Electronic Cigarette Exposure: Inflammatory and Immunological Implications in Structural Lung Cells and the Potential Public Health Consequences

by Jack Edward Bozier

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of Professor Brian G.G. Oliver and Hui Chen

University of Technology Sydney
Faculty of Science

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Jack Bozier declare that this thesis, is submitted in fulfilment of the requirements for the award of *Doctor of Philosophy*, in the *School of Life Science, Faculty of Science* at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Production Note:
Signature: Signature removed prior to publication.

Date: 24/02/21

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I hereby state that this submission is a thesis by compilation.

Awards, Presentations and Posters Arising from This Work

Awards

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Award to attend ERS 2018 Annual Scientific Meeting to present two abstracts in the form of posters. Award was based on the merit of the submitted abstracts.

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Award to attend ERS 2018 Annual Scientific Meeting to present two abstracts in the form of posters. Award was based on the merit of the submitted abstracts.

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Award to attend TSANZ 2017 Annual Scientific Meeting to present two abstracts in the form of a plenary presentation and a poster. Award was based on the merit of the submitted abstracts.

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Awarded to attend the American Thoracic Society Annual Scientific Meeting to present an abstract in the form of an online presentation. Award was based on the merit of the submitted abstracts.

Posters and Presentations

TSANZ Poster Presentation **October 2017**

Presentation of a Poster on "Toxic and Inflammatory Effects of E-cigarette Vapour are Power Dependant" at the Thoracic Society Australia & New Zealand Clinical Update and Annual Scientific Meeting

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Presentation of a Poster on "Increasing Operating Voltage Increases the Toxic and inflammatory effects of E-cigarette Vapour" at the Thoracic Society Australia & New Zealand Clinical Update and Annual Scientific Meeting

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Poster presentation titled "'Airway Smooth Muscle Cells from COPD patients are Hyper-Responsive to E-cigarette Vapour" for Woolcock Student Research Symposium 2018

Poster presentation **March 2018**

Poster Presentation titled "'Cigarettes or E-cigarettes': Young Peoples Beliefs about Emerging Tobacco Products" for Thoracic Society of Australia and New Zealand Annual Scientific Meeting.

Plenary Presentation TSANZ 2018 **March 2018**

Oral presentation on "E-cigarette Vapour Stimulates Greater Inflammatory Mediator Production in COPD Airway Smooth Muscle Cells than Non-COPD Smooth Muscle Cells" at the Thoracic Society Australia & New Zealand Clinical Update and Annual Scientific Meeting.

Poster Presentation European Respiratory Society ASM **September 2019**

Presentation of two abstracts: "Lung cells from people with COPD are hyper responsive to E-cigarette vapour" and "A new era of smoking: Young people's perceptions of emerging tobacco products" at the European Respiratory Society Annual Scientific Meeting in Paris, France.

Poster Presentation American Thoracic Society ASM **May 2019**

Poster Presentation titled "'To vape or not to vape': Young Peoples Beliefs about Emerging Tobacco Products" for American Thoracic Society Annual Scientific Meeting in Dallas, Texas.

Poster Presentation American Thoracic Society ASM **May 2019**

Rapid Fire poster presentation on "E-cigarette Aerosol Stimulates Changes in Structural Proteins of the Lung" at the American Thoracic Society Annual Scientific Meeting in Dallas, Texas.

Invited Speaker – Centre for Inflammation Seminar Series **April 2019**

Oral presentation on the negative health effects of Electronic cigarette use to promote research collaboration between Australian medical research institutes.

Invited Speaker – Respiratory Seminar Series, RPAH **July 2019**

Oral presentation and clinical update for respiratory physicians on the negative health effects of Electronic cigarettes.

Invited Speaker – Woolcock Seminar Series, WIMR **August 2020**

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Statements of Contributions of Authors

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Jack Bozier, Razia Zakarya, David G. Chapman, and Brian G. Oliver.

J.B, R.Z, D.G.C, B.G.O.

J.B, R.Z, D.G.C and B.G.O conceived the idea for a review updating literature on E-cigarettes, J.B and R.Z drafted the manuscript, All authors provided critical feedback, edited and approved the final version of the manuscript.

By signing this document, I declare that Jack Bozier has contributed to a level that justifies first authorship on this manuscript

Jack Bozier

Signed Production Note: Signature removed prior to publication. Date 30/11/2020

Razia Zakarya

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

David G. Chapman

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Brian G. Oliver

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

The Evolving Landscape of e-Cigarettes: A Systematic Review of Recent Evidence

Jack Bozier, Emily K. Chivers, David G. Chapman, Alexander N. Larcombe, Nicole A. Bastian, Jorge A. Masso-Silva, Min Kwang Byun, Christine F. McDonald, Laura E. Crotty Alexander, Miranda P. Ween.

J.B, E.K.C, D.G.C, A.N.L, N.A.B, J.A.M.S, M.K.B, C.F.M, L.E.C.A, M.P.W.

J.B and E.K.C contributed to the manuscript equally. M.P.W performed literature review and assigned literature to each author. J.B, E.K.C, D.G.C, A.N.L, N.A.B, J.A.M.S, M.K.B, C.F.M, L.E.C.A, M.P.W critically appraised literature and summarised literature, drafted sections of the manuscript. J.B, E.K.C, M.P.W summarised and formatted literature submitted by all authors into a standardised format for the final version of the manuscript, J.B, E.K.C, D.G.C, A.N.L, N.A.B, J.A.M.S, M.K.B, C.F.M, L.E.C.A, M.P.W edited the manuscript and approved the final version to be published.

By signing this document, I declare that Jack Bozier has contributed to a level that justifies first authorship on this manuscript

Jack Bozier

Signed Production Note: Signature removed prior to publication. Date 23/11/2020

Emily K. Chivers

Signed Production Note: Signature removed prior to publication. Date 04/12/2020

David G. Chapman

Signed Production Note: Signature removed prior to publication. Date 27/11/2020

Alexander N. Larcombe

Signed Production Note: Signature removed prior to publication. Date 26/11/2020

Nicole A. Bastian

Signed Production Note: Signature removed prior to publication. Date 30/11/2020

Jorge A. Masso-Silva

Signed Production Note: Signature removed prior to publication. Date 26/11/2020

Min Kwang Byun

Signed Production Note: Signature removed prior to publication. Date 27/11/2020

Christine F. McDonald

Signed Production Note: Signature removed prior to publication. Date 08/12/2020

Laura E. Crotty Alexander

Signed Production Note: Signature removed prior to publication. Date 28/11/2020

Miranda P. Ween

Signed Production Note: Signature removed prior to publication. Date 27/11/2020

Survey of Young Australians Attitudes Towards Emerging Tobacco Products

Jack Bozier, Brian G. Oliver, and Juliet M Foster.

J.B, B.G.O, J.M.F.

Author contribution statement: J.B collected the data, carried out the analysis, and drafted the manuscript. J.M.F assisted with data analysis. J.B, B.G.O, and J.M.F contributed to the study design, interpretation and editing of the manuscript.

By signing this document, I declare that Jack Bozier has contributed to a level that justifies first authorship on this manuscript

Jack Bozier

Signed Production Note: Signature removed prior to publication. Date 23/11/20

Brian G. Oliver

Signed Production Note: Signature removed prior to publication. Date 03/12/20

Juliet M. Foster

*Due to unforeseen health concerns Dr Juliet Foster was unable to confirm authorship on this document. This has been confirmed with the Graduate Research School.

Heightened Response to E-cigarettes in COPD

Jack Bozier, Sandra Rutting, Dia Xenaki, Matthew Peters, Ian Adcock, and Brian G. Oliver.
J.B, S.R, D.X, M.P, I.A, B.G.O.

J.B and B.G.O conceived and planned the experiments; J.B, D.X and S.R carried out the experiments; J.B, M.P, I.A and B.G.O contributed to interpretation of the results, J.B drafted the manuscript, All authors provided critical feedback, helped with study design, edited and approved the final version of the manuscript.

By signing this document, I declare that Jack Bozier has contributed to a level that justifies first authorship on this manuscript

Jack Bozier

Signed Production Note: Signature removed prior to publication. Date 23/11/2020

Sandra Rutting

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Dia Xenaki

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Matthew Peters

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Ian Adcock

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Brian G. Oliver

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

E-cigarette Vapor Induces Cellular Senescence in Lung Fibroblasts and may Contribute to Lung Pathology

Jack Bozier*, Roy R. Woldhuis*, Baoming Wang, Irene H. Heijink, Maaike de Vries, Maarten van den Berge, Wim Timens, Corry-Anke Brandsma, Brian G.G. Oliver

R.W J.B, B.W, I.H.H, M.D.V, W.T, C.A.B, B.G.G.O.

Authors' contributions:
Conception and design: RRW, JB, BGGO
Acquisition and analysis of data: RRW, JB, BW, BGGO
Interpretation of data: RRW, JB, IHH, MvdB, WT, CAB, BGGO
Drafting the manuscript: RRW, JB
All authors reviewed, edited, and approved the final manuscript.
RRW and JB contributed equally.

By signing this document, I declare that Jack Bozier has contributed to a level that justifies first authorship on this manuscript

Jack Bozier

Production Note:
Signed Signature removed prior to publication. Date 23/11/2020

Roy R. Woldhuis

Production Note:
Signed Signature removed prior to publication. Date 27/11/2020

Baoming Wang

Production Note:
Signed Signature removed prior to publication. Date 27/11/2020

Irene H. Heijink

Production Note:
Signed Signature removed prior to publication. Date 27/11/2020

Maaike de Vries

Production Note:
Signed Signature removed prior to publication. Date 08/12/2020

Maarten van den Berge

Signed Production Note: Signature removed prior to publication. Date 27/11/2020

Wim Timens

Signed Production Note: Signature removed prior to publication. Date 27/11/2020

Corry-Anke Brandsma

Signed. Production Note: Signature removed prior to publication. Date 08/12/2020

Brian G. G. Oliver

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Combined E-cigarette and cigarette use reduces efficacy of dexamethasone to attenuate neutrophilic inflammatory markers

Jack Bozier, Roy R. Woldhuis, Karosham D. Reddy, Ian M. Adcock, Brian G.G. Oliver

J.B, R.W, K.D.R, I.M.A, B.G.G.O.

Authors' contributions:
Conception and design: J.B, I.M.A and B.G.G.O Conceived and designed the experiments in this study; J.B, R.R.W, K.D.R, B.G.G.O performed experiments, J.B analysed the data; Interpretation of data: J.B, I.M.A, BGGO interpreted the data; J.B Drafted the manuscript; all authors reviewed, edited, and approved the final manuscript.

By signing this document, I declare that Jack Bozier has contributed to a level that justifies first authorship on this manuscript

Jack Bozier

Signed Production Note: Signature removed prior to publication. Date 30/11/20

Roy R. Woldhuis

Signed Production Note: Signature removed prior to publication. Date 03/12/20

Karosham D. Reddy

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Ian M. Adcock

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

Brian G. G. Oliver

Signed Production Note: Signature removed prior to publication. Date 03/12/2020

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Abstract

Electronic cigarettes have rapidly become the consumer preferred alternative to tobacco cigarettes, but very little is known about the harms associated with their use. Electronic cigarettes are often proposed as a cessation device from a harm reduction standpoint, but this overlooks the lack of evidence for reduced harms and the numerous new vapers who have never smoked that are exposed to harms they otherwise would have avoided. Studies within this thesis provide essential evidence in the harm reduction debate.

In Chapter 3 we surveyed perceptions of young Australians towards E-cigarettes. We hypothesised that they would believe E-cigarettes to be less harmful than tobacco cigarettes, and that they would be misinformed about E-cigarette regulations in Australia due to a lack of education from regulatory bodies. In Chapters 4, 5 and 6 of this thesis we used *in vitro* models of exposure to determine potential health risks associated with E-cigarette use. Chronic obstructive pulmonary disease (COPD) patients have been identified as a high-risk population of E-cigarette users, hence our studies focused on the potential effect E-cigarette exposure may have on mechanisms related to the underlying pathophysiology of COPD.

In Chapter 4, we developed an *in vitro* E-cigarette exposure model to determine the cytotoxic and inflammatory effects of E-cigarette exposure in COPD and non-COPD primary human airway smooth muscle cells. In this study we confirmed earlier suspicions on cytotoxicity and provided the first evidence that COPD cells are hyper-responsive to E-cigarettes. In Chapter 5 we provided the first evidence that E-cigarettes have the potential to induce cellular senescence. This finding gives further support to avoiding use of E-cigarettes in COPD patients, given the role cellular senescence plays in COPD pathophysiology. In Chapter 6 we provided evidence that combined cigarette and E-cigarette use is significantly more harmful than using either product alone. Furthermore, we found that the inflammatory response induced by dual exposure was glucocorticoid resistant. Glucocorticoid

resistance is one of the hallmarks of COPD, and thought to contribute to uncontrolled inflammation in pre-COPD (symptomatic smokers) so dual use should be avoided.

Importantly, this thesis elucidates pathological harms associated with E-vapour exposure. The evidence provided in the studies within this thesis should be used to inform clinicians, researchers and patients on the harms associated with E-cigarette use to improve clinical outcomes in terms of morbidity and mortality in COPD.

List of Abbreviations

AKT - Protein kinase B

ALI – Air liquid interface

APS – Ammonium persulfate

ASM – Airway smooth muscle

ASMCs – Airway smooth muscle cells

BALF – Bronchoalveolar lavage fluid

BME - β-mercaptoethanol

cDNA – Complementary DNA

COPD – Chronic obstructive pulmonary disease

CFTR – Cystic fibrosis transmembrane conductance regulator

CS – Cigarette smoke

CSE – Cigarette smoke extract

CXCL1 – chemokine (C-X-C motif) ligand-1

CXCL2 – chemokine (C-X-C motif) ligand-2

CXCL8 – chemokine (C-X-C motif) ligand-8 or Interleukin-8

DNA – Deoxynucleic acid

DMEM – Dulbecco's modified eagle medium

ELISA – Enzyme linked immunosorbent assay

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

EVE – E-vapour extract

FBS – Fetal bovine serum

FEV1 – Forced expiration volume in 1 second

FEF – Forced expiratory flow

FOXO1 – Forkhead box protein O1

FVC – Forced vital capacity

GOLD – Global Initiative for Chronic Obstructive Lung Disease

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HBECs – Human bronchial epithelial cells

HR – Heart rate

HRP – Horseradish peroxidase

HREC – Human research ethics committee

IL-1 – Interleukin-1

IL-1α - Interleukin-1 alpha

IL-1 β – Interleukin-1 beta

IL-6 – Interleukin-6

IL-8 – Interleukin-8

LABA – Long acting beta agonist

LAMA – Long acting antimuscarinic

MCP-1 – Monocyte chemoattractant protein-1

MMP – Matrix metalloproteinase

mTOR – Molecular target of rapamycin

MTT - 3-4,5- dimethylthiazol-2-(yl)-2,5-disphenyltetrazolium

NAC – N-acetyl cystine

NASEM – National Academies of Science, Engineering and Medicine

NDSHS – National drug strategy household survey

NE – Neutrophil elastase

NF-κB – Nucleation factor-kappa B

NIH – National Institute of Health

NLR – Neutrophil to lymphocyte ratio

NRT – Nicotine replacement therapy

NSCLC – Non-small cell lung carcinoma

p38 MAPK - p38 mitogen-activated protein kinases

PAHs – Polycyclic aromatic hydrocarbons

PBS – Phosphate buffered saline

PI3K – Phosphoinositide 3-kinase

PG – Propylene glycol

PM – Particulate matter

PMNs – Polymorphonuclear cells

PQ - Paraquat

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Room temperature

RT – qPCR – Real time quantitative polymerase chain reaction

SASP – Senescence associated secretory phenotype

SA- βGal – Senescence associated beta-galactosidase staining

T-PBS – Phosphate buffered saline supplemented with 0.05% Tween (v/v)

T-TBS – Tris Buffered Saline supplemented with 0.05% Tween

TAFE - Technical and Further Education

TEMED - N,N,N',N'-Tetramethyl ethylenediamine

TGF- β – Transforming growth factor beta

TLR – Toll like receptor

TMV – Terminal mucous velocity

TNFα - Tumor necrosis factor alpha

TSANZ – Thoracic Society of Australia and New Zealand

TSNAs – Tobacco specific nitrosamines

VAPI – Vaping associated pulmonary injury

VG – Vegetable glycerin

VOCs – Volatile organic compounds

Chapter 1 Introduction

1.1 History of Tobacco use

Tobacco smoking has been a common habit since the tobacco plant was introduced to Spain and England by early explorers, who were responsible for its discovery and later re-exportation to the rest of the world during eras of colonisation. There are more than sixty species of tobacco plant, most of which are native to America with the exception of a few native to Australia [1, 2]. The harms associated with tobacco smoking are well documented today, but this has not always been the case. Medicinal use of tobacco was prolific during its early appearance in Europe. At the time it was believed to have therapeutic potential to treat colds, fevers, aid in digestion and treat hunger or thirst among many other ailments [3]. The medical community continued to use the tobacco leaf or extracts from the plant for treatment of medical conditions up to the 20th century, when our understanding of the harms associated with tobacco use started to outweigh the perceived therapeutic benefits of the time [4].

The first evidence of documented harms of tobacco smoking were published in 1602 by an Elizabethan doctor named Philaretes [5, 6]. In this publication Philaretes expresses concern for the use of medicinal tobacco, discussing many points that have later been proven true though scientific studies. Due to tobaccos popularity and rapid uptake globally succeeding its exportation worldwide, it wasn't long before the scientific community started to debate its safety [6-9]. In particular, the increasing incidence of lung cancer in the 1940's-1950's was recognized as a consequence of cigarette smoking. Towards the end of the 19th century hand rolled and manufactured cigarettes resulted in greater access to tobacco globally, resulting in a spike in tobacco usage rates for the decades following [7]. The industrial scale production of cigarettes combined with a lack of knowledge of associated harms contributed to Australian smoking rates of 72% in adult males and 26% in females in 1945 [10].

Highly successful marketing of cigarettes by tobacco companies resulted in the global lung cancer epidemic of the 1940's-1950's, but recognition of cigarettes as a causative agent for lung cancer and other chronic diseases was met with hostile denialism by the tobacco industry [11]. The campaign by the tobacco industry to present tobacco as an innocuous product worked in their favour, with more than two thirds of doctors considering there to be insufficient evidence and the proposed harms to be unfounded as late as the 1960's [11].

Despite the success of pro-tobacco propaganda by the tobacco industry, the Surgeon General's first report on smoking and health was published in 1964 to challenge many of the claims of big tobacco and present evidence of the harms associated with smoking [12]. This report summarized the available literature at the time and concluded that cigarette smoking was associated with increased deaths from lung cancer, coronary heart disease, chronic bronchitis and emphysema. The growing body of evidence has resulted in a global push towards lower smoking rates with mixed success. Even with this regulatory push towards reducing smoking rates, smoking related diseases are responsible 7 million deaths a year globally [13].

1.2 Harmful effects of cigarette smoke exposure

Tobacco smoke is a carcinogenic mix of more than 5000 chemicals formed from complete and incomplete combustion of cigarettes. Analysis of mainstream smoke chemical composition identifies 542 chemicals that smokers expose themselves to, of which 98 have been identified to have an inhalation risk above thresholds of toxicological concern [14]. Inhalation of the harmful compounds identified in the Talhout et al.'s study results in an increased oxidative burden and inflammatory response in smokers, with potential damage and structural changes in the lung as an adjunct to these disease mechanisms. The effects of cigarette smoking are not isolated to the lung, with an increased risk of cardiovascular disease, stroke, diabetes and a range of cancers [15].

The airway epithelium is the first barrier of defence against the inhaled particulate matter (PM) and noxious gases in cigarette smoke [16]. Airway epithelial cells form tight junctions between cells to form a protective layer between outer environments and cells within the airways [17]. In healthy airway epithelium there 5 main cell types that work together to maintain homeostasis; ciliated cells, goblet cells, club cells, serous cells and basal cells [18]. Mucous and airway fluid are produced by goblet cells and serous cells to capture pathogens, keep the airways hydrated and provide further defence through solubilised antioxidants that reduce damage from inhaled ROS [19-21]. Ciliated cells beat in a metachronal wave pattern to clear the airways of inhaled PM, pathogens and mucous to prevent obstruction from mucous plugging [22]. Chronic exposure to noxious gases and PM in the form of cigarette smoke, results in a loss of mucous homeostasis and ciliary function [18, 23-25].

The noxious particulate matter and gases in cigarette smoke interact with epithelial cells, airway smooth muscle cells, airway fibroblasts and parenchymal fibroblasts to stimulate a proinflammatory response through the production of cytokines and chemokines. An increased production of TNFα, IL-6, IL-8, IL-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) contributes towards the recruitment and activation of immune cells in the airways and alveolar tissue [26-29]. Neutrophils and macrophages are the most numerous innate immune cells in the airways of smokers [30, 31]; and once activated they secrete cytokines, chemokines, proteases and endogenous cell-derived reactive oxygen species (ROS) that further contribute to tissue damage and immune cell recruitment in the lung [32-35].

Oxidative stress resulting from acute and chronic cigarette smoke exposure has been studied in depth and has many implications for smokers. In addition to the 98 harmful compounds listed in Talhout et al.'s study [14], each puff of cigarette smoke contains 10^{14} free radicals that cause damage to epithelial cells and lung tissue [36]. These inhaled ROS contribute to tissue destruction to a degree that is directly

correlated with the concentration a smoker is exposed to. Endogenous ROS are produced by host structural and inflammatory cells in response to cigarette smoke exposure [37], further contributing to the highly oxidative environment in the lungs of smokers. As a result, there is a disrupted resolution of inflammatory response that persists long after smoking cessation in long-term smokers [36]. Multiple studies have shown upregulation of oxidative stress markers from acute and chronic exposure to cigarette smoke [38-40], with some markers being correlated with tissue damage, disease onset or exacerbation of disease in long-term smokers [39]. Endogenous ROS are produced by inflammatory cells and through mitochondrial respiration in response to cigarette smoke and chronic inflammation that persists from cigarette smoke exposure.

Oxidative stress drives cellular senescence, which is a cellular phenotype associated with smoke exposure and may contribute to the accelerated aging phenomenon seen in Chronic Obstructive Pulmonary Disease (COPD). Cellular senescence can occur DNA damage from exogenous and endogenous ROS resulting activation of tumour suppressor p53 [41]. Exposure to cigarette smoke results in an increase in cellular senescent markers cyclin-dependent kinase inhibitor p16 and cyclin-dependent kinase inhibitor p21 which initiate cell cycle arrest and contribute to the accumulation of senescent cells in COPD [42]. Senescent cells do not divide but they are still metabolically active, contributing to chronic inflammation in the lung through the senescence associated secretory phenotype (SASP) by activation of NF-kB resulting in increased production of proinflammatory cytokines and chemokines. The continuous exposure to noxious particles in cigarette smoke initiates innate and adaptive immune responses in smokers [43, 44]. Chronic cigarette smoke exposure results in an infiltration of polymorphonuclear cells (PMNs), macrophages, natural killer cells and mast cells in the airway wall and parenchyma [43]. The infiltration of immune cells is assisted by an increased permeability of the epithelium after smoke exposure, allowing noxious particles to interact with tissue that would be protected under normal healthy conditions [45].

Both acute and chronic cigarette exposure result in an increase in neutrophils in the Bronchoalveolar lavage fluid (BALF) of smokers [46-49]. Neutrophils contribute to tissue damage and proteolysis in the lung and play a key role in the pathophysiology of COPD [43]. Early detection of increased activated neutrophils in the lung may indicate risk of COPD development prior to symptom onset and disease progression [50, 51].

1.3 Chronic Obstructive Pulmonary Disease

COPD is a disease of progressive airflow limitation resulting in persistent shortness of breath [44]. Development of COPD is most commonly attributed to cigarette smoke exposure, but chronic exposure to any noxious particulate matter or gasses can drive disease progression. COPD is currently reported as the third leading cause of death worldwide [52], with approximately $1 billion AUD is spent annually on treatment and management of the disease in Australia [53]. Cigarette smoking is the most common cause of COPD in Australia, prompting a major focus on smoking cessation support and anti-tobacco legislation to try to reduce the burden of disease. Daily tobacco smoking has declined in Australians 14 years or older from 24.3% in 1991 to 12.8% in 2013. We have seen a smaller decline in recent years with the most recent data reporting 11% smoking rate in 2019 [54], suggesting that legislation changes are having a lesser effect on smoking rates compared to previous interventions.

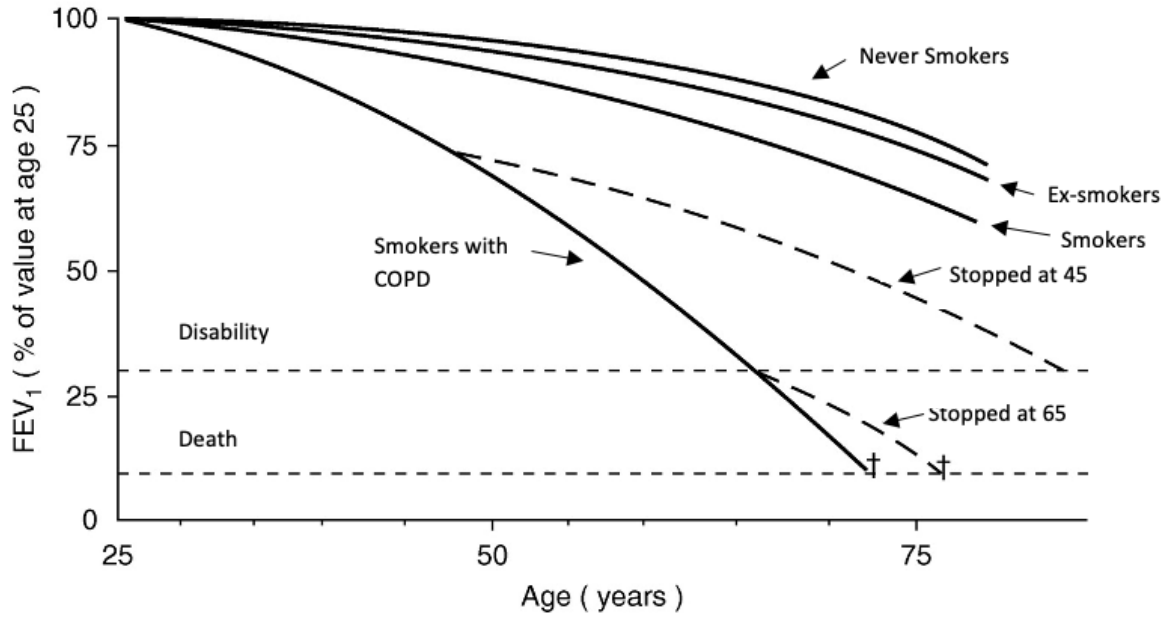

Figure 1.1 – Decline of FEV1 in susceptible smokers with COPD, non-susceptible smokers, ex-smokers and non-smokers. Adapted from Fletcher and Peto, 1977 [55].

The cornerstone study by Fletcher and Peto provided data on lung function in the early stages of COPD and evidence that some smokers aren't susceptible to developing COPD [55]. In this epidemiological study they followed 792 men aged 30-59 for eight years with bi-annual assessment of lung function, bronchial infections and mucus production. There are two major findings of this study that impacted our understanding of COPD: smokers can be categorized as either susceptible or non-susceptible to the rapid lung function decline in COPD that is associated with cigarette smoke exposure. Secondary to susceptibility, it was also discovered that smokers with COPD who ceased cigarette smoking slowed their decline in lung function. These findings crucially adapted our understanding of smoking and susceptibility in the aetiology of COPD, highlighting the importance of smoking cessation in disease treatment. Further studies on prevalence and incidence of COPD in smokers have identified that approximately 20% of smokers are susceptible to the pathophysiological processes of COPD that result in lung function decline [56].

1.4 Symptoms of COPD

COPD can remain asymptomatic for up to twenty years in smokers due to the progressive nature of disease [57]. Symptoms of obstruction and airflow limitation appear after significant lung function decline, it is important to detect changes is immune response and accumulation of components of the innate immune system early as they are a key biomarker for disease development and prognosis for young smokers. The chronic inflammatory response, resulting tissue damage and tissue remodelling manifests in the triad of symptoms experienced by most COPD patients: Chronic cough, excessive mucus production and dyspnoea [44, 58]. These symptoms are progressive in nature and worsen with disease severity, often coinciding with more frequent and longer lasting bronchial infections. As seen in Figure 1.1 the resulting decline in lung function of COPD patients will result in disability and eventually death. As symptoms progress COPD patients will experience a lower exercise capacity due to the increased demand for gas exchange in the lung. The discomfort caused by physical exertion often results in COPD patients becoming less physically active, which may have further adverse health effects [59].

1.5 Diagnosis

Due to the delayed onset of symptoms in COPD there is a risk of underdiagnosis in patients. If a patient begins to display mild symptoms and has prior exposure to noxious gases or particulate matter known to be a risk factor for disease spirometry should be utilized as a diagnostic tool. Spirometry measures the flow and volume of air that enters and exits the lungs, and is useful in detecting restrictive or obstructive breathing patterns compared to predicted normal values [60]. Spirometry requires patients to forcibly exhale into a mouthpiece allowing measurement of their functional lung capacity and the rate that air can be exhaled from the lung. The two lung function parameters most utilized in the diagnosis of COPD are forced expiratory volume over 1 second (FEV1) and forced vital capacity (FVC). These two measures of lung function are then used to calculate the FEV1/FVC ratio, a useful diagnostic value in COPD. An FEV1/FVC ratio < 0.70 combined with

symptoms and a history of exposure to COPD risk factors results in a positive diagnosis. Regular spirometry can also be used to monitor disease progression as the disease is categorized into 4 stages of GOLD 1-4 [44]. GOLD 1 is mild COPD with FEV1 ≥ 80% predicted, GOLD 2 is moderate COPD with 50% ≤ FEV1 ≤ 79% predicted, GOLD 3 is severe COPD with 30% ≤ FEV1 ≤ 49% and GOLD 4 is very severe with FEV1 ≤ 29% predicted. The categorical scoring of disease severity is used alongside a respiratory symptom questionnaire and the patient's exacerbation history to determine overall disease burden and maintenance. Recent studies have proposed forced oscillation technique (FOT) as a diagnosis tool to be used alongside spirometry for early detection of COPD [61, 62]. Ribeiro et al found FOT significantly improved the early diagnosis of mild COPD with a high level of accuracy [61]. Early diagnosis gives clinicians an advantage in treating patients with COPD, particularly in convincing smokers to quit smoking.

1.6 COPD Pathophysiology

Chronic inflammation as a result of long-term exposure to noxious particles is hypothesized to be central to pathophysiology in COPD. There are 3 main components that contribute to the symptom burden experienced by COPD patients: Chronic bronchitis; consisting of chronic airway inflammation and mucus hypersecretion, emphysematous damage to parenchymal tissue, and lastly small airway destruction and remodelling [43].

Increased production of mucus by goblet cells, goblet cell hyperplasia and ineffective mucocilliary clearance by ciliated cells results in obstruction from inflammatory mucosal exudate in the central airways (> 4 mm in diameter) [63, 64]. The clinical pathology of mucous airway obstruction paired with inflammation is referred to as chronic bronchitis [65]. The Reid index [66] was developed to determine the ratio of mucosal glandular tissue to airway size as a diagnostic tool, but more recent studies have questioned the accuracy of the ratio with regards to diagnosing chronic bronchitis [67-69]. The current school of thought is that a combination of enlarged mucosal cells and an overproduction of mucus work in

tandem to cause the mucus plugging in COPD [70]. Mucus obstruction is paired with a loss of epithelial barrier function and infiltration of inflammatory cells, which contribute to tissue damage and ineffective repair though extracellular matrix (ECM) remodelling.

Chronic bronchitis is often alternatively referred to as airway obstruction, this can be somewhat misleading as it is more common for obstruction to occur in the small airways (< 2 mm in diameter) rather than the central airways [71]. A plethora of studies have found structural abnormalities in the small airways that contribute to obstruction and ultimately their destruction with more severe stages of COPD [72-76]. Numerous immunological and inflammatory changes precede the structural changes in the small airways, all in relation to cigarette smoke exposure. An increased infiltration of neutrophils, activation of macrophages and activation of T-lymphocytes is seen in the airways of COPD patients [77, 78]. Epithelial cells in the airways exhibit a more active secretory phenotype in COPD, producing inflammatory mediators TNF-α, IL-1β, IL-6, GM-CSF and IL-8 [79]. Chronic production and secretion of these mediators orchestrates the aberrant inflammatory response in COPD patients. Epithelial cells also express TGF- β, which induces fibrosis within the airways as an ineffective repair mechanism to combat the damage done by inflammatory processes. The number of active immune cells is also greater in COPD patients compared to smokers without COPD, multiple studies have investigated the molecular mechanisms behind this increase as potential therapeutic targets [80-84].

Emphysematous damage to alveolar tissue can be directly attributed to the increased active immune cells in the parenchymal tissue and BALF. Neutrophil elastase (NE) secreted by neutrophils is an elastolytic enzyme involved in the degradation of elastic alveolar attachments that hold alveoli open [85, 86]. Other serine proteases such as cathepsin G, Proteinase 3, and MMP's -2, -8, -9, -12 are secreted by macrophages and neutrophils, likely further contributing to tissue damage seen in emphysema [87-89].

1.7 COPD Treatment

Current treatment of COPD varies depending on national guidelines, but predominantly includes an inhaled corticosteroid, inhaled long acting beta agonist (LABA), long acting antimuscarinic (LAMA) or a combination of the three [90]. In Australia we have adopted the COPD-X guidelines which consist of:

- **C**ase finding and diagnosis
- **O**ptimise function
- **P**revent deterioration
- **D**evelop a plan of care
- Manage e**X**acerbations

Under these guidelines focus is given to educating patients on their disease and how to best use therapies to prevent disease progression. Health practitioners are also expected to monitor their patient's condition and adapt their treatment plan accordingly. Inhaled corticosteroids are suggested to be used in a stepwise approach under the COPD-X guidelines, as their efficacy can be varied depending on the severity of disease. Mechanisms of steroid insensitivity in COPD are not well understood but some mechanisms have been proposed [91].

Smoking cessation remains an integral facet of the treatment of COPD, since Fletcher and Peto's initial observational study [55] a multitude of supporting studies still identify quitting smoking as the most important factor for improved patient outcomes [92]. There are a range of psychological, behavioural and pharmacological treatment options available for patients with ranging levels of effectiveness. Behavioural and psychological therapies include group or individual counselling, exercise therapy and aversion therapy. Aversion therapy involves pairing the patients pleasurable association of smoking with an unpleasant stimulus, there is little evidence for its efficacy in smoking cessation [93]. Exercise therapy involves getting the patient to exercise to alleviate some of the psychological factors that often lead to relapse [94], there is still insufficient data to support it as a treatment option [95]. Counselling to specifically target smoking cessation is the current gold

standard of behavioural therapies and it is currently used in Australia under the smoking cessation guidelines for general practice [96].

The above guidelines also suggest the use of pharmacotherapy in combination with behavioural therapy for best results. Current approved pharmacotherapy options include nicotine replacement therapy (NRT), varenicline and bupropion. The preferred combination treatment with NRT, varenicline and behavioural therapy is almost three times more effective than control groups using no therapeutic support. Often a lack of compliance drastically reduces the efficacy of these treatments, leaving patients looking for alternatives to the currently approved therapies.

The smokers preferred alternative to tobacco smoking in recent years has been the electronic cigarette. Electronic cigarettes are an alternative source of nicotine for smokers, they aerosolize E-liquids at high temperatures delivering an aerosol containing nicotine to the lungs. A preference for this as a nicotine replacement product lies in its ability to mimic the sensation of smoking whilst delivering nicotine to the patient. Current guidelines strongly recommend against the use of E-cigarettes as a smoking cessation aid but this is the most common reason for use [97].

1.8 How harmless are E-cigarettes: effects in the pulmonary system

Included below is a broad literature review on the potential harms associated with E-cigarette use. In this publication we covered some background on the devices, their evolution towards modern E-cigarettes and the demographics of users internationally. I have also addressed some of the major concerns around E-cigarette use including the immunological changes associated with their use, pulmonary and cardiovascular effects of use, the effects of flavours included in E-liquids and the risks of inhaling them in a superheated aerosol. This literature review identifies evidence gaps that will be addressed in the later experimental chapters of this thesis.

REVIEW

How harmless are E-cigarettes? Effects in the pulmonary system

Jack Bozier[a,b], *Razia Zakarya*[a,b], *David Geoffrey Chapman*[c,d], *and Brian Gregory George Oliver*[a,b]

Purpose of review
Electronic cigarettes have quickly risen to be the leading alternative nicotine source to tobacco. E-cigarette use is hard to research and regulate because of the novelty and rapid evolution of the devices and E-liquids. Epidemiological data on long-term usage is currently lacking, but in smaller cohort studies we are starting to understand the usage patterns and demographics of users, which differ depending on where the study takes place and the regulatory environment. The present review describes the current knowledge of the effects of E-cigarettes on the pulmonary system and knowledge of their usage patterns worldwide.

Recent findings
E-cigarette use is continuing to rise in young adults in United States and Canada, but not in United Kingdom. These suggest that regulation is influencing uptake in young adults. If E-cigarettes are to be considered as a harm minimisation smoking cessation product, use in young never smokers must be factored into the risk assessment. A recent surge in cases of lung injury associated with vaping in America has resulted in the definition of vaping associated pulmonary injury, although the exact cause remains unknown.

Summary
It is our opinion that E-cigarettes can no longer be defined as harmless. Further studies are needed to determine the risks for all populations as it is evident that a large proportion of E-cigarette users are never-smokers, meaning they cannot only be considered from a harm reduction perspective.

Keywords
E-cigarettes, inflammation, respiratory disease, smoking cessation

INTRODUCTION

The idea of an electronic nicotine delivery device as an alternative to tobacco was first conceived in 1963, but the first patented design came to market in 2003 [1]. Hon Lik originally designed the 'cigalike' devices as an alternative to combustible tobacco and their popularity has been rapidly increasing since their introduction to America and Europe in 2007 [2]. The devices have evolved to more advanced customisable products, but a shift towards Pod style devices in recent years suggests discretion and convenience is preferred by users. A good analogy to the different types of E-cigarette devices is home coffee machines. Some people prefer to roast, grind, and brew their own coffee, whereas others opt for the convenience of pre-packed coffee pods. The regulation surrounding E-cigarettes varies significantly throughout the world, although the development of devices and E-liquids by hundreds of different international manufacturers has left an insurmountable task for regulators to tackle [3]. It seems that it is universally agreed that they should not be sold to minors, but beyond that opinions differ drastically on the harm versus benefit of E-cigarettes in different countries. In the United Kingdom, a pro-E-cigarette position has been taken by Public Health England, starting with the endorsement of Nutt *et al*. paper [4], proposing that E-cigarettes are 95% more healthy than tobacco cigarettes. The present study has been

[a]Respiratory Cellular and Molecular Biology Group, Woolcock Institute of Medical Research, [b]Respiratory Cellular and Molecular Biology Group, School of Life Sciences University of Technology Sydney, [c]Translational Airways Group, School of Life Sciences, University of Technology Sydney and [d]Airway Physiology & Imaging Group and Emphysema Centre, Woolcock Institute of Medical Research, Sydney, Australia

Correspondence to Jack Bozier, Level 3, 431 Glebe Point Road, Glebe 2037, Australia. Tel: +61 02 9114 0363; fax: +61 9114 0010; e-mail: Jack.Bozier@sydney.edu.au

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KEY POINTS

- E-cigarettes can no longer be considered harmless, and should be considered in their own light without comparison to tobacco cigarettes.
- There is evidence for immunological, respiratory, and cardiovascular complications from short or long-term E-cigarette use.
- Regulations on E-cigarettes have an impact on the demographics that use them, hence further restrictions are needed to protect nonsmokers and at-risk populations.

criticised for making conclusions without evidence to support their claims.

E-CIGARETTE UPTAKE INTERNATIONALLY

In the United States and Canada, a lack of regulation has contributed to an epidemic of E-cigarette use in young people. Recent data show that E-cigarette use has significantly increased in young adults aged 16–19 between 2017 and 2018 [5▪]. E-cigarette use of 15 or more days in the last 30 days increased from 3.0 to 5.2% in United States and 2.1 to 3.6% in Canada. They also looked at changes in a cohort of 16- to 19-year olds from the United Kingdom, but no significant changes in prevalence of E-cigarette use were found. The United Kingdom was quicker to act on regulating E-cigarettes and United States waited on a congressional hearing about increasing prevalence of youth E-cigarette use, where it was revealed that Juul Labs was offering financial incentives to talk to students about the safety of e-cigarettes [6]. Australia is currently much further behind in the regulation of E-cigarettes, with regulation only allowing the sale of nonnicotine containing E-liquids in as nicotine is a registered poison with the Therapeutic Goods Administration.

Given the vast differences in international regulation of e-cigarettes, it is important to evaluate recent evidence as to their effectiveness as a smoking cessation tool. A recent study by Hajek *et al.* [7] compared the smoking cessation success of two cohorts, with one using nicotine replacement therapy (NRT) under the current therapeutic guidelines and the other using e-cigarettes as a replacement. They found that E-cigarettes were more effective as a cessation aid than NRT (18 vs. 9.9%). They also found that 80% of the E-cigarette cohort was still using their cessation product at a 52-week follow up, suggesting that they had not beaten their nicotine dependency. Conversely, a study by Gomajee *et al.* [8] found that E-cigarette use in former smokers is associated with smoking relapse, suggesting that E-cigarettes efficacy as a cessation aid could be more complicated than looking at short-term quit rates.

E-CIGARETTES VERSUS TOBACCO

The ongoing debate on whether E-cigarettes are more or less harmful than traditional tobacco cigarettes is delaying progress on regulation and distracting from the other major public health questions, particularly understanding the long-term population effects of E-cigarette use. It is clear that a lack of regulation is resulting in rapid uptake in younger non and never smokers, which may result in a rise in smoking rates among these populations [9,10]. One of the big unanswered questions is will the potential harm minimisation from E-cigarette use in current smokers outweigh the rate of smoking relapse and uptake of smoking by never smokers? The National Academies of Science, Engineering, and Medicine (NASEM) released a report in 2018 concluding that E-cigarette use results in reduced toxins, suggesting potential harm minimization [11].

Furthermore, the question on whether E-cigarettes are harmful as a stand-alone product, without comparison to cigarettes is still being debated. In human clinical trials and in real-life pragmatic studies the different combinations of machine, machine operating parameters, liquids, and usage time initially led to a lot of confusion. In-vivo and in-vitro models have also been complicated by the lack of standard models, and the potential for models to be developed by those with commercial interests is worrying. However, even with this turbidity, it is hard to ignore the clear majority of studies concluding that E-cigarettes are cytotoxic, pro-inflammatory, genotoxic, and effect respiratory function of users after a single session.

Higham *et al.* [12] exposed Calu-3 cells and primary human bronchial epithelial cells in air–liquid interface (ALI) culture to E-cigarette vapour extract resulting in an increase in proinflammatory cytokines interleukin (IL)-6 and CXCL8. The E-cigarette vapor extract was also cytotoxic to human bronchial epithelial cells (HBECs). A similar study using Beas-2Bs epithelial cell line in ALI found that E-cigarette exposure was not cytotoxic, and relatively inert when looking at inflammatory mediator production [13]. Of the 10 inflammatory mediators measured only IL-6 was upregulated. Contradictory studies are regularly found because of the lack of a standardized E-cigarette exposure model. The study by Anthérieu *et al.* outlines in their methods that they bubble a defined amount of vapor, 16 × 3 s puffs through cell culture media, but Higham *et al.*

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state their dose as an OD value. Anthérieu *et al.* also received funding for their study from Innova, a French E-cigarette company, which should be considered as a conflict of interest. Without standard exposure times, volumes of E-vapor, clarity on the devices, and E-liquids used we move further away from an answer on the health effects of using E-cigarettes. Regular publication of unreproducible data further adds to the confusion and delays action by regulatory bodies.

IMMUNOLOGICAL IMPACT OF E-CIGARETTES

Although the examples above point out some flaws in current E-cigarette research, it is evident that E-cigarettes have clear detrimental immunological effects in humans and on cells of the respiratory tract, cardiovascular system, and immune system. Lugg *et al.* [14] exposed primary alveolar macrophages to E-cigarette condensate finding upregulation of IL-6, TNFα, CXCL8, MCP-1, and MMP-1. They also found that both nicotine and nicotine-free vapor condensates were pro-apoptotic. Furthermore, addition of N-acetyl cystine (NAC) ameliorated the apoptosis, suggesting ROS were responsible for the cytotoxicity. Ween *et al.* [15] had similar findings in THP-1 macrophage cell line, with increased inflammatory markers after E-cigarette exposure and reduced phagocytotic ability. Poststimulation with E-cigarette vapor there was a reduction in expression of phagocytic surface receptors SR-A1 and TLR-2. Macrophages play a pivotal role in maintaining homeostasis within the lung, phagocytosing invading pathogens and apoptotic/necrotic host cells. The loss of macrophage function could result in increased infection and ineffective turnover and recycling of dead cells. It has also been shown that macrophages from e-cigarette exposed mice had significantly reduced ability to phagocytose bacteria than the macrophages of control mice [16], confirming that macrophage function is effected in the lung and in culture. This study also looked at viral infection in mice after E-cigarette exposure, with a significant decrease in viral clearance and an increase in morbidity and mortality. Furthermore, a study exposing mice to a range of flavored E-cigarettes both with and without nicotine looked at the effects on features of house dust mite-induced allergic [17]. All nicotine containing E-cigarettes suppressed airway inflammation but did not alter airway hyper responsiveness or airway remodeling. Compared to room air, nicotine-free cinnacide flavored E-cigarette exposure resulted in reduced total leukocytes and eosinophils, with an increase in airway hyper responsiveness. Banana pudding E-cigarette exposure resulted in increased soluble lung collagen. These findings suggest that different E-liquid flavors may have a negative impact on users with allergic airways disease.

Higham *et al.* [18] exposed primary human neutrophils to E-cigarette vapor to determine activation and inflammatory response. There was an increase in activation markers CD11b and CD66b after E-cigarette exposure and increased secretion of MMP-9, neutrophil elastase, and CXCL-8. These findings suggest that E-cigarette use would result in an increase in neutrophilic activation and recruitment. Dysregulated neutrophilic inflammation contributes to disorder in chronic obstructive pulmonary disease through increased matrix metalloproteinase (MMP) activity, suggesting a plausible mechanistic link between e-cigarette use and long-term respiratory disease. Clapp *et al.* [19] also found altered neutrophil function and cytokine production after E-cigarette exposure. There was a decrease in phagocytosis from several different E-liquid extract exposures, further supporting the increased risk of infection because of e-cigarette use. The impact of increased MMP activity could be explained in Ghosh *et al.* [20] study, where E-cigarette users, smokers and nonsmokers received bronchial brushings to determine any changes in bronchial epithelial protein expression. They found 113 uniquely altered proteins in E-cigarette users, and 78 proteins altered in both E-cigarette users and smokers. Furthermore, E-cigarette users and smokers both experienced frequent coughing, poor tolerance for the bronchoscope and had erythematous airway mucosa, with more irritability and redness in the e-cigarette users.

PULMONARY AND CARDIOVASCULAR COMPLICATIONS FROM E-CIGARETTE USE

Mucocilliary dysfunction after E-cigarette exposure has been found in an *ex vivo* model of mucocilliary transport using Bullfrog Pallets [21]. There were similar findings in HBECs and *in vivo* in a Sheep model [22▪]. Exposure to E-cigarette vapor reduced airway surface liquid hydration and increased mucus viscosity of HBECs in a nicotine-dependent manner. In both *ex vivo* and *in vivo* models, E-cigarettes caused a reduction in tracheal mucous velocity (TMV). Cystic fibrosis transmembrane conductance regulator ion channel (CTFR) is also functionally altered after E-cigarette vapor exposure [23]. CTFR dysfunction is a proposed mechanism for mucus obstruction in chronic bronchitis and is also found in cystic fibrosis. These studies suggest that E-cigarettes could worsen symptoms and lung function for patients with lung diseases. There is currently no data on E-cigarette use in patients with

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cystic fibrosis, but there has been a steady increase in use in patients with COPD or long-term smokers at risk of developing COPD [24]. The use of E-cigarettes in the at risk and COPD groups was associated with worse pulmonary related health outcomes such as increased prevalence of chronic bronchitis or a greater likelihood of COPD disease progression. Primary human airway smooth muscle cells from COPD patients had increased production of proinflammatory mediators compared to cells from healthy patients [25]. E-cigarettes could exacerbate the chronic inflammatory environment in the lungs of patients with COPD and should not be considered as a well tolerated option for smoking cessation. A maternal E-cigarette exposure model using mice measured inflammation and DNA methylation in mothers exposed to E-cigarettes and their offspring who were exposed second-hand when feeding from the mother [26▪▪]. Mothers had increased TNFα, IL-6, IL1β measured after weening of pups, whereas offspring had increased TNFα and suppressed IL1β. They also found a global increase in methylation. Although there is current paucity of literature on intergenerational impacts of maternal e-cigarette use [27], in-utero nicotine exposure has been shown to cause epigenetic aberrations and induce asthmatic pathology in offspring that persisted into the third generation [28]. This is most pertinent as smoking women have been shown to swap to e-cigarettes during pregnancy, believing it is a healthier alternative to smoking [29], therein propagating not yet identified health risks to further generations.

E-cigarettes have been shown to effect respiratory mechanical function of young healthy male study participants [30]. A cohort of 30 E-cigarette users performed a range of lung function tests and their results were compared against 30 healthy non-E-cigarette users. They found a pattern indicative of peripheral airway obstruction or small airways dysfunction, confirming that E-cigarette use had acute effects on lung function. Conversely another study [31] found that E-cigarette use had no effect on lung function in both asthma patients and healthy controls. In this study participants completed a 1 h vaping session of a high-grade and contaminant-free mixture of propylene glycol and glycerol, with no flavourings or nicotine. This study is not representative of normal E-cigarette use should be repeated to include an E-liquid containing nicotine to show any harm minimisation potential if used as a smoking cessation device.

There has been a recent surge in hospital admissions in the United States because of lung injury associated with e-cigarette use. This has resulted in the definition of vaping associated pulmonary injury (VAPI) [32▪▪], which the USA Center for Disease Control and Prevention defines as e-cigarette use in the 90 days prior to symptoms, presence of pulmonary infiltrates on chest computed tomography imaging and no evidence of alternative diagnoses such as pulmonary infection or nonpulmonary disease [33]. Four chest imaging patterns associated with VAPI have been identified; acute eosinophilic pneumonia, diffuse alveolar damage, organizing pneumonia, and lipoid pneumonia [34]. VAPI has been suggested to be characterized by lipid-laden alveolar macrophages [35▪▪], consistent with the recent finding that mice exposed to e-cigarettes had accumulation of lipids within alveolar macrophages and epithelial cells, altered surfactant homeostasis, and impaired viral immunity [36]. However, another study did not show lipid accumulation in macrophages in those with VAPI [34]. No common compound/ingredient has been identified, although the majority of patients reported use of Tetrahydrocannabinol or Cannabidiol [37]. However, given the large number of users of e-cigarette liquids containing cannabis [38], the relatively small number of cases, and the lack of regulation of e-cigarette liquid manufacturing in United States, there remains uncertainty as to the underlying cause of VAPI.

The health effects of E-cigarettes extend beyond the respiratory system, with growing concerns for cardiovascular health of E-cigarette users. The Health eHeart study [39] looked at how E-cigarette, cigarette, and dual use influenced cardiopulmonary symptoms in patients. E-cigarette only use was associated with higher occurrence of chest pain, palpitations, coronary heart disease, and arrhythmia. This study only has a low proportion of E-cigarette only users (1.4%) and did not identify whether any of these reported cardiovascular symptoms were from preexisting conditions. Osei *et al.* [40▪▪] pooled data from the behavioral risk factor surveillance system, finding that E-cigarette users and concurrent smokers who use E-cigarettes were at greater risk of cardiovascular disease. Further studies need to be done to identify how E-cigarettes affect the cardiovascular health of users versus non users. Lee *et al.* [41] found that exposure to E-cigarette liquid caused endothelial cell dysfunction which is a precursor to myocardial infarction and other cardiovascular diseases. The exposure to un-vaporised E-liquid is not a physiologically relevant model as endothelial cells would only come into contact with the E-liquid in this form if it was ingested

E-LIQUID FLAVORS

A regular focus point of *in vitro* studies is how flavor additives effect immunological response and

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cytotoxicity. It was last estimated in 2014 that there were more than 7500 different E-liquids available for purchase, this number is likely much larger today [42]. It is clear that the constituents of E-liquids affect the response from cells after exposure, including the flavor molecules in these liquids. Current regulation does not require manufacturers to test how harmful flavour molecules are on respiratory cells, or whether they are safe to inhale after vaporization at high temperature. Krüsemann *et al.* [42] proposed a new classification system for E-cigarette flavors with 13 main categories and 90 subcategories. They generated the categories from current E-liquid flavors names found in a literature search grouping them on common linking flavor groups they belonged to. A universal categorical system for E-cigarette flavours is a starting point for E-cigarette research with ingredients already known to have harmful effects, such as diacetyl [43], being banned from use. But much more needs to be done by the scientific community to set appropriate parameters on research expectations. A lack of universal *in vitro* and *in vivo* exposure models makes it difficult to compare two different studies. There is also very little transparency in the methodology used by researchers, further complicating the comparison of research to date.

One of the biggest dangers with E-cigarette use is its inherent novelty. Not only does it heighten its appeal, and make it hard to research and regulate; but also means there is a current dearth of epidemiological data on long-term usage consequences. Identifying any possible health risks is made more arduous by the comparison to cigarettes, which draws scientists and regulators into primarily assessing cigarette smoked induced changes and pathology as outcomes in studies. However, the basic constituents of E-cigarettes and cigarettes are radically different, and therefore E-cigarettes are likely to cause novel diseases. For example, inhalation of silicates in the form of asbestos causes radically different diseases to the inhalation of quartz [44]. Thereby we advocate for it to be of utmost importance that the scientific and medical communities are on high alert for novel pathological manifestations attributable to long-term E-cigarette use.

CONCLUSION

It is our opinion that E-cigarettes can no longer be defined as harmless. Furthermore, the toxicity profile of e-cigarettes should not only be compared to tobacco cigarettes but considered in their own light, that is as compared to never users. If e-cigarettes were prescription only smoking quit aids their utility and relative risk in the community would be very different to their use as recreational drug (nicotine) delivery system. When evaluating risks, differing points of view has led authors into presenting the data in ways to support their respective argument. We recommend further debate and studies to thoroughly discern the risks for all populations. In particular, studies focused on at-risk populations, such as teenagers, pregnant women, children, and people with preexisting medical conditions need to be defined separately from those investigating risks associated with E-cigarette use as a quit aid or tobacco replacement product.

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Conflicts of interest

There are no conflicts of interest.

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1.9 The Evolving Landscape of E-cigarettes: A Systematic Review of Recent Evidence

This systematic review of literature was conceived as a side project while concurrently producing the Thoracic Society of Australia and New Zealand (TSANZ) position paper on electronic cigarettes. In this review we break down the potential harms and health outcomes that may arise from use with a focus on at risk populations. This project was conceived as an evidence update to follow up the National Academies of Science, Engineering and Medicine review on the public health consequences of E-cigarettes [98]. An important focus of this systematic review was to breakdown the harm reduction debate into key sub-arguments. We aimed summarise evidence from a balanced standpoint to elucidate the harms or benefits associated with E-cigarette use.

CHEST

The Evolving Landscape of e-Cigarettes

A Systematic Review of Recent Evidence

Check for updates

Jack Bozier, BSc(Hons); Emily K. Chivers, BSc(Hons); David G. Chapman, PhD; Alexander N. Larcombe, PhD; Nicole A. Bastian, BSc(Hons); Jorge A. Masso-Silva, PhD; Min Kwang Byun, MD, PhD; Christine F. McDonald, PhD, FCCP; Laura E. Crotty Alexander, MD, PhD; and Miranda P. Ween, PhD

Smoking continues to be a burden to economies and health-care systems across the world. One proposed solution to the problem has been e-cigarettes; however, because they are a relatively new product in the market, little is known about their potential health impacts. Furthermore, e-cigarettes continue to evolve at a rapid rate, making it necessary to regularly review and summarize available studies. Although e-cigarettes are marketed as a smoking cessation tool by some manufacturers, the reality is that many nonsmokers, including youth, are using them. This review focuses on two major demographic groups (smokers and nonsmokers) and evaluates the most recent data (early 2017 to mid 2019) regarding the potential health effects of e-cigarettes. We assessed peer-reviewed studies on the health impacts of e-cigarettes, with a particular focus on common questions asked by policy makers, clinicians, and scientists: (1) What are the effects of e-cigarettes compared with air/not smoking?; (2) Is there any direct evidence of harm or benefit to humans?; (3) Is there a risk from secondhand exposure?; (4) What are the risks and/or benefits of e-cigarettes compared with tobacco cigarette use?; (5) Are there risks or benefits to specific populations (eg, people with COPD or asthma, pregnant women [and their offspring])?; (6) What are the effects of flavoring chemicals?; (7) What are the effects of including nicotine in e-liquids?; (8) How often is nicotine concentration labeling incorrect?; and (9) What are the risks when e-cigarettes explode?

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Modern e-cigarettes were commercially developed in 2003 as an alternate nicotine delivery device for tobacco smokers with the aim of smoking abstinence.[1] Since then, the use of e-cigarettes worldwide has grown exponentially, with prevalence particularly

ABBREVIATIONS: EVALI = e-cigarette, or vaping, product use-associated lung injury; HR = heart rate; NASEM = National Academies of Sciences, Engineering and Medicine; NRT = nicotine replacement therapy

AFFILIATIONS: From the School of Life Sciences (Mr Bozier and Dr Chapman), University of Technology Sydney, Sydney, NSW, Australia; Woolcock Institute of Medical Research (Mr Bozier and Dr Chapman), University of Sydney, Sydney, NSW, Australia; Telethon Kids Institute (Ms Chivers and Dr Larcombe), Perth, WA, Australia; School of Public Health (Dr Larcombe), Curtin University, Perth, WA, Australia; Discipline of Obstetrics and Gynaecology (Ms Bastian), School of Medicine, Robinson Research Institute, The University of Adelaide, Adelaide, SA, Australia; School of Medicine (Dr Ween), University of Adelaide, Adelaide, SA, Australia; Department of Medicine (Drs Masso-Silva, Byun, and Crotty Alexander), Division of Pulmonary Critical Care & Sleep, University of California San Diego, San Diego, CA; Division of Pulmonology (Dr Byun), Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea; Department of Respiratory and Sleep Medicine (Dr McDonald), Austin Health, Heidelberg, VIC, Australia; Institute for Breathing and Sleep (Dr McDonald), Heidelberg, VIC, Australia; School of Medicine (Dr McDonald), University of Melbourne, VIC, Australia; Pulmonary Critical Care Section (Dr Crotty Alexander), Veterans Affairs San Diego Healthcare System, San Diego, CA; and the Department of Thoracic Medicine (Dr Ween), Royal Adelaide Hospital, Adelaide, SA, Australia.

Mr Bozier and Ms Chivers contributed equally to this manuscript.

CORRESPONDENCE TO: Miranda Ween, PhD, AHMS Bldg N Terrace, University of Adelaide, Adelaide, SA, 5000, Australia; e-mail: miranda.ween@adelaide.edu.au

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high in North America[2,3] and England,[3,4] whereas monthly use among adolescents in Poland has been estimated at 35%.[5] (Table 1 presents the prevalence of current e-cigarette use among the general population [tobacco smokers and nonsmokers] in different regions/countries.[2-12]) It is generally accepted that e-cigarette vapor (ie, the cloud of aerosol/mist/fog released by an e-cigarette) contains fewer toxicants than tobacco smoke; however, it still contains numerous toxicants due to the presence of nicotine (in the majority of e-liquids), the humectants propylene glycol and glycerin, flavor additives, and the presence of metal contaminants.[13,14]

In the absence of data, tobacco regulatory officials have argued for various positions along a spectrum, from promotion of e-cigarettes for harm minimization[15] to a regulatory approach that prevents expansion of the nicotine market and favors proven smoking cessation techniques.[16] This has led to a vastly heterogeneous regulatory approach to e-cigarettes,[17] with greater regulatory restriction corresponding to lower prevalence of e-cigarette use among tobacco smokers.[18]

e-Cigarettes are used by nonsmokers (including never smokers), smokers who have switched to e-cigarettes,

TABLE 1] Prevalence of Current e-Cigarette Use Among the General Population (Tobacco Smokers and Nonsmokers) in Different Regions/Countries

Region/Country	Prevalence of e-Cigarette Use Among General Population	Population (Date of Data Collection)	Reference
United States	5.5% of adults (> 18 y old)[a] 5.2% of youth (16-19 y old)[b]	32,320 adults from the Population Assessment of Tobacco and Health (PATH) study (2013-2014) 4,045 youth from the International Tobacco Control Youth Tobacco and Vaping Survey (2018)	Kasza et al, 2017[2] Hammond et al, 2019[3]
England	5.5% of adults (≥ 16 y old)[c] 2.2% of youth (16-19 y old)[b]	81,063 adults (2014-2017) 3,902 youth from the International Tobacco Control Youth Tobacco and Vaping Survey (2018)	Kock et al, 2019[4] Hammond et al, 2019[3]
Canada	2.9% of adults (≥ 15 y old)[d] 3.6% of youth (16-19 y old)[b]	30,291 adults from The Canadian Tobacco, Alcohol and Drugs Survey (2017) 3,853 youth from the International Tobacco Control Youth Tobacco and Vaping Survey (2018)	Reid et al, 2019[6] Hammond et al, 2019[3]
Central and Eastern Europe Poland only	2.9% of adults[d] 35% of adolescents (15-19 y old)[e]	14,352 University students in the Young People E-Smoking Study (2017-2018) 1,978 secondary and technical school students (2015-2016)	Brozek et al, 2019[7] Smith et al, 2019[5]
Australia	1.2% of adults (≥ 18 y old)[f]	22,354 adults in the National Drug Strategy Household Survey	Chan et al, 2019[8]
New Zealand	2.1% of adults (≥ 15 y old)[e]	3,854 adults in the Health and Lifestyles Survey (2016)	Oakly et al, 2019[9]
China	1.2% of adolescents (11-17 y old)[e]	155,117 middle school students in the Global Youth Tobacco Survey (GYTS) China Project (2013-2014)	Xiao et al, 2019[10]
Japan	1.9% of adults (17–71 y old)[e]	4,217 adults (2017)	Tabuchi et al, 2018[11]
Mexico	1% of adolescents (12-17 y old)[g] 1.1% of adults (18-65 y old)[g]	12,436 adolescents and 44,313 adults from the Encuesta Nacional de Consumo de Drogas, Alcohol y Tabaco (translation National Survey of Drugs, Alcohol and Tobacco Use) (ENCODAT) survey (2016)	Zavala-Arciniega et al, 2018[12]

[a]Current use was assessed as use of e-cigarettes every day or some days.
[b]Current use was assessed as use on ≥ 15 days in the past 30 days.
[c]Current use was assessed as indication of e-cigarette in response to the question "Can I check, are you using any of the following?"
[d]Current use was assessed as the answers "Yes, I use e-cigarettes" or "Yes, I smoke traditional cigarettes and e-cigarettes."
[e]Current use was assessed as e-cigarette use (including dual use) within the past 30 days.
[f]Current use was assessed as 'less than monthly' or more regularly.
[g]Current use was assessed as e-cigarette use on a "daily or less than daily" basis.

ex-smokers who have taken up vaping, and those using both conventional cigarettes and e-cigarettes. Regarding e-cigarette use as a smoking cessation tool, a recent randomized controlled trial reported that smoking cessation was achieved in more participants using e-cigarettes than in those using conventional nicotine replacement but with the caveat that participants in both groups had regular face-to-face meetings with clinicians.[19] This form of support is not provided to the majority of those seeking to quit smoking, and in particular medical support and knowledge are not provided for e-cigarette use. Furthermore, only 18% of participants using e-cigarettes achieved smoking abstinence, suggesting that e-cigarettes are not the "cure" for tobacco smoking. In addition, 80% of the e-cigarette users who were tobacco abstinent were continuing to use e-cigarettes 12 months later, suggesting that e-cigarettes may promote continued nicotine dependence. Evidence also suggests that many e-cigarette users continue to smoke cigarettes,[20] and the extent of harm minimization, if any, in dual users (ie, user of both cigarettes and e-cigarettes) is unclear. Even more worrisome, use of e-cigarettes may contribute to relapse of smoking in ex-smokers[21,22] and may encourage initiation of tobacco smoking among nonsmokers.[21] Systematic reviews on the role of e-cigarettes in smoking cessation have been previously published.[23,24]

Much-needed clarity was brought to the debate surrounding e-cigarettes by the 2018 report of the National Academies of Sciences, Engineering and Medicine (NASEM),[25] which summarized and drew conclusions based on the current understanding of e-cigarettes at the time. However, the e-cigarette landscape continues to evolve rapidly, with constant development of new devices and an exponentially growing body of scientific literature. The current review provides a comprehensive update of data on the potential health effects of e-cigarettes since the NASEM report. We have provided focused discussion of the scientific literature that will help inform the general public, health-care practitioners, and policy makers of the effects of e-cigarette use on health.

Materials and Methods

The authors conducted a PubMed search in the title or abstract for "electronic cigarette" OR "e-cigarette" OR "electronic nicotine delivery system" OR "personal vaporiser" OR "personal vaporizer"' published between February 2017 until end of May 2019 as well as "e-liquid" AND "nicotine content" until end of May 2019 as these were not covered in the NASEM report. This initial series of searches identified 2,687 unique results. Items were removed if they were a duplicate, not in English, or meeting abstracts, reviews, editorials, or comments on articles. The authors then screened for articles that specifically answered these common questions: (1) What are the effects of e-cigarettes compared with air/not smoking?; (2) Is there any direct evidence of harm or benefit to humans?; (3) Is there a risk from secondhand exposure?; (4) What are the risks and/or benefits of e-cigarettes compared with tobacco cigarette use?; (5) Are there risks or benefit to specific populations (eg, people with COPD or asthma, pregnant women [and their offspring])?; (6) What are the effects of flavoring chemicals?; (7) What are the effects of including nicotine in e-liquids?; (8) How often is nicotine concentration labeling incorrect?; and (9) What are the risks when e-cigarettes explode?

A total of 225 unique results were included in this review. The authors conducted reviews of the literature and categorized the study types as follows: 0, chemical analysis of e-liquid or vapor; 1, in vitro work; 2, ex vivo work from human samples; 3, animal model; 4, case study; or 5, human study. The authors had a specific focus on elucidating study design for reproducibility in future studies, study results, and any limitations to the studies including any stated conflict of interest. Where authors had authored an article to be assessed, an author who did not participate in the study independently reviewed the study.

The Harms of Vaping to Nonsmokers

Numerous studies have investigated the health effects of using e-cigarettes compared with breathing air/not smoking as well the effects of secondhand exposure to people, animals, or cells naive to tobacco smoke (e-Tables 1 and 2; Table 2[26-66]).

Progression to Tobacco Smoking

When addressing the role of e-cigarettes in harm minimization for smokers, consideration must also be given to any effect e-cigarettes may have in promoting the uptake of tobacco cigarettes among nonsmokers; that is, the "gateway effect."[21] An early meta-analysis of nine studies containing data from 17,389 adolescents and young adults showed that e-cigarette use was associated with an increased risk of subsequent initiation of tobacco smoking (OR, 3.5).[67] The evidence from more recent studies is conflicting. Temporal analysis of youth tobacco smoking and e-cigarette use suggested that at a population level, increasing prevalence of e-cigarette use in the United States was associated with a faster decline in tobacco smoking.[68] In contrast, Barrington-Trimis et al[69] and Chaffee et al[70] reported that among never smokers, adolescents who used e-cigarettes were more likely to transition to tobacco cigarette smoking, whether as a dual user or sole tobacco

TABLE 2] Studies Addressing Research Question 2: Is There Any Direct Evidence of Harm or Benefit to Humans?

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
Abafalvi et al, 2019[26]	5 General health	**Survey** Self-reported health effects of vaping among Hungarian adults Participants were either current smokers (tobacco cigarettes only), ex-smokers who had switched to exclusive e-cigarette use, or dual users of both tobacco and e-cigarettes. Smoking and e-cigarette use was graded according to frequency of use and nicotine content Exclusion criteria: < 18 y old, never smokers, invalid/incomplete responses Less than one-quarter of e-cigarette users (single + dual) reported AEs related to use but the majority perceived improvements in several health outcomes such as breathing, quality of sleep, and general physical status. Dual users were more likely to report AEs than exclusive e-cigarette users (26.2% vs 11.8%). More than 80% of exclusive e-cigarette users reported improved breathing and overall physical status. More than 65% of dual users reported improvements in breathing and overall physical status Self-reported improvements were significantly higher among individuals exclusively using e-cigarettes for > 1 y and people who were past heavy smokers (smoked ≥ 20 cigarettes per day)	Self-reported effects Recruited through online e-cigarette forum websites, which may add bias Confined to 14 specified acute events and 10 physiological functions No data on generation/type of e-cigarette No assessment of health effects in e-cigarette users who have never smoked	1,042 unique responders Dual users = 183 e-Cigarette-only users = 858 e-Cigarette-only users had higher odds of reporting benefits in breathing (OR [95% CI], 3.39 [2.15-5.33]), general physical status (2.28 [1.56-3.32]), mood (2.09 [1.48-2.96]), and quality of sleep (1.70 [1.21–2.41])
Ahmed et al, 2018[27]	4 Cardiovascular	**Case report** A 41-year-old woman with no significant medical history except daily e-cigarette use developed SCAD while breastfeeding following an uncomplicated delivery. Patient recovered following treatment	No mention of device, e-juice, or nicotine level used Postpartum hormonal changes are a known risk factor for SCAD, and the use of e-cigarettes may only "potentially" increase the risk	N = 1

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
Al-Aali et al, 2018[28]	5 Oral health	**Human study** Group 1: Current vapers who reported e-cigarette use for ≥ 1 y Group 2: Never smokers (no tobacco in any form) Exclusion: current cigarette smokers, water-pipe smokers, smokeless tobacco smokers Comparison of dental health around dental implants in male e-cigarette users vs nonusers (no tobacco of any form). e-Cigarette users had more bone loss around the implant but had less bleeding on probing. Inflammation (TNF-α and IL-1β) was considerably higher in sulcular fluid from e-cigarette users	Participants in vaper group may have previously smoked Only studied male subjects Less than one-half in each group brushed twice per day No mention of nicotine or flavors	e-Cigarette users = 47 Nonusers = 45
AlQahtani et al, 2018[29]	5 Oral health	**Human study** Group 1: Current e-cigarette users Group 2: Current cigarette users Group 3: Never-smokers (control) Participants had ≥ 1 dental implant that had been in service for ≥ 3 y Plaque index, probing depth, and mesial and distal bone loss were all higher in e-cigarette users than in control subjects, whereas bleeding on probing was lower than in control subjects. Bone loss and probing depth were higher in cigarette users than in e-cigarette users. Peri-implant sulcular fluid, TNFα, IL-6, and IL-1β were increased in e-cigarette users compared with nonusers. IL-6 was higher in cigarette users than in e-cigarette users	Lack of information on what defined an e-cigarette user. May have been dual users rather than exclusive e-cigarette users Although full-mouth periodontal examination was performed, their recordings are not presented in the study	n = 40 per group
Ang et al, 2018[30]	5 Poisonings	**Analysis of telephone enquiries** The UK National Poisons Information Service received 278 enquiries relating to e-cigarette liquid exposure in children between April 2008 and March 2016 There has been a consistent and substantial increase in calls regarding e-liquid exposure in children since 2012, with > 100 calls in	Complete follow-up data unavailable	278 enquiries Symptoms present in 63/278 cases

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		2018; 80% of cases involved children aged < 4 y. Most cases were minor and asymptomatic, and there were no fatalities. In symptomatic cases, the most common symptoms were vomiting and tachycardia		
Arter et al, 2019[31]	4 Respiratory	**Case report** An 18-year-old woman presented with fever, cough, difficulty breathing, and pleuritic chest pain. She developed hypoxic respiratory failure and was diagnosed with acute eosinophilic pneumonia. Cause was attributed to e-cigarette use as she had begun using e-cigarette with nicotine (6%) 2 mo prior. She vaped for 30 min, 5 times/d	No clear link between e-cigs and acute eosinophilic pneumonia e-Cigarette use was implicated because there was a lack of other causative irritants	N = 1
Agustin et al, 2018[32]	4 Respiratory	**Case report** A 33-year-old man presented with hemoptysis and subacute respiratory failure. He was later diagnosed with diffuse alveolar hemorrhage syndrome, likely induced by aggressive vaping. Blood was found in BAL but little inflammation in serum. No microbiological growth. The patient recovered following steroid treatment and has ceased using e-cigarettes. e-Liquid used in this case was predominantly PG-based, with nicotine (quantity not specified) and flavorings	Was treated with antibiotics for pneumonia 2 weeks prior to visit with minimal improvement Implied that it was caused by "experiment[ing] with new flavors" but no causality No mention of flavors used or nicotine level	N = 1
Bardellini et al, 2018[33]	5 Oral health	**Human study** Group 1: Former smokers (daily/almost daily use, ≥ 100 lifetime cigarettes, quit between 6 mo and 2 y ago) Group 2: Current e-cigarette users (≥ 6 mo of use) e-Cigarette was associated with more oral mucosal lesions than former cigarette use. Significant increases in rates of nicotine stomatitis, hairy tongue, and hyperplastic candidiasis were seen in e-cigarette users compared with former smokers. One case of	Definition of smokers is only "smoked more than 100 cigarettes in lifetime"; not very representative of real smokers; could include people who smoked for a short period against heavy e-cigarette users No details of use frequency of participants e-Cigarette users weren't restricted to same device/e-liquid, which could contribute to variation e-Cigarette group was almost all men	n = 45 former smokers, 45 e-cigarette users

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		squamous cell carcinoma was seen in former smokers but none in e-cigarette users. No differences in terms of precancerous oral mucosal lesions (lichen planus, leukoplakia) were found between the two groups		
Cant et al, 2017[34]	4 Oral health	**Case report** A 72-year-old man presented with a severe, necrotic looking oral ulcer attributed to a severe burn caused by e-cigarette use. He gave a history of a painful area appearing after inhaling strongly on his e-cigarette and suffered extreme discomfort immediately afterward. The area eventually healed completely	Patient had smoked 20 conventional cigarettes per day for 30 y	N = 1
Caponnetto et al, 2017[35]	5 Neurological	**Human study** Thirty-four smokers (using ≥ 15 cigarettes/d for ≥ 10 y) abstained from smoking for ≥ 12 h before using one of four e-cigarettes or their usual tobacco cigarette. First- and second-generation devices tested, 0 or 24 mg/mL nicotine, mint or tobacco flavor An initial 15-min session was followed by ad libitum use throughout the day No effect was seen on cognitive performance (attention, executive function, and working memory)	COI: Research supported by Happy Liquid Current smokers; thus no true control session was available due to nicotine withdrawal	34 regular tobacco smokers
Carter et al, 2017[36]	4 Respiratory	**Case report** A 35-year-old woman presented to the ED with sudden-onset dyspnea, extensive pattern of suspected chemical injury was noted in her airways; her injuries were likely suffered secondary to use of an ENDS Reported daily use of ENDS with 25 mg/mL nicotine	Case report Patient had several chronic conditions associated with obesity	N = 1
Chatterjee et al, 2019[37]	3 Vascular 5 Systemic/blood marker	**Human study** Ten healthy nonsmokers Device name: E-puffer eco-disposable Device power settings: 3.7 V 2.7Ω Puff length: 2 s Puff frequency:	Cell lines used for ROS experiments; would have been better to measure ROS in participants (although not enough oxidant may have been available)	N = 10

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TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		Puff volume in mL: No. of puffs: 16-17 Total exposure time: Nicotine concentration (if any): 0 mg/mL List flavors tested: PG/VG ratio: 70:30 Increased serum CRP and ICAM1 after single vape session (blood from participants) Decreased nitric oxide was also found in serum (sign of endothelial dysfunction) Increased ROS in HPMVEC treated with EVE		
Chaumont et al, 2018[38]	5 Cardiovascular Systemic/ blood marker	**Human study** 25 occasional smokers used an e-cigarette containing 50:50 PG:VG, 0 or 3 mg/mL nicotine for ~12 min. A "last-generation high-power vaping device" was used with the following settings: Power settings, 60 W (0.4 Ω dual coil). Puff number and frequency, 25 × 4 s puffs, at 30 s intervals. Vaping nicotine free PG/VG mix did not alter microcirculatory function, arterial stiffness, or oxidative stress. Using the nicotine-containing e-cigarette decreased microcirculatory endothelial-dependent function, increased arterial stiffness, increased BP and heart rate, and increased plasma myeloperoxidase (oxidative stress)	All participants were occasional smokers Blinding not possible Low nicotine level, not representative of common levels	25 participants N = 25
Cho, 2017[39]	5 Oral health	**Survey** Responses from the Twelfth Korean Youth Risk Behavior Web-based Survey were analyzed. Responses were divided into never, former, past month, and daily e-cigarette users. e-Cigarette users were further divided into nicotine and nicotine-free groups. Compared with never users, daily e-cigarette use was associated with an increased odds of having a cracked or broken tooth in the past 12 mo and tongue/inside-cheek pain.	Low numbers of non-nicotine e-cigarette users yielded very wide CIs	65,528 students Daily users = 297 Past month users = 1,259 Former users = 3,484 *Cracked tooth* Never use: no = 53,605, yes = 6,519 Former: no = 3,202, yes = 646 (OR, 1.16 [1.04-1.30])[a] Past month: no = 1,005, yes = 254 (OR, 1.26 [1.06-1.51])[a]

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TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		Odds were adjusted for potential confounders such as tobacco smoking, economic status, obesity, and carbonated drink consumption. ORs were only significant in nicotine e-cigarette users		Daily user: no = 216, yes = 81 (OR, 1.65 [1.19-2.27])[b] *Tongue/inside-cheek pain* Never use: no = 53,549, yes = 6,575 Former: no = 3,417, yes = 431 (OR, 0.98 [0.86 ± 1.11]) Past month: no = 1,087, yes = 172 (OR, 1.26 1.08 (0.88 ± 1.33) Daily user: no = 238, yes = 59 (OR, 1.54 [1.05 ± 2.26])[a]
Demir and Topal, 2018[40]	4 Auditory	**Case report** A 6-year-old girl presented to the emergency clinic after ingesting a bottle of e-liquid (1.2 mg/mL nicotine) 3 to 4 h previously. The patient was experiencing nausea and vomiting. Her estimated nicotine intake was 8.4 mg. She experienced sudden sensorineural hearing loss (defined as 30+ dB bilateral or unilateral sensorineural hearing loss at 3 consecutive frequencies within 72 h) 24 h after consuming the e-liquid. In the absence of any other significant history or abnormal test results, the authors theorize that the hearing loss is related to the ingestion of e-liquid. The patient's hearing did not recover, and now uses bilateral hearing devices	Case report Only subjective evidence of normal hearing loss prior to exposure Methylprednisolone was given although it is unclear as to whether immunologic causes were ruled out	N = 1
Flower et al, 2017[41]	4 Respiratory	**Case report** A 33-year-old man experienced respiratory bronchiolitis interstitial lung disease. The patient was a dual user of tobacco and e-cigarettes, vaping 10 to 15 times/d in addition to smoking 10 tobacco cigarettes. After 3 mo of e-cigarette use, the patient had poorly defined pulmonary nodules with fluffy parenchyma opacification along the terminal bronchovascular units on their chest CT scan.	No mention of nicotine Patient "continued to smoke 10 cigarettes per day" suggests tobacco smoking did not change with e-cigarettes. Therefore, could be caused by increased exposure Diagnosis of mixed germ cell cancer may reduce generalizability to all e-cigarette users	N = 1

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TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		The abnormalities resolved after the patient ceased using the e-cigarettes		
Franzen et al, 2018[42]	5 Cardiovascular	**Human study** Crossover study of 15 smokers using an e-cigarette with nicotine, an e-cigarette without nicotine, and tobacco cigarettes in three separate sessions (48 h washout between) Device name, DIPSE, eGo-T CE4 vaporizer Device power settings, 3.3 V, 1.5 Ω 7.26 W Puff length, 4 s Puff frequency, 1 puff per 30 s Puff volume in ml, NA No. of puffs, 10 Duration and frequency of exposure, Nicotine concentration (if any), 0 and 24 mg/mL nicotine List flavors tested (if any), tobacco flavor PG/VG ratio, 55/35 Participants were followed up for 2 h after smoking a cigarette or vaping an e-cigarette Nicotine-containing EV had similar effects to cigarettes N-EV increased systolic BP for 45 min after exposure. CS increased SBP for 15 min after exposure. NF-EV did not affect SBP N-EV increased heart rate for 45 min after exposure. CS increased SBP for 30 min following exposure. NF-EV did not affect SBP Elevation of pulse wave velocity was independent from mean arterial pressure as well as HR in the N-EV and CS groups	Small group sizes Only used 10 puffs of e-cigarette	n = 4-6 per group
Fuller et al, 2018[43]	5 Urine marker	**Human study** 13 non-smoking (≥ 6 mo) e-cigarette users (> 24 times per week) 10 nonsmoking, non-e-cigarette using control subjects Analysis of e-cigarette user urine revealed the presence of two carcinogenic	Small group sizes Not all participants in e-cigarette group were long-term nonsmokers (> 12 mo) No comparison with levels in current smokers Limited information about e-cigarette use	Nonsmoker nonvaper = 10 e-Cigarette user = 13

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TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		compounds, o-toluidine and 2-naphthylamine, at a mean 2.3- and 1.3-fold higher concentration		
Goniewicz et al, 2018[44]	5 Systemic/blood marker	**Human study** PATH Study data were used. Exclusive e-cigarette users, tobacco-only users, and dual users were identified and biomarkers of nicotine exposure and other tobacco-related toxicants (eg, PAHs, VOCs, metals) were assessed Concentrations of nicotine and toxicants were lower among exclusive e-cigarette users compared with tobacco smokers. Dual users exhibited higher concentrations of exposure to almost all the biomarkers compared with exclusive cigarette smokers and exclusive e-cigarette smokers	No data on generation/type of e-cigarette	The final analytic sample size was 5,105 participants. Smokers = 2,411 e-Cigarette-only users = 247 Dual users = 792 Never users = 1,655
Govindarajan et al, 2018[45]	5 Poisonings	**e-Cigarette exposure report analysis** Reports of liquid nicotine exposure data from the NPDS for January 2012 through April 2017 were analyzed. 8269 exposures occurred among children aged < 6 y. The number of exposures has fallen since January 2015, which may be related to introduction of child-resistant packing (federal law requiring child-resistant packaging was introduced after July 26, 2016). 20.3% of children experienced a minor effect, 1.67% a moderate effect, and 0.1% a major effect; 35.1 % were treated and/or evaluated and released; and 1.4 % were admitted to the hospital. There was one death of a child aged 1 y	Calls to poisons centers are voluntary; therefore, the NPDS likely underestimates the true incidence of exposures to liquid nicotine Differentiation of liquid nicotine exposures due to e-cigarette themselves vs e-liquids was uncertain, thus limiting the ability to assess impact of child-resistant packaging laws that only apply to e-cigarette liquid containers	
Hughes and Hendrickson, 2019[46]	4 Poisonings	**Review of case reports** 265 calls to the Oregon Poison Center related to e-cigarettes were assessed. Cases were followed up in 4 h to re-evaluate symptoms of the affected individual Of the 265 incidents, 193 involved children and 72 involved adults	Not all patients or practitioners were able to identify the brand of e-cigarette solution or the concentration Poison center reporting is voluntary; their data likely underestimate the total number of e-cigarette exposures and associated adverse clinical effects	n = 265

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TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		72% of the pediatric cases and 61% of the adult cases involved e-liquid refill containers or liquid 56% of pediatric exposures involved ingestion of refill liquid. Of these, 32% exhibited symptoms after ingesting e-liquid. Only 2 children who were asymptomatic during the initial call became symptomatic on follow-up. Most symptomatic patients were no longer symptomatic on follow-up. 71 specific products/brands were identified as being involved in the incidents. These products had nicotine concentrations ranging from 0 to 60 mg/mL. A variety of flavors of e-liquid were involved, many of which with names that may be attractive to children		
Itoh et al, 2018[47]	4 Respiratory	**Case report** A 46-year-old healthy man developed respiratory distress, night sweats, fever, and weight loss after 1 mo of e-cigarette use. He was admitted to the hospital after 2 mo and diagnosed with acute alveolitis (intra-alveolar fibrosis accompanied with exudate containing abundant lipid-laden macrophages, eosinophils, and neutrophils). This was attributed to e-cigarette- induced acute lung injury following other testing. Glycerin could be attributed to the abundant lipid-laden macrophages. The patient ceased e-cigarette use, and the acute lung injury was resolved following pharmacologic treatment	Single case report, not representative of how all patients would respond, although the author cites two other case studies of acute eosinophilic pneumonia related to e-cigarette use	N = 1
Khan et al, 2018[48]	4 Respiratory	**Case report** 40 -year-old African-American woman who presented with acute hypoxemic respiratory failure and was diagnosed with organizing pneumonia secondary to e-cigarette use following 1 mo of symptoms	No evidence given as to why e-cigarettes were decided to be the cause beyond coincidental timing of symptom onset and switching from tobacco cigarettes to e-cigarettes	

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		History: smoked one-half a pack a day for > 10 y; 1 mo ago, she switched to e-cigarettes to help her quit		
Lappas et al, 2018[49]	5 Respiratory	**Human study** Participants (current dual e-cigarette and cigarette users) with and without mild asthma used an e-cigarette. Device name: NA Device power settings: 1.6 Ω, 3.7V Puff length: 4 s Puff frequency: 30 s puff interval Puff volume in mL: NA No. of puffs: 10 Duration and frequency of exposure: Nicotine concentration (if any): 12 mg/mL List flavors tested (if any): tobacco PG/VG ratio: 46:34 PG:VG **Asthma groups** Both "healthy" and "mild asthmatic" groups exhibited increased total impedance and resistance immediately following e-cigarette session. Mild asthmatic group had higher baseline values and more prominent effects immediately following the e-cigarette session. F_{ENO_2} decreased significantly in both groups; "asthmatic" group took an additional 15 min to return to baseline levels (≥ double 2-fold "healthy" group time).	Participants were all dual e-cigarette and cigarette users Participants were all smokers. No smoke group comparison	N = 54 smokers
Marasco et al, 2018[50]	4 Respiratory	**Case report** A 17-year-old male subject experienced dyspnea, shortness of breath, and painful swallowing, and was found to have spontaneous pneumomediastinum (mediastinal emphysema) from e-cigarette use. The patient claimed this was his first and only e-cigarette use. Patient was discharged and referred to general thoracic surgery department outpatient clinic after discharge because a 48 h chest radiograph showed no progression/complication. At the	Single case report	

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		2-month follow-up, the condition appeared to have resolved, and the patient had stopped vaping		
Meo et al, 2019[51]	5 Respiratory	**Human study** e-Cigarette users defined as using nicotine-containing e-liquid daily for N at least the past 6 mo with current or former cigarette smokers, shisha smokers, and users of other tobacco products excluded. Nonusers defined as never tried e-cigarettes, regular cigarettes, or shisha The lung function test parameters that were found to be significantly decreased in e-cigarette users compared with their control group were FEV_1, FEV_1/FVC, FEF25%, FEF50%, FEF75%, FEF25%–75%, and FEF75%–85%. No significant difference in $FENO_2$, FVC, and PEF between the 2 groups. The reduced pattern of lung function test parameters exhibits peripheral obstructive airway impairment	Only male subjects assessed Only 6 mo of e-cigarette exposure Were not able to calculate the dose response of e-cigarettes as there was no set "dose" for use	Nonusers = 30 Daily users = 30
Miler and Hajek, 2017[52]	4 Oral health/ infection	**Case report** A never smoker who became a vaper experienced a complete resolution of chronic tonsillitis and a marked improvement in tonsil stones after 8 mo of e-cigarette use	Self-reported diagnosis and improvement rather than medical records	N = 1
Miler and Hajek, 2018[53]	4 Respiratory/ infection	**Case report** A never smoker adopted an e-cigarette that his wife was using. After a few weeks of vaping liquids containing vegetable glycerin with low levels of nicotine (3 mg/mL), he experienced a complete resolution of chronic nasal *Staphylococcus aureus* infections. Patient periodically exhaled the vapor through nostrils. No VG in e-liquids	Single patient with no investigation as to what changed in the nasal passages, and no discussion about what else was ruled out as the cause. Authors admit it could be merely coincidental	N = 1
Mokeem et al, 2018[54]	5 Oral health/ infection	**Human study** Group 1: cigarette smokers (≥ 100 cigarettes during their lifetime and reported smoking daily)	Only male participants Narrow range of nicotine concentrations in e-liquids. No comparison with nicotine-free e-liquid	Smokers = 24 Water-pipe smokers = 33 e-Cigarette users = 30 Never smokers = 32

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TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		Group 2: water-pipe smokers (smoked water pipe daily for ≥ 15 min for 12 mo and had never smoked other tobacco products) Group 3: e-cigarette users (vaping ≥ 6 times daily since 12 mo) Group 4: never smokers (never used any form of tobacco product) Determination of oral *Candida* species was performed, followed by identification of yeast species. Microbial colonies were subcultured and then on each sample, PCR was performed without DNA extraction to generate PCR products for 3 different *Candida* species: *Candida tropicalis*, *Candida parapsilosis*, and *Candida guilliermondii* Overall, the oral *Candida* carriage rate was the highest among cigarette smokers, water-pipe smokers, and e-cigarette users compared with never smokers, with *C albicans* being the most commonly isolated oral yeast species from all groups. The percentage of patients colonized by *C albicans* was the highest for cigarette smokers, followed by water-pipe smokers, e-cigarette users, and never smokers. Prevalence of *C albicans* was significantly higher in cigarette smokers, water-pipe smokers, and e-cigarette users compared with never smokers Following stratification for age and among individuals with up to 6 missing teeth, there was no significant differences in oral yeast carriage between cigarette smokers, water-pipe smokers, and e-cigarette users, but the oral yeast carriage for these groups was significantly higher than never smokers. Following stratification for daily frequency of tooth brushing and UWSFR, there was no	Self-perceived oral symptoms taken into account; may not be accurate Mean number of missing teeth was > 4 across all groups, suggesting low dental hygiene in this population. No assessment of oral health/hygiene performed by a dental clinician. History of oral health not known	

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		significant difference in oral yeast carriage between individuals in all groups.		
Morley et al, 2017[55]	4 Poisonings	**Case report** A 32-year-old man ingested nicotine-containing e-liquid while under the influence of alcohol. His ingested approximately 1,440 mg of nicotine by drinking 20 mL of e-liquid. The patient suffered brain hypoxia caused by prolonged CPR. He died following 3 days in intensive care	Single case report	N = 1
Motooka et al, 2018[56]	4 Respiratory/ general health	**Case report analysis** The authors analyzed 7,348,357 cases of AEs in the US Food and Drug Administration Adverse Event Reporting System. Twenty-seven cases were identified in which e-cigarettes were designated as the primary source of the AE. Causes of AE included dizziness, dyspnea, nausea, chest pain, cough, and wheeze. Other, non-MedDRA labels included chills, seventh nerve paralysis, and productive cough (1 each)	May be limited by underreporting and/or miss-classification in the reporting system. No adjustment for confounding and/or patient medical history	Reporting ORs not calculated for e-cigarettes
Noble et al, 2017[57]	4 Poisonings	**Case report** A 6-year-old girl with severe toxicity who required intubation following ingestion of e-cigarette liquid containing nicotine. It was estimated that the child ingested approximately 703 mg (35 mg/kg) of nicotine. The patient survived	The e-cigarette liquid was stored in an ibuprofen bottle and was (unknowingly) given to the girl by her father	N = 1
Paik et al, 2018[58]	4 Poisonings/ cardiovascular	**Case report** A male orally ingested a high concentration of liquid bought for e-cigarette use with the intention to commit suicide. The patient presented with bradycardia and hypotension, together with impaired consciousness. He recovered following treatment with atropine and a vasopressor		N = 1
Park and Min, 2018[59]	4 Poisonings/ cardiovascular	**Case reports** A 27-year-old man who ingested about 23 mg/kg of nicotine and a 17-year-old female subject who ingested about 30 mg/kg of nicotine. Both patients presented		

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		seizure-like movement and cardiac arrest. They had metabolic acidosis and transient cardiomyopathy. They were ultimately discharged with a cerebral performance category of 2 and 4, respectively		
Polosa et al, 2017[60]	5 Respiratory	**Human study** Prospective study of vapers (regular daily for ≥ 3 mo) (who had previously not smoked cigarettes) to assess a range of health outcomes, including respiratory parameters, over 3.5 y. No significant differences in lung function, including eNO and eCO, over 3.5 y in vapers and in vapers compared with nonsmokers. No worsening in spirometry findings (ie, lung function); no development of respiratory symptoms; no changes in markers of lung inflammation in exhaled air; no signs of early lung damage on high-resolution CT imaging	COI: R.P. has previously received funding or fees from Philip Morris and other pro-e-cigarette associations Only 9 subjects completed study. Young subjects, short term follow-up. Potential for selection bias if those who failed to return for follow-up may have been experiencing adverse effects	N = 9
Pywell et al, 2018[61]	5 Vascular	**Human study** Measured hand microcirculation in smokers/nonsmokers using a Doppler probe, after using a 0- or 24-mg/mL nicotine e-cigarette (5-min session). As quitting smoking attributed to 41% reduction in complications following hand surgery, use of e-cigarettes to decrease hand surgery complications was assessed. Nicotine containing e-cigarette significantly reduces hand microcirculation of smokers, with no change in nonsmokers; thus should be used as a safe replacement prior to surgery	No information on e-cigarette used Smoking protocol was not ad libitum, followed a 10-inhalation protocol over 5 min, with no reference to why these times were chosen other than more sessions made some participants feel nauseous N is low	Smokers = 7 Nonsmokers = 8
Richmond et al, 2018[62]	5 Poisonings/respiratory	**Survey** A total of 220 cases of harm to children and adolescents reported by survey. A total of 135 cases of adolescents seeking treatment for nausea, vomiting, cough, throat irritation, or acute nicotine toxicity after inhalation of e-cigarette vapor. Eighty-five cases of children presenting to the ED with nausea, vomiting cough, or	Data are self-reported, from survey COI reported "AM reports grants from Canadian Institutes of Health Research during the conduct of the study"	

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
		respiratory irritation following e-liquid ingestion		
Samburova et al, 2018[63]	5 Respiratory/ cancer markers	**Human study** Measured exhaled aldehydes in breath and retention of aldehydes in respiratory tract of 12 e-cigarette users. All participants used BLU e-cigarettes, and their own personal devices for comparison (CE4, V2, Sigelei, Aspire Cleito). Menthol, Classic, Red tobacco, Bubble-gum, Watermelon, Fruit mix, Butterspot, Vanilla, Snozberry, Vanilla + Fruit flavors used. Puff durations for participants were 3 ± 1 s for each participant. Aldehydes were measured in straight mainstream extracts from e-cigarette minus exhaled aldehydes to determine respiratory tract retention. Results showed increased concentrations of exhaled carbonyls following e-cigarette use (ie, formaldehyde, acetaldehyde, propionaldehyde, benzaldehyde, glyoxal, methyl ethyl ketone). Also showed approximately 97% formaldehyde retention and 91.6 ± 10% acetaldehyde retention in participants. Highest total aldehyde exposure was (14.2 μg/puff), (53.2 μg/puff), and (12.8 μg/puff) Range of formaldehyde and acetaldehyde exposure was 0.33-24.4 μg/puff Acrolein exposure was seen in 12 of 19 samples at a range of 0.01 and 1.4 μg/puff	No smoking histories listed; participants were asked to abstain from e-cigarette use for 2 h prior; no mention of smoking abstinence Small sample size Method for determining retention is not accurate, topography of all participants would be different so cannot assume they all inhale the same amount as a mainstream sample	n = 12 participants, 19 samples
Sommerfeld et al, 2018[64]	4 Respiratory	**Case report** An 18-year-old female subject with mild intermittent asthma experienced hypersensitivity pneumonia and ARDS. She had started to use e-cigarettes over preceding 2 to 3 weeks and had been using them 1 to 2 days prior to onset of symptoms. She was intubated and required vasopressor support but responded rapidly to corticosteroids	Suggestive but not confirmed diagnosis	

(Continued)

TABLE 2] *(Continued)*

Reference	Study Type	Text Summary (Methodology, Findings, Conclusions)	Limitations	Summative Data, Including No. of Participants, Pooled ORs
Spindle et al, 2017[65]	5 Cardiovascular	**Human study** Experienced e-cigarette users used an e-cigarette (either ad libitum or directed) with or without a "mouthpiece-based computerized systems" used to measure puff topography. Researchers assessed whether presence of the mouthpiece influenced nicotine delivery and other acute effects of e-cigarette use (eg, heart rate). e-Cigarette use altered heart rate and plasma nicotine content and had subjective effects. Mouthpiece-based computerized systems had no effect on outcomes		N = 29 experienced e-cigarette users, each with a session with and without mouthpiece addition
van der Meer et al, 2017[66]	4 Poisonings	**Case report** A 42-year-old man was admitted to the intensive care ward due to cardiac arrest. The patient had ingested highly concentrated liquid nicotine, originating from a vial with liquid for e-cigarettes. When the ambulance personnel found the patient, he did not have a pulse; his pulse returned following CPR and administration of adrenaline. Upon admission, the patient's plasma nicotine level was high at 3.0 mg/L (reference values for a smoker, 0.01-0.05 mg/L) and the patient's neurological function was poor. The patient was treated symptomatically but eventually died of a postanoxic encephalopathy	Case report	N = 1

Study types are categorized as: 0, chemical analysis of e-liquid or vapor; 1, in vitro work; 2, ex vivo work from human samples; 3, animal model; 4, case study; or 5, human study. AE = adverse event; COI = conflict of interest; CPR = cardiopulmonary resuscitation; CRP = C-reactive protein; CS = cigarette smoke; eCO = exhaled carbon monoxide; ENDS = electronic nicotine delivery system; eNO = exhaled nitric oxide; EV = e-cigarette vapor; EVE = e-cigarette vapor extract; F_{ENO} = fractional exhaled nitric oxide; HPMVEC = human primary pulmonary microvascular endothelial cells; ICAM1 = intercellular adhesion molecule 1; MedDRA = The Medical Dictionary for Regulatory Activities; NA = not available; N-EV = nicotine containing e-cigarette vapor; N-EVE = nicotine containing e-cigarette vapor extract; NF-EV = nicotine free e-cigarette vapor; NF-EVE = nicotine free e-cigarette vapor extract; NPDS = US National Poison Data System; PAHs = polycyclic aromatic hydrocarbons; PCR = polymerase chain reaction; PG = propylene glycol; ROS = reactive oxygen species; SBP = systolic blood pressure; SCAD = spontaneous coronary artery dissection; TNF = tumor necrosis factor; UWSFR = unstimulated whole salivary flow rate; VG = vegetable glycerin; VOCs = volatile organic compounds. See Table 1 legend for expansion of other abbreviation.

[a] $P < .05$.

[b] $P < .01$.

cigarette smoker. This finding suggests that the effect of e-cigarettes may be different at the population level and the individual level.

Inflammation and Immune Response

e-Cigarette-induced toxicity in a range of cells (including lung cells) was well established in the NASEM report,[25] and other studies have reinforced these findings,[71-75] with many studies suggesting increased oxidative stress as a cause of toxicity.[71,76-78] Studies have begun to explore beyond toxicity, many with a focus on inflammation. This focus has proven an interesting area of research as some in vitro/ex vivo studies have shown that e-cigarettes can cause the release of pro-inflammatory cytokines,[71,79-81] whereas others have shown that the levels of some cytokines can decrease with certain flavors or exposure times.[74,75,82-84] Mouse studies also yielded conflicting cytokine data.[85,86] Human studies showed unchanged cytokines in the gingival crevicular fluid[87] or saliva[88] of e-cigarette users, whereas several cytokines were increased in the sulcular fluid of e-cigarette users.[28,29]

e-Cigarette exposure also impaired in vitro/ex vivo viral responses[74] and bacterial clearance by macrophages[71,89] and neutrophils.[83,90] Additional studies showed increased adhesion and colonization of bacteria.[90,91] These findings suggest that use of e-cigarettes may impair the ability to fight infection.

Clinical Analysis of Cardiorespiratory Changes

e-Cigarette users have an increased risk of respiratory symptoms compared with never smokers, and there is a significantly increased risk in ex-smokers who currently use e-cigarettes compared with ex-smokers who do not.[92] Although lung function, as assessed by using spirometry, has been shown to be impaired in tobacco-naive e-cigarette users compared with never smokers,[51] a prospective study (with a conflict of interest) of tobacco-smoking-naive e-cigarette users showed no change in lung function, respiratory symptoms, and inflammation over 3.5 years of use.[60] The airways of e-cigarette users appear more friable and erythematous during bronchoscopy compared with those of nonusers.[93] Short-term e-cigarette use elevated heart rate (HR) in humans,[65,94] whereas in mice, chronic exposure decreased HR and elevated BP.[80] There are conflicting reports as to whether e-cigarette use is associated with cardiovascular disease in population studies.[95,96] Although a very small study in humans (N = 9) suggested that e-cigarette use does not affect the metabolic activity of aortic wall tissue,[97] another showed that e-cigarette use increased arterial stiffness.[98] Murine models have shown that e-cigarette exposure leads to increased aorta stiffness and constrictor responses,[99] increased angiogenesis in heart tissue,[100] increased endothelial cell markers,[98] and decreased vasodilation.[99] In rodents, long-term e-cigarette exposure leads to emphysematous lung destruction, loss of pulmonary capillaries,[101] reduced small airway function, and airway hyperresponsiveness.[102] Furthermore, acute exposure in guinea pigs causes transient bronchoconstriction due to activation of vagal bronchopulmonary C-fiber afferents.[103]

There have been a number of case studies in which e-cigarette use is believed to be the cause of respiratory disease (Table 2). Recently, there has been an outbreak of lung injuries associated with e-cigarette use in the United States that has been labeled EVALI (e-cigarette, or vaping, product use associated lung injury)/vaping-associated pulmonary illness. Almost 2,300 cases of EVALI had been reported to the Centers for Disease Control and Prevention by late November 2019, with 47 deaths confirmed.[104,105] Cases including one death have now been reported from Canada, Great Britain, Malaysia, Argentina, and Malaysia. Many of the respiratory case reports in Table 2 may also have been early cases of EVALI. Although the specific chemicals responsible for the lung damage have yet to be determined with proof of causation, these reports highlight the fact that heating and inhalation of the wrong substance into the lungs can cause serious lung damage and even death.[105-107]

Dental Health

Reports suggest that, compared with never smokers, e-cigarette users are more likely to have gum disease, bone loss around teeth, and broken teeth.[39,108-110] In people with dental implants, e-cigarette use was associated with bone loss around the implant, increased inflammation, and higher plaque index and probing depth.[28,29] However, other studies report no difference in tooth health or oral inflammation.[54,87] Conflicting results have also been reported regarding the effect of e-cigarette use on the oral microbiome, with studies showing higher oral *Candida* carriage rates[54] and greater *Streptococcus mutans* colonization on the enamel surface,[111] or no difference in the oral microbiome.[112]

Developmental Effects

e-Cigarette exposure reduced proliferation and altered morphology in healthy human bone marrow-derived mesenchymal stem cells[78] as well as reduced placental trophoblast invasion and tube formation.[113] In a frog model, flavored e-cigarette exposure with and without nicotine during embryonic development led to craniofacial defects.[114] Similarly, exposure of pregnant mice to e-cigarettes without nicotine led to heavier offspring with more fat mass and body fat percentage, suggesting that in utero exposure may lead to metabolic dysfunction in the offspring.[115] In addition, maternal e-cigarette exposure also increased brain neuropeptide Y and inducible nitric oxide synthase in offspring,[115] leading to impaired short-term memory and hyperactivity.[116]

Harms and/or Benefits of Vaping for Smokers

e-Cigarettes are proposed as a harm reduction tool for tobacco smokers wishing to quit. Systematic reviews suggest a lack of clear efficacy of e-cigarettes in smoking cessation.[23,24] Although a recent randomized controlled trial reported a higher quit rate with e-cigarettes compared with nicotine replacement in committed quitters, 80% of those in the e-cigarette group were still vaping at 12 months.[19] It is therefore vital to determine the potential risks and/or benefits of transitioning to e-cigarettes from tobacco cigarettes (e-Table 3). Studies have compared the toxicant exposure between e-cigarettes and tobacco cigarettes, assessed by systemic and salivary tobacco-specific nitrosamines, toxicants, and metals.[44,117-119] Levels of nitrosamine *N*-nitrosonornicotine, carbon monoxide, and nicotine-derived nitrosamine ketone (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol were all lower in e-cigarette users than in smokers, suggesting a reduced risk of harm from toxicant exposure if smokers were to switch to e-cigarettes. Goniewicz et al[44] found that toxicant exposures were highest in dual users but were reduced in e-cigarette users compared with smokers. Smokers and e-cigarette users both had increased toxic metals in urine and blood, but the metals detected in each group were different.[44,118,120] Data from the Behavioral Risk Factor Surveillance System (BRFSS) study showed that smokers and dual users, but not e-cigarette only users, were at increased risk of cardiovascular disease compared with nonsmokers.[96]

Alzahrani et al[95] analyzed data from the 2014 and 2016 National Health Interview Surveys and found that daily e-cigarette use resulted in an increased odds of myocardial infarction, suggesting that switching to e-cigarettes may not alleviate risk of cardiovascular disease. In contrast, studies have suggested that switching from tobacco cigarettes to e-cigarettes may improve oral health,[54,77,87,88,121,122] as well as improved BP, HR, exhaled carbon monoxide, exhaled nitric oxide, and voice shimmer.[123-126] However, it should be noted that in the majority of the studies in which healthy control subjects were compared with e-cigarette users, these markers were higher than nonsmoking, highlighting that long-term switching to e-cigarettes instead of smoking may not be superior to smoking cessation using nicotine replacement therapy (NRT) (e-Table 3). Furthermore, numerous studies have shown e-cigarettes to be pro-inflammatory in vitro and in vivo, suggesting that lung pathology in smokers related to a dysregulated inflammation would not necessarily be resolved by switching to e-cigarettes. Therefore, studies comparing e-cigarettes with the more traditional NRT should be considered to identify the best methods to begin the healing process that has been shown to occur in smokers who quit entirely as soon as possible and avoid further damage.

Harms and/or Benefits of e-Cigarette Use in High-Risk Populations

Populations that are especially vulnerable to the effects of tobacco smoking include people with COPD and asthma, and pregnant women and their offspring. Recent studies on the effects of e-cigarettes in these vulnerable populations were therefore reviewed (e-Table 4).

People With COPD

People with COPD who are struggling to quit smoking may consider e-cigarette use as an alternative smoking cessation tool, and some patients with COPD may have already transitioned to dual use or e-cigarette only use. The NASEM review[25] concluded that results were unclear regarding whether e-cigarette use in patients with COPD would be beneficial, neutral, or harmful. Recent studies have not further elucidated whether switching to e-cigarettes from traditional tobacco cigarettes would reduce lung inflammation or disease progression in these patients. Traditional NRT is currently the safest option for patients with COPD, as research to date suggests that e-cigarettes dysregulate inflammation and have adverse effects on the airways of users,[93] and thus a negative impact on patients with COPD cannot be ruled out. Furthermore, these patients

are at significant risk of cardiovascular comorbidity that may be worsened by e-cigarette use given its known association with increased cardiovascular events.[95,96]

People With Asthma

The prevalence of e-cigarette use in adults with asthma has continued to increase.[127] Currently, some clinicians and researchers advocate that smokers with asthma switch to e-cigarettes to ameliorate the effect of smoking on asthma exacerbations.[128,129] However, the health outcomes of e-cigarette use in people with asthma are unclear. e-Cigarette use is more prevalent in adolescents with asthma than without asthma.[130-133] e-Cigarette use has been associated with asthma diagnosis and exacerbations,[127,134,135] even with secondhand exposure.[136] A study in dual users, nonatopic smokers, and smokers with mild, intermittent, and well-controlled asthma showed that even a single session of e-cigarette use (using standardized puffing settings) induced pro-inflammatory markers and impaired respiratory mechanics.[49] Importantly, the effects were exaggerated in smokers with asthma. These findings suggest that the negative effects of e-cigarettes may be exaggerated in people with asthma[133] and highlight an important area for further research.

Pregnant Woman and Their Offspring

Many women perceive e-cigarettes to be safer than tobacco smoking during pregnancy.[137] However, there are currently no human experimental or epidemiologic studies that assess the potential for maternal e-cigarette use to impact the health of the developing human fetus. Research using animal models[85,86,116,138] has shown that maternal exposure to e-cigarette vapor can have significant impacts on offspring health, particularly with respect to neurodevelopment.[85,116,138] In most animal studies, exposures continue after the offspring are born, and thus clear conclusions regarding in utero effects alone are impossible. Regardless, studies have shown that exposure to e-cigarette vapor during pregnancy can alter behavior and cognition in offspring[116,138] and that these changes are often unrelated to the nicotine content of the e-cigarette used.

Health Effects of Flavor Additives in e-Cigarettes

The "generally recognized as safe" classification of flavorings is based on ingestion into the GI system, not heating until vaporization and inhalation into the lungs.[139] Fruit, candy/dessert, and tobacco-based flavorings are the most popular among e-cigarette users.[140] Additional chemical compounds are generated during the vaporization process, and studies suggest adducts may form over time,[141] complicating the issue further. Much of the flavoring research to date has focused on toxicity in a range of cells, with cinnamon in particular being singled out for its toxic effects[83,84,142-145] (e-Table 5). Several in vitro/ex vivo studies have shown that flavors could also affect cellular function, including phagocytosis[82,83,146] and cytokine production.[82-84,147]

One of the biggest flaws to date with studies into flavorings is the lack of clarity regarding the components of each e-liquid, making reproducibility an issue. The specific ingredients and quantities are rarely listed on the bottles and are often not accurate when they are listed (e-Table 6). It is therefore important for future research to include identification of the flavor compounds in the tested e-liquids. Studies that have used mass spectrometry have identified up to 28 mg/mL of total flavoring in some e-liquids and found that the total amount of flavoring correlated to toxicity.[73,148]

Effects of Nicotine vs Nicotine-Free e-Cigarettes

Most commercial e-cigarettes/e-liquids include nicotine. The more recent e-cigarettes use nicotine salts to deliver high nicotine levels up to 59 mg/mL. It is therefore important to understand the contribution of nicotine to the health effects of e-cigarettes (e-Table 7). In a large-scale population-based sample, depressive symptoms were associated with e-cigarette use and nicotine concentration.[149] Several human studies have shown that e-cigarettes containing nicotine have greater effects than nicotine-free e-cigarettes. In particular, inhaled vaporized nicotine via an e-cigarette was shown to increase HR, arterial stiffness, and flow resistance,[98] and in another study to decrease microcirculatory endothelial-dependent function, increase arterial stiffness, and increase BP, HR, and plasma myeloperoxidase[38] in occasional smokers. In healthy nonsmokers, inhalation of unflavored, nicotine-containing vapor increased HR variability, a measure of cardiac sympathetic nerve activity.[150] However, these and other in vitro, ex vivo, and animal studies showed other effects regardless of whether nicotine was present.[82,83,86,143] In conclusion, future studies should continue to investigate the effects of heated, vaporized nicotine because it has been shown to have effects outside of the other ingredients in e-cigarettes.

Incorrect Nicotine Concentration Labeling

Many studies have shown that nicotine concentrations in e-liquids are often considerably different from the concentrations listed on the labels[151-163] (e-Table 6). There is no consistent trend in measured concentrations being higher or lower than on the label, yet variation beyond 10% is commonplace.[164] These inaccuracies are unsurprising due to lack of quality control in the e-liquid manufacturing industry, which already suffers from the entrance to the market of poor-quality counterfeit versions of major brands. Most alarmingly, in numerous instances, nicotine has been detected in e-liquids that are labeled as "nicotine-free."[153-155,160,161,163] Nicotine in these e-liquids is often at trace amounts, although levels in excess of 20 mg/mL have been reported.[151,160,163] This finding has implications for health, from a legal standpoint, whereby nicotine-containing e-liquids are sold in jurisdictions where the practice is illegal (eg, Australia[153]) and, from an addiction standpoint, whereby "vapers" (ie, e-cigarette user) may unwittingly be exposing themselves to an addictive substance.

Harm From Exploding e-Cigarettes

Another concern regarding e-cigarette use is the potential of these devices to spontaneously explode and cause harm to users, with one reported death.[165] Currently, the frequency of e-cigarette explosions remains unclear, although many cases have been reported (e-Table 8). These explosions are largely attributed to the overheating of lithium ion batteries in e-cigarettes,[166] which could be due to faulty batteries or user modification of batteries. Furthermore, storing the battery in contact with metal objects could create an external short circuit that could also lead to explosions. Case studies have reported e-cigarette explosions in the mouth during use, resulting in oral and facial injuries, including tooth avulsions and fractured facial bones.[167-170] Numerous case studies have also reported significant burns due to e-cigarette explosions while being held[171,172] or while in pants or breast pockets.[166,173-178] In some cases, the severity of burns required surgery or skin grafts to aid wound healing.

Relation Between e-Cigarette Use and Cancer Risk

Many questions exist about whether e-cigarettes pose a similar, lesser, or greater cancer risk than cigarette smoking. Known carcinogens, formaldehyde, and acrolein have been found in e-cigarette vapor[179-181] at lower levels than cigarette smoke,[182,183] but it remains unclear if the levels produced are enough to contribute to cancer development. Decreased levels of carcinogens were observed in e-cigarette users vs smokers in two studies; however, no healthy control subjects were assessed.[119,184] Schaal et al[185] found increased epithelial-mesenchymal transition markers, increased spheroid formation, increased wound healing, proliferation, and increased Sox2 expression in NSCLC cells; Tommasi et al[186] found a similar downregulation of tumor suppressor genes in oral cells of both e-cigarette users and smokers. Previous animal studies into the effects of nicotine on cancer development found no evidence of tumorogenicity[187-189]; however, the delivery methods used did not involve inhalation of heated nicotine, and existing e-cigarette animal studies were too acute for tumorigenicity studies. Dodmane et al[190] found that nicotine ingestion caused changes that could lead to bladder cancer, and Fuller et al[43] found bladder carcinogenic compounds were increased in the urine of e-cigarette users vs healthy control subjects (many were ex-smokers). Canistro et al[191] reported that urine from e-cigarette-exposed rats caused bacterial mutagenicity according to the Ames assay. Further studies are clearly needed to determine the effects of inhalation of heated nicotine, glycerin, glycols, and flavors on cancer development.

Conclusions

The findings in this review established via in vitro, ex vivo, and animal models that e-cigarette exposure/use leads to distinct immunologic alterations that may contribute to an increased susceptibility to infection. Although the presence of nicotine contributes to the detrimental effects of e-cigarettes, recent research has highlighted the potential toxicity of flavor additives. Furthermore, flavor-specific findings highlight the need for human studies to consider whether varied flavor use among e-cigarette users may unwittingly conceal outcomes. e-Cigarette use in humans has been shown to affect the cardiopulmonary system, with evidence of reduced lung function and increased BP, HR, and arterial stiffness compared with never smoker/never vapers. This review did find evidence suggesting that smokers who switch to e-cigarettes may experience harm reduction, particularly in relation to cardiopulmonary health, but we were unable to find evidence suggesting that these clinical measures returned to the levels of a nonsmoker. In addition, much remains unknown about the effects of e-cigarette use, in particular in the long term, and there is evidence that

smokers do not "quit" with e-cigarettes but rather "switch" to e-cigarette use. Of great concern are the latest studies which show that dual use of e-cigarettes and tobacco cigarettes may put users at increased cardiovascular disease risk over smoking or e-cigarette use alone.

There is currently a lack of evidence as to the effects of e-cigarette use in vulnerable populations, such as people with respiratory disease and pregnant women. However, the evidence to date suggests that e-cigarette use may worsen asthma and that maternal use may negatively affect the development of the child. Additional studies are needed in both humans and animal models to determine what health impacts e-cigarettes may have on the many groups who may use them. Overall, this review adds to the conclusion of the NASEM report,[25] which indicated that there is increasing emerging evidence that e-cigarette use is not risk free for nonsmokers, and that use in smokers as a cessation aid is not preferential to NRT from a health impact perspective.

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1.10 Outline of Chapters

As described in both literature reviews the research focusing on the harms associated with E-cigarette is very novel, dynamic and leaves many questions yet to be answered. There is still a great deal of discussion regarding the use of E-cigarettes from a harm reduction standpoint, this thesis intended to elucidate some of the questions raised in this debate. At the inception of this project we had minimal data on E-cigarette use and user demographics in Australia, hence the design of our survey with the intent to provide pilot data and validate survey questions for assessing the current attitudes and opinions of Australians towards E-cigarettes. This study is included as Chapter 3 of this thesis, unfortunately there were unforeseen circumstances that delayed the publication of this Chapter, but it is currently being written up to be submitted alongside this thesis.

A recent study identified a potential for e-cigarettes to be used as a cessation device that outperforms traditional NRT [99]. One of the by-products of this increased cessation efficacy is an increased continuation and dependency on the E-cigarette as a source of nicotine. Although this may still reduce the level of toxicants that users expose themselves to, it still leaves many questions that need to be answered. Our first study addresses this question by measuring the immunomodulating and cytotoxic potential of E-cigarettes when operating the device under different power settings. Further to this primary human airway smooth muscle (ASM) cells from COPD and non-COPD patients were used in this study, allowing us to measure the inflammatory response to E-cigarette vapour stimulation. It has been shown previously that COPD cells were hyper responsive to cigarette stimulation with respect to inflammatory mediator production, so it was important to determine whether they respond in a similar fashion to E-vapour.

Following this we investigated whether E-cigarettes had the potential to induce cellular senescence, a disease mechanism that is hypothesized to contribute to the pathophysiology of COPD. The relationship between cigarette smoke exposure and cellular senescence has been studied in depth, although mechanisms of senescence

are yet to be elucidated it has been concluded that cigarette smoke is a known inducer of cellular senescence. This study is of great importance in the harm reduction debate, as its findings may impact the recommendation of E-cigarette use as a cessation device in high-risk patients with COPD.

The final study in this thesis investigates whether E-cigarettes when combined with cigarette smoke has an effect on the efficacy of dexamethasone, an oral corticosteroid used in the treatment of COPD exacerbations, with respect to reducing markers of inflammation. Prior to this studies conception dual users of E-cigarettes and tobacco cigarettes had been identified as a subpopulation of E-cigarette users through demographic studies. Although both stimuli had been studied in depth at this point, there is very little data on how cells respond to combination treatments. Investigating whether exposure to E-cigarettes and cigarette smoke in tandem has an effect on steroid efficacy also adds to the harm reduction debate. Steroid insensitivity is an issue in COPD patients and without other effective treatment options it is of great importance to preserve the effectiveness of treatments available to patients.

1.11 Aims and Hypothesis

Chapter 3:

E-cigarette use in Young Australians: perceptions of harms or benefits of emerging tobacco products

Jack Bozier, Juliet Foster, Brian G. Oliver

Aims:

- To determine the perceived harms or benefits of E-cigarette use in young Australians.
- To determine young Australians opinions of current regulation and safety of E-cigarettes.
- To determine whether young Australians believe nicotine containing E-cigarettes are more harmful than non-nicotine containing E-cigarettes.

Hypotheses:

- Young Australians will perceive E-cigarette use to be harmful, but less harmful than traditional tobacco cigarettes.
- Young Australians will perceive nicotine containing E-cigarettes as more harmful than non-nicotine containing E-cigarettes.

Chapter 4:

Heightened response to e-cigarettes in COPD

Jack Bozier, Sandra Rutting, Dia Xenaki, Matthew Peters, Ian Adcock, Brian G. Oliver

European Respiratory Journal Open Research 2019

DOI: 10.1183/23120541.00192-2018

Aims:

- To evaluate dose–response relationships of E-cigarette stimulation of primary airway smooth muscle cells (ASMCs) from people with and without COPD under realistic physiological conditions.
- To determine whether increasing the power settings of E-cigarettes has an effect on cytotoxicity.

Hypotheses:

- That cells from patients with COPD will produce more proinflammatory mediators than cells from patients without COPD
- E-vapour produced at higher power settings will be more cytotoxic to cells than E-vapour produced at lower power settings.

Chapter 5:

E-cigarette vapour induces cellular senescence in lung fibroblasts and may contribute to lung pathology

Jack Bozier*, Roy R. Woldhuis*, Baoming Wang, Irene H. Heijink, Maaike de Vries, Maarten van den Berge[4], Wim Timens, Corry-Anke Brandsma, Brian G.G. Oliver

* Co-first authors

Submitted to the European Respiratory Journal - November 2020

DOI: N/A

Aims:

- To Determine whether E-cigarette stimulation results in an upregulation of cellular senescence markers p16 and p21 in primary human lung fibroblasts.
- To determine whether E-cigarette stimulation results in an increased SA-βGal positive staining as a marker of cellular senescence
- To determine E-cigarette stimulation will result in inflammatory and functional responses of cells associated with cellular senescence.

Hypotheses:

- EVE stimulation will result in increased p16 and p21 expression due to increased cellular senescence
- EVE stimulation will result in increased SA-βGal positive staining due to increased cellular senescence
- After induction of senescence by EVE, primary human lung fibroblasts will produce greater IL-6 and IL-8 which are known SASP's. Furthermore, there will be an impaired wound healing capacity in the EVE stimulated cells compared to unstimulated.

Chapter 6:

Dual E-cigarette and cigarette use reduces dexamethasone sensitivity in-vitro

Jack Bozier, Roy Woldhuis, Diren K. Reddy, Ian M. Adcock, Brian G.G. Oliver

Submitted to the American Journal of Physiology - Lung Cellular and Molecular Physiology – Submitted December 2020

DOI: N/A

Aims:

- To determine the effect of E-cigarette vapour extract (EVE) and cigarette smoke extract (CSE) both alone and in combination on inflammatory mediators.
- To determine corticosteroid sensitivity through pre-treatment with dexamethasone.

Hypotheses:

- Combination treatment with EVE and CSE will elicit a greater production of inflammatory mediators than either stimulus alone.
- A greater concentration of dexamethasone will be needed to attenuate inflammatory mediator production when stimulated with EVE and CSE in combination than either treatment alone.

Chapter 2

Methods

2.1 Checklist for Reporting Results of Internet E-Surveys (CHERRIES)

Table 2.1 – CHERRIES Checklist

Checklist Item	Explanation	Response
Describe survey design	Describe target population, sample frame. Is the sample a convenience sample? (In "open" surveys this is most likely.)	See methods section in Chapter 3. Target population was 18-30 year old young adults in Australia. Participants were contacted through university email lists (Health, Science and Business) and all UTS societies listed on UTS database. Social media posts on Facebook targeting the demographic above and posters around TAFE Ultimo and UTS were also used. Non-probability sampling was used as some targeted posts on E-cigarette specific subreddits related to E-cigarette use in Australia were used to stratify number of current and past E-cigarette user responses.
IRB approval	Mention whether the study has been approved by an IRB.	Ethics approval was given for this study by the University of Technology Sydney Human Research Ethics Committee. **HREC # - ETH17-1589**

Informed consent	Describe the informed consent process. Were the participants told the length of time of the survey, which data were stored and where and for how long, who the investigator was, and the purpose of the study?	See appendix at the end of Chapter 3 for a copy of the Participant information sheet. Participants were required to read a participant information sheet and confirm they had understood what they had read and agree to take part in the study. Information sheet outlined the approximate time to completion, the type of data collected, de-identification of this data, investigators in the study and the purpose of the study. Consent was considered after selecting confirm on 'By ticking this box I confirm I have read the patient information sheet in full'.
Data protection	If any personal information was collected or stored, describe what mechanisms were used to protect unauthorized access.	All survey data was collected through SurveyMonkey and access was only provided to study investigators.
Development and testing	State how the survey was developed, including whether the usability and technical functionality of the electronic questionnaire had been tested before fielding the questionnaire.	Survey was tested among 5 PhD students at the Woolcock Institute of Medical Research. Typos and technical errors were corrected to improve flow of questions based on the participants' responses.

Open survey versus closed survey	An “open survey” is a survey open for each visitor of a site, while a “closed survey” is only open to a sample which the investigator knows (password-protected survey).	Open Survey – As part of the distribution of this survey participants were encouraged to share the link with peers that fit the demographic of the study.
Contact mode	Indicate whether or not the initial contact with the potential participants was made on the Internet. (Investigators may also send out questionnaires by mail and allow for Web-based data entry.)	Contact was made via email, social media posts or advertisement posters
Mandatory/voluntary	Was it a mandatory survey to be filled in by every visitor who wanted to enter the Web site, or was it a voluntary survey?	Survey response was not mandatory, participants were informed that they did not need to complete responses and could drop out of the study at any time.
Incentives	Were any incentives offered (eg, monetary, prizes, or non-monetary incentives such as an offer to provide the survey results)?	A random prize draw of 5 double movie passes was outlined in participant information sheet.

Time/Date	In what timeframe were the data collected?	September 2017 – September 2018
Adaptive questioning	Use adaptive questioning (certain items, or only conditionally displayed based on responses to other items) to reduce number and complexity of the questions.	Conditionally displayed questions were used based on the participants' responses. Some responses made following questions redundant, so they were skipped if this was the case.
Number of Items	What was the number of questionnaire items per page? The number of items is an important factor for the completion rate.	41 Questions
Number of screens (pages)	Over how many pages was the questionnaire distributed? The number of items is an important factor for the completion rate.	42 screens – 1 per screen + consent
Completeness check	It is technically possible to do consistency or completeness checks before the questionnaire is submitted. Was this done, and if "yes",	A completeness check was not performed. Of 378 respondents that started the survey 270 gave complete responses

	how (usually JAVAScript)? An alternative is to check for completeness after the questionnaire has been submitted (and highlight mandatory items).	
Review step	State whether respondents were able to review and change their answers (eg, through a Back button or a Review step which displays a summary of the responses and asks the respondents if they are correct).	Participants had the option to return to previous questions
Unique site visitor	If you provide view rates or participation rates, you need to define how you determined a unique visitor. There are different techniques available, based on IP addresses or cookies or both.	Unique site visitors are determined through SurveyMonkey detecting IP addresses of respondents.
Handling of incomplete questionnaires	Were only completed questionnaires analysed? Were	Incomplete questionnaires were not included for analysis, some questions allowed 'not sure' responses so smaller

	questionnaires which terminated early (where, for example, users did not go through all questionnaire pages) also analysed?	numbers of participants are included in those analyses
Questionnaires submitted with an atypical timestamp	Some investigators may measure the time people needed to fill in a questionnaire and exclude questionnaires that were submitted too soon. Was there a cut-off point?	Timeframe was not used as a cut-off for this study, most responses were very close to the average time for completion with few outliers.
Statistical correction	Indicate whether any methods such as weighting of items or propensity scores have been used to adjust for the non-representative sample; if so, please describe the methods.	No weighting of responses or statistical correction was used.

2.2 Primary Cell Isolation

Human research ethics committee (HREC) approval for the collection of lung tissue and isolation of primary cells was provided by Sydney Local Health District for Roy Prince Alfred Hospital and St Vincent's Hospital HREC. HREC approval numbers were as follows:

- Royal Prince Alfred Hospital - X05-0295, X09-0373, X14-0045, X15-0285 and HREC/15/RPAH/383
- St. Vincent's Hospital - HREC/15/SVH/351

Primary human parenchymal fibroblasts and ASMCs were isolated from explanted lungs and lung tissue from patients undergoing resection for thoracic malignancies. This method was optimised and described previously by Krimmer et al for fibroblasts [100] and Chen et al for ASMCs [101]. Parenchymal fibroblasts were isolated after making an incision in lung proximal tissue containing airways smaller than 1 mm. A small piece of tissue approximately 1 cm x 1 cm was washed in Hanks balanced salt solution (Hanks) (Invitrogen, Carlsbad, USA) and then minced into 0.5 – 1 mm pieces. The minced tissue was then returned to sterile Hanks and centrifuged at 1000 rpm for 5 minutes. The supernatant was then aspirated, and the tissue pellet was resuspended in Dulbecco's modified eagle medium (DMEM) (Gibco, Grand Island, USA) supplemented with 10% (v/v) Fetal bovine serum (FBS), 1% antibiotic-antimycotic (Gibco). 10 ml of fibroblast cell suspension was added to each T75 tissue culture flask (Thermo Fisher Scientific, Waltham, USA) and cells were grown for 2 weeks before passaging.

Airway smooth muscle cells were isolated from bronchial airways approximately 1 – 2 cm in diameter. Airways were isolated from surrounding tissue and cut longitudinally to allow airways to be washed in Hanks three times before a 3 second wash in 70% ethanol (v/v), following this the airway was returned to Hanks prior to microdissection. The airway was then pinned out on a dissection dish and placed under a dissection microscope. Airway epithelium was collected for future experiments and storage in the Woolcock Institute Lung Biobank.

ASM bands were collected using forceps by pinching the band at one edge of the airway and pulling in the direction of the band to collect it in one piece. Isolated ASM bands were placed in Hanks until microdissection was finished. Upon completion, ASM was centrifuged at 1000 rpm for 5 minutes and the supernatant was aspirated. Following this ASM was resuspended in DMEM supplemented with 10% (v/v) Fetal bovine serum (FBS), 1% (v/v) antibiotic-antimycotic. 3 ml of ASMCs suspension was added to each T25 tissue culture flask provided there were a minimum of six pieces of ASM in each flask.

2.3 Cell Culture

Primary parenchymal lung fibroblasts were grown in T75 and T175 tissue culture flasks depending on the number of cells required for experimental setup. Cells were grown in DMEM supplemented with 5% FBS, 1% antibiotic- antimycotic at 37 °C in 5% CO_2. Cells were observed microscopically to confirm normal growth and morphology of cells prior to experimental setup. Cells were included in experiments between passages 2-7, providing there were no abnormalities in cell culture flasks. When cells had reached confluency, flasks were washed with Hanks and trypsin was used to detach cells from the flask. The detached cells were washed with 7 ml DMEM with 5% FBS, 1% antibiotic-antimycotic and collected in a 15 ml falcon tube. The cell suspension was centrifuged at 1000rpm for 5 minutes and the supernatant was aspirated. The cell pellet was then resuspended in 5ml DMEM 5% FBS, 1% antibiotic-antimycotic and 10 μl was collected for determining the concentration of cells per millilitre. 10 μl of cell suspension was combined with 10 μl trypan blue (Sigma Aldrich, St. Louis, USA) to stain for cell viability prior to counting using a hemocytometer on a light microscope. Once the cell suspension solution concentration of cells per millilitre was known, the desired concentration of either 40,000 cells/ml or 60,000 cells/ml was made up to the volume required with a minimum of $1x10^6$cells being returned to a T175 cell culture flask. Cells were then plated on the required tissue culture plates and incubated for 2-3 days at 37 °C in 5% CO_2 before serum starvation or stimulation, depending on the study protocol.

Primary ASMCs underwent the same steps as above but were cultured in flasks in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic. When resuspending ASMCs for tissue culture plates media was switched to DMEM supplemented with 5% FBS, 1% antibiotic-antimycotic.

2.4 E-vapour Extract Generation

E-vapour extract (EVE) was generated using a KangerTech Nebox 3rd generation electronic cigarette (KangerTech, Shenzen, China) containing 18mg/ml nicotine or 0mg/ml nicotine E-liquid (VaperEmpire, Australia) of 80:20 Propylene Glycol (PG): Vegetable Glycerin (VG) base in tobacco or menthol flavour. E-vapour was bubbled through 25ml of DMEM in a T175 tissue culture flask using peristaltic pump to remove air from the flask and draw in E-vapour. The E-cigarette was used in 5 second bursts with 30 seconds cool down between each activation. A total of 100 seconds of E-vapour was collected before the flask was sealed with parafilm (Livingstone, Australia) and left to solubilise for 15 minutes. Once solubilised EVE was diluted to working concentrations for experimental stimulations. Weighing the E-cigarette device before and after EVE generation determined the weight of E-liquid vapourised, which is the equivalent of two standard tobacco cigarettes based on nicotine content.

2.5 Cigarette Smoke Extract Generation

Cigarette smoke extract (CSE) was generated using a modified protocol described previously [102]. One filtered high tar commercial tobacco cigarette (Marlboro, USA) was bubbled through 25 ml of DMEM in a T175 tissue culture flask using the peristaltic pump used in EVE generation. Once the cigarette was smoked down to lettering above the filter the flask was sealed and left for 15 minutes to solubilise. Once solubilised CSE was diluted to working concentrations for experimental stimulations.

2.6 MTT Assay

At required time-point post stimulation 10 µl of 0.5% (w/v) 3-4,5- dimethylthiazol-2-(yl)-2,5-disphenyltetrazolium (MTT dye) (Sigma Aldrich) in PBS water was added to each well of a 96 well plate. Stimulations were done in triplicates vertically with no-cell controls to match each stimulation. Plates were then incubated for 4 hours at 37 °C in 5% CO_2. 100 µl of 10% (w/v) Sodium Dodecyl Sulfate (SDS) was added to each well and the plates were incubated overnight to dissolve crystalline dye. The following morning plates were read on a SpectraMax M2 spectrophotometer (Molecular Devices, San Jose, USA) at 570nm with a background reading at 630nm.

2.7 Enzyme Linked Immunosorbent Assay – ELISA

ELISA was performed on cell free supernatants to determine IL-6 and IL-8 concentrations produced by primary parenchymal fibroblasts, primary ASMCs and BEAS2B cell line (used in optimization studies). Purified Rat Anti-human IL-6 (#554543, BD Biosciences, San Jose, USA) was diluted in in 0.1M Na_2HPO_4 1:1000 and Purified Mouse Anti-Human IL-8 (#554716, BD Biosciences) was diluted in PBS 1:500. 100 µl of Antibody solution was added to each well of a 96 well Nunc MaxiSorp™ flat-bottom ELISA plate (Thermo Fisher), plates were sealed with parafilm and stored at 4 °C overnight on an orbital shaking platform. The next day Plates were washed three times with Phosphate buffered saline supplemented with 0.05% Tween (v/v) (T-PBS) and tapped dry before 200 µl of 1% BSA (v/v) in phosphate buffered saline (PBS) was added to each well to block for non-specific binding. Plates were incubated with 1% BSA PBS at room temperature (RT) for 1 hour on an orbital shaking platform before being washed three times with T-PBS.

After plates were tapped dry 100 µl of cell free supernatant was then added in duplicates side by side down the plate from column 3-12. Standards were prepared by performing serial dilutions giving concentration ranges of 2000- 31.25 µg/ml for IL-6 and 1000 – 15.625 µg/ml for IL-8. Standards were added in duplicates side by side in columns one and two with the highest concentration in row A and lowest in row G, with blank DMEM in row H. Plates containing standards and supernatants

were covered and incubated either at RT for 2 hours or at 4 °C overnight on an orbital shaking platform. Secondary antibody solutions were prepared during this incubation period prior to the next wash step. Biotin Rat Anti-Human IL-6 (#554546, BD Biosciences) secondary antibody was diluted in 1% BSA T-PBS 1:1000 and Biotin Mouse Anti-Human IL-8 (#554718, BD Biosciences) was diluted in 0.1% BSA Tris Buffered Saline supplemented with 0.05% Tween (T-TBS) 1:1000.

Next plates were washed four times with T-PBS, tapped dry and 100 μl of secondary antibody solution was added to each well. Plates were incubated at RT for 1 hour on an orbital shaking platform. Biotinylated streptavidin horseradish peroxidase (HRP) was diluted 1:200 in 1% BSA T-PBS for IL-6 and 0.1% T-TBS for IL-8 for the next step. Plates were washed six times with T-PBS, tapped dry and 100 μl of HRP solution was added to each well. Plates were then incubated in the dark for 30 minutes at RT on an orbital shaking platform. Following this, plates were washed eight times with T-PBS to wash away excess HRP and tapped dry. 100 μl of TMB chromogen solution (Thermo Fisher) was then added to each well as a substrate and plates were mixed by hand. Development time for plates ranged from 5 – 15 minutes, when developed completely the reaction was stopped by adding 100 μl 1 M H_3PO_4 and plates were imaged on a Spectramax M2 spectrophotometer at 450 nm with a background reading of 570 nm. Standard curves were created on excel of concentration vs absorbance and unknown values were calculated by interpolating absorbance values.

2.8 Western Blot

Western Blots were used to analyse protein levels of proinflammatory signalling factors related to steroid insensitivity. Cells were lysed in protein lysis buffer including protease and phosphatase inhibitors 30 minutes post stimulation with EVE or CSE alone or in combination, then collected and stored at -20 °C.
Polyacrylamide gels were made in advance of western blotting with the following volumes:

Table 2.2 – Reagent volumes for polyacrylamide gels used in western blotting

	Analysis Gel (10% Acrylamide)		**Analysis Gel (6% Acrylamide)**		**Stacking Gel (4% Acrylamide)**		
Milli Q	2.5 ml	4.9 ml	2.75 ml	5.4 ml	**Milli Q**	1.63 ml	3.25 ml
SDS/Tris pH8.8	1.25 ml	2.5 ml	1.25 ml	2.5 ml	**SDS/Tris pH6.8**	0.63 ml	1.25 ml
Acrylamide 40%	1.25 ml	2.5 ml	1 ml	2 ml	**Acrylamide 40%**	0.25 ml	0.5 ml
TEMED	5 µl	10 µl	5 µl	10 µl	**TEMED**	2.5 µl	5 µl
10% APS	50 µl	100 µl	50 µl	100 µl	**10% APS**	2.5 µl	50 µl
Total	**5 ml**	**10 ml**	**5 ml**	**10 ml**	**Total**	**2.5 ml**	**5 ml**

Table 2.3 – Buffers and reagents for Western blots

Buffer	Contents	Volume	PH
Cell Lysis Buffer	200 mM Tris-HCl pH 7.4 (1 ml), 1 M NaCl (1 ml), 0.1 M Na_2EDTA (100 µl), 0.1 M EGTA (100 µl), 0.1 M NaF (100 µl), 0.1 M $Na_4P_2O_7$ (2 ml), 0.1M Na_3VO_4 (200 µl), 10% Triton X-100 (1 ml), 50% Glycerol (2 ml), 10% SDS (100 µl), 10% Sodium Deoxycholate (500 µl), Milli-Q Water (1.4 ml)	10 ml	Not measured
5x Loading Buffer	0.312 M Tris-HCl pH 6.8 (3.125 g), Tris-Base (1.893 g), 50% Glycerol (10 ml), SDS (2.5 g), DTT (3.875 g), Bromophenol blue (2.5 mg)	50 ml	Not Measured
10x Running Buffer	0.25 M Tris-Base (30.2 g), 1.92 M Glycine (144 g), SDS (10 g)	1 L	Not Measured
Transfer Buffer	Tris-Base (58 g), Glycine (29 g)	1 L	Not Measured
5x Stripping buffer	Tris-Base (38.17 g) - 2% (w/v) SDS added fresh to 1x buffer, 210 µl β-mercaptoethanol/30 ml 1x buffer	1 L	pH 6.8

Table 2.4 – Primary and secondary Antibodies used for Western blots

Catalogue No (Supplier)	**Antibody (Species)**	**Protein size KDa (Bands)**	**Blocking Buffer**	**Diluent**	**Ratio**
9211S (Cell Signalling)	p38 MAPK	**38**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
9212S (Cell Signalling)	Phospho-p38 MAPK	**38**	5% w/v BSA, 1X TBS 0.1% Tween	1X TBST with 5% w/v skim milk	**1:1000**
8242S (Cell Signalling)	NF-kB p65, D14E12 (Rabbit)	**65**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
3033S (Cell Signalling)	Phospho-NFKb, Ser536 (Rabbit)	**65**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
2880S (Cell Signalling)	FoxO1, C29H4 (Rabbit)	**78-82**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
9461S (Cell Signalling)	Phospho-FoxO1, Ser256 (Rabbit)	**82**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
2983S (Cell Signalling)	mTOR, 7C10 (Rabbit)	**289**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
5536S (Cell Signalling)	Phospho-mTOR, Ser2448 (Rabbit)	**289**	1X TBST with 5% w/v skim milk	5% w/v BSA, 1X TBS 0.1% Tween	**1:1000**
CB1001 (Merck Millipore)	GAPDH (Mouse)	**36**	1X TBST with 5% w/v skim milk	1% w/v BSA, 1X TBS 0.1% Tween	**1:5000**
AP160P (Merck Millipore)	Rabbit Anti-Mouse HRP Conjugated	***Secondary**	N/A	1% w/v BSA, 1X TBS 0.1% Tween	**1:10,000**
P0448 (Dako)	Goat Anti-Rabbit HRP Conjugated	***Secondary**	N/A	1% w/v BSA, 1X TBS 0.1% Tween	**1:2000**

Gels were cast using Mini-PROTEAN® 0.75mm integrated spacer plates (Bio-Rad) and gel stands. First, add Milli Q water, SDS/Tris pH8.8 and Acrylamide 40% in a Falcon tube and mix well; this is the base of the analysis gel. Once casting plates and gel stands have been checked, add ammonium persulfate (APS) and N,N,N',N'-Tetramethyl ethylenediamine (TEMED) to the falcon tube and mix. Add the acrylamide solution to the centre of the casting plates quickly as APS and TEMED initiate polymerisation. Add 500 µl of butanol to the centre of the casting plates on top of the acrylamide solution to flatten the top edge of the gel. Once set, excess butanol was poured off and the gel was washed twice with Milli Q water. Next, the analysis gel was mixed in a falcon tube and added to the casting plates with the plastic well comb was added. Once set the gels were added into running tanks and submerged in running buffer and stored at 4 °C until samples were prepared.

Samples were defrosted and stored on ice prior to centrifuging at 12,000 rpm for 7 minutes at 4 °C. In fresh tubes 20 µl of sample was combined with 5 µl of loading buffer. Tubes with the new sample + loading buffer were heated at 95 °C for 10 minutes then returned to ice to cool before loading. 20 µl of sample + loading buffer was loaded into each lane on 10-lane gels and 10 µl was added to each lane of 15-lane gels. 7.5 µl and 3.75 µl of standards were added to lane one of 10 well and 15-well gels, respectively. Tanks were then sealed and connected to a PowerPac power supply (BioRad) and ran at 90V constant voltage for 30 minutes until dye front entered analysis gel, then 120V for a further 60-120 minutes dependant on desired separation.

Gels were then removed from glass casting moulds and stacking gel was discarded. 0.45 µm pore PVDF membrane was cut to 8.5 cm x 6.75 cm and placed in 100% methanol for 1 – 2 minutes to activate the membrane, use forceps and avoid touching the membrane with gloves. A transfer stack was then made inside plastic stacking moulds in the following order, clear side of the stack down in transfer buffer:

Clear → Sponge → 2 x Filter → Membrane → Gel → 2 x Filter → Sponge → Black
Transfer stack was placed in the transfer stack and topped with transfer buffer; an ice block was added to keep the buffer cold during transfer. Transfer tank was connected to a PowerPac power supply and ran for 1 hour at 100V.

Transfer stacks were then opened, and membranes were placed in the required blocking buffer from Table 3. Membrane was blocked in 25 ml of blocking buffer for 30 mins at RT on an orbital shaking platform. Primary antibody solutions from Table 3 were made during this blocking step. Following this membranes were washed three times with T-TBS and placed in 5ml of antibody solution overnight in 50ml Falcon tubes. Tubes containing membranes and antibody solution were placed on a roller tube tilt mixer (Ratek, VIC, Australia) at 4°C overnight to incubate. The next day membranes were washed four times for 5 minutes in T-TBS. Secondary antibody solutions from Table 2 were mixed during the wash steps and added to membranes after washing was finished. Membranes were incubated at RT on an orbital shaker for 1 hour and then washed four times for 5 minutes in T-TBS.

Clarity Enhanced Chemiluminescence substrate (BioRad) was mixed 1:1 and membranes were placed protein side down in the substrate mixture for 2 minutes. Following this, membranes were lifted with forceps and excess substrate was removed before placing on the imaging tray of a ChemiDoc Imager (BioRad). Images were captured in 10-second increments to avoid overexposure. Image Lab software (BioRad) was used densitometry of detected bands and protein levels were normalised to GAPDH. If required Blots were stripped for 30 mins in sealed containers at 55°C in a water bath following four 5 minute washes with T-TBS.

2.9 RNA isolation and purification

Ribonucleic acid (RNA) was isolated from parenchymal lung fibroblasts and ASMCs and purified using the ISOLATE II RNA Mini kit (Bioline) according to the manufacturer's instructions.

Cells were washed twice with 2 ml of cold PBS per well, once PBS was aspirated 300 µl of RNA Lysis buffer (RLY) supplemented with 1% β-mercaptoethanol (BME) was added to each well and left for 5 minutes to lyse cells. Plates were inspected on the light microscope to confirm cell lysis and lysates were collected in RNase + DNase free Eppendorf tubes then stored at -20°C. RNA was then purified according to ISOLATE II RNA Mini kit protocol and eluted RNA was measured on the NanoDrop™ 2000 Spectrophotometer (Thermo Fisher) to determine RNA concentration. Tubes were kept on ice while measuring concentration and then stored at -20°C until cDNA synthesis.

2.10 cDNA Synthesis

cDNA was synthesised using the SensiFAST™ cDNA synthesis Kit (Bioline) according to the manufacturer's instructions. In eight strip tubes RNA was diluted in RNase free water to give a total of 200ng RNA for each reaction, following the completion of these dilutions the tubes were sealed and spun for 30 seconds using a benchtop minifuge. A master mix of 4 µl 5x TransAmp buffer and 1 µl Reverse Transcriptase per reaction was mixed in a 1.7ml Eppendorf tube. 5 µl of mastermix was added to each tube then tubes were sealed and spun again for 30 seconds using the benchtop minifuge. The tubes were then placed in the Mastercycler® X50 (Eppendorf, NSW, Australia) and ran under the following thermal cycling conditions:

- 25 °C for 10 minutes (primer annealing)
- 42 °C for 15 minutes (reverse transcription)
- 85 °C for 5 minutes (inactivation)
- 4 °C temperature hold

Tubes were then diluted in RNase free water up to a total volume of 100 µl for a concentration of 20 ng/µl.

2.11 Real Time Quantitative Polymerase Chain Reaction - RT – qPCR

RT-qPCR was performed using the SensiFast™ Hi-ROX probe Kit according to the manufacturer's instructions. TaqMan® gene expression assays were used to quantify target gene expression for the below genes:

Table 2.5 - TaqMan® **Gene expression Assays**

Gene	Assay ID	Quencher
CXCL8	Hs00174103_m1	FAM-MGB
IL6	Hs00174131_m1	FAM-MGB
IL1α	Hs00174092_m1	FAM-MGB
CXCL1	Hs00236937_m1	FAM-MGB
CXCL2	Hs00601975_m1	FAM-MGB

Triplicates of each sample were prepared in eight strip tubes containing the following quantities:

5 µl RNase free water

16.5 µl 2x Probe Hi-ROX Mix

1.7 µl 20 x 18S Assay (endogenous control)

1.7 µl 20 x TaqMan Assay (from Table 5)

8.25 µl cDNA (5ng/well)

Then 10 µl cDNA-Assay mixture was added in triplicates on MicroAmp™ Fast Optical 96-well plates (Thermo Fisher). Plates were sealed with MicroAmp™ Optical Adhesive Film and centrifuged on short setting up to 1000rpm. Plates were then placed in the StepOnePlus™ RT-PCR Machine or stored at 4°C until the machine was ready for use. The StepOnePlus was run using the following protocol:

Table 2.6 – Hi-ROX qPCR Protocol

Cycles	Temperature	Time	Purpose
1	95 °C	2 minutes	Polymerase Activation
40	95 °C 60°C	10 seconds 20 seconds	Denaturation Annealing/extension

After all cycles were complete the data was exported and analysed using StepOnePlus™ v2.3 software.

2.12 Senescence Induction by Paraquat

Senescence induction was required as a positive control for the study in Chapter 5. The protocol for senescence induction has been optimized and described previously [103]. Primary Parenchymal Fibroblasts and ASMCs were grown at 37°C 5% CO_2 in 6-well cell culture plates for 48-72 hours depending on required confluency for experiments. At required confluency cells were treated with 250 µM Paraquat (Sigma Aldrich) in DMEM with 5% FBS 1% antibiotic 1% antimycotic for 24 hours, then washed once with 2 ml Hanks. After aspirating Hanks 2ml DMEM with 5% FBS 1% antibiotic 1% antimycotic was added to cells and plates were incubated for 4 days at 37°C 5% CO_2.

2.13 Wound Healing Assay

Wound Healing assays were performed on confluent primary parenchymal lung fibroblasts that had been treated with paraquat, CSE or EVE in Chapter 5. A scratch wound was inflicted on the cell layer using a P200 pipette tip from top to bottom through the centre of the well on a 6-well plate. Plates were washed with 2ml Hanks to remove detached and damaged cells, before replacing cells in 2ml DMEM supplemented with 0.5% FBS 1% antibiotic 1% antimycotic for serum starvation. Images of the wound were captured at 0 hours as a baseline for wound size on Nikon Eclipse Ti microscope at 40x magnification. XYZ overview saves the well

locations of each capture to keep consistency in the images between timepoints. Images were then captured at 18 hours, 24 hours, 42 hours, 48 hours, 66 hours and 72 hours. Images were analysed using ImageJ software (NIH) to measure wound size at three locations on the most uniform section of the wound.

2.14 Senescence Associated β-Galactosidase Staining - SA-βGal staining

Senescence associated β-Galactosidase staining was used to stain for senescent positive cells in Chapter 5. This protocol was optimised and described previously [103]. Cells were stained 4 days post stimulation as optimisation with positive control paraquat at 250 µM revealed this as the optimal timepoint.

Cells were washed twice with 1 ml PBS prior to fixing for 5 minutes at RT in 2% (v/v) formaldehyde with 0.2% (w/v) glutaraldehyde in PBS. Fixing solution was aspirated and wells were washed 3 times with PBS. SA-β-Gal staining solution was made fresh with the below contents:

Table 2.7 - SA-β-Gal Staining solution

Component	Volume	Final Concentration
20 mg/ml X-gal in dimethylformamide	1 ml	1 mg/ml
0.2M citric acid/Na phosphate buffer, pH = 6.0	4 ml	40 mM
100 mM potassium ferrocyanide	1 ml	5 mM
100 mM potassium ferricyanide	1 ml	5 mM
5 M sodium chloride	0.6 ml	150 mM
1 M magnesium chloride	40 µl	2 mM
Milli-Q Water	12.4 ml	-

2 ml of SA-β-Gal staining solution was added to each well and plates were sealed with parafilm before incubating at 37 °C in a heating oven. Wash three times with PBS the next morning 12-16 hours later to remove the staining solution. 1ml of 70% (v/v) glycerol in PBS was added to each well and plates were stored at 4 °C before capturing on a Nikon Eclipse Ti microscope at 40x magnification. Total cell counts vs SA-β-Gal positive cells were calculated using ImageJ software.

2.15 Mouse model of E-cigarette and cigarette smoke exposure

The mouse model of E-cigarette exposure and cigarette smoke exposure was described previously [104]. Female Balb/c mice were exposed to either air, E-cigarette vapour of tobacco smoke for six weeks prior to mating until weaning of pups for a total of nine weeks. After the pups had weaned, the mother's lungs were harvested, and RNA lysates were made from lung tissue. These lysates were used for RT-qPCR to determine gene expression levels of cellular senescence markers p16 and p21 in Chapter 5.

2.16 Dexamethasone Pre-treatment

Dexamethasone pre-treatment was used in Chapter 6 to determine the steroid sensitivity of cells stimulated with CSE and EVE alone and in combination. Concentrations have been optimised and described previously in our laboratory [105]. Primary parenchymal fibroblasts were treated with 1 nM, 10 nM, 100 nM and 1 µM concentrations. Dexamethasone solutions were made by dissolving 58.9mg of water-soluble dexamethasone (Sigma Aldrich) in water and filtering through a 0.2 µm syringe top filter. This solution was then serially diluted in DMEM and added to aspirated wells 1 hour before stimulation with CSE and EVE.

Chapter 3

E-cigarette use in Young Australians: perceptions of harms or benefits of emerging tobacco products

Authors and affiliations: Jack Bozier[1,2], Brian G. Oliver[1,2], Juliet M. Foster[1],

[1]Woolcock Institute of Medical Research, University of Sydney

[2]University of Technology Sydney

Corresponding author details: Jack Bozier – jack.bozier@sydney.edu.au

Declaration of interest: Authors have no conflicts of interest to declare

Introduction

Since their inception in 2004 E-cigarettes have become increasingly popular as an alternative to tobacco smoking. In particular, young adults are proving to be a major user of E-cigarettes with the Surgeon General declaring an epidemic of Youth E-cigarette use in 2019 [106]. Most lifelong tobacco habits begin with smoking in adolescence, hence further research is needed to understand the attitudes, perceptions and use of emerging tobacco products in young Australians [107]. Smoking rates have been consistently declining in Australian young adults but preliminary evidence shows that E-cigarette use may be growing in this demographic [54].

Declining smoking rates is indicative of an understanding of the harms associated with smoking, but there currently no data on the attitudes of young adults towards E-cigarettes [108]. According to the Australian National Drug Strategy Household Survey (NDSHS) of 24,000 respondents 31% reported ever trying electronic cigarettes, and 4.4% of respondents were current users [109]. Within this survey no questions were asked about perceptions of risks and harms associated with E-cigarette use. The current estimates of E-cigarette use in Australia are much lower than USA and most European countries, but there is little regulation around e-cig production and sale which could result in similar usage rates in the future.

Our study was designed to address research gaps on E-cigarettes, focusing on a younger demographic as they are more likely to use E-cigarettes overseas. We assessed a range of demographic characteristics, respondent's perceptions of harms or risks from using E-cigarettes and we also designed our questions to address nicotine containing and nicotine free e-cigarettes. Current legislation only addresses E-liquids containing nicotine, which could mislead consumers to believe that E-cigarettes without nicotine are less harmful and safe to use.

The aim of the present study was to determine the opinions and perceptions of young Australians towards E-cigarettes and to explore whether nicotine content was considered a greater risk factor in E-cigarette use.

Methods

Study design

We carried out a cross-sectional online survey of young Australians attitudes toward e-cigarettes following the Checklist for Reporting Results of Internet E-Surveys (CHERRIES) [110]. Data were collected between September 2017 and September 2018 inclusive. Ethics approval was obtained from the Human Research Ethics Committee, UTS HREC ETH17-1589, NSW. All participants provided informed consent before completing the survey.

Inclusion criteria and recruitment

The target population was Australians aged 18 to 30 years. Participants were required to read the participant information sheet on the first page and give informed consent by ticking that they had read and understood the information sheet. A copy of the participant information sheet can be found in the appendix of this Chapter. Participants did not receive reimbursement for completing the survey but were offered the option of entering a prize draw for one of five double movie vouchers.

Non-probability sampling was used to recruit respondents through University of Technology Sydney faculty(Health, Science and Business) and society mailing lists, poster advertisements at the University of Technology Sydney and Technical and Further Education (TAFE) Ultimo Sydney, online posts on Facebook, and posts for enrichment of e-cigarette users on Reddit.

Questionnaire

The survey instrument was designed based on a review of all scientific publications on youth attitudes to tobacco and e-cigarette use between 2010 and 2017 and by a panel of experts on electronic cigarettes, health behaviour and survey design. The survey was pilot tested in 5 people and some questions were amended to resolve minor difficulties reported in question comprehension. The survey took approximately 10 minutes to complete.

The final questionnaire comprised 40 questions structured as binary (e.g. yes/no), multiple choice (allowing single or multiple responses i.e. tick all that apply), Likert scale or hierarchical ranking (e.g. Rate the risk the following: 1= least harmful; 7 = most harmful) questions. The questions covered 8 topics: Reason for e-cigarette uptake, Understanding of Australian e-cig regulation, Tobacco and e-cigarette use, Family and friend smoking history, E-cigarette safety, E-cigarettes as a cessation tool, Illicit drug and alcohol consumption and Respondent demographics. At the beginning of the survey a screening question "Have you ever heard of electronic cigarettes (also known as e-cigarettes, vaping, electronic nicotine delivery systems or personal vaporisers)? (tick one)" was asked to ascertain eligibility for the survey. A final version of the survey can be found in the appendix of this Chapter.

Statistical analysis

Analyses were restricted to respondents aged 18-30 years who had complete demographics data, smoking and E-cigarette use, and harm perceptions of nicotine and non-nicotine containing E-cigarettes. Simple descriptive statistics are presented related to smoking status (current, past, never), E-cigarette use (current, past, never), Age, Gender, Education and E-cigarette use in relation to smoking status. Chi-square analyses were used to compare relationships between categorical variables. All analyses were performed using SPSS Statistics 26.0 (IBM).

Results

A total of 384 respondents began the survey of whom 287 had heard of electronic cigarettes and were eligible to complete the survey.

Table 3.1 - Characteristics of complete survey respondents

Participant Characteristics	**n=287**
Present/Past/Never E-cigarette Users	44 (15) / 62(22) / 181(63)
Age, mean (range)	22.2 (18-30)
Male / Female	108 (38) / 179 (62)
Asthmatic	74 (26)
Highest Completed Education:	
Year 10 High School	4 (1)
Year 12 High School	150 (52)*
TAFE	40 (14) †
Bachelor's degree or Higher	93 (32)
Living in socially disadvantaged area	90 (31.2%)¶

All data n (%) except where indicated
*Year 12 High School: 139 of 150 (93%) currently at university
†TAFE: 24 of 40 (60%) currently at university
¶ ''Disadvantaged'' Socio-Economic Indexes For Area (SEIFA) quintile <3, ''Advantaged'' SEIFA quintile: 4-5

Table 3.2 – E-cigarette use dependant on smoking status

Cigarette Smoking status	**Present E-cig use**	**Past E-cig use**	**Never E-cig use**
Current Smokers, n=31	5 (13.3%)	17 (56.7%)	9 (30%)
Past Smokers, n=90	40 (44.4%)	31 (21.1%)	31 (34.4%)
Never smokers, n=166	0 (0%)	26 (15.7%)	152 (87.5%)

All data n (%)
Chi-square analysis found a significant relationship between Cigarette smoking and E-cigarette use in respondents ($p<0.0001$).

E-cigarette use was most commonly reported by respondents as out of curiosity (58%), because they are not as bad for your health as cigarettes (45%), or to help quit smoking (41%). Chi-square analyses were used to determine whether there was a relationship between nicotine and non-nicotine containing e-cigarettes and the potential harms or benefits related to their use. Respondents believed that nicotine containing E-cigarettes were useful as a cessation aid but non-nicotine containing E-cigarettes were not useful (p=0.276). Respondents reported no difference between nicotine and non-nicotine E-cigarettes in relation to the following parameters: E-cigarettes are bad for your health (p<0.0001), E-cigarettes less harmful to the lungs than smoking (p<0.0001), E-cigarette use is associated with increased lung cancer risk (p<0.0001). Respondents also reported E-cigarette use to be less harmful than fast food consumption, irrespective of nicotine content (p<0.0001).

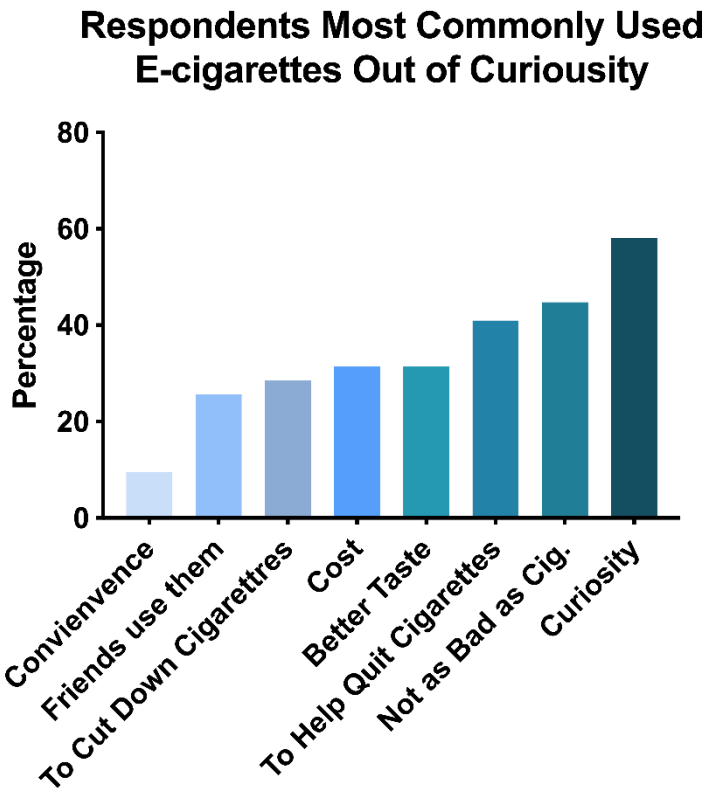

Figure 3.1 – Respondents first introduction to E-cigarettes.

56% of respondents heard of E-cigarettes through friends first, 52% through social media first, while 48% of respondents heard of E-cigarettes on the internet first.

Q: *Where did you first hear about electronic cigarettes? (tick all that apply).* (n=287)

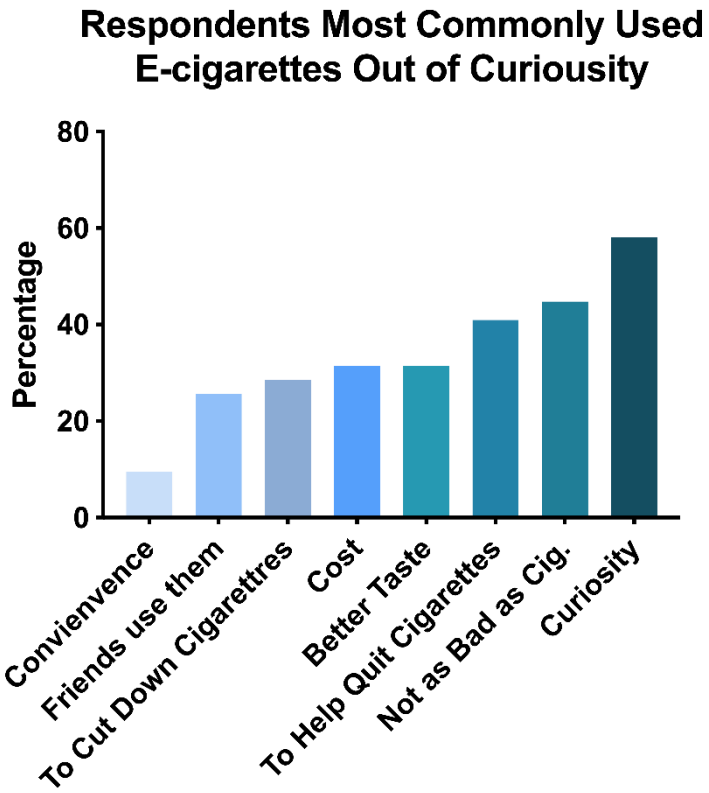

Figure 3.2 – Respondents reasons for E-cigarette use

Respondents reported using E-cigarettes out of curiosity (58%), because they aren't as bad for your health as cigarettes (45%), or to help quit smoking (41%) as the most common reasons for use Q: *What are your main reasons for currently using an electronic cigarette?* (tick all that apply) (n=287)

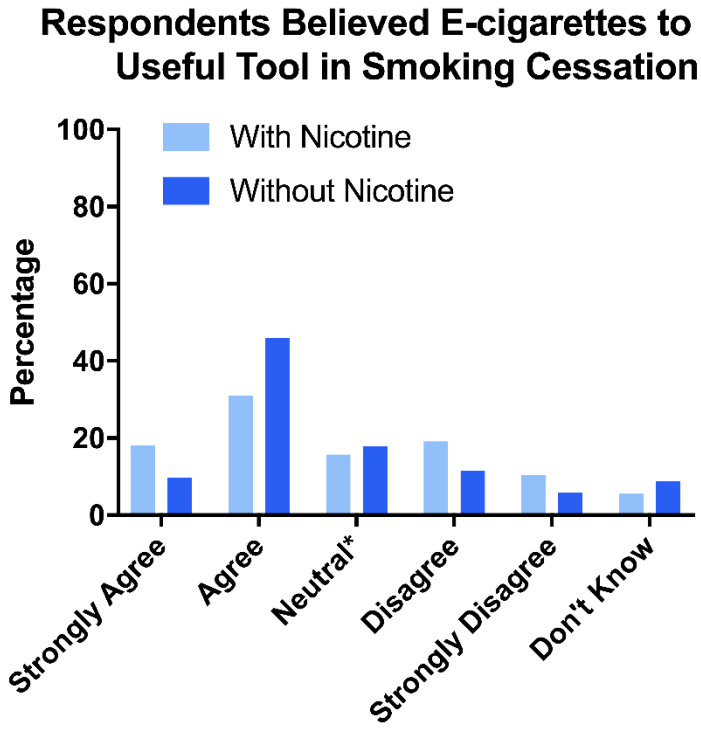

Figure 3.3 – Respondents opinion of nicotine and non-nicotine containing E-cigarettes as a cessation tool.

Likert scales were recoded to strongly agree, agree and neutral = agree; disagree, strongly disagree = Disagree. Chi-square analyses revealed no association between responses related to the usefulness of nicotine and non-nicotine containing E-cigarettes as a cessation aid (p= 0.276). Q*: Electronic cigarettes that contain nicotine are useful for people trying to quit smoking: (tick one)* Q: *Electronic cigarettes that do not contain nicotine are useful for people trying to quit smoking: (tick one) * Neutral was worded as neither agree nor disagree in original question.* (n=254)

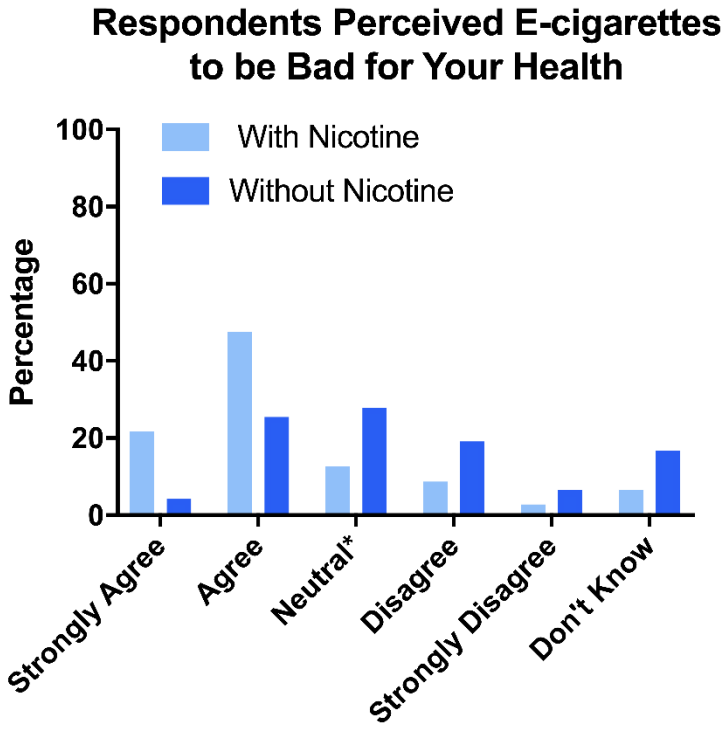

Figure 3.4 – Respondents opinion of whether nicotine and non-nicotine containing E-cigarettes are bad for your health.

Likert scales were recoded to strongly agree + agree + neutral = agree; disagree + strongly disagree = disagree. Chi-square analysis revealed an association between respondents' opinions of nicotine and non-nicotine containing E-cigarettes health effects ($p<0.0001$). Q: *Electronic cigarettes that contain nicotine are bad for your health: (tick one),* Q: *Electronic cigarettes that do not contain nicotine are bad for your health: (tick one) * Neutral was worded as neither agree nor disagree in original question.* (n=236)

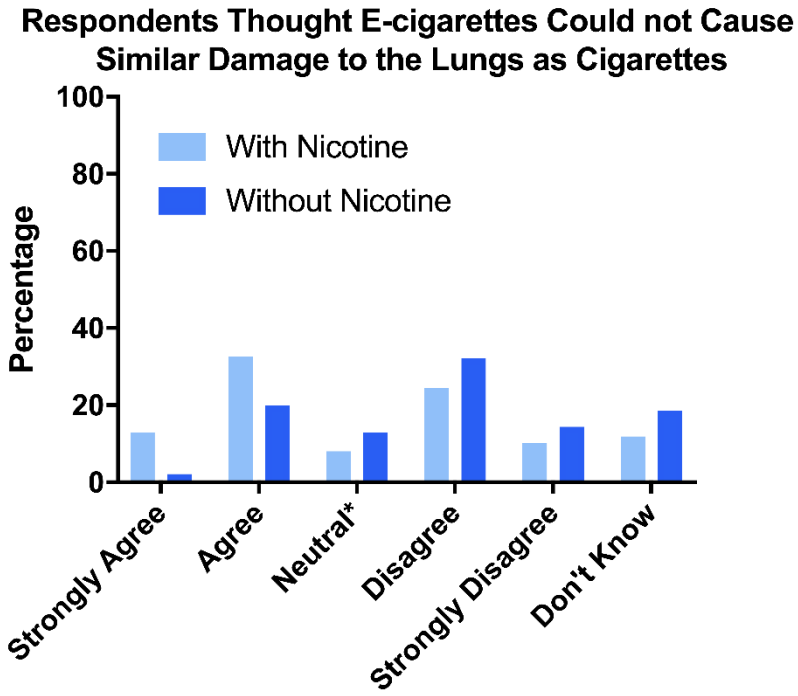

Figure 3.5 – Respondents opinion of whether nicotine and non-nicotine containing E-cigarettes can cause similar damage to the lungs as cigarettes.

Likert scales were recoded to strongly agree + agree + neutral = agree; disagree + strongly disagree = disagree. Chi-square analysis revealed an association between respondents' opinions of nicotine and non-nicotine containing E-cigarettes damage to the lungs compared to cigarettes ($p<0.0001$). Q: *Electronic cigarettes that contain nicotine can cause similar damage to your lungs as cigarettes: (tick one),* Q: *Electronic cigarettes that do not contain nicotine can cause similar damage to your lungs as cigarettes: (tick one), * Neutral was worded as neither agree nor disagree in original question.* (n=225).

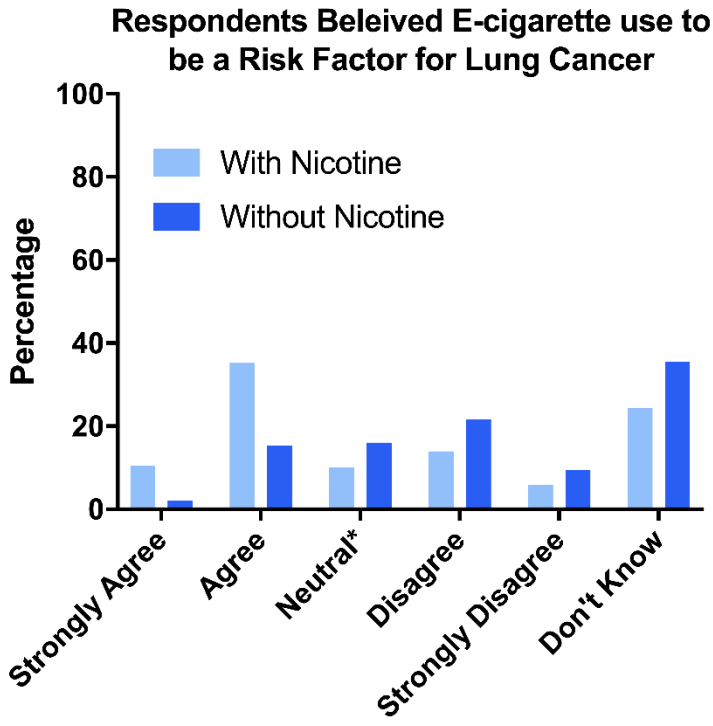

Figure 3.6 – Respondents opinion of whether use of nicotine and non-nicotine containing E-cigarettes had an increased risk for lung cancer.

Likert scales were recoded to strongly agree + agree + neutral = agree; disagree + strongly disagree = disagree. Chi-square analysis revealed an association between respondents' opinions of nicotine and non-nicotine containing E-cigarettes risk of lung cancer with use ($p<0.0001$). Q: *Electronic cigarettes that contain nicotine can cause lung cancer: (tick one),* Q: *Electronic cigarettes that do not contain nicotine can cause lung cancer: (tick one) * Neutral was worded as neither agree nor disagree in original question* (n=217)

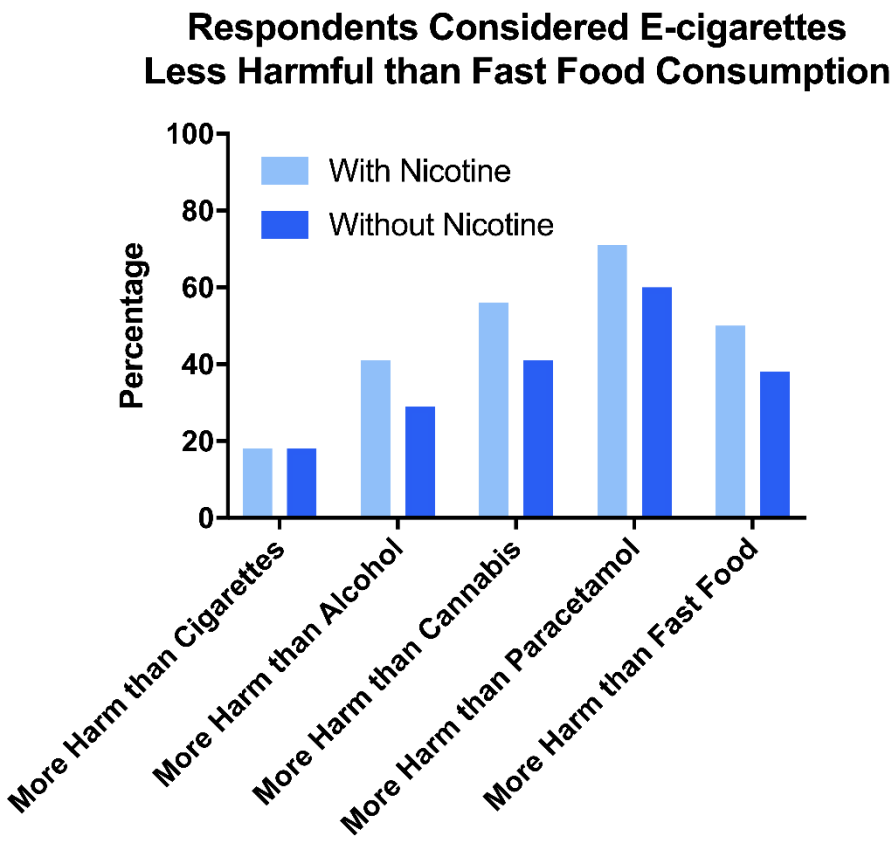

Figure 3.7 – Respondents rating of health risks related to E-cigarette use compared to common habits.

Respondents thought E-cigarettes without nicotine were less harmful than Fast food (62%), Cannabis (59%). Q: *Please rate the health risk of regular use of the following: Cigarettes; Electronic cigarettes that contain nicotine; Electronic cigarettes that do not contain nicotine; Alcohol; Cannabis; Paracetamol; Fast food A: 1=least harmful; 7=most harmful* (n=287)

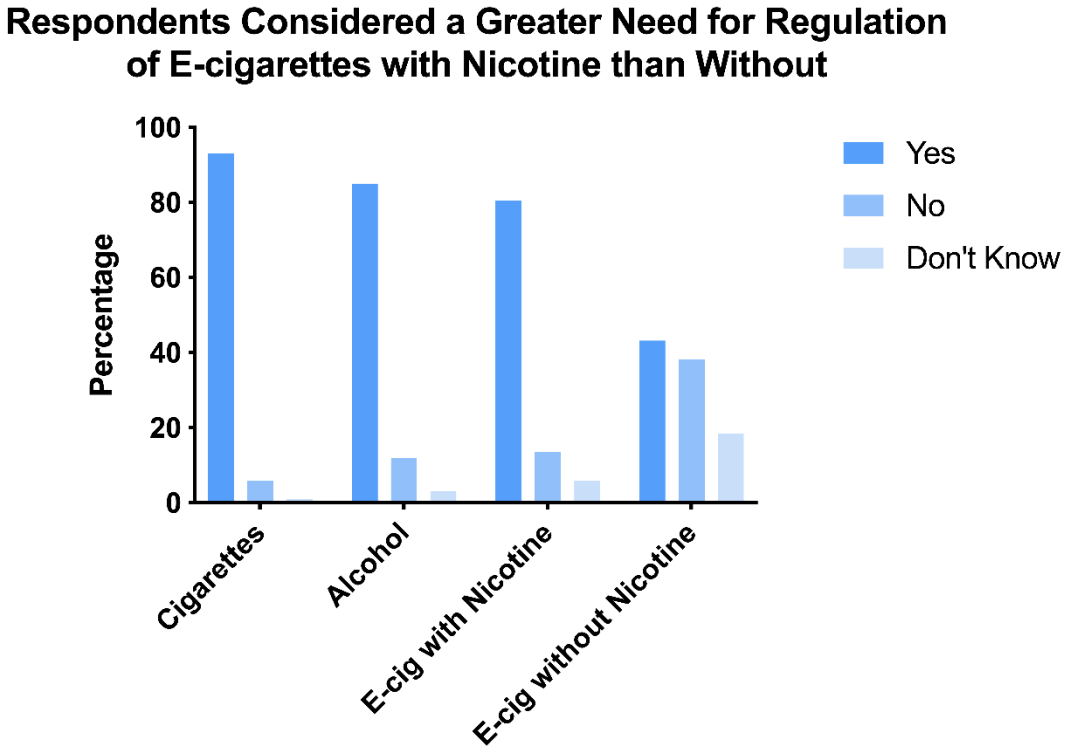

Figure 3.8 – Respondents opinions on whether cigarettes, alcohol, nicotine E-cigarettes or non-nicotine E-cigarettes should be regulated.

Q: *Should these substances be regulated by government (regulation often includes sales laws, taxation, advice on reducing use or quitting)? (answer all) (tick one)*

(n=287)

Discussion:

Respondents most commonly first heard of E-cigarettes through friends (56%), Social Media (52%) and the Internet (48%) (**Fig. 3.1**). The main reasons for E-cigarette use were curiosity (58%), because they are not as bad for your health as cigarettes (45%) and to help quit cigarettes (41%) (**Fig. 3.2**). The majority of current E-cigarette users (75%) thought that nicotine containing E-liquids were regulated the same as non-nicotine E-liquids. E-cigarettes were perceived to be useful as a cessation aid if they did not contain nicotine (**Fig. 3.3**). No differences in perception of nicotine and non-nicotine containing E-cigarettes in the remaining harm perception questions (**Fig. 3.4-3.6**). Respondents thought E-cigarettes without nicotine were less harmful than consuming fast food, but E-cigarettes containing nicotine were more harmful than fast food (**Fig. 3.7**). Lastly, 36% of respondents knew that regulations were stricter for nicotine containing E-cigarettes compared to non-nicotine containing E-cigarettes, and the remaining 64% thought regulations were the same (**Fig. 3.8**).

In contrast to our findings, a longitudinal study between 2013 and 2014 found that curiosity was not a predictor for extended use of electronic cigarettes, but other factors such as the low cost and prior smoking history were predictors for extended E-cigarette use [111]. 31 % of respondents in our study reported using E-cigarettes because they were cheaper than tobacco cigarettes. Current legislation does not tax the sale of E-cigarettes and E-cigarette related products allowing them to be sold substantially cheaper than traditional tobacco cigarettes [112]. The increasing cost of tobacco due to tobacco tax is associated with significant reduction in smoking rates [113]. Australia has developed many approaches to reduce smoking rates, our understanding of reducing tobacco use in Australia should be applied to E-cigarettes to stop the rapid increase in E-cigarette use that has occurred in the United States of America [96, 114].

The flavours of E-liquids have regularly been debated as to whether they are marketed towards a younger demographic, thus contributing to lifelong nicotine addiction and tobacco use. Within our respondents 61 % of current E-cigarette users and 31 % of past E-cigarette users reported tasting better than cigarettes as a reason for use. This suggests that flavours may play a role in attracting young Australians to use E-cigarettes. In 2009 the FDA banned the sale of fruit, candy or clove (excluding menthol) flavoured tobacco to reduce initiation of tobacco smoking [115]. A national study of adolescents in USA found that respondents were more likely to report interest in trying E-cigarettes if they were menthol or fruit flavoured than tobacco [116], and a survey of young Australians had similar findings [117]. Unfortunately, we did no collect data on the flavours of E-liquids used by respondents, but this data should be collected in future studies.

A parliamentary inquiry in 2017 led to a ban on the sale of nicotine containing E-liquids, a ban on advertisement of E-cigarettes and liquids, as well as sale restrictions to people 18 years and older [112, 118]. The ban on importing E-liquids containing nicotine is exempt with a prescription from a medical practitioner providing it is less than a 3 month supply [119]. Online retailers do not require a prescription to be presented upon sale making this legislation redundant. In June 2020 it was announced that a prohibition on the import of e-cigarette products containing vaporiser nicotine would come into place, this has since been deferred pending the outcome of a separate scheduling for nicotine [120]. The new legislation requested by the therapeutic goods administration will still allow for the import of nicotine containing E-cigarette products with a doctor's prescription, but how this will be policed and implemented is yet to be elucidated. We believe that future legislation to deter young adults and non-smokers from purchasing E-cigarettes should be implemented, including a ban on the sale of flavoured E-cigarette products that may be appealing to younger demographics and non-tobacco smokers.

Our study aimed to determine the attitudes of young Australians towards nicotine and non-nicotine containing E-cigarettes, and whether current legislation might be guiding their opinions on safety. Jongenelis et al provided evidence that majority of young Australian E-cigarette users prefer E-liquids containing nicotine, suggesting that current regulations aren't stopping users from importing nicotine containing E-liquids illegally [117]. Respondent's opinions on nicotine and non-nicotine containing E-cigarettes suggests a lack of education on the products. This could be attributed to the mass of contradictory information available to consumers, and the differing opinions of international health bodies on the harms of E-cigarettes[121, 122].

Our study was limited by its small sample size, but we believe it highlights a lack of education around emerging tobacco products, and a lack of research on young people's perceptions of these products. Furthermore, non-probability sampling can potentially reduce how representative the responses of our study are compared to the general population. A future nationwide study would help to further understand the attitudes of young Australians towards emerging tobacco products and give a better estimate of current E-cigarette usage rates. Cancer Council NSW surveyed 1001 Australians aged 18-64 on their awareness, opinions and use of E-cigarettes in 2015 [123]. This is a good starting point, but it lacks several questions that are essential for understanding E-cigarettes in Australia. Utilising the questions validated in this study will add further understanding of how Australians perceive e-cigarettes and the harms related to their use.

To conclude, the wide range of products and the ability to import from overseas with little to no regulation presents a possible future public health crisis. Our study suggests that young Australians consider E-cigarettes to be considerably less harmful than tobacco cigarettes. Although this is still up for debate it does not take into consideration that rate of transition to tobacco smoking from E-cigarette use, suggesting a normalisation of smoking in a younger demographic. Smoking normalisation will result in an increase in smoking rates that have been on a steady

decline for decades due to crucial public health intervention in tobacco control. Further regulatory action needs to take place soon to keep moving forward with tobacco control in Australia instead of taking steps backwards.

Appendix

Participant Information Sheet

Opinions of young Australians on the use of current and emerging tobacco products

(1) What is this study about?

This study aims to explore the perceptions and attitudes of young Australians towards current and emerging tobacco products. You have been invited to participate in this study because you are between the ages of 18-30. This Participant Information Statement includes information relevant to the study. Please read this information sheet carefully as you will be required to confirm that you have read all the information before you can complete the survey.

Any further questions related to the study can be addressed to Jack Bozier (Phone: 02 [redacted]; Email: jack.e.bozier@student.uts.edu.au). Participation in this research study is voluntary, and by giving your consent to take part in this study you are telling us that you:

a) Understand what you have read.
b) Agree to take part in the research study as outlined below.

(2) Who is running the study?

The study is being carried out by the following researchers:

A/Prof Brian GG Oliver (BSc Hons, MSc, PhD), Dr Juliet Foster (BSc Hons, PhD), Mr Jack E Bozier (BSc Hons)

(3) What will the study involve for me?

This study will involve completing a survey of less than 40 questions about your opinions on tobacco products and their regulation in Australia. Participants who provide their email address will be entered into a prize draw to win one of five double movie vouchers.

(4) How much of my time will the study take?

The survey should take no more than 15 minutes to complete.

(5) Who can take part in the study?

Anyone between the ages of 18-30 can take part in this study

(6) Do I have to be in the study? Can I withdraw from the study once I've started?

Participation in this study is voluntary, you do not have to take part and you are free to withdraw at any time. Should you wish to complete the survey at a later time, you can resubmit as long as the survey is still open. Submitting your survey responses is an indication of your consent to participate in the study.

(7) Are there any risks or costs associated with being in the study?

This study will only take up to 15 minutes of your time, there will be no other risks or costs associated with participating.

(8) Are there any benefits associated with being in the study?

The information gathered from this study may act as a precursor to a nationwide study assessing Australia's attitudes to current and emerging tobacco products.

(9) What will happen to information about me that is collected during the study?

The following types of information will be collected and used as part of this study:

- Basic demographic data (i.e. Your age, Postcode, Academic qualifications)
- Your perceptions and attitudes about tobacco and emerging tobacco products

This information will not be linked to any details that might identify you. For example, your email address will be separated from your answers to the survey keeping them unidentifiable. Results from this study may be published in scientific journals, presented at conferences or used to support further research projects on similar topics.

(10) Can I tell other people about the study?

Yes, you are also welcome to invite others to participate in this study.

(11) What if I would like further information about the study?

If you have any further questions after reading this information sheet, or at any point during this study, please contact Jack Bozier – Phone: 02 [redacted] Email: jack.e.bozier@student.uts.edu.au

(12) Will I be told the results of the study?

We are unable to provide the survey results of any individual participant but if you would like a copy of any publication arising from this research please contact Jack Bozier – Phone: 02 [redacted] Email: jack.e.bozier@student.uts.edu.au

(13) What if I have a complaint or any concerns about the study?

Please contact Jack Bozier – Phone: 02 [redacted] Email: jack.e.bozier@student.uts.edu.au

Questionnaire

Q1 (By ticking this box I confirm I have read the patient information sheet in full)
Confirm

Q2 If you would like to be entered into the prize draw please enter your email below:
(Open Response)

Q3 How old are you today (in years)?
(Open Response)

Q4 Are you male or female?
Male
Female

Q5 Have you ever smoked cigarettes or tobacco? (tick one)
I currently smoke
I don't smoke now but I have in the past
I have never smoked

Q6 For how long have you smoked? (tick one)
Less than a year
1 to 5 years
More than 5 years

Q7 How often do you smoke? (tick one)
Daily
More than 3 days per week
Weekly
Monthly
Less than monthly
Not at all

Q8 Which of these people have ever smoked? (tick all that apply)
My mother
My father
Both my mother and father
Another family member
My friend/s, My work colleague/s
None of these

Q9 In which of the following places can cigarettes be legally smoked in Australia? (tick all that apply)

In restaurants
In public places outdoors
In public places indoors
On public transport
In outdoor dining areas
In designated smoking areas
In some Australian states
In all Australian states

Q10 Have you ever heard of electronic cigarettes (also known as e-cigarettes, vaping, electronic nicotine delivery systems or personal vaporisers)? (tick one)

Yes
No

Q11 Where did you first hear about electronic cigarettes? (tick all that apply)

Social media
YouTube video
Internet
Newspaper
Television
Radio
Friends
Family
Other

Q12 Which of these people have ever used an electronic cigarette? (tick all that apply)

My mother
My father
Both my mother and father
Another family member
My friend/s
My work colleague/s
None of these.

Q13 In which of the following places can an electronic cigarette be legally used in Australia? (tick all that apply)

In restaurants
In public places outdoors
In public places indoors
On public transport
In outdoor dining areas
In designated smoking areas
In some Australian states
In all Australian states

Q14 In Australia are the regulations about places where an electronic cigarette can be legally used the same for nicotine-containing and non- nicotine containing electronic cigarettes? (tick one)

Regulations are the same
Regulations are stricter for nicotine-containing electronic cigarettes

Q15 Have you ever used an electronic cigarette? (tick one)

I currently use an electronic cigarette
I don't use an electronic cigarette now but I have in the past
I have never used an electronic cigarette

Q16 How often do you use an electronic cigarette? (tick one)

Daily
More than 3 days per week
Weekly
Monthly
Less than monthly

Q17 What concentration of nicotine is your current E-liquid? (tick one)

24mg/ml
18mg/ml
12mg/ml
6mg/ml
3mg/ml
0mg/ml (I use an E-liquid without nicotine)
Other (please specify)/mg/ml

Q18 How much e-liquid do you use in your electronic cigarette per week? (tick one)

1 to 10 ml per week
11 to 20ml per week
More than 21 ml per week
Don't Know

Q19 What are your main reasons for currently using an electronic cigarette? (tick all that apply)

To cut down on the number of cigarettes I smoke
To help me quit smoking cigarettes
Because they taste better than cigarettes
Because they are not as bad for your health as cigarettes
Because they are cheaper than cigarettes
So I can smoke in places where smoking cigarettes is not allowed
Because it's safer to smoke without nicotine
Curiosity
To improve my health
Because my friends use them
Other (please specify)

Q20 Why did you start using an electronic cigarette? (tick all that apply)

To cut down on the number of cigarettes I smoke
To help me quit cigarettes
Because they taste better than cigarettes
Because they are not as bad for your health as cigarettes
Because they are cheaper than cigarettes
So I can smoke in places where smoking cigarettes is not allowed
Because it's safer to smoke without nicotine
Curiosity
To improve my health
Because my friends use them
Other (please specify)

Q21 Electronic cigarettes that contain nicotine are useful for helping people give up smoking: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q22 Electronic cigarettes that do not contain nicotine are useful for helping people give up smoking: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q23 Electronic cigarettes that contain nicotine are bad for your health: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q24 Electronic cigarettes that do not contain nicotine are bad for your health: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q25 Electronic cigarettes that contain nicotine can cause similar damage to your lungs as cigarettes: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q26 Electronic cigarettes that do not contain nicotine can cause similar damage to your lungs as cigarettes: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q27 Electronic cigarettes that contain nicotine can cause lung cancer: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q28 Electronic cigarettes that do not contain nicotine can cause lung cancer: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't Know

Q29 Please rate the health risk of regular use of the following (1= least harmful; 7 = most harmful)

Cigarettes
Electronic cigarettes that contain nicotine
Electronic cigarettes that do not contain nicotine
Alcohol
Cannabis
Paracetamol
Fast food

Q30 Taking drugs recreationally is dangerous because you don’t really know what you are taking: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't know

Q31 Taking drugs recreationally is OK if you are careful and you know what you are doing: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't know

Q32 Most young people who use drugs recreationally come to little harm: (tick one)

Strongly agree
Agree
Neither agree nor disagree
Disagree
Strongly disagree
Don't know

Q33 Should these substances be regulated by government (regulation often includes sales laws, taxation, advice on reducing use or quitting)? (answer all)

Cigarettes
Electronic cigarettes that contain nicotine
Electronic cigarettes that do not contain nicotine
Alcohol

Q34 How often do you use illicit or prescription drugs recreationally (e.g. Cannabis, Ecstasy, MDMA, Valium, Adderall)? (tick one)

Every day
once a week or more
about once a month
Every few months
once or twice a year
I used them in the past but not now
Never
I'd rather not say

Q35 How often do you have a drink containing alcohol? (tick one)

Every day
5-6 days a week
3-4 days a week
1-2 days a week
2-3 days a month
about 1 day a month
Less than monthly

Q36 Have you ever been told by a health professional that you have asthma? (tick one)

Yes
No

Q37 What is your postcode? (fill in)

(Open Response)

Q38 Do you usually speak a language other than English at home? (tick one)

Yes
No

Q39 If yes, which language(s) (fill in)

(Open Response)

Q40 What is your highest completed level of education? (tick one)

Completed primary school
Completed year 10 of secondary school
Completed year 12 of secondary school
Completed TAFE
Completed Bachelor degree or higher (Masters or PhD)
Other (please specify)

Q41 If you are you are a university student, which faculty do you belong to? (tick one)

I am not a university student
Science faculty
Health faculty
FASS faculty
DAB faculty
Engineering faculty
Other (please specify)

Chapter 4

Heightened Response to E-cigarettes in COPD

Authors and affiliations: Jack Bozier [1,2], Sandra Rutting[1], Dia Xenaki[1], Matthew Peters[3], Ian Adcock[4,5], Brian G. Oliver[1,2]

[1] Respiratory Cellular and Molecular Biology, Woolcock Institute of Medical Research, University of Sydney, Australia.

[2]School of Life Sciences, University of Technology Sydney, Australia.

[3] Concord Hospital, University of Sydney, Australia.

[4] Airway Diseases Section, National Heart and Lung Institute, Imperial College London, London, UK.

[5] Biomedical Research Unit, Royal Brompton and Harefield NHS Trust, London, UK; Section of Respiratory Diseases.

Corresponding author details: Jack Bozier – jack.bozier@sydney.edu.au

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ORIGINAL RESEARCH LETTER

Heightened response to e-cigarettes in COPD

To the Editor:

E-cigarettes are used as an alternative to cigarette smoking, and as nicotine replacement therapy, with suggestions that they are markedly less harmful than cigarettes to the user. The confusion around the safety of e-cigarettes stems from contradictory findings in which variations in experimental methodology and the testing of different devices has not been accounted for. The harms associated with their use are not well understood and this is commonly misconceived as meaning that they are a healthy alternative to smoking. This misconception is further exacerbated by physicians and public health bodies which have made recommendations without strong scientific evidence [1]. Multiple studies have concluded that e-cigarette vapour exposure could lead to inflammation [2–5], emphysema [4] and a greater risk of bacterial and viral infection [3]. The lack of a defined model for e-cigarette exposure for both *in vitro* cellular and *in vivo* animal studies has led to contradictory findings between studies. Such differences can be attributed to different devices (first *versus* fourth generation), vaporisation temperature, different E-liquids or the concentration of e-cigarette vapour used. The confusion caused by contradictory findings leaves consumers and clinicians to form their own opinions about e-cigarettes safety which may lead to further public health issues in the future. Furthermore, no studies have compared responses in cells from people with chronic obstructive pulmonary disease (COPD), a disease state where the use of e-cigarettes is particularly attractive. However, COPD lung cells are known to be hyperresponsive to a range of environmental stimuli including cigarette smoke and pollution, and therefore might also respond differently to e-cigarette vapour.

The aim of this study was to evaluate dose–response relationships of e-cigarette stimulation of primary airway smooth muscle cells (ASMCs) from people with and without COPD under realistic physiological conditions. ASMCs were chosen for this study because of their contribution to pathological processes in COPD. Not only is smooth muscle bulk increased in COPD airways, ASMCs have been shown to secrete increased inflammatory mediators and chemokines compared with cells from smokers without COPD [6], suggesting their response to inflammatory stimuli might contribute to lung inflammation and/or disease progression in COPD. We have previously shown that ASMCs and airway fibroblasts from people with COPD are hyperresponsive to cigarette smoke [6, 7], and hypothesised that they would also be hyperresponsive to e-cigarettes. We also hypothesised that the increased toxic by-product formation seen at higher vaporisation temperatures would result in greater cytotoxicity in ASMCs [8].

ASMCs from 22 patients were included in this study, of which nine had a diagnosis of COPD (forced expiratory volume in 1 s (FEV1) <80%, FEV1/forced vital capacity <0.7). The non-COPD patients had normal lung spirometry. E-cigarette vapour extract (EVE) was made by bubbling 20×5 s bursts of E-vapour through 25 mL of DMEM supplemented with 0.1% Fetal Bovine Serum (FBS) (JRH Biosciences, Melbourne, Australia), 2.5 $\mu g \cdot mL^{-1}$ amphotericin B, 20 $\mu g \cdot mL^{-1}$ streptomycin and 20 $U \cdot mL^{-1}$ penicillin (Invitrogen, Mount Waverly, Australia). CXCL8 release was measured using an ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cell viability was assessed by measuring mitochondrial activity. 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (Sigma Aldrich, Castle Hill, Australia) (0.005% w/v in sterile PBS) was added to each well, followed by incubation for 6 h at 37°C in 5% carbon dioxide.

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E-cigarettes induce greater inflammatory mediators from COPD lung cells; therefore, the risks of e-cigarette use in COPD might be greater than in people without COPD http://ow.ly/xmnN30nzDhX

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To investigate if lung cells from people with COPD are hyperresponsive to e-cigarettes we stimulated COPD and non-COPD cells with increasing concentrations of tobacco- and menthol-flavoured EVEs containing 18 mg nicotine·mL^{-1} (n=7 COPD, n=7 non-COPD) or 0 mg nicotine·mL^{-1} (n=9 COPD, n=6 non-COPD).

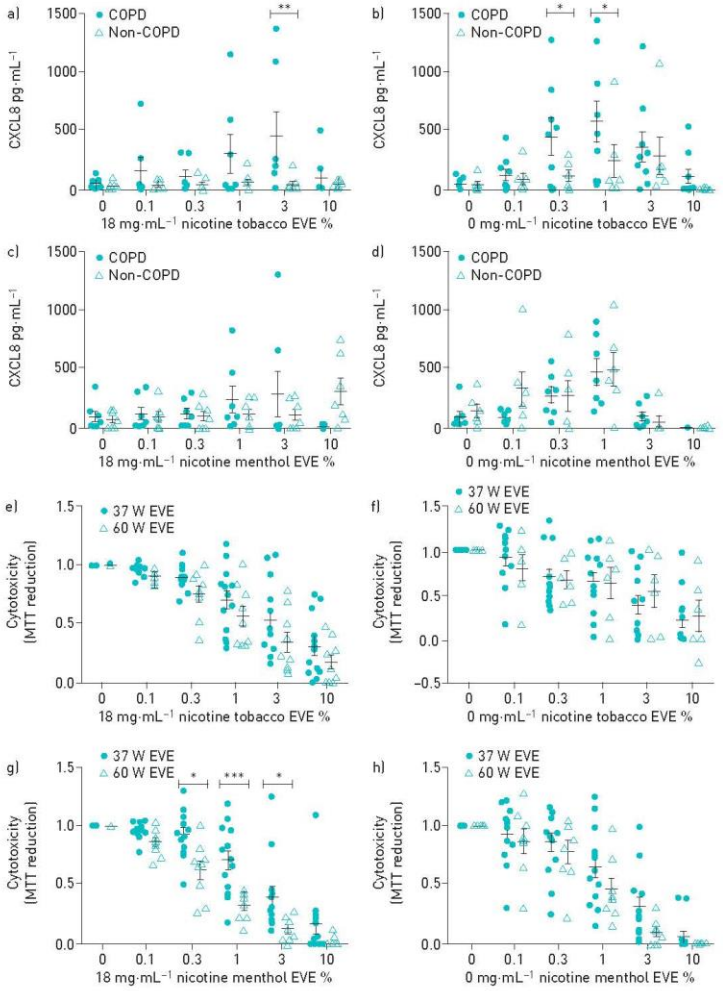

FIGURE 1 Primary human airway smooth muscle cells were stimulated with different concentrations of a, e) 18 mg·mL^{-1} nicotine tobacco-flavoured e-cigarette vapour extract (EVE), b, f) 0 mg·mL^{-1} nicotine tobacco-flavoured EVE, c, g) 18 mg·mL^{-1} nicotine menthol-flavoured EVE and d, h) 0 mg·mL^{-1} nicotine menthol-flavoured EVE for 24 h. The control well contained 0.1% FBS DMEM. a–d) CXCL8 release production was measured in supernatant using ELISA. Error bars represent ±SEM. Two-way ANOVA with Tukey's post-test was used for statistical analysis, significance is represented as *: p≤0.05; **: p≤0.01. e–h) Cytotoxicity was determined post-stimulation using an MTT assay. Data are normalised to unstimulated cells and error bars represent ±SEM. Two-way ANOVA with Tukey's post-test was used for statistical analysis, significance is represented as *: p≤0.05; ***: p≤0.001. COPD: chronic obstructive pulmonary disease; MTT: 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

We selected these flavours as they proved to be more popular in smokers attempting to abstain from smoking [9]. Both tobacco EVEs induced greater CXCL8 production in COPD cells compared with non-COPD cells (figure 1a and b); however, this was not observed with the menthol EVEs (figure 1c and d). Next, we investigated the effects of vaporisation temperature on toxicity, and found that menthol EVE containing nicotine was more cytotoxic when generated at 60 W, with a trend towards increased toxicity with menthol flavoured nicotine-free EVE (figure 1g and h). In contrast, tobacco-flavoured EVE had no effect on cytotoxicity when generated at higher temperatures (figure 1 e and f).

Cytotoxicity and inflammation are aetiological in COPD as they both contribute to pathological processes of the disease. Emphysema is caused by alveolar cell death and ineffective repair, resulting in an enlargement of the alveolar spaces [10]. The cytotoxicity of cigarette smoke is well documented and considered a contributing factor to disease progression. We have previously shown that cigarette smoke extract is cytotoxic to primary human ASMCs using methodology similar to that used with EVEs here [6]. This method is representative of what happens in the lung tissue, as only soluble components of the smoke or e-vapour will permeate through the epithelial layer to the underlying mesenchymal cells.

The power settings of the e-cigarette used were chosen according to the manufacturer's guidelines and users' preferences. The maximum power recommended for this coil (60 W) was used, as users often prefer to use their device at the high settings. Interestingly at the higher power setting, we found that that there was a significant increase in cytotoxicity with 18 mg nicotine·mL^{-1} menthol e-vapour extract. Coils were inspected before and after each extract generation and the tanks were refilled with e-liquid to confirm no dry wicking was taking place.

Patients with COPD, or smokers, might switch to e-cigarettes as an alternative nicotine source, believing that they are safer. All e-cigarette aerosols increased CXCL8 production in ASMCs irrespective of flavour or nicotine concentration. This suggests that e-cigarettes would stimulate lung neutrophilic inflammation. Furthermore, our data suggests that e-cigarette aerosol stimulates COPD cells in a similar manner to cigarette smoke, resulting in an increased production of CXCL8 from COPD cells compared with non-COPD cells.

Overall, our data suggests that COPD patients should avoid using e-cigarettes as a smoking cessation aid as they have a similar ability to stimulate inflammation and lung damage as cigarette smoke, and thus potentially accelerate their disease progression.

Jack Bozier[1,2], Sandra Rutting[1], Dia Xenaki[1], Matthew Peters[3], Ian Adcock[4,5] and Brian G. Oliver[1,2]
[1]Respiratory Cellular and Molecular Biology, Woolcock Institute of Medical Research, University of Sydney, Sydney, Australia. [2]School of Life Sciences, University of Technology Sydney, Sydney, Australia. [3]Concord Hospital, University of Sydney, Sydney, Australia. [4]Airway Diseases Section, National Heart and Lung Institute, Imperial College London, London, UK. [5]Biomedical Research Unit; Section of Respiratory Diseases, Royal Brompton and Harefield NHS Trust, London, UK.

Correspondence: Brian G. Oliver, Woolcock Institute of Medical Research, Level 3 Cell Biology, 431 Glebe point road, Glebe, NSW, Australia 2037. E-mail: brian.oliver@uts.edu.au

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Chapter 5

E-cigarette vapour induces cellular senescence in lung fibroblasts and may contribute to lung pathology

Authors and affiliations: Jack Bozier [1,2*], Roy R. Woldhuis[1,2,3,6*], Baoming Wang[1,2], Irene H. Heijink[3,4,6], Maaike de Vries[5,6] Maarten van den Berge[4,6], Wim Timens[3,6], Corry-Anke Brandsma[3,6], Brian G.G. Oliver[1,2]

* Co-first authors

[1]*Woolcock Institute of Medical Research, Respiratory Cellular and Molecular Biology Group.*
[2]*University of Technology Sydney, School of Life Sciences.*
[3]*University of Groningen, University Medical Centre Groningen, Department of Pathology and Medical Biology.*
[4]*University of Groningen, University Medical Centre Groningen, Department of Pulmonary Diseases.*
[5]*University of Groningen, University Medical Centre Groningen, Department of Epidemiology.*
[6]*Groningen Research Institute for Asthma and COPD (GRIAC).*

Corresponding Author:
Brian G.G. Oliver
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E-cigarette vapour induces cellular senescence in lung fibroblasts and may contribute to lung pathology

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Complete List of Authors:	Woldhuis, Roy; Woolcock Institute of Medical Research, Respiratory Cellular and Molecular Biology Group; University of Technology Sydney, School of Life Sciences; University Medical Centre Groningen, Pathology and Medical Biology; University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC) Bozier, Jack; Woolcock Institute of Medical Research, Respiratory Cellular and Molecular Biology Group; University of Technology Sydney, School of Life Sciences Wang, Baoming; Woolcock Institute of Medical Research, Respiratory Cellular and Molecular Biology Group; University of Technology Sydney, School of Life Sciences Heijink, Irene; University Medical Centre Groningen, Pathology and Medical Biology; University Medical Centre Groningen, Pulmonary Diseases; University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC) de Vries, Maaike; University Medical Centre Groningen, Epidemiology; University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC) van den Berge, Maarten; University Medical Centre Groningen, Pulmonary Diseases; University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC) Timens, Wim; University Medical Centre Groningen, Pathology and Medical Biology; University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC) Brandsma, Corry-Anke; University Medical Centre Groningen, Pathology and Medical Biology; University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC) Oliver, Brian; Woolcock Institute of Medical Research, Respiratory Cellular and Molecular Biology Group; University of Technology Sydney, School of Life Sciences
Key Words:	E-cigarettes, E-vapour, Cellular senescence, COPD, lung fibroblasts, Tissue repair
Abstract:	

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E-cigarette vapour induces cellular senescence in lung fibroblasts and may contribute to lung pathology

Roy R. Woldhuis[1,2,3,6]*, Jack Bozier[1,2]*, Baoming Wang[1,2], Irene H. Heijink[3,4,6], Maaike de Vries[5,6] Maarten van den Berge[4,6], Wim Timens[3,6], Corry-Anke Brandsma[3,6], Brian G.G. Oliver[1,2]

* Co-first authors

Woolcock Institute of Medical Research, Respiratory Cellular and Molecular Biology Group[1], University of Technology Sydney, School of Life Sciences [2], University of Groningen, University Medical Centre Groningen, Department of Pathology and Medical Biology[3], Department of Pulmonary Diseases[4], Department of Epidemiology[5] Groningen Research Institute for Asthma and COPD (GRIAC)[6]

Corresponding Author:
Brian G.G. Oliver
Level 3 Cell Biology
Woolcock Institute of Medical Research
431 Glebe point road, Glebe, NSW, Australia 2037
E-mail: brian.oliver@uts.edu.au

Take home message: E-cigarette vapour induces cellular senescence in lung fibroblasts in a dose-dependent manner, which should serve as a warning to avoid use as a safe alternative to cigarette smoking or as cessation device and excessive and long-term use should be avoided.

Keywords: E-cigarettes, E-vapour, Cellular senescence, COPD, lung fibroblasts, tissue repair

Word count: 1141

To the editor,

Introduction

COPD is a progressive inflammatory lung disease caused by exposure to noxious gases, in particular cigarette smoke. Chronic exposure to cigarette smoke (CS) causes inflammation and eventually leads to lung tissue damage. Ultimately, these pathologic events lead to airway fibrosis and airflow limitation, as well as alveolar breakdown and lack of tissue repair in the parenchyma, i.e. emphysema [1].

Cellular senescence has been recognized to play a role in the pathophysiology of COPD [2]. Cellular senescence is defined as an irreversible cell cycle arrest to prevent cell death or abnormal growth. Induction of cellular senescence can be caused by multiple mechanisms, including oxidative stress and DNA damage, both known to result from chronic CS exposure [3]. Accumulation of senescent cells in lung tissue can result in chronic inflammation and tissue dysfunction [4] and as such contribute to COPD pathology.

Electronic cigarettes have been proposed as a safer alternative to cigarettes. However, evidence of the harms related to E-cigarette use is growing. Similar to CS, E-cigarette exposure causes a pro-inflammatory response after acute and chronic exposure in multiple structural lung cells *in vitro*, *in vivo* in mouse lungs and in human clinical studies [5, 6], suggesting prolonged use may also contribute to COPD. Furthermore, E-cigarette vapour has been shown to induce DNA damage and reduce DNA damage repair in lung epithelial cell lines [7]. Oxidative stress from E-cigarette exposure has been studied to a greater extent *in vitro* and *in vivo,* with the majority demonstrating a high oxidative burden from E-vapour exposure [8, 9]. Although E-cigarette use may upregulate known senescence inducers, DNA damage and oxidative stress, it is unknown whether E-vapour can induce cellular senescence. Therefore, we investigated whether E-vapour exposure induces cellular senescence in primary lung fibroblasts and whether this affects their tissue repair function.

Methods

Primary parenchymal lung fibroblasts (n=11) were grown in DMEM (Gibco) supplemented with 5% foetal bovine serum (FBS) as described previously [10]. At passage 5-6, cells were seeded on 6 well plates (for collection of supernatants, RNA & SA-β-gal staining) and on 12 well plates (for wound healing assay). Cells were serum-starved in 0.5% FBS DMEM after 48 hours, and 24 hours later cells were stimulated with 250 μM Paraquat (PQ; positive control for senescence induction), 5% cigarette smoke extract (CSE), 1.5% (Lo) or 2% (Hi) nicotine-containing (18mg/ml) tobacco-flavoured E-cigarette Vapour extract (EV), or 1.5% (Lo) or 2% (Hi) nicotine-free tobacco-flavoured E-cigarette Vapour extract (NF EV) as described previously [11]. A cytotoxic dose for CSE (10%) and both EV (5%) was used as a positive control for the stimuli (data not shown). Supernatants (for IL-8 ELISA, data not shown) and RNA extracts were collected after 24 hours of stimulation, whilst remaining plates were refreshed to 5% FBS DMEM to enable cell proliferation for 3 days. Cellular senescence was assessed by SA-β-gal staining, cell proliferation inhibition, and p16 and p21 gene expression as described

previously [12]. Wound healing capacity was assessed 4 days after stimulation using the scratch assay by measuring wound closure after 48 and 72 hours of scratching stimulated and untreated fibroblasts. A wound in the cell layers was made by scratching with a p200 pipet tip in the middle of the well from top to bottom, wells were washed twice with Hanks buffer and DMEM + 0.5% FBS was added to enable wound closure, which was captured on a Nikon Eclipse Ti microscope at total magnification of 40x. Wound sizes after 48 hours and 72 hours were measured and percentages of wound closure were calculated.

Results

To confirm the stimulatory response of the different stimuli on primary lung fibroblasts, IL-8 secretion was measured, which was significantly induced by all stimuli compared to untreated fibroblasts (data not shown). Cellular senescence was induced by PQ and CSE with an increase in p21 expression (Fig. 1A), increased percentages of SA-β-gal positive cells (Fig. 1B), and reduced cell proliferation (Fig. 1C). For both no increase in p16 expression was found (data not shown). CS is a known risk factor for COPD and both stimuli are known to induce senescence, confirming our model's validity.

Upon stimulation with nicotine-containing E-vapour extract (EV), cellular senescence was induced with significant differences in the same senescence markers as PQ and CSE, which was dose-dependent (Fig. 1A-C). This induction of cellular senescence by E-vapour appeared to be nicotine-independent as stimulation with nicotine-free E-vapour extract (NF EV) also increased p21 expression and the percentages of SA-β-gal positive cells, and reduced cell proliferation. Only upon the low dose of NF EV stimulation, no significant p21 increase was observed (Fig. 1A). Similar to PQ and CSE, no increase in p16 expression was found after NF EV and EV stimulations.

Upon senescence induction by PQ and CSE, impaired tissue repair in a wound healing model occurred after 48 hours (Fig. 1D) and 72 hours (data not shown). Low dose EV impaired wound healing capacity with reduced wound closure after both 48 hours (Fig. 1D) and 72 hours (data not shown), which again appeared to be nicotine independent as similar results were found in NF EV treated cells. No significant reduction in wound closure was found upon high dose of EV stimulation, due to one donor with improved wound closure upon stimulation.

To confirm our *in vitro* results *in vivo*, gene expression of p21 was measured in a previously performed mouse model [13], where p21 expression seemed higher upon exposure compared to non-exposed controls (mean fold change + SEM: 1.3+0.09 (EV) and 1.2+0.2 (NF EV) vs SHAM). However, no significant differences were found as this pilot study was underpowered, thus larger *in vivo* studies should be done to confirm our *in vitro* findings.

Discussion

This study is the first to identify E-cigarette vapours' potential to induce cellular senescence in primary lung cells, which is a known contributing factor to disease in COPD [2]. The findings of our study further add to the identified risks of E-cigarette use [5]. E-cigarette harms are

often compared to cigarettes in relation to harm reduction, but this study focused on the standalone risk for E-cigarette users. These risks are not isolated to COPD patients with other E-cigarette users like young never-smokers, more likely to develop lung pathology from long-term use.

In the current study, we did not directly investigate the mechanisms of senescence induction by E-vapour extract, but we hypothesize that DNA damage and oxidative stress may be involved as previous studies demonstrated that E-cigarette vapour exposure can result in DNA damage and oxidative stress [5, 7-9]. Future studies should elucidate the mechanisms involved in EV-induced senescence and whether specific components of E-liquids are directly up-regulating these mechanisms.

E-cigarettes' potential to induce cellular senescence, alongside other previously identified risks, should serve as a warning to avoid use as a safe alternative to cigarette smoking or as a cessation device. Considering senescence induction was dose-dependent indicates excessive and long-term use should be avoided.

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Woolcock Institute of Medical Research[1]

Authors' contributions:
Conception and design: RRW, JB, BGGO
Acquisition and analysis of data: RRW, JB, BW, BGGO
Interpretation of data: RRW, JB, IHH, MdV, MvdB, WT, CAB, BGGO
Drafting the manuscript: RRW, JB
All authors reviewed, edited, and approved the final manuscript.
RRW and JB contributed equally.

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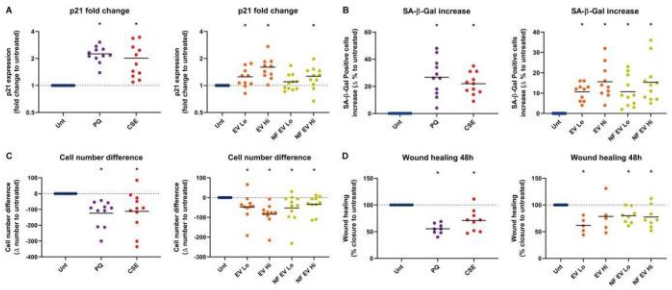
Figure legends

Figure 1: Cellular senescence induction upon stimulation with known senescence-inducers and upon E-vapor extract stimulation. Differences in p21 gene expression (A) (24h post stimulation), percentages of Senescence-associated beta-galactosidase (SA-β-gal) positive cells (B) (4d post stimulation), total cell numbers (C) (4d post stimulation) and percentages of wound closure after 48 hours (D) are shown compared to untreated (Unt, blue) upon stimulation with 250µM paraquat (PQ, purple) 5% cigarette smoke extract (CSE, red) and low (Lo = 1.5%) and high (Hi = 2%) doses of nicotine-containing (EV Lo and Hi, orange) and nicotine-free E-vapor (NF EV Lo and Hi, yellow) extracts. N=11 per group. Lines represent means and dotted lines represent levels of untreated. Significant differences between stimulated and untreated fibroblasts were tested using One-Way ANOVA with Fisher's LSD tests, * means P-value < 0.05 compared to untreated.



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Appendix – Full size figure from cellular senescence manuscript

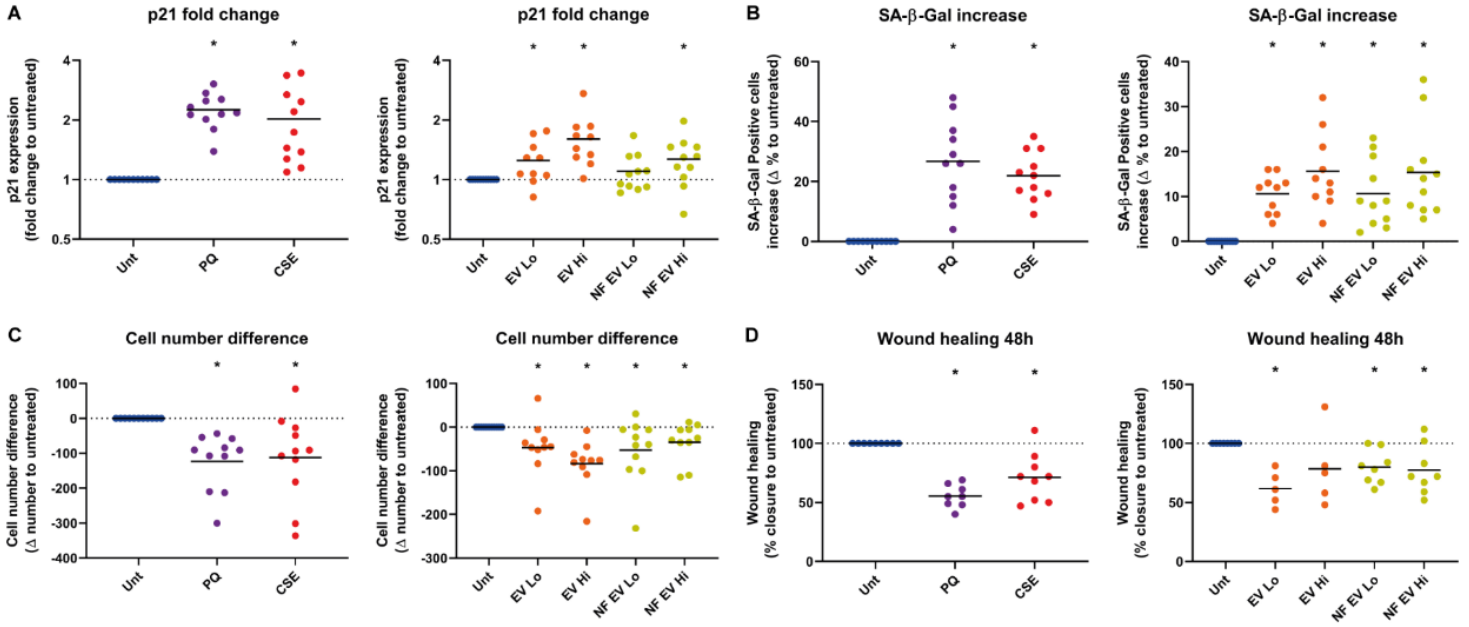

Figure 1: Cellular senescence induction upon stimulation with known senescence-inducers and upon E-vapor extract stimulation. Differences in p21 gene expression (A) (24h post stimulation), percentages of Senescence-associated beta-galactosidase (SA-β-gal) positive cells (B) (4d post stimulation), total cell numbers (C) (4d post stimulation) and percentages of wound closure after 48 hours (D) are shown compared to untreated (Unt, blue) upon stimulation with 250µM paraquat (PQ, purple) 5% cigarette smoke extract (CSE, red) and low (Lo = 1.5%) and high (Hi = 2%) doses of nicotine-containing (EV Lo and Hi, orange) and nicotine-free E-vapor (NF EV Lo and Hi, yellow) extracts. N=11 per group. Lines represent means and dotted lines represent levels of untreated. Significant differences between stimulated and untreated fibroblasts were tested using One-Way ANOVA with Fisher's LSD tests, * means P-value < 0.05 compared to untreated.

Chapter 6

Dual E-cigarette and cigarette use reduces dexamethasone sensitivity in-vitro

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Dual E-cigarette and cigarette use reduces dexamethasone sensitivity in-vitro

Jack Bozier[1,2], Roy R. Woldhuis[1,2,3,4], Karosham D. Reddy[1,2], Ian M. Adcock[5,6], Brian G Oliver[1,2]

[1]Respiratory Cellular and Molecular Biology Group, Woolcock Institute of Medical Research, Sydney, Australia

[2]Respiratory Cellular and Molecular Biology Group, University of Technology Sydney, Australia

[3]University of Groningen, University Medical Centre Groningen, Department of Pathology and Medical Biology

[4]University of Groningen, University Medical Centre Groningen, Groningen Research Institute for Asthma and COPD (GRIAC)

[5]National Heart and Lung Institute, Imperial College London, London, UK

[6]NIHR Imperial Biomedical Research Centre, London, UK

Corresponding Author

Jack Bozier, Respiratory Cellular and Molecular Biology Group, Woolcock Institute of Medical Research, The University of Sydney, NSW, Australia

Email: jack.bozier@sydney.edu.au

Authorship Statement: J.B., R.R.W., and K.D.R. performed experiments; J.B. analyzed data, J.B. and B.G.O. interpreted results of experiments; J.B. prepared figures and drafted manuscript; J.B., R.R.W., K.D.R., I.M.A. and B.G.O. edited and revised manuscript; J.B., R.R.W., K.R., I.M.A., and B.G.O. approved final version of manuscript; and J.B. and B.G.O. conceived and designed research.

Keywords: E-cigarettes, Cigarette smoke, Dexamethasone, Steroid insensitivity, COPD

Abbreviations: AKT, COPD, CXCL8, CSE, EVE, FOXO1, IL-6, IL1α, NF-κB, NRT, mTOR, PI3k, p38 MAPK.

Abstract

E-cigarettes are an alternative to tobacco smoking, with many people utilizing them as a cessation aid and as a result often using them in combination with tobacco cigarettes (dual use). Little is known about the toxicity or inflammatory potential of dual use, nor the effects on clinically relevant endpoints such as glucocorticoid sensitivity. We investigated these effects *in vitro* in primary human lung fibroblasts.

Fibroblasts were stimulated with non-cytotoxic doses of cigarette smoke extract (CSE) and/or E-vapor extract (EVE) for 24 hours and CXCL8, IL1α, CXCL1 and CXCL2 mRNA levels and IL6 and CXCL8 protein secretion were measured. Glucocorticoid sensitivity was assessed by pre-treatment for 1 hour with increasing concentrations of dexamethasone (1nM-1μM). Signaling pathway activation was measured using Western blot analysis of phosphorylated and total protein.

CSE and EVE induced CXCL8 production, and this was inhibited by 1nM and 100nM dexamethasone, respectively. CXCL8 production from dual stimulation was significantly greater than occurred from either alone suggesting synergy between the two stimuli. Importantly, the enhanced CXCL8 protein production induced by dual stimulation was not inhibited by 1μM dexamethasone. FOXO1 and p38 MAPK signaling were increased in dual stimulation and were not inhibited by dexamethasone, suggesting a potential mechanism of glucocorticoid insensitivity.

This study suggests a greater risk of using E-cigarettes and cigarettes in combination, with a significant risk in COPD patients who are susceptible to exacerbations driven by neutrophilic inflammation and glucocorticoid insensitivity.

Abstract word count: 234 words

Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death globally, with a current trajectory to be the third leading cause of death in the next decade [52]. COPD is caused by chronic inhalation of noxious gas or particulate matter resulting in airway remodeling and parenchymal tissue destruction [43]. Cigarette smoking is considered the greatest risk factor for the development of COPD, followed by other inhaled toxicants and irritants that carry potential of chronic exposure through occupational risks or burning of biomass fuels with poor ventilation [124]. Fletcher and Peto's observational epidemiological study discovered different subgroups of smokers, with some being susceptible to COPD and some not [55].

Since it was found to slow the onset of symptoms in susceptible smokers in Fletcher and Peto's study, smoking cessation has remained an integral facet of the treatment of COPD. Furthermore a multitude of supporting studies have identified quitting smoking as the most important factor for improved patient outcomes [92]. Typically, as part of routine care practitioners are required to provide COPD patients with the most effective available tools to help them quit smoking. The 'Five A' model of ask, advise, assess, assist, and arrange is the current GOLD standard for identifying smokers who may be at risk for COPD and implementation of strategies to help the patient quit smoking. Current approaches to treatment and support involve a combination of psychological and pharmacological support strategies. Pharmacological therapies involve administration of nicotine replacement therapy (NRT), varenicline or bupropion either alone or in combination. Evidence still flavors them as an effective treatment [125], but this hasn't stopped smokers from turning to alternative options for NRT [126]. Since their inception in 2006, E-cigarettes have been the consumer preferred alternative to smoking when it comes to nicotine consumption. The rapid uptake of E-cigarettes has left governments and regulatory bodies behind when it comes to safety testing and regulations around the sale of these products [127, 128]. Smoking cessation remains such an important factor in the management of COPD due to the limited tools available to treat patients.

Inhaled corticosteroids are prescribed routinely but most patients do not respond to treatment, only showing benefit in reduced exacerbation frequency but not in markers of inflammation [91].

Different subsets of E-cigarettes users have been identified, with COPD patients and smokers utilizing them as a cessation aids, or tobacco replacements, and both are considered at-risk groups [129]. Cigarette smoking is correlated with a greater risk of exacerbation in COPD patients, which coincides with a worsening of chronic inflammation in the lung, greater neutrophil numbers, and disease progression [83, 130, 131].

E-cigarettes are often proposed as a harm reduction approach to smoking cessation, but it is yet to be elucidated whether they are a safer alternative to smoking. E-cigarettes have been shown to be proinflammatory, with greater inflammatory response in COPD cells compared to non-COPD cells [132]. COPD patients have been identified as an at-risk group of E-cigarette users [97] but little is known about how E-cigarettes may impact their disease. Neutrophilic inflammation is known to play an important role in both the frequency and severity of exacerbations of COPD [50, 133]. It is important, therefore, that we understand how E-cigarettes effect inflammation in COPD patients.

Thus, the aims of this study were to determine the inflammatory potential of E-cigarettes and cigarette smoke both alone and in combination; and whether stimulation results in dysregulation of glucocorticoid induced reduction of inflammatory mediators particularly those implicated in neutrophil recruitment.

Methods

Study Subjects

Human lung tissue was collected following written informed consent. Primary human lung fibroblasts were isolated from subjects with COPD or no obstructive lung disease. Patient demographics can be found in Table 1

Cell Isolation and Culture

Primary human lung fibroblasts were isolated from the parenchyma of explanted lungs or resectioned lung tissue from patients with thoracic malignancies as described previously [134]. All cell culture reagents were from Gibco (Gaithersburg, USA) unless specifically stated. Primary lung fibroblasts were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Gibco) supplemented with 5% Fetal bovine serum (FBS), 25 mM HEPES buffer (Gibco), and 1% antibiotic-antimycotic at 37°C/5% CO_2. After 72 hours cells were serum staved in 0.1% FBS DMEM for 24 hours at 37°C/5% CO_2. Cells were pre-treated with increasing concentration of dexamethasone (1nM-1µM) for 1 hour before stimulation with either 1.5% E-vapor extract EVE) or 5% cigarette smoke extract (CSE) alone or in combination. Cells were stimulated for 30 mins before protein lysates were collected for western blot analyses of transcription factors. Separate plates were stimulated for 24 hours prior to RNA lysate collection and cell-free supernatant collection.

EVE Generation

E-vapor was generated using a KangerTech NEBOX 3rd generation device, filled with 80:20 PG:VG Tobacco flavored 18mg nicotine E-liquid. 20 x 5 second puffs were bubbled through 25ml of un-supplemented DMEM cell culture media in a T175 flask with 30 second rest between puffs to prevent overheating. The flask was sealed and left to rest for 15 minutes before diluting the extract down to an optimized non-cytotoxic working concentration of 1.5%.

CSE Generation

One Marlboro Red tobacco cigarette was bubbled through 25ml of non-supplemented DMEM cell culture media in a T175 flask. The flask was sealed and left to rest for 15 minutes before diluting the extract down to an optimized non-cytotoxic working concentration of 5%.

Gene expression analyses

To analyze gene expression, RNA extract and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed as described previously [135]. Taqman® assays for IL1α, CXCL1, CXCL2, IL6 and CXCL8 (LifeTechnologies, Carlsbad, USA) were used to quantify gene expression compared to 18s endogenous control. Data from the reactions was quantified using StepOne Software v 2.3 (Applied Biosystems, Waltham, USA).

Secreted Protein Analyses

Cell-free supernatants were collected 24 hours post stimulation and stored at -20°C prior to ELISA analysis. Secreted IL-6 and CXCL8 protein was measured using Human DuoSet ELISA kit (R&D Systems, Minneapolis, USA) following the manufacturer's instructions.

Western Blot Analysis

Western Blots were used to analyze protein levels of proinflammatory signaling factors related to steroid insensitivity. Cells were lysed in protein lysis buffer including protease and phosphatase inhibitors 30 minutes post stimulation with EVE or CSE alone or in combination, then collected and stored at -20°C. SDS-PAGE gels were prepared using 40% acrylamide solution (BioRad Laboratories, Hercules, USA) and stored in electrophoresis buffer overnight. Proteins were separated on 10% SDS-PAGE gels (p38 MAPK, phospho-p38 MAPK, NF-κB, and phospho-NF-κB) and 6% SDS-PAGE gels (MTOR, phospho-MTOR, FOXO1 and phospho-FOXO1). Separated proteins were transferred onto 0.4µm PVDF membrane using standard wet transfer conditions. Membranes were blocked 5% non-fat skim milk powder in T-TBS for 1

hour, except for phospho-p38 MAPK which was blocked in 5% BSA T-TBS for 1 hour. Membranes were then incubated overnight at 4°C with primary antibodies p38 MAPK, phospho-p38 MAPK, NF-κB, phospho-NF-κB, MTOR, phospho-MTOR, FOXO1, phospho-FOXO1 (all 1:1000, Cell Signaling Technologies, Danvers, USA). GAPDH (1:5000, Merck Millipore, Burlington, USA) was used as a loading control. Secondary antibodies conjugated with HRP (Goat anti Rabbit (1:2000, Dako, Glostrup, Denmark) or Rabbit anti Mouse (1:10000, Merck Millipore) were incubated at room temperature for 1 hour followed by visualization with Clarity Enhanced Chemiluminescence substrate (Bio-Rad Laboratories) on a ChemiDoc Imager (Bio-Rad Laboratories). Image Lab software (Bio-Rad Laboratories) was used densitometry of detected bands and protein levels were normalized to GAPDH.

Statistical Analysis

GraphPad Prism 8 software (GraphPad) was used for statistical analyses. Normality was assessed and a one-way ANOVA was used to detect differences in gene expression, secreted protein and signaling proteins between treatments. Tukey's post-test analysis was performed and *$p<0.05$ was considered significant.

Results

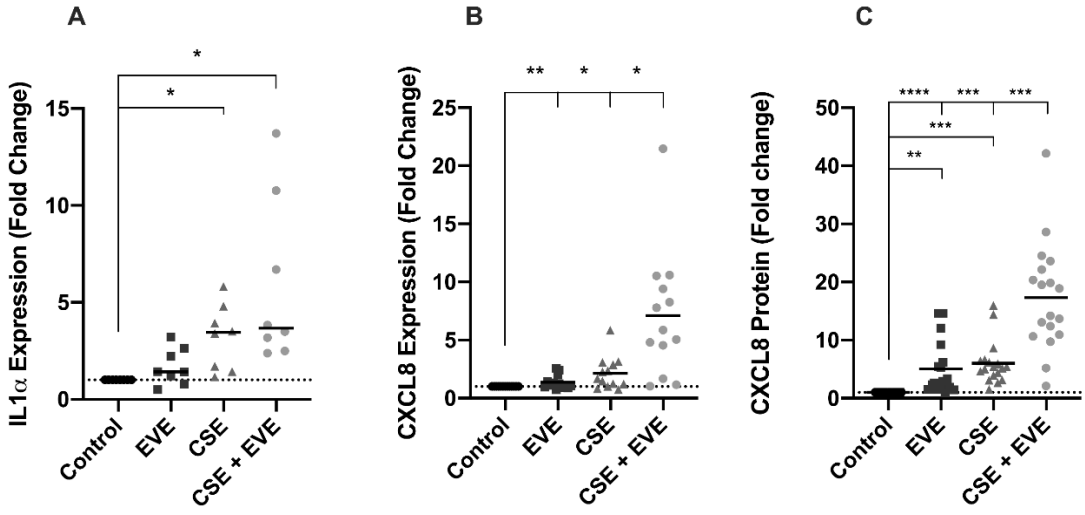


Figure 1: IL1α gene expression, CXCL8 gene expression and CXCL8 release from cigarette smoke extract (CSE) and E-vapor extract (EVE) exposed cells. Primary human lung fibroblasts were untreated (Control) or stimulated with EVE or CSE both alone and in combination for 24 hours. RNA lysates were collected for gene expression of IL1α (A)(n=8) and CXCL8 (B)(n=13) using RT-qPCR. Cell free supernatants were collected determine CXCL8 production(C)(n=18) using ELISA. Data is expressed as fold change of untreated, line at mean. All columns are compared to each other using one-way ANOVA with Tukey's post-hoc test. Significance is represented as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001; lines identify the columns being compared.

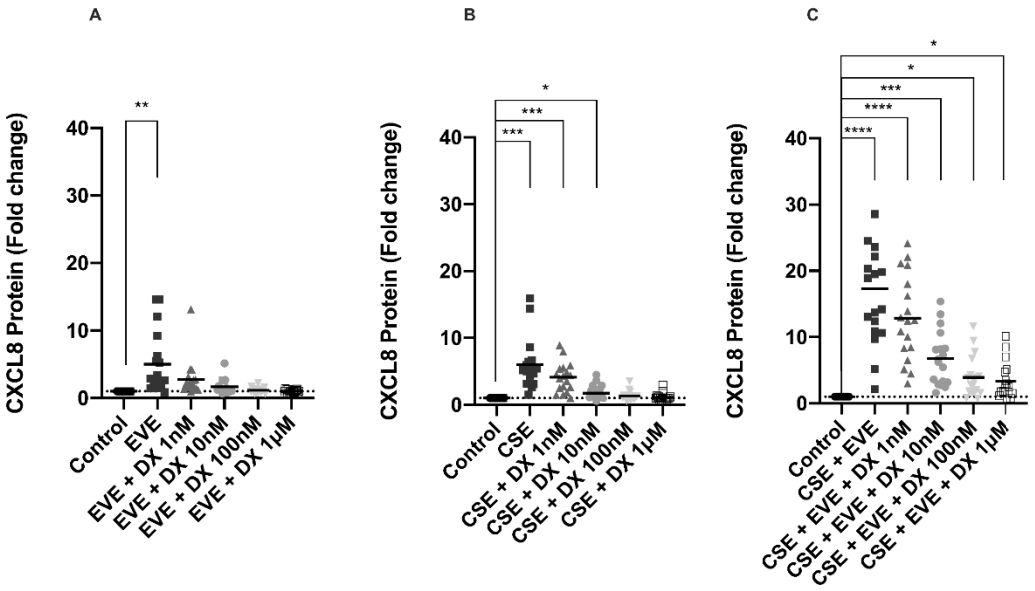


Figure 2: Concentration-dependent effect of dexamethasone (DX) on CXCL8 release from cigarette smoke extract (CSE) and E-vapor extract (EVE) exposed cells. Primary human lung fibroblasts (n=18) were untreated (Control) or stimulated with EVE (A) or CSE (B) both alone and in combination (C). Pre-treatment with DX at increasing concentrations (1nM-1µM) 1 hour prior to stimulation was included to determine the relative steroid response. Cell free supernatants were collected after 24 hours and ELISA was used to determine CXCL8 production. Data is expressed as fold change of untreated, line at mean. All columns are compared to each other using one-way ANOVA with Tukey's post-hoc test. Significance is represented as *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001; lines identify the columns being compared.

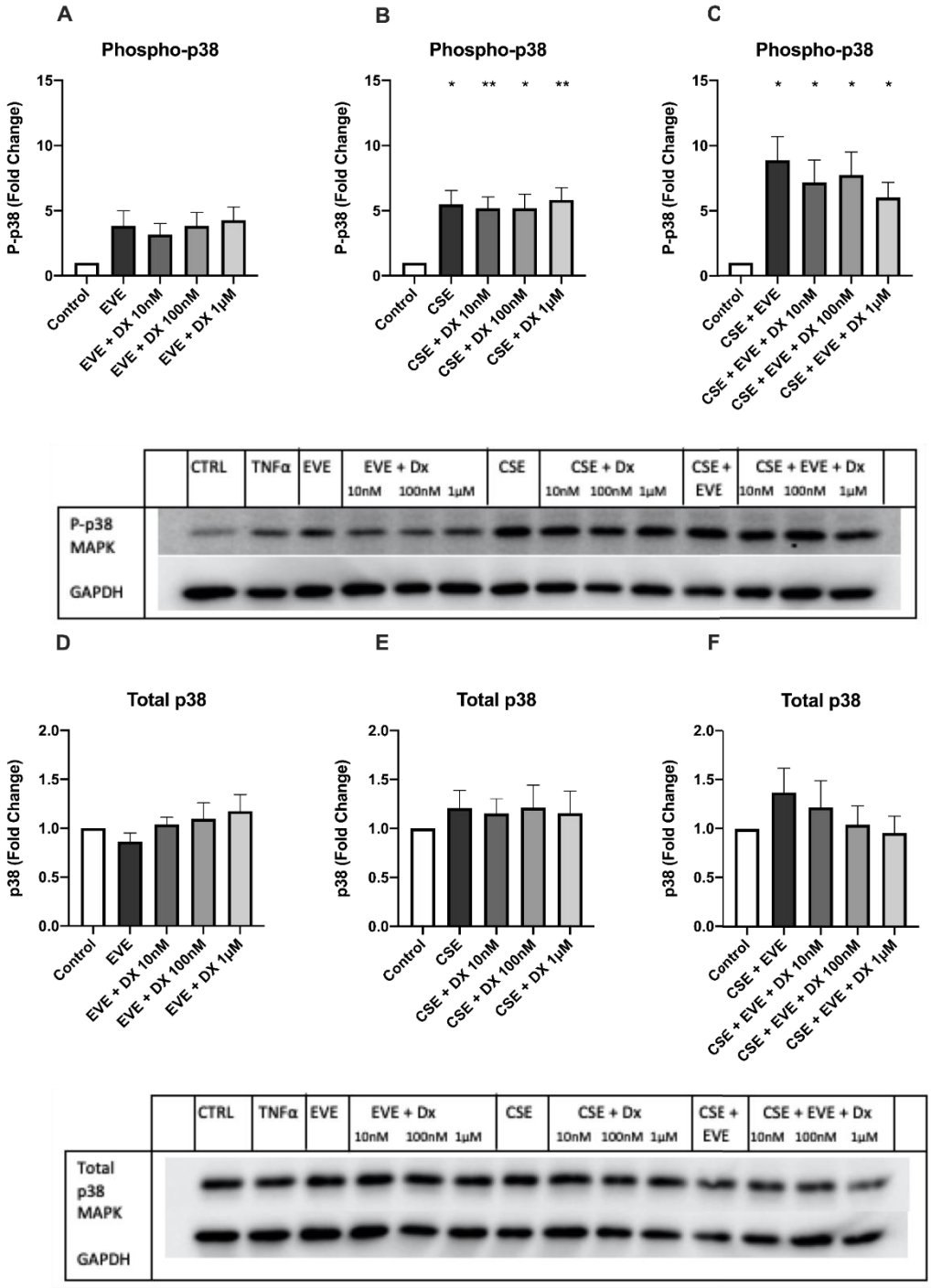


Figure 3: Activation of p38 MAPK after exposure of primary human lung fibroblasts to cigarette smoke extract (CSE) and/or E-vapor extract (EVE). Primary human lung fibroblasts (n=8) were untreated (Control) or stimulated with EVE (A) or CSE (B) both alone and in combination (C) for 30 minutes. Pre-treatment with dexamethasone (Dx) at 10nM, 100nM and 1μM concentrations 1 hour prior to stimulation was included to determine the relative steroid response. TNF-α was

included as a positive control for stimulation. Whole cell lysates were collected, and western blots were used to quantify phosphorylated p38 MAPK (P-p38 MAPK) and total p38 MAPK. Densitometry was performed and all values were normalized to GAPDH as a loading control. Images of Western blots are representative of n=9 independent experiments and data is expressed as fold change of untreated, mean +/- standard error of the mean. All columns were compared to control using one-way ANOVA with Tuckey's post-hoc test. Significance is represented as *$p \leq 0.05$, **$p \leq 0.01$.

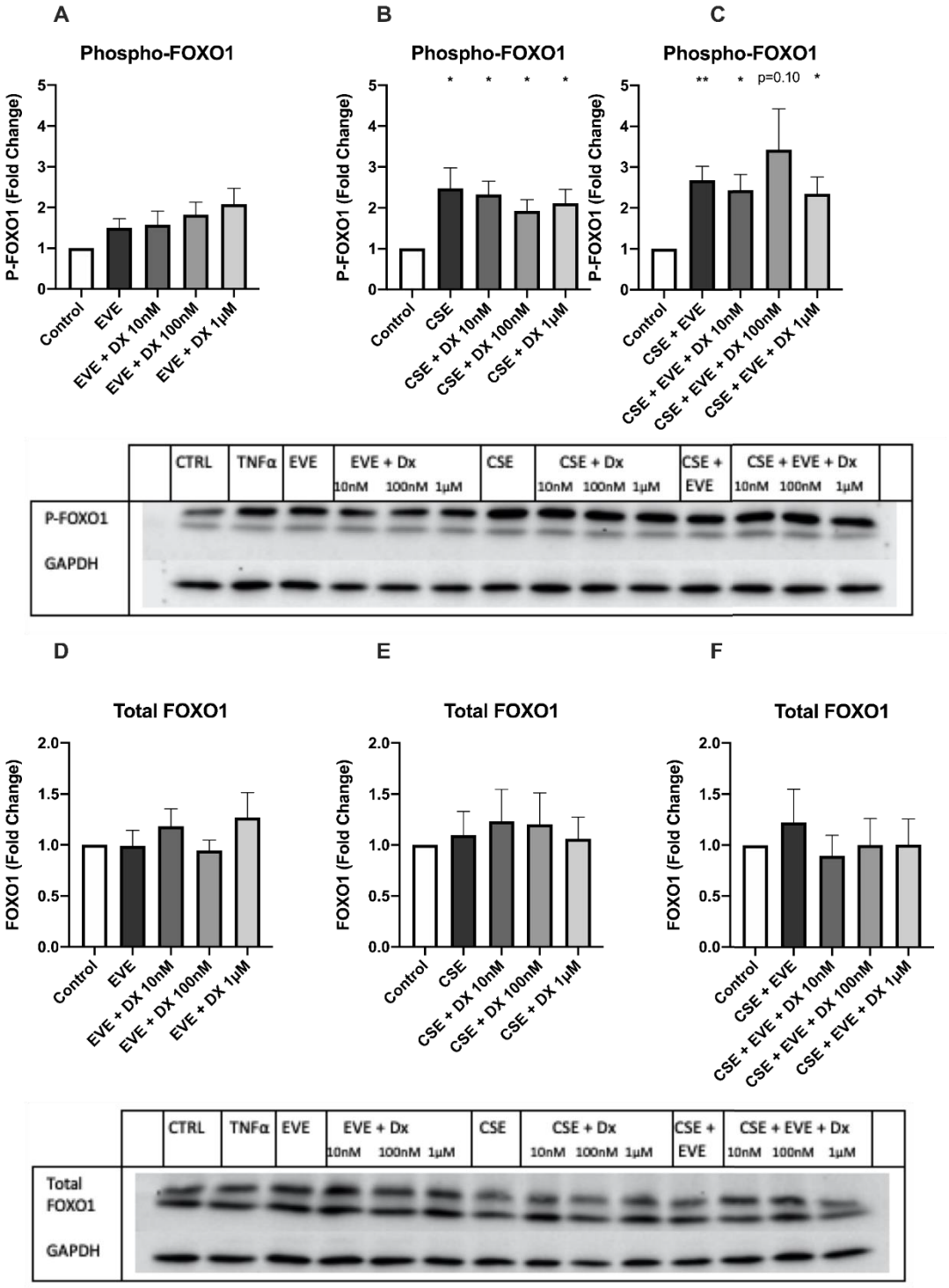


Figure 4: Activation of FOXO1 after exposure of primary human lung fibroblasts to cigarette smoke extract (CSE) and/or E-vapor extract (EVE). Primary human lung fibroblasts (n=8) were untreated (Control) or stimulated with EVE (A+D) or CSE (B+E) both alone and in combination (C+F) for 30 minutes. Pre-treatment with increasing concentration of dexamethasone (Dx, 10nM-1µM) 1 hour prior to stimulation was included to determine relative steroid response. TNF-α was

included as a positive control for stimulation. Whole cell lysates were collected, and western blots were used to quantify phosphorylated FOXO1 (P-FOXO1) and total FOXO1. Densitometry was performed and all values were normalized to GAPDH as a loading control. Images are representative of n=9 independent experiments are data is expressed as fold change of untreated, mean +/- standard

Dual stimulation with CSE and EVE results in greater CXCL8 gene expression and protein production than either stimulation alone.

It was shown previously that CSE exposure stimulates increased IL1α and CXCL8 gene expression in primary lung cells [136] but little is known about EVE exposure or exposure to CSE and EVE in combination. We found that EVE stimulation alone did not increase IL1α, CXCL8 (**Fig.1A-B**), CXCL1 or CXCL2 gene expression after 24 hours (**Supplementary Figure S1**). However, EVE alone did increase CXCL8 protein release after 24 hours (**Fig. 2A-C**). In contrast, CSE stimulation significantly increased IL1α and CXCL8, but not CXCL1 or CXCL2, gene expression at 24 hours (**Supplementary Figure S1**). CSE also significantly increased CXCL8 protein release after 24 hours compared to untreated cells (**Fig. 2A-C**). Combined CSE + EVE stimulation significantly increased CXCL8 and IL1α gene expression after 24 hours compared to untreated cells (**Fig. 1A-B**). In addition, combined CSE + EVE stimulation significantly increased CXCL8 protein production at 24 hours (**Fig. 2A-C**). Furthermore, dual stimulation with CSE + EVE resulted in a significant increase in CXCL8 gene expression and protein production compared to CSE or EVE stimulation alone (**Fig.1A-C)**.

Stimulation with CSE and EVE in combination causes steroid insensitive CXCL8 production

To determine the inflammatory response of primary lung fibroblasts ELISA was used to determine IL-6 and CXCL8 production. We found no changes in IL6 production after stimulation with CSE and EVE alone or in combination (**Supplementary Figure S2**). IL1α protein levels released by primary lung fibroblasts were under the level of detection (data not shown). As indicated above, CXCL8 production was increased by CSE, EVE and CSE + EVE stimulation after 24 hours (**Fig. 2A-C**). EVE-induced CXCL8 production was attenuated by pre-treatment with 1nM dexamethasone (**Fig. 2A**) but CSE stimulation required pre-treatment with 100nM dexamethasone to reduce CXCL8 to similar levels as seen in control unstimulated cells (**Fig. 2B**). CXCL8 production from CSE + EVE stimulation required 1μM dexamethasone pre-treatment to reduce levels to those similar to control unstimulated cells (**Fig. 2C**).

Phospho-p38 MAPK is increased by CSE and CSE + EVE stimulation

To determine signaling factors related to steroid insensitive inflammation, activation of p38 MAPK was analyzed by measuring phosphorylated p38. EVE stimulation alone for 30 minutes did not significantly increase phosphorylated p-38 MAPK (**Fig. 3A**). In contrast, phosphorylated p38 MAPK was increased after 30 minutes stimulation with CSE (**Fig. 3B**) and with CSE and EVE combined (**Fig. 3C**). Pre-treatment with dexamethasone for 1 hour did not reduce p38 MAPK phosphorylation under any conditions (**Fig. 3A-C**). Total p38 MAPK did not significantly increase with EVE alone (**Fig. 3D**), CSE alone (**Fig. 3E**) or with combined EVE and CSE (**Fig. 3F**). In addition, dexamethasone had no effect on total p38 MAPK expression (**Fig. 3D-F**).

Phospho-FOXO1 is increased following CSE and CSE + EVE stimulation

To determine signaling factors related to steroid insensitive inflammation, inactivation of FOXO1 was analyzed by measuring phosphorylated FOXO1. EVE stimulation for 30 minutes did not significantly increase phosphorylated FOXO1 (**Fig. 4A**). However, phosphorylated FOXO1 was increased after 30 minutes stimulation with CSE (**Fig. 4B**) and with CSE and EVE combined (**Fig. 4C**). Pre-treatment with 1μM dexamethasone for 1 hour did not reduce FOXO1 phosphorylation in either CSE- or combined CSE and EVE-stimulated cells (**Fig. 4A-C**). Total FOXO1 did not significantly increase with EVE alone (**Fig. 4D**), CSE alone (**Fig. 4E**) or with combined EVE and CSE (**Fig. 4F**). In addition, dexamethasone had no effect on total FOXO1 expression (**Fig. 4D-F**).

No effect of EVE or CSE on NF-κB or mTOR activation

We found no effect of EVE alone, CSE alone or the combination of the two stimuli on NF-κB (**Supplementary Figure S3**) or mTOR (**Supplementary Figure S4**) pathway activation measured at 30 minutes. In addition, dexamethasone did not affect the expression of phospho- or total NF-κB (**Supplementary Figure S3**) or of phospho- or total mTOR (**Supplementary Figure S4**) at this time point.

Discussion:

This is the first study to investigate whether dual stimulation with CSE and EVE has an effect on the inflammatory response of primary lung fibroblasts. It is also the first study to determine whether cells stimulated with CSE and EVE respond to corticosteroid attenuation of inflammatory mediators. We found that both IL1α and CXCL8 gene expression was increased with both CSE and combined CSE and EVE stimulation after 24 hours. This increase in inflammatory gene expression was attenuated by pre-treatment with dexamethasone at 1nM concentrationCXCL8 protein production after stimulation with a combination of CSE and EVE was significantly greater than either stimulus alone. Furthermore, CXCL8 production from CSE and EVE in combination was two-fold of the combined CXCL8 production from separate CSE and EVE stimulations indicating a synergistic interaction between the two stimuli. Pre-treatment with 1nM dexamethasone attenuated CXCL8 production from EVE stimulation and 10nM dexamethasone pre-treatment attenuated CXCL8 production from CSE stimulation. Interestingly, combined stimulation with CSE and EVE resulted in CXCL8 production that was not attenuated by dexamethasone pre-treatment at an order of magnitude higher (1μM) than the concentration required to reduce CSE induced CXCL8 production. Using western blots, we identified p38 MAPK and FOXO1 as potential mediators of steroid insensitive inflammation.

Potential mechanisms for glucocorticoid insensitivity have previously been identified in both structural and immune cells of the lung. There is sufficient evidence that p38 MAPK signaling is involved in glucocorticoid resistance, with inhibition of p38 MAPK signaling resulting in a reversal of steroid insensitivity in COPD [137-140]. Studies have demonstrated that CSE stimulation can induce dexamethasone insensitive CXCL8 production in alveolar macrophages from participants with COPD, but this has not been measured in structural cells of the lung [141, 142]. Lastly, the PI3K/AKT/mTOR pathway has been identified as an important therapeutic target in steroid insensitivity, with Phosphorylation of AKT

resulting in activation of mTOR and inactivation of FOXO1 resulting in downstream effects on steroid sensitivity [143].

The findings of the above studies suggested potential involvement of mTOR, FOXO1 and NF-κB transcription factors within the PI3k pathway, or p38 MAPK signaling in the dysregulation of glucocorticoid response in primary lung fibroblasts. We found that CSE and CSE combined with EVE stimulation resulted in significantly increased p38 MAPK phosphorylation after 30 minutes. This activation of p38 MAPK was not affected by pre-treatment with dexamethasone (0.01-1μM). A recent study in human lung fibroblasts has identified that combined pre-treatment with dexamethasone and p38 MAPK inhibitor BIRB significantly decreased CXCL8 production in primary lung fibroblasts [144], supporting p38 MAPK signaling as a potential mechanism for the increased CXCL8 production.

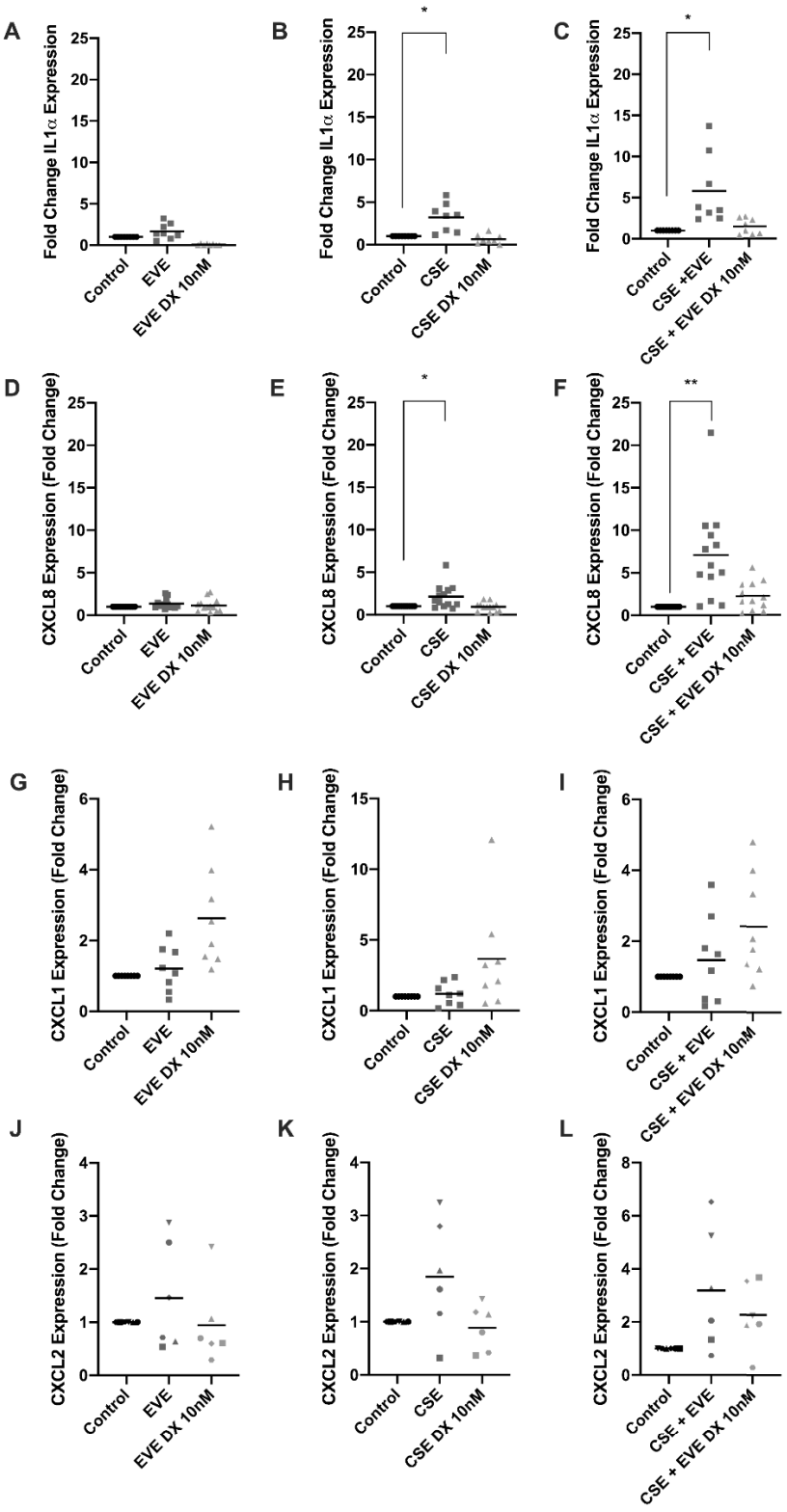
FOXO1 undergoes inhibitory phosphorylation by phospho-AKT and phospho-p38 MAPK [145], causing it to translocate from the nucleus resulting in reduced transcriptional activity. We found that CSE and CSE combined with EVE stimulation both resulted in an increase in FOXO1 phosphorylation. In the current study, pre-treatment with dexamethasone did not significantly reduce FOXO1 phosphorylation.

There were no changes found in either NF-κB or mTOR phosphorylation at the 30-minute time point studied (**Supplementary figures S3-S4**). This suggests that the p38 MAPK and FOXO1 signaling pathways are more likely to be mechanisms for the reduced efficacy of dexamethasone in this study. This mechanism is supported by the findings of other studies as mentioned above, but further research is needed to elucidate the exact mechanism of action. CSE and EVE are both complex stimuli to study with both extracts containing a range of cytotoxic, oxidative and proinflammatory molecules as shown in previous studies [14, 146]. The highly stimulatory nature of both CSE and EVE is likely to activate multiple pathways, but recent evidence in primary lung fibroblasts suggests p38 MAPK inhibition combined

with glucocorticoid treatment is likely to improve sensitivity and reduce inflammation [144]. We didn't perform a time course for signaling pathway activation and instead based our time points on previously optimized experiments within our lab [147], so there is potential that we missed differences that occurred earlier or later than 30 minutes. Future studies using knockdowns and pharmacological intervention could confirm FOXO1 and p38 MAPK involvement in steroid insensitivity from EVE and CSE stimulation.

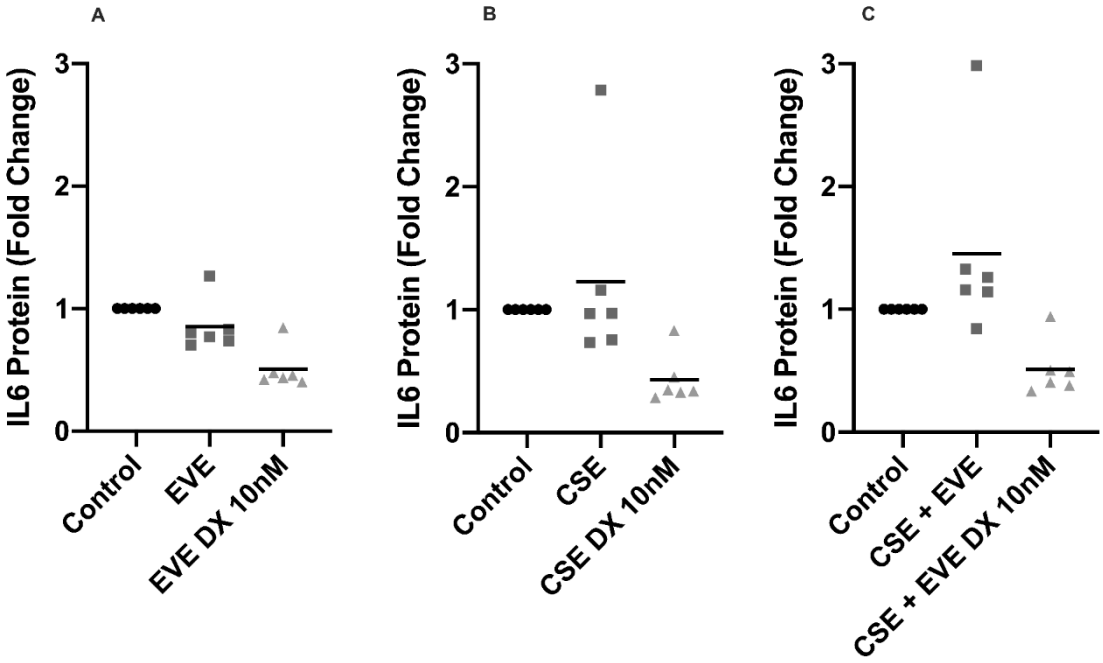
Considering COPD patients have been identified as a high risk group of E-cigarette users [97], the results of this study highlight further risks that may be associated with dual use of tobacco cigarettes and E-cigarettes in this group. Dual use of tobacco cigarettes and E-cigarettes is becoming increasingly common as a patient-preferred way to replace nicotine or to navigate strict tobacco restrictions [148, 149] A longitudinal study following smokers, e-cigarette users and dual users identified dual use as a greater risk of developing respiratory disease compared to using either product alone [150]. Other studies have also identified use of E-cigarette as a cessation device may result in long term use of E-cigarettes instead of cessation and correlation between relapsing to smoking and E-cigarette use [99, 151]. The effectiveness of E-cigarettes as a tool in smoking cessation is yet to be elucidated, but at this point all harms should be considered before use. In particular, the findings of this study identify that dual use should be avoided at all cost and never considered safe in COPD patients who are susceptible to exacerbations driven by neutrophilic inflammation and glucocorticoid insensitivity.

Data Supplement

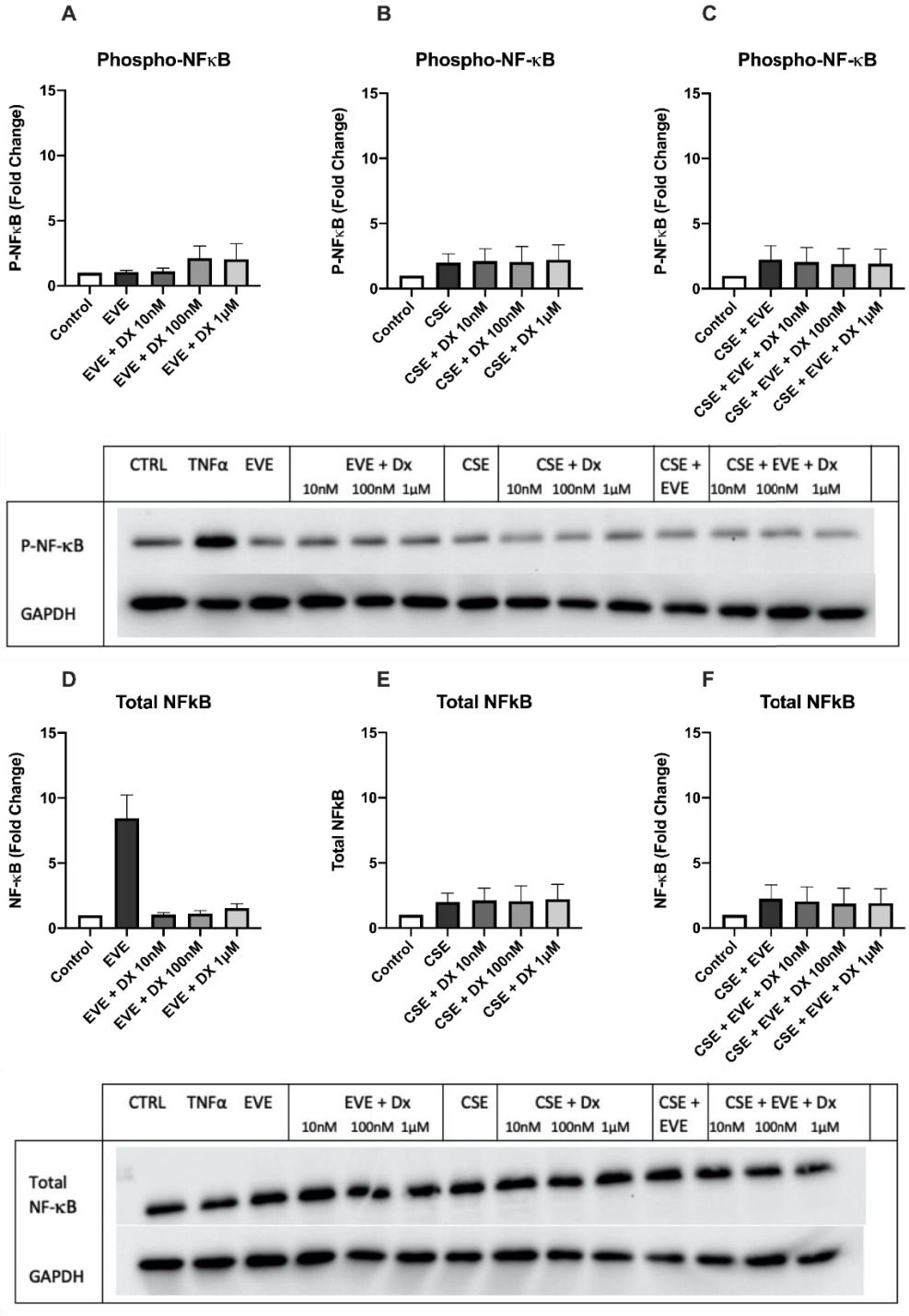


Supplementary Figure S1. IL1α, CXCL8, CXCL1 and CXCL2 gene expression from cigarette smoke extract (CSE) and E-vapor extract (EVE) exposed cells.

Primary human lung fibroblasts (n=8) were untreated (Control) or stimulated with EVE alone (A+D+G+J), CSE alone (B+E+H+K) or in combination (C+F+I+L) for 24 hours. Pre-treatment with DX (10nM) 1 hour prior to stimulation was included to determine the relative steroid response. Gene expression of IL1α (A-C), CXCL-8 (E-F), CXCL1 (G-I) and CXCL2 (J-L) was measured by RT-qPCR. Data is expressed as fold change of untreated, line at mean. All columns are compared to control using one-way ANOVA with Tukey's post-hoc test. Significance is represented as *p≤0.05, **p≤0.01.

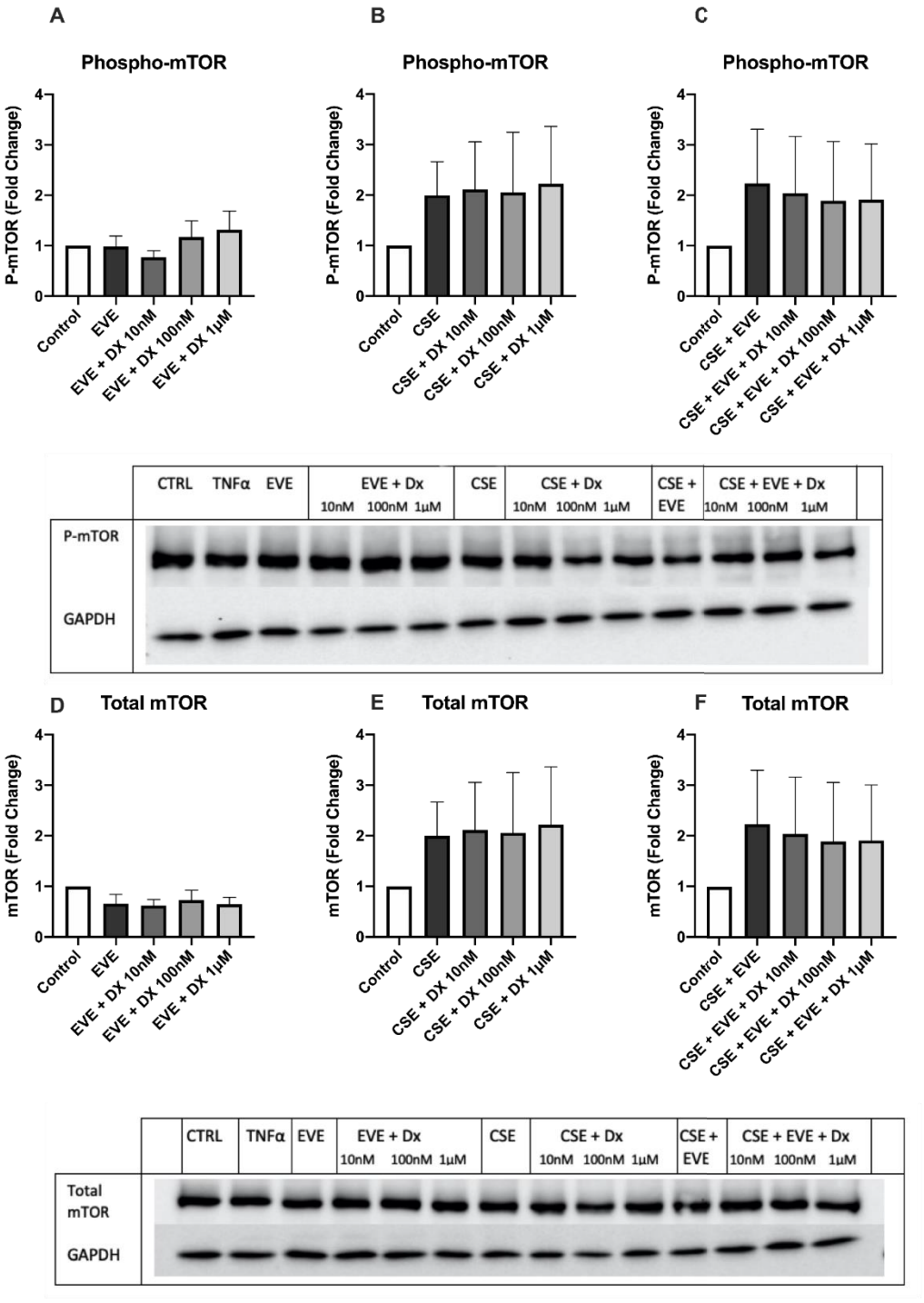


Supplementary Figure S2. Effect of dexamethasone (DX) on IL6 protein release from cigarette smoke extract (CSE) and E-vapor extract (EVE) exposed cells.
Primary human lung fibroblasts (n=18) were untreated (Control) or stimulated with EVE (A) or CSE (B) both alone and in combination (C). Pre-treatment with DX (10nM) for 1 hour prior to stimulation was included to determine the relative steroid response. Cell free supernatants were collected after 24 hours and ELISA was used to determine IL6 production. Data is expressed as fold change of untreated, line at mean. All columns are compared to control using one-way ANOVA with Tukey's post-hoc test.



Supplementary Figure S3: Activation of NF-κB after exposure of primary human lung fibroblasts to cigarette smoke extract (CSE) and/or E-vapor extract (EVE). Primary human lung fibroblasts (n=8) were untreated (Control) or stimulated with EVE (A+D) or CSE (B+E) both alone and in combination (C+F) for 30 minutes. Pre-

treatment with increasing concentration of dexamethasone (Dx, 1nM-100nM) 1 hour prior to stimulation was included to determine relative steroid response. TNF-α was included as a positive control for stimulation. Whole cell lysates were collected, and western blots were used to quantify phosphorylated NF-κB (P-NF-κB) and total NF-κB. Densitometry was performed and all values were normalized to GAPDH as a loading control. Images are representative of n=9 independent experiments are data is expressed as fold change of untreated, mean +/- standard error of the mean. Data was analyzed using one-way ANOVA with Tuckey's post-hoc test. No significant differences were found.



Supplementary Figure S4. Activation of mTOR after exposure of primary human lung fibroblasts to cigarette smoke extract (CSE) and/or E-vapor extract (EVE). Primary human lung fibroblasts (n=8) were untreated (Control) or stimulated with EVE (A+D) or CSE (B+E) both alone and in combination (C+F) for 30 minutes. Pre-treatment with increasing concentration of dexamethasone (Dx, 1nM-100nM) 1

hour prior to stimulation was included to determine relative steroid response. TNF-α was included as a positive control for stimulation. Whole cell lysates were collected, and western blots were used to quantify phosphorylated mTOR (P-mTOR) and total mTOR. Densitometry was performed and all values were normalized to GAPDH as a loading control. Images are representative of n=9 independent experiments are data is expressed as fold change of untreated, mean +/- standard error of the mean. Data was analyzed using one-way ANOVA with Tuckey's post-hoc test. No significant differences were found.

Chapter 7

7.1 Discussion, Conclusions and Future Directions

The rapid evolution and uptake of electronic cigarettes combined with a slow response from regulatory bodies has resulted in a widely used product with uncertain harms or benefits. The lack of regulation has made E-cigarettes easily available to at risk populations who may not understand the risks associated with their use, influencing the E-cigarette epidemic in American young adults [152]. It has been many years since a public health topic has been as divisive between practitioners, researchers and the public as E-cigarettes have been.

The debate around the safety of E-cigarettes is in constant flux trying to find a balance between harm minimisation for smokers and reducing uptake or recreational use in non-smokers. The NASEM publication covered the public health consequences of E-cigarettes from a balanced and impartial standpoint [146]. We provided an update on the evidence presented in the NASEM review in the form of a systematic review included in the introduction of this thesis [129].

The main focus points in the NASEM review and our evidence update published in Chest were:

- E-cigarette, E-liquids and aerosol constituents and the potential health effects associated with their use
- Human health effects
- Initiation and cessation of tobacco smoking attributable to E-cigarette use
- Harm reduction associated with E-cigarette use
- Risks or benefits to specific populations (e.g. people with COPD or asthma, pregnant women, adolescents)

Both documents identified substantial gaps in current evidence that need to be filled before we can understand the right approach to regulating E-cigarettes to best serve the public and any potential users of the ever-changing alternative tobacco product. This thesis contains research aimed to address the gaps outlined by NASEM and our review.

At the time of its inception, our study in Chapter 3 of this thesis was the first to address young Australians attitudes, opinions and patterns of use of E-cigarettes. With the epidemic of E-cigarette use in American youth being reported in detail, we were left with a dearth of literature on how young Australians were using E-cigarettes. With the progressive nature of E-cigarette research, we were constantly adapting our research to best address gaps in the literature. A follow up study to Chapter 3 addressing how smokers with and without COPD perceive and use electronic cigarettes is still required but time constraints did not allow for this to be completed within the time frame of this project.

The overarching aim for this thesis was to evaluate the inflammatory, immunomodulatory and pathological effects that arise from E-cigarette use. We addressed this aim using primary human lung cell exposure models, with a particular focus on COPD. COPD patients have been identified as a high-risk population of E-cigarette users due to the pre-existing lung-damage, the hyper-responsiveness to noxious stimuli, and the progressive nature of the disease. The studies in Chapter 4, Chapter 5 and Chapter 6 identified the potential for E-cigarettes to differentially and disproportionately effect COPD patients. Experiments in this thesis resulted in novel evidence of a differential inflammatory response of lung mesenchymal cells from COPD patients compared to non-COPD patients. Furthermore, we identified E-cigarettes as a potential inducer of senescence; and the increased risk of dual tobacco and e-cigarette use compared to either product alone. The final experiments also identified potential implications for COPD patient's prognosis and exacerbation risk.

7.2 Perceptions of harm and usage patterns of E-cigarettes in Australia

Our pilot study into the opinions of young Australians towards E-cigarettes provided evidence that a lack of information and regulation may contribute to misinformation around the safety and legality of E-cigarettes. Since the initial design and delivery of our survey, multiple other studies have assessed how Australians perceive and use E-cigarettes [117, 153-156]. The inclusion of questions related to

E-cigarette use in the National Drug Strategy Household Survey (NDSHS) from 2016 to 2019 is a starting point for understanding how Australians are using E-cigarettes [157, 158]. Considering E-cigarettes have been used by Australians for up to 10 years prior to the first inclusion of these survey questions we have plenty of ground to make-up in understanding how and why they are used in Australia [159]. Longitudinal data from 22,000+ respondents gives insight into how E-cigarettes are used across all demographics and should be used to influence policy decisions around E-cigarettes.

Our survey in Chapter 3 indicated some concerning findings about safety and legislative perceptions that young Australians had of E-cigarettes. The majority of respondents (64%) were misinformed about legislation restricting the purchase of nicotine containing E-cigarette products in Australia. Considering how accessible E-cigarettes are through online stores and the delay on a government crackdown on their importation, Australians are potentially illegally importing nicotine containing E-cigarettes unknowingly [160]. This finding alone exemplifies the need for better education on E-cigarette products and tighter restrictions to stop uptake in non-smokers. A multitude of longitudinal studies found that E-cigarette use is correlated with a greater likelihood of future tobacco smoking [161, 162], highlighting the need to restrict access to E-cigarette products for non-smokers.

Nicotine variability and harmful components in E-liquids are two safety considerations that can be controlled through stricter safety testing and quality control standards. Nicotine variability has been identified in multiple studies, with the labelled nicotine content being drastically different to the measured nicotine content [146, 163-167]. Some E-liquids labelled nicotine free have also been found to contain nicotine [168, 169], with NSW health Identifying many E-liquids sold in Australia to contain nicotine when they should not [170]. Regular testing of E-liquids purchased from tobacco and E-cigarette retailers in Australia is useful in understanding how closely E-cigarette manufacturers and retailers are following legislation around nicotine, but testing should come prior to sale of the product.

The public would be better protected by testing of E-liquids for nicotine prior to the registration and sale of E-cigarette products. We can then build a database of manufacturers that are transparent in their pursuit of providing a safer alternative to help smokers quit tobacco and refuse to register and sell products that are not attempting to offer safer alternatives.

The current legislation of nicotine containing E-cigarettes requiring a doctor's prescription should be extended to all E-cigarette products [171]. This would drastically reduce the uptake of E-cigarette use in non-smokers, and as a result reduce subsequent transition to tobacco smoking in this population. E-cigarette flavours have been linked to increased appeal in younger populations [116, 172, 173] and some flavours have also been shown to illicit greater harmful effects [174-176]. The findings of these studies suggest that E-liquid flavourings should be heavily restricted, leaving only tobacco flavoured and non-flavoured E-liquids to reduce the appeal for any use other than smoking cessation. Our experiments in Chapter 4 [132] revealed that increasing the power settings used to vaporise E-liquids further increased the cytotoxicity of the EVE. This finding is supported by other studies that found more cytotoxic compounds in E-vapour extracts generated at higher settings [177].

Registering E-cigarettes as a therapeutic device remains a controversial topic considering the identified harms associated with their use [129, 146, 171]. Greater effort is needed to inform practitioners, E-cigarette users and young adults of the risks associated with E-cigarette use. If a smoker requests a prescription to use an E-cigarette, practitioners should also come up with a cessation plan for E-cigarette use.

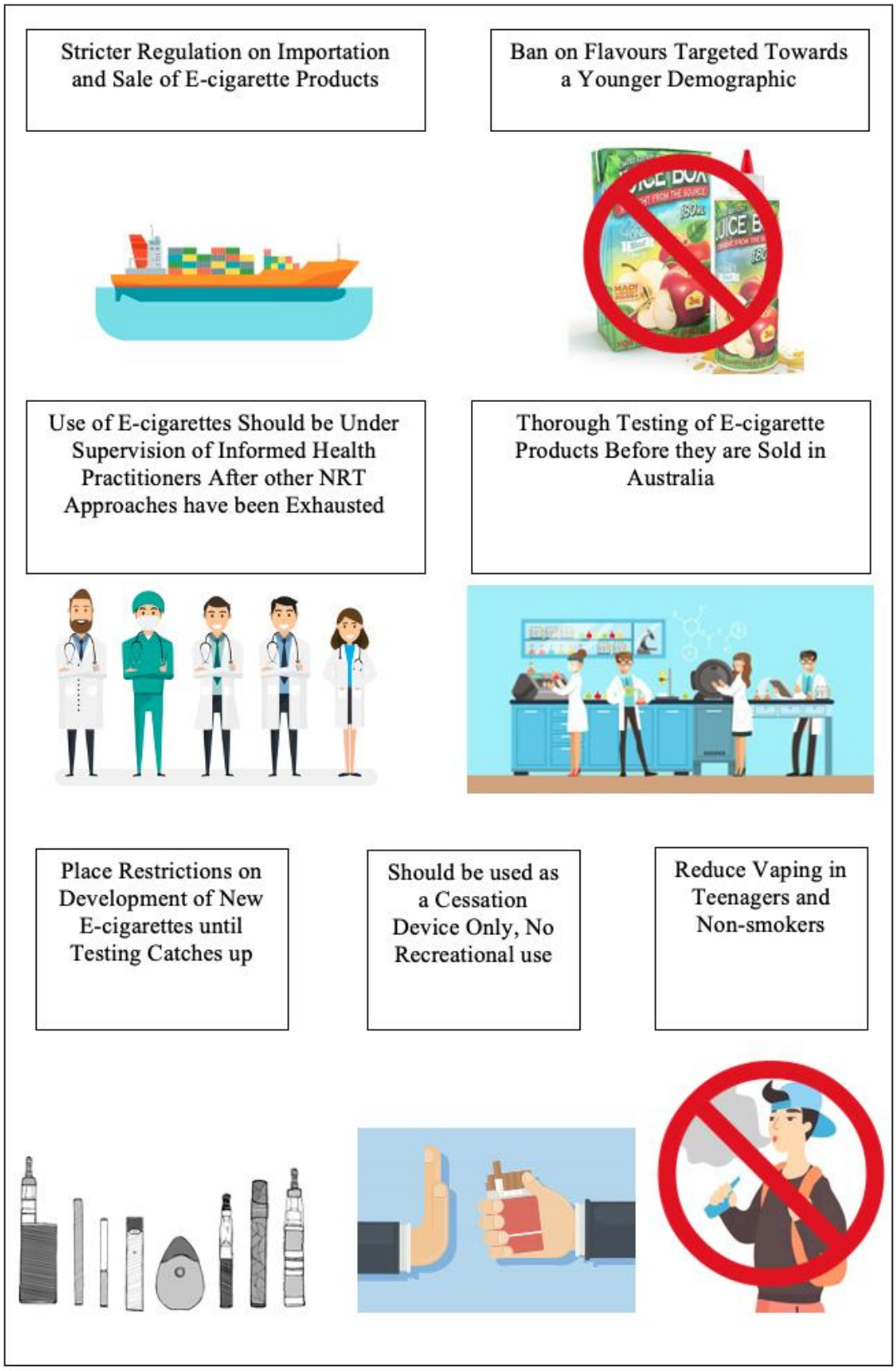


Figure 7.1 – Suggestion of new approaches to regulation of E-cigarettes if they are still going to be sold within Australia

7.3 Harms Associated with E-cigarette use in COPD Patients

In Chapters 4, 5 and 6 we designed our studies to identify pathophysiological processes that may be up regulated through E-cigarette use. At the time of publication, Chapter 4 was the first study to identify a differential response of COPD cells compared to non-COPD cells. Hypersecretion of pro-inflammatory mediators from primary COPD ASMCs and fibroblasts from after CS exposure has been measured previously in our lab, suggesting that COPD cells are primed to respond greater irrespective of the stimulus in this case[100, 178]. An increased secretion of IL-8 in COPD cells after EVE exposure is concerning considering the central it plays as a neutrophil chemokine orchestrating the chronic inflammatory state experienced by COPD patients[28, 179]. Furthermore, in Chapter 6 we found that combined stimulation with CSE and EVE had a synergistic effect on IL-8 production, with a significantly greater production than with either treatment alone. IL-8 is a major attractant of neutrophils which are recruited to the site of IL-8 secretion where they secrete inflammatory mediators and proteases that contribute to tissue damage and a resulting progression of airflow limitation [43, 180]. Number of neutrophils and IL-8 concentration in the lung are both correlated with disease severity [181-183]. Neutrophils have also been linked to mucus hypersecretion in COPD patients, which can contribute to exacerbation of disease [184, 185]. More recently, Neutrophil to lymphocyte ratio (NLR) has been identified as a useful biomarker of inflammation in COPD. An increased NLR is associated with reduced lung function and an increased risk of COPD [186]. Any stimulus that increases IL-8 should thus be avoided by COPD patients to reduce risk of accelerating disease progression. The underlying mechanisms of inflammation from E-cigarette use are yet to be elucidated, but it is hypothesized that it is related to oxidative stress through exposure to oxidants and other chemicals in the heated and aerosolized E-liquid inhaled by users [187, 188]. The findings of EVE effects on Inflammation and cytotoxicity are summarised in figure 7.2.

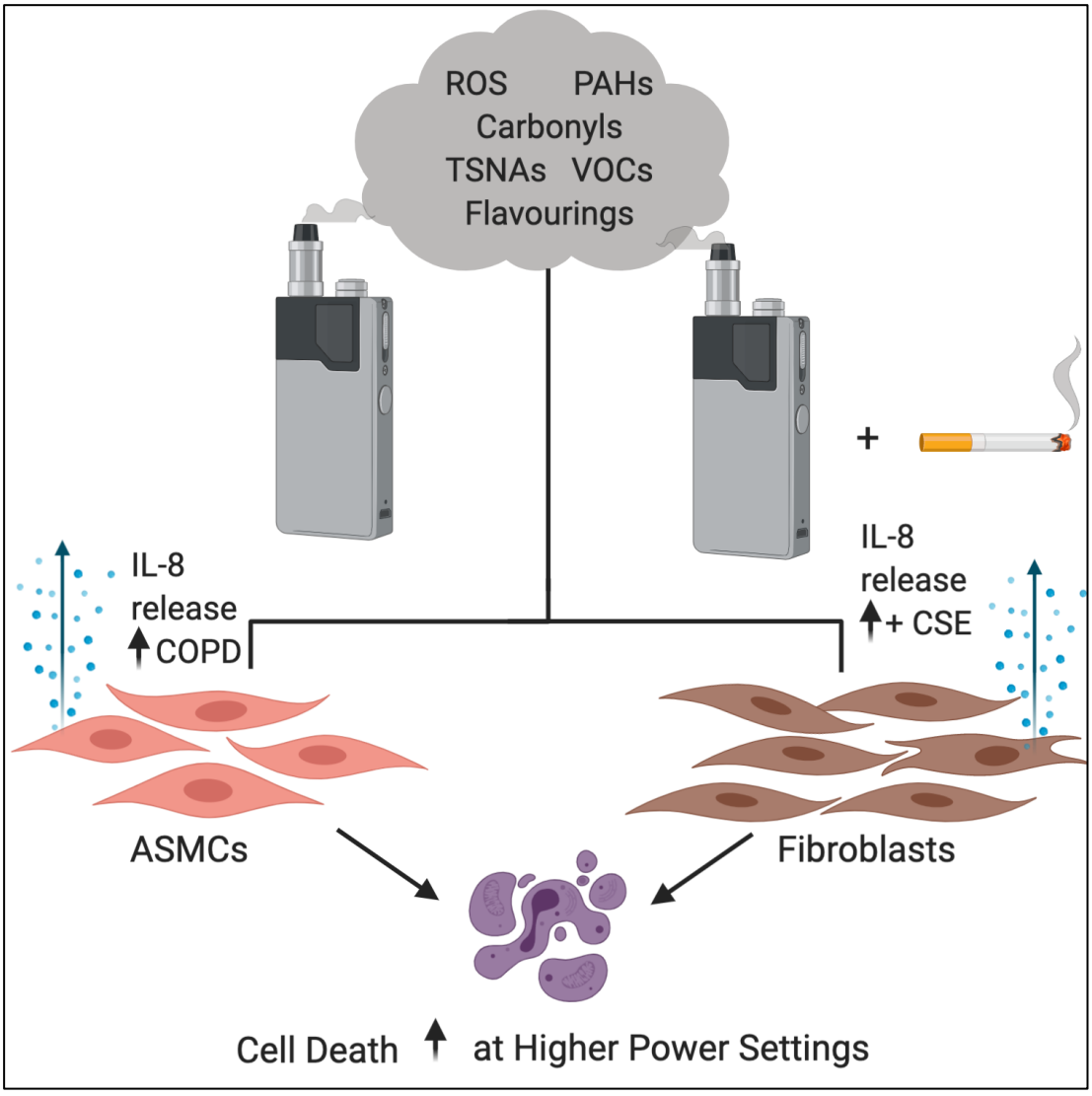


Figure 7.2 – EVE stimulates IL-8 release from primary ASMCs and Fibroblasts.
COPD ASMCs hyper-secrete IL-8 after EVE stimulation compared to non-COPD cells. IL-8 production is significantly greater with CSE + EVE combined stimulation, suggesting synergy between the stimuli. Cytotoxic effect of EVE is increased when generated at higher power settings, supported by previous studies that show higher concentration of toxic compounds at higher power settings [189, 190]
Abbreviations: PAHs-Polycyclic aromatic hydrocarbons; ROS-Reactive oxygen species; TSNAs-Tobacco specific nitrosamines; VOCs-Volatile organic compounds.

In Chapter 5 we designed experiments to test our hypothesis that E-cigarette vapour stimulation would induce a cellular senescent phenotype. There is growing recognition that accelerated aging and cellular senescence may play a role in the pathophysiology of COPD [191, 192]. As mentioned in Chapter 5, cellular senescence is a state of cell cycle arrest in which senescent cells are protected from cell death resulting in an accumulation in affected tissues [193]. Senescent cells have a dysfunctional metabolism and increased secretion of mediators that have both an autocrine and paracrine effect on themselves and surrounding cells, this is referred to as the SASP [194, 195]. We tested whether EVE induced cellular senescence through SA β-gal staining and by measuring gene expression of cell cycle arrest markers p16 and p21, all of which are known to be increased by CSE stimulation in structural lung cells [196, 197].

EVE with and without nicotine was found to increase p21 gene expression in primary lung fibroblasts after 24 hours in a dose dependant manner. No changes in p16 gene expression were measured after CSE or EVE stimulation, suggesting cell cycle arrest was a p21 dependant mechanism. Senescence induction was confirmed at 4 days with increased SA β-gal staining after EVE stimulation irrespective of nicotine content. We also identified a decreased wound healing capability in fibroblasts 4 days after stimulation with EVE which could be a functional consequence of senescence induction. The findings in this Chapter are particularly concerning for COPD patients considering the role cellular senescence plays in COPD pathophysiology. With E-cigarettes often proposed as a harm reduction tool for smokers, but smokers susceptible to COPD are at risk of progression of lung function decline [198]. Considering the poor tools available to detect COPD susceptibility smokers should instead utilise other evidence based NRT options and the support of clinicians in smoking cessation [96, 199]. The major findings of Chapter 5 are summarised in figure 7.3.

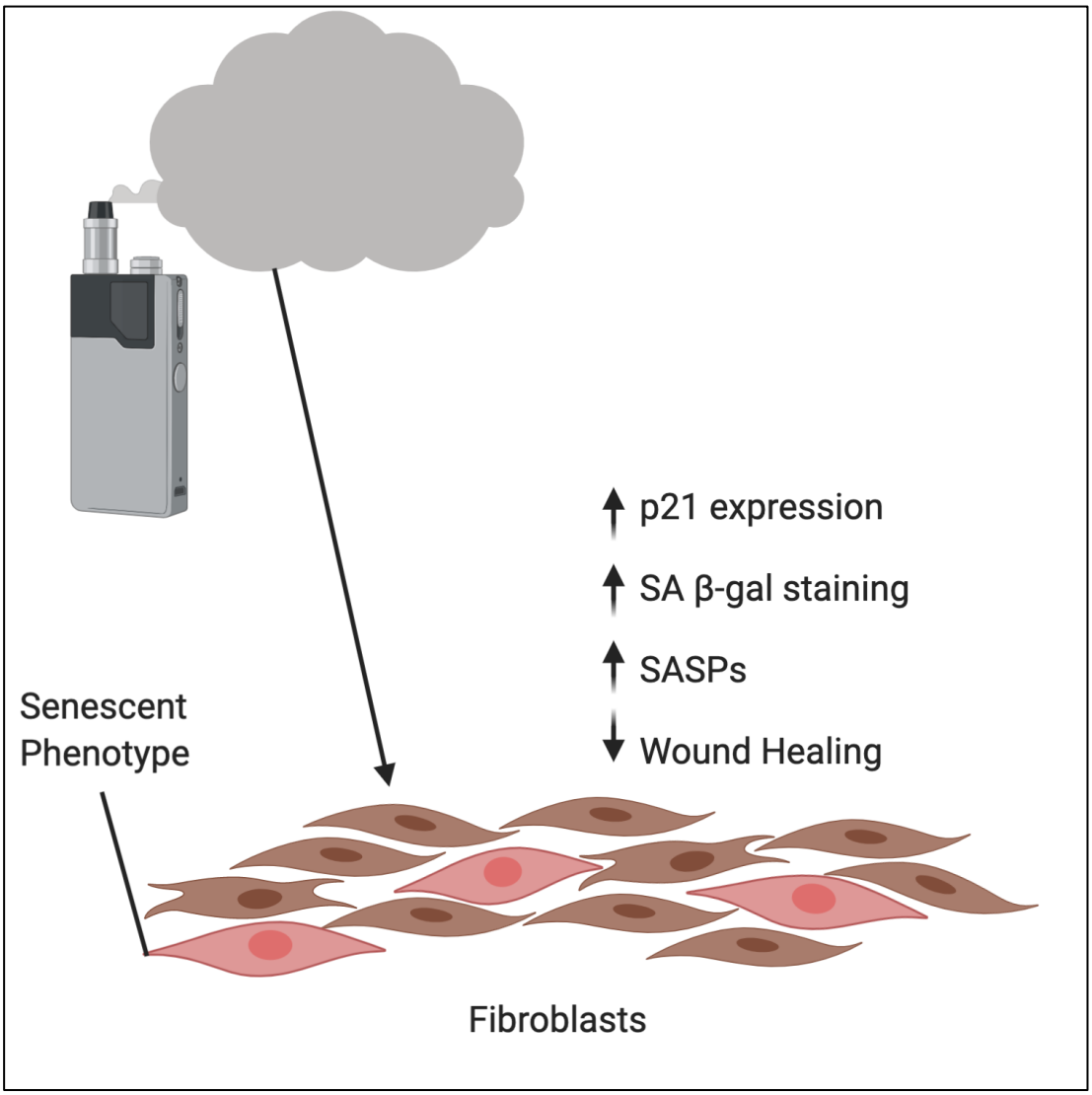


Figure 7.3 – E-vapour stimulates induction of cellular senescence

Cellular senescence was induced in lung fibroblasts after EVE exposure. After induction of senescence fibroblasts had an increased production of senescence associated secretory phenotype proteins (SASPs) and a decreased wound healing capacity.

In Chapter 6 we measured the effects of dual CSE and EVE exposure on primary human lung fibroblasts. Alongside this we pre-treated fibroblasts with dexamethasone to determine whether cells were responsive to corticosteroids after stimulation. Corticosteroid resistance is common in COPD and is a major barrier to effective treatment for patients as we have limited pharmacological tools to combat disease progression [91, 200]. Corticosteroids are still given as standard treatment in COPD and although ineffective in reducing chronic inflammation in disease they reduce exacerbation frequency and improve health status of COPD patients [44, 96, 201].

We found an increase in IL-1α and IL-8 gene expression after stimulation with CSE and a combination of CSE and EVE. Interestingly there was no change in IL-8 gene expression from EVE stimulation, but a change in IL-8 protein production suggests that the change in gene expression may occur before or after our selected time point in these experiments. We selected a panel of neutrophilic inflammatory mediators for their importance in COPD pathophysiology, but found no changes in CXCL1, CXCL2 and IL-6 gene expression. The steroid insensitive production of IL-8 protein after combined stimulation with CSE and EVE (Chapter 6) is likely to involve p38 MAPK signalling, which was still significantly activated with the highest dose of dexamethasone. FOXO1 was also found to be significantly phosphorylated after pre-treatment with the highest dose of dexamethasone. FOXO1 and p38 MAPK signalling have both been identified as potential contributors to steroid insensitivity in COPD but further work is needed to elucidate the exact mechanism behind the insensitivity seen in our experiments [144, 192, 202-205]. The major findings from Chapter 6 are summarised in figure 7.4.

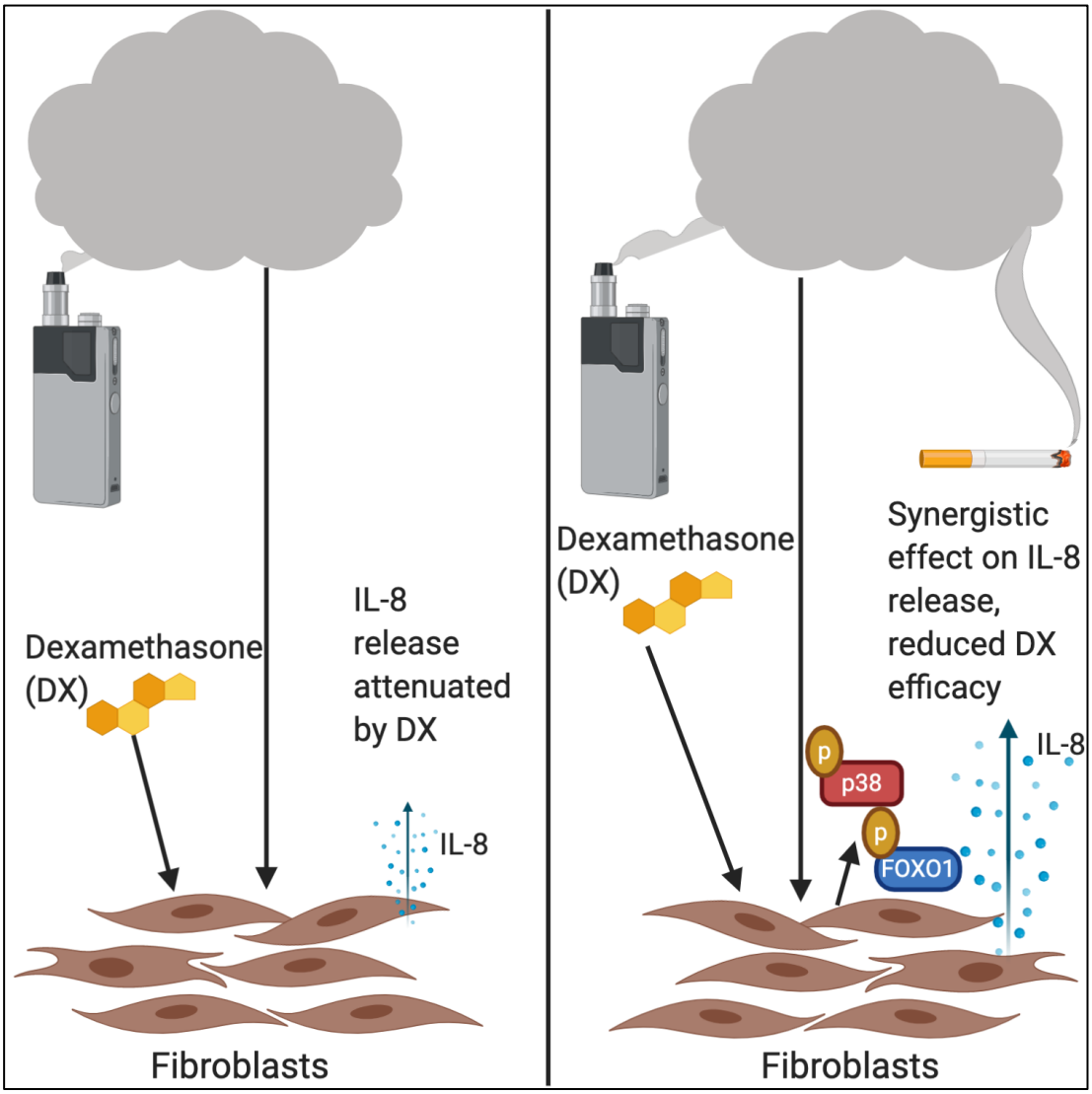


Figure 7.4 – Combined EVE and CSE stimulation results in steroid insensitive inflammation

Combined stimulation of primary lung fibroblasts with CSE and EVE had a synergistic effect on IL-8 production, with significantly greater IL-8 produced than either stimulation alone. Dexamethasone pre-treatment at the highest concentration of 1μM did not attenuate the significant increase in IL-8 production. Phosphorylated p38 MAPK and phosphorylated FOXO1 were found to be increased after 30 minutes stimulation with CSE and EVE. Pre-treatment with 1μM dexamethasone did not reduce phospho-p38 MAPK or phospho-FOXO1 protein levels.

7.4 Limitations and Future Directions of this Research

Although the studies included in this thesis primarily used primary human lung cells, future studies to confirm these observations in vivo are required to fully understand the clinical implications for E-cigarette users. One of the most important limitations in E-cigarette research is the lack of a standardised exposure model. In earlier research studies opted to use un-vaporised E-liquid [143, 206], which is not representative of exposures that E-cigarette users experience making these findings incomparable to our own. We optimised our EVE to be the equivalent to the nicotine in 2 cigarettes based on the weight/volume labelled nicotine concentration of our liquids. All stimulatory concentrations for experiments were based on dose-response curves using cytotoxicity or inflammatory response as a measure of the activity of the extract. In my systematic review of E-cigarette literature, we summarised each studies method for generating E-vapour extracts. Often their method for generating E-vapour extracts was missing or drastically different to our own, all information is included in the online supplement of my publication [129].

We standardised EVE generation at the start of our *in vitro* experimental work to keep consistency across this thesis but we understand that other studies may find different results to our own based on experimental differences. Future studies should adopt a standardised stimulation for E-cigarette research that accounts for device and E-liquid variability. Ideally the standardised stimulation for E-cigarette research would be modelled off the standard CSE exposure model [207]. The tobacco industry has regularly been involved in their own research to disprove the harms of tobacco, including the development of their own exposure systems [208, 209]. This clear conflict of interest must be avoided and a standard exposure model for E-cigarette research must be designed and agreed upon by researchers with no conflict of interest.

In Chapter 4 we measured disease specific differences in response to EVE as a stimulus. It is possible that data may be skewed by sampling differences in dissection and the cell culture process, but we avoid this by following our optimised protocol for lung microdissection as described in Chapter 2. We isolated fibroblasts from a distal portion of parenchymal tissue and ASMCs were isolated by longitudinally dissecting airways under a dissection microscope. Similar studies have stained for markers such as smooth muscle α-actin and calponin alongside microscopic confirmation of morphology to validate this method of microdissection of airways. Fibroblast and ASM cultures have been shown to be approximately 98% pure using similar protocols to our own [210, 211].

In Chapter 6 we measured the proinflammatory effects of E-cigarettes and cigarette smoke in combination. Within this study I found that combined treatment with CSE and EVE resulted in steroid insensitive inflammation. We identified p38 MAPK signalling and FOXO1 as potential mediators of this phenomenon, but future studies using inhibitors are required to confirm this finding. CSE and EVE have both been shown to contain a multitude of stimulatory compounds, so it is very likely multiple pathways are involved in this process and the use of inhibitors may not direct us to a specific pathway to explain our findings. Our findings are well supported by recent studies published indicating that p38 MAPK inhibitors restored efficacy of dexamethasone to inhibit IL-8 production [144].

To further elucidate the risks of E-cigarette use longitudinal studies following cohorts of non-smokers, smokers who aren't susceptible to COPD, and COPD-susceptible smokers. This will allow us to answer the common questions that perpetuate the harm reduction debate by determining:

- Is smoking more harmful than E-cigarette use?
- Does E-cigarette use effect disease progression and lung function decline in COPD?
- Do E-cigarettes cause harm in smokers who aren't susceptible to COPD?

- Do E-cigarette users who have never smoked experience different pathology to smokers?

Clinical measurements of disease severity such as spirometry and exacerbation frequency would allow us to determine whether E-cigarettes are providing any benefit from a harm reduction standpoint. It must be remembered that smokers and COPD patients are the only groups with some potential for harm reduction, any others who use E-cigarettes are exposing themselves to a harm they otherwise would not be exposed to. The harm reduction debate is ongoing, and without longitudinal studies to differentiate between harms experienced by smokers and E-cigarette users it will continue to no end.

7.5 Final Conclusion

In conclusion, the studies within this thesis provide new evidence on the potential harms related to E-cigarette use. At the time of its conception, the study in Chapter 3 provided the first evidence of a lack of education on E-cigarettes in young Australians, which could have grave implications for the future of tobacco control and health in Australia if left unaddressed. In Chapter 4 we provided the first evidence that COPD cells produce a differential response to E-cigarette vapour compared to non-COPD cells. Within this study we also confirmed earlier suspicions that E-cigarettes used at higher power settings would be more harmful through cytotoxicity measurements. In Chapter 5 we identified E-cigarette vapour as a potential inducer of senescence, suggesting it could further contribute to pathophysiology if COPD patients use E-cigarettes as a replacement or cessation device. In Chapter 6 we found that combined use of E-cigarettes and cigarette smoking was significantly more stimulatory than either stimulus alone. This finding is particularly concerning given the increase in the number of dual users globally. Combination stimulation also resulted in dexamethasone insensitive stimulation which could be particularly important for COPD patients that opt for dual use to reduce the number of cigarettes they smoke, potentially progressing disease and increasing their risk of exacerbations. The combination of the findings in this thesis

provide an important base for future studies to expand upon, and the findings alone should be used to develop public health messaging around the use of E-cigarettes.

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