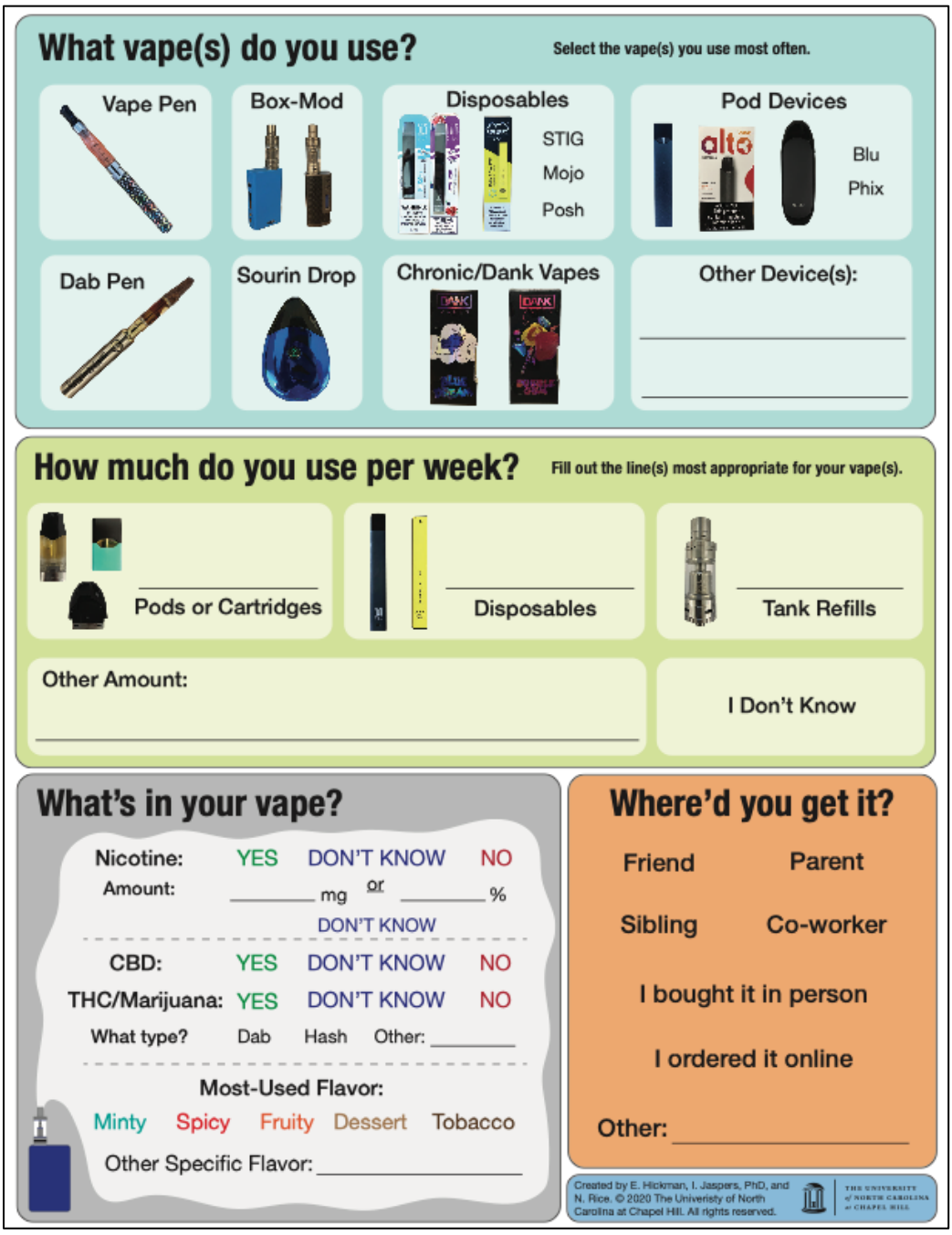
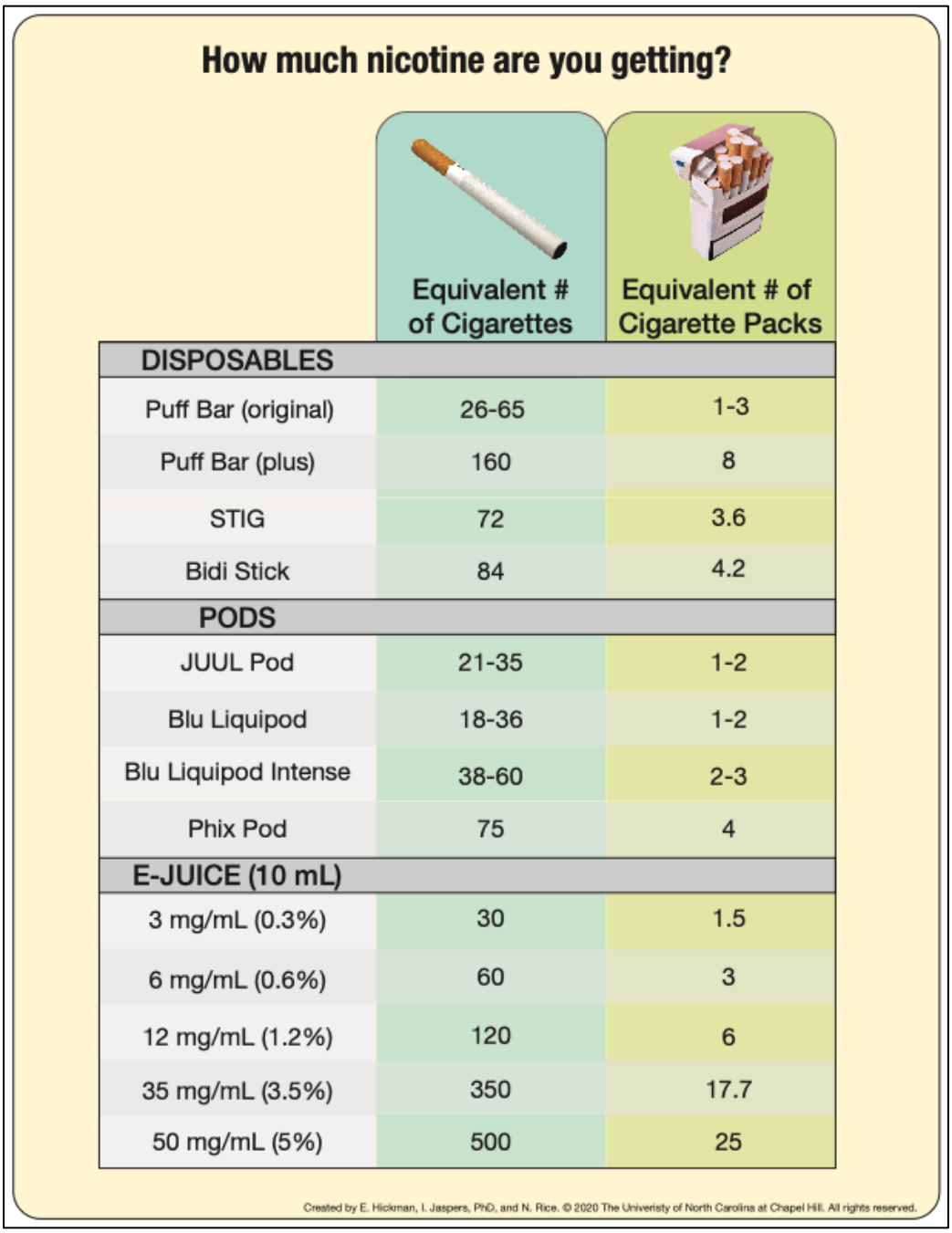


Figure 7-10. Front of vaping questionnaire.



How much nicotine are you getting?

	Equivalent # of Cigarettes	Equivalent # of Cigarette Packs
DISPOSABLES		
Puff Bar (original)	26-65	1-3
Puff Bar (plus)	160	8
STIG	72	3.6
Bidi Stick	84	4.2
PODS		
JUUL Pod	21-35	1-2
Blu Liquipod	18-36	1-2
Blu Liquipod Intense	38-60	2-3
Phix Pod	75	4
E-JUICE (10 mL)		
3 mg/mL (0.3%)	30	1.5
6 mg/mL (0.6%)	60	3
12 mg/mL (1.2%)	120	6
35 mg/mL (3.5%)	350	17.7
50 mg/mL (5%)	500	25

Figure 7-11. Back of vaping questionnaire.

REFERENCES

1. **Creamer MR, Wang TW, Babb S, Cullen KA, Day H, Willis G, Jamal A, and Neff L**. Tobacco Product Use and Cessation Indicators Among Adults - United States, 2018. *MMWR Morb Mortal Wkly Rep* 68: 1013-1019, 2019.

2. **Park-Lee E, Ren C, Sawdey MD, Gentzke AS, Cornelius M, Jamal A, and Cullen KA**. Notes from the Field: E-Cigarette Use Among Middle and High School Students - National Youth Tobacco Survey, United States, 2021. *MMWR Morb Mortal Wkly Rep* 70: 1387-1389, 2021.

3. **Wang TW, Neff LJ, Park-Lee E, Ren C, Cullen KA, and King BA**. E-cigarette Use Among Middle and High School Students - United States, 2020. *MMWR Morb Mortal Wkly Rep* 69: 1310-1312, 2020.

4. **Obisesan OH, Osei AD, Uddin SMI, Dzaye O, Mirbolouk M, Stokes A, and Blaha MJ**. Trends in e-Cigarette Use in Adults in the United States, 2016-2018. *JAMA Intern Med* 180: 1394-1398, 2020.

5. **Hair EC, Barton AA, Perks SN, Kreslake J, Xiao H, Pitzer L, Leventhal AM, and Vallone DM**. Association between e-cigarette use and future combustible cigarette use: Evidence from a prospective cohort of youth and young adults, 2017–2019. *Addictive Behaviors* 112: 106593, 2021.

6. **Loukas A, Marti CN, and Harrell MB**. Electronic nicotine delivery systems use predicts transitions in cigarette smoking among young adults. *Drug Alcohol Depend* 231: 109251, 2021.

7. **Hammond D, Reid JL, Cole AG, and Leatherdale ST**. Electronic cigarette use and smoking initiation among youth: a longitudinal cohort study. *Cmaj* 189: E1328-e1336, 2017.

8. **O'Brien D, Long J, Quigley J, Lee C, McCarthy A, and Kavanagh P**. Association between electronic cigarette use and tobacco cigarette smoking initiation in adolescents: a systematic review and meta-analysis. *BMC Public Health* 21: 954, 2021.

9. **U.S. Department of Health and Human Services**. E-Cigarette Use Among Youth and Young Adults. A Report of the Surgeon General Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2016.

10. **Machluf Y, Gelbart H, Ben-Dor S, and Yarden A**. Making authentic science accessible-the benefits and challenges of integrating bioinformatics into a high-school science curriculum. *Brief Bioinform* 18: 145-159, 2017.

11. **Wefer SH, and Sheppard K**. Bioinformatics in high school biology curricula: a study of state science standards. *CBE Life Sci Educ* 7: 155-162, 2008.

12. **National Research Council**. *A Framework for K-12 Science Education: Practices, Crosscutting Concepts, and Core Ideas*. Washington, DC: The National Academies Press, 2012.

13. *Next Generation Science Standards: For States, By States*. Washington, DC: The National Academies Press, 2013.

14. **College Board**. AP Biology Course and Exam Description 2020.

15. **Vapring Prevention Resource**. https://vapingprevention.org/science.

16. **Manduca CA, and Mogk DW**. Using Data in Undergraduate Science Classrooms Carlton College, 2002.

17. **Hotaling L, McDonnell J, Ferraro C, Florio K, and Lichtenwalner S**. Educating with Data. In: *Exemplary Practices in Marine Science Education: A Resource for Practitioners and Researchers*, edited by Fauville G, Payne DL, Marrero ME, Lantz-Andersson A, and Crouch F. Cham: Springer International Publishing, 2019, p. 207-223.

18. **Bybee RW**. 5E Instructional Model: Creating Teachable Moments 2015.

19. **Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I**. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278-L292, 2017.

20. **Hadland SE, and Chadi N**. Through the Haze: What Clinicians Can Do to Address Youth Vaping. *J Adolesc Health* 66: 10-14, 2020.

21. **LeLaurin JH, Theis RP, Thompson LA, Tan ASL, Young-Wolff KC, Carter-Harris L, Shenkman EA, and Salloum RG**. Tobacco-Related Counseling and Documentation in Adolescent Primary Care Practice: Challenges and Opportunities. *Nicotine Tob Res* 22: 1023-1029, 2020.

22. **Pepper JK, Gilkey MB, and Brewer NT**. Physicians' Counseling of Adolescents Regarding E-Cigarette Use. *J Adolesc Health* 57: 580-586, 2015.

23. **Gorukanti A, Delucchi K, Ling P, Fisher-Travis R, and Halpern-Felsher B**. Adolescents' attitudes towards e-cigarette ingredients, safety, addictive properties, social norms, and regulation. *Prev Med* 94: 65-71, 2017.

CHAPTER 8: CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS[3]

Framing the E-Cigarette Debate

Key questions in the e-cigarette debate

E-cigarettes have created a highly complex public health challenge. Their uncontrolled entry into the United States market and subsequent expansion to thousands of different products, alongside their popularity with young, never smokers, has raised many questions regarding their overall impact on society. This debate is further complicated by the political and financial power of the tobacco industry (1-4), which, given the decrease in cigarette smoking over time (5), is highly motivated to see e-cigarettes succeed so that the industry can survive. Some of the central topics that are important to consider in determining whether e-cigarettes overall are "good" or "bad" are 1) can e-cigarettes be used as a tool for harm reduction and/or smoking cessation? 2) does using e-cigarettes result in adverse health effects? And 3) how do e-cigarettes influence tobacco initiation and prevalence of tobacco use in the future?

While the focus of this dissertation was to address whether e-cigarettes cause adverse health effects, it is important to acknowledge these other societal questions when considering e-cigarettes holistically and to put the research presented in this dissertation into societal context. E-cigarettes were originally marketed as a safer alternative to conventional cigarette smoking (6-8), and many proponents of e-cigarettes argue that the benefits of e-cigarettes in facilitating smoking cessation and in reducing exposure to harmful chemicals outweigh their negative health effects and tobacco product initiation in youth (9, 10).

[3] Parts of this chapter previously appeared as part of an article in *Tobacco Control.* Reproduced from "Evolving chemical landscape of e-cigarette, 2021" by Hickman, E. and Jaspers, I., Copyright 2021 with permission from the BMJ Publishing Group Ltd.

E-cigarettes as a tool for smoking cessation

Studies assessing the association between e-cigarette use and smoking cessation have found mixed results. In a recently published longitudinal study using data from the U.S. Population Assessment of Tobacco and Health (PATH) study, smokers using e-cigarettes had an increased likelihood of quitting smoking in comparison with smokers who did not use e-cigarettes (11). However, this study did not compare e-cigarette use to other smoking cessation tools (nicotine replacement therapy (NRT), smoking cessation medication) and did not assess nicotine dependence or smoking relapse. Other longitudinal analyses of PATH data have demonstrated that smokers who switched to e-cigarettes or any other tobacco product when trying to quit smoking had higher relapse rates than those who did not use any tobacco products to quit (12), that smokers who used e-cigarettes did not have a higher rate of smoking abstinence than non-e-cigarette users (13), and that smokers who use e-cigarettes had significantly lower nicotine abstinence rates than those who did not use e-cigarettes (13). Additional longitudinal and cross-sectional studies within the U.S. support these data and demonstrate that using e-cigarettes to quit smoking is not associated with smoking reduction or cessation (14-16) and may be associated with higher nicotine consumption (16). Studies conducted in the United Kingdom suggest that e-cigarettes may be slightly more effective than NRT and less effective than smoking cessation medication for smoking cessation, but whether this improvement is significant varies across studies (17, 18).

Randomized controlled trials in the United States, the United Kingdom, Australia, and New Zealand have shown that subjects who were assigned to nicotine vaping had increased smoking abstinence in comparison with NRT and/or non-nicotine vaping (19-22); however, the difference between nicotine vaping and nicotine patches was not statistically significant in one study (21), and a higher percentage of the nicotine vaping product group was still using the treatment at follow up compared with the NRT group (19). Notably, the randomized controlled trial that showed the greatest difference between smoking abstinence in the e-cigarette group in comparison with the NRT group provided all participants with weekly behavioral support for at least four weeks (22), suggesting that behavioral support is a key component in facilitating smoking cessation with e-cigarettes.

Overall, these studies show that e-cigarettes can aid in smoking cessation, but they are not significantly more effective than existing tools for smoking cessation, particularly in the United States, and their use for smoking cessation may result in continued nicotine dependence and smoking relapse. Therefore, the benefits of e-cigarette use for smoking cessation, in the context of already available treatments, may not outweigh the public health cost of youth tobacco product initiation.

Comparing e-cigarettes with cigarettes

E-cigarettes generally expose users to fewer cancer-causing chemicals than cigarettes (23-25). Because of this, the question "Do e-cigarettes cause adverse health effects?" is often presented in the context of asking "Are e-cigarettes safer than smoking?". While this is a relevant perspective to consider when assessing harm reduction, it interferes with assessment of e-cigarettes in comparison with the healthiest alternative – not smoking or not using any tobacco/nicotine products. Chronic smoking is a highly toxic exposure associated with many pathologies, including cancer, reproductive effects, chronic obstructive pulmonary disease, and cardiovascular disease (26, 27), so determining whether vaping is safer than smoking is not a health-protective benchmark. It is also important to consider that the chemical exposure created by e-cigarettes is unique from the chemical exposure created by cigarettes (28-33). Thus, it is likely that the effects of e-cigarettes are unique from cigarettes, rather than on the same spectrum of effects, making it difficult to compare their inhalation toxicities. This concept is supported by our findings in Chapters 3 and 4, where we demonstrated that the respiratory microbiome and central airway biomarkers are shifted in e-cigarette users and cigarette smokers differently in comparison with non-smokers/non-vapers.

Effects of E-Cigarettes *In Vitro* on Human Neutrophils and Macrophages

Contribution of this dissertation

The respiratory tract and lungs represent biologically complex tissues comprised of many different cell types, including airway epithelial cells, connective tissue cells, and immune cells (34) . Determining the effects of e-cigarettes and their chemical component on the different cells in the respiratory tract and the immune cells that patrol the airways is of great interest because knowing how e-cigarette chemicals affect these cell types is informative in understanding the pathologies that may arise from e-cigarette use. Cells of airway host defense, such as macrophages and neutrophils, are particularly important due to their roles in mediating the inflammatory state in the airways and in responding to inhaled pathogens (35).

Prior to 2019, only two studies had directly assessed the effect of e-cigarettes on neutrophil function *in vitro* (36). Hwang et al. demonstrated nicotine-dependent impairment in human neutrophil antimicrobial activity against methicillin-resistant *Staphylococcus aureus* following e-cigarette condensate exposure (36), and Clapp et al. demonstrated cytotoxicity and impairment in human neutrophil phagocytosis and NETosis following exposure to flavored, non-nicotine e-liquids, particularly those containing the flavoring chemical cinnamaldehyde (37). In Chapter 2, we expanded upon these studies to investigate whether specific flavoring chemicals commonly found in e-liquids affected neutrophil function (38). We found that benzaldehyde and benzaldehyde PG acetal impaired neutrophil phagocytosis of *S. aureus* and that cinnamaldehyde, ethyl vanillin, benzaldehyde, and benzaldehyde PG acetal impaired neutrophil oxidative burst at sub-cytotoxic doses (38). Importantly, all of the flavoring chemicals that impaired neutrophil function were aromatic aldehydes or their derivatives, suggesting that e-cigarette flavoring chemicals could be tested and regulated by chemical class. Since this study was published, additional studies have demonstrated the potential for e-cigarettes and their chemical components to adversely affect neutrophil function (39, 40), complementing the findings presented in Chapter 2.

Altogether, this body of research indicates that e-cigarettes can impair human neutrophil function, which could result in increased susceptibility to airway infections and altered airway immune homeostasis, both potential contributors to airway disease. However, additional research is needed to understand the mechanisms underlying these functional impairments and how the effects of individual chemical components (flavors, nicotine) synergize when cells are exposed to the complex chemical mixture found in e-liquids and e-cigarette aerosols. Furthermore, the data presented in Chapter 6 demonstrating sex-dependent differences in neutrophil phagocytosis and correlation between phagocytosis and bioenergetic parameters emphasize the need for future studies. Additional investigations into sex-dependent differences in neutrophil responses to toxicants such as e-cigarette aerosol and investigations into mechanisms underlying these observed sex differences are warranted.

Relative to neutrophils, the effects of e-cigarettes on macrophages *in vitro* have been studied more extensively, with many studies demonstrating altered cellular function following exposure to e-liquid, e-liquid components, or e-cigarette condensate (37, 40-43). However, these studies used cell lines and/or murine cells, which do not always accurately recapitulate human macrophage phenotypes. These studies also used earlier generation e-cigarettes (vape pens, box mods), and none of these studies exposed cells to e-cigarette aerosol directly at air-liquid interface. Therefore, we set out to expose human bronchoalveolar lavage cells to e-cigarette aerosol from newer, nicotine-salt containing e-cigarettes and to assess cellular effects. The COVID-19 pandemic limited our ability to obtain cells for these experiments, necessitating the use of human monocyte-derived macrophages (hMDMs). However, in developing a protocol to culture hMDMs, we found a lack of standardized protocols and thorough baseline characterization of polarized hMDMs in the literature, with only one study evaluating a wide range of endpoints to assess polarization (44). In Chapter 5, we thoroughly characterized polarized hMDMs, including assessment of bioenergetic parameters, which allowed us to compare our polarized hMDMs to primary human bronchoalveolar lavage and induced sputum macrophages (45). Importantly, phenotypes were similar in hMDMs differentiated on Transwell membranes, confirming that these cells could be used for *in vitro* experiments with e-cigarette aerosol. Although our polarized hMDMs recapitulated many

phenotypes observed in primary human airway macrophages, they were not a perfect model. Two recent studies describe the development of culturing protocols for murine cells that more accurately recapitulate resident lung macrophages (46, 47), and there is a need to develop similar protocols for human cells.

Limitations of this dissertation and future needs

In vitro studies are an invaluable tool to understand the effects of e-cigarettes and their chemical components on different cell types because they allow for controlled investigation of dose-response and cause-and effect-relationships. Progress has been made in understanding the chemical composition of e-liquids (31, 32, 48-50), whether these chemicals transfer to the aerosol (48, 51), and to what extent degradation products and new chemicals are generated through aerosolization (33, 52-60), which is highly useful when selecting exposures for *in vitro* studies. However, one of the most significant limitations in designing these studies and interpreting the data generated from them is that the dose of e-cigarette aerosol deposited in different regions of the respiratory system during vaping is poorly characterized. Without knowing what dose of e-cigarette aerosol or concentration of individual chemical components cells are exposed to *in vivo*, it is difficult to extrapolate findings demonstrated *in vitro* to human health effects *in vivo.* Thus, future studies are needed to establish the amount of e-cigarette aerosol deposited in human airways during a session of vaping. It is likely that deposition varies widely between users and is dependent on device, e-liquid composition, use frequency, and puffing topography; however, understanding biologically relevant doses, even if this includes a wide range of possible doses, will be critical to ensure maximal impact of future *in vitro* studies.

The cell types used in this dissertation (primary human blood neutrophils and monocyte-derived macrophages) were chosen to mimic *in vivo* biology as closely as possible; however, following up on the study presented in Chapter 2 with human blood neutrophils primed as they would be before they extravasate into the airway from circulation (e.g., with a chemoattractant such as IL-8) would improve biological relevance. Similarly, exposing human bronchoalveolar lavage macrophages and induced sputum cells to e-cigarette aerosol at air-liquid interface would improve biological relevance in

comparison with monocyte-derived macrophages, though these primary airway cells can be difficult to obtain regularly and in sufficient numbers to perform controlled experiments.

Effects of E-Cigarettes *In Vivo* on Human Respiratory Immune Homeostasis

Contribution of this dissertation

Previous studies demonstrated that e-cigarettes impair respiratory host defense and increase bacterial adherence *in vitro* and *in vivo* (37, 38, 61-64), but the effects of e-cigarette use on the respiratory microbiome and host-microbiota interactions had not been studied. In Chapter 3, we present the first study to determine whether e-cigarette use is associated with respiratory microbiome dysbiosis using samples from the nasal cavity. Our study is also unique in the analysis approach we took, which was designed with the high dimensional, sparse datasets characteristic of microbiome studies and our relatively small sample size in mind. We found that e-cigarette use is associated with a shift in the composition of the nasal microbiome and that this shift is unique from that observed in smokers. Some of the taxa that were enriched in e-cigarette users, such as *Lactobacillus* and *Staphylococcus*, have been previously associated with airway diseases (65-67), though studies of the nasal microbiome and direct influence of specific taxa on human health are limited. We then found that nasal microbial dysbiosis was associated with shifts in the balance of immune proteins involved in host-microbe interaction, such as lactoferrin, neutrophil elastase, and IL-8 in matched nasal lavage fluid. Notably, we also observed striking sex differences in the nasal microbiome, which have not been previously reported. Overall, these results provide evidence that e-cigarette use is associated with altered microbial communities in the respiratory tract; however, additional studies are needed to determine cause-and-effect relationships (e.g., do e-cigarettes directly impact the microbiome, host, or both to result in our observed effects), to determine specific implications for human health (e.g., increased risk for infection or microbiome-mediated chronic disease), and to assess the lower airway microbiome in the context of e-cigarette exposure.

Noting the heterogeneity in device types in our samples collected from e-cigarette users and the observation that previous studies on biomarkers of respiratory immune homeostasis included only

subjects who used 3rd generation e-cigarettes such as vape pens and box mods (40, 68), we next wanted to understand how currently popular devices such as JUUL and disposables (4th generation) may affect the respiratory immune system differently from previous generations of e-cigarettes. In Chapter 4, we demonstrated that users of 4th generation e-cigarettes have unique changes in induced sputum cell composition and soluble mediator milieu in comparison with 3rd generation e-cigarette users, non-smokers/non-vapers, and smokers. These differences were the most pronounced in the comparison between 3rd and 4th generation e-cigarette users, and 4th generation e-cigarette users had significantly lower levels of many soluble mediators, including sICAM-1, CRP, IFN-γ, uteroglobin, and MCP-1, indicative of suppressed immune responses. These findings provide striking evidence that the respiratory effects of 4th generation e-cigarettes are unique from 3rd generation e-cigarettes, which has implications both for future studies of e-cigarette toxicity and for the regulation of e-cigarettes. Additionally, data in Chapter 6 demonstrate that central airway macrophages from 4th generation e-cigarette users have increased mitochondrial membrane potential in comparison with non-smokers/non-vapers *ex vivo*, potentially indicative of disrupted cellular function.

A key characteristic of both human *in vivo* studies presented in this dissertation (Chapters 3 and 4) is that the analyses approaches employed were carefully selected to answer the biological questions of interest while accounting for influence of demographic variables and ensuring appropriate statistical treatment of the large number of variables measured per sample. Traditional approaches that test significant differences between experimental groups for each endpoint one-by-one run the risk of missing important features in the data, such as sex differences or overall shifts in biomarker milieu, which may not be evident when using a traditional analysis approach. For example, in our study of the nasal microbiome (Chapter 3), exposure group differences were not apparent until sex differences were adjusted for. Had we not explored the impact of demographic variables on the nasal microbiome, we would have missed an important observation of sex differences (which are not frequently reported), and this effect would have confounded our assessment of the effects of e-cigarettes and cigarettes on the nasal microbiome. In this study, we also approach data analysis from a ratio perspective, in which ratios of

abundance of specific taxa to each other or concentrations of proteins are the primary variables of interest. This approach attempts to account for the compositional nature of microbiome data (in which all taxa are measured relative to other taxa) and reflect the complex interactions of signaling molecules within the mucosa. In Chapter 4, we included a machine learning approach alongside a variable-by-variable approach, which provided additional evidence that the overall soluble immune mediator milieu in 4th generation e-cigarette users was unique from all other groups. Machine learning models can detect patterns and structure in data that are not always immediately evident when manually inspecting data, and this can be particularly important when considering complex biological systems and datasets that contain a high amount of variability (69). Given the increasing ease and affordability of measuring many biological endpoints in a single sample, analyses such those described in this dissertation will become increasingly necessary to extract patterns from high-dimensional clinical datasets, consider novel hypotheses, and improve robustness of analyses.

Limitations of this dissertation and future needs

Clinical studies using samples from human subjects are incredibly powerful in understanding the effects of e-cigarettes, particularly due to challenges in accurately modeling e-cigarette exposures *in vitro* and with rodent models. However, clinical studies present unique challenges, including recruiting and retaining subjects, accurately characterizing e-cigarette and other substance use, and accounting for demographic differences between smoking, e-cigarette, and non-smoking/non-vaping populations. Interindividual variation is much higher in human clinical studies than in controlled *in vitro* and rodent *in vivo* studies, which can make it difficult to detect significant changes in endpoints of interest. There is also significant variation in device and flavor usage among e-cigarette users, making it difficult to assess associations between specific use parameters in the cohort sizes used in Chapters 3 and 4. Therefore, larger cohort studies are needed in the future to ensure statistical power and to elucidate the relationship between e-cigarette use history (e.g., flavor, frequency of use, length of use, prior smoking) and biological endpoints. Additionally, Chapters 3 and 4 are cross-sectional studies and are only able to

demonstrate associations. Therefore, longitudinal studies of e-cigarette users are needed to assess cause-and-effect relationships between e-cigarette use and changes in biomarkers of host defense directly. It is entirely possible that, given the short period of time that e-cigarettes have been available and popular, most e-cigarette users have not used e-cigarettes for a sufficient length of time to begin experiencing overt pathologies associated with their e-cigarette use. Finally, dual use of cannabis (vaping or smoking) and e-cigarettes is highly prevalent, particularly in younger populations (70-73), so future studies that both adjust for this additional exposure and explore the potential for augmented biological effects associated with dual cannabis and e-cigarette use are needed.

Other Key Knowledge Gaps and Challenges in E-Cigarette Toxicology

E-cigarettes and susceptible subpopulations

Chapters 2, 3, and 4 used samples collected from healthy adult subjects. However, people with diseases such as asthma and COPD, which already involve disrupted respiratory immune homeostasis, may have different responses to e-cigarette aerosol in comparison with people who do not have a pre-existing respiratory disease. Current e-cigarette research is largely focused on understanding the effects of e-cigarettes and the mechanisms underlying these effects in healthy adult populations, but there is a need to expand study populations to include those with pre-existing respiratory diseases and to younger populations, as these responses may be different from those observed in healthy adult cells or populations.

E-cigarette toxicokinetics

Additionally, little is known about the toxicokinetics and biotransformation of the chemicals in e-cigarette aerosol once they are deposited in the airways. A large body of literature exists that characterizes nicotine pharmacokinetics (74, 75), but there are few studies evaluating absorption and detoxification of other chemicals found in e-cigarette aerosols, such as PG/VG, flavoring chemicals, and organic acids. Interestingly, one study demonstrated that CYP2A6, which metabolizes nicotine, is inhibited by aromatic aldehyde flavoring chemicals found in e-liquids (particularly benzaldehyde and cinnamaldehyde),

suggesting that nicotine metabolism could be impaired in e-cigarette users who vape e-liquids containing those flavoring chemicals (76). Understanding these toxicokinetics will be informative in predicting long-term effects of e-cigarette exposure, selecting biologically relevant doses for *in vitro* studies using e-cigarettes, understanding the interactions between e-liquid chemical components and detoxification enzymes, and assessing the potential for systemic effects of these chemicals.

Emerging vaping products and chemical components

The e-cigarette market is fast-moving, which presents challenges for researchers investigating health effects associated with vaping. Recently, cannabis vaping (both THC and CBD) has become increasingly popular, and little is known about the effects of cannabis vaping on respiratory health. Recent studies have shown that cannabinoids can modulate immune responses (77, 78), and additional studies that address the health effect associated with cannabis vaping are critically needed.

Furthermore, the increased popularity of disposable e-cigarettes following the FDA's restrictions on flavored pod-based e-cigarettes has also added complexity to assessing the respiratory toxicity of e-cigarettes. A recent study by Omaiye et al. showed that both mint and menthol JUUL (pod) and Puff (disposable) e-cigarettes contain high concentrations of menthol; however, Puff e-cigarettes had higher levels of synthetic coolants and pulegone, a compound found in mint oil, suggesting that Puff products have the potential to be toxicologically "worse" than JUUL (31). These chemicals may contribute to the palatability of e-cigarettes and may augment their toxicity, so these chemicals, and others that may serve similar functions, will need to be included in any future e-cigarette toxicity testing and regulations.

Overall Impact and Convergence Science Approach to Toxicology

The research presented in this dissertation demonstrates that e-cigarettes can disrupt respiratory immune homeostasis and that e-cigarette device type is an important consideration when assessing the respiratory toxicity of e-cigarettes. This dissertation has yielded scholarly products relevant to computational biologists, toxicologists, immunologists, policy makers, clinicians, K-12 educators, and the

public. The research presented here is unique in that spanned the development of high school biology lessons and a vaping questionnaire (Chapter 7) to cutting-edge high dimensional data analysis to address the important public health challenges surrounding vaping. This dissertation serves as an example for how others in biomedical science can approach their research topics not only from a scientific perspective but also with the goal of ensuring their research reaches the groups that their research directly impacts. One way to do this is through an approach known as convergence (79). Convergence science is the expansion of collaborations beyond traditional multi- and inter-disciplinary interactions to achieve significant progress in a specific area of societal need (Figure 8-1). Convergence science will be integral to pushing toxicology and public health forward, and for addressing ongoing needs related to e-cigarettes. Researchers from fields such as biomedicine, sociology, and science communication must work together to ensure that e-cigarette research reaches key stakeholders and that progress is made in controlling the vaping epidemic by making health-protective decisions.

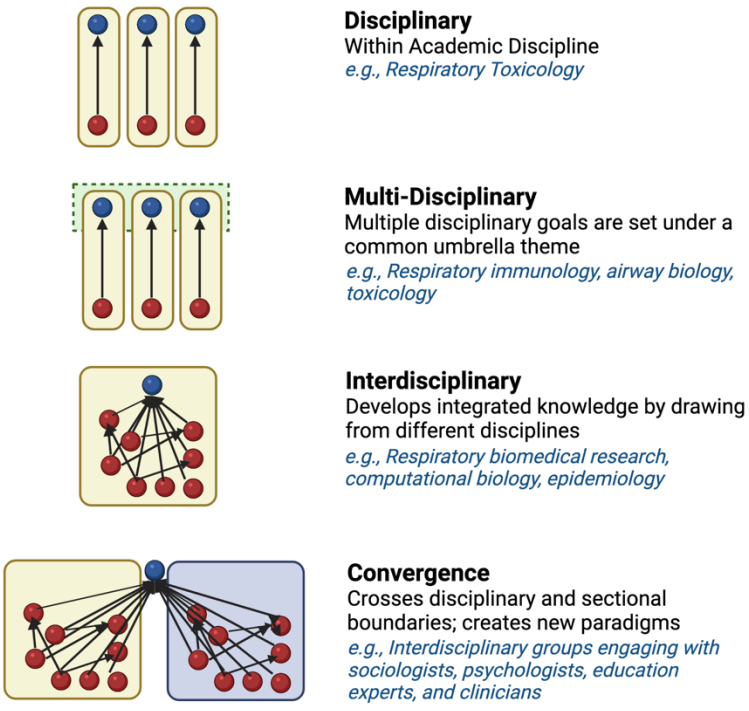


Figure 8-1. Convergence approach to problem solving and knowledge development. Examples of the convergence approach in the context of e-cigarettes are provided in blue. Schematic developed by Ilona Jaspers with modifications by Elise Hickman. Created with biorender.com.

E-Cigarette Regulation: Where to go from here?

An increasing number of studies, including those in this dissertation, support the notion that e-cigarettes are not without health effects. In response to this body of scientific work and the youth vaping epidemic (80), the FDA has attempted to regulate e-cigarettes through bans on flavored e-cigarette pods (81). However, the complexity of e-liquid formulations and rapid evolution of different e-cigarette devices, coupled with regulations focused on narrowly defined e-cigarette products, has resulted in exploitation of regulatory loopholes and few substantive changes in overall e-cigarette product availability, appeal, and use. Future regulations governing e-cigarette devices, flavors, and chemical components will need to be rigorous and thorough, with careful consideration of ways in which companies may attempt to evade regulations by making small changes to their products. This foresight will be needed to prevent the constant emergence of new device types and formulations, which presents a significant challenge in assessing toxicity of e-cigarettes and understanding their public health impact. Ultimately, a paradigm shift in the way e-cigarettes are brought to the market is needed so that assessment of potential e-cigarette toxicity is not constantly lagging behind consumer behavior. Like most other consumer products brought to market, the chemical composition and potential toxicity of e-cigarettes should be evaluated prior to public sale of these devices, allowing the public to be informed about the risks of e-cigarette use and preventing newer, potentially more toxic products to gain traction.

REFERENCES

1. **Kaplan B, Cherukupalli R, Welding K, Kennedy RD, and Cohen JE**. The youth e-cigarette epidemic: New estimates of JUUL Labs' revenue from youth users in the US. *Tob Induc Dis* 19: 33, 2021.

2. **U.S. Department of Health and Human Services**. Preventing tobacco use among youth and young adults. Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention, 2012.

3. **Holden C, and Lee K**. Corporate Power and Social Policy: The Political Economy of the Transnational Tobacco Companies. *Glob Soc Policy* 9: 328-354, 2009.

4. **Savell E, Gilmore AB, and Fooks G**. How does the tobacco industry attempt to influence marketing regulations? A systematic review. *PLoS One* 9: e87389, 2014.

5. Cigarette Smoking Among U.S. Adults Hits All-Time Low. United State Centers for Disease Control 2019.

6. **Richardson A, Ganz O, Stalgaitis C, Abrams D, and Vallone D**. Noncombustible Tobacco Product Advertising: How Companies Are Selling the New Face of Tobacco. *Nicotine & Tobacco Research* 16: 606-614, 2014.

7. **Grana RA, and Ling PM**. "Smoking revolution": a content analysis of electronic cigarette retail websites. *Am J Prev Med* 46: 395-403, 2014.

8. **Paek HJ, Kim S, Hove T, and Huh JY**. Reduced harm or another gateway to smoking? source, message, and information characteristics of E-cigarette videos on YouTube. *J Health Commun* 19: 545-560, 2014.

9. **Balfour DJK, Benowitz NL, Colby SM, Hatsukami DK, Lando HA, Leischow SJ, Lerman C, Mermelstein RJ, Niaura R, Perkins KA, Pomerleau OF, Rigotti NA, Swan GE, Warner KE, and West R**. Balancing Consideration of the Risks and Benefits of E-Cigarettes. *American Journal of Public Health* 111: 1661-1672, 2021.

10. Do the Benefits of Electronic Cigarettes Outweigh the Risks? *Can J Hosp Pharm* 71: 44-47, 2018.

11. **Glasser AM, Vojjala M, Cantrell J, Levy DT, Giovenco DP, Abrams D, and Niaura R**. Patterns of E-cigarette Use and Subsequent Cigarette Smoking Cessation Over 2 Years (2013/2014-2015/2016) in the Population Assessment of Tobacco and Health Study. *Nicotine Tob Res* 23: 669-677, 2021.

12. **Pierce JP, Chen R, Kealey S, Leas EC, White MM, Stone MD, McMenamin SB, Trinidad DR, Strong DR, Benmarhnia T, and Messer K**. Incidence of Cigarette Smoking Relapse Among Individuals Who Switched to e-Cigarettes or Other Tobacco Products. *JAMA Netw Open* 4: e2128810, 2021.

13. **Chen R, Pierce JP, Leas EC, White MM, Kealey S, Strong DR, Trinidad DR, Benmarhnia T, and Messer K**. Use of Electronic Cigarettes to Aid Long-Term Smoking Cessation in the United States: Prospective Evidence From the PATH Cohort Study. *Am J Epidemiol* 189: 1529-1537, 2020.

14. **Selya AS, Dierker L, Rose JS, Hedeker D, and Mermelstein RJ**. The Role of Nicotine Dependence in E-Cigarettes' Potential for Smoking Reduction. *Nicotine Tob Res* 20: 1272-1277, 2018.

15. **Grana RA, Popova L, and Ling PM**. A longitudinal analysis of electronic cigarette use and smoking cessation. *JAMA Intern Med* 174: 812-813, 2014.

16. **Bowler RP, Hansel NN, Jacobson S, Graham Barr R, Make BJ, Han MK, O'Neal WK, Oelsner EC, Casaburi R, Barjaktarevic I, Cooper C, Foreman M, Wise RA, DeMeo DL, Silverman EK, Bailey W, Harrington KF, Woodruff PG, and Drummond MB**. Electronic Cigarette Use in US Adults at Risk for or with COPD: Analysis from Two Observational Cohorts. *J Gen Intern Med* 32: 1315-1322, 2017.

17. **McDermott MS, East KA, Brose LS, McNeill A, Hitchman SC, and Partos TR**. The effectiveness of using e-cigarettes for quitting smoking compared to other cessation methods among adults in the United Kingdom. *Addiction* 116: 2825-2836, 2021.

18. **Brown J, Beard E, Kotz D, Michie S, and West R**. Real-world effectiveness of e-cigarettes when used to aid smoking cessation: a cross-sectional population study. *Addiction* 109: 1531-1540, 2014.

19. **Bonevski B, Manning V, Wynne O, Gartner C, Borland R, Baker AL, Segan CJ, Skelton E, Moore L, Bathish R, Chiu S, Guillaumier A, and Lubman DI**. QuitNic: A Pilot Randomized Controlled Trial Comparing Nicotine Vaping Products With Nicotine Replacement Therapy for Smoking Cessation Following Residential Detoxification. *Nicotine Tob Res* 23: 462-470, 2021.

20. **Cobb CO, Foulds J, Yen MS, Veldheer S, Lopez AA, Yingst JM, Bullen C, Kang L, and Eissenberg T**. Effect of an electronic nicotine delivery system with 0, 8, or 36 mg/mL liquid nicotine versus a cigarette substitute on tobacco-related toxicant exposure: a four-arm, parallel-group, randomised, controlled trial. *Lancet Respir Med* 9: 840-850, 2021.

21. **Bullen C, Howe C, Laugesen M, McRobbie H, Parag V, Williman J, and Walker N**. Electronic cigarettes for smoking cessation: a randomised controlled trial. *Lancet* 382: 1629-1637, 2013.

22. **Hajek P, Phillips-Waller A, Przulj D, Pesola F, Myers Smith K, Bisal N, Li J, Parrott S, Sasieni P, Dawkins L, Ross L, Goniewicz M, Wu Q, and McRobbie HJ**. A Randomized Trial of E-Cigarettes versus Nicotine-Replacement Therapy. *The New England journal of medicine* 380: 629-637, 2019.

23. **Stephens WE**. Comparing the cancer potencies of emissions from vapourised nicotine products including e-cigarettes with those of tobacco smoke. *Tob Control* 2017.

24. **Shahab L, Goniewicz ML, Blount BC, Brown J, McNeill A, Alwis KU, Feng J, Wang L, and West R**. Nicotine, Carcinogen, and Toxin Exposure in Long-Term E-Cigarette and Nicotine Replacement Therapy Users: A Cross-sectional Study. *Ann Intern Med* 166: 390-400, 2017.

25. **Shein M, and Jeschke G**. Comparison of Free Radical Levels in the Aerosol from Conventional Cigarettes, Electronic Cigarettes, and Heat-Not-Burn Tobacco Products. *Chemical research in toxicology* 32: 1289-1298, 2019.

26. **Onor IO, Stirling DL, Williams SR, Bediako D, Borghol A, Harris MB, Darensburg TB, Clay SD, Okpechi SC, and Sarpong DF**. Clinical Effects of Cigarette Smoking: Epidemiologic Impact and Review of Pharmacotherapy Options. *Int J Environ Res Public Health* 14: 2017.

27. **Office of the Surgeon General (US), and Office on Smoing and Health (US)**. The Health Consequences of Smoking: A Report of the Surgeon General Atlanta (GA): Centers for Disease Control and Prevention (US), 2004.

28. **Aherrera A, Olmedo P, Grau-Perez M, Tanda S, Goessler W, Jarmul S, Chen R, Cohen JE, Rule AM, and Navas-Acien A**. The association of e-cigarette use with exposure to nickel and chromium: A preliminary study of non-invasive biomarkers. *Environmental research* 159: 313-320, 2017.

29. **Kosmider L, Sobczak A, Prokopowicz A, Kurek J, Zaciera M, Knysak J, Smith D, and Goniewicz ML**. Cherry-flavoured electronic cigarettes expose users to the inhalation irritant, benzaldehyde. *Thorax* 71: 376-377, 2016.

30. **Zhu SH, Sun JY, Bonnevie E, Cummins SE, Gamst A, Yin L, and Lee M**. Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tob Control* 23 Suppl 3: iii3-9, 2014.

31. **Omaiye EE, Luo W, McWhirter KJ, Pankow JF, and Talbot P**. Flavour chemicals, synthetic coolants and pulegone in popular mint-flavoured and menthol-flavoured e-cigarettes. *Tobacco Control* tobaccocontrol-2021-056582, 2021.

32. **Omaiye EE, McWhirter KJ, Luo W, Tierney PA, Pankow JF, and Talbot P**. High concentrations of flavor chemicals are present in electronic cigarette refill fluids. *Sci Rep* 9: 2468, 2019.

33. **Escobar YH, Nipp G, Cui T, Petters SS, Surratt JD, and Jaspers I**. In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System. *Chemical research in toxicology* 2020.

34. **Franks TJ, Colby TV, Travis WD, Tuder RM, Reynolds HY, Brody AR, Cardoso WV, Crystal RG, Drake CJ, Engelhardt J, Frid M, Herzog E, Mason R, Phan SH, Randell SH, Rose MC, Stevens T, Serge J, Sunday ME, Voynow JA, Weinstein BM, Whitsett J, and Williams MC**. Resident cellular components of the human lung: current knowledge and goals for research on cell phenotyping and function. *Proceedings of the American Thoracic Society* 5: 763-766, 2008.

35. **Iwasaki A, Foxman EF, and Molony RD**. Early local immune defences in the respiratory tract. *Nat Rev Immunol* 17: 7-20, 2017.

36. **Hwang JH, Lyes M, Sladewski K, Enany S, McEachern E, Mathew DP, Das S, Moshensky A, Bapat S, Pride DT, Ongkeko WM, and Crotty Alexander LE**. Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria. *J Mol Med (Berl)* 94: 667-679, 2016.

37. **Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I**. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278-L292, 2017.

38. **Hickman E, Herrera CA, and Jaspers I**. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chemical research in toxicology* 32: 982-985, 2019.

39. **Corriden R, Moshensky A, Bojanowski CM, Meier A, Chien J, Nelson RK, and Crotty Alexander LE**. E-cigarette use increases susceptibility to bacterial infection by impairment of human

neutrophil chemotaxis, phagocytosis, and NET formation. *Am J Physiol Cell Physiol* 318: C205-c214, 2020.

40. **Ghosh A, Coakley RD, Ghio AJ, Muhlebach MS, Esther CR, Jr., Alexis NE, and Tarran R**. Chronic E-Cigarette Use Increases Neutrophil Elastase and Matrix Metalloprotease Levels in the Lung. *Am J Respir Crit Care Med* 2019.

41. **Muthumalage T, Prinz M, Ansah KO, Gerloff J, Sundar IK, and Rahman I**. Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine. *Front Physiol* 8: 1130, 2017.

42. **Scott A, Lugg ST, Aldridge K, Lewis KE, Bowden A, Mahida RY, Grudzinska FS, Dosanjh D, Parekh D, Foronjy R, Sapey E, Naidu B, and Thickett DR**. Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages. *Thorax* 73: 1161-1169, 2018.

43. **Ween MP, Whittall JJ, Hamon R, Reynolds PN, and Hodge SJ**. Phagocytosis and Inflammation: Exploring the effects of the components of E-cigarette vapor on macrophages. *Physiol Rep* 5: e13370, 2017.

44. **Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, and Fantino E**. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am J Respir Cell Mol Biol* 53: 676-688, 2015.

45. **Lavrich KS, Speen AM, Ghio AJ, Bromberg PA, Samet JM, and Alexis NE**. Macrophages from The Upper and Lower Human Respiratory Tract Are Metabolically Distinct. *Am J Physiol Lung Cell Mol Physiol* 2018.

46. **Gorki AD, Symmank D, Zahalka S, Lakovits K, Hladik A, Langer B, Maurer B, Sexl V, Kain R, and Knapp S**. Murine ex vivo Cultured Alveolar Macrophages Provide a Novel Tool to Study Tissue-Resident Macrophage Behavior and Function. *Am J Respir Cell Mol Biol* 2021.

47. **Luo M, Lai W, He Z, and Wu L**. Development of an Optimized Culture System for Generating Mouse Alveolar Macrophage-like Cells. *J Immunol* 207: 1683-1693, 2021.

48. **El-Hellani A, El-Hage R, Baalbaki R, Salman R, Talih S, Shihadeh A, and Saliba NA**. Free-Base and Protonated Nicotine in Electronic Cigarette Liquids and Aerosols. *Chemical research in toxicology* 28: 1532-1537, 2015.

49. **Omaiye EE, McWhirter KJ, Luo W, Pankow JF, and Talbot P**. High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations. *Chemical research in toxicology* 32: 1058-1069, 2019.

50. **Tierney PA, Karpinski CD, Brown JE, Luo W, and Pankow JF**. Flavour chemicals in electronic cigarette fluids. *Tob Control* 25: e10-15, 2016.

51. **Erythropel HC, Davis LM, de Winter TM, Jordt SE, Anastas PT, O'Malley SS, Krishnan-Sarin S, and Zimmerman JB**. Flavorant-Solvent Reaction Products and Menthol in JUUL E-Cigarettes and Aerosol. *Am J Prev Med* 57: 425-427, 2019.

52. **Uchiyama S, Noguchi M, Sato A, Ishitsuka M, Inaba Y, and Kunugita N**. Determination of Thermal Decomposition Products Generated from E-Cigarettes. *Chemical research in toxicology* 33: 576-583, 2020.

53. **Sleiman M, Logue JM, Montesinos VN, Russell ML, Litter MI, Gundel LA, and Destaillats H**. Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ Sci Technol* 50: 9644-9651, 2016.

54. **Son Y, Weisel C, Wackowski O, Schwander S, Delnevo C, and Meng Q**. The Impact of Device Settings, Use Patterns, and Flavorings on Carbonyl Emissions from Electronic Cigarettes. *Int J Environ Res Public Health* 17: 2020.

55. **Reilly SM, Bitzer ZT, Goel R, Trushin N, and Richie JP**. Free Radical, Carbonyl, and Nicotine Levels Produced by Juul Electronic Cigarettes. *Nicotine Tob Res* 21: 1274-1278, 2019.

56. **Li Y, Burns AE, Tran LN, Abellar KA, Poindexter M, Li X, Madl AK, Pinkerton KE, and Nguyen TB**. Impact of e-Liquid Composition, Coil Temperature, and Puff Topography on the Aerosol Chemistry of Electronic Cigarettes. *Chemical research in toxicology* 34: 1640-1654, 2021.

57. **Cirillo S, Urena JF, Lambert JD, Vivarelli F, Canistro D, Paolini M, Cardenia V, Rodriguez-Estrada MT, Richie JP, Jr., and Elias RJ**. Impact of electronic cigarette heating coil resistance on the production of reactive carbonyls, reactive oxygen species and induction of cytotoxicity in human lung cancer cells in vitro. *Regulatory toxicology and pharmacology : RTP* 109: 104500, 2019.

58. **Klager S, Vallarino J, MacNaughton P, Christiani DC, Lu Q, and Allen JG**. Flavoring Chemicals and Aldehydes in E-Cigarette Emissions. *Environmental Science & Technology* 51: 10806-10813, 2017.

59. **Azimi P, Keshavarz Z, Lahaie Luna M, Cedeno Laurent JG, Vallarino J, Christiani DC, and Allen JG**. An Unrecognized Hazard in E-Cigarette Vapor: Preliminary Quantification of Methylglyoxal Formation from Propylene Glycol in E-Cigarettes. *Int J Environ Res Public Health* 18: 2021.

60. **Park HR, Vallarino J, O'Sullivan M, Wirth C, Panganiban RA, Webb G, Shumyatcher M, Himes BE, Park JA, Christiani DC, Allen J, and Lu Q**. Electronic cigarette smoke reduces ribosomal protein gene expression to impair protein synthesis in primary human airway epithelial cells. *Sci Rep* 11: 17517, 2021.

61. **Martin EM, Clapp PW, Rebuli ME, Pawlak EA, Glista-Baker E, Benowitz NL, Fry RC, and Jaspers I**. E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 311: L135-144, 2016.

62. **Clapp PW, Lavrich KS, van Heusden CA, Lazarowski ER, Carson JL, and Jaspers I**. Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *Am J Physiol Lung Cell Mol Physiol* 316: L470-l486, 2019.

63. **Miyashita L, Suri R, Dearing E, Mudway I, Dove RE, Neill DR, Van Zyl-Smit R, Kadioglu A, and Grigg J**. E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells. *Eur Respir J* 51: 2018.

64. **Madison MC, Landers CT, Gu BH, Chang CY, Tung HY, You R, Hong MJ, Baghaei N, Song LZ, Porter P, Putluri N, Salas R, Gilbert BE, Levental I, Campen MJ, Corry DB, and Kheradmand F**. Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine. *J Clin Invest* 129: 4290-4304, 2019.

65. **Kumpitsch C, Koskinen K, Schöpf V, and Moissl-Eichinger C**. The microbiome of the upper respiratory tract in health and disease. *BMC Biol* 17: 87, 2019.

66. **Liu CM, Price LB, Hungate BA, Abraham AG, Larsen LA, Christensen K, Stegger M, Skov R, and Andersen PS**. Staphylococcus aureus and the ecology of the nasal microbiome. *Science Advances* 1: e1400216, 2015.

67. **Sakr A, Brégeon F, Mège J-L, Rolain J-M, and Blin O**. Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. *Frontiers in microbiology* 9: 2419-2419, 2018.

68. **Reidel B, Radicioni G, Clapp PW, Ford AA, Abdelwahab S, Rebuli ME, Haridass P, Alexis NE, Jaspers I, and Kesimer M**. E-Cigarette Use Causes a Unique Innate Immune Response in the Lung, Involving Increased Neutrophilic Activation and Altered Mucin Secretion. *Am J Respir Crit Care Med* 197: 492-501, 2018.

69. **Goecks J, Jalili V, Heiser LM, and Gray JW**. How Machine Learning Will Transform Biomedicine. *Cell* 181: 92-101, 2020.

70. **Westling E, Rusby JC, Crowley R, and Light JM**. Electronic Cigarette Use by Youth: Prevalence, Correlates, and Use Trajectories From Middle to High School. *J Adolesc Health* 60: 660-666, 2017.

71. **Wills TA, Leventhal AM, Sargent JD, and Pagano I**. Concurrent Use of E-cigarettes, Combustible Cigarettes, and Marijuana. *Pediatrics* 148: 2021.

72. **Boykan R, Messina CR, Chateau G, Eliscu A, Tolentino J, and Goniewicz ML**. Self-Reported Use of Tobacco, E-cigarettes, and Marijuana Versus Urinary Biomarkers. *Pediatrics* 143: 2019.

73. **Trivers KF, Phillips E, Gentzke AS, Tynan MA, and Neff LJ**. Prevalence of Cannabis Use in Electronic Cigarettes Among US Youth. *JAMA Pediatr* 172: 1097-1099, 2018.

74. **St Helen G, Havel C, Dempsey DA, Jacob P, 3rd, and Benowitz NL**. Nicotine delivery, retention and pharmacokinetics from various electronic cigarettes. *Addiction* 111: 535-544, 2016.

75. **Benowitz NL, Hukkanen J, and Jacob P, 3rd**. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handbook of experimental pharmacology* 29-60, 2009.

76. **Winters BR, Kochar TK, Clapp PW, Jaspers I, and Madden MC**. Impact of E-Cigarette Liquid Flavoring Agents on Activity of Microsomal Recombinant CYP2A6, the Primary Nicotine-Metabolizing Enzyme. *Chemical research in toxicology* 33: 1689-1697, 2020.

77. **Nguyen Long C, Yang D, Nicolaescu V, Best Thomas J, Gula H, Saxena D, Gabbard Jon D, Chen S-N, Ohtsuki T, Friesen John B, Drayman N, Mohamed A, Dann C, Silva D, Robinson-Mailman L, Valdespino A, Stock L, Suárez E, Jones Krysten A, Azizi S-A, Demarco Jennifer K, Severson William E, Anderson Charles D, Millis James M, Dickinson Bryan C, Tay S, Oakes Scott**

A, Pauli Guido F, Palmer Kenneth E, Consortium The National CCC, Meltzer David O, Randall G, and Rosner Marsha R. Cannabidiol inhibits SARS-CoV-2 replication through induction of the host ER stress and innate immune responses. *Science Advances* 0: eabi6110.

78. **Nichols JM, and Kaplan BLF**. Immune Responses Regulated by Cannabidiol. *Cannabis Cannabinoid Res* 5: 12-31, 2020.

79. **Committee on Key Challenge Areas for Convergence and Health; Board on Life Sciences; Division on Earth and Life Studies; National Research Council**. Convergence: Facilitating Transdisciplinary Integration of Life Sciences, Physical Sciences, Engineering, and Beyond. Washington (DC): National Academies Press 2014.

80. **U.S. Department of Health and Human Services**. E-Cigarette Use Among Youth and Young Adults. A Report of the Surgeon General Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2016.

81. FDA finalizes enforcement policy on unauthorized flavored cartridge-based e-cigarettes that appeal to children, including fruit and mint U.S. Food and Drug Administration. https://www.fda.gov/news-events/press-announcements/fda-finalizes-enforcement-policy-unauthorized-flavored-cartridge-based-e-cigarettes-appeal-children.

APPENDIX 1: SUPPLEMENTAL FIGURES

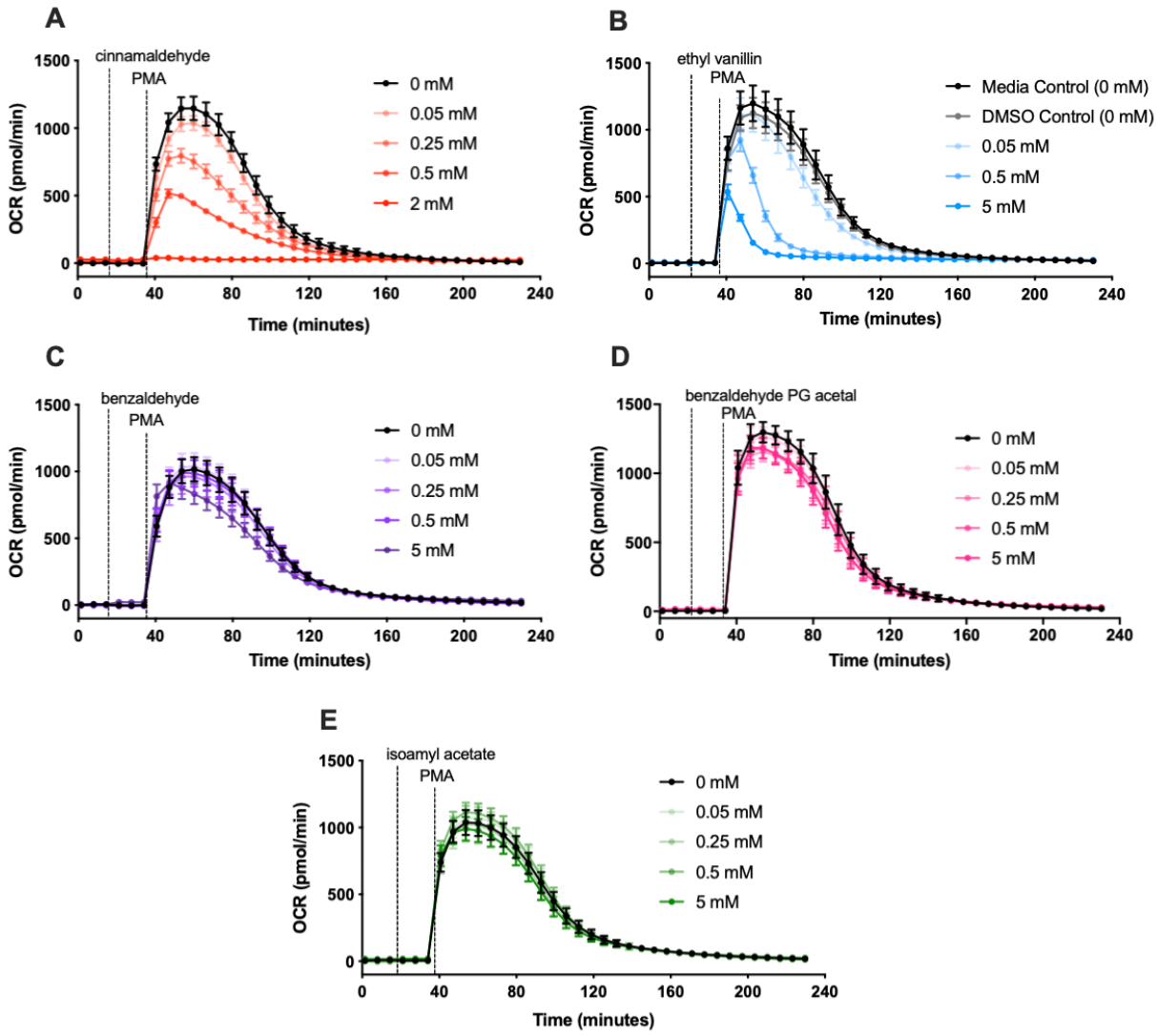


Figure S2-1. Rate graphs of neutrophil oxidative burst following exposure to flavoring chemicals. Effects of flavoring chemicals cinnamaldehyde (A), ethyl vanillin (B), benzaldehyde (C), benzaldehyde propylene glycol acetal (D), and isoamyl acetate (E) on PMA-stimulated neutrophil oxidative burst as measured by oxygen consumption rate (OCR, pmol/min).

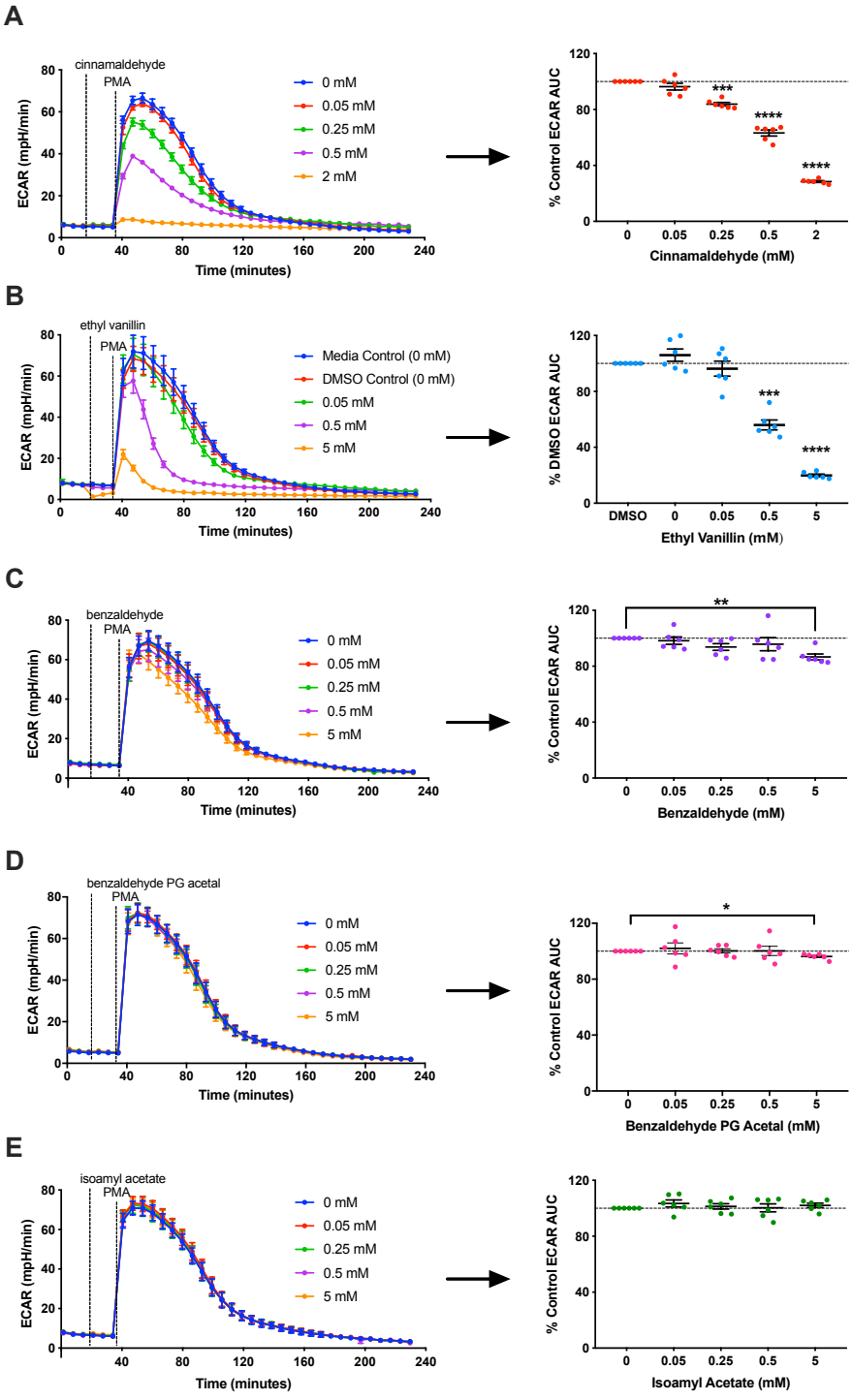


Figure S2-1. Neutrophil glycolysis during oxidative burst following exposure to flavoring chemicals. Effects of flavoring chemicals cinnamaldehyde (A), ethyl vanillin (B), benzaldehyde (C), benzaldehyde PG acetal (D), and isoamyl acetate (E) on PMA-stimulated neutrophil glycolysis during oxidative burst as measured by extracellular acidification rate (ECAR, mpH/min). Total glycolysis was calculated by integrating the oxygen consumption rate (ECAR, mpH/min) of PMA-stimulated neutrophils over time. Data represents mean ± SEM with n = 6. * $p < 0.05$, ** $p < 0.01$

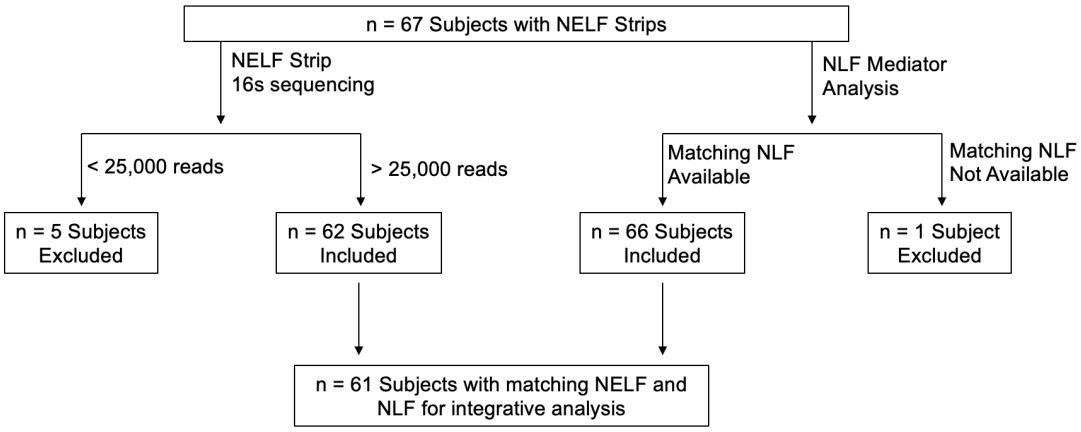


Figure S3-1. Flow chart showing inclusion and exclusion criteria for NELF microbiome component, NLF component, and integrative analysis.

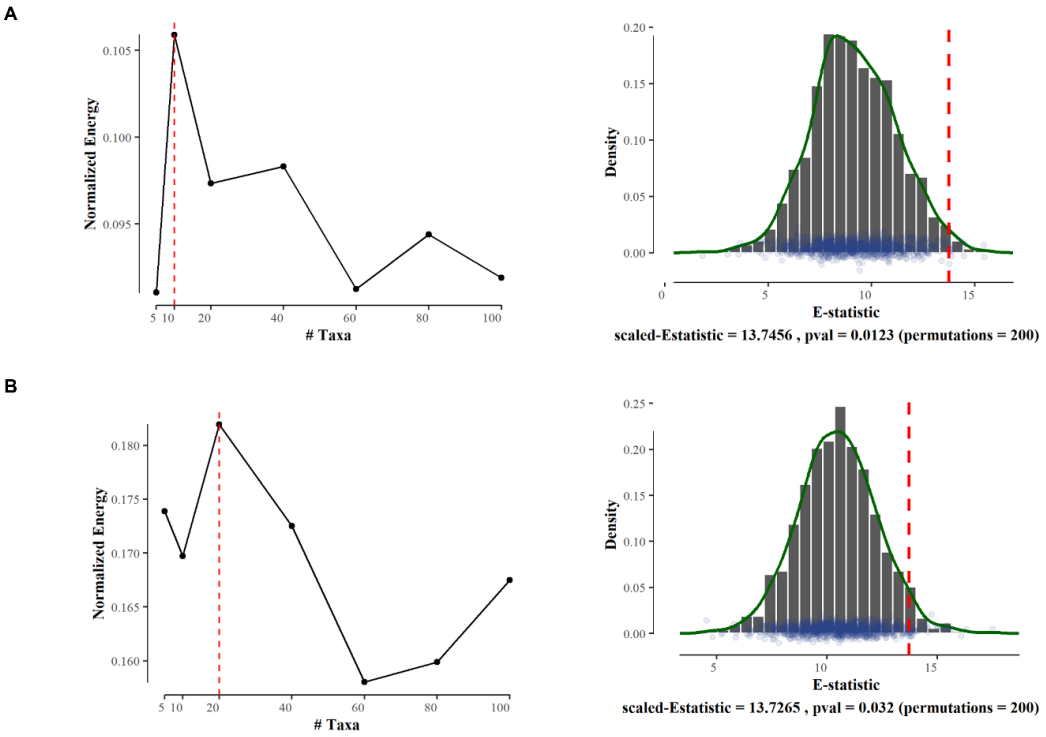


Figure S3-2. SelEnergyPerm taxa subset selection and significance results. (A) Left - Selection of number of taxa (t = 10) by normalized energy maximization to test in the final by Sex microbial log ratio signature. Right – SelEnergyPerm by Sex microbial signature significance via permutation testing. (B) Left - Selection of number of taxa (t = 20) by normalized energy maximization to test in the final by Subject microbial log ratio signature. Right – SelEnergyPerm by Exposure group microbial signature significance results via permutation testing.

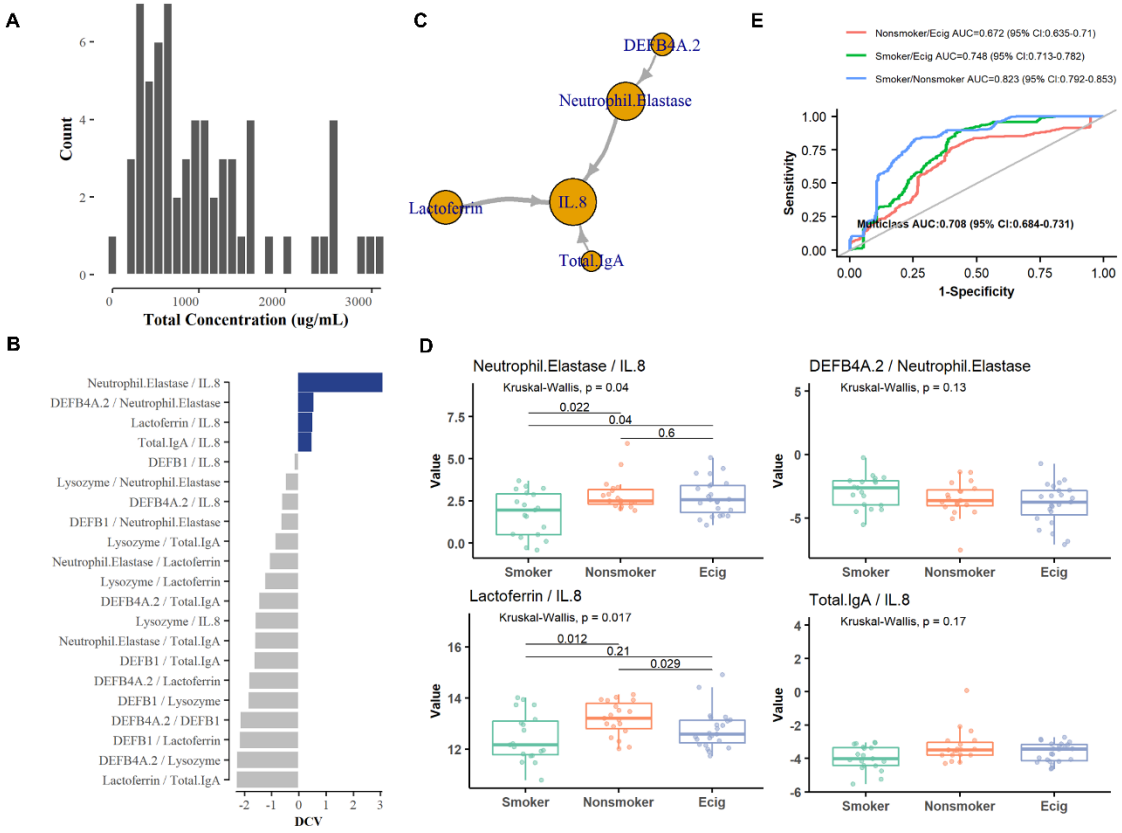


Figure S3-3. NLF Mediator Analysis. (A) Histogram of total NLF mediator concentrations by sample. (B) DCV scoring of NLF mediator log ratios. Grey: DCV < 0; Blue: DCV ≥ 0 (C) Graph representation of DCV derived key NLF mediators. (D) Log ratio values of key NLF mediator log ratios by exposure group with subsequent Wilcoxon Rank-Sum test pairwise comparison displayed. (E) NLF mediator exposure group discrimination via ROC curve displaying the multi-class AUC results of 50 repeats of 10-fold cross-validation using a 2-component PLS-DA model.

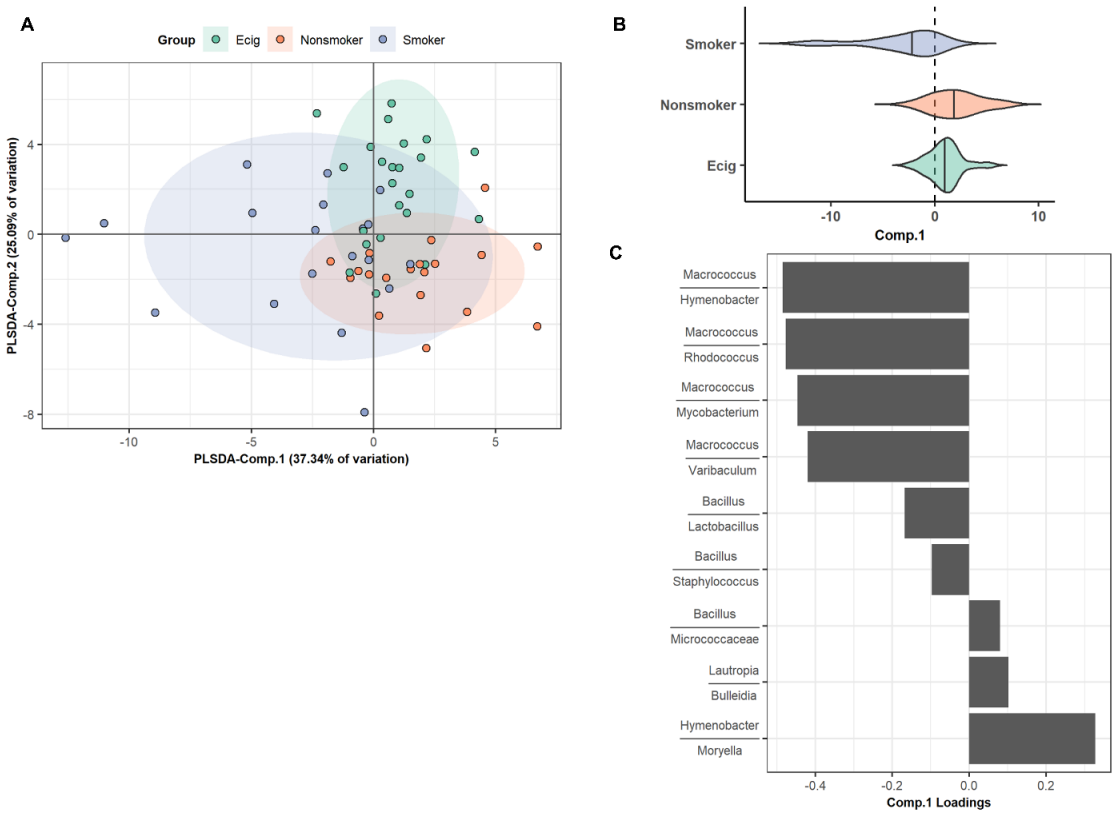


Figure S3-4. By Exposure group Nasal Microbial Signature Latent Space Analysis. (A) 2-Component PLS-DA Biplot (B) Violin plot with means showing the distribution of first PLS-DA component scores by exposure group (C) PLS-DA loadings on the first component.

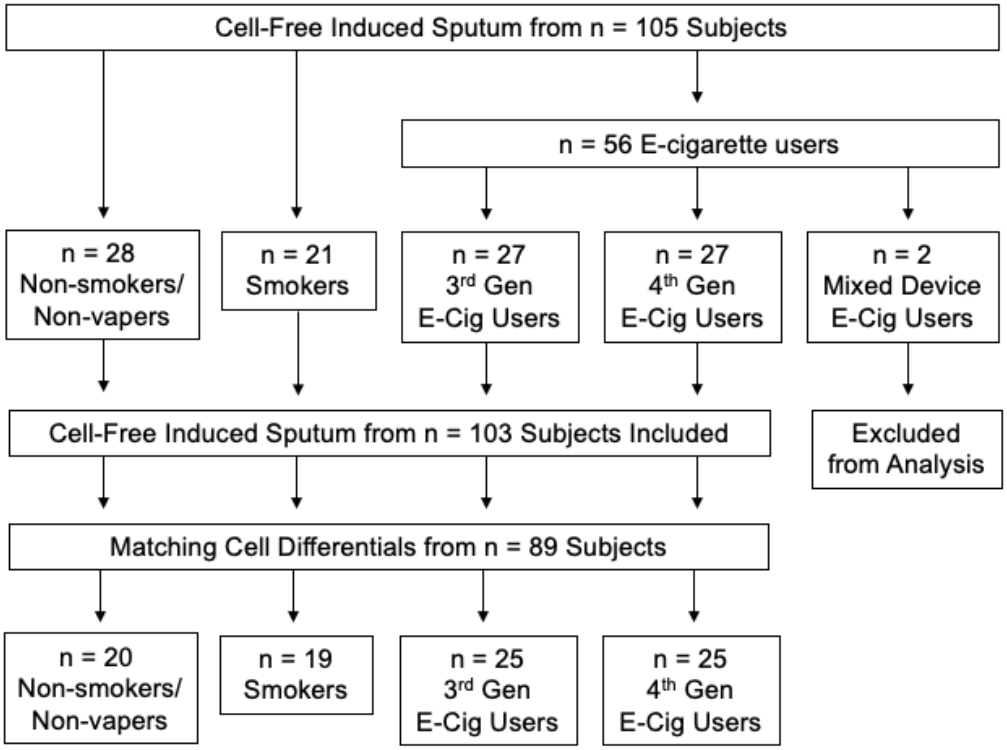


Figure S4-1. Flow chart showing inclusion and exclusion of clinical study subjects by device type.

APPENDIX 2: SUPPLEMENTAL TABLES

Table S2-1. Maximum oxygen consumption rate (OCR, pmol/min) for each flavoring chemical as determined via Seahorse assay, expressed as a percent of the media or vehicle control. n = 3 males and n = 3 females per chemical and dose. CA = cinnamaldehyde; EV = ethyl vanillin; BZ = benzaldehyde, BZPGA = benzaldehyde PG acetal, IA = isoamyl acetate. Data are presented as mean ± standard error. No data = n.d. * indicates statistical significance in comparison with the media or vehicle control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

	Dose (mM)						
Flavoring	**0**	**DMSO**	**0.05**	**0.25**	**0.5**	**2**	**5**
CA	100 ± 0.0	n.d.	93 ± 5.5	70 ± 2.7***	46 ± 2.0****	4.2 ± 0.8****	n.d.
EV	106 ± 4.1	100 ± 0.0	100 ± 6.1	n.d.	84 ± 4.7	n.d.	48 ± 3.9*
BZ	100 ± 0.0	n.d.	103 ± 3.8	93 ± 5.3	98 ± 5.2	n.d.	90 ± 2.8
BZPGA	100 ± 0.0	n.d.	98 ± 2.3	89 ± 1.8**	91 ± 1.3**	n.d.	91 ± 1.8*
IA	100 ± 0.0	n.d.	104 ± 3.1	97 ± 6.6	109 ± 4.2	n.d.	96 ± 6.0

Table S2-2. Time to maximum oxygen consumption rate (pmol/min) as determined via Seahorse assay, expressed as time in minutes from the start of the assay. (t = 0 at the start of the assay). n = 3 males and n = 3 females per chemical and dose. CA = cinnamaldehyde; EV = ethyl vanillin; BZ = benzaldehyde, BZPGA = benzaldehyde PG acetal, IA = isoamyl acetate. Data is presented as mean ± standard error. No data = n.d. * indicates statistical significance in comparison with the media or vehicle control group. * $p < 0.05$

	Dose (mM)						
Flavoring	**0**	**DMSO**	**0.05**	**0.25**	**0.5**	**2**	**5**
CA	57 ± 1.5	n.d.	58 ± 2.2	53 ± 1.2	49 ± 1.4	67 ± 30	n.d.
EV	52 ± 1.4	51 ± 1.5	49 ± 2.2	n.d.	46 ± 1.0	n.d.	41 ± 0.1*
BZ	59 ± 2.0	n.d.	58 ± 3.2	58 ± 2.2	56 ± 2.8	n.d.	48 ± 2.6*
BZPGA	55 ± 2.0	n.d.	55 ± 2.0	53 ± 2.0	51 ± 2.2	n.d.	52 ± 1.4
IA	57 ± 2.8	n.d.	58 ± 2.8	56 ± 2.8	55 ± 3.1	n.d.	55 ± 3.1

Table S2-3. Maximum extracellular acidification rate (ECAR, mpH/min) for each flavoring chemical as determined via Seahorse assay, expressed as a percent of the media or vehicle control. n = 3 males and n = 3 females per chemical and dose. CA = cinnamaldehyde; EV = ethyl vanillin; BZ = benzaldehyde, BZPGA = benzaldehyde PG acetal, IA = isoamyl acetate. Data is presented as mean ± standard error. No data = n.d. * indicates statistical significance in comparison with the media or vehicle control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

	Dose (mM)						
Flavoring	**0**	**DMSO**	**0.05**	**0.25**	**0.5**	**2**	**5**
CA	100 ± 0.0	n.d.	98 ± 3.1	83 ± 1.6***	58 ± 1.3****	13 ± 0.7****	n.d.
EV	104 ± 5.6	100 ± 0.0	102 ± 5.8	n.d.	84 ± 2.4**	n.d.	32 ±1.8****
BZ	100 ± 0.0	n.d.	98 ± 2.7	99 ± 1.6	93 ± 3.4	n.d.	91 ± 2.2
BZPGA	100 ± 0.0	n.d.	102 ± 4.3	101 ± 1.1	101 ± 3.3	n.d.	101 ± 1.7
IA	100 ± 0.0	n.d.	105 ± 4.2	102 ± 1.3	100 ± 3.6	n.d.	103 ± 1.8

Table S2-4. Time to maximum extracellular acidification rate (ECAR, mpH/min) as determined via Seahorse assay, expressed as time in minutes from the start of the assay. (t = 0 at the start of the assay). n = 3 males and n = 3 females per chemical and dose. CA = cinnamaldehyde; EV = ethyl vanillin; BZ = benzaldehyde, BZPGA = benzaldehyde PG acetal, IA = isoamyl acetate. Data is presented as mean ± standard error. No data = n.d. * indicates statistical significance in comparison with the media or vehicle control group. * $p < 0.05$

	Dose (mM)						
Flavoring	**0**	**DMSO**	**0.05**	**0.25**	**0.5**	**2**	**5**
CA	53 ± 1.1	n.d.	51 ± 2.2	47 ± 0.1	48 ± 1.1	45 ± 1.4*	n.d.
EV	49 ± 1.4	47 ± 1.6	47 ± 1.6	n.d.	45 ± 1.4	n.d.	41 ± 0.1
BZ	53 ± 2.0	n.d.	51 ± 2.2	53 ± 1.1	51 ± 2.2	n.d.	45 ± 1.4
BZPGA	48 ± 2.0	n.d.	50 ± 2.2	48 ± 2.0	46 ± 1.1	n.d.	46 ± 1.1
IA	50 ± 2.8	n.d.	52 ± 2.8	49 ± 2.7	50 ± 2.2	n.d.	49 ± 2.7

Table S3-1. Commercially available ELISA kits used to measure mediators of host-microbiota interaction.

Mediator	Limit of Detection	Company	Company Location
Neutrophil Elastase	0.8 ng/mL	Thermo Fisher Scientific (Invitrogen)	Waltham, MA
Total IgA	1.6 ng/mL	Thermo Fisher Scientific (Invitrogen)	Waltham, MA
Lactoferrin	156.3 pg/mL	Abcam	Cambridge, UK
Lysozyme	31.25 pg/mL	Abcam	Cambridge, UK
IL-8	3.1 pg/mL	BD Biosciences	San Diego, CA
Beta-Defensin 1	7.8125 pg/mL	LifeSpan Biosciences	Seattle, WA
Beta-Defensin 2	7.8125 pg/mL	LifeSpan Biosciences	Seattle, WA

Table S4-1. Levels of serum biomarkers of liver injury by group. Data are presented as mean (SEM).

	NS/NV (N=21)	Smoker (N=21)	3rd Gen (N=26)	4th Gen (N=12)
ALP (U/L)				
Mean (SEM)	75.7 (6.80)	78.9 (5.57)	73.8 (4.57)	87.2 (7.12)
N > 147 U/L	1 (4.8%)	0 (0%)	0 (0%)	0 (0%)
ALT (U/L)				
Mean (SEM)	19.2 (3.68)	15.5 (1.72)	29.8 (6.00)	21.2 (2.82)
N > 30 U/L	2 (9.5%)	1 (4.8%)	6 (23%)	1 (8.3%)
AST (U/L)				
Mean (SEM)	22.9 (1.82)	18.7 (0.986)	25.8 (3.31)	20.4 (1.51)
N > 34 U/L	3 (14.3%)	0 (0%)	4 (15.4%)	0 (0%)
GGT (U/L)				
Mean (SEM)	27.6 (11.5)	26.9 (4.17)	36.2 (6.95)	27.8 (4.05)
N > 64 U/L	1 (4.8%)	2 (9.5%)	3 (11.5%)	1 (8.3%)
DBIL (mg/dL)				
Mean (SEM)	0.244 (0.0164)	0.175 (0.0157)	0.230 (0.0297)	0.253 (0.0377)
N > 0.2 mg/dL	16 (76.2%)	7 (33.3%)	8 (30.78%)	6 (50%)
TBIL (mg/dL)				
Mean (SEM)	0.624 (0.0511)	0.457 (0.0362)	0.662 (0.102)	0.675 (0.103)
N > 1 mg/dL	2 (9.5%)	0 (0%)	3 (11.5%)	2 (16.7%)
ALB (g/L)				
Mean (SEM)	4.90 (0.130)	4.77 (0.121)	4.92 (0.108)	5.35 (0.154)
N < 3.5 g/L	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table S4-2. P-values output by ANCOVA for effects of device, sex, age, and race on expression of soluble mediators in induced sputum.

	Device	Sex	Age	Race
Albumin	0.2082	0.03	0.1462	0.3214
bFGF	0.0845	0.0402	0.7378	0.8089
CRP	0.0367	0.2173	0.047	0.8506
dsDNA	0.2503	0.6202	0.1294	0.3717
Eotaxin	0.3628	0.9251	0.0027	0.8337
Eotaxin-3	0.9277	0.0249	0.5807	0.7037
Flt1	0.0138	0.3114	0.0514	0.6962
GM-CSF	0.2602	0.7785	0.9186	0.347
IFN-γ	0.0266	0.8528	0.8552	0.6624
IL-10	0.0877	0.657	0.1332	0.3651
IL12p40	0.819	0.4636	0.1964	0.6435
IL12p70	0.7122	0.5052	0.98	0.8067
IL-13	0.1922	0.4041	0.0289	0.2269
IL-15	0.7775	0.3013	0.1088	0.6615
IL-16	0.1007	0.553	0.1285	0.1855
IL-17	0.477	0.4003	0.8594	0.0978
IL-1α	0.3385	0.0968	0.1722	0.0352
IL-1β	0.3290	0.5362	0.2755	0.4424
IL-2	0.1223	0.223	0.5227	0.496
IL-4	0.2633	0.8331	0.2997	0.4732
IL-5	0.5339	0.1543	0.6403	0.5193
IL-6	0.0232	0.316	0.1334	0.4723
IL-7	0.1333	0.2347	0.075	0.7845
IL-8	0.4295	0.4568	0.1054	0.8938
IP-10	0.1405	0.7454	0.0309	0.1902
MCP-1	0.0057	0.0966	0.0037	0.867
MIP-1α	0.9751	0.1657	0.019	0.6023
MIP-1β	0.0183	0.2838	0.0274	0.4631
MMP-2	0.0184	0.3625	0.1694	0.1418
MMP-9	0.4125	0.6823	0.3035	0.5889
MPO	0.7323	0.5668	0.1054	0.6872
NE	0.1311	0.5209	0.1301	0.5846
PIGF	0.0866	0.0401	0.3215	0.9739
SAA	0.079	0.8876	0.3538	0.7345
sICAM1	0.0008	0.1692	0.0153	0.5665
sVCAM1	0.0023	0.0576	0.2387	0.6263
TARC	0.2081	0.0898	0.0281	0.3016
Tie2	0.0419	0.9833	0.537	0.9571
TNF-α	0.4261	0.465	0.1538	0.4918
Uteroglobin	0.02	0.1542	0.2425	0.2556
VEGF	0.0213	0.2653	0.0491	0.6691
VEGFC	0.301	0.9191	0.227	0.4324
VEGFD	0.1089	0.5429	0.0559	0.463

Table S4-3. Soluble mediators measured in induced sputum in pg/mL. Data are presented as mean (standard error). Groups were compared using the Kruskal-Wallis test followed by Dunn's test for non-parametric multiple comparisons. Comparisons between NS/NV and all other exposure groups, and comparisons between 3rd and 4th gen e-cig users, are indicated with bolded font and symbols: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ in comparison with NS/NV; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ in comparison with 3rd gen e-cig users. Additional significant comparisons can be viewed using R code: https://github.com/ehickman0817/dissertation-ch4-devicecomparison.

	NS/NV (N=28)	Smoker (N=21)	3rd Gen (N=27)	4th Gen (N=27)	P-value
Albumin	156000 (70700)	147000 (30300)	138000 (35100)	**130000 (75800) #**	**0.003**
bFGF	14.9 (2.83)	**30.8 (4.68) ***	27.3 (5.71)	21.6 (6.13)	**0.013**
CRP	1030 (622)	621 (152)	561 (129)	**113 (41.8) *, ##**	**<0.001**
dsDNA	3080000 (531000)	3730000 (515000)	4020000 (506000)	3430000 (549000)	0.185
Eotaxin	60.2 (32.6)	73.2 (28.8)	100 (43.7)	87.3 (40.0)	0.556
Eotaxin-3	3.71 (1.48)	4.00 (1.65)	6.84 (2.10)	10.3 (4.48)	0.61
Flt1	180 (35.6)	**329 (52.4) ***	253 (41.6)	142 (48.3)	**<0.001**
GM-CSF	1.11 (0.219)	1.84 (0.353)	0.988 (0.270)	1.35 (0.914)	**0.024**
IFN-γ	31.4 (16.4)	154 (95.0)	164 (80.9)	0.776 (0.143)	0.277
IL-10	0.636 (0.0709)	0.731 (0.0967)	0.656 (0.0620)	0.857 (0.0963)	0.203
IL12p40	87.4 (58.6)	34.6 (8.93)	35.3 (5.55)	46.2 (13.5)	0.702
IL12p70	0.690 (0.118)	1.20 (0.371)	1.06 (0.303)	0.715 (0.101)	0.735
IL-13	133 (24.2)	191 (27.9)	154 (22.3)	155 (23.7)	0.264
IL-15	0.882 (0.321)	1.15 (0.454)	0.890 (0.320)	0.364 (0.137)	0.446
IL-16	911 (172)	807 (165)	1250 (150)	1220 (296)	0.106
IL-17	5.40 (1.69)	9.58 (3.44)	3.99 (1.62)	9.85 (3.55)	0.562
IL-1α	401 (65.0)	**697 (122) ***	609 (104)	414 (71.3)	**0.012**
IL-1β	61.0 (10.4)	91.9 (14.9)	82.7 (13.9)	77.2 (18.2)	0.102
IL-2	1.63 (0.249)	2.82 (0.521)	2.04 (0.289)	2.30 (0.275)	0.0924
IL-4	0.376 (0.108)	4.78 (3.12)	4.63 (3.81)	**0.156 (0.0313) #**	**0.030**
IL-5	1.00 (0.255)	1.47 (0.341)	1.25 (0.336)	0.758 (0.236)	0.371
IL-6	27.5 (5.72)	**127 (35.0) ****	61.4 (14.8)	47.6 (10.1)	**0.007**
IL-7	13.7 (3.56)	12.8 (2.31)	15.4 (3.51)	**7.12 (2.11) #**	**0.018**
IL-8	3550 (683)	3730 (702)	3930 (503)	4240 (957)	0.357
IP-10	2190 (1500)	937 (253)	1200 (290)	875 (349)	0.128
MCP-1	151 (32.4)	**386 (119) ***	**231 (48.3) ***	**75.0 (18.9) ###**	**<0.001**
MIP-1α	35.4 (6.96)	39.7 (10.3)	46.1 (9.01)	45.9 (17.5)	0.607
MIP-1β	139 (42.7)	69.8 (35.8)	131 (31.1)	**81.2 (36.1) #**	**0.009**
MMP-2	105000 (16900)	57300 (12500)	130000 (11400)	**79900 (15500) #**	**0.002**
MMP-9	344000 (78700)	351000 (60900)	435000 (76200)	504000 (112000)	0.506
MPO	1690000 (297000)	1950000 (260000)	2120000 (326000)	1800000 (379000)	0.272
NE	358000 (93500)	**525000 (79400) ***	405000 (47800)	**316000 (77600) #**	**0.005**
PlGF	0.759 (0.123)	1.30 (0.293)	0.630 (0.123)	0.750 (0.118)	0.136
SAA	13400 (3140)	**75400 (54800) ***	70400 (50000)	19200 (4510)	**0.026**

	NS/NV (N=28)	Smoker (N=21)	3rd Gen (N=27)	4th Gen (N=27)	P-value
sICAM1	39400 (15800)	74600 (39800)	30900 (7100)	**7230 (3020)** *, ####	**<0.001**
sVCAM1	3880 (925)	5670 (932)	5080 (1240)	**2030 (732)** ##	**<0.001**
TARC	19.1 (4.47)	19.9 (4.79)	23.3 (4.91)	**8.39 (2.21)** #	**0.038**
Tie2	156 (70.5)	157 (72.9)	**466 (118)** *	**69.0 (27.1)** #	**0.028**
TNF-α	5.96 (0.955)	6.94 (0.993)	7.62 (0.854)	7.01 (1.53)	0.155
Uteroglobin	39300000 (11900000)	20300000 (5290000)	36600000 (6400000)	**21500000 (6260000)** #	**0.031**
VEGF	1080 (223)	**1500 (203)** *	1220 (209)	**663 (137)** #	**<0.001**
VEGFC	155 (56.5)	150 (130)	151 (65.8)	348 (81.0)	**0.018**
VEGFD	417 (111)	382 (94.4)	394 (76.4)	**114 (48.6)** #	**0.018**

Table S4-4. By class and overall performance metrics for predictive modeling of separation between exposure groups.

Model Predictors Included	Accuracy	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Balanced Accuracy
(A) Multinomial Logistic Regression						
Soluble mediators						
Non-smokers/Non-vapers	0.5200	0.5200	0.6690	0.3617	0.7967	0.5945
Smokers	0.3200	0.3200	0.8822	NaN	0.8380	0.6011
3rd Generation E-Cig Users	0.2000	0.2000	0.8120	0.1757	0.7449	0.5060
4th Gen E-Cig Users	0.6200	0.6200	0.8577	0.5914	0.8708	0.7388
Overall	0.4206	0.4150	0.8052	0.3763	0.8126	0.6101
Soluble mediators + covariates						
Non-smokers/Non-vapers	0.4800	0.4800	0.8296	0.5676	0.8125	0.6548
Smokers	0.4200	0.4200	0.8925	0.5167	0.8581	0.6562
3rd Generation E-Cig Users	0.4200	0.4200	0.7479	0.3794	0.7895	0.5839
4th Gen E-Cig Users	0.7333	0.7333	0.8827	0.6829	0.9086	0.8080
Overall	0.5160	0.5133	0.8382	0.5366	0.8422	0.6757
(B) Quadratic Discriminant Analysis						
Soluble mediators						
Non-smokers/Non-vapers	0.3733	0.3733	0.7743	0.3829	0.7668	0.5738
Smokers	0.1400	0.1400	0.9159	0.2333	0.8064	0.5280
3rd Generation E-Cig Users	0.6067	0.6067	0.6825	0.4219	0.8284	0.6446
4th Gen E-Cig Users	0.6000	0.6000	0.8719	0.6410	0.8641	0.7359
Overall	0.4397	0.4300	0.8111	0.4198	0.8164	0.6206

Table S5-1. Concentrations of mediators secreted by hMDMs in pg/mL were measured using multiplex ELISA. Concentrations are reported as mean (standard error). n = 4 subjects (1 male, 3 females). [a] at least $p < 0.05$ in comparison with M0; [b] at least $p < 0.05$ in comparison with M1; [c] at least $p < 0.05$ in comparison with M2 by either one-way ANOVA with Tukey's multiple comparisons test or Friedman test with Dunn's multiple comparisons test.

	M0	M1	M2
Eotaxin	94.5 (7.92)[b]	209 (14.7)[a,c]	110 (2.32)[b]
Eotaxin-3	41.5 (3.27)[b]	207 (30.1)[a]	250 (78.6)
GM-CSF	0.486 (0.152)	20.0 (6.74)	0.580 (0.229)
IL-1α	0.180 (0.180)[b]	15.9 (9.19)[a]	0.825 (0.379)
IL-1β	4.13 (0.274)	228 (159)[c]	2.72 (0.857)[b]
IL-2	5.57 (0.139)[b]	16.6 (1.80)[a,c]	3.34 (1.19)[b]
IL-5	0.119 (0.0824)	0.106 (0.0556)	0.0110 (0.00652)
IL-6	12.5 (3.17)[b]	2430 (292)[a,c]	13.9 (3.43)[b]
IL-7	0.436 (0.0850)	0.200 (0.0899)	0.423 (0.0193)
IL-8	9770 (1390)	111000 (30000)[c]	7320 (2130)[b]
IL-10	5.77 (1.12)	1300 (384)	4.62 (1.15)
IL-12p40	0.306 (0.117)[b]	577 (184)[a]	1.09 (0.534)
IL-12p70	2.67 (0.397)[b]	15.6 (2.32)[a]	4.61 (1.03)
IL-13	60.2 (4.38)[b]	90.5 (6.29)[a]	46.7 (11.3)
IL-15	0.517 (0.0853)[b]	5.33 (0.494)[a,c]	0.359 (0.0484)[b]
IL-16	16.6 (4.31)	50.7 (17.0)	32.2 (15.5)
IL-17	0 (0)	2.77 (1.38)	0.0152 (0.0152)
IP-10	315 (153)	18700 (5360)	550 (231)
MCP-1	4000 (70.0)	4100 (51.5)	4060 (76.1)
MCP-4	54.4 (4.21)[b,c]	106 (9.04)[a,c]	3110 (334)[a,b]
MDC	1330 (579)	1070 (214)	14900 (8490)
MIP-1α	80.0 (6.44)	1830 (1830)	305 (100)
MIP-1β	384 (207)	11700 (3200)	2010 (1070)
TARC	22.0 (9.23)[c]	53.7 (5.69)	1070 (505)[a]
TNF.α	14.0 (2.11)[b]	3260 (33.1)[a,c]	17.4 (6.49)[b]
TNF.β	0.0300 (0.0300)[b]	4.84 (2.92)[a]	0.0912 (0.0372)
VEGF	43.1 (17.7)	514 (129)[c]	5.40 (2.17)[b]