Pulmonary and systemic effects of electronic cigarette use

Thèse

Ariane Lechasseur

Doctorat en sciences cliniques et biomédicales
Philosophiæ doctor (Ph. D.)

Québec, Canada

RESUME

Le marché de la cigarette électronique est en constante expansion. La majorité des utilisateurs de cigarette électronique sont également des fumeurs de cigarette de tabac. Un nombre croissant de jeunes commencent à utiliser la cigarette électronique, sans avoir fumé la cigarette de tabac au préalable. Outre le propylène glycol et le glycérol, le liquide de la cigarette électronique peut contenir différentes concentrations de nicotine et se décliner dans près de 15 000 mélanges de saveurs. Le glycérol est un substrat métabolique impliqué dans la production de glucose en période de jeûne, et de lipides en période d'excès de glucides. Les impacts de la cigarette électronique sur la santé restent à déterminer. **L'hypothèse générale de cette thèse est que l'utilisation de la cigarette électronique perturbe la biologie pulmonaire et métabolique.**

Le premier objectif de cette thèse était d'évaluer les effets de la variation des paramètres physiques de la cigarette électronique ainsi que la composition du liquide de vapotage sur la taille des particules d'aérosols générée. L'extension e-cigarette InExpose (SCIREQ) a été utilisée. Différentes concentrations de nicotine, saveurs et proportions de propylène glycol et glycérol ont été utilisées. La taille des particules des vapeurs de cigarette électronique a été analysée par un Scanning Mobility Particle Sizer spectrometer (SMPS 3080, TSI Inc). Nous avons montré qu'une puissance de la cigarette électronique plus élevée augmente la taille des particules émises. Nous avons également montré qu'une plus grande proportion de glycérol, la présence de nicotine et de vanilline augmente la taille des particules. Ces changements modifient la déposition pulmonaire prédite des particules de cigarette électronique.

Le deuxième objectif de cette thèse était d'investiguer les effets pulmonaires de la double exposition aux vapeurs de cigarette électronique et à la fumée de cigarette. L'exposition de souris BALB/c femelles à la fumée de cigarette 3R4F a été effectuée dans un système automatisé de type « whole-body » (SIU24, Promech Lab AB). L'exposition aux vapeurs de cigarette électronique a été réalisée dans un système « whole-body » développé dans notre laboratoire, et en utilisant un liquide de vapotage sans saveurs et sans nicotine. Pour les deux types d'expositions, les souris ont été exposées successivement 2h/jour, 5

jours/semaine pendant 8 semaines. Nous avons montré que les souris exposées aux vapeurs de cigarette électronique et à la fumée de cigarette présentent des changements dans l'expression de gènes impliqués dans la régulation du cycle circadien. Nous avons montré une augmentation de la fréquence des cellules dendritiques, macrophages, neutrophiles, lymphocytes B ainsi que lymphocytes T CD4+ et CD8+ au poumon comparativement aux souris exposées seulement à la fumée de cigarette. L'exposition aux vapeurs de cigarette électronique a également modulé les niveaux d'immunoglobulines dans le lavage bronchoalvéolaire et le sérum. Une augmentation de la résistance des voies aériennes a été observée pour les souris exposées aux vapeurs de cigarette électronique, avec ou sans exposition concomitante à la fumée de cigarette.

Le troisième objectif de cette thèse était de caractériser les effets de l'inhalation de vapeurs de glycérol sur le métabolisme énergétique hépatique. Les souris ont été exposées aux vapeurs de glycérol en utilisant notre système d'exposition de type « whole-body ». Des souris C57BL/6 mâles et femelles ont été exposées de manière aigüe pour une exposition de 6h. Bien que des changements mineurs ont été observés suivant l'exposition aigüe, l'exposition aux vapeurs de glycérol semble prévenir les effets métaboliques du jeûne. Par la suite, des souris C57BL/6 mâle et femelle, âgées de 6 ou 12 semaines, ont été exposées 2h/jour, 5 jours/semaine pour 9 semaines. Aucun changement dans le poids ou la composition en tissu adipeux n'a été observé. Nous avons montré une diminution de la tolérance au glucose chez jeunes souris mâle et femelle. Nous avons également observé une augmentation de la concentration hépatique de triglycérides et de phosphatidylcholine chez les souris femelles, sans augmentation chez les souris mâles. Aucun changement dans les marqueurs d'inflammation, de remodelage ou de stress du réticulum endoplasmique n'a été observé dans les tissus hépatiques.

Les travaux présentés dans cette thèse mettent en lumière les effets de la cigarette électronique sur la santé pulmonaire et métabolique. Davantage d'études sur les effets des composantes de la cigarette électronique sont nécessaires afin de caractériser les mécanismes responsables de ces changements.

ABSTRACT

The electronic cigarette market is in constant expansion. A majority of electronic cigarette users are also tobacco cigarette smokers though an increasing number of young people are starting to use electronic cigarettes without having to smoke tobacco cigarettes first. In addition to propylene glycol and glycerol, vaping liquids in electronic cigarettes contain different concentrations of nicotine and nearly 15,000 flavours are available. Glycerol is a metabolic substrate involved in the production of glucose during fasting and lipids after feeding. The impacts of electronic cigarettes on health remain to be determined. **The general hypothesis of this thesis is that the use of electronic cigarettes disrupts lung and metabolic processes.**

The first objective of this thesis was to evaluate the effects of the variation in the electronic cigarette model as well as the composition of the vaping liquid on the size of the emitted particles generated. Using the InExpose e-cigarette extension (SCIREQ), different concentrations of nicotine, flavours and proportions of propylene glycol and glycerol were assessed. The particle size of electronic cigarette aerosols was analyzed by a Scanning Mobility Particle Sizer spectrometer (SMPS 3080, TSI Inc). We have shown that increasing electronic cigarette power increases the size of the particles emitted. We have also shown that a greater proportion of glycerol or the presence of nicotine and vanillin led to increased particle size. These changes alter the predicted pulmonary deposition of e-cigarette particles.

The second objective of this thesis was to investigate the pulmonary effects of dual exposure to electronic cigarette aerosols and cigarette smoke. Exposure of female BALB/c mice to 3R4F cigarette smoke was performed in an automated whole-body system (Promech Lab AB SIU24). Exposure to electronic cigarette aerosols was carried out in a whole-body system developed in our laboratory, using a flavourless and nicotine-free vaping liquid. For both types of exposure, mice were exposed successively 2 hours/day, 5 days/week for 8 weeks. We showed that mice exposed to electronic cigarette aerosols and cigarette smoke exhibit changes in the expression of genes involved in the regulation of the circadian rhythm. We found increases in the frequency of dendritic cells, macrophages, neutrophils, B

lymphocytes as well as CD4+ and CD8+ T lymphocytes in lung tissue compared to mice exposed only to cigarette smoke. Exposure to electronic cigarette aerosols also modulated immunoglobulin levels in the bronchoalveolar lavage and serum. An increase in airway resistance was observed in mice exposed to electronic cigarette aerosols, with or without concomitant exposure to cigarette smoke.

The third objective of this thesis was to characterize the effects of glycerol vaping liquid aerosol inhalation on energy metabolism. Mice were exposed to glycerol aerosols using our whole-body exposure system. Male and female C57BL/6 mice were acutely exposed for 6 hours. Although only minor changes were observed, acute exposure to glycerol aerosols appears to prevent the metabolic effects of fasting. Separately, male and female C57BL/6 mice of 6- or 12-week-old, were exposed for 2 hours/day, 5 days/week for 9 weeks. No change in weight or body fat composition was observed. We showed decrease glucose tolerance of young male and female mice. We also observed an increase in hepatic triglyceride and phosphatidylcholine concentration in female mice, without effect in male mice. No changes in markers of endoplasmic reticulum stress, inflammation, or remodeling were observed in liver tissue.

The work presented in this thesis highlights the effects of electronic cigarettes on lung and metabolic health. More studies on the effects of the components of electronic cigarettes are needed to further characterize the mechanisms involved in these changes.

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ABBREVIATIONS

ACC1	Acetyl-CoA carboxylase 1
ACS	Acyl-CoA synthases
ADP	Adenosine diphosphate
ADP	Guanosine diphosphate
AGPAT	Acylglycerol-3-phosphate acyltransferase
Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
Apo	Apolipoprotein
AQP7	Aquaporin 7
AQP9	Aquaporin 9
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein 1
ASCL	Long chain acyl-CoA synthetase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
C/EBPα/β	CCAAT-enhancer-binding proteins
CAT	COPD assessment test
CCL	Chemokine (C-C motif) ligand
CD	Cluster differenciation
cDNA	Complementary deoxyribonucleic acid
CDP-DG	Cytidine diphosphate-diacylglycerol
ChREBP	CCAAT-enhancer-binding proteins
CLOCK	Circadian locomotor outpout cycles kaput
CoA	Coenzyme A
COPD	Chronic obstructive pulmonary disease
CPT1A	Carnitine palmitoyltransferase 1A
CREB	Cyclic AMP-responsive element binding protein
CRP	C-reactive protein
CXCL	C-X-C motif chemokine
DF	Total deposition
DFAL	Alveolar deposition fraction
DFHA	Head airway deposition fraction
DFTB	Tracheobronchial deposition fraction
DGAT	Diacylglycerolacyltransferase
DHAP	Dihydroxyacetone-P
dp	Particle size
EAA	Extrinsic allergic alveolitis
ENDS	Electronic nicotine delivery system
ETF	Electron transferring flavoprotein
EVALI	Electronic cigarette or vaping product use-associated lung injury
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein

FBP1	Fructose 1,6-bisphosphatase
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in the first second
FFA	Free fatty acids
FOXO	Forkhead box O
FVC	Forced vital capacity
G3P	Glycerol-3-phosphate
G6PC	Glucose-6-phosphatase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GK	Glycerol kinase
GLT-1	Glutamate transporter 1
GLUT	Glucose transporter
Gly	Glycerol
Gly	Glycerol
GPAT	Glycerol-3-photsphate-acyltransferase
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
HBE	Human bronchial epithelial cells
HDAC	Histone deacetylases
HDL	High density lipoprotein
HDM	House dust mite
HFD	High fat diet
HFL	Human lung fibroblast
Hprt	Hypoxanthine-guanine phosphoribosyltransferase protein coding gene
HSL	Hormone sensitive lipase
HUEVC	Human umbilical vein endothelial cell
ICAM-1	Intercellular Adhesion Molecule 1
ICRP	International Commission on Radiology Protection model
IF	Inhalable fraction
Ig	Immunoglobulin
IL	Interleukine
IRS	Insulin receptor substrate
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LPM	Litre per minute
LXR	Liver X receptors
MGL	Monoacylglycerol lipase
MMP	Matrix metallopeptidase
MSC	Bone marrow-derived mesenchymal stem cells
MSRA	Multi-Resistant Streptococcus aeruginosa
mTOR	Mammalian target of rapamycin
MUC5AC	Mucin 5AC
nAChR	Nicotinic acetylcholine receptor
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide plus hydrogen
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

NET	Neutrophil extracellular trap
NK-κb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK1	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NR1D1	Nuclear receptor subfamily 1/group D/member 1
NR1D2	Nuclear receptor subfamily 1/group D/member 2
NTHI	Non-typeable Heamophilus influenzae
PBS	Phosphate-buffered saline
PC	Pyruvate carboxylase
PDC	Mitochondrial pyruvate dehydrogenase complex
PDC	Pyruvate dehydrogenase complex
PDK	3-phosphoinositide-dependent protein kinases
PEPCK	Phosphoenolpyruvate carboxykinase
PER	Period
PG	Propylene glycol
PGK1	Phosphoglycerate kinase
PHI	Phosphohexose isomerase
pIgR	Polymeric immunoglobulin receptor
PIP2	Phosphatidylinositol 4,5-niphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKCλ/ζ	Atypical protein kinase C λ and ζ
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator-activated receptor
PTEN	Lipid phosphatase phosphatase and tension homologue deleted on chromosome 10
PTP	Protein tyrosine phosphatase
qPCR	Quantitative polymerase chain reaction
RLEC	Rat lung epithelial cells
ROS	Reactive oxygen species
Rplp0	60S acidic ribosomal protein P0 coding gene
SH2	Src homology 2
SIRT1	Sirtuin-1
SP	Surfactant protein
SREBP	Sterol regulatory element binding transcription factor
STAT	Signal transducer and activator of transcription
TAG	Triacylglycerol
TBE	Tracheobronchoal epithelial cells
TCA	Tricarboxylic acid cycle
THC	Tetrahydrocannabinol
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion protein
VLDL	Very low density lipoprotein
xCT	Cystine/glutamate transporter

Pass on what you have learned. Strength, mastery.
But weakness, folly, failure also.
Yes, failure, most of all.
The greatest teacher, failure is.
Luke, we are what they grow beyond.
That is the true burden of all masters.

Master Yoda
Star Wars: The Last Jedi

À mon père
Pour m'avoir montré l'exemple

À mes petites sœurs, Maude et Hélène
Pour qui j'espère en être un.

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[1] *Ndlr* : 9 et 11 ans nous séparent. C'est ça la blague.

FOREWORD

The results presented in this thesis comprise projects designed and realized by myself, under the supervision of Dr Mathieu Morissette. This thesis presents an overview of the current knowledge on electronic cigarette use, its content and its effect on biological systems. Chapter 1 (published review) presents a literature review of the effects of electronic cigarette on pulmonary health. Chapter 2 (published original article) addresses the impact of vaping liquid constituents on electronic cigarette emitted particle size and deposition. Chapter 3 (published original article) presents the effects of electronic cigarette and tobacco cigarette dual use on lung inflammation and respiratory functions. Chapter 4 (submitted original article) focuses on the effects of glycerol aerosols emitted by an electronic cigarette on energy metabolism. Additional information is given in the beginning of each chapter.

INTRODUCTION

Tobacco leaves have been smoked for over a thousand years, with a peak in the 1960s [1]. Smoking was a sign of elevated social status and wealth as well as a social link between individuals [1]. Often depicted in movies, television and video games as exciting, glamorous and safe, the tobacco industry used to spend billions of dollars every year to ensure that this pristine image remains [2]. Even with a sharp decrease in tobacco smoking over the past 50 years, there are still 1.1 billion smokers worldwide, with 80% of them residing in low- and middle-income countries [3]. As of 2019, 12% of Canadians are current tobacco cigarette smokers, representing 3.7 million people [4]. Of the Canadians who took part in the survey, 9% of them were daily smokers [4].

Tobacco cigarettes take a huge toll on the lives of smokers, killing as much as 8 million people per year [3]. In Canada, this number rises to just over 45,000 smoking-related deaths per year, representing 18% of nationwide mortality [5]. Smoking-related deaths and illnesses are a great economic burden, costing a striking yearly 300 billion dollars in the United States [6] and 20 billion dollars in Canada [5].

Early efforts have been made to modify tobacco cigarettes to promote a “healthier” product. Nicotine delivery was essential to the development of the modern cigarette in the twentieth century, as nicotine was thought to be addicting and thus vital to retaining customers [7]. In the 1910s, the Camel brand and American Tobacco developed new cigarettes with high nicotine content but with additives that made for smoother smoking [7]. As the market grew, advertisements for major brands routinely included health-related statements and testimonials from physicians [7]. This shows that early modifications to the cigarette were made so that it was more palatable, had a higher nicotine delivery and uptake, and could be marketed as “safe” [7].

Over the years, the tobacco industry used multiple methods to reduce the machine-tested yields of tar and nicotine in cigarettes as a way to claim “healthier” cigarettes [7]. New products introduced in the 1990s or later included modified tobacco cigarettes, cigarette-like products, also called cigalikes and smokeless tobacco products. Studies have shown that smokers are interested in trying novel “reduced-exposure” products and perceive them to

have lower health risks, even when advertising messages do not make explicit health claims [8, 9].

An early approximation of the current electronic cigarette was patented in August 1965 by Herbert A. Gilbert [10]. The application was for a "smokeless non-tobacco cigarette," with the aim of providing "a safe and harmless means for a method of smoking" by replacing burning tobacco and paper with heated, moist, flavoured air. A battery-powered heating element would heat the flavour elements without combustion [10]. This invention did not generate interest from the industry at the time and was never commercialized. The first electronic cigarette modern device was patented in the United States by Chinese pharmacist Lik Hon in 2003 [11]. The aim was to provide a nicotine delivery system without tar and mimicking the tobacco cigarette smoking habits [11]. In 2007, electronic cigarettes were introduced to North America and have become a growing market ever since. Tobacco companies have diversified by developing electronic cigarette models of their own [7]. Interestingly, online and vape shop-based electronic cigarette companies are now more prevalent than tobacco company-owned electronic cigarette brands [12]. From 2012 to 2016, electronic cigarette sales in the United States increased by 132% [6], reaching 1.7 billion US dollars in 2018 [13].

The following sections will focus on electronic cigarette use trends in Canada and worldwide, its harm perception, its use as a smoking cessation tool and its effect on pulmonary and systemic health.

Electronic Cigarette Use

Electronic cigarette use spans many demographics, with a significant number of people in all age groups reporting to have tried electronic cigarette at least once. These subsets of users have various reasons to initiate electronic cigarette use and differ in the ways they use the product. Recent surveys indicate that 16% of Canadians over 15 years old have tried electronic cigarette at least once, and 5% in the last month [4]. Similar trends are found in Europe and Asia [14, 15]. The vast majority of adult electronic cigarette users are dual users, with 80% of them being active or former tobacco smokers [4, 16]. Such numbers are also found in Europe, with 90% of Spaniards being dual users [17]. Understanding the potential

interaction between electronic cigarette aerosols and tobacco smoke on pulmonary health is of crucial importance.

Harm Perception

Harm perception of electronic cigarettes has changed over the past few years. Data collected from 2014 to 2015 indicates that electronic cigarette was perceived as relatively harmless [18]. As electronic cigarette research has progressed, more information regarding its impact on health has started to shift public opinion [19]. In 2017, 40% of Canadians classified using electronic cigarettes regularly as a high-risk behaviour [20]. However, electronic cigarettes were and are still perceived as a safer alternative to combustible cigarettes [18-23]. Interestingly, fruity and sweet flavours are perceived as less harmful than tobacco flavours [24]. This change of heart in the public's harm perception highlights the necessity for more research on electronic cigarettes.

Electronic Cigarette as a Smoking Cessation Tool

One of the main reasons to initiate electronic cigarette use in adults is to reduce or quit tobacco cigarette smoking [17, 18, 22, 25]. In 2014-2015, most of electronic cigarette users are former smokers who use these devices as a medium for smoking cessation [26, 27]. In 2019, 35% of Canadians reported having tried to quit tobacco cigarettes using an electronic cigarette device [20]. However, smokers who quit combustible cigarettes using electronic cigarettes often continue using their electronic cigarette and do not plan on quitting vaping [22]. A 2019 survey indicated 70% of Canadians have never tried to quit vaping in the previous year [20]. Electronic cigarettes have been shown to help reduce tobacco cigarette smoking [26, 28-30], leading to improved pulmonary symptoms as coughing and phlegm production [28, 31]. While there were no changes in lung functions, a 48-subject cohort of chronic obstructive pulmonary disease (COPD) patients using electronic cigarettes showed smoking reduction, fewer exacerbations, and improvement of 6-minute-walk performance and chronic obstructive pulmonary disease assessment test (CAT) scores [32].

There is no current scientific consensus on the efficacy of electronic cigarettes as a complete smoking cessation tool [33], with studies showing generally low long-term rates of smoking cessation with electronic cigarette use [30, 34]. Compared to nicotine patches, using

electronic cigarettes does not improve cessation rates [34]. Over the course of 24 months, 19% (43/299 subjects) of electronic cigarette users quit all nicotine products, compared to 18% (84/480 subjects) of tobacco cigarette smokers only [30]. Most of electronic cigarette users continued vaping (42% or 97/229 subjects) and a great proportion of them relapsed to tobacco cigarette smoking (30% or 70/229 subjects) [30]. Cessation rates are lower for dual users (14% or 32/223 subjects), with 11% (26/223 subjects) switching from tobacco cigarettes to electronic cigarettes and 57% (128/223 subjects) quitting electronic cigarettes and reverting to tobacco cigarettes [30]. Moreover, users who gradually decrease the nicotine concentration in their vaping liquid often increase their e-liquid intake, therefore reaching the same nicotine intake levels [22, 35]. This shows that even if electronic cigarettes help reduce tobacco cigarette smoking, it is not an efficient nicotine cessation tool. A recent Cochrane study indicated that as of now, most studies show underwhelming effects of electronic cigarettes on smoking cessation [36]. Most of them have small cohorts and short cessation periods. These limitations prevent us from grasping the potential for electronic cigarettes as a smoking cessation tool. As the chronic impact of electronic cigarette aerosol exposure remains to be fully elucidated, traditional cessation tools and counselling remain a more effective way to cease nicotine use.

Other Motivations for Electronic Cigarette Use

Electronic cigarette users have described their vaping experience as a hobby, with “smoke tricks” being very popular online [18]. The main appealing aspect of electronic cigarettes is the multitude of flavours that can customize the consumer experience [25, 37]. Most electronic cigarette users prefer fruity, sweet and/or minty flavours [38]. Tobacco-flavoured liquids are also common, especially with adult consumers [38]. An interesting trend on flavour preference was found over time: 60% of adult users first initiated electronic cigarette use with a tobacco-flavoured vaping liquid but after four years, this proportion fell to 30%, the remaining having switched to a fruit- or sweet-flavoured liquids [39]. This trend is particularly strong in electronic cigarette only users, as electronic cigarette and tobacco cigarette dual users continue using tobacco-flavoured vaping liquid over the course of the study [39]. This echoes the phenomenon observed in tobacco cessation studies using electronic cigarettes. It seems people who try to quit smoking with electronic cigarettes

continue to use their electronic cigarettes after cessation, because of their nicotine addiction and because electronic cigarettes are enjoyable by their diverse flavours.

Adolescents and Young Adults

Electronic cigarette use has more than tripled between 2014 and 2018 among adolescents and young adults [40-42]. In Canada, adolescent ever-e-cigarette users range from 4 to 10% of surveyed students, with ever-tobacco smokers ranging from 5 to 20% [43-45]. Of the electronic cigarette only users, most adolescents have tried it once, with over 75% of surveyed student having used it under 10 times [46]. In Europe, 34% of adolescents tried electronic cigarettes at least once, with 1.5% using them on a daily basis [47].

In North America, adolescent electronic cigarette users have surpassed tobacco cigarette smokers in number [46, 48, 49]. In Asia, where tobacco smoking is particularly prevalent, electronic cigarette use is still lower than tobacco smoking in younger populations [50, 51]. Dual use of tobacco and electronic cigarettes is also prevalent in secondary school students in Canada as well as in other countries [44, 46, 52]. Moreover, electronic cigarette use has a direct link to tobacco cigarette initiation [45]. A study associated tobacco and electronic cigarette dual use with increased frequency of cannabis use [43]. This shows a growing trend of electronic cigarette use in youths, whose chronic health impacts are still to be fully understood. More importantly, electronic cigarette use could act as a gateway to subsequent or concomitant tobacco cigarette use.

Contrary to adults, the majority of adolescents prefer fruit-flavoured vaping liquids, followed by menthol, candy and desserts [17, 48, 49]. Tobacco-flavoured vaping liquids only represent 5-10% of vaping liquids preferred by adolescents [48]. In Europe, of the teenagers that use electronic cigarettes on a monthly or weekly basis, most of them use vaping liquids containing nicotine [47] and 20% did not know the nicotine content of their vaping liquid nicotine [47].

Young consumers are very susceptible to trends in the electronic cigarette market. First commercialized in 2015, the JUUL brand has taken over the electronic cigarette market in the United States and, just 3 years later, accounts for 40% of the total retail sales [53]. JUUL represents 60% of electronic cigarette brands that are used by adolescents [49]. Discreet,

these vape pods can be sneaked into class, with “stealth vaping” on the rise in youths [54]. In fact, devices that do not resemble a classic cigarette have hit the market in recent years, with products similar to asthma inhalers, travel mugs, car keys, MP3 music players, backpacks and hoodies are now being sold as vaping devices. Low density emission vaping liquids, with increased propylene glycol percentage over glycerol, are also being commercialized with discreet vaping in mind [54]. Sold in youth-friendly flavours, JUUL contains high levels of nicotine, reaching as high as 59 mg/ml, compared to the average 24 mg/ml in adult users [53]. This increase in nicotine accessibility in youths is concerning.

Section summary: Why people use electronic cigarettes

- Most electronic cigarette users are former or current tobacco smokers, and most of them consider electronic cigarettes to be safer than tobacco cigarettes.
- The wide range of flavours and marketing are very appealing to never smokers, especially among youth. The onset of a new habit throughout demographics is concerning, especially when the health impacts of electronic cigarette aerosol inhalation are still emerging.
- Electronic cigarette use is promoted as a tobacco cigarette cessation tool. However, evidence regarding its efficacy compared to existing methods is weak and remains to be confirmed in robust clinical trials.

Knowing that most users are also tobacco smokers, investigating the impact of dual use on pulmonary health is critical.

Chemical Composition of Electronic Cigarette Aerosols

Although electronic cigarettes mimic traditional tobacco cigarettes by allowing nicotine to be delivered to the respiratory tract, in many ways the resemblance ends there. With electronic cigarette use, the delivery method for nicotine changes from conventional cigarettes, as there is no combustion *per se*, rather a heating process that aerosolizes propylene glycol, glycerol, nicotine and flavours. Several electronic components are involved in the creation of electronic cigarette emissions (Figure A). A microchip is activated by the push of a button or following a pressure change after inhalation, triggering the heating coil. The liquid, also called e-liquid, is dragged through the heating coil, leading the atomization of the vaping liquid.

Electronic cigarette liquids contain mainly propylene glycol and glycerol, to which different nicotine concentrations and flavours are added. As of 2018, over 15,000 different e-liquid flavour blends are commercially available, each flavour being a specific blend of flavouring additives that differ between brands [12]. There are also several nicotine concentrations available to users, ranging from 0 mg/ml to as high as 59 mg/ml, achievable using a nicotine salt to increase solubility [55]. This thesis addresses the impact of these components on the aerosol composition and size that are emitted by electronic cigarettes, as well as the pulmonary and systemic impact of their inhalation.

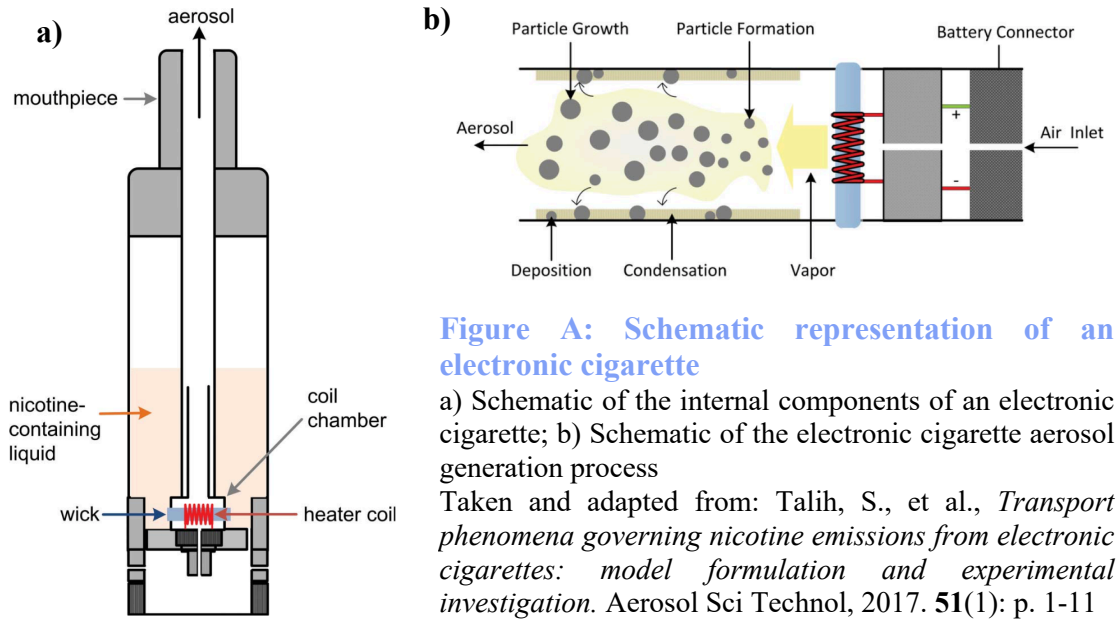

Figure A: Schematic representation of an electronic cigarette

a) Schematic of the internal components of an electronic cigarette; b) Schematic of the electronic cigarette aerosol generation process

Taken and adapted from: Talih, S., et al., *Transport phenomena governing nicotine emissions from electronic cigarettes: model formulation and experimental investigation.* Aerosol Sci Technol, 2017. **51**(1): p. 1-11

Aerosol Composition

Electronic cigarette aerosols may appear safer than tobacco cigarette smoke. Early research indicates that tobacco cigarette smoke is composed of 3000 organic compounds [56], including toxic chemicals such as polynuclear aromatic hydrocarbons [57], *N*-nitrosamines [58, 59], dioxins [60] and acrylamide [61]. The ingredient list for electronic cigarette vaping liquids is short, with only propylene glycol, glycerol, nicotine and flavourings listed. However, upon heating, these ingredients can transform into several by-products, some of which are harmful when inhaled. The next sections detail the chemical compounds found in electronic cigarette-generated aerosols.

Propylene Glycol and Glycerol

Nuclear magnetic resonance (NMR) analysis on a single puff of electronic cigarette aerosols revealed that heated propylene glycol and glycerol generate dozens of by-products (**Figure B**) [62]. Upon heating, glycerol oxidizes into acrolein and propylene glycol oxidizes into methylglyoxal, then formaldehyde and acetaldehyde [63, 64]. Other volatile aldehydes are generated through electronic cigarette vaporization, such as acetone and propionaldehyde [65, 66]. In general, these molecules are at a lower concentration than what is found in tobacco cigarette smoke [67-70].

These organic carbonyls have been known for several decades to be very reactive, having the ability to cross-link to proteins and covalently bind to nucleic acids [71]. **Acrolein** is a reactive aldehyde primarily used as an intermediate in chemical manufacturing and as a biocide in water treatment plants [72]. It is also an abundant component of airborne pollution, the average adult inhaling around 26 µg of acrolein per day [73]. Relatively, tobacco cigarette smoking represents the major source of acrolein for humans, with cigarettes producing between 2.4 µg and 62 µg of acrolein per cigarette [73]. Secondhand and side-stream smoke exposure is also a great source of acrolein exposure [72]. The adverse effects of acrolein exposure range from ocular, respiratory tract and gastrointestinal mucosa irritation [72, 73] as well as protein cross-linking, nucleotide binding and oxidative stress [74-77]. Acrolein can cause apnea, shortness of breath, cough, airway obstruction and mucus secretion through the activation of TRPA1, a receptor present on the sensory neurons innervating the airways

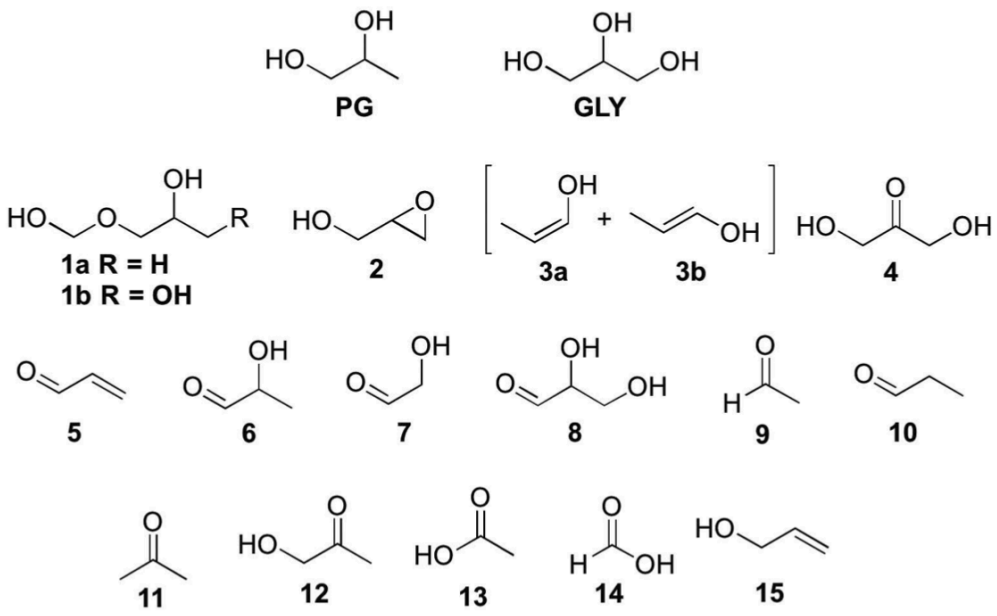

Figure B: Compounds identified in electronic cigarette aerosols

PG = propylene glycol; GLY = glycerol; 1a = propylene glycol hemiformal (major isomer); **1b** = glycerol hemiformal (major isoform); **2** = glycidol; **3a** = (Z)-prop-1-en-1-ol; **3b** = (E)-prop-1-en-1-ol; **4** = dihydroxyacetone; **5** = acrolein; **6** = lactaldehyde; **7** = glycoaldehyde; **8** = glyceraldehyde; **9** = acetaldehyde; **10** = propanal; **11** = acetone; **12** = hydroxyacetone (acetol); **13** = acetic acid; **14** = formic acid; **15** = allyl alcohol.

Taken and adapted from: Jensen, R.P., R.M. Strongin, and D.H. Peyton, Solvent Chemistry in the Electronic Cigarette Reaction Vessel. Sci Rep, 2017. 7: p. 42549.

whose activation increases vascular permeability and thus leukocyte extravasation [78, 79]. It can also cross alveolar-capillary membrane, cause inflammation and contribute to cardiovascular disease [73, 80]. Acrolein can increase macrophage foam cell formation [81], a common feature observed following chronic tobacco cigarette smoke exposure [82-84]. Exposure to acrolein has been extensively researched and leads to several adverse effects. The fact that it can be found in electronic cigarette aerosols, while in lower concentrations than what is found in tobacco cigarette smoke, is of concern.

Formaldehyde has tissue fixating abilities and exposure is associated with several adverse effects, notably during pregnancy, where exposure to more than 2 $\mu g/m^3$ increases the risk of fetal malformations or spontaneous abortions [85]. Once inhaled, formaldehyde is deposited and absorbed in the upper respiratory tract [86]. Formaldehyde can then cross-link

proteins and nucleic acids [85]. It can be further metabolized into formate through the formaldehyde dehydrogenase using glutathione [85]. Formaldehyde inhalation of over 2 ppm inhibits mucocilliary clearance and glutathione-mediated metabolism is saturated when a 4 ppm exposure is reached, preventing its further metabolism [87]. These examples are relevant in a work environment and are the result of acute exposures to airborne formaldehyde. In normal use conditions, vaping 3 g of e-liquid generates 32% less formaldehyde than smoking 20 tobacco cigarettes [88, 89].The impact of chronic low-dose exposures to formaldehyde in the context of electronic cigarette use remains to be assessed.

Electronic cigarette power parameters can change the way propylene glycol and glycerol are aerosolized. Using a 50/50 propylene glycol and glycerol liquid, Talih *et al.* showed that more propylene glycol mass can be recovered at lower battery power settings [90]. This could be explained by the fact that propylene glycol and glycerol have different vaporization temperatures and, therefore, different vaporization rates [90]. More energy is required to aerosolize glycerol compared to propylene glycol, meaning a higher temperature is needed for glycerol vaporization [90]. This preferential vaporization has an impact on the chemical composition of electronic cigarette emitted propylene glycol and glycerol. Glycerol-based vaping liquids produce more aerosol mass than propylene glycol-based e-liquids [91]. Propylene glycol only e-liquids generate more acetaldehyde, acetone, acrolein and formaldehyde than glycerol only e-liquids [91]. Acrolein, acetaldehyde, propionaldehyde, acetone and methylglyoxal are also found in lesser concentrations in electronic cigarette aerosols than in tobacco smoke [92].

Electronic cigarette power settings also affect the chemical composition of emitted aerosols. Acetaldehyde, acetone and methylglyoxal levels are increased at higher power settings [92, 93]. High concentrations of formaldehyde were found in vaping conditions that are far from desirable for electronic cigarette users such as ‘dry-puffing’, the act of engaging the heating coil with little to no e-liquid in the cartridge [88]. Knowing there are currently over 250 brands of electronic cigarettes on the market [12], the way power settings can change the chemical composition of electronic cigarette aerosols is of great interest as it can impact which molecules are delivered to the airways and in what proportions.

Nicotine

Nicotine is a natural alkaloid in tobacco leaves where it acts as a botanical insecticide [94, 95]. The nicotine in tobacco is mainly the (*S*)-nicotine isomer, with only 0.1 to 0.6% being the (*R*)-isomer [96]. N-Nitroso derivatives of tobacco alkaloids are created by the action of nitrous acid on nicotine [97]. Eight tobacco-specific nitrosamines have been identified, among which *N*'-Nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK1), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are the most carcinogenic [98]. Cotinine is the main metabolite derived from nicotine. Produced by the liver, cotinine acts as a biomarker for tobacco smoking when found in urine and blood [99].

When tobacco smoke reaches the small airways and the alveoli of the lung, nicotine is rapidly absorbed [96]. After a puff, nicotine reaches the brain in 10 to 20 seconds, faster than with intravenous administration, producing a rapid behavioural reinforcement through the activation of the dopaminergic reward system [99, 100]. The speed of the nicotine rise enables the smoker to regulate puff by puff the nicotine dose needed to fulfill the desired effect [100, 101]. Knowing that the nicotine concentration can be modified, the ability for electronic cigarette users to regulate their nicotine intake is even greater.

The nicotine concentration in vaping liquids markedly increased in recent years. From 2013 to 2015, most of vaping liquids sold in the United States had a nicotine concentration of 1 to 3% [13]. In 2018, 66% of vaping liquids sold had a 5-6% nicotine concentration [13]. As a reference, nicotine concentration in tobacco cigarettes is approximately 1-2% [102]. This significant increase in vaping liquid nicotine concentration is correlated with the commercialization of JUULs, pod-like devices containing very high nicotine concentrations [13].

The addition of nicotine can change the chemical composition of electronic cigarette aerosols. Nicotine increases acetaldehyde and acrolein levels but decreases formaldehyde and propanal levels [68]. Nicotine in vaping liquids also increases polycyclic aromatic hydrocarbons in aerosols [68]. As mentioned previously, propylene glycol vaporization rate is higher than for glycerol, meaning less energy is needed for its aerosolization. Talih *et al.* demonstrated that the nicotine delivery is governed by the rate at which the solvents vaporize

[90]. This means that propylene glycol-rich vaping liquids result in a greater nicotine flux than the glycerol-rich liquids [90, 103]. Similarly, increased battery power also increases nicotine yield in electronic cigarette aerosols [90, 103-105].

Flavours

Tens of thousands of different flavour blends in vaping liquids are available to users [12]. The chemical composition of vaping liquid flavours is diverse [106]. While absent from the ingredient list, many e-liquids contain glucose, fructose and sucrose to enhance the flavour profile [107]. Multiple flavour compounds in different ratios can produce a single flavour profile, unique to each brand [89, 108, 109]. Some of these flavour compounds are known to be harmful or irritants [106]. Flavour molecules such as vanillin, ethyl maltol, ethyl vanillin, menthol and piperonal are of the most common flavour constituents in vaping liquids [66, 106, 110]. Inhalation these flavour chemicals found in electronic cigarette liquids remains to be investigated.

Menthol is a common compound found in mint-flavoured electronic cigarette liquids [63, 64, 66]. Menthol has long been used in the tobacco industry to create an illusion of reduced health risk of cigarette smoke [111]. Its cooling and anesthetic effects counteract the harshness of nicotine and smoke inhalation, creating a smooth smoking experience [112]. Often used as a nasal decongestant and for cough relief in aromatherapy, menthol alters the perception of breathing patterns, increasing the perceived freeness of breath [113, 114]. This effect is possible through the calcium-modulating action of menthol on thermoreceptors on free nerve endings, activating the sensation of cold [115]. Increased inhaled volumes while using menthol-flavoured tobacco cigarettes have also been reported, leading to increased nicotine absorption [111]. While menthol is highly regulated in medicinal products, there are no specific product standards for tobacco and electronic cigarettes [111]. The impact of chronic use and extensive toxicological studies of menthol-flavoured electronic cigarettes remain to be investigated.

Diacetyl is a chemical found in a wide range of flavours, such as caramel, butterscotch, coffee, maple, vanilla, alcohol, nut and fruit flavours [116]. Diacetyl has previously been linked to respiratory issues, later named ‘popcorn lung’. In the early 2000s, several former

employees of a popcorn processing and packaging plant were hospitalized for bronchiolitis obliterans, marked by irreversible loss of lung function [117]. Further investigations indicated a 2-fold increase in chronic cough, shortness of breath, asthma and chronic bronchitis, and a 10-fold increase in prevalence of airway obstruction for plant workers [117]. Mixed with colouring agents, salt and oil, diacetyl was aerosolized in mixing rooms through heat, generating inhalable particles [118]. Given its ubiquitous nature in food flavourings, Allen *et al.* investigated its presence in electronic cigarette aerosols [116]. On the 51 flavours analyzed, 39 had detectable diacetyl levels, with 'cocktail' flavours reaching the highest concentrations [116]. While there exists diacetyl exposure threshold for adult workers, there are no standards for the general public [116]. In animal models, a 6-hour acute diacetyl aerosol exposure leads to epithelial necrosis and inflammation in both the head and bronchial airways [119]. Chronic inhalation of diacetyl induced lung fibrosis in rodent experiments [120].

Taken together, it seems that regulation of chemicals found in e-liquid flavourings known to be linked to pulmonary diseases must be implemented to protect electronic cigarette users. Flavour compounds present in vaping liquids affect the chemical composition of electronic cigarette aerosols. Flavoured electronic cigarette aerosols had different levels of formaldehyde, acetaldehyde and acrolein compared to unflavoured aerosols [121-123]. Nevertheless, all three compounds were in significantly lower concentration than the recommended exposure limit by the National Institute of Occupational Safety and Health (NIOSH) [121].

Free Radicals and Heavy Metals

While electronic cigarette aerosol formation does involve combustion like for tobacco smoke, e-liquid is still aerosolized through heat, leading to free radical formation. Increased radical formation is associated with higher glycerol-containing liquids, higher coil temperature and higher battery power [124]. The level of free radicals found in electronic cigarette aerosols can also be changed by the type of flavourings found in the liquid, as some flavours generate more free radicals, some less and some about the same as propylene glycol and glycerol alone [125]. On the same note, some flavours have a greater oxidative potency,

such as linalool (floral/spicy scent), piperonal (cherry/vanilla flavour), or citral (citrus flavour) [125].

Since the heating coil of the electronic cigarette is metallic, different metals such as arsenic, chronium, copper, iron, manganese, nickel and lead can be found in electronic cigarette aerosols [126, 127]. A recent study did not detect any lead in plastic bottled e-liquids in Canada or the United States [128]. However, lead was found in products containing e-liquid, such as JUULs and other cartridge models, purchased one year prior to the study, suggesting interaction between the liquid and the metal components in the electronic cigarettes over time [128]. Factors such as electronic cigarette power can increase metal concentration found in electronic cigarette aerosols [126], while the addition of nicotine does not change heavy metal levels detected in electronic cigarette aerosols [129].

The chemical composition of electronic cigarette vaping liquid in turn changes the chemical composition of the emitted aerosols. The electronic cigarette model and power settings also affect the nature of the particles. Once generated, these particles make their way into the respiratory tract. The next section outlines the path leading to aerosol deposition in the lung.

Aerosol Deposition in the Respiratory Tract

An aerosol is defined as a system of solid or liquid particles that is dispersed in a gaseous form, is able to remain suspended in the gaseous state for a long time and has a high surface area to volume ratio [130]. Mathematical models have been designed to predict the lung deposition of inhaled particles according to their size (**Figure C**) [131]. They take into account the physics of aerosol particles, the anatomy of the respiratory tract and respiratory physiology [130]. Calculated breathability for particles larger than 100 µm is unknown and is thus considered not to be inhalable [130].

The human respiratory tract can be separated into two main regions, extra-thoracic and intra-thoracic regions (**Figure C**) [130]. The extra-thoracic, or head airway region, includes the nose, mouth, pharynx and larynx [130]. At rest or during mild exercise, this part of the respiratory tract is the first to have contact with inhaled particles [130]. The oral airways are usually involved during exercise or nasal blockage [130]. Inhaled cigarette smoke and

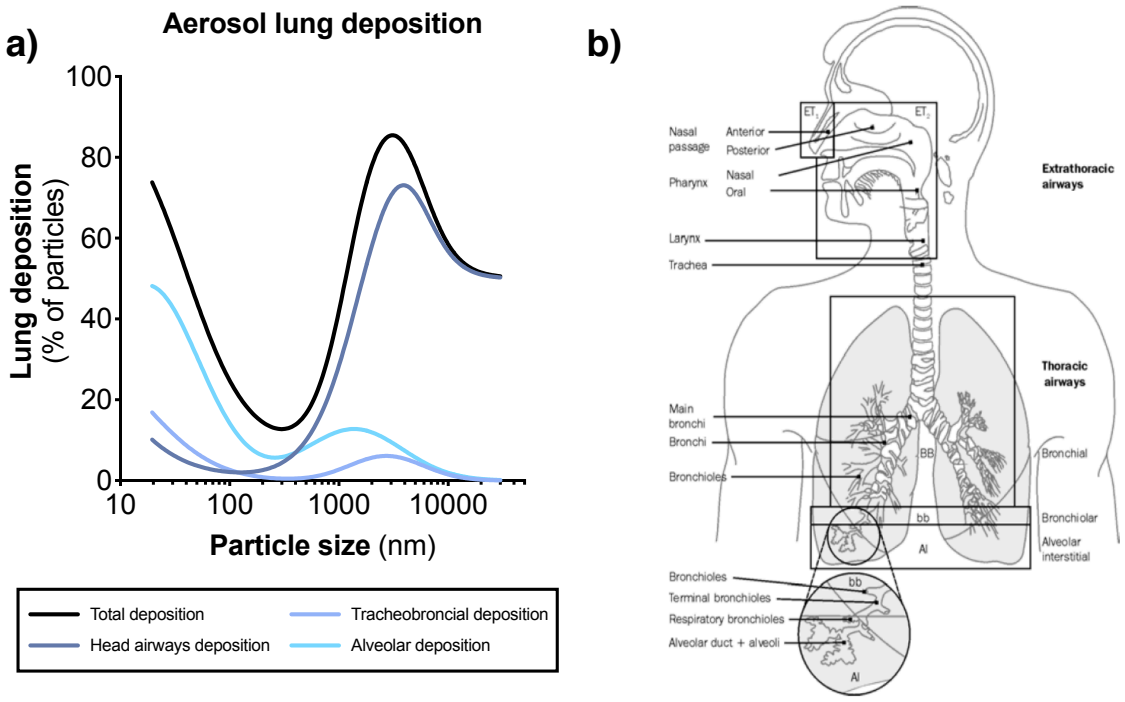

Figure C: Particle deposition in the lung according to size using the ICRP model

a) Lung particle deposition by particle size. Taken and adapted from: ICRP, *Human Respiratory Tract Model for Radiological Protection*. 1994, Internatinoal Commission on Radiological Protection. p. 492.

b) Schematic representation of human airways and the different subregions for aerosol deposition. Taken and adapted from: Connelly, H. and R. Jackson, *Review of Respirable Particle Size Range*. 2013, AMEC: Oxfordshire, United Kingdom. p. 30.

electronic cigarette aerosols flow in the airways through the oral airways [130]. The intra-thoracic region includes the tracheobronchial and the alveolar regions [130]. The tracheobronchial region begins at the top of the trachea to the terminal bronchioles while the apex of the respiratory tract is the alveolar region [130]. The average adult human lung has 3 x 10^8 alveoli and a total fluid surface area of 40 m^2 [130].

Electronic cigarette aerosol chemical content can be modulated by battery power settings, propylene glycol and glycerol ratio, as well as nicotine and flavour content. This change in particle chemical composition could lead to changes in particle size formation, thus affecting aerosol deposition in the airway tract. Knowing all the different cell types present along the respiratory tract, investigating the impact of electronic cigarette parameters on pulmonary responses is of great interest. The original article presented in **CHAPTER 2** demonstrated that alterations in power, propylene glycol and glycerol ratio, nicotine and flavour

components do change particle size formation and lead to differential lung deposition of emitted particles.

Lung Physiology

Inhaled particles can affect several cell subtypes on their way to the alveoli, each with unique and important functions [130]. The tracheobronchial region is lined with the bronchial epithelium, composed of basal cells, ciliated cells and secretory cells [132]. Ciliated cells beat together to clear and maintain the cleanliness of the liquid layer surrounding the epithelial surface, thus acting as the first step in the innate immune response [132]. The bronchial epithelium also acts a physical barrier against irritants and pathogens [132]. In pathologies such as asthma, the integrity of the epithelial lining is lost, leading to airway remodeling [132]. Another barrier against pathogens is the mucus layer lining the bronchial airways [132]. Mucus is a gel composed of 97% water and 3% solids, namely mucins, non-mucin proteins, salts, lipids and cellular debris [133]. There are 17 mucins encoded in the human genome, five of which have terminal cysteine-rich domains that allow for polymer formation that imparts the gelatinous properties of mucus [133]. Mucin 5AC and 5B (MUC5AC, MUC5B) are the most highly expressed in the airways [134, 135] and several pathologies have been linked to impaired mucus clearance, namely cystic fibrosis, asthma, and COPD [133].

The alveoli host a large cell type diversity. The alveolar epithelium is composed of type I and type II alveolar epithelial cells and represents 99% of the surface of the lung [136]. They have an important structural function, acting as a physical barrier against pathogens and by producing the surfactant layer [136]. Produced by type II alveolar epithelial cells, pulmonary surfactant is a complex layer composed of 90% lipids, mainly the phospholipid phosphatidylcholine [136]. The remaining proportion of the surfactant is composed of specific surfactant proteins (SP-A, SP-B, SP-C and SP-D [136]. While SP-B and SP-C play a role in surfactant structure and recycling, SP-A and SP-D are members of the collectin family of host-defence proteins that bind microbial pathogens [137].

Type I alveolar epithelial cells are involved in the pro-inflammatory response through their proximity to alveolar macrophages in the alveoli [136]. Alveolar epithelial cells express toll-

like receptors (TLR) at their surface, enabling them to recognize pathogens and initiate an immune response by secreting of pro-inflammatory cytokines [138, 139]. Alveolar macrophages are the sentinel phagocytic cells of the pulmonary innate immune system [140]. Normally, resident alveolar macrophages account for approximately 95% of the airspace leukocytes, with 1 to 4% lymphocytes and only 1% neutrophils [140]. Dendritic cells, B lymphocytes and T lymphocytes are key players in the adaptive immune defence of the lungs [141]. The following section and **CHAPTER 1** address the impact of each electronic cigarette constituents on pulmonary health.

Section summary: Aerosol composition of electronic cigarette aerosols

- Electronic cigarette aerosols contain a wide variety of molecules and the composition of the emitted molecules can be affected by the electronic cigarette puffing parameters as well as nicotine and flavour content.
- Some molecules found in electronic cigarette aerosols, such as formaldehyde, acrolein and diacetyl, are known to have adverse effects. In some cases, effects of chronic inhalation of these compounds remain unknown. Similarly, the impact of chronic inhalation of flavouring compounds is also unknown.
- Differences in aerosol size change the lung deposition distribution and could impact the way these chemical compounds are distributed in the airways.

Knowing how much electronic cigarette parameters and e-liquid composition change the chemical composition of the aerosols generated, it is of great interest to assess how these factors influence the particle size distribution of electronic cigarette aerosols and thus their deposition in the respiratory tract.

Biological Effects of Electronic Cigarette Use

Impact of Electronic Cigarette on Pulmonary Health

Glycerol and propylene glycol, two main components of the vaping liquids, have been used in the food industry as food additives and sweeteners for decades [142, 143]. While they are considered safe for ingestion, the impact of inhaling glycerol and propylene glycol on pulmonary health were unclear at the beginning of electronic cigarette commercialization. Flavours and nicotine are also key components of vaping liquids. Electronic cigarette vaping liquids come in thousands of flavours [12] and the impact of inhaling these molecules remains mostly unknown.

Lung Functions and Molecular Processes

CHAPTER 1 is a literature review written and published during my thesis in September 2020. It depicts the current knowledge on the pulmonary impact of electronic cigarette aerosols. We focused this review on the different compounds found in electronic cigarette liquids, namely propylene glycol, glycerol, nicotine and flavours. We also divided this review into different levels of biological complexity, with *in vitro* and animal models as well as in clinical studies.

Impact of Electronic Cigarette on Systemic Health

Impacts of electronic cigarette exposure can go beyond the lungs. As mentioned previously, propylene glycol and glycerol are two metabolites that can be utilized in several energy metabolism pathways. Nicotine also enters the circulation rapidly and can affect various systems [96]. As for studies presented in **CHAPTER 1**, the next sections are divided into the specific effects of propylene glycol and glycerol, nicotine and flavours, with the final section including studies investigating the combined effects of all four major vaping liquid constituents.

Propylene Glycol and Glycerol

Cardiovascular System

As of today, very few studies have assessed the cardiovascular impact of propylene glycol and glycerol aerosols. A recent study found that 60-week exposure of male mice to propylene

glycol and glycerol electronic cigarette aerosols increased systolic and diastolic pressure [144]. Mice also developed hypertension and heart hypertrophy, as well as increased oxidative stress and aortic thickness after 60 weeks of exposure [144].

Energy Metabolism

In vitro exposure of lung bronchiolar epithelial cells to propylene glycol and glycerol reduced glucose uptake and mitochondrial ATP production [145]. Chronic exposure to propylene glycol and glycerol electronic cigarette aerosols of *Apoe*$^{-/-}$ mice, prone to develop atherosclerosis and metabolic syndrome, did not alter blood total cholesterol, chylomicron, HDL, LDL or VLDL cholesterol levels [146]. Also, a 12-week exposure to electronic cigarette aerosols did not induce changes in glucose metabolism when assessed by glucose tolerance test, nor did it induce changes in circulating insulin levels [147].

Nicotine

Nicotine rapidly enters the blood circulation and has an effect on multiple organs. Based on human autopsies, nicotine has the highest affinity for the liver, followed by the kidneys, spleen and the lungs, with the lowest for adipose tissues [148]. Electronic cigarettes being a new method for nicotine delivery, several studies investigated the role of nicotine aerosols on extrapulmonary systems.

Cardiovascular System

Nicotine-containing electronic cigarette aerosol exposure in *Apoe*$^{-/-}$ mice decreases chylomicron, LDL and VLDL cholesterol levels, while hematological parameters such as erythrocyte count or hemoglobin levels remain unchanged [146]. Concordantly, nicotine containing electronic cigarette aerosols did not alter atherosclerosis plaque formation [146]. While two studies reported no changes in heart function and tissue morphology [149, 150], another group reported increased heart fibrosis following chronic nicotine inhalation in CD-1 mice [151]. In fact, this particular study also reported fibrosis onset in the kidneys and the liver as well [151]. Some arterial stiffness was noted following nicotine exposure; however, it remains lower than the arterial stiffness observed following tobacco smoke exposure [146]. In humans, acute electronic cigarette emissions with nicotine causes elevated plasma endothelial microparticle levels, suggesting endothelial activation of injury [152]. 60-week

exposure of male mice to propylene glycol and glycerol increases systolic and diastolic pressure [144]. As for propylene glycol and glycerol alone, mice developed hypertension and heart hypertrophy as well as increased oxidative stress and aortic thickness after 60 weeks of exposure. Interestingly, these cardiac abnormalities were proportional to the nicotine concentration in the vaping liquid [144].

Neurological System

Nicotine exposure reduces the expression of glial glutamate transporters GLT-1 and xCT [153]. Nicotine exposure also increases nicotinic acetylcholine receptor (nAChR) protein levels, with increased α-3 nAChR [153], α-7 nAChR [153] and α4β2 nAChR [154] in the brain tissue of electronic cigarette aerosol-exposed mice. This indicates that nicotine found in electronic cigarette aerosols increases protein expression of proteins implicated in nicotine dependence pathways.

Energy Metabolism

Nicotine exposure decreases glycogen storage but does not change liver cholesterol or triglyceride storage [150]. Liver morphology is not impacted by nicotine exposure [150].

Reproductive Health and Development

Exposure of dams to nicotine containing electronic cigarette aerosols in early pregnancy impairs embryo attachment, with gene expression changes in major pathways implicated in uterine receptivity such as integrin, prostanoid biosynthesis, proliferation, JAK, and chemokine signalling [155]. Nicotine exposure in breeding mice does not, however, significantly change pup number per litter or pup weight [155]. Progesterone levels remain unchanged in female mice exposed to nicotine containing electronic cigarette aerosols [155]. Exposure *in utero* to nicotine containing electronic cigarette aerosols has no effect on male reproductive functions in electronic cigarette-exposed mice[155].

Flavours

Energy metabolism

Tobacco-flavoured electronic cigarette aerosols does not lead to triglyceride accumulation [156]. Independently from nicotine, oxygen consumption rate analyses show that exposure

to cinnamaldehyde containing electronic cigarette aerosols impaired mitochondrial respiration and glycolysis, leading to decreased ATP intracellular levels [157].

Reproductive health and development

Exposure to tobacco-flavoured electronic cigarette aerosols *in utero* and during lactation could alter the development of the central nervous system [158].

Indistinguishable and Additive Effects of Electronic Cigarette Components

As discussed in **CHAPTER 1**, the majority of preclinical and clinical studies investigating electronic cigarette aerosol exposure effects on biology use vaping liquids containing nicotine and flavours. These studies render impossible to decipher the independent effect of propylene glycol, glycerol, nicotine and flavours. However, they remain relevant as they depict real-life conditions of electronic cigarette use.

Cellular Processes

Treatment of bone marrow-derived mesenchymal stem cells (MSC) with strawberry-flavoured, nicotine-containing electronic cigarette extract reduces osteogenic differentiation, decreases osteogenic markers, inhibits cell-cell communication and decreases mineralization [159]. In humans, of the 597 immunology-related genes assessed by the Nanostring gene expression analysis, 543 are differentially expressed in nasal cells isolated from electronic cigarette users compared to never users [160]. These genes are involved in major immunological pathways, such as cytokine-cytokine receptor interaction, TLR signalling, Jak-STAT signalling, natural killer cell-mediated cytotoxicity, immune network for IgA production, apoptosis, T cell receptor signalling, NOD-like receptor signalling, RIG-I-like receptor signalling and complement/coagulation cascades [160]. Interestingly, expression of 53 of these deregulated genes are also altered in tobacco smoking subjects [160]. Urine analysis of active electronic cigarette users shows increased bladder carcinogens, namely o-toluidine and 2-naphthylamine [161]. These two carcinogens are found to be elevated in active smokers compared to non-smokers [162]. Acute use of tobacco-flavoured electronic cigarette does not change circulating leukocyte levels [163].

Cardiovascular System

Chronic exposure to cappuccino-flavoured electronic cigarette aerosols containing nicotine increases arterial stiffness of mice to levels similar to combustible tobacco cigarette smoke. While it does not change heart weight or heartbeat rate, this exposure protocol impaired vascular reactivity in response to a methacholine challenge [164]. In humans, acute use of tobacco-flavoured electronic cigarette with nicotine increased arterial stiffness and oxidative stress burden; however, to a lesser extent than combustible tobacco cigarette use [165]. After a month, it was found that active smokers who also use electronic cigarettes have reduced arterial stiffness, possibly due to reduced tobacco cigarette smoking in dual users [165]. Clinical investigations showed short-term use of cherry- or tobacco-flavoured electronic cigarette containing nicotine does not lead to changes in blood pressure or heart rate [166]. Long-term electronic cigarette use increased LDH oxidizability, suggesting increased cardiovascular oxidative stress and risk in humans [167].

Energy Metabolism

Energy and liver metabolism are affected by nicotine and flavouring molecules contained in vaping liquids. In lung fibroblasts, tobacco-flavoured nicotine containing electronic cigarette aerosol exposure increases mitochondrial ROS levels compared to controls, with reduced stability of electron chain transport complex IV cytochrome C oxidase subunit [168]. These results suggest an inefficient transfer of electrons leading to electron leak, thus enhancing mitochondrial ROS formation [168]. Menthol-flavoured vaping aerosols with nicotine decrease mitochondrial respiration in lung epithelial cells, with increased proton leak as well as decreased all mitochondrial complex protein levels [169]. On the other hand, tobacco-flavoured electronic cigarette aerosols containing nicotine does not change mitochondrial respiration rates but increases mitochondrial complex protein levels [169]. Exposure to tobacco-flavoured electronic cigarette aerosols with nicotine reduces the ability for mouse primary cortical neuronal cells to internalize glucose in a ischemic stroke model [170]. This impairment is caused by the reduction of glucose transporter 1 and 3 in brain tissue (GLUT1, GLUT3) [170]. These studies indicate that flavoured electronic cigarette aerosols alter metabolic processes occurring at the cellular level. While interesting, *in vitro* studies are often

executed over short periods to ensure cell viability and typically investigate effects on a single cell type.

As of today, few studies have investigated the impact of electronic cigarette aerosols on energy metabolism *in vivo*. Mice exposed to tobacco-flavoured electronic cigarette aerosols containing nicotine show hepatic mitochondrial dysfunction [171]. Exposure to tobacco-flavoured electronic cigarette aerosols containing nicotine increased the triglyceride accumulation in the liver in *Apoe* $^{-/-}$ mice fed a Western diet (high-fat, high-cholesterol) compared to controls [156]. While there was no immune cell infiltration nor any sign of steatosis, an increase in oxidized lipids was found in the liver of these mice, along with increased hepatocyte apoptosis [156], a finding also reported in rats [172]. Gene array analysis of *Apoe* $^{-/-}$ mice found that electronic cigarette aerosol exposure altered gene expression of lipid metabolism, cholesterol biosynthesis and circadian rhythm pathways [156]. There is little to no evidence of the impact of chronic electronic cigarette use on human metabolic health. Using the National Health and Nutrition Examination Survey (NHANES) database, it was found that electronic cigarette use did not change glucose metabolism rate or insulin secretion [147].

Section summary: Systemic impact of electronic cigarette aerosols

- Propylene glycol and glycerol inhalation induce pulmonary and systemic changes.
- Nicotine and flavours contained in electronic cigarette aerosols affect various biological systems.
- Few studies investigated the impact of propylene glycol and glycerol on systemic health, especially on their impact on energy metabolism.

Assessing the role of propylene glycol and glycerol aerosols on biological systems is crucial in order to understand the potential harms of electronic cigarette use.

Metabolic Impact of Glycerol Contained in Electronic Cigarette Vapours

Electronic cigarette liquids contain glycerol and propylene glycol as a vehicle for delivering nicotine and flavours. Propylene glycol and glycerol can both be metabolized. **Propylene glycol**, or propane-1,2-diol, is commonly administered to ruminants to prevent acid ketosis during lactation [173] and can be transformed into lactaldehyde, methylglyoxal and finally into D-lactate [174]. Biologically, D-lactate is generated during fermentation processes that transport pyruvate into D-lactate to be excreted [175, 176]. Approximately 55% of the ingested propylene glycol is metabolized, the remaining 45% being excreted unchanged by the kidneys [175]. Healthy humans dispose of exogenous D-lactate predominantly through efficient oxidation to pyruvate, although it remains a minor contributor to gluconeogenesis [177]. Elevated circulating propylene glycol levels can lead to lactic acidosis [177, 178]. Being a direct substrate in both fasting and post-absorptive state pathways, this thesis focused on the effects of inhaled **glycerol** on energy metabolism. Glycerol is crucial for triglyceride synthesis in post-absorptive state [179]. During fasting, it can be used to produce glucose through the gluconeogenesis pathway [179]. The following sections will detail glycerol transport and its utilization in glucose and lipid metabolism.

Glycerol Biological Sources and Pharmacology

Glycerol, or 1,2,3-propanetriol, is produced and distributed in cells in low concentrations, ranging from 0.05 to 0.3 mM in humans [180]. At these concentrations, 30% to 79% of glycerol is transformed into glucose via the gluconeogenesis pathway in the liver, with trace amounts being secreted in the urine [180, 181]. If glycerol concentration increases, the kidneys reabsorb the majority of glycerol filtered by the liver and it is excreted through the urine [180, 182, 183].

Glycerol Transport

Aquaporins transport small uncharged molecules such as water, glycerol, urea, purines and pyrimidines [184, 185]. Aquaporins can be found almost ubiquitously across tissues, some aquaporins being expressed in brain, gastrointestinal tract, skin, or lung tissues [186](Table A). Of the 13 mammalian aquaporins that have been identified, 4 have been categorized as

Table A: Mammalian aquaporins and their distribution

Taken and adapted from: Li, C. and W. Wang, *Molecular Biology of Aquaporins.* Adv Exp Med Biol, 2017. **969**: p. 1-34

Name	Transport	Distribution
Aquaporins		
AQP0	Water	Eye
AQP1	Water	Brain, eye, kidney, heart, lung, gastrointestinal tract, salivary gland, liver, ovary, testis, muscle, erythrocytes, spleen
AQP2	Water	Kidney, ear, ductus deferens (male reproductive system)
AQP4	Water	Brain, kidney, salivary gland, heart, gastrointestinal tract, muscle
AQP5	Water	Salivary gland, lung, gastrointestinal tract, ovary, eye, kidney
AQP6	Water, urea, ammonia	Brain, kidney
AQP8	Water, urea, ammonia	Water, urea, ammonia
Aquaglyceroporins		
AQP3	Water, urea, glycerol, ammonia	Kidney, heart, ovary, eye, salivary gland, gastrointestinal tract, respiratory tract, brain, erythrocyte
AQP7	Water, urea, glycerol, ammonia	Testis, heart, kidney, ovary, fat
AQP9	Water, urea, glycerol	Liver, spleen, testis, ovary, leukocyte
AQP10	Water, urea, glycerol	Gastrointestinal tract
Superaquaporins		
AQP11	Water	Testis, heart, kidney, ovary, muscle, gastrointestinal tract, leukocytes, liver, brain
AQP12	Unknown	Pancreas

aquaglyceroporins, proteins that transport water as well as glycerol [187]. Of them, AQP7 and AQP9 are particularly crucial to energy homeostasis. During fasting, glycerol leaves adipocytes after lipolysis through **AQP7**. AQP7 is abundantly expressed in white and brown adipose tissues and allows glycerol to exit adipocytes [185, 188]. Glycerol enters the hepatocytes through **AQP9,** expressed in the liver as well as in leukocytes [188]. The effects of metabolic state and regulators of glycerol uptake are presented in **Table B**.

Implication of Glycerol in Glucose Production

Glucose regulation is central in energy homeostasis. Food is digested in the gastrointestinal tract, where glucose and amino acids are absorbed into the bloodstream and transported to the liver through the portal vein [189]. Blood glucose enters hepatocytes via the glucose transporter 2 (GLUT2) and can subsequently be transformed into glycogen or fatty acids [189]. In short-term fasting conditions, glycogen and proteins are broken down in skeletal muscle tissues, releasing lactate and alanine [179, 189]. In fasting states or during exercise, adipose tissues release non-esterified fatty acids and glycerol [189]. Glycerol, lactate and alanine act as substrates for hepatic glucose synthesis via the gluconeogenesis pathway [189]. The next section describes the implication of glycerol in glucose synthesis.

Gluconeogenesis Pathway

During a prolonged fasting period, glucose is synthesized in the liver using lactate, pyruvate and glycerol as a substrate for the **gluconeogenesis** pathway (Figure D) [189]. In mouse metabolism, glycerol is responsible for 90% of hepatic glucose production during the fasting state and for 50% in post-absorptive state [190]. Glycerol is an excellent and efficient gluconeogenic substrate compared to other three-carbon molecules, as it lacks a charge or a nitrogen moeity that would have to be altered in order be converted into glucose [191].

Glycerol enters the liver through AQP9 and is phosphorylated into glycerol-3-phosphate (**G3P**) by the glycerol kinase (**GK**). To note, the main glycerol kinase activity occurs in the liver and the kidneys, with a small activity level in the skeletal muscle and intestinal mucosal tissues [180]. It is then converted into dihydroxyacetone-P (**DHAP**), then into glyceraldehyde-3-phosphate (Figure D) [189, 191]. Glucose can also be produced from pyruvate through the conversion of cytoplasmic oxaloacetate into phosphoenolpyruvate via the rate-limiting enzyme, phosphoenolpyruvate carboxykinase (**PCK1**) [192, 193]. Through a series of steps, phosphoenolpyruvate is then converted into fructose-6-biphosphate by the fructose-1,6-biphosphatase. Fructose-6-phosphate is converted into glucose-6-phosphate and transported to the endoplasmic reticulum, to finally be dephosphorylated into **glucose** by the glucose-6-phosphatase (**G6Pc**) (Figure D) [189].

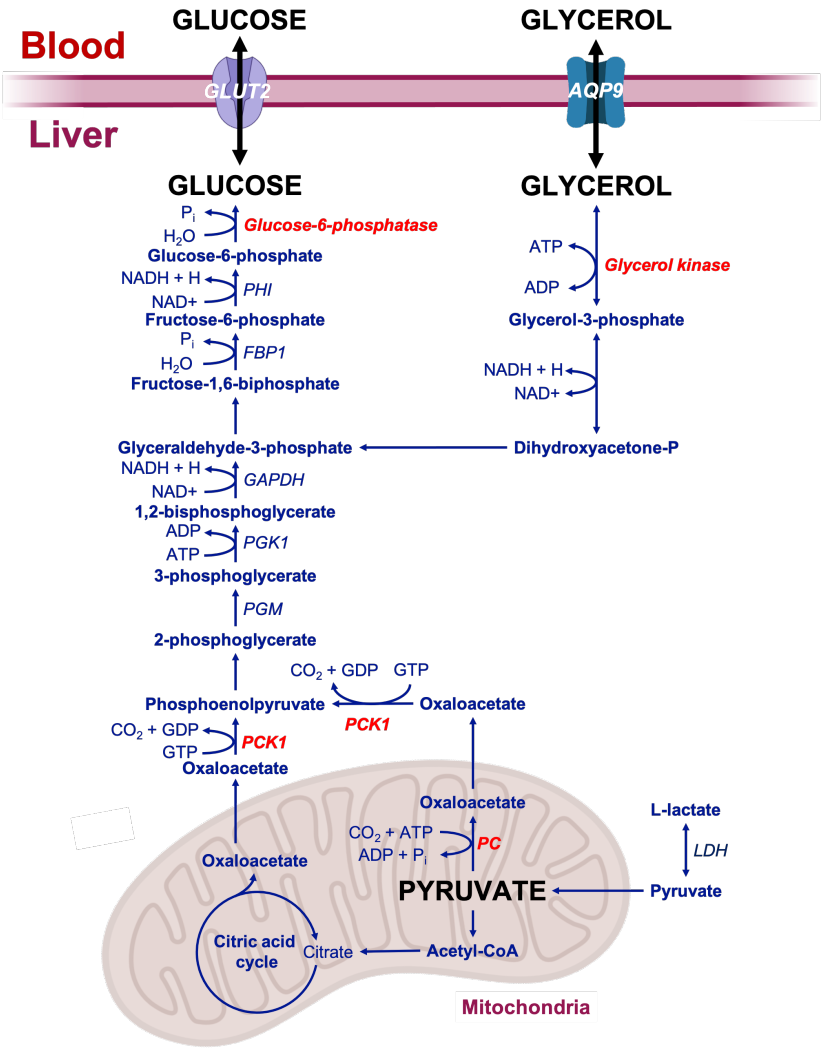

Figure D: Gluconeogenesis pathway

Glycerol has been shown to be the main carbon contributor for glucose synthesis through the gluconeogenesis pathway [194]. However, other substrates can be used for gluconeogenesis. Lactate can be oxidized into pyruvate by the lactate dehydrogenase (**LDH**), then transported into the mitochondria where it can be converted in oxaloacetate by the pyruvate carboxylase (**PC**) [189]. Amino acids can also be converted into α-ketoacids through deamination reactions, then enter the citric acid cycle to be converted into oxaloacetate (Figure D) [189].

Table B: Effects of metabolic state on hormones and mediators implicated in energy metabolism pathways

Name	Fed state	Fasted state
Glucocorticoids		Increased levels in fasted state [195] Activates gluconeogenesis [195] by promoting deacetylation of HSP90 by HDAC6, inducing a HSP90-GR complex [196] Activates lipolysis [197]
Insulin	Activates lipogenesis [198-200], by increasing SREBP-1c [201] and LXRα expression [202], mTORC1 activation by Akt [203, 204] Suppresses lipolysis [205] Suppresses glycerol uptake [184, 188, 206] Suppresses gluconeogenesis by promoting the phosphorylation of FOXO1, FOXO3, FOXO4 and FOXO6 [207-210], the phosphorylation of PGC-1α by Akt [211, 212]	

Gluconeogenesis is an important pathway in energy homeostasis and is tightly regulated. Effects of metabolic state and post-transcriptional modifications involved in the gluconeogenesis pathway are presented in **Table B**. Post-transcriptional modifications and hormonal regulators implicated in gluconeogenesis are presented in **Table C** and **Table D**.

Implication of Glycerol in Lipid Production

Lipid metabolism starts with the intestinal absorption of dietary fats [179], which constitute the main source of lipids and fatty acids. Dietary fatty acids are digested in the small intestine then absorbed by the enterocytes [213]. Regardless of the source, fatty acids are repackaged into triglycerides before being secreted into the gastric lumen through chylomicrons [213]. Non-esterified fatty acids, being hydrophobic, bind to albumin in the chylomicrons which allows them to be soluble in the circulation [213]. The chylomicrons then journey through the vascular system where they lose two apolipoproteins (apoA-1 and apoA-IV), which are replaced by apoE and apoC-II crucial to their further procession [179]. Lipoprotein lipase (**LPL**) is activated by apoC-II, enabling the digestion of the chylomicron triacylglycerols into fatty acids and glycerol [179, 213]. They can then enter hepatocytes through CD36 or fatty acid transport proteins (FATP2 or FATP5) [213].

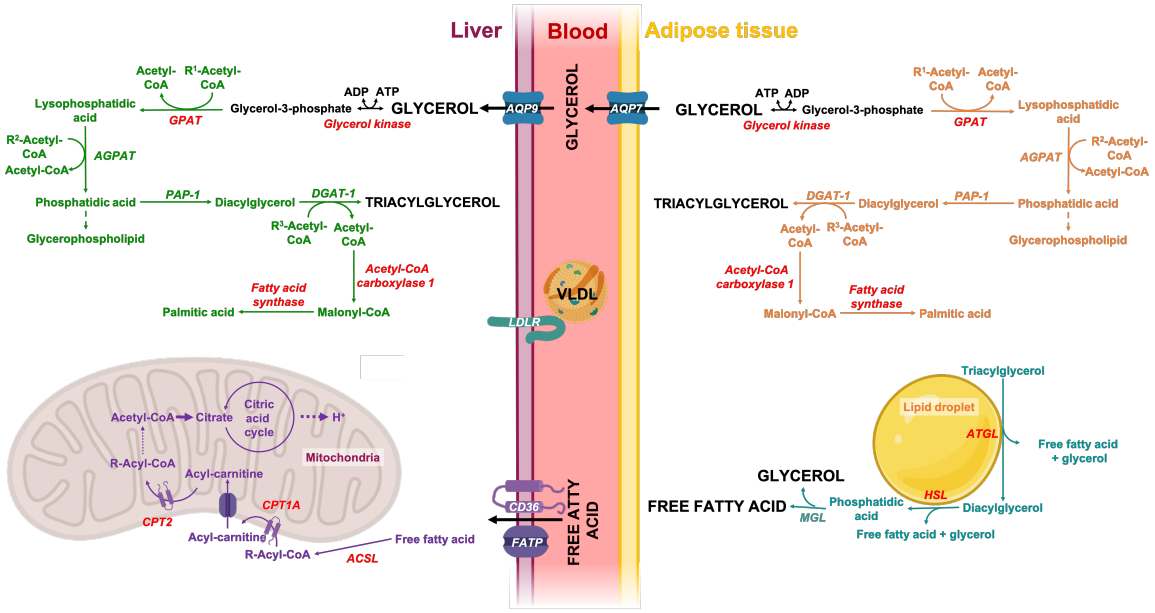

Figure E: Implication of glycerol in lipid metabolism

Fatty Acid Sources

When carbohydrates are abundant, the liver converts glucose into fatty acids through *de novo* **lipogenesis** (Figure E) [214]. The first step of lipogenesis pathway is catalyzed by ATP-citrate lyase (**ACLY**), which converts citrate to acetyl-CoA that is then carboxylated to malonyl-CoA by acetyl-CoA carboxylase 1 (**ACC1**). A series of reactions convert malonyl-CoA into palmitate. Fatty acid synthase (**FAS**) is the key rate-limiting enzyme in palmitate synthesis. Palmitate can be modified by elongases and desaturases to generate a variety of fatty acid species [214]. Under normal conditions, hepatic *de novo* lipid synthesis is not very common. Among the TAG found in VLDL, less than 5% contain fatty acids derived from *de novo* lipogenesis [214]. This process is linked to pathological conditions, with *de novo* fatty acid synthesis being increased under high carbohydrate diets [215] and in patients suffering from obesity or non-alcoholic fatty liver disease (NAFLD) [216, 217].

Another source of fatty acids derives from **lipolysis** in adipose tissue. During fasting, lipolysis provides glycerol and fatty acids necessary for energy production (in teal in Figure E). Within the lipid droplets of adipocytes, the rate-limiting adipose triglyceride lipase (**ATGL**) hydrolyzes triacylglycerols into diacylglycerol, which is in turn hydrolyzed into monoacylglycerol by the hormone sensitive lipase (**HSL**) [214]. Finally, monoacylglycerol lipase (MGL) cleaves monoacylglycerol into glycerol and fatty acids [214].

Fatty Acid β-oxidation

During fasting, fatty acids derived from hydrolysis of hepatic TAGs, circulating lipids or *de novo* lipogenesis can by oxidized and transformed into energy (in purple in Figure E) [214]. Long-chain fatty acids enter hepatocytes through fatty acid transport proteins (**FATP**) and **CD36**. Intracellular fatty acids undergo thio-esterification to become their respective CoA derivates. This process is catalyzed by acyl-CoA synthases (**ACS**s) and results in the formation of acyl-CoA products, the activated form of intracellular fatty acids [214, 218].

Hepatic fatty acids are mainly oxidized in the mitochondrial β-oxidation pathway, including short- (<C4), medium- (C4-C12), and long-chain (C12-C20) fatty acids [214]. β-oxidation of very long- (C20-C26) and branched-chain fatty acids takes place in the peroxisomes. Branched fatty acids can be oxidized by α-oxidation [218]. α-oxidation and ω-oxidation take place within the peroxisomes in the endoplasmic reticulum, respectively [218]. The mitochondrial membrane is not permeable to acyl-CoAs and they must therefore be conjugated to carnitine to enter the mitochondria (in purple in Figure E) [219]. Carnitine forms an ester bond with long-chain fatty acids by the action of carnitine palmitoyl transferase 1 (**CPT-1**), located in the outer mitochondrial membrane, generating acylcarnitines [218]. Acylcarnitines are then translocated across the inner mitochondrial membrane by the carnitine acylcarnitine translocase [218]. Once inside mitochondria, carnitine palmitoyl transferase 2 (CPT-2), located in the inner mitochondrial membrane, removes carnitine from acylcarnitines and regenerates acyl-CoAs [218].

Once inside the mitochondria, mitochondrial β-oxidation of saturated fatty acyl-coA esters takes place in repeated rounds of 4 biochemical reactions: oxidation, hydration, second oxidation, and thiolysis [218]. During each cycle, acetyl-CoA is removed from the acetyl-CoA esther chain, shortening it by two carbon atoms. For example, palmitoyl-CoA (C16:0) generates eight molecules of acetyl-CoA [218]. The produced reducing equivalents are transferred to the respiratory chain via the electron transferring flavoprotein (**ETF**), to which flavin adenine dinucleotide (**FAD**) is attached and acts as an electron acceptor [218]. Electrons are then passed to ubiquinone through the ETF dehydrogenase, producing ubiquinol. Finally, ubiquinol donates the electrons to the complex III of the mitochondrial respiratory chain [218].

Table C: Effects of metabolic state on the proteins implicated in energy metabolism pathways

Name	Fed state	Fasted state
Glycerol transport		
Aquaporin 7 AQP7	Downregulated by insulin [220]	Increased expression [220, 221]
Aquaporin 9 AQP9	Downregulated by insulin [220]	Increased expression [220]
Gluconeogenesis		
Glucose-6-phosphatase catalytic subunit G6pc	Downregulated [195]	Increased expression [195] by CREB [222-224], FOXO1 and FOXO3 [207, 209, 225].
Glycerol kinase Gk	Reduce expression [220]	Increased expression [220, 226, 227]
Phosphoenol-pyruvate carboxykinase 1 PCK1	Decreased expression [195, 220] by insulin [220] and glucose-induced actelytation [193, 228]	Increased expression during fasting [195, 220, 229] by CREB [222-224] and deacetylation by SIRT1 and SIRT2 [193, 228]
Lipid synthesis		
ACC1 Acetyl-CoA carboxylase 1	Increased expression in the liver [230]	Inhibited [231] Phosphorylated by AMPK [231]
ATGL Adipose triglyceride lipase	Reduced adipose tissue expression [232] Inhibited by increased long-chain acyl-CoA levels [233]	Increased expression [227, 232] Phosphorylated [234]
CPT1A Carnitine palmitoyltransferase 1A	Inhibited by increased malonyl-CoA levels [235]	Increased expression by PPARα [236], phosphorylation [237]
GPAT Glycerol-3-phosphate acyltransferase	Increased expression in the liver [232] by ChREBP, SREBP-1c, LXRα and PPARγ [198, 238]	Reduced hepatic expression [232] by phosphorylation by AMPK [239]
FAS Fatty acid synthase	Increased expression [227, 240]	Reduced expression [240]
HSL Hormone sensitive lipase	Inhibited by increased long-chain acyl-CoA levels [241]	Increased expression [205] Phosphorylated by PKA [242]

Table D: Effects of metabolic state on the transcription factors and cofactors implicated in energy metabolism pathways

Name	Fed state	Fasted state
C/EBPα/β CCAAT-enhancer-binding proteins		Increases gluconeogenesis [222].
ChREBP Carbohydrate-response element-binding protein	Increased expression due to glucose [227, 230, 243, 244], its acetylation by p300 [245] and its translocation following complex formation with G6P [230, 246] Activates lipogenesis [198, 247]	Inhibited translocation by its phosphorylation by the cAMP/PKA pathway [246]
CREB Cyclic AMP-responsive element binding protein		Increased levels during fasting [248] Activates gluconeogenesis [224, 248]
LXRs Liver X receptors	Activates lipogenesis [198, 249] by binding to SREBP [250] and ChREBP [251] Inhibits gluconeogenesis [252]	
PGC-1α Peroxisome proliferator-activated receptor gamma coactivator 1 **α**	Reduced expression after feeding [195] Inhibited by its phosphorylation by Akt [212]	Increased expression in the liver [195, 229, 253] Activates gluconeogenesis [189]
PPARα Peroxisome proliferator activated receptor alpha	Reduced expression in the liver [232]	Increased expression liver in the [226, 227, 232] by deacetylation by SIRT1[254] Activates fatty acid β-oxidation [236, 255, 256]
PPARγ Peroxisome proliferator activated receptor gamma	Increased expression [227, 232] Activates fatty acid uptake and lipogenesis [257]	Decreased expression [227, 232] Increases glycerol uptake [258]
SREBP-1c Sterol regulatory element binding transcription factor	Increased expression in adipose tissue and liver [230, 232] following ER stress [259], low cholesterol and phosphatidylcholine levels [260, 261], its coactivation with PGC-1b [262], its phosphorylation by mTORC1 [263] Activates lipogenesis [198]	Reduced expression [232] Inhibited by its phosphorylation by AMPK [264] and deacetylation by SIRT1 [265, 266]

Triglyceride Synthesis

In post-absorptive state, glycerol and fatty acids are converted into TAGs for storage (in green and orange in Figure E). The first and rate-limiting step of TAG synthesis is the esterification of long-chain acyl-CoA to glycerol-3-phosphate, catalyzed by the mitochondrial and microsomal glycerol-3-phosphate acyltransferase (**GPAT**) [214]. The lysophosphatidic acid molecules produced by this step are then acetylated in endoplasmic reticulum into phosphatidic acid by the acylglycerol-3-phosphate acyltransferase (**AGPAT**) [214]. Phosphatidic acid is further transformed into cytidine diphosphate-diacylglycerol (**CDP-DG**) which is a substrate for the synthesis of certain glycerophospholipids and cardiolipins [214]. CDP-DG can be dephosphorylated by phosphatidate phosphohydrolase to form diacylglycerol, which serves as precursor molecules for the synthesis of TAG as well as glycerophospholipids, such as phosphatidylcholine and phosphatidylethanolamine [214]. Diacylglycerol acyltransferase (**DGAT**) catalyzes the acylation of diacylglycerol, constituting the final step of TAG synthesis [218].

Summary Box D: Propylene glycol and glycerol metabolism

- Glycerol and free fatty acids are derived from lipolysis in the adipose tissue in fasting state.
- Glycerol can be used during fasting to produce glucose in hepatocytes.
- Free fatty acids can be oxidized in the mitochondria to produce energy.
- When carbohydrate levels are high, glycerol and fatty acids can be used for triglyceride and glycerophospholipid synthesis.

Knowing the biological uses of glycerol, assessing the effects of acute and chronic glycerol electronic cigarette aerosol inhalation on energy metabolism is of great interest.

OBJECTIVES AND HYPOTHESES

Research on the health effects of electronic cigarettes is still in its infancy. The wide range of electronic cigarette products makes the study of their biological effects particularly challenging. Moreover, electronic cigarette use is increasingly popular across all ages, in spite of the lack of evidence regarding health effects of vaping. Generally considered safe, the chronic effects of the inhalation of propylene glycol, glycerol, nicotine and flavours delivered through electronic cigarette systems remain to be fully understood. **The general aim of this thesis is to characterize the effects of electronic cigarette aerosols and its constituents on biological systems.**

Figure F: Schematic representation of the different themes presented in this thesis.

CHAPTER 2: Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by electronic cigarettes.

When passing through the heating coil, the propylene glycol, glycerol, nicotine and flavours contained in the vaping liquid aerosolize to produce electronic cigarette aerosols. Once generated, airborne particles deposit into the respiratory tract differently according to their size. We hypothesized that the power/temperature settings, the propylene glycol to glycerol ratio, the nicotine content and the presence of flavours would affect the particle size

distribution of emitted aerosols. We also hypothesized that these changes in particle size would change the predicted lung deposition of electronic cigarette aerosols.

We aimed to investigate the impact of vaping liquid constituents on the particle size distribution and their lung deposition. In that regard, we specifically aimed to:

- Characterize the effects of the power/temperature settings, the propylene glycol to glycerol ratio and the nicotine and flavour compositions on particle size distribution.
- Investigate if the differences in particle size change the predicted deposition of electronic cigarette aerosols.

CHAPTER 3: Exposure to nicotine-free, flavour-free e-cigarette aerosols modifies the pulmonary response to tobacco cigarette smoke in female mice

The vast majority of electronic cigarette users are active or former smokers of tobacco cigarettes. Cigarette smoking has a huge human and economical toll and is a major risk factor is multiple cancers and other chronic illnesses. With electronic cigarette use on the rise, it is crucial to investigate the respiratory effects of electronic cigarette use. Previous investigations during my master's degree revealed that propylene glycol and glycerol electronic cigarette aerosol exposure leads to changes in the expression of circadian rhythm regulatory genes in the lung. Disruptions in the circadian rhythm can have a wide range of effects on multiple biological processes and can have a significant impact on immune function.

We hypothesized that exposure to electronic cigarette aerosols could alter lung circadian rhythm, immunity and pulmonary function in mice. We hypothesized that electronic cigarette aerosols have the potential to change inflammatory response to tobacco cigarette smoke.

We aimed to investigate the effects electronic cigarette aerosol exposure on lung circadian rhythm, inflammation, immune cell populations and respiratory functions. Therefore, we specifically aimed to:

- Assess the effects of electronic cigarette aerosols, alone or with cigarette smoke exposure, on the lung circadian rhythm.

- Investigate the effects of electronic cigarette aerosols, alone or with cigarette smoke exposure, on the immune cells present in bronchoalveolar lavage and lung tissue.
- Measure the effects of electronic cigarette aerosols, alone or with cigarette smoke exposure, on lung function parameters.

CHAPTER 4: Glycerol contained in electronic cigarette aerosols affects energy metabolism in a sex-dependent manner

Propylene glycol and glycerol are the common ingredients in all vaping liquids. Glycerol is a fundamental substrate used for energy homoeostasis. During fasting, glycerol acts as a substrate for gluconeogenesis to produce glucose. In post-absorptive state, glycerol is used for triglyceride and phospholipid synthesis.

We hypothesized that glycerol electronic cigarette aerosols reach the circulation and can be metabolized. We also hypothesized that chronic exposure to glycerol electronic cigarette aerosols could change glycerol, glucose and lipid metabolism in mice.

We aimed to investigate the effects electronic cigarette aerosol exposure on glycerol, glucose and lipid metabolism. Specifically, we sought to:

- Assess the kinetics of inhaled glycerol electronic cigarette aerosols.
- Investigate the effects of chronic glycerol electronic cigarette aerosol exposure on glycerol, glucose and lipid metabolism.

CHAPTER 1: PULMONARY EFFECTS OF VAPING WITH A FOCUS ON THE CONTRIBUTION OF EACH MAJOR VAPING LIQUID CONSTITUENT

1.1 Foreword

The review article presented is Chapter I, called '*The fog, the attractive and the addictive: pulmonary effects of vaping with a focus on the contribution of each major vaping liquid constituent*' has been published in *European Respiratory Review* in 2020 by Ariane Lechasseur and Mathieu C Morissette. I did the bibliography review and data representation and Mathieu C Morissette and I did the manuscript preparation.

The Fog, The Attractive and The Addictive: Pulmonary Effects of Vaping with A Focus on The Contribution of Each Major Vaping Liquid Constituent

Ariane Lechasseur[1,2] and Mathieu C Morissette[1,3].

[1]Quebec Heart and Lung Institute - Université Laval, [2]Faculty of Medicine, Université Laval, [3]Department of Medicine, Université Laval.

Take home message: A review of what is currently known of the pulmonary effects of vaping with special emphasis on the specific effects of each e-liquid constituents.

Corresponding author:
Mathieu C Morissette, PhD
Quebec Heart and Lung Institute
Department of Medicine, Université Laval
2725 Chemin Sainte-Foy
Québec, QC, Canada, G1V 4G5
Phone: +1 418 656 8711 ext. 5394
Fax: +1 418 656 4509
Email: mathieu.morissette@criucpq.ulaval.ca

1.2 Résumé

Le vapotage est en constate croissance durant la dernière décennie. Cette revue de littérature présente les effets biologiques de l'inhalation de vapeurs de cigarettes électroniques, plus spécifiquement sur ses effets pulmonaires. Une attention particulière a été consacrée à fournir les effets documentés spécifiques à chaque ingrédient majoritaire, à savoir le propylène glycol et le glycérol, la nicotine et les agents aromatisants. Pour chaque ingrédient, les résultats sont divisés en fonction de la méthodologie utilisée, qu'il s'agisse d'études in vitro, d'études animales et d'études cliniques. Enfin, nous fournissons des réflexions et des idées sur l'état actuel de la compréhension des effets pulmonaires du vapotage ainsi que de nouvelles pistes et méthodologies de recherche.

1.3 Abstract

Vaping has become increasingly popular over the past decade. This pragmatic review presents the published biological effects of electronic cigarette aerosol inhalation with a focus on the pulmonary effects. Special attention has been devoted to providing the documented effects specific to each major ingredient, namely propylene glycol/glycerol, nicotine and flavouring agents. For each ingredient, findings are divided according to the methodology used, being *in vitro* studies, animal studies and clinical studies. Finally, we provide thoughts and insights on the current state of understanding of the pulmonary effects of vaping as well as novel research avenues and methodologies.

1.4 Introduction

The use of electronic cigarettes or devices, also known as vaping, has become increasingly prevalent over the past 10-12 years (1). Initially marketed as a cessation tool for tobacco smoking, electronic cigarettes are now being used by never smokers, being highly popular among adolescents and young adults (2). Electronic cigarette use has surpassed combustible tobacco cigarettes use in adolescents in the United States (3). The disconnect between the number of e-cigarette users and the scientific knowledge on its pulmonary effects is astonishing. While unravelling the complete nature of the pulmonary effects of vaping represents a daunting task, research groups around the world have tackled this scientific problem and continue to uncover the short- and long-term health effects of vaping. In this review, we present specific effects of each major vaping liquid ingredients, as well as the undistinguishable effects. We also share thoughts on conceptual and methodological avenues that are/could be taken by scientists to pursue basic and translational research on the pulmonary effects of vaping.

1.5 Effects Specific to Each Vaping Liquid Constituent

Electronic cigarettes allow nicotine and flavouring agents to be delivered into the lungs through a heat-mediated vaporization process. When activated, a battery-powered heating coil rapidly heats a liquid, often called e-liquid or e-juice, leading to its atomization and easy inhalation. The main constituents of e-liquids are propylene glycol and glycerol, which act as vehicles for various concentrations of nicotine and different types of flavouring agents. During their shelf live and through the atomization process, e-liquid constituents are likely to interact, oxidize and generate potentially harmful by-products (4). Some of these chemicals have been identified, such as free radicals, heavy metals, acrolein, carbonyls, formaldehyde and acetaldehyde (5-7). However, the sheer number of commercial e-liquids makes hard to identify and study the entire chemical compounds present in electronic cigarette vapours, and the majority of them remain unknown. We thus address in this review the physiological impact of electronic cigarette based on the formulation of vaping liquids, not vapors, with the main chemicals being propylene glycol, glycerol, nicotine and flavour molecules.

1.5.1 The Fog - Propylene Glycol and Glycerol

Propylene glycol (propane-1,2-diol) and glycerol (vegetable glycerine) are the common denominator of e-liquids. Propylene glycol and glycerol allow for the solubilization nicotine and flavours into a homogenous solution and are also responsible for the fog-like nature of e-cigarette vapours. Both compounds have long being used by the food industry as sweeteners or stabilizing agents (8). While they are considered safe to ingest (9), the consequences of propylene glycol and glycerol inhalation remain unknown despite worldwide availability of electronic cigarettes. Earlier reports indicated increased upper airway symptoms and lower lung functions for entertainment workers chronically exposed to theatrical fogs, composed of propylene glycol and other glycols (10). The next sections will focus on the impact of propylene glycol and glycerol exposure alone, without nicotine or flavourings.

1.5.1.1 In vitro Studies

Cell viability and gene expression profile - Several studies reported no changes in cell viability when exposing lung epithelial cells (11-13), lung fibroblasts (14), embryonic stem cells (14) or monocytes (15) to propylene glycol and/or glycerol. Other studies reported increased cell death following exposure to propylene glycol vapours (16, 17), glycerol vapours (17) or both (18, 19). It also triggers an oxidative stress response, with elevated expression of antioxidant enzymes Glutathione peroxidase 2 (GPX2), NAD(P)H Quinone Dehydrogenase 1 (NQO1) and Heme oxygenase-1 (HO-1) (17, 20). Glycerol vapours alone increased mRNA levels of HO-1, cellular stress-related genes, as well as carbonylated protein levels, suggesting increased cellular stress (16). Genome-wide expression arrays conducted in primary human bronchial epithelial cells exposed to propylene glycol and glycerol for 24 hours indicated minimal changes overall, with variations in Ribosomal Protein S8 (RPS8), Zinc Finger Protein 721 (ZNF721), Cytochrome P450 Family 1 Subfamily A Member 1 (CYP1A1), Cytoplasmic Polyadenylation Element Binding Protein 1 (CPEB1), Zinc Finger Protein 275 (ZNF275), Mucin 5AC (MUC5AC), Serpin Family A Member 3 (SERPINA3) and DNA Topoisomerase II Alpha (TOP2A) gene expression. Proteins encoded by these genes are involved in diverse biological pathways, suggesting a broad effect of propylene glycol and glycerol vapours on cellular mechanisms (21).

Inflammatory response and immune cell function - Air-liquid interface exposure to propylene glycol vapours has been reported to increase the release of pro-inflammatory cytokine IL-6 by lung epithelial cells (15, 16). Lung fibroblast exposure to propylene glycol and glycerol exposure does not induce IL-8 production (19). Exposure to propylene glycol or glycerol does not appear to alter THP-1 macrophages' ability to phagocytose Non-typeable *Heamophilus influenzae* bacteria (NTHI), with no SR-A1 scavenger receptor or Toll-like receptors 2 and 4 (TLR2, TLR4) change expression (15). Culture of phorbol 12-myristate 13-acetate (PMA)-treated human neutrophil cells with propylene glycol and glycerol reduced neutrophil extracellular trap (NET) formation, suggesting alteration of neutrophil antibacterial abilities (22). Consistent with these results, human blood macrophages and neutrophils exposed to propylene glycol or glycerol showed reduced antimicrobial functions against Multi-Resistant *Streptococcus aeruginosa* (MSRA) (18).

In vitro viability tests suggest glycerol and propylene glycol added to the culture media had little to no cytotoxic effects, while exposure to vapours of glycerol and propylene glycol can have cytotoxic effects, possibly through oxidative stress mechanisms. Vapours of propylene glycol and glycerol can also trigger immune response in epithelial cells and/or affect the functionality of immune cells such as neutrophils and macrophages.

1.5.1.2 Animal Studies

Physiology - Propylene glycol or glycerol exposure does not induce body weight changes in male or female mice (23, 24). Interestingly, neonatal exposure to propylene glycol and/or glycerol can decrease weight gain in offspring (25, 26). However, this change in body weight does not translate in a behavioural change in adult mice that were exposed *in utero* to propylene glycol and glycerol (26).

Lung functions and histology - As of now, acute exposure to propylene glycol and glycerol does not seem to impact lung functions (27). More prolonged exposures does alter lung functions, at steady state, following methacholine challenge to assess airway hyperresponsiveness or when combined with tobacco smoke exposure (27-30). No changes on lung histology in adult mice (28) or mice exposed during the neonatal stage were reported (25). However, exposure to propylene glycol vapours increase mucocilliary clearance (31),

as well as the number of MUC5AC positive cells (27). Propylene glycol and glycerol exposure also modulate the lung lipid mediators, with increased levels of 2-arachidonoylglycerol (2-AG) and 12-Hydroxyeicosatetraenoic acid (12-HETE) (30).

Inflammatory response – Only a few studies investigated the inflammatory response to propylene glycol and glycerol, finding no increase in total bronchoalveolar lavage (BAL) cell numbers in mice following up-to 8 weeks of exposure (29, 32, 33). In the house dust mite (HDM) asthma model, exposure to propylene glycol and glycerol does not appear to affect the immune response, as BAL cell numbers remained unchanged compared to control mice (23). Another study indicated decreased total BAL cell levels, mainly driven by decreased macrophage numbers (34). These results concur with other findings indicating no changes in pro-inflammatory cytokine secretion (32) or total BAL protein levels (27) following propylene glycol and glycerol exposure. Propylene glycol and glycerol exposure, alone or with tobacco smoke, also changes lung leukocyte cell frequency (29). These findings were confirmed in another study, with increased lung dendritic cells, B cells and CD4+ T cells (30).

Circadian rhythm - Propylene glycol and glycerol exposure modulate the expression of key circadian regulatory genes in lung and peripheral organs, alone or combined to tobacco smoke exposure (29, 32, 35). As circadian rhythm plays a role in a wide array of mechanisms, from immunological to metabolic pathways [reviewed in (36-38)], these findings show that even nicotine-free and flavour-free electronic cigarette vapours have the potential to modulate a wide set of pulmonary and systemic biological processes.

Overall, in vivo animal studies suggest that the adverse pulmonary effects of inhaled propylene glycol and glycerol vapours are relatively limited. However, propylene glycol can affect mucociliary clearance and both propylene glycol and glycerol affect the pulmonary expression of genes regulating the circadian rhythm, suggesting these two compounds could change the normal and optimal pulmonary response to pathogens, irritants and allergens.

1.5.1.3 Clinical Studies

Lung functions – Boulet *et al.* reported that acute propylene glycol and glycerol inhalation (1h) do not change lung functions parameters assessed by spirometry and forced

oscillometry, in both healthy and asthmatic subjects, nor does it induce respiratory symptoms (39). Chaumont *et al.* reported reduced transcutaneous O_2 tension in young smokers and subtle changes in forced expiratory volume in the first second (FEV_1) and FEV_1/forced vital capacity (FVC) ratio (40).

Inflammatory markers - A recent study reported no change in BAL immune cells or pro-inflammatory mediator levels in never smokers and never electronic cigarette users following a 2-week use compared to the baseline (41). Acute propylene glycol and glycerol inhalation (1h) does not elevate fractional exhaled nitric oxide (FeNO) or circulating C-reactive protein (CRP) (39). Acute electronic cigarette use causes a slight increase in circulating club-cell secretory protein (CCSP) and surfactant protein D (SPD) levels (40). Acute propylene glycol and glycerol vaping use does not induce endothelial microparticle formation, a hallmark of vascular damage (42). Acute propylene glycol and glycerol exposure increases circulating c-reactive protein (CRP) in humans and soluble intercellular adhesion molecule (sICAM) (43).

Gene expression profile – A 4-week use of propylene glycol and glycerol does not appear to significantly alter mRNA and miRNA levels in bronchial epithelial cells of healthy volunteers (41). Another gene expression study in acute propylene glycol and glycerol users (20 puffs) reported changes in expression of 65 genes in small airway epithelial cells and 61 genes in alveolar macrophages (42). Interestingly, this study reported altered expression of key circadian regulatory genes were found in small airway epithelial cells (42), reinforcing previous preclinical findings (32, 35). Clinical investigations so far are very limited but support propylene glycol and glycerol having little impact on lung functions as well as no significant inflammatory effects. There is no doubt inhaling propylene glycol and glycerol is unusual with regard to lung homoeostasis, suggesting effects other than changes in lung functions and inflammation should be observed. Considering alterations in normal lung rhythmicity may reveal insightful for future research. To the best of our knowledge, there are currently no clinical studies assessing the pulmonary impact of chronic propylene glycol and glycerol use, in absence of nicotine or flavourings.

Summary Box 1.1 – Pulmonary effects of propylene glycol and glycerol

- *In vitro* studies suggest vapours of propylene glycol and glycerol could have cytotoxic effects on lung epithelial cells and other cell types, however, animal and clinical studies do not report signs of extensive lung damage or lung function alteration.
- While minor signs of inflammation have been reported in some *in vitro*, in animal and clinical studies, the general consensus is that exposure to vapours of propylene glycol and glycerol do not trigger a meaningful inflammatory response. However, alterations of the normal function of immune cells have been reported.
- While exposure to vapours of propylene glycol and glycerol appears to be well processed by the lungs, more subtle effects such as disruption in lung circadian rhythmicity and mucocilliary clearance could impact the response to concomitant exposure to pollutants, allergens and pathogens.

1.5.2 The Attractive - Flavours

Unravelling the effects of vaping flavours on lung biology is by far the most intricate matter in vaping research. As of 2018, over 15 000 different flavour blends are commercially available (44). Investigating the biological impact of flavouring inhalation is a daunting task, as most flavours are often a unique combination of tens, even hundreds, of different molecules. This great variety of flavours is one of the most cited reasons people choose to initiate electronic cigarette use (45, 46). While some flavours are relatively straight forward, such as Vanilla, Strawberry or Menthol, some have names that do not evoke any particular flavour, such as “Treasury”, “Highlander Grog” or “California Blues” (18). With that being said, even the simplest flavour names can have a very complex chemical composition (47, 48). Studying the biological impact of flavouring molecules upon vaporization is of utmost importance, as pulmonary effects remain largely unknown. In this section, we detail the impact of exposure to electronic cigarette liquids or vapours containing flavouring molecules, independently of nicotine.

1.5.2.1 In Vitro Studies

Cellular viability and integrity - Flavour chemicals in e-liquids generate fragmented molecules with oxidative and inflammatory potential (6, 49). Some flavours are cytotoxic (12, 50). For instance, cinnamaldehyde, responsible for cinnamon flavour, is cytotoxic to endothelial HUVEC/Tert2 cells with increased cell lysis and almost complete cessation of cell metabolic activity (51). However, other flavours have been shown to only have limited cytotoxic effects (11, 52). Exposure to 2,5-dimethypyrazine, flavouring compound for chocolate flavour, alters ion conductance in immortalized human bronchial epithelial cells (16HBE14o-), a phenomenon attributed to protein kinase A-dependent activation of the cystic fibrosis transmembrane conductance regulator (CFTR) ion channel (53). While tobacco flavoured electronic cigarette vapour does not change barrier electrical resistance in primary human small airway epithelial cells (54), cinnamaldehyde exposure decreases ciliary beat frequency in a dose dependent manner in human bronchial epithelial cells (55).

Inflammatory response - No change in pro-inflammatory mediator secretion following tobacco- or grape-flavoured e-liquid exposure of human airway epithelial cells (H292) was reported (19). Other studies showed increased IL-6 production with tobacco flavoured liquid (52, 54). Exposure to cinnamon-flavoured e-liquid increases IL-8 and reactive oxygen species production by human foetal lung fibroblasts (19). Chemicals such as acetoin and diacetyl (butter flavour), maltol (sweet flavour) and o-vanillin (vanilla flavour) induce IL-8 secretion in bronchial epithelial cells and lung fibroblasts (47).

Immune function – Tobacco-flavoured e-liquid with or without nicotine increases human rhinovirus load in human epithelial cells, associated with a decreased expression in short palate, lung, and nasal epithelial clone-1 (SPLUNC1) receptor (52). At high concentrations, cinnamaldehyde (cinnamon flavour), ethyl vanillin (vanilla flavour) and benzaldehyde (almond or cherry flavour) decrease PMA-stimulated neutrophil capacity to phagocytose *Staphylococcus aureus* (56), with a decrease in oxygen consumption rate of PMA-stimulated neutrophils. Exposure to isoamyl acetate (banana flavour) having no effect did not change phagocytosis functions or oxygen consumption rates in neutrophils (56). Apple-flavoured electronic cigarette extract decreases THP-1 macrophages phagocytosis of NTHi, with a

reduction in TNFα, IL-6, IL-1b, CCL3 and CCL4 secretion as well as lower surface expression of SR-A1 ad TLR2 (15). Of interest, exposure of *Streptococcus pneumoniae* to strawberry-flavoured vapour extract changes the expression levels of genes involved in sugar transport and metabolism (57). Another study reported an increase in pneumococcal penetration of lung epithelial cells following exposure to tobacco-flavoured electronic cigarette extract (58).

This shows that several compounds found in electronic cigarette vapours derived from flavour molecules can have adverse cytotoxic and inflammatory effects. More studies are needed to assess the specific chemicals that trigger these effects.

1.5.2.2 Animal Studies

Physiology - While exposure to American tobacco-flavoured electronic cigarette vapours does not affect body weight (59), adult mice that were exposed *in utero* to tobacco-flavoured electronic cigarette vapours showed deficits in short-term memory, reduced anxiety and hyperactivity (60).

Lung functions and physiology - Tobacco-flavoured electronic cigarette vapours increase hyper-responsiveness to methacholine, with decreased mucus positive cells in the epithelium compared to air controls (59). Exposure to vanilla-flavoured vapours increased lung tissue resistance following a methacholine challenge (30). 'Black Liquorice'- and 'Kola'-flavoured vapours superimposed to HDM exposure does not change lung reactivity, while 'Banana Pudding'-flavoured vapours leads to airway remodelling (23). Exposure to 'Cinnacide'-flavoured vapours increases tissue elastance (23).

Inflammatory response - Exposure to flavoured electronic cigarette vapours (5 tested) does not significantly change BAL total cellularity (23, 59). Exposure to vanilla-flavoured vapours does not change the number of macrophages within the alveoli (30). Female mice exposed to tobacco-flavoured electronic cigarette vapours showed increased pulmonary IL-1b, IL-6 and TNFα levels, and adult offspring of these females also exhibited increased lung TNFα (61). Exposure to strawberry-flavoured electronic cigarette vapour does not aggravate *Streptococcus pneumoniae*-induced pneumonia in mice (57).

Very few animal studies tackled the pulmonary effects of flavouring agents in vaping and those who did were only able to address a handful of them. Considering these pre-clinical studies act as a proof-of-principle for the potential effects of vaping flavours, it is impossible to deny that these flavour chemicals can have significant effects on lung homoeostasis.

1.5.2.3 Clinical Studies

Very few clinical studies use specific e-liquid flavours, or even disclose the flavours used in the study. In fact, only one did and used hazelnut nicotine-free e-liquid to assess acute use of electronic cigarette vapours on lung functions. Ferrari *et al*. indicated acute hazelnut-flavoured electronic cigarette use decreased FEV_1 and forced expiratory flow at 25% of FVC (FEF 25%) in smokers, with no changes in non-smokers (62). (62)(61)(58)(Ferrari, Zanasi et al. 2015)(Ferrari, Zanasi et al. 2015)(56)(56)(56)(56)However, this study assessed lung functions following a 5-minute electronic cigarette use, which is more representative of a unique and single use than the normal use regiment of electronic cigarette users.

Altogether, our current understanding of the pulmonary effects of vaping flavours is negligible. Data available are highly affected by the monstrous number of flavours on the market, making it easier to perform *in vitro* testing than preclinical and clinical research. However, the pioneers in vaping research definitely established that inhaling flavouring agents can be detrimental to the lungs. We simply do not know which ones and how severe will the consequences.

Summary Box 1.2 – Pulmonary effects of vaping flavours

- Great disproportion towards *in vitro* studies *versus* preclinical and clinical research.
- Established potential for flavourings causing cellular cytotoxicity, triggering inflammatory responses, impairing immune function and altering lung functions.

1.5.3 The Addictive - Nicotine

Nicotine delivery to the lungs was the original purpose when electronic cigarettes were brought on the market. Lately, reports showed growing sales of e-liquids containing very high nicotine concentrations (≥50 mg/ml (63)). Given that the biological effects of nicotine have been studied for decades, this review will be presenting only the new data generated in the context of vaping. In fact, knowing the specific impact of chronic and high-dose nicotine inhalation through electronic cigarette vapours is of utmost importance and could potentially diverge from what is already known.

1.5.3.1 In Vitro Studies

Cell viability and gene expression profile - Studies show increased epithelial cell cytotoxicity following nicotine exposure in a dose dependent manner (12, 13). Nicotine-containing vapours also decrease epithelial barrier integrity (28, 64, 65) and lower ciliary beat frequency in epithelial cells (28). A 24h exposure of human bronchial epithelial (HBE) cells to electronic cigarette extract with nicotine modulated the expression of 57 genes involved in diverse pathways such as cell cycle, response to hypoxia, response to organic substance, apoptosis, MAP kinase signalling, acute inflammatory pathways as well as phospholipid and fatty acid/triacylglycerol metabolism (21). Solleti *et al.* also identified 571 miRNAs that were differentially expressed when adding nicotine-containing e-liquid to the culture medium of NHBE cells (20).

Immune function – THP-1 macrophages exposed to nicotine-containing extract showed decreased phagocytic abilities, with decreased secretion of CCL3 and CCL4. They also expressed less SR-A1 and TLR2, suggesting a decreased ability to detect pathogens (15). Presence of nicotine in electronic cigarette extract aggravated the reduction in antimicrobial function against MRSA in mouse alveolar MH-S macrophages and human blood neutrophils (18). Nicotine vapour extract also affected the virulence of MRSA on its own, with greater biofilm formation and increased adherence and invasion of epithelial cells (18). Nicotine vapours also directly affect gingival strains of bacteria, with an impact on growth and biofilm formation of oral commensal *Streptococcus* strains (66).

So far, *in vitro* studies on nicotine in the context of vaping confirmed several findings that were previously observed, mainly impacts on epithelial cell viability and permeability as well as its immune-modulatory effects.

1.5.3.2 Animal Studies

Physiology - Some reports show that exposure to nicotine vapours does not affect weight gain in male (24, 67) or female mice (23, 24). Others show weight loss in adult male mice (23, 64, 68) and in adult males exposed to nicotine vapours at the neonatal stage (25, 26). Exposure to nicotine vapours also changes adult mice behaviour, with increased anxiety-like behaviour in male mice, as shown increased number of marbles buried when subjected to the marble burring tests (67). Nicotine vapours also decrease grip strength and swimming abilities in mice (69). Adult mice exposed *in utero* to nicotine show behavioural changes as well, with either increased locomotor activity (24) or increased cognitive flexibility when subjected to the water maze test (26).

Lung physiology - Exposure to nicotine vapour does not cause lung histologic abnormalities in adult mice (18, 69). However, nicotine vapours can affect mucus production in mice, with increased MUC5AC positive cells and expression in lung tissue (28). Exposure to nicotine vapours during the neonatal stage increases mean linear intercept (MLI) (25). The latter observation appears to be caused by a decreased alveolar cell proliferation and lung growth impairment rather than lung damage (25). Both increase (28) and lack of effect (27) on lung resistance following a methacholine challenge were reported.

Inflammatory response - Nicotine vapours alone do not generate an influx of immune cells in the mouse lung (18, 23, 27, 28, 33). Another study reported increased lung tissue neutrophils and $CD8^+$ T lymphocytes in female mice (34). In a HDM asthma model, exposure to nicotine vapours reduces BAL total cell number, mainly driven by a reduction in eosinophils and macrophages, but increased neutrophils (23). Mixed reports on inflammatory mediator levels, increase (18, 28) or no change (33, 34), can be found. Wang *et al.* showed a sex-dependent increase in BAL cytokine levels, with elevated levels in male but not female mice (34). Nicotine vapours also increase neutrophil myeloperoxidase (MPO) activity in BAL fluid in mice (34) as well as intracellular phospholipid levels in BAL cells (33).

Immune function - Nicotine vapours alter neutrophil functions. *In vitro* culture of PMA-activated human neutrophil incubated with nicotine, propylene glycol and glycerol decrease NET formation as well as phagocytosis of *Staphylococcus aureus* and *Escherichia coli* (22). Nicotine vapours also reduce innate immune response to *Pseudomonas aeruginosa*, with an increase in peritoneal bacteria burden and decrease in peritoneal neutrophils (22). Nicotine vapours can also affect pulmonary macrophages. Macrophages harvested from the lungs of mice exposed to nicotine vapours have reduced levels of TNF-α, IL-1β, NOS2, CD86, CD80, and TLR7 mRNA. Influenza A infection is more severe in mice exposed to nicotine vapours, with a decrease in survival rates, increase weight loss, and aggravated lung tissue damage (33).

Nicotine vapours from an electronic cigarette have a significant impact on lung development and lung physiology. In vivo animal studies confirm that nicotine is not pro-inflammatory per se, but ratter has significant immune-modulatory properties.

1.5.3.3 Clinical Studies

Very few clinical studies investigated the specific pulmonary effects of nicotine delivered by an electronic cigarette without flavours. A genome-wide gene expression study in small airway epithelial cells and alveolar macrophages from never smokers following acute electronic cigarette use with nicotine found modulation in 71 genes and 27 genes, respectively (42). Of interest, several genes belonged to the p53 pathway in small airway epithelial cells, but no clear pathways were found in alveolar macrophages (42).

Nicotine found in vaping liquids appears to maintain previously documented properties on lung physiology and immune system. Nicotine concentrations in vaping liquid can greatly vary; from none to over 50 mg/ml. Nicotine poisoning is a well-documented phenomenon in electronic cigarette users, and a deeper understanding of the dose dependent physiological and biological effects on the lung, especially at high doses, may provide novelty.

Summary Box 1.3 – Pulmonary effects of nicotine

- Advanced scientific evidence based on decades of research on tobacco smoking
- *In vitro* studies show vapours of nicotine have cytotoxic effects on lung epithelial cells and can impair epithelial barrier integrity. While, animal studies do not report signs of extensive lung damage or lung function alteration, early exposure to nicotine may be detrimental to lung development.
- No significant evidence of significant inflammatory effects but well documented immune-modulatory effects and alterations of the normal antimicrobial function of immune cells.

1.5.4 Indistinguishable and Additive Effects of Electronic Cigarette Components

Several studies on vaping do not assess the respective impact of the vehicle, nicotine and flavours. This leads to very interesting observations that, while being caused by electronic cigarette, cannot be attributed to a specific constituent. The following section details the effects of combined but indistinguishable e-liquid constituents.

1.5.4.1 In Vitro Studies

Cellular viability - In a study investigating 35 different liquids with different flavours and nicotine concentrations, Bahl *et al.* showed that e-liquid potency varied greatly depending on cell type used, with greater sensibility using stem cells than pulmonary fibroblasts (14). There was no correlation between cytotoxicity and nicotine concentration, the most cytotoxic flavour tested being cinnamon (14). The degree of cytotoxicity appears to be highly variable within a given flavour group (i.e. fruits, tobacco) (14). Tobacco-flavoured electronic cigarette vapours or extract consistently reduces cell viability (17, 19, 70-73). Leigh *et al.* also found that tobacco, piña colada, menthol, coffee and strawberry flavours decreases cell viability and metabolic activity (74). Several studies report increased oxidative stress following cell incubation with tobacco-flavoured electronic cigarette vapour extract with nicotine (70-73) and with strawberry-flavoured extract with nicotine (75). Other studies indicate no changes

in antioxidant response in epithelial cells exposed to menthol- and tobacco-flavoured electronic cigarette vapours (11, 54).

Epithelial cell function - Combined nicotine and flavouring exposure leads to dose dependent loss of epithelial cell barrier integrity (76, 77). Highly concentrated nicotine tobacco- and menthol-flavoured electronic cigarette vapours also decreases epithelial cell barrier integrity (78). Inhibition of Rho kinase and the addition of sphingosine-1 phosphate receptor 1 (S1P1) agonist restores barrier integrity, suggesting a protective role of S1P1 on epithelial barrier integrity in these conditions (76). E-liquid induces morphological changes in human lungs and gingival fibroblasts, suggesting a wide range effect of nicotine and/or flavours on several cell types (19, 71). Exposure to berry- and menthol-flavoured electronic cigarette extract with nicotine do not affect surfactant pressure sustaining proprieties. However, it increases the area between surfactant lipid multilayers, suggesting a potential disruption in surfactant functions (79). Exposure to tobacco-flavoured electronic cigarette vapours with nicotine alters expression of genes involved in metabolic processes, response to organic substances, apoptosis and hypoxia (77).

Inflammatory response - Tobacco-flavoured liquids with nicotine increase IL-6 production (52, 74) and, at high concentrations, decrease MUC5AC production by epithelial cells (77). Strawberry- and coffee-flavoured vapours with nicotine also induce IL-1β, IL-6, IL-10, CXCL1, CXCL2 and CXCL10 secretion (74). Another study indicates that menthol-flavoured electronic cigarette vapours can increase COX2, S100A8, RAGE and γH2A.X protein levels in gingival ligament fibroblasts and gingival epithelium, suggesting broad range of inflammatory pathway activation across the respiratory tract (80). Exposure to tobacco-flavoured electronic cigarette extract with nicotine increases platelet activation, complement protein expression and deposition, as well as platelet aggregation (72, 81). Tobacco-flavoured electronic cigarette extract containing nicotine increases neutrophil activation in blood neutrophils, with an increase in MMP9 levels and CXCL8 secretion, coupled with p38 MAPK activation (82).

Immune function - Tobacco-flavoured electronic cigarette extracts with nicotine decrease antimicrobial functions of blood neutrophils against MRSA (18) and increase pneumococcal

penetration of lung epithelial cells. Strawberry-flavoured electronic cigarette extracts with nicotine change expression levels of genes involved in stress response and metabolism in *Streptococcus pneumoniae* (57), suggesting an impact on pathogens themselves.

1.5.4.2 Animal Studies

Physiology - Liquorice, cinnamon and tobacco-flavoured electronic cigarette vapours prevent normal weight gain over time (23, 83). Mice exposed to cinnamon-flavoured electronic cigarette vapours containing nicotine before conception and during pregnancy give smaller pups at birth (84). Adult mice exposed *in utero* to tobacco-flavoured electronic cigarette vapours with nicotine show deficits in short-term memory, reduced anxiety and hyperactivity (60), as well as higher locomotor activity (83).

Lung physiology - While causing no apparent alterations observable on lung histology (27, 85), tobacco-flavoured electronic cigarette exposure containing nicotine increases MUC5AC positive cells (27), as well as lung resistance following a methacholine challenge (27). Cinnamon-flavoured electronic cigarette vapours containing nicotine increase lung elastance in dams (84), with their offspring having increased airspace enlargement at birth; a defect associated with alterations in expression of several genes involved in the *Wnt* pathway (84).

Inflammatory response - Animal modelling shows mixed results regarding pulmonary inflammation induced by flavours and nicotine. Some studies report no changes in BAL pro-inflammatory mediator levels (27, 86), while others report higher levels of pro-inflammatory mediator secretion (19, 61). Similarly, some studies indicate no changes in BAL cellularity (19), while others indicate increased BAL cellularity (27, 86). With all three studies using whole-body exposures, similar nicotine concentrations and exposure period (Table 2), these results can likely be explained by the different e-liquid flavours or types of electronic cigarette used.

Immune functions - Mice inoculated with *Streptococcus pneumoniae* and exposed for the previous 2h to strawberry-flavoured electronic cigarette vapour extract containing nicotine do not show any worsening of pneumonia compared to unexposed controls (57). On the other hand, mice exposed to menthol-flavoured electronic cigarette vapours with nicotine show decreased abilities to resolve *Streptococcus pneumoniae* infection, with more BAL colony

forming units (CFU) and less intracellular CFU (86). Increased nasopharyngeal carriage of *Streptococcus pneumoniae* is also found in mice exposed to tobacco flavoured electronic cigarette extract containing nicotine (58). Mice exposed to menthol-flavoured electronic cigarette vapours with nicotine show greater weight loss and mortality following Influenza H1N1 infection, with a greater neutrophil recruitment to the airways (86).

1.5.4.3 Clinical Studies

Several clinical studies assessing electronic cigarette health effects had subjects using their own electronic cigarette containing both flavoured and nicotine; others providing electronic cigarettes for the subjects most commonly used tobacco-flavoured e-liquids or unflavoured ones (please refer to Table 3). Short-term electronic cigarette use does not change pulmonary function parameters in healthy individuals (87-89). Electronic cigarette users have increased sputum and bronchial epithelial MUC5AC levels (90, 91).

Studies assessing the combined but indistinguishable effects of the propylene glycol, glycerol, nicotine and flavours made more or less the same observations as those who investigated the specific compounds. However, without proper control groups, it remains difficult to assess which e-liquid constituent is responsible for the observed effects.

Summary Box 1.4 – Pulmonary effects of vaping that cannot specifically be attributable to a given e-liquid component

- Clear detrimental effects of flavoured-e-liquids with nicotine on lung epithelial cell viability and cell function *in vitro*, with most of the studies using tobacco flavours.
- Adverse effects on airway resistance and lung development.
- The nature of the inflammatory response triggered by flavoured e-liquids with nicotine is not clear, with mixed results from animal models.
- Impaired response to bacterial and viral lung infections with worsened outcomes

1.6 EVALI: What Have We Learned from the 2019 Epidemic?

In Summer and Fall 2019, a series of reports published in the New England Journal of Medicine reported 78 cases of "e-cigarette, or vaping, associated lung injury" (EVALI) over the course of just a few mounts (92-95). These reports raised awareness of the potential harm of acute electronic cigarette use, as most cases were young men (mean age of 19 years old), who mainly vaped for only a few months or years prior to the events. As of February 2020, there has been 2807 hospitalizations associated with EVALI in the United States, of which 50% of cases reported Tetrahydrocannabinol (THC), main psychoactive constituent of cannabis (96). In Canada, current data reveal that of the 20 cases of EVALI reported as of April 2020, only 5 cases reported using THC in their vaping liquids (97). This highlights the difference between the spike in EVALI in September 2019 in the United States, and the rest of the cases previously and subsequently reported in North America. The presence of vitamin E acetate in illegally sourced e-liquids has been the main culprit of the 2019 cases in the United-States (98). To this day, few *in vitro* and animal studies assessed the impact of vitamin E acetate on pulmonary health. A very brief study exposed mice to vitamin E acetate for two weeks and showed increased BAL albumin levels and CD45+ cells in the lungs, suggesting increased lung inflammation. Bhat *et al.* also noted the presence of neutral lipid droplets in BAL and lung tissue cells (99), finding similar to previous case reports [reviewed in (100)]. Another study assessed the chemical composition of THC-containing counterfeit electronic cigarette products retrieved from EVALI patients. Interestingly, they did not find vitamin E acetate in all cartridges, but found hydrocarbons, silicates and aldehydes in both liquid and vapour phases of electronic cigarette cartridges (101). Further analysis from this group showed that air-liquid interface exposure of epithelial cells to vapour from counterfeit cartridges induces IL-6 and IL-8, and that vitamin E acetate alone did not have this inflammatory effect (102). Similar effects were found in mice with increased BAL lipid-laden macrophages, neutrophils and CD4+ T cells, but not in vitamin E acetate controls (102). This case is definitely not closed, and more studies are necessary to find causation and not correlation with electronic cigarette liquid constituents as EVALI cases caused by legal vaping liquid use are still diagnosed in North America.

1.7 Thoughts on The Future of Vaping Research

Our group has been conducting research on vaping for the past 5 years. We were able to identify serious experimental and conceptual limitations and difficulties associated with basic, translational and clinical research on vaping that need to be addressed rapidly to advance our knowledge on the pulmonary and systemic effects of this now not-so-new habit. We humbly make three suggestions to help current and future researchers in their research on vaping.

The need to investigate e-liquid ingredients separately - This review focuses on highlighting the independent pulmonary effects of each major e-liquid constituent. While many studies used combinations of different e-liquids with and without nicotine and/or flavours, allowing to pinpoint the constituent responsible for the effects, others did not and used a single or several e-liquids with nicotine and flavour. If we want to identify the constituents with potential detrimental effects to pulmonary health and decipher the underlying mechanisms, we need to know exactly what each constituent can do. This is relatively easy for propylene glycol, glycerol and nicotine, but much more complex for flavours due to their great molecular diversity. In addition, it is much easier to investigate a myriad of different compounds and conditions in *in vitro* settings than in *in vivo* animal models or clinical settings. This represents a significant experimental challenge that requires our full attention.

Interactions with other pulmonary conditions and diseases - Electronic cigarette users are not only vaping, they smoke, have asthma, are exposure to air pollution, have allergies, catch pneumonia, and have comorbidities (i.e. diabetes, cardiovascular diseases). Pioneer researchers already started looking at interactions between vaping and other lung diseases and extra-pulmonary conditions in animal models. These complex but critical investigations will help enlighten how vaping can impact and interact with other lung irritants and pathogens as well as comorbidities.

Thinking outside the 'smoking' box - From chemical and biological point of view, the only similarity between tobacco cigarettes and electronic cigarettes is the actual name 'cigarette' and the presence of nicotine. Everything else in electronic cigarettes has nothing to do with tobacco smoking. So why keep looking for smoking-like effects on the lungs when

investigating the pulmonary effects of vaping? Why should vaping cause emphysema or chronic inflammation? Is it possible that vaping may impact lung homeostasis in other ways? Well, it is very likely. Broader omics approaches will likely help unveil unexpected pulmonary effects of vaping. Moreover, the constant comparison between exposure to tobacco cigarette smoke and electronic cigarette vapours by assessing biological variable we know are affected by smoking introduce a great bias in favour of supporting the likely false sense of harmlessness of vaping. Meaning that we definitely need to think outside the 'smoking' box.

1.8 Final Remarks

While there is far more to learn on the effects of electronic cigarette use and the underlying mechanisms, it is safe to say that vaping is not innocuous. More properly controlled studies at the cellular, animal and clinical level must be made to assess and regulate the yet to be discovered impact of propylene glycol, glycerol, nicotine and flavours present in electronic cigarette liquids.

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Table 1.1: Methodological characteristics of *in vitro* studies

Cell culture models		
Electronic cigarette vapour exposure		
Air-liquid interface		(11, 12, 16, 17, 19, 21, 28, 34, 54, 55, 65, 66, 71, 73, 74, 77, 78, 103)
E-liquid mixed with cell medium or electronic cigarette vapour extracts		(13-15, 18, 20, 22, 47, 50-53, 56-58, 70, 72, 75, 76, 79, 81, 104)
Cell types		
Lung epithelial cells	16HBE (human)	(16, 53, 64)
	hTBE (human)	(52)
	A549 cells (human)	(12, 13, 18, 50, 58, 64, 73)
	NHBE (human)	(17, 20, 28, 65, 73)
	Beas-2B (human)	(11, 13, 19, 47, 55, 58, 76)
	H292 (human)	(19, 47, 74)
	Primary bronchial epithelial cells (human)	(16, 21, 55)
	Primary small airway epithelial cells (human)	(54)
	3D epithelial cell model (human)	(34, 78)
	Primary tracheal epithelial cells (mouse)	(53)
	RLEC (rat)	(76)
Nasal epithelial cells	HBEpC	(58)
	3D epithelial cell model from healthy donors (human)	(77)
Immune cells	White blood cells (human)	(18)
	Blood neutrophils (human)	(22, 56, 82)
	Blood monocyte-derived dentritic cells (human)	(104)
	THP-1 monocytic cells (human)	(15)
	Kupffer cells (rat)	(72)
	Platelets	(81)
Endothelial cells	HUVEC (human)	(51, 70)
	Primary human microvascular cells-lung derived	(76)
	MLEC (mouse)	(76)

Stem cells	Embryonic stem cells (human)	(14)
	Bone marrow-derived mesenchymal stem cells (human)	(75)
	Neural stem cells (mouse)	(14)
Lung fibroblasts	Human pulmonary fibroblasts	(14)
	HFL-1 (human)	(19, 47, 103)
Skin cells	HaCaTs (human)	(12, 18)
	Primary gingival epithelial cells (human)	(71, 80)
	Periodontal ligament fibroblasts (human)	(80)
Lung surfactant	Infasurf (calf)	(79)
Bacteria	*Streptococcus pneumoniae*	(57)
	Streptococcus gordonii	(66)
	Streptococcus intermedius	(66)
	Streptococcus mitis	(66)
	Streptococcus oralis	(66)
Nicotine		
Low nicotine (< 6 mg/ml)		(14, 19, 55, 57, 70, 76)
Medium nicotine (6 – 18 mg/ml)		(11, 14, 15, 19, 21, 47, 52, 75, 77, 81, 103-105)
High nicotine (> 18 mg/ml)		(13, 14, 17, 19, 20, 22, 28, 47, 50, 54, 58, 66, 71-74, 78-80, 82, 104, 106)
No nicotine		(13, 14, 16, 17, 19-21, 28, 47, 53, 54, 56-58, 66, 72, 73, 79-82)
Unspecified concentration		(12)
Flavours		
Tobacco flavours		(11, 14, 19, 52, 54, 58, 70-74, 76-78, 80-82, 103, 105)
Menthol		(11, 14, 74, 78-80)
Cinnamon		(55)
Coffee		(47)
Fruit flavours		(14, 15, 19, 50, 53, 57, 74, 75, 79)
Desert and sweet drink flavours		(14, 19, 53, 74, 76)
Other		(12)
Targeted flavour molecules		(19, 47, 51, 53, 56)
Unflavoured		(11, 13, 14, 16, 19-22, 28, 55, 66, 76, 79, 104, 106)

Table 1.2: Methodological characteristics of animal studies

Animals models		
Electronic cigarette vapour exposure		
Nose only		(18, 50, 64, 65)
Whole body		(19, 23-35, 59-61, 67-69, 83-86, 107-109, 111)
Electronic cigarette vapour extract		(58)
Electronic cigarette vapour exposure period		
Up to 4 days		(19, 26, 27, 34, 35, 50, 58, 64)
Between a week and a month		(18, 22-25, 31, 68, 69, 83, 84, 86, 107, 109)
Over a month		(27-30, 32, 33, 59-61, 65, 67, 83, 85, 108, 111)
Nicotine		
Low nicotine (< 6 mg/ml)		(69, 76)
Medium nicotine (6 – 18 mg/ml)		(15, 18, 19, 23, 24, 27, 28, 50, 59, 60, 67, 85, 86, 107, 111)
High nicotine (> 18 mg/ml)		(22, 25, 26, 31, 33-35, 58, 61, 65, 68, 83, 84, 108, 109)
No nicotine		(15, 23, 24, 26, 27, 29, 30, 32-35, 61, 69, 108)
Flavours		
Tobacco flavours		(18, 19, 27, 58, 60, 61, 76, 83, 107)
Menthol		(86)
Fruit flavours		(15, 18, 23, 50)
Desert and sweet beverage flavours		(18, 23, 30, 76, 108)
Cinnamon		(23, 84)
Coffee		(18, 85)
Unflavoured		(18, 22, 24-35, 59, 65, 67-69, 109, 111)
Animals		
Mice	BALB/c	(23, 29, 32, 59-61, 67, 84)
	C57BL/6	(19, 22, 25-27, 30, 31, 33-35, 50, 57, 65, 68, 85, 86, 107, 109)
	CD-1	(18, 24, 58, 64, 65, 69)
	A/J	(28)
	FVBN	(111)
	ApoE -/- (C57BL/6 background)	(83, 108)
Rats	Sprague-Dawley	(64)
Sex (all species)		
Male only		(27, 31, 50, 67, 83, 86, 107, 111)

Female only	(18, 22, 24, 25, 29, 30, 32, 33, 58-60, 65, 85, 108)
Both	(23, 26, 34, 35, 61, 68, 84, 109)
Unspecified	(19, 28, 57)

Table 1.3: Methodological characteristics of clinical studies

Clinical studies	
Electronic cigarette use status	
Never users	(39-43, 58, 88, 89)
Active users	(58, 90, 91)
Tobacco cigarette smoking status	
Never smokers	(39, 41-43, 62, 87, 91)
Active smokers	(40, 62, 87-90)
Former smokers	(58, 90)
Health status	
Healthy	(39-43, 58, 62, 87, 88, 90, 91)
Asthmatic	(39, 40)
Period of electronic cigarette use in the study	
Up to 1 hour inclusively	(39, 40, 42, 43, 62, 87)
1 hour to 1 day inclusively	-
1 day to 1 week inclusively	(88)
1 week to 1 month inclusively	(41, 90)
1 to 6 months inclusively	(89)
6 months to 1 year inclusively	
Over 1 year	-
Undisclosed or self-reported	(58, 91)
Number of study participants	
Under 50	(39-43, 58, 62, 87)
Between 51 and 100	(91)
Over 100	(88, 89)
Flavour used	
Tobacco	(87, 89)
Menthol	(89)
Dessert flavours	(62)
Unflavoured	(39-43)
User's choice (multiple flavours)	(58, 90, 91)
Nicotine concentration used	
Low nicotine (< 6 mg/ml)	(40, 89)
Medium nicotine (6 – 18 mg/ml)	(87)
High nicotine (> 18 mg/ml)	(88)
No nicotine	(39-41, 43, 62)
User's choice (multiple nicotine concentration)	(58, 90, 91)
Unknown	(42)
Tissue collected or analysis performed	
Bronchoalveolar lavage	(41, 42)
Nasal epithelial cells	(58)
Sputum	(90)

Bronchial brushings	(41, 42, 91)
Lung function assessment	(39, 40, 42, 62, 87-89)
Cardiac function assessment	(88)
Blood analysis	(40, 43, 87, 89)
Urine analysis	(41, 42)

CHAPTER 2: VARIATIONS IN COIL TEMPERATURE/POWER AND E-LIQUID CONSTITUENTS CHANGE SIZE AND LUNG DEPOSITION OF PARTICLES EMITTED BY AN ELECTRONIC CIGARETTE

2.1 Foreword

The original article presented is Chapter II, called 'Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette' has been published in *Physiological Reports* in 2017 by Ariane Lechasseur, Simon Altmejd, Natalie Turgeon, Giorgio Buonanno, Lidia Morawska, David Brunet, Caroline Duchaine, Mathieu C Morissette. I designed the experimental settings and conducted the experiments, data and statistical analysis. The manuscript was redacted by me and Mathieu C Morissette. Simon Altmejd and David Brunet from Scireq provided the electronic cigarette and other apparatus. Natalie Turgeon and Caroline Duchaine provided expertise for the particle collection and analysis. Giorgio Buonanno, Lidia Morawska and Caroline Duchaine helped revise the manuscript.

2.2 Résumé

La cigarette électronique utilise le propylène glycol et le glycérol pour véhiculer de la nicotine et des saveurs aux voies respiratoires. On dénombre actuellement des centaines de marques de cigarette électronique différentes, des milliers de saveurs disponibles et une multitude de concentrations de nicotine présent dans les liquides de vapotage. Il est donc plausible que ces paramètres de la cigarette électronique et la composition du liquide de vapotage affectent la distribution de taille des particules émises, et leur déposition pulmonaire.

Nous avons utilisé l'extension e-cigarette inExpose pour étudier deux modes de fonctionnement distincts de la cigarette électronique, à savoir à puissance contrôlée et à température contrôlée. Nous avons également évalué plusieurs e-liquides en fonction des concentrations de propylène glycol et de glycérol, de la teneur en nicotine et de certaines saveurs monomoléculaires (menthol, vanilline et maltol). La distribution de la taille des particules a été mesurée en utilisant un *Condensation Particle Counter* et un *Scanning Mobility Particle Sizer*. La déposition pulmonaire des particules a été prédit à l'aide du modèle de la *International Commission on Radiological Protection*.

Pour chaque résistance d'élément chauffant, l'augmentation de la puissance a généré des particules plus grosses. L'augmentation de la température de l'élément chauffant a généré des particules plus petites. L'augmentation de la concentration de glycérol a conduit à la génération de particules plus grosses. En ce qui concerne les arômes, la vanilline augmente considérablement la taille des particules, avec des changements mineurs pour le menthol et le maltol. La présence de nicotine a également augmenté la taille des particules émises. Enfin, les particules émises par la cigarette électronique se sont principalement déposées dans les alvéoles et les conditions générant des particules de plus grande taille ont conduit à une réduction de la déposition pulmonaire prédite.

Cette étude montre que la température de l'élément chauffant, les concentrations de propylène glycol et de glycérol, la présence de nicotine et d'arômes affectent la taille des particules émises par une cigarette électronique, affectant directement le dépôt pulmonaire prévu de ces particules.

2.3 Abstract

Electronic cigarette uses propylene glycol and glycerol to deliver nicotine and flavors to the lungs. Given the hundreds of different brands, the thousands of flavors available and the variations in nicotine concentrations, it is likely that electronic cigarette settings and e-liquid composition affect the size distribution of particles emitted, and ultimately pulmonary deposition.

We used the inExpose e-cigarette extension to study two separate modes of operation of electronic cigarettes, namely power-controlled and the temperature-controlled. We also assessed several e-liquids based on propylene glycol and glycerol concentrations, nicotine content and selected monomolecular flavoring agents (menthol, vanillin and maltol). Particle size distribution was measured using a Condensation Particle Counter and a Scanning Mobility Particle Sizer spectrometer. Lung deposition was predicted using the International Commission on Radiological Protection model.

For all resistance coils, increase in power delivery generated larger particles while maintaining a higher coil temperature generated smaller particles. Increase in glycerol concentration led to the generation of larger particles. With regard to flavors, we showed that despite minor effect of menthol and maltol, vanillin dramatically increased particle size. Presence of nicotine also increased particle size. Finally, particles emitted by the electronic cigarette were predicted to mainly deposit in the alveoli and conditions generating larger particle sizes led to a reduction in predicted lung deposition.

This study shows that coil temperature, propylene glycol and glycerol concentrations, presence of nicotine and flavors affect the size of particles emitted by an electronic cigarette, directly affecting predicted lung deposition of these particles.

Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette

Ariane Lechasseur[1,2], Simon Altmejd[3], Natalie Turgeon[1], Giorgio Buonanno[4,5], Lidia Morawska[5], David Brunet[3], Caroline Duchaine[1,6], Mathieu C Morissette[1,7]

[1]Quebec Heart and Lung Institute - Université Laval, [2]Faculty of Medicine, Université Laval, [3]SCIREQ Scientific Respiratory Equipment Inc., [4]University of Cassino and Southern Lazio, Italy, [5]Queensland University of Technology, Australia, [6]Department de Medicine, Université Laval, [7]Departement of biochemistry, microbiology and bioinformatics, Université Laval

Corresponding author:
Mathieu C Morissette, PhD
2725 Chemin Sainte-Foy
Québec, QC, Canada
G1V 4G5
Tel: 1+ 418 656 8711 ext. 5394
Fax: 1+ 418 656 4509
mathieu.morissette@criucpq.ulaval.ca

Running head: E-cigarette parameters on particle size and lung deposition

Keywords: Electronic cigarette, vaping, e-liquid, particle size, lung distribution, nicotine

2.4 Introduction

Electronic cigarette (e-cigarette) use has markedly increased over the past years. A recent study conducted in the United States showed that e-cigarette use has even surpassed tobacco cigarette use among middle-school and high-school students (Singh et al., 2016). The market for e-cigarettes is greatly diversified. In 2017, 433 brands of e-cigarettes were commercially available (Hsu et al., 2018, Zhu et al., 2014). Moreover, e-liquids are available in over 7000 flavors, with nicotine concentrations ranging from 0 mg/ml to 24 mg/ml (Hsu et al., 2018, Zhu et al., 2014, Goniewicz et al., 2013).

Upon inhalation, the e-liquid, a mixture of propylene glycol (PG), glycerol (Gly), nicotine and/or flavors, is dragged through a heating coil, which leads to its aerosolization (Brown and Cheng, 2014, Talih et al., 2017). The popularity of e-cigarettes is mainly due to the impression of safety surrounding its use (Camenga et al., 2015, Majeed et al., 2017, Farsalinos et al., 2015). However, studies have shown that e-cigarette vapors contain several oxidants, carcinogens and irritants, such as formaldehyde, acetaldehyde, acrolein, methylglyoxal and other free radicals (Bekki et al., 2014, Margham et al., 2016, Farsalinos et al., 2017). It has been shown that increasing puff duration and coil power can increase the generation of these hazardous components (Farsalinos et al., 2017, Gillman et al., 2016). Moreover, addition of nicotine and flavorings also increases the number of potential irritants that are inhaled (Bitzer et al., 2018, Khlystov and Samburova, 2016). However, we currently do not know how e-cigarette settings and e-liquid constituents specifically impact the size of the particle generated and, consequently, lung deposition.

Particle aerodynamic diameter is the main predictor of where inhaled particles will deposit into the lungs and in what proportion (ICRP, 1994). Since variations in aerosolization conditions can very likely impact particle size and ultimately lung deposition, it is critical to assess the impact of the multiple product variation of e-cigarettes (i.e. coil power, PG/Gly ratios, flavors, nicotine content) on particle size and determine how it affects lung deposition. In this study, we assessed the impact of power, temperature, PG/Gly ratios, flavors and nicotine content on the size of particles emitted by an e-cigarette using a single brand of e-cigarette and found that all modifiable aspects of e-cigarette settings tested or e-liquid constituents directly affect particle size and lung deposition.

2.5 Methods

2.5.1 Electronic Cigarette and Aerosol Generation

The inExpose e-cigarette extension (SCIREQ, Montreal, PQ) was used in this study. The inExpose e-cigarette extension is composed of a *Joyetech eVIC-VTC Mini* e-cigarette connected to a computer-controlled system that automates the e-cigarette activation and standardizes the vaping conditions for research purposes.

The inExpose system bypasses the native battery of the *eVIC-VTC,* thereby eliminating aerosol output variations associated with battery drainage. The inExpose puff profiles were configured to a half-sinusoidal shape with a volume of 70 ml, applied every 30 seconds. Total puff run time was of 4.2 seconds. The inExpose system also provided a 2 LPM bias flow to push the e-cigarette vapor into the 45L dilution chamber. The *eVIC-VTC* can be configured in two distinct modes: power-controlled and temperature-controlled. When configured in power-controlled mode, a preset power value is selected by the user (0.5 Ω range 15W – 60W ; 1.5 Ω range : 10W – 25W). During a puff, the power delivered to the coil stays relatively constant during the puff cycle. The constant power translates into a steady increase of the coil's temperature throughout the puff cycle. In the temperature-controlled mode, the power transferred to the coil is regulated with a feedback mechanism. This closed-loop control aims to maintain a constant temperature throughout the puff. The temperature set point is adjustable (range 200°C - 250°C) and configured by the user.

During experiments under the power-controlled mode (50% PG/50% Gly ratio), two different stainless-steel coils were used with respective resistance of 0.5 Ω and 1.5 Ω. Each of these coils was tested at three different power levels (0.5 Ω coil at 24 W, 37.5 W and 51 W and 1.5 Ω coil at 13.2 W, 18 W and 22.8 W). Experiments using the temperature-controlled mode (50% PG/50% Gly ratio) were also conducted. The temperature-controlled experiments were carried out with a coil made of nickel, using the following set points: 210°C, 225°C and 250°C. To assess the impact of PG/Gly ratios, flavors and nicotine, the temperature-controlled setting was used at 210°C. The e-liquids used in the study were composed of 100% PG/0% Gly, 70% PG/30% Gly, 30% PG/70% Gly or 0% PG/100% Gly, with or without 18 mg/ml of nicotine. Menthol (10 mg/ml), vanillin (10 mg/ml) or maltol (5 mg/ml) were added

to a 70% PG/30% Gly or 30% PG/70% Gly e-liquid, in concentrations based on previous studies (Tierney et al., 2016). Flavors and nicotine were added to a 70% PG/30% Gly e-liquid.

2.5.2 Instrumentation and Aerosol Sampling

A new heating coil was used for each parameter investigated. To avoid dry puffing, 5 puffs (70 ml puff, 2 per minute) were made outside the collection system. A total of 5 puffs (70 ml puff, 2 per minute with a 2L/min bias flow) were generated and collected. Vapors diluted with a 40L/min airflow were collected in a 45L barrel placed in a biosafety cabinet to avoid room air particles from being sampled. Measures for each experimental condition were performed in triplicate.

2.5.3 E-Cigarette Particle Size Distribution Analyses

Measurements of particle size distribution were carried out by a Condensation Particle Counter (CPC 3787, TSI Inc.) and a Scanning Mobility Particle Sizer spectrometer (SMPS 3080, TSI Inc). Particle size range was fixed at 20.9 nm to 881.7 nm. Data collection was performed during a scan time of 120 seconds with a sheath flow of 2 LPM and an aerosol flow of 0.2 LPM. Each data acquisition was made in triplicate. Each curve represents the proportion of each particle diameter normalized to the total number of particles analyzed (% of total particles analyzed).

2.5.4 E-Cigarette Particle Lung Deposition Analyses

Lung deposition was calculated using the International Commission on Radiology Protection model (ICRP). Total, head airway region, tracheobronchial airway region and alveolar airway region deposition were assessed according to previously published work (ICRP, 1994):

The head airway deposition fraction DF_{HA} is

$$DF_{HA}=IF\left(\frac{1}{1+e^{6.84+1.183\ln d_p}}+\frac{1}{1+e^{0.924-1.885\ln d_p}}\right)$$

where d_p is the particle size in μm and IF is the inhalable fraction, given by

$$IF = 1-0.5\left(1- \frac{1}{1+0.00076d_p^{2.8}}\right)$$

The tracheobronchial deposition fraction DF_{TB} is

$$DF_{TB} = \left(\frac{0.00352}{d_p}\right)\left(e^{-0.234\left(\ln d_p + 3.40\right)^2} + 63.9e^{-0.819\left(\ln d_p - 1.61\right)^2}\right)$$

The alveolar deposition fraction DF_{AL} is

$$DF_{AL} = \left(\frac{0.0155}{d_p}\right)\left(e^{-0.416\left(\ln d_p + 2.84\right)^2} + 19.11e^{-0.482\left(\ln d_p - 1.392\right)^2}\right)$$

The total deposition DF is the sum of the regional depositions, or

$$DF = IF\left(0.0587 + \frac{0.911}{1+ e^{4.77 + 1.485 \ln d_p}}\right) + \left(\frac{0.943}{1+ e^{0.508 - 2.58 \ln d_p}}\right)$$

Each curve represents the deposition in each lung region multiplied by the emitted relative proportion of each particle diameter analyzed.

2.5.5 Statistical Analyses

Particle size distribution between two experimental groups was assessed by a Kolmogorov-Smirnov test (Table S1). Lung particle deposition between two experimental groups was also assessed using a Kolmogorov-Smirnov test (Table S2). Resulting p-values are indicated in Table S1 and Table S2, a p-value < 0.05 indicating a significantly different distribution between the two compared groups. Statistical analyses were made using GraphPad Prism Software (v. 8, La Jolla California USA).

2.6 Results

2.6.1 E-Cigarette Particle Size Increases in a Coil Power-Dependent Manner.

A large variety of e-cigarette brands are commercially available, meaning numerous possible combinations in coil resistance, power settings and temperature. We first assessed the impact of the coil power on the e-cigarette particle size distribution. A 50% PG/50% Gly e-liquid without flavors or nicotine was used. For the 0.5 Ω coil, we found that increased coil power led to the generation of larger particles (Figure 2.1A; Table S2.1). Similar trends were found for the 1.5 Ω coil (Figure 2.1B; Table S2.1). Intriguingly, while the two lowest temperatures of the temperature-controlled mode generated similar particle size distribution, smaller particles were emitted while using the highest temperature setting (Figure 2.1C; Table S2.1).

2.6.2 A Greater Proportion in E-liquid Glycerol Leads to Larger E-cigarette Particle Size.

We investigated the impact of different PG/Gly ratios on the e-cigarette particle emission. We found that higher Gly proportion, with and without nicotine, led to the generation of larger particles (Figure 2.2A-C; Table S2.1). This phenomenon was also shown in menthol and vanillin containing e-liquids, as larger particles were generated in flavor-containing 30% PG/70% Gly e-liquid compared to the 70% PG/30% Gly e-liquid (Figure 2.3A-C; Table S2.1).

2.6.3 Nicotine Changes E-Cigarette Particle Size Distribution

Addition of nicotine in e-liquids is very common, with concentrations ranging from 0 mg/ml to 24 mg/ml (Tierney et al., 2016). We therefore assessed the impact of nicotine on e-cigarette particle size distribution. Regardless of PG/Gly ratios, addition of nicotine to flavor-free e-liquid increased emitted particle size (Figure 2.2A-C; Table S2.1). However, adding nicotine to flavored e-liquid (menthol, vanillin or maltol) did not affect particle size distribution (Figure 2.4A-C; Table S2.1).

2.6.4 Vanillin Increase E-Cigarette Particle Size

Several flavonoids are used to reproduce the 7000 flavors in which e-liquids are sold. We further assessed the impact of flavors on particle size distribution. We found that adding menthol or maltol to the e-liquid did not change the particle size distribution compared to the unflavored e-liquid (Figure 2.3A-B; Table S2.1). However, adding vanillin drastically increased the e-cigarette emitted particle size (Figure 2.3A-B; Table S2.1).

2.6.5 Variations in E-cigarette Components and E-liquid Composition Affect the Predicted Lung Deposition

We observed several effects of e-cigarette settings and e-liquid constituents on particle size and distribution. Using pre-established lung deposition equations for head airways, tracheobronchial airways and alveoli, we calculated how variations in particle size distribution affects predicted lung deposition. Particles generated by the e-cigarette at any setting and with any e-liquid were predicted to mainly deposit in the alveoli. Conditions that led to an increase in particle size generated by the e-cigarette, such as increase in power and e-liquid glycerol proportion as well as presence of nicotine and vanillin in the e-liquid, led to a reduction in alveolar deposition (Figure 2.5; Table S2.2).

2.7 Discussion

This study is one of the first to document that changing e-cigarette settings and e-liquid composition has an impact on particle size distribution. Consequently, this variation in particle size is also predicted to change how particles emitted by the e-cigarette deposit in the lungs.

In this study, we were able to modulate the power of the heating coil using a single e-cigarette brand. Under the power-controlled setting, we have shown that increased heating coil power leads to increased particle size. This phenomenon was not reproduced when using the temperature-controlled setting. This could be explained by the fact that, since having a fixed power instead of a fixed endpoint temperature, the power-controlled coil reaches greater temperatures than the temperature-controlled coil. Gillman *et al.* assessed the difference between different brands of e-cigarette, showing that greater coil power led to the generation of greater e-cigarette aerosol mass and formaldehyde, acetaldehyde and acrolein levels (Gillman et al., 2016). This shows that changes in e-cigarette model, and therefore coil power, can not only change the particle size distribution but can also change the composition of the aerosols that will be delivered to the lungs.

E-liquids can be sold in several PG/Gly ratios and in a wide range of nicotine concentrations. We found that PG/Gly ratio can impact particle size distribution, as higher Gly concentration increases particle size, as has previously been observed in other studies (Baassiri et al., 2017, Larcombe et al., 2017). This phenomenon could be explained by the fact that PG has a higher volatility than Gly (NCBI, 2018a, NCBI, 2018b). Upon heating, PG is aerosolized at a lower temperature, and thus faster than Gly. Until Gly reaches its aerosolization temperature, condensation is formed in the e-cigarette, leading in time to the formation of larger particles. Real-time assessment of e-cigarette aerosol particle composition and size distribution could help elucidate this phenomenon.

A wide range of nicotine concentrations and flavors in e-liquids are available on the market. We showed that the addition of nicotine also increases particle size. Others have shown that addition of nicotine increases the number of particles that are generated (Fuoco et al., 2014, Manigrasso et al., 2015), as well as their size (Larcombe et al., 2017, Laube et al., 2017).

Although we do not fully understand this phenomenon, it appears consistent across experimental settings that presence of nicotine leads to higher numbers and larger particles and, consequently, a reduced lung deposition.

We also assessed the impact of adding laboratory grade menthol, maltol and vanillin, three flavour molecules frequently found in e-liquids (Tierney et al., 2016), on e-cigarette particle size. In their study, Fuoco *et al.* did not report any changes in particle size when using selene-flavored, strawberry-flavored or two different tobacco-flavored e-liquids (Fuoco et al., 2014). Here, we show that, while menthol and maltol had mild impact on particle size, the addition of vanillin increased particle size. This shows that flavors can have different effects on particle size and that findings made using a given flavor cannot be easily extrapolated to another. Chemical properties of certain flavor molecules could facilitate the generation of larger particle compared to others. However, this remains to be confirmed experimentally.

Changes in power, PG/Gly ratios, nicotine concentration and flavors can change the e-cigarette emitted particle size distribution, potentially affecting lung deposition. Using the ICRP deposition model, we estimated how changes in particle size affected lung deposition (Figure 2.5; Table S2.2). Changes were observed in the total deposition fraction by changes in coil power (Figure 2.5A; Table S2.2), nicotine concentration (Figure 2.5B; Table S2.2), PG/Gly ratios (Figure 2.5C; Table S2.2) and the addition of vanillin (Figure 2.5D; Table S2.2). While few changes in deposition were observed in the head airway region and tracheobronchial airway region, drastic changes in alveolar airway deposition were observed in each variable analyzed. For e-cigarette users, these differences suggest changes in the nicotine deposition, as well as the lung deposition of aforementioned harmful chemical compounds such as formaldehyde, acetaldehyde, acrolein and other free radicals.

Overall, this study shows that changing the e-cigarette setting and e-liquid composition can alter e-cigarette particle size distribution, leading to changes in lung deposition. This may affect the amount of nicotine that is absorbed, and how much PG/Gly and flavors interact with the alveoli. It also highlights how flavoring agents can drastically alter the physicochemical nature of e-liquids. The physiological impacts of these changes remain to be investigated.

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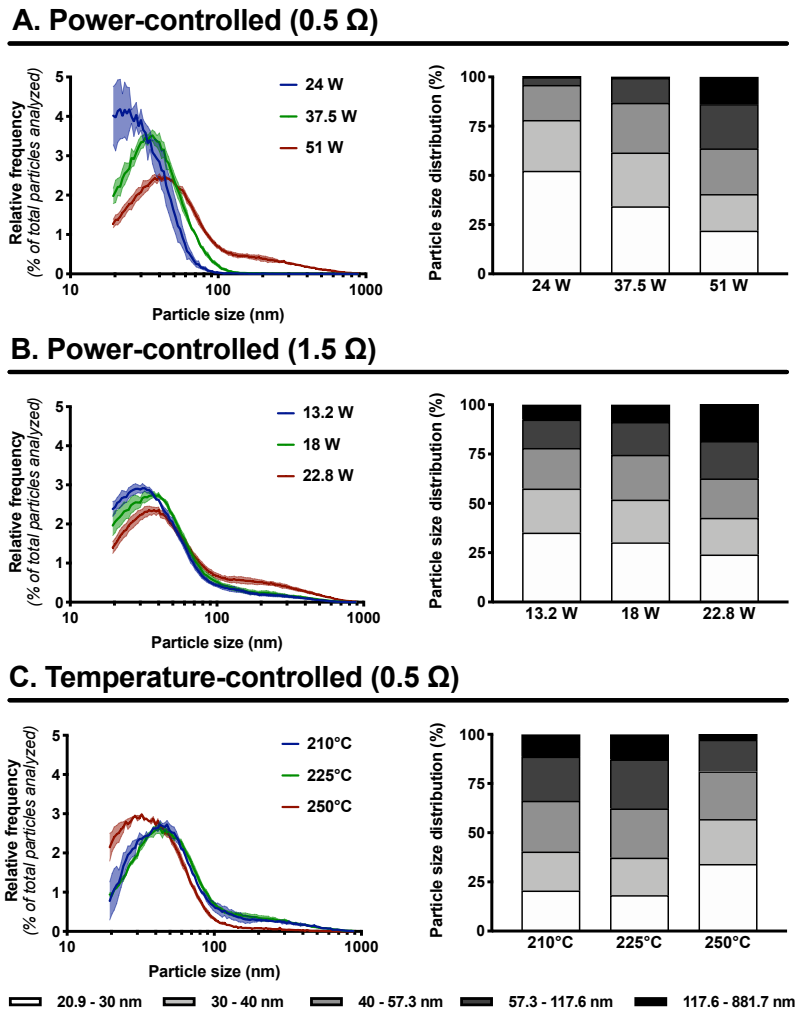

Figure 2.1. Impact of coil power and temperature on size distribution of particles emitted by an e-cigarette.

Size distribution and size intervals of particles emitted by an e-cigarette under (A) a power-controlled setting with a 0.5 Ω coil, (B) a power-controlled setting with a 1.5 Ω coil, and (C) a temperature-controlled setting with a 0.5 Ω coil. In all cases, a 50% PG/50% Gly e-liquid ratio was used, with no nicotine or flavors. Mean (hard line) of 3 replicates per condition ± standard error mean (shade). For each replicate, particle diameter frequencies were normalized to the total number of particles analyzed.

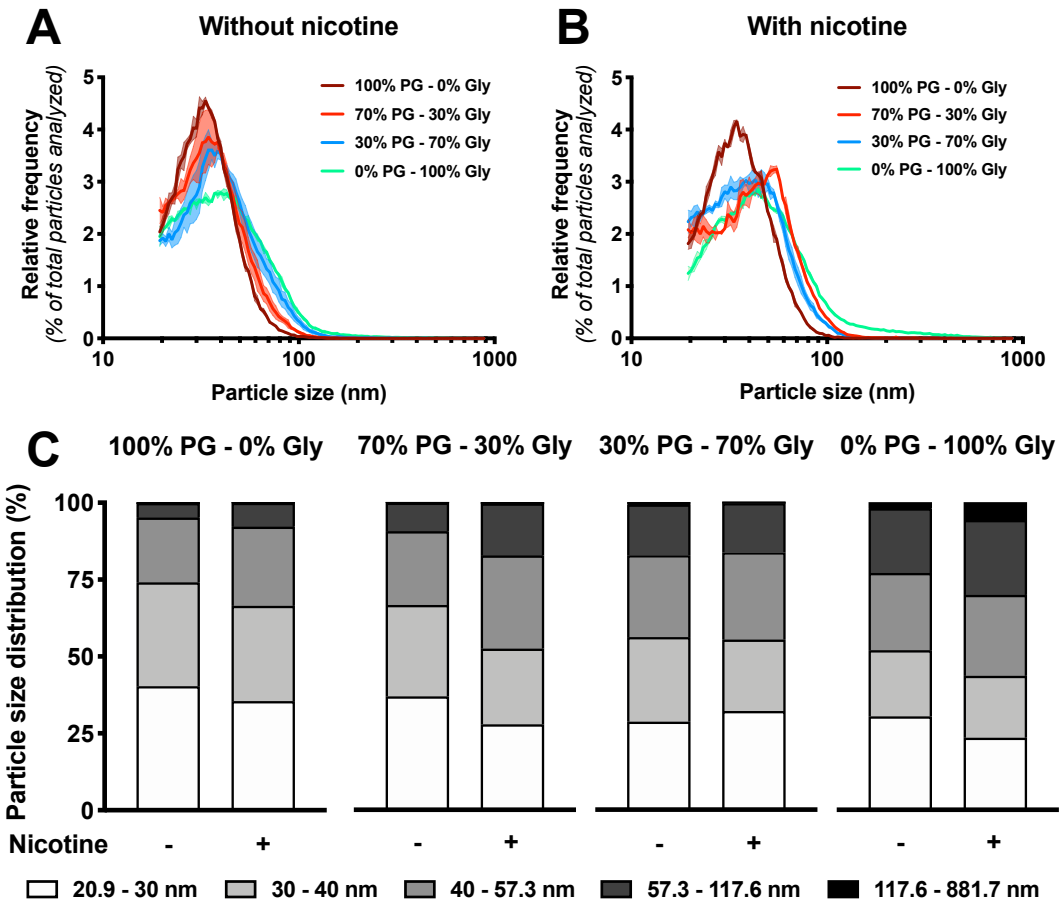

Figure 2.2. Impact of PG/Gly ratios and nicotine on size distribution of particles emitted by an e-cigarette.

Size distribution of particles emitted by an e-cigarette under temperature-controlled set at 210 °C with e-liquid containing (**A**) 0 mg/ml of nicotine or (**B**) 18 mg/ml of nicotine. In both cases, e-liquids made of 100% PG/0% Gly (maroon line), 70% PG/30%Gly (red line), 30% PG/70% Gly (blue line) or 0% PG/100% Gly (teal line) were used, all without flavors. (**C**) Size intervals of particles emitted are presented. Mean (hard or dotted lines) of 3 replicates per condition ± standard error mean (shade). For each replicate, particle diameter frequencies were normalized to the total number of particles analyzed.

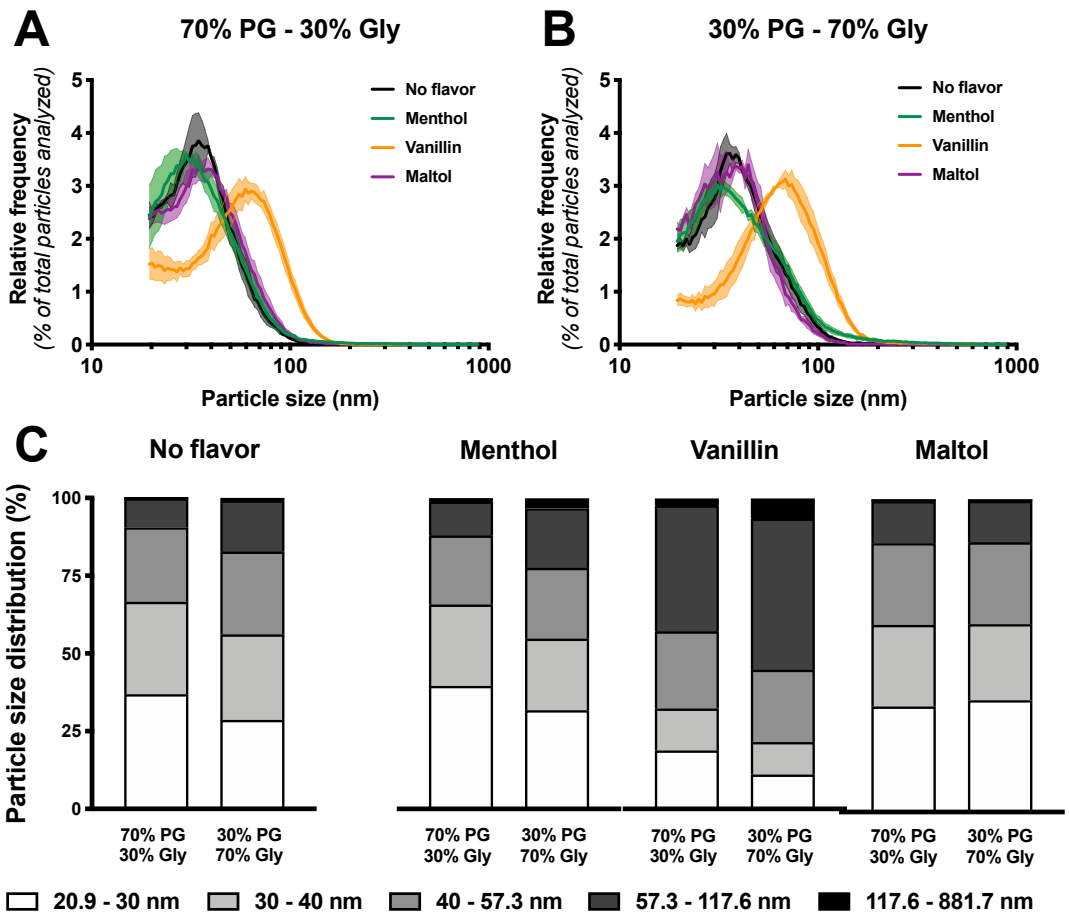

Figure 2.3. Impact of menthol, vanillin or maltol on size distribution of particles emitted by an e-cigarette.

Size distribution of particles emitted by an e-cigarette under temperature-controlled set at 210 °C with (**A**) 70% PG/30% e-liquid or (**B**) 30% PG/70% Gly e-liquid containing no flavor (black line), menthol (green line), vanillin (orange line) or maltol (purple line). (**C**) Size intervals of particles emitted are presented. Mean (hard lines) of 3 replicates per condition ± standard error mean (shade). For each replicate, particle diameter frequencies were normalized to the total number of particles analyzed.

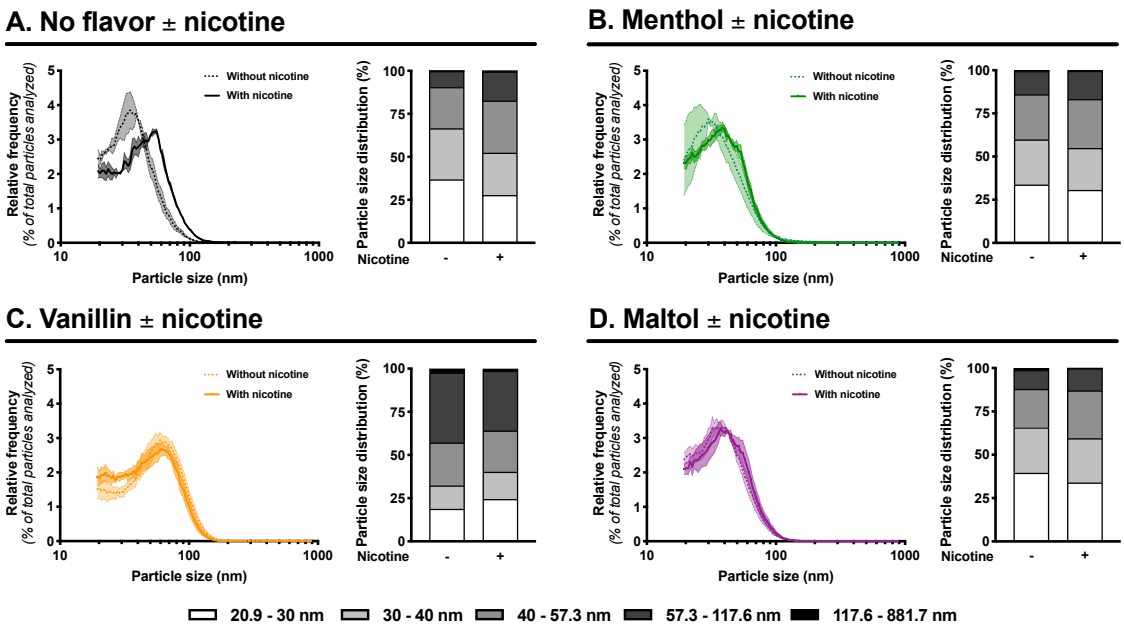

Figure 2.4. Impact of nicotine with menthol, vanillin or maltol on size distribution of particles emitted by an e-cigarette.

Size distribution and size intervals of particles emitted by an e-cigarette under temperature-controlled set at 210 °C with 70% PG/30% Gly e-liquid with (**A**) no flavor, (**B**) menthol (**C**), vanillin or (**D**) maltol without nicotine (dotted line) or with 18 mg/ml of nicotine (hard line). Mean (lines) of 3 replicates per condition ± standard error mean (shade). For each replicate, particle diameter frequencies were normalized to the total number of particles analyzed. For comparisons purposes, Figure 2.4 presents controls without nicotine that were also presented in Figure 2.3A.

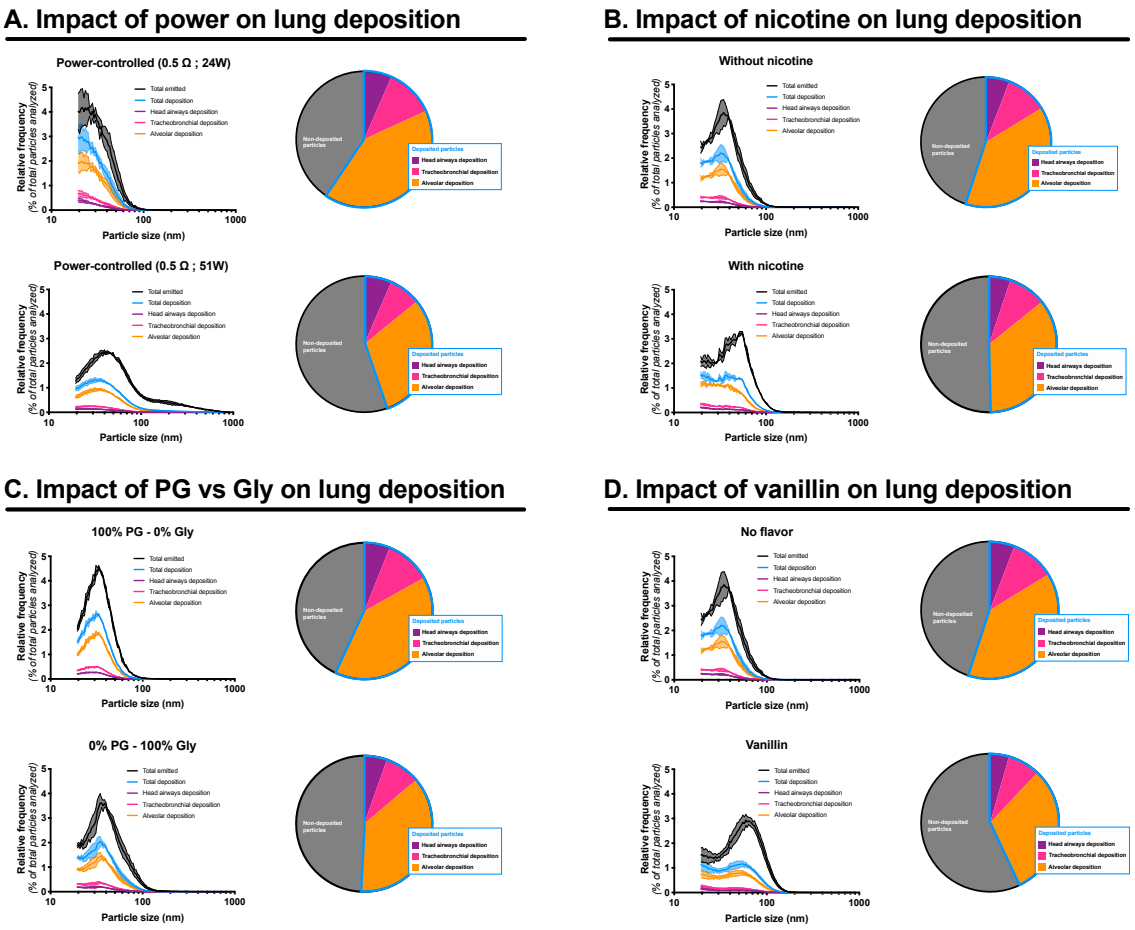

Figure 2.5. Impact of variations in e-cigarette settings and e-liquid constituents on lung deposition of emitted particles.

Lung deposition of particles emitted by the e-cigarette was calculated according to the International Commission on Radiology Protection (ICRP) model. Impact of (**A**) e-cigarette power, (**B**) presence of nicotine in the e-liquid, (**C**) PG-based or Gly-based e-liquid and (**D**) presence of vanillin in the e-liquid are presented. Mean (hard line) of 3 replicates per condition ± standard error mean (shade). Each pie chart represents the percentage of total deposited particles that are specifically deposited in the head region (purple section), tracheobronchial region (pink section) and alveolar region (orange section). For each replicate, particle diameter frequencies were normalized to the total number of particles analyzed.

2.9 Supplementary Material

Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette

Ariane Lechasseur[1,2], Simon Altmejd[3], Natalie Turgeon[1], Giorgio Buonanno[4,5], Lidia Morawska[5], David Brunet[3], Caroline Duchaine[1,6], Mathieu C Morissette[1,7]

[1]Quebec Heart and Lung Institute - Université Laval, [2]Faculty of Medicine, Université Laval, [3]SCIREQ Scientific Respiratory Equipment Inc., [4]University of Cassino and Southern Lazio, Italy, [5]Queensland University of Technology, Australia, [6]Department de Medicine, Université Laval, [7]Departement of biochemistry, microbiology and bioinformatics, Université Laval

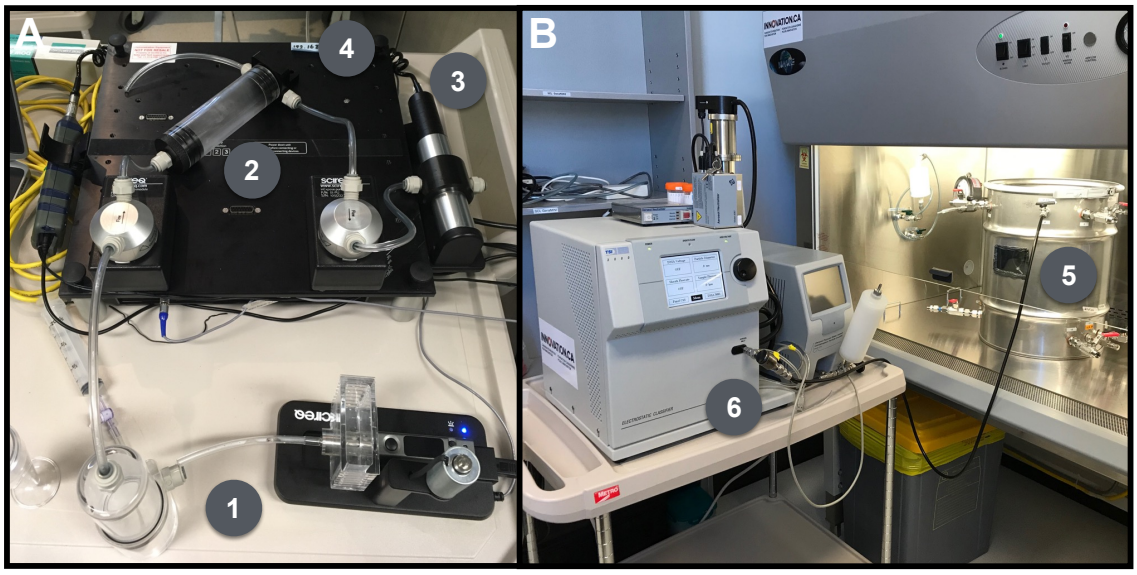

Figure S2.1. Pictures of the vapour-generating device, dilution drum and particle analysing system.

A) 1] the Scireq electronic cigarette module connected to an overflow and condensation collection chamber. **2]** Pump #1 generating the puffs and pump #2 responsible for maintaining the bias flow. **3]** Particle density analyser. **4]** Output to the 45L dilution drum. **B) 5]** Dilution drum. **6]** SMPS particle analyser.

Table S2.1. Statistical analysis of the impact of electronic cigarette settings and e-liquid constituents on particle size distribution

Power-controlled *(50%PG/50%Gly)*	0.5 Ω	24W			
	24W	–	37.5W		
	37.5W	**0.0004**	–	51W	
	51W	**<0.0001**	**<0.0001**	–	
Power-controlled *(50%PG/50%Gly)*	1.5 Ω	13.2W			
	13.2W	–	18W		
	18W	0.6239	–	22.5W	
	22.5W	**0.0022**	**0.0008**	–	
Temperature-controlled *(50%PG/50%Gly)*	0.15 Ω - 70W	210°C †			
	210°C †	–	225 °C		
	225 °C	0.5115	–	250 °C	
	250 °C	**<0.0001**	**<0.0001**	–	
Propylene glycol/Glycerol Ratio *(T°-controlled 210°C)*		100% PG - 0% Gly			
	100% PG - 0% Gly	–	70% PG/30% Gly †		
	70% PG/30% Gly †	**0.0143**	–	30% PG - 70% Gly	
	30% PG - 70% Gly	**<0.0001**	**0.0013**	–	0% PG/100% Gly †
	0% PG/100% Gly †	**<0.0001**	**<0.0001**	**0.0476**	–
Nicotine *(T°-controlled 210°C)*		No vs With Nicotine			
	100% PG - 0% Gly	0.996			
	70% PG/30% Gly †	**0.0476**			
	30% PG - 70% Gly	**0.0217**			
	0% PG/100% Gly †	**0.0092**			
Flavors *(T°-controlled 210°C)*		Without vs With Flavor		Without vs With Nicotine	
		70% PG - 30% Gly	30% PG - 70% Gly		
	Menthol	**0.0003**	**0.0013**	**0.0003**	
	Vanillin	0.1342	**0.0036**	0.0685	
	Maltol	**0.0217**	0.3189	0.5115	

† Data collected in duplicates

Kolmogorov-Smirnov tests were performed - p-values < 0.05 (bold) indicate that compared distributions are significantly different

Table S2.2. Statistical analysis of the impact of electronic cigarette settings and e-liquid constituents on predicted lung deposition of aerosolized particles

Power-controlled *(50%PG/50%Gly)*	**0.5 Ω**	Deposition region	24W			
	24W	Total	–			
		Head airway region	–			
		Tracheobronchial airway region	–			
		Alveolar airway region	–	37.5W		
	37.5W	Total	**0.0004**	–		
		Head airway region	**< 0.0001**	–		
		Tracheobronchial airway region	**0.0004**	–		
		Alveolar airway region	**0.0004**	–	51W	
	51W	Total	**< 0.0001**	**< 0.0001**	–	
		Head airway region	**< 0.0001**	**< 0.0001**	–	
		Tracheobronchial airway region	**< 0.0001**	**< 0.0001**	–	
		Alveolar airway region	**<0.0001**	**< 0.0001**	–	
Power-controlled *(50%PG/50%Gly)*	**1.5 Ω**	Deposition region	13.2W			
	13.2W	Total	–			
		Head airway region	–			
		Tracheobronchial airway region	–			
		Alveolar airway region	–	18W		
	18W	Total	0.1827	–		
		Head airway region	**< 0.0001**	–		
		Tracheobronchial airway region	0.5115	–		
		Alveolar airway region	0.1342	–	22.8W	
	22.8W	Total	**0.0013**	**0.0058**	–	
		Head airway region	**< 0.0001**	**< 0.0001**	–	
		Tracheobronchial airway region	**0.0036**	**0.0092**	–	
		Alveolar airway region	**0.0004**	**0.0013**	–	
Temperature-controlled *(50%PG/50%Gly)*	**0.15 Ω - 70W**	Deposition region	210°C †			
	210°C †	Total	–			
		Head airway region	–			
		Tracheobronchial airway region	–			
		Alveolar airway region	–	225 °C		
	225 °C	Total	**0.0143**	–		
		Head airway region	**< 0.0001**	–		
		Tracheobronchial airway region	**0.0217**	–		
		Alveolar airway region	**0.0143**	–	250 °C	
	250 °C	Total	**0.0004**	**< 0.0001**	–	
		Head airway region	**< 0.0001**	**< 0.0001**	–	
		Tracheobronchial airway region	**0.0008**	**< 0.0001**	–	
		Alveolar airway region	**0.0001**	**< 0.0001**	–	
Propylene glycol/Glycerol Ratio *(T°-controlled 210°C)*		Deposition region	100% PG - 0% Gly			
	100% PG - 0% Gly	Total	–			
		Head airway region	–			
		Tracheobronchial airway region	–			
		Alveolar airway region	–	70% PG/30% Gly †		
	70% PG/30% Gly †	Total	**0.0143**	–		
		Head airway region	**0.0143**	–		
		Tracheobronchial airway region	**0.0143**	–		
		Alveolar airway region	**0.0143**	–	30% PG - 70% Gly	
	30% PG - 70% Gly	Total	**< 0.0001**	**< 0.0001**	–	
		Head airway region	**< 0.0001**	**< 0.0001**	–	
		Tracheobronchial airway region	**< 0.0001**	**0.0003**	–	
		Alveolar airway region	**< 0.0001**	**< 0.0001**	–	0% PG/100% Gly †
	0% PG/100% Gly †	Total	**< 0.0001**	**< 0.0001**	**0.0022**	–
		Head airway region	**< 0.0001**	**< 0.0001**	**0.0004**	–
		Tracheobronchial airway region	**< 0.0001**	**< 0.0001**	**0.0092**	–
		Alveolar airway region	**< 0.0001**	**< 0.0001**	**0.0022**	–
Nicotine *(T°-controlled 210°C)*		Deposition region	Without vs With Nicotine			
	100% PG - 0% Gly	Total	0.8435			
		Head airway region	0.6239			
		Tracheobronchial airway region	0.6239			
		Alveolar airway region	0.8435			
	70% PG/30% Gly †	Total	**0.0143**			
		Head airway region	**0.0013**			
		Tracheobronchial airway region	0.0685			
		Alveolar airway region	**0.0143**			
	30% PG - 70% Gly	Total	**0.0092**			
		Head airway region	**< 0.0001**			
		Tracheobronchial airway region	**0.0092**			
		Alveolar airway region	**0.0092**			
	0% PG/100% Gly †	Total	**0.0013**			
		Head airway region	**< 0.0001**			
		Tracheobronchial airway region	**0.0004**			
		Alveolar airway region	**0.0008**			
Flavors *(T°-controlled 210°C)*		Deposition region	Without vs With Flavor		Without vs With Nicotine	
			70% PG - 30% Gly	30% PG - 70% Gly		
	Menthol	Total	**< 0.0001**	**< 0.0001**	**< 0.0001**	
		Head airway region	**< 0.0001**	**< 0.0001**	**< 0.0001**	
		Tracheobronchial airway region	**0.0003**	**0.0013**	**0.0013**	
		Alveolar airway region	**0.0036**	**0.0001**	**< 0.0001**	
	Vanillin	Total	**0.0324**	**< 0.0001**	0.0685	
		Head airway region	**< 0.0001**	**< 0.0001**	**0.0022**	
		Tracheobronchial airway region	**0.0217**	**0.0003**	**0.0324**	
		Alveolar airway region	0.0968	**< 0.0001**	0.0324	
	Maltol	Total	**0.0036**	**0.0022**	**0.0968**	
		Head airway region	**< 0.0001**	**< 0.0001**	**0.0324**	
		Tracheobronchial airway region	**0.0036**	**0.0217**	0.5115	
		Alveolar airway region	**0.0013**	**0.0143**	0.4086	

† Data collected in duplicates

Kolmogorov-Smirnov tests were performed - p-values < 0.05 (bold) indicate that compared distributions are significantly different

CHAPTER 3: EXPOSURE TO NICOTINE-FREE AND FLAVOR-FREE E-CIGARETTE VAPORS MODIFIES THE PULMONARY RESPONSE TO TOBACCO CIGARETTE SMOKE IN FEMALE MICE

3.1 Foreword

The original article presented is Chapter III, called '*Exposure to nicotine-free and flavor-free e-cigarette vapors modifies the pulmonary response to tobacco cigarette smoke in female mice'* has been published in *American Journal of Physiology - Lung Cellular and Molecular Physiology* in 2020 by Ariane Lechasseur, Carole-Ann Huppé, Maude Talbot, Mélanie Hamel-Auger, Joanie Routhier, Sophie Aubin, Marie-Josée Beaulieu, Marie-Ève Paré, Caroline Duchaine, David Marsolais et Mathieu C Morissette. I designed the experimental settings and conducted the experiments, data and statistical analysis. Carole-Ann Huppé, Maude Talbot, Mélanie Hamel-Auger, Joanie Routhier, Sophie Aubin, Marie-Josée Beaulieu and Marie-Ève Paré helped with mice euthanasia and experiments. The manuscript was redacted by me and Mathieu C Morissette. Caroline Duchaine and David Marsolais helped revise the manuscript.

3.2 Résumé

Contexte. La plupart des utilisateurs de cigarettes électroniques (e-cigarette) fument également des cigarettes de tabac. Dû à la nouveauté de l'utilisation de e-cigarette, l'impact du vapotage sur la santé pulmonaire demeure encore méconnu, encore moins les effets de l'interaction de la double utilisation de la e-cigarette et de la cigarette de tabac.

Méthodes. Nous avons utilisé des modèles murins bien établis pour étudier l'impact de la double exposition aux vapeurs de cigarettes électroniques et à la fumée de cigarette de tabac sur l'homéostasie pulmonaire. Des groupes de souris femelles BALBC/c ont été exposés à l'air ambiant, à la fumée de tabac uniquement, uniquement aux vapeurs de cigarette électronique (sans arôme et sans nicotine) ou encore à la fumée de tabac et aux vapeurs de cigarette électronique. De plus, étant donné que la fumée de tabac et les vapeurs de cigarettes électroniques affectent les processus circadiens dans les poumons, des groupes de souris ont été euthanasiés à deux moments distincts au cours de la journée.

Résultats. Nous avons constaté que la double exposition à la fumée de cigarette et aux vapeurs de e-cigarette avaient modifié l'expression du gène circadien pulmonaire par rapport aux souris exposées à la fumée de tabac seule. Les souris doublement exposées présentaient également des fréquences différentes de cellules dendritiques, de macrophages et de neutrophiles dans le tissu pulmonaire par rapport aux souris exposées à la fumée de tabac seule. Des résultats similaires ont été observés pour les lymphocytes B et les lymphocytes T CD4 + et CD8 +. L'exposition aux vapeurs de cigarettes électroniques a également eu un impact sur les niveaux d'immunoglobulines dans le lavage bronchoalvéolaire et le sérum. Enfin, la cigarette électronique et la double exposition ont augmenté la résistance des voies respiratoires par rapport aux souris exposées à l'air ambiant ou à la fumée de tabac uniquement, respectivement.

Discussion. Ces résultats suggèrent que les vapeurs de cigarettes électroniques, sans nicotine ni saveurs, pourraient affecter la réaction des poumons à l'exposition à la fumée de cigarette de tabac chez les utilisateurs doubles, modifiant potentiellement l'évolution pathologique du tabagisme.

3.3 Abstract

Background. Most of electronic cigarette (e-cigarette) users are also smoking tobacco cigarettes. Due to the relative novelty of this habit, very little is known on the impact of vaping on pulmonary health, even less on the potential interactions of dual e-cigarette and tobacco cigarette use.

Methods. Therefore, we used well-established mouse models to investigate the impact of dual exposure to e-cigarette vapors and tobacco cigarette smoke on lung homeostasis. Groups of female BALB/c mice were exposed to room air, tobacco smoke only, nicotine-free flavor-free e-cigarette vapors only or both tobacco smoke and e-cigarette vapors. Moreover, since tobacco smoke and electronic cigarette vapors both affect circadian processes in the lungs, groups of mice were euthanized at two different time points during the day.

Results. We found that dual-exposed mice had altered lung circadian gene expression compared to mice exposed to tobacco smoke alone. Dual-exposed mice also had different frequencies of dendritic cells, macrophages and neutrophils in the lung tissue compared mice exposed to tobacco smoke alone, an observation also valid for B-lymphocytes and $CD4^+$ and $CD8^+$ T lymphocytes. Exposure to e-cigarette vapors also impacted the levels of immunoglobulins in the bronchoalveolar lavage and serum. Finally, e-cigarette and dual exposures increased airway resistance compared to mice exposed to room air or tobacco smoke alone, respectively.

Discussion. Taken together, these data suggest that e-cigarette vapors, even without nicotine or flavors, could affect how the lungs react to tobacco cigarette smoke exposure in dual users, potentially altering the pathological course triggered by smoking.

Exposure to nicotine-free and flavor-free e-cigarette vapors modifies the pulmonary response to tobacco cigarette smoke in female mice

Ariane Lechasseur[1,2], Carole-Ann Huppé[1,2], Maude Talbot[1,2], Joanie Routhier[1], Sophie Aubin[1], Marie-Josée Beaulieu[1], Caroline Duchaine[1,3], David Marsolais[1,4] and Mathieu C Morissette[1,4].

[1]Quebec Heart and Lung Institute - Université Laval, [2]Faculty of Medicine, Université Laval, [3]Departement of Biochemistry, Microbiology and Bioinformatics, Université Laval, [4]Department of Medicine, Université Laval.

Corresponding author:
Mathieu C Morissette, PhD
2725 Chemin Sainte-Foy
Québec, QC, Canada
G1V 4G5
Tel: 1+ 418 656 8711 ext. 5394
Fax: 1+ 418 656 4509
mathieu.morissette@criucpq.ulaval.ca

Running head: Dual exposure to electronic and tobacco cigarettes

Take home message: Impact of dual exposure to electronic and tobacco cigarettes on the lungs is different from exposure to tobacco cigarette alone; including differences in pulmonary circadian regulatory gene expression, immune cell frequencies, lung functions and immunoglobulins levels.

Funding sources: National Sanitarium association (Dr Morissette); Fondation of the Quebec Heart and Lung Institute (Dr Morissette)

3.4 Introduction

Important research is being conducted worldwide to better understand how vaping and its specific constituents can impact pulmonary health. Meanwhile, the majority of adult users of electronic cigarette (e-cigarette) remain active tobacco smokers (19). This dual use can be temporary or sustained (24). Since tobacco smoking can cause pulmonary health issues and lung pathologies such as emphysema and chronic obstructive pulmonary disease (COPD), dual use could disturb or at least interact with the ongoing pulmonary immune and cellular processes triggered by tobacco smoking and alter the pathological course of developing lung diseases.

While a growing number of clinical and pre-clinical studies are reporting detrimental effects of vaping on pulmonary health, very few were designed to discern the effects of each e-liquid constituent, namely propylene glycol, glycerol, nicotine and flavors. Recent preclinical investigations conducted by Madison *et al*. showed that nicotine-free and flavor-free vapors altered the immune response to influenza virus infection, suggesting the vehicle alone can disturb immune processes in the lungs (18). Madison *et al.* also found that propylene glycol and glycerol exposure impacted pulmonary lipid homeostasis, independently of nicotine and flavors (18). This is of outmost relevance since tobacco smoke has been shown to disrupt pulmonary lipid homeostasis, promoting processes involved in smoking-related lung diseases (14, 20). Moreover, tobacco smoking as well as nicotine-free and flavor-free e-cigarette vapors have been shown to change expression cycles of circadian rhythm regulatory genes (11, 15, 17, 27). Changes in circadian rhythm gene expression have a great impact on the pulmonary immune response (1, 8). These studies provide solid scientific evidence suggesting the vehicle used in e-cigarette could change the nature of the pulmonary response to smoking, possibly interfering with the pathological course of smoking-associated lung diseases in dual users. Moreover, as every e-cigarette user inhales propylene glycol and/or glycerol vapors, evidence that these two e-liquid constituents can impact lung biology in non-smokers and smokers, independently of nicotine or flavors, is of outmost relevance.

In this study, we hypothesized that daily exposure to glycerol and propylene glycol vapors generated by an e-cigarette would change the pulmonary response to tobacco smoke exposure at the immunological and physiological levels. Established *in vivo* mouse models of e-

cigarette (17) and tobacco smoke exposure (14, 29) allowed dissecting the distinct and cumulative impact of the two exposures. Since e-cigarette vapors affect pulmonary markers of circadian rhythmicity (15, 17) and considering the practicality of the euthanasia and data/sample collection procedures, we designed protocols allowing to investigate potential time-dependent phenomena by including two specific euthanasia timepoints, one in the morning and on in the afternoon. We found that nicotine-free flavor-free e-cigarette vapors altered the expression of genes controlling the circadian molecular clock in tobacco smoke-exposed mice and also led to changes in dendritic cells, macrophages, neutrophils and T lymphocyte populations in the lung tissue of tobacco smoke-exposed mice without affecting the inflammatory response in the airway lumen. Circulating and pulmonary immunoglobulin levels were also affected by e-cigarette exposure. Finally, changes in airway resistance were observed in all groups exposed to e-cigarette vapors. Therefore, this study suggests that dual use of tobacco smoking and e-cigarette modifies several immunological and physiological outcomes induced by tobacco smoking alone.

3.5 Methods

3.5.1 Experimental Design

Due to the number of mice included in each group (10 mice/group, 4 AM groups and 4 PM groups), it was impossible to euthanize and process that many animals at the same time. With all the assessments performed, only 20 mice could be processed in the AM and 20 in the PM. Since the main scientific question for this study was to investigate the impact of vaping on the 'normal' lungs (*room air vs E-cig*) and on the lungs of cigarette smoke-exposed mice (*tobacco smoke vs dual use*), we made the choice to emphasize the comparisons between 'room air' and 'E-cig' groups and 'tobacco smoke' and 'dual use' groups. Moreover, 2 separate experimental protocols had to be made, meaning 2 protocols of 8 weeks with euthanasia sequence inverted between the groups (see Figure 3.1A). Thus, to maintain the scientific rigor to a maximum, this means that comparisons between 'room air' *vs* 'tobacco smoke' groups and 'vaping' *vs* 'dual use' groups cannot be made. In this study, our goal was not to investigate the impact of tobacco smoke on the normal lung. The comparison between 'E-cig' *vs* 'dual use' groups is the price that had to be paid to make the study possible. To prevent false comparisons from being made, 'room air' and 'vaping' groups are separated from the 'tobacco smoke' and 'dual use' groups.

3.5.2 Tobacco Smoke and Electronic Cigarette Vapors Exposure

Female 6-8-week BALB/c mice were obtained from Charles River (St-Constant, PQ, Canada). Mice were housed in 12:12 light/dark cycles (light periods from 6 AM to 6 PM) with access to food and water *ad libitum*. Mice were exposed to the mainstream smoke of 24 3R4F research cigarettes with the filter removed, 8 puffs/cigarette, over 2 consecutive hours (University of Kentucky, Lexington, KY, USA) using the Promech SIU24 whole-body exposure system (Promech Lab AB, Vintrie, Sweden) as described previously (12-14). Mice were exposed from 9AM to 11AM, 5 days a week, for 8 weeks (Figure 3.1A).

Exposure to electronic cigarette vapors took place between 1PM and 3PM using a whole-body exposure system (Figure 3.1A) (15). A pump and pinch valve are controlled by a programmable automated system (InExpose control board; SCIREQ Scientific Respiratory Equipment Inc, Montreal, PQ, Canada) to take a 70 ml puff every 20 seconds for 2h from a

refillable commercial e-cigarette (7's hybrid vision, SS Choice LLC; unknown power and coil resistance). The puffs are then mixed with room air at a bias flow of 3L/min and sent in the whole-body exposure chamber by laminar flow where mice breathe freely the vapors. Old vapor can freely exit the exposure chamber at the bottom when new vapor is forced into the chamber at the top to create a continuous laminar flow. E-liquid was made using high-grade USP 70% propylene glycol and 30% glycerol, representative of most e-liquids commercially available. No nicotine or flavor agents were added to the e-liquid. Mice were exposed 2 consecutive hours per day in the afternoon, 5 days a week, for 8 weeks.

Mice were housed according to the Canadian Council for Animal Care (CCAC) guidelines and Université Laval's Animal Research Ethics Board approved all procedures (Animal utilization protocol #2014121-2).

3.5.3 Lung Function Measurement

Mice were euthanized the day following the last exposure. Mice were euthanized at two time points, the first at 7AM corresponding to the morning time point, the second at 1PM corresponding to the afternoon time point.

Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Lung function parameters were attested by FlexiVent (Scireq, Montréal, PQ, Canada). Mice were tracheotomized with an 18-gage blunted needle, mechanically ventilated at a respiratory rate of 150 breaths/min and a tidal volume of 10 mL/kg, with a pressure limit of 30 cmH_2O. Muscle paralysis was achieved using pancuronium (2 mg/kg, Sandoz, Boucherville, PQ, Canada) to prevent respiratory efforts during the measurement. The following sequence of measures was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3 and Pressure/Volume-loop to obtain lung resistance, compliance and elastance, Newtonian resistance, tissue resistance, tissue elastance, a pressure-volume curve, inspiratory capacity, and hysteresis.

3.5.4 Lung Harvesting and Processing

Blood was collected from the retro-orbital vein and serum was isolated (10 min, 12 000 g). Mice were then euthanized by exsanguination by severing the descending aorta. Lungs were

removed from the thoracic cavity and the trachea was cannulated. A part of the right lobe was tied up, dissected, snap frozen in liquid nitrogen and stored at -80°C for transcriptional analyses, or kept on ice until flow cytometry processing. The left lobe was lavaged with 250 µl and 200 µl of cold PBS sequentially. Cells were then pelleted (10 min, 800 g, 4°C) and suspended in PBS for cytology analyses. Cytospins were stained using Hema3 (Fisher Scientific, Ottawa, ON, Canada) for differential counts, for which 300 cells per sample were counted using the ImageJ software (v. 1.47). BAL macrophage size was determined by measuring the surface of 30 macrophages per mice, again using the ImageJ software, and was expressed as a percentage of the average macrophage surface for room air exposed group, which was set to 100%.

3.5.5 Flow Cytometry

Lungs were minced and digested with 0.1% collagenase IV for 90 min at 37°C, then passed through a 70 µm cell strainer to obtain a single cell suspension. Red blood cells were lysed, and single-cell suspensions were passed through a 70 µm cell strainer second time. Data were acquired using a FACS Diva-driven LSR Fortessa (BD Biosciences, Franklin Lake, NJ). Results were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA). Primary antibodies raised against CD45 (30-F11), CD11c (N418), MHCII (M5/114.15.3), GR-1 (RB6-8C5), CD90.2 (30-H12), CD4 (RM4-5), CD8 (53-6.7) (Biolegend, San Diego, CA, USA), CD11b (M1/70; eBioscience, Waltham, MA, United States) and CD19 (1D3; Abcam, Cambridge, UK) were used.

From the CD45+ cells, frequency of autofluorescent–, CD11b+, CD11c+, MHCII+ dendritic cells; autofluorescent+, CD11c+ macrophages; autofluorescent–, CD11b+, GR-1+ neutrophils; autofluorescent–, CD19+ CD90.2– B cells; autofluorescent– CD19– CD90.2+ CD4+ T cells; and autofluorescent– CD19– CD90.2+ CD8+ T cells were obtained (Figure 3A, 4A). Since macrophages' autofluorescence increases with tobacco smoke exposure, fluorescence minus one analysis (FMO) were performed separately for room air and electronic cigarette groups and for tobacco smoke and dual exposed groups. Frequencies of cell subsets were expressed as a percentage of CD45+ cells.

3.5.6 Cytokine and Immunoglobulin Quantification

BAL fluid CCL2 and IL-1α concentrations were assessed using the mouse CCL2/JE/ MCP-1 and Mouse IL-1 alpha/IL-1F1 DuoSet® ELISA (R&D systems, Minneapolis, MN, USA) respectively, according to the manufacturer's instructions. BAL and serum IgA, IgG, and IgM concentrations were assessed using the mouse IgA, IgG and IgM ELISA kit (Invitrogen – Thermofisher Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions.

3.5.7 Quantitative PCR

Total RNA was extracted using TRIzol reagent (Fisher Scientific). RNA quantification and purity were assessed with the Synergy H1 plate reader and the Gen5 software (BioTek, VT, USA). RNA integrity was assessed by gel electrophoresis. 1 µg of RNA was converted into cDNA using the iScript Advanced cDNA synthesis kit (Bio-rad). qPCR analyses were performed using SsoAdvanced Universal SYBR Green Supermix (Bio-rad) and primers (IDT, Coralville, IA, USA) at 300 nM (See Table 1 for primer information). qPCRs were performed using a CFX384 Touch qPCR System (Bio-rad) as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 55-60°C for 30 s followed by a melt curve to assure specificity. For each gene, a temperature gradient was made to define the ideal annealing temperature. A calibration curve was also made to determine the PCR efficiency and a r^2. All qPCR efficiencies were between 90 and 110%, with r^2 values ranging between 0.97–1.00. Data were acquired and analyzed with the CFX Manager software (version 3.1). For each gene, C_q values were determined as the intercept of each amplification curve with the threshold establish in the calibration curve. All reactions were performed in triplicate (SD < 0.3). Gene expression levels were assessed using *hprt* and *rplp0* reporter genes using the ΔΔCq method.

3.5.8 Statistical Analysis

For two-group comparisons in Figures 3.1, 3.2, 3.3, 3.4, and 3.6, two-sided t-tests were performed. For Figure 3.5, since the distribution of immunoglobulin levels was not normal according to Shapiro-Wilk statistical test, Mann-Whitney tests were performed for two-group

comparisons. Statistically significant differences were considered if $p < 0.05$. All statistical analyses were performed using Prism 8 from GraphPad Software, Inc. (La Jolla, CA, USA).

3.6 Results

3.6.1 Exposure to Nicotine-Free Flavor-Free E-Cigarette Vapors Modifies the Effects of Tobacco Smoke Exposure on The Pulmonary Transcript Levels of Circadian Regulatory Genes

Our group and others previously showed that exposure to e-cigarette and tobacco smoke changes the expression of key circadian regulatory genes (15, 17). We first investigated the impact of dual exposure to e-cigarette vapors and tobacco smoke on the expression of *arntl*, *nr1d1*, *nr1d2*, *per1*, *per2* and *per3*. Mice were exposed to tobacco smoke for 2h/day in the morning and to e-cigarette vapors for 2h/day in the afternoon, 5 days/week for 8 weeks. Mice were euthanized in the morning or afternoon (Figure 3.1A). Compared to room air, exposure to e-cigarette vapors led to significant decrease in the expression of *nr1d2* ($p<0.05$) and a tendency for increased expression for *arntl* ($p=0.074$) and decreased expression for *per1* ($p=0.11$) in the morning. No changes in expression were found in the afternoon (Figure 3.1B). Compared to tobacco smoke alone, dual exposure led to significant changes mainly noticeable in the afternoon, with increased expression for *arntl* and decreased expression for *nr1d1*, *nr1d2*, *per2* and *per3* (Figure 3.1C). These observations emphasize how exposure to e-cigarette vapors can alter ongoing circadian regulatory adjustments in the lung.

3.6.2 Exposure to Nicotine-Free Flavor-Free E-Cigarette Vapors Does Not Change Tobacco Smoke-Induced Inflammation in The Bronchoalveolar Lavage

Tobacco smoke exposure rapidly induces an immune response in the lungs often characterized by an influx in neutrophils, increased macrophages size and the release of cytokines and chemokines such as CCL2 and IL-1α in the airway lumen (reviewed in (4, 26)). Since tobacco smoke-induced lung inflammation has been linked to several detrimental pulmonary outcomes, we investigated the impact of e-cigarette vapors on classical features of tobacco smoke-induced lung inflammation. As previously reported (6, 10, 16, 18, 21, 31), vapors from nicotine-free flavor-free e-cigarette vapors did not induce cellular inflammation in the BAL, with no noticeable increase in neutrophils or mononuclear cells compared to room air exposure (Figure 3.2A). A slight reduced size of alveolar macrophages (Figure 3.2B) and increase in BAL IL-1α (Figure 3.2C) were detected. A reduction in *icam1*, *vcam1*

and *pigr* mRNA levels in the lung tissue was also found, with no changes in *muc5ac* levels (Figure 3.2D). With regard to the impact of e-cigarette vapors on tobacco smoke exposure, we did not observe any difference in BAL cell numbers (Figure 3.2E) or in CCL2 levels or IL-1α levels (Figure 3.2G). W observed a reduction in the size of alveolar macrophages in the dual exposure group compared to tobacco smoke alone (Figure 3.2F). Lung tissue *icam1*, *vcam1* and *pigr* mRNA levels were reduced in the dual exposure compared to the tobacco smoke exposure group, with no changes in *muc5ac* mRNA levels (Figure 3.2G). In light of these results, exposure to e-cigarette vapors does not exacerbate classical features of tobacco smoke-induced lung inflammation but leads to lower *icam1*, *vcam1* and *pigr* mRNA levels and smaller alveolar macrophages.

3.6.3 Exposure to Nicotine-Free Flavor-Free E-Cigarette Vapors Causes Changes in Lung Tissue Immune Cell Populations and Modifies the Effects of Tobacco Smoke Exposure

Tobacco smoke exposure causes changes in immune cell populations in the lung tissue (reviewed in (4, 5)). Therefore, we used flow cytometry (Figure 3.3A and 3.4A) to assess the impact of e-cigarette vapors on key immune cell populations in the lung parenchyma, namely macrophages, neutrophils, dendritic cells and B and T lymphocytes. Exposure to e-cigarette vapors alone decreased the frequency of neutrophils in the afternoon and tend to decrease macrophage and increase neutrophil frequencies in the morning (Figure 3.3B). In the context of tobacco smoke exposure (Figure 3.3C), e-cigarette exposure reduced macrophage proportion in the afternoon and increased dendritic cell proportions (p=0.062) in the morning. Exposure to e-cigarette vapors alone significantly increased the frequency of B lymphocytes and decreased the frequency T lymphocyte populations in the morning (Figure 3.4B). In the context of tobacco smoke exposure, additional exposure to e-cigarette vapors reduced the proportion of $CD4^+$ and $CD8^+$ T lymphocytes in the afternoon (Figure 3.4C). The fact that immune cell frequencies were changed only in the morning or the afternoon again emphasizes the circadian nature of immune changes caused by tobacco smoke and e-cigarette vapors.

3.6.4 Exposure to Nicotine-Free Flavor-Free E-Cigarette Vapors Affects Pulmonary and Circulating Immunoglobulin Levels in Normal and Tobacco Smoke Exposure Conditions

We observed that exposure to e-cigarette vapors changes the proportion of B lymphocytes in the lungs, suggesting that it could also affect immunoglobulin levels. Moreover, tobacco smoke exposure is known to affect pulmonary and circulating immunoglobulin levels (7). Therefore, we assessed the impact of e-cigarette vapors on IgG, IgM and IgA immunoglobulin levels in both BAL fluid and serum. We observed that e-cigarette alone significantly reduced IgM levels in the BAL of mice euthanized in the morning without affecting circulating immunoglobulins (Figure 3.5A and 3.5B). Interestingly, e-cigarette vapors did not significantly affect BAL immunoglobulins in tobacco smoke-exposed animals but markedly reduced circulating IgM levels in mice euthanized in the afternoon (Figure 3.5A and 3.5B). These observations show that exposure to e-cigarette vapors can have local and systemic effects on immunoglobulin production and localization, also interacting with tobacco smoke to modulate this biological process.

3.6.5 Exposure to Nicotine-Free Flavor-Free E-Cigarette Vapors Increases Airway Resistance

Tobacco smoke exposure alters lung functions and cause chronic lung pathologies. Clinical and preclinical studies have shown that e-cigarette vapors exposure can affect lung functions but effects are dependent on nicotine content in e-liquids and animal exposure models (2, 6, 16, 18, 22, 25). Exposure to e-cigarette vapors did not change the profile of the pressure-volume (P-V) loop compared to room air-exposed as the average curve of both groups overlap (Figure 3.6A-B). Yet, exposure to e-cigarette vapors alone increased Newtonian resistance (upper airway resistance) and reduced tissue damping, with a tendency to reduce elastance in mice euthanized in the afternoon (Figure 3.6A). No change was observed in inspiratory capacity, hysteresis, compliance, resistance, and tissue elastance in both morning and afternoon (Figure 3.6A). Exposure to e-cigarette vapors in the context of tobacco smoke exposure did not affect the profile of the pressure-volume (P-V) loop compared to those of mice exposed to tobacco smoke alone, as, again, the average curve of both groups overlap (Figure 3.6B). Dual exposure led to a higher airway resistance and Newtonian resistance in the morning, with no changes in the afternoon (Figure 3.6B). No significant change was

observed in inspiratory capacity, hysteresis, compliance, elastance, tissue elastance and tissue damping in both morning and afternoon (Figure 3.6B). Exposure to e-cigarette vapors appears to affect airway resistance in normal and tobacco smoke exposure conditions, a phenomenon that appears to vary during the daytime.

3.7 Discussion

In this study, we aimed to conduct preclinical investigations on the effects of nicotine-free flavor-free e-cigarette vapors in non-pathological conditions. Seeing as most e-cigarette users are tobacco cigarette smokers as well, we investigated the impact of e-cigarette vapors when superimposed to tobacco smoke exposure. To do so, we used established preclinical models of e-cigarette vapors and tobacco smoke exposure. Of great interest, we found that, in the context of tobacco smoke exposure, exposure to nicotine-free flavor-free e-cigarette vapors altered the pulmonary circadian rhythm regulatory gene expression, did not change the inflammatory response in the airway lumen but did affect the proportion of dendritic cells, macrophages, neutrophils and T lymphocytes in the lung tissue, markedly reduced circulating IgM levels and, finally, increased airway resistance. This is the first study reporting biological interactions between tobacco smoke and e-cigarette exposures.

Tobacco smoke exposure has been shown to disrupt the pulmonary expression of genes controlling the circadian molecular clock. Moreover, key circadian molecular clock genes *arntl* and *nr1d1* are involved in the pulmonary response to tobacco smoke (11, 28). Our group previously found that e-cigarette exposure induced changes in the expression of circadian regulatory genes in various tissues (17), which has since been confirmed by other groups (15). In the present study, an important new finding is that exposure to e-cigarette vapors causes changes in the expression of genes controlling the circadian molecular clock in the context of tobacco smoke exposure. This suggests that the disrupting effects of tobacco smoking on pulmonary circadian rhythmicity can be further altered by vaping. While the mechanisms linking vaping and circadian rhythm alterations remain to be deciphered, they appear to be powerful enough to interfere with the effects of tobacco smoke on these same genes. It has been suggested that tobacco smoke reduces Sirtuin1 (SIRT1) levels in the lungs, leading to increased *arntl* acetylation and degradation (11). It is therefore possible that propylene glycol and glycerol vapors could also affect the SIRT1-ARNTL axis and change the effects of tobacco smoke on circadian rhythmicity. However, aside from potential mechanisms, repercussions of altered circadian rhythmicity in the lungs can be observed on immunity and lung functions, providing a valid rationale for pathology-modifying effects of

nicotine-free flavor-free vaping that have been observed, notably on the response to influenza infection (18).

As previously documented by our group and others, exposure to nicotine-free flavor-free e-cigarette vapors does not cause any noticeable inflammatory reaction in the lungs (6, 10, 16, 18, 21, 31), as opposed to tobacco smoke exposure (14, 29). Moreover, in the present study, we found that exposure to nicotine-free flavor-free e-cigarette vapors does not modify the mononuclear and neutrophilic response to tobacco smoke, nor does it appear to cause major alterations in the inflammatory process happening in the airway lumen. One recurrent observation was that exposure to nicotine-free flavor-free e-cigarette vapors causes alveolar macrophages to become slightly smaller and, in the context of tobacco smoke exposure, prevents alveolar macrophages from getting as big as they would get under tobacco smoke exposure alone (20). Moreover, this observation appears to be rhythmic, again suggesting implication of the circadian molecular clock. This effect of vaping on macrophages is somehow in contradiction with recently published data by Madison *et al.* suggesting that nicotine-free flavor-free e-cigarette vapors can cause macrophages to become enlarged and accumulate neutral lipids (18). However, Madison *et al.* did not provide a thorough quantification of the size of alveolar macrophages, which does no guarantee that the overall size of macrophages increased. Our data therefore suggest that nicotine-free flavor-free vaping does not lead to increased alveolar macrophage size, rather leads to a timely decreased size compared to room air exposure or tobacco cigarette smoke exposure.

It is well established that immune cell trafficking is under circadian control and there is evidence that circadian processes influence immune cell migration in the lungs (23). We found that e-cigarette exposure alone or with cigarette smoke exposure led to reduced levels of *icam1*, *vcam1,* integrins and a receptor highly important in circadian immune cell recruitment from the periphery suggesting potential alterations in cell recruitment (23). Interestingly, this decrease in expression was matched by reduced proportions of dendritic cells, macrophages, neutrophils as well as B and T lymphocytes in the lung of e-cigarette-exposed mice. The fact that exposure to e-cigarette vapors can affects these immune cell populations is therefore of outmost relevance. Indeed, neutrophils and macrophages are extremely important cell types in the development of COPD, and there is data suggesting that

CD4 and CD8 T lymphocytes may also play an important role in COPD pathogenesis (reviewed in (4, 5, 26). We also found decreased levels of pulmonary and circulating IgM immunoglobulin levels. Concurrently, we observed decreased expression levels of *pigr*, a receptor implicated in IgM and IgA transcytosis into the airway lumen (30). This shows that e-cigarette vapors alter mechanisms involved in leukocyte trafficking, resulting in changes in lymphoid and myeloid cell frequencies in the lung tissue. While we currently do not know if these effects are positive or negative, vaping may have the ability to alter both the pulmonary innate and adaptive response to tobacco smoke and other inflammatory stimuli.

Potential impact of e-cigarette use on lung functions is a critical aspect. Alongside circadian and immunological changes, e-cigarette exposure, alone or during dual exposure to tobacco smoke, did alter some lung function parameters such as Newtonian resistance and tissue damping, but left the dynamic of the pressure-volume (P-V) loop unchanged. Others have reported increased airway resistance in e-cigarette exposure models (16). This increase in upper airway resistance was not accompanied by an increase in mucus secretion, as *muc5ac* expression remained unchanged following e-cigarette exposure. Interestingly, alterations in airway resistance vary during the day, suggesting that processes interconnecting circadian rhythmicity and inflammatory processes might be involved. Active inflammatory processes in large and smaller airways have been linked to increased resistance of the respiratory system, such as in asthma (3, 9). Mechanisms behind propylene glycol and/or glycerol-mediated increased airway resistance remain to be further investigated.

Limitations. The present study does have some limitations. E-cigarette and tobacco smoke exposure models, as well as the sequence of exposure during the day, may not reflect exactly what happens in humans. Due to technical limitations, only two time points of euthanasia were selected, limiting the data resolution needed to assess the full circadian changes induced by e-cigarette. However, despite these limitations, data clearly show that propylene glycol and glycerol can affect lung biology and change how the lungs respond to smoking. Only female mice were used in this study. Considering sex differences in lung biology, these observations cannot be extrapolated to male mice. The experimental design does not allow for direct comparison between ‘room air’ and ‘tobacco smoke’ groups or between ‘E-cig’ and ‘dual use’ groups. While numerous studies document the impact of tobacco smoke on

the lungs, the impossibility to directly compare the ‘E-cig’ group and the ‘dual use’ group is a minor but clear limitation of the study.

This study clearly shows that the lungs of mice exposed to both tobacco smoke and e-cigarette vapors have different features than the lungs from mice exposed to tobacco smoke only. While the biological mechanisms leading to this phenomenon remain to be identified, our findings, along with others, support that the vehicle contained in e-cigarettes, glycerol and propylene glycol, are not inert and could possibly have diseases-modifying effects in dual users who are smoking and vaping.

3.8 References

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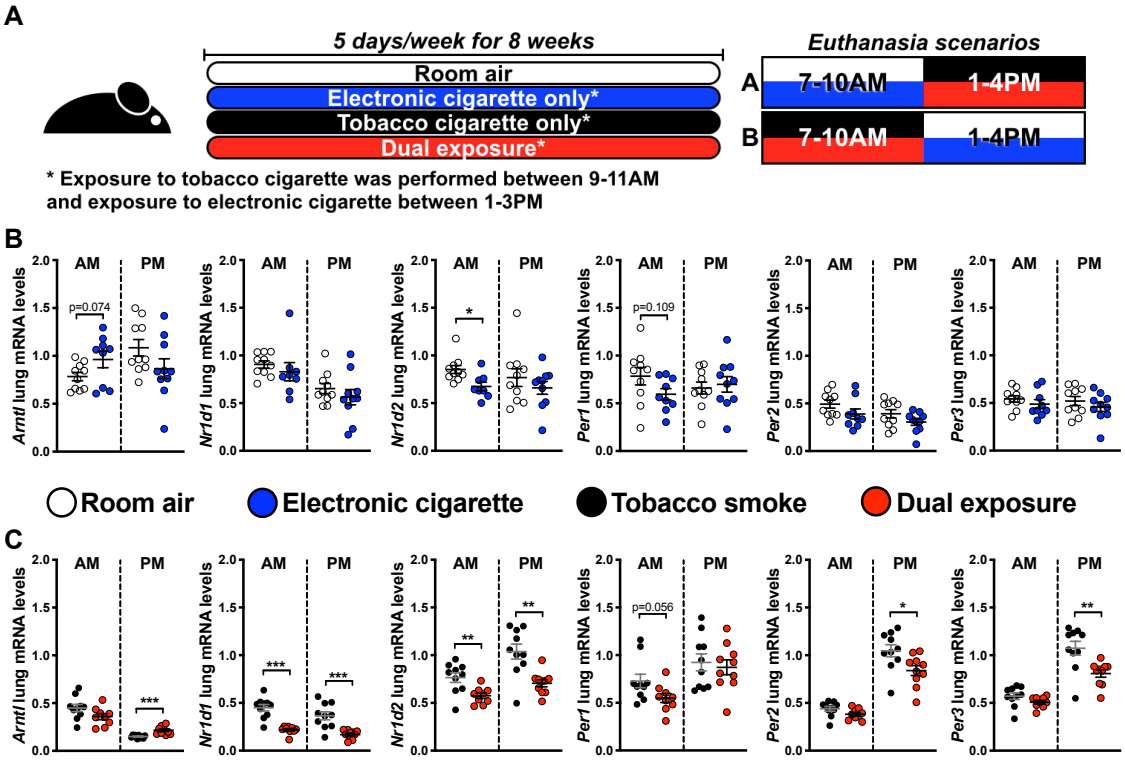

Figure 3.1. Impact of e-cigarette and dual exposure on pulmonary circadian rhythm regulatory genes.

(A) Six to eight-week-old mice were exposed to e-cigarette vapors from 9AM to 11AM and/or tobacco smoke from 1PM to 3PM, for eight weeks (n=9-10/group). Mice were euthanized in the morning or in the afternoon. (B) Lung expression of circadian regulatory genes was assessed for room air and e-cigarette exposed mice as well as for (C) cigarette smoke and dual-exposed mice. Data are presented as mean ± SEM. Two-sided Student T-tests were performed. *p < 0.05; **p < 0.01; ***p < 0.001.

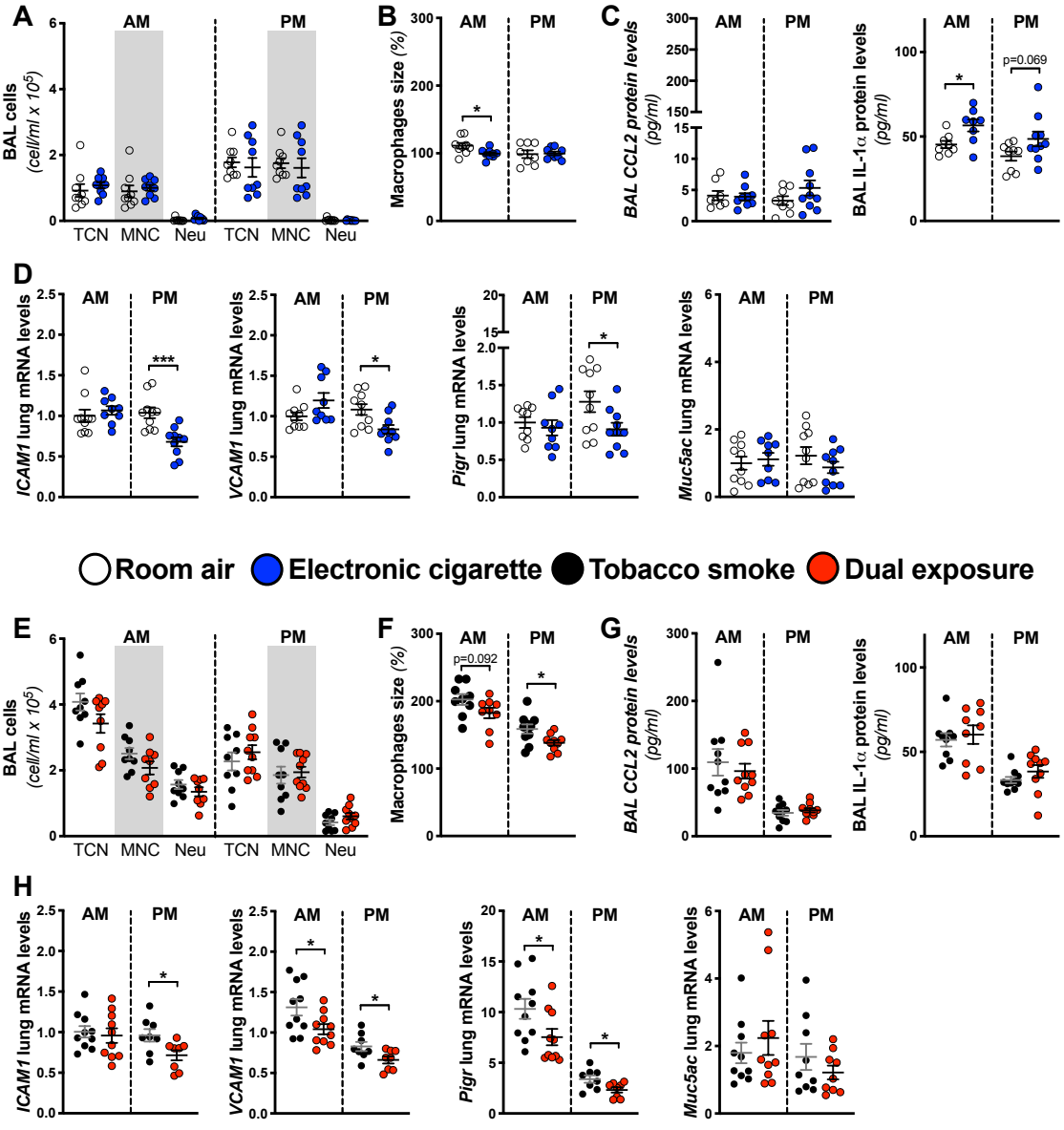

Figure 3.2. Impact of e-cigarette and dual exposure on bronchoalveolar lavage inflammation.

Six to eight-week-old mice were exposed to e-cigarette vapors from 9AM to 11AM and/or tobacco smoke from 1PM to 3PM, for eight weeks (n=9-10/group). Mice were euthanized in the morning or in the afternoon. Bronchoalveolar lavage (BAL) total and differential cell number were assessed (A, E). Pulmonary macrophage size measurements were assessed (B, F). BAL CCL2, G-CSF and IL-1α protein levels were assessed by ELISA (C, G). Lung expression of *icam1*, *vcam1, pigr* and *muc5ac* was assessed for room air and e-cigarette exposed mice (D) as well as for cigarette smoke and dual-exposed mice (H). TCN = total cell number; MNC = mononuclear cell number; Neu = Neutrophil cells number. Data are presented as mean ± SEM. Two-sided Student T-tests were performed. *p < 0.05; ***p < 0.001.

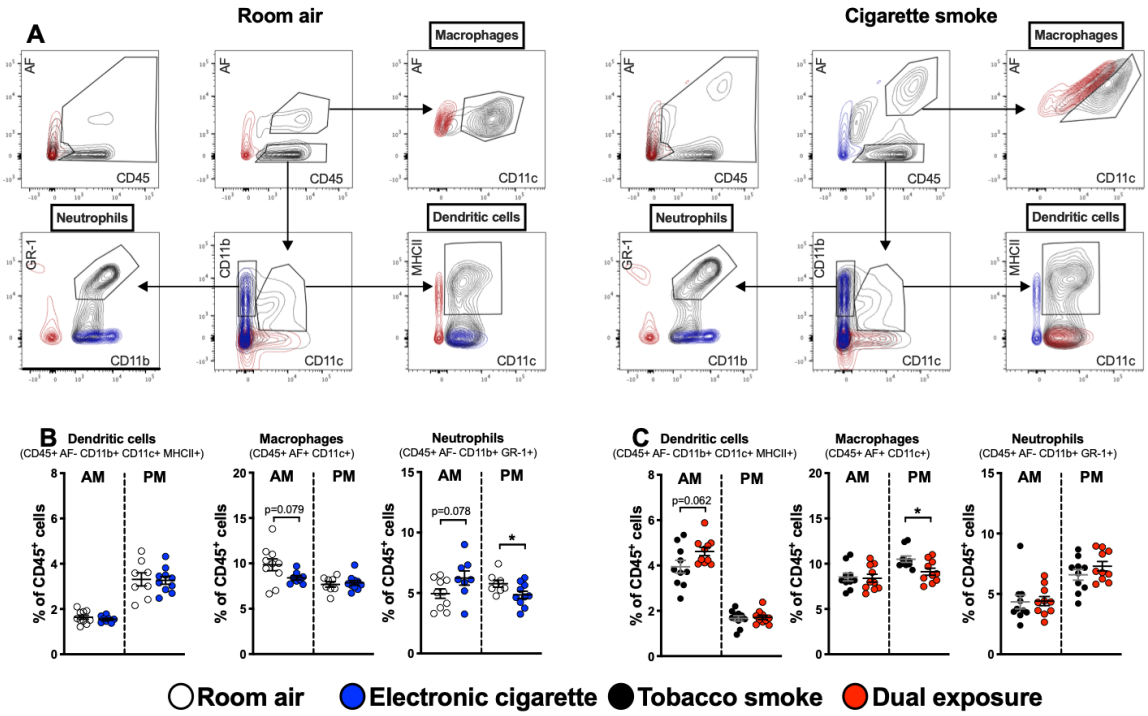

Figure 3.3. Impact of electronic cigarette dual exposure on myeloid cell frequencies.
Six to eight-week-old mice were exposed to e-cigarette vapors from 9AM to 11AM and/or tobacco smoke from 1PM to 3PM, for eight weeks (n=9-10/group). Mice were euthanized in the morning or in the afternoon. (A) Dendritic cells, macrophages and neutrophils were assessed by flow cytometry; black contour lines denote the fully-stained samples, blue contour lines and red contour lines represent the FMO controls for the X and Y axes parameters, respectively. Frequencies of cell subsets are expressed as a percentage of CD45+ cells for room air and e-cigarette exposed mice (B), as well as for cigarette-exposed and dual exposed mice (C). Data are presented as means ± SEM. Two-sided parametric Student T-tests were performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

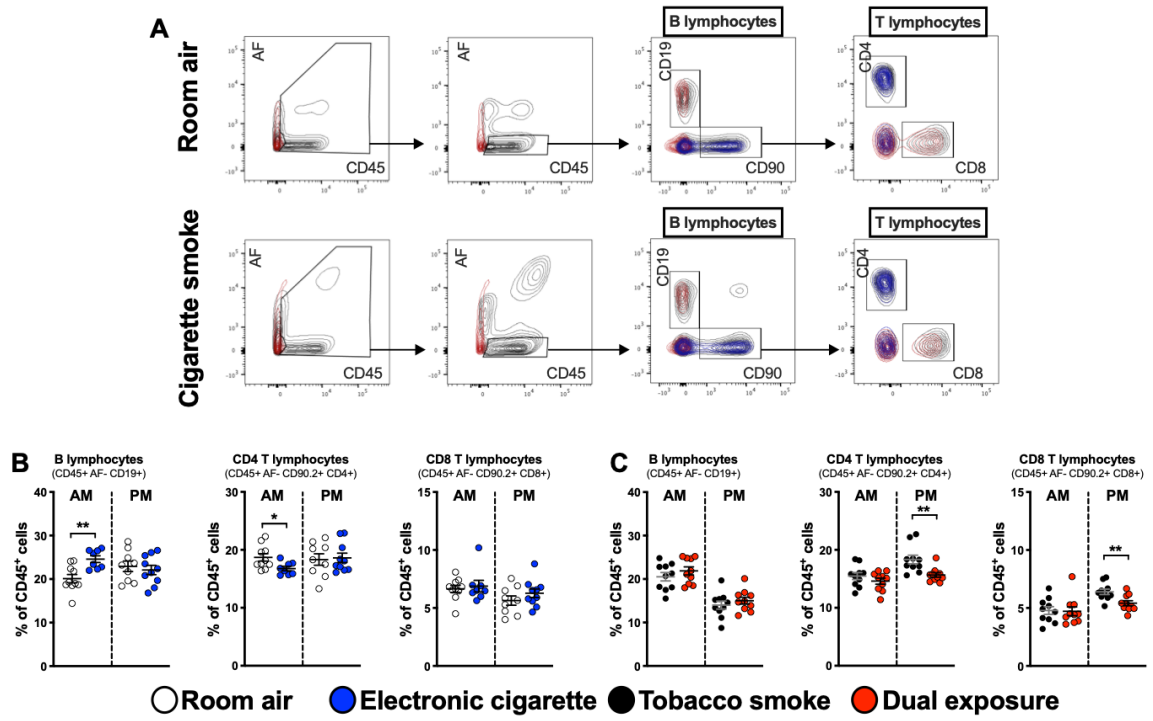

Figure 3.4. Impact of electronic cigarette dual exposure on lymphocyte cell frequencies. Six to eight-week-old mice were exposed to e-cigarette vapors from 9AM to 11AM and/or tobacco smoke from 1PM to 3PM, for eight weeks (n=9-10/group). Mice were euthanized in the morning or in the afternoon. (A) B cell, T cell, CD4+ T cell and CD8+ T cell populations were assessed by flow cytometry; black regions are a representative sample, blue and red regions are FMOs for staining on the X and Y axis respectively. Frequencies of cell subsets were expressed as a percentage of CD45+ cells for room air and e-cigarette exposed mice (B), as well as for cigarette-exposed and dual exposed mice (C). Data are presented as mean ± SEM. Two-sided parametric Student T-tests were performed. *$p < 0.05$, **$p < 0.01$.

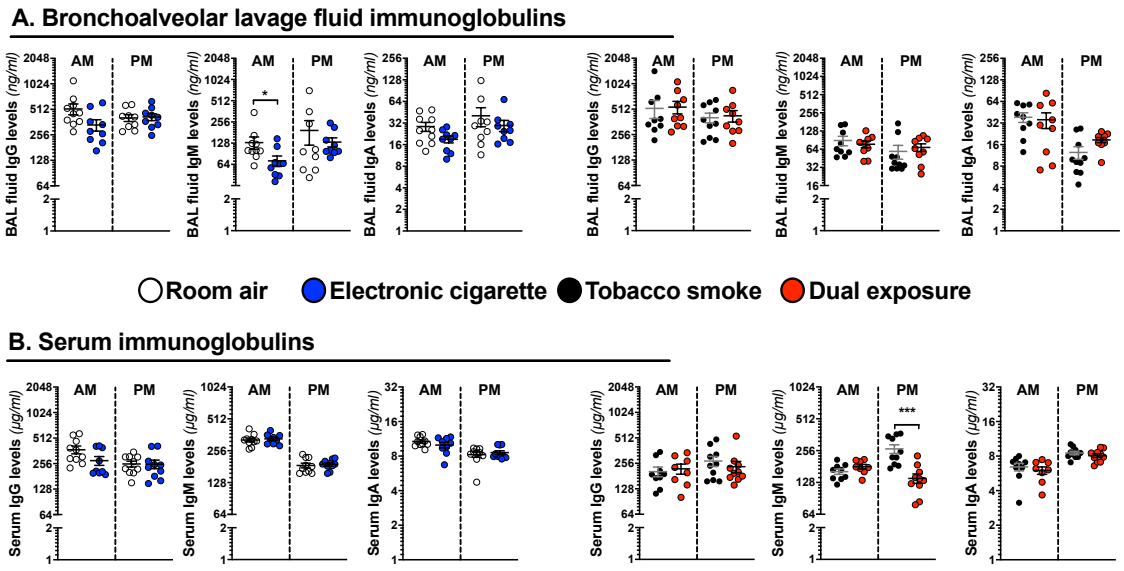

Figure 3.5. Impact of e-cigarette and dual exposure on pulmonary and circulating immunoglobulins.

Six to eight-week-old mice were exposed to e-cigarette vapor from 9AM to 11AM and/or tobacco smoke from 1PM to 3PM, for eight weeks (n=9-10/group). Mice were euthanized in the morning or in the afternoon. IgA, IgG and IgM levels were assessed by ELISA in the bronchoalveolar lavage (A) and serum (B) by ELISA. Data are presented as mean ± SEM. Mann-Whitney non-parametric tests were performed. *$p < 0.05$; ***$p < 0.001$.

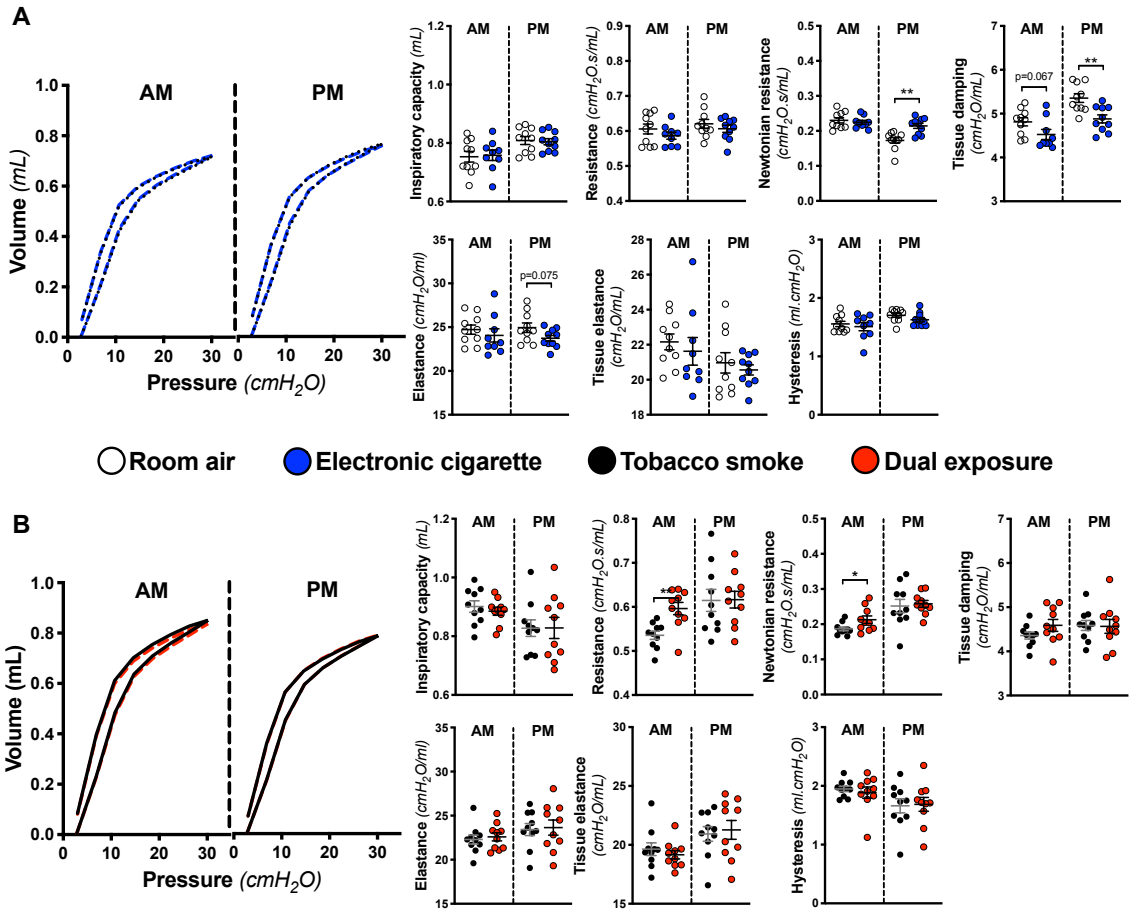

Figure 3.6. Chronic exposure to e-cigarette vapors and cigarette smoke affects lung resistance.

Six to eight-week-old mice were exposed to e-cigarette vapors from 9AM to 11AM and/or tobacco smoke from 1PM to 3PM, for eight weeks (n=9-10/group). Mice were euthanized in the morning or in the afternoon. Lung function parameters were assessed by FlexiVent for room air and e-cigarette exposed mice (A) as well as for cigarette smoke and dual exposed mice (B). Data are presented as mean ± SEM. Two-sided parametric Student T-tests were performed. *p < 0.05; **p< 0.01.

Table 3.1. Primer sequences

Gene symbol	Sequence accession number	Amplicon size (pb)	Exon	Primer sequences	Annealing temperature (°C)
Arntl	NM_007489	100	9-10	For: CGG TCA CAT CCT ACG ACA AAC Rev: CAG AAG CAA ACT ACA AGC CAA C	57
Hprt	NM_013556	125	2-3	For: AGC AGG TCA GCA AAG AAC T Rev: CCT CAT GGA CTG ATT ATG GAC A	57
Icam1	NM_10493.3	121	2-3	For: AGC AGG TCA GCA AAG AAC T Rev: CTG TGC TTT GAG AAC TGT GG	57
Nr1d1	NM_145434	101	1-2	For: GAG CCA CTA GAG CCA ATG TAG Rev: CCA GTT TGA ATG ACC GCT TTC	57
Nr1d2	NM_011584	112	3-4	For: ACA GTT CTC ATT CTT CAG GCA Rev: GGC ATC AGG ATT CCA CTA TGG	57
Per1	NM_011065	133	19-20	For: CTT TGC TTT AGA TCG GCA GTG Rev: CTT CCT CAA CCG CTT CAG A	57
Per2	NM_011066	118	8-10	For: TGA GGT AGA TAG CCC AGG AG Rev: GCT ATG AAG CGC CTA GAA TCC	57
Per3	NM_011067	114	4-6	For: CTC TTC TCT CTG TCT CCA CCT Rev: TCC AAC TCA GCT TCC TTT CTG	57
Rplp0	NM_007475	96	5-6	For: ATC ACA GAG CAG GCC CTG CA Rev: CAC CGA GGC AAC AGT TGG GT	57
Vcam1	NM_11693.3	115	5-6	For: GCA AAG GAC ACT GGA AAA GAG Rev: TGT GCA GTT GAC AGT GAC A	57

Table 3.2. Overview of the observed impacts of E-cig on the normal and smoking lungs

E-cig* vs room air	°Mild impact on the expression of genes controling the circadian molecular clock (AM)
	°No major effects on lung inflammation but marked reduction in ICAM1, VCAM1 and PIGR (PM)
	°Slight alterations in lung neutrophil (PM), B and CD4+ lymphocyte (AM) populations
	°Reduced IgM levels in the BAL (AM)
	°Increased Newtonian resistance (PM) and reduced tissue damping (PM)
Dual use vs tobacco	°Very significant impact on the expression of genes controling the circadian molecular clock (AM and PM)
	°No major effects on lung inflammation but marked reduction in ICAM1, VCAM1 and PIGR (AM and PM)
	°Reduced lung macrophage, CD4+ and CD8+ lymphocyte populations (PM)
	°Reduced serum IgM levels (PM)
	°Increased Resistance and Newtonian resistance (AM)

* E-cig refers to vapors from nicotine-free and flavor-free e-liquid

CHAPTER 4: GLYCEROL CONTAINED IN ELECTRONIC CIGARETTE AEROSOLS AFFECTS ENERGY METABOLISM IN A SEX-DEPENDENT MANNER

4.1 Foreword

The original article presented is Chapter III, called '*Glycerol contained in electronic cigarettes affects the liver and aspects of energy homeostasis in a sex-dependent manner'* has been submitted in *American Journal of Physiology - Endocrinology and metabolism* in 2021 by Ariane Lechasseur, Mathilde Mouchiroud, Félix Tremblay, Gabrielle Bouffard, Nadia Milad, Marie Pineault, Michaël Maranda-Robitaille, Joanie Routhier, Marie-Josée Beaulieu, Sophie Aubin, Mathieu Laplante et Mathieu C Morissette. I designed the experimental settings and conducted the experiments, data and statistical analysis. Mathilde Mouchiroud, Félix Tremblay, Gabrielle Bouffard, Nadia Milad, Marie Pineault, Michaël Maranda-Robitaille, Joanie Routhier, Marie-Josée Beaulieu, Sophie Aubin helped with mice euthanasia and experiments. The manuscript was redacted by me and Mathieu C Morissette. Mathieu Laplante helped revise the manuscript.

4.2 Résumé

Contexte : La cigarette électronique (e-cigarette) est de plus en plus populaire chez les jeunes et les adultes. Les liquides de vapotage contenus dans les e-cigarettes sont principalement composés de propylène glycol et de glycérol, auxquels s'ajoutent de la nicotine et des arômes. Parmi plusieurs autres processus biologiques, le glycérol est un substrat métabolique utilisé pour la synthèse des lipides après un repas, ainsi que pour la synthèse du glucose lors du jeûne. Dans cette étude, nous avons étudier les effets de l'exposition aux aérosols de glycérol émis par la cigarette électronique sur certains aspects de l'homéostasie du glycérol et du glucose.

Méthodes : Des souris C57BL/6 adultes et jeunes, mâles et femelles, ont été exposées à des aérosols de cigarette électronique contenant un liquide de vapotage composé de 100% de glycérol de qualité USP en utilisant notre système d'exposition de type « whole body ». Les souris ont été exposées de manière aiguë (exposition unique de 2 heures) ou chronique (2 h/jour, 5 jours/semaine pendant 9 semaines). Les concentrations de glycérol et de glucose circulants ont été mesurées et des tests de tolérance au glycérol et au glucose ont été réalisés. Le foie a également été étudié afin d'évaluer les changements histologiques, dans la teneur en lipides, ou bien sur les niveaux d'inflammation et de marqueurs de stress. Les fonctions pulmonaires ont également été évaluées, ainsi que l'expression de l'ARNm hépatique des gènes contrôlant le rythme circadien.

Résultats : Une exposition aiguë aux aérosols de glycérol générés par une cigarette électronique a augmenté les taux de glycérol circulant chez les souris femelles. Une augmentation des concentrations hépatiques de triglycérides et de phosphatidylcholine a été observée chez les souris femelles, sans toutefois d'augmentation de l'ALT circulante ou de signe d'inflammation, de fibrose ou de stress du réticulum endoplasmique. L'exposition chronique aux aérosols de cigarettes électroniques au glycérol a eu un impact modéré sur le test de tolérance au glucose chez les jeunes souris mâles et femelles. Les niveau de glycérol, glucose et d'insuline à jeun sont restés inchangés. Une résistance pulmonaire accrue a été observée chez les jeunes souris mâles. Des changements dans l'expression des gènes régulateurs circadiens hépatiques ont été observés chez les jeunes souris mâles et femelles.

Discussion : Cette étude exploratoire montre que le glycérol contenu dans les liquides de cigarettes électroniques peut affecter le foie ainsi que des aspects de l'homéostasie du glucose et du glycérol. Des travaux supplémentaires sont nécessaires pour traduire ces observations aux humains et déterminer les impacts biologiques et potentiellement pathologiques de ces découvertes.

4.3 Abstract

Background: Electronic cigarette (e-cigarette) is increasingly popular among the young and adult population. Vaping liquids contained in e-cigarette are mainly composed of propylene glycol and glycerol, to which nicotine and flavors are added. Among several biological processes, glycerol is a metabolic substrate used for lipid synthesis in fed state as well as glucose synthesis in fasting state. We aimed to investigate the effects of glycerol e-cigarette aerosol exposure on aspects of glycerol and glucose homeostasis.

Methods: Adult and young male and female C57BL/6 mice were exposed to electronic cigarette aerosols with 100% USP-grade glycerol as vaping liquid using an established whole-body exposure system. Mice were exposed acutely (single 2-hour exposure) or chronically (2 h/day, 5 days/week for 9 weeks). Circulating glycerol and glucose levels were assessed and glycerol as well as glucose tolerance tests were performed. The liver was also investigated to assess changes in the histology, lipid content, inflammation, and stress markers. Lung functions were also assessed as well as hepatic mRNA expression of genes controlling the circadian rhythm.

Results: Acute exposure to glycerol aerosols generated by an electronic cigarette increased circulating glycerol levels in female mice. Increased hepatic triglyceride and phosphatidylcholine concentrations were observed in female mice with no increase in circulating ALT or evidence of inflammation, fibrosis or endoplasmic reticulum stress. Chronic exposure to glycerol electronic cigarette aerosols mildly impacted glucose tolerance test in young female and male mice. Fasting glycerol, glucose and insulin remained unchanged. Increased pulmonary resistance was observed in young male mice. Changes in hepatic circadian regulatory gene expression were found in both young male and female mice.

Discussion: Taken together, this exploratory study shows that the glycerol contained in electronic cigarette liquids can affect the liver as well as aspects of glucose and glycerol homeostasis. Additional work is required to translate these observations to humans and determine the biological and potentially pathological impacts of these findings.

Glycerol contained in electronic cigarette aerosols affects energy metabolism in a sex-dependent manner

Ariane Lechasseur[1,2], Mathilde Mouchiroud[1,2], Félix Tremblay[1,2], Gabrielle Bouffard[1,2], Nadia Milad[1,2], Marie Pineault[1,2], Michaël Maranda-Robitaille[1,2], Joanie Routhier[1], Marie-Josée Beaulieu[1], Sophie Aubin[1], Mathieu Laplante[1,3] et Mathieu C Morissette[1,3].

[1]Quebec Heart and Lung Institute - Université Laval, Quebec, Canada; [2]Faculté de médecine, Université Laval, Quebec, Canada; [3]Department of Medicine, Université Laval, Quebec, Canada.

Running title: Glycerol in vaping liquids affects liver and energy homeostasis

Disclosures: The authors have no relevant disclosures or conflict of interest

Corresponding Author:

Dr. Mathieu Morissette

Québec Heart Lung Institute – Université Laval

2725 chemin Sainte-Foy

Québec, QC, Canada, G1V 4G5

Phone: +1 418 656 8711 ext. 5394

Email: mathieu.morissette@criucpq.ulaval.ca

4.4 Introduction

Electronic cigarette (e-cigarette) use, also known as vaping, is now a widespread habit about which we know very little of the safety and biological effects. In North America, e-cigarette use has surpassed tobacco cigarette smoking among adolescents and young adults (3, 4, 11). Cases of severe lung injury, called 'Electronic cigarette or vaping product use-associated lung injury' or EVALI, suggest asymptomatic as well as symptomatic biological effects of vaping are to be expected. Therefore, as vaping is now common across many demographic groups, the need to identify the biological effects of this habit and the specific role of each constituent is crucial.

The major constituents of vaping liquids are propylene glycol, glycerol, nicotine and a wide variety of flavoring chemicals. This liquid is aerosolized upon contact with a battery-powered heat-generating atomizer and inhaled by the user. The biological effects of nicotine are well established, thanks to decades of research to tobacco smoking, and flavoring agents greatly vary from a vaping liquid to another (36). Propylene glycol and/or glycerol are the common constituents of every vaping liquid, which act as vehicles for nicotine and flavors and also facilitate aerosolization.

Main focus has been placed on investigating pulmonary effects of e-cigarette emissions, since its aerosols are inhaled. However, a study by our research group showed that both glycerol and propylene glycol have the ability to change the expression of genes controlling the circadian rhythm in the lungs, but also in the liver, kidneys and skeletal muscles, suggesting systemic effects independent of nicotine or flavors (25).

While propylene glycol is a man-made chemical, glycerol is found in large quantities in living organisms and is involved in numerous metabolic processes. Among others, glycerol acts as a substrate for gluconeogenesis during fasting periods to support glucose synthesis in the liver and maintain glycemia. It also acts as a building block for several lipid species including triglycerides and phospholipids [reviewed in: (32)]. To this day, only a few studies investigated the impact of glycerol vaping on energy metabolism, and none included both male and female mice of various ages.

In this study, we aimed at investigating in male and female mice the impact of glycerol vaping on the liver and aspects of energy homeostasis in a well-established model of e-cigarette exposure. We found that glycerol can accumulate in the blood of female mice exposed to glycerol aerosols generated by an e-cigarette, a phenomenon not observed in males. We also found that long-term exposure increases hepatic triglyceride and phosphatidylcholine contents in younger and older female mice, but not in their male counterparts. We also found mild alterations in glucose and glycerol tolerance tests, also showing sexual dimorphism. Interestingly, these metabolic effects were happening in the absence of major changes in lung functions, suggesting metabolic effects of glycerol vaping can precede its effects on lung physiology. Finally, this study suggests that glycerol vaping can impact liver and energy metabolism, especially in females.

4.5 Methods

4.5.1 Glycerol E-cigarette Aerosol Exposure

Male and female young (6-week-old) and adult (12-week-old) C57bl/6 mice were purchased from Charles River (St-Constant, PQ, Canada). Mice were housed in 12:12 light/dark cycles (light periods from 6 AM to 6 PM) with access to food and water *ad libitum*. Mice were housed according to the Canadian Council for Animal Care (CCAC) guidelines and Université Laval's Animal Research Ethics Board approved all procedures (Animal utilization protocol #2014121-2).

Exposure to e-cigarette aerosols took place using a whole-body exposure system as previously described (24, 25). A pump and pinch valve are controlled by a programmable automated system (InExpose control board; SCIREQ Scientific Respiratory Equipment Inc, Montreal, PQ, Canada) to take two 70 ml puffs per minute from a commercial e-cigarette. The puffs are then mixed with room air (bias flow of 3L/min) and sent in the whole-body exposure chamber by laminar flow where mice freely breathe the aerosols. E-cigarette device used was a draw-activated UWELL by Caliburn, with a refillable open pod cartridge. Coil resistance was of 1.4 Ω and battery power was of 11 W. 100% USP grade glycerol was used as e-liquid, with no nicotine or flavoring added. Mice were exposed acutely (a single 2-hour exposure) or chronically for two consecutive hours between 1300 and 1500, 5 days a week, for 9 weeks.

4.5.2 Blood Glycerol Assessment Following Inhalation and Gavage

Mice were fasted for 12 hours (from 2000 to 0800) prior to experiments. Mice (n = 3-4/group) were then subjected to a 2-hour glycerol e-cigarette aerosol exposure and blood was drawn before, at mid-exposure, after the exposure and 30 as well as 60 minutes following the end of the exposure. Other groups also received glycerol by gavage (2, 0.7, 0.2, 0.07 or 0 g/kg of glycerol in water). Blood was collected from the tail vein before and at 30, 60, 90, 120 minutes following the gavage. Glucose was measured using a glucometer (Roche, Accu-Chek Performa).

4.5.3 Glycerol and Glucose Tolerance Tests

To assess the impact of glycerol aerosol exposure on glucose and glycerol circulating levels, glucose and glycerol tolerance tests were conducted following 6 and 7 weeks of exposure, respectively. Mice were fasted for 12 hours (from 2000 to 0800) prior to experiments. For glycerol tolerance tests, all mice were subjected to a 2 mg/kg glycerol intraperitoneal injection. Glucose was measured using a glucometer (Roche, Accu-Chek Performa) and blood was collected from the tail vein before and at 30, 60, 90, 120-minute post-injection. For glucose tolerance tests, all mice were injected with 1 g/kg of D-glucose. Glucose was measured using a glucometer (Roche, Accu-Chek Performa) before and at 15, 30, 45, 60, 90, 120-minute post-injection. Blood serum was isolated and treated with Carrez Clarification Reagent Kit (ab202373, Abcam, Cambridge, United Kingdom). Free glycerol was measured according to the manufacturer's instructions (ab65337, Abcam).

4.5.4 Lung Function Measurement

Mice were fasted at 2000 on the day of the last exposure. Starting at 0800 the next day, mice were weighted, and glucose was measured using a glucometer (Accu-Chek Performa, Roche). Mice were then anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Lung function parameters were attested by FlexiVent (Scireq, Montréal, Canada). Mice were tracheotomized with an 18-gage blunted needle, mechanically ventilated at a respiratory rate of 150 breaths/min and a tidal volume of 10 mL/kg, with a pressure limit of 30 cmH_2O. Muscle paralysis was achieved using pancuronium (2 mg/kg, Sandoz, Boucherville, PQ, Canada) to prevent respiratory efforts during the measurement. The following sequence of measures was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3 and Pressure/Volume-loop to obtain lung resistance, compliance and elastance, Newtonian resistance, tissue resistance, tissue elastance, a pressure-volume curve, inspiratory capacity, and hysteresis.

4.5.5 Sample Harvesting and Processing and Histology Assessment

In anesthetized mice, blood was collected from the retro-orbital vein to obtain serum (incubated at 37°C for 60 min then spun 10 min at 12 000 g). Mice were then euthanized at random by exsanguination by severing the descending aorta. Liver and adipose tissue

(ovarian/epididymal, inguinal and retroperitoneal) were harvested, weighted and snap frozen for further analysis. A portion of the liver was placed in 10% formalin for 3 days prior transfer to 70% ethanol and paraffin-embedding. Liver sections were stained with hematoxylin and eosin (H&E).

4.5.6 Triglyceride, Phosphatidylcholine, Insulin and ALT Measurements

Liver lipids were extracted from tissues as described by Folch *et al.* (12) and resuspended in isopropanol. Hepatic triglyceride levels were determined with a standard assay kit (TR22421, Thermo Fisher Scientific) according to the manufacturer's instructions. Hepatic phosphatidylcholine levels were measured with a standard assay kit (STA-600, Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Fasting insulin was assessed (Ultra Sensitive Mouse Insulin ELISA Kit, Crystal Chem, Elk Grove Village, IL, USA). Blood alanine aminotransferase (ALT) activity was assessed according to the manufacturer's instructions (ALT Activity Assay, MAK052, Sigma Aldrich).

4.5.7 Quantitative PCR

Total RNA was extracted using TRIzol reagent (Fisher Scientific). RNA quantification and purity were assessed with the Synergy H1 plate reader and the Gen5 software (BioTek, VT, USA). RNA integrity was assessed by gel electrophoresis. 1 µg of RNA was converted into cDNA using the iScript Advanced cDNA synthesis kit (Bio-rad). qPCR analyses were performed using SsoAdvanced Universal SYBR Green Supermix (Bio-rad) and primers (IDT, Coralville, IA, USA) at 300 nM (See Table 1 for primer information). qPCRs were performed using a CFX384 Touch qPCR System (Bio-rad) as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 57-60°C for 30 s followed by a melt curve to assure specificity. For each gene, a temperature gradient was made to define the ideal annealing temperature. A calibration curve was also made to determine the PCR efficiency and a r^2. All qPCR efficiencies were between 90 and 110%, with r^2 values ranging between 0.97–1.00. Data were acquired and analyzed with the CFX Manager software (version 3.1). For each gene, C_q values were determined as the intercept of each amplification curve with the threshold establish in the calibration curve. All reactions were performed in triplicate (SD

< 0.3). Gene expression levels were assessed using *hprt* and *rplp0* reporter genes using the ΔΔCq method.

4.6.8 Statistical Analysis

Two-sided T-tests were performed for two-group comparisons. Two-way ANOVA comparing experimental groups to control group (Šídák's multiple comparisons post-test) were performed for multiple group analysis. For blood glycerol and glucose measurements, more detailed statistical analyzes were carried out on certain groups using two-sided T-tests. Statistically significant differences were considered if $p < 0.05$. All statistical analyses were performed using Prism 9 from GraphPad Software, Inc. (La Jolla, CA, USA).

4.6 Results

4.6.1 Glycerol E-Cigarette Aerosol Exposure Impacts Circulating Glycerol Levels

We first sought to investigate the impact of an acute glycerol e-cigarette aerosol exposure as well as glycerol gavage on circulating glycerol and glucose levels. Mice were fasted 12 hours prior and blood was drawn at different time points to assess glucose and glycerol concentration. Female mice exposed to glycerol e-cigarette aerosols showed a significant increase in serum glycerol, peaking at the 60-minute mark and returning to baseline levels at 150 minutes, or 30 minutes following the end exposure (Figure 4.1A). Interestingly, no increase in blood glycerol was detected in male mice following exposure (Figure 4.1C). Blood glucose remained unchanged for female (Figure 4.1B) and male (Figure 4.1D) mice. Gavage of 2 g/kg of glycerol increased blood glycerol in female and male mice (Figure 4.1E, 1G), and subsequently increased blood glucose in both sexes (Figure 4.1F, 4.1H). While we cannot statistically compare these separate experiments, this shows that exposure to glycerol e-cigarette aerosols differently affects female and male mice circulating glycerol levels while gavage leads to similar outcomes in both sexes.

4.6.2 Exposure to Glycerol E-Cigarette Aerosols Does Not Affect Body Weight

Studies have shown that age can change the response to diets, with mice being less susceptible to gaining weight when initiated early to a high-fat diet (10, 13). As glycerol can be used as a source of energy, we investigated the impact of chronic 9-week glycerol e-cigarette aerosol exposure on weight gain, considering sex as well as age at exposure onset as variables. Overall, we found no changes in weight gain between controls and mice exposed to glycerol e-cigarette aerosols (Figure 4.2A, 4.2C, 4.2E, 4.2G). In addition, glycerol e-cigarette aerosol exposure did not affect adipose tissue mass in all four groups (Figure 4.2B, 4.2D, 4.2F, 4.2H).

4.6.3 Exposure to Glycerol E-Cigarette Aerosols Increases Hepatic Triglycerides and Phosphatidylcholine Concentrations in Female Mice

Since the liver is a key organ in glycerol metabolism, livers were collected following the chronic 9-week exposure to glycerol e-cigarette aerosols. Liver weight remained similar between control and exposure groups, even when accounting for body weight (Figure 4.3A, 4.3E, 4.3I, 4.3M). Hepatic triglyceride concentrations were increased in glycerol e-cigarette aerosol-exposed young and adult female mice (Figure 4.3B, 4.3F), but not in male mice (Figure 4.3J, 4.3N). Similarly, hepatic phosphatidylcholine levels were increased in glycerol e-cigarette aerosol-exposed young and adult female mice (Figure 4.3C, 4.3G), but not in male mice (3K, 3O). This increased hepatic lipid accumulation in female mice was not associated with marked histologic changes (Figure 4.3D, 4.3H, 4.3L, 4.3P). Trying to identify potential transcriptional changes in key genes involved in lipid metabolism, we found no changes in liver mRNA levels for *ldlr,* involved in lipid transport, *agpat9*, involved in triglyceride synthesis, or *acaca*, involved in *de novo* lipogenesis. While there were no changes for females or for young males, adult male mice exposed to glycerol e-cigarette aerosols showed increased liver mRNA for *cpt1a*, involved in mitochondrial β-oxidation (Figure S4.1).

4.6.4 Exposure to Glycerol E-Cigarette Aerosols Does Not Induce Classical Pathogenic Inflammatory or Stress Markers in the Liver

To determine if triglyceride accumulation induced by chronic exposure to glycerol e-cigarette aerosols in the liver of female mice induced pathogenic processes, we assessed hepatic inflammatory markers as well as markers of endoplasmic reticulum stress known to be associated with hepatic steatosis. Activity for circulating ALT, a biomarker for liver damage, remained unchanged in female mice and adult male mice (Figure 4.4A, 4.4E, 4.4I), even slightly lower in young male mice (Figure 4.4M). Hepatic mRNA levels for the pro-inflammatory mediators C-C Motif Chemokine Ligand 2 (*ccl2*) and tumour necrosis factor (*tnf*) were similar between control and exposed groups (Figure 4.4B, 4.4F, 4.4J, 4.4N). Hepatic mRNA levels for endoplasmic reticulum stress markers activating transcription factor 6 (*atf6*) and DNA damage-inducible transcript 3 protein (*ddit3*) were also similar (Figure 4.4C, 4.4G, 4.4K, 4.4O). Hepatic mRNA levels for connective tissue growth factor (*ctfg*) was higher in adult males (Figure 4.4L) but not in other groups (Figure 4.4D, 4.4H, 4.4P).

4.6.5 Exposure to Glycerol E-Cigarette Aerosols Does Not Change Fasting Glycerol and Glucose Metabolism

We then investigated if repeated exposure to inhaled aerosols of glycerol generated by an e-cigarette could affect fasting glycerol and glucose circulating levels. Following 9 weeks of exposure, fasting blood glycerol levels remained similar between control and exposed groups (Figure 4.5A, 4.5D, 4.5G, 4.5J). Exposed young female mice showed slightly reduced fasting glucose levels compared to controls (Figure 4.5E), with similar fasting glucose between exposed and control adult female mice and male mice of both age groups (Figure 4.5B, 4.5H, 4.5K). Insulin concentrations were similar between all sex and age control and exposed groups (Figure 4.5C, 4.5F, 4.5I, 4.5L). Trying to identify potential transcriptional changes in key genes involved in glycerol uptake and metabolism, we found no variations in hepatic mRNA levels for *aqp9* and *gk* in adult and young female mice (Figure S4.1). However, exposed young and adult male mice showed a slight increase in *aqp9* liver mRNA levels compared to control groups. Both exposed adult and young male mice showed *gk* liver mRNA levels similar to the control groups (Figure S4.1). Hepatic mRNA levels for gluconeogenic genes *g6p6* and *pck1* remained similar between exposed and control mice for all four sex and age groups (Figure S4.1).

4.6.6 Exposure to Glycerol E-Cigarette Aerosols Changes Glycerol and Glucose Tolerance

We further assessed the impact of chronic exposure to glycerol e-cigarette aerosols on glycerol metabolism. We first evaluated the ability of mice to process glycerol using a glycerol tolerance test. Based on a previously published study (21), all mice were fasted for 12 hours after 6 weeks of glycerol e-cigarette aerosol exposure. Mice were injected with 2 g/kg of glycerol and blood was drawn to assess blood glycerol and glucose levels. We found young female mice exposed to glycerol e-cigarette aerosols metabolized injected glycerol differently than room air controls, with close to significant differences in adult female mice (Figure 4.6A, 4.6D). Adult male mice showed increased glycerol concentrations at the 120-minute mark, with similar trends in young male mice (Figure 4.6G, 4.6J). In all groups,

changes found in glycerol concentration did not transpose into different glucose concentrations during the glycerol tolerance test (Figure 4.6B, 4.6E, 4.6H, 4.6K).

We next sought to investigate if glycerol e-cigarette aerosol exposed mice had normal glucose tolerance. After 7 weeks of glycerol e-cigarette aerosol exposure, mice were fasted for 12 hours. Mice were injected with 1 g/kg of D-Glucose and blood glucose concentration were measured. Adult female and male did not show changes in glucose tolerance (Figure 4.6C, 4.6I). Young female mice showed increased blood glucose concentration in the first hour of the procedure, later to be decreased at the 120-minute mark (Figure 4.6F). Young male mice showed decreased blood glucose concentration in the first hour of the procedure, later to return to similar levels as of room air exposed mice (Figure 4.6L).

4.6.7 Glycerol E-Cigarette Aerosol Exposure Alters Pulmonary Functions in Young Male Mice

To investigate how exposure to glycerol e-cigarette aerosols was affecting the lungs, we assessed multiple parameters of lung functions *in situ*. While there were no changes between exposed and control female or adult male mice, young male mice exposed to glycerol e-cigarette aerosols showed increased lung resistance and tissue damping, with decreased tissue damping (Figure S4.1A, S4.1B, S4.1C, S4.1D).

4.6.8 Exposure to Glycerol E-Cigarette Aerosols Changes the Expression of Genes Regulating the Circadian Rhythm in the Liver of Young Male and Female Mice

Since we observed several impacts of sex and age on the liver and aspect of energy homeostasis, we wanted to revisit how the expression of genes regulating the circadian rhythm was affected in the liver. We found no differences between adult male and female mice (Figure S4.2A, 2C). Interestingly, we found than young mice exposed to glycerol e-cigarette aerosols, both females and males, depicted changes in circadian gene expression, with changes for *arntl, per2* and *per3* for females (Figure S4.2B) and *nr1d1, nr1d2* for males (Figure S4.2D).

4.7 Discussion

In this study, we aimed to investigate the effects of e-cigarette aerosols on the liver and aspects of energy homeostasis in non-pathological conditions using mice. More specifically, as glycerol is a direct substrate for several metabolic processes, we assessed the effects of inhaled glycerol aerosols generated by an e-cigarette on the liver itself as well as circulating glucose and glycerol homeostasis. We found that inhaling aerosolized glycerol can affect circulating glucose and glycerol levels, as well as liver triglyceride concentration. We also found that young female mice were more susceptible to these effects. This is the first study investigating the specific impact of glycerol contained in electronic cigarette liquids on the liver and aspects of energy homeostasis.

Free glycerol levels are largely maintained by triglyceride hydrolysis (lipolysis) in adipose tissue during fasting (31). In this study, we found that inhaled glycerol e-cigarette aerosols likely enter the blood stream, leading to a transient elevation of circulating glycerol levels in females but not in males, at least not at this concentration of aerosols. However, increase in blood glycerol following gavage is very similar between females and males, as well as the associated elevation in glucose. This suggests that the buffering capacity of males and females may be similar when glycerol is adsorbed through the digestive system but that, when entering from the lungs, sex differences can be observed. Aquaporins (AQP) such as AQP9 in the liver or AQP7 in adipose tissue facilitate glycerol transport through cellular membranes (22). Obese and insulin-resistant mice show increased *aqp7* and *aqp9* expression, in spite of hyperglycemia (17, 22). The lung also expresses several aquaporins: AQP1 in microvascular endothelia, AQP3 in large airways, AQP4 in large- and small-airway epithelia, and AQP5 in type I alveolar epithelial cells (38). While AQP1, AQP4 and AQP5 function as selective water channels, AQP3 has been shown in human skin to transport glycerol (7, 15). Glycerol can also passively permeate across membranes (28, 39). This suggests that less inhaled glycerol is ‘retained’ by the lungs of female mice compared to male mice, leading to increased glycerol leakage to the blood stream in females. Additional research is required to better understand the fate of inhaled glycerol in the lungs.

In this study, we show that mice exposed to glycerol aerosols generated by an e-cigarette do not gain nor lose significant weight, with no changes in adipose tissue weight or distribution.

Glycerol uptake is regulated by insulin and is suppressed in fed state (16, 20, 30). In fasting state, glycerol is a key substrate for glucose production through the gluconeogenesis pathway (5, 23). In post-absorptive state, glycerol is used for lipid synthesis, such as triglycerides and phospholipids (31, 36). High-fat diets induce weight gain, mainly attributed to increased adipose tissue weight (33, 35). Given that weights remained similar between exposed and control mice and that adipose tissues remained similar between groups, it does not appear that exposure to glycerol vaping would impact fat mass. However, further studies are needed to assess if early glycerol aerosol exposure can change food intake and energy expenditure.

High-fat diet promotes non-alcoholic fatty liver disease (NAFLD), characterized by increased liver weight largely due to triglyceride accumulation (2, 19, 35). Insulin resistance is also associated with NAFLD, further increasing pathological fatty acid metabolism (18). Interestingly, hepatic AQP9 protein levels, the main hepatic glycerol aquaporin, are inversely associated with the severity of hepatic steatosis, suggesting glycerol homeostasis affects and/or is affected by liver steatosis (29). In this study, we found that exposure to glycerol e-cigarette aerosols mildly affects glucose tolerance in young female and male mice. Moreover, we also found that female mice presented increased hepatic triglyceride and phosphatidylcholine levels despite no changes in liver weight, inflammation, remodeling or endoplasmic reticulum stress. With unchanged fasting blood glucose, this hepatic lipid accumulation phenotype suggests that excess glycerol could be converted into lipids by the liver and stored there, representing an adaptive mechanism to maintain normal circulating glycerol levels. Interestingly, no increase in adipose tissue weight was observed, supporting that the adipose tissue is not the main site dealing with glycerol excess. The reason why this phenomenon is only observed in females remains unknown, however the amount of glycerol reaching the circulation from the lungs could provide some insight. While the liver phenotype observed in female mice does not appear to be pathogenic and males appear to be resistant, longer exposures over several months may be required to reach a pathogenic state in females and break the resistance in males. Moreover, investigating the impact of inhaled glycerol aerosolized by an electronic cigarette in mice developing and with an established NAFLD may show how vaping glycerol can affect prevalent liver diseases.

Circadian rhythm regulates a multitude of biological pathways, from our sleep and awake cycle, to our immune response to infection and to metabolic processes (1, 6, 34). In recent years, studies have associated the rise of metabolic syndrome prevalence with increased circadian rhythm deregulation due to light pollution, reduction in quality and quantity of sleep as well as jet lag due to work shifts or travel (8). Among many other factors, it appears circadian rhythm plays an important role in the pathogenesis of metabolic syndrome (14). Our group reported that nicotine-free and flavor-free propylene glycol and/or glycerol aerosols induce changes in circadian rhythm regulatory gene expression in multiple organs in female BALB/c mice (24, 25). In this study, we also found that young but not older female and male C57BL/6 mice present hepatic changes in circadian gene expression levels. This suggests that the age of initiation has an impact on circadian changes observed, since the length of exposure for adult and young mice was the same. While it is possible that interactions between the circadian regulation of energy homeostasis and exposure to inhaled glycerol may be interacting more deeply in younger mice, our understanding of this interaction is currently very limited. Nevertheless, this finding is of great interest knowing how e-cigarette use is highly prevalent in adolescents (9, 27, 37).

Our group previously reported that an 8-week exposure to flavor-free and nicotine-free propylene glycol and glycerol e-cigarette aerosol increases larger airway resistance in female BALBC/c mice (24). Others also evaluated the impact of flavor-free and nicotine-free e-cigarette aerosols on lung functions [*reviewed in* (26)]. However, previous studies did not assess specifically age and sex differences. In the present study, we found that lung functions from young males were more affected by exposure to glycerol emission of electronic cigarettes, with no changes observed in adult male mice or both female groups. This highlights that, along with our findings on the liver and energy homeostasis, age and sex differences are crucial variables in studying the effects of vaping, no matter to organ or the biological function studied. Also, the marked disconnection between the metabolic (females more affected) and the respiratory effects (young mice more affected) of glycerol-based electronic cigarette emissions suggest pulmonary involvement does not mean systemic effects, and vice versa.

This study shows that the glycerol contained in vaping liquids can affect the liver and aspects of energy homeostasis in a sex-dependent manner without necessarily affecting lung functions. While additional studies are definitely needed to deepen our understanding or these observations, it shows how complex the biological impacts of vaping can be.

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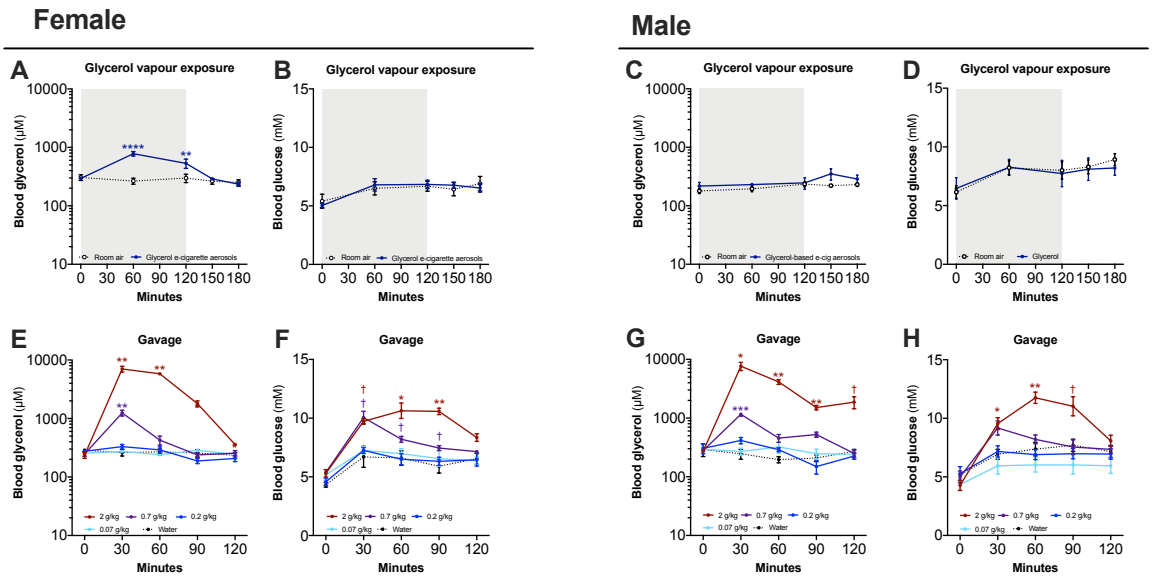

Figure 4.1. Impact of glycerol e-cigarette aerosol inhalation and glycerol gavage on blood glycerol and glucose concentration.

Six-week-old female and male mice (n = 3-4) were administered glycerol in different forms. Mice were exposed for two hours to glycerol e-cigarette aerosols (blue circles) or room air (white open circles). Exposure period is represented by the shaded region. Blood glycerol (*A, C*) and blood glucose (*B, D*) concentrations were measured. Mice received a glycerol gavage containing 2 g/kg (red), 0.7 g/kg (purple), 0.2 g/kg (dark blue), 0.07 g/kg (light blue) or water (white open circles). Blood glycerol (*E, G*) and blood glucose (*F, H*) concentrations were measured. Data are presented as mean ± SEM. Two-way ANOVA with Šídák's multiple comparison post-test were performed comparing experimental groups to control group: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Two-sided Student T-tests were performed for two group comparisons: † $p < 0.05$; † $p < 0.01$.

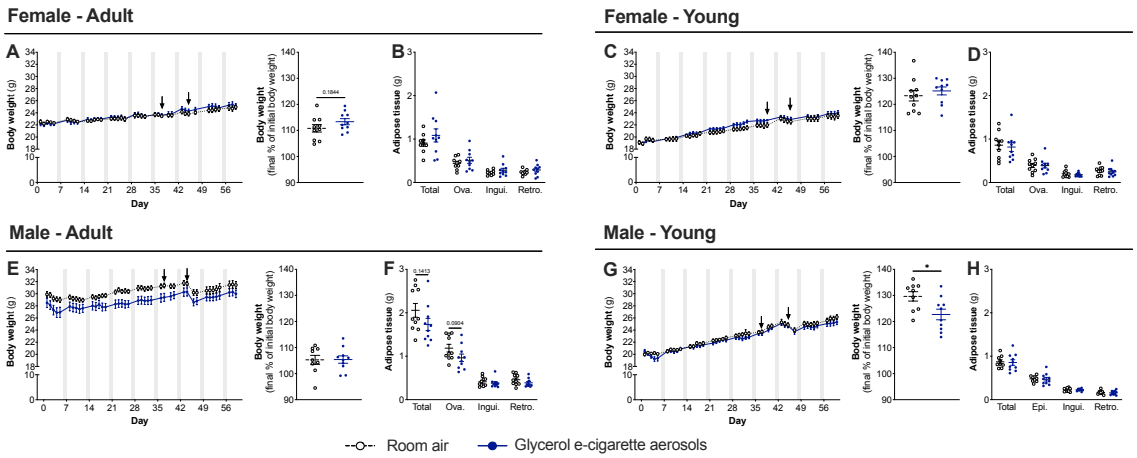

Figure 4.2. Glycerol e-cigarette aerosol exposure does not change body weight.
Twelve-week-old (adult) and six-week-old (young) female and male mice (n = 9-10) were exposed to room air (white open circles) or glycerol e-cigarette aerosols (blue circles) for 2 hours a day, 5 days a week for 9 weeks. Mice were weighted every morning at the same time to ensure reproducibility (*A*, *C*, *E*, *G*). Upon euthanasia, ovarian (Ova)/epididymal (Epi), inguinal (Ingui) and retroperitoneal (Retro) adipose tissue were weighted for room air (black and gray boxes) and glycerol e-cigarette aerosol exposed mice (blue boxes) (*B*, *D*, *F*, *H*). Arrows represent fasting period mice underwent for glycerol and glucose tolerance tests. Gray shaded regions represent weekends, where no exposure took place. Data are presented as mean ± SEM. Two-way ANOVA with Šídák's multiple comparison post-test were performed for body weight curves. Two-sided Student T-tests were performed for two group comparisons. *p < 0.05.

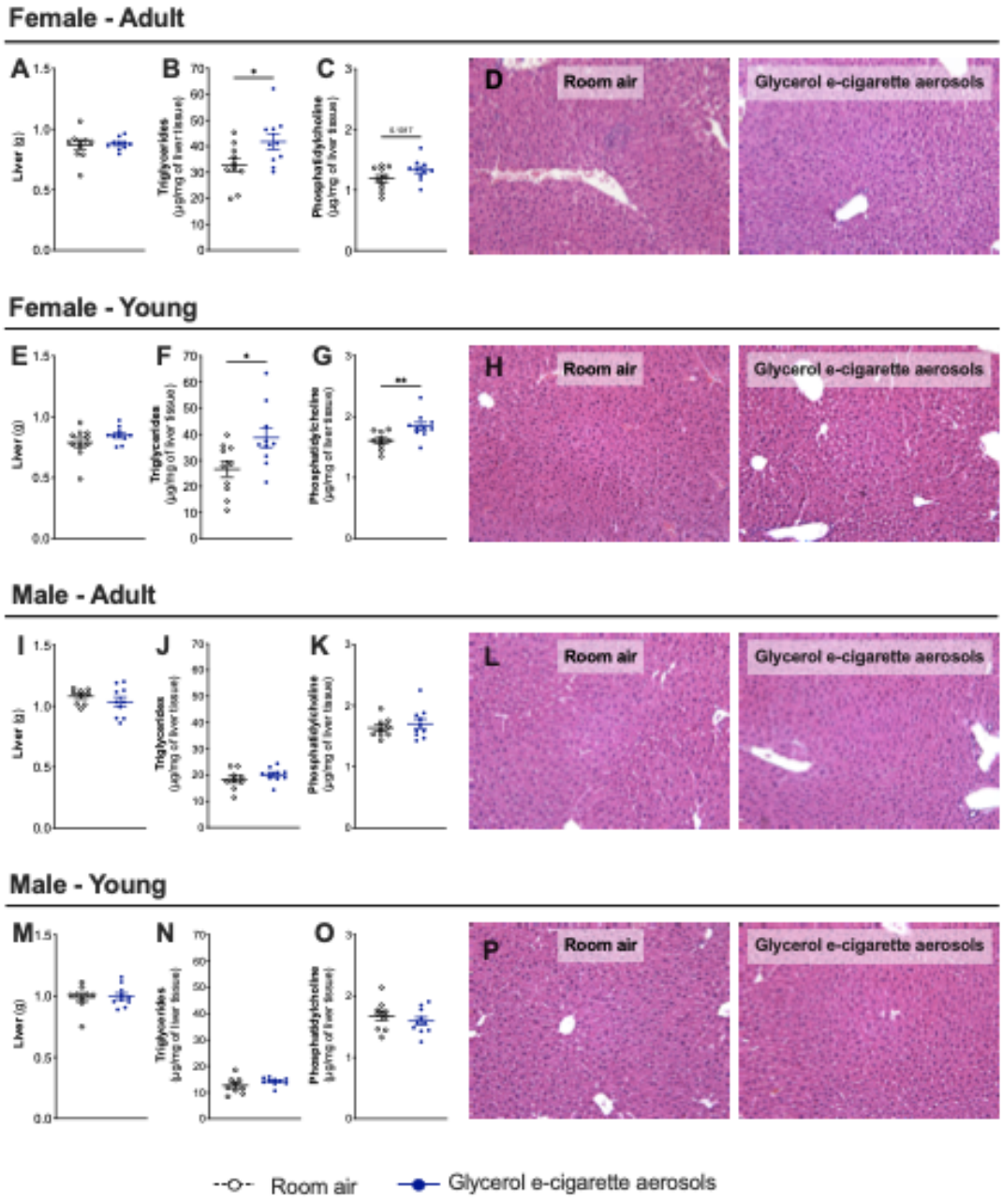

Figure 4.3. Glycerol e-cigarette aerosol exposure increases hepatic triglyceride and phosphatidylcholine content in female mice.

Twelve-week-old (adult) and six-week-old (young) female and male mice (n = 9-10) were exposed to room air (white open circles) or glycerol e-cigarette aerosols (blue circles) for 2 hours a day, 5 days a week for 9 weeks. Upon euthanasia, liver weight was measured (*A*, *E*, *I*, *M*). Hepatic triglycerides (*B*, *F*, *J*, *N*) and phosphatidylcholine (*C*, *G*, *K*, *O*) levels were measured. Hematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded liver sections were made (*D*, *H*, *L*, *P*). Data are presented as mean ± SEM. Two-sided Student T-tests were performed for two group comparisons. *$p < 0.05$; **$p < 0.01$.

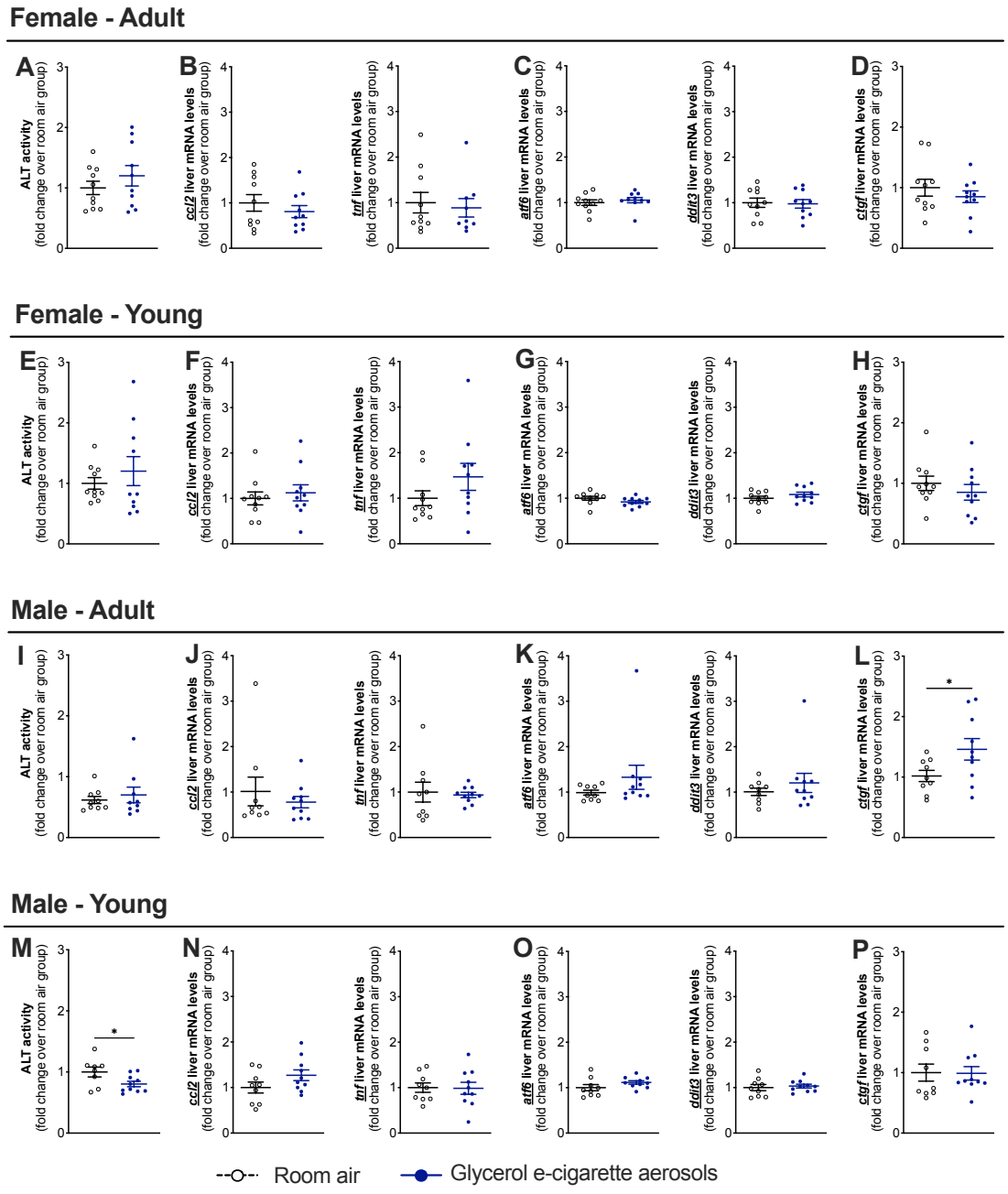

Figure 4.4. Impact of glycerol e-cigarette aerosol exposure on liver inflammation, endoplasmic reticulum stress and remodeling.

Twelve-week-old (adult) and six-week-old (young) female and male mice (n = 9-10) were exposed to room air (white open circles) or glycerol e-cigarette aerosol (blue circles) for 2 hours a day, 5 days a week for 9 weeks. Blood alanine aminotransferase activity (ALT) was measured (*A*, *E*, *I*, *M*). Hepatic expression level for inflammatory markers *ccl2* and *tnf* (*B*, *F*, *J*, *N*), endoplasmic reticulum stress markers *atf6*, *ddit3* (*C*, *G*, *K*, *O*) and remodeling marker *ctgf* were measured by qPCR analysis (*B*, *E*, *H*, *K*). Data are presented as mean ± SEM. Two-sided Student T-tests were performed for two group comparisons. *p < 0.05.

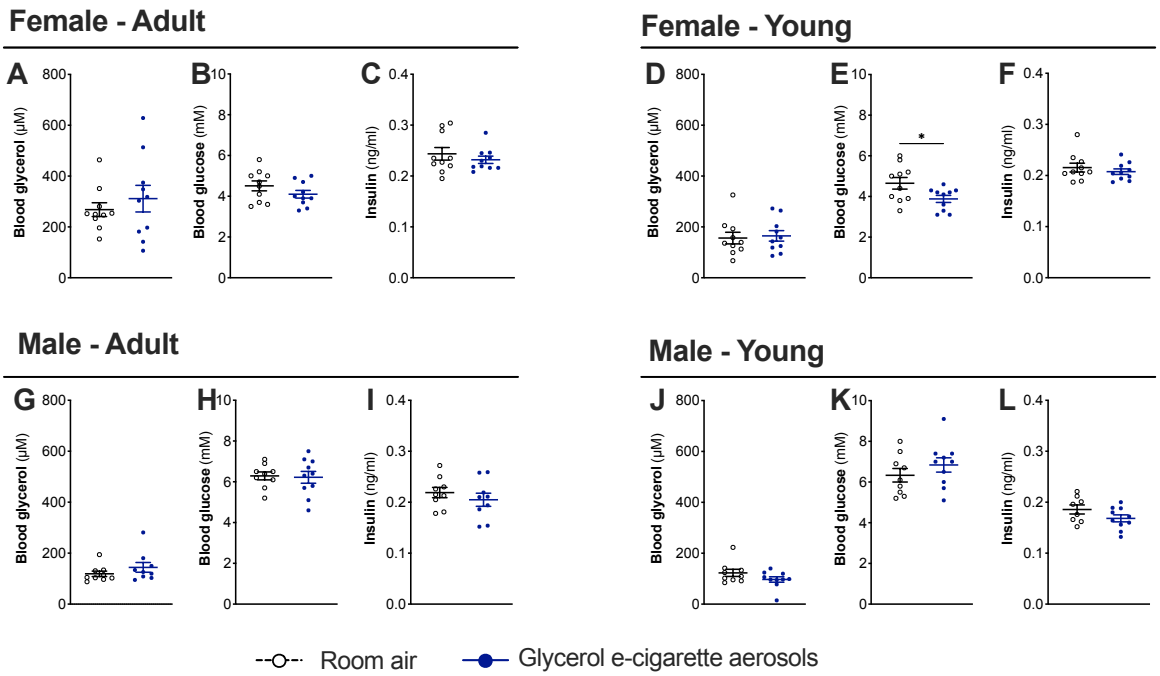

Figure 4.5. Impact of glycerol e-cigarette aerosol exposure on fasting glycerol, glucose and insulin concentrations.

Twelve-week-old (adult) and six-week-old (young) female and male mice (n = 9-10) were exposed to room air (white open circles) or glycerol e-cigarette aerosol (blue circles) for 2 hours a day, 5 days a week for 9 weeks. Upon euthanasia, fasting blood glycerol (*A*, *D*, *G*, *J*), glucose (*B*, *E*, *H*, *K*) and insulin (*C*, *F*, *I*, *L*) were assessed. Data are presented as mean ± SEM. Two-sided Student T-tests were performed for two group comparisons. *p < 0.05.

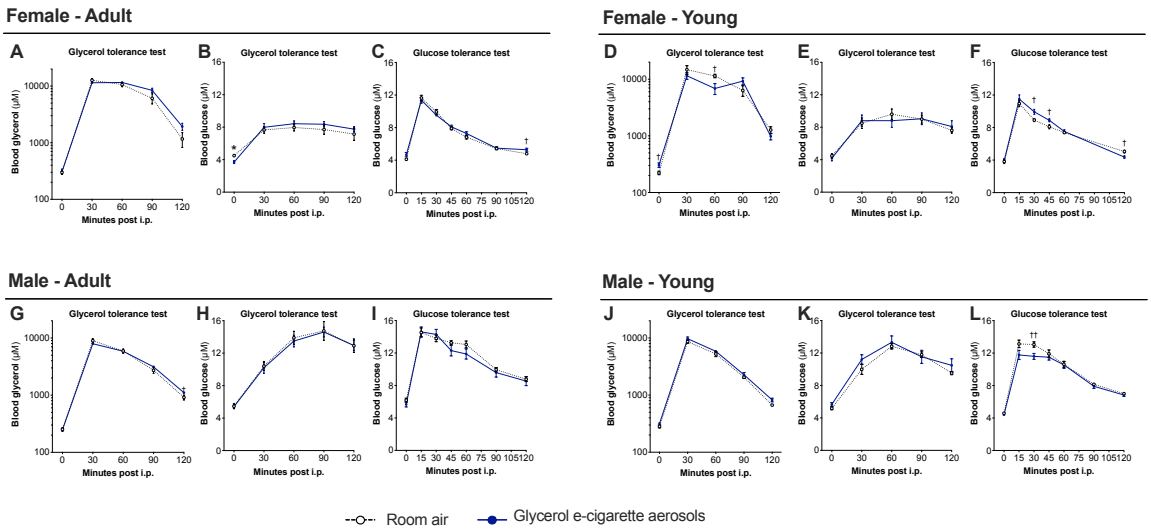

Figure 4.6. Impact of glycerol e-cigarette aerosol exposure on glycerol and glucose tolerance.

Twelve-week-old (adult) and six-week-old (young) female and male mice (n = 9-10) were exposed to room air (white open circles) or glycerol e-cigarette aerosol (blue circles) for 2 hours a day, 5 days a week for 9 weeks. After 6 weeks of exposure, mice were injected with 2 g/kg of glycerol in a saline solution and blood glycerol (*A*, *D*, *G*, *J*) and blood glucose (*B*, *E*, *H*, *K*) were assessed. After 7 weeks of exposure, mice were injected with 1 g/kg of D-glucose in a saline solution and blood glucose (*C*, *F*, *I*, *L*) were assessed. Data are presented as mean ± SEM. Two-way ANOVA with Šídák's multiple comparison post-test were performed for tolerance curves: *$p < 0.05$; **$p < 0.01$. Two-sided Student T-tests were performed for two group comparisons at each time point: † $p < 0.05$; †† $p < 0.01$.

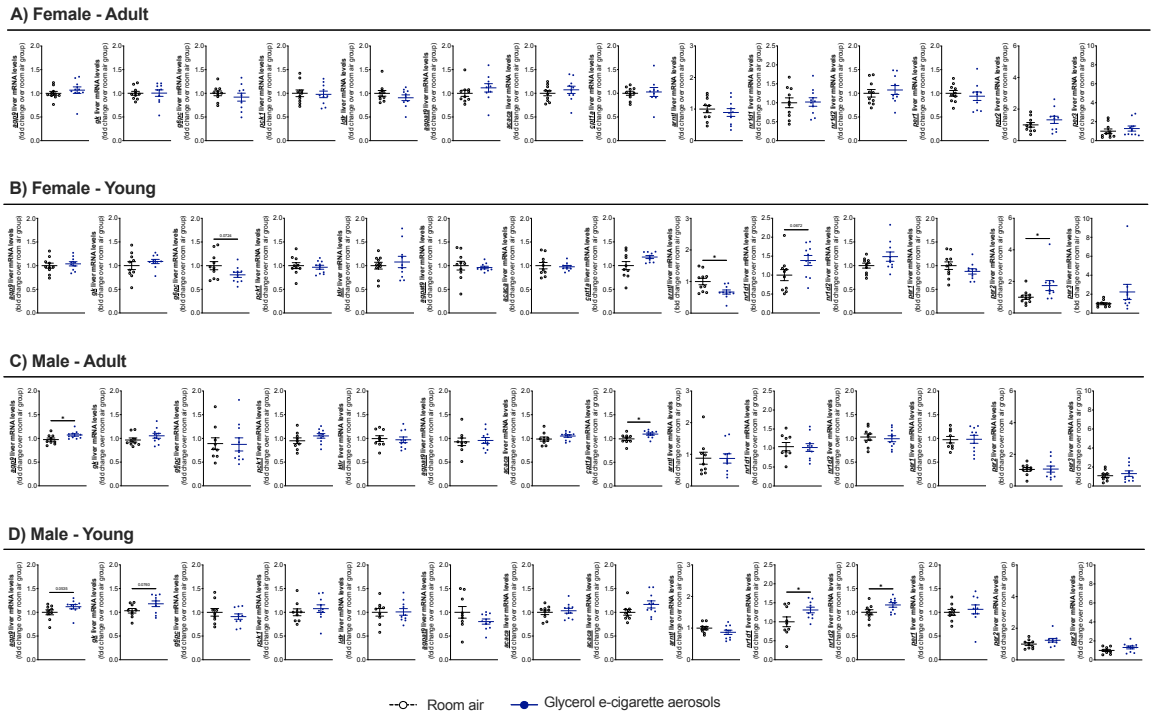

Figure S4.1. Impact of glycerol e-cigarette aerosol exposure on mRNA levels of genes involved in glycerol metabolism, glucose metabolism, lipid metabolism and circadian rhythm regulatory genes.

Hepatic *aqp9*, *gk*, *g6pc*, *pck1*, *ldlr*, *gpat3*, *acaca*, *cpt1a*, *arntl*, *nr1d1*, *nr1d2*, *per1*, *per2* and *per3* mRNA levels were measured by qPCR analysis (*A-D*). Data are presented as mean ± SEM. Two-sided Student T-tests were performed for two group comparisons. *p < 0.05.

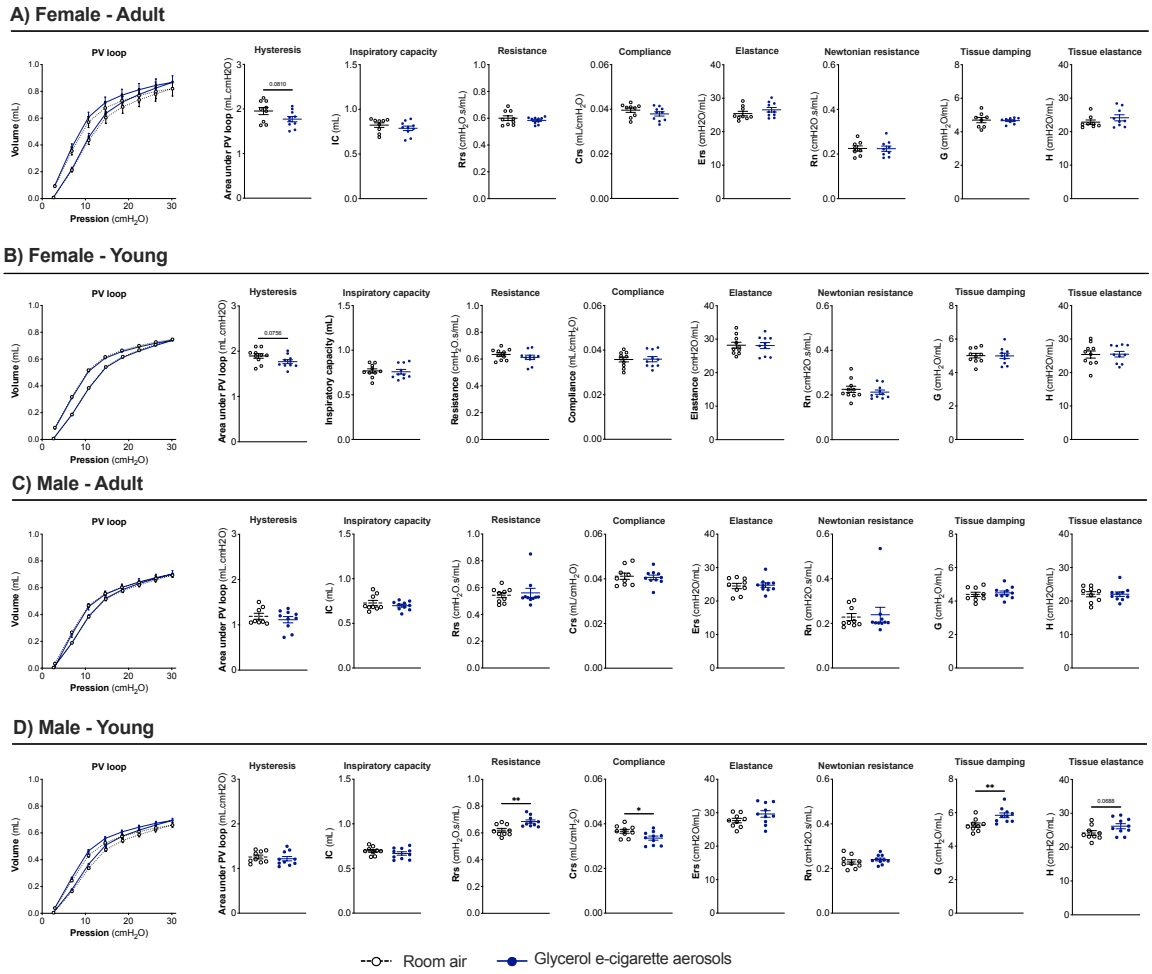

Figure S4.2. Impact of chronic glycerol e-cigarette aerosol exposure on lung function.
Twelve-week-old (adult) and six-week-old (young) female and male mice (n = 9-10) were exposed to room air (white open circles) or glycerol e-cigarette aerosol (blue circles) for 2 hours a day, 5 days a week for 9 weeks. Lung function parameters were assessed by FlexiVent (*A-D*). Data are presented as mean ± SEM. Two-sided Student T-tests were performed for two group comparisons. *p < 0.05; **p < 0.01.

Table 4.1: Primer sequences

Gene symbol	Sequence accession number	Amplicon size (pb)	Primer sequences	Annealing temperature (°C)
Acaca	NM_133360	93	For: AAC ATC CCC ACG CTA AAC AG	59
			Rev: GTC CAA CAG AAC ATC GCT GA	
Agpat9	NM_172715	148	For: ACC ATA ACA AGC AGT ACA GAC C	59
			Rev: GCT CTC TGA ATG ATC CCC ATC	
Atf6	NM_001081304	204	For: GAA CTT CGA GGC TGG GTT CA	60
			Rev: TCC AGG GGA GGC GTA ATA CA	
Aqp9	NM_022026	123	For: TCA CGG GAG AAA ATG GAA CG	59
			Rev: TGG CAA AGA CAA TCA GAA GGA	
Arntl	NM_007489	100	For: CGG TCA CAT CCT ACG ACA AAC	60
			Rev: CAG AAG CAA ACT ACA AGC CAA C	
Ccl2	NM_011333.3	142	For: AAC TAC AGC TTC TTT GGG ACA	57
			Rev: CAT CCA CGT GTT GGC TCA	
Cpt1a	NM_013495	119	For: CAG CAA GAT AGG CAT AAA CGC	59
			Rev: AGT GTC CAT CCT CTG AGT AGC	
Ctgf	NM_001901	140	For: TTG ACA GGC TTG GCG ATT	60
			Rev: GTT ACC AAT GAC AAT ACC TTC T	
Ddit3	NM_007837	176	For: TGC AGA TCC TCA TAC CAG GC	60
			Rev: CCA GAA TAA CAG CCG GAA CCT	
G6pc	NM_008061	108	For: GGA GGC TGG CAT TGT AGA TG	57
			Rev: TCT ACC TTG CTG CTC ACT TTC	
Gk	NM_008194	124	For: CCA ACG AAG TTT CAC TGC AC	57
			Rev: TGA CCT AAG AAC CCA GTC TAC T	
Hprt	NM_013556	125	For: AGC AGG TCA GCA AAG AAC T	57
			Rev: CCT CAT GGA CTG ATT ATG GAC A	
Ldlr	NM_001252659	132	For: TGC ATT TTC CGT CTC TAC ACT	57
			Rev: CAA CGC AGA AGC TAA GGA TGA	
Nr1d1	NM_145434	101	For: GAG CCA CTA GAG CCA ATG TAG	57
			Rev: CCA GTT TGA ATG ACC GCT TTC	
Nr1d2	NM_011584	112	For: ACA GTT CTC ATT CTT CAG GCA	57
			Rev: GGC ATC AGG ATT CCA CTA TGG	
Pck1	NM_011044	144	For: GCG AGT CTG TCA GTT CAA TAC C	57
			Rev: GGA TGT CGG AAG AGG ACT TTG	
Per1	NM_011065	133	For: CTT TGC TTT AGA TCG GCA GTG	57
			Rev: CTT CCT CAA CCG CTT CAG A	
Per2	NM_011066	118	For: TGA GGT AGA TAG CCC AGG AG	57
			Rev: GCT ATG AAG CGC CTA GAA TCC	
Per3	NM_011067	114	For: CTC TTC TCT CTG TCT CCA CCT	60
			Rev: TCC AAC TCA GCT TCC TTT CTG	
Rplp0	NM_007475	96	For: ATC ACA GAG CAG GCC CTG CA	57
			Rev: CAC CGA GGC AAC AGT TGG GT	
Tnf	NM_013693	145	For: AGA CCC TCA CAC TCA GAT CA	57
			Rev: TCT TTG AGA TCC ATG CCG TTG	

DISCUSSION

A great effort by electronic cigarette companies has been made to convince the public of the safety of their product. This thesis does not focus on comparing electronic cigarette use and tobacco smoking, nor does it investigate vaping as a cigarette smoking cessation tool. Though frequently compared, the two products have little to nothing in common, from the composition and size of the aerosols generated to their biological effects. Instead, we aimed to identify some of the biological effects of specific vaping liquid components, primarily propylene glycol and glycerol, on many aspects of pulmonary and extra-pulmonary health. We have shown that propylene glycol and glycerol inhalation can cause broad range biological changes, from altering circadian rhythm regulatory gene expression, leukocyte circulating patterns and sex-dependent lipid accumulation in the liver. We have also shown that vaping liquid composition can alter the particle size distribution and deposition of electronic cigarette aerosols. Here, I discuss future research avenues to pursue investigating the biological effects electronic cigarette use and the mechanisms behind these changes.

Can Electronic Cigarette Constituents Alter Immune Response?

Proper immune function is key in maintaining homoeostasis. The relationship between immunity and metabolism is bidirectional. Inflammation plays a role in the pathogenesis of metabolic disorders such as diabetes, obesity and metabolic syndrome. On the other hand, several metabolic factors regulate immune cell functions [267]. Results presented in **CHAPTER 3** show that propylene glycol and glycerol aerosol exposure induce changes in the percentage of CD4+ T cells in the lungs of female mice. This phenomenon was also found following dual exposure to electronic cigarette aerosols and tobacco cigarette smoke, with changes in both CD4+ and CD8+ T cell levels. We have shown in **CHAPTER 4** that glucose tolerance test is changed in young male and female mice following chronic electronic cigarette aerosol exposure. Nutrient environment induced by glycerol electronic cigarette aerosol exposure could change the T cell activation and could change the pathophysiological progression of an array of diseases.

Activated T Cells Drastically Change Their Metabolism

T cell activation requires two major stimulatory signals to induce an immune response. Firstly, T cell receptors (TCRs) recognize the major histocompatibility complex (MHC) on the antigen presenting cells. Secondly, a co-stimulatory signal engages T cells, such as CD28 which triggers IL-2 production, in turn sustaining T cell activation [268]. In general, T cell activation increases glucose uptake and increases glycolytic flux. As T cells do not store large quantity of glycogen, glucose import through GLUT1 receptors is required to meet their metabolic needs [268-271]. Pyruvate and citrate production are reduced, leading to decreased oxidative phosphorylation of glucose and increased glycolysis intermediates for biosynthesis [272]. Pyruvate and citrate are redirected for *de novo* fatty acid, triglyceride, amino acid and nucleotide synthesis, all of which are required for T cell proliferation [273, 274]. Cholesterol transport is suppressed, and its synthesis is increased by SREBP1 and mTOR [275, 276]. Lipid rafts, enriched in cholesterol, facilitate signalling by stabilizing the TCR and other co-activators that form the immunological synapse [272, 277].

Inhaled glycerol from electronic cigarette aerosols could change the nutrient milieu and thus affect T cell activation. It has previously been shown that glycerol uptake increases CD8+ T cells longevity [273]. The major glycerol transporter, AQP9, has a unique temporal expression pattern in virus specific CD8+ T cells: with low expression levels in naïve T cells which progressively increase during T cell proliferation [273]. On the other hand, AQP9 deficiency in T cells reduces their longevity, TAG synthesis, glycolysis and ATP production [273]. As presented in **CHAPTER 4**, we did not detect any significant increase in blood glycerol during glycerol aerosol exposure in female or male C57BL/6 mice. Data from the glycerol gavage experiments suggest that inhaled glycerol doses are sufficiently low to be rapidly eliminated from the circulation and maintain homeostatic levels. To note, we did not quantify the glycerol concentration present in bronchoalveolar lavage fluid. Nevertheless, both local pulmonary and circulating immune cells are exposed to changing glycerol concentration in their environment. Knowing that glycerol can prolong T cell activation, it is plausible that inhaled glycerol exposure may impact T cell immune functions. Concomitant infection and glycerol aerosol exposure could demonstrate the effect of glycerol aerosols on lung inflammation and the rate of pathogen clearance.

Nutrient Environment Dictates Disease Progression

Since T cell activation involves several metabolic changes, it is unsurprising that alterations in the nutrient milieu of immune cells can affect their activation and function. T cells are responsive to insulin, as they express insulin receptors. Hyperinsulinemia leads to increased T cell activation by increasing glucose uptake, amino acid transport and lipid metabolism [278-280]. Hence, in type 2 diabetes, where glucose and insulin tolerances are impaired, T cell activation is increased, promoting a pro-inflammatory environment [278, 281, 282]. Nutrient environment of immune cells also changes their function in respiratory diseases. COPD subjects with different disease stages show progressive increase of systemic leptin, an adipose tissue-derived pro-inflammatory molecule [283]. At high concentrations, this increased leptin concentration is associated with impaired lung function and capacity of T cells to engage in glycolysis [283]. Obese COPD subjects with high leptin levels show decreased capacity to generate regulatory T cells [283, 284]. Nutrient environment also plays a role in asthma severity, as obesity worsens asthma symptoms in adults and children [285, 286]. A shift in T cell-mediated response occurs in obese asthmatic patients, with higher Th1/Th2 T cell response ratio compared to normal-weight subjects [287]. In obese asthmatic patients, worsened lung inflammation, bronchial responsiveness and airway obstruction is associated with insulin resistance and alteration in lipid metabolism [287, 288]. Results presented in **CHAPTER 4** indicate that glucose tolerance is altered in young female and male mice acutely exposed to electronic cigarette aerosols. While we did not find any significant change in fasting insulin levels, we did not determine whether insulin signalling in T cells is changed following glycerol exposure. Further studies could assess the impact of glycerol aerosol exposure on nutrient-mediated T cell activation on pulmonary pathologies. Clinical investigations could assess if electronic cigarette users diagnosed with chronic respiratory disease such as COPD and asthma have worst clinical presentations and symptoms.

The tumoral microenvironment can be modulated by tumours and alter T cell function. Cancer cells are able to increase glucose uptake and glycolysis, leading to a decrease of intra-tumoral glucose levels [289, 290]. The reduced glucose availability in the tumoral environment reduces T cell activity and prevents tumor immune destruction [291]. Another

way cancer cells can change the nutrient environment is *via* glycerol metabolism. Glycerol intake is increased in tumour cells due to increased energetic need of cancer cells [292]. AQP3, expressed in epithelial cells, participates in increased metabolic flux of cancer cells in a multitude of tissues such as brain [293], cervical [294], bladder [295], colorectal [296], liver [297], pancreas [298], renal [299], lung [300, 301], oesophageal [302] and ovarian [303] tumor cells. This suggesting glycerol contributes to tumour growth and proliferation by providing phospholipid synthesis substrate and as an intermediate for ATP production. Further studies are needed to investigate if glycerol intake *via* electronic cigarette aerosols is beneficial in the antitumoral functions of T cells by increasing their activity, or rather detrimental by increasing tumour proliferation.

Taken together, this shows that the nutrient environment is crucial to maintain cellular homoeostasis and proper immune cell functions. The periodic increase in glycerol concentration in the lung, circulation and in extrapulmonary tissues could change immune response to pathogens as well as the pathological course of chronic pulmonary and metabolic diseases. More studies are needed to assess the effects of glycerol electronic cigarette aerosol inhalation on the nutrient environment of immune cells and its effect on immune processes.

Nicotinic Acetylcholine Receptors Have Anti-Inflammatory Properties

A third of electronic cigarette users over 15 years old are non-tobacco smokers [20]. This trend is especially predominant in youth, 74% of past-30-day vape users among youth aged 15 to 19 years old being never smokers [20]. Another small study indicated that 63% (12/19 subjects) of never smokers have nicotine in their vaping liquids [304]. While sampling is small, this highlights that electronic cigarette users, especially young users, inhale nicotine without prior or current tobacco cigarette smoking. Lung deposition of electronic cigarette aerosols is greater than of tobacco cigarette smoke, due to the smaller size of generated aerosols [305, 306]. Combined with high levels of nicotine in vaping liquids, this indicated that electronic cigarette users are exposed to great levels of nicotine. As mentioned previously, we found in **CHAPTER 3** that propylene glycol and glycerol aerosol exposure, alone or with tobacco cigarette smoke, changed macrophage, B cell and T cell lung populations. Nicotine delivery being the primary goal of electronic cigarettes, further

research could investigate the interactions of nicotine and other electronic cigarette constituents on immune cell function through nicotine acetylcholine receptor (nAChR).

Once in the bloodstream, nicotine rapidly crosses the blood/brain barrier and activates nAChRs on brain endothelial cells [307]. In the brain, activation of nAChRs is involved in many behavioural pathways, such as psychostimulation, reward, stress and anxiety reduction, learning, memory, motor control and analgesia [308, 309]. nAChRs are also expressed in bronchial, oral and gastrointestinal epithelial cells, as well as lymphocytes, macrophages, vascular endothelium and muscle fibres [310, 311]. The α7 subunit of nAChR is of particular interest in the regulation of inflammation, as it is expressed in the brain [312], as well in lymphocytes, macrophages and monocytes [313, 314]. α7 nAChR activation leads to anti-inflammatory effects, mediated by Ca^{2+} influx and subsequent PI3K/Akt pathway activation [315]. Nicotine-mediated activation of α7 nAChR inhibits NF-κB and attenuates pro-inflammatory mediator production [314, 316]. α7 nAChR also plays a role in adaptive immunity response by downregulating B cell activation and function [317, 318]. This reduced inflammatory state can be beneficial in several autoimmune diseases. Nicotine improves central nervous system inflammation in multiple sclerosis, where fewer dendritic cells lead to reduced demyelination and axonal damage [319, 320]. Nicotine also improves the clinical score for rheumatoid arthritis patients, showing reduced synovial inflammation [321-323]. nAChRs agonists have been used in asthma models and reduce eosinophil function and proinflammatory cytokine release [324, 325]. Nicotine contained in electronic cigarette aerosols decrease macrophage and neutrophil response to bacterial infection [326-329]. Exposure to nicotine through electronic cigarette aerosols led to worsened influenza A infection in mice [330]. In asthma mouse models, the addition of nicotine in vaping liquids reduced BAL eosinophil and macrophage recruitment but increased neutrophil count compared to room air controls [331]. Taken together, this highlights that nicotine intake through electronic cigarette aerosols could have broad range anti-inflammatory effects. More studies are needed to fully understand the effects of nicotine in electronic cigarette aerosols on bacterial and viral infection mechanisms as well as in pathological conditions.

How Do Electronic Cigarette Constituents Cause EVALI?

From September 2019 to January 2020, surge in E-cigarette or Vaping Product Use-Associated Lung Injury (EVALI) occurred in the United States. As of February 2020, 2,807 cases have been reported in the United States, with 68 deaths confirmed [332]. The majority of patients were under young men under 25 years-old, with 15% being under 21 years old [332]. The most recent data available indicate only 20 cases of EVALI were reported as of August 2020 [333]. A first European case of EVALI has been reported in German in early 2021 [334]. Since clinical presentations are similar to COVID-19 [335-338] and it is not yet a reportable disease in Quebec [339], EVALI has arguably been underdiagnosed and underreported of late.

Clinical Presentation of EVALI

Patients suffering from EVALI present with systemic symptoms such as fever, chills, fatigue, dry cough, dyspnea, myalgia, vomiting, diarrhea, weight loss and/or tachycardia [334, 336-338, 340-347]. EVALI patients often exhibit blood oxygen desaturation [334, 336, 337, 342, 344-347] and radiological anomalies, including multifocal ground-glass opacities (GGO) and bilateral interstitial infiltrates [334, 336-338, 340-344, 346] (**Figure G**). These radiological presentations can be accompanied by decreased lung function [334] and increased BAL infiltration of eosinophils [345] or neutrophils [334, 341, 347]. Foamy, lipid-laden alveolar macrophages have also been reported [341, 342, 344, 345]. Neutrophil-predominant leukocytosis is also common, often coupled with lymphopenia [336, 338, 340, 343-347]. High C-reactive protein levels and liver enzymes (AST and ALT) are also found in EVALI patients [343, 346, 347].

It has been proposed that the presence of tetrahydrocannabinol (THC) in vaping liquids is linked to the development of EVALI [334, 336, 341-345, 347, 348]. Most recent data indicate 50% of American EVALI cases reported THC use [332]. In Canada, of the 20 reported cases, only 5 reported using THC in their vaping liquids [333]. Vitamin E acetate in BAL fluid as

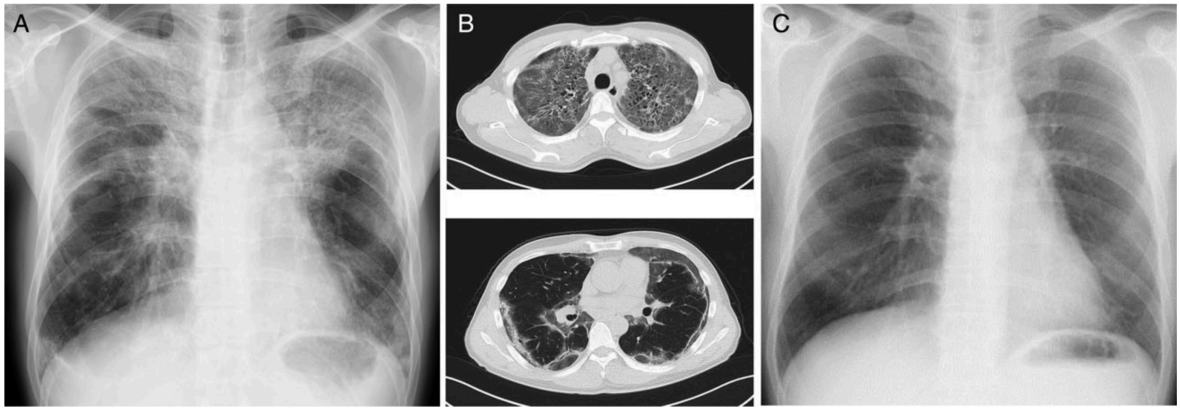

Figure G: Chest radiography of EVALI patient
A) Radiography at admission revealed extensive ground-glass opacity (GGO) in the bilateral upper lung fields, and GGO predominantly on the lateral segments of the lungs. B) Computed tomography at the same time of examination. Extensive GGO is seen in the upper lobes, accompanied with traction bronchiectasis. Non-segmental GGO is seen in the lateral segments of the lungs bilaterally, and curvilinear shadow can be seen. C) Chest radiography taken after 4 weeks of treatment. The opacity has almost disappeared.
Taken and adapted from: Itoh, M., et al., *Lung injury associated with electronic cigarettes inhalation diagnosed by transbronchial lung biopsy.* Respirol Case Rep, 2018. **6**(1): p. e00282.

well as in vaping liquids is thought to be the causal agent of EVALI, by incorporating into the pulmonary surfactant and preventing it from reducing the alveolar surface tension [349]. Biophysical analysis showed that vitamin E acetate reduces the elastic properties of synthetic pulmonary surfactant lipids [350]. This membrane softening could promote pulmonary surfactant failure following expiration, triggering lung dysfunction [350]. As mentioned in **CHAPTER 1**, preclinical investigations on vitamin E alone exposure did not trigger lung inflammation [351] and chemical analysis of vaping liquids inducing EVALI revealed that not all contain vitamin E [352]. Therefore, the underlying causes for EVALI remain to be fully understood.

EVALI: A Hypertensivity Response to Flavour Molecules?

One hypothesis is that EVALI could be a hypersensitivity response to ingredients found in electronic cigarette aerosols. EVALI patients present clinical characteristics that resemble antigen-induced hypersensitivity pneumonitis, also known as extrinsic allergic alveolitis (**EAA**) [353]. EAA is a progressive disease marked by interstitial and alveolar inflammation [353] that is influenced by the period and amount of antigen inhaled [353]. CT scans show ground glass shadowing [354, 355] with restricted lung volumes [356], impaired gas

diffusion, hypoxemia and airway obstruction [353]. EAA patients exhibit increased BAL neutrophils in the first 48h following exposure to the causal agent, followed by progressive inflammation decline once the allergen is absent [357, 358]. BAL lymphocyte infiltration is a common trait for all stages of EAA, with levels reaching up to 50% of total BAL leukocytes. This infiltration is persistent, as levels remain high even 7 days after the final antigen exposure [357, 358].

More than 200 antigens have already been identified as causal agents of EAA and these can be found in the workplace, at home, and in recreational activities. The majority are derived from fungi, bacteria, protozoa, and animal proteins. A small proportion of EAA-inducing molecules are low molecular weight chemical compounds, such as those found in polymers, paints, adhesives and resins [353, 359-363].The majority of chemical allergens are associated with skin sensitization and allergic contact dermatitis [364]. In their native state, low molecular weight chemicals are not immunogenic and fail to provoke an immune response [365]. To be effective sensitizers, inhaled molecules must first reach the epithelium, then need to form a protein complex called a hapten. Chemicals that are unable to associate effectively with proteins will fail to stimulate an immune response [366]. To induce sensitization, hapten-protein conjugates must cause sufficient trauma to stimulate the production of pro-inflammatory mediators thus stimulating dendritic cells to present the antigenic complex to T cells in the lymph nodes [366]. In general, chemicals capable of inducing EAA are more lipophilic and are more likely to be protein cross-linkers compared to agents associated with occupational asthma [367]. The mechanisms behind chemically induced EAA are not fully understood, but it is true that sensitization to chemicals can induce pulmonary symptoms similar to those observed in EVALI patients.

A potential root cause for EVALI could be hapten formation following electronic cigarette aerosol exposure. To address this question, we aimed to assess the chemical diversity of vaping liquids made and sold in Quebec. Preliminary data presented in **Figure H** shows the chemical composition of these products in liquid form, without any aerosolization. It is important to mention that the heating processes involved in electronic cigarette aerosol formation likely transform these molecules into by-products, multiplying the potential causal molecules. While the majority of these molecules remain to be identified, these results show

that vaping liquids contain a huge diversity of molecules. Further investigations on the chemical composition of vaping liquids could target molecules with strong hapten-forming potential. Identifying the most abundant molecules in each flavour (red regions in Figure H-a) could help direct research towards discovering which molecules induce biological changes. Chemical analysis of EVALI-inducing vaping liquids from hospitalized patients could also help to identify causal agents. These preliminary results also indicate that the most abundant chemical compounds are very different between flavours (Figure H-b). In the hopes of regulating electronic cigarette use and prevent vaping initiation in youth, countries and provinces have started to restrict certain flavours of electronic cigarettes, especially fruity

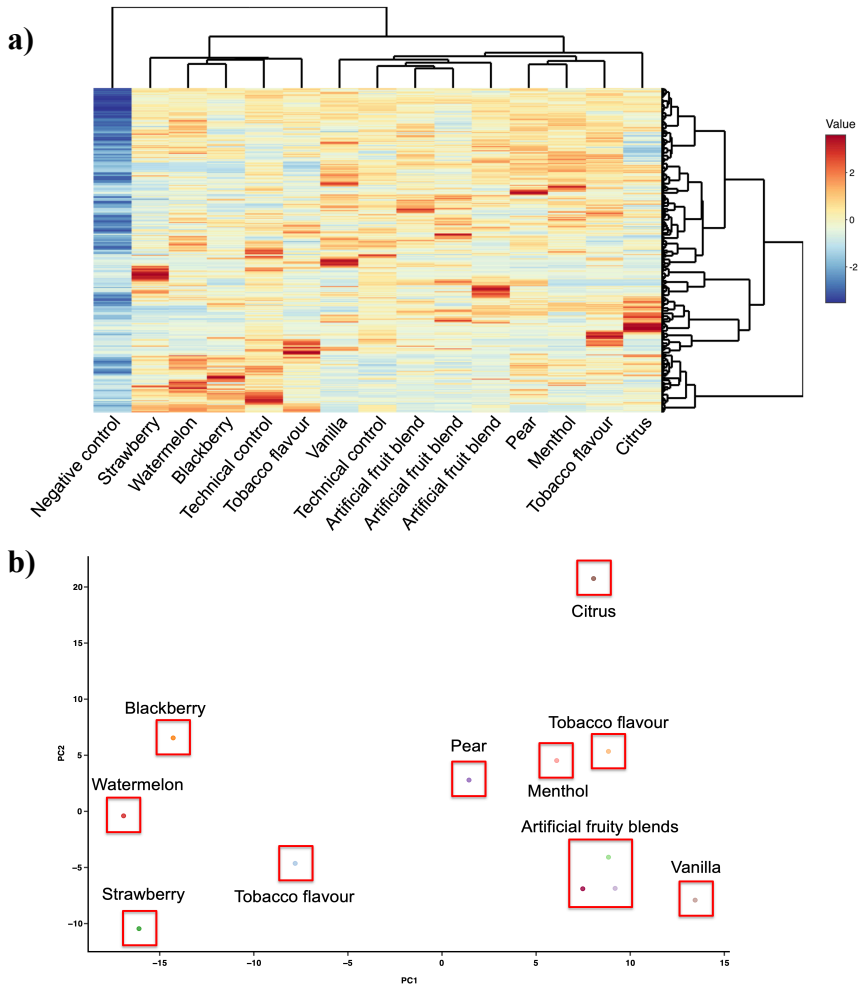

Figure H: Chemical diversity in vaping liquids

a) Hierarchical clustering analysis illustrating. Red regions represent molecules that are in high abundance and are unique to a particular flavour.

b) Principal component analysis illustrating the chemical composition of 12 vaping liquids containing nicotine sold in Quebec.

and sweet flavours. It seems that excluding these chemicals from vaping liquids altogether may be more prudent, in order to limit the adverse effects of vaping in the adult population as well. Furthermore, knowing tobacco cigarette smoke also contains potential protein reactive chemicals, it is possible that dual use of electronic cigarettes and tobacco cigarettes could increase hapten formation and increase EVALI incidence. Clinical studies investigating vaping liquid chemical composition, vaping habits and co-usage of tobacco cigarettes and other substances could help us better understand risk factors for developing pulmonary symptoms associated with electronic cigarette use.

Can Electronic Cigarette Constituents Interfere With Metabolic Disorders?

Aside from pulmonary health, electronic cigarette use could affect other extra-pulmonary conditions, especially those involving energy homoeostasis imbalance. Obesity prevalence has been on the rise since the 1980s [368]. Recent epidemiology studies indicate that 25% of the adult Canadian population is considered obese and 35% are overweight [368]. Although the criteria for metabolic syndrome differ between guidelines, it is generally characterized by the presence of at least two of the following: elevated fasting blood glucose or insulin, increased waist circumference, high blood triglycerides and cholesterol and/or high blood pressure [369, 370]. These risk factors are associated with several co-morbidities, such as type 2 diabetes or cardiovascular diseases [369, 370]. Considering the prevalence of obesity and the rise of electronic cigarette use, it is pertinent to investigate the potential relationship between the electronic cigarette aerosol exposure and metabolism. We found in **CHAPTER 4** that glycerol aerosols from electronic cigarette induced changes in the glucose tolerance as well as increased hepatic triglycerides and phosphatidylcholine in young and adult female mice. These changes did not seem to be pathological, as there were no changes in fasting glucose or insulin levels. We did not observe changes in body weight nor in adipose tissue weight. Similarly, liver weight remained unchanged, and no hepatic inflammation was detected. Nevertheless, we do not know how these changes in glucose tolerance and hepatic lipid levels following glycerol electronic cigarette aerosol inhalation could affect the pathogenesis of metabolic syndrome, obesity or type 2 diabetes.

Glycerol Transport Metabolism Is Altered in Metabolic Diseases

Glycerol transport is central to energy homoeostasis and chronic glycerol intake via electronic cigarette aerosols could adversely affect these processes. Obese and insulin-resistant mice show increased AQP7 and AQP9 expression, despite their hyperglycemia [371]. Diabetic animals exhibit increased glycerol release from adipose tissues into the circulation, thus activating gluconeogenesis [220, 372]. This shows that elevated blood glycerol and increased glycerol intake can contribute to diabetes pathogenesis. We show in **CHAPTER 4** that fasting expression levels for *Aqp9* and *Gk* as well as blood glycerol levels remain unchanged following a 9-week exposure to glycerol electronic cigarette aerosols. More studies are needed to assess the effects of chronic glycerol exposure on the protein level and activity of AQP9 and Gk in order to better understand the impact of electronic cigarette smoke exposure on glycerol uptake.

AQP9 localization differs between male and female rodents: it is more homogeneously expressed in the liver of male rats in perivascular regions of hepatocytes than for female rats [373]. Male and female rodents also respond differently to starvation, with increased *Aqp9* expression, glycerol permeability and blood glycerol levels in starved male but not in female rats [374]. Interestingly, ovariectomized-female rats exhibited a starvation response pattern similar to male rats, suggesting a role for estrogen in glycerol transport regulation [374]. Results presented in **CHAPTER 4** show that glycerol electronic cigarette aerosols induce a transient change in circulating glycerol, with detectable levels in female mice but not in male mice, while AQP9 expression is indeed greater male mice compared to female mice. This suggests a sexual dimorphism in hepatic glycerol uptake by AQP9. Further clinical investigation measuring the glycerol levels in women and men during electronic cigarette use could elucidate whether this sexual dimorphism is also present in humans.

Gluconeogenesis is Increased in Diabetes and Obesity

Diabetes mellitus has reached epidemic proportions. In 2009, it was estimated that 285 million people had type 2 diabetes, now reaching 463 million people a decade later [375]. Type 2 diabetes is most prevalent in older populations, with 20% of adults over 60 years old being affected worldwide [375]. Ninety percent of diabetic patients suffer from type 2

diabetes, the remaining 10% can be accounted for type 1 diabetes, an autoimmune response-mediated destruction of the pancreatic β cells, resulting in insulin deficiency [375, 376].

In non-pathological conditions, the rise of blood glucose following eating induces insulin secretion by pancreatic β cells. Normoglycaemia is maintained by the balanced interplay between insulin action of different molecular pathways and insulin secretion [377]. The ability of insulin to increase glucose transport in skeletal muscle is mediated by the translocation of glucose transporter 4 (**GLUT4**) from intracellular vesicles to the plasma membrane [378]. Briefly, insulin signalling involves a cascade of events initiated by insulin binding to the insulin receptor (**IR**) at the cell surface. Through a series of phosphorylation steps involving the PI3K/Akt pathway, downstream signalling molecules promote the translocation of GLUT4 to the plasma membrane (**Figure I**) [379-385]. Type 2 diabetes is characterized by insulin resistance and associated with impaired insulin secretion that develops in multiple organs, including skeletal muscle, liver, adipose tissue and heart [376]. Insulin resistance is caused by impaired insulin transduction signalling and decreased skeletal muscle activity of PI3K [386, 387]. We found in **CHAPTER 4** that fasting glucose and insulin levels were not affected by electronic cigarette aerosol exposure the time of euthanasia, while glucose tolerance was affected in young female and male mice. Since blood

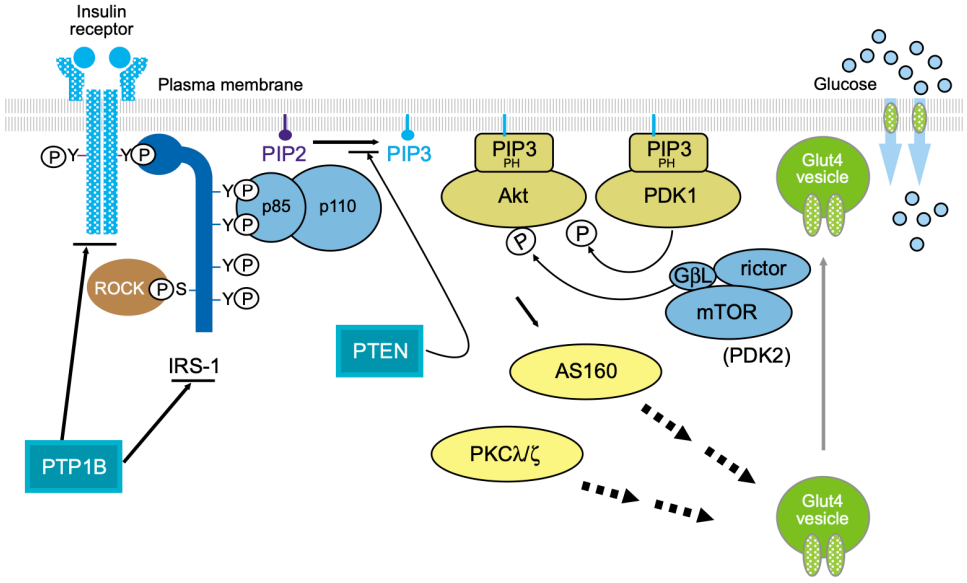

Figure I: Insulin signalling pathway
Taken and adapted from: Choi, K. and Y.B. Kim, *Molecular mechanism of insulin resistance in obesity and type 2 diabetes.* Korean J Intern Med, 2010. **25**(2): p. 119-29.

glucose levels in glycerol-exposed mice remain unchanged compared to room air controls during the glycerol tolerance test, it is possible insulin signalling is increased to compensate for glycerol conversion into glucose. Further studies could investigate the effects of chronic glycerol exposure on insulin signalling mechanisms immediately during after a vaping session. Clinical studies could assess the impact of electronic cigarette use on fasting glucose levels or insulin resistance in chronic users.

Elevated circulating glycerol produced via lipolysis contributes to diabetes via increased activity and expression of gluconeogenesis enzymes [388]. Murine models of type 1 and type 2 diabetes have shown elevated hepatic PCK1 expression [195, 220, 389, 390]. Similar trends are found in high-fat diet models, where rodents exhibit elevated PCK1 protein and mRNA expression [192, 195]. In human subjects, PCK1 expression levels correlated with fasting blood glucose [192]. This phenomenon may be due to several downstream mediators implicated in insulin signalling. Downregulation of PI3K in insulin resistance increases gluconeogenesis, as its inhibition increases PCK1 and G6Pc expression [210, 391-393]. Results presented in **CHAPTER 4** show that glycerol electronic cigarette aerosol exposure does not induce changes in *Pck1* or *G6pc* expression following 9 weeks of exposure at the time of euthanasia. It is possible that glycerol electronic cigarette aerosol exposure does not alter constitutive *Pck1* or *G6pc* expression while the activity of these key enzymes could be increased by chronic glycerol exposure. Knowing gluconeogenesis pathway regulation is impaired in diabetes, it would be interesting to investigate the impact of excess circulating glycerol via glycerol electronic cigarette aerosol inhalation on the pathogenesis of diet-induced type 2 diabetes.

Fatty Acids and Triglycerides is Deregulated in Liver Disease

Under normal physiological conditions, there is a significant import and export of triglyceride and fatty acids in the liver in response to fasting and feeding. However, the liver is not a fat storage depot, and hepatic lipid accumulation is the result of pathological mechanisms [213]. Non-alcoholic fatty liver disease (**NAFLD**) is the most common liver disease, with a prevalence of 25% of the world population [394]. NAFLD is a broad term used to describe a range of related disorders (**Figure J**) [395]. The earliest stage is hepatic steatosis, characterized by the deposition of triglycerides as lipid droplets in the cytoplasm of

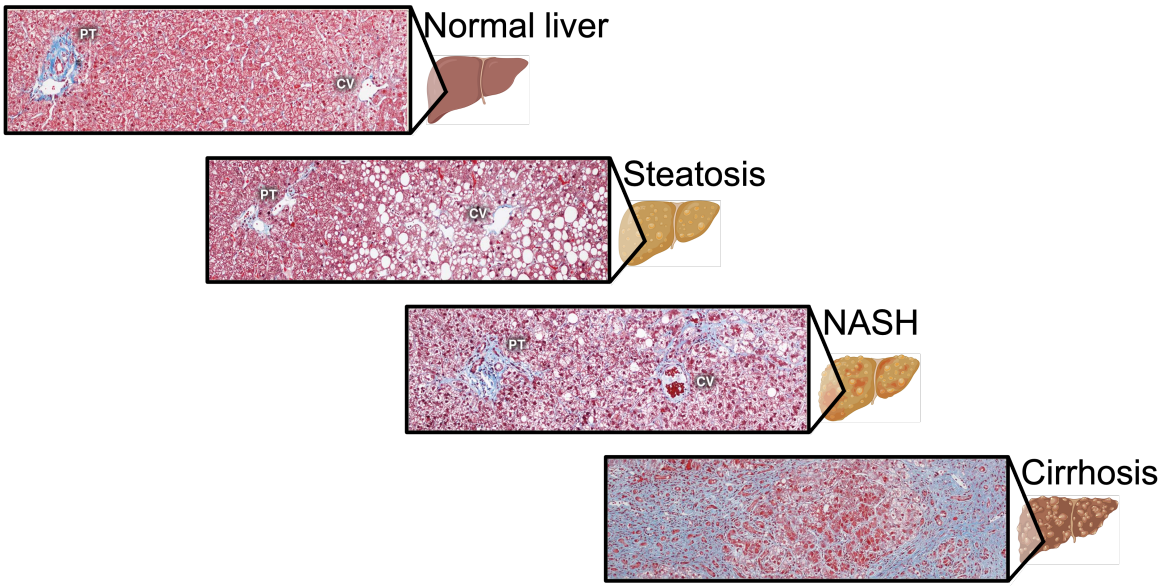

Figure J: Disease spectrum of non-alcoholic liver disease
The accumulation of TG within lipid droplets in hepatocytes causes steatosis. Steatosis associated with inflammation, cell death, and fibrosis is referred to as NASH, which can progress to cirrhosis. Histologic sections illustrating normal liver, steatosis, NASH, and cirrhosis. Collagen fibres are stained blue with Masson's trichrome stain. The portal triad (PT), which consists of the hepatic artery, portal vein, and bile duct, and the central vein (CV) are shown.
Taken and adapted from: Cohen, J.C., J.D. Horton, and H.H. Hobbs, *Human fatty liver disease: old questions and new insights.* Science, 2011. **332**(6037): p. 1519-23.

hepatocytes [213]. Steatosis is defined as hepatic triglyceride levels exceeding the 95th percentile of lean, healthy subjects, or as the presence of triglyceride droplets in more than 5% of hepatocytes [396]. Hepatic steatosis can progress into non-alcoholic steatohepatitis (**NASH**). NASH is different from simple steatosis by the presence of liver injury, inflammatory infiltrates and collagen deposition [395, 397]. In turn, NASH can progress into cirrhosis, where hepatocytes are replaced by scar tissue composed of type 1 collagen [395]. As shown in **CHAPTER** 4, histologic analysis and triglyceride quantification in liver tissues suggest only mild triglyceride accumulation following glycerol exposure. We also found increased expression for *ctgf* in adult male mice, suggesting that pathological processes of fibrosis could be initiated. Prolonged exposure protocols are needed to investigate if the lipid accumulation in females could progress into greater steatosis and if male mice would eventually present with sings of steatohepatitis.

Fatty acid uptake by the liver plays a role in NAFLD pathogenesis. Plasma non-esterified fatty acid concentrations are elevated in NAFLD subjects, an effect linked to increased fatty

acid release from adipose tissue [217, 398-400]. NAFLD is indeed strongly associated with obesity, where increased fat mass directly contributes to greater fatty acid release from adipose tissue [401]. Peripheral insulin resistance in NAFLD patients also contributes to increased plasma fatty acid and correlates with steatosis severity [402-404]. Mice fed a high-fat diet and obese subjects develop hepatic steatosis and present increased mRNA levels for CD36, translocase protein that facilitates the transport of long-chain fatty acids [404-407]. This increased intracellular fatty acid trafficking stimulates pathological triglyceride storage, in turn promoting steatosis [408-410]. In the study presented in **CHAPTER 4**, we did not assess the specific fatty acid composition. It is possible that fatty acid uptake could be increased to compensate for the increased glycerol uptake in the liver. It could be a way to prevent glucotoxicity and converting glycerol into lipids instead of glucose. Further studies could investigate the fatty acid content present in the liver. We could also assess if hepatic fatty acid metabolism is increased flowing glycerol exposure, contributing to lipid accumulation in the liver derived from glycerol intake.

Fatty acid synthesis and lipid export are also altered in NAFLD. *De novo* synthesis of fatty acids from acyl-CoA is increased in NAFLD patients, even when fasting [217, 400, 411]. The impaired inactivation of *de novo* lipogenesis in NAFLD can be explained by the deregulation SREBP1c, which is activated by insulin and in turn promotes the downstream expression of ACC and FAS [201, 397, 407, 411, 412]. Elevated blood insulin concentrations also promote lipid export through VLDL, an ApoB-100 containing lipoprotein composed of a core of triglycerides and cholesterol esters surrounded by a monolayer of phospholipids and unesterified cholesterol [213]. Despite the increased VLDL secretion observed in NAFLD patients [399], this overproduction does not compensate for the excess intra-hepatic lipid levels. Instead, this environment induces ER stress and ApoB-100 degradation, further reducing triglyceride secretion and worsening steatosis [413]. As presented in **CHAPTER 4**, expression for *Acaca* remains unchanged at the time of euthanasia, suggesting that perhaps *de novo* fatty acid synthesis is not induced following glycerol exposure. It would be pertinent to quantify the substrates involved in fatty acid synthesis to assess whether the activity of these enzymes is upregulated in the context of chronic glycerol inhalation. We also noted no hepatic inflammation nor markers of ER stress following the 9 weeks of glycerol electronic cigarette aerosol exposure. On its own, glycerol exposure seems to initiate metabolic changes

in the liver that, over time or in combination with other factors, could lead to liver disease. It would be interesting to determine whether glycerol exposure could change the pathological course of steatosis using a diet-induced NAFLD mouse model, such as high-fat or methionine- and choline-deficient diets. In a clinical setting, the impact of electronic cigarette use on the incidence of NAFLD and liver inflammation could be assessed.

Besides the vaping liquid vehicle, other electronic cigarette constituents could affect hepatic health. Nicotine modulates the pathological course following high-fat diet intake, resulting in blunted weight gain, hepatic triglyceride accumulation, hepatocyte apoptosis and worsened steatosis [414-416]. This phenomenon is mediated through decreased AMPK phosphorylation, leading to increased hepatic expression of FAS and SREBP1c [414, 415]. Nicotine also has detrimental effects on liver injury repair, with direct treatment in mice being associated with increased liver fibrosis, inflammation and oxidative stress [417]. Furthermore, tobacco cigarette smoking has been shown to be associated with NAFLD and liver fibrosis severity [418-420]. Taken together, these studies suggest that other electronic cigarette constituents, such as nicotine, or dual tobacco and electronic cigarette use may impact hepatic metabolism and NAFLD pathogenesis. Epidemiological studies looking into electronic cigarette use in current and never smokers could help us determine whether dual use is associated with increased NAFLD prevalence.

Strengths and Limitations

CHAPTER 2. As mentioned throughout this thesis, electronic cigarette research is limited by multiple cofounding factors such as electronic cigarette device, propylene glycol to glycerol ratio, nicotine concentration and added flavours. We were able in this study to use a model of electronic cigarette where different power settings can be used, limiting the variability between brands. Using a single electronic cigarette power and using vaping liquids made with lab-grade constituents, we have shown that these variables influence electronic cigarette particle size distribution. One limitation of this study is that the results are not presented as an absolute number of particles, rather as a percentage of total particles for each analyzed particle size. Using a closed-system method, the sampling alone progressively decreased the number of particles, resulting in significant differences between technical replicates. However, since we aimed to investigate particle size distribution, I believe the data representation method used is acceptable and sufficiently highlights the effects of electronic cigarette variables on emitted particles and their deposition.

CHAPTER 3. To the best of my knowledge, this study is the first to investigate the pulmonary effects of tobacco and electronic cigarette dual use. We conducted a robust study with two time points to better assess the circadian rhythm changes and their effects on lung immunity and respiratory function. Due to technical limitations, only two time points were possible, preventing the assessment of pulmonary changes across 24 hours. Furthermore, we were not able to proceed to the exposure and sample processing of all 80 mice in a single day, limiting the statistical analysis between groups. Nevertheless, we were able to show the effects of nicotine- and flavour-free electronic cigarette aerosols on pulmonary immunity and function, alone or with tobacco cigarette smoke.

CHAPTER 4. This study represents the first study to investigate the metabolic effects of glycerol electronic cigarette aerosols in wild-type mice. We conducted an extensive study using male and female mice to address the sexual dimorphisms in energy metabolism. We also used young and adult mice to assess the effects of age on metabolic changes induced by glycerol electronic cigarette aerosol exposure. Due to ethical and technical limitations, it was not possible to harvest blood at time points closer than 30 minutes apart during glycerol tolerance tests, meaning some resolution on the glycerol blood kinetics was lost. Another

limitation is that we did not control the food consumption across the 9 weeks of the exposure period. Since mice are housed in groups of 3 for males and 5 for females, measuring the mean food consumption per cage would not have been particularly relevant. Furthermore, housing mice individually would have multiplied the costs dramatically and could have induced stress to the animals. I believe that despite these limitations, we have effectively shown that glycerol electronic cigarette aerosols can induce metabolic changes in mice.

General limitations. Some general limitations can be found across our studies. Firstly, we used a mouse model of electronic cigarette aerosol exposure. The use of mice to assess potential electronic cigarette aerosol exposure and its impact on health is required in order to explore this nascent field of research. However, insights from animal models must be applied with caution, as results found in mouse models often fail to translate to humans. Extensive clinical research is needed in order to fully understand the effects of electronic cigarette use in humans. Another limitation is our exposure system. While it allows for easy exposure of many mice simultaneously, the whole-body exposure system used in our studies does not only expose the respiratory tract to the electronic cigarette emitted aerosols, but also the eyes, skin and fur. It is possible that glycerol permeated through the skin or ingested through grooming could impact our results. Lastly, mice were always exposed during the daytime, corresponding to their resting period. In an attempt to control for this, we subjected room air mice to disruptions similar to those experienced by electronic cigarette aerosol exposed mice: they were removed from their housing racks, brought into the room where the exposure system is found, and therefore exposed to similar light, sounds and vibrations. In doing so, we have shown that the impact of electronic cigarette aerosols on pulmonary and metabolic processes is due to exposure rather than a disruption in sleeping schedule and routine.

CONCLUSION

The studies presented in this thesis are among the first studies to investigate the biological effects of electronic cigarette aerosols. As research on electronic cigarette use is still in its infancy, we prioritized the broad characterization of aerosols emitted by electronic cigarettes, as well as its pulmonary and metabolic effects. Firstly, the chemical composition of vaping liquids as well as the impact of electronic cigarette parameters on aerosol particle size distribution was shown to affect their lung deposition. Secondly, exposure to electronic cigarette aerosols was shown to modify lung circadian rhythm, immunity, and pulmonary function. In addition, electronic cigarette aerosol exposure led to changes in the inflammatory response to tobacco cigarette smoke. Finally, we found that glycerol electronic cigarette aerosols reach the circulation and change energy metabolism in mice. Male and female mice, as well as young and adult mice, did not respond in the same way to chronic glycerol exposure. Overall, this thesis shows the importance of vaping liquid chemical composition on aerosol lung deposition and highlights the important pulmonary and systemic health impacts of flavour-free and nicotine-free electronic cigarette aerosols.

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