

City Research Online

City St George's, University of London

Citation: Effah, F. (2023). The Pharmaco-Toxicological Effects of E-cigarette Flavours on Human Bronchial Epithelial Cells. (Unpublished Doctoral thesis, St George's, University of London)

This is the accepted version of the paper.

This version of the publication may differ from the final published version. To cite this item please consult the publisher's version.

Permanent repository link: https://openaccess.city.ac.uk/id/eprint/37133/

Copyright and Reuse: Copyright and Moral Rights remain with the author(s) and/or copyright holders. Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge, unless otherwise indicated, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way. For full details of reuse please refer to City Research Online policy.

City Research Online: http://openaccess.city.ac.uk/ publications@citystgeorges.ac.uk

The Pharmaco-Toxicological Effects of E-cigarette Flavours on Human Bronchial Epithelial Cells

Thesis by

Felix Effah

Submitted in part-fulfilment of the requirements for the degree of *Doctor of Philosophy*

Institute of Medical and Biomedical Education,

St. George's, University of London

October 2023





DECLARATION OF ORIGINALITY

The thesis titled *The toxicological effects of E-cigarette flavours on bronchial epithelial cells* and its work is a result of my independent research. Any ideas, data, images, or text taken from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text or bibliography. I declare that this thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has every right to submit my work to the plagiarism detection service TurnitinUK for originality checks.

Felix Effah

ABSTRACT

This study investigates the potential adverse effects of flavoured e-liquids on the cellular components of the respiratory system, addressing a critical gap in understanding the adverse health consequences associated with electronic cigarette (EC) use. While ECs are deemed less hazardous than conventional tobacco smoking, concerns persist, especially regarding the inhalation of flavouring chemicals. Though these flavourings are generally recognised as safe for ingestion, their impacts upon inhalation, particularly when heated, remain ambiguous.

We evaluated 53 nicotine-free flavoured e-liquids, focusing on their direct and aerosolized impacts on submerged cultures and air-liquid interface (ALI) exposures. Employing *in vitro* endpoint assays, including cell count, cell viability, and cytotoxicity tests facilitated via a high-throughput 96-well plate system, we identified adverse effects associated with four specific flavours: cinnamon, hazelnut, vanilla tobacco, and menthol. These e-liquids demonstrated decreased mitochondrial membrane potential and increased reactive oxygen species (ROS) production, surpassing the effects of PG/VG vehicle control.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis unveiled the presence of potentially toxic volatile organic compounds, including estragole, biphenyls, and 1,3-dioxane in these e-liquids. Aerosol exposure studies in ALI-differentiated HBEC-3KT cells illustrated a unique toxicological profile for each of the four flavours, with cinnamon emerging as the most toxic. Notably, all flavours and, more markedly, cinnamon elicited a heightened genetic response in oxidative stress, marked by the significant stimulation of ddit3 and hmox1 genes. However, morphological changes to the lung epithelium were less pronounced than the 3R4F cigarette control. These findings contribute valuable insights to the ongoing discourse on EC safety. They

provide empirical data that can inform regulatory decisions concerning restricting or banning specific e-liquid flavours due to their toxicological profiles. The nuanced understanding of flavour-specific adverse effects underscores the need for comprehensive evaluations of all e-liquid constituents to ensure public health safety amidst the growing popularity of ECs.

DEDICATION

I want to dedicate this Ph.D. thesis to my mother and father, Mr Samuel Godfred Effah and Mrs Augustina Adubeah Effah, for being my source of academic inspiration. Also, I dedicate this thesis to my younger brothers, Ebenezer Effah and Christian Agyapong Effah, for being the fantastic brothers they are to me. I thank my dearest friend Maria-Rosa Boasiako for her support throughout this journey. Lastly, I dedicate this thesis to myself for not giving up and fighting through the hard times.

ACKNOWLEDGEMENTS

This work was sponsored by NIHR (HPRU) and UK Health Security Agency (UKHSA). Firstly, I would like to thank God for his protection and guidance over me during these 3 years of my PhD. Secondly, I would like to express my sincere gratitude to my supervisors, Dr Tim Marczylo, Dr Alexis Bailey, and Prof. Deborah Baines, for their support, encouragement, and advice throughout my PhD. I would also like to thank Prof. Terry Gordon (New York University), Prof. Tim Gant, Dr Emma Marczylo, Dr Abigail Dalzell, Emma-Jane Goode (Emma was an excellent support whenever I hit rock bottom), and my fellow PhD. Colleagues John Adragna (NYU), David Luglio (NYU), Lorna Jones, and Rachel Bowsher offered their time to provide expert advice and support. This gratitude is extended to the rest of the Toxicology team at UKHSA, especially Natalie Blowfield, for her outstanding admin support. A special thanks to my family.

“A journey of a thousand miles begins not just with a step but with the very thought of it. Through reflection, prayer, and action, I've achieved my Ph.D. goals. God has been my guide.”

~ Felix Effah

TABLE OF CONTENTS

Contents	Page
DECLARATION OF ORIGINALITY	**II**
ABSTRACT	**III**
DEDICATION	**V**
ACKNOWLEDGEMENTS	**VI**
TABLE OF CONTENTS	**VIII**
LIST OF FIGURES	**XIV**
LIST OF TABLES	**XVIII**
LIST OF PUBLICATIONS	**XX**
LIST OF PUBLICATIONS IN PREPARATION	**XXI**
LIST OF ABSTRACTS SUBMITTED AT CONFERENCES	**XXII**
LIST OF AWARDS	**XXIV**
CHAPTER 1 GENERAL INTRODUCTION	**1**
1.1 HISTORY OF TOBACCO SMOKING	1
1.1.1 TOBACCO SMOKING TOXICITY	2
1.2 PREVALENCE OF SMOKING	9
1.2.1 NICOTINE ADDICTION & PERIPHERAL EFFECTS	10
1.2.2 TOBACCO SPECIFIC NITROSAMINES	15
1.3 SMOKING AND PULMONARY DISORDERS	16
1.3.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASES PATHWAYS	17
1.3.2 MITOCHONDRIAL IMPAIRMENTS IN SMOKING-INDUCED LUNG DISEASE	19
1.4 LUNG CANCERS	21
1.4.1 SMOKING-INDUCED LUNG CANCER PATHWAYS	21
1.4.2 THE ROS- AND RNS-MEDIATED PATHWAY	22
1.4.3 THE IMMUNE CELL-MEDIATED PATHWAYS	24
1.5 HARM REDUCTION AND SMOKING CESSATION POLICIES IN THE UK	*27*
1.6 ELECTRONIC CIGARETTES	*29*
1.6.1 ELECTRONIC CIGARETTE USE, RECENT TRENDS, EFFECTIVENESS & POTENTIAL HAZARDS	32
1.6.2 ELECTRONIC CIGARETTE FLAVOURINGS	36
1.7 THESIS QUESTION, HYPOTHESIS, AND AIMS	*42*
1.7.1 RESEARCH QUESTION:	42
1.7.2 THE HYPOTHESES:	42

1.7.3 THE AIMS: 42

CHAPTER 2 PULMONARY EFFECTS OF ELECTRONIC CIGARETTES FLAVOURS: A SYSTEMATIC REVIEW 44

2.1 INTRODUCTION 45

2.2 METHODS 46

2.2.1 DATA SOURCES AND SEARCHES 46

2.2.2 SELECTION AND EXCLUSION CRITERIA 47

2.3 RESULTS 48

2.4 DISCUSSION 83

2.4.1 ADVERSE EFFECTS OF FLAVOURED E-LIQUIDS 84

2.4.2 THE POTENTIAL ADDITIVE EFFECTS OF NICOTINE AND PG/VG ON FLAVOUR-INDUCED TOXICITY 90

2.4.3 RECOMMENDED APPROACH TO REDUCE UNCERTAINTY IN EC INVESTIGATION *IN VITRO* AND *IN VIVO* MODELS. 94

2.5 CONCLUSION 99

CHAPTER 3 IN VITRO HIGH-THROUGHPUT TOXICOLOGICAL ASSESSMENT OF E-CIGARETTE FLAVOURS ON HUMAN BRONCHIAL EPITHELIAL CELLS AND THE POTENTIAL INVOLVEMENT OF TRPA1 IN CINNAMON FLAVOUR-INDUCED TOXICITY 100

3.1 INTRODUCTION 101

3.1.1 AIM 102

3.1.2 HYPOTHESES: 102

3.1.3 OBJECTIVES: 102

3.2 MATERIALS AND METHODS 103

3.2.1 CELL CULTURE 103

3.2.2 CELL EXPOSURE TO E-LIQUIDS 103

3.2.3 PRELIMINARY STUDIES WITH NICOTINE 107

3.2.4 MECHANISTIC STUDIES 108

3.2.5 TRPA1 INHIBITOR STUDIES 110

3.2.6 CHEMICAL ANALYSIS 114

3.2.7 STATISTICAL ANALYSIS 115

3.3 RESULTS 116

3.3.1 THE EFFECTS OF NICOTINE ON HBEC-3KT CELL COUNT, VIABILITY, AND TOXICITY 116

3.3.2 HIGH-THROUGHPUT SCREENING 118

3.3.3 MECHANISTIC STUDIES 141

3.3.3.1 GROWTH CURVES 141
3.3.4 GC-MS/MS ANALYSIS OF VOCS FROM CINNAMON, HAZELNUT, AND VANILLA E-LIQUIDS 148
3.3.5 TRPA1 INHIBITOR STUDIES 150
3.4 DISCUSSION 162
3.4.1 STUDY LIMITATIONS 171
3.4.2 CONCLUSION 172
3.4.3 FUTURE STUDIES & REGULATIONS OF FLAVOURS 173

CHAPTER 4 TOXICOLOGICAL ASSESSMENT OF E-CIGARETTE FLAVOURED E-LIQUIDS AEROSOLS USING CALU-3 CELLS: A 3D LUNG MODEL APPROACH 175

4.1 INTRODUCTION 176
4.1.1 AIR-LIQUID INTERFACE 177
4.1.2 AIMS AND HYPOTHESIS 182
4.1.3 HYPOTHESES: 182
4.1.4 OBJECTIVES: 183
4.2 METHODS 183
4.2.1 CELL CULTURE 183
4.2.2 CHARACTERISATION OF CALU-3 CELLS AS A PHYSIOLOGICALLY RELEVANT 3D MODEL 185
4.2.3 TOXICOLOGICAL ASSESSEMENT OF CINNAMON, HAZELNUT AND VANILLA TOBACCO E-LIQUIDS ON CALU-3 CELLS 190
4.2.4 EC AEROSOL SIZE DISTRIBUTION CHARACTERISATION 192
4.2.5 STATISTICAL ANALYSIS 193
4.3 RESULTS 194
4.3.1 VALIDATION OF CALU-3 CELLS AT ALI 194
4.3.2 LDH AND TEER IN CALU-3 IN SUSPENSION CULTURE 196
4.3.3 ROS PRODUCTION IN CALU-3 IN SUBMERGED EXPOSURES 197
4.3.4 LDH AND TEER IN CALU-3 ALI POST-EXPOSURE TO EC AEROSOL 198
4.3.5 PARTICLE SIZE DISTRIBUTION CHARACTERIZATION 199
4.4 DISCUSSION 201
4.4.1 CONCLUSION 211

CHAPTER 5 ESTABLISHING A SUITABLE PHYSIOLOGICALLY REPRESENTATIVE *IN VITRO* AIRWAY MODEL TO INVESTIGATE THE TOXICOLOGICAL EFFECTS OF EC AEROSOL IN LUNG EPITHELIAL CELLS (A549, BEAS-2B, HBEC-3KT, AND RPMI 1640) 212

5.1 INTRODUCTION 213
5.1.2 RPMI 2650 AND A549 CELL LINES 214
5.1.3 COMPARISON OF RPMI 2650, CALU-3, AND A549 AS 3D LUNG MODELS AT THE AIR-LIQUID INTERFACE 214
5.1.4 HBEC-3KT AND BEAS-2B CELL LINES 215
5.1.5 COMPARISON OF HBEC-3KT AND BEAS-2B AS 3D LUNG MODELS AT THE AIR-LIQUID INTERFACE 216
5.1.6 COMPARISON BETWEEN PRIMARY, IMMORTALISED, NORMAL AND CANCEROUS CELLS 218
5.1.7 AIMS AND HYPOTHESIS 219
5.1.8 THE HYPOTHESES 219
5.1.9 THE OBJECTIVES 219
5.2 METHODS 220
5.2.1 CELL LINES AND CELL CULTURE 220
5.2.2 CELL PASSAGING 220
5.2.3 COLLAGEN COATING 221
5.2.4 CELL SEEDING OPTIMISATION 221
5.2.5 TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER) 222
5.2.6 RNA EXTRACTION AND CDNA SYNTHESIS 222
5.2.6 RT-qPCR 223
5.2.7 STATISTICAL ANALYSIS 223
5.3 RESULTS 224
5.3.1 GENETIC MARKERS OF BASAL CELLS AT ALI 224
5.3.2 GENETIC MARKERS OF MUCUS-PRODUCING CELLS 227
5.3.3 GENETIC MARKERS OF CILIATED CELLS 231
5.3.4 GENETIC MARKERS OF TIGHT JUNCTION PROTEIN 232
5.3.5 TIGHT JUNCTION FORMATION 233
5.3.6 THE MORPHOLOGICAL CHANGES IN HBEC-3KT, BEAS-2B, A549 and RPMI 2650 OVER 28 DAYS AT ALI 235
5.4 DISCUSSION 240
5.4.1 CONCLUSIONS 245
CHAPTER 6 UTILISING THE CULTEX EXPOSURE SYSTEM TO ASSESS THE TOXICITY ASSOCIATED WITH FLAVOURS IN E-CIGARETTE AEROSOLS: A PHYSIOLOGICALLY RELEVANT APPROACH USING 3D HBEC-3KT LUNG MODELS 246
6.1 INTRODUCTION 247
6.1.2 AIMS AND HYPOTHESIS 248
6.1.3 THE HYPOTHESES: 248

6.1.4 THE OBJECTIVES: 248
6.2 MATERIALS AND METHODS 249
6.2.1 CELL CULTURE 249
6.2.2 AEROSOL EXPOSURE AT AIR LIQUID INTERFACE (AE-ALI) EXPOSURE SYSTEM 249
6.2.3 TIME OF EXPOSURE & SYSTEM OPTIMISATION 255
6.2.4 HBEC-3KT EXPOSURES TO AEROSOLS 255
6.2.5 AEROSOL CHARACTERISTICS 257
6.2.7 CELL CYTOTOXICITY: LDH ASSAY 262
6.2.8 RANSEPITHELIAL ELECTRICAL RESISTANCE (TEER) 262
6.2.9 qRT-PCR ANALYSIS 262
6.3 RESULTS 268
6.3.1 ASSESSING THE EFFECTS OF VARIED AIR EXPOSURE DURATIONS ON HBEC-3KT CELLS. IMPLICATIONS FOR CYTOTOXICITY, GENETIC MARKERS FOR CELL STRESS, AND AIRWAY DIFFERENTIATION 268
6.3.2 EC AEROSOL PARTICLE SIZE DISTRIBUTION 273
6.3.3 CYTOTOXICITY AND BARRIER DISRUPTION IN HUMAN BRONCHIAL EPITHELIAL CELLS AT ALI FOLLOWING EXPOSURE TO FLAVOURED E-CIGARETTE AEROSOL 276
6.3.4 DIFFERENTIAL EFFECTS OF E-CIGARETTE AEROSOLS ON TRANSCRIPT LEVELS OF AIRWAY DIFFERENTIATION MARKERS IN HUMAN BRONCHIAL EPITHELIAL CELLS 278
6.3.5 THE EFFECTS OF FLAVOURED E-CIGARETTE AEROSOLS ON GENETIC MARKERS OF CELL STRESS 283
6.3.6 THE EFFECTS OF FLAVOURED E-CIGARETTE AEROSOLS ON mRNA LEVELS OF CYTOKINES 285
6.3.7 DIFFERENTIAL EFFECTS OF 28, 42, & 56 PUFFS OF E-CIGARETTE AEROSOL ON PROTEIN LEVELS 288
6.3.8 DIFFERENTIAL EFFECTS OF 28, 42, & 56 PUFFS OF E-CIGARETTE AEROSOLS ON GALECTIN-3 LEVELS AND DNA DAMAGE MARKER 8-OHdG 290
6.3.9 THE EFFECTS OF FLAVOURED E-LIQUID AEROSOLS ON THE MORPHOLOGY OF HBEC-3KT EPITHELIUM 292
6.4 DISCUSSION 296
6.4.1 ELECTROSTATIC PRECIPITATOR'S IMPACT ON CELLULAR STRESS AND TOXICITY IN ALI-CULTURED HBEC-3KT CELLS 296
6.4.2 EXPLORING THE VARIABILITY IN PARTICLE SIZE AND COUNT IN E-CIGARETTE AEROSOLS: INFLUENCE OF E-LIQUID COMPOSITION AND METHODOLOGY 297

6.4.3 DIFFERENTIAL EFFECTS OF E-CIGARETTE FLAVOURINGS AND CIGARETTE SMOKE EXTRACT ON EPITHELIAL INTEGRITY AND CYTOTOXICITY 298
6.4.5 COMPLEX MODULATION OF AIRWAY EPITHELIAL GENE EXPRESSION BY FLAVOURED E-LIQUID AEROSOLS: IMPLICATIONS FOR RESPIRATORY HEALTH AND HOMEOSTASIS 299
6.4.6 UNDERSTANDING THE CELLULAR STRESS RESPONSES INDUCED BY FLAVOURED E-LIQUIDS' AEROSOLS 301
6.4.7 THE EFFECTS OF FLAVOURED E-LIQUIDS' AEROSOLS ON INFLAMMATORY CYTOKINES MRNA TRANSCRIPT LEVELS 303
6.4.8 THE ABSENCE OF SIGNIFICANT INFLAMMATORY RESPONSES AT THE PROTEIN LEVEL POST E-CIGARETTE EXPOSURE 304
6.4.9 UNALTERED GALECTIN-3 AND 8-OXODG LEVELS: EVALUATING THE IMPACT OF E-CIGARETTE FLAVOURS ON INFLAMMATION AND DNA DAMAGE 304
6.4.10 MORPHOLOGICAL ALTERATIONS IN CELLS EXPOSED TO DIFFERENT E-CIGARETTE FLAVOURS AND CIGARETTE SMOKE: INSIGHTS INTO CELLULAR INTEGRITY AND RESPONSES 305
6.4.11 STUDY LIMITATIONS 306
6.5 CONCLUSIONS 307
CHAPTER 7 GENERAL DISCUSSION 310
7.1 DISCUSSION 311
7.2 GENERAL SUMMARY OF FINDINGS AND STUDY LIMITATIONS 311
7.3 CONTRIBUTION AND IMPACT TO PUBLIC HEALTH 315
7.4 FUTURE DIRECTIONS 315
7.5 CONCLUSION 320
REFERENCES 321

LIST OF FIGURES

Figure	Title	Page
1. 1	Health risks associated with smoking	4
1. 2	Metabolic Pathways of Nicotine in Smokers	14
1. 3	The potential metabolomic pathway of TSNA formation	16
1. 4	Alterations in gas exchange may alter MMP	19
1. 5	The microenvironment of lung cancer	26
1. 6	A schematic diagram of the main components of an EC	29
1. 7	E-liquid composition	30
1. 8	Different generations of EC devices	31
1. 9	The flavour wheel	40
2. 1	PRISMA flowchart used to conduct the systematic review	49
2. 2	The potential pathways of flavour toxicty	88
2. 3	The effects of flavours in different experimental models	98
3. 1	The high throughput approach in flavour toxicity screening	106
3. 2	The effect of nicotine on PG/VG toxicity	118
3. 3	The cytotoxic effects of PG/VG (60/40) and PG alone	119
3. 4	The effects of cinnamon, hazelnut, and vanilla tobacco on cell count	121
3. 5	The effects of cinnamon, hazelnut, and vanilla tobacco on cell viability	130
3. 6	The effects of cinnamon, hazelnut and vanilla tobacco on LDH release	132
3. 7	The effects of PG/VG, cinnamon, hazelnut, and vanilla tobacco on HBEC-3KT proliferation	142
3. 8	The effects of PG/VG, cinnamon, hazelnut, and vanilla tobacco on MMP	145
3. 9	The effects of PG/VG, cinnamon, hazelnut, and vanilla tobacco on ROS production	146
3. 10	The chemical structure of cinnamaldehyde	151
3. 11	Characterising the IC_{50} of AP-18	152
3. 12	Effects of AP-18 on cinnamon e-liquid	155
3. 13	Effects of AP-18 on cinnamaldehyde	157

3. 14 Comprehensive gene stability of the housekeeping genes 159
3. 15 Images taken after 24h treatment with AP-18 and/or cinnamon and qPCR analysis to determine the mRNA levels of TRPA1 in the treatment groups 161
3. 16 Potential toxicity mechanisms of PG/VG in e-liquid. 164
3. 17 The The potential pharmacological actions of AP-18 (10 mM) in combination with cinnamon (cinnamaldehyde). 168
3. 18 Summary of the pathways via which cinnamon, hazelnut and vanilla tobacco induced toxicity. 174
4. 1 The multipotent abilities of pulmonary basal cells 178
4. 2 The ALI process 185
4. 3 Quality score for the housekeeping genes 188
4. 4 Comprehensive gene stability of the housekeeping genes 188
4. 5 Establishment of Calu-3 epithelium using ALI cultivation 196
4. 6 The effects of cinnamon, hazelnut, and vanilla tobacco e-liquids on TEER measurement and LDH levels in Calu-3 cells in suspension cultures 197
4. 7 The effects of cinnamon, hazelnut, and vanilla tobacco e-liquids on ROS production in Calu-3 cells in suspension cultures 198
4. 8 The effects of cinnamon, hazelnut, and vanilla tobacco aerosols on TEER measurements and LDH in Calu-3 at ALI 201
5. 1 Diagram to depict the airway cell lines validated and compared at the air-liquid interface 218
5. 2 mRNA levels of genetic markers of basal cells differentiation in HBEC-3KT, Beas-2b, A549 and RPMI 2650 226
5. 3 mRNA levels of genetic markers mucus-producing cells differentiation in HBEC-3KT, Beas-2b, A549 and RPMI 2650 230
5. 4 mRNA levels of genetic marker of ciliogenesis in HBEC-3KT, Beas-2b, A549 and RPMI 2650 232
5. 5 mRNA levels of genetic marker of tight junction protein 1 formation in HBEC-3KT, Beas-2b, A549 and RPMI 2650 233
5. 6 TEER measurements in Beas-2b, A549, RPMI 2650 and HBEC-3KT 234
5. 7 Images of HBEC-3KT taken on day 0, 14 and 28 236
5. 8 Images of Beas-2b cells taken on day 0, 14 and 28 237

5. 9	Images of A549 cells taken on day 0, 14 and 28	238
5. 10	Images of RPMI 2650 cells taken on day 0, 14 and 28	239
6. 1	A diagram of the “directed flow” ALI exposure technique utilised in the CULTEX	256
6. 2	Typical puff particle number count profile for PG/VG, cinnamon, hazelnut, vanilla tobacco, and menthol	258
6. 3	Schematic diagram of the ALI system used for *in vitro* cell exposure	260
6. 4	Images to depict the aerosol exposure settings and the CULTEX system used to expose HBEC-3KT cells	261
6. 5	Quality score for the housekeeping genes	264
6. 6	Comprehensive gene stability of the housekeeping genes	264
6. 7	Effect of different air exposure durations on HBEC-3KT Cells	273
6. 8	Aerosol particle size distribution for PG/VG, cinnamon, vanilla tobacco, hazelnut, and menthol e-liquids	276
6. 9	The effect of increasing concentrations of flavoured-e-liquids (vanilla tobacco, cinnamon, menthol & hazelnut) aerosols on LDH release and TEER measurements in HBEC-3KT cells	278
6. 10	mRNA transcript levels of markers of differentiation following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol	282
6. 11	mRNA transcript levels of cellular stress following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol	284
6. 12	mRNA transcript levels of cytokines following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol	288
6. 13	Cytokine levels following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol e-liquids	290
6. 14	Characterising the dose-dependent (28 puffs, 42 puffs, and 56 puffs) effects of cinnamon, vanilla tobacco, hazelnut, and menthol on Galectin-3 and 8-OHdG in HBEC-3KT	292

6. 15 images of HBEC-3KT cells following exposure to PG/VG, vanilla tobacco, cinnamon, hazelnut, menthol, and cigarette smoke extract 295

LIST OF TABLES

Table	Title	Page
1. 1	List of some of 93 harmful chemicals in tobacco smoke	5
1. 2	Flavours in e-liquid and some of the main flavouring chemicals they are composed of	38
2. 1	*In vitro* studies where toxicological effects of flavoured e-liquids were investigated with primary epithelial cells alone or together with immortalised normal pulmonary cells.	50
2. 2	*In vitro* studies where toxicological effects of flavoured e-liquids were carried out on immortalised pulmonary normal epithelial cells alone or together with carcinoma pulmonary cells.	57
2. 3	*In vitro* studies where toxicological effects of flavoured e-liquids were investigated in pulmonary carcinoma cells alone or together with primary pulmonary cells.	69
2. 4	Chemical composition analyses of flavoured EC.	77
2. 5	In vivo studies of toxicological effects of EC flavours.	79
3. 1	pH measurements of the exposure conditions for the TRPA1 inhibitor studies.	111
3. 2	The primer sequences for the 4 endogenous controls (gapdh, hprt1, 18sRNA and beta-actin) and trpa1.	113
3. 3	Cell count analysis for all 53 flavoured e-liquids vs. media-only controls and their respective PG/VG controls.	122
3. 4	LDH analysis for all 53 flavored e-liquids vs. media-only controls and their respective PG/VG controls.	133
3. 5	Tukey's multiple comparisons test to characterize the dose-response relationship within the toxic hits on cell count, % of viable cells, and LDH release.	140
3. 6	Tukey post-hoc comparisons for Figure 3.9.	143
3. 7	Tukey's multiple comparisons test to characterize the dose-response relationship within the toxic hits on MMP and ROS generation.	147
3. 8	List of volatile organic compounds detected in Cinnamon, Hazelnut, and Vanilla. Compounds are listed in order of elution.	149

4. 1 Primer sequences for the six primers of differentiation and their primer efficiency for Calu-3 cells. 187

6. 1 Details of the experimental design, e-liquids used per exposure and the amount of aerosols deposited onto cells in the CULTEX. 251

6. 2 Primer sequences. For the primer sequences for the epithelial differentiation and the HK genes, see Table 4.1 in Chapter 4 265

LIST OF PUBLICATIONS

1 - Authors: Felix Effah, Benjamin Taiwo, Deborah Baines, Alexis Bailey, and Tim Marczylo

DOI: 10.1080/10937404.2022.2124563

Title: Pulmonary effects of e-liquid flavors: a systematic review

Status: Published in the Journal of Toxicology and Environmental Health, Part B

2 - Authors: Felix Effah, Atallah Elzein, Benjamin Taiwo, Deborah Baines, Alexis Bailey, and Tim Marczylo

DOI: **https://doi.org/10.1016/j.tox.2023.153617**

Title: *In Vitro* high-throughput toxicological assessment of E-cigarette flavors on human bronchial epithelial cells and the potential involvement of TRPA1 in cinnamon flavor-induced toxicity.

Status: Published in Toxicology

3 - Authors: Felix Effah, John Adragna, David Luglio, Deborah Baines, Alexis Bailey, Tim Marczylo and Terry Gordon

Title: Toxicological assessment of e-cigarettes flavored aerosols using calu-3 cells: A 3D lung model approach

DOI: https://doi.org/10.1016/j.tox.2023.153683

Status: Published in Toxicology

LIST OF PUBLICATIONS IN PREPARATION

1 - Authors: Felix Effah, John Adragna, David Luglio, Deborah Baines, Alexis Bailey, Tim Marczylo and Terry Gordon

Title: Aerosols from PG/VG, Cinnamon, Hazelnut, and Vanilla tobacco e-liquids induce comparative toxicity than cigarette smoke in 3D Calu-3 lung model

Status: Ready for Submission to Toxicology

2 - Authors: Rachel Bowsher, Emma-Jane Goode, Felix Effah, Tim Marczylo, Emma Marczylo

Title: Establishing a physiologically representative *in vitro* airway model: a comparison of commercially available lung epithelial cells

Status: In experimentation and writing

LIST OF ABSTRACTS SUBMITTED AT CONFERENCES

Gordon Research Conferences, **Andover, USA** 12-18 August 2023

Authors: Felix Effah, Atallah Elzein, Ben Taiwo, Deborah Baines, Alexis Bailey, Tim Marczylo

Title: In vitro high-throughput toxicological assessment of e-cigarette flavors in human bronchial epithelial cells and the role of TRPA1 in cinnamon flavor-induced toxicity

Role: Oral presentation and Poster presentation

The British Toxicology Society, Birmingham, UK 17-19 April 2023

Authors: Felix Effah, Atallah Elzein, Ben Taiwo, Deborah Baines, Alexis Bailey, Tim Marczylo

Title: In vitro high-throughput toxicological assessment of e-cigarette flavors in human bronchial epithelial cells and the role of TRPA1 in cinnamon flavour-induced toxicity

Role: Poster presentation

NIHR HPRU Annual Meeting London, **UK** 15-17 November 2022

Authors: Felix Effah, Ben Taiwo, Deborah Baines, Alexis Bailey, Tim Marczylo

Title: In vitro high-throughput toxicological assessment of e-cigarette flavours in human bronchial epithelial cells and the role of TRPA1 in cinnamon flavor-induced toxicity

Role: Oral presentation and Poster

UK Health Security Agency Leeds 18-19 October 2022

Authors: Felix Effah, Ben Taiwo, Deborah Baines, Alexis Bailey, Tim Marczylo

Title: In vitro high-throughput toxicological assessment of e-cigarette flavours in human bronchial epithelial cells

Role: Oral presentation and Poster

LIST OF AWARDS

1. **NIHR Fourteenth Annual Doctoral Training Camp**: Best Patient and Public Involvement Application Award 2023
2. **Royal Society of Chemistry and British Toxicology Society**: Best Poster Award 2023
3. **British Pharmacological Society**: Schachter Award for New York
4. **St**. **George's University of London:** Turing Travel Award for New York

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORY OF TOBACCO SMOKING

Nicotiana tobacum (NT) is a herbaceous plant from which tobacco for use in cigarettes, cigars and other products is derived. NT produces nicotine as a secondary metabolite to defend against insects; nicotine was subsequently used globally as a pesticide until it was banned in the 1960s (Russo *et al.*, 2011). Tobacco smoking has been and continues to be a popular pastime. The first archaeological evidence for tobacco use arguably came from a clay pipe from the Banda region of Ghana in West Africa, dating to the 19th century BCE, as supported by gas chromatography/mass spectrometry (GC– MS) of the pipe residues, which found peaks identical to those observed with samples of pure nicotine (Rafferty, 2002; Stahl, 1994). However, it is more generally accepted that NT is native to the Americas and was used by the Native Americans from around 6000 BCE (Pearsall, 1992). Tobacco came to Europe in the late 15th century after the *discovery* of the Americas by Christopher Columbus. Still, tobacco smoking did not become widespread until the First World War (1914-1918), when cigarettes were included in the daily rations of American soldiers. Tobacco smoke induces psychological benefits of reduced stress (O'Neill and Parrott, 1992), increased stimulation (Parrott, 1993), and an improved mood (Parrott, 1994). As a result, healthcare professionals once encouraged the use of tobacco for medicinal purposes (Charlton, 2004). Concerns about the health implications of cigarette smoking commenced in the mid-20th century following epidemiological studies linking smoking to the incidence of cancer (for review, see (Russo *et al.*, 2011). In 1955 the UK officially recognised the causal link between smoking and cancer following human studies conducted by Doll and Hill, (1950).

1.1.1 TOBACCO SMOKING TOXICITY

According to the World Health Organization, there are approximately 1.1 billion smokers worldwide. Ever-increasing research reporting detrimental health effects, and the consequences of raised taxes, pictorial warning labels, advertisement bans, and government-imposed restrictions on public smoking have significantly contributed to the decline in cigarette consumption in Great Britain (ASH, 2022b) and many other developed countries. However, in other parts of the world, such as China and some Eastern European countries, the prevalence of smoking remains high. Irrespective of their knowledge of the adverse health implications of tobacco smoking, many people still refuse to give smoking up or fail in their attempts to quit smoking with or without the help of smoking cessation experts in the NHS stop smoking service (NHS-SSS). The difficulty in quitting smoking is due to the addictive nature of the nicotine content in tobacco. For decades, tobacco smoking has remained the primary cause of preventable diseases and deaths globally (WHO, 2022b). In England, approximately 77,800 deaths and 489,300 hospital admissions were attributed to smoking in 2018 (NHS, 2020, p. 20), with 5,500 deaths each year in Wales, 10,000 in Scotland, and 2,300 in Northern Ireland (NHS, 2019).

Tobacco smoke comprises approximately 8,000 different chemicals, of which 93 are identified as toxicants (**Table 1.1**) or potential toxicants that cause direct/indirect harm through inhalation, ingestion, or absorption into the body (FDA, 2012). Some of these toxicants are responsible for the onset of life-threatening medical conditions (**Figure 1.0)** that affect the cardiovascular (i.e., angina, myocardial infarction, atherosclerosis, etc.) (Frost-Pineda *et al.*, 2011), digestive (Doll *et al.*, 2005; El-Zayadi, 2006) and respiratory system (Buist *et al.*, 2008; Vestbo *et al.*, 2013). Common respiratory health risks of smoking include but are not limited to, chronic obstructive pulmonary diseases

(COPD), and cancer (Buist *et al.*, 2008; Vestbo *et al.*, 2013). In addition, second-hand smoke (passive smoking) can also result in a range of health problems (stroke, nasal irritation, lung cancer, coronary heart disease) in non-smokers, especially children, the elderly, and individuals with pre-existing health complications (CDC, 2014; McEvoy and Spindel, 2017; Olivieri *et al.*, 2019). Moreover, chemicals in tobacco smoke can alter and promote the growth of aggressive microbes and kill the 'good' microbes in the oral and pulmonary microflora and the gut microbiota (Huang and Shi, 2019). Because commensal microbes contribute to host immunity and regulation of several physiological mechanisms, especially neurochemicals, via the gut-brain axis (Biedermann *et al.*, 2014; Crumeyrolle-Arias *et al.*, 2014; Cryan and Dinan, 2012), recent evidence suggests that changes to the microbiota may be involved in tobacco smoking-induced adverse health effects (Biedermann *et al.*, 2014; Huang and Shi, 2019).

The tobacco-related chemicals cancer-promoting and carcinogenic effects are the polycyclic aromatic hydrocarbons (PAHs) (i.e., Benzo[a]pyrene) (Hecht, 2003; Kalabus *et al.*, 2012; Mantey *et al.*, 2014; Melendez-Colon *et al.*, 1999) and tobacco-specific nitrosamines (TSNAS), such as 4-(methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) (Cheng *et al.*, 2014; Zhang *et al.*, 2016) and N-nitrosonornicotine (NNN) (Balbo *et al.*, 2013; Stepanov *et al.*, 2009).

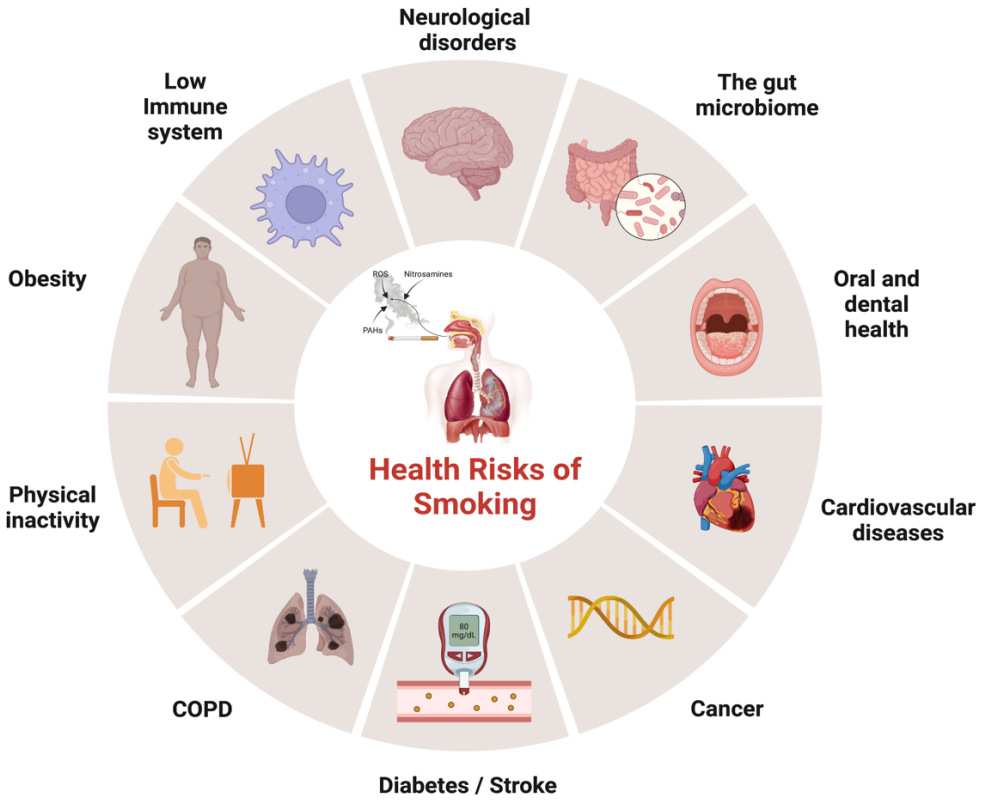


Figure 1. 1 Health risks associated with smoking

Table 1. 1 List of some of 93 chemicals and chemical compounds identified by FDA as harmful and potentially harmful constituents in tobacco smoke

Chemical	Toxicity
Acetaldehyde	Carcinogen, Respiratory Toxicant, Addictive
Acetamide	Carcinogen
Acetone	Respiratory Toxicant
Acrolein	Respiratory Toxicant, Cardiovascular Toxicant
Acrylamide	Carcinogen
Acrylonitrile	Carcinogen, Respiratory Toxicant
Aflatoxin B1	Carcinogen
4-Aminobiphenyl	Carcinogen
1-Aminonaphthalene	Carcinogen
2-Aminonaphthalene	Carcinogen
Ammonia	Respiratory Toxicant
Anabasine	Addictive
o-Anisidine	Carcinogen
Arsenic	Carcinogen, Cardiovascular Toxicant, Reproductive or Developmental Toxicant

A-α-Carotene	Carcinogen
Benzene	Carcinogen, Respiratory Toxicant
Benzo[a]pyrene	Carcinogen
Benzo[b]fluoranthene	Carcinogen
Benzo[j]fluoranthene	Carcinogen
Benzo[k]fluoranthene	Carcinogen
Benzo[a]anthracene	Carcinogen
Benzo[b]naphtho[2,3-d]thiophene	Carcinogen
Benzo[a]pyrene	Carcinogen
Benzo[e]pyrene	Carcinogen
Benzo[ghi]perylene	Carcinogen
Benzo[1,2,3-cd]pyrene	Carcinogen
Benzylamine	Respiratory Toxicant
Beryllium	Carcinogen, Respiratory Toxicant
1,3-Butadiene	Carcinogen, Respiratory Toxicant
Cadmium	Carcinogen, Cardiovascular Toxicant, Reproductive or Developmental Toxicant

Carbon monoxide	Respiratory Toxicant, Cardiovascular Toxicant
Crotonaldehyde	Respiratory Toxicant
p-Cresidine	Carcinogen
Crotonaldehyde	Respiratory Toxicant
p-Cresidine	Carcinogen
2,3-Dimethyl-2-butene	Respiratory Toxicant
1,1-Dimethylhydrazine	Carcinogen
Ethyl carbamate	Carcinogen
Ethylbenzene	Respiratory Toxicant
Ethylene dibromide	Carcinogen, Respiratory Toxicant
Ethylene oxide	Carcinogen, Respiratory Toxicant
Formaldehyde	Carcinogen, Respiratory Toxicant
Furan	Carcinogen
Hydrazine	Carcinogen, Respiratory Toxicant
Hydrogen cyanide	Respiratory Toxicant
Indeno[1,2,3-cd]pyrene	Carcinogen

Isoprene	Respiratory Toxicant
Lead	arcinogen, Cardiovascular Toxicant, Reproductive or Developmental Toxicant
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	Carcinogen, Addictive
Naphthalene	Carcinogen, Respiratory Toxicant
Nickel	Carcinogen, Respiratory Toxicant
4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone	Carcinogen
N-Nitrosonornicotine	Carcinogen
N-Nitrosodiethylamine	Carcinogen
N-Nitrosodimethylamine	Carcinogen
N-Nitrosopyrrolidine	Carcinogen
Phenol	Respiratory Toxicant
Polonium-210	Carcinogen
Propionaldehyde	Respiratory Toxicant
Propylene oxide	Carcinogen, Respiratory Toxicant
Quinoline	Carcinogen

Styrene	Carcinogen, Respiratory Toxicant
Toluene	Respiratory Toxicant
2-Toluidine	Carcinogen
o-Toluidine	Carcinogen
1,2,3-Trichloropropane	Carcinogen
Urethane	Carcinogen
Vinyl acetate	Carcinogen, Respiratory Toxicant
Vinyl chloride	Carcinogen, Respiratory Toxicant
Xylene	Respiratory Toxicant

1.2 PREVALENCE OF SMOKING

The prevalence of smoking, nicotine addiction, and the use of smoking cessation aids varies across different world regions and populations. In the United Kingdom, smoking prevalence has fallen from 51% of men and 41% of women in 1972, to 15.1% of men and 11.5% of women in 2021 (NICE, 2023c). This equates to 13.3% of the UK adult population (around 6.6 million people), which is a 6.9 percent reduction since 2011 (NICE, 2023c). This significant decrease in prevalence is likely due to public health campaigns and policies to reduce tobacco use. In the United States, smoking has declined from 20.9% of adults in 2005 to 11.5% adults in 2021 (CDC, 2023). This means an estimated 28.3 million adults in the United States currently smoke cigarettes (Cornelius E. *et al.*, 2023). More than 16 million Americans live with a smoking-related

disease (CDC, 2023). Like the UK, smoking in the US has been declining in recent years, but smoking remains a significant public health concern. Despite some progress in reducing smoking rates in Europe over the last decade, more than one in five adults still smoked daily in 2018 on average across EU countries (OECD Health statistics, 2020). The proportion of adults who smoke daily varies more than three-fold across countries. In some countries, such as Sweden and Norway, smoking prevalence is relatively low, with less than 10% of the population reporting that they are smokers. In other countries, such as Bulgaria (27%), Turkey (27%), and Greece (35%), the prevalence of smoking is considerably higher (OECD Health statistics, 2020). This marked difference is likely due to a combination of reasons including smoking prevention policies of each country such as plain packaging, point-of-sale legislation, use of pictorial health warnings) and the availability of tobacco alternatives i.e., snus (McNeill *et al.*, 2022, 2018).

1.2.1 NICOTINE ADDICTION & PERIPHERAL EFFECTS

Nicotine addiction is a significant contributor to the prevalence of smoking. Nicotine is the primary psychoactive substance in tobacco (Benowitz, 2010; Benowitz *et al.*, 2009). When a person smokes, the chemical structure of nicotine plays a role in its rapid absorption into the bloodstream (Benowitz *et al.*, 2009). Nicotine is an alkaloid molecule; its solubility is influenced by factors like temperature and pH. At room temperature, it has moderate solubility in water and is more readily soluble in various organic solvents like glycerine and propylene glycol. This property is attributed to its relatively small size and ability to engage in hydrogen bonding and dipole-dipole interactions. (**Figure 1.2)**. This structure allows it to be easily absorbed through the skin (Gorsline *et al.*, 1993), oral mucosa (Benowitz and Iii, 1984), lungs (Russell, 1980), and

the gastrointestinal tract (Gourlay and Benowitz, 1997). Once absorbed, nicotine is rapidly transported to the brain, where it binds to and activates nicotinic acetylcholine receptors (nAChRs), releasing neurotransmitters, such as dopamine, which produce a highly reinforcing feeling of pleasure and relaxation (Tiwari *et al.*, 2020). This rapid onset of action is one of the reasons that nicotine is highly addictive. The amount of nicotine absorbed from tobacco smoke depends on factors such as the depth and duration of inhalation, the cigarette's pH, the nicotine levels, and individual biological factors (Benowitz *et al.*, 2009). Apart from the brain, nicotine and its metabolites affect various biological functions. High doses of nicotine can induce toxicity in several organs. Mainly, prolonged exposures to nicotine may negatively contribute to cardiovascular diseases by stimulating the release of catecholamines (predominantly noradrenaline), which, in turn, induce tachycardia. Also, hypertension, vasoconstriction, gene expression alterations (such as DNA methylation (Silva and Kamens, 2021), KRAS and TP53 gene mutations (Blackford *et al.*, 2009), 15q24 gene alterations (Pray, 2008)) and hormonal secretion regulation (such as arginine vasopressin, oxytocin (Tweed *et al.*, 2012), cortisol (Funabashi *et al.*, 2005), and prolactin (Fuxe *et al.*, 1989)) are all adverse health effects elicited by long-term exposure to nicotine (Benowitz *et al.*, 2009; Benowitz and Iii, 1984; Russell, 1980). Nicotine metabolism is complex and involves several enzymes, primarily in the liver. The major metabolite of nicotine in humans is cotinine (70-80%), which is further metabolized to trans-3'-hydroxycotinine (3-HC). Among the other nicotine metabolites are nicotine-N'-oxide (4-7%) and nornicotine a N-demethylated metabolite of nicotine (Tiwari *et al.*, 2020). Cotinine, 3-HC, nicotine-N'-oxide, and nornicotine are each metabolized by the CYP2A6 enzyme, which belongs to the cytochrome P450 superfamily of enzymes (Murphy, 2021). Cotinine metabolites are used as biomarkers to determine nicotine exposure because of their longer half-lives

compared to nicotine itself (Tiwari *et al.*, 2020). Of note, there is a lack of evidence to suggest the direct carcinogenic properties of nicotine and its metabolites. Nonetheless, nicotine is a precursor of tobacco-specific nitrosamines (TSNA), associated with carcinogenesis (Balbo *et al.*, 2013)

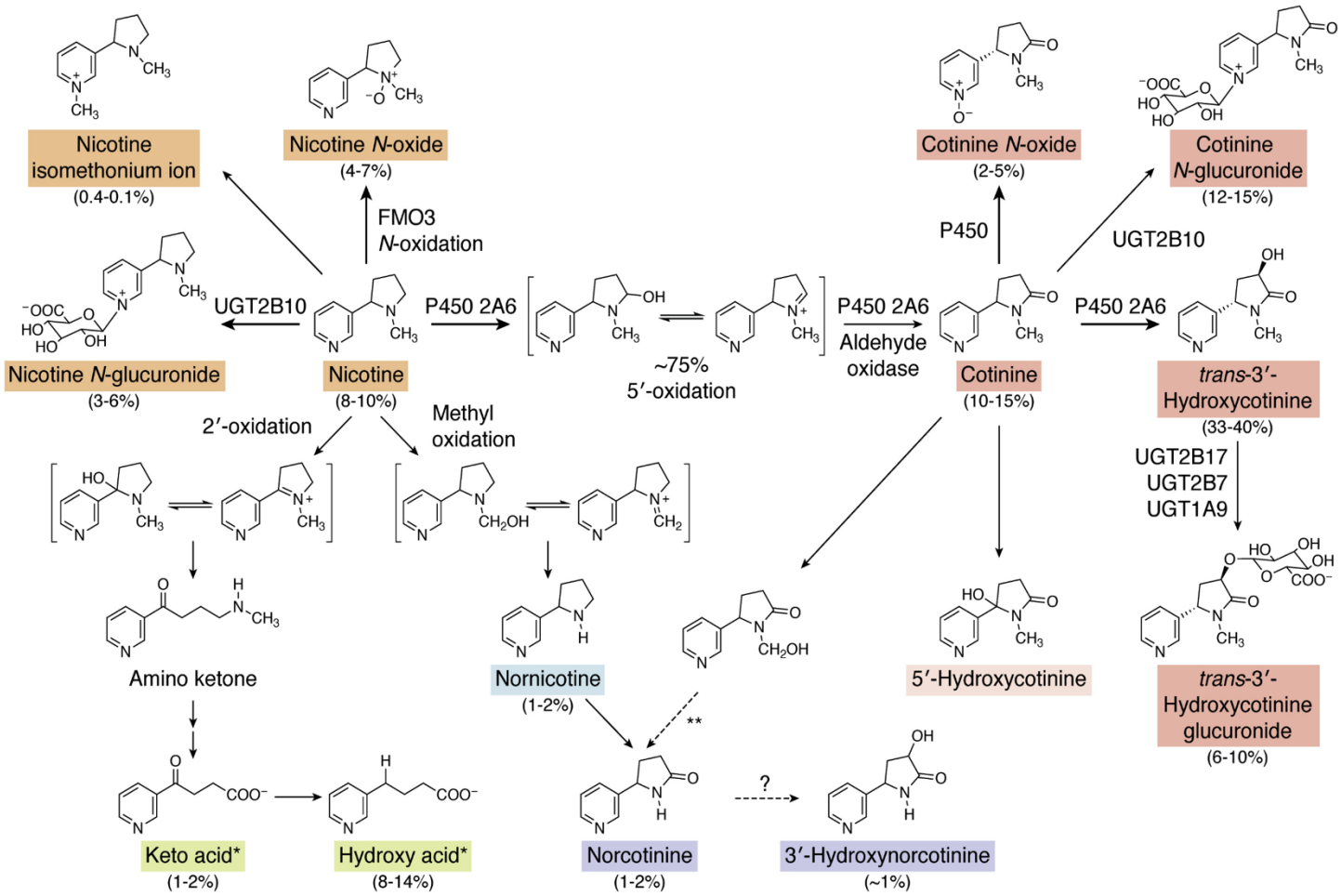

Nicotine isomethonium ion
(0.4-0.1%)
Nicotine N-oxide
(4-7%)
FMO3
N-oxidation
Nicotine N-glucuronide
(3-6%)
UGT2B10
Nicotine
(8-10%)
P450 2A6
~75%
5′-oxidation
2′-oxidation
Methyl
oxidation
Amino ketone
Keto acid*
(1-2%)
Hydroxy acid*
(8-14%)
Nornicotine
(1-2%)
**
Norcotinine
(1-2%)
?
3′-Hydroxynorcotinine
(~1%)
P450 2A6
Aldehyde
oxidase
Cotinine
(10-15%)
P450
Cotinine N-oxide
(2-5%)
5′-Hydroxycotinine
UGT2B10
Cotinine
N-glucuronide
(12-15%)
P450 2A6
trans-3′-
Hydroxycotinine
(33-40%)
UGT2B17
UGT2B7
UGT1A9
trans-3′-
Hydroxycotinine
glucuronide
(6-10%)

Figure 1. 2 Metabolic Pathways of Nicotine in Smokers: Key compounds, identified by boxed labels, represent the urinary metabolites detected in smokers. These percentage estimations pertain to smokers who do not exhibit deficiencies in either P450 2A6 or UGT2B10 activities. Noteworthy mentions include: 4-hydroxy-4-(3-pyridyl) butanoic acid, also known as hydroxy acid, 4-oxo-4-(3-pyridyl) butanoic acid, referred to as keto acid, 4-(methylamino)-1-(3-pyridyl)-1-butanone, termed aminoketone. Norcotinine is a resultant of the P450 2A6–driven metabolism of cotinine. However, it is important to note that norcotinine was not detected in the urine samples from individuals to whom cotinine was administered. Image taken from (Murphy, 2021)

1.2.2 TOBACCO SPECIFIC NITROSAMINES

TSNAs, or Tobacco-Specific Nitrosamines, are cancer-causing compounds that develop during the curing and processing of tobacco leaves. They are produced through the nitrosation of nicotine and related tobacco alkaloids. The primary TSNAs of concern include NNK, its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and NNN (**Figure 1.3**). NNK and NNN are categorized as Group 1 probable human carcinogens by the International Agency for Research on Cancer (IARC) (Yach, 2005). NNK and its metabolite NNAL are particularly potent for causing lung cancer in tobacco users (Akopyan and Bonavida, 2006). Studies have demonstrated their lung carcinogenicity in various animal models, including mice (International Agency for Research on Cancer (IARC), 2004), rats (Balbo *et al.*, 2013; International Agency for Research on Cancer (IARC), 2004), and hamsters (International Agency for Research on Cancer (IARC), 2004). NNN is also considered a probable carcinogen for the lungs, oesophagus, and oral cavity (Hecht, 2003). There is a significant association between urinary biomarkers of NNN and smokers' risk of oesophageal cancer development (Yuan *et al.*, 2011). *In vivo* animal experiments have shown the development of oesophageal and nasal tumours in rats (Murphy *et al.*, 2000; Wong *et al.*, 2005; Yuan *et al.*, 2011) and pulmonary tumours in mice (Murphy *et al.*, 2000; Wong *et al.*, 2005; Yuan *et al.*, 2011) due to exposure to these carcinogenic compounds.

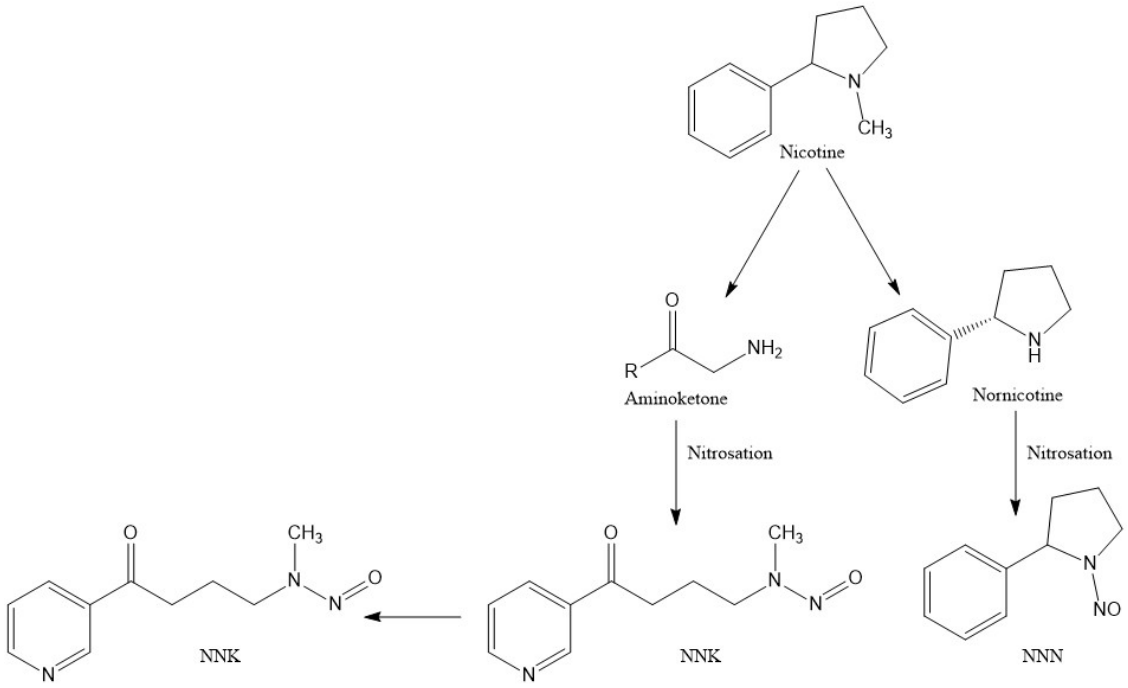


Figure 1. 3 The potential metabolic pathway of TSNA formation following nitrosation of nicotine

1.3 SMOKING AND PULMONARY DISORDERS

The lungs and the mouth are the first sites of contact for the numerous harmful substances in tobacco smoke—for example, the incomplete combustion of organic materials in tobacco yields carbon monoxide (CO). CO from tobacco smoke has adverse effects on the body. While its primary and most well-known impact is on the cardiovascular system (Raub *et al.*, 2000), it also indirectly affects the lungs. The primary effect of CO is its ability to bind to haemoglobin in red blood cells, forming carboxyhaemoglobin (Freeman and Perks, 1990). This reduces the amount of oxygen that the blood can carry. This decrease in oxygen-carrying capacity can compromise the oxygenation of tissues throughout the body, including the lung tissues (McDonough and Moffatt, 1999).

Smoking is the leading cause of COPD (Buist *et al.*, 2008; Karadogan *et al.*, 2018); CO exposure can exacerbate COPD symptoms. While the development of COPD is

primarily attributed to other components in tobacco smoke, the reduction in oxygen delivery due to CO can worsen this condition (McDonough and Moffatt, 1999). Although rare and more commonly seen in cases of prolonged exposure to very high concentrations of CO (like in house fires), there is a possibility of lung oedema (fluid accumulation) due to the heart's inability to pump oxygen-deprived blood effectively.

1.3.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASES PATHWAYS

COPD is the medical term used to describe a set of obstructive lung diseases characterised by poor airflow. These diseases include emphysema (damage to alveolar walls resulting in larger alveoli that are less efficient at transporting oxygen), refractory asthma (tightening and swelling of airways that are irreversible by therapeutic agents), and chronic bronchitis (loss of cilia from bronchi). Although the pathophysiology of COPD is multifactorial and complex, cigarette smoking is the most commonly encountered risk factor for COPD (Buist *et al.*, 2008; Karadogan *et al.*, 2018). Combustion of tobacco generates reactive oxygen species (ROS), nitrogen oxygen species (NOS), PAHs, and toxicants that can induce an inflammatory response (Brody and Spira, 2006) in the central airways, peripheral airways, and lung parenchyma (Angelis *et al.*, 2014). Repeated exposure to these noxious particles in tobacco smoke can trigger the release of chemokines (Wang *et al.*, 2015) and cytokines to initiate inflammatory responses in the airways (Strzelak *et al.*, 2018). This can result in inflammatory cells such as macrophages, neutrophils, and eosinophils infiltrating the lungs (**Figure 1.4**) (Aldonyte *et al.*, 2015; O'Farrell *et al.*, 2021; Strzelak *et al.*, 2018).

Chemotactic mediators such as CXCL1 (growth-related oncogene alpha), CXCL2 (macrophage inflammatory protein 2-alpha), and CXCL10 (interferon-gamma-induced protein 10) may mediate the smoking-induced infiltration of inflammatory cells.

CXCL1, CXCL2, and CXCL10 are produced by various pulmonary cells, including epithelial cells, macrophages, and neutrophils. CXCL1 and CXCL2 bind to the CXCR2 receptor on the surface of neutrophils (Zhou *et al.*, 2023a). CXCL10 release occurs in response to IFN-γ (interferon-gamma), and it has roles in chemoattraction for monocytes/macrophages, T cells, natural killer (NK) cells, and dendritic cells (Zhou *et al.*, 2023a). CXCL10 binds to CXCR3 receptor, which is expressed on monocytes, macrophages, T cells (T helper cells, CD8+ cytotoxic T lymphocytes and regulatory T cells), and B cells (Tokunaga *et al.*, 2018).

In contrast, interleukins (IL-1β, IL-6, IL-10, IL-13, IL-33) and tumour necrosis factor α (TNF-α) are released by cells in the epithelial lining of the airways as well as the immune cells by tobacco smoking and they can modulate the inflammatory response (She *et al.*, 2021) leading to the inhibition or recruiting and accumulation of neutrophil granulocytes, macrophages, and lymphocytes, such as natural killer cells, T cells, and B cells (Aldonyte *et al.*, 2015; Karadogan *et al.*, 2018; Soriano *et al.*, 2018) (**Figure 1.4)**. Activating these pro-inflammatory cells, in turn, initiates the release of more pro-inflammatory mediators, TNF-α, ROS, and C-reactive protein. Inflammatory processes in the airways can stimulate the overproduction of mucus, specifically the mucins MUC5AC and MUC5B (Donaldson *et al.*, 2005). Cigarette smoke can lead to the loss of cilia, the hair-like structures that help move mucus and trapped particles out of the airways (O'Byrne and Postma, 1999). This loss of cilia further impairs the clearance of mucus and debris from the lung ultimately leading to inefficient gas exchange in the alveoli, emphysema, and the decline of lung function (Tantucci and Modina, 2012) (**Figure 1.4)**. Alterations in gas exchange may in turn alter mitochondrial membrane potential (MMP) locally in the lungs and systematically.

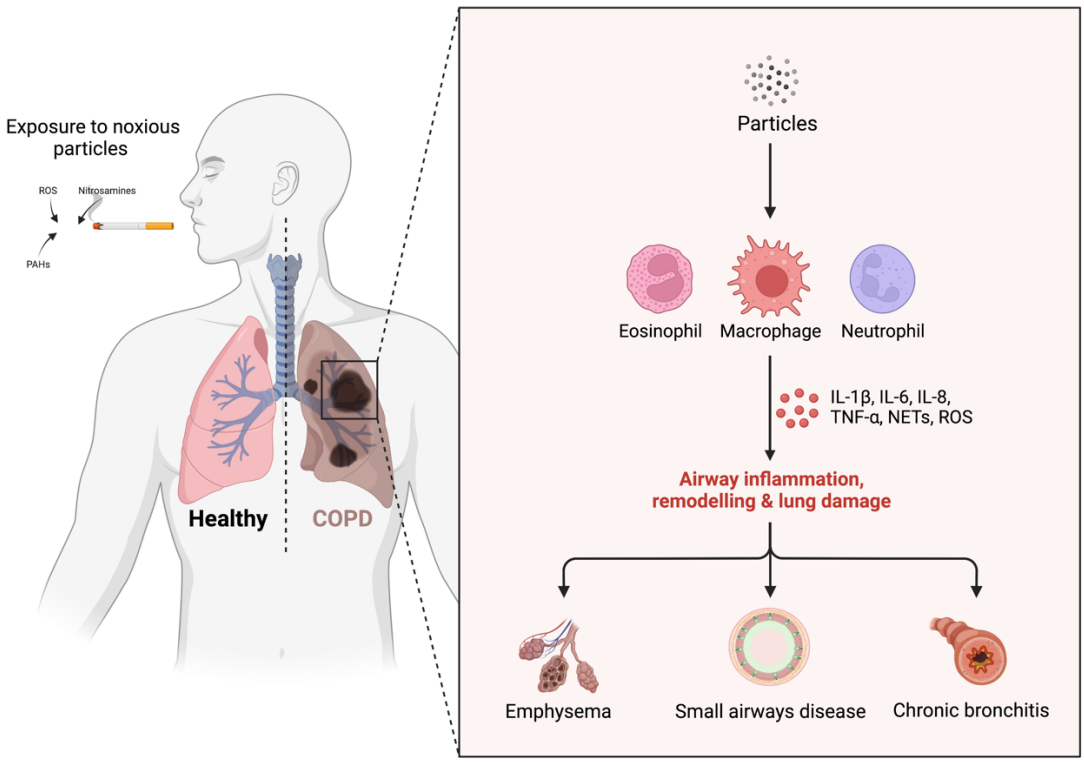


Figure 1. 4 Alterations in gas exchange may in turn alter mitochondrial membrane potential (MMP) locally in the lungs and systematically. The particles formed from tobacco smoke include ROS, PAHs, and nitrosamines.

1.3.2 MITOCHONDRIAL IMPAIRMENTS IN SMOKING-INDUCED LUNG DISEASE

MMP drives the production of adenosine triphosphate (ATP). Therefore, alterations in MMP may lead to alterations in ATP levels. MMP generated by proton pumps (Complexes I, III, and IV) is essential in energy storage during oxidative phosphorylation (Zorova *et al.*, 2018). The proton gradient forms the transmembrane potential of hydrogen ions harnessed to make ATP (Poburko *et al.*, 2011). The levels of MMP and ATP in the cell are kept relatively stable, although there are little fluctuations of both these factors reflecting regular physiological activity. However, sustained changes in both factors may be deleterious (Zorova *et al.*, 2018). A long-lasting drop or

rise in MMP vs. normal levels may impact the electron transport chain and ATP production, which, in turn, may induce alterations in cytosolic and mitochondrial calcium regulation (Prakash *et al.*, 2017), unwanted loss of cell viability resulting from apoptosis (Li, 2003) or necrosis (Lemasters *et al.*, 1999, 2002) These cascading events may give rise to several medical conditions in different human biological systems (Kroemer *et al.*, 2007; Tsujimoto *et al.*, 2006).

Cigarette smoke is known to cause mitochondrial dysfunction in lung cells, leading to cellular senescence (Sundar *et al.*, 2019). This, together with cytosolic oxygen reduction by NADPH oxidases (NOX), can lead to undesirable levels of ROS (Vermot *et al.*, 2021). Recent evidence has highlighted new roles for ROS as secondary messenger molecules in various cell signalling pathways as part of both normal physiology and pathophysiology (Magnani and Mattevi, 2019). ROS mediates processes at low to intermediate concentrations, leading to cell growth, cell migration, and apoptosis (Redza-Dutordoir and Averill-Bates, 2016). The ability of ROS to destroy invading pathogens in human biological systems implies that cellular constituents forming the first line of defence in various organs will generate ROS as part of an immune response to invading pathogens and/or xenobiotics (Magnani and Mattevi, 2019; Seriani *et al.*, 2021). Human biological systems differentially express NOX enzymes based on their functionalities (Vermot *et al.*, 2021). Inhibition of ROS can lead to chronic granulomatous disease (CGD), an immunodeficiency disorder which increases the susceptibility of the body to infections (Roos, 2016). In contrast, overactivation of the constitutively active NOX 4, which leads to increased ROS production, has been linked to the development of lung fibrosis in mouse studies (Carnesecchi *et al.*, 2011). Also, prolonged exposure to high ROS concentration induced by cigarette smoke can lead to non-specific damage to proteins, lipids, and nucleic acids, which, in turn, may lead to a

disease state (i.e., cancers and pulmonary, cardiovascular, and neurodegenerative diseases) (Aggarwal *et al.*, 2019; Brieger *et al.*, 2012; Dimauro *et al.*, 2016; Morsch *et al.*, 2019).

1.4 LUNG CANCERS

Cancer is a prominent cause of premature death globally. Although the lung is under constant threat by potentially injurious xenobiotics and/or pathogenic insults, it is equipped with proficient defence mechanisms to counteract the adverse health effects of these and noxious particles. Nonetheless, the lung is the leading site of worldwide cancer-related mortality in men and second in women (behind breast cancer) (Torre *et al.*, 2015), with a combined toll of 1.59 million associated premature deaths annually (WHO, 2022a). This is because prolonged exposure to pulmonary irritants may induce inflammation and jeopardize the resolution capacity of the lungs to fight other invading particles. This may lead to DNA damage and, eventually, lung cancer development. The primary and most avoidable risk factor for lung cancer is smoking, which is responsible for approximately 70% of non-small cell lung cancer (NSCLC) and 90% of small cell lung cancer (SCLC) instances (Hecht, 2003). Moreover, smoking increases the risk of developing many other cancers, including but not limited to oro- and hypopharyngeal, oesophageal, larynx, bladder, and stomach cancers (Doll *et al.*, 2005).

1.4.1 SMOKING-INDUCED LUNG CANCER PATHWAYS

While the link between tobacco smoking and lung cancer is well established, the specific mechanisms by which tobacco smoke causes cancer are still not fully understood. Some theories suggest that the chemicals in tobacco smoke damage DNA directly, while others propose that they cause lung inflammation, leading to cancer development over time

(**Figure 1.5)**. Regardless of the precise mechanisms by which tobacco smoke causes cancer, the evidence is clear that smoking is a significant risk factor for lung cancer. Quitting smoking can significantly reduce an individual's risk of developing the disease, and public health efforts to reduce smoking rates are critical for preventing lung cancer.

1.4.2 THE ROS- AND RNS-MEDIATED PATHWAY

Tobacco combustion releases high concentrations of free radicals (Foronjy and D'Armiento, 2006; Hecht, 2003). Tobacco combustion involves the pyrolysis of tobacco and the generation of smoke. TSNAs can form DNA adducts in urine samples of smokers and, therefore induce cancer (Carmella *et al.*, 1993; Cheng *et al.*, 2014; Yuan *et al.*, 2011). Free radicals are produced during this process, including ROS and reactive nitrogen species (RNS). The highly reactive species such as superoxide (O2-), hydroxyl (OH-), and nitric oxide (NO-) can react with other compounds in the smoke, leading to the formation of even more reactive species, including hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), and peroxyl radicals (ROO-) (Valavanidis *et al.*, 2009). In addition to the free radicals generated by the incomplete combustion of tobacco, the presence of additives and contaminants in tobacco and cigarette paper can also produce free radicals. For example, additives, such as phenols, can act as pro-oxidants, increasing the generation of free radicals in the tobacco smoke (Valavanidis *et al.*, 2009). Similarly, heavy metals in tobacco, such as cadmium, can also contribute to producing free radicals (Jaishankar *et al.*, 2014; Milnerowicz *et al.*, 2015).

Antioxidative enzymes are an essential defence mechanism in the body against oxidative stress. Oxidative stress refers to an imbalance between ROS production and the body's ability to counteract or detoxify its harmful effects through neutralisation by antioxidants (Jones, 2008). Prolonged oxidative stress can lead to pathologies, including

chronic inflammation (Chung-man Ho *et al.*, 2001). Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are among the most well-known and studied antioxidative enzymes (Asaduzzaman Khan *et al.*, 2010; Sun *et al.*, 1989). SOD converts superoxide radicals into hydrogen peroxide, which is then detoxified by catalase and GPx (Pigeolet *et al.*, 1990; Weydert and Cullen, 2010). Inflammation is characterised by an increase in reactive oxygen species (ROS) and a decrease in antioxidative enzymes, which leads to tissue damage, DNA adducts (including 8-oxo-2'-deoxyguanosine (8-oxodG)), and DNA mutations in urine (Chelombitko, 2018; Mittal *et al.*, 2014; Weimann *et al.*, 2001). Several studies have shown that smokers have higher levels of 8-oxodG adducts in their DNA than non-smokers (Herrero and Barja, 1999; Munnia *et al.*, 2017; Phillips, 2002; Phillips and Venitt, 2012; Zhou *et al.*, 2023b). These adducts result in increased cell cycle arrest by activating molecules such as cyclins and by altering apoptotic pathways such as the caspase 3/7 activity (Pezzuto *et al.*, 2019). DNA adducts from cigarette smoke have been associated with an increased risk of cancer and other smoking-related diseases (Mittal *et al.*, 2014). High levels of malondialdehyde, a marker for lipid peroxidation, have been associated with cigarette smoking (Lykkesfeldt *et al.*, 2004). Additionally, exposure to second-hand smoke increases the levels of 8-oxodG adducts in non-smokers, indicating that tobacco smoke exposure can cause DNA damage in both smokers and non-smokers (Munnia *et al.*, 2017).

I*n vitro* studies have shown that the overexpression of antioxidative enzymes such as SOD and GPx can inhibit inflammation and reduce oxidative stress (Ho *et al.*, 2013; Younus, 2018). Additionally, in animal models of inflammatory diseases such as rheumatoid arthritis and colitis, increased expression of antioxidative enzymes has been

associated with decreased inflammation and tissue damage (Ho *et al.*, 2013). Therefore, the accumulation of high levels of free radicals may eventually overwhelm the antioxidative system involved in scavenging and detoxifying smoking chemicals, which may lead to respiratory and cardiovascular diseases and cancer.

1.4.3 THE IMMUNE CELL-MEDIATED PATHWAYS

The immune system plays a critical role in recognizing and eliminating cancer cells. Immune cells, such as T cells, natural killer cells, and macrophages, can detect and destroy cancer cells through various mechanisms, including releasing cytotoxic molecules and activating programmed cell death (Cendrowicz *et al.*, 2021; Wu *et al.*, 2020). However, chronic exposure to cigarette smoke can suppress these immune-mediated pathways and promote the development and progression of cancer (Sopori, 2002). PAHs can stimulate the production of pro-inflammatory cytokines, such as TNF-α and IL-6, by immune cells (Coppack, 2001; Popko *et al.*, 2010). These cytokines can promote the recruitment of immune cells to the site of tissue damage, but chronic exposure to TNF-α and IL-6 can also lead to chronic inflammation and tissue damage, increasing the risk of cancer development (Furman *et al.*, 2019; Neurath and Finotto, 2011; Popa *et al.*, 2007).

Chronic exposure to cigarette smoke can also lead to the upregulation of immunosuppressive pathways in the immune system. For example, cigarette smoke can increase the expression of programmed cell death ligand-1 (PD-L1) on cancer cells and immune cells (Shimoji *et al.*, 2016; Yeo *et al.*, 2019). PD-L1 can bind to programmed cell death protein-1 (PD-1) on T cells and suppress T cell activation, leading to an

immunosuppressive microenvironment that can promote cancer growth and progression (**Figure 1.5**) (Angelis *et al.*, 2014; Diskin *et al.*, 2020).

Chronic exposure to cigarette smoke leads to the accumulation of regulatory T cells (Tregs) in the lung tissue. Tregs are a subset of T cells that play a critical role in suppressing immune responses and maintaining self-tolerance. However, Tregs can also inhibit anti-tumour immune responses and promote the growth and metastasis of cancer cells. This highlights the importance of smoking cessation and the development of effective strategies to reverse the immune dysfunction induced by cigarette smoke exposure. Immunotherapies that target the immune-mediated pathways in lung cancer development, such as PD-1/PD-L1 inhibitors and Treg-targeted therapies, may also provide new opportunities for treating smoking-related cancers (**Figure 1.5**) (Yeo *et al.*, 2019).

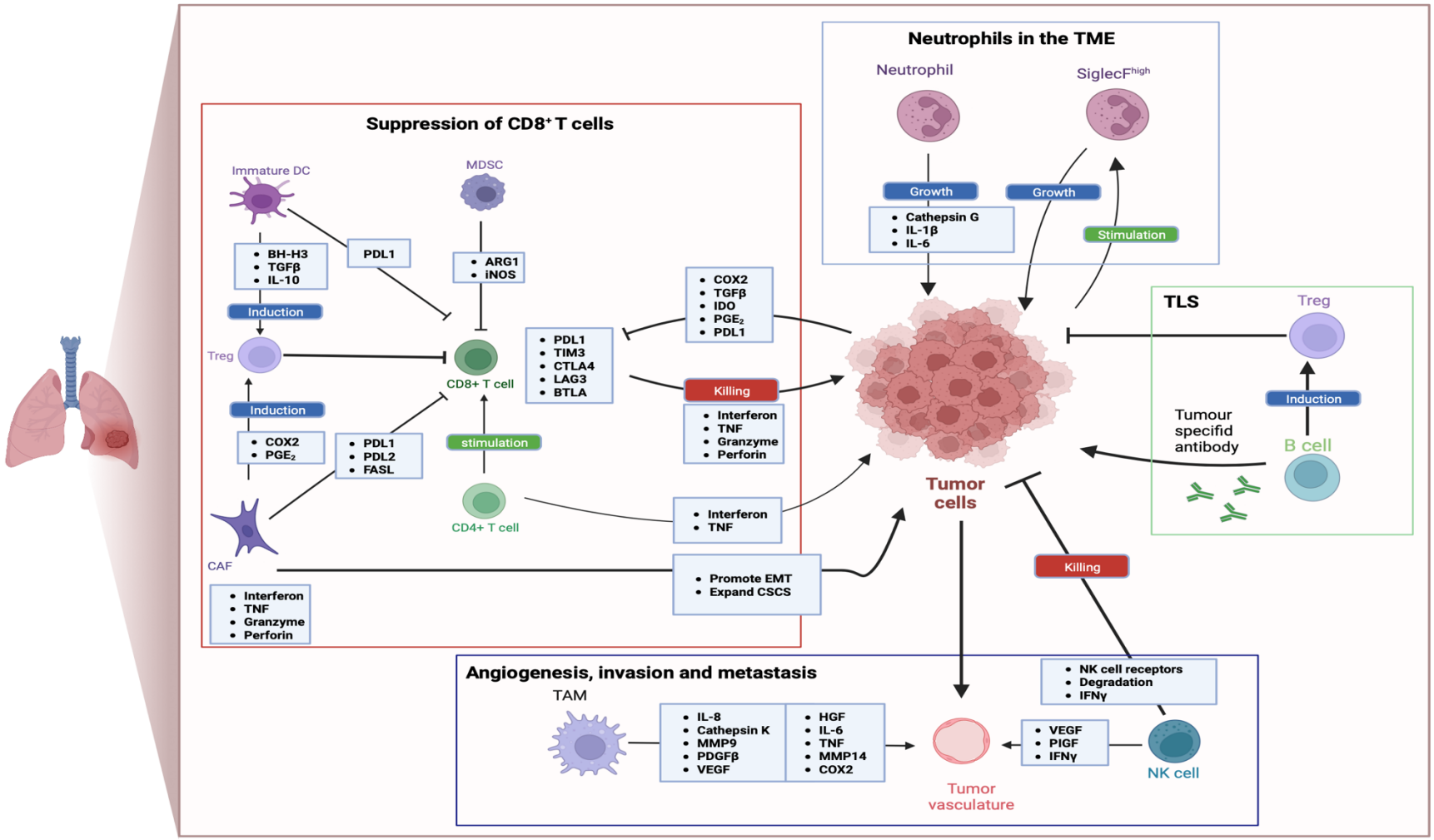


Figure 1. 5 The microenvironment of lung cancer and the heterogeneity of cells involved in the body's response to remove tumour cells.

Abbreviations: BTL A, B and T lymphocyte attenuator; CSCs, cancer stem cells; CTL A4, cytotoxic T lymphocyte-associated antigen 4; EMT, epithelial-to-mesenchymal transition; FGF4, fibroblast growth factor 4; GAS6, growth arrest-specific protein 6; HGF, hepatocyte growth factor; IGF1R, insulin-like growth factor 1 receptor; IGF2, insulin-like growth factor 2; L AG3, lymphocyte activation gene 3; PDGFβ, platelet-derived growth factor- β; PlGF, placental growth factor; STAT, signal transducer and activator of transcription; TIM3, T cell membrane protein 3; TNF, tumour necrosis factor. Figure adapted from Altorki *et al.*, 2019).

1.5 HARM REDUCTION AND SMOKING CESSATION POLICIES IN THE UK

As previously discussed, tobacco smoking remains a leading cause of preventable deaths worldwide, with significant health and economic burdens. Non-smokers are also at risk. Exposure to second-hand smoke has been linked to respiratory diseases and sudden infant death syndrome, among other health issues (Cuthbertson and Britton, 2010). The National Health Service (NHS) incurs significant costs treating smoking-related illnesses, estimated at around £2.6 billion annually (NICE, 2023b). As such, the United Kingdom has implemented harm reduction and smoking cessation policies to reduce these burdens.

The Department of Health and Social Care has outlined a tobacco control plan to reduce smoking prevalence among adults, youths, and pregnant women (Department of Health and Social Care, 2020). Also, the UK introduced plain packaging and larger health warnings on cigarette packs to make them less attractive and inform potential users of the risks (MHRA, 2015). Smoking in enclosed public places and workplaces has been prohibited since 2007, reducing exposure to harmful second-hand smoke (Centre for public impact, 2019). There are 3 main pharmacotherapies prescribed for treating smoking dependence in England: Nicotine Replacement Therapy, Bupropion (Zyban),

and Varenicline (Champix). The NHS has endorsed and provided various nicotine replacement therapy (NRT) options, including patches, gums, and inhalers, to help smokers quit (NICE, 2023a). Nicotine patches (highest amount of nicotine is 25 mg) and gums (highest amount of nicotine is 4 mg) are a form of NRT. They deliver a controlled amount of nicotine to the body, helping to reduce withdrawal symptoms and cravings when someone stops smoking (NICE, 2018; Stead *et al.*, 2016). Unlike other NRT methods, the nicotine inhaler mimics the hand-to-mouth action of smoking, considering the behavioural component of smoking when trying to quit (Steinberg, 2009). Another smoking cessation aid is the electronic cigarette (EC) or electronic (non) nicotine delivery system (E(N)NDS). ECs are the most successful smoking cessation aid in the UK (ASH, 2023). Public Health England, The Royal College of Physicians (London) and The Royal College of Practitioners have embraced EC as a tool to reduce smoking incidence in the UK, and as a consequence, reduce the morbidity and mortality associated with smoking (Marczylo, 2020). ECs are recognised as being 95% less harmful than tobacco and have been promoted as a safer alternative and a tool for smoking cessation (McNeill *et al.*, 2022, 2018). However, although randomised clinical trials have demonstrated the efficacy of ECs for smoking cessation (Hajek *et al.*, 2019), they have also found that most participants did not stop using ECs after quitting smoking. Although continued use may be an effective means to prevent relapse, there is no current safety data for prolonged use of ECs.

1.6 ELECTRONIC CIGARETTES

Nicotine is responsible for tobacco dependence; thus, nicotine replacement therapies (NRT), void of most of the 93 chemicals of concern, are used as smoking cessation aids. However, traditional NRTs have protracted nicotine absorption profiles which means they take much longer to reach peak plasma concentrations than traditional cigarettes, creating an unpopular choice for cigarette smoking cessation aids. Alternatively, ECs or ENDS are battery-powered devices designed to vaporize a nicotine-containing solution (known as e-liquid) for a relatively rapid and efficient nicotine delivery to the brain, which is more comparable to traditional cigarettes.

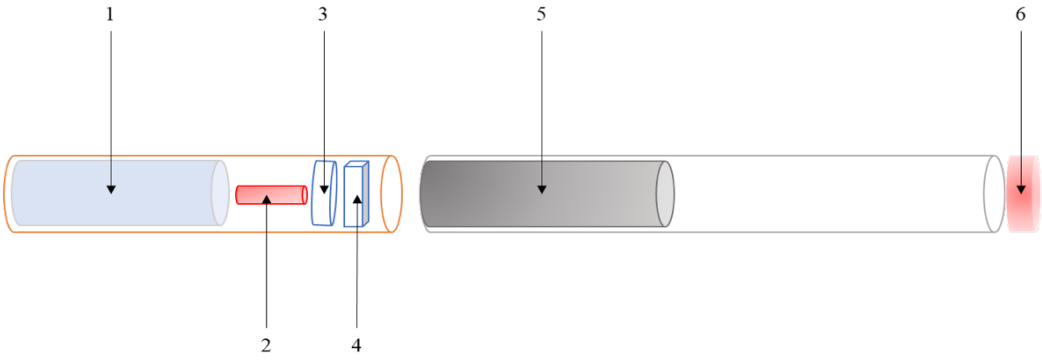


Figure 1. 6 A schematic diagram of the main components of a generic first-generation EC device; (1) cartridge/ tank containing the e-liquid, (2) heating element/ filament used to vaporize the e-liquid, (3 & 4) the sensor and microprocessor detect, activate, and control the atomizer, (5) battery, (6) LED light used to indicate inhalation.

E-liquids contain mostly propylene glycol (PG) and/or vegetable glycerine (VG) and may contain nicotine and/or flavours and other additives (**Figure 1.7**). ECs, provide reinforcing sensory and behavioural cues in addition to nicotine, which (1) aids in alleviating withdrawal symptoms, (2) reduces nicotine craving, and (3) decreases relapse to smoking (Wadgave and Nagesh, 2016). Nicotine in e-liquids may exist in two

distinctive forms: free-base nicotine or nicotine salts, and the state of one or the other is determined by the pH of the e-liquid (Duell *et al.*, 2019). Interestingly, the pharmacokinetics of ECs with nicotine salts typically match the absorption profile of a traditional pyrogenic cigarette, thus making them the most utilized non-tobacco nicotine delivery systems in the UK (ASH, 2022b).

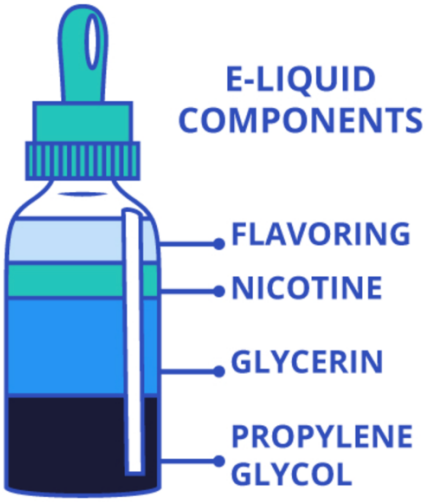
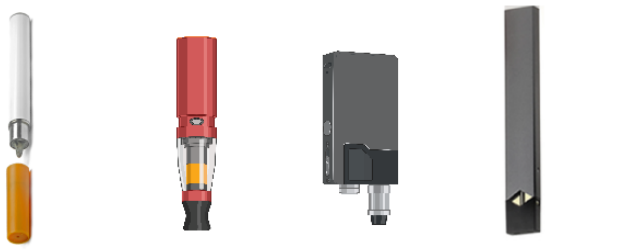


Figure 1. 7 E-liquid composition

Many variations of EC devices on the market are characterised into 4 generations based on the device's structure, reusability, and voltage (**Figure 1.8**). First-generation ECs are disposable devices that resemble cigarettes. Certain types resemble pens, screwdrivers, or hookah tips, aiming to imitate the conventional way of smoking (Hiemstra and Bals, 2016). Compared to the first generation, the second generation is slightly larger in shape with a higher voltage lithium battery and comprised of a heating element called an atomizer used to heat e-liquids into vapour (i.e., aerosols) and a replaceable cartridge or tank to hold refill solutions (Talih *et al.*, 2015). Third-generation EC devices are comparable to second-generation; however, these provide the user more freedom to customize components of the device (i.e., low resistance 'sub-ohm' heating coils) and

adjustable temperature and power. Low resistance, high power settings enable vapers to 'cloud-chase'— consuming greater volumes of e-liquid to generate higher amounts of aerosol. Finally, the fourth generation of ECs is referred to as pod mods, which resemble some of the first EC devices in shape; however, these items are refillable and rechargeable using a USB cable. These e-liquids predominantly contain nicotine salts, permitting quicker delivery to the brain.



	First generation (Cig-a-Like)	Second generation (Vape-Pen)	Third generation (Box Mod)	Fourth generation (Pod Mod)
Design	**CC Style**	**Pen Style**	**Tank Style**	**Pod Mods**
Battery Voltage	**~ 3.7 - 4.2 V**	**~ 3.7 - 6 V**	**~ 6 - 8 V**	**~ 3 - 8 V**
Disposable	**√**	**X**	**X**	**X**
Refillable cartridge	**X**	**√**	**√**	**√**
Components customization	**X**	**X**	**√**	**X**
Voltage-Wattage setup	**X**	**X**	**√**	**X**

Figure 1. 8 Different generations of EC devices and their main differences

1.6.1 ELECTRONIC CIGARETTE USE, RECENT TRENDS, EFFECTIVENESS & POTENTIAL HAZARDS

In 2023, after 17 years of commercial availability, an estimated 4.3 million adults comprising ex-smokers (57%) or dual users (41.7%) and relatively few never-smokers (1.3%) were EC consumers in Great Britain (ASH, 2022b). An increase in the proportion of ex-smokers and a fall in current smokers using EC over time is thought to be due to the general perception that ECs are a safer alternative to tobacco smoking and therefore used as a smoking cessation aid (ASH 2020). A review commissioned by Public Health England of available evidence suggested that ECs are at least 95% less harmful to health than tobacco smoking (McNeill *et al.*, 2018). Therefore, smokers who switched entirely to EC-only use are expected to improve their long-term health compared with those who continue to smoke (McNeill *et al.*, 2022, 2018). While there was a decrease in EC users who were ex-smokers from 65% to 56% from 2021 to 2023; in contrast, there has been an increase from 4.9% to 6.7% from 2021 to 2023 of never-smokers who are EC users (ASH, 2023).

Although ECs are considered less hazardous than cigarettes, it is less clear how harmful EC use is compared to not using EC, especially in former and never smokers. EC users avoid or reduce exposure to most of the 93 toxicants in combusted tobacco smoke. It is considered that approximately 80% of cigarette smoke-induced cardiovascular toxicity is attributed to the first 1-3 cigarettes per day (Gallucci *et al.*, 2020). This is likely in contrast to vaping because carbon monoxide (CO) and many other toxicants are not generated during vaping. However, in addition to nicotine, EC users are also exposed to PG, VG, and flavour and other additive compounds, and both degradation and condensation by-products formed during the heating of e-liquid to generate an inhalable aerosol which may cause toxicity (Schick *et al.*, 2017). It appears that the composition

of the e-liquid and repeated dosing or vaping "behaviour" may be responsible for EC-related toxicity. Burgwardt *et al.*, (2020) reported that Valsalva vaping may enhance exposure to harmful constituents such as flavouring in the EC aerosol vaper. Valsalva vaping is achieved when EC users over-inhale and then forcefully exhale to eliminate the vapour, which is usually produced in greater quantities than that generated by a conventional cigarette (Burgwardt *et al.*, 2020). Concerns exist that EC use is associated with the onset of various adverse health effects related to both cardiovascular (Bhatnagar, 2016; Schweitzer *et al.*, 2017) and lung diseases (Chun *et al.*, 2017; Moazed and Calfee, 2017). Data in animals suggest that short-term and long-term exposures to EC aerosols might result in cardiovascular remodelling driven by PG/VG vehicles, with nicotine potentially playing a protective role (Neczypor *et al.*, 2022). Analyses of the variety and concentrations of EC aerosol toxicants infer that EC aerosol is less harmful than tobacco smoke (Azzopardi *et al.*, 2016); however, lower concentrations of tobacco-related toxicants, and the presence of non-tobacco-related toxicants indicate these aerosols are not likely to be risk-free.

Several investigators have reported the presence of hazardous carbonyl compounds such as formaldehyde, acetaldehyde, glyoxal, and acrolein in EC aerosol (Bekki *et al.*, 2014; Etter *et al.*, 2013; Ogunwale *et al.*, 2017; Schober *et al.*, 2014) and TSNA in e-liquids and their aerosols (Farsalinos *et al.*, 2015). Acetaldehyde (classified by the International Agency for Research on Cancer (IARC) as Group 2B, which means it is possibly carcinogenic to humans) and formaldehyde (Classified by the IARC as Group 1, carcinogenic to humans) are recognised carcinogens (Feron *et al.*, 1991; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006; Roe and Wood, 1992), and acrolein is a cardiovascular toxicant (Arman and İşisağ Üçüncü, 2020; DeJarnett *et al.*, 2014). TSNAs are Group 1 carcinogens (IARC, 2004; Xia *et al.*, 2021) formed from

the curing and processing of tobacco leaves through the nitrosation of nicotine and related tobacco alkaloids (Bush *et al.*, 2001; Wang *et al.*, 2017). There is conflicting evidence (Farsalinos *et al.*, 2015; Goniewicz *et al.*, 2014a; Kim and Shin, 2013; Laugesen Murray, 2008; U.S. Food and Drug Administration, 2009) to suggest that ECs are a source of TSNA exposures; however, it is physiologically possible that endogenous formation might occur from nicotine through nitrite exposure in the saliva (Knezevich *et al.*, 2013), acidic stomach, or microbiome-mediated nitrosation at different sites (Ziebarth, 1997) Indeed, human biomarker studies conducted by Shahab *et al.*, (2017) and Martin *et al.*, (2016) revealed low levels of NNAL in the urine samples of EC users albeit at much lower concentrations compared to cigarette users. Traces of toxic metals such as cadmium, nickel, and lead potentially originating from EC devices (Goniewicz *et al.*, 2014b) are also found in the blood and urine of EC users (Aherrera *et al.*, 2017; Badea *et al.*, 2018; COT, 2019; Goniewicz *et al.*, 2018; Jain, 2019). A recent report by the BBC reported that vapes gathered at Baxter College in Kidderminster could expose the children using them to more than twice the daily safe amount of lead and nine times the safe amount of nickel. Some vapes also contained harmful chemicals like those in cigarette smoke (BBC, 2023). Lab tests revealed the presence of high levels of carbonyls in these illegal vapes, which can break down into chemicals such as formaldehyde and acetaldehyde when the e-liquid heats up (BBC, 2023). Young children are particularly vulnerable to the toxic effects of lead and can suffer profound and permanent adverse health impacts, particularly on the development of the brain and nervous system (WHO, 2023). Lead also causes long-term harm in adults, including increased risk of high blood pressure, cardiovascular problems, kidney damage, nasal and lung cancers (Jaishankar *et al.*, 2014; WHO, 2023). However, the devices found to have these toxicants were not registered with MHRA and are not compliant with UK legislation on e-liquid contents.

Further, EC refills and aerosols contain volatile organic chemical compounds like benzene, toluene, xylene, and styrene (BTXS) (Schober et al. 2014), albeit at substantially lower concentrations than in tobacco smoke (Margham *et al.*, 2016). Previous epidemiological studies found that individuals who are chronically exposed occupationally to BTXS (individually or mixtures) exhibit a lower than normal peak expiratory flow [PEF, litre per second (L/s)] and 25–75% forced expiratory flow (FEF25∼75%, L/s) (Liao *et al.*, 2022). In addition, occupational exposure to BTXS is linked to increased risks of prostate (Blanc-Lapierre *et al.*, 2018) and lung cancer (Warden *et al.*, 2018). In addition to this evidence, concerns over increasing utilization by young individuals and the potential for youth EC use to progress to tobacco smoking have led to regulations restricting their use in many countries. In the UK alone, there has been a 3% rise in EC use among young people between the ages of 11 – 17 from 2020 to 2022 (ASH, 2022a). Although there are some regulations regarding the use of EC in the EU, the Tobacco Products Directive 2014/14/EU (TPD) introduced new restrictions on nicotine concentration and size of EC refill containers (this directive has been adopted in the UK post-Brexit under Tobacco and Related Products Regulations 2016 (Part 6 Electronic cigarettes)); the government should ensure existing laws are enforced and identify where regulations could be extended.

1.6.2 ELECTRONIC CIGARETTE FLAVOURINGS

The flavouring compounds used in EC e-liquids are generally regarded as safe (GRAS) for ingestion and are used extensively in the food industry in the USA. While the GRAS system in the U.S. allows for self-affirmed or FDA-reviewed designations of safety for food substances based on consensus among experts and scientific evidence, the EU's Novel Food Regulation requires a more formal pre-market approval process, often demanding more comprehensive data and studies. The differences between these two systems reflect varying approaches to food safety and regulation in different regions.

However, there are concerns about EC flavouring toxicity when inhaled using EC because the evidence for safety following inhalation has not been extensively investigated (Farsalinos *et al.*, 2013b; Khlystov and Samburova, 2016; Leigh *et al.*, 2016a; Sundar *et al.*, 2016a). In 2014, there were an estimated 8000 flavoured e-liquids on the market, which has likely increased (Zhu *et al.*, 2014) significantly. Initially, the most popular EC flavours in the UK mimicked the flavours of tobacco products (e.g., cigarettes, cigars, or pipe tobacco) and menthol (ASH, 2023). However, preferences have shifted more recently to fruity flavours, and the popularity of other flavours (desserts, candy, beverages, etc.) has also increased (ASH, 2023). Unfortunately, some product names and flavours provide little information about the flavour category (e.g., unicorn blood, truth serum, snake oil, etc.), let alone their likely constituents. The vast number of products and the presence of hard-to-categorize product names led the Dutch National Institute for Public Health and the Environment (RIVM) to develop a flavour wheel (**Figure 1.9**, Krüsemann *et al.*, 2019), which groups e-liquids into 13 flavour categories: candy, dessert, other sweets, fruit, alcohol, coffee/tea, other beverages, spices, nuts, menthol/mint, tobacco, and unflavoured (**Figure 1.9**), based upon the

chemical composition of the e-liquid (In Table 1.1, we have reported the main flavouring chemicals in several flavours in e-liquids).

Table 1. 2 Flavours in e-liquid and some of the main flavouring chemicals they are composed of.

Flavouring chemical	Common flavours with chemical agents	References
Cinnamaldehyde, 2-methoxycinnamaldehyde	Cinnamon	(Behar *et al.*, 2014a; Gerloff *et al.*, 2017)
3,4-Dimethoxybenzaldehyde	Cherry	(Kavvalakis *et al.*, 2015)
2,5-Dimethylpyrazine	Chocolate	(Kavvalakis *et al.*, 2015)
Maltol, ethyl maltol	Caramel, Vanilla	(Gerloff *et al.*, 2017; Tierney *et al.*, 2016)
Menthol, Menthone	Mint	(Berkelhamer *et al.*, 2019)
p-Anisaldehyde	Anise	(Behar *et al.*, 2018a; Berkelhamer *et al.*, 2019; Noël *et al.*, 2020b)
Benzaldehyde	Almond	(Behar *et al.*, 2018a; Berkelhamer *et al.*, 2019)
Vanillin, ethyl-vanillin	Vanilla	(Behar *et al.*, 2014b; Gerloff *et al.*, 2017; Muthumalage *et al.*, 2018)
Hexyl acetate, 3-Hexen-1-ol	Apple	(Kerasioti *et al.*, 2020; Kim *et al.*, 2018)
Isoamyl acetate	Banana	(Fetterman *et al.*, 2018)
Methyl cinnamate, furaneol, α-ionone	Balsamic, Strawberry, raspberry	(Berkelhamer *et al.*, 2019; Leigh *et al.*, 2016b)
Methyl anthranilate	Berry	(Tierney *et al.*, 2016)

Other flavouring agents less known: Damascenone (α or β), 3-methyl-1,2-cyclopentanedione, acetamide, linalool, terpineol, citral, corylon, anisaldehyde, trimethylpyrazine, eugenol, benzaldehyde, limonene	Miscellaneous	(Gerloff *et al.*, 2017; Tierney *et al.*, 2016)

Flavour additives in food may undergo enzymatic metabolism, producing fewer toxic metabolites due to phase I and II metabolism (del Olmo *et al.*, 2017). In contrast, exposure to GRAS-like substances by inhalation after thermal degradation may produce active compounds that cause severe adverse health effects (Fedan *et al.*, 2006). For example, occupational diacetyl inhalation exposure can lead to bronchiolitis obliterans (BO), more commonly referred to as "popcorn lung" (Rose, 2017). BO causes severe coughing, wheezing, and shortness of breath, similar to the symptoms of COPD (Aguilar *et al.*, 2016). An epidemiological study performed in the Netherlands suggests a causal link between BO and chemical workers producing diacetyl for food flavourings (van Rooy *et al.*, 2007). The British Medical Association (BMA) believes flavours' safety should be closely monitored as evidence for potential adverse effects are emerging after heating and inhalation of e-liquid aerosol (Sassano *et al.*, 2018). The medicines and healthcare products regulatory agency (MHRA) collects UK reports of EC's harmful effects and safety concerns through their yellow-card scheme (https://yellowcard.mhra.gov.uk/). Moreover, EU TPD established a priority list of 15 potentially harmful additives (Havermans *et al.*, 2022) that should not be contained in EC based on the published literature. These include flavouring additives and chemicals such as guaiacol, geraniol, menthol, ethyl maltol, and diacetyl. Since 2016, diacetyl has

also been banned from e-liquids in the UK, whereas flavours containing menthol and ethyl maltol are still available.

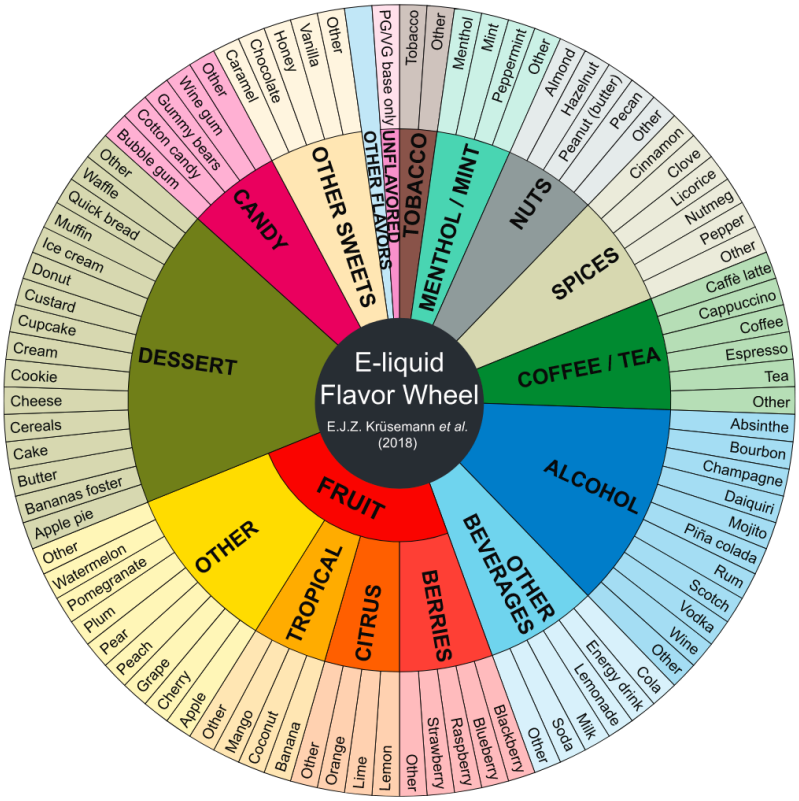


Figure 1. 9 The flavour wheel. Taken from Krüsemann et al., (2019).

Flavours in e-liquids, as mentioned earlier, may contain pharmacologically active compounds (Clapp *et al.*, 2019a). For example, mint flavours contain menthol or menthone (Pereira *et al.*, 2013), and candy flavours include vanillin and cinnamaldehyde (Krüsemann *et al.*, 2019). Fruit flavours can have 3-hexen-1-ol (apple) or methyl anthranilate (berry), and so on (Tierney *et al.*, 2016) (see Table 1). Menthone is the ketone analogue of menthol. Menthol (l-menthol) is a naturally occurring agonist for the cold transient receptor potential melastatin 8 (TRPM8), a calcium channel which is functionally expressed in the airways (Sabnis *et al.*, 2008). Due to the efficacy of menthol in inhibiting cigarette-induced cough via the TRPM8, it was added to tobacco

as a ‘flavouring’ (Lin *et al.*, 2018). Moreover, the masking of the harshness and irritancy of tobacco underpins the initiation of more intense and protracted smoking (Wickham, 2020). This effect contributes to the inclusion of menthol in the EU TPD list of potentially harmful tobacco additives, yet there is a widespread use of menthol in e-liquids. The committee on toxicity of chemicals in food, consumer products, and environment (COT) in the UK reviewed data published on the four main chemical components in the flavoured e-liquids: vanillin, menthone, menthol and cinnamaldehyde and concluded that although they may activate receptors in the airways, on the skin and eye to cause some irritation, none of them were mutagenic. COT concluded that these chemicals do not pose severe adverse health effects (COT, 2019).

Vanillin and cinnamaldehyde are TRPV1 and TRPA1 agonists, respectively, which are also functional in the airways (Wang *et al.*, 2019). TRPV1 and TRPA1 are Ca^{2+} -permeable cation channels functionally expressed in various organs, including the lungs, and they are activated by a wide spectrum of endogenous and exogenous chemicals, including vanillin and cinnamaldehyde, the main chemical component of vanilla and cinnamon flavours (Talavera *et al.*, 2020). Furthermore, alterations in TRPA1 expression and/or activation are involved in the pathophysiology of COPD and asthma (Talavera *et al.*, 2020). Therefore, activation of TRPM8, TRPA1, and TRPV1 through EC usage may alter normal physiological responses of the airways. The effects of other chemical constituents of flavours, such as 3-hexen-1-ol and methyl anthranilate, warrant further investigation. Unsurprisingly, the largest category on the flavour wheel, given its popularity in the UK, is fruit, which RIVM subcategorized into groups (tropical, citrus, berries, and others) in the flavour wheel (Krüsemann *et al.*, 2019). Not explicitly covered by the flavour wheel, tobacco e-liquids can be sub-divided into those that extract

flavour compounds directly from tobacco and those using synthetic flavourings to simulate tobacco taste.

1.7 THESIS QUESTION, HYPOTHESIS, AND AIMS

The evidence provided in this chapter reveals the damaging effects of tobacco smoking on health. However, smokers fail several attempts to quit smoking. This is because of the addictive nature of nicotine. Evidence suggests EC are a safer alternative to tobacco smoking. However, there is little to no direct evidence of the toxicological effects of EC flavours in humans. To date, studies of EC flavour toxicity are primarily *in vitro* studies in human-derived cell lines, with a handful of studies conducted *in vivo*. Nonetheless, the literature presents discrepancies among studies. Therefore, this research project aims to investigate and evaluate a wide range of flavours to identify the ones that elicit toxic effects on pulmonary bronchial cells.

1.7.1 RESEARCH QUESTION:

- Do all flavours in EC pose a potential health risk to the pulmonary bronchial cells at physiologically relevant concentrations?

1.7.2 THE HYPOTHESES:

Toxicity of EC flavours varies depending on nature of flavour and manner of exposure

1.7.3 THE AIMS:

- Conduct a systematic literature review (**Chapter 2)** to identify what is known about EC flavours and determine the gaps in the literature.
- Screen a wide range of flavours in submerged cultures in a high-throughput manner to identify the toxic ones (**Chapter 3).**
- Explore the potential mode of toxicological actions of the toxic flavours (**Chapter 3**)
- Validate and establish 3D Calu-3 air-liquid interface (ALI) model (**Chapter 4**)

for further toxicity testing of the toxic flavours identified in the submerged high throughput system (**Chapter 4)**.

- Validate different human pulmonary cells grown at ALI and to select the most suitable one for a dose-response exposure to aerosols of the toxic flavours (**Chapter 5)**

CHAPTER 2

PULMONARY EFFECTS OF ELECTRONIC CIGARETTES FLAVOURS: A SYSTEMATIC REVIEW

Part of this chapter has been published in the Journal of Environmental Health and Toxicology Part B - DOI: 10.1080/10937404.2022.2124563.

2.1 INTRODUCTION

As discussed in **Chapter 1**, The advent of electronic cigarettes (e-cigarettes) has profoundly impacted tobacco consumption trends worldwide (WHO, 2022c). Marketed as a safer alternative to conventional smoking, e-cigarettes have witnessed a surge in popularity, especially among younger populations in the UK (ASH, 2022a). Central to this appeal is the variety of available flavours, ranging from classic tobacco to a plethora of fruit, dessert, and novelty profiles (Krüsemann *et al.*, 2019) (Figure 1.9). While these flavours enhance the user experience, there is growing concern about their potential pulmonary health implications, given that the inhalation route exposes the respiratory tract directly to these flavouring agents (see **Chapter 1**).

E-cigarettes aerosolise a mixture of nicotine, PG/VG, and flavouring chemicals. Research suggests that while e-cigarettes contain fewer toxic substances than traditional cigarettes, they are not risk-free (COT, 2019; McNeill *et al.*, 2022). Among the components of e-cigarette liquid, flavours have come under scrutiny for their potential adverse effects on pulmonary health. Studies on pulmonary cells provide insights into the cellular and molecular consequences of exposure to these flavouring compounds (see **Chapter 1**). While some evidence suggests potential cytotoxic effects on pulmonary cells, inflammation, and impaired lung function (Khlystov and Samburova, 2016; O'Farrell *et al.*, 2021; Sundar *et al.*, 2016a), other studies report minimal (Czekala *et al.*, 2019a, 2019b) or no adverse effects.

The contrasting evidence in the literature regarding the effects of e-cigarette flavours on pulmonary cells necessitates a systematic review. It remains essential to understand whether certain flavours are more harmful than others and whether the observed discrepancies are due to differences in methodologies, concentrations, or other

confounding factors. Therefore, we conducted a review to systematically synthesise and critically evaluate published data on flavoured e-liquids and their effects on the respiratory system to identify the EC flavours and flavour chemicals most associated with adverse respiratory effects. A secondary aim was to investigate whether the outcomes observed *in vitro* are reflected by adverse effects reported *in vivo*. Finally, we propose a potential 'gold standard' approach for future EC investigations regarding the experimental method, which may help reduce inter-study discrepancies. This is the first systematic review to focus specifically on the toxicity of EC flavours on the respiratory system. It will help researchers identify the essential EC flavours and chemicals that contribute to toxicity and could be the focus of further investigations, including risk assessments that may advise regulators on meaningful and appropriate e-liquid ingredient regulation. The review focuses on the effects on the pulmonary system specifically. Moreover, although the review summarises the published data, we have endeavoured to be more critical and questioning of the published data, including 1) the contributions of nicotine and inconsistencies across studies, 2) the effect of vehicle/PG/VG, and the lack of appropriate controls in many studies 3) potential additive effects of nicotine.

2.2 METHODS

2.2.1 DATA SOURCES AND SEARCHES

A literature search was completed in November 2020, which was updated in April 2023, using Scopus and healthcare database advanced search (HDAS) (including CINAHL, EMBASE, MEDLINE, PsychINFO, PubMed). Retrieved articles on *in vitro* and *in vivo* studies were published between 2006 and 2021. Keywords included terms to capture concepts associated with e-cigarettes, flavours, and the respiratory system. The search

stream here pasted was used for HDAS search – (((((electronic cigarette OR electronic nicotine delivery system OR electronic non-nicotine delivery system OR e cigarette OR e-cigarette OR e-cig OR e cig) AND (flavour OR flavor OR flavouring OR flavoring OR flavouring agents OR Flavoring agents OR Flavoured OR Flavored)) AND (lung OR alveolar OR bronchial OR pulmonary)).title, abstract, key. Whereas the search stream below was used for our Scopus search – ((TITLE-ABS-KEY ("electronic cigarette")) OR (TITLE-ABS-KEY ("electronic nicotine delivery system")) OR (TITLE-ABS-KEY ("electronic (non) nicotine delivery system")) OR (TITLE-ABS-KEY ("e cigarette")) OR (TITLE-ABS-KEY ("e-cigarette")) OR (TITLE-ABS-KEY ("e-cig")) OR (TITLE-ABS-KEY ("e cig"))) AND ((TITLE-ABS-KEY (flavour)) OR (TITLE-ABS-KEY (flavor)) OR (TITLE-ABS-KEY (flavouring)) OR (TITLE-ABS-KEY (flavoring)) OR (TITLE-ABS-KEY ("Flavouring agents")) OR (TITLE-ABS-KEY ("Flavoring agents")) OR (TITLE-ABS-KEY ("Flavoured")) OR (TITLE-ABS-KEY ("Flavored")) AND (TITLE-ABS-KEY (lung)) OR (TITLE-ABS-KEY (alveolar))) AND ((TITLE-ABS-KEY (lung)) OR (TITLE-ABS-KEY (alveolar)) OR (TITLE-ABS-KEY (bronchial)) OR (TITLE-ABS-KEY (pulmonary))) AND (LIMIT-TO (DOCTYPE , "ar")) AND (LIMIT-TO (LANGUAGE , "English")))))).

2.2.2 SELECTION AND EXCLUSION CRITERIA

Retrieved articles were screened, duplicates were eliminated, and remaining citations were organized in Zotero (George Mason University, Virginia) following Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines. Only primary articles published in English were included. After a manual screening of titles and abstracts within the 225 and 74 records from HDAS and Scopus, respectively, 37 primary articles

met the inclusion criteria for this systematic review (**Figure 2.1**). One additional manuscript was identified during data extraction from the primary articles.

2.3 RESULTS

The search stream included 28 *in vitro* studies where human cell lines were exposed to flavoured e-liquids, 5 articles were e-liquid chemical composition studies, and 5 reported studies where rodents (mice and rats) were exposed to flavoured e-liquids. Analysis of the 38 included reports and their reported adverse effects of flavours are summarized in **Tables 2.1, 2.2, 2.3, 2.4, & 2.5**.

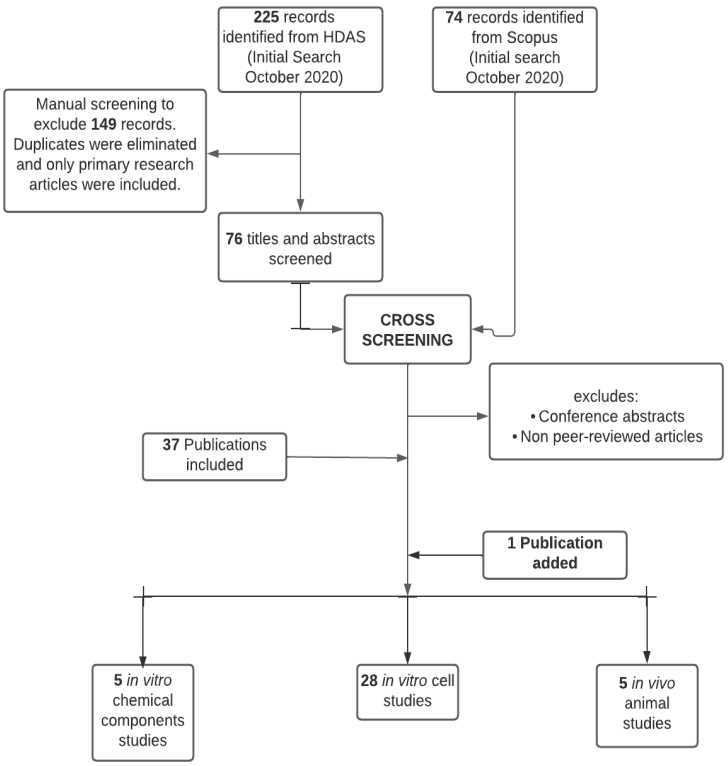


Figure 2. 1 PRISMA flowchart. Articles were retrieved from HDAS and Scopus databases. Primary research articles that met the selection criteria were included irrespective of the year of publication. The figure was created with Lucidchart.com

Most *in vitro* investigations on cells were carried out on at least two different cell lines belonging to one of primary (5 articles), immortalized normal (15 articles), or cancerous pulmonary cells (13 articles). Therefore, we have subdivided the reporting of these papers based on the type of cell lines used in **Tables 2.1, 2.2** & **2.3**. Mint/menthol (10 articles) was the most frequently reported e-liquid flavour to demonstrate harmful effects, followed by cinnamon (9 articles) and strawberry (5 articles) in cytotoxicity assays, metabolic activity assays, and pro-inflammatory biomarkers assays.

Similarly, cinnacide (cinnamon-based), black liquorice, banana pudding, vanilla, and tobacco-flavoured e-liquids-induced differential toxicity *in vivo* (**Table 2.5**.). Adverse effects observed *in vivo* include, but are not limited to, increased levels of pro-inflammatory cells in bronchial alveolar lavage (BAL), increased MUC5AC production, and increased oxidative stress markers. MUC5AC is the predominant mucin glycoprotein produced by respiratory epithelium that normally protects against infection but when over-expressed is associated with respiratory diseases like asthma and COPD. There were no human studies where the effects of flavours on the respiratory system were investigated.

Table 2. 1 In vitro studies where toxicological effects of flavoured e-liquids were investigated with primary epithelial cells alone or together with immortalized normal pulmonary cells.

Cell type	Flavours or flavouring chemicals investigated	Exposure (Time & flavour dose)	E-liquid Nicotine Dose	Nicotine Control	Main findings	Reference
Primary human bronchial epithelial (hBE), immortalised bronchial epithelial cells (BEAS-2B)	• Hot Cinnamon Candies, • 'Sinicide', • Kola (Each containing cinnamaldehyde) • Cinnamaldehyde alone at 5 different concentrations (0.05, 0.25, 0.5 & 5 mM).	0, 20, 40, 60, 80, 100, 120 min exposure to aerosol at ALI	No nicotine	N/A	• Since aerosol transiently suppressed ciliary beat frequency (CBF), and cinnamaldehyde recapitulated this and other differential toxic effects in both cell lines following metabolic and glycolysis assays using a Seahorse XFe21 extracellular flux analyser. • Cinnamaldehyde impaired mitochondrial respiration and glycolysis dose-dependently in BEAS-2B. • Reduction of Intracellular ATP levels dose-dependently in differentiated hBE and BEAS-2B cells.	(Clapp *et al.*, 2019a)

Human bronchial epithelial cells (hBE)	• Apple (3 different variants)	Puff duration of 2.6 s, 50×3 s puffs with 5 s in between were bubbled through 10 mL of culture medium to create EC extract. Cells were exposed to 100% of this media. •	• 0, 18 mg/ mL	• Nicotine controls present	• Each apple flavour increased necrosis and apoptosis. • Each apple flavour reduced the secretion of TNF-α, IL-6, IP-10, MIP-1α, and MIP-1β because of flavour-effects. • Nicotine alone induced a decrease in pro-inflammatory cytokines (IP-10 & MIP-1β) similar to cigarette smoke extract (CSE) and increased % of macrophages efferocytosing hBE cells but had no effect when in combination with e-liquid.	(Ween *et al.*, 2020a)

Primary human 24-h alveolar macrophages, neutrophils, and natural killer (NK) cells.	• Kola • Hot Cinnamon • Candies • Menthol • Banana pudding • Menthol tobacco • Banana • 'Sinicide'	Adherent alveolar macrophages were challenged in triplicate • with cell culture medium containing 1, 0.5, or 0.25% flavoured e-liquid for 1 h	• 0, 0.6, 1.2, 1.8, and 2.4 mg/ml	• No nicotine controls present	• Hot Cinnamon Candies, and Kola decreased phagocytosis and viability of macrophages, neutrophils, and NK cells. • Hot Cinnamon Candies, and Kola mediated suppression of alveolar macrophage phagocytosis. • No nicotine dose-dependent effects were observed. • No nicotine x flavour interaction observed.	(Clapp *et al.*, 2017a)
Primary human bronchial epithelial cells	• Diacetyl (DA)	• 1-h ALI aerosol exposure to 12, 25, and 50 mM of diacetyl.	• No nicotine	• N/A	• LDH increased significantly with 50 mM DA- compared to PBS, 12- and 25-mM DA-exposed cultures. • Transient decrease in trans-epithelial electrical	(McGraw *et al.*, 2020)

					resistance in all DA-exposed cultures. • At 25 and 50 mM, airway basal cell injury with keratin five ubiquitination and decreased p63 expression were observed. The ubiquitin-proteasome-pathway partially mediates airway basal cell repair after acute DA exposure.	
16HBE14o- airway epithelial cell line (16HBE), Normal human bronchial epithelial cells (NHBE), THP-1 differentiated macrophages & primary alveolar macrophages (AM).	• Cappuccino • Chocolate • Peppermint	• E-cigarette vapor extracts (EVE) generated using 50 x 3s puffs and exposed to cells for 24-h	• 18 mg/mL (No nicotine-free e-liquids)	• Nicotine alone controls present	• Cappuccino, Chocolate and Peppermint caused cellular cytotoxicity by inducing morphological changes and cell shedding in 16HBE. • All 10 flavours induced significant LDH release (chocolate highest and nicotine alone significantly higher) in 16HBE. • Chocolate > cappuccino >peppermint > banana induced apoptosis. Nicotine and PG/VG did not induce apoptosis in 16HBE and	(Ween *et al.*, 2020b)

			primary alveolar macrophages, the other cells were not specified). • Chocolate flavour most cytotoxic following an LDH assay in NHBE in inducing necrosis and apoptosis. Nicotine showed significant toxicity but lower than chocolate, whereas PG:VG showed no effect. • Mango, Tobacco, Banana and Chocolate caused decreased macrophage phagocytic capacity. PG:VG and nicotine alone showed a significant effect on efferocytosis. • Chocolate > Banana > nicotine induced increase in IL-8 secretion by NHBE. Decrease in IL-1β by all flavours as well as nicotine.

					• Effects are linked to concentration and benzene-ring containing chemicals.	
Human bronchial epithelial cells of COPD models, immortalized 16HBE cells	• Virginia Tobacco (Flavour from tobacco extract) • Menthol	Intense puffing regime of 55 mL over 4 s, with 30 s interval) for 30 min at ALI	5 mg/ml	No nicotine alone control	• Virginia tobacco-induced significantly higher LDH levels compared to menthol in the COPD cells • Menthol in 16HBE induced a significantly lower LDH release when compared to air-only control. • No difference in DNA damage in either cell line in Virginia tobacco or menthol when compared to cigarette controls. • In COPD cells, cigarette, and Virginia tobacco-induced a significant decrease in IL-8 production. No alteration in IL-6 release compared to air-only controls.	(O'Farrell *et al.*, 2021)

			• In 16HBE, IL-8 production was significantly higher in all groups compared to air-only controls. Tobacco significantly decreased IL-6 release compared to cigarette.

Table 2. 2 In vitro studies where toxicological effects of flavoured e-liquids were carried out on immortalized pulmonary normal epithelial cells alone or together with carcinoma pulmonary cells.

Cell type	Flavours or flavouring chemicals investigated	Exposure (Time & flavour dose)	E-liquid Nicotine Dose	Nicotine Control	Main findings	Reference
Human bronchial epithelial cell line (16HBE14o)	• 2,5-dimethylpyrazine (chocolate, nutty flavour) • Damascenone (apple, citrus, wine-like) • Linalool (floral, spice) • α-ionone (fruity, raspberry) • Ethyl maltol (caramel) • Furaneol (strawberry, sweet) • Vanillin (vanilla)	24-h direct exposure (flavouring chemical)	No nicotine	N/A	• 2,5-Dimethylpyrazine reduces the physiological response to signalling molecules important in aspects of airway epithelial cell innate immunity. • 2,5-Dimethylpyrazine increases airway epithelial ion conductance. • 2,5-Dimethylpyrazine activates apical ion efflux via cystic fibrosis transmembrane conductance regulator (CFTR). • Vanillin and 2,5-Dimethylpyrazine significantly altered airway epithelial cellular	(Sherwood and Boitano, 2016)

					physiology indicative of cellular signalling events. • Linalool, α-ionone, damascenone, ethyl maltol, and furaneol, no significant effects?	
Immortalized human bronchial epithelial cells (16-HBE, BEAS-2B)	• Cool Cucumber • Mango • Strawberry coconut • Classic Menthol • Caffe Latte	66 puffs during 22 minutes with a three-second puff duration at 1.6 L/min flow rate and an inter puff interval of approximately 17s.	50 mg/mL nicotine	No nicotine control	• Cool cucumber, classic menthol, caffe latte induced pro-inflammatory biomarkers (IL-1ß, IFN-γ, IL-17, etc.) depending on the cell line as 16-HBE and BEAS-2B present specific physiological differences. It is unclear whether these effects are in part influenced by nicotine content due to the lack of nicotine control samples. • Classic Menthol induced the most significant mitochondrial ROS production in 16-HBE.	(Muthumalage *et al.*, 2019)

					• Prostaglandin production was induced in BEAS-2B, not in 16-HBE, by strawberry coconut. • IL-8 production was induced in 16-HBE, but not in BEAS-2B, by cool cucumber, classic menthol, strawberry coconut, and caffe latte.	
Normal human bronchial epithelial (NHBE) cells	• Flavouring chemicals diacetyl and 2,3-pentanedione (Concentrations of diacetyl and 2,3-pentanedione were not specified)	24-h chemical exposure after media dilution in an ALI model	No nicotine	N/A	• Cytoskeletal and cilia processes among common genes (142 genes) were perturbed by both diacetyl and 2,3-pentanedione. • The expression of multiple genes involved in cilia biogenesis was significantly downregulated by diacetyl and 2,3-pentanedione. • The number of ciliated cells significantly decreased.	(Park *et al.*, 2019)

BEAS-2B and human pulmonary fibroblast (HPF)	• Ethyl maltol • Menthol	48-h chemical (diluted liquid) direct exposure	No nicotine	N/A	• Both flavour chemicals were highly cytotoxic following a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at concentrations 30 (menthol) and 100 times (ethyl maltol) lower than the highest concentrations in the refill fluids. BEAS-2B cells (IC50 = 0.15 mg/ml) were more sensitive to ethyl maltol than HPF (IC50 = 0.28 mg/ml). • No difference in sensitivity to menthol between HPF and BEAS-2B.	(Omaiye *et al.*, 2019a)
BEAS-2B	• Menthol • Virginia Tobacco	30 min x 3 (With a 12-h interval) aerosol exposure	5% nicotine (Specific mg/ml of nicotine not specified)	No nicotine control	• Menthol- mitochondrial effects: proton leakage, a decrease in coupling efficiency, and a reduction of complex I, II, and IV	(Lamb *et al.*, 2020)

					• 24-h post-menthol exposure reduction of basal respiration, maximal respiration, spare capacity, and complex I. • Tobacco had no significant effects on mitochondrial respiration, but immediately post final exposure increased complex I, IV, and V. Whether this effect is synergistic with nicotine was unclear.	
Human pulmonary fibroblasts (hPF)	• Cinnamon Ceylon	48-h e-liquid direct exposure to 1% and 0.3% doses.	Different concentrations of nicotine, but not specified.	No nicotine control	• Nicotine concentration did not correlate with cytotoxicity in MTT assays. • Cinnamon Ceylon cytotoxicity effect following MTT assay was dose-dependent. • Cinnamaldehyde and 2-methoxycinnamaldehyde (2-MOCA) were the	(Behar *et al.*, 2014b)

					major components of cinnamon flavours. • Cinnamaldehyde and 2-MOCA standards were highly cytotoxic in MTT assay.	
Human lung fibroblasts (hPF)	• Bubble gum • Butterscotch • Caramel • Butterfinger • Menthol Arctic • Wisconsin frost • Domestic • JC Original • French vanilla • Vanilla Tahiti • Tennessee cured • Island • Valencia • Mint chocolate • Swiss Dark • Caramel • Espresso • Mercado • Simply strawberry	48-h direct e-liquid exposure to 0.001%, 0.01%, 0.03%, 0.1%, 0.3%, and 1% v/v.	0 to 24 mg/ml	No nicotine alone control	• Most significant cytotoxicity following MTT assay was observed with cinnamon and menthol flavours which did not contain nicotine. • Levels of nicotine were not correlated with the degree of MTT cytotoxicity. • The humectants vegetable glycerol (VG) and propylene glycol (PG) were non-cytotoxic for the HPF.	(Bahl *et al.*, 2012)

	• Arctic Menthol • Summer Peach • Black Cherry • Chocolate truffle • Cinnamon Ceylon					
Human airway epithelial cells (H292) & human lung fibroblast cells (HFL-1 cell line)	• Tobacco • Cinnamon roll • Grape	30 s interval with a 4 s pulse for different time durations 5, 10, and 15 minutes at ALI	0, 6, 16, 18 and 24 mg	No nicotine alone control present	• Tobacco and cinnamon roll-flavoured e-liquid increased the secretion of inflammatory cytokines IL-6 and IL-8 in H292 and HFL-1. • The addition of nicotine gave a striking, dose-dependent increase only in IL-8 secretion in HFL-1.	(Lerner *et al.*, 2015)
Normal human bronchial epithelial (NHBE) cells	• Menthol • Tobacco • Vanilla • Blueberry	24-h direct e-liquid exposure	Different dilutions (0.03125%, 0.0625%, 0.125%, 0.25%, 0.5%, 1%, 2% & 3% of 24 mg/ml e-liquids.	No Nicotine control	• Only menthol elicited dose-dependent cytotoxic effects on activating G0/G1cycle arrest, decreasing glutathione concentration, activation of caspase 3/7 activity, increasing nuclear factor-kB (NF-kB), &	(Czekala *et al.*, 2019a)

			increased mitochondrial mass. • Tobacco, vanilla, and menthol decreased mitochondrial membrane potential dose-dependently. • Glutathione content decreased after exposure to tobacco, blueberry, and vanilla, but only above 1%. • No nicotine-free e-liquids were tested. Therefore, it is unclear whether these effects are due to flavours alone or a combination of flavour plus nicotine. • Base liquids (PG/VG), with or without nicotine, and flavoured, nicotine-containing e-liquids, had little or no effect on cell viability, cell count, mitochondrial mass, and

					potential and oxidative stress.	
Pulmonary fibroblasts (HFL-1)	• A mixture of tobacco, coconut, vanilla, and cookie e-liquids	24-h direct e-liquid exposure 0.1%, 0.25%, 0.5% & 1%.	3 mg/ml (No nicotine-free group included)	Nicotine alone control present at 0.25% and 0.5%.	• A dose-dependent decrease in cell viability using the mixture of e-liquids. • An increase in IL-8 release induced only by 0.25% flavour mix. • Elevated senescence-associated beta-galactosidase (SA-β-gal) activity in a dose-response fashion. • Transforming growth factor-β1 (TGF-β1) induced myofibroblast differentiation was inhibited by flavour mix, decreasing α-smooth muscle actin and fibronectin protein levels	(Lucas *et al.*, 2020)

					at only one concentration 0.5%. • No significant nicotine effect was observed.	
Human bronchial epithelial cell lines (BEAS-2B, H292)	• Crème brûlée-flavour	5s puff duration every 30s for 1-h to aerosol at ALI	50 mg/mL	No nicotine control	1. Aerosol decreased cell viability (≥50%) of BEAS-2B cells alone and increased nitric oxide (NO) production (≥30%), as well as iNOS gene expression. 2. In H292 cells, aerosol increased the production of reactive oxygen species (ROS). • Aerosol dysregulated expression of several genes related to biotransformation, inflammation, and airway remodelling, including CYP1A1, IL-6, and MMP12 in both cell lines. • Nicotine-free e-liquids were not investigated, it	(Pinkston *et al.*, 2020)

					is unclear whether nicotine has any synergistic effects	
BEAS-2B, Human cystic fibrosis cell line (IB3-1), and Calu3	• Apple, • Cherry, • Tobacco, • Strawberry	24-h exposure to condensates (50%, 25%, and 12.5% diluted with media)	0, 6, 12 and 18 mg/mL	No nicotine alone control	• Cherry- similarly cytotoxic to the C38 and BEAS-2B • In BEAS-2B, tobacco-decreased cell viability at all dilutions tested. • Strawberry reduced cell viability in BEAS-2B cells was significantly greater than apple, cherry, and tobacco. • CALU-3 showed no cytotoxic response to any flavour. • There was no nicotine concentration-dependent effect.	(Leslie *et al.*, 2017)

Human pulmonary fibroblasts (hPF), alveolar epithelial cells (A549)	• Vanilla • Mint/Menthol • Butterscotch • Caramel • Butterfinger	E-fluid (0.001%, 0.01%, 0.03%, 0.1%, 0.3%, 1.0%) and aerosol exposures. For each batch of aerosol, 24 puffs were collected into 4 mL of culture medium	18 mg/ml	No nicotine control	• Vanilla (creamy/buttery) and Butterscotch (creamy/buttery) were cytotoxic using MTT assay both as fluid and as aerosol. • Mint/Menthol was cytotoxic using MTT assay only as e-liquid. • Butterfinger (creamy/buttery) and caramel aerosol were the most toxic in a dose-dependent fashion of all flavours using MTT. • It is unclear whether the effects observed are influenced by nicotine as there are no nicotine controls.	(Behar *et al.*, 2018a)

Table 2. 3 In vitro studies where toxicological effects of flavoured e-liquids were investigated in pulmonary carcinoma cells alone or together with primary pulmonary cells.

Cell type	Flavours or flavouring chemicals investigated	Exposure (Time & dose)	E-liquid Nicotine Dose	Nicotine Control	Main findings	Reference
Human bronchial epithelial cells (H292)	• Strawberry • Tobacco • Pina colada • Menthol • Coffee	3 s puff duration, every 30 s, with a 55 mL puff volume. 30 min was used at ALI.	0, 6, 12, 18, or 24 mg/mL of nicotine	Nicotine control present.	• Strawberry was most cytotoxic using neutral red assay. • Exposure to each aerosol decreased metabolic activity and cell viability. • Coffee increased the release of IL-6, CXCL1, and CXCL2. • Strawberry increased the release of IL-1ß IL-10, CYCL1, CXCL2, and CXCL10. • No significant differences were observed between the different concentrations of nicotine and air control for both metabolic activity and viability. • Unflavoured e-liquids significantly decreased	Leigh et al., 2016)

					IL-1β, CXCL1, and CXCL2 in response to 18 mg/mL nicotine aerosol versus air controls. • Unflavoured e-liquids significantly increased IL-6 in response to 24 mg/mL nicotine aerosol versus air control. • Metabolic activity was significantly decreased in cells exposed to PG/VG and VG-only, but not PG-only aerosols compared to air controls.	
Primary bronchial epithelial cells and human distal lung epithelial cells (NCI-H441)	• Mix-1: raspberry, orange, lemon, and lime • Mix-2: ripe strawberry, sweet apples, and tart kiwi	40 ml/puff, 3s puff duration, 30 s puff interval, 10 puffs/ session at ALI	0, 3 mg/ml	No nicotine alone control present	• Exposure to mix-1 (nicotine-free) significantly increased transcript expression of pro-inflammatory cytokines CXCL8, IL6, NFKB1, TNF (not specified whether α or β) by more than three-fold and IL-1ß by 44-fold.	(Ganguly *et al.*, 2020)

					• Mix-2 (nicotine-free) PI3 transcript was reduced two-fold. • The presence of nicotine in mix-1 significantly reduced the release of inflammatory markers of IL-1ß, 1L-10, IL-13, and IL-6. • The presence of nicotine in mix-2 increased the transcript of TNF (not specified whether α or β).	
H292 cells	• Cinnamon • Butter	2-h/day for 1 or 3 consecutive days aerosol exposure. Using sub-ohm EC	• mg/ml	No nicotine control	• 1 Day of exposure is cytotoxic, alters gene expression, and increases ROS (cinnamon> butter). • After 3 days of exposure, cinnamon is more cytotoxic using LDH assay and causes oxidative damage to a greater extent than butter e-cig aerosol. • Cinnamon-flavoured aerosol induced pro-oxidant effects, e.g.,	(Noël *et al.*, 2020b)

					increased 8-hydroxy-2-deoxyguanosine levels. • It is unclear whether the effects are influenced by nicotine as there are no nicotine controls.	
A549 (3D) co-culture of alveolar and H441 cells	• Menthol • Cinnamon • Tobacco	24-h exposure to condensed aerosol (puff regime was set to 55 mL puff volume, 3 s draw, 60 s 200 puffs)	0 mg/ mL & 18 mg/ mL	No Nicotine control present	• Cell viability significantly decreased in mono- and 3D culture by mint and cinnamon. • Significant IL-8 release only in monoculture after Cinnamon exposure. • No increase in MCP-1 release • E-liquids containing nicotine did not alter cytotoxicity or pro-inflammatory effects relative to nicotine-free.	(Bengalli *et al.*, 2017)
CALU-3 airway epithelial cells	• Banana Pudding (Southern Style) • Kola	24-h direct e-liquid exposure (0%, 0.1%, 0.3%, 1%, 3.0%, 6.0%, 10% (v/v))	0 mg/mL and 12 mg/mL of nicotine	Nicotine alone control present	• Hot cinnamon candies and menthol tobacco show cytotoxicity (cell and mitochondrial viability) in the absence of nicotine.	(Rowell *et al.*, 2017)

	• Hot Cinnamon Candies • Menthol Tobacco • Solid Menthol • Captain black cigar • Peanut butter cookies • Popcorn • Black Licorice • Vanilla Tobacco	• Nicotine control decreases cell proliferation and viability dose-dependently and is cytotoxic compared to the PG/VG controls. • Nicotine (12 mg/mL) containing captain black cigar e-liquid showed more cytotoxicity (MTT assay) compared to captain black cigar alone. • Nicotine-free e-liquids inhibit cell proliferation and viability in a dose-dependent manner. • Nicotine-free kola, hot cinnamon candies, and menthol tobacco significantly decrease viability (calcein assay) than vehicle control.

Human adenocarcinoma alveolar basal epithelial cells (A549 cells)	• Balsamic flavour (with and without nicotine)	50 min aerosol exposure followed by analysis at 2-h, 6-h, 24-h.	Nicotine concentration not specified	No nicotine alone control present	• Nicotine-free balsamic aerosol caused a significant, progressive, time-dependent loss of viability and increased cytotoxicity (LDH assay). • Nicotine-containing e-liquids compared to flavour alone caused a significant loss in viability and increased LDH release. Quantitative and qualitative responses are superimposable to that of cigarette smoke exposure. • Humectants alone did not cause cytotoxicity even 24 hours after treatment.	(Cervellati *et al.*, 2014)
Human airway epithelial cells (H292) and human lung fibroblast cells (HFL-1)	• Tobacco • Cinnamon roll • Grape	30 s interval with a 4 s puff for 5, 10, and 15 minutes	0, 6, 16, 18 and 24 mg	No nicotine alone control present	• Nicotine-free tobacco and cinnamon roll- e-liquid increased the secretion of inflammatory cytokines IL-6 and IL-8 in H292 and HFL-1. • Nicotine-contained tobacco e-liquid	(Lerner *et al.*, 2015)

					significantly increase IL-8 secretion dose-dependently in HFL-1 but not other markers.	
Human adenocarcinoma alveolar basal epithelial cells (A549)	• Tobacco • Menthol	Six puffs were dissolved per 1 mL of A549 culture medium, referred to as 6 total-puff-equivalents (TPE) of aerosol, and grown in control or treatment medium for 3–8 days.	48 mg/ml (No nicotine free e-liquids present)	No nicotine control	• Both EC liquids and aerosols induced epithelial-to-mesenchymal transition (EMT) • Both tobacco and Menthol flavours alter cell-to-cell junctions induced an EMT, internalization of E-cadherin, increased motility, and upregulation of EMT markers. • The EMT was concurrent with the plasma membrane to nuclear translocation of active β-catenin. • Due to the lack of nicotine controls, it is unclear whether the	(Zahedi *et al.*, 2018)

					effects observed are a result of flavour alone. • No cytotoxicity was measured.	
Primary human epithelial cells (HBEC), cancerous human bronchial epithelial cells CALU-3	• Banana Pudding (BP)	5–25 puffs	0 and 12 mg/ml nicotine	No nicotine alone control, but 0 mg/mL BP	• BP acutely elevates cytosolic Ca^{2+} in both pulmonary cell types in no nicotine samples. • No adverse effects induced by PG/VG in HBEC and CALU-3. • Nicotine increased endoplasmic reticulum Ca^{2+} in CALU-3. Effect of nicotine on HBEC was not characterized.	(Rowell *et al.*, 2020)

Table 2. 4 Chemical composition analyses of flavoured EC.

E-liquid flavour	Experimental approach	E-liquid Nicotine Dose	Main findings	Reference
Menthol, Vanillin, Maltol	Particle size distribution	18 mg/ mL of nicotine	• Vanillin and nicotine increase e-cigarette particle size distribution. • Increasing the glycerol (VG) proportion in e-liquid increases EC particle size.	(Lechasseur *et al.*, 2019)
Menthol	GC−MS Analysis of Liquid, Vapor, and Particulate Phases, FTIR Spectrometry	18 mg nicotine/mL	• EC emissions contained acetaldehyde, acrolein, benzaldehyde, benzene, toluene, ethylbenzene, and xylene • The concentrations of these chemicals increased with increasing VG composition. • Benzaldehyde, naphthalene, diphenyl ether, menthol, and nicotine were also detected in the aerosol condensates.	(Ooi *et al.*, 2019)
Strawberry, Cinnamon, Sweet cream	Hydroxyl radicals in reaction mixes were measured based on the formation of 2-hydroxyterephthalic acid (2OHTA)	3, 12, 24 and 36 mg/mL	• VG e-liquids generated higher •OH levels than PG e-liquids, as did flavoured e-liquids relative to nonflavoured e-liquids (cinnamon>sweet cream>strawberry>non-flavoured). • E-vapor, combined with ascorbic acid, can also induce •OH formation. • The levels of •OH in 3 and 12 mg/ml nicotine solutions were 19.6% and 10.0% lower than that in the equivalent nicotine-containing-vapor solutions. Conversely, 24 and 36 mg/ml nicotine solution induced 40% more •OH than the equivalent e-vapor solutions.	(Son *et al.*, 2019)
51 flavors	Mass spectrometry	5% nicotine	• At least one flavoring chemical was detected in 47 of 51 unique flavors tested.	(Allen *et al.*, 2016)

		• Diacetyl was detected above the laboratory limit of detection in 39 of the 51 flavours tested, ranging from below the limit of quantification to 239 µg/EC. • 2,3-Pentanedione and acetoin were detected in 23 and 46 of the 51 flavours tested at concentrations up to 64 and 529 µg/EC, respectively.	
Mango, Banana, Tobacco, Cappuccino, Chocolate, Bubblegum, Peppermint, Cinnamon, Cherry, Apple	GC-MS	• Vanillin was the most frequently detected flavouring chemical. It was found in banana, tobacco, cappuccino, chocolate, bubblegum, and cinnamon. • Chocolate and cinnamon e-liquids had the most complex flavouring profile, with 14 and 15 flavouring peaks, respectively. • Tobacco flavour had the least complex flavouring profile with only of vanillin alone.	(Ween *et al.*, 2020b)

Table 2. 5 In vivo studies of toxicological effects of EC flavours.

Strain/sex	Exposure	E-liquid Nicotine Dose	Flavour(s) investigated	Main findings	Reference
Adult Balb/c/ (male and female)	30 min twice daily, 6 days/week exposed for 18 days (aerosol exposure)	0 mg/mL &12 mg/mL	• Black liquorice (BL) • Kola • Banana Pudding • Cinnacide	• House dust mite (HDM) challenged mice exposed to nicotine-free cinnacide reduced airway inflammation and increased peripheral airway hyperresponsiveness compared to air-only controls. • HDM-challenged mice exposed to flavoured EC without nicotine had significant but heterogeneous effects on features of allergic airway disease compared to air controls. • Nicotine-free BL increased macrophage and eosinophils count in bronchoalveolar lavage (BAL). • Nicotine in all flavours induced an increase in macrophages and eosinophils in BAL compared to nicotine-free. • Sex-difference in toxicity were not explored.	(Chapman *et al.*, 2019)
C57BL/6 /female	2 h a day, 7 days a week, for 6 weeks	No nicotine	• Vanilla	• Exposures to VG/PG + vanilla increased lung tidal and minute volumes and tissue damping. • Increased number of dendritic cells, CD4+ T cells, and CD19+ B cells in the lungs in	(Szafran *et al.*, 2020)

				the VG/PG-exposed group compared to air, irrespective of the presence of vanilla. • A >3-fold increase of 2-arachidonoylglycerol (2-AG), an anti-inflammatory mediator, and a 2-fold increase of 12-hydroxyeicosatetraenoic acid (12-HETE), an inflammatory mediator, following VG/PG exposure, with or without vanilla. • The PG/VG EC aerosol dysregulated genes related to biotransformation (Aldh8a1), transcription factors expressed in pulmonary surfactant (F2), synuclein-alpha (snca), which interacts with phospholipids and proteins, as well as IL-6 compared to air controls.	
Balb/c/Pregnant and offspring	14 –31 days exposure. Vaping topography profile of 3-s puff duration and a 55-mL puff volume every 30 s.	36 mg/ml Nic (No 0 mg/mL available)	• Cinnamon-flavour	• Preconception and prenatal exposures to EC aerosols significantly decreased the offspring birth weight and body length compared to non-exposed controls. • Preconception exposure caused downregulation of 7 inflammation-related genes. Four genes were common to both dams and foetuses. Preconception EC exposure led to offspring with significantly	(Noël *et al.*, 2020a)

				increased lung tissue fraction at birth compared to unexposed controls. • Increase in nicotinic receptors gene expression (α7nAChR) in the lungs.	
C57BL6/male	Whole-body exposure for 3 days or 4 weeks. Puff volume was 20 ml. Mice were exposed to CS or EC 4 times a day with 30-min smoke-free intervals.	18 mg/ml (No 0 mg/mL control)	• Tobacco flavour	• Increased BAL cellularity, MUC5AC production, and oxidative stress markers in BAL and lung compared to unexposed controls. • In many cases, increases were more than for CS. • BAL markers increased significantly in PG/VG + tobacco flavour + nicotine compared to PG/VG + nicotine.	(Glynos *et al.*, 2018)
Crl:CD (Sprague-Dawley) rats/male and female	90-day nose-only inhalation to 3.2, 9.6 and 32 mg/kg/day of aerosol, which are referred to as low, mid, and high, respectively.	20 mg Nic	• PG:VG control (77% PG: 23% VG) • Formulation 1 (22.5% VG, 75.5% PG, 20 mg Nic) • Formulation 2 (18.1% VG, 62.3% PG, 20 mg NIC and 17.6%	• The inflammatory effects (increase in neutrophils, lymphocytes) in a dose-related manner were observed in the PG:VG control in both males and female groups. • No flavour-dependent effect was observed for pulmonary toxicity. • No nicotine-related pulmonary effects were observed.	(Werley *et al.*, 2016)

	proprietary flavour)

2.4 DISCUSSION

This systematic review screened literature to determine which e-liquid flavours and flavouring agents (if any) elicit toxicological effects in the airways. The studies identified several flavour categories demonstrating toxicity (**Tables 2.1, 2.2, 2.3, 2.4 & 2.5**), suggesting that several flavour chemicals could adversely affect respiratory cells. The most frequently reported e-liquids eliciting adverse responses were cinnamon, menthol, and strawberry flavours. However, it is unclear whether these flavours are the most frequently reported because they are the most investigated or *vice versa*. Moreover, it is unclear which flavouring chemical(s) plays the pivotal role in the toxicity of strawberry-induced toxicity in pulmonary cells; nonetheless, cinnamaldehyde and menthol are proposed as the primary components of cinnamon and mint/menthol flavours, respectively (Clapp *et al.*, 2017b; Omaiye *et al.*, 2019b), which are TRPs agonists. They induce oxidative stress and inflammation and disrupt pulmonary barrier functionalities (Behar *et al.*, 2014a; Clapp *et al.*, 2017b, 2019a; Lamb *et al.*, 2020; Muthumalage *et al.*, 2018, 2019). The mode of toxicological actions for many of the flavoured e-liquids is elusive. Still, the evidence suggests that flavours such as cinnamon, menthol, strawberry, tobacco, and others either induce one or more of the following adverse effects: mitochondrial dysfunction, cell death, ROS production, and dysregulation of inflammatory cytokines (**Table 2.1, 2.2, 2.3, & 2.5**). Given that cinnamaldehyde and menthol can activate TRPA1 and TRPM8, respectively, the toxic effects observed with cinnamon and menthol e-liquids may occur via these TRPs. Nonetheless, we identified a few omissions from many of the studies, such as the absence of appropriate controls, limited dose response data, lack of validation for the experimental model, and justification for concentrations used, which may impact inter-study comparisons and variability and hinder the accurate

interpretation of results. As such, we will recommend experimental approaches to reduce discrepancies in the toxicity testing of EC flavourings in future studies (see **section 2.4.3**).

2.4.1 ADVERSE EFFECTS OF FLAVOURED E-LIQUIDS

The evidence presented in this review suggests that flavours in e-liquids contribute to their toxicity. However, this evidence might be biased or affected due to the limited number of appropriately conducted toxicological studies. The most frequently reported adverse effects are reduced cell count and viability, altered pro-inflammatory biomarkers, cytokine release, and increased oxidative stress (see **Figure 2.2** for a schematic overview). These effects mentioned above are consistent across pulmonary cell types, including normal and cancerous cells, large and small airway epithelial cells, alveolar epithelial cells, pulmonary smooth muscle, fibroblasts, and alveolar macrophages. Although there are no studies where the sensitivity to different flavoured e-liquids across primary, normal immortalized, and cancerous cells are simultaneously probed; nonetheless, in this review, primary cells and normal immortalized cells are seemingly more susceptible to the adverse effects of flavoured e-liquid exposures than cancerous cell lines (**Table 2.2 & 2.3**) (Leslie *et al.*, 2017; Pinkston *et al.*, 2020).

Immortalized human cell lines are normal cells artificially manipulated to proliferate indefinitely. In contrast, cancer cell lines are immortalised malignant cells with mutations in oncogenes and tumour suppressor genes typical of the originating tumour. There is evidence to suggest that cancer cells have increased levels of antioxidant enzymes such as superoxide dismutase and catalases compared to healthy cells, potentially as a defence mechanism against oxidative stress or to promote DNA damage and cancer progression (Asaduzzaman Khan *et al.*, 2010; Chung-man Ho *et*

al., 2001; Sun *et al.*, 1989). Given the differential expression of antioxidant enzymes in cancer cells vs. primary and normal cells, this may contribute to the inter-study variability in EC flavour toxicity. Therefore, caution should be taken in interpreting and drawing conclusions about results obtained from experimental models that may not reflect normal human cellular physiology.

Currently, no standardized protocol exists for determining physiologically relevant EC exposure dosage ranges that reflect actual use for either *in vitro* or *in vivo* studies. Many studies carried out dose-response investigations for total e-liquid, condensates or aerosol but not specifically for flavours (Behar *et al.*, 2018a; Bengalli *et al.*, 2017; Ganguly *et al.*, 2020; Leslie *et al.*, 2017; Lucas *et al.*, 2020; Rowell *et al.*, 2017). Decreases in cell proliferation and viability are indicative of increased cytotoxicity. A dose-dependent decrease in cell proliferation and cell viability was observed (**Table 2.1, 2.2 & 2.3**) with a mixture of tobacco, coconut, vanilla, and cookie (Lucas *et al.*, 2020), or hot cinnamon, menthol tobacco, and black cigar (Rowell *et al.*, 2017), butterfinger and caramel (Behar *et al.*, 2018a) following cells exposure. Moreover, a combination of single-dose and multiple-dose studies reported that menthol (Czekala *et al.*, 2019a; Lamb *et al.*, 2020; Muthumalage *et al.*, 2019), blueberry and tobacco (Czekala *et al.*, 2019a), cinnamon (Behar *et al.*, 2014a; Rowell *et al.*, 2017), menthol tobacco and kola (Rowell *et al.*, 2017), and creamy/buttery and caramel (Behar *et al.*, 2018b) demonstrated the most significant effects on mitochondrial dysfunction in airway epithelial cells suggesting some role for induction of mitochondrial-induced apoptosis. In addition, cinnamon (Noël *et al.*, 2020b), menthol (Muthumalage *et al.*, 2019), and crème brûlée flavours (Pinkston *et al.*, 2020) showed the most significant increase in the production of markers of oxidative stress such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) in bronchial epithelial cells (see

Figure 2.2). ROS and RNS are highly reactive chemical molecules intrinsic to cellular functioning when maintained at low normal cell levels. Still, excess ROS and RNS formation can cause damage to cellular macromolecules, including DNA (O'Farrell *et al.*, 2021), adversely affecting normal cell functions.

Several studies investigated the ability of flavoured e-liquids to induce differential expression of pro-inflammatory cytokines in pulmonary epithelial cells (Clapp *et al.*, 2019a; Pinkston *et al.*, 2020; Ween *et al.*, 2020a), basal cells (Zahedi *et al.*, 2018), and lung fibroblasts (Behar *et al.*, 2014a; Lucas *et al.*, 2020; Omaiye *et al.*, 2019a). Although Bahl et al. (2012); Leigh et al. (2016); Lucas et al. (2020); Rowell et al. (2017) explored different ranges of flavoured e-liquid concentration, none of them justified the relevance of the applied doses for human exposure. Lucas et al (Lucas *et al.*, 2020) investigated the dose-dependent effects on IL-8 release but found that the highest concentrations of flavoured e-liquid (0.5% and 1%) did not induce an increase in IL-8, whereas 0.25% did. It is unclear why this 'bell-shaped' effect was observed with a mixture of tobacco, coconut, vanilla, and cookie. However, it might be that higher doses failed to induce an increase in IL-8 because of the increased cytotoxicity (Lucas *et al.*, 2020). IL-8 is a potent chemokine that is pivotal in leukocyte infiltration during inflammation. Its concentration was increased by chocolate, banana, cinnamon, tobacco, and a mixture of tobacco, coconut, vanilla, and cookie flavoured e-liquids in lung epithelial cells and pulmonary fibroblast. In **Tables 2.2, 2.3 & 2.4**, menthol, cinnamon, cool cucumber, classic menthol, and caffe' latte (Lucas *et al.*, 2020; Muthumalage *et al.*, 2019; Pinkston *et al.*, 2020; Ween *et al.*, 2020a, 2020b) flavoured e-liquids induced the release of pro-inflammatory biomarkers (IL-1ß, IFN-γ, IL-17) in lung epithelial cells (**Table 2.2, 2.3 & 2.4**). Coffee e-liquid increased the release of IL-6, CXCL1, and CXCL2, whereas strawberry increased the release of IL-1ß, IL-10,

CYCL1, CXCL2, and CXCL10 (IP-10). IL-1ß, CXCL1, and CXCL2 are pro-inflammatory cytokines (Leigh *et al.*, 2016a). IL-10 is an anti-inflammatory cytokine, and IL-6 can function as either pro- or anti-inflammatory depending on the phase of inflammation at which it is released (**Figure 2.2**). In contrast, in a separate study, apple flavour reduced the pro-inflammatory cytokines secretion of TNF-α, IL-6, IP-10, MIP-1α, and MIP-1β (Ween *et al.*, 2020a). In **Tables 2.1, 2.2, & 2.3**, EC flavours increased pro-inflammatory biomarkers, mainly in pulmonary epithelial and fibroblast cells.

The release of cytokines by the lung epithelium leads to the recruitment, activation, and differentiation of innate and adaptive inflammatory cells: macrophages, neutrophils, B cells, and T cells, among others. Although not many articles assessed flavour-induced pro-inflammatory cytokines in immune cells *in vitro*, cinnamon was found to suppress alveolar macrophage phagocytosis *in vitro* (Clapp *et al.*, 2017a), suggesting prolonged exposure to cinnamon flavour may impact the resolution of lung inflammation. The mechanism behind this effect is elusive but likely via one of its predominant constituents, cinnamaldehyde. The latter has been shown to inhibit the toll-like receptor (TLR) subtype 4 activation (Youn *et al.*, 2008), which, in turn, might play a pivotal role in macrophage phagocytosis (Skjesol *et al.*, 2019). In alveolar macrophages, phagocytosis is critical for the defence against invading pathogens, the removal of dead cells or foreign particles, and the resolution of inflammatory responses and tissue remodelling, processes that are mediated by various surface receptors, including TLRs (Micera *et al.*, 2016; Paradowska-Gorycka, 2015). This evidence suggests that the resolution of respiratory inflammation may be altered in users of cinnamon flavoured EC.

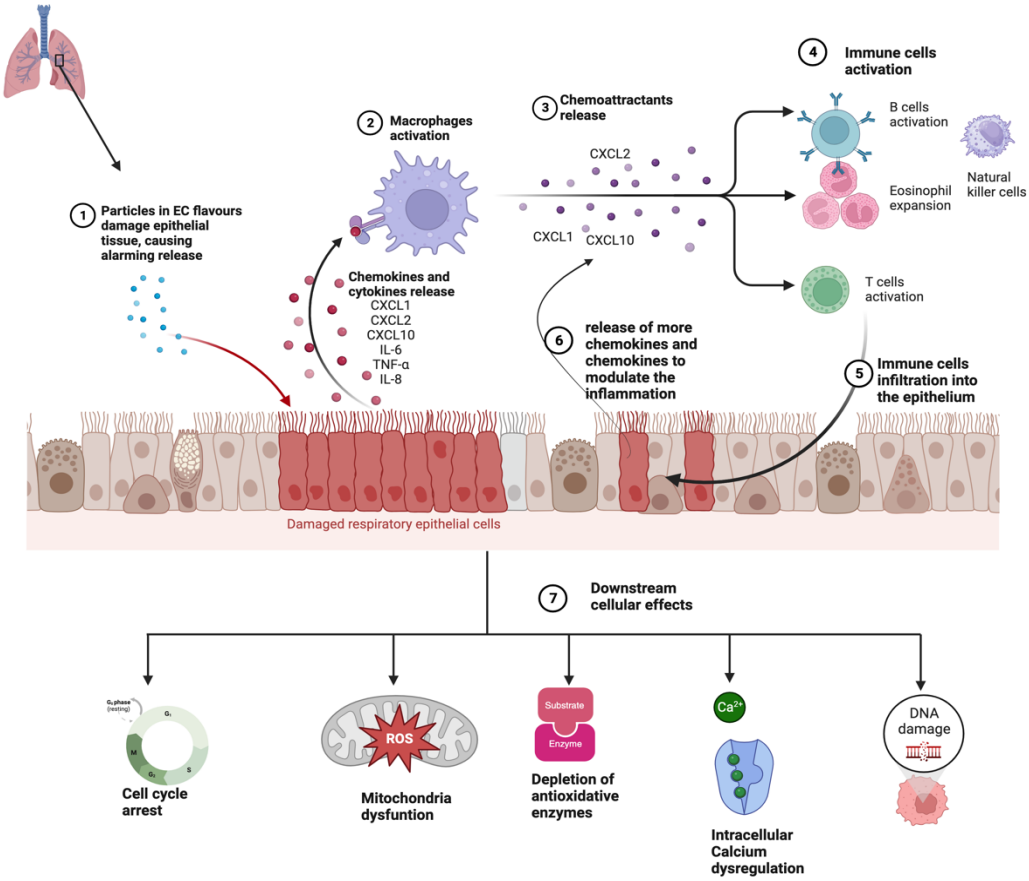


Figure 2. 2 The potential pathways via which flavours in EC exert their toxicological effects on bronchial epithelium.

In vitro observations following flavoured-e-liquid exposures were mirrored in the few *in vivo* studies on EC toxicity. Cinnacide, whose active flavour ingredient is cinnamaldehyde, and black liquorice caused alterations in airways inflammation *in vivo* (Chapman *et al.*, 2019) (**Table 2.5**) by lowering numbers of eosinophils and increasing macrophages, respectively, in BAL in an allergenic airway disease adult Balb/c mouse model (males and females). In contrast, vanilla increased dendritic cells, CD4+ cytotoxic T cells, and CD19+ B cells infiltration in the C57BL/6 mouse (female) lungs (Szafran *et al.*, 2020). Moreover, the effects of cinnamon-flavour exposure in pregnant Balb/c mice and their offspring showed that preconception e-

liquid flavour exposure increases lung tissue fraction at birth compared to non-exposed groups (Noël *et al.*, 2020a), suggesting that cinnamaldehyde may be teratogenic. Glynos et al. (2018) showed a flavour effect on bronco-alveolar lavage fluid (BALF) markers of effect, such as an increase in malondialdehyde and BALF protein carbonyls, increased BALF macrophages, elevated IL-1β and IL-6 levels, and increase BALF total cell count in an acute C57BL/6 male mouse exposure model. Werley et al. (2016) showed that the addition of flavour (flavour type unknown) did not impact pulmonary toxicity in a chronic Sprague-Dawley rat (female and male) exposure study; however, the effects observed were driven by the PG/VG vehicle (**Table 2.5**). All the aforementioned studies included PG/VG controls to isolate flavour-specific effects *in vivo*. Altogether, the reported *in vivo* studies suggest potential adverse pulmonary effects of at least some flavoured-e-liquids in rodents irrespective of strain and/or sex.

Nonetheless, caution must be taken in interpreting the *in vivo* studies on EC toxicity (Marczylo, 2020) where whole-body exposures were performed. This does not mimic human exposure models as the animals are exposed through both inhalation and oral routes via grooming. Moreover, further *in vivo* studies into the effects of different flavours are warranted to allow for comparisons between flavours.

Altogether, the above evidence points towards potential harm from exposure to flavoured EC. Some e-liquids can induce oxidative stress, the release of pro-inflammatory mediators, and cytotoxicity (**Figure 2.3**), which may have implications for healthy lungs and could exacerbate inflammatory-mediated lung diseases such as COPD (O'Farrell *et al.*, 2021), and pneumonia. However, some uncertainties need addressing. Some studies do not provide enough detail on the exposure dose, nicotine concentrations, and exposure durations to enable investigations to be reproduced.

Moreover, most studies (**Table 2.1, 2.2, 2.3, 2.4, & 2.5**) were not designed to include appropriate controls (PG/VG, nicotine) to allow an evaluation of the specific contributions of flavour components to observed toxicity. These are important because studies, where appropriate PG/VG and nicotine controls were included, have shown that these alone have some toxicological effects that may account for at least some of the observed toxicities of the flavoured e-liquids (see **section 2.4.2** for a detailed discussion).

2.4.2 THE POTENTIAL ADDITIVE EFFECTS OF NICOTINE AND PG/VG ON FLAVOUR-INDUCED TOXICITY

Nicotine is a potent stimulator of cell proliferation (Dasgupta, 2006; Khalil *et al.*, 2013; Lee *et al.*, 2005). It is an agonist for the nicotinic acetylcholine receptors (nAChRs), which are functionally expressed in the neuronal and non-neuronal tissues of the lung (Chernyavsky *et al.*, 2015; Improgo *et al.*, 2011). There are more than a dozen different nAChR subunit proteins, subdivided into α (10 different types: α1 through α10) , β (4 different types: β1 through β4) , γ (1 type), δ (1 type), & ε (1 type) subfamilies (Ho *et al.*, 2020), which form pentameric ion channels consisting of either a single type of α subunit (homopentamers) or a combination of α and β subunits (heteropentamers) (Shahsavar *et al.*, 2016). As ligand-gated ion channels, nAChRs undergo complex allosteric changes in response to binding to either the endogenous ligand acetylcholine or exogenous ligands, including nicotine. nAChRs are classically linked to the plasma membrane depolarization required for neurotransmission; however, non-neuronal nAChRs in the lung act most frequently as calcium channels. Nicotine has been shown to activate numerous cellular pathways through the binding to either adrenergic receptors, nicotinic receptors, or by direct action within the

cytoplasm (Wen *et al.*, 2011), which can control cell proliferation. Nicotine alone is not a carcinogen but is a tumour promoter (International Agency for Research on Cancer (IARC), 2004; Ping Wu, 2019). High doses of nicotine can induce multi-organ toxicity and perhaps death from paralysis of respiratory muscles via the nAChRs (Mishra *et al.*, 2015).

Most commercially available e-liquids contain nicotine; therefore, it is understandable that most studies report using nicotine-containing products. There is conflicting evidence regarding the additive effects of nicotine on flavour-induced toxicity. Only 4 (Bahl *et al.*, 2012; Leigh *et al.*, 2016a; Rowell *et al.*, 2017; Ween *et al.*, 2020b) out of the 28 *in vitro* studies in Tables 2, 3, & 4 that reported adverse flavour effects in nicotine-containing e-liquids had included appropriate controls for both nicotine content and PG/VG to isolate flavour-effects. Leigh et al (Leigh *et al.*, 2016a) and Bahl et al (Bahl *et al.*, 2012) showed that nicotine had no additive effects on strawberry, coffee (Leigh *et al.*, 2016a), menthol, and cinnamon (Bahl *et al.*, 2012) flavour-dependent toxicity in H929 cells and hPF cells, respectively. In contrast, Rowell *et al.*, (2017) and Ween *et al.*, 2020b) revealed that nicotine-alone inhibited cell proliferation, decreased cell viability (Rowell *et al.*, 2017), and induced necrosis (measured by Sytox Green) (Ween *et al.*, 2020b) in Calu3 and NHBE cells, respectively. Different countries have different legal regulations on the maximum amount of nicotine that an e-liquid could contain. For example, in the UK, an e-liquid cannot legally contain more that 20 mg/ml, whereas in America, there is no legal regulations on nicotine in e-liquids. As such, studies that use e-liquid dilutions or aerosols from e-liquids that contain 50 mg/mL of nicotine or more and those that use lower concentrations without nicotine controls may present discrepancies. These

discrepancies may arise from 1) variability in nicotine concentrations and/or 2) differential expression of nAchRs or β-adrenergic receptors in the submerged models used. Because nAChR activation often leads to a positive feedback loop that induces receptor expression, high-concentration stimulation of nAChRs could lead to channel desensitization and decreased activity. Thus, elucidating functional roles for nAChRs is particularly complex and requires consideration of subunit composition, dose response, and duration of ligand stimulation. Moreover, undifferentiated bronchial epithelial cells in submerged cultures can be considered uniformly basal. In contrast, validated ALI models of bronchial epithelium represent a model of differentiated airway cells, including ciliated epithelial cells and goblet cells. Therefore, the lack of a nicotine effect in submerged cell cultures may be attributed to low expression levels or the absence of cell surface receptors, including nAChRs and β-adrenergic receptors. Ganguly et al. (2020) reported additive effects of nicotine in reducing inflammatory cytokines of IL-1ß, 1L-10, IL-13, and IL-6 in a validated NCI-H441 ALI model, which probably expressed nAChRs. In contrast, (Wang *et al.*, 2020) have shown that *in vivo* EC exposure with or without nicotine affected lung inflammation, repair responses, and extracellular matrix remodelling mediated by the α7 nicotinic acetylcholine receptor (nAChRα7) in a sex-dependent manner. In addition, Glynos et al. (2018) showed no nicotine-dependent effects on inflammatory markers (IL-6, IL-1β, TNF-α) release and MUC5AC score in acute or chronic C57BL/6 mouse exposure models; however, they did observe nicotine plus flavour-dependent effects suggesting that e-liquid toxicity may not be associated with nicotine but rather the flavour content. Moreover, no nicotine-related adverse pulmonary effects were observed in male or female Sprague-Dawley rats following chronic exposures, but a PG/VG effect was observed (Werley *et al.*, 2016). Although there is contrasting evidence on the adverse

health effects of nicotine in *vitro* against *in vivo* EC studies, the non-harmful effects of nicotine via inhalation were shown in a two-year survey in humans by Waldum et al. (1996). Therefore, nicotine may promote EC-related adverse pulmonary effects when initiated by the flavour or vehicle component of the e-liquid.

Five studies allowed the evaluation of PG/VG components alone by including a vehicle control. PG/VG-alone decreased metabolic activity in H929 cell line derived from B lymphocytes from a plasmacytoma myeloma patient (Leigh et al., 2016a), In H441 adenocarcinoma, and HBECs (Woodall et al., 2020) PG/VG alone elicits its toxic effects via a mechanism that likely involves the blockade of glucose uptake by glucose transporters (GLUT1, GLUT2, and GLUT10). In comparison, Bahl et al. (2012), Leigh et al. (2016), and Ween et al. (2020) reported that PG/VG elicits no adverse effects on the pulmonary cells. Therefore, based on the contrasting evidence provided above, potentially stemming from methodological differences including the choice of cell line and dose, attributing the adverse effects observed with flavoured nicotine-containing e-liquids to the presence of nicotine by comparison to their nicotine-free e-liquid equivalents (**Table 2.1, 2.2, 2.3, 2.4, & 2.5**), without appropriate nicotine- and PG/VG-alone control may be misleading.

As such, future studies on flavours and/or flavouring ingredients of e-liquids would be improved by including appropriate controls: cell-only for submerged exposures and air-only controls for air-liquid interface (ALI) exposures, PG/VG/vehicle alone controls, and PG/VG + nicotine controls to better inform on the contributions of the flavour compounds. Additionally, justifications for e-liquid dosages would improve the interpretation of the relevance of the findings for vapers' health. Establishing an appropriate dosimetry for ALI inhalation studies is paramount as the number of puffs

is not directly proportional to the amount of aerosols deposited on cells and not consistent across different ALI exposure systems. However, none of the studies that exposed ALI cultures to EC aerosols provided an estimate of deposited aerosols. These would lead to a better understanding of the relative contributions of flavour compounds, vehicles, and nicotine to the adverse effects of e-liquids, may aid interpretation of results across studies, and will aid the ongoing debate over the safety and banning of flavoured-e-liquids.

2.4.3 RECOMMENDED APPROACH TO REDUCE UNCERTAINTY IN EC INVESTIGATION *IN VITRO* AND *IN VIVO* MODELS.

In this review, we noticed 1) a lack of appropriate controls in many studies to allow for interpretation of data for identification of hazards and risks due to flavour components, 2) ambiguity over physiologically relevant doses of e-liquid/flavour used, and 3) lack of assessments of dose-dependent relationships. Therefore, this section proposes an approach to reduce discrepancies among EC investigations, whether *in vitro* or *in vivo*.

In vitro submerged culture models are extensively used in EC toxicity screening. Evidence suggests that cytotoxicity of e-liquids in submerged models accurately predicts aerosol cytotoxicity 74% of the time (Behar *et al.*, 2018b; Sassano *et al.*, 2018). Behar *et al.*, (2018b) carried out a cytotoxicity study on 35 e-liquids. The patterns of data were characterized as follows: (1) both the refill fluid and its aerosol were non-cytotoxic (7 of 35=20%) (2) both the refill fluid and its aerosol were cytotoxic (19 of 35=54%) (3) the refill fluid was cytotoxic, but the aerosol was not (1 of 35=3%) (4) the aerosol was cytotoxic, but the refill fluid was not (8 of 35=23%)

Collectively when combining patterns (1) and (2), 74% of the 35 unheated refill fluids correctly predicted the cytotoxicity of their corresponding aerosol. The remaining 26% did not predict cytotoxicity because (1) certain flavours (i.e., menthol arctic) were cytotoxic in submerged cultures but not in their aerosol exposures in the ALI model (2) other flavours are cytotoxic in their aerosolized form (i.e., butterfinger and caramel) but not in their liquid form (Behar *et al.*, 2018b). Although primary human bronchial epithelial cells in submerged cultures are basal cells and do not recapitulate the microenvironment of *in vivo* lung epithelial structures, using e-liquid with submerged models with the inclusion of appropriate controls: nicotine-alone, PG/VG-alone, and physiologically relevant range of flavour doses may help high-throughput screening of the extensive library of flavoured e-liquids for toxicity. Subsequently, the most revealing e-liquids can be progressed into aerosol exposures using air-liquid interface (ALI) systems with standardized puff profiles suggested by CORESTA (CORESTA, 2015) to eliminate potential variability within EC aerosol toxicity.

In contrast to the submerged models, ALI lung cell models that have been validated for adequate transepithelial membrane resistance (TEER) measurements, expression of differential markers for goblet cells (eg, MUC5AC), ciliated cells (eg, FOXJ1), Clara cells (eg, SCGB1A1), and basal cells (eg, KrT5), can be used to investigate physiological and pathophysiological responses of the respiratory tract, molecular events, modes of action and interactions of the different cell types. Potentially, they mimic the *in vivo* microenvironment of cells in the respiratory tract more closely, namely, being apically exposed to air. Also, they generate airway surface liquid (ASL), and the apical surface can be exposed directly to EC vapor. It is probably a better model than adding e-liquid in submerged basal cell culture. Such ALI models

focus either on anatomical regions of the lung such a bronchiolar, or molecular pathways (for example the Calu-3 cells grown at ALI express the cystic fibrosis (CFTR) gene) and aim to model relevant processes *in vivo*. Moreover, establishing a dose-response relationship for e-cigarette flavor toxicity is crucial. By establishing a dose-response relationship, researchers can determine the level of exposure at which these flavours become harmful and better understand the potential health risks associated with their use. Also, a dose-response relationship can help inform these regulations by providing a basis for setting safe exposure limits for different flavors. Subsequently, e-cigarette manufacturers can use the information about the dose-response relationship to develop safer products. For example, if a certain flavor is found to be particularly toxic, manufacturers may choose to reduce or eliminate it from their products to minimize the potential health risks for consumers (like it has been seen with cinnamon flavour in **Chapter 3**). Pairing this with analytical approaches to identify potential chemical compositions and concentrations (especially in flavours where toxicity is observed) for further investigation may eventually lead to limitations on specific flavour chemical concentrations or outright bans from use in e-liquids. In addition, studies assessing the toxicity of e-cigarettes require data from conventional cigarettes before concluding on the relative safety of these devices. This could be a gold-standard protocol to screen e-liquids toxicity that minimizes uncertainties.

To date, no human studies have explored the toxicological effects of flavours in e-liquids on the respiratory system. Nonetheless, observations (inflammatory biomarkers, gene expression, cytotoxicity, and many others) from both *in vitro* studies (especially where the experimental model used closely recapitulates the microenvironments of the 3D human lung eg. ALI, lung-on-chip, etc.) and *in vivo* models may help extrapolation of EC toxicity to humans (**Figure 2.3**) if relevant

dosage and exposures are employed. Due to the lack of reporting of deposited aerosols to allow better comparisons across studies, we propose that future studies measure the deposition of EC aerosols by incorporating a disposition efficiency system that enables them to quantitatively estimate the aerosols reaching the surface area of the cells at ALI (See **Chapter 6.2 for detailed protocol**)

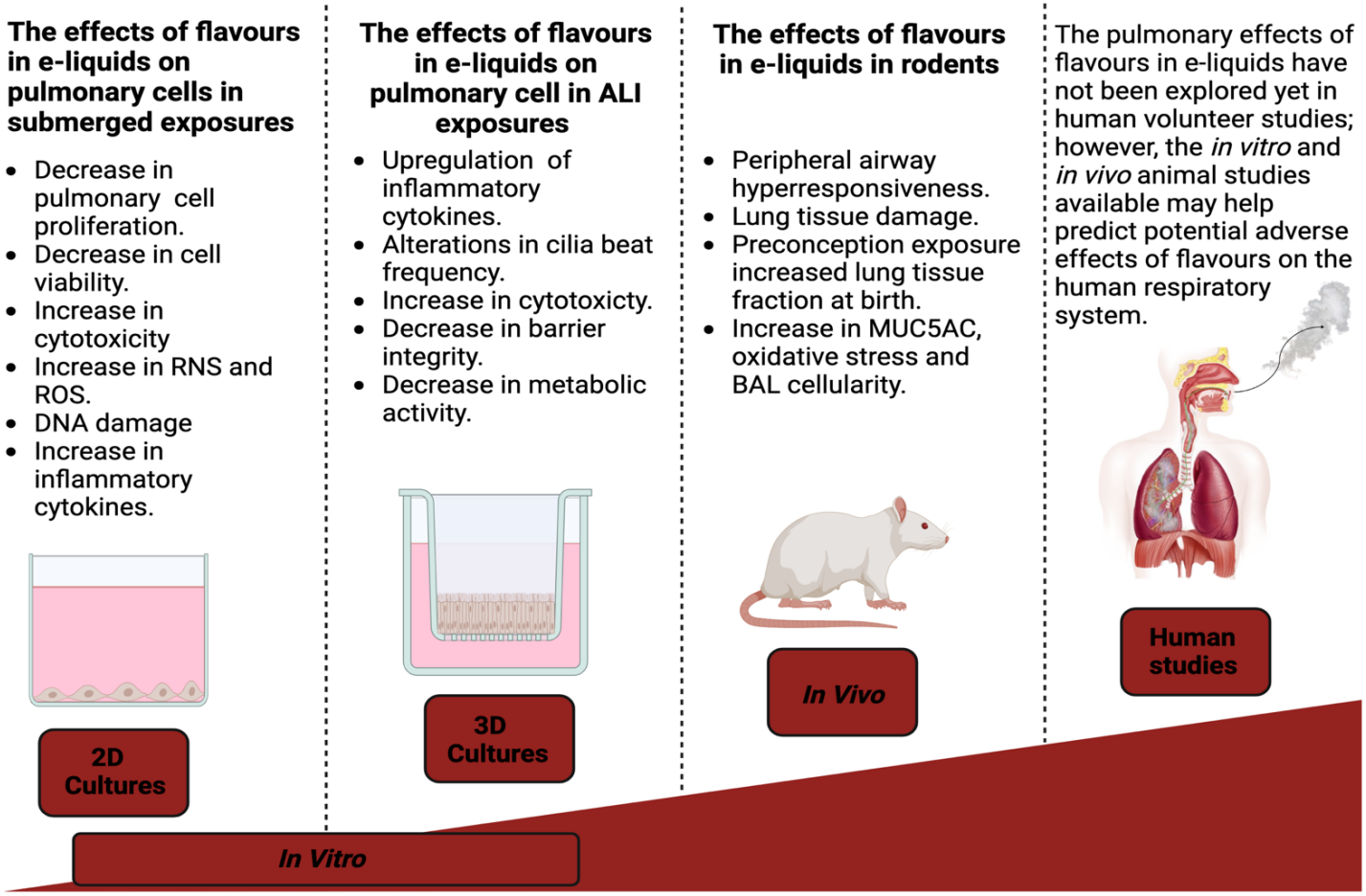


Figure 2. 3 The effects of flavours in e-liquids reported in different experimental models

2.5 CONCLUSION

Some flavours in e-liquids may elicit pulmonary toxicity. However, flavours are generally believed to attract long-time tobacco cigarette smokers to EC and to help them switch to EC use. As such, imposing tight restrictions on the use of flavours in EC based on the reported hazard may be counterproductive. This is because 1) several *in vitro* studies have shown EC toxicity to be related to PG/VG and nicotine rather than flavours per se, 2) not all flavours demonstrate toxicity, and 3) few studies report the actual flavour chemicals present in the e-liquid and their concentrations 4) more evidence-based on risk assessments can be used for those flavours that demonstrate some toxicity to set concentration limits to reduce the potential for causing harm in humans. No human studies have investigated potential adverse pulmonary effects of flavoured e-liquids. To enable public health authorities to extrapolate data from *in vitro* studies to predict the risks posed by EC flavours to human health, a robust toxicological screening of a wide range of flavours is warranted including all appropriate controls. Nonetheless, findings in this study shed light on the intricate nature of e-liquid toxicity. Notably, the observed effects do not align consistently with specific flavour categories, suggesting that toxicity may not be attributed to a singular flavouring agent. Even more intriguing is the observation that e-liquids with similar flavour profiles, potentially containing the same chemical constituents, do not uniformly exhibit the same level of toxicity. This variability in toxicity may be influenced by the concentration of flavourings within the e-liquid. As such, it is imperative to consider both the chemical composition and concentration when evaluating the potential toxic effects of e-liquids, emphasizing the complexity of drawing definitive conclusions on e-liquid safety based solely on flavour categorisation.

CHAPTER 3

IN VITRO HIGH-THROUGHPUT TOXICOLOGICAL ASSESSMENT OF E-CIGARETTE FLAVOURS ON HUMAN BRONCHIAL EPITHELIAL CELLS AND THE POTENTIAL INVOLVEMENT OF TRPA1 IN CINNAMON FLAVOUR-INDUCED TOXICITY

Part of this chapter has been published in the journal "Toxicology"

https://doi.org/10.1016/j.tox.2023.153617

3.1 INTRODUCTION

As reviewed in **Chapter 2**, the flavouring compounds used in EC e-liquids are generally considered safe (GRAS) for ingestion and are used extensively in the food industry but, there are concerns about EC flavouring toxicity when inhaled using EC (Farsalinos *et al.*, 2013a; Khlystov and Samburova, 2016; Kim *et al.*, 2018; Leigh *et al.*, 2016c; Lucas *et al.*, 2020; Madison *et al.*, 2019; Russell *et al.*, 2018; Sundar *et al.*, 2016b). In Chapter 2, we reported the primary research on potential adverse health effects on pulmonary cells from exposure to flavouring compounds in e-liquids, including but not limited to growth inhibition, cytotoxicity, apoptosis, reactive oxygen species (ROS) generation, and mitochondrial dysfunction (see **Chapter 2**), for extensive review). Nonetheless, we concluded that there was; 1) a lack of appropriate controls that could allow the identification of hazards due specifically to flavour components, 2) uncertainty over whether physiologically relevant doses of flavour/e-liquid have been used, and 3) an absence of multiple dosing to establish a dose-dependent relationship (for an extensive review see (Effah *et al.*, 2022) and Chapter 2). This study provides a high-throughput experimental approach to screening cytotoxicity in a wide range of flavoured, nicotine-free e-liquids (n=53). It incorporates at least three flavoured e-liquids per flavour category, identified using the flavour wheel (**Figure 1.9**) generated by Krüsemann and colleagues (Krüsemann *et al.*, 2019). We hypothesized that some flavours, but not all, may elicit dose-dependent adverse effects on pulmonary cells. Subsequently, mechanisms by which those 'toxic hits' in e-liquids demonstrate dose-dependent cytotoxicity will be further examined by investigating cell proliferation, ROS generation, and mitochondrial membrane potential (MP).

3.1.1 AIM

To use a high-throughput experimental approach to screen flavoured, nicotine-free e-liquids (n=53) for adverse effects incorporating at least three flavoured e-liquids per flavour category (Krüsemann *et al.*, 2019).

3.1.2 HYPOTHESES:

- Nicotine induce proliferation in HBEC-3KT
- All flavoured e-liquids elicit dose-dependent, adverse effects in HBEC3KT cells.
- Cinnamaldehyde in cinnamon induces its toxicity via TRPA1

3.1.3 OBJECTIVES:

- To expose HBEC-3KT cells to 53 commercially available flavoured e-liquids across a 3-point concentration range (0.25% v/v, 0.5% v/v or 1% v/v) for 48 hours. To assess cell growth, cytotoxicity (lactate dehydrogenase, LDH), and cell viability to identify the flavours that may pose adverse health risks to EC users.
- To investigate how those toxic flavours in e-liquids demonstrate dose-dependent toxicity by further investigating cell proliferation, ROS generation, and mitochondrial membrane potential (MMP) using high throughput cell painting techniques.

3.2 MATERIALS AND METHODS

3.2.1 CELL CULTURE

Two vials of normal human bronchial epithelial cells immortalized with CDK4 and hTERT (HBEC-3KT; ATCC CRL-4051) at passage 3 were kind gifts from Dr. Martin Leonard (RCE, UKHSA, Chilton). HBEC-3KT cells were cultured in complete bronchial/tracheal growth media (BTEGm, ThermoScientific, UK) under standard airway epithelial cell growth conditions (37 ^{0}C, 95% humidity, and 5% CO_2). Unless specified, all studies in HBEC-3KT cells were performed using cells between passages 10 and 15.

3.2.2 CELL EXPOSURE TO E-LIQUIDS

3.2.2.1 FLAVOURED E-LIQUIDS IN SUBMERGED EXPOSURE

Each of the 53 nicotine-free flavoured e-liquids was purchased from online EC sales websites (**Table 3.1**). E-liquids were stored in opaque boxes at 4 ^{0}C and diluted for each experiment. Not all flavoured e-liquids were available at the same PG/VG ratio; therefore, each plate contained PG/VG (ThermoScientific, UK) controls reflecting the PG/VG ratio in the flavoured-e-liquids. E-liquids were diluted with BTEGm immediately before use in each experiment for final concentrations of 1%, 0.5%, or 0.25 % (v/v).

3.2.2.2 FLAVOURED-E-LIQUIDS WORKING CONCENTRATION VALIDATION

The choice of concentrations used for this investigation is based on estimating the maximum concentration of e-liquid deposited per surface area of bronchial epithelial cells of an EC user adjusted to the surface area of the 96 wells that would be exposed in a day. Real-life deposition of e-liquid condensates in the lung might be lower in the airways and airways surface area (trachea and bronchi); as such, the final concentrations used are probably an overestimate.

Given an adult human lung surface area of 50 - 75 m^2 (Fröhlich *et al.*, 2016) and a mean of 62.5 m^2 with an average daily e-liquid consumption of 3.35 mL (ASH, 2022b).

Therefore, 3350 µL/ 625000 cm^2 = **5.36×10^{-3} µL/cm^2**

5.36×10^{-3} µL/cm^2 is the amount of e-liquid that a whole lung would be exposed to if the deposition is 100% and uniform across airways. The surface area for confluent cells in 96 well plates is 0.32 cm^2/well.

Therefore, 1 cm^2 : **5.36×10^{-3} µL/cm^2** = 0.32 cm^2 : X

X = 5.36×10^{-3} µL/cm^2 x 0.32 cm^2 = **1.72×10^{-3} µL**

Assuming that e-liquids are exposed directly to only the lower respiratory cells in their liquid state, **1.72×10^{-3} µL** would be the maximum quantity of e-liquid a lung's surface area equivalent to the surface area of a 96 well plate would be exposed. However, the maximum depth of mucus gel and mucus that cover human bronchial epithelial cells in the lower respiratory is 13 µm (Fahy and Dickey, 2010). In contrast, the 96 well is about 14 mm:

1.72×10^{-3} µL:13 µm = X:14000 µm

X = 1.85 µL

Therefore, confluent HBEC-3KTs in a 96-well plate need to be exposed to **1.85 µL** of e-liquid/well to recapitulate the microenvironment of the lungs, assuming 100% deposition and even distribution in an average UK EC user consuming the average amount of e-liquid. Therefore, we chose 2 µL of e-liquid in 200 µL of media (1%) as the maximum concentration (v/v) and decided on two other sub-concentrations (0.5% and 0.25 % v/v) based on our preliminary studies to establish whether the flavoured e-liquids elicit their effects in a dose-dependent manner. Moreover, because differently flavoured eliquids contained different ratios of PG/VG (50/50, 60/40, and 70/30), we included appropriate PG/VG controls in each experiment. To the best of our knowledge, we are the first to provide validation for the doses used in e-liquids investigations by using a mathematical estimation model. The 1% maximum e-liquid dose we estimated agrees with Bishop *et al.*, (2023) who observed that any dose greater than 1% formulation exceeded physiological osmolarity ranges (280-400 mOsm), thus potentially causing cells to burst (an artefact of the direct e-liquid exposure methodology rather than chemically induced toxicity). Therefore, the response observed beyond the 1% e-liquid range cannot be solely attributed to the potential cellular toxicity of the e-liquid alone.

3.2.2.3 SCREENING ASSAYS

3.2.2.3.1 CELL COUNT AND PERCENTAGE OF VIABLE CELLS

HBEC-3KTs were plated at 2 x 10^4 cells/well in 200 µL of growth medium into Greiner 96-well/clear plates. The outer wells were filled with Dulbecco's phosphate saline (DPBS) to avoid the so-called 'edge effects'. Cells were placed into the environmental control at 37 ^{0}C, 95% humidity, and 5% CO_2 for 24 hours to allow cellular adherence before e-liquid exposure. A 48h incubation period for the high-throughput screening

(cell count, percentage of viable cells, and LDH) was chosen as the optimum period to detect e-liquid toxicity, if any, based on preliminary experiments of 24, 48 and 72h. After the incubation period, a 200 µL PBS solution containing 6 µM Hoechst 33342 (DAPI) (ThermoScientific, UK) and 6 µM calcein-AM (FITC-C) (Sigma Aldrich, UK) dyes were added to each cell-containing well for analysis of cell count and cell viability, respectively. Plates were left for 0.5 hours inside the environmental control of ImageXpress PICO (PICO) (Molecular Devices, UK) at 37°C, 5% CO2, and 98% humidity (**Figure 3.1A)**. Live-cell image acquisition and analyses of plates were performed using the Pico aided by CellReporterXpress software. A preconfigured 2-channel analysis was chosen for cell count and live cells with DAPI and FITC-C (**Figure 3.1B**). The whole surface area of the plates was analysed with a 4x Plan Fluor objective.

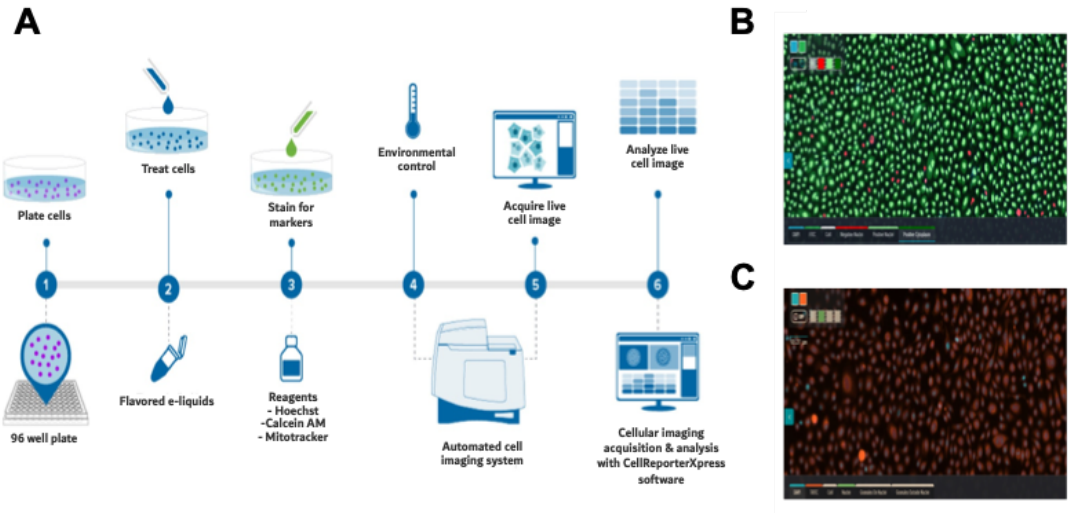


Figure 3. 1 A) The high throughput approach taken to assess the toxic effects of the 53 flavors B) preconfigured 2-channel analysis chosen for cell count and live cells with calcein AM and Hoechst dye C) preconfigured 2-channel analysis chosen for mitochondrial health with Hoechst dye and TRIT-C. Figure 3.2A courtesy of Molecular Devices.

3.2.2.3.2 CYTOTOXICITY: LDH ENDPOINT ASSAY

Before cells were stained with Hoechst 33342 and calcein-AM for cell count and viability, spent media was removed and retained for lactase dehydrogenase (LDH) assay using a LDH cytotoxicity kit (Roche, UK) according to the manufacturer's protocol. LDH is an enzyme found in nearly all living cells, which is responsible for converting lactate to pyruvate and vice versa. LDH catalyses the reversible conversion of lactate to pyruvate, along with the conversion of NAD+ to NADH (nicotinamide adenine dinucleotide, reduced form). Since LDH is released from cells upon membrane damage or cell lysis, an increase in LDH activity in the extracellular environment is indicative of cell damage or death. Plate absorbance was measured at 490nm and 680nm using the BIO-TEK synergy HT absorbance reader (Bio-Tek, USA). The plotted LDH values are relative LDH levels obtained by dividing the absorbance of each well by their respective cell count.

3.2.3 PRELIMINARY STUDIES WITH NICOTINE

Several studies in **Chapter 2**, attributed the toxicity of flavoured e-liquid to the flavour component alone without considering the potential additive or protective effects of nicotine. Therefore, this study was conducted to understand whether nicotine alone had any impact on HBEC-3KTs. L-Nicotine (99.8% pure nicotine, ThermoScientific UK) was serially diluted in either BTEGm or BTEGm containing 1% PG/VG (50/50 ratio) (ThermoScientific, UK) for final concentrations of 10, 30, 60, 180, 360 and 600 mg/ml. The maximum concentration of nicotine legally available in e-liquids in UK, Canada and the EU is 20 mg/ml whereas there is no legal upper limit in the USA and some other regions, therefore, we chose a range of concentrations spanning across

these concentrations. Subsequently, the exposure conditions were the same as described in the section below.

3.2.4 MECHANISTIC STUDIES

3.2.4.1 GROWTH CURVES IN HBEC-3KT

The ability of human bronchial epithelial cells to proliferate is paramount to repair processes following injurious insults from environmental xenobiotics. We tracked the impact of e-liquids testing positive in the toxicity screening, on HBEC-3KT cell proliferation over 42 hours. 8 x 10^3 cells/well in 200 µL of serum-free airways epithelial growth media (AEGM) (PromoCell, UK) were seeded in 96 Greiner well plates x7. To avoid the so-called 'edge effects', the outer wells were filled with Dulbecco's phosphate buffered saline (DPBS). Evidence suggests that serum-free or low-serum growth media 24 hours before compound treatments synchronize cells to be in the same cell cycle phase. This is a gold standard procedure to allow for: 1) a better understanding of the toxic effects of compounds on cell growth and 2) to increase data reproducibility. Plates were challenged with 0.25%, 0.5%, and 1% (v/v) of the e-liquids. Cells were counted at different time points, 0h, 6h, 12h, 18h, 24h, 30h, 36h, and 42h, with 0h being cell count immediately before cell treatment. Transmitted light cell count analysis was performed on the PICO aided by CellReporterXpress software with a 4x Plan Fluor objective.

3.2.4.2 REACTIVE OXYGEN SPECIES

Increased ROS generation in human bronchial epithelial cells can cause damage at the molecular and cellular level and can induce local inflammation, which may, in turn, lead to systemic inflammation. Therefore, in this assay, we aimed to characterize

whether e-liquids stimulate ROS generation in HBEC-3KT cells. The cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (ThermoFisher Scientific, USA) is a chemically reduced form of fluorescein used as an indicator for ROS levels within cells. 24h after seeding $2x10^4$ HBEC-3KT cells into black-walled 96 well plates with clear bottoms, they were treated with 1.5 mM H_2DCFDA and incubated at 37°C, 5% CO_2, and 98% humidity for 0.5h. Subsequently, cells were washed twice with DPBS and challenged with diluted e-liquids (diluted with DMEM/F-12, HEPES, no phenol red (Gibco, UK)) or 2% hydrogen peroxide (positive control) for 3h. Fluorescence intensity was measured using the BIO-TEK synergy HT fluorescence reader at 485 nm excitation and 528 nm emission wavelengths.

3.2.4.3 MITOCHONDRIA MEMBRANE POTENTIAL (MMP)

MMP drives the production of adenosine triphosphate (ATP). Therefore, alterations in MMP may lead to alterations in ATP levels. This experiment aimed to characterise the impact of e-liquids on MMP. HBEC-3KT cells at 8 x 10^3 cells/well were seeded in 96-well plates and allowed to adhere. HBEC-3KT cells at 8 x 10^3 cells/well were seeded in 96-well plates and allowed to adhere. Cells were challenged with the flavoured e-liquids at different concentrations and incubated for 8h. Subsequently, media containing dilutions of e-liquids were discarded. 200 µL PBS solution containing 6 µM Hoechst 33342 (DAPI) (Thermo Scientific, UK) 0.2 µM MitoTracker™ Orange CMTMRos (TRIT-C) (Sigma Aldrich, UK) dye was added to each cell-containing well for cell count and mitochondrial function, respectively. The esterase reaction of a MitoTracker Orange CMTMRos is a biochemical process where the MitoTracker molecule, which is initially non-fluorescent, is modified by esterase enzymes inside a living cell to form the fluorescent compound that accumulates in the mitochondria.

This process is crucial for the MitoTracker's role in labelling mitochondria in live cells for fluorescence microscopy. Plates were incubated for 0.5h inside the environmental control of the PICO (**Figure 3.1A**) at 37°C, 5% CO2, and 98% humidity. Before analysis, cells were fixed with 4% paraformaldehyde for 0.5h at room temperature. A preconfigured 2-channel analysis for cell count and MMP with DAPI and TRIT-C (**Figure 3.1C**) was chosen, and exposures were set at 20ms and 300ms, respectively. 10% surface area of plates was analysed with a 20x Plan Fluor objective aided by CellReporterXpress software.

3.2.5 TRPA1 INHIBITOR STUDIES

Cinnamaldehyde, the main cinnamon flavour chemical component in e-liquids, is an agonist for the transient receptor protein ankyrin 1 (TRPA1). Therefore, in this experiment, we aimed to characterise whether the exposure to HBEC-3KTs to cinnamon e-liquid impacted TRPA1 transcript levels and whether the TRPA1 inhibitor blocked the adverse effects of the cinnamon-flavoured e-liquid. HBEC-3KT cells at 5 x 10^3 cells/well were seeded in 96-well plates and were allowed to adhere. Cells were pre-treated with 10 μM of AP-18 (selective and reversible TRPA1 antagonist) for 2 hours before exposing them to different concentrations of cinnamon-flavoured (0.25%, 0.5%, and 1%) e-liquid or cinnamaldehyde (2.5, 5, and 10 mM) for another 12 h or 24 h. 10 μM of AP-18 was established to be the non-toxic IC_{50} in preliminary studies. 12h and 24h post-exposure, the supernatant was collected for LDH assay (see **section 3.2.1** for detailed protocol). Cells were stained to assess cell count and % of viable cells (see **section 3.2.1** for detailed protocol). Analyses were performed using the PICO (**Figure 3.1A)** aided by CellReporterXpress software.

3.2.5.1 qRT-PCR ANALYSIS FOR TRPA1 EXPRESSION

HBEC-3KT seeded in 6 well plates (Costar, UK) were grown to ~ 85% confluence. Cells were treated for 12h and 24h. Before treatment, the pH of all the exposure solutions was measured (see **Table 3.1**) because variations in pH can differentially activate TRPA1s.

Table 3. 1 pH measurements of the exposure conditions for the TRPA1 inhibitor studies.

Exposure condition	pH
Media-only	7.50
DMSO	7.48
AP-18 (10 µM)	7.47
AP-18 (10 µM) + 1% Cinnamon	7.52
1% Cinnamon	7.53

3.2.5.2 RNA EXTRACTION

At 12 h or 24 h post exposure, 700µl QIAzol lysis reagent was added to HBEC-3KT cells in the transwell chamber for 3 min, the lysate was collected and homogenized using a 5mL syringe, and 25-gauge needle by aspiration, repeated 10 times. The homogenate was incubated at room temperature for 5 min to promote cell dissociation of nucleoprotein complexes. Then, 140µl chloroform was added, vortexed, and incubated for a further 2 min at room temperature. The cell suspension was then centrifuged at 12,000g, for 15 min at 4^0C. The upper colourless aqueous phase was transferred to a 2 mL Qiagen collection tube. The following steps were performed by

the QIAcube, (Qiagen, Venlo, Netherlands). 525µl of 100% ethanol was added and subsequently vortexed. 700µl of the sample was transferred into a RNeasy mini spin column and centrifuged at 8,000g for 15 min at 20^0C. This step was repeated. Subsequently, 500µl RPE buffer was added to the RNeasy mini spin column and was centrifuged for 2 min at 8,000g. The RNeasy mini spin column was transferred to a new 1.5 ml collection tube, and 50 µl RNase-free water was added and subsequently centrifuged for 1 min at 8,000g. RNA purity was calculated using the nanodrop 2000/c spectrophotometer (Thermo-Scientific) from the 260/280 nm and 260/230 nm absorbance ratios.

3.2.5.3 SYNTHESIS OF cDNA

cDNA was synthesized using a deoxyribose containing nucleoside triphosphate (dNTP) solution-based protocol using the cDNA synthesis kit, from Applied Biosystems (Thermo-Scientific).

3.2.5.4 RT-qPCR

Primer validation and mRNA concentration optimisation assays were carried out before the experiment. cDNA was synthesized from total RNA (200 ng) using the High-Capacity RNA-to-cDNA kit (Thermo Scientific, UK). Expression was analysed in triplicate 10µl reactions containing 1µl cDNA, 5µl Fast SYBR Green Master Mix (Applied Biosystems), 3µl nuclease-free water and 0.5µl of 10µM forward and reverse primers. Expression analysis was carried out by quantitative real-time polymerase chain reaction (qRT-PCR) using a Life Technologies QuantStudio 6 Flex instrument. The primer, probe sequences (see **Table 3.2**), and concentrations (100 mM) for *gapdh*

(93% primer efficiency), *b*-actin, HPRT1, 18srRNA, and *trpa1* (91% primer efficiency) were prepared according to the manufacturer's instructions (ThermoFischer Scientific). When calculating results, mRNA expression levels were first normalized against *gapdh* mRNA levels and then against media-only control.

Table 3. 2 The primer sequences for the 4 endogenous controls (gapdh, hprt1, 18sRNA and beta-actin) and trpa1.

Gene	Sequence (forward and reverse)
gapdh	F – 5' – CTGACTTCAACAGCGACACC – 3'
	R– 5'– TCGTTGTCATACCAGGAAATGA – 3'
Hprt1	F – 5′- CCTGGCGTCGTGATTAGTGAT– 3'
	R– 5'– AGACGTTCAGTCCTGTCCATAA – 3'
18sRNA	F – 5' – GGCCCTGTAATTGGAATGAGTC – 3'
	R– 5'– CCAAGATCCAACTACGAGCTT – 3'
Beta-actin	F – 5' – TCATGAAGTGTGACGTGGACATC – 3'
	F – 5' – CAGGAGGAGCAATGATCTTGATCT – 3'
Trpa1	F – 5' – TCCTCTCCATCTGGCAGCAAAG – 3'
	R – 5' – GGACGCATGATGCAAAGCTGTC–3'

3.2.5.5 IDENTIFICATION OF STABLE HOUSEKEEPING GENE

4 housekeeping genes were included in our experiments and screened for their stability, namely *gapdh*, *hprt1*, *beta-actin*, and *18s rRNA* (**Table 3.2**). Protocol detailed in section 3.2.5.3 were used for the qPCR analysis.

3.2.6 CHEMICAL ANALYSIS

Three e-liquid flavours Cinnamon, Hazelnut, and Vanilla tobacco were analysed by gas chromatography (Agilent 7890B) triple quadrupole mass spectrometry (Agilent 7010B) (GC-MS/MS) to identify the volatile organic compounds (VOCs) emitted from the e-liquids after heating. A headspace SPME (HS-SPME) method was used to extract the gas phase chemicals from each e-liquid. In this study we chose the SPME fibre Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) from Supelco (1 cm length, StableFlex, 50/30 μm thickness, 23 Ga needle, operating temperature 230-270°C) due to its suitability to extract VOCs, and Semi-VOCs. The fibre was first conditioned before use for 30 min at 270 °C in the autosampler conditioning unit (PAL RTC 120). A volume of 0.6 mL of each e-liquid was added into a separate 2 mL vial for HS-SPME analysis. Each sample was first incubated at 50°C for 2 min with agitation followed by insertion of the SMPE fibre 22 mm into the 2 ml autosampler vial and incubated at 50°C for a further 15 min for extraction of VOCs without agitation. Each sample was analysed twice on the GC-MS/MS and compared to blanks and quality control (QC) samples (empty vials, water spiked with P-cresol at 300 ng mL^{-1}). P-cresol samples were injected 8 times during the sample sequence to evaluate the fibre extraction efficiency and analytical method repeatability and they showed an RSD of 17 %. Chemical compounds were separated on a Rxi-SVOCms fused silica GC capillary column (Restek, low polarity phase, 5% diphenyl / 95% dimethyl polysiloxane, 30 m length, 0.25 mm diameter, 0.25 μm film thickness) in a constant flow of Helium at 1 mL min^{-1}. The SPME fibre was injected and thermo-desorbed for 2 min in the GC inlet liner (Agilent, 0.75 mm ID straight SPME liner) in splitless mode at 260 °C and purge flow of 40 mL min^{-1} at 2.1 min. After each sample analysis the fibre was reconditioned for 3 min at 270 °C. The GC initial oven

temperature was set at 50 °C for 2 min, increasing to 170 °C at a heating rate of 30 °C/min, followed by 10 °C/min to 300 °C and held for 3 min to ensure the elution of all analytes from the column. The mass spectrometer was operated in MS2 scan mode and mass range from 40 to 350 amu. The MS transfer line was set at 300°C, dwell time 20 ms, and the ion source operated in EI mode at 70 eV, 230°C. Untargeted analysis of VOCs was performed using the ‘‘Agilent MassHunter Unknowns Analysis” software. Annotated compounds were selected based on best ion peaks shape of a component and best hit compound that have a match factor between 80 and 100 % of mass spectral match against the NIST2020 database. Compounds were identified at signal-to-noise ratio of >3 and after blank substruction.

3.2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using one-way ANOVA for each experiment. Where one-way ANOVA revealed significance, a Levenes' variance test was performed to test for differences in variance and to inform the choice of Tukey's HSD *post-hoc* test corrected for multiple comparisons. To establish the dose-response relationship within flavours and to analyse growth curves a two-way ANOVA was performed, followed by Tukey's HSD *post-hoc* test corrected for multiple comparisons using python statistics modules (statsmodel and scipy). Graphs were generated using GraphPad Prism 9. Initial toxicity screening was carried out once with three technical replicates for each experimental condition. After detecting the toxic hits (cinnamon, vanilla tobacco, and hazelnut), assays were repeated at least twice with six technical replicates on different days. Data are expressed as mean ± SD, and the p-value was set at $p < 0.05$.

3.3 RESULTS

3.3.1 THE EFFECTS OF NICOTINE ON HBEC-3KT CELL COUNT, VIABILITY, AND TOXICITY

PG/VG (60/40) carrier alone reduced cell count compared to non-treated controls (Figure 3.4A). Nicotine alone from 10 μg/mL to 600 mg/mL had no effect compared to untreated controls but significantly increased HBEC-3KT cell counts compared to their respective concentrations in combination with 1% PG/VG (**Figure 3.2A**). For cell viability, low-concentration nicotine (10 and 30 μg/mL) did not alter cell viability compared to media alone control though nicotine in 1% PG/VG increased cell viability compared to 1% PG/VG alone, albeit not significant. Above 180 μg/mL nicotine either with or without PG/VG significantly (P) decreased viability compared to respective controls (**Figure 3.2B**) Furthermore, at 180 μg/mL the nicotine alone significantly decreased cell viability compared to 1% PG/VG + 180 μg/mL nicotine (**Figure 3.2B**). The cytotoxic concentration for nicotine alone was lower (180 μg/mL) than nicotine in combination with 1% PG/VG (360 mg/mL) (**Figure 3.2B**). Interestingly, for LDH, high nicotine concentrations did not increase release vs either control or PG/VG.

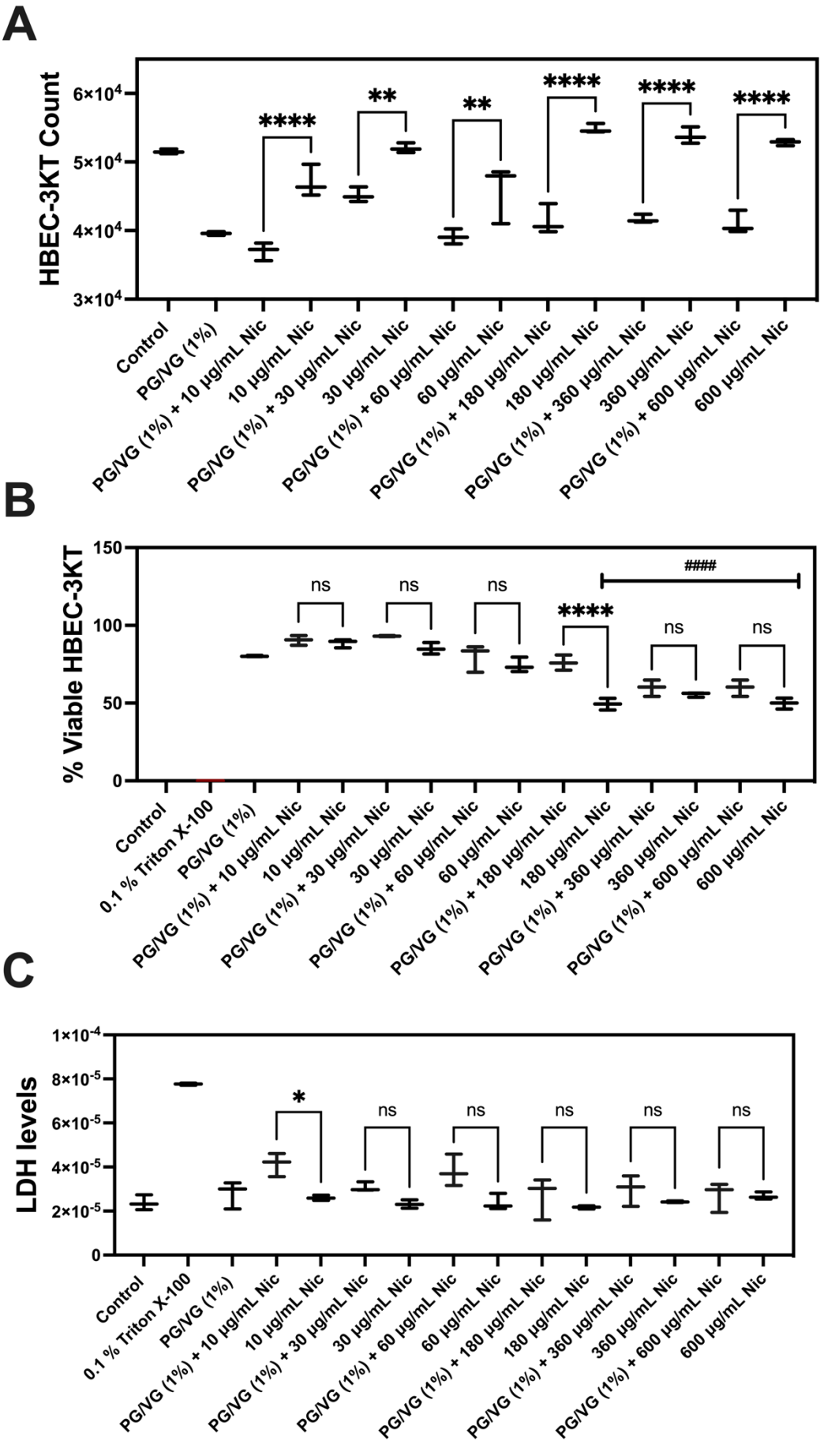

A
HBEC-3KT Count
6×10^4
5×10^4
4×10^4
3×10^4
****
**
**
****
****
****
Control
PG/VG (1%)
PG/VG (1%) + 10 µg/mL Nic
10 µg/mL Nic
PG/VG (1%) + 30 µg/mL Nic
30 µg/mL Nic
PG/VG (1%) + 60 µg/mL Nic
60 µg/mL Nic
PG/VG (1%) + 180 µg/mL Nic
180 µg/mL Nic
PG/VG (1%) + 360 µg/mL Nic
360 µg/mL Nic
PG/VG (1%) + 600 µg/mL Nic
600 µg/mL Nic
B
% Viable HBEC-3KT
150
100
50
0
ns
ns
ns
****
####
ns
ns
Control
0.1 % Triton X-100
PG/VG (1%)
PG/VG (1%) + 10 µg/mL Nic
10 µg/mL Nic
PG/VG (1%) + 30 µg/mL Nic
30 µg/mL Nic
PG/VG (1%) + 60 µg/mL Nic
60 µg/mL Nic
PG/VG (1%) + 180 µg/mL Nic
180 µg/mL Nic
PG/VG (1%) + 360 µg/mL Nic
360 µg/mL Nic
PG/VG (1%) + 600 µg/mL Nic
600 µg/mL Nic
C
LDH levels
1×10^-4
8×10^-5
6×10^-5
4×10^-5
2×10^-5
0
*
ns
ns
ns
ns
ns
Control
0.1 % Triton X-100
PG/VG (1%)
PG/VG (1%) + 10 µg/mL Nic
10 µg/mL Nic
PG/VG (1%) + 30 µg/mL Nic
30 µg/mL Nic
PG/VG (1%) + 60 µg/mL Nic
60 µg/mL Nic
PG/VG (1%) + 180 µg/mL Nic
180 µg/mL Nic
PG/VG (1%) + 360 µg/mL Nic
360 µg/mL Nic
PG/VG (1%) + 600 µg/mL Nic
600 µg/mL Nic

Figure 3. 2 The effect of PG/VG + different concentrations of nicotine and different concentrations of nicotine alone on HBEC-3KT (A) cell counts, (B) % of viable cells HBEC-3KT, and (C) relative LDH release after a 48h exposure. A one-way ANOVA with multiple comparisons was corrected using Tukey to determine statistical significance. Data are presented as box and whiskers min-max (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ####p<0.0001 vs 1% PG/VG. All groups were significantly different compared to the 0.1% Triton X-100 control (p<0.0001). Abbreviations: PG/VG; propylene glycol/vegetable glycerine ; nic; nicotine

3.3.2 HIGH-THROUGHPUT SCREENING

The high-throughput screening of all 53 flavours (**Table 3.2**) clearly demonstrated that the PG/VG (60/40) component of e-liquids alone induced significant toxicity. This PG/VG effect was dose-dependent in decreasing cell count (**Figure 3.3A**) but not in reducing cell viability (**Figure 3.3B**) or LDH release (**Figure 3.3C**). Albeit not dose-dependent, some flavours (flavour categories) significantly ($p<0.01$) decreased cell count and increased LHD release at 1% (v/v) vs. PG/VG control: mint (menthol/mint), charger (berries), energy drink (other beverages), apple (fruit/other), coffee (coffee/tea), mojito (alcohol), heisenberg (candy). We noticed no specific flavour category-related toxicity; nonetheless, e-liquids containing PG alone as carriers (5 e-liquids: Belgian cocoa, menthol ice, Turkish tobacco, black diamond, café mocha, **Table 2**) showed no decrease in either cell count, % of viable cells, or LDH release. Moreover, PG alone only altered cell count at 1% (v/v) (**Figure 3.3D**); nonetheless, it neither altered cell viability (**Figure 3.3E**) nor LDH release (**Figure 3.3F**) vs. media-only control. The only flavours that showed a consistent flavour-specific (significantly different from PG/VG vehicles) and dose-dependent effect vs their respective PG/VG control across cell count and LDH release were cinnamon,

hazelnut, and vanilla tobacco (**Figure 3.4, Figure 3.5, Figure 3.6**). Noteworthily, none of the 53 flavours screened elicited adverse effects on % of viable cells except cinnamon (**Figure 3.6A**).

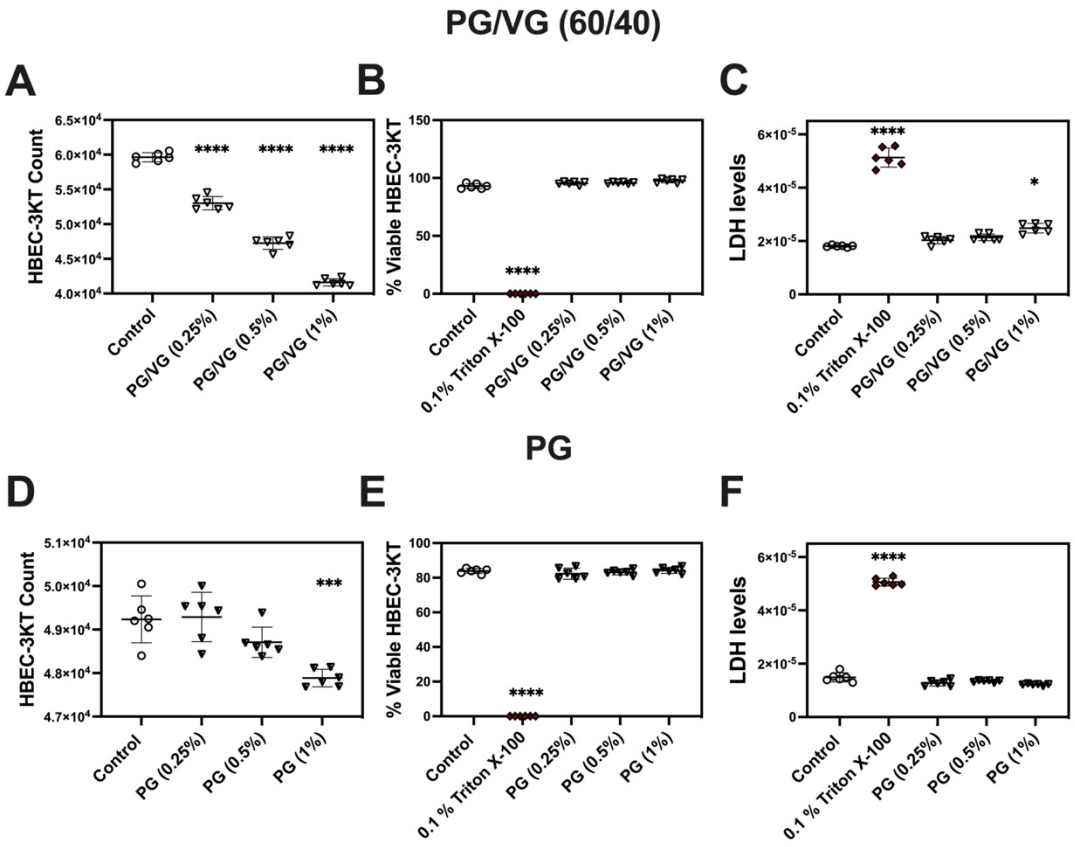


Figure 3. 3 The effects of PG/VG (60/40) at 0.25%, 0.5% and 1% (v/v) on A) HBEC-3KT cell count B) percentage of viable HBEC-3KT cells, and C) relative LDH release after a 48h exposure. The effects of PG alone at 0.25%, 0.5% and 1% (v/v) on D) HBEC-3KT cell count E) percentage of viable HBEC-3KT cells, and F) relative LDH release after a 48h exposure. A one-way ANOVA with multiple comparisons, corrected using Tukey's was used to determine statistical significance. Data are expressed mean ± SD (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs media-only control. All groups were significantly different (p<0.0001) compared to the 0.1% Triton X-100 control. Abbreviations: PG/VG; propylene glycol/vegetable glycerine, PG; propylene glycol.

3.3.2.1 CELL COUNT

Xenobiotics that impair pulmonary basal cell proliferation may cause long-term harm to normal physiological functions of the lung. Cell counts were reduced significantly by almost all 53 flavoured e-liquids compared to media-only controls across the 3-point concentration range **Table 3.2**). However, correlation analyses revealed that the toxicity observed in 85% of all flavoured e-liquids positively correlated with their matched PG/VG controls ($R^2 > 90\%$). Only 3/53: cinnamon (**Figure 3.4A**), hazelnut (**Figure 3.4B**), and vanilla tobacco (**Figure 3.4C**) revealed a flavour-isolated, dose-dependent reproducible reduction in cell count relative to PG/VG controls.

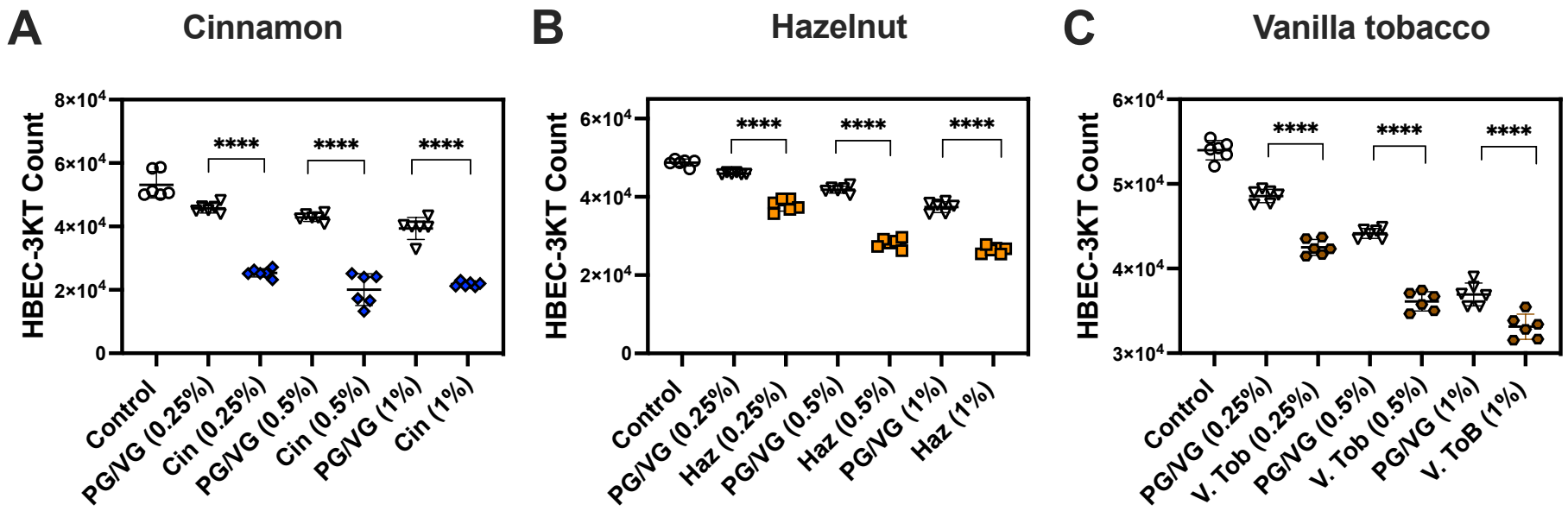


Figure 3. 4 HBEC-3KT count of after exposure to A) cinnamon, B) hazelnut and C) vanilla tobacco at ranging concentration from 0.25%, 0.5% and 1% for 48 hours. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as SD (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs PG/VG. All groups were significantly different compared to incubator control

Table 3. 3 Cell count analysis for all 53 flavoured e-liquids vs. media-only controls and their respective PG/VG controls.

E-liquid name		Flavours vs. media-only control			Correlation vs PG/VG controls (R^2)	Simple Linear regression (R^2)	PG/VG ratio	Flavour Category	Manufacturer
		0.25%	0.5%	1%					
1	**Apple**	<0.0001	<0.0001	<0.0001	0.9848	0.9724	**60/40**	**Fruit/Other**	**V4POUR**
2	**Artic Fruit**	0.0268	<0.0001	<0.0001	0.9748	0.9853	**60/40**	**Fruit/Citrus**	**VAMPIRE VAPE**
3	**Aniseed**	0.0004	0.0002	<0.0001	0.9868	0.8992	**60/40**	**Spices**	**V4POUR**
4	**Heisenberg**	0.0026	<0.0001	<0.0001	0.9824	0.9926	**60/40**	**Candy**	**VAMPIRE VAPE**
5	**Rollie**	ns	<0.0001	<0.0001	0.9560	0.9891	**60/40**	**Tobacco**	**V4POUR**
6	**Cool Cig**	ns	ns	<0.0001	0.9082	0.6990	**60/40**	**Tobacco**	**V4POUR**
7	**Cool Mint**	<0.0001	<0.0001	<0.0001	0.9642	0.9774	**60/40**	**Menthol/**	**V4POUR**

8	**Energy Drink**	<0.0001	<0.0001	<0.0001	0.9875	0.9719	**60/40**	**Mint Other Beverages**	**V4POUR**
9	**Wine Gummies**	<0.0001	<0.0001	<0.0001	0.9965	0.9700	**60/40**	**Candy**	**V4POUR**
10	**Butterscotch**	<0.0001	<0.0001	<0.0001	0.9735	0.9891	**60/40**	**Other Sweets**	**V4POUR**
11	**Grape**	0.0019	<0.0001	<0.0001	0.9944	0.9695	**60/40**	**Fruit/Other**	**V4POUR**
12	**NRG Charger**	<0.0001	<0.0001	<0.0001	0.9760	0.9617	**60/40**	**Other Beverages**	**V4POUR**
13	**Virginia**	<0.0001	<0.0001	<0.0001	0.9992	0.9937	**60/40**	**Tobacco**	**V4POUR**
14	**Lemon & Lime**	<0.0001	<0.0001	<0.0001	0.9886	0.9643	**60/40**	**Fruit/Citrus**	**V4POUR**

15	**Black jack**	<0.0001	<0.0001	<0.0001	0.9793	0.9692	**60/40**	**Candy**	**V4POUR**
16	**Blueberry**	0.0043	<0.0001	<0.0001	0.9311	0.9774	**60/40**	**Fruit/Berries**	**V4POUR**
17	**Raspberry**	ns	<0.0001	<0.0001	0.9992	0.8997	**60/40**	**Fruit/Berries**	**VAMPIRE VAPE**
18	**Rhubarb Crumble**	ns	<0.0001	<0.0001	0.9222	0.9833	**60/40**	**Dessert**	**V4POUR**
19	**Banana**	ns	ns	0.0015	0.8608	0.7071	**60/40**	**Fruit/Tropical**	**V4POUR**
20	**Cola**	ns	0.0016	<0.0001	0.9405	0.9769	**60/40**	**Other Beverages**	**V4POUR**
21	**Toffee**	ns	0.0052	<0.0001	0.9624	0.9555	**60/40**	**Other Sweets**	**V4POUR**
22	**Custard**	0.0010	<0.0001	<0.0001	0.9922	0.9634	**60/40**	**Dessert**	**V4POUR**

23	**Coffee**	<0.0001	<0.0001	<0.0001	0.9778	0.9848	**60/40**	**Coffee/Tea**	**V4POUR**
24	**Menthol**	0.0003	0.0041	<0.0001	0.8075	0.6661	**60/40**	**Menthol/Mint**	**VAMPIRE VAPE**
25	**Peach**	ns	0.0005	<0.0001	0.9955	0.8196	**60/40**	**Fruit/Other**	**V4POUR**
26	**Pineapple**	<0.0001	<0.0001	<0.0001	0.7459	0.7607	**60/40**	**Fruit/Other**	**V4POUR**
27	**Mint**	<0.0001	<0.0001	<0.0001	0.9562	0.9694	**60/40**	**Menthol/Mint**	**V4POUR**
28	**Bat Juice**	ns	ns	<0.0001	0.9204	0.9321	**60/40**	**Fruit/Berries**	**V4POUR**
29	**Charger**	<0.0001	<0.0001	<0.0001	0.9034	0.8644	**60/40**	**Fruit/Berries**	**VAMPIRE VAPE**
30	**Fruit Salad**	<0.0001	<0.0001	<0.0001	0.9703	0.9595	**60/40**	**Fruit**	**V4POUR**
31	**Menthol Tobacco**	0.0319	<0.0001	<0.0001	0.9972	0.9937	**60/40**	**Crossed Flavours**	**VAMPIRE VAPE**

32	**Vanilla tobacco***	<0.0001	<0.0001	<0.0001	0.8309	0.7666	**60/40**	**Crossed Flavours**	**VAMPIRE VAPE**
33	**Strawberry Menthol**	<0.0001	<0.0001	<0.0001	0.9676	0.9612	**60/40**	**Crossed Flavours**	**V4POUR**
34	**Vanilla**	<0.0001	<0.0001	<0.0001	0.9995	0.9869	**60/40**	**Other Sweets**	**VAMPIRE VAPE**
35	**Belgian Cocoa**	ns	ns	<0.0001	0.9897	0.8641	**PG only**	**Fruit/Tropical**	**V4POUR**
36	**Menthol Ice**	ns	ns	ns	0.9960	0.8869	**PG only**	**Menthol/Mint**	**V4POUR**
37	**Turkish Tobacco**	ns	ns	0.0020	0.9999	0.9642	**PG only**	**Tobacco**	**V4POUR**
38	**Black Diamond**	ns	ns	0.0001	1.000	0.9656	**PG only**	**Unknown**	**Vapeshop**

39	**Café Mocha**	ns	<0.0001	<0.0001	0.9382	0.8537	**PG only**	**Coffee/Tea**	**V4POUR**
40	**Mango**	0.0006	<0.0001	<0.0001	0.9885	0.9352	**60/40**	**Fruit**	**Vapemate**
41	**Cappuccino**	0.0010	<0.0001	<0.0001	0.9684	0.9798	**60/40**	**Coffee/Tea**	**Vapeshop**
42	**Dark rum**	0.0005	<0.0001	<0.0001	0.8137	0.7583	**60/40**	**Alcohol**	**Motivape**
43	**Cinnamon***	<0.0001	<0.0001	<0.0001	0.7891	0.9123	**70/30**	**Spices**	**Vapekit**
44	**Mojito**	0.0001	<0.0001	<0.0001	0.9452	0.9366	**60/40**	**Alcohol**	**Vapekit**
45	**Watermelon**	<0.0001	<0.0001	<0.0001	0.9510	0.7412	**60/40**	**Fruit/Other**	**Vampirevape**
46	**Coconut**	ns	<0.0001	<0.0001	0.9996	0.8840	**60/40**	**Fruit/Tropical**	**Vapemate**
47	**Hazelnut***	<0.0001	<0.0001	<0.0001	0.8428	0.7477	**50/50**	**Nuts**	**Mist**

48	**Energy**	0.0004	<0.0001	<0.0001	0.9736	0.9930	**60/40**	**Other Beverages**	**VAMPIRE VAPE**
49	**Cherry, mint & menthol**	0.0057	<0.0001	<0.0001	0.9401	0.8494	**60/40**	**Crossed Flavours**	**Vapourlites**
50	**Blueberry forest**	0.0012	<0.0001	<0.0001	0.9996	0.9741	**60/40**	**Fruit/Berries**	**Vapourlites**
51	**Almond**	0.0010	<0.0001	<0.0001	0.9969	0.9741	**60/40**	**Nuts**	**Nucig**
52	**Lemon, Strawberry & Aniseed**	<0.0001	<0.0001	<0.0001	0.9626	0.9953	**60/40**	**Crossed Flavours**	**Vapourlites**
53	**Strawberry, vanillin & Custard**	<0.0001	<0.0001	<0.0001	0.9986	0.9521	**60/40**	**Crossed Flavours**	**Vapourlites**

3.3.2.2 CELL VIABILITY

Cell viability is a recognized cell health parameter. Live cells are distinguished by ubiquitous intracellular esterase activity determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein (Méry *et al.*, 2017). 98% of all flavoured e-liquids showed no significant effect on the % of viable cells following exposure compared to media-only or their PG/VG controls. Nonetheless, cinnamon-flavoured e-liquid revealed a significant dose-dependent decrease in % of viable HBEC-3KTs compared to both media-only and PG/VG controls with a 95% decrease in cell viability at 1% (v/v) concentration (**Figure 3.5A**).

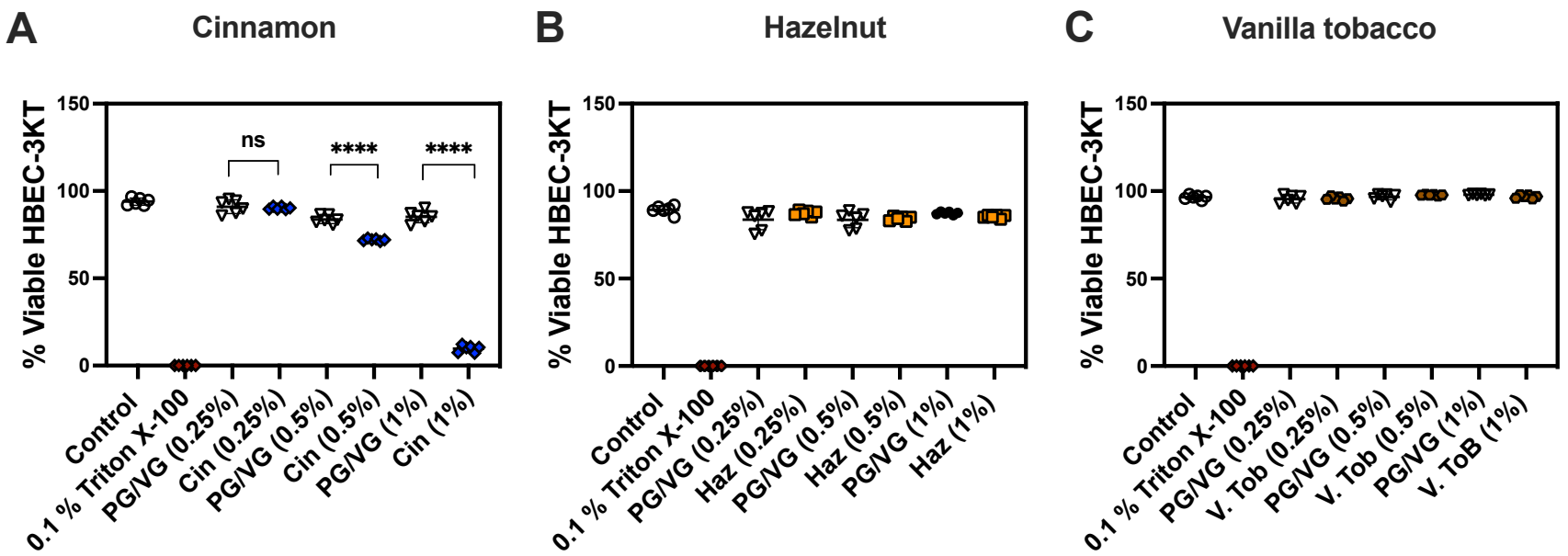


Figure 3. 5 Percentage of viable HBEC-3KT count of after 48h exposure to A) cinnamon, B) hazelnut and C) vanilla tobacco at ranging concentration from 0.25%, 0.5% and 1%. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean SD (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs PG/VG. All groups were significantly different compared to the 0.1% Triton X-100 control apart from the 1% cinnamon group.

3.3.2.3 CYTOTOXICITY: LDH ASSAY

Compared to the media-only control, PG/VG alone showed a significant increase in LDH release at 1% (v/v) concentration (**Table 3.5**). LDH release in most flavours positively correlated with the PG/VG vehicle ($R^2 > 0.90$). Interestingly, cinnamon (**Figure 3.6A**), hazelnut (**Figure 3.6B),** and vanilla tobacco (**Figure 3.6C**) e-liquids showed a significant increase in LDH release compared to both media-only and their respective PG/VG controls. 1% (v/v) of cinnamon showed LDH release, with values even exceeding those of the 0.1% Triton X-100, the LDH positive control (**Figure 3.6A**). Besides cinnamon, which showed a dose-response in LDH release (**Table 3.5**), hazelnut or vanilla tobacco did not elicit dose-dependent effects on LDH release (**Table 3.5**).

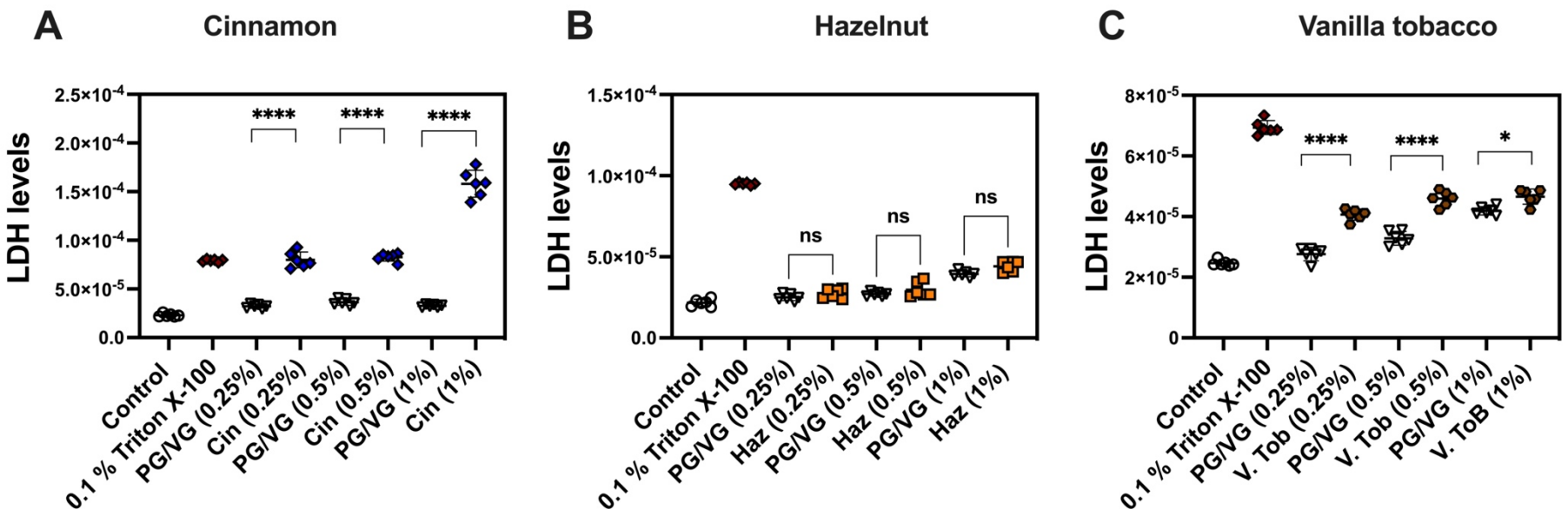


Figure 3. 6 Relative LDH levels after 48h exposure to A) cinnamon, B) hazelnut and C) vanilla tobacco at ranging concentration from 0.25%, 0.5% and 1%. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean SD (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs PG/VG. All groups were significantly different compared to the 0.1% Triton X-100 control apart from the 0.5% and 1% cinnamon groups.

Table 3. 4 LDH analysis for all 53 flavored e-liquids vs. media-only controls and their respective PG/VG controls.

	E-liquid name	Flavors vs media-only control			Correlation vs PG/VG controls (R^2)	Simple Linear regression (R^2)	Flavour Category
		0.25%	0.5%	1%			
1	**Apple**	ns	ns	< 0.05	0.857	0.08624	**Fruit/Other**
2	**Artic Fruit**	ns	ns	< 0.01	0.923	0.8288	**Fruit/Citrus**
3	**Anised**	ns	ns	ns	0.999	0.1957	**Spices**
4	**Heisenberg**	ns	ns	< 0.0001	0.999	0.6662	**Candy**
5	**Rollie**	ns	ns	< 0.0001	0.675	0.5542	**Tobacco**
6	**Cool Cig**	ns	ns	< 0.0001	0.981	0.1216	**Tobacco**
7	**Cool Mint**	ns	ns	< 0.05	0.810	0.3988	**Menthol/Mint**

8	**Energy Drink**	ns	ns	< 0.0001	0.194	0.8752	**Other Beverages**
9	**Wine Gummies**	ns	< 0.05	< 0.001	0.874	0.3222	**Candy**
10	**Butterscotch**	ns	ns	ns	0.887	0.3043	**Other Sweets**
11	**Grape**	ns	< 0.001	< 0.0001	0.974	0.6702	**Fruit/Other**
12	**NRG Charger**	ns	ns	< 0.0001	0.996	0.7080	**Other Beverages**
13	**Virginia**	ns	ns	< 0.001	0.999	0.3545	**Tobacco**
14	**Lemon & Lime**	ns	ns	< 0.001	0.999	0.5448	**Fruit/Citrus**
15	**Blackjack**	ns	ns	< 0.001	0.999	0.7071	**Candy**
16	**Blueberry**	ns	< 0.05	< 0.001	0.9999	0.1982	**Fruit/Berries**

17	**Raspberry**	ns	< 0.01	< 0.001	0.9633	0.6978	**Fruit/Berries**
18	**Rhubarb Crumble**	ns	< 0.01	< 0.001	0.9739	0.5255	**Dessert**
19	**Banana**	ns	ns	< 0.01	0.3958	0.4489	**Fruit/Tropical**
20	**Cola**	ns	ns	< 0.001	0.1568	0.9769	**Other Beverages**
21	**Toffee**	ns	ns	ns	0.1242	0.9555	**Other Sweets**
22	**Custard**	ns	ns	ns	0.7625	0.1115	**Dessert**
23	**Coffee**	ns	< 0.05	< 0.0001	0.9854	0.9100	**Coffee/Tea**
24	**Menthol**	ns	ns	ns	0.155	0.550	**Menthol/Mint**

25	**Peach**	ns	ns	ns	0.762	0.712	**Fruit/Other**
26	**Pineapple**	ns	ns	< 0.001	0.867	0.547	**Fruit/Other**
27	**Mint**	ns	ns	< 0.001	0.998	0.847	**Menthol/Mint**
28	**Bat Juice**	ns	ns	ns	0.997	0.381	**Fruit/Berries**
29	**Charger**	ns	ns	ns	0.366	0.287	**Fruit/Berries**
30	**Fruit Salad**	< 0.05	< 0.05	< 0.0001	0.933	0.611	**Fruit**
31	**Menthol Tobacco**	< 0.05	< 0.001	< 0.0001	0.997	0.705	**Crossed Flavours**
32	**Vanilla tobacco***	< 0.0001	< 0.0001	< 0.0001	0.772	0.556	**Crossed Flavours**

33	**Strawberry Menthol**	< 0.001	< 0.001	< 0.001	0.997	0.701	**Crossed Flavours**
34	**Vanilla**	< 0.001	< 0.001	< 0.001	0.906	0.721	**Other Sweets**
35	**Belgian Cocoa**	ns	ns	ns	-0.342	0.0303	**Fruit/Tropical**
36	**Menthol Ice**	ns	ns	ns	0.593	0.312	**Menthol/Mint**
37	**Turkish Tobacco**	ns	ns	ns	-0.187	0.0921	**Tobacco**
38	**Black Diamond**	ns	ns	ns	-0.820	0.0921	**Unknown**
39	**Café Mocha**	ns	ns	ns	0.643	0.443	**Coffee/Tea**
40	**Mango**	ns	ns	ns	-0.576	0.769	**Fruit**

41	**Cappuccino**	ns	ns	< 0.0001	-0.460	0.820	**Coffee/Tea**
42	**Dark rum**	ns	ns	ns	-0.954	0.115	**Alcohol**
43	**Cinnamon***	<0.0001	<0.0001	< 0.0001	-0.3244	0.8863	**Spices**
44	**Mojito**	ns	ns	<0.0001	-0.0695	0.7117	**Alcohol**
45	**Watermelon**	ns	ns	ns	-0.4978	0.5519	**Fruit/Other**
46	**Coconut**	ns	ns	ns	-0.9408	0.0375	**Fruit/Tropical**
47	**Hazelnut***	< 0.001	<0.001	< 0.001	0.930	0.569	**Nuts**
48	**Energy**	< 0.05	< 0.05	< 0.001	0.694	0.144	**Other Beverages**

49	**Cherry, mint & menthol**	ns	ns	ns	-0.302	0.849	**Crossed Flavours**
50	**Blueberry forest**	ns	ns	ns	-0.534	0.995	**Fruit/Berries**
51	**Almond**	ns	ns	<0.0001	-0.576	0.630	**Nuts**
52	**Lemon, Strawberry & Aniseed**	ns	ns	< 0.05	-0.343	0.952	**Crossed Flavours**
53	**Strawberry, vanillin & Custard**	ns	ns	< 0.0001	-0.486	0.981	**Crossed Flavours**

Table 3. 5 Tukey's multiple comparisons test to characterize the dose-response relationship within the toxic hits on cell count, % of viable cells, and LDH release.

Tukey's multiple comparisons tests	Dose-response	HBEC-3KT count (Adjusted P value)	% of Viable HBEC-3KT (Adjusted P value)	LDH release (Adjusted P value)
PG/VG (60/40)	0.25% vs. 0.5%	$p < 0.0001$	ns	ns
	0.25% vs. 1%	$p < 0.0001$	ns	$p < 0.0001$
	0.5% vs. 1%	$p < 0.0001$	ns	$p < 0.0001$
Cinnamon	0.25% vs. 0.5%	$p = 0.4302$	$p < 0.0001$	ns
	0.25% vs. 1%	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	0.5% vs. 1%	$p = 0.0002$	$p < 0.0001$	$p = 0.0110$
Hazelnut	0.25% vs. 0.5%	$p < 0.0001$	ns	ns
	0.25% vs. 1%	$p < 0.0001$	ns	ns
	0.5% vs. 1%	$p = 0.0009$	ns	ns
Vanilla Tobacco	0.25% vs. 0.5%	$p < 0.0001$	ns	ns
	0.25% vs. 1%	$p < 0.0001$	ns	ns
	0.5% vs. 1%	$p = 0.0019$	ns	ns

Abbreviations: PG/VG, propylene glycol/vegetable glycerine; ns, non-significant

3.3.3 MECHANISTIC STUDIES

3.3.3.1 GROWTH CURVES

A decrease in cell count can occur due to either cell death: apoptosis, necrosis, or a reduction in cellular proliferation. The ability of HBECs to proliferate and repair is paramount to lung physiology. Following the initial end-point screening, we further investigated the effects of cinnamon, hazelnut, and vanilla tobacco-flavoured e-liquids. We assessed the impact of these three 'toxic hits' on HBEC-3KT proliferation in real time. In **Figure 3.7**, we demonstrate the time-dependent effects on cell proliferation induced by the e-liquids and the PG/VG control. In the first 12h post-exposure, there was no significant effect with any of the challenged cells compared to the media-only control (**Table 3.6, Figure 3.7**). At 18h, although cinnamon and vanilla tobacco significantly decreased cell proliferation compared to the media-only control, only cinnamon significantly reduced cell proliferation compared to the PG/VG control (**Table 3.6**). From 24h post-exposure, each flavoured e-liquid, and the PG/VG control, had significantly decreased cell proliferation compared to the media-only control. (**Table 3.6**). At 30h, cinnamon and vanilla tobacco were significantly decreased compared to PG/VG control (**Figure 3.7**). At 36h and 42h, all flavours had significantly reduced cell proliferation compared to the PG/VG control (**Table 3.6**). Regarding inter-flavour comparisons, cinnamon vs. hazelnut were significantly different after 18h. Cinnamon reduced cell proliferation significantly more than vanilla tobacco at 24h. Finally, the impact of vanilla tobacco on HBEC-3KT proliferation was statistically decreased vs hazelnut after 36 hours of exposure (**Table 3.6**).

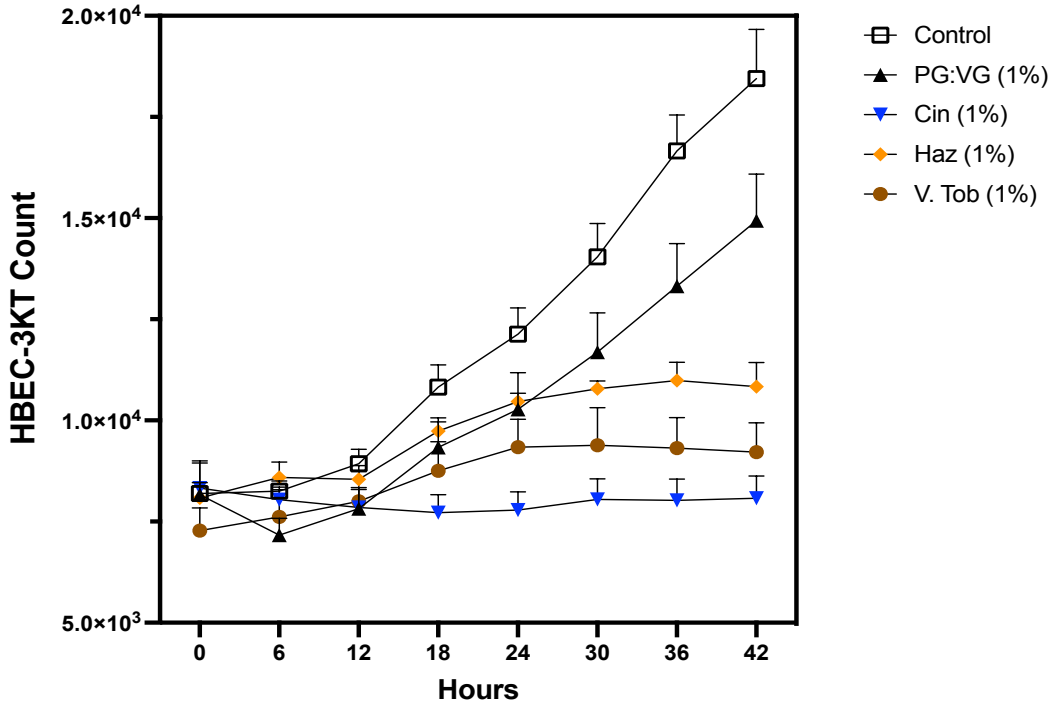


Figure 3. 7 The effect of 1% (v/v) of PG/VG, cinnamon, hazelnut, and vanilla tobacco on HBEC-3KT proliferation for 42h post-exposure. A two-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Significance between all groups can be seen in Table 3.6. Data are expressed as mean ± SD (n=6 per group). Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Cin, cinnamon; Haz, hazelnut; V. Tob, vanilla tobacco.

Table 3. 6 Tukey post-hoc comparisons for Figure 3.9.

Experimental Group	0 hours	6 hours	12 hours	18 hours	24 hours	30 hours	36 hours	42 hours
Control vs. PG/VG (1%)	ns	ns	ns	ns	$p = 0.0076$	$p = 0.0003$	$p < 0.0001$	$p < 0.0001$
Control vs. Cin (1%)	ns	ns	ns	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Control vs. Haz (1%)	ns	ns	ns	ns	$p = 0.0238$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Control vs. V. Tob (1%)	ns	ns	ns	$p = 0.0020$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
PG/VG (1%) vs. Cin (1%)	ns	ns	ns	$p = 0.0312$	$p = 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
PG/VG (1%) vs. Haz (1%)	ns	ns	ns	ns	ns	ns	$p = 0.0003$	$p < 0.0001$
PG/VG (1%) vs. V. Tob (1%)	ns	ns	ns	ns	ns	$p = 0.0004$	$p < 0.0001$	$p < 0.0001$
Cin (1%) vs. Haz (1%)	ns	ns	ns	$p = 0.0027$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Cin (1%) vs. V. Tob (1%)	ns	ns	ns	ns	$p = 0.0439$	ns	ns	ns
Haz (1%) vs . V. Tob (1%)	ns	ns	ns	ns	ns	ns	$p = 0.0231$	$p = 0.0307$

Abbreviations: PG/VG, propylene glycol/vegetable glycerine; Haz, hazelnut; Cin, cinnamon; V. Tob, vanilla tobacco.

3.3.3.2 MITOCHONDRIAL HEALTH

Mitochondrial function is a crucial indicator of cell health, which can be assessed by monitoring changes in mitochondrial membrane potential (MMP). Mitochondrial depolarization is an early signal for hypoxic damage or oxidative stress. The esterase reaction of a MitoTracker Orange CMTMRos is a biochemical process where the MitoTracker molecule, which is initially non-fluorescent, is modified by esterase enzymes inside a living cell to form the fluorescent compound that accumulates in the mitochondria. We observed that vanilla tobacco at 0.25%, and each flavour at 0.5% and 1%, (**Figure 3.8**) significantly decreased average granule (mitochondria) intensity compared to the media-only control and PG/VG vehicle. PG/VG did not substantially alter MMP (**Figure 3.8**). Inter-flavour concentration comparison and dose-response relationship is observed in **Table 3.7**.

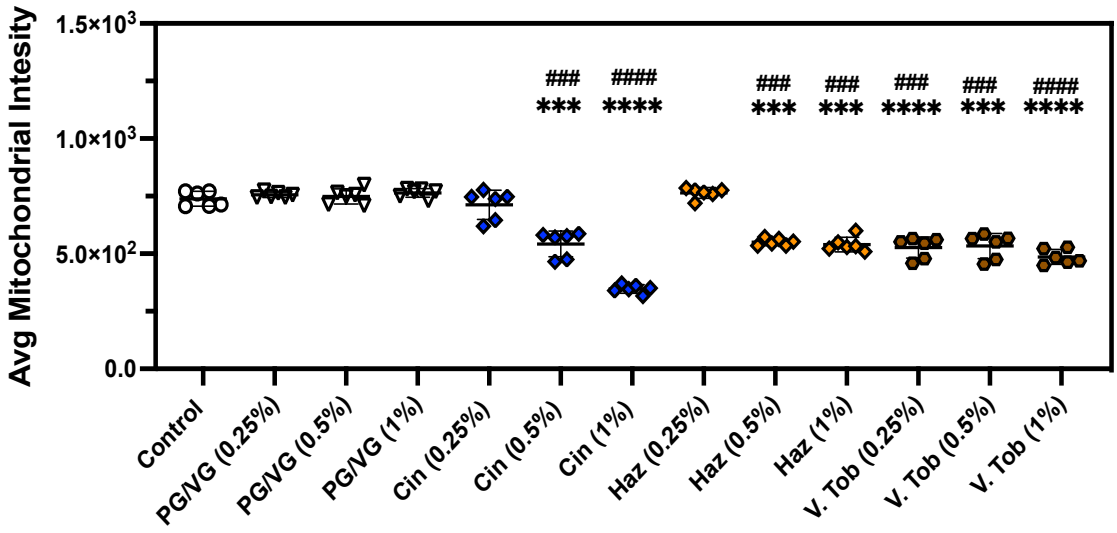


Figure 3. 8 The effect of 0.25%, 0.5% and 1% (v/v) of PG/VG, cinnamon, hazelnut, and vanilla tobacco on mitochondrial membrane potential after 8h exposure. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Significance between concentrations can be seen in Table 3.7. Data are expressed as mean ± SD (n=6 per group). ***p<0.001; ****p<0.0001 vs control. ###p<0.001; ####p<0.0001 vs PG/VG. Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Cin, cinnamon; Haz, hazelnut; V. Tob, vanilla tobacco.

3.3.3.3 REACTIVE OXYGEN SPECIES GENERATION

ROS analysis (**Figure 3.9**) revealed a significant increase in ROS generation with hazelnut at all concentrations and vanilla tobacco at 1% (v/v) vs. media-only and PG/VG controls. PG/VG vehicle controls did not significantly affect ROS production. In contrast, cinnamon slightly decreased ROS levels, albeit not significantly. Inter-flavour concentration comparisons and dose-response relationships are observed in **Table 3.7**.

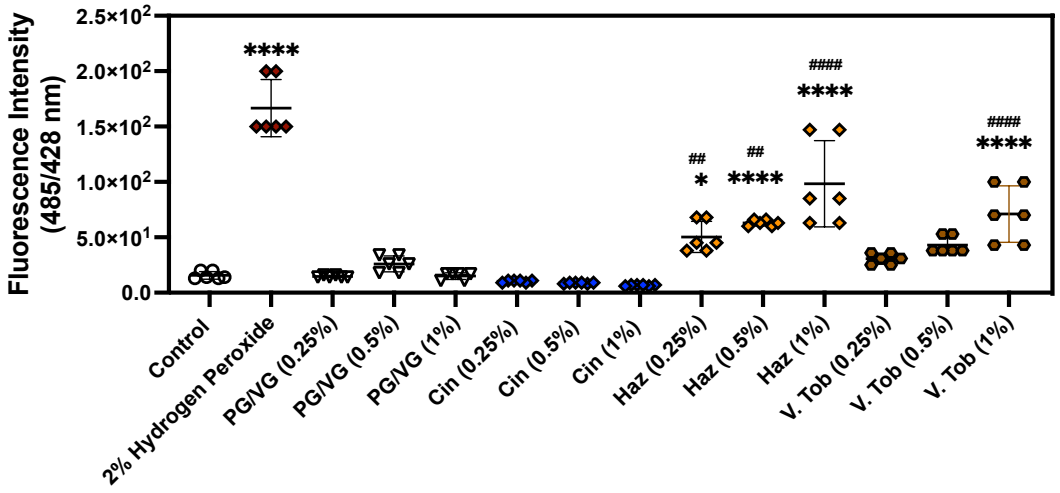


Figure 3. 9 The effect of 0.25%, 0.5% and 1% (v/v) of PG/VG, cinnamon, hazelnut, and vanilla tobacco on ROS production after 3h exposure. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Significance between concentrations can be seen in Table 3.7. Data are expressed as mean ± SD (n=6 per group). *p<0.05; ****p<0.0001 vs control. ##p<0.01; ####p<0.0001 vs PG/VG. Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Cin, cinnamon; Haz, hazelnut; V. Tob, vanilla tobacco.

Table 3. 7 Tukey's multiple comparisons test to characterize the dose-response relationship within the toxic hits on MMP and ROS generation.

Tukey's multiple comparisons tests	Dose-response	MMP	ROS
PG/VG (60/40)	0.25% vs. 0.5%	ns	ns
	0.25% vs. 1%	ns	ns
	0.5% vs. 1%	ns	ns
Cinnamon	0.25% vs. 0.5%	$p = 0.0061$	ns
	0.25% vs. 1%	$p < 0.0001$	ns
	0.5% vs. 1%	$p = 0.0004$	ns
Hazelnut	0.25% vs. 0.5%	$P = 0.0002$	ns
	0.25% vs. 1%	$p = 0.0003$	$p < 0.0001$
	0.5% vs. 1%	ns	$p = 0.0044$
Vanilla Tobacco	0.25% vs. 0.5%	ns	ns
	0.25% vs. 1%	ns	ns
	0.5% vs. 1%	ns	$p < 0.05$

Abbreviations: PG/VG, propylene glycol/vegetable glycerine; ns, non-significant

3.3.4 GC-MS/MS ANALYSIS OF VOCS FROM CINNAMON, HAZELNUT, AND VANILLA E-LIQUIDS

The untargeted analysis of volatile organic compounds from Cinnamon, Hazelnut, and Vanilla e-liquids showed more than 1000 components for each e-liquid sample, while the best hit components (match factor 80-100%) varied between 50 and 70 compounds. In **Table 4,** we only present the VOCs showing a good peak intensity and shape for the extracted ion peaks (EIC). The major VOCs emitted from cinnamon e-liquid are α-pinene (pine, earthy), *p*-cymene (citrus, woody), 3-carene (earthy, citrus, pine), cinnamaldehyde (cinnamon, spice), and ethyl maltol (sweet, fruity). Hazelnut e-liquid showed a high response for 2-methoxy-6-methylpyrazine (nutty type flavouring), ethyl octanoate (fruity, floral, apricot), benzenemethanol α-methylacetate, piperonal (cherry, vanilla), hexyl benzoate (woody, balsamic), and benzyl benzoate (floral, fruity). Vanilla tobacco e-liquid also showed components such as 2-methoxy-6-methylpyrazine (nutty), vinyl benzoate, 2-methoxyphenol (smoky), menthol (mint), estragole (liquorice, spice), and vanillin (vanilla). Most of the VOCs shown in **Table 4** are generally emitted from flavouring agents, used as flavour enhancers, or are plant metabolites. However, there are also some unexpected VOCs potentially detected that long-term exposures **Table 4** may have toxicological impacts on human health, such as respiratory and cardiovascular diseases. In cinnamon e-liquid, for example, we detected biphenyl (used as a food preservatives or heat transfer fluid), caryophyllene oxide and benzyl benzoate (used as insecticides). Hazelnut and Vanilla e-liquids contained other VOCs that can be harmful to human health when inhaled such as dioxane, 2-methoxyphenol, benzyl benzoate in Hazelnut flavour, and 2-methoxyphenol and toluene in Vanilla tobacco flavour.

Table 3. 8 List of volatile organic compounds detected in Cinnamon, Hazelnut, and Vanilla. Compounds are listed in order of elution.

Compound	Formula	Match Factor (%)	Retention time (min)
Cinnamon			
α-pinene	$C_{10}H_{16}$	87	5.88
p-Cymene	$C_{10}H_{14}$	83	6.52
3-Carene	$C_{10}H_{16}$	92	6.74
γ-terpinene	$C_{10}H_{16}$	87	6.92
Dichloro methylbenzene	$C_7H_6Cl_2$	88	7.19
Ethyl benzoate	$C_9H_{10}O_2$	90	7.46
Methyl salicylate	$C_8H_8O_3$	87	7.66
Ethyl maltol	$C_7H_8O_3$	92	7.69
Cinnamaldehyde	C_9H_8O	95	7.83
Copaene	$C_{15}H_{24}$	82	8.98
Biphenyl	$C_{12}H_{10}$	88	9.05
γ-Muurolene	$C_{15}H_{24}$	88	9.89
Isoledene	$C_{15}H_{24}$	89	10.04
Cis-calamenene	$C_{15}H_{22}$	90	10.10
Caryophyllene oxide	$C_{15}H_{24}O$	84	10.75
3-phenylpropanoic anhydride	$C_{17}H_{18}O_2$	85	10.79
Stilbene	$C_{14}H_{12}$	93	11.81
Benzyl benzoate	$C_{14}H_{12}O_2$	86	12.26
Hazelnut			
1,3-Dioxane	$C_6H_{12}O_2$	83	4.66
Ethyl butyrate	$C_6H_{12}O_2$	85	4.71
2,3-dimethyl Pyrazine	$C_6H_8N_2$	81	5.70
2-Methoxy-6-methylpyrazine	$C_6H_8N_2O$	89	6.23
2-Methoxyphenol	$C_7H_8O_2$	90	6.93
Ethyl octanoate	$C_{10}H_{20}O_2$	87	7.53
Benzenemethanol, α-methyl-acetate	$C_{10}H_{20}O_2$	85	7.56

Piperonal	$C_8H_6O_3$	96	8.68
β-Phenylethyl butyrate	$C_{12}H_{16}O_2$	89	9.33
Ethyl Vanillin	$C_9H_{10}O_3$	92	9.50
Allyl cinnamate	$C_{12}H_{12}O_2$	83	10.30
Hexyl Benzoate	$C_{13}H_{18}O_2$	90	10.47
Vanillin propylene glycol acetal	$C_{11}H_{14}O_4$	90	11.34
Benzyl Benzoate	$C_{14}H_{12}O_2$	93	12.25
Vanilla tobacco			
Ethyl butyrate	$C_6H_{12}O_2$	84	4.74
2,3-dimethyl Pyrazine	$C_6H_8N_2$	86	5.74
2-Methoxy-6-methyl pyrazine	$C_6H_4O_3$	83	6.23
p-Cymene	$C_{10}H_{14}$	88	6.52
Vinyl benzoate	$C_9H_8O_2$	93	6.82
2-Methoxyphenol	$C_7H_8O_2$	83	6.93
Maltol	$C_6H_6O_3$	81	7.16
Toluene	C_7H_8	84	7.18
Dichloro methylbenzene	$C_7H_6C_{l2}$	85	7.23
Menthol	$C_{10}H_{20}O$	90	7.55
Estragole	$C_{10}H_{12}O$	92	7.66
Anethole	$C_{10}H_{12}O$	86	8.02
Piperonal	$C_8H_6O_3$	94	8.69
Vanillin	$C_8H_8O_3$	91	9.11
Vanillin propylene glycol acetal	$C_{11}H_{14}O_4$	82	11.34

3.3.5 TRPA1 INHIBITOR STUDIES

Following the toxic effects of cinnamon e-liquid, we carried out TRPA1 inhibitor studies to unravel the mode of toxicological actions of cinnamon. Cinnamon flavour primarily comprises cinnamaldehyde (**Figure 3.10**), an agonist of the transient protein receptor ankyrin 1 (TRPA1) (Hoi *et al.*, 2020).

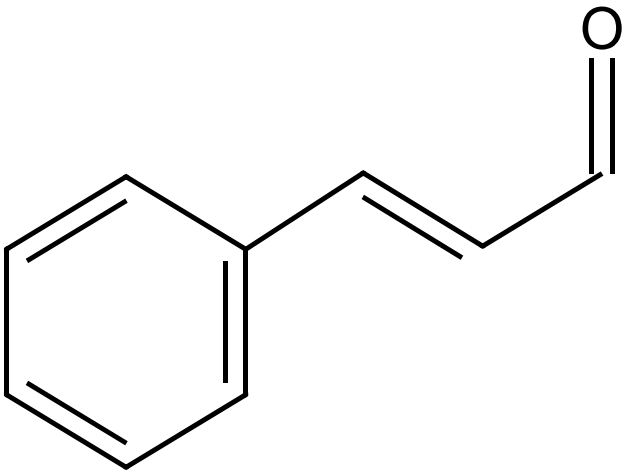


Figure 3. 10 The chemical structure of cinnamaldehyde

Therefore, we hypothesized that the toxic effects observed in cinnamon-flavoured e-liquid might be mediated via TRPA1. To test our hypothesis, we pharmacologically blocked TRPA1 with AP-18 (10 μM). Use of AP-18 (10 μM) was decided upon based upon preliminary data following 48 h exposure to a range of AP-18 concentrations (**Figure 3.11**).

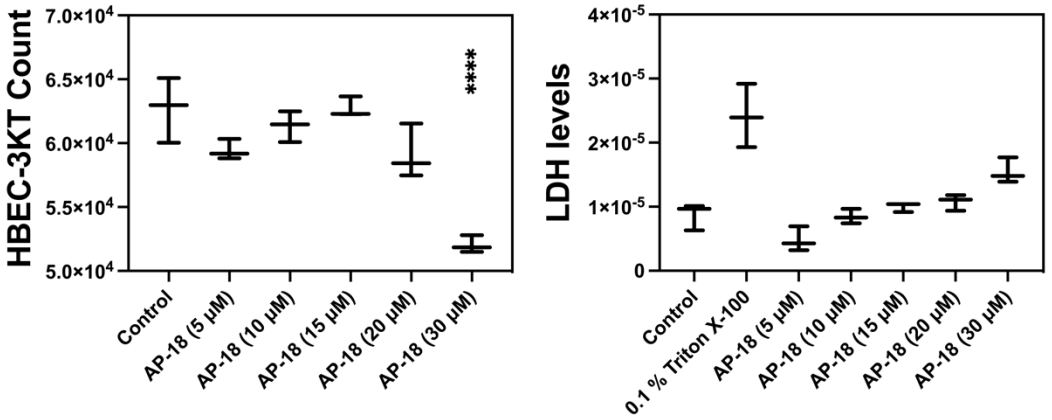


Figure 3. 11 Characterising the effects of AP-18 on HBEC-3KT cells for A) cell count and B) relative LDH release. AP-18 at (10 μM) was chosen as the concentration to have had similar cell count and LDH levels to incubator only control. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are presented as box and whiskers min-max (n=6 per group). ****p<0.0001 vs control.

In the 12h exposure, cinnamon alone significantly decreased cell count vs. media-only control only at 1% (v/v) (**Figure 3.12A)**. No effect on cell viability was seen (**Figure 3.12A B)**. LDH release was significantly increased at 0.5% and 1% (v/v) of cinnamon alone vs. media-only control (**Figure 3.12A C)**. In contrast, the combination of AP-18 (10 μM) and cinnamon (0.25%, 0.5%, and 1%) (v/v) decreased cell count significantly vs. media-only control. Further, the addition of AP-18 (10 μM) to 0.5% (v/v) and 1% significantly reduced cell count vs. cinnamon 0.5% and 1% (v/v) alone (**Figure 3.12A)**. AP-18 (10 μM) + 0.5% cinnamon and AP-18 (10 μM) + 1% cinnamon significantly decreased cell viability vs their respective cinnamon flavour only. (**Figure 3.12B**). There was no difference in the AP-18 (10 μM) + cinnamon vs. cinnamon alone treatments in the LDH assay (**Figure 3.12C**). In comparison to the 12h cinnamon effects on cell count (**Figure 3.12D**), cell viability (**Figure 3.12E**), and LDH release (**Figure 3.12F**), we observed that 24h exposure on cell count (**Figures 3.12A)**, cell viability (**Figures 3.12B)**, and LDH release (**Figures 3.12C)** were more pronounced. AP-18 (10 μM) did not have any effect on cell count when combined with cinnamon vs. cinnamon alone (**Figure 3.12D**); however, AP-18 (10 μM) + cinnamon 1% (v/v) significantly decreased cell viability (**Figure 3.12E**) and LDH release (**Figure 3.12F)** vs. cinnamon 1% (v/v) alone. In **Figure 3.15**, the effects of cinnamon alone (**Figure 3.15E**) and AP-18 (10 μM) + cinnamon (**Figure 3.15D**)

treatments altered cell morphology by rounding them off and detaching them from the plate when compared to the media-only control (**Figure 3.15A**), DMSO (**Figure 3.15B**) and AP-18 (10 μM) (**Figure 3.15C**)

Figure 5

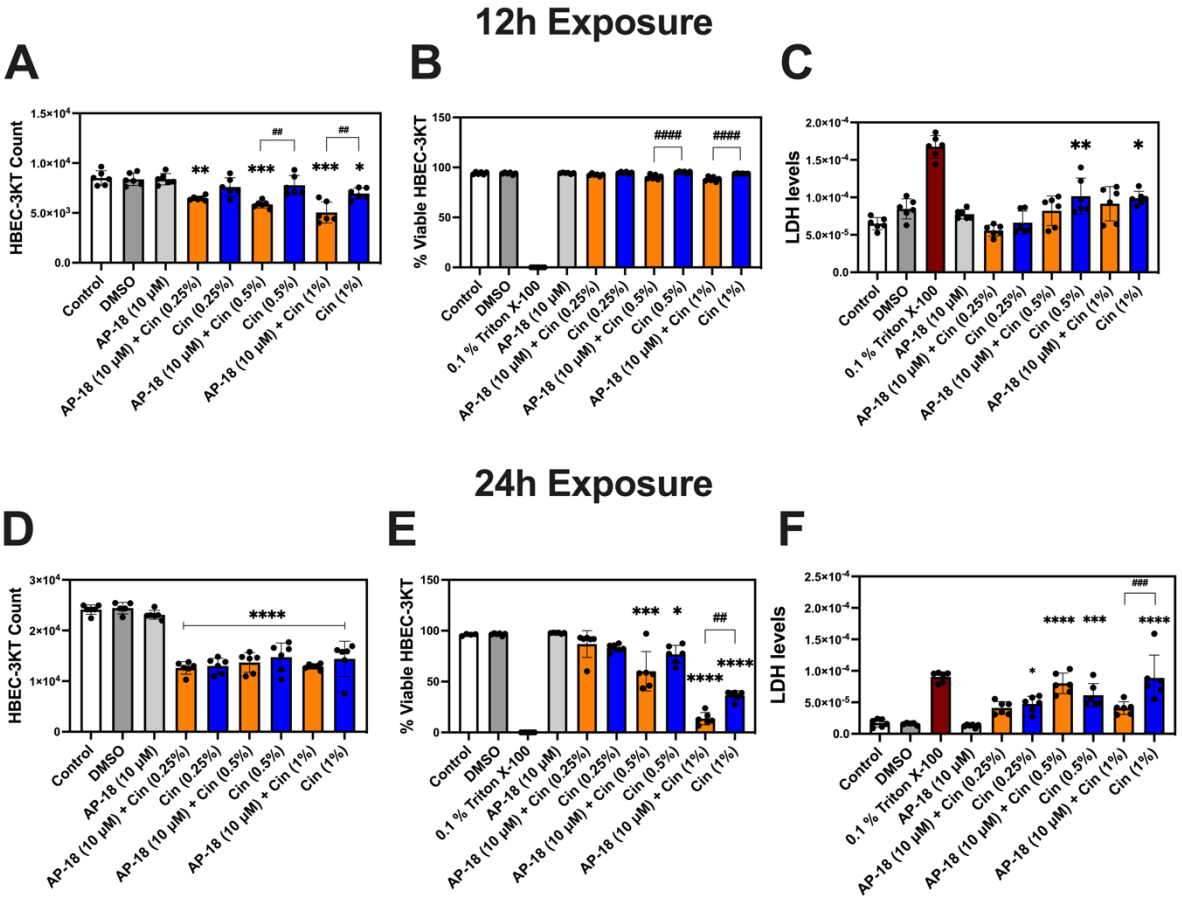

12h Exposure
A
B
C
HBEC-3KT Count
% Viable HBEC-3KT
LDH levels
24h Exposure
D
E
F
Control
DMSO
0.1 % Triton X-100
AP-18 (10 µM)
AP-18 (10 µM) + Cin (0.25%)
Cin (0.25%)
AP-18 (10 µM) + Cin (0.5%)
Cin (0.5%)
AP-18 (10 µM) + Cin (1%)
Cin (1%)

Figure 3. 12 Effects of AP-18 (10 µM) on cinnamon e-liquid (0.25%, 0.5%, and 1%) on cell count, cell viability and LDH release in HBEC-3KT following 12h and 24h exposure. Combined exposures to AP-18 and cinnamon were pre-exposed to AP-18 for 2 hours prior to cinnamon addition. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean ± SD (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs control; #p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001 vs AP-18 (10 µM) + cin. All groups were significantly different compared to the 0.1% Triton X-100 control apart from the relative LDH released in AP-18 (10 µM) + Cin (0.5%) and 1% cinnamon at 24h. All groups were significantly different compared to the 2% hydrogen peroxide. Abbreviations: DMSO, dimethyl sulfoxide; Cin, cinnamon e-liquid.

To understand whether these effects are primarily driven by cinnamaldehyde in cinnamon-flavoured e-liquid, we repeated these studies with cinnamaldehyde-alone (**Figure 3.13**). A similar pattern of effects was observed when cinnamaldehyde was used in place of cinnamon-flavoured e-liquid. (**Figure 3.13A, B & C**), albeit the effects were more remarkable in the cell viability assays vs. cinnamon e-liquid.

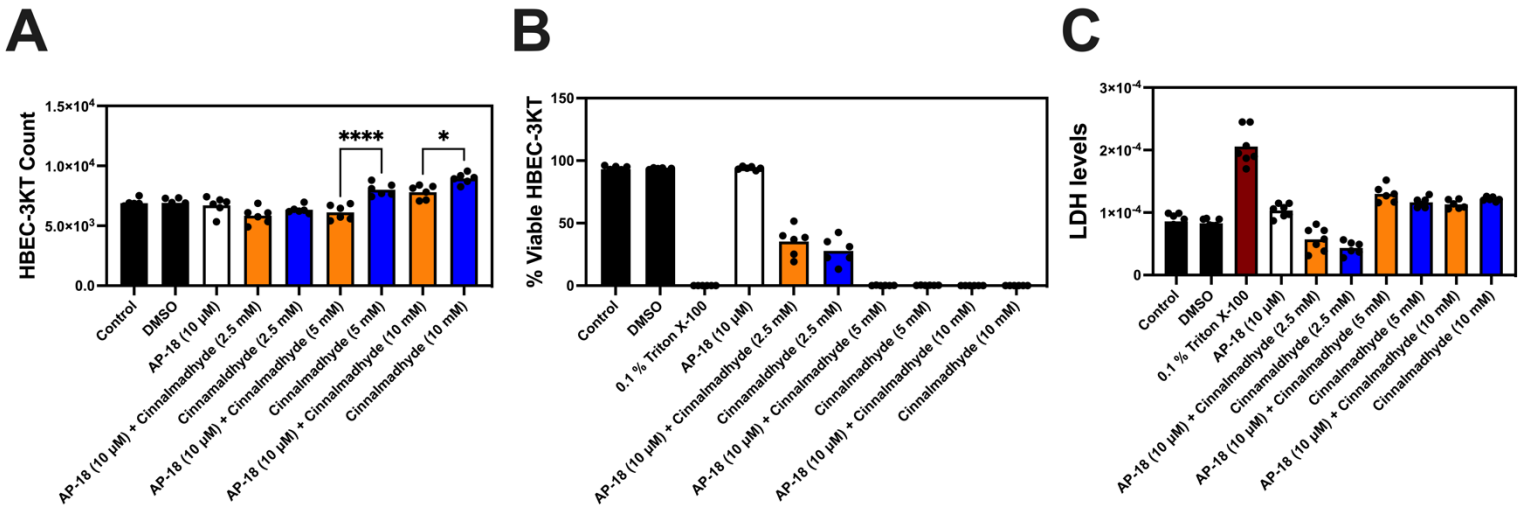


Figure 3. 13 Effects of AP-18 (10 µM) on cinnamaldehyde (0.25%, 0.5%, and 1%) on HBEC-3KT A) cell count, B) cell viability and C) LDH release in HBEC-3KT following 24h exposure. Combined exposures to AP-18 and cinnamaldehyde were pre-exposed to AP-18 for 2 hours prior to cinnamon addition. A one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean ± SD (n=6 per group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs AP-18 (10 µM) + cinnamaldehyde. All groups were significantly different compared to the 0.1% Triton X-100 control apart from the relative LDH released in AP-18 (10 µM) + Cin (0.5%) and 1% cinnamon at 24h. All groups were significantly different compared to the 2% hydrogen peroxide. Abbreviations: DMSO, dimethyl sulfoxide

3.3.5.1 QRT-PCR EXPRESSION OF TRPA1 POST CINNAMON FLAVOUR EXPOSURE

The $^{\Delta\Delta}$Ct method (Haimes and Kelley, 2014) was used in the calculations for the gene expression analysis. After gene analysis, we observed that our housekeeping genes were significantly affected by the AP-18 and Cinnamon treatments. These findings suggest that these genes may not be suitable as reference genes under the specific conditions of our study. We consulted the geNorm website (https://genorm.cmgg.be/), a widely accepted tool for assessing the stability of housekeeping genes. According to the geNorm analysis (**Figure 3.14**), gapdh consistently exhibited the most stable expression among the screened genes in our experimental context after repeating the experiments at least twice.

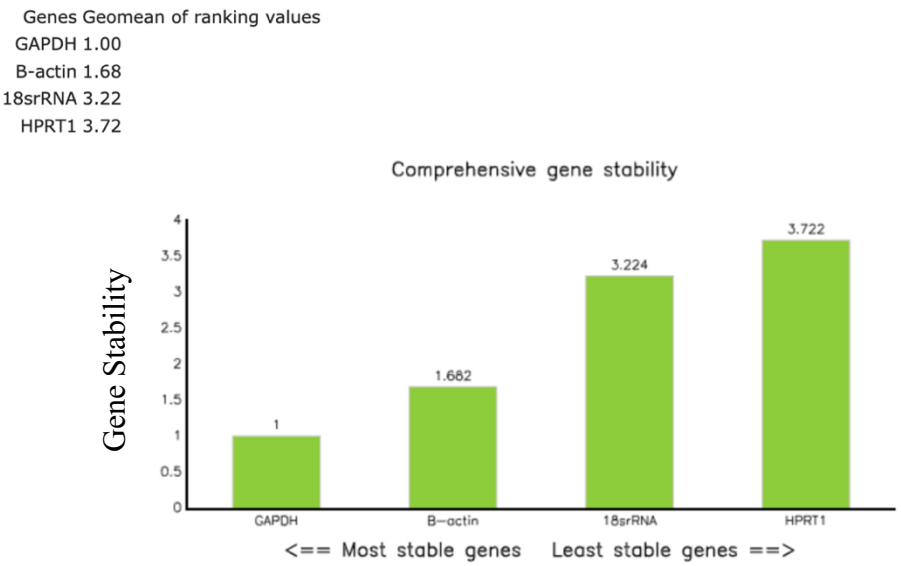


Figure 3. 14 Comprehensive gene stability of the housekeeping genes tested for the conditions of our study. Only GAPDH was not affected by AP-18 (10 µM) alone and AP-18 (10 µM) + cinnamon exposure. The other housekeeping genes, namely HPRT1, beta-actin, and 18s rRNA, was significantly affected by the AP-18 and Cinnamon treatments. These findings suggest that these genes may not be suitable as reference genes under the specific conditions of our study.

AP-18 inhibition studies failed to mitigate cinnamon's adverse effects. Nonetheless, exposure to 1% (v/v) cinnamon e-liquid significantly increased the expression of *trpA1* at the mRNA level compared to media-only control. The presence of AP-18 (10 µM) with the 1% (v/v) cinnamon e-liquid (**Figure 3.15F)** almost completely blocked this increase in *trpA1* expression. AP-18 (10 µM) alone and DMSO did not alter *trpA1* mRNA expression.

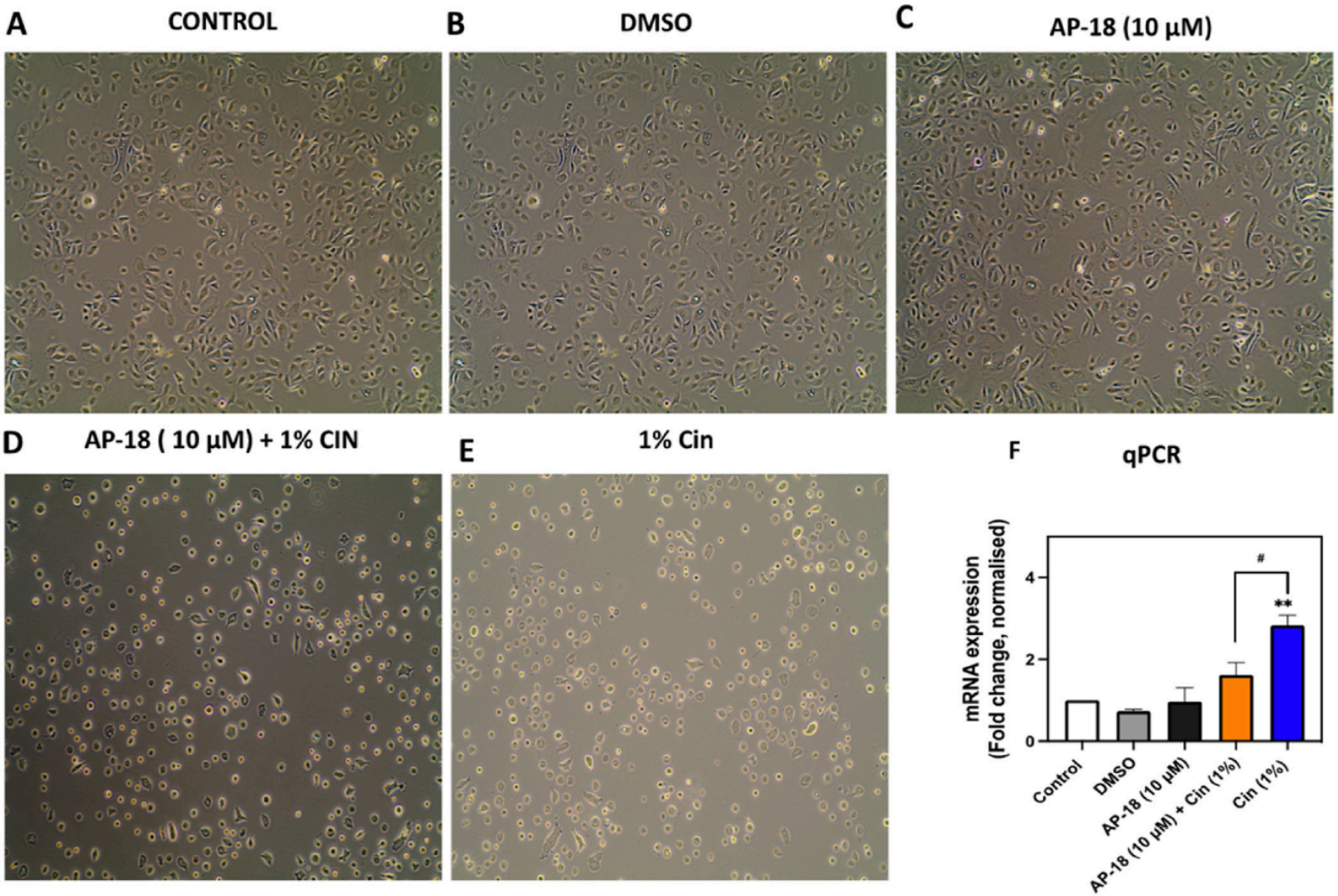

A
CONTROL
B
DMSO
C
AP-18 (10 µM)
D
AP-18 (10 µM) + 1% CIN
E
1% Cin
F
qPCR
mRNA expression
(Fold change, normalised)
0
2
4
#
**
Control
DMSO
AP-18 (10 µM)
AP-18 (10 µM) + Cin (1%)
Cin (1%)

Figure 3. 15 A – E) Images taken using a light microscope at 10x after 24h treatment with AP-18 (10 µM) and/or 1% cinnamon e-liquid. F) qPCR analysis to determine the mRNA levels of TRPA1 in the treatment groups A-E. For E), a one-way ANOVA with multiple comparisons, corrected using Tukey was used to determine statistical significance. Data are expressed as mean ± S.E.M (n=3 per group). **p<0.01; vs control; $^{\#}p$<0.05; vs AP-18 (10 µM) + 1% cinnamon. Abbreviations: DMSO, dimethyl sulfoxide; Cin, cinnamon.

3.4 DISCUSSION

This study was conducted in response to the discrepancies around the reported toxicity of flavoured e-liquids identified in our systematic review (Chapter 2, Effah *et al.*, 2022). E-liquid toxicity is affected by PG/VG ratios with nicotine as a co-contributor increasing toxicity in the absence of any flavour (Sassano *et al.*, 2018). Our data did not agree with the literature on the proliferative power of nicotine on human cells *in vitro*, which has been associated with the stimulation of DNA synthesis (Villablanca, 1998). However, because evidence of exposure of nicotine to human bronchial epithelial cells decreased their expression of the IL-22 gene, hampering the regenerative and repair power of IL-22 (Nguyen *et al.*, 2020a), these interactions between nicotine and e-liquid components meant that we decided to investigate nicotine-free e-liquids to investigate the effect of flavours more easily.

Our data indicate that the PG/VG component alone in e-liquids can reduce cell growth dose-dependently. There is contrasting evidence for the toxicity of PG/VG. Some studies showed that PG/VG-alone decreased metabolic activity in *in vitro* studies in primary HBECs (Sassano *et al.*, 2018) and human H441 airway epithelial cells (Woodall *et al.*, 2020). While others reported that PG/VG alone exerted no marked adverse effects on human pulmonary fibroblasts (Bahl *et al.*, 2012), NCI-H292 pulmonary cell line (Leigh *et al.*, 2016d), and 16HBE cells (Ween *et al.*, 2020a). These discrepancies may stem from methodological differences, including the choice of the cell line, dosimetry, and exposure models (for a more extensive review, see **Chapter 2**, **Table 2.1, 2.2, 2.3, & 2.4**). Our data confirm that PG/VG alone induces toxicity in HBEC 3KT bronchial epithelial cells in submerged culture. Although we found that PG/VG attenuated cell proliferation, it did not impact MMP or ROS generation. Interestingly, Woodall and colleagues (Woodall *et al.*, 2020) showed that:

3% PG/VG decreases glucose uptake and metabolism significantly in human bronchial epithelial cells (H441 airway epithelial cell) (**Figure 3.16)**. Therefore, PG/VG (1%) could reduce cell proliferation by a similar mechanism without affecting cell viability. Moreover, PG and VG can permeate membranes. In particular, high VG concentration was shown to modify membrane structure by direct interaction with the lipid components in the plasma membrane (Biondi *et al.*, 1991; Biondi and Disalvo, 1990; Madison *et al.*, 2019; Woodall *et al.*, 2020), potentially rendering cell membrane more permeable. This may partly account for the increased toxicity of PG/VG compared to PG alone. Based on the evidence provided here, we propose that repeated exposure to PG/VG alone can potentially contribute to adverse health effects in the airways, which may lead to pulmonary diseases in EC users. A better understanding of the dose of PG/VG depositing in airways following e-cigarette use, would aid any risk assessment.

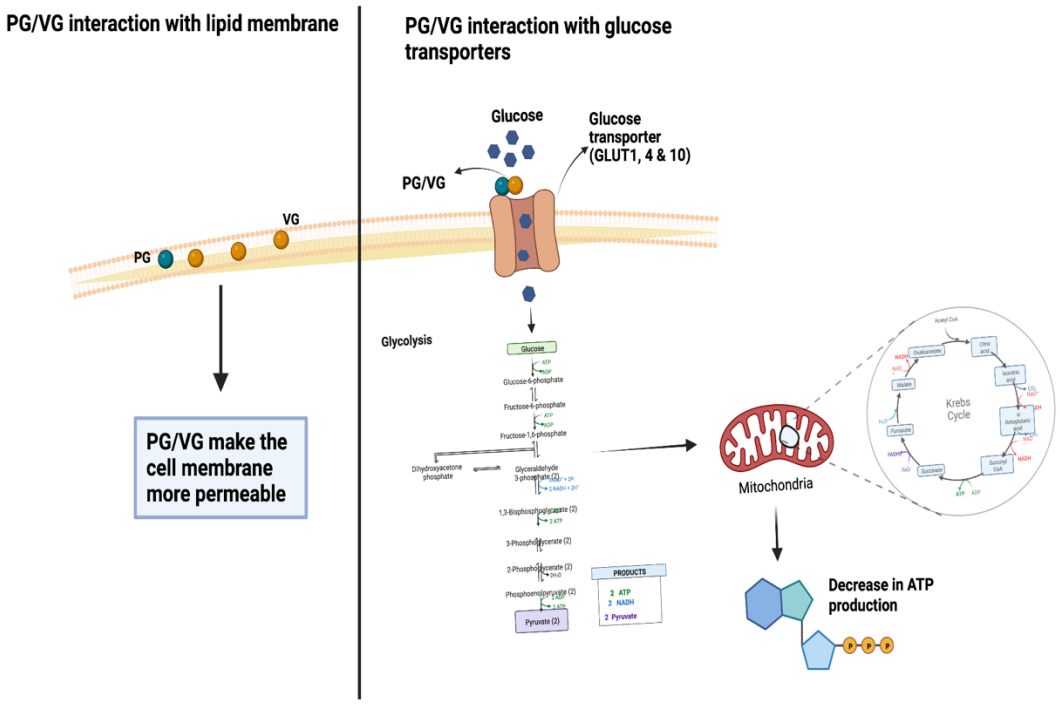


Figure 3. 16 Potential toxicity mechanisms of PG/VG in e-liquid. The figure illustrates two potential pathways by which the PG/VG components of e-liquid can induce cellular toxicity. The first pathway suggests a direct interaction of PG/VG with the cell's lipid bilayer, increasing its permeability and subsequently disrupting cellular homeostasis. The second pathway posits that PG/VG might interfere with glucose transporters (GLUT1, 4 & 10), potentially attenuating glucose uptake. This could result in reduced ATP production and consequent cytotoxicity

In addition to the adverse effects of PG/VG, it is crucial to characterise any potentially harmful flavours in e-liquids that might either potentiate PG/VG toxicity or individually induce toxicity. This is because there is a widespread use of flavours in EC and flavours in e-liquids may interact with PG/VG, and they may either enhance their toxic effects or create new toxic effects. Therefore, understanding the potential interactions between flavours and PG/VG can help in assessing the overall toxicity of e-liquids.

Consistent with the literature, cinnamon was revealed to be the most toxic among the 53 flavours screened (Behar *et al.*, 2014b; Clapp *et al.*, 2017b, 2019a; Effah *et al.*, 2023). To confirm and add to the pre-existing evidence, we show that cinnamon flavour in EC decreased cell count, increased LDH release and was the only flavour that elicited a dose dependent-decrease in cell viability. The pathways via which cinnamon might induce its toxicity are complex. Cinnamon at 0.5% (v/v) and 1% (v/v) significantly decreased MMP, which has been reported to decrease the production of ATP (Poburko *et al.*, 2011), vital to several cellular signalling pathways for homeostasis (Novak, 2003; Zorova *et al.*, 2018). Evidence suggests that ROS can interfere with cell membranes to cause lipid peroxidation, which, in turn, may lead to apoptosis and elevated LDH release (Molavian *et al.*, 2016). Surprisingly, however, in our studies cinnamon did not affect ROS generation. The chemical composition analysis revealed that cinnamon flavoured e-liquid contains VOCs such as dichloromethylbenzene, stilbene, γ-muurolene, isoledene, and insecticide chemicals like benzyl benzoate; however, cinnamaldehyde (match factor 95%) was the major flavour constituent followed by stilbene (match factor 93%). Although there is no study evaluating the effects of stilbene on pulmonary cells, there is some evidence to suggest the anti-proliferative and ROS scavenging effects of stilbene (Kimura and Okuda, 2010; Medrano-Padial *et al.*, 2019). Therefore, this may explain the absence of ROS production in cinnamon e-liquid-exposed HBEC-3KTS in this study. Further studies are warranted to test this hypothesis. Several studies show that cinnamaldehyde, is an agonist of the TRPA1 receptor (Camacho *et al.*, 2015; Hoi *et al.*, 2020; Tamura *et al.*, 2012) that is functionally expressed in HBEC-3KTs (Nguyen *et al.*, 2020b). The blockade of TRPA1 with a selective and reversible antagonist, AP-18, failed to mitigate the adverse effects of cinnamon in our study suggesting that other

chemical components in cinnamon e-liquid may be contributing to its toxicity. Quantitation of TRPA1 mRNA levels however, revealed that 1% cinnamon e-liquid significantly increased *trpA1* expression without any changes to external pH (a known activator of TRPA1). To the best of our knowledge, this is the first-time an e-liquid has been shown to induce TRPA1 transcript levels. This could be a direct induction of *trpA1* transcription by cinnamaldehyde or an induction via other signalling pathways activated by cellular defence mechanisms stimulated by cinnamaldehyde. Evidence suggests that TRPA1 expression and lung activation assume protective effects against noxious xenobiotics and play a pivotal role in the onset of pulmonary inflammation by recruiting immune cells (Viana, 2016). Thus, the elevation of *trpa1* may be a protective response to cinnamon exposure. It is unclear why the combination of AP-18 + cinnamon significantly decreased the percentage of viable HBEC-3KTs. However, we hypothesised that when AP-18 occupies TRPA1, the cinnamaldehyde in cinnamon e-liquid may now activate other membrane-bound receptors and so can exert its effects through TRPA1-independent mechanisms or may directly permeate the cell membrane to interact with necrotic or apoptotic pathways (**Figure 3.17**). These signalling pathways may not be fully understood, and the blockade of TRPA1 alone may not be sufficient to prevent the downstream effects of cinnamaldehyde. Moreover, because *trpa1* expression may not necessarily be due to cinnamon flavour toxicity. Further research is warranted to test this hypothesis.

There is evidence to support the toxicological impacts of diacetyl- and cinnamaldehyde-containing e-liquids following human inhalation **(Chapter 2;** Effah *et al.*, 2022) but whereas diacetyl is banned in the UK, cinnamaldehyde-containing e-liquids are still available on the British market. Interestingly, the market already appears to be moving away from cinnamon flavoured e-liquids/e-cigarettes with no

cinnamon-flavoured product registered with the Medicines and Healthcare products Regulatory Agency (MHRA), the competent authority in the UK for e-cigarettes (personal communication). We (UKHSA) have initiated conversations with The Office for Health Improvement and Disparities (OHID) on whether increased regulation is required. The COT in the UK reviewed data published on four main chemicals in e-liquids including cinnamaldehyde and concluded that although they may activate receptors in the airways and cause irritation and hypersensitivity, it was not mutagenic (COT, 2020).

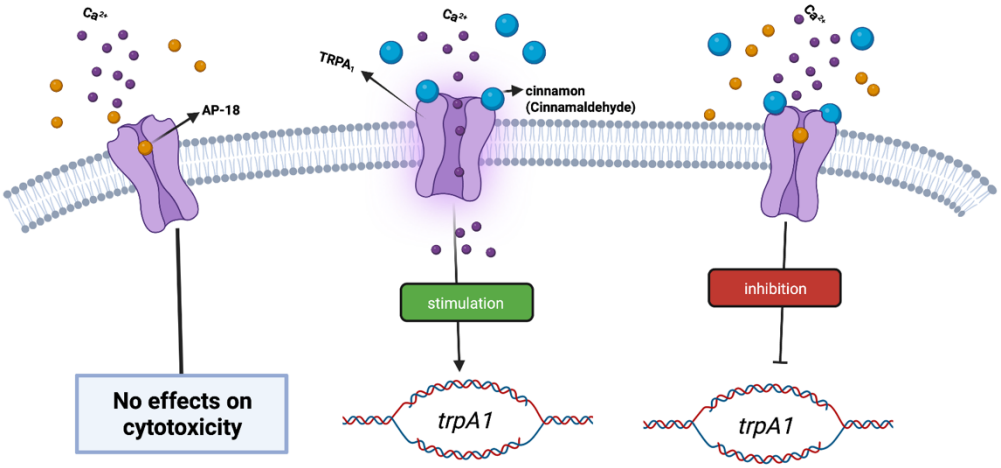


Figure 3. 17 The The potential pharmacological actions of AP-18 (10 mM) in combination with cinnamon (cinnamaldehyde). TRPA1 is activated by cinnamon, which, in turn increases the influx of calcium ions into the cytosol. Increased levels of intracellular calcium may trigger an increase in trpA1 transcript levels. Nonetheless, the blockade of TRPA1 by AP-18 inhibits the expression of TRPA1 transcript levels.

While the toxic effects of cinnamon have been reported in several studies, to our knowledge, the toxic potential of hazelnut has not. Most of the main chemical VOC components of hazelnut-flavoured e-liquid were flavouring agents such as piperonal and ethyl vanillin and condensation products such as vanillin propylene glycol acetal. The VOCs also contained toxic compounds i.e., 1, 3-dioxane and chemicals used in insecticides such as benzyl benzoate. Although their toxicity has not been tested on HBECs, a 90-day exposure to 25 mg kg^{-1} body weight and 100 mg kg^{-1} body weight benzyl benzoate induced increased blood percentage of monocytes, neutrophils and lymphocytes in rats (Kılıç Süloğlu *et al.*, 2022). Also, the International Agency for Research on Cancer (IARC) has determined that 1,4-dioxane is possibly carcinogenic

to humans when inhaled in large concentrations (ATSDR, 2015). Hazelnut e-liquid induced a significant dose-dependent decrease in cell count and decreased cell proliferation. Moreover, it was the only flavour that reduced MMP and increased ROS generation, even at the lowest concentrations tested. Evidence suggests that impaired mitochondrial functions can lead to excessive generation of ROS, which, in turn, can induce more ROS generation in the so-called ROS-induced ROS-release process (Zorov *et al.*, 2014). Therefore, the adverse effects of hazelnut e-liquid may be via an effect on mitochondrial function. However, because cytosolic oxygen reduction by NADPH oxidases (NOX), most especially NOX2, is the primary source of ROS production and not oxidative phosphorylation by the mitochondria (Vermot *et al.*, 2021; Zhang and Wong, 2021), further work is needed to shed light on the main chemical components in hazelnut and what modes of action mediate these toxicological effects.

Vanilla tobacco flavoured e-liquid decreased cell count dose-dependently and increased LDH release but showed no effect on % of viable cells. Further mechanistic investigation revealed that even at the lowest concentrations, vanilla tobacco e-liquid induced a very marked decrease in cell proliferation in a time-dependent manner, a significant decrease in MMP and a dose dependent increase in ROS generation. As mentioned earlier, alterations in molecular systems paramount to normal cellular physiology may underpin the adverse effects observed in the HBEC-3KTs as a consequence of e-liquid exposure. Regardless that no effect was observed with vanilla tobacco on % of viable cells in this study; seeing the adverse effects on ROS generation and MMP, we assume that prolonged exposure to this flavoured-e-liquid might induce adverse effects such as apoptosis or necrosis. Vanilla tobacco contained vanillin, ethyl vanillin, ethyl butyrate, and other flavouring agents. Interestingly, it

contained estragole, a suspected carcinogen and genotoxic chemical according to the European Union Committee on Herbal Medicinal Products (European Medicines Agency, 2023). Although there are several previous *in vitro* and *in vivo* studies reporting toxicity of vanilla and tobacco-flavoured e-liquids, there is limited evidence on the toxicity of vanilla tobacco-flavoured e-liquids (Effah *et al.*, 2022). Nonetheless, our data agree with the one published study on vanilla tobacco (Rowell *et al.*, 2017). However, that study did not undertake any investigation of potential modes of action (Rowell et al 2017). More studies are warranted to delineate the main chemical components of vanilla tobacco and the pathways they interact with to induce the observed adverse effects in pulmonary cells. It is possible that the flavouring components of vanilla and tobacco flavours in combination may act synergistically or additively by dysregulating calcium homeostasis via the TRPs. Vanillin is an agonist for transient protein receptor vanilloid 1 (TRPV1) (Wu *et al.*, 2017). Moreover, TRPV1 has been shown in another bronchial epithelial cell line (Beas-2B) to mediate several cytotoxic effects such as cell death, proinflammatory cytokine production, and increased calcium influx via a mechanism that involves translocation of the existing receptor from the endoplasmic reticulum to the plasma membrane (Johansen *et al.*, 2006). Our data indicate that vanilla tobacco flavoured e-liquid could increase ROS levels and impair ATP production, which, in turn, could lead to or exacerbate pulmonary inflammatory disorders like COPD, cystic fibrosis, and asthma.

Although the majority of flavoured e-liquids did not demonstrate convincing evidence of flavour-dependent toxicity, further research is needed to inform public health and regulatory bodies on those flavours that do.

3.4.1 STUDY LIMITATIONS

There are limitations to this study. Firstly, testing a representative sample of all the available e-liquids was not practicable. However, by selecting flavours from each category of the flavour wheel (Krüsemann *et al.*, 2019), we have attempted to make our choices less random and as such is one of the most extensive studies of this kind to date. Secondly, choosing relevant doses of e-liquids in cell culture studies is complicated, especially when exposure by vaping depends on frequency and duration of use. We have attempted to justify the doses used using some simple measures. At these doses, we identified e-liquids (5.7%) with adverse effects, but this may increase with repeated exposures, unfortunately this was beyond the remit of this study. Finally, for practical reasons, we decided to use the e-liquid directly and not generate a condensate from vaping the liquids. The effect of heating e-liquids during vaping may release additional toxicants such as VOCs, breakdown products, and condensation products that could increase adversity. We also used nicotine-free e-liquids to compare the effects of the different flavourings more readily without the added potential impacts of co-exposure with nicotine. Another shortfall is the need for a tobacco cigarette control to establish the relative toxicity of these e-liquids. Finally recent media coverage has included the widespread use of illegal e-cigarette devices, especially by school-aged vapers (BBC, 2023). Our study focused only on e-liquids available from reputable manufacturers and that are registered through MHRA. The latter was because we initially had considered including nicotine-containing e-liquids for comparison and so we selected only products available in both nicotine-free and nicotine containing formulations However, given the number of nicotine concentrations available, and use of different nicotine salts we decided that this would make this initial study overly complicated. Most non-nicotine containing e-cigarette

products fall outside of the scope of the EU Tobacco Products Directive (TPD, https://health.ec.europa.eu/tobacco/product-regulation/implementing-tobacco-products-directive-directive-201440eu/tobacco-products-directive-2021-application-report_en) and its UK replacement Tobacco and Related Products Regulations 2016. Instead, they fall under general product safety and do not need to submit information on their contents (liquid and aerosol) to MHRA. It is possible that more products would have demonstrated toxicity if we had investigated products not regulated under TPD.

Our purpose was to screen flavoured e-liquids to identify those with the highest toxicity. However, HBEC-3KTs are basal cells and do not fully recapitulate the microenvironment of *in vivo* lung epithelial structures and although the cytotoxicity of e-liquids in submerged models predicts aerosol cytotoxicity 74% of the time (Behar *et al.*, 2018b; Sassano *et al.*, 2018) there is the potential that we have miss-characterised some e-liquids in our set. Subsequently, we aim to progress those e-liquids demonstrating adverse effects in this study into aerosol exposures using an air-liquid interface (ALI) system, a much more robust model recapitulating the lung microenvironment *in vivo.* The ALI will also be amenable to direct e-cigarette aerosol exposure and for comparison against tobacco smoke.

3.4.2 CONCLUSION

We hypothesized that nicotine exerts additive proliferative effects on e-liquid-induced toxicity on human bronchial epithelial cells. Indeed, high levels of nicotine significantly increased HBEC-3KT proliferation and caused cell death. Secondly, we hypothesized that some e-liquids, but not all, elicit dose-dependent adverse effects on HBEC-3KT cells. Most e-liquids demonstrated toxicity related to PG/VG content.

PG/VG vehicle alone exerts adverse effects in HBEC-3KT cells. Additional dose-dependent toxicity was only seen in 3 flavours: cinnamon, hazelnut, and vanilla tobacco. As such, flavours contributed little to e-liquid toxicity. Our last hypothesis was that cinnamaldehyde in cinnamon (the most toxic among the investigated e-liquids) flavour, induced its toxicity via TRPA1. Our data suggested that cinnamon significantly increased TRPA1 expression, which AP-18 almost completely blocked. In the high-throughput experiments, we noticed condensation on the plate lid over the wells exposed to menthol. This led us to hypothesize that menthol or menthone present in the e-liquid might be evaporating, causing an underestimation of its toxic effects. To explore this further, we examined menthol using the CULTEX inhalation system, as detailed in Chapter 6.

3.4.3 FUTURE STUDIES & REGULATIONS OF FLAVOURS

Flavours in e-liquids contain pharmacologically active compounds such as cinnamaldehyde, ethyl vanillin, and vanillin. that are capable of eliciting effects in pulmonary cells. Therefore, future studies are needed to quantitatively assess the emissions from flavoured e-liquids by measuring EC components in both the gas and aerosol phases using high-resolution mass spectrometry such as the GC-quadrupole-Time of Flight-MS or the GC-quadrupole-Orbitrap-high resolution MS. Flavours in e-liquids are believed to attract long-time tobacco cigarette smokers to switch to EC use and tobacco naïve non-smokers to take up e-cigarettes. Therefore, identifying harmful flavours will help inform public health authorities in the UK and elsewhere about which EC flavours may need monitoring and/or restricting.

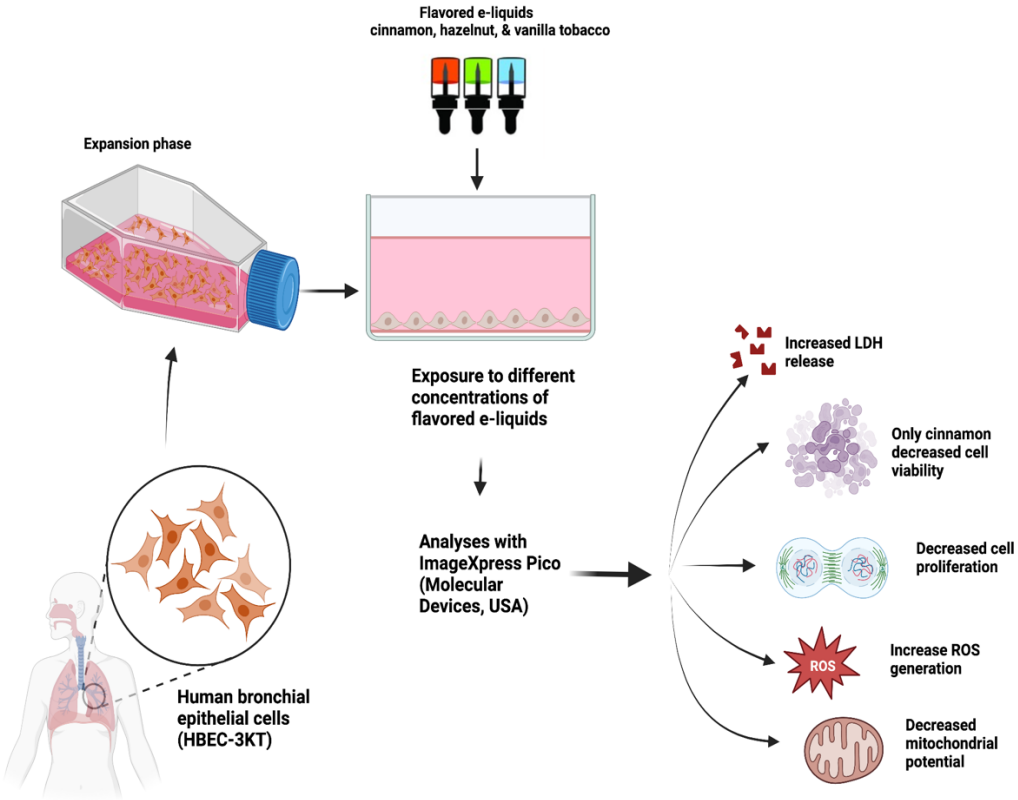


Figure 3. 18 Summary of the pathways via which cinnamon, hazelnut and vanilla tobacco induced toxicity.

CHAPTER 4

TOXICOLOGICAL ASSESSMENT OF E-CIGARETTE FLAVOURED E-LIQUIDS AEROSOLS USING CALU-3 CELLS: A 3D LUNG MODEL APPROACH

4.1 INTRODUCTION

Evidence emerging on the potential toxicity of EC use in human volunteer studies suggests that EC use is associated with the presence of harmful substances in urine samples, including but not limited to NNAL, acrolein, and acrylamide (see **Chapter 1**), albeit in lower concentrations compared to conventional cigarette use. Whether seeking to encourage or prohibit EC use, it is morally irresponsible to wait decades for possible chronic toxicities from flavourings in ECs in human to become evident. Therefore, several studies have reported potential health risks from exposure to flavoured e-liquids in *in vivo* models (**Chapter 2**, **Table 2.5**) to help predict pulmonary toxicity in humans. Nonetheless, although animal studies have the advantage of mimicking the human respiratory environment more closely and evaluating systematic effects, they have several limitations. These include the ethical use of animals (3Rs principles: replacement, reduction, refinement), the costs and time associated with conducting the studies, and the potential for inter-species-specific differences (Lewis, 2019; MacArthur Clark, 2018; Vitale and Ricceri, 2022). For example, while basal cells are found throughout the human airways, they are only found within the trachea of mice (Boers *et al.*, 1998; Evans *et al.*, 2001). Inversely, clara cells are present throughout the murine airways but are predominantly confined to the most distal human bronchioles (Rawlins *et al.*, 2009).

One way to implement the 3Rs principle is by using alternative methods to animal use such as *in vitro* cell culture models, which can be used to study the effects of substances on cells in a controlled laboratory setting. 2D submerged cell cultures, which consist of cells grown in a monolayer, have been widely used in pharmaco-toxicology research to study the effects of substances on cellular functions. These models have several advantages, including easily manipulating the cells and using

well-established cell lines. However, 2D submerged cultures do not accurately recapitulate the *in vivo* microenvironment of the respiratory epithelium as they are basal cells and do not reflect the structural and functional properties of the airways. Additionally, the cells in these cultures can undergo gene expression and cellular behaviour changes that differ from cells in the body (da Silva da Costa *et al.*, 2021; Kapałczyńska *et al.*, 2016). Moreover, it is challenging to expose submerged cells meaningfully to gas, aerosol, or particulate exposures especially where these are immiscible or poorly dissolved in media, rendering the model less predictive for *in vivo* situations (Barrila *et al.*, 2010).

4.1.1 AIR-LIQUID INTERFACE

3D lung models, which include, but are not limited to lung-on-a-chip (Bennet *l.*, 2021; Shrestha *et al.*, 2020) and air-liquid-interface (ALI) (Agraval *et al.*, 2022; Guénette *et al.*, 2022) models, have been developed to mimic the human respiratory epithelium more accurately and to better reflect its structural and functional properties. In these models, cells are grown on a porous membrane that enables the apical surface to be exposed to air while the cells are maintained through basal exposure to liquid cell culture media (**Figure 4.2**). This experimental condition delivers more oxygen and shifts cellular respiration into a more aerobic state. Consequently, oxidative cellular metabolism is accelerated which enables the differentiation of basal cells to functional epithelium including formation of cilia and mucus production. Pulmonary ALI models have been used to study a variety of substances, including therapeutic agents (Lee *et al.*, 2021), environmental toxicants and respiratory viruses (Upadhyay and Berg, 2018). Primary, immortalised normal, and cancerous basal cell lines from various upper and lower respiratory tract locations can be used in lung ALI models, each with

its own advantages and limitations (discussed below). Basal cells isolated from the bronchi grown at ALI have the pluripotent capabilities to differentiate into several types of airway cells depending on the conditioning media (Delgado *et al.*, 2011). This suggests that an appropriate validation and optimisation of the ALI method could potentially serve as a robust model to characterise the effects of substances on a wide range of airway cells and their interactions under the same conditions.

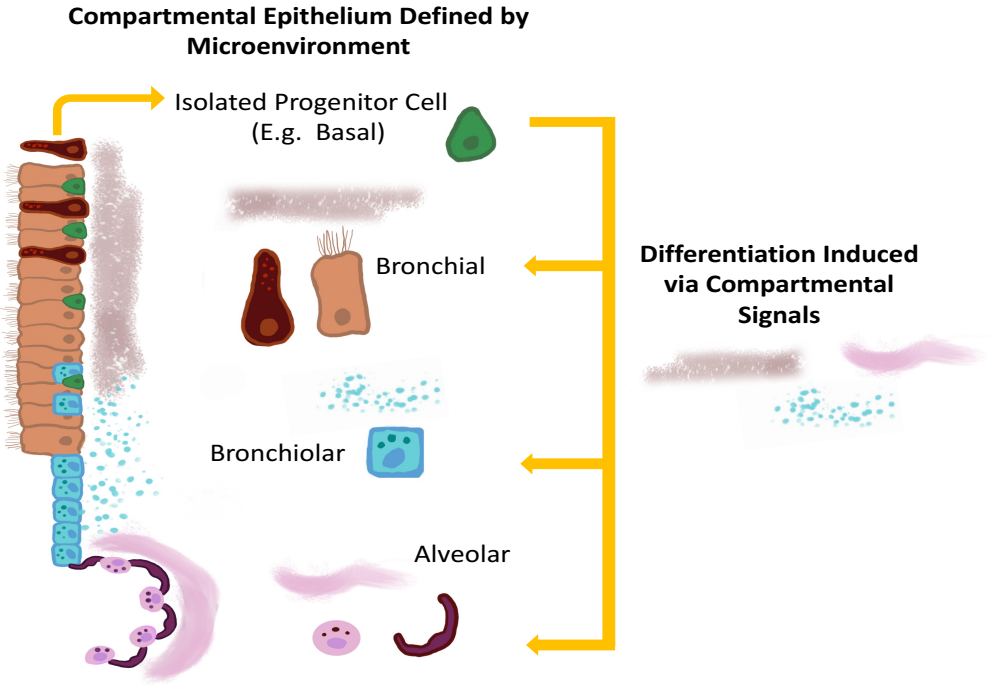


Figure 4. 1 Basal cells isolated from the human bronchi can differentiate into bronchial, bronchiolar, and alveolar cells depending in the conditional media and the culture conditions.

The Calu-3 cell line, derived from a human bronchial adenocarcinoma submucosal glands and akin to respiratory epithelia, is a widely used *in vitro* model in academic and industry research for examining the pharmaco-toxicology of inhaled drugs (Ong *et al.*, 2013). This cell line has been extensively employed to study the transport and

metabolism of numerous therapeutic compounds, especially those for cystic fibrosis, due to its relatively high expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Haws *et al.*, 1994) and environmental inhalation toxicology studies (Foster *et al.*, 2000). A key advantage of using Calu-3 cells, as demonstrated in this study, is their ability to replicate the biological behavior of the human respiratory tract upon exposure to airborne chemicals. Thus, Calu-3 cells are apt for screening respiratory irritation and toxicity (Jeong *et al.*, 2019; Ji *et al.*, 2016; Pasman *et al.*, 2021; Sakagami, 2020) because they can form tight junctions with robust barrier integrity, essential for creating a relevant lung model.

Here, we used Calu-3 cells grown at air-liquid interface (ALI) to assess the toxicological effects of the three flavours we identified in the previous high throughput submerged culture study (**Chapter 3**). Although several lung cell culture models are available, none are considered perfect models for studying respiratory tract effects. There are no studies where the sensitivity to different flavoured e-liquids across primary, normal immortalized, and cancerous cells are simultaneously probed; nonetheless, in the systematic review conducted in **Chapter 2,** primary cells and normal immortalized cells are seemingly more susceptible to the adverse effects of flavoured e-liquid exposures than cancerous cell lines. Ji *et al.*, (2022) showed that Calu-3 cells grown at ALI for 8 days already reveal a pseudostratified structure and express tight junction proteins ZO-1 and adherent junction proteins E-cadherins. Moreover, they used this model to evaluate the toxicity of two flavour extracts (flavour-type not specified) and 6 extracts of tobacco. They concluded that the flavour extract decreased cellular integrity by reducing transepithelial electrical resistance (TEER) measurements as well as a decrease in ZO-1 and E-cadherins. Several studies

have validated Calu-3 cells grown at ALI for 21 days as an appropriate 3D lung model for pharmaco-toxicological studies by measuring the TEER, the expression of tight junction proteins, and mucus production as markers for differentiation. Nonetheless, none of the studies have investigated them beyond this timeframe or investigated the time course of changes that occur within the genes that encode these markers over the 4 weeks the cells are grown at ALI. This is because there is a consensus that the longer pulmonary cells are left at ALI, the more the basal cells differentiate into more specialised cells (Bukowy-Bieryłło, 2021). Nonetheless, the high susceptibility of ALI cultures to infections means that they are mostly only taken to 8, 14 or a maximum of 21 days at ALI. Therefore, this study had two main aims. The first aim was to evaluate Calu-3 cells beyond the typical 21 days at ALI to understand whether pronounced morphological and genetic changes occur beyond 21 days by 1) measuring TEER, 2) measuring the expression of genetic markers of differentiation for goblet cell (*muc5ac* and *muc5b*), the expression of ciliated cell markers (*foxj1*), and tight junction (*tjp1*), and basal markers (*trp63, krt5* and *itga6*) (Hewitt and Lloyd, 2021; Rock *et al.*, 2009) using qRT-PCR and how these change over 28 days at ALI, and 3) by measuring immunofluorescence for mucin (MUC5AC) production with digital confocal microscopy after 21 days of ALI.

In **Chapter 3**, we adopted a high-throughput experimental approach to screening cytotoxicity in a wide range of flavoured, nicotine-free e-liquids (n=53) (Effah *et al.*, 2023). In that study, we identified that out of 53 nicotine-free e-liquids, only 3 (i.e. cinnamon, hazelnut and vanilla tobacco) revealed dose-dependent toxicity independent of the PG/VG component in human bronchial epithelial cells (HBEC-3KT) in submerged cultures. In this chapter, we exposed Calu-3 cells grown at ALI to

both flavoured e-liquid and aerosols as recommended in **Chapter 2**, **section 4** to characterize whether their e-liquid toxicity correlates with the aerosol toxicity following protocols from The Cooperation Center for Scientific Research Relative to Tobacco (CORESTA). This is because heating e-liquids during vaping may release additional toxicants such as volatile organic compounds, breakdown products, and condensation products that could increase toxicity (**Chapter 1**).

The CORESTA protocol for EC studies includes various recommended methods and standards for the generation, collection, and analysis of EC aerosols. One of the key protocols is the CORESTA Recommended Method No. 81 (CRM 81), which specifically addresses EC aerosol generation and collection requirements for analytical testing purposes (CORESTA, 2015). The CRM 81 method provides standardized conditions and procedures for evaluating e-cigarette aerosols, ensuring better comparability and reproducibility of research findings. This protocol is essential for conducting reliable and accurate studies on e-cigarette aerosols and their potential effects. In addition to CRM 81, CORESTA has developed other resources and guides for e-cigarette research. For example, Guide No. 18 provides technical guidance for the sample handling of e-cigarettes and e-vapour products before analysis, including guidance for sample aerosol collection and storage (CORESTA, 2021). These resources contribute to the advancement of scientific knowledge and understanding in the field of e-cigarette research.

In addition, we include 3R4F reference cigarette as a positive control. 3R4F cigarettes are commonly used as a reference for conventional cigarettes in research (Kärkelä *et al.*, 2022). By comparing the effects of EC aerosols to those of 3R4F cigarette smoke

extract, we can better understand the potential harm reduction or harm equivalence of EC compared to traditional cigarettes (Azzopardi *et al.*, 2016). Also, using a standardised reference product like 3R4F cigarettes allows for better reproducibility and comparisons between studies (Simonavicius *et al.*, 2019). This is particularly important in the rapidly evolving field of EC, where various devices and e-liquid formulations are being introduced. Electronic cigarette aerosols have been shown to induce significantly less cytotoxicity than 3R4F cigarette smoke (Azzopardi *et al.*, 2016). Therefore, by including 3R4F cigarette smoke controls, we can quantitatively assess the differences in toxicity and potentially identify specific components or mechanisms that contribute to the observed effects. Lastly, comparing the effects of EC to those of 3R4F cigarettes can provide valuable information for regulatory agencies when evaluating the potential risks and benefits of these products (Uguna and Snape, 2022).

4.1.2 AIMS AND HYPOTHESIS

To use a validated 3D Calu-3 lung model approach to screen the aerosols of the three toxic flavours identified in **Chapter 3**.

4.1.3 HYPOTHESES:

- The genetic markers of basal cells (*trp63, krt5* and *itga6*) will be downregulated in a time-dependent fashion in 3D Calu-3 cells grown at ALI.
- The genetic markers for mucus-producing cells (*muc5ac & muc5b*), ciliated cells (foxj1) and tight-junction proteins (*tjp1*) will be upregulated in a time-dependent fashion in 3D Calu-3 cells grown at ALI.
- Cinnamon, hazelnut, and vanilla tobacco e-liquid (1% v/v) and aerosols will elicit toxic effects in both 2D and 3D Calu-3 models.

- E-liquid effects will be lower compared to those of 3R4F cigarette smoke.

4.1.4 OBJECTIVES:

36 To culture Calu-3 cells at ALI for 28 days to characterise how the changes that occur in genetic markers of differentiation correlate with the development of a pseudostratified 3D structure.

- To expose differentiated Calu-3 cells grown at ALI to e-cigarette aerosol generated from cinnamon, hazelnut and vanilla tobacco flavoured e-liquids following CORESTA protocols.
- Compare the toxicity of e-cigarette aerosols to 3R4F cigarette smoke using LDH levels and TEER measurements.
- To investigate the relationship between e-cigarette aerosol particle size distribution and toxicity.

4.2 METHODS

4.2.1 CELL CULTURE

The human bronchial epithelial Calu cell line (ATCC) was cultured in advanced Dulbecco's modified eagle medium (DMEM) with 10% foetal bovine serum (FBS, GIBCO), 1% non-essential amino acids (NEAA, GIBCO), 1% antibiotic-antimycotic (GIBCO) and 10 mM Hepes. Cells were cultured at 37 ^{o}C with 5% CO_2. 0.25% Trypsin EDTA (GIBCO) was added during passaging, and cells incubated at 37^{o}C with 5% CO_2 for 3-5 minutes. The agitation of the flask detached the cells, which could be observed using the light microscope. Phosphate buffered saline (PBS) (GIBCO) containing 5% FBS (GIBCO) was added to neutralise the Trypsin EDTA before the cell suspension was centrifuged at 1000 rpm for 5 minutes. After discarding the supernatant, the cell pellet was resuspended in the appropriate growth medium. A

small sample was added to trypan blue (Sigma-Aldrich) in a 1:1 ratio and placed into a haemocytometer for a viable cell count. Cells were seeded at 2 x 10^6 per T175 flask (ThermoFischer Scientific, US) for the next passage.

4.2.1.1 CALU-3 ALI CULTURE

6 well PET transwells plates were used for ALI exposures (ThermoFischer Scientific, US). Calu-3 cells were seeded at passage 10-15 into PET transwells with 0.4 µm pores and 24 mm thickness (Corning) at 1.2x10^5 cells/well. The cell medium used to culture cells during expansion was as previously described (section 4.2.1). Confluence was observed using a light microscope after 72h. All apical media was removed from cells once confluence was reached. Once at an ALI, the growth medium in the basal compartment was replaced every 2-3 days whilst the transwells were incubated at 37°C with 5% CO_2.

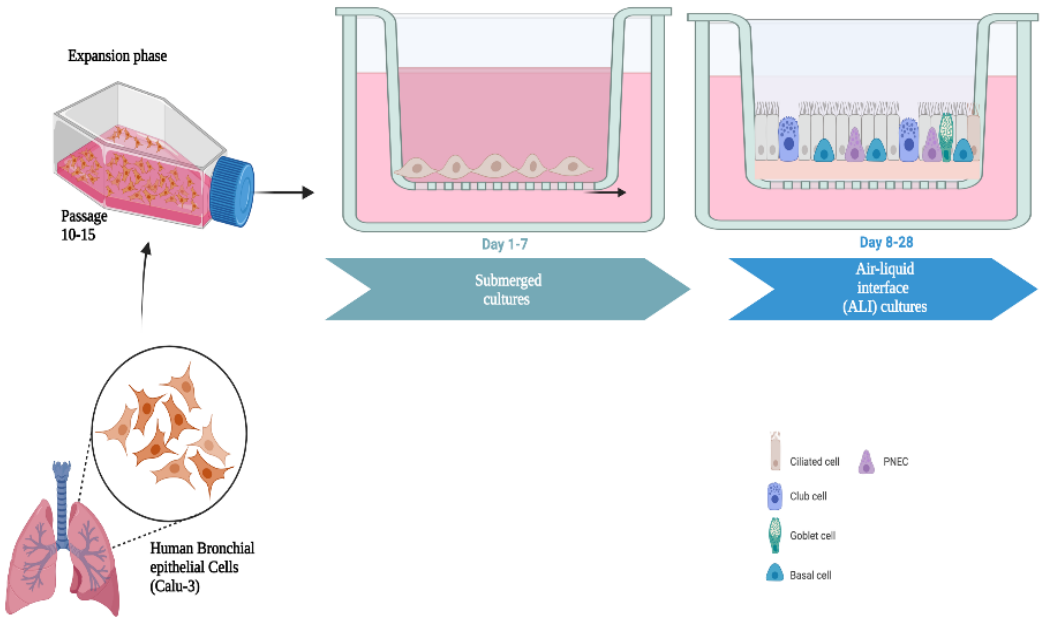


Figure 4. 2 The ALI process. Calu-3 Cell Culture and Preparation: Calu-3 cells, between passages 10-15, were expanded in a DMEM growth medium contained in tissue culture flasks. These cells were subsequently seeded at a density of 1.2x105 cells/well across 6 transwell inserts and cultured in submerged conditions for a duration of one week. Post this period, the apical media was aspirated while the basal media underwent replacement every 2-3 days. Following this procedure, the cells were exposed to an air-liquid interface for two distinct durations: 21 days (utilized for experimental procedures) and 28 days (employed for 3D lung model characterisation).

4.2.2 CHARACTERISATION OF CALU-3 CELLS AS A PHYSIOLOGICALLY RELEVANT 3D MODEL

4.2.2.1 RNA EXTRACTION

The expression of genetic differentiation markers was assessed to validate this 3D model of the microenvironment of the human lung. Every 7 days for 28 days, 700µl QIAzol lysis reagent was added to Calu-3 cells in a transwell chamber for 3 min, the lysate was collected and homogenized using a 5mL syringe, and 25-gauge needle by aspiration, repeated 10 times. The homogenate was incubated at room temperature for 5 min to promote cell dissociation of nucleoprotein complexes. Then, 140µl chloroform was added, vortexed, and incubated for a further 2 min at room temperature. The suspension was then centrifuged at 12,000g, for 15 min at 4^0C. The upper colourless aqueous phase was transferred to a 2 mL Qiagen collection tube. The following automated steps were performed using a QIAcube, (Qiagen, Venlo, Netherlands). 525µl of 100% ethanol was added and subsequently vortexed. 700µl of the sample was transferred into a RNeasy mini spin column and centrifuged at 8,000g for 15 min at 20^0C. This step was repeated. Subsequently, 500µl RPE buffer was added to the RNeasy mini spin column and was centrifuged for 2 min at 8,000g. The RNeasy

mini spin column was transferred to a new 1.5 ml collection tube, and 50 µl RNase-free water was added and subsequently centrifuged for 1 min at 8,000g to elute the RNA. RNA purity was calculated using a nanodrop 2000/c spectrophotometer (Thermo-Scientific) from the 260/280 nm and 260/230 nm absorbance ratios.

4.2.2.2 SYNTHESIS OF CDNA AND QRT-PCR

Primer validation and mRNA concentration optimization assays were carried out before the experiment. cDNA was synthesized from total RNA (200 ng) using a High-Capacity RNA-to-cDNA kit (Thermo Scientific, UK). Initially, serial cDNA dilutions were used to validate the primers for the marker genes and the genes of interest. Marker genes were used to indicate the differentiation of Calu-3 cells to specific cell types in the ALI cultures. Expression was analysed in triplicate 10µl reactions containing 1µl cDNA, 5µl Fast SYBR Green Master Mix (Applied Biosystems), 3µl nuclease-free water and 0.5µl of 10µM forward and reverse primers of the appropriate endogenous control (*gapdh, hprt1, ywaz, tata,* see **Figure 4.3** for quality score of endogenous controls), tight junctions (*tjp1*), basal cell (*itga6* and *trp63*) goblet cell (*muc5ac* and *muc5b*) or ciliated cell markers (*foxj1*). All primers were purchased from Thermo-Scientific. We consulted the geNorm website (https://genorm.cmgg.be/), a widely accepted tool for assessing the stability of housekeeping genes. According to the geNorm analysis all housekeeping genes consistently exhibited stable expression among the screened genes in our experimental context after repeating the experiments at least twice. The $^{\Delta\Delta}$Ct method (Haimes and Kelley, 2014) was used in the calculations of expression levels. The primers of differentiation markers are presented in **Table 4.1**.

Table 4. 1 Primer sequences for the six primers of differentiation and their primer efficiency for Calu-3 cells.

Gene	Sequence (forward and reverse)	Primer efficiency
gapdh	F – 5' – CTGACTTCAACAGCGACACC – 3'	93
	R– 5'– TCGTTGTCATACCAGGAAATGA – 3'	
tjp1	F – 5' – TCTATCCATGCAGAGAAGCCT– 3'	96
	R– 5'– TCCACAGCTGAAGGACTCAC– 3'	
muc5ac	F – 5' – GCTGTGTACCATAGGAGCCT– 3'	96
	R– 5'– AGCGAGTACATGGAAGAGCT– 3'	
muc5b	F – 5' – CCTGTCAAGTCCGCATCAAC– 3'	104
	R– 5'– CTGCTGAGTACTTGGACGCT– 3'	
itga6	F – 5' – TGCGAGCCTTCATTGATGTG– 3'	106
	R– 5'– ACACAGTCACTCGAACCTGA– 3'	
trp63 (tp63)	F – 5' – ACTCCATGGATGATCTGGCA – 3'	111
	R– 5'– AAGGGGAGGAGAATTCGTGG – 3'	
foxj1	F – 5' – CCTGTCGGCCATCTACAAGT – 3'	107
	R– 5'– CGGATTGAATTCTGCCAGGT – 3'	
Krt5	F – 5' – GGAGCTCATGAACACCAAGC – 3'	75
	R– 5'– CACTGAGTCTGCATTCCTCG – 3'	
Tata	F – 5' – TGCTGCGGTAATCATGAGGA – 3'	98
	R– 5'– TTCTTCACTCTTGGCTCCTGT – 3'	
YWAZ	F – 5' – CTGGTGATGACAAGAAAGGGAT – 3'	103
	R– 5'– GGATGTGTTGGTTGCATTTCC – 3'	
Hprt1	F – 5' – CCTGGCGTCGTGATTAGTGAT – 3'	95
	R– 5'– AGACGTTCAGTCCTGTCCATAA – 3'	

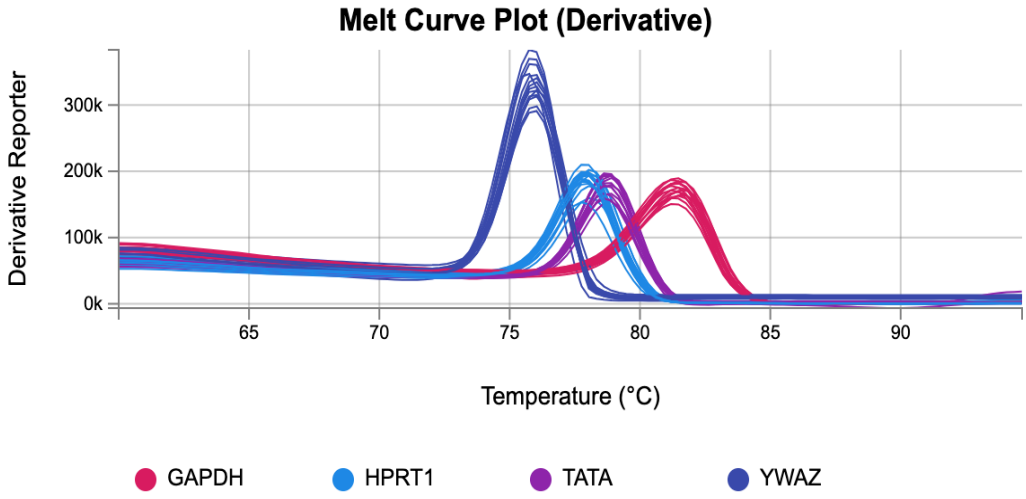


Figure 4. 3 Quality score (melt curve plot) for the housekeeping genes used as endogenous controls GAPDH, HPRT1, TATA, and YWAZ in Calu-3.

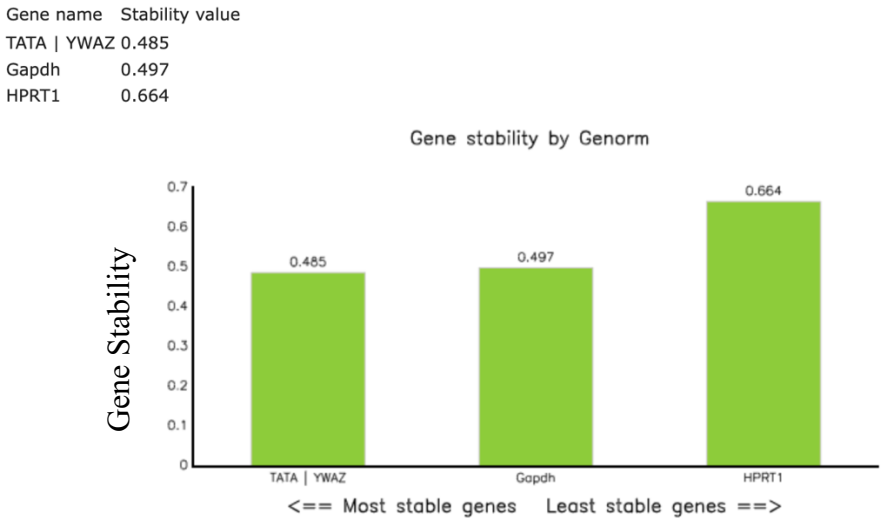


Figure 4. 4 Comprehensive gene stability of the housekeeping genes tested for the conditions of our study. All 4 tested genes were stable for the condition of our study. We determined the expression stability of these 4 genes using geNORM qBasePlus software

4.4.2.3 TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)

Transepithelial Electrical Resistance (TEER) barrier integrity is a measure of tight junction formation between cells (Srinivasan, *et al*., 2015). Basal media was removed every 2-3 days, and PBS was placed in both the apical and basal compartments, allowing TEER measurements to be recorded using an Evo meter (World Precision Instruments). Adhering to the manufacturer's protocol, the PBS was warmed to equilibrate in the incubator before measurements were recorded. Values were corrected by subtracting the measurement of the blank transwell (PBS without cells) and multiplying by the effective membrane area (4.67 cm^2), resulting in measurements related to Ωcm^2 (Srinivasan *et al.*, 2015). PBS was removed after TEER measurements, and ALI culture conditions were restored with ALI Medium in the basal compartment.

4.2.2.4 IMMUNOFLUORESCENCE WITH MUC5AC AND HOECHST 33342 (DAPI)

On day 21 of Calu-3 grown at ALI, cells were fixed in 4% paraformaldehyde for 0.5h. The cells were permeabilized by incubating with 0.5% Triton X-100 for 0.25h. Cells were washed 3 times with PBS. Cells were blocked with 2% BSA (diluted in PBS) for 1h at room temperature. Subsequently, cells were washed 3x with PBS. Cells were incubated with MUC5AC primary antibody (mouse monoclonal antibody, ThermoScientific, UK) at 2 μg/mL concentration/dilution in 0.5% BSA (diluted in PBS), for 3h at room temperature. Cells were washed 3x with PBS. Cells were incubated with a secondary antibody (rabbit polyclonal antibody, Alexa Fluor 488, ThermoScientific, UK) at 1:1000 concentration/dilution in 5% BSA (diluted in PBS), for 1h at room temperature. The antibody solution was aspirated, and cells were

counter-stained with 6 μM Hoechst 33342 (DAPI) (ThermoScientific, UK). The cells were washed 3x with PBS, and images were taken with excitation/emission of 499/520 nm with the Z-stacking option on an ImageXpress Pico digital confocal microscope (Molecular Devices, UK) aided by CellReporterXpress software (Molecular Devices, UK).

4.2.3 TOXICOLOGICAL ASSESSEMENT OF CINNAMON, HAZELNUT AND VANILLA TOBACCO E-LIQUIDS ON CALU-3 CELLS

4.2.3.1 CELL EXPOSURE TO EC E-LIQUID, AEROSOLS AND 3R4F KENTUCKY CIGARETTE

After Calu-3 cells were validated for 28 days, the data from the genetic markers of differentiation revealed the peak expression of *foxj1*, *tjp1* and *muc5ac*. Also, TEER values had been stabilised well before day 21. In contrast, the second phase of genetic and morphological changes began at day 24 to day 28, which presented a non-stable window which could have impacted on the predictive power of the model in this phase. Therefore, we utilised Calu-3 cells grown at ALI for 21 days to assess the toxicity of cinnamon, vanilla tobacco, and hazelnut flavored e-liquids. They were exposed to either 1% (v/v) of the e-liquid diluted with ALI maintenance media for 1.5h (dose validation is provided in **Chapter 3**) or to the aerosols of these for 0.5h following CORESTA protocol (CORESTA, 2015). EC aerosols were generated using an in-house electronic cigarette aerosol generator (ECAG, eAerosols, Central Valley, NY) following the CORESTA protocol of a 5s puff with a 30s interval for 0.5h totalling 60 puffs. The EC aerosol generator was set to puff at 12W, and the coil resistance of the cartridges used for studies varied between 1.8 and 2.0Ω. Cigarette smoke was generated with a smoking machine (Heinr. Borgwaldt, Germany). ALI cultures were

placed in a Thermolyne incubator (37ºC), where aerosol emitted from the ECAG was diluted with HEPA-filtered air (4L/min) and dispersed in the incubator's atmosphere, allowing the particles to impact on the apical surface. The amount of aerosol reaching the apical region of ALI cultures was characterized by measuring the mass difference (between pre-exposure and post-exposure) of a glass slide placed in an empty transwell at the same level as the cells. The masses of e-liquids deposited were: PG/VG (60/40, 1.21 mg), cinnamon (0.11 mg), hazelnut (0.16 mg), and vanilla tobacco (0.8 mg). One entire cigarette was used to generate cigarette smoke (~12 puffs) and exposed to cells for 0.5h.

4.2.3.2 TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)

For exposures, TEER readings were taken using an Evo meter (World Precision Instruments) before and after either the 1.5 h exposure to e-liquid in suspension culture or the 0.5h aerosol exposure at ALI. Adhering to the manufacturer's protocol, the PBS was warmed to equilibrate in the incubator before measurements were recorded. Values were corrected by subtracting the measurement of the blank transwell (PBS without cells) and multiplying by the effective membrane area (4.67 cm^2), resulting in measurements related to Ωcm^2 (Srinivasan *et al.*, 2015).

4.2.3.3 REACTIVE OXYGEN SPECIES

In this assay, we aimed to characterize whether cinnamon, hazelnut and vanilla tobacco-flavored e-liquids stimulate reactive oxygen species (ROS) generation in Calu-3 cells in submerged cultures. Calu-3 cells were seeded at $6x10^4$ cells in black-walled 96 well plates with clear bottoms (ThermoaFisher Scientific, USA). Once cells reached confluence, they were treated with 1.5 mM of cell-permeant 2',7'-

dichlorodihydrofluorescein diacetate (H_2DCFDA) (ThermoFisher Scientific, USA) and incubated at 37°C, 5% CO_2, and 98% humidity for 0.5h. Subsequently, cells were washed 2x with Dulbecco's phosphate buffer saline (DPBS) and challenged with diluted e-liquids (1%, 0.5% and 0.25% diluted with DMEM/F-12, HEPES, no phenol red (Gibco, UK)) or 2% hydrogen peroxide (positive control) for 3h. Fluorescence intensity was measured using the Spectramax M2e microplate reader (Molecular Devices, San Jose, California) at 495 nm excitation and 530 nm emission wavelengths.

4.2.3.4 CYTOTOXICITY: LDH ENDPOINT ASSAY

After 1.5h of hazelnut, vanilla tobacco and cinnamon flavored-e-liquid direct treatment (1% v/v) 5h of aerosol exposure to ALI cultures, maintenance medium was replaced, and cells were incubated at 37°C, 5% CO_2, and 98% humidity for 24h. Subsequently, spent media was removed from the basal region of the transwells and retained for lactase dehydrogenase (LDH) assay using the LDH cytotoxicity kit (Roche, UK) according to the manufacturer's protocol. Plate absorbance was measured at 490 nm and 680nm using the BIO-TEK synergy HT absorbance reader (Bio-Tek, USA).

4.2.4 EC AEROSOL SIZE DISTRIBUTION CHARACTERISATION

As detailed here, a sampling train was used to characterise the particle size distribution of the e-cig aerosols. The exhaust from an e-cigarette aerosol generator (ECAG, eAerosols, Central Valley, NY) was attached to an inlet of an airtight, plastic exposure chamber. A Mercer-style impactor (Model SMEIMA7, CH Technologies, Westwood, NJ, USA), in-line with a Gast pump (Model 1023-101Q-6Q608X, Benton Harbor, MI, USA), was attached to another open port of the chamber. The pump's flow rate with

the impactor was set to 4.0 L/min utilizing an external valve and checked with a DryCal air flow calibrator (Mesa Laboratories, Lakewood, CO, USA). The EC cartridges were loaded with each flavor [i.e. PG/VG (60/40), cinnamon, hazelnut and vanilla tobacco]. The ECAG was set to puff every 30 s at 12W, and the coil resistance of the cartridges used for studies varied between 1.8 and 2.0Ω. The machine puffed into the chamber for 2 minutes before the Gast pump was turned on for 2 minutes. Each stage of the Mercer impactor was weighed before and after each sample in a humidity- and temperature-controlled weighing chamber (MTL, Minneapolis, MN, USA) with a microbalance (Model MT5, 1 μg readability; Mettler-Toledo, Columbus, OH). Each aerosol's size distribution was calculated from the mass distribution collected across each impactor stage.

4.2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using one-way ANOVA for each experiment. Where one-way ANOVA revealed significance, a Levenes' variance test was performed to test for differences in variance and to inform the choice of Bonferroni HSD *post-hoc* test corrected for multiple comparisons. Graphs were generated using GraphPad Prism 9. Cinnamon, vanilla tobacco, and hazelnut assays were repeated at least twice with six technical replicates on different days. Data are expressed as mean ± SD, and the *p*-value was set at $p \leq 0.05$.

4.3 RESULTS

4.3.1 VALIDATION OF CALU-3 CELLS AT ALI

The genes of differentiation that were characterized were normalized against four housekeeping genes: gapdh, hprt1, ywaz and tata (see **Figure 4.3** for the quality score of the housekeeping genes and **Figure 4.4** for the stability of the genes). The genes characterized were basal cell markers (*itga6, krt5* and *trp-63* **Figure 4.5A)** mucus-production (*muc5ac*, **Figure 4.5B**; *muc5b* **Figure 4.5C**), a tight junction marker (*tjp1*, **Figure 4.5D)**, and cilia formation (*foxj1*, **Figure 4.5E)**. We observed a significant time-dependent increase in *trp63* (3-fold increase from day 0 to day 28), *muc5ac* (~13-fold increase from day 0 to day 28), *foxj1* (~4-fold increase from day 0 to day 28), and *tpj1* (~2.5-fold change from day 0 to day 28) gene expression, with the highest expression on day 21 or 28 at ALI. Whilst *Muc5b* increased about 400-fold from day 0 to day 7, it was followed by a subsequent 200-fold decline from day 7 to day 14, whence levels were maintained till day 28. In contrast, we observed a decrease in *krt5 from day 0* till day 7, which then remained stable till day 28. Interestingly, *itga6* mRNA levels remained constant throughout the 28 days. The presence of *muc5ac*-producing mucins was observed on day 28 at ALI (**Figure 4.5G**).

TEER measurements (**Figure 4.5F**) increased significantly from less than 1000 Ωcm^2 to nearly 2500 Ωcm^2 over the first 6 days at ALI. Between days 6 and 24, no further change in TEER was observed until between days 24 and 28, when there was a second rapid increase in TEER, from 1800 Ωcm^2 to 3000 Ωcm^2.

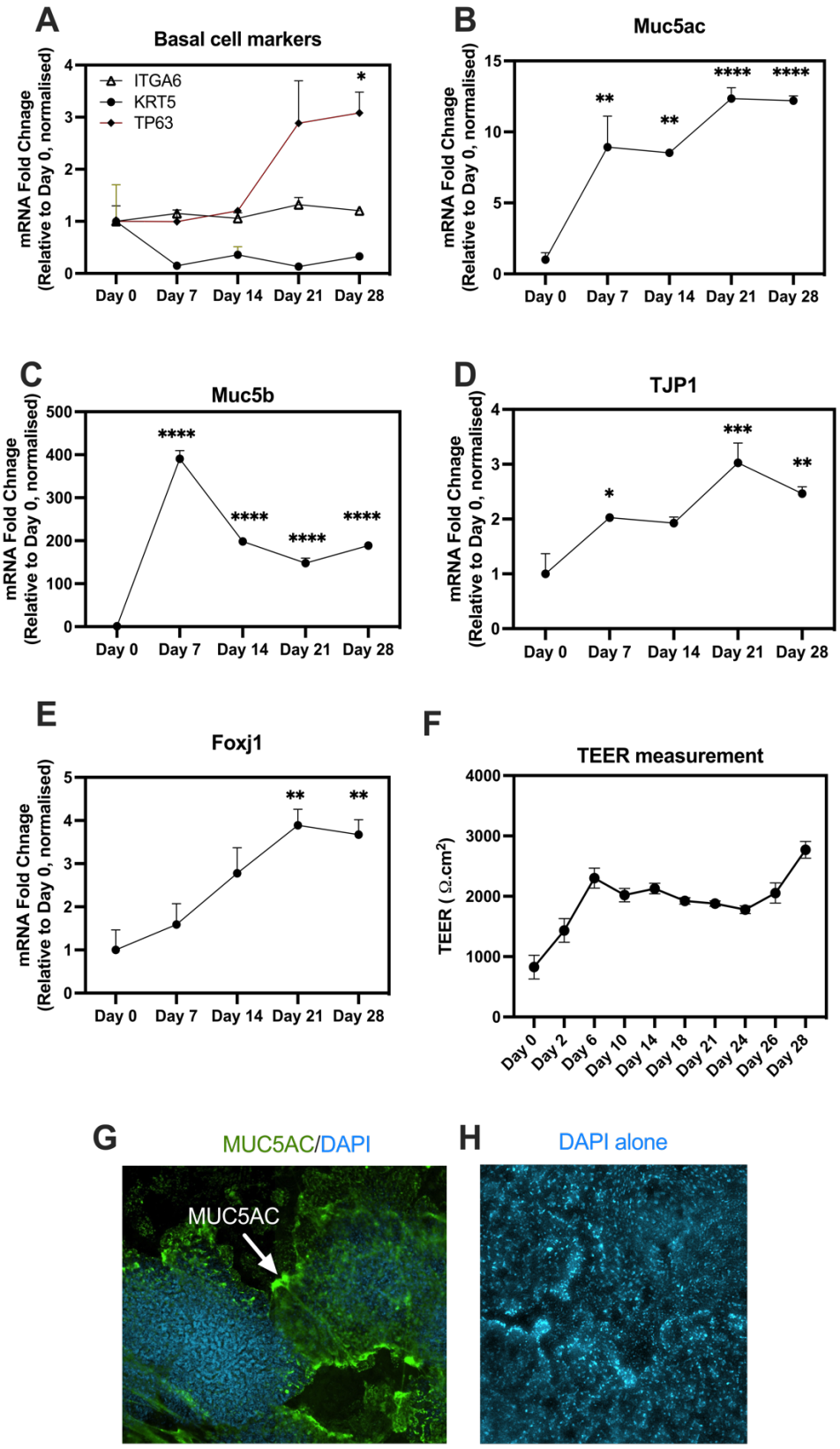

A
Basal cell markers
mRNA Fold Chnage (Relative to Day 0, normalised)
ITGA6
KRT5
TP63
Day 0
Day 7
Day 14
Day 21
Day 28
B
Muc5ac
C
Muc5b
D
TJP1
E
Foxj1
F
TEER measurement
TEER (Ω.cm²)
Day 0
Day 2
Day 6
Day 10
Day 14
Day 18
Day 21
Day 24
Day 26
Day 28
G
MUC5AC/DAPI
MUC5AC
H
DAPI alone

Figure 4. 5 Establishment of Calu-3 epithelium using ALI cultivation. Markers of differentiation were characterised every week for 28 days. The mRNA expression of (A) basal cell markers (krt5, tp63, and itga6), (B) muc5ac, (C) muc5b (D) tjp1 (E) foxj1 was investigated using qPCR. (F) TEER values were measured every 2-3 days with an EVOM 2 voltammeter from Day 0 (before the apical medium was aspirated). (G) Digital confocal microscopy was used to qualitatively examine the Calu-3 cells after MUC5AC (green) counterstained with DAPI (light blue) and H) DAPI alone and revealed the expression of MUC5AC mucins on day 21. A one-way ANOVA with multiple comparisons, corrected using Bonferroni's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 per each day group for qPCR gene expression, n = 9 for each TEER day group). Each time point for TEER measurements was statistically significant ($p<0.0001$) compared to day 0. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$ vs day 0.

4.3.2 LDH AND TEER IN CALU-3 IN SUSPENSION CULTURE

The TEER measurements in **Figure 4.6A** are the relative TEER value obtained from the difference between post-exposure TEER and pre-exposure TEER after ALI-cultured Calu-3 cells were exposed directly to dilutions of e-liquids for 1.5h on the apical region. Interestingly, the relative TEER for PG/VG ($p<0.01$), cinnamon ($p<0.001$), and vanilla tobacco ($p<0.05$), all at 1% (v/v) dilution (justification behind this dilution choice is provided in our most recent publication Effah *et al.*, (2023)), were significantly reduced from the relative TEER of media-only control and had decreased compared to pre-treatment. Hazelnut, however, showed no difference compared to media-only control but was significantly higher than PG/VG control having increased following treatment. Although the 24h LDH release in all groups was slightly higher than in the media-only control, there were no significant differences between groups.

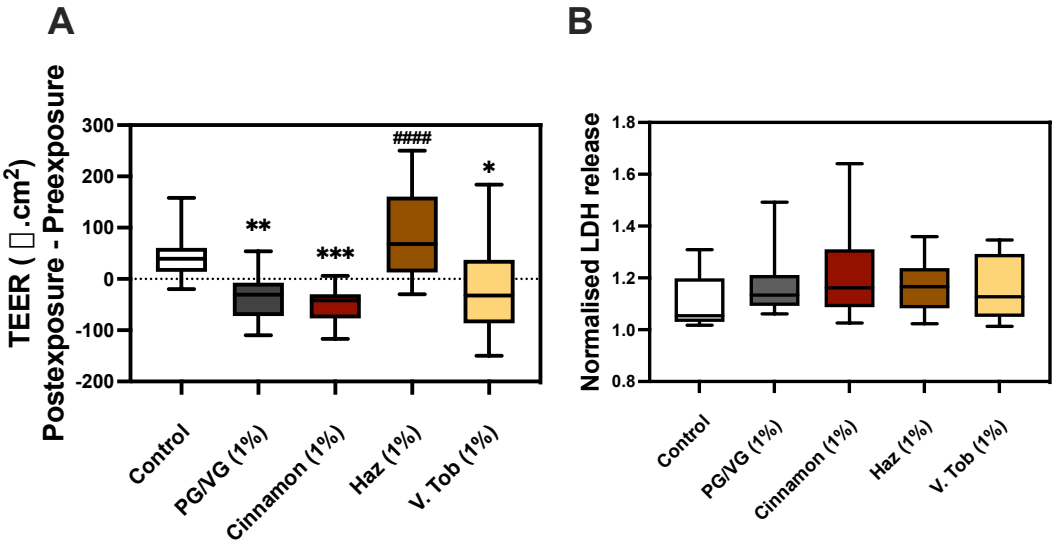


Figure 4. 6 Toxicological assessment of cinnamon, hazelnut, and vanilla tobacco flavored-e-liquids on Calu-3 cells. (A) Relative change in TEER and (B) LDH levels in Calu-3 cells grown at ALI and exposed for 1.5h to diluted e-liquids. A one-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SD (n=6 for each group). *p<0.05; **p<0.01; ****p<0.0001 vs Control; ####p<0.0001 vs PG/VG. All groups were significantly lower compared to the 2% hydrogen peroxide. Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Vanilla Tob., vanilla tobacco

4.3.3 ROS PRODUCTION IN CALU-3 IN SUBMERGED EXPOSURES

The ROS analysis (**Figure 4.7**) showed no significant differences between PG/VG, vanilla tobacco, and hazelnut at 0.25, 0.5, and 1% vs media-only control. In contrast, cinnamon significantly and dose-dependently decreased ROS generation at 0.25% ($p<0.05$), 0.5% ($p<0.001$), and 1% ($p<0.001$) vs media-only and at 0.5% ($p<0.05$) and 1% ($p<0.01$) vs PG/VG controls.

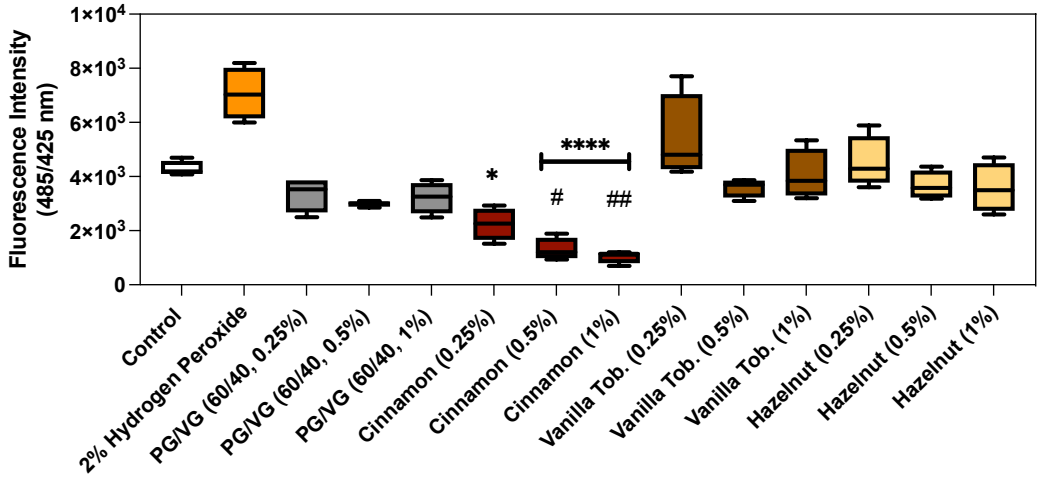


Figure 4. 7 Toxicological assessment of cinnamon, hazelnut and vanilla tobacco flavored-e-liquids on Calu-3 cells. ROS production in Calu-3 cells grown in monolayer exposed to diluted e-liquids for 3h. A one-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are presented as box and whiskers min-max (n=6 for each group). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs Control; #p<0.05; ##p<0.01 vs PG/VG. All groups were significantly lower compared to the 2% hydrogen peroxide. Abbreviations: PG/VG; propylene glycol/vegetable glycerine; Vanilla Tob., vanilla tobacco.

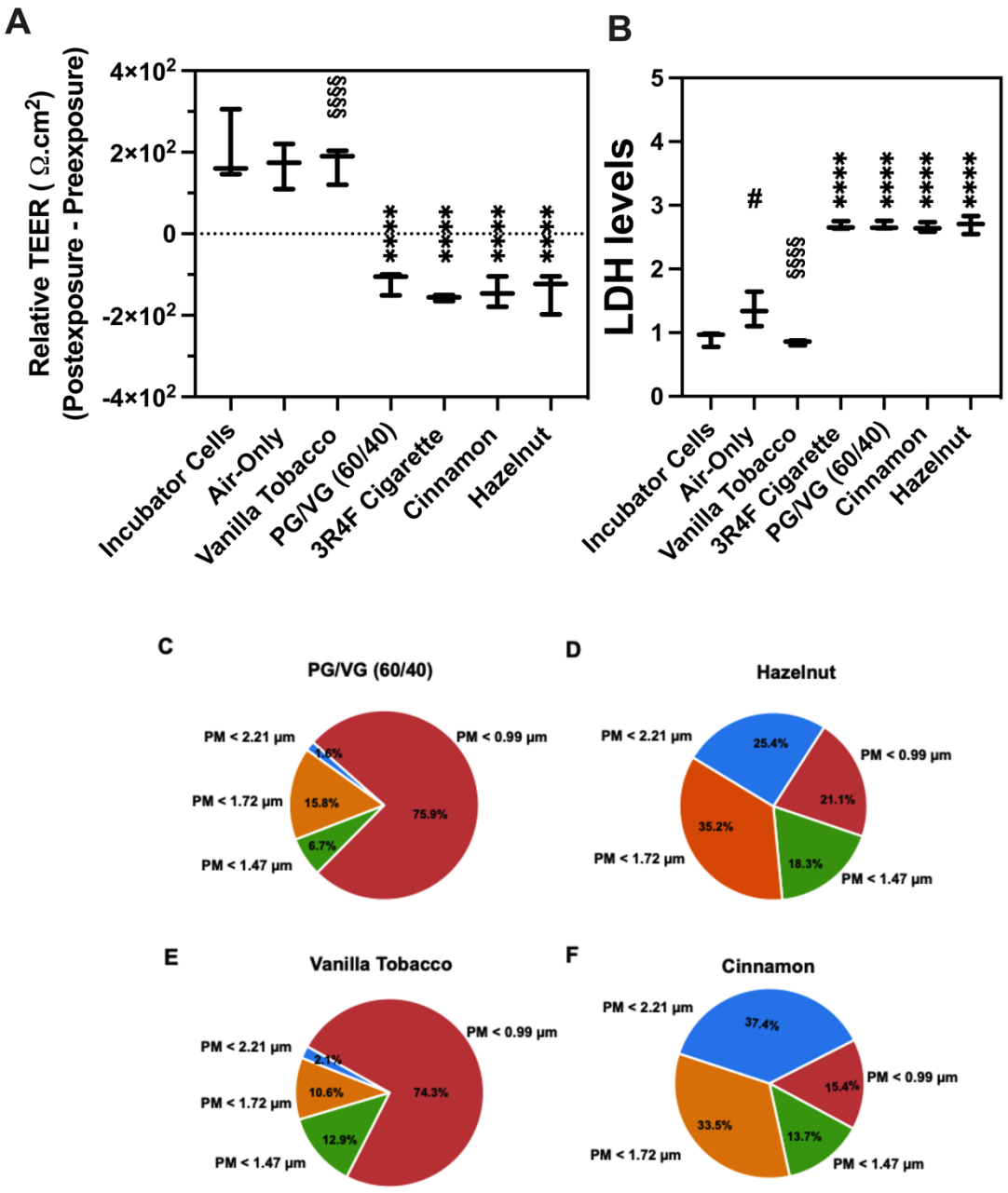
4.3.4 LDH AND TEER IN CALU-3 ALI POST-EXPOSURE TO EC AEROSOL

This study investigated the relative toxicity of cinnamon, hazelnut, and vanilla tobacco-flavored e-cigarette aerosols on 21-day ALI-cultured Calu-3 cells compared to the emissions from a 3R4F Kentucky reference cigarette positive control. Moreover, a PG/VG vehicle and an air-only control were included for a comprehensive assessment. As expected, we noted that the air-only control had no significant impact on the TEER measurements (i.e., a maintained cell barrier integrity), thus underlining the appropriateness of the exposure period (**Figure 4.8A**). TEER measurements for the vanilla tobacco-flavored aerosol exposure group were unaffected. However, the PG/VG

vehicle, cinnamon, and hazelnut-flavoured aerosols each exhibited a significant decrease in TEER measurements ($p<0.0001$) compared to the incubator and air-only controls. These results were not statistically distinguishable from the TEER reduction induced by the 3R4F Kentucky cigarette control. LDH release at 24h hours, a marker of cellular damage and cytotoxicity, was used to ascertain further the effects of e-cig aerosol exposure. It was noted that all groups except for the vanilla tobacco exhibited a significant two-fold increase in LDH release (**Figure 4.8B**) compared to incubator and air-only controls, thus corroborating the TEER measurements. The increase in LDH levels in the flavors and PG/VG aerosols were indistinguishable from that of cigarette aerosols.

4.3.5 PARTICLE SIZE DISTRIBUTION CHARACTERIZATION

We hypothesized that particle size distribution might play a key role in explaining why vanilla tobacco e-liquid but not aerosol adversely affected TEER. Specifically, it was postulated that vanilla tobacco might have a distinct particle size distribution that limits deposition when compared to the cytotoxic e-liquid aerosols (cinnamon and hazelnut). Vanilla tobacco (**Figure 4.8E**) demonstrated a particle size distribution profile similar to that of the PG/VG (60/40) vehicle (**Figure 4.8C**), with the highest percentage of particle size being particles with an aerodynamic size <0.99 μm at 74.3% and 75.9% respectively. The particle size distribution for hazelnut (**Figure 4.8D**) and cinnamon-flavored (**Figure 4.8F**) aerosols was larger particles with the majority of PM being >1μm for hazelnut (79.9%) and cinnamon (84.6%), respectively. This suggests that flavouring in the e-liquid affects the size distribution of the generated aerosol.


A
Relative TEER (Ω.cm²)
(Postexposure - Preexposure)
4×10²
2×10²
0
-2×10²
-4×10²
§§§§
****
Incubator Cells
Air-Only
Vanilla Tobacco
PG/VG (60/40)
3R4F Cigarette
Cinnamon
Hazelnut
B
LDH levels
5
4
3
2
1
0
#
§§§§
****
Incubator Cells
Air-Only
Vanilla Tobacco
3R4F Cigarette
PG/VG (60/40)
Cinnamon
Hazelnut
C
PG/VG (60/40)
PM < 2.21 µm
1.6%
PM < 0.99 µm
PM < 1.72 µm
15.8%
75.9%
6.7%
PM < 1.47 µm
D
Hazelnut
PM < 2.21 µm
25.4%
PM < 0.99 µm
21.1%
35.2%
PM < 1.72 µm
18.3%
PM < 1.47 µm
E
Vanilla Tobacco
PM < 2.21 µm
2.1%
PM < 0.99 µm
PM < 1.72 µm
10.6%
74.3%
12.9%
PM < 1.47 µm
F
Cinnamon
PM < 2.21 µm
37.4%
15.4%
PM < 0.99 µm
33.5%
13.7%
PM < 1.72 µm
PM < 1.47 µm

PFigure 4. 8 Characterizing the toxic effects of cinnamon, hazelnut, and vanilla tobacco in Calu-3 to EC aerosols. (A) TEER and (B) LDH levels in Calu-3 cells grown at ALI exposed EC aerosols generated with the ECAG for 0.5. Particle size distribution of (C) PG/VG (60/40), (D) hazelnut, (E) vanilla tobacco, and (F) cinnamon. Smaller particle sizes less than 2.5 mm have the potential to reach the alveolar region and may adversely interfere with normal gas exchange. For Figure 3A & 3B, a one-way ANOVA with multiple comparisons, corrected using Bonferroni was used to determine statistical significance. Data are presented as box and whiskers min-max (n=6 for each group). ****p<0.0001 vs air-only; #p<0.05 vs incubator control; §§§§p<0.05 vs PG/VG control. Abbreviations: PG/VG; propylene glycol/vegetable glycerine, PM; particulate matter.

4.4 DISCUSSION

This study aimed to evaluate the utility of the Calu-3 human bronchial epithelial cell culture model as a pre-screening mechanism for the inhalation toxicity of EC aerosols. The lung lining, lining fluids and constituents situated on the respiratory tract surface often serve as the initial protective barrier against respiratory xenobiotics (LeMessurier *et al.*, 2020).

Our study aimed at developing an *in vitro* model to predict the acute inhalation toxicity of three flavored-e-liquids and their aerosols using Calu-3 epithelium. The study began with validating the cell culture model to confirm that the air-liquid interface (ALI) cultured cell layers display the expected morphological and bioelectrical characteristics. Under ALI culture conditions, mucus secretion (at day 21) and tight junction formation (as early as day 6) were confirmed. We observed a time-dependent significant increase in TEER values. The optimum window of studying bioelectric properties in several Calu-3 studies has differed; for example, Ji *et al.*, (2016), after culturing Calu-3 cells at ALI for 22 days, observed this stable window between days 8 to 16. Jeong *et al.*, (2019)

only cultured the Calu-3 cells for 8 days and observed the highest TEER values around day 8, whereas Foster *et al.*, (2000) cultured Calu-3 at ALI for 14 days and observed an increase in TEER from day 6 till 14. The TEER readings in our study were biphasic. In the first phase, it agrees with Ji *et al.*, (2016) as we observed an increase in TEER measurement levels, which initially peaked at day 6 and then fell by about 20% until day 24. Interestingly, in the second phase, we observed a rapid increase in TEER values between day 24 and 28. Lee *et al.*, (2021) also cultured Calu-3 cells at ALI for 28 days and observed the highest TEER at day 28, although the increment in TEER value was strictly time-dependent in their study. It is unclear why there were discrepancies among these studies. However, we assume that factors that may have influenced these results are the passage number of the cells used at ALI, culture medium, seeding density and transwell inserts.

TEER values offer insight into the physical structure and quality of the cells on the porous membrane by gauging the transcellular and paracellular resistance across the membrane, which amplifies with cell-substrate and cell-cell interaction (Chen *et al.*, 2015). Therefore, the expression of high TEER values observed in this study may be a result of the formation of a pseudostratified structure, which, in turn, may have resulted from the time-dependent increase in the *tjp1*, *muc5ac*, *muc5b* and *foxj1* genes. There was a decrease *in krt5 gene*; whereas *itga6* gene remained constant from day 0 to day 28. The expression of *itga6* is a biomarker to measure the stemness of isolated basal cells from human airway epithelium and murine trachea (Rock *et al.*, 2009). Basal cells are pivotal for forming and repairing the pseudostratified epithelium of the human lung following injurious insults. However, the evidence provided in this study suggests that more types of basal cells are expressed within the lung epithelium recapitulated with

Calu-3 cells at ALI, with each playing a distinctive role in maintaining the pseudostratified structure. For example, the krt5-expressed basal cells may be the ones regenerating differentiated cells, whereas the itga6-expressed basal cells maintain a steady state level for epithelial repair, recapitulating the physiology of the human lung. In contrast, the expression *trp63* followed an interesting pattern. It remained unchanged from day 0 till day 14, followed by a significant increase in expression till day 28. The tumour-suppressor gene *trp63* encodes for the transformation-related protein 63 (p63), which is pivotal for cell differentiation and development such as in basal cells where it is important in generating and maintaining pseudostratified bronchial epithelium *in vitro* (Arason *et al.*, 2014). Moreover, human bronchial epithelial cells, where *trp63* gene was knocked down, failed to differentiate into goblet cells and showed non-phenotypical bronchial basal cells when grown at ALI (Arason *et al.*, 2014). As such, the initial no-change in levels in *trp63* observed in this study might have occurred during differentiation from basal cells into more specialized bronchial epithelial cells, as demonstrated by the increased expressions of *muc5ac* (**Figure 4.5B**), *muc5b* (**Figure 4.5C**), *tjp1* (**Figure 4.5D**), and *foxj1* (**Figure 4.5E**). This may also corroborate the second rapid increase in TEER values from day 24 to 28 **Figure 4.5F**). Indeed, our findings agree with Felix *et al.*, (2021) who showed an increase in TEER values in caco-2 cells cultured at a liquid-liquid interface, followed by a decrease during differentiation and an increase after developing a full barrier. Taken together, the results from these differentiation genes and the TEER values indicate that the longer Calu-3 cells are grown at ALI, the more they differentiate into several other bronchial epithelial cell types (i.e submucosal glands, goblet cells, ciliated cells, basal cells) to enable the recapitulation of the *in vivo* microenvironment of human lung epithelium. No study has reported beating cilia in Calu-3 cells grown at ALI. Although we observed a 4-fold increase in

the *foxj1* gene on day 28 compared to day 0, no visible cilia were observed under light microscopy. Conversely, functional cilia have been shown in primary human bronchial epithelial cells grown at ALI for 28 days (Leung *et al.*, 2020). This may indicate that functional cilia are not expressed in Calu-3 cells because of their submucosal. gland origin, or a better visualization technique is required to view them. Moreover, the high expression of *muc5b* compared to *muc5ac* over the 28 days agrees with the literature, as Calu-3 cells derive from human bronchial submucosal glands, the main source of MUC5B (Liegeois and Fahy, 2022). In contrast, goblet cells primarily secrete MUC5AC proteins (Liegeois and Fahy, 2022). While several studies have studied the expression of ZO-1, MUC5AC, and E-cadherins proteins in Calu-3 cells grown at ALI (Jeong *et al.*, 2019; Ji *et al.*, 2022b; Lee *et al.*, 2021; Pasman *et al.*, 2021), this is the first time a time-dependent variation in the genes encoding these proteins has been validated for Calu-3 cells. Moreover, this is the first time a differential expression of genetic markers of basal genes in Calu-3 cells recapitulates the *in vivo* pattern of genetic expression. This study may help future studies investigating lung epithelium repair and other pulmonary disorders understand which basal type to target. Moreover, it will help as a robust screening model to screen pulmonary toxicants and their mode of toxicological actions. Here, we used this validated model to characterize the potential toxicity of flavors in e-liquid.

Currently, more than 8000 flavored e-liquids from various brands are on the UK market (Effah *et al.*, 2022). Although carcinogens appear to be reduced or eliminated in ECs compared to cigarette smoke (Amatngalim *et al.*, 2016), the inhalation toxicity of EC aerosols with flavoring chemicals, and flavor enhancers is poorly understood based on toxicological effects on the lungs. Several *in vitro* studies have shown the link between

EC and e-liquids to lung tissue damage. The most frequently reported adverse effects are reduced cell count and viability, altered pro-inflammatory biomarkers, cytokine release, and increased oxidative stress *in vitro* (Balharry *et al.*, 2008; Behar *et al.*, 2018b; Clapp *et al.*, 2019b; Sundar *et al.*, 2016b). These effects mentioned above are consistent across pulmonary cell types, including normal and cancer-derived cells, large and small airway epithelial cells, alveolar epithelial cells, pulmonary smooth muscle, fibroblasts, and alveolar macrophages (for a detailed review, see Effah *et al.*, (2022b)). Although the flavoring chemicals used are generally regarded as safe (GRAS) for oral ingestion, their GRAS status post-inhalation remains elusive, and the impact of specific flavoring chemicals on airway epithelial cells remains unclear (Clapp *et al.*, 2019b; Neilson *et al.*, 2015; Omaiye *et al.*, 2022). *In vitro* assessment of inhalation toxicology has also been applied to the EC liquids (Khlystov and Samburova, 2016; Leigh *et al.*, 2016a). Nevertheless, information on flavoring-specific inhalation toxicity and the relative toxicity of flavored-e-liquids vs tobacco smoking needs further examination. In this study, we tested the acute inhalation toxicity of 3 previously identified flavored-e-liquids that demonstrated toxicity in submerged bronchioepithelial cells (Effah *et al.*, 2023) and compared the toxicity as e-liquids and as EC aerosols to conventional cigarette emissions.

We used TEER as the main indicator of tissue stress by measuring the integrity of cellular tight junctions and, thus, epithelial permeability. LDH levels were used as a proxy to measure cytotoxicity and overall cell health. PG/VG, vanilla tobacco, and cinnamon e-liquids at 1% (v/v), but not hazelnut, significantly decreased TEER values in suspension culture. In contrast, no effect was observed on LDH release. Interestingly, with aerosol exposures, vanilla tobacco was the only flavored-e-liquid which did not

impact on TEER value or LDH release, with PG/VG, hazelnut, cinnamon and 3R4F cigarette reducing TEER measurements and increasing LDH levels by 2-3 fold, respectively compared to air only exposure. Surprisingly, TEER values and LDH levels induced by aerosols from PG/VG alone did not differ from the flavoured-e-liquids. This may suggest that 60 puffs of PG/VG alone may be enough to exacerbate the toxicological response of bronchial epithelial cells to an extent that the additive toxicity of flavors may not be significant *in vitro*. Therefore, further studies are required to establish whether the toxicity of aerosolized PG/VG is dose dependent. Nonetheless, this contrasted with our expectations, given the common perception of e-liquids as a safer alternative and the contrasting evidence on PG/VG toxicity in the literature (for an extensive review see **Chapter 2**).

EC aerosol contains fewer and significantly lower concentrations of toxic chemicals than conventional cigarettes (Dusautoir *et al.*, 2021; Margham *et al.*, 2016). However, only a few studies assessing the toxicity of EC flavors compared to conventional cigarettes. Czekala *et al.*, (2019) have shown that aerosols from blueberry flavored-e-liquids induced less toxicity than conventional cigarettes in a 3D *in vitro* human respiratory model developed from primary cells. However, they only screened one flavor and it did not justify choosing that particular flavor. In contrast, Glynos *et al.*, (2018) showed that EC aerosols, especially those containing flavor (flavor type not specified), increased bronchoalveolar lavage fluid (BALF) cellularity, Muc5ac production, as well as BALF and lung oxidative stress markers in mice at least comparably and in many cases more than did conventional cigarettes. Conversely, we observed no flavor-specific additive toxic effects in our study. Taken together, our study suggests that the potentially adverse health effects associated with flavoured e-liquid aerosols may be heavily dependent on

PG/VG and not on flavor type as not all flavors exert added toxicity (as observed in **Chapter 3** as well). Therefore, we strongly advocate for increased user awareness on the potential toxicity of PG/VG and rigorous regulatory scrutiny of potentially toxic flavours to protect public health.

A discrepancy between the toxicity observed for e-liquid or as an aerosol agrees with previous observations. Behar et al. (2018), carried out a pattern of cytotoxicity studies on 35 e-liquids and their aerosols in A549 and human pulmonary fibroblasts using the MTT assay as follows: (1) both the refill fluid and its aerosol were non-cytotoxic (7 of 35=20%); (2) both the refill fluid and its aerosol were cytotoxic (19 of 35=54%); (3) the refill fluid was cytotoxic, but the aerosol was not (1 of 35=3%); or (4) the aerosol was cytotoxic, but the refill fluid was not (8 of 35=23%). Collectively when combining patterns (1) and (2), 74% of the 35 unheated refill fluids correctly predicted the cytotoxicity of their corresponding aerosol. The remaining 26% did not predict cytotoxicity because: (1) certain flavours (i.e., menthol artic) were cytotoxic in submerged cultures but not in their aerosol exposures in the ALI model and (2) other flavours are cytotoxic in their aerosolized form (i.e., butterfinger and caramel) but not in their liquid form (Behar et al. 2018). A technical difference between our studies and this study is that we employed ALI exposure to aerosol (0.5h exposure to 60 puffs) whereas in this study, aerosol was collected by bubbling 24 puffs through 4 mL of cell media which was applied to the submerged cells at doses of 0.0006 to 6 TPE (TPE = total puff equivalents, which is the number of puffs/mL of culture medium).

To justify why the aerosolised vanilla tobacco elicited no toxic effects even though it was the flavour with the highest most deposited mass (0.8 mg) compared to cinnamon (0.11 mg), and hazelnut (0.16 mg), we hypothesized that it might have a different particle size distribution compared to cinnamon and hazelnut. Therefore, we characterized the particulate matter (PM) size produced from the EC aerosol. Hazelnut and cinnamon showed similar patterns in their particle size distribution, which reinforces the findings regarding TEER reduction and increased LDH release in the cells exposed to these e-liquid aerosols. In contrast, vanilla tobacco and PG/VG showed a similar pattern with the greatest particle size distribution below 0.99 microns. Smaller particles tend to have a larger surface area relative to their mass, which can increase their reactivity and ability to interact with biological systems (Hewitt *et al.*, 2020); however, vanilla tobacco showed no cytotoxicity in its aerosolized form. Therefore, this suggests that factors beyond particle size distribution may contribute to the toxicity of cytotoxic PG/VG vehicle, cinnamon, and hazelnut. Although more studies are warranted to delineate why vanilla tobacco aerosol showed no toxicity in Calu-3 cells grown at ALI, Rowell *et al.*, (2017) showed that vanilla tobacco reduced cell proliferation and decreased cell viability dose-dependently in submerged Calu-3 cells exposed to vanilla tobacco aerosol bubbled through culture medium. Therefore, differences in cell model may account for the differences in toxicological outcome in our study and that of Rowell *et al.*, 2017. In addition, we showed in our previous study that vanilla tobacco and hazelnut may elicit their toxic effect by decreasing mitochondrial membrane potential and inducing ROS production in HBEC-3KT (**Chapter 3**). Nonetheless, hazelnut and vanilla tobacco e-liquid did not adversely affect Calu-3 cells at the doses tested compared to PG/VG controls. Interestingly, while cinnamon e-liquid showed no effect on ROS production in direct diluted e-liquid exposure to submerged HBEC-3KTs, it induced a dose-dependent

decrease in ROS production compared to media-only and PG/VG vehicle controls in Calu-3 cells. We hypothesise that the differential responses in HBEC-3KT and Calu-3 cells for ROS generation may be rooted in the differential expression of antioxidative stress enzyme in cancerous (Calu3) and immortalised (HBEC-3KT) normal cells (Perillo *et al.*, 2020). Conversely, cinnamon e-liquids have been shown to induce oxidative stress and increase ROS production in osteoblast-like MG-63 (Wavreil and Heggland, 2020).

The observed decrease in ROS production in Calu-3 cells exposed to diluted cinnamon e-liquids could be attributed to the presence of stilbene (**Chapter 3**), a compound found in cinnamon e-liquid. Stilbene have been found to exhibit antioxidant properties and act as ROS scavengers (Frombaum *et al.*, 2012; Hamadouche *et al.*, 2021; Reinisalo *et al.*, 2015), leading to a dose-dependent decrease in ROS levels in this cell line.

This suggests that the effects of cinnamon e-liquids on ROS production may be influenced by the specific composition of the e-liquid. Moreover, it is important to note that the antioxidant activity of stilbene compounds can be context dependent. In certain situations, these compounds may also exhibit prooxidative effects, depending on the cellular environment and concentration (Kładna *et al.*, 2021). Therefore, further research is needed to fully understand the mechanisms underlying the effects of cinnamon e-liquids on ROS production and the potential role of stilbene in this process.

The evidence provided in this study suggests that not only is the mode of exposure of flavoured-e-liquids to pulmonary cells important, but also the cell line used for screening may influence the model's predictive power.

One important factor of Calu-3 ALI characteristics is the formation of a mucus layer, which has a significant impact in the deposition and dissolution of inhaled drugs (Silva *et al.*, 2023). Permeation profiles obtained with a PennCenturyTM Dry Powder Insufflator in Calu-3 cell layers in ALI conditions revealed that mucus facilitates the dissolution and permeability of poorly soluble drugs (indomethacin), but restrains the absorption of drugs with high solubility (salbutamol) (Cingolani *et al.*, 2019).

PG is already highly soluble in water and various solvents due to its diol structure, which allows it to form hydrogen bonds with polar molecules. VG, on the other hand, is also water-soluble but has a higher viscosity and lower volatility compared to PG. The solubility of PG and VG in e-liquids is primarily determined by their interactions with other components, such as water, flavors, and nicotine. Therefore, we hypothesised that other pulmonary cells (cancerous, immortalized or primary) grown at ALI that do not present a thick mucus layer like Calu-3 cells exposed to aerosolized e-liquids in *in vitro* inhalation systems such as the CULTEX system (Aufderheide *et al.*, 2013) may be a more robust experimental approach to screen for flavour toxicity in ECs. During the high-throughput studies in **Chapter 3**, menthol demonstrated a unique characteristic. It formed condensation on the lid of the plate. This peculiarity led to its inclusion in this aerosol study. However, we observed a distinctive behaviour of menthol e-liquid; when aerosolised and introduced into the exposure chamber, it formed clouds that lingered in the atmosphere without settling on the cells. This observation prompted a detailed investigation into the effects of menthol within a controlled inhalation system, ensuring direct contact with cells. The outcomes of this in-depth analysis are elaborated in **Chapter 6**.

4.4.1 CONCLUSION

Flavours in EC are believed to attract long-time tobacco cigarette smokers to switch to EC use and tobacco naïve non-smokers to take up e-cigarettes; they also potentially may exert adverse effects on airways. In this study, we tested our hypothesis on the toxicity of PG/VG alone aerosols exerts adverse effects in Calu-3 cells in ALI cultures comparable to the positive cigarette controls. Nonetheless, the hypothesis that in Calu-3 suspension cultures, hazelnut and vanilla tobacco elicit ROS production was not achieved. Also, the hypothesis that the aerosols of flavoured e-liquids may exert additive toxicity was not achieved. Interestingly, none of the flavored-e-liquids that showed toxicity comparable to conventional cigarettes significantly differed from their PG/VG vehicle control. In contrast, while vanilla tobacco elicited adverse effects in suspension culture, it was non-toxic in its aerosol form. This suggests that the toxicity associated with aerosols of flavored-e-liquids may be due to the vehicle components and not the flavours themselves. This study suggests that the toxicity associated with aerosolised e-liquids heavily depends on the PG/VG component. As we identified in our systematic review (**Chapter 3**), cancerous cells may be less sensitive to e-liquids and their aerosols. Therefore, future work needs to focus on building robust and validated lung models with primary or immortalised cells grown at ALI that are sensitive enough to predict the toxicity of aerosols and the potential additive effects of flavouring components to the toxic effects of PG/VG. This will help inform public health authorities in the UK and elsewhere about which EC flavours may need monitoring and/or restricting.

CHAPTER 5

ESTABLISHING A SUITABLE PHYSIOLOGICALLY REPRESENTATIVE *IN VITRO* AIRWAY MODEL TO INVESTIGATE THE TOXICOLOGICAL EFFECTS OF EC AEROSOL IN LUNG EPITHELIAL CELLS (A549, BEAS-2B, HBEC-3KT, AND RPMI 1640)

This work was done in collaboration with colleagues at UKHSA: Dr Emma Marczylo, Emma-Jane Goode and Rachel Bowsher. I contributed to the cell culture, Primer design, RNA extraction, and qPCR analysis.

5.1 INTRODUCTION

In toxicology, pharmacology, and environmental health research, pulmonary exposure studies assess the potential effects of chemicals, drugs, or other substances on the respiratory system. As discussed in Chapter 4 (section 4.1), the species-specific differences between humans and rodents means that inhalation pharmacology and toxicology studies are moving from animal studies, including larger animals, to in vitro toxicology studies with organotypic models that closely recapitulate the human lung microenvironment of target. Such organotypic models include air-liquid interface models of the lung. In Chapter 4, we validated Calu-3 cells at ALI to screen the aerosols of 3 flavoured e-liquids identified to be toxic in submerged HBEC-3KT models (Chapter 3).

While the evaluation of Calu-3 cells after ALI differentiation as a predictive model for cytotoxicity was performed with 38 positive and 22 negative chemicals of acute inhalation drugs (Jeong *et al.*, 2019). The drugs were diluted in phosphate-buffer saline, dimethyl sulfoxide (up to 5%) or mineral oil. At 5% drug concentration, the assays predicted toxic effects with high sensitivity (82 %) and accuracy (78%) (Jeong *et al.*, 2019). Nonetheless, in our studies (**Chapter 4**), Calu-3 cells did not suggest flavoured e-liquid aerosol toxicity. However, there are question marks around the sensitivity of Calu-3 cells for testing EC toxicity as observed in **Chapter 4**, there was no flavour specific effects as hypothesized. Therefore, we validated RPMI 2650, A549, Beas-2b and HBEC-3KT cells at ALI for 28 days by characterising the genes of differentiation over this time course as well as tight junction formation and morphological changes.

5.1.2 RPMI 2650 AND A549 CELL LINES

RPMI 2650 and A549 are basal cells derived from cancerous tissue from the nasal septum and alveolar regions of human donors, respectively (Figure 5.1) (Binder *et al.*, 2022; He *et al.*, 2004; Ladel *et al.*, 2019; Mercier *et al.*, 2018). RPMI 2650 are derived from a human nasal septum squamous cell carcinoma; the RPMI 2650 cell line is commonly used to study nasal epithelium and drug transport (Mercier *et al.*, 2018). They form tight junctions and produce mucus, making them helpful in investigating the mucociliary clearance mechanism (Gonçalves *et al.*, 2016). Nonetheless, there is no evidence for the presence of cilia in RPMI 2650 cells grown at ALI. The A549 cell line was established from human lung carcinoma, the A549 cell line is an epithelial-like cell line often used to model alveolar type II cells. They are widely employed in lung cancer, inflammation, and drug transport studies. Many studies suggest that these cells are unable to polarize and lack functional tight junctions, although they seem to express certain tight-junction proteins such as occludin and E-cadherin (Baldassi *et al.*, 2021).

5.1.3 COMPARISON OF RPMI 2650, CALU-3, AND A549 AS 3D LUNG MODELS AT THE AIR-LIQUID INTERFACE

Physiological relevance: Calu-3 cells closely resemble the human respiratory epithelium in terms of mucus secretion, tight junction formation; however, for this cell line the data for cilia expression appear to be contradictory (Fiegel, 2003; Patel *et al.*, 2002; Shen *et al.*, 1994). Patel *et al.*,which Calu-3 cells produce cilia, whereas Fiegel et al., and Shen et al., similar to us observed no cilia production. Therefore, we hypothesise that these discrepancies might be related to the number of cell passages, the type of growth media and transwell used. RPMI 2650 cells more closely represent the nasal epithelium, while A549 cells serve as a model for alveolar type II cells which are

concerned with protective roles like response to damage and immune response (Baldassi *et al.*, 2021).

Barrier function: Calu-3 and RPMI 2650 cells both form tight junctions, contributing to the development of strong barrier function in 3D lung models. A549 cells, on the other hand, have been reported to exhibit weaker barrier properties due to their lower expression of tight junction proteins. Both Calu-3 and RPMI 2650 cells, but not A549 cells, also produce mucus, which is crucial for studying mucociliary clearance mechanisms (Baldassi *et al.*, 2021).

Disease modelling: Calu-3 and A549 cells are more commonly used in modelling respiratory diseases due to their origin from lung adenocarcinoma and lung carcinoma, respectively. In contrast, RPMI 2650 cells are predominantly used to study nasal epithelium and drug transport (Baldassi *et al.*, 2021).

5.1.4 HBEC-3KT AND BEAS-2B CELL LINES

Immortalised normal cell lines (HBEC-3KT and Beas-2b) are genetically modified bronchial basal cells that can divide indefinitely and grow in cell culture (Figure 5.1). They exhibit genetic homogeneity and provide reproducibility of results. These have been used as ALI models to study cellular functions, aerosol toxicity testing, and inhalation drug permeability testing (Candeias *et al.*, 2021; Delgado *et al.*, 2011; Juarez Facio *et al.*, 2022; Kaisani *et al.*, 2014). The HBEC-3KT cell line is derived from human bronchial epithelial cells that have been immortalized using a combination of CDK4 and hTERT (Ramirez *et al.*, 2004). These cells maintain many of the characteristics of

primary bronchial epithelial cells, such as the ability to differentiate into a pseudostratified mucociliary epithelium under air-liquid interface conditions (Leslie Fulcher *et al.*, 2004). The Beas-2B cell line was derived from human bronchial epithelium transformed with an adenovirus 12-SV40 hybrid (Reddel *et al.*, 1993). These cells have epithelial morphology and are often used to study lung carcinogenesis, inflammation, and drug transport.

5.1.5 COMPARISON OF HBEC-3KT AND BEAS-2B AS 3D LUNG MODELS AT THE AIR-LIQUID INTERFACE

Physiological relevance: HBEC-3KT cells maintain many characteristics of primary human bronchial epithelial cells, making them a more physiologically relevant model than Beas-2Bs for studying airway biology and diseases (Leslie Fulcher *et al.*, 2004). Beas-2B cells, on the other hand, have been transformed using viral oncogenes, which may impact their physiological relevance (Reddel *et al.*, 1993). Viral oncogenes can alter the expression of various genes in the host cell. SV40, for instance, encodes for the T-antigen, which can interfere with cell cycle control by binding to proteins like p53 and Rb. As a result, the cell's normal regulatory pathways might be disrupted, which can lead to altered behavior in the cells.

Differentiation potential: HBEC-3KT cells can differentiate into a well-differentiated, pseudostratified, mucociliary epithelium under air-liquid interface conditions, which closely resembles the human airway epithelium (Leslie Fulcher *et al.*, 2004). Beas-2B cells can also differentiate under air-liquid conditions but display a more limited

differentiation potential compared to HBEC-3KT cells (Silva *et al.*, 2023; Stewart *et al.*, 2012)

Barrier function: Both HBEC-3KT and Beas-2B cells form tight junctions, which are essential for maintaining the integrity of the airway epithelial barrier (Silva *et al.*, 2023; Stewart *et al.*, 2012). However, HBEC-3KT cells exhibit a stronger barrier function due to their closer resemblance to primary human bronchial epithelial cells.

Disease modelling: HBEC-3KT cells are more suitable for modelling airway diseases due to their closer resemblance to primary human bronchial epithelial cells (Leslie Fulcher *et al.*, 2004). Beas-2B cells, while useful for studying lung carcinogenesis and inflammation, may be less relevant for modelling certain airway diseases due to their transformed nature with viral oncogenes (Reddel *et al.*, 1993).

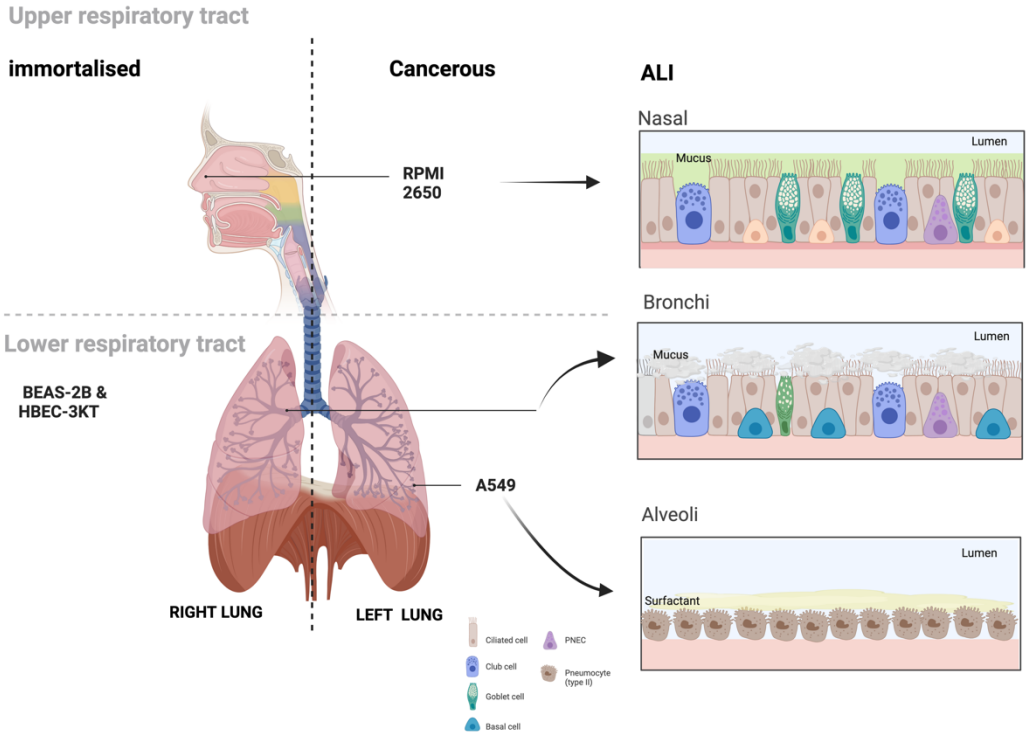


Figure 5. 1 Diagram to depict the primary airway cells and airway cell lines validated and compared at the air-liquid interface. We assessed 2 cancerous cell lines (RPMI 2650 and A549) and two immortalised cell lines (Beas-2b and HBEC-3KT).

5.1.6 COMPARISON BETWEEN PRIMARY, IMMORTALISED, NORMAL AND CANCEROUS CELLS

Evidence from Chapter 2 suggests a greater toxicological or pharmacological predictive power for EC toxicity compared of primary cells compared to immortalized cells or the cancerous ones grown at ALI. Primary airway cells grown at ALI are arguably the preferred model for ALI models to explore the anatomy and physiology of normal human airways, they are expensive, often hard to get, short-lived (only few passages) and labour-intensive. As such, most studies that use cell lines, either cancerous or immortalized at ALI investigations, fail to provide appropriate justification for their

choice of cell line. This lack of justification may stem from a need for standardised protocols and validation studies for their potential relevance as normal or diseased airway models. The choice of model may also be determined by the research question under investigation e.g., screening of potential chemotherapeutics in cancerous cell lines. As such, in this study, not only did we validate several of the commercially available cell lines previously used for ALI models: cancerous (A549 and RPMI 2650), immortalized (HBEC-3KT, Beas-2b), but we provided evidence for structural, genetic, and bioelectrical differences of these models. By comparing all of these airway models, we provide evidence to inform researchers on the best fit for purpose lung model for their specific investigations. This study will help researchers with detailed protocols for pulmonary ALI models and justifications for the use of appropriate model to address their specific research questions.

5.1.7 AIMS AND HYPOTHESIS

This study aims to provide evidence to help choose the best representation of airway models for aerosol exposure.

5.1.8 THE HYPOTHESES:

- Immortalised and cancerous epithelial cells (**Figure 5.1**) grown at ALI express different levels of differentiation markers suggesting different suitability for different studies.

5.1.9 THE OBJECTIVES:

- To grow all cell lines at ALI for 28 days.

- To assess differentiation at ALI using qPCR to analyse the expression of marker genes for goblet, ciliated, and basal cells.
- To assess tight junction formation and membrane integrity of ALI cultures every week with the Evo metre.

5.2 METHODS

5.2.1 CELL LINES AND CELL CULTURE

The human bronchial epithelial cell line HBEC-3KT (ATCC) was cultured in airway epithelial growth media and respective supplement mix (PromoCell). The human nasal squamous cell carcinoma cell line RPMI 2650 (ECACC) was cultured in Eagle's Minimum Essential Medium (EMEM, GIBCO) supplemented with 10% foetal bovine serum (FBS, GIBCO, UK) and 1% non-essential amino acids (NEAA, GIBCO, UK). The human alveolar basal epithelial adenocarcinoma cell line A549 (ECACC) was cultured in F-12 nutrient mix (GIBCO, UK) supplemented with 10% FBS (GIBCO, UK). The normal bronchial epithelial cell line BEAS2B (ATCC) was cultured in the Lonza Bullet Kit containing basal media and the recommended supplements (Lonza).

5.2.2 CELL PASSAGING

During cell passaging, 0.25% Trypsin EDTA (GIBCO, UK) was added, and the flask was incubated at 37^{o}C with 5% CO_2 for 3-5 minutes. Agitation of the flask detached the cells, which could be observed using the light microscope. Phosphate buffered saline (PBS) (GIBCO, UK) containing 5% FBS (GIBCO, UK) was added to the flask to neutralise the Trypsin EDTA before the cell suspension was placed into a centrifuge tube and centrifuged at 1000 rpm for 5 minutes. After discarding the supernatant, the cell pellet was resuspended in the appropriate growth media. A small sample was added

to trypan blue (Sigma-Aldrich) in a 1:1 ratio and placed into a haemocytometer for a viable cell count. For the next passage, cells were seeded at 2 x 10^6 per T175 flask.

5.2.3 COLLAGEN COATING

Before any ALI culturing, all transwell inserts were coated in 10μg/cm^2 collagen (Advanced BioMatric PureCol bovine collagen solution, Type 1). When 24 well transwells were used for assays, the inserts in the external wells were removed to avoid any edge effect.

5.2.4 CELL SEEDING OPTIMISATION

A549 and RPMI cells were seeded at passage 12 in 24-well PET transwells with 0.4uM pores and 12mm thickness (Corning) at various cell densities ($2x10^5$; $5x10^5$; $8x10^5$; $1x10^6$ cells/well). HBEC-3KT cells were seeded at passage 12 in a 24-well transwell at various seeding densities ($4x10^4$; $6x10^4$; $8x10^4$; $1x10^5$; $5x10^5$ cells/well). BEAS2B cells were seeded at $4x10^4$ in 24-well transwells and a single 12-well polycarbonate transwell with 12mm thickness and 0.4μm pores (Corning). The media used to culture all cells during expansion was previously described (section 2.2). Confluence was observed using a light microscope and was achieved after 24h for A549 and RPMI, and 48h for HBEC-3KT and BEAS2Bs cells. All media was removed from cells once confluence was reached. PneumaCult ALI Media, containing Basal Medium (STEMCell), hydrocortisone, heparin, PneumaCult ALI Maintenance Supplement (100X) and PneumaCult ALI 10X Supplement, was placed into the basal compartment exposing the apical region to the air. Once at an ALI, the PneumaCult ALI Media in the basal compartment was replaced every 2-3 days whilst the transwells were incubated at 37^{o}C

with 5% CO_2. ALI cultures were maintained for up to 28 days with images captured using a light microscope and cells harvested with Qiazol (stored at -80°C until RNA extraction) every seven days.

5.2.5 TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)

Transepithelial Electrical Resistance (TEER) helps to identify the presence of tight junctions. A higher TEER value demonstrates an increased resistance across the epithelial barrier and therefore, a greater barrier integrity (Srinivasan, et al., 2015).

Every 7 days basal media was removed, and PBS was placed in both the apical and basal compartments allowing TEER measurements to be recorded using the Evo meter (World Precision Instruments). Adhering to the manufacturers protocol, the PBS was warmed to equilibrate in the incubator before measurements were recorded. Values were corrected by subtracting the measurement of the blank transwell (PBS without cells) and multiplying by the effective membrane area resulting in measurements related to Ωcm^2 (Srinivasan, et al., 2015). PBS was removed after TEER measurements and ALI culture conditions were restored with ALI Medium in the basal compartment.

5.2.6 RNA EXTRACTION AND CDNA SYNTHESIS

RNA was extracted from all cells using Qiazol and RNeasy Mini Kit (Qiagen) following the manufacturers protocol. RNA samples were analysed in triplicate using a mySPEC spectrophotometer (VWR) and stored short-term at -20°C. After thawing, RNA was converted to cDNA using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturers protocol.

5.2.6 RT-qPCR

5.2.6.1 PRIMER DESIGN

Primers were designed manually with Ensembl, NCBI Primer-blast and Primer3 and supplied by Integrated DNA Technologies. Sequences can be found in **Chapter 4**, **Table 4.1**.

5.2.6.2 mRNA EXPRESSION

Initially, serial cDNA dilutions were used to validate the primers of various marker genes and genes of interest (GOI). Marker genes were used to indicate the presence of specific cell types to help determine the levels of differentiation in the ALI cultures. Expression was analysed in triplicate 10µl reactions containing 1µl cDNA, 5µl Fast SYBR Green Master Mix (Applied Biosystems), 3µl nuclease-free water and 0.5µl of 10µM forward and reverse primers of the appropriate endogenous control *(gapdh*), tight junctions (*tjp1*), basal cell (*krt5*, *itga6* and *tp63*) goblet cell (*muc5ac* and *muc5b*) or ciliated cell markers (*foxj1*). *gapdh, ywaz* and *tata* were used to normalise multiple qPCR plates and calculate the fold change using the Pfaffl method (Pfaffl, 2001). All graphs were created using GraphPad PRISM V9.

5.2.7 STATISTICAL ANALYSIS

TEER (n=6 unless otherwise stated) and gene expression data (n=3) was expressed as mean±SEM. RT-qPCR fold change data for all the cells were assessed with a 2-way ANOVA and a post-hoc test with Tukey's adjustment. $P<0.05$ was considered significantly different. All statistical analysis was undertaken using RStudio or GraphPad PRISM V9 (Dotmatics).

5.3 RESULTS

5.3.1 GENETIC MARKERS OF BASAL CELLS AT ALI

The analysis of the expression of the krt5 gene across four distinct cell lines, namely Beas-2B, RPMI 2650, A549 cells and HBEC-3KT, unveiled a noticeable decline in all cases. Among the four, Krt5 gene expression declined across all cell lines (**Figure 5.2A)**, with the most significant decreases in HBEC-3KT cells. HBEC-3KT cells showed a time-dependent 6-fold decrease in krt5 expression, which was significant at day 7 ($p<0.01$), and more pronounced at day 21 ($p<0.001$) and day 28 ($p<0.001$) versus day 0. A549 cells exhibited a 2-fold decrease in krt5, which was significant at day 21 ($p<0.01$) and day 28 ($p<0.05$) relative to day 0. RPMI 2650 and Beas-2b cells displayed non-significant decreases in krt5 over 28 days compared to day 0.

In regard to tp63 (**Figure 5.2B**), HBEC-3KT cells exhibited a significant time-dependent decrease in tp63 expression at days 14 ($p<0.01$), 21 ($p<0.001$), and 28 ($p<0.001$) compared to day 0, similar to the reduction seen in krt5. Beas-2b and A549 cells showed no statistically significant change in tp63 gene expression over time. RPMI 2650 cells displayed a significant increase in tp63 expression at days 7 ($p<0.05$), 14 ($p<0.05$), and 21 ($p<0.05$) compared to day 0. However, by day 28 the difference versus day 0 was no longer significant.

For itga6 (**Figure 5.2C)**, in HBEC-3KT cells, itga6 expression showed a significant time-dependent decrease at day 7 ($p<0.05$), day 14 ($p<0.001$), day 21 ($p<0.001$), and day 28 ($p<0.001$) compared to day 0. Beas-2b cells exhibited a time-dependent decrease in tp63 expression that was only significant at day 28 ($p<0.01$) relative to day 0. RMPI 2650 cells showed no significant change in tp63 expression over time, although there was an apparent time-dependent increase. A549 cells displayed a time-dependent

increase in itga6 expression at day 7 ($p<0.05$), day 14 ($p<0.001$), day 21 ($p<0.001$) and day 28 ($p<0.01$) compared to day 0

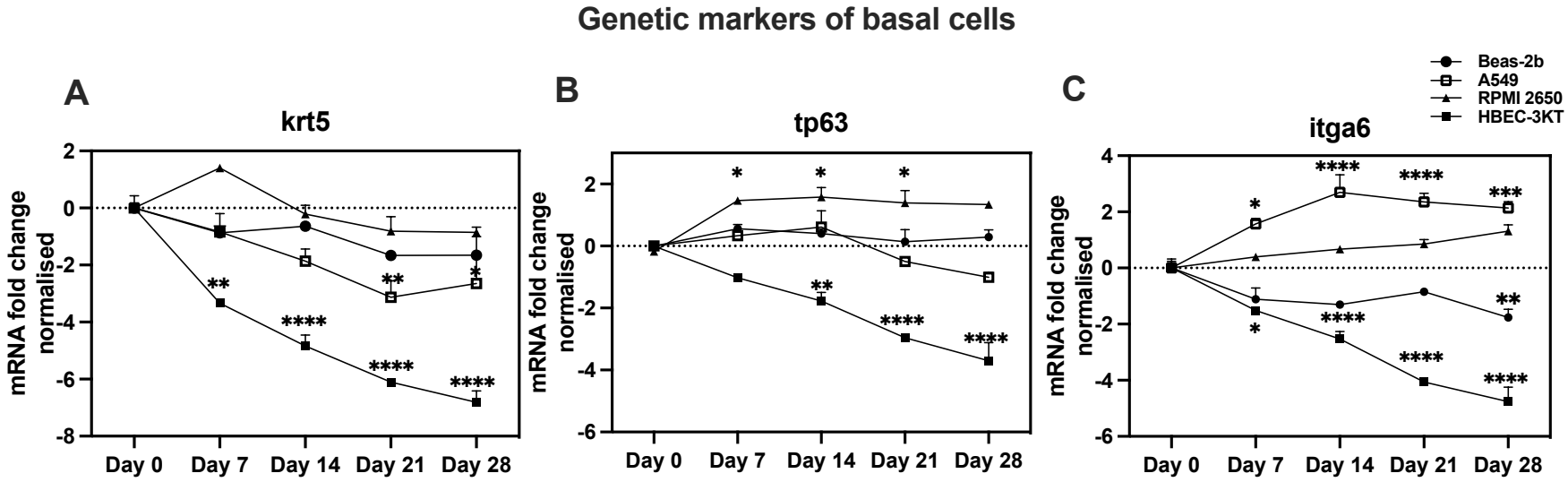


Figure 5. 2 Establishment of commercially available pulmonary cell lines epithelium using ALI cultivation. mRNA levels of genetic markers of differentiation A) krt5, B) tp63 and C) itga6 in HBEC-3KT, Beas-2b, A549 and RPMI 2650 were characterised every week for 28 days. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 per each day group for qPCR gene expression). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs day 0.

5.3.2 GENETIC MARKERS OF MUCUS-PRODUCING CELLS

Expression of mucin genes *muc5ac* was examined over 28 days across the 4 pulmonary cell lines: HBEC-3KT, Beas-2b, A549, and RPMI 2650. In Beas-2b cells, muc5ac expression significantly decreased at day 7 ($p<0.0001$) versus day 0, with no significant changes at other timepoints despite an apparent increase (**Figure 5.3A)**. RPMI 2650 cells displayed a significant increase in muc5ac expression at day 7 ($p<0.01$) and day 14 ($p<0.05$), but no significant change at day 21 or 28, though an apparent decrease was seen (**Figure 5.3A)**. Both HBEC-3KT and A459 cells exhibited significant increases in muc5ac mRNA across all days examined compared to day 0 ($p<0.0001$). At day 28, muc5ac expression in HBEC-3KT cells increased ~8-fold versus day 0 (**Figure 3.5A)**. In A549 cells at day 28, muc5ac increased ~ 4-fold versus day 0. Muc5ac expression was significantly upregulated over time in HBEC-3KT and A549 cells, transiently increased then normalized in RPMI 2650 cells, and initially declined then recovered in Beas-2b cells (**Figure 3.5A)**.

Expression of the muc5b was examined over 28 days in four pulmonary cell lines (**Figure 5.3B**). RPMI 2650 cells showed no statistically significant change in *muc5b* expression over time. The other three cell lines (HBEC-3KT, A549, and Beas-2b) exhibited significant increases in muc5b mRNA at all timepoints tested. HBEC-3KT displayed the highest fold-increase, with ~15-fold higher muc5b expression at day 28 vs day 0 (**Figure 5.3B**). A549 cells showed a ~5-fold increase in muc5b at day 28 compared to day 0. Beas-2b cells also had a ~5-fold change increase in muc5b expression at day 28 relative to day 0. Muc5b was significantly upregulated over 28 days in HBEC-3KT, A549, and Beas-2b cells, with the greatest increase in HBEC-3KT cells. Muc5b expression in RPMI 2650 cells remained unchanged over time

(**Figure 5.3B**).

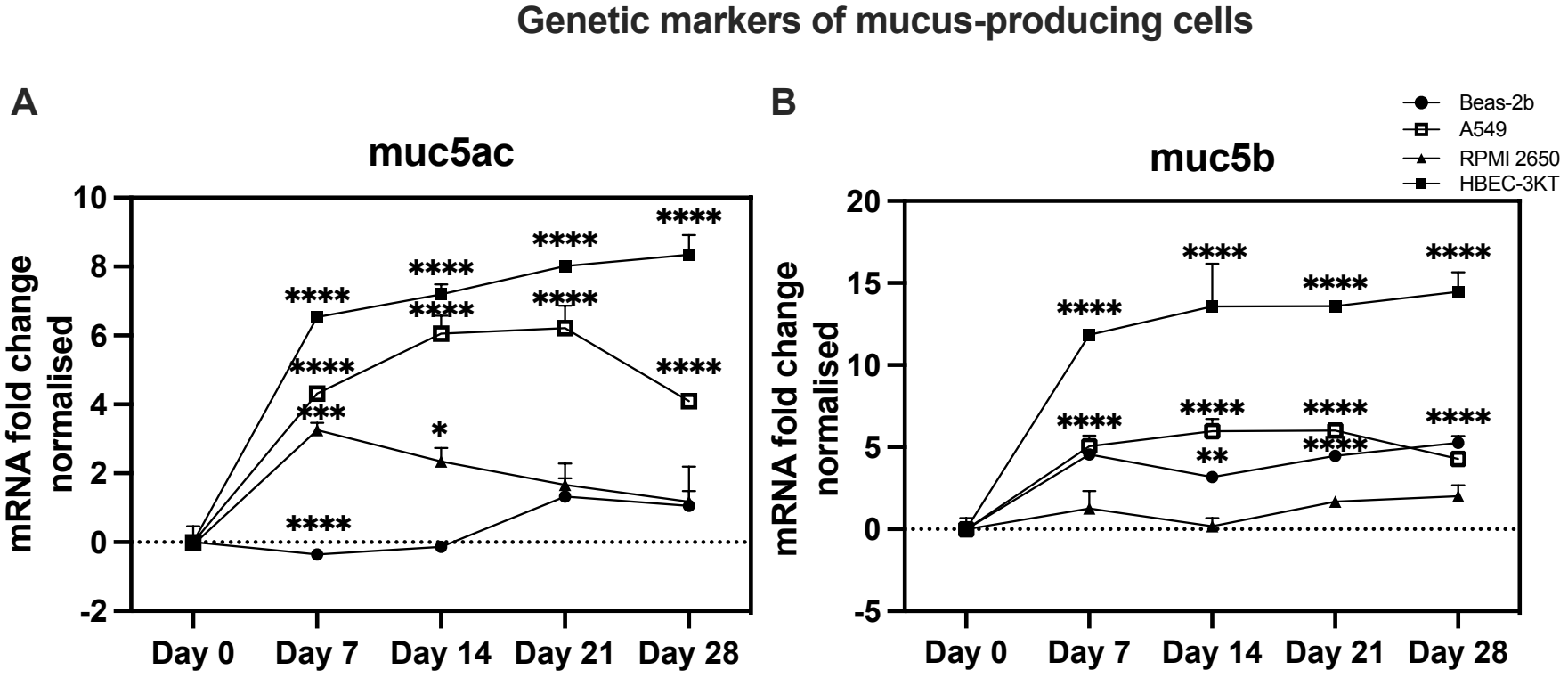

Genetic markers of mucus-producing cells
A
muc5ac
mRNA fold change normalised
10
8
6
4
2
0
-2
Day 0
Day 7
Day 14
Day 21
Day 28
B
muc5b
mRNA fold change normalised
20
15
10
5
0
-5
Beas-2b
A549
RPMI 2650
HBEC-3KT

Figure 5. 3 Establishment of commercially available pulmonary cell lines epithelium using ALI cultivation. mRNA levels of genetic markers of differentiation A) muc5ac and B) muc5b HBEC-3KT, Beas-2b, A549 and RPMI 2650 were characterised every week for 28 days. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 per each day group for qPCR gene expression). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs day 0.days. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 per each day group for qPCR gene expression). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs day 0.

5.3.3 GENETIC MARKERS OF CILIATED CELLS

Expression of the foxj1 gene was examined over 28 days in four cell lines: HBEC-3KT, Beas-2b, A549, and RPMI 2650 (**Figure 4.5**). We observed a time-dependent decrease in foxj1 in A549 cells albeit significant only at day 14 ($p<0.01$) and day 21 ($p<0.001$) vs day 0 (**Figure 4.5**). No changes occurred in RPMI 2650's foxj1 levels as it remained constant over the 28 days at ALI. Interestingly, both Beas-2b and HBEC-3KT cells significantly increased foxj1 levels as early as day 7 ($P<0.001$) and increased even more over the 28 days. HBEC-KT cells at day 28 increased foxj1 expression about 12-fold compared to day 0. In contrast, foxj1 levels increase in Beas-2b cells for about 5-fold at day 28 when compared to day 0 (**Figure 4.5**).

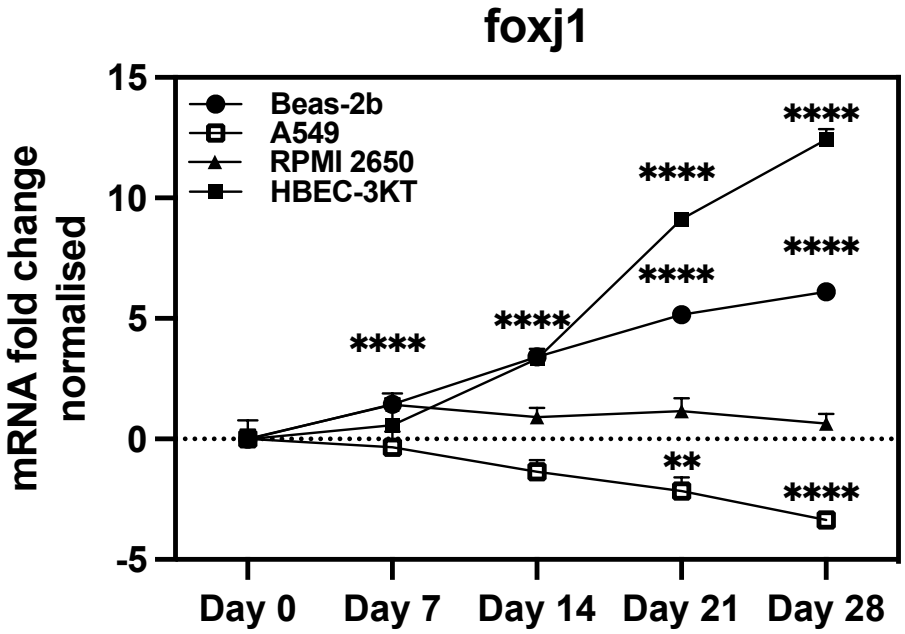


Figure 5. 4 Establishment of commercially available pulmonary cell lines epithelium using ALI cultivation. mRNA levels of genetic marker of ciliogenesis in HBEC-3KT, Beas-2b, A549 and RPMI 2650 was characterised every week for 28 days. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 per each day group for qPCR gene expression). **p<0.01 ***p<0.001; ****p<0.0001 vs day 0.

5.3.4 GENETIC MARKERS OF TIGHT JUNCTION PROTEIN

Expression of the tjp1 gene was examined over 28 days in four cell lines: HBEC-3KT, Beas-2b, A549, and RPMI 2650. HBEC-3KT cells exhibited a time-dependent decrease in tjp1 mRNA levels that reached significance at day 28 ($p<0.001$) compared to day 0 **(Figure 5.5**). In contrast, tjp1 expression in A549, RPMI 2650, and Beas-2b cells remained fairly consistent over the 28-day period. No significant changes in tjp1 mRNA levels were observed in A549, RPMI 2650, or Beas-2b cells across the time course.

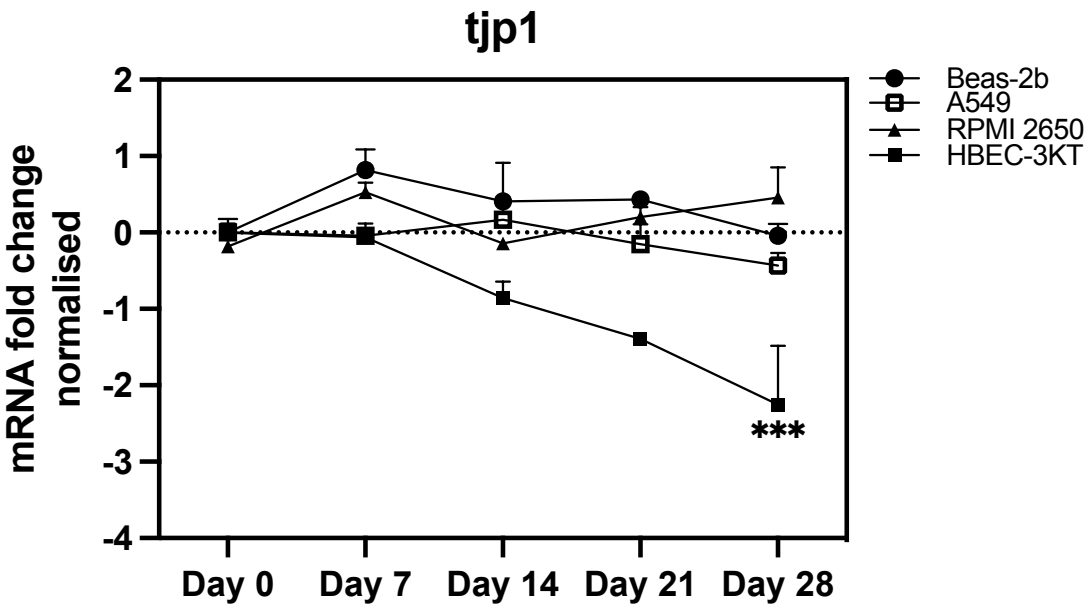


Figure 5. 5 Establishment of commercially available pulmonary cell lines epithelium using ALI cultivation. mRNA levels of genetic marker of tight junction protein 1 in HBEC-3KT, Beas-2b, A549 and RPMI 2650 was characterised every week for 28 days. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 per each day group for qPCR gene expression). ***p<0.001 vs day 0.

5.3.5 TIGHT JUNCTION FORMATION

In this study, we assessed the changes in transepithelial electrical resistance (TEER) values for HBEC-3KT, RPMI 2650, Beas-2b, and A549 cells at air-liquid interface (ALI) over a 28-day period. TEER is a widely accepted quantitative technique to measure the integrity of tight junctions and evaluate the barrier function of epithelial cells. We observed that A549, Beas-2b, and RPMI 2650 cells did not show any significant difference in TEER values over the 28 days, they maintained a TEER value between 100- 250 $\Omega.cm^2$ indicating a stable barrier integrity for these cell lines. However, HBEC-3KT cells, although initially showing no significant difference in

TEER compared to day 0, exhibited a significant increase in TEER from day 14 to 21 ($p<0.001$), which further increased at day 28 ($p<0.001$). These findings suggest that HBEC-3KT cells undergo maturation and develop a more robust barrier function over time at ALI, while the other cell lines maintain a stable barrier integrity. The observed increase in TEER values for HBEC-3KT cells may be attributed to the development of tight junctions and improved cell-cell interactions, leading to a more effective barrier against paracellular transport

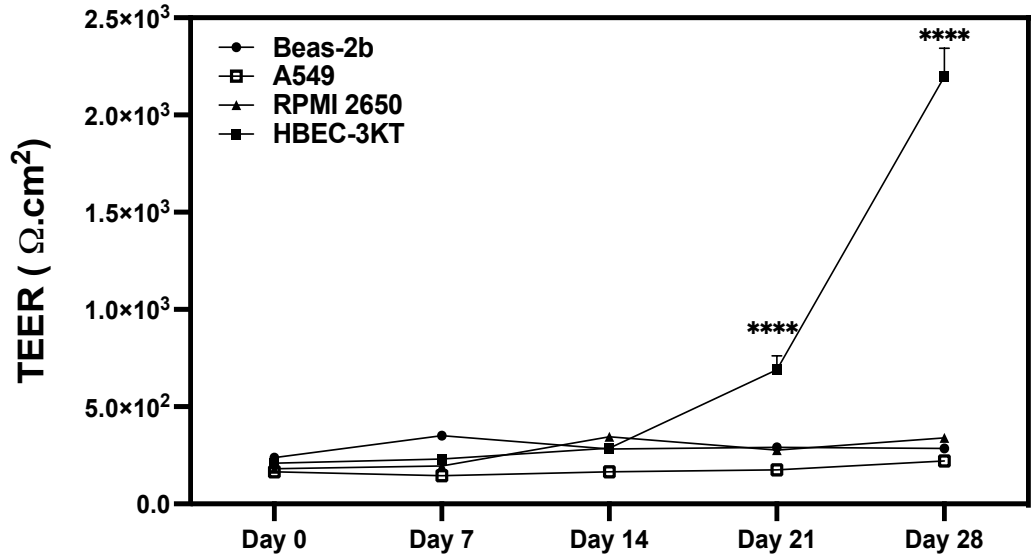


Figure 5. 6 Establishment of commercially available pulmonary cell lines Beas-2b, A549, RPMI 2650 and HBEC-3KT epithelium using ALI cultivation. TEER measurements were carried out in each cell line every week for 4 weeks. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=6 per each day group). ****p<0.0001 vs day 0.

5.3.6 THE MORPHOLOGICAL CHANGES IN HBEC-3KT, BEAS-2B, A549 AND RPMI 2650 OVER 28 DAYS AT ALI

The morphological changes that occur in images of HBEC-3KT (**Figure 5.7**), Beas-2b (**Figure 5.8**) , A549 (**Figure 5.9**), and RPMI 2650 (**Figure 5.10**) cultured at the air-liquid interface (ALI) from day 0 to day 14 and day 28 can provide insights into their differentiation and functional properties. At the beginning of the ALI culture, on day 0, HBECs appeared as a monolayer of cells with a flattened morphology, almost similar to that of 2D cultures (**Figure 5.7**) unlike Beas-2b, A549 and RPMI 2650 that showed cells growing on top of each other. From day 0, the cells are in contact with the culture medium at the basal side and exposed to air at the apical side, mimicking the *in vivo* airway epithelium. By day 14, the mucociliary differentiation of HBECs at the ALI became more pronounced with increased mucus production (**Figure 5.7**). In contrast, this is not a phenomenon easily observed under light microscopy due to the thick overcrowding layer of cells preventing the penetration of light. HBEC-3KT cells exhibit a more columnar and polarised morphology, resembling the *in vivo* airway epithelium. After four weeks of ALI culture, the HBECs continue to mature and maintain their differentiated phenotype (**Figure 5.6)**. In contrast, the other cell lines' layer grew even thicker. Morphologically, HBEC-3KT cells at day 28 was critically different from day 0 and 14 providing a functional and structurally relevant model for studying airway biology and diseases

.

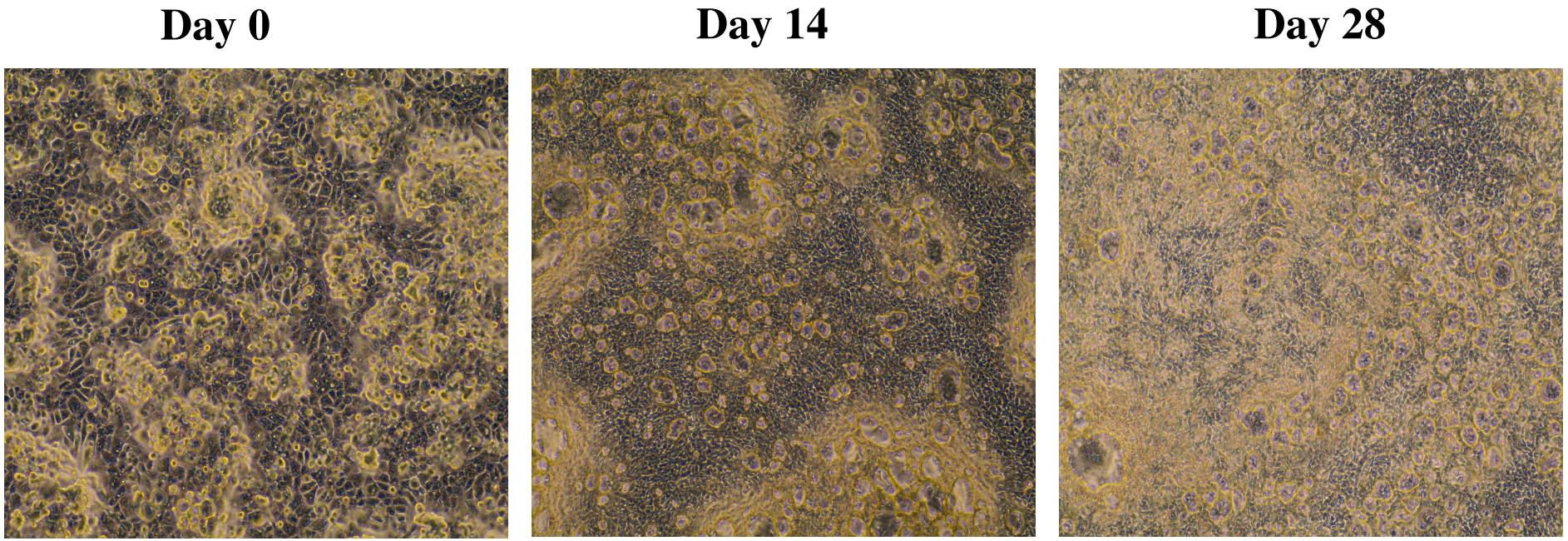


Figure 5. 7 Light microscopy images of HBEC-3KT taken on day 0, 14 and 28 with ImageXpress Pico at 10X. Genetic analysis for genes of differentiation together with morphological observation underpinned the choice to select HBEC-3KT as the most appropriate model for the flavoured aerosol exposures in Chapter 6.

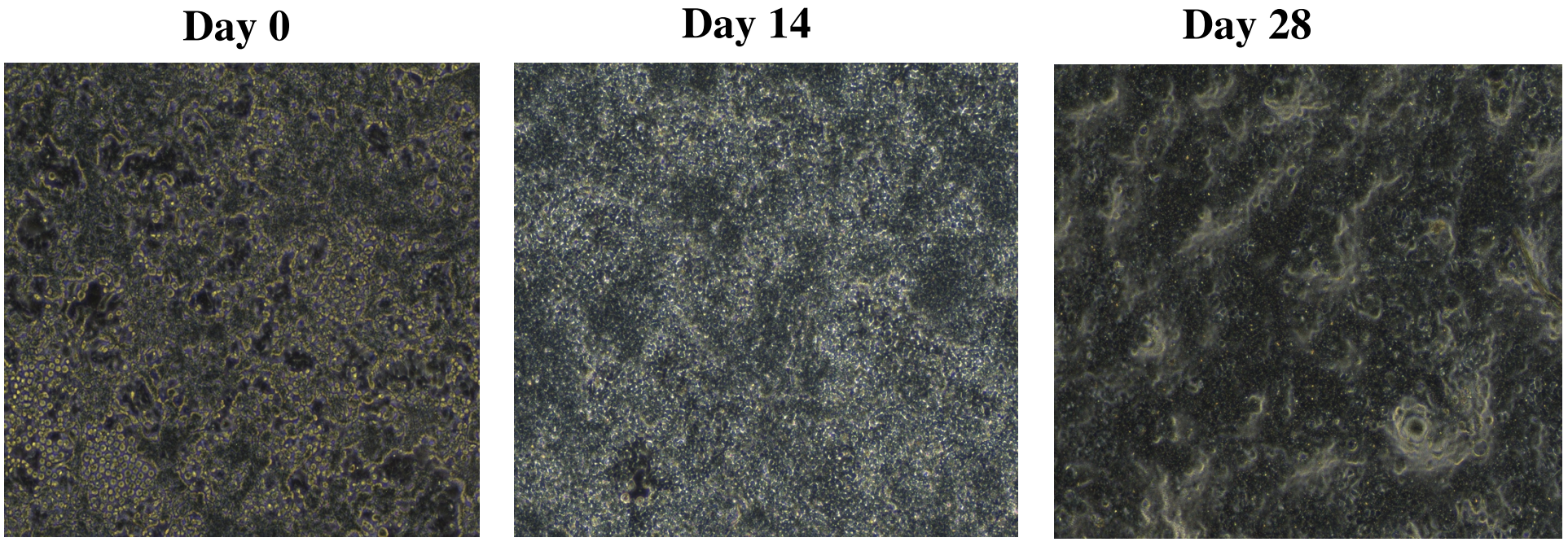


Figure 5. 8 Light microscopy images of Beas-2b cells taken on day 0, 14 and 28 with ImageXpress Pico at 10X.

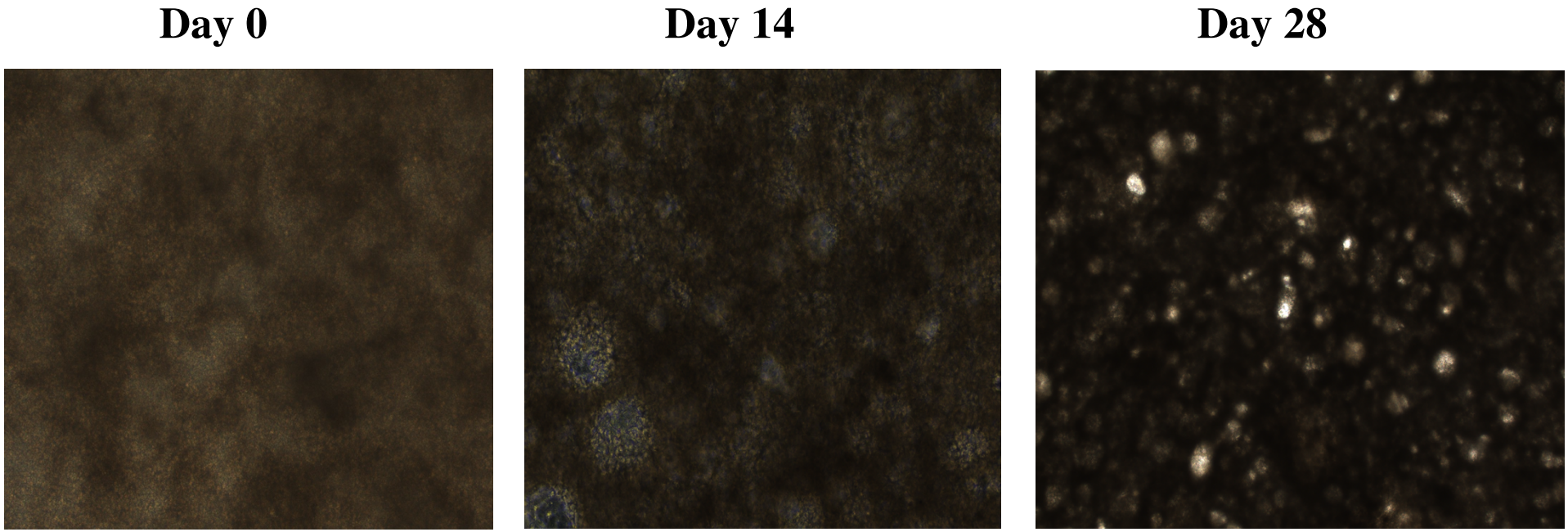


Figure 5. 9 Light microscopy images of A549 cells taken on day 0, 14 and 28 with ImageXpress Pico at 10X.

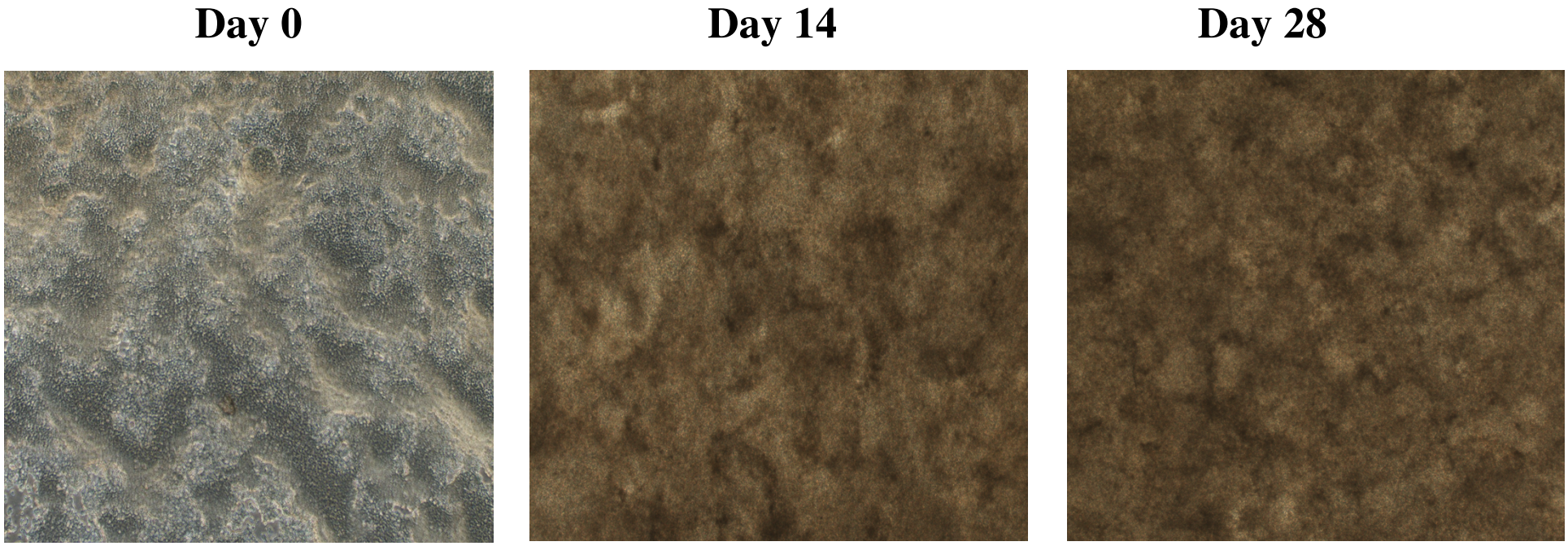


Figure 5. 10 Light microscopy images of RPMI 2650 cells taken on day 0, 14 and 28 with ImageXpress Pico at 10X.

5.4 DISCUSSION

This study aimed to evaluate four commercially available cell lines commonly used in toxico-pharmacological investigations in academia and the pharmaceutical industry. The cell lines under investigation were Beas-2b, HBEC-3K, RPMI 2650, and A549. Although there are studies in which each of these cell lines have been validated at ALI for different days, there is no study where all four cell lines have been evaluated and compared to each other. The objective was to determine which of these cell lines could best mimic the human lung microenvironment and, at the same time, be sensitive enough to predict the potential toxicity of aerosols from flavoured e-liquids to humans. We assessed various genetic markers of airway differentiation, including basal cells (krt5, tp63, itga6), mucus-producing cells (muc5ac and muc5b), tight junction protein (tjp1), and ciliated cells (foxj1). Additionally, the formation of tight junctions was measured by evaluating the trans-epithelial electrical resistance (TEER) values. This study was conducted in response to the lack of sensitivity observed in the Calu-3 cells used in **Chapter 4**.

The comprehensive analysis of the gene expression dynamics of krt5, tp63, itga6, muc5ac, muc5b, and tjp1, as well as the assessment of transepithelial electrical resistance (TEER) in Beas-2B, RPMI 2650, A549, and HBEC-3KT cell lines, provided pivotal insights into their distinctive cellular behaviours and responsiveness.

The decline of KRT5 protein expression across all cell lines, particularly pronounced in HBEC-3KT cells, echoes findings from Peters-Hall *et al.*, (2018), who highlighted the decline of krt5 expression may be associated with the transition of basal cells to more differentiated cell types, such as ciliated or goblet cells. HBEC-3KT's pronounced 6-fold decrease underscores its potential role in studying cellular dynamics under various

stress conditions, including exposure to aerosol toxicants (Delgado *et al.*, 2011). The non-significant decreases in RPMI 2650 and Beas-2B align with observation of these cell lines' relative resilience. This may be attributed to several factors including retinoic acid (RA) signalling. Krt5 gene expression has been shown to be negatively regulated by RA signalinghttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC5348080/ (Abashev *et al.*, 2017). The non-significant decreases in krt5 expression in RPMI 2650 and Beas-2B cells may be due to the absence or low levels of RA signaling in these cell lines, which could contribute to their relative resilience. However, this hypothesis warrants further investigation.

The specific implications of a decline or increase in the tp63 gene in cells grown at the ALI are not well-documented. However, Arason *et al.*, (2014) reported that in a tp63 knocked-out HBECs, a pseudostratified epithelium failed to form. However, understanding the general role of the TP63 gene and its protein product, tumor protein p63, can provide some insights. TP63 has been linked to chromatin remodeling and enhancer reprogramming in epidermal differentiation and squamous cell carcinoma development (Yi *et al.*, 2020). This suggests a potential role for TP63 in regulating cellular processes related to epithelial differentiation. Therefore, the differential expression of this gene in the pulmonary cell lines may be linked to more intricate factors like pulmonary provenance and nature (whether cancerous or immortalised).

The decline in the ITGA6 gene in immortalized cells Beas-2B and HBEC-3KT, and the increase in this gene in the cancerous cell lines A549 and RPMI 2650, may have different implications due to the distinct characteristics and behaviors of these cell lines. ITGA6 encodes the alpha-6 integrin subunit, which is involved in cell adhesion and signaling (Hu *et al.*, 2016). The decline in ITGA6 gene expression in Beas-2B and HBEC-3KT

cells grown at ALI may indicate a shift towards a more differentiated state, as integrin expression is often associated with undifferentiated or proliferative cells (Yang *et al.*, 2019). In contrast, the increase in ITGA6 gene expression in A549 and RPMI 2650 cancerous cell lines may reflect their more aggressive and less differentiated nature, as integrins are known to play a role in cancer cell invasion and metastasis (Hamidi and Ivaska, 2018).

Cilia and mucociliary clearance are the most important physiological defense mechanism of the airway epithelium, affecting nasal and pulmonary penetration of inhalation toxicants (Gizurarson, 2015). We observed no cilia in any of the pulmonary cells investigated. However, there is evidence to suggest that RPMI 2650 at ALI do not produce cilia (Salib *et al.*, 2005; Silva *et al.*, 2023). Therefore, the no-change in foxj1 we observed over the 28 days was not surprising. In contrast,

There are conflicting reports on whether A549 cells grown at the air-liquid interface (ALI) produce cilia. Some sources suggest that A549 cells do not form cilia under ALI conditions (Silva *et al.*, 2023), while others mention that these cells can replicate functional properties of primary cells, which may include cilia production (Munis *et al.*, 2021). The significant downregulation of foxj1 in A549 in our study and the lack of visible cilia beat agrees with Silva et al.

In contrast to the cancerous cells, because HBEC-3KT and Beas-2b cells are immortalised, we expected them to produce cilia at ALI. Surprisingly both cell lines lacked physical cilia beat but showed a time-dependent upregulation of foxj1 compared to day 0, suggestive of ciliogenesis. Evidence suggest that neither Beas-2b or HBEC-3KT cells grown at ALI produce cilia (Delgado *et al.*, 2011; Stewart *et al.*, 2012). It is

unclear why these cells differentiate genetically but fail to physically differentiate. Other than cilia, the presence of cilia is paramount for mucociliary clearance to occur. Mucus, produced by goblet cells and submucosal glands, is a viscoelastic gel formed by water, salts, high molecular weight glycoproteins (mucins), other proteins (i.e., lysozyme, lactotransferrin, secretory immunoglobulins and albumin) and lipids (Silva *et al.*, 2023). According to the Human Genome Organization gene nomenclature committee, to date, 21 mucin-type glycoproteins (*MUC* gene family) were identified (Wagner *et al.*, 2018). Here, we have assessed the changes that occur over 28 days in two of the 21 genes that encode mucin-type glycoproteins, namely muc5ac and muc5b genes, in HBEC-3KT, RPMI 2650, A549 and HBEC-3KT. The muc5ac expression patterns revealed in our study correlate with the evidence provided in **Chapter 4** and existing literature emphasising the differentiation of goblet cells and mucus-producing cells in basal cells cultured at ALI (Ji *et al.*, 2022b; Silva *et al.*, 2023; Young *et al.*, 2007). MUC5AC and MUC5B proteins have been stained in HBEC-3KT cells and A549 cells grown at ALI (Liegeois and Fahy, 2022; Okuda *et al.*, 2019; Silva *et al.*, 2023; Wu *et al.*, 2018). Moreover, a study conducted by Liegeois and Fahy, (2022) reveals that the presence of MUC5B is a prerequisite for a physiological lung function. Therefore, the significant time-dependent increase in muc5ac and muc5b gene in HBEC-3KT and A549 cells, and the time-dependent increase in muc5b in Beas-2b, as early as the first week at ALI as well as visible mucus covering the apical of the epithelium, indicates that these the three cell lines recapitulate the *in vivo* airway epithelium's microenvironment. RPMI 2650 cell showed no change in muc5b across the 28-day period; however, it increased muc5ac gene in the first two weeks at ALI, followed by a decline. This pattern in muc5ac gene may explain the low levels of MUC5AC protein in RPMI 2650 cells grown at day 21 vs porcine respiratory primary cells grown at ALI for 21 days (Ladel *et al.*, 2019). We

observed a downregulation of muc5ac gene in Beas-2b compared to the other cell lines. This evidence is in concordance Stewart *et al.*, (2012) who showed that Beas-2b cells at ALI do not express MUC5AC, suggesting an impairment in their mucociliary clearance ability. Taken together, this suggests that mucin production is expressed heterogeneously by airway epithelial cells.

For tight junction formation and membrane integrity evaluation, the lack of change in tjp1 gene expression over 28 days in A549, RPMI 2650, and Beas-2b correlates with the consistent TEER measurements over the 28-day period. This evidence is agreement with Silva *et al.*, (2023) who reported that A549, RPMI 2650, and Beas-2b cells at ALI do not form robust tight junction irrespective of the length of culture time. However, for HBEC-3KT cells, we observed that whiles there was a significant decrease in tjp1 gene, there was an increase in TEER values, showing a temporal disconnection. It is unclear why this phenomenon occurred; however, this may be due to post-transcriptional regulation. Gene expression is a multi-step process that involves transcription (the synthesis of mRNA from DNA) and translation (the synthesis of protein from mRNA). While gene expression can be regulated at the transcriptional level, it can also be regulated at the post-transcriptional level, such as mRNA stability, processing, and transport (Bryois et al., 2017). In some cases, a decrease in gene expression may be compensated by increased mRNA stability or enhanced translation efficiency, leading to an increase in protein levels. Another possibility may be stochasticity in gene networks. Gene expression is a complex and stochastic process, and time-dependent changes in protein levels can be influenced by the inherent stochasticity of gene networks (Innocentini *et al.*, 2018). Stochastic mRNA and protein synthesis can lead to time-dependent fluctuations in protein levels, even in the absence of significant changes in gene expression. These are hypotheses currently under investigation in our lab.

5.4.1 CONCLUSIONS

With aerosol toxicants posing significant health risks, selecting an appropriate cell line for studying their impacts is critical. As hypothesised, there is clearly differential expression of markers of ALI differentiation in the 4 cell lines. However, A549 and HBEC-3KT cells among the 4 cell lines revealed as potential candidates for our inhalation toxicology study. Our study concords with the evidence that HBEC-3KT cells possess a multipotent capacity to differentiate into several cell types of the lung epithelium, they have a dynamic gene expression (Delgado *et al.*, 2011). They also enhanced barrier function over time which suggest their suitability for assessing long-term impacts of aerosol exposure. In contrast, A549's moderate gene expression changes indicate its balanced responsiveness, making it a suitable model for capturing realistic in vivo-like responses without overstating effects (Wu *et al.*, 2018). Nonetheless, the suitability of this cell line is controversial because 95 % of alveolar epithelial surface cells are type I, instead of type II, as referred in **section 5.2**. A549 cells were initially considered inappropriate to detect chemically-induced toxicity, due to an inability to distinguish pulmonary toxins from non-toxins (O'Brien *et al.*, 1987; Winton *et al.*, 1998). Also, because A549 cells are cancerous cells and do not recapitulate normal lung microenvironment, they may not be as sensitive as HBEC-3KT cells in predicting EC aerosol toxicity (**Figure 5.6**).

Whereas primary HBECs currently under investigation in our laboratory (data not shown in this thesis) show a thorough recapitulation of the bronchial microenvironment with visible cilia beat as well as mucus production, our immortalised HBECs, Beas-2b and HBEC-3KTs did not show cilia beat. However, we chose HBEC-3KT cells as the most suitable and sensitive model to take forward for our EC inhalation toxicology study in **Chapter 6**.

CHAPTER 6

UTILISING THE CULTEX EXPOSURE SYSTEM TO ASSESS THE TOXICITY ASSOCIATED WITH FLAVOURS IN E-CIGARETTE AEROSOLS: A PHYSIOLOGICALLY RELEVANT APPROACH USING 3D HBEC-3KT LUNG MODELS

6.1 INTRODUCTION

ALI cell culture models have become essential tools in toxicology and environmental research, enabling investigation into the effects of airborne substances on human health (see **Chapters 4** and **5** for thorough review). Due to its unique design and capabilities, the CULTEX system has emerged as a prominent choice of ALI exposure system (Aufderheide *et al.*, 2013). The CULTEX system closely mimics the physiological conditions encountered by cells in the respiratory tract and other barrier tissues. The ALI setup allows cells to be exposed directly to airborne substances while maintaining an interface with the culture medium, mimicking the *in vivo* microenvironment (Aufderheide *et al.*, 2017). ALI cultures allow for high-throughput screening of multiple substances with relatively small quantities, reducing the need for extensive animal testing and the associated ethical concerns (Guénette *et al.*, 2022; Leung *et al.*, 2020). The CULTEX system offers precise control over exposure parameters, such as airflow rates, temperature, and humidity, maintaining consistent and reproducible experimental conditions (Aufderheide *et al.*, 2017). This level of control is crucial in enhancing the model's predictive power and facilitates the comparison of results between different studies.

There are growing concerns about the safety profiling of flavours in EC (**Chapter 1**). Although some evidence suggests that EC aerosols may induce the release of pro-inflammatory cytokines, including TNF-alpha, IL-8, and IL-10, there are discrepancies between studies (**Chapter 2**). As such, we utilised the CULTEX system to expose differentiated HBEC-3KT to aerosols of the 3 toxic e-liquids identified in **Chapter 3** (cinnamon, hazelnut, and vanilla tobacco) as demonstrating toxicity above that of PG/VG controls and menthol flavoured e-liquids *in vitro*. As stated in **Chapter 3** & **4**,

we observed a peculiar characteristic of menthol. Therefore, we assessed the effects of menthol in a close system of exposure with the CULTEX to prevent evaporation.

6.1.2 AIMS AND HYPOTHESIS

This study aims to expose differentiated HBEC-3KT cells to 3 different doses (28, 42 and 56 puffs) of cinnamon, hazelnut, vanilla tobacco, and menthol flavoured aerosols to assess their dose-dependent pulmonary toxicity.

6.1.3 THE HYPOTHESES:

- Cinnamon flavour will elicit the greatest cytotoxicity (increased LDH and reduced TEER measurements) among the flavours tested
- Cigarette smoke condensate will yield greater cytotoxicity than the flavoured e-liquids
- Particle size distribution of flavours will correlate with their toxicity, with smaller particles associated with greater toxicity.
- Changes to inflammatory cytokine mRNA and protein levels correlate with flavour-induced toxicity.

6.1.4 THE OBJECTIVES:

- To mimic human flavoured aerosol exposure by exposing HBEC-3KT cells differentiated at ALI for 14 days to EC aerosols using the CULTEX inhalation system.
- To assess dose-dependent cellular toxicity of EC aerosols with end-point assays such as LDH and TEER measurements using Evo metre.

- To assess the dose-dependent effects of flavoured EC aerosols on expression of genetic markers of airway differentiation, cytokines, DNA damage and oxidative stress by using qRT-PCR analysis.
- To assess the dose-dependent effects of the flavoured EC aerosols on protein levels of cytokines (including IL-1β, TNF-α, IL-6 and IL-10), DNA damage marker (8-OHdG), and cytotoxicity (Galectin-3).

6.2 MATERIALS AND METHODS

6.2.1 CELL CULTURE

Cell culture procedure for HBEC-3KT in **Chapter 5, section 5.2** was used to differentiate HBEC-3KT cells at ALI in this chapter. ALI cultures were maintained for up to 14 days.

6.2.2 AEROSOL EXPOSURE AT AIR LIQUID INTERFACE (AE-ALI) EXPOSURE SYSTEM

Aerosols were produced using a 3rd-generation E-Cigarette Aerosol Generator (E-CAG, Innokin T18E), which utilises positive pressure and coil voltage control to generate realistic aerosols with a customisable puff profile from e-cigarette liquid in particle-free air using commercial EC tanks. All aerosols were produced using a 3rd-generation EC refillable tanks (Innokin T18E) containing freshly filled e-liquid and the CORESTA vaping cycle (3 s puff of 55mL at 30 s puff interval) and a coil voltage of 3.7 V using new coils. To produce the 55 ml puff, the flowrate of air pushed through the EC was set to 1.1 L/min using a Gilian® Gilibrator® 3 air flow calibrator (Sensidyne, St Petersburg, Florida, USA). A separate tank was used for each e-liquid, details of e-liquids used are given in **Table 6.1**.

The EC aerosols were puffed into a continuous stream of particle-free, humidified dilution air at 1.1 L/min spiked with 5% CO_2. The CO_2 percentage in the dilution gas was monitored continuously using a CARBOCAP® carbon dioxide probe (GMP251(check number), Vaisala, Vantaa, Finland) with the CO2 flow adjusted if necessary to maintain the desired concentration. The relative humidity (RH) and the dilution gas temperature were also continuously measured using a Humidity and Temperature Meter, (model HMT330, Vaisala, Vantaa, Finland). Throughout all exposures, relative humidity was > 75% and temperatures were between 21 and 22˚C.

Table 6. 1 Details of the experimental design, e-liquids used per exposure and the amount of aerosols deposited onto cells in the CULTEX.

Flavour	Repeat	Wells exposed n	Time min	Puffs	EC liquid usage mg/puff	Av. PEAK PNC #/cm^3	Std. Dev	Avg. PNC #/cm^3	Std. Dev	Deposited dose ug/cm^2	Deposited dose rate ug/cm^2/puff
Air only control	1	6	14	n/a	n/a	n/a	n/a	4.00E+00	5.70E+01	0	0
	2	6	21	n/a	n/a	n/a	n/a	1.00E+00	9.00E+00	0	0
	3	6	28	n/a	n/a	n/a	n/a	2.00E+00	3.50E+01	0	0
PG/VG	1	6	14	28	2.067	3.92E+08	6.27E+07	5.70E+07	1.10E+08	6.22	0.22

	2	6	21	42	2.159	2.04E+08	6.29E+07	3.06E+07	6.01E+07	7.74	0.18
	3	6	28	56	1.737	1.99E+08	5.92E+07	3.02E+07	5.80E+07	10.11	0.18
Vanilla tobacco	1	6	14	28	3.200	4.30E+08	2.86E+07	6.63E+07	1.25E+08	11.62	0.42
	2	6	21	42	4.136	4.48E+08	3.55E+07	6.93E+07	1.32E+08	27.25	0.68
	3	6	28	56	4.136	6.39E+08	4.28E+07	9.86E+07	1.90E+08	42.14	0.75

Cinnamon	1	6	14	28	4.000	4.45E+08	8.51E+07	7.03E+07	1.35E+08	14.62	0.52
	2	6	21	42	5.552	4.43E+08	1.23E+08	6.53E+07	1.35E+08	21.36	0.70
	3	6	28	56	4.500	5.17E+08	6.09E+08	7.94E+07	1.52E+08	39.25	0.55
Hazelnut Latte	1	6	14	28	1.533	3.13E+08	7.26E+07	4.46E+07	8.89E+07	1.04	0.04
	2	6	21	42	0.909	3.36E+08	6.95E+07	4.68E+07	9.53E+07	2.15	0.03

	3	6	28	56	0.678	1.71E+08	5.55E+07	2.38E+07	4.85E+07	2.07	0.01
Menthol	1	6	14	28	3.667	4.49E+08	3.08E+07	6.86E+07	1.31E+08	22.92	0.82
	2	6	21	42	4.205	3.76E+08	6.10E+07	5.83E+07	1.13E+08	35.67	0.85
	3	6	28	56	3.948	5.80E+08	3.24E+07	8.67E+07	1.69E+08	51.11	0.91

6.2.3 TIME OF EXPOSURE & SYSTEM OPTIMISATION

Firstly, we carried out optimisation for the exposure time at the CULTEX HBEC-3KT grown at ALI were exposed to increasing periods (5, 10, 15, 30, 60, 15 min) of lab-filtered air or 15 min of lab-filtered air with the electrostatic precipitator turned on. We observed that apart from lab-filtered air with the electrostatic precipitator, there were no significant differences in either the cytotoxic biomarkers (LDH or TEER), the genetic markers of airway differentiation (*foxj1, tjp1, and muc5ac*) or in genetic markers of cellular stress (*hmox1 and ddit3*).

6.2.4 HBEC-3KT EXPOSURES TO AEROSOLS

For cell exposures, the conditioned aerosol was delivered to a CULTEX® Radial Flow System-Compact (RFS-C) (Cultex Technology GmbH, Hanover, Germany). The CULTEX© RFS-C is a 6-well *in vitro* exposure system designed for the homogeneous exposure of cultivated cells at ALI to aerosols and gases. Detailed descriptions and validation of the CULTEX© radial flow systems can be found in (Aufderheide *et al.*, 2013, 2017; Tsoutsoulopoulos *et al.*, 2019). Both the sampling module containing the wells and the aerosol guiding module above are heated to 37°C. Cells Airway epithelial cell basal medium (5 mL) was added to each of the 6 wells and preconditioned for 5 min at 37 ^{0}C, 95% humidity, and 5% CO2 before placement of the cell-populated transwell inserts within the sampling module, and the unit assembled ready for exposure (**Figure 6.3 & Figure 6.4**)

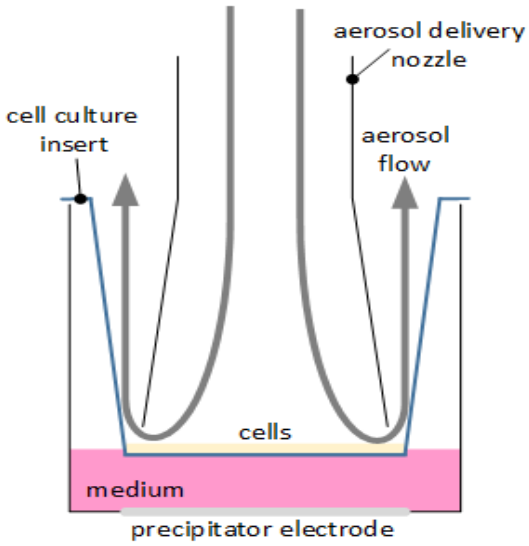


Figure 6. 1 A diagram of the "directed flow" ALI exposure technique which is utilised in the CULTEX. In this study, we did not use the electrostatic precipitator in the aerosol exposure study.

Within the CULTEX RFS-C, the continuous aerosol flow is directed to each well in the sampling module individually, being drawn vertically/downwards towards and over the cells by the aerosol guiding module, the flow pattern of which is designed to maximise contact (and therefore deposition) between the test aerosol and cells (**Figure 6.1**). While the CULTEX RFS-C is equipped with an electrostatic precipitator to enhance deposition, this was not used due to sufficient dosing being achieved without it and the changes in LDH and TEER induced by its use (**Figure 6.7**). To minimise any negative effects of the flow being directed towards the cells, the aerosol flowrate to each well was set to 5 ml/min, controlled individually using mass flow controllers (MFC-1-3: iQ-flow, MCF: EL-Flow, Bronkhorst, Netherlands). The excess aerosol flow was passed through a bypass aerosol line to the aerosol characterisation equipment and exhaust. The balance of the aerosol not directed to the characterisation equipment is drawn through the system using a vacuum pump, the flowrate of which was controlled using an MFC (MFC-1-3:

iQ-flow, MCF: EL-Flow, Bronkhorst, Netherlands) and set to balance the flows being pushed into and drawn through and to minimise pressure in the CULTEX (**Figure 6.3 and 6.4**). All exhausts were filtered through carbon and HEPA filter capsules and exhausted into a fume hood.

6.2.5 AEROSOL CHARACTERISTICS

6.2.5.1 AEROSOL PARTICLE NUMBER CONCENTRATION (PNC) MEASUREMENT

Real-time aerosol particle number concentration (PNC) measurements and average aerosol mass concentration measurements were collected during each exposure. Separate measurements of a typical aerosol size distribution for each of the EC flavours investigated were also made. Aerosol PNC with 1 s resolution was measured using a condensation particle counter (CPC, Model 3775, TSI Inc., Minnesota, USA). Due to the high concentration of the aerosol, the aerosol was diluted prior to measurement using a Palas VKL 100 dilution system (Palas, Karlsruhe, Germany). Measurement of the dilution ratio was carried out using laboratory air pre- and post- each exposure set. The average dilution ratio across all exposures was 225 ± 25, which was used to adjust all PNCs reported. The average aerosol mass concentration was determined gravimetrically. The aerosol was drawn through 47mm glass microfibre filters (Pallflex® EMFAB^{TM} filters, TX40HI20WW) held in a stainless steel 47mm inline filter holder (Cytiva (formally Pall Lab)) at 0.2 lpm using a Gilian GilAir Plus Personal Air Sampling Pump (Sensidyne, St Petersburg, Florida, USA) for the duration of the exposure from a tee piece at the bypass aerosol exhaust of the CULTEX RFS-C. Due to the volatility and hygroscopicity of the EC aerosols, the standard method of measuring the deposited mass after equilibrating in atmospheric conditions for 24 hours was not

possible, therefore the "deposited" aerosol mass concentration was determined by weighing filter as soon as the exposure ended. Aerosol mass concentration, Cm (mg/m^3) is calculated (**Table 6.1**) from the total mass deposited on the filter, M (mg) and volume of air drawn through the filter:

V (m3) using $C_m = M/V$.

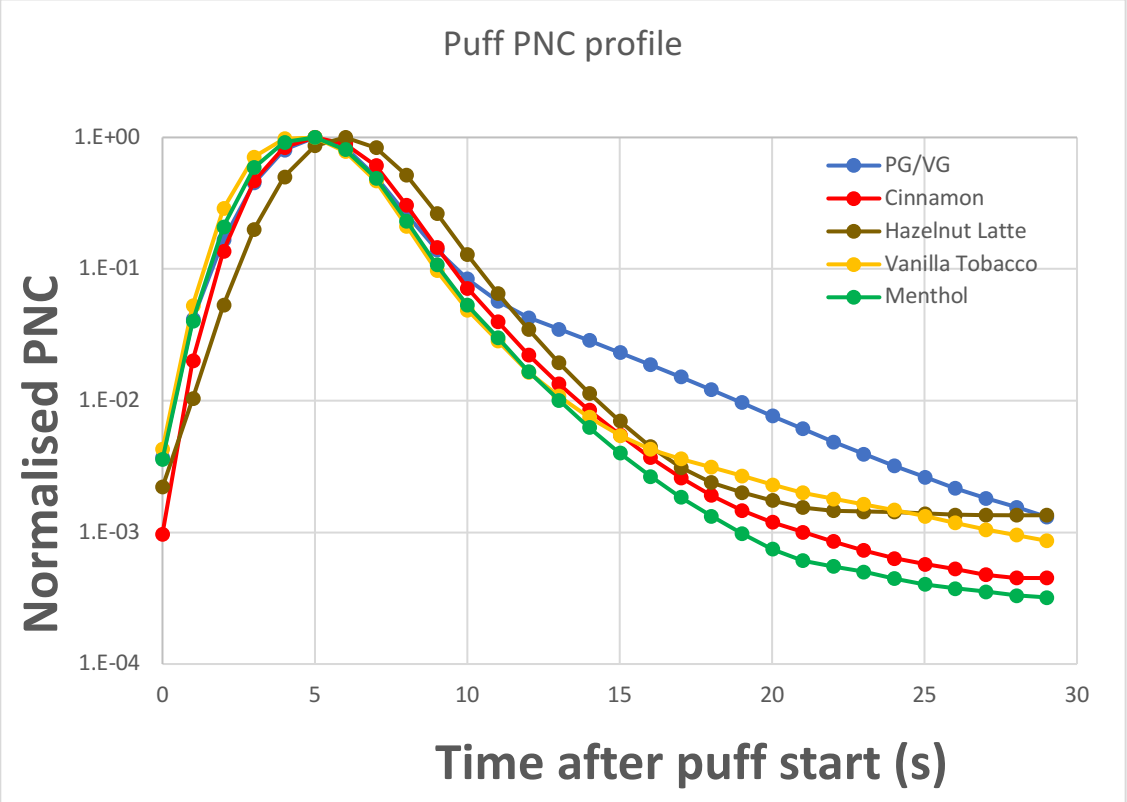


Figure 6. 2 Typical puff particle number (PNC) count profile for PG/VG, cinnamon, hazelnut, vanilla tobacco, and menthol.

6.2.5.2 AEROSOL SIZE DISTRIBUTION MEASUREMENT

Representative number-weighted mobility equivalent size distribution measurements of the aerosol produced by each EC liquid investigated were measured using a Differential Mobility Spectrometer (Cambustion DMS500 Mk2). Due to the high flow required by the instrument, these measurements were made separately from the exposures. The DMS500 has a measurement size range of 5 – 1000 nm and a time response of 10 Hz (measurements every 0.1 s) allowing the short, 3 s puff profile to be measured. Due to the high concentration of the EC aerosols, internal dilution of 12:1 was required which has been corrected for in the measurements reported. For each of the 4 flavours investigated, 3 sequential puffs were measured.

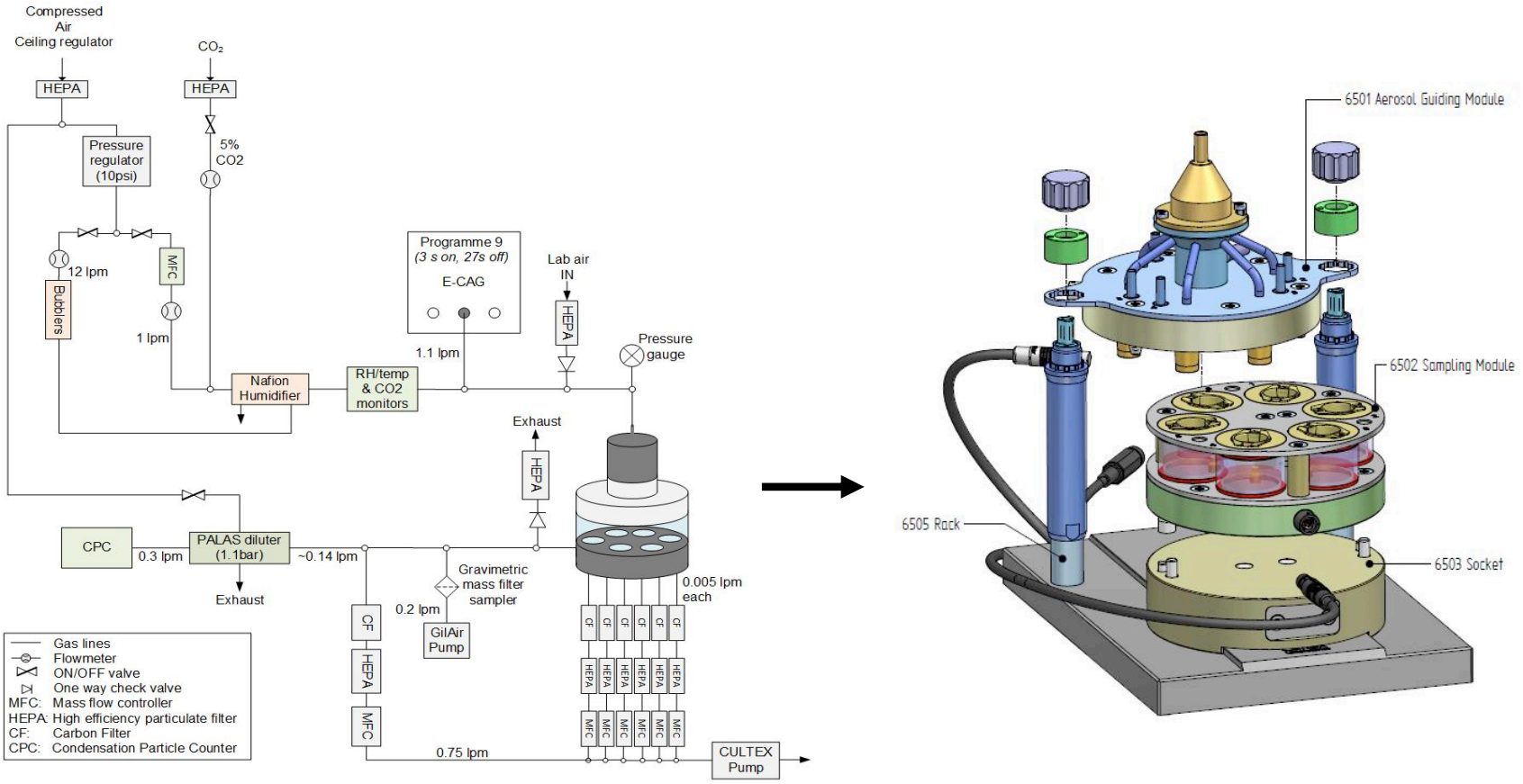


Figure 6. 3 | Schematic diagram of the ALI system used for in vitro cell exposure. The test atmosphere during system operation was kept at a temperature of 22ºC ± 2ºC, and relative humidity of 85% ± 5%.

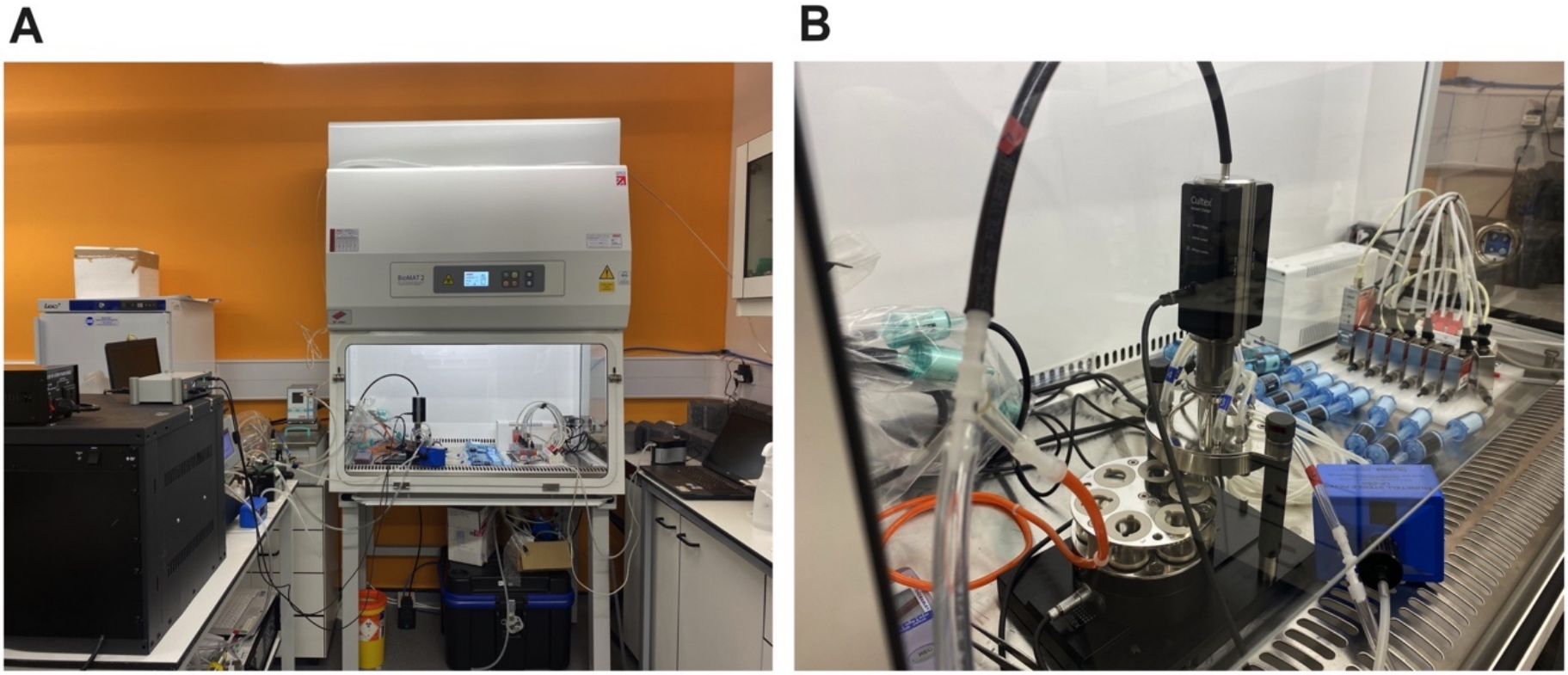


Figure 6. 4 A) Image to depict the aerosol exposure settings, B) the CULTEX system used to expose HBEC-3KT cells grown at ALI to EC aerosol

6.2.7 CELL CYTOTOXICITY: LDH ASSAY

After aerosol exposure to ALI cultures, maintenance media was replaced, and cells were incubated at 37°C, 5% CO_2, and 98% humidity for 24h. Subsequently, spent media was removed from the basal region of the transwells and retained for lactate dehydrogenase (LDH) assay using the LDH cytotoxicity kit (Roche, UK) according to the manufacturer's protocol. Also, the apical region was washed with fresh media (200 μL) for 1 min and collected for LDH. Plate absorbance was measured at 490 and 680nm using the BIO-TEK synergy HT absorbance reader (Bio-Tek, USA).

6.2.8 RANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)

TEER recordings were done following the protocol in **Chapter 5**, section **5.2.5**.6.2.9

6.2.9 QRT-PCR ANALYSIS

6.2.9.1 RNA EXTRACTION

RNA extraction was performed following the protocol in **Chapter 4**, **section 4.2.2.1**.

6.2.9.2 SYNTHESIS OF CDNA

Synthesis of cDNA was performed following protocol in **Chapter 4**, **section 4.2.2.2**.

6.2.9.3 QRT-PCR

Gene expression was analyzed using qRT-PCR employing the SYBR green fluorescent dye method and the QuantStudio™ 7 Flex Real-Time PCR system. The transcript sequence for each primer was found in the Ensemble database. The primers were 80-120 base pairs, 40-60% GC content, and spanning across exon-exon junctions of the mRNA to avoid amplifying genomic DNA. The primer specificity was assessed using NCBI primer blast. Primers were designed manually with Ensembl, NCBI Primer-blast and Primer3 and supplied by Integrated DNA Technologies. Sequences

can be found in **Table 6.2.** A serial dilution was used to determine optimal cDNA concentration for gene expression analysis (1 µg, 200 ng, 40 ng, 8 ng, 1.6 ng, 0.32 ng) and amplification between Ct 18-25 was selected. Four genes: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta, (YWAZ), TATA box binding protein (TBP), and Hypoxanthine Phosphoribosyltransferase 1 (HPRT1) were selected following assessment using the geNorm housekeeping gene selection kit protocol and evaluated to be used as housekeeping genes based on the literature. For the primer sequence for the genes of epithelial differentiation (tjp1, muc5ac, muc5b, itga6, tp63, foxj1, and krt5), please see **Table 4.1** in **Chapter 4**. Amplification of cDNA was performed under the following conditions: 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 s, and 60ºC for 30 s. The $^{\Delta\Delta}$Ct method (Haimes and Kelley, 2014) was used in the calculations.

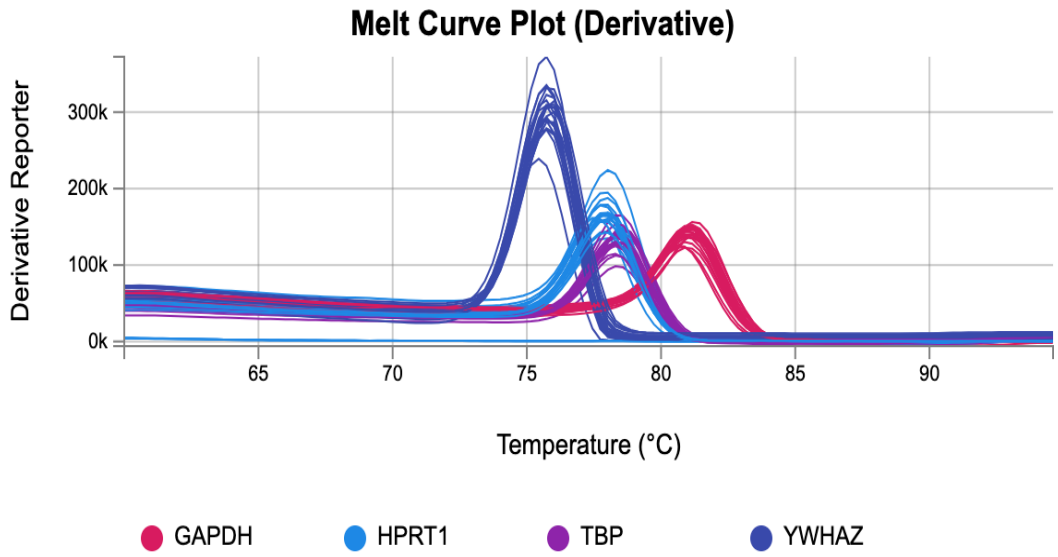


Figure 6. 5 Quality score (melt curve plot) for the housekeeping genes used as endogenous controls GAPDH, HPRT1, TATA, and YWAZ in HBEC-3KT for all the exposure conditions tested.

Gene name	Stability value
HPRT1 | YWHAZ	0.293
TBP	0.444
GAPDH	0.487

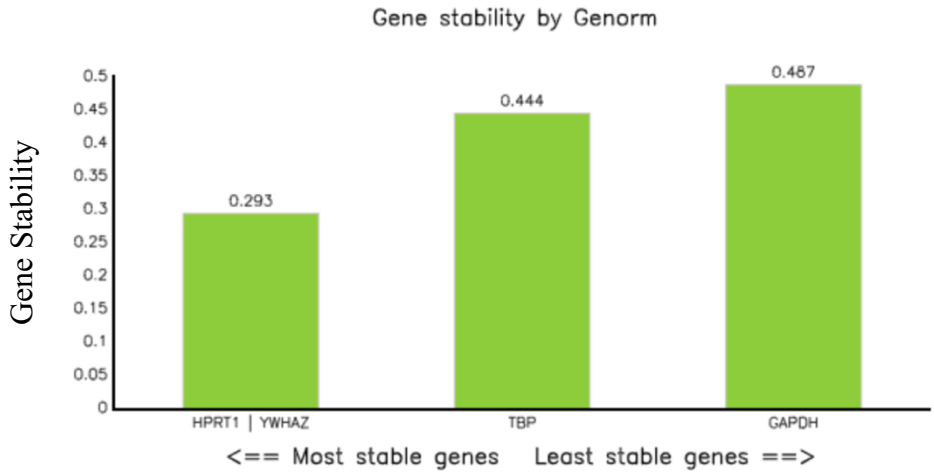


Figure 6. 6 Comprehensive gene stability of the housekeeping genes (HPRT1, YWHAZ, TBP and GAPDH tested for the conditions of our study. All 4 tested HK genes were stable for the condition of our study. We determined the expression stability of these 4 genes using geNORM qBasePlus software.

Table 6. 2 Primer sequences. For the primer sequences for the epithelial differentiation and the HK genes, see Table 4.1 in Chapter 4

Gene	Sequence (forward and reverse)
DDIT3	GGAGCTGGAAGCCTGGTATG GGTGAAACATAGGTACCCCCA
HMOX1	TCACTGTGTCCCTCTCTC ATTGCCTGGATGTGCTTT
IL-8	GCGCCAACACAGAAATTATTGTA ACTTCTCCACAACCCTCTGC
IL-6	TGTGAAAGCAGCAAAGAGGC ACCAGGCAAGTCTCCTCATT
TNF-α	CCCAGGGACCTCTCTCTAATC GGGTTCGAGAAGATGATCTGAC
IL-6	TGTGAAAGCAGCAAAGAGGC ACCAGGCAAGTCTCCTCATT
IL-1β	TACCTGTCCTGCGTGTTGAA TGGGATCTACACTCTCCAGC

6.2.9.4 ELISA ASSAY FOR CYTOKINES, GALECTIN-3 AND 8-OHDG QUANTIFICATION

Post-exposure (24h) to the respective environmental stimuli, the spent media were meticulously collected from the basal compartment from 3 wells of the ALI cultures. This ensured that the cytokines, galectin-3 and 8-OHdG released from the cells into the medium were effectively captured for subsequent analysis. Enzyme-Linked Immunosorbent Assay (ELISA) was conducted to quantify IL-10, TNF-α, IL-1β, IL-6, galectin-3, and 8-OHdG (all ELISA were purchased from ThermoScientific, UK) in the collected media. The assays were executed according to the manufacturer's specified protocols to ensure accuracy and reliability of the results. All the required reagents, including ELISA plates, antibodies, and standards were prepared and brought to room temperature. The standards were serially diluted to create a standard curve for each analyte. 50 µl of each sample and standard was pipetted into the respective wells of the ELISA plate. The plates were then sealed and incubated for a specified period, typically 2 hours at room temperature, to facilitate the binding of antigens present in the samples and standards to the immobilized antibodies. The plates were washed thoroughly to remove any unbound substances. Washing was done using washing buffer, and the step was repeated three times to ensure complete removal of non-specific bindings. A biotinylated secondary antibody specific for each analyte was added and the plate which was then incubated for an additional hour. This secondary antibody was used to detect the bound antigens. The plates were again washed to remove excess secondary antibodies. A substrate solution was added to each well to initiate a colorimetric reaction catalysed by the enzyme linked to the secondary antibody. The plates were incubated in the dark for a period, generally not exceeding 30 minutes. The reaction was terminated by adding a stop solution to each well. This

resulted in a change in colour intensity, proportional to the concentration of the analytes in the samples. The absorbance of each well was read at 405 nm using the Synergy plate reader (Biotek, USA). Owing to the absence of a 450 nm wavelength in the Synergy plate reader, the construction of an accurate standard curve, in adherence to the established protocol, was impeded. Consequently, the standard curve rendered was characterised by a suboptimal slope, compromising the reliability of protein concentration extrapolation. In light of this limitation, we utilised the absorbance values directly as a quantitative metric for assessing protein expression levels. Furthermore, to validate the efficacy of the assay under the adapted conditions, we incorporated positive controls supplied with the kits into our experimental design. The inclusion of these controls was instrumental in assessing the reliability and accuracy of our findings, offering a comparative benchmark to evaluate the performance of the assay amidst the modified operational parameters.

6.2.9.5 STATISTICAL ANALYSIS

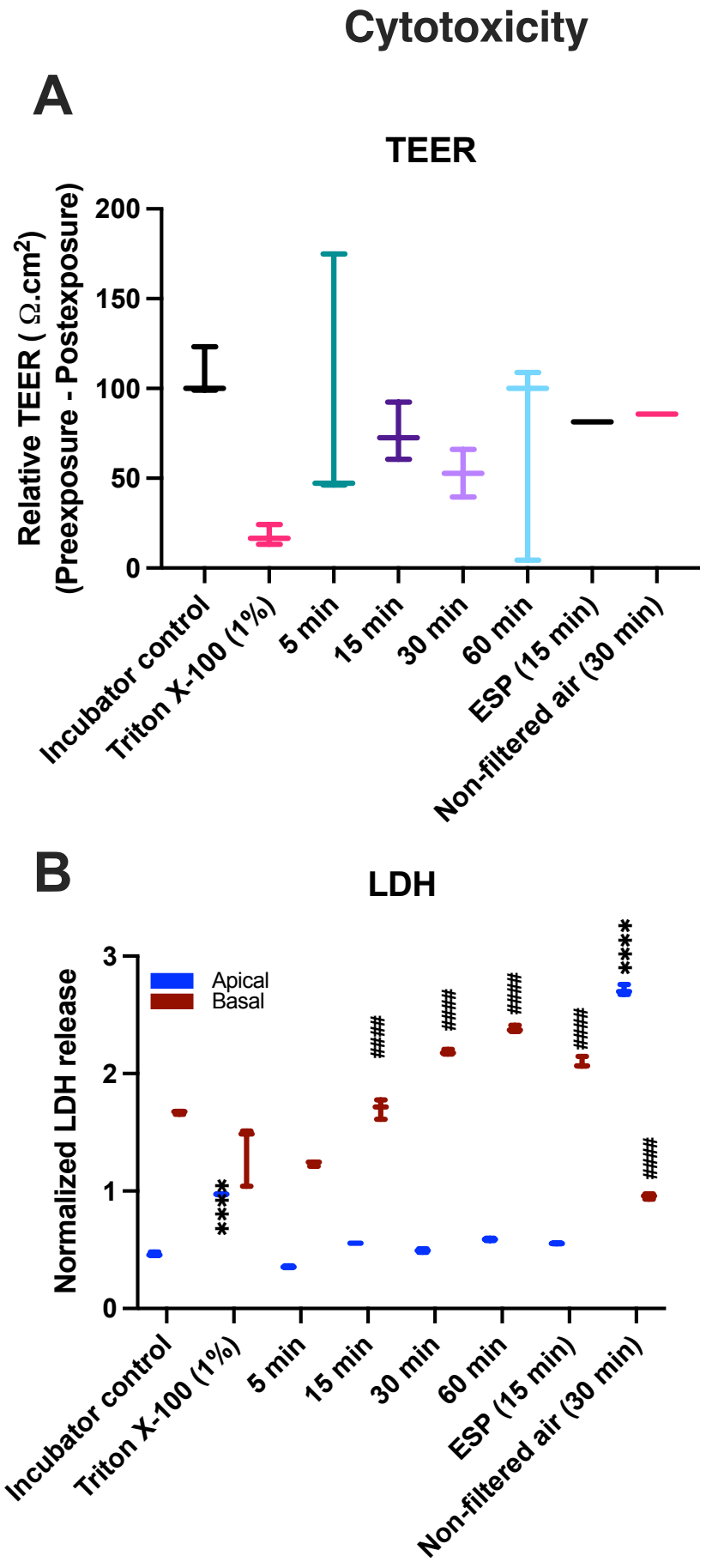
Statistical analysis was performed using a two-way ANOVA to establish the dose-response relationship within flavours and dose effects, followed by Tukey's HSD *post-hoc* test corrected for multiple comparisons using python statistics modules (statsmodel and scipy). Graphs were generated using GraphPad Prism 9. All experiments were carried out using 6 technical replicates for each experimental condition. Data are expressed as mean ± SEM, and the p-value was set at $p < 0.05$.

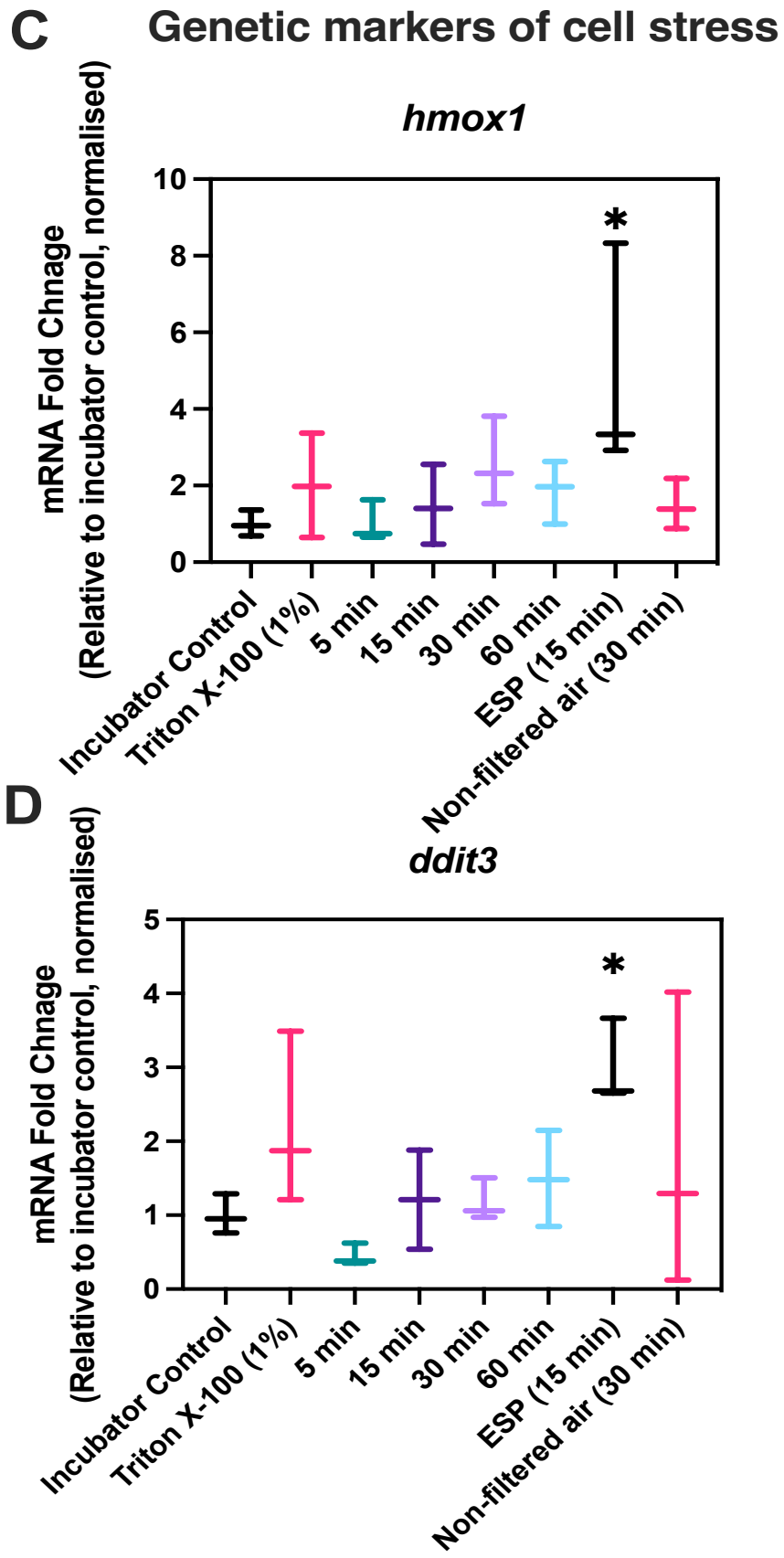
6.3 RESULTS

6.3.1 ASSESSING THE EFFECTS OF VARIED AIR EXPOSURE DURATIONS ON HBEC-3KT CELLS. IMPLICATIONS FOR CYTOTOXICITY, GENETIC MARKERS FOR CELL STRESS, AND AIRWAY DIFFERENTIATION

The primary aim of this study was to assess the tolerability of HBEC-3KT cells, cultured at the air-liquid interface (ALI), to varying durations of air exposure to establish appropriate exposure durations without imparting toxicity to the cells due to CULTEX use alone. Following exposure to air for periods of 5, 15 (with and without an electrostatic precipitator), 30, and 60 minutes, we observed no significant changes in transepithelial electrical resistance (TEER) values between incubator control and any of the exposure groups at 24 hours post-exposure (**Figure 6.7A**), suggesting maintained epithelial integrity across these time points. An assessment of cytotoxicity, utilising LDH release, revealed a significant increase in LDH levels in the basal media following 30 minutes, 60 minutes, and 15 minutes + electrostatic precipitator air exposures as compared to the incubator control ($p<0.0001$) (**Figure 6.7B)**. In contrast, non-filtered lab air LDH levels in the basal media was significantly decreased compared to incubator-only control ($p<0.0001$) (**Figure 6.7B**). Interestingly, for the LDH released in the apical region, only the triton x-100 positive control ($p<0.0001$) and non-filtered lab air ($p<0.0001$) significantly induced increased LDH levels compared to incubator control, implying a distinctive response to these specific conditions. Our examination of the genetic markers of cell stress, *hmox1* (**Figure 6.7C**), and *ddit3* (**Figure 6.7D**), revealed that 15 minutes of air exposure in conjunction with an electrostatic precipitator significantly increased their transcript levels compared to the incubator control. This increase suggests that this exposure

condition elicited a cellular stress response. In contrast, when investigating markers of airway differentiation such as *tjp1* (**Figure 6.7E**), *muc5ac* (**Figure 6.7F**), and *foxj1* (**Figure 6.7G**), the 30 min of non-filtered lab air exposure resulted in a significant upregulation in *tjp1* ($p<0.05$), *muc5ac* ($p<0.0001$), and *foxj1* ($p<0.0001$), indicative of an influence on cell differentiation under this exposure condition. In summary, these results show differential responses to varied air exposures in terms of cytotoxicity and cell differentiation. Our findings highlight the importance of characterising control conditions in *in vitro* models of air exposure. These data provided valuable insights into the time-points used for the EC aerosol exposures.


Cytotoxicity
A
TEER
Relative TEER (Ω.cm²)
(Preexposure - Postexposure)
200
150
100
50
0
Incubator control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)
B
LDH
Normalized LDH release
Apical
Basal
3
2
1
0
****
####
Incubator control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)


C
Genetic markers of cell stress
hmox1
mRNA Fold Chnage
(Relative to incubator control, normalised)
10
8
6
4
2
0
*
Incubator Control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)
D
ddit3
mRNA Fold Chnage
(Relative to incubator control, normalised)
5
4
3
2
1
0
*
Incubator Control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)

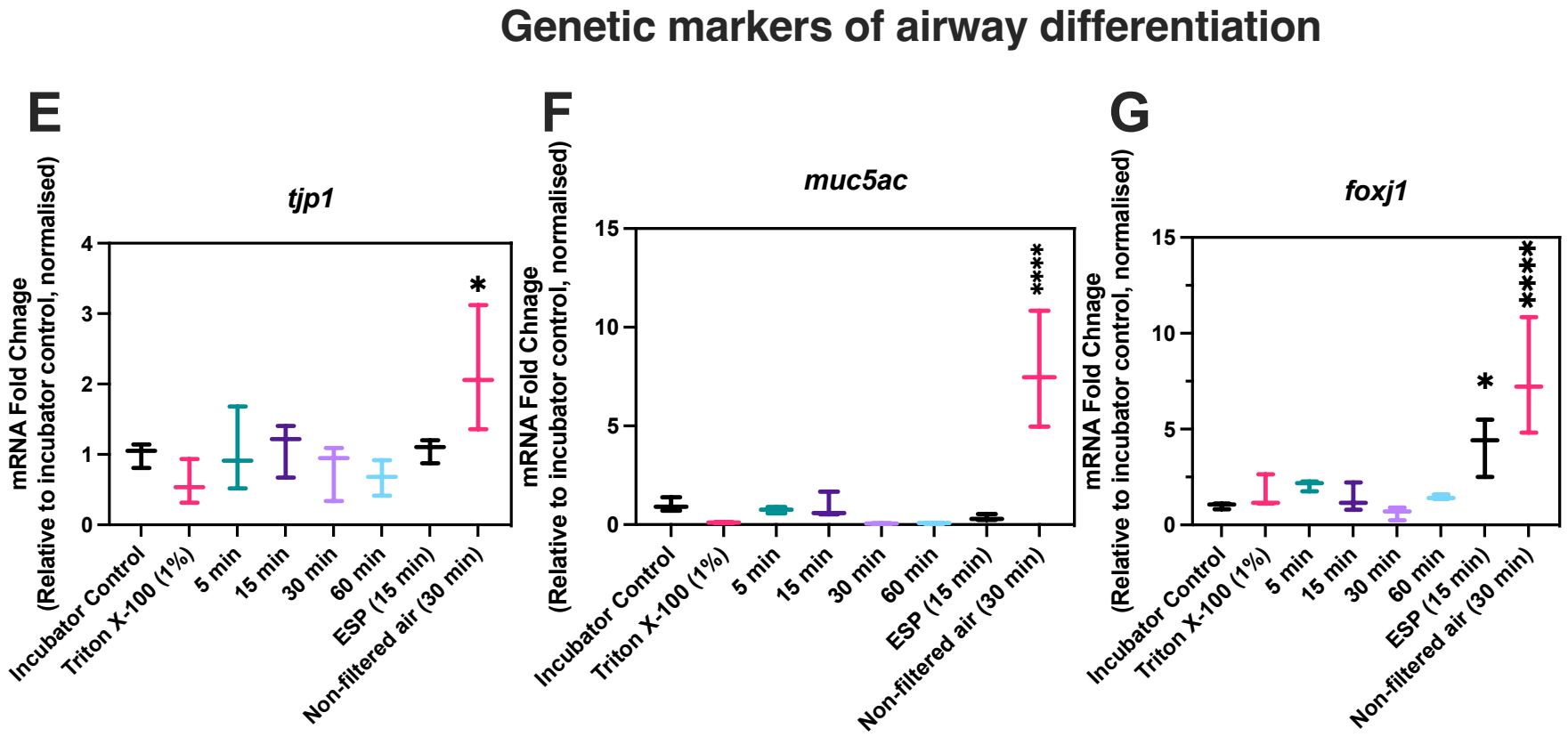
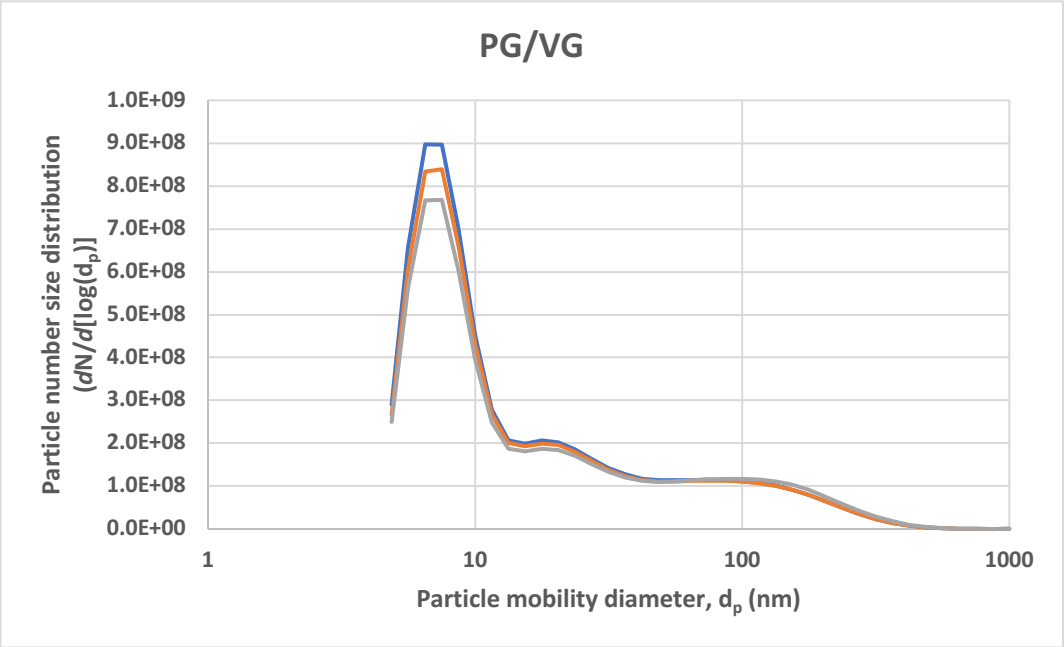

Genetic markers of airway differentiation
E
tjp1
mRNA Fold Chnage
(Relative to incubator control, normalised)
4
3
2
1
0
*
Incubator Control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)
F
muc5ac
mRNA Fold Chnage
(Relative to incubator control, normalised)
15
10
5
0
****
Incubator Control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)
G
foxj1
mRNA Fold Chnage
(Relative to incubator control, normalised)
15
10
5
0
*
****
Incubator Control
Triton X-100 (1%)
5 min
15 min
30 min
60 min
ESP (15 min)
Non-filtered air (30 min)

Figure 6. 7 Effect of Different Air Exposure Durations on HBEC-3KT Cells (5 min, 15 min, 30 min, 60 min, 15 min with electrostatic precipitator and 30 min of non-filtered lab air) grown at ALI for 14 days. The endpoints assessed are A) TEER measurement B) LDH and C) & D) genetic markers of cellular stress, and E) – G) genetic markers of airway differentiation. A one-way ANOVA with multiple comparisons, corrected using Tukey's was used to determine statistical significance. Data are presented as box and whiskers min-max (n=3 with 3 biological replicates for qPCR gene expression, n=6 per group for TEER and LDH). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs. incubator control (for LDH, vs incubator control apical LDH). ####p<0.0001 vs. incubator control basal LDH. $$$$p<0.0001 vs. 15 min basal LDH. Abbreviations: ESP, electrostatic precipitator.

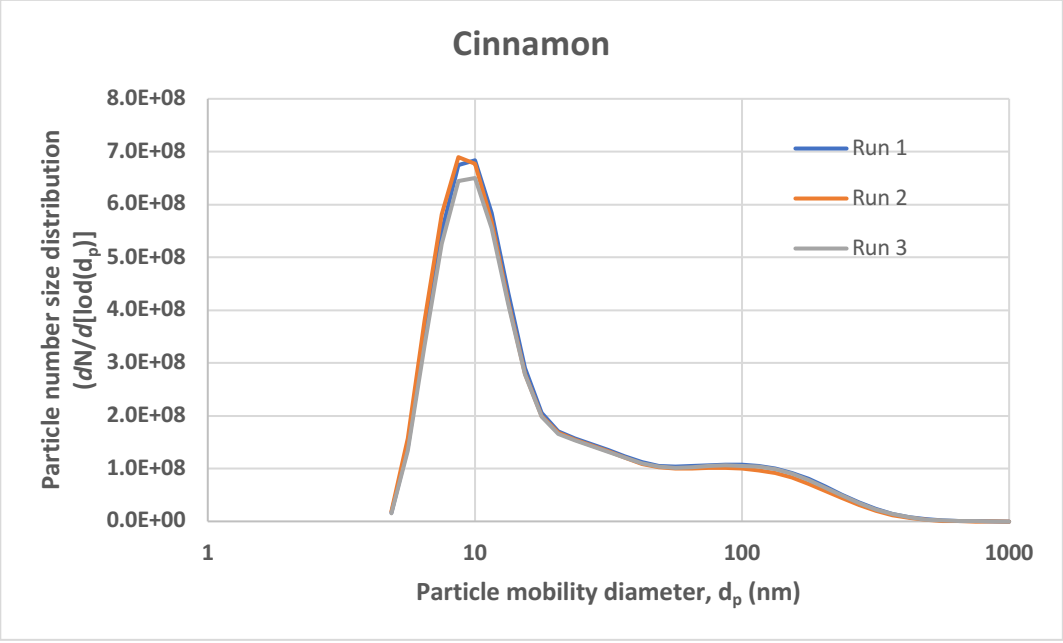
6.3.2 EC AEROSOL PARTICLE SIZE DISTRIBUTION

In our assessment of particle size distribution across different e-liquids, we observed discernible variations in the size and number of particles produced by each e-liquid. Regarding particle size, PG/VG was found to produce the smallest particles among all e-liquids tested with a particle mobility diameter (d_p) of ~ 8 nm (**Figure 6.8A**). Cinnamon (**Figure 6.8B**) and menthol (**Figure 6.8E**) particles were slightly larger than those of PG/VG but smaller than the other e-liquids with a d_p of ~ 9 nm. Hazelnut (**Figure 6.8D**) and vanilla tobacco (**Figure 6.8C**) had the largest d_p of about ~ 20 nm. In terms of particle numbers, PG/VG had the highest with approximately 9.0E+08 particles. Cinnamon followed with a particle count of approximately 7.0E+08. Vanilla tobacco, hazelnut, and menthol exhibited similar particle counts, each registering at around 5.0E+08 particles. These findings provide a comparative view of particle size distribution across the tested e-liquids, highlighting the variability in both size and particle number across different flavourings and compositions.

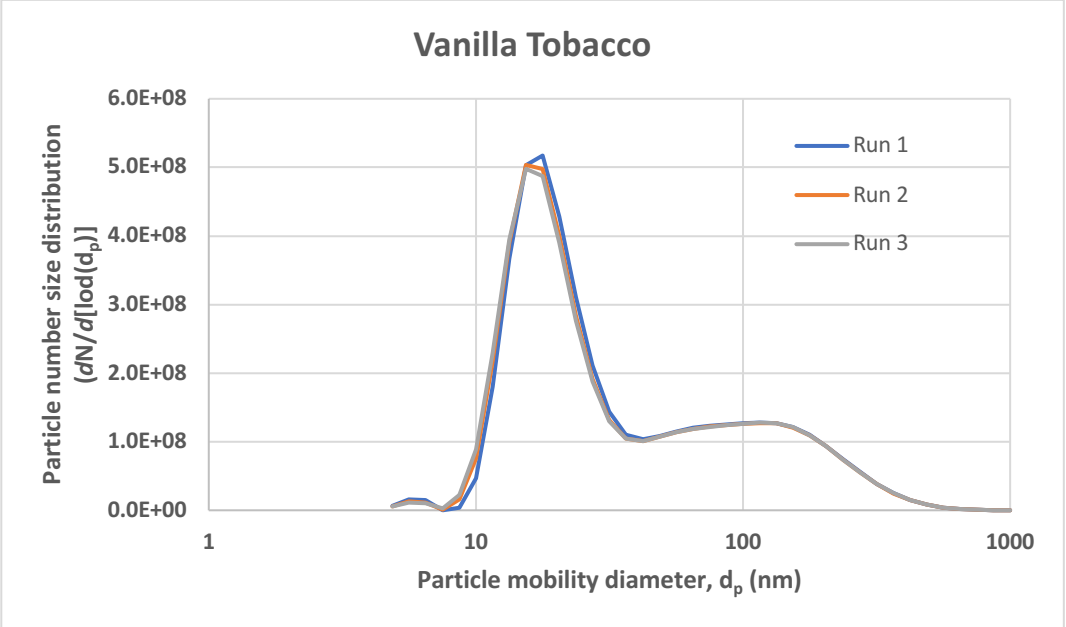
A


PG/VG
Particle number size distribution (dN/d[log(dp)])
1.0E+09
9.0E+08
8.0E+08
7.0E+08
6.0E+08
5.0E+08
4.0E+08
3.0E+08
2.0E+08
1.0E+08
0.0E+00
1
10
100
1000
Particle mobility diameter, dp (nm)

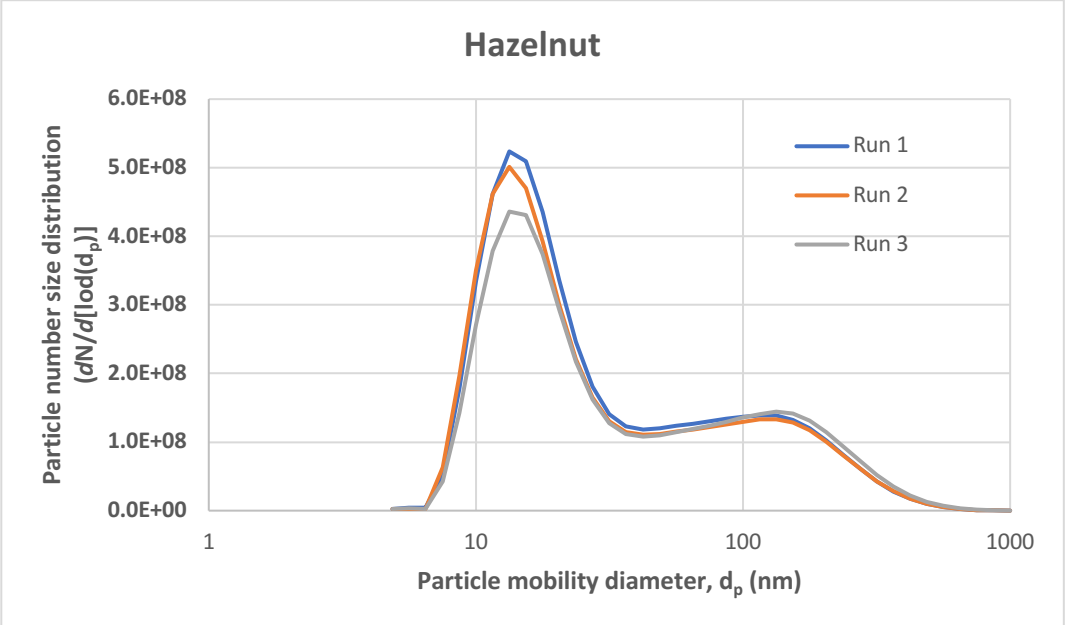
B


Cinnamon
Particle number size distribution (dN/d[lod(dp)])
8.0E+08
7.0E+08
6.0E+08
5.0E+08
4.0E+08
3.0E+08
2.0E+08
1.0E+08
0.0E+00
1
10
100
1000
Particle mobility diameter, dp (nm)
Run 1
Run 2
Run 3

C


Vanilla Tobacco
Particle number size distribution (dN/d[log(dp)])
6.0E+08
5.0E+08
4.0E+08
3.0E+08
2.0E+08
1.0E+08
0.0E+00
1
10
100
1000
Particle mobility diameter, dp (nm)
Run 1
Run 2
Run 3

D


Hazelnut
Particle number size distribution (dN/d[log(dp)])
6.0E+08
5.0E+08
4.0E+08
3.0E+08
2.0E+08
1.0E+08
0.0E+00
1
10
100
1000
Particle mobility diameter, dp (nm)
Run 1
Run 2
Run 3

E

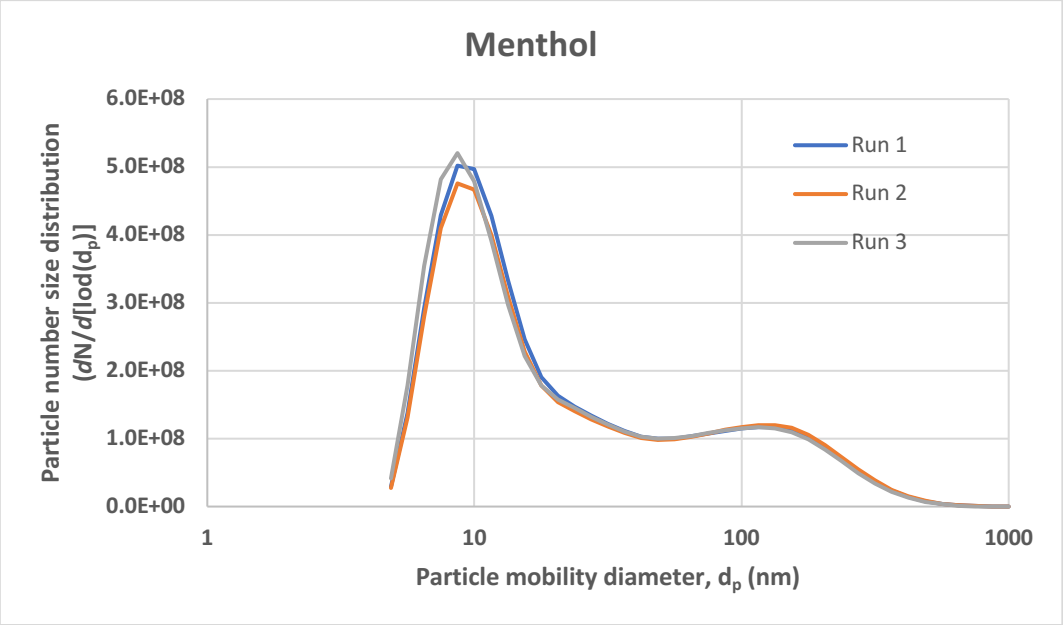


Figure 6. 8 Aerosol particle size distribution for A) PG/VG B) cinnamon C) vanilla tobacco D) hazelnut and E) menthol e-liquids taken from 3 puffs from the ECAG using a differential mobility spectrometer (DMS500). DMS500 has a measurement size range of 5 – 1000 nm and a time response of 10 Hz (measurements every 0.1s) allowing the short, 3s puff profile to be measured.

6.3.3 CYTOTOXICITY AND BARRIER DISRUPTION IN HUMAN BRONCHIAL EPITHELIAL CELLS AT ALI FOLLOWING EXPOSURE TO FLAVOURED E-CIGARETTE AEROSOL

Our findings revealed a significant increase in LDH levels at the highest dose (56 puffs) of cinnamon and hazelnut compared to PG/VG at 56 puffs. Surprisingly, the diluted cigarette smoke extract did not induce significant LDH release in HBEC-3KT cells (**Figure 6.9A**). For TEER measurements, cinnamon at 56 puffs significantly decreased ($p<0.001$) relative TEER values compared to controls. In contrast, Menthol

at 42 and 65 puffs, significantly increased relative TEER measurements ($p<0.0001$). Again, the 3R4F cigarette extract did not affect relative TEER values (**Figure 6.9B**)

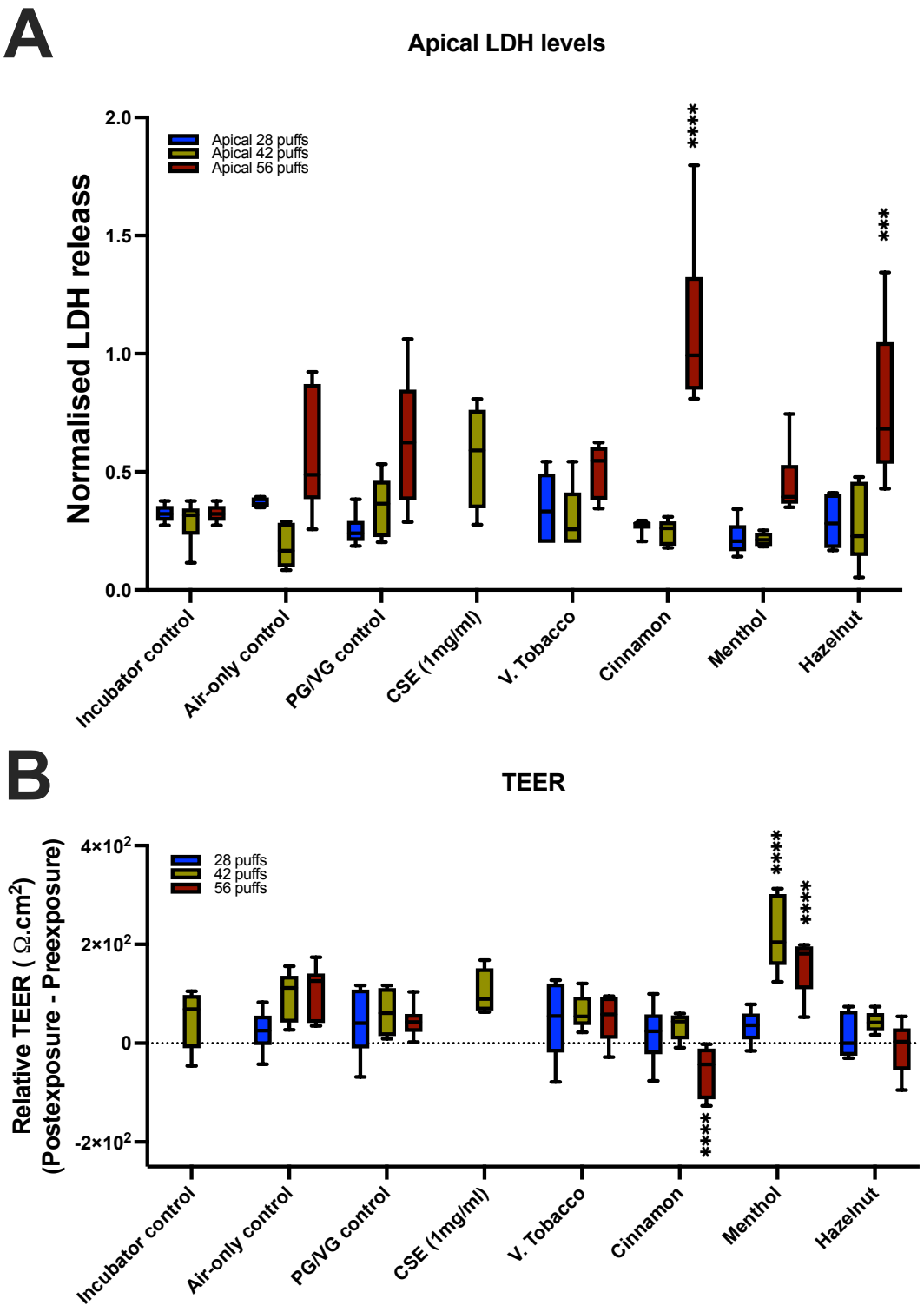


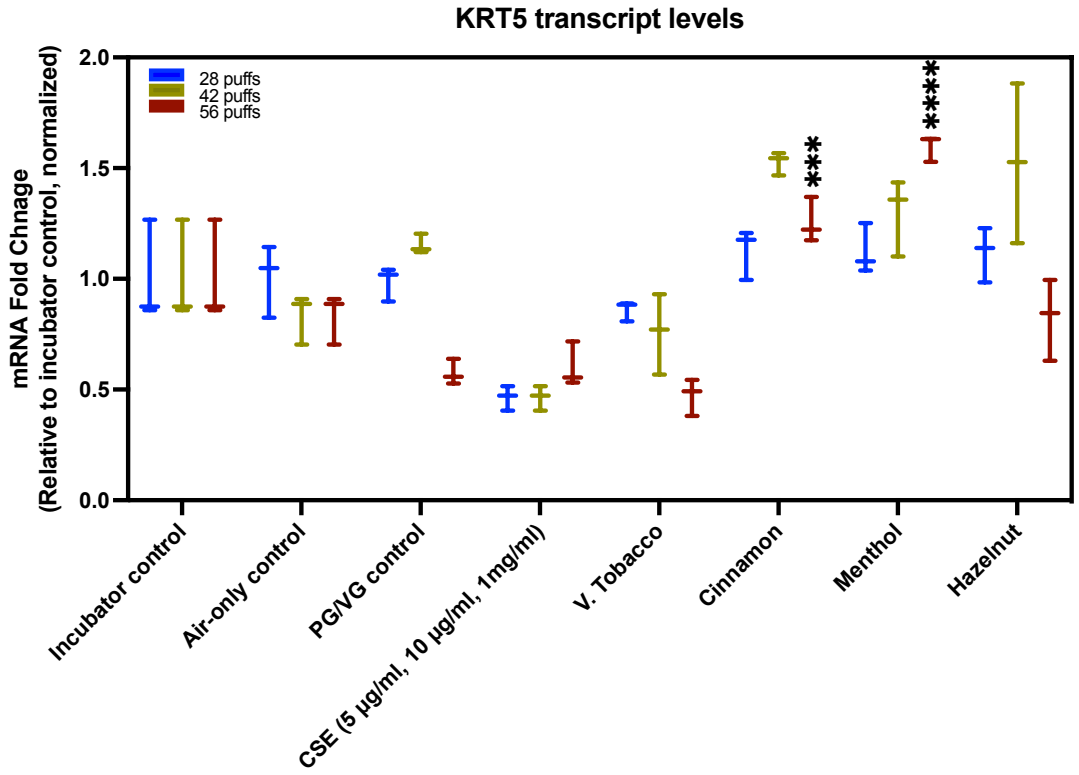
Figure 6. 9 The effect of different flavoured-e-liquids (vanilla tobacco, cinnamon, menthol & hazelnut) aerosols at increasing concemtrations (28, 42, and 56 puffs) on HBEC-3KT cells grown at ALI for 14 days. A) LDH releases and B) TEER measurements. Cigarette smoke extract at 1 mg/mL was included as a comparative measure. A two-way ANOVA with multiple comparisons, corrected using Tukey's test, was used to determine statistical significance. Data are presented as box and whiskers min-max (n=3 with 3 biological replicates for qPCR gene expression). Abbreviations: incu, incubator; PG/VG; propylene glycol/vegetable glycerine, V. tobacco, vanilla tobacco; CSE, cigarette smoke extract.

6.3.4 DIFFERENTIAL EFFECTS OF E-CIGARETTE AEROSOLS ON TRANSCRIPT LEVELS OF AIRWAY DIFFERENTIATION MARKERS IN HUMAN BRONCHIAL EPITHELIAL CELLS

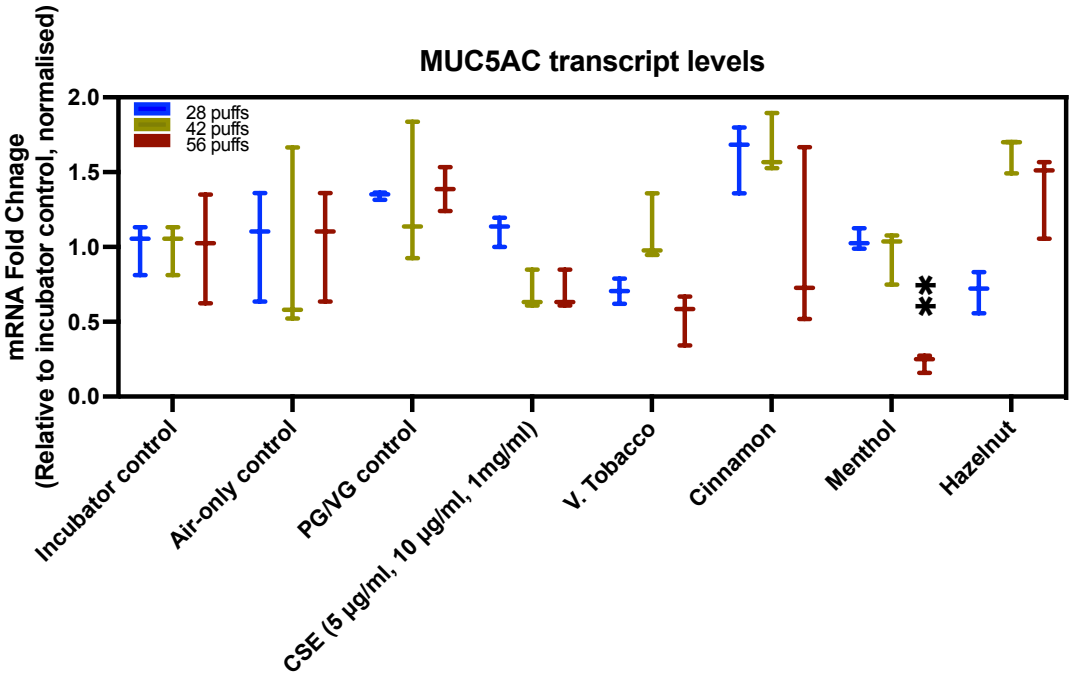
We characterised the effects of three different doses of aerosols (28 puffs, 42 puffs, and 56 puffs) of flavoured e-liquids on genetic markers of airway differentiation, including *krt5* (**Figure 6.10A**), *muc5ac* (**Figure 6.10B**), *muc5b* (**Figure 6.10C**), *foxj1* (**Figure 6.10D**), *tjp1* (**Figure 6.10E**), and *tp63* (**Figure 6.10F**) in human bronchial epithelial cells (HBEC-3KT). Cinnamon, and menthol at 56 puffs significantly increased *krt5* transcript levels ($p<0.01$ and $p<0.0001$, respectively) compared to the PG/VG 56 puffs control. Only menthol at 56 puffs significantly decreased *muc5ac* mRNA levels compared to the PG/VG 56 puffs control. Vanilla tobacco, menthol, and hazelnut at 28 puffs all significantly reduced *muc5b* transcript levels compared to 28 puffs of PG/VG. At 42 puffs, cinnamon significantly increased *muc5b* levels ($p<0.001$) compared to the PG/VG control. Whereas at 56 puffs, only hazelnut induced a significant increase in *muc5b* transcript levels compared to the PG/VG control. With 28 puffs, only vanilla tobacco decreased foxj1 transcript levels compared to the

PG/VG control ($p<0.05$). No significant effect was observed with any flavoured e-liquids tested at 42 puffs but with 56 puffs, both cinnamon ($p<0.001$) and menthol ($p<0.01$) significantly decreased foxj1 transcript levels compared to the PG/VG control. We observed no treatment-related significant changes in tjp1 transcript levels across all the concentrations or e-liquids tested. Interestingly, for the air-only control, we observed a significant decrease in tp63 transcript levels at both 42 and 56 puffs compared to the incubator-only control. In contrast, 42 puffs of cinnamon, or menthol significantly increased tp63 expression ($p<0.01$ and $p<0.001$, respectively) compared to the PG/VG control. At 56 puffs, cinnamon significantly decreased *tp63* transcript levels compared to the PG/VG control.

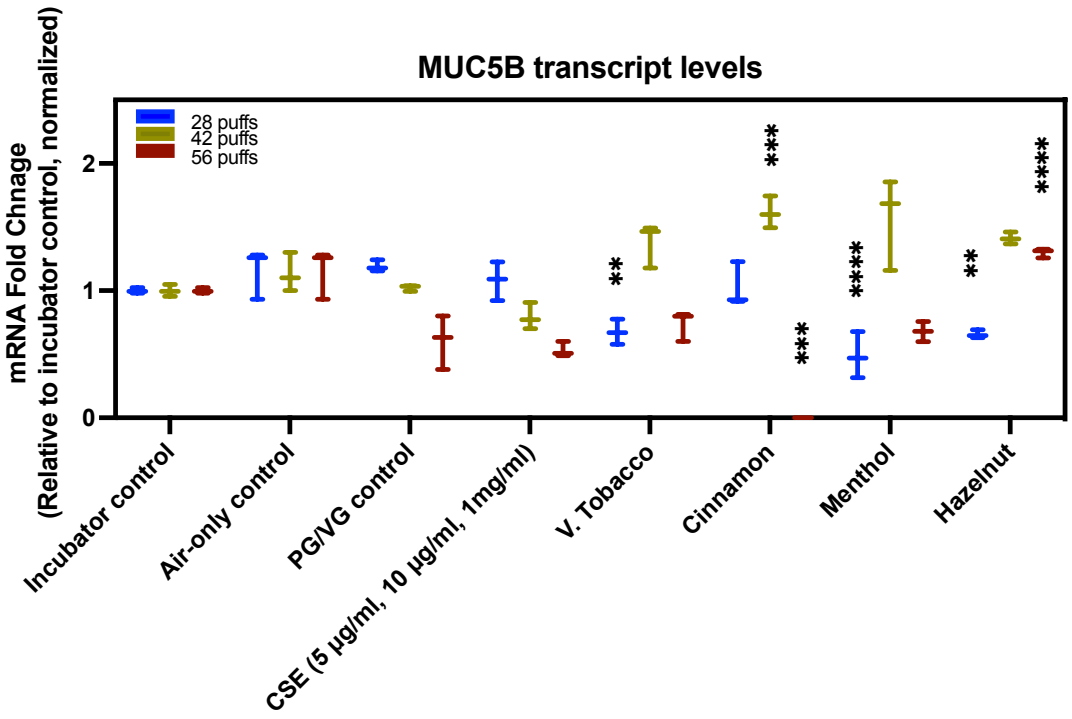
A



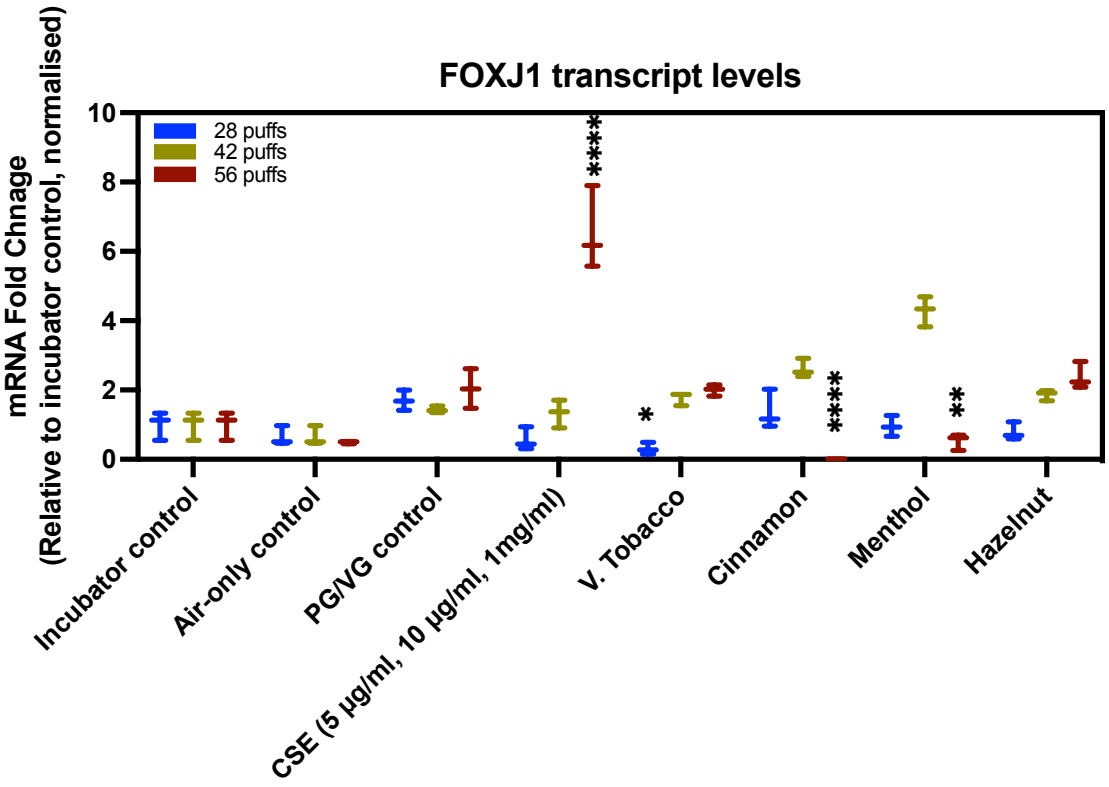
B


MUC5AC transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
2.0
1.5
1.0
0.5
0.0
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

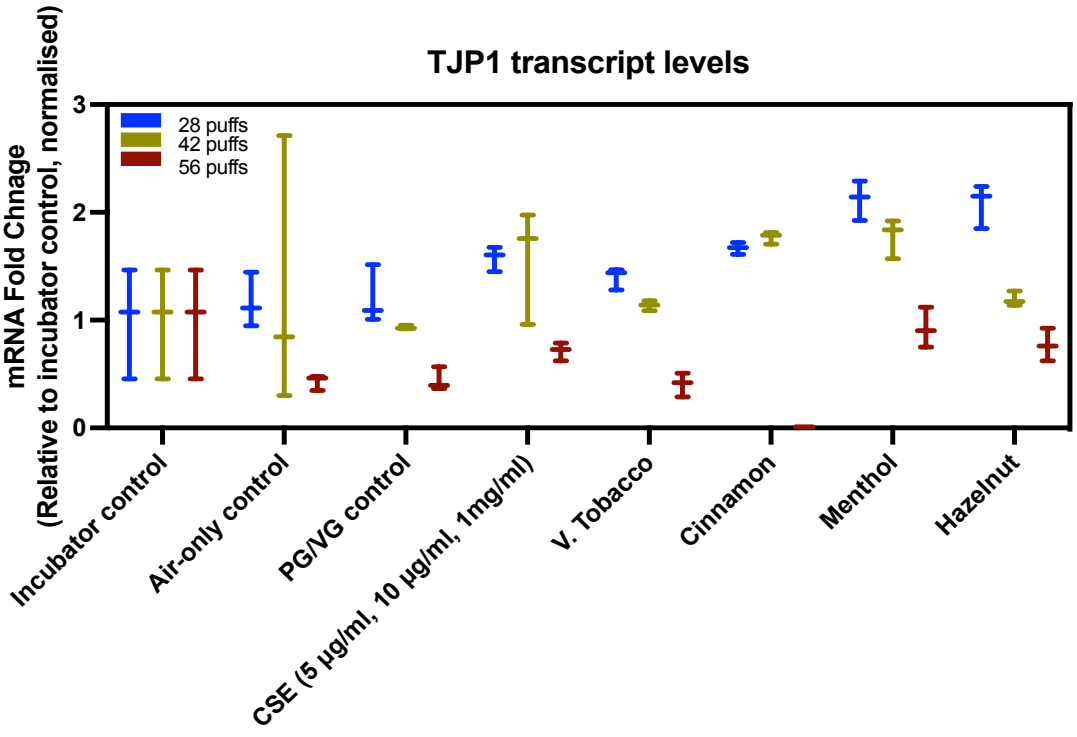
C


MUC5B transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalized)
28 puffs
42 puffs
56 puffs
2
1
0
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

D


FOXJ1 transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
10
8
6
4
2
0
****
*
****
**
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

E


TJP1 transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
3
2
1
0
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

F

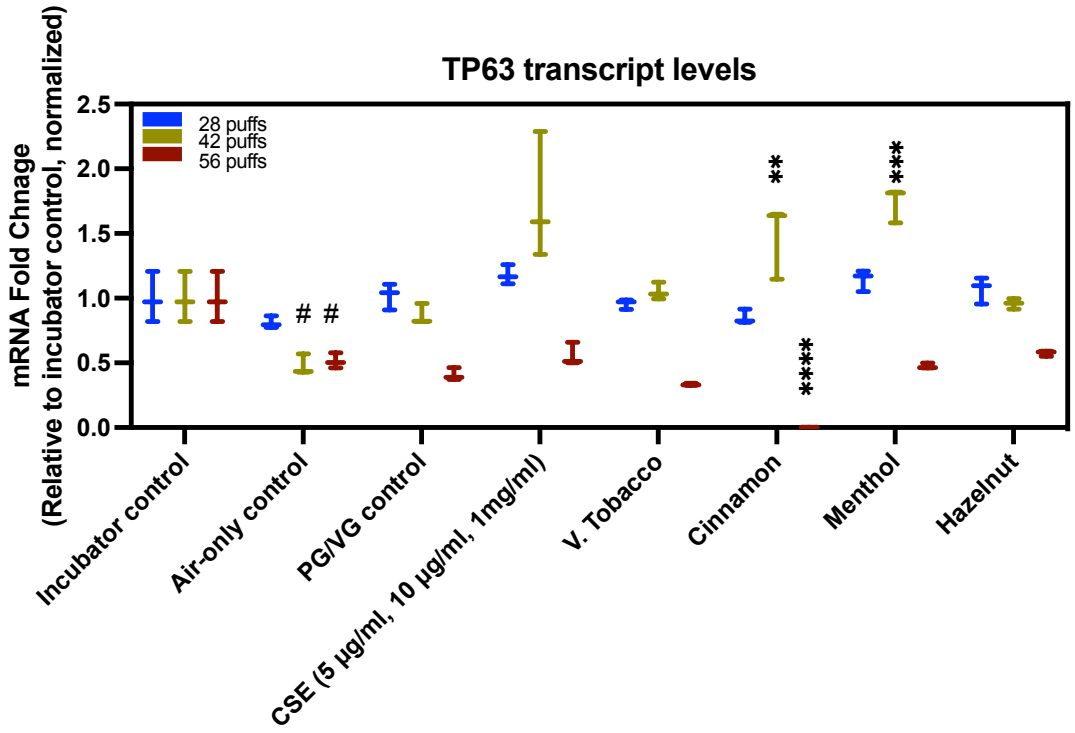
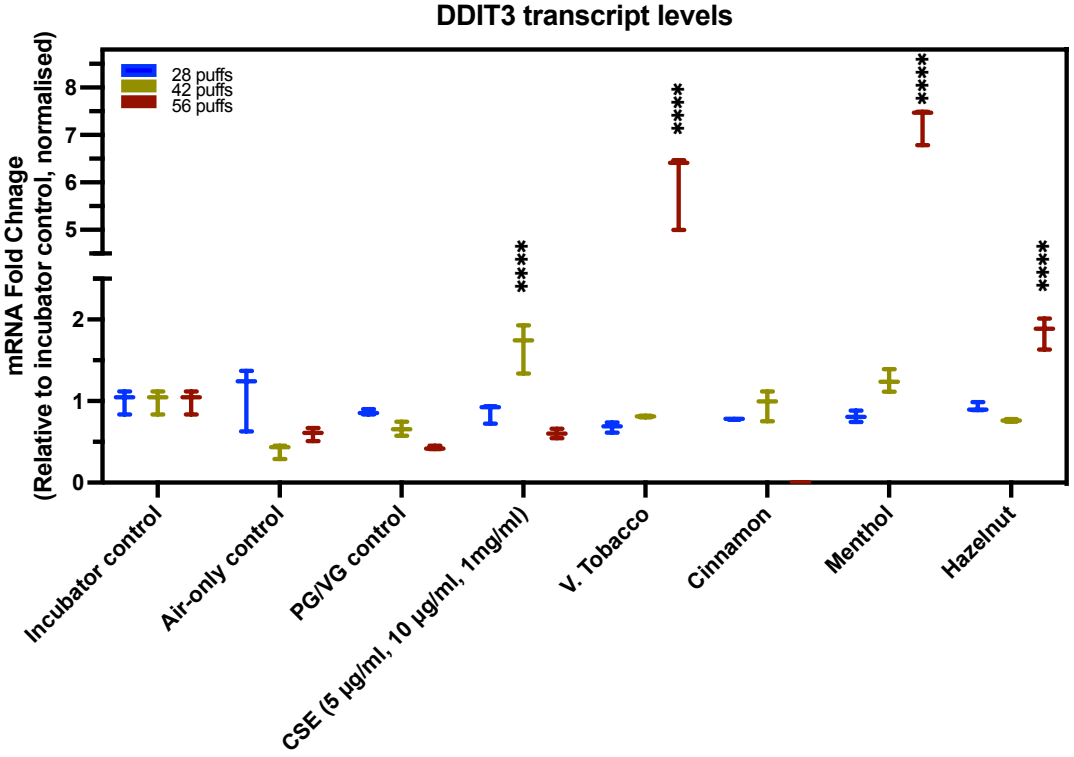


Figure 6. 10 mRNA transcript levels of markers of differentiation following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol. A) & F) basal cells (*krt5* and *tp63*), B) & C) mucus-producing cells (*muc5ac* and *muc5b*), D) ciliogenesis (*foxj1*), and E) tight junction formation (*tjp1*) in 14-days differentiated HBEC-3KT cells at ALI. A two-way ANOVA with multiple comparisons, corrected using Tukey's test, was used to determine statistical significance. Data are presented as box and whiskers min-max(n=3 with 3 biological replicates for qPCR gene expression). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs day 0. $^{\#}p$<0.05 vs incubator control.

6.3.5 THE EFFECTS OF FLAVOURED E-CIGARETTE AEROSOLS ON GENETIC MARKERS OF CELL STRESS

DDIT3 gene expression was only significantly increased by 56 puffs of vanilla tobacco, menthol, and hazelnut (all at $p<0.001$) (**Figure 6.11A)**. Cinnamon at 56 puffs showed no e DDIT3 expression, which is possibly due to cell death. Cigarette smoke extract at 10µg/ml induced a significant increase in expression ($p<0.001$), but not the highest dose of 1mg/ml. All flavoured aerosols at 42 and 56 puffs significantly increased HMOX1 transcript levels. Cigarette smoke extract only showed a significant increase at 10µg/ml, but not the highest concentration of 1mg/ml. The PG/VG vehicle showed no significant effect compared to air- or incubator-only control conditions in both experiments.

A



B

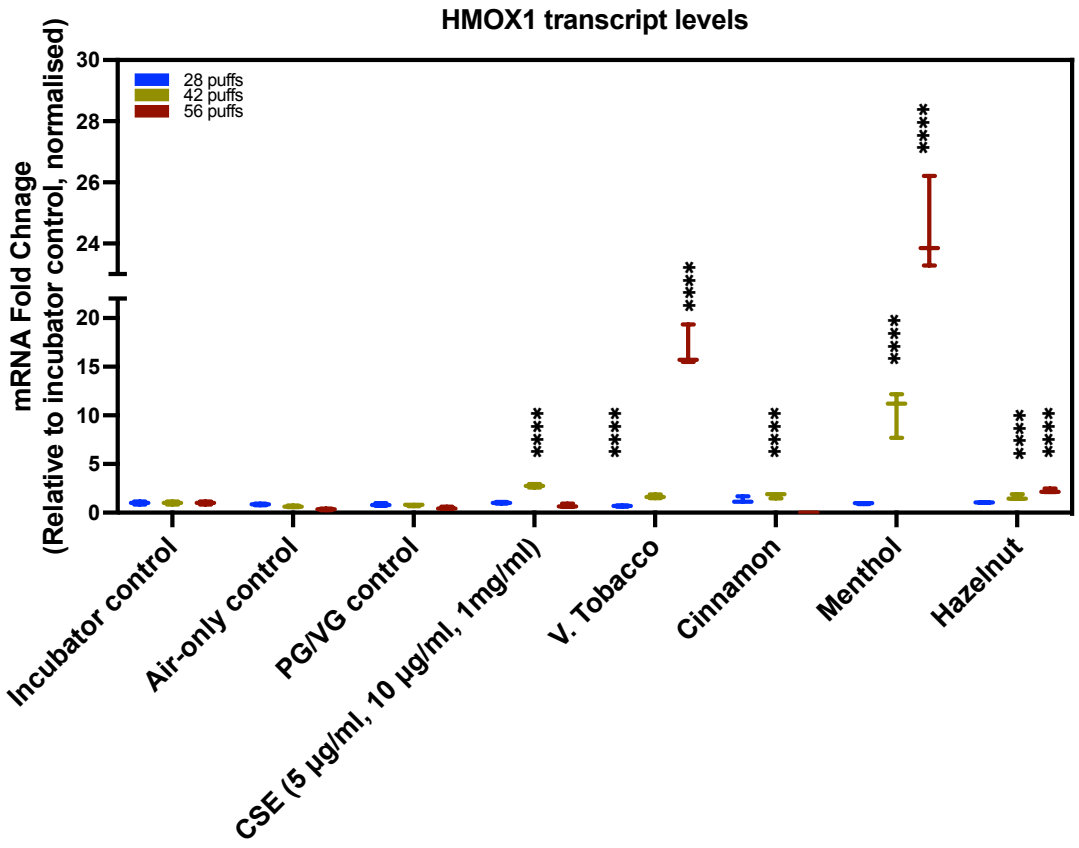
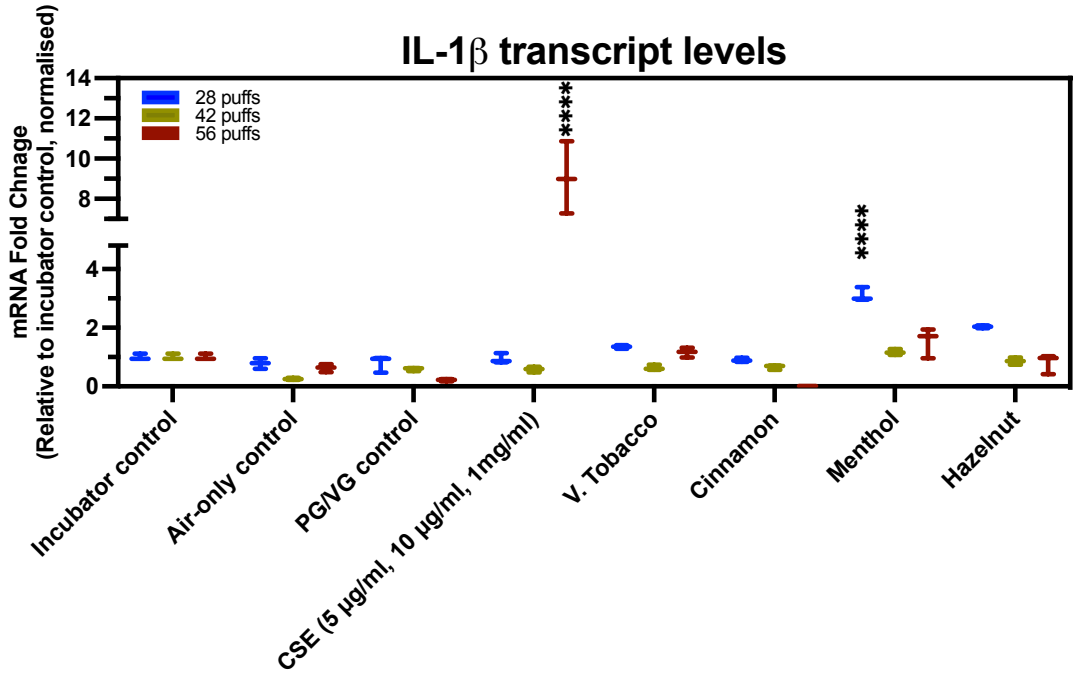


Figure 6. 11 mRNA transcript levels of cellular stress following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol. A) DDIT3 and B) HMOX1 in 14-days differentiated HBEC-3KT cells at ALI. A two-way ANOVA with multiple comparisons, corrected using Tukey's test, was used to determine statistical significance. Data are presented as box and whiskers min-max(n=3 with 3 biological replicates for qPCR gene expression). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs day 0. $^{\#}p$<0.05 vs incubator control.

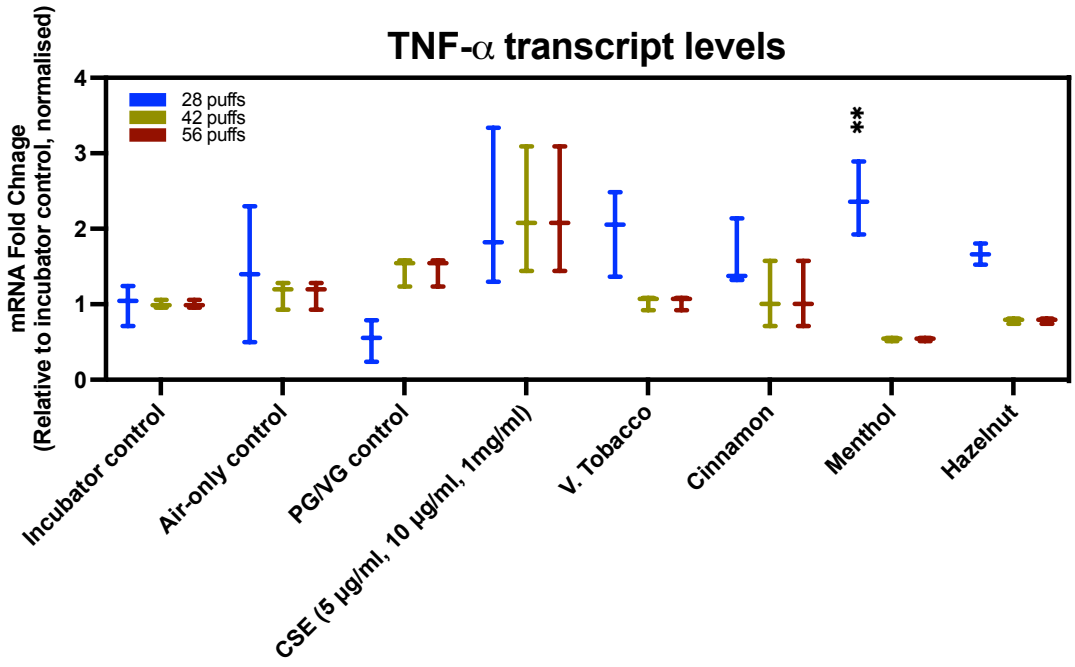
6.3.6 THE EFFECTS OF FLAVOURED E-CIGARETTE AEROSOLS ON MRNA LEVELS OF CYTOKINES

Employing a two-way ANOVA for the analysis of the obtained data, no statistically significant difference was discerned in the levels of IL-1β, TNF-α, IL-8, and IL-6 between cells retained in the incubator control, exposed to air-only, or to PG/VG or the majority of EC aerosols, across the three different puff intensities (**Figure 6.12A-D**). A marked increase in IL-1β levels was noted upon exposure to 1mg/ml of cigarette smoke extract ($p<0.0001$) and 28 puffs of menthol ($p<0.0001$), compared to PG/VG (**Figure 6.12A**). 28 puffs of menthol also resulted in a substantial amplification in TNF-α transcript levels ($p<0.01$) relative to PG/VG (**Figure 6.12B**). IL-8 presented a unique pattern, with 42 puffs of air-only significantly augmenting transcript levels in contrast to the incubator control (**Figure 6.12C**). This effect was mirrored with 42 puffs of vanilla tobacco and hazelnut ($p<0.001$) . Interestingly, menthol delineated a 'bell' effect, attaining significance at 28 ($p<0.05$) and 56 puffs ($p<0.0001$) in comparison to PG/VG. IL-6 increased with 42 puffs of PG/VG and hazelnut exposures, albeit without reaching statistical significance (**Figure 6.12D**). Vanilla tobacco, cinnamon, and menthol in contrast showed a dose-dependent decrease in IL-6 transcript reaching significance at 28 puffs of vanilla tobacco and menthol ($p<0.05$) relative to PG/VG.

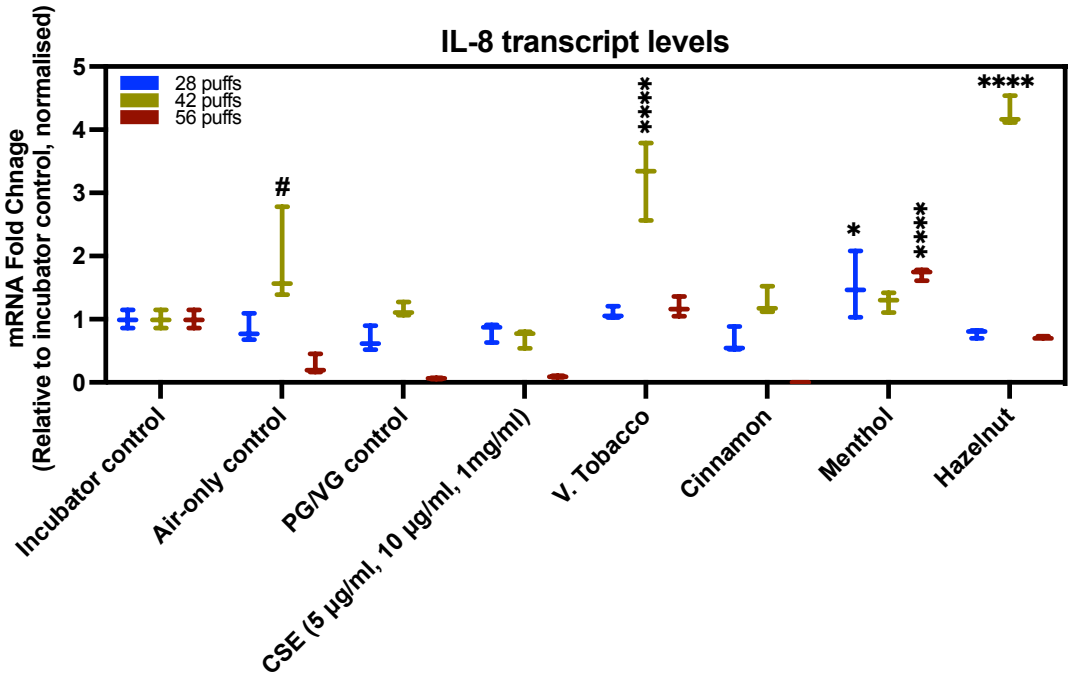
A


IL-1β transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
14
12
10
8
4
2
0
****
****
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

B


TNF-α transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
4
3
2
1
0
**
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

C


IL-8 transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
0
1
2
3
4
5
#
*
****
****
****
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

D

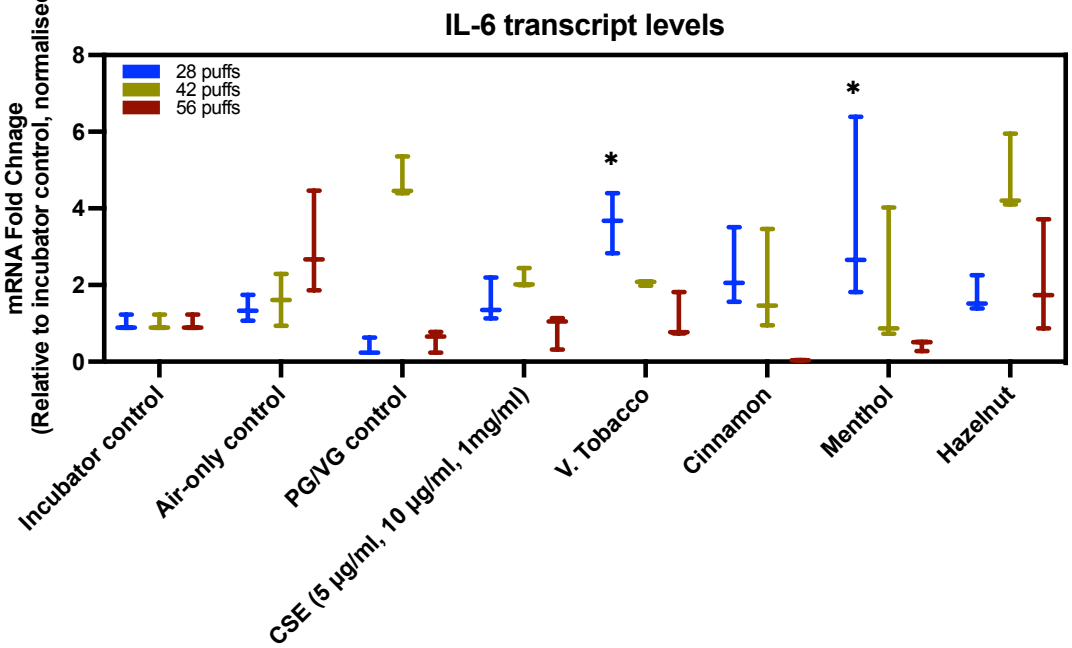
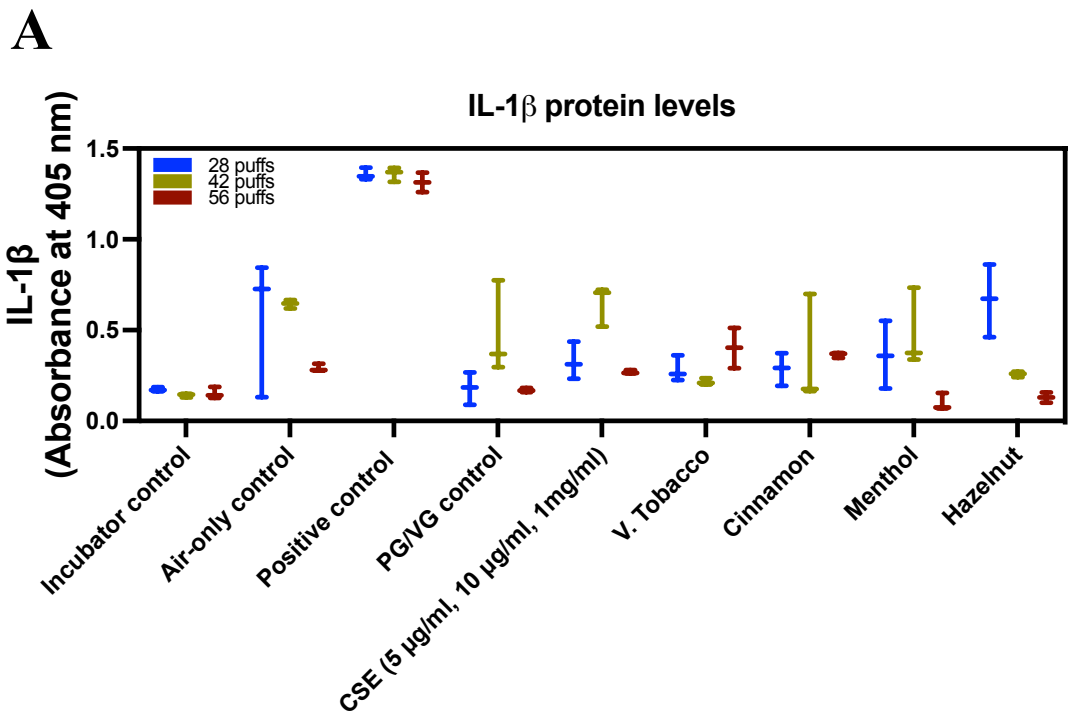

IL-6 transcript levels
mRNA Fold Chnage
(Relative to incubator control, normalised)
28 puffs
42 puffs
56 puffs
0
2
4
6
8
*
*
*
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

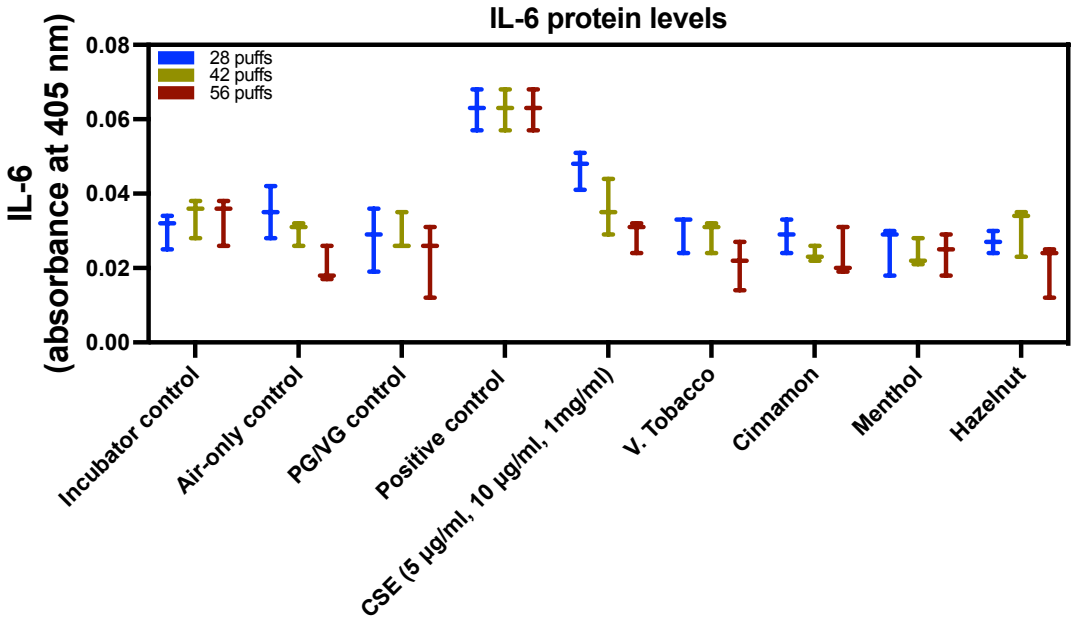
Figure 6. 12 mRNA transcript levels of cytokines following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol. A) IL-β, B) TNF-α, C) IL-10, and D) IL-6 in 14-days differentiated HBEC-3KT cells at ALI. A two-way ANOVA with multiple comparisons, corrected using Tukey's test, was used to determine statistical significance. Data are presented as box and whiskers min-max(n=3 with 3 biological replicates for qPCR gene expression). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 vs day 0. $^{\#}p$<0.05 vs incubator control.incubator control.

6.3.7 DIFFERENTIAL EFFECTS OF 28, 42, & 56 PUFFS OF E-CIGARETTE AEROSOL ON PROTEIN LEVELS

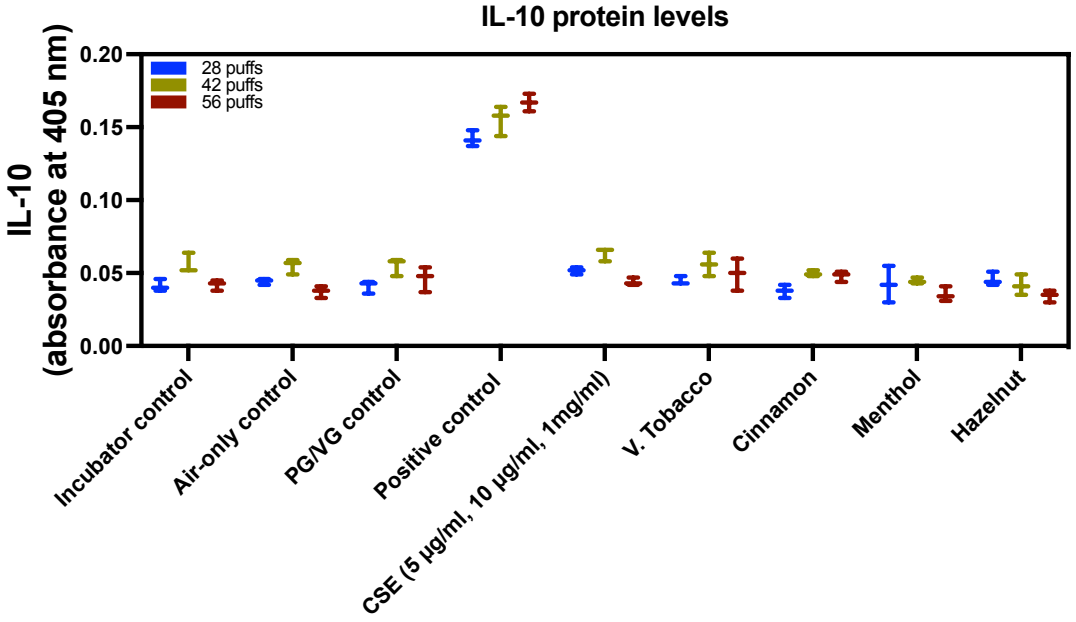
Contrary to anticipation, at the doses investigated, neither the e-liquid flavour nor cigarette smoke condensate caused statistically significant changes to the protein levels of IL-1β (**Figure 6.13A**), IL-6 (**Figure 6.13B**), IL-10 (**Figure 6.13C),** and TNF-α) (**Figure 6.13D).**



B


IL-6 protein levels
IL-6 (absorbance at 405 nm)
0.08
0.06
0.04
0.02
0.00
28 puffs
42 puffs
56 puffs
Incubator control
Air-only control
PG/VG control
Positive control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

C


IL-10 protein levels
IL-10 (absorbance at 405 nm)
0.20
0.15
0.10
0.05
0.00
28 puffs
42 puffs
56 puffs
Incubator control
Air-only control
PG/VG control
Positive control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

D

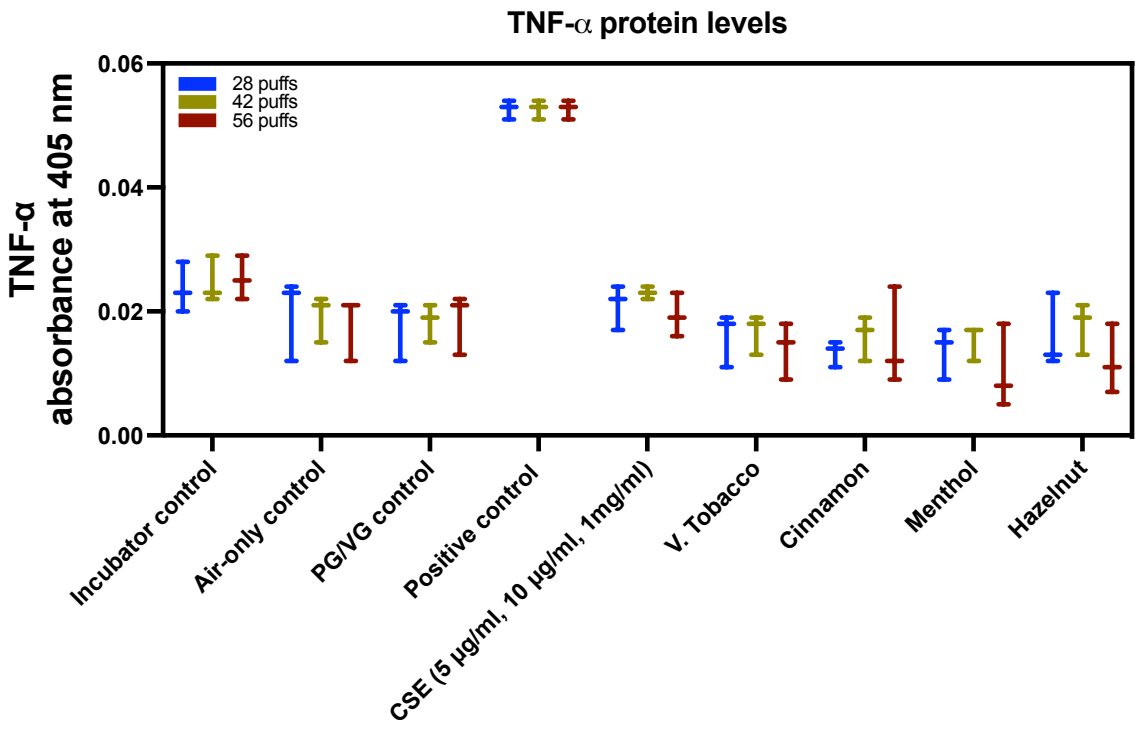
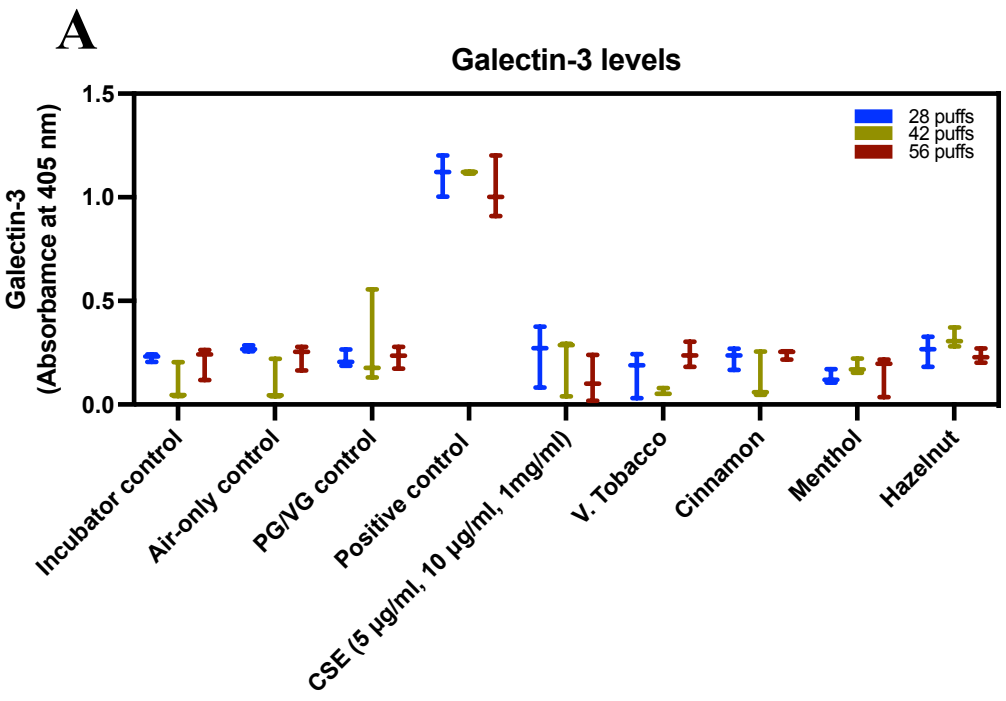


Figure 6. 13 Cytokine levels following exposure to EC aerosol (28 puffs, 42 puffs, and 56 puffs) generated from cinnamon, vanilla tobacco, hazelnut, and menthol e-liquids. A) IL-β, B) IL-6, C) IL-10 and D) TNF-α, in 14-days differentiated HBEC-3KT cells at ALI. A two-way ANOVA with multiple comparisons, corrected using Tukey's test, was used to determine statistical significance. Data are presented as box and whiskers min-max (n=3 with 3 biological replicates for qPCR gene expression). All groups were statistically significant compared to their respective positive controls (p<0.0001).

6.3.8 DIFFERENTIAL EFFECTS OF 28, 42, & 56 PUFFS OF E-CIGARETTE AEROSOLS ON GALECTIN-3 LEVELS AND DNA DAMAGE MARKER 8-OHDG

There were no statistically significant changes elicited by 28, 42, and 56 puffs of e-cigarette aerosols on either galectin-3 (**Figure 6.14A**) or the DNA damage marker 8-OHdG (**Figure 6.14B**) on 14-day ALI differentiated HBEC-3KT cells.


A
Galectin-3 levels
Galectin-3
(Absorbamce at 405 nm)
1.5
1.0
0.5
0.0
28 puffs
42 puffs
56 puffs
Incubator control
Air-only control
PG/VG control
Positive control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

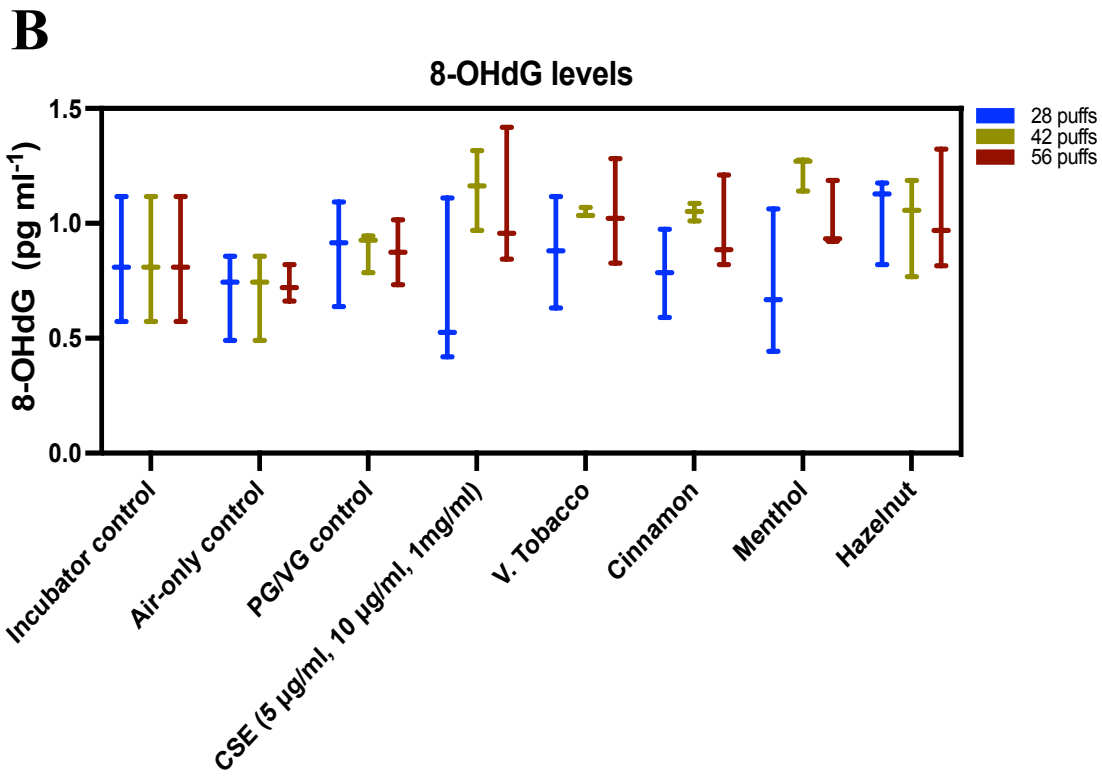

B
8-OHdG levels
8-OHdG (pg ml-1)
1.5
1.0
0.5
0.0
28 puffs
42 puffs
56 puffs
Incubator control
Air-only control
PG/VG control
CSE (5 µg/ml, 10 µg/ml, 1mg/ml)
V. Tobacco
Cinnamon
Menthol
Hazelnut

Figure 6. 14 Characterising the dose-dependent (28 puffs, 42 puffs, and 56 puffs) effects of cinnamon, vanilla tobacco, hazelnut, and menthol on the protein levels of A) Galectin-3 and B) DNA damage marker 8-OHdG in 14-days differentiated HBEC-3KT cells at ALI. A two-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are expressed as mean ± SEM (n=3 with 3 biological replicates). All groups were statistically significant compared to positive controls (p<0.0001).

6.3.9 THE EFFECTS OF FLAVOURED E-LIQUID AEROSOLS ON THE MORPHOLOGY OF HBEC-3KT EPITHELIUM

Microscopic evaluation reveals that the cellular and morphological integrity of the epithelial layer remained largely unaffected in incubator, air-only, and PG/VG-exposed cells. However, exposure to specific aerosolised substances resulted in distinct morphological alterations. In vanilla tobacco-exposed cells (**Figure 6.16D**), a notable scaliness was observed (change in colour towards orange colour), though it was more pronounced in cinnamon-exposed cells (**Figure 6.16E**), indicating a substantial alteration in cellular morphology and potentially impacting barrier function. The hazelnut-exposed cells (**Figure 6.16F**) were characterised by the presence of a mucus layer (grey viscoelastic gel) enveloping the apical region, indicative of a possible protective or reactive cellular response. Conversely, menthol exposure (**Figure 6.16G**) resulted in the absence of this mucus layer, leading to an apparent clarity of the epithelial surface under microscopic examination. This phenomenon could imply an inhibitory effect on mucus production or an acceleration in its clearance.

The most substantial impact was observed in cells exposed to cigarette smoke extract (**Figure 6.16H**). The epithelial layer exhibited distinct disruptions, evidenced by visible gaps indicating cellular damage and compromised epithelial integrity.

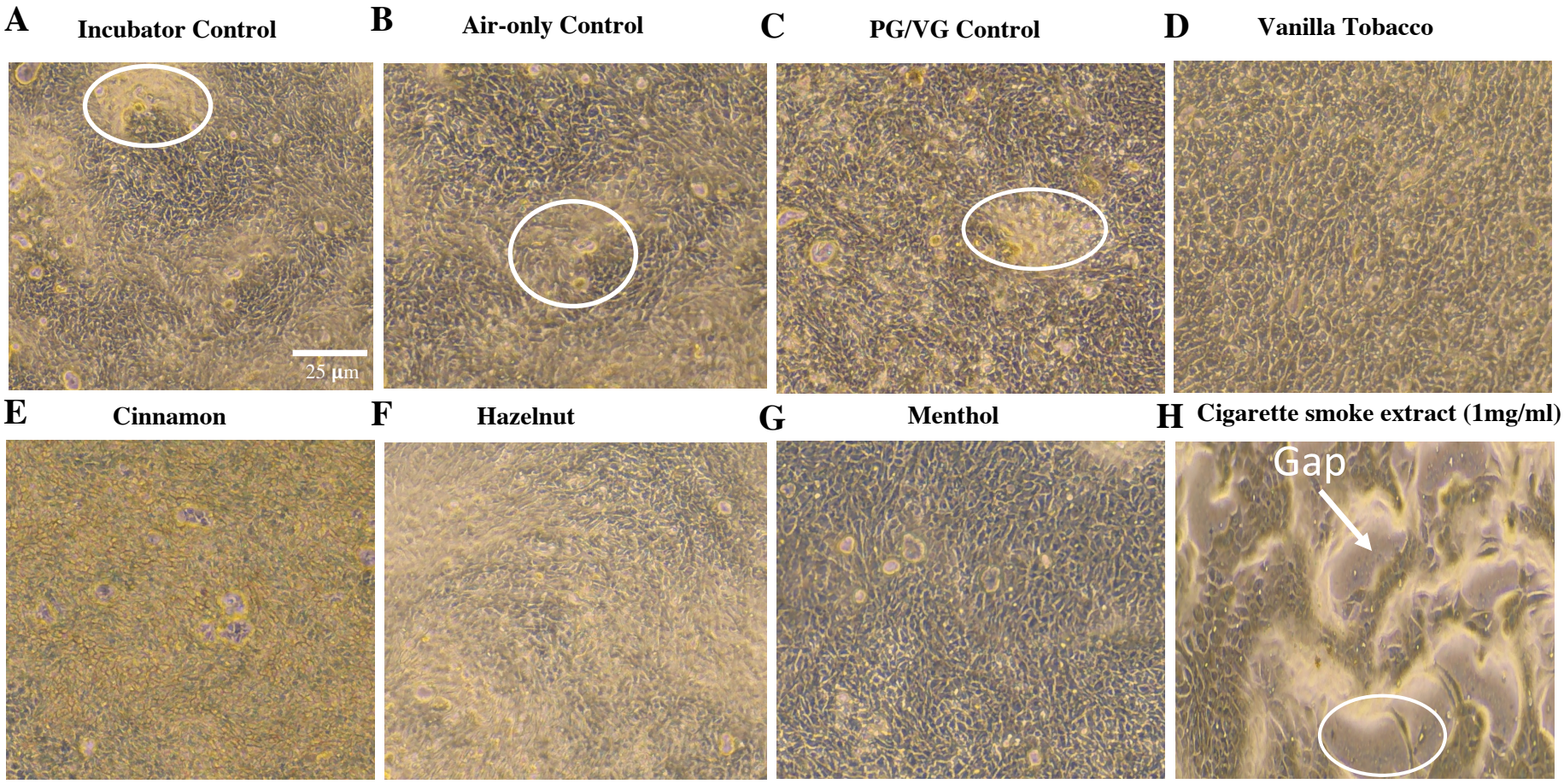

A
Incubator Control
B
Air-only Control
C
PG/VG Control
D
Vanilla Tobacco
25 µm
E
Cinnamon
F
Hazelnut
G
Menthol
H
Cigarette smoke extract (1mg/ml)
Gap

Figure 6. 15 Light microscopy 10x images of 14-days ALI differentiated HBEC-3KT cells either maintained in the A) incubator or exposed to 56 puffs of B) air-only C) PG/VG, D) vanilla tobacco, E) cinnamon, F) hazelnut, G) menthol, H) cigarette smoke extract (1mg/ml). The oval shape added on top of images show mucus.

6.4 DISCUSSION

In this study, we conducted a toxicological assessment of 4 flavoured e-liquids mimicking the *in vivo* exposure of these flavours to differentiated human bronchial epithelium. We initially optimised the exposure time in the CULTEX system, which helped reduce system-based artifacts as much as possible in the experimental design.

6.4.1 ELECTROSTATIC PRECIPITATOR'S IMPACT ON CELLULAR STRESS AND TOXICITY IN ALI-CULTURED HBEC-3KT CELLS

The electrostatic precipitator, a key component of the CULTEX® exposure system, offers a sophisticated approach to depositing aerosol particles onto cells cultured at the ALI. Utilizing the principles of electrostatics, the precipitator enhances the deposition of airborne particles onto the cell culture surface, ensuring a controlled and reproducible exposure scenario that is attractive for toxicity studies (Aufderheide *et al.*, 2011, 2013; Aufderheide and Mohr, 2000). There are concerns about the introduction of cellular stress or toxicity by the electric fields generated yet several *in vitro* inhalation studies have shown that using the electrostatic precipitator does not significantly increase cytotoxicity (LDH) or measures of cellular inflammation (IL-8 protein, hmox-1 and cyclooxygenase-2 transcript levels) in either normal human bronchial epithelial cells obtained from human volunteers (Volckens *et al.*, 2009) or A549 airway epithelial cells (De Bruijne *et al.*, 2009). In our study, we observed that 15 min exposure of HBEC-3KT cells at ALI to air-only using the electrostatic precipitator significantly increased LDH release in the basal media and increased hmox-1 and ddit3 transcript levels compared to 15 min without. Factors like experimental conditions, cell type, and exposure durations may explain these

divergent observations. Therefore, we chose not to incorporate electrostatic precipitation.

6.4.2 EXPLORING THE VARIABILITY IN PARTICLE SIZE AND COUNT IN E-CIGARETTE AEROSOLS: INFLUENCE OF E-LIQUID COMPOSITION AND METHODOLOGY

The particle size distribution (PSD) and number of e-cigarette aerosols are important factors to consider in terms of respiratory dosimetry and potential health effects, as they can influence the deposition and distribution of particles in the respiratory system (Jiang *et al.*, 2023). The particle size of e-cigarette aerosols can vary depending on the composition of the ratio of humectant and the flavourings used (Stefaniak *et al.*, 2022). In this study, PG/VG was found to produce the smallest particles, followed by cinnamon and menthol, while hazelnut and vanilla tobacco had the largest particles. These findings are consistent with previous research, which has also reported submicron-sized particles in e-cigarette aerosols (Lalo *et al.*, 2020).

Nonetheless, Stefaniak *et al.*, (2022) reported that aerosol particles from PG/VG were significantly larger than from vanillin flavoured e-liquids. Although like us, this study used the ECAG system with the same power and coil resistance for aerosol generation, we used a real-time PSD able to collect VOCs and smaller particles, whereas Stefaniak et al. utilised a low flow cascade impactor. Interestingly, even when we utilised a low flow cascade impactor to characterise PSD, PG/VG had the highest proportion (75.9%) of PM<0.99 microns. The most plausible explanation for this discrepancy is that Stefaniak et al., used 70/30 PG/VG and we used 60/40 PG/VG. The small particle size of e-cigarette aerosols may allow them to penetrate deep into the respiratory system, potentially reaching the alveolar region. The number of particles emitted by e-cigarettes can also vary depending on the e-liquid composition and the flavourings

used. In this study, PG/VG had the highest particle count, followed by cinnamon, vanilla tobacco, hazelnut, and menthol (Jiang *et al.*, 2023) suggesting the particle number is not solely determined by the particle size, but also the e-liquid composition including the flavourings.

Submicron-sized particles, have a greater probability of deposition in the alveolar region compared to larger particles (Jiang *et al.*, 2023). We initially hypothesised that the high particle number of e-cigarette aerosols, particularly those with small particle size, may result in a higher deposition of particles in the respiratory system; therefore PG/VG and menthol will have the highest deposition onto the cells and potentially increasing the exposure to harmful substances. Nonetheless, the deposition dose calculated in our study and elsewhere (Stefaniak *et al.*, 2022) revealed no correlation between particle size or number and the amount deposited on the cells.

6.4.3 DIFFERENTIAL EFFECTS OF E-CIGARETTE FLAVOURINGS AND CIGARETTE SMOKE EXTRACT ON EPITHELIAL INTEGRITY AND CYTOTOXICITY

Our study found a significant increase in LDH levels at the highest dose (56 puffs) of cinnamon and hazelnut compared to PG/VG 56 puffs control. Surprisingly, the diluted cigarette smoke extract did not induce significant LDH release. This suggests that certain e-cigarette flavourings may have a greater cytotoxic effect on bronchial epithelial cells than others and that the cytotoxicity of EC may not be solely attributed to nicotine or traditional tobacco components. For TEER measurements, cinnamon at 56 puffs significantly decreased relative TEER values compared to controls, indicating a disruption in the epithelial barrier function. In contrast, menthol at 42 and 56 puffs significantly increased relative TEER measurements. A potential explanation is the

absence of a mucus layer on menthol-exposed cells. Mucus is a viscoelastic gel formed by water, salts, high molecular weight glycoproteins (mucins), and other proteins, which can decrease TEER values (Silva *et al.*, 2023). While this effect may seem protective on the epithelial barrier, it is rather due to the absence of mucus or as a compensatory mechanism for the absence of mucus to increase epithelial protection.

These findings highlight the variability in the effects of different e-cigarette flavourings on the integrity of the respiratory epithelium. Surprisingly, 1 mg/ml of diluted cigarette smoke extract exposed to HBEC-3KT cells for 28 min at ALI did not affect relative TEER values. It is unclear why the formation of gaps in the middle of the epithelium failed to reduce TEER; nonetheless, we hypothesize that basal cells underneath the epithelial cells can affect TEER values. Basal cells can form tight junctions with the epithelial cells and contribute to the overall barrier function (Srinivasan *et al.*, 2015)

6.4.5 COMPLEX MODULATION OF AIRWAY EPITHELIAL GENE EXPRESSION BY FLAVOURED E-LIQUID AEROSOLS: IMPLICATIONS FOR RESPIRATORY HEALTH AND HOMEOSTASIS

The impact of various doses of flavoured e-liquid aerosols on specific genetic markers associated with airway differentiation in HBEC-3KT cells is intricate and underscores the critical need for rigorous assessment and regulation of e-liquids. Each flavour and dose revealed distinct patterns of modulation on gene expression, shedding light on their potential implications in airway health and disease. Increased *krt5* transcript levels, instigated by 56 puffs of cinnamon and menthol, are noteworthy. *krt5* is a marker for basal cells, which are progenitor cells in the airway epithelium (see **Chapter 5**). An upregulation may suggest enhanced basal cell activity or proliferation,

potentially implicating a repair mechanism response to injury caused by e-liquid exposure. However, whether this upregulation is a protective or detrimental response is a subject warranting further investigation. The absence of a mucus gel layer on the microscopic images corroborates the significant downregulation of muc5ac and muc5b by menthol at 56 puffs. Similarly, the significant upregulation of muc5ac and muc5b in hazelnut translated into high mucus production as observed in the microscopic images. Muc5ac is associated with mucus production and is often upregulated in respiratory diseases like asthma and COPD (**Chapter 1)**. Therefore, this suggest that prolonged exposure to hazelnut may increase mucus production and may eventually lead respiratory diseases like asthma and COPD. In contrast, the absence or reduction in physiological levels of mucus on the epithelium could imply potential attenuation of mucus production, which could compromise epithelial barrier defense mechanisms against xenobiotics (Zhang *et al.*, 2023). The *foxj1* gene, associated with ciliated cell differentiation, was downregulated with 56 puffs of cinnamon and menthol, raising concerns on impaired cilia function, which is essential for mucus clearance and respiratory health (see **Chapter 5**). Conversely, cigarette increased foxj1 remarkably. Evidence suggests that toxicants in tobacco smoke paralyze cilia and eventually destroy them, removing an important protection from the respiratory system (Leopold *et al.*, 2009). Moreover, clinical studies have shown that cilia length is reduced in healthy smokers and that long-term exposure to cigarette smoke leads to reduced numbers of ciliated cells (Aufderheide *et al.*, 2015). Therefore, although no visible cilia were present, the upregulation of foxj1 by cigarette smoke may have occurred as a defense and compensatory mechanism for more cilia to be formed. We also observe a small increase in PG/VG, vanilla tobacco, and hazelnut in foxj1. We hypothesize that this may be associated with the irritant effect of PG-

contained e-liquids in EC users. While most EC users can inhale PG without issues, some may experience sensitivity or allergic reactions. Noteworthily, even EC users who have used PG-based liquids without issues can develop a sensitivity or allergy over time. The impact on tp63 expression, especially its reduction in the air-only control and modulation marked increase in cinnamon and menthol at the middle dose but not at the lowest or highest dose, reveals the complexity beneath the mechanism of actions of these flavours while also revealing the potential effects on basal cell and epithelial homeostasis. Although it is unclear why this phenomenon occurred, it is possible that the effect of cinnamon and menthol exposure on tp63 transcript levels in ALI cells follows a non-linear dose-response relationship. Therefore, it is possible that the middle dose of cinnamon exposure was within the range that caused an effect on tp63 transcript levels, while the lowest and highest doses were outside of that range. However, this hypothesis warrants further investigation.

6.4.6 UNDERSTANDING THE CELLULAR STRESS RESPONSES INDUCED BY FLAVOURED E-LIQUIDS' AEROSOLS

The most significant effects of flavoured e-liquids and cigarette smoke extract in this study were the significant increases in *ddit3* and *hmox1* mRNA, The significant increase in DDIT3 gene expression induced by high doses of vanilla tobacco, menthol, and hazelnut aerosols is indicative of the cellular stress response. DDIT3 (also known as CHOP) plays a pivotal role in the endoplasmic reticulum (ER) stress response and apoptosis (Hu *et al.*, 2019; Yang *et al.*, 2017). We attribute the absence of this response following 56 puffs of cinnamon to cytotoxicity (Behar *et al.*, 2014b). This raises questions on the safe concentration thresholds for cinnamon and other similar e-liquid

flavours. The response of DDIT3 to cigarette smoke extract at a moderate concentration but not at the highest concentration echoes the complex dose-response relationship as stated earlier. It suggests the existence of a threshold beyond which cellular damage is sufficient to reduce this stress response markers. This complexity underscores the need for a granular understanding of dose-response relationships to inform regulatory guidelines. The uniform upregulation of hmox1 across all flavoured aerosols at higher doses highlights a pronounced oxidative stress response. Hmox1 is a crucial component of cellular defence mechanisms against oxidative stress. Its universal upregulation indicates that irrespective of flavour, e-liquid aerosols at higher doses induce oxidative stress, aligning with findings that flavoured e-cigarette vapour can instigate oxidative stress (Lerner *et al.*, 2015).

The response pattern of hmox1 and ddit3 to cigarette smoke extract mirrors that of ddit3, again hinting at a cytotoxic effect at higher concentrations. The induction of hmox1 and ddit3 may be associated with the cytotoxic effects observed in Chapter 3, where cinnamon, hazelnut, and vanilla tobacco decreased mitochondrial membrane potential and increased ROS production (apart from cinnamon). Also, the inert response of cells to PG/VG vehicle confirms our findings in Chapter 3 that PG/VG's cytotoxicity does not occur through an oxidative stress mechanism (Leigh *et al.*, 2016d; Ween *et al.*, 2020b). This evidence underscores that the flavours and other additives predominantly contribute to the cellular stress responses observed.

6.4.7 THE EFFECTS OF FLAVOURED E-LIQUIDS' AEROSOLS ON INFLAMMATORY CYTOKINES MRNA TRANSCRIPT LEVELS

The effects of differently flavoured EC aerosols on the mRNA transcript levels of IL-1β, TNF-α, IL-8, and IL-6 are mixed. The significant increases in mRNA levels of IL-1β, a pivotal cytokine involved in the inflammatory process and immune response, due to exposure to cigarette smoke extract and menthol aerosols (28 puffs) aligns with previous research that associates cigarette smoke and certain e-liquid flavours with inflammatory responses (see **Chapter 2**). TNF-α is similarly significantly amplified by menthol exposure supporting menthol's impact on the inflammatory pathway. TNF-α plays a role in systemic inflammation and is associated with various inflammatory and autoimmune diseases (Bradley, 2008). For the mRNA levels of IL-8, there was an increase at 42 puffs in vanilla tobacco and hazelnut above air-only. IL-8 is known for its role in neutrophil recruitment and activation during inflammation (Maslanik *et al.*, 2012). This implies that the inflammatory response might be exclusively associated with specific flavours like vanilla tobacco and hazelnut. IL-6's mRNA levels, we observed a dose-dependent increase in air-only and an increase in the middle dose of PG/VG and hazelnut, albeit not significant. It is unclear why this occurs. But this contributes to the complexity associated with the interpretation of flavour data and is even more difficult to conclude from these.

6.4.8 THE ABSENCE OF SIGNIFICANT INFLAMMATORY RESPONSES AT THE PROTEIN LEVEL POST E-CIGARETTE EXPOSURE

We also investigated protein levels of key cytokines (IL-1β, IL-6, IL-10, and TNF-α) following flavoured EC exposures but there were no significant changes at the protein level despite marked changes at the transcript level. These findings seemingly contradict earlier research that suggested potential inflammatory responses, including elevated cytokine levels, associated with EC use (**Chapter 2**). Several factors could account for the observed lack of any significant effects. One plausible explanation might be the cellular adaptation or resilience developed in ALI-differentiated cells. A comparative analysis with existing literature reveals disparities in biological responses induced by e-cigarettes. While some studies in **Chapter 2** have reported elevated inflammatory markers, others indicate minimal to no significant impact. Our study suggests flavoured e-liquid aerosols do not induce an inflammatory response in ALI-differentiated HBEC-3KT cells.

6.4.9 UNALTERED GALECTIN-3 AND 8-OXODG LEVELS: EVALUATING THE IMPACT OF E-CIGARETTE FLAVOURS ON INFLAMMATION AND DNA DAMAGE

We observed no statistically significant increase in either the levels of galectin-3 and 8-oxodG, regardless of the e-cigarette flavour and puff intensities. Galectin-3, a β-galactoside-binding lectin, is implicated in various biological processes, including inflammation, cancer diagnosis, cell growth and differentiation, and apoptosis (Sciacchitano *et al.*, 2018)(Henderson et al., 2018). Because flavours caused no change in the protein levels of inflammatory cytokines, the lack of significant changes in galectin-3 was not surprising, suggesting a minimal inflammatory response, at least within the parameters of our experimental design. Similarly, although we observed a

high expression of mRNA transcript levels of ddit3 and hmox1induced by flavoured e-liquid aerosols, no effect was observed in 8-OHdG, a well-established marker of oxidative DNA damage (Valavanidis *et al.*, 2009). Ddit3 is related to endoplasmic reticulum (ER) stress and is involved in the unfolded protein response (UPR) (Li *et al.*, 2021). ER stress can lead to DNA damage, and ddit3 has been shown to be involved in this process (Li *et al.*, 2021). Hmox1 is associated with DNA damage and repair signaling (Otterbein *et al.*, 2011). Additionally, a study found that excessive hmox1 levels are correlated with aggravation of photoreceptor degeneration, which is related to DNA damage (Li *et al.*, 2021). Therefore, the no change in 8-OHdG levels may indicate a functional DNA repair system in the HBEC-3KT cell line.

6.4.10 MORPHOLOGICAL ALTERATIONS IN CELLS EXPOSED TO DIFFERENT E-CIGARETTE FLAVOURS AND CIGARETTE SMOKE: INSIGHTS INTO CELLULAR INTEGRITY AND RESPONSES

Cells at ALI exposed to air-only, and PG/VG, as seen in **Figures 6.16A-C**, maintained cellular and morphological integrity. This observation supports the notion that baseline conditions and PG/VG vehicles do not induce significant cellular morphological changes, consistent with previous findings that PG/VG base e-liquids have minimal cytotoxic effects (Bahl et al., 2012; Leigh et al., 2016; Ween et al., 2020). However, the exposure to EC aerosols presented distinct cellular morphological alterations. The scaliness observed following exposure to vanilla tobacco and cinnamon-flavoured aerosols, **Figures 6.16D-E**, suggests a notable alteration in cellular integrity. This could impact the barrier function of the epithelium. Studies have reported that flavourings in e-cigarettes can induce cytotoxicity and impair cellular functions (Chapter 2), aligning with our observations. The presence of a

mucus layer enveloping the apical region of hazelnut-exposed cells (**Figure 6.16F**) could be interpreted as a protective or reactive cellular response. This observation invites comparisons with findings that some e-liquid flavourings can induce mucus hypersecretion, potentially as a defence mechanism. In contrast, the clarity of the epithelial surface in menthol-exposed cells (**Figure 6.16G**) indicates an inhibitory effect on mucus production, which is corroborated by the downregulation of muc5ac and muc5b gene. This observation could be aligned with findings that menthol can induce changes in mucus consistency and clearance rates. The marked disruption to the epithelial layer of ALI exposed to cigarette smoke extract (**Figure 6.16H**) underscores the damaging effects of combustible tobacco. This finding is consistent with studies documenting the harmful impacts of cigarette smoke on cellular integrity, leading to various respiratory diseases.

6.4.11 STUDY LIMITATIONS

In this study, we endeavoured to simulate human *in vivo* exposure to flavoured aerosols but were met with several unavoidable limitations. One significant limitation pertained to our cell culture model. Ideally, primary human bronchial cells would have provided a more accurate representation; however, due to their high cost, limited availability, high susceptibility to infections, and restricted passage numbers (primary cells cannot be used beyond passage 5), we had to rely on HBEC-3KT cells.

Regarding the dosage selection, we recognise that the highest dose of 56 puffs in 28 min of EC might not accurately reflect typical human exposure. This dose was, however, deemed essential for discerning the potential impacts of high doses of EC. We simulated the human experience by diluting aerosols with air to avoid inducing comprehensive cytotoxicity in the HBEC-3KT cells, even at these elevated doses.

Another limitation arose in our attempt to quantify protein levels using absorbance measurements. The lack of an appropriate wavelength to measure protein absorbance in ELISA prevented us from constructing a standard curve for extrapolating precise protein concentrations. As a result, our data presentation is in terms of absorbance rather than protein concentration, which may not provide a direct insight into the biological implications of exposure to flavoured aerosols.

Furthermore, including a nicotine control was overlooked in our experimental design. Since the cigarette smoke extract used as a positive control contained nicotine, incorporating a nicotine control would have been instrumental in delineating whether nicotine exerts a protective effect. This oversight limits our ability to draw conclusive insights regarding the specific role and impact of nicotine in the context of cigarette smoke extract toxicity.

These limitations notwithstanding, our findings contribute valuable insights and underscore the need for further in-depth studies, especially those employing primary human bronchial cells and incorporating nicotine controls when cigarette smoke extracts are used, to comprehensively elucidate the biological effects and potential health risks associated with flavoured aerosol exposure in comparison to cigarette smoke.

6.5 CONCLUSIONS

These findings could be attributed to multiple factors. The resilience or adaptability of HBEC-3KT cells, the specific experimental conditions, chosen puff intensities, and the 14-days ALI differentiation period might have influenced the outcomes. It’s also conceivable that the cellular responses might be more subtle and complex, requiring more sensitive assays or a broader spectrum of biomarkers to be fully elucidated. In

the context of existing literature, these results offer a counter-narrative to studies documenting pronounced inflammatory responses associated with EC use (**Chapter 2**). It emphasises the importance of a multifaceted approach to understanding ECS' biological impacts, incorporating various experimental conditions, cellular models, and biomarkers.

The comprehensive investigation into the impacts of various e-cigarette flavourings and cigarette smoke extracts on human bronchial epithelial cells (HBEC-3KT) has yielded complex and multifaceted results that offer insights into the cellular and molecular implications of e-cigarette use and exposure. The study confirmed that cinnamon flavour elicited more cytotoxicity, as evidenced by increased LDH levels and reduced TEER measurements, than other tested flavours. The cigarette smoke extract did not induce significant LDH release or affect TEER values as much as some e-liquids, especially cinnamon. This challenged the second hypothesis that cigarette smoke would induce more cytotoxicity than the flavoured e-liquids. This may be attributed to the protective effect of nicotine and the absence of a substantial amount of VOCs in the cigarette smoke extracts. The particle size distribution did not consistently correlate with cytotoxicity. Though smaller particles were initially expected to be more toxic due to greater surface area/mass ratio, no direct correlation was found between particle size and number with cellular deposition. The study presented intricate patterns of cellular and molecular responses, with some e-liquids modulating the expression of transcript levels of inflammatory cytokines. However, the uniformity in protein levels of key cytokines across varying doses and flavours contradicts the anticipation of fluctuating levels aligning with different e-liquids, which partially refutes the fourth hypothesis. The novelty of this study is the

remarkable increase of oxidative stress genes ddit3 and hmox1. This implies that flavours tested in this study may cause toxicity via an oxidative stress-based mechanism and not through cytokine-induced inflammation

.

CHAPTER 7
GENERAL DISCUSSION

7.1 DISCUSSION

The safety of flavours is well-established in the context of food consumption, where they are typically recognised as safe. However, the assurance of their safety becomes ambiguous when these flavours are subjected to thermal degradation and introduced into the respiratory system via inhalation, as seen in the case of ECs. Since ECs have been in the consumer market for only 14 years, there is a paucity of conclusive evidence on the impact of inhaled flavours on lung health. This thesis explores this gap through a series of *in vitro* studies.

7.2 GENERAL SUMMARY OF FINDINGS AND STUDY LIMITATIONS

Chapter two of this thesis employed the PRISMA model to systematically evaluate the existing literature on the toxicological impacts of e-liquid flavours. However, due to methodological shortcomings and inconsistencies in the available studies, we discovered inconclusive evidence linking flavours like cinnamon, menthol, and strawberry to toxicological effects on pulmonary cells. Recognising these gaps, we proposed 'gold-standard' recommendations, a robust methodology to strengthen future research in this field.

In chapter three, we conducted an in-depth toxicological assessment of 53 nicotine-free flavours on HBEC-3KT cells in submerged exposures. We provide robust calculations to justify why e-liquid direct exposure studies should not exceed 1% (v/v). This is the first time a study has justified the usage of e-liquid doses. Our findings indicated that PG/VG base, commonly considered innocuous, is not entirely harmless, with PG/VG displaying notable toxicity. Among the 53 flavours assessed, cinnamon, hazelnut, and vanilla tobacco elicited distinct and dose-dependent toxic effects independent of the PG/VG base. Further investigations revealed cytostatic effects,

reductions in cell viability (specifically for cinnamon), decreased MMP, and increased ROS production. Chemical analysis of the aerosols from the three toxic hits revealed the presence of volatile organic compounds known to be toxic to humans, such as estragole, 1,3-dioxane, and biphenyls. We also observed the presence of reaction products of unknown toxic profiles, such as vanillin propylene glycol acetal, ethyl maltol, and ethyl vanillin. Because such compounds can be pharmacologically active, it is unsurprising that they potentially induce toxicity in the bronchial cells by interacting with membrane-bound proteins or permeating the membrane to interact with cytoplasmic signalling pathways. Indeed, we show that the cytotoxicity of cinnamon may, in part, be mediated by TRPA1, a calcium channel that plays a pivotal protective role in human bronchial epithelial cells. Therefore, exposure to cinnamaldehyde may cause calcium imbalance, leading to the remarkable adverse effects seen in cinnamon-exposed cells. We acknowledge that the exposure model utilised in this chapter does not recapitulate the human *in vivo* exposure to flavoured e-liquids. This is because the HBEC-3KTs cells are largely basal cells exposed to direct dilutions of e-liquids. However, this experimental approach is paramount to assess a wide range of flavoured e-liquids across the flavour wheel to determine the individual toxic flavours and characterise any flavour-category-dependent toxicity in e-liquids. Also, we recognise that this approach may imply that certain potentially toxic flavors only in their aerosol forms might be overlooked; nonetheless, this study proposes an approach that is the most comprehensive way to increase the chances of identifying the highly toxic flavours in an ever-increasing flavouring market. Subsequently, the aerosols of these identified toxic flavours can be assessed with 3D cell culture models that mimic the microenvironment of the lung epithelium, as we have done in this study.

Chapter 4 validates ALI-differentiated Calu-3 cells by characterising genetic markers of airway differentiation over 28 days. Calu-3 differentiated at ALI are extensively used as a model for inhalation drug permeation and delivery studies. Although Calu-3 cells proved to be a robust ALI model by forming tight junctions and producing mucus, the model did not reveal flavour-specific additive effects to PG/VG when used to assess the three toxic hits identified in **Chapter 3**. Consistent with their liquid form, aerosols from PG/VG vehicles revealed cytotoxicity comparable to 3R4F cigarette smoke. This led to the hypothesis that Calu-3 cells may not be a good model to study flavored e-liquids due to the lack of sensitivity to flavoured e-liquid aerosols. Moreover, we hypothesized that the experimental approach used in this study may not be robust enough to mimic the human *in vivo* exposure. This is because in this study vapour from the flavoured liquids was puffed in an incubator (used as the exposure chamber) where the ALI-differentiated Calu-3 cells were placed and were allowed to settle on the cells. While several studies have taken this approach in **Chapter 2** to assess aerosols toxicity, we believe limiting factors such as humidity in the exposure chamber, particle shape and density, and particle electrical charges impact the aerodynamics of flavoured aerosol particles: hence, impacting the predictiveness of the model and increasing uncertainties around flavour toxicity. Therefore, the discrepancies among studies observed in **Chapter 2** may be improved or eliminated if robust experimental approaches are taken.

In **Chapter 5**, we evaluated and validated four commercially available pulmonary cell lines within an ALI setting, namely Beas-2b, HBEC-3KT, A549, and RPMI 2650, in response to the lack of sensitivity in the ALI-differentiated calu-3 cells. In this detailed scrutiny, we characterised various genetic markers pivotal for differentiating basal cells, mucus-producing cells, ciliated cells, and tight-junction proteins. Moreover,

TEER measurements were considered to ensure a thorough evaluation. The morphological transformations that each cell line underwent during the differentiation phase were closely monitored and documented. Our intensive evaluation and literature backing converged to spotlight HBEC-3KT cells as the most promising candidate multipotent basal cells, exhibiting superior attributes in mirroring the epithelial microenvironment. Consequently, this cell line was chosen as the subject for the in-depth inhalation study presented in **Chapter 6**, offering valuable insights into the interactions between these particular cells and the flavours under toxicological assessment.

Chapter 6 details the toxicological evaluation of increasing concentrations of aerosols in various flavours: cinnamon, hazelnut, vanilla tobacco, and menthol on differentiated HBEC-3KT cells. During the high-throughput studies in **Chapter 3**, menthol demonstrated a unique characteristic. It formed condensation on the lid of the plate. This peculiarity led to its inclusion in this aerosol study conducted in **Chapter 4**. However, we observed yet another distinctive behaviour of menthol e-liquid; when aerosolised and introduced into the exposure chamber, it formed clouds that lingered in the atmosphere without settling on the cells. This observation prompted a detailed investigation into the effects of menthol within a controlled inhalation system, ensuring direct contact with cells here in **Chapter 6**.

For comparison, 3R4F cigarette smoke extract was incorporated as a positive control. A notable observation was the disparate effects of these flavours on genetic markers associated with epithelial differentiation, cellular stress, and cytokine production. However, these fluctuations did not translate into significant alterations in the protein levels of inflammatory markers. This dichotomy alludes to a scenario where acute exposure to EC flavours might not be immediately detrimental at the protein level.

Interestingly, we observed a persistent increase in oxidative stress genes ddit3 and hmox1 expression in all flavours, consistent with the ROS production increase in **Chapter 3.** Therefore, although no change was observed in protein levels of cytokines, inflammatory marker galectin 3, and DNA damage marker 8-OHdG, we hypothesize that persistently increased levels of oxidative stress signalling could lead to increased levels of inflammation, which could induce DNA damage.

While all the flavours distinctively induced cytotoxicity, Cinnamon's toxicity stood out as the only flavour to decrease membrane integrity and increase LDH release significantly. Moreover, it was the only flavour to induce a remarkable morphological change in the epithelium. A peculiar observation was made regarding the 3R4F cigarette smoke extract. Contrary to expectations, CSE did not induce significant alterations in the biological markers of inflammation. However, its detrimental effect was visually evident as exposure to CSE resulted in epithelial tearing, manifesting as observable gaps under light microscopy. This anomaly underscores the complexity of the toxicological landscape associated with smoke and vapour exposure, suggesting that cellular and visual assessments can sometimes yield divergent insights. This study presents several limitations regarding its direct translation to *in vivo* human exposures to flavours. Here, we mimic acute exposure to increasing flavoured aerosols without nicotine. Nonetheless, EC users are mostly exposed to nicotine-contained flavoured-e-liquid aerosols. Therefore, the combinatory effects of nicotine and flavours, especially the toxic ones identified in this study, are unknown. More studies are warranted to delineate the influence of nicotine on flavour-induced toxicity.

7.3 CONTRIBUTION AND IMPACT TO PUBLIC HEALTH

This body of work will substantially impact the scientific community, policymakers,

and the general public.

Determining a gold standard, physiologically relevant *in vitro* model system to study flavour safety is one of the most impactful contributions in this study. For too long, many studies on flavour toxicity have relied on inappropriate models that do not accurately reflect real-world human exposures. This has not only led to a waste of resources but has also led to questionable and potentially misleading conclusions about flavour safety. Some key problems with past *in vitro* studies on flavor toxicity include using excessively high flavor concentrations that far exceed real-world human exposure levels. Concentrations of over 2% (v/v) are common. Nonetheless, this work justifies that concentrations above 1% (v/v) in e-liquid direct exposure are not physiologically relevant. Indeed, the 1% maximum e-liquid dose we estimated agrees with Bishop *et al.*, (2023) who observed that any dose greater than 1% formulation exceeded physiological osmolarity ranges (280-400 mOsm), thus potentially causing cells to burst (an artefact of the direct e-liquid exposure methodology rather than chemically induced toxicity). Therefore, the response observed beyond the 1% e-liquid range cannot be solely attributed to the potential cellular toxicity of the e-liquid alone, calling into question the relevance of any observed toxic effects.

Another pitfall of several studies addressed in this study is the failure to use human-relevant cell types or models, for example, the use of pulmonary cells from rodents and/or *in vivo* animal studies to study inhalation toxicity. While *in vivo* animal studies may be a valuable tool in predicting the impact of flavours on humans generally, due to species-specific differences in the human pulmonary system and rodents, data from animal studies may not be directly translatable for human exposures. Moreover, there are ethical concerns about the use of animals in research. This study provides an *in vitro* approach to implementing the 3Rs in animal testing.

Although there are no human volunteer studies yet on the impact of flavours on the lungs and other biological systems, we propose that robust *in vitro* aerosol exposure models such as the ALI, organ on a chip, and human on-chip studies in combination with robust experimental design, maybe a better predictive tools to extrapolate the potential adverse effects of flavours to human lungs. This work proposes a study design that considers the complex chemical mixtures and aerosol physics involved in real-world flavour inhalation.

The development of advanced *in vitro* systems such as the CULTEX that expose human lung cell cultures at the air-liquid interface (ALI) to aerosols from EC or flavour mixtures under realistic conditions has been a major advance. Compared to older methods, these systems more closely model real human exposures to vaping. Our study using ALI systems with appropriate controls has shown that flavours such as cinnamon, hazelnut, vanilla tobacco, and menthol can especially exhibit cytotoxicity and oxidative stress effects on lung cells at physiologically relevant concentrations. Moreover, this raises valid concerns about the safety of these flavors via inhalation.

Our study will contribute to the general public's understanding of the implications of EC flavours on their health, especially pertinent for smokers seeking cessation and never-smoker young adults who have started vaping illegal EC and flavoured e-liquids due to the general perception that they are harmless. Our study provides a new perspective, suggesting that flavours in EC are not harmless and their toxicity is not flavor-category-specific. All flavours and brands of e-liquid could have a distinct risk profile, deviating from the more uniform risk associated with conventional cigarettes. Nonetheless, cinnamon flavour’s pulmonary toxicity has been consistent across different lines of investigation and experimental models. Therefore, if not an

immediate ban on cinnamon flavour, we propose close monitoring of cinnamaldehyde-contained flavoured e-liquids in the UK and elsewhere. Moreover, because PG/VG alone is not harmless, we propose that less harmful alternatives must be tested and used. Potential candidates may be propanediol derived from corn and safe for inhalation (Scott *et al.*, 2005). Our findings underscore the importance of detailed ingredient disclosure on e-liquid packaging and suggest potential dangers from unlisted chemicals formed during aerosolisation. This research offers valuable insights for regulatory bodies such as the MHRA, healthcare professionals, and policymakers, particularly in evolving EC regulations and the growing popularity of vaping as a smoking cessation aid. Although our data supports the need for continued surveillance and research to understand the long-term effects of flavours in ECs, especially considering the varied toxicological profiles of different flavours, it is worth noting that the majority of flavours tested were relatively much safer, at least in submerged exposures.

7.4 FUTURE DIRECTIONS

The harmonisation of methodologies and standards in e-liquid flavour toxicity research is a salient issue that warrants urgent address. The diversity in experimental designs, as evidenced in chapters two through six, underscores the compelling need for a unified approach to generate consistent, comparable, and actionable insights.

The role of *in vivo* studies in ascertaining the toxicological impacts of e-liquid flavours cannot be understated. While the 3Rs principle underscores the ethical imperative to minimise animal testing, a persistent need exists to correlate *in vitro* findings with *in vivo* responses for comprehensive insights. Mouse/rat models could offer crucial insights into systemic, sub-cellular, and organ-specific impacts of e-liquid flavours, laying the groundwork for intricate understandings of the human biological and

physiological responses. A debate ensues: can we wholly rely on *in vitro* models, or is there an irrefutable need for *in vivo* studies to bridge the knowledge gaps and offer holistic insights into the multi-dimensional toxicological landscapes?

The combination of robust *in vitro* investigations and bioinformatics may provide enough toxicological evidence to not rely on animal testing. In the future, scientists investigating flavour toxicity should utilise these improved physiologically relevant *in vitro* models with aerosol exposure. Appropriate controls, such as unflavoured carrier liquids, are critical to isolate the effects of specific flavours. Testing should focus on lung cell types at realistic exposure levels. Flavoured e-liquids and aerosols should be evaluated, not just individual chemicals in the solution. Reliance on older, less relevant models has propagated misconceptions that all flavour chemicals are universally safe via inhalation. We advocate for developing a machine learning algorithm meticulously trained with data encompassing flavouring chemicals proven in *in vitro* inhalation studies to be toxic to human lungs. The primary objective of this innovative tool would be to rigorously analyse and map the chemical composition of new e-liquids entering the market. Our proposition entails a stringent regulatory measure where if 5% or more of the chemicals in a new e-liquid align with the toxic chemical library, it should be flagged as potentially hazardous. Consequently, the MHRA would be advised to bar such products from the British market, reinforcing a proactive stance in safeguarding public health against the potential perils associated with harmful constituents in e-liquids. This approach underscores the imperative of intertwining advanced technology with regulatory oversight to elevate safety standards in the rapidly evolving EC landscape.

Furthermore, the longitudinal assessment of e-liquid exposure impacts necessitates robust observational and epidemiological studies. Human-focused research can unveil

nuanced insights into the long-term health implications, behavioural patterns, and psychosocial dynamics associated with e-liquid consumption. These studies can provide real-world data, encapsulating the complexities of human biology, behaviour, and environment, thereby offering a comprehensive panorama of e-liquid impacts beyond the cellular and molecular realms.

To foster a balanced discourse, we must juxtapose the ethical, practical, and scientific facets of e-liquid research. Thus, a multi-pronged approach, harmonised methodologies, and collaborative international efforts are pivotal to unearthing the comprehensive toxicological, behavioural, and health landscapes associated with e-liquid flavours, driving informed regulatory and public health interventions. The synthesis of *in vitro*, bioinformatics data, and human studies can offer an unparalleled depth and breadth of insights, serving as the cornerstone for evidence-based policies, public health strategies, and individual choices in the evolving e-liquid landscape.

7.5 CONCLUSION

The findings of this thesis underscore the urgent need for a standardised methodology for assessing the safety of flavours in ECs *in vitro*. The nuanced variations in the toxicological impacts of different e-liquid flavours point to the necessity of individualised risk assessments for each product. Although limitations exist, our work indicates that the inhalation of EC flavours, in general, may pose a lesser threat to the pulmonary system than traditional cigarette smoking. Nonetheless, establishing harmonised guidelines for safety assessments will be instrumental in advancing public health policies, fostering the development of safer EC products, and enriching our comprehension of their toxicological profiles.

REFERENCES

Abashev, T. M., Metzler, M. A., Wright, D. M. and Sandell, L. L. (2017) Retinoic acid signaling regulates *Krt5* and *Krt14* independently of stem cell markers in submandibular salivary gland epithelium: Retinoic Acid Represses *KRT5* in epithelium, *Developmental Dynamics*, 246 (2), pp. 135–147. DOI:10.1002/dvdy.24476.

Aggarwal, V., Tuli, H., Varol, A., Thakral, F., Yerer, M., Sak, K., *et al.* (2019) Role of Reactive Oxygen Species in Cancer Progression: Molecular Mechanisms and Recent Advancements, *Biomolecules*, 9 (11), pp. 735. DOI:10.3390/biom9110735.

Aguilar, P. R., Michelson, A. P. and Isakow, W. (2016) Obliterative Bronchiolitis, *Transplantation*, 100 (2), pp. 272–283. DOI:10.1097/TP.0000000000000892.

Aherrera, A., Olmedo, P., Grau-Perez, M., Tanda, S., Goessler, W., Jarmul, S., *et al.* (2017) The association of e-cigarette use with exposure to nickel and chromium: A preliminary study of non-invasive biomarkers, *Environmental Research*, 159, pp. 313–320. DOI:10.1016/j.envres.2017.08.014.

Akopyan, G. and Bonavida, B. (2006) Understanding tobacco smoke carcinogen NNK and lung tumorigenesis, *International Journal of Oncology*, 29 (4), pp. 745–752.

Aldonyte, R., Bagdonas, E., Raudoniute, J. and Bruzauskaite, I. (2015) Novel aspects of pathogenesis and regeneration mechanisms in COPD, *International Journal of Chronic Obstructive Pulmonary Disease*, pp. 995. DOI:10.2147/COPD.S82518.

Allen, J. G., Flanigan, S. S., LeBlanc, M., Vallarino, J., MacNaughton, P., Stewart, J. H. and Christiani, D. C. (2016) Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-

Pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes, *Environmental Health Perspectives*, 124 (6), pp. 733–739. DOI:10.1289/ehp.1510185.

Altorki, N. K., Markowitz, G. J., Gao, D., Port, J. L., Saxena, A., Stiles, B., McGraw, T. and Mittal, V. (2019) The lung microenvironment: an important regulator of tumour growth and metastasis, *Nature Reviews Cancer*, 19 (1), pp. 9–31. DOI:10.1038/s41568-018-0081-9.

Amatngalim, G. D., Broekman, W., Daniel, N. M., Van Der Vlugt, L. E. P. M., Van Schadewijk, A., Taube, C. and Hiemstra, P. S. (2016) Cigarette Smoke Modulates Repair and Innate Immunity following Injury to Airway Epithelial Cells, *PLOS ONE*, 11 (11), pp. e0166255. DOI:10.1371/journal.pone.0166255.

Angelis, N., Porpodis, K., Zarogoulidis, P., Spyratos, D., Kioumis, I., Papaiwannou, A., *et al.* (2014) Airway inflammation in chronic obstructive pulmonary disease, *Journal of Thoracic Disease*, 6 Suppl 1, pp. S167-172. DOI:10.3978/j.issn.2072-1439.2014.03.07.

Arason, A. J., Jonsdottir, H. R., Halldorsson, S., Benediktsdottir, B. E., Bergthorsson, J. T., Ingthorsson, S., *et al.* (2014) deltaNp63 Has a Role in Maintaining Epithelial Integrity in Airway Epithelium, *PLoS ONE*, 9 (2), pp. e88683. DOI:10.1371/journal.pone.0088683.

Arman, S. and İşisağ Üçüncü, S. (2020) Cardiac toxicity of acrolein exposure in embryonic zebrafish (Danio rerio), *Environmental Science and Pollution Research*, 27 (18), pp. 22423–22433. DOI:10.1007/s11356-020-08853-7.

Asaduzzaman Khan, Md., Tania, M., Zhang, D. and Chen, H. (2010) Antioxidant enzymes and cancer, *Chinese Journal of Cancer Research*, 22 (2), pp. 87–92. DOI:10.1007/s11670-010-0087-7.

ASH (2019) Use of e-cigarettes (vaporisers) among adults in Great Britain. Available from: https://ash.org.uk/wp-content/uploads/2019/09/Use-of-e-cigarettes-among-adults-2019.pdf

ASH (2022a) *Fears of growth in children vaping disposables backed up by new national survey*. Available from: https://ash.org.uk/media-and-news/press-releases-media-and-news/fears-of-growth-in-children-vaping-disposables-backed-up-by-new-national-survey/

ASH (2022b) Use of e-cigarettes (vapes) among adults in Great Britain. Available from: https://ash.org.uk/uploads/Use-of-e-cigarettes-vapes-among-adults-in-Great-Britain-2022.pdf?v=1661865959 [Accessed

ASH (2023) *Use of e-cigarettes (vapes) among adults in Great Britain*. Available from: https://ash.org.uk/uploads/Use-of-e-cigarettes-among-adults-in-Great-Britain-2023.pdf

ATSDR (2015) *Public Health Statement for 1,4 Dioxane*. Available from: https://wwwn.cdc.gov/TSP/PHS/PHS.aspx?phsid=953&toxid=199#:~:text=The%20International%20Agency%20for%20Research%20on%20Cancer%20%28IARC%29,1%2C4-dioxane%20is%20likely%20to%20be%20carcinogenic%20to%20humans.

Aufderheide, M., Halter, B., Möhle, N. and Hochrainer, D. (2013) The CULTEX RFS: A Comprehensive Technical Approach for the *In Vitro* Exposure of Airway Epithelial

Cells to the Particulate Matter at the Air-Liquid Interface, *BioMed Research International*, 2013, pp. 1–15. DOI:10.1155/2013/734137.

Aufderheide, M., Heller, W.-D., Krischenowski, O., Möhle, N. and Hochrainer, D. (2017) Improvement of the CULTEX ® exposure technology by radial distribution of the test aerosol, *Experimental and Toxicologic Pathology*, 69 (6), pp. 359–365. DOI:10.1016/j.etp.2017.02.004.

Aufderheide, M. and Mohr, U. (2000) CULTEX — an alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface, *Experimental and Toxicologic Pathology*, 52 (3), pp. 265–270. DOI:10.1016/S0940-2993(00)80044-5.

Aufderheide, M., Scheffler, S., Ito, S., Ishikawa, S. and Emura, M. (2015) Ciliatoxicity in human primary bronchiolar epithelial cells after repeated exposure at the air–liquid interface with native mainstream smoke of K3R4F cigarettes with and without charcoal filter, *Experimental and Toxicologic Pathology*, 67 (7–8), pp. 407–411. DOI:10.1016/j.etp.2015.04.006.

Aufderheide, M., Scheffler, S., Möhle, N., Halter, B. and Hochrainer, D. (2011) Analytical in vitro approach for studying cyto- and genotoxic effects of particulate airborne material, *Analytical and Bioanalytical Chemistry*, 401 (10), pp. 3213–3220. DOI:10.1007/s00216-011-5163-4.

Azzopardi, D., Patel, K., Jaunky, T., Santopietro, S., Camacho, O. M., McAughey, J. and Gaça, M. (2016) Electronic cigarette aerosol induces significantly less cytotoxicity than tobacco smoke, *Toxicology Mechanisms and Methods*, 26 (6), pp. 477–491. DOI:10.1080/15376516.2016.1217112.

Badea, M., Luzardo, O. P., González-Antuña, A., Zumbado, M., Rogozea, L., Floroian, L., *et al.* (2018) Body burden of toxic metals and rare earth elements in non-smokers, cigarette smokers and electronic cigarette users, *Environmental Research*, 166, pp. 269–275. DOI:10.1016/j.envres.2018.06.007.

Bahl, V., Lin, S., Xu, N., Davis, B., Wang, Y. and Talbot, P. (2012) Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models, *Reproductive Toxicology*, 34 (4), pp. 529–537. DOI:10.1016/j.reprotox.2012.08.001.

Balbo, S., James-Yi, S., Johnson, C. S., O'Sullivan, M. G., Stepanov, I., Wang, M., *et al.* (2013) (S)-N'-Nitrosonornicotine, a constituent of smokeless tobacco, is a powerful oral cavity carcinogen in rats, *Carcinogenesis*, 34 (9), pp. 2178–2183. DOI:10.1093/carcin/bgt162.

Baldassi, D., Gabold, B. and Merkel, O. M. (2021) Air−Liquid Interface Cultures of the Healthy and Diseased Human Respiratory Tract: Promises, Challenges, and Future Directions, *Advanced NanoBiomed Research*, 1 (6), pp. 2000111. DOI:10.1002/anbr.202000111.

Balharry, D., Sexton, K. and BéruBé, K. A. (2008) An in vitro approach to assess the toxicity of inhaled tobacco smoke components: Nicotine, cadmium, formaldehyde and urethane, *Toxicology*, 244 (1), pp. 66–76. DOI:10.1016/j.tox.2007.11.001.

Barrila, J., Radtke, A. L., Crabbé, A., Sarker, S. F., Herbst-Kralovetz, M. M., Ott, C. M. and Nickerson, C. A. (2010) Organotypic 3D cell culture models: using the rotating wall vessel to study host–pathogen interactions, *Nature Reviews Microbiology*, 8 (11), pp. 791–801. DOI:10.1038/nrmicro2423.

BBC (2023) *Vaping: High lead and nickel found in illegal vapes*. Available from: https://www.bbc.co.uk/news/health-65614078

Behar, R. Z., Davis, B., Wang, Y., Bahl, V., Lin, S. and Talbot, P. (2014a) Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids, *Toxicology in Vitro*, 28 (2), pp. 198–208. DOI:10.1016/j.tiv.2013.10.006.

Behar, R. Z., Davis, B., Wang, Y., Bahl, V., Lin, S. and Talbot, P. (2014b) Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids, *Toxicology in Vitro*, 28 (2), pp. 198–208. DOI:10.1016/j.tiv.2013.10.006.

Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F. and Talbot, P. (2018a) Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids, *Scientific Reports*, 8 (1), pp. 8288. DOI:10.1038/s41598-018-25575-6.

Behar, R. Z., Wang, Y. and Talbot, P. (2018b) Comparing the cytotoxicity of electronic cigarette fluids, aerosols and solvents, *Tobacco Control*, 27 (3), pp. 325–333. DOI:10.1136/tobaccocontrol-2016-053472.

Bekki, K., Uchiyama, S., Ohta, K., Inaba, Y., Nakagome, H. and Kunugita, N. (2014) Carbonyl Compounds Generated from Electronic Cigarettes, *International Journal of Environmental Research and Public Health*, 11 (11), pp. 11192–11200. DOI:10.3390/ijerph111111192.

Bengalli, R., Ferri, E., Labra, M. and Mantecca, P. (2017) Lung Toxicity of Condensed Aerosol from E-CIG Liquids: Influence of the Flavor and the In Vitro Model Used, *International Journal of Environmental Research and Public Health*, 14 (10), pp. 1254. DOI:10.3390/ijerph14101254.

Benowitz, N. L. (2010) Nicotine addiction, *The New England Journal of Medicine*, 362 (24), pp. 2295–2303. DOI:10.1056/NEJMra0809890.

Benowitz, N. L., Hukkanen, J. and Jacob, P. (2009) Nicotine Chemistry, Metabolism, Kinetics and Biomarkers, in: Henningfield, J. E., London, E. D., and Pogun, S. (eds.) *Nicotine Psychopharmacology*. Berlin, Heidelberg: Springer Berlin Heidelberg,192, pp. 29–60.

Benowitz, N. L. and Iii, P. J. (1984) Daily intake of nicotine during cigarette smoking, *Clinical Pharmacology and Therapeutics*, 35 (4), pp. 499–504. DOI:10.1038/clpt.1984.67.

Berkelhamer, S. K., Helman, J. M., Gugino, S. F., Leigh, N. J., Lakshminrusimha, S. and Goniewicz, M. L. (2019) In Vitro Consequences of Electronic-Cigarette Flavoring Exposure on the Immature Lung, *International Journal of Environmental Research and Public Health*, 16 (19), pp. 3635. DOI:10.3390/ijerph16193635.

Bhatnagar, A. (2016) E-Cigarettes and Cardiovascular Disease Risk: Evaluation of Evidence, Policy Implications, and Recommendations, *Current Cardiovascular Risk Reports*, 10 (7), pp. 24. DOI:10.1007/s12170-016-0505-6.

Biedermann, L., Brülisauer, K., Zeitz, J., Frei, P., Scharl, M., Vavricka, S. R., *et al.* (2014) Smoking Cessation Alters Intestinal Microbiota: Insights from Quantitative Investigations on Human Fecal Samples Using FISH, *Inflammatory Bowel Diseases*, 20 (9), pp. 1496–1501. DOI:10.1097/MIB.0000000000000129.

Binder, S., Rastak, N., Karg, E., Huber, A., Kuhn, E., Dragan, G. C., *et al.* (2022) Construction of an In Vitro Air–Liquid Interface Exposure System to Assess the

Toxicological Impact of Gas and Particle Phase of Semi-Volatile Organic Compounds, *Toxics*, 10 (12), pp. 730. DOI:10.3390/toxics10120730.

Biondi, A. C. and Disalvo, E. A. (1990) Effect of glycerol on the interfacial properties of dipalmitoylphosphatidylcholine liposomes as measured with merocyanine 540, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1028 (1), pp. 43–48. DOI:10.1016/0005-2736(90)90263-N.

Biondi, A. C., Féliz, M. R. and Disalvo, E. A. (1991) Surface changes induced by osmotic stress and its influence on the glycerol permeability in lipid bilayers, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1069 (1), pp. 5–13. DOI:10.1016/0005-2736(91)90097-R.

Bishop, E., East, N., Miazzi, F., Fiebelkorn, S., Breheny, D., Gaca, M. and Thorne, D. (2023) A contextualised e-cigarette testing strategy shows flavourings do not impact lung toxicity in vitro, *Toxicology Letters*, 380, pp. 1–11. DOI:10.1016/j.toxlet.2023.03.006.

Blackford, A., Parmigiani, G., Kensler, T. W., Wolfgang, C., Jones, S., Zhang, X., *et al.* (2009) Genetic Mutations Associated with Cigarette Smoking in Pancreatic Cancer, *Cancer Research*, 69 (8), pp. 3681–3688. DOI:10.1158/0008-5472.CAN-09-0015.

Blanc-Lapierre, A., Sauvé, J.-F. and Parent, M.-E. (2018) Occupational exposure to benzene, toluene, xylene and styrene and risk of prostate cancer in a population-based study, *Occupational and Environmental Medicine*, 75 (8), pp. 562–572. DOI:10.1136/oemed-2018-105058.

Boers, J. E., Ambergen, A. W. and Thunnissen, F. B. J. M. (1998) Number and Proliferation of Basal and Parabasal Cells in Normal Human Airway Epithelium, *American Journal of Respiratory and Critical Care Medicine*, 157 (6), pp. 2000–2006. DOI:10.1164/ajrccm.157.6.9707011.

Brieger, K., Schiavone, S., Miller, Jr. and Krause, K. (2012) Reactive oxygen species: from health to disease, *Swiss Medical Weekly*. DOI:10.4414/smw.2012.13659.

Brody, J. S. and Spira, A. (2006) State of the art. Chronic obstructive pulmonary disease, inflammation, and lung cancer, *Proceedings of the American Thoracic Society*, 3 (6), pp. 535–537. DOI:10.1513/pats.200603-089MS.

Bryois, J., Buil, A., Ferreira, P. G., Panousis, N. I., Brown, A. A., Viñuela, A., *et al.* (2017) Time-dependent genetic effects on gene expression implicate aging processes, *Genome Research*, 27 (4), pp. 545–552. DOI:10.1101/gr.207688.116.

Buist, A. S., Vollmer, W. M. and McBurnie, M. A. (2008) Worldwide burden of COPD in high- and low-income countries. Part I. The burden of obstructive lung disease (BOLD) initiative, *The International Journal of Tuberculosis and Lung Disease: The Official Journal of the International Union Against Tuberculosis and Lung Disease*, 12 (7), pp. 703–708.

Bukowy-Bieryłło, Z. (2021) Long-term differentiating primary human airway epithelial cell cultures: how far are we?, *Cell Communication and Signaling*, 19 (1), pp. 63. DOI:10.1186/s12964-021-00740-z.

Burgwardt, S., Huskic, A., Schwartz, G., Mason, D. P., Tapias, L. and Podgaetz, E. (2020) Spontaneous pneumomediastinum secondary to electronic cigarette use,

Baylor University Medical Center Proceedings, 33 (2), pp. 229–230. DOI:10.1080/08998280.2020.1717407.

Bush, L. P., Cui, M., Shi, H., Burton, H. R., Fannin, F., Lei, L. and Dye, N. (2001) Formation of tobacco-specific nitrosamines in air-cured tobacco, *Rec. Adv. Tob. Sci.*, 27, pp. 23–46.

Camacho, S., Michlig, S., de Senarclens-Bezençon, C., Meylan, J., Meystre, J., Pezzoli, M., Markram, H. and le Coutre, J. (2015) Anti-Obesity and Anti-Hyperglycemic Effects of Cinnamaldehyde via altered Ghrelin Secretion and Functional impact on Food Intake and Gastric Emptying, *Scientific Reports*, 5 (1), pp. 7919. DOI:10.1038/srep07919.

Candeias, J., Schmidt-Weber, C. B. and Buters, J. (2021) Dosing intact birch pollen grains at the air-liquid interface (ALI) to the immortalized human bronchial epithelial cell line BEAS-2B, *PLOS ONE*, 16 (11), pp. e0259914. DOI:10.1371/journal.pone.0259914.

Carmella, S. G., Akerkar, S. and Hecht, S. S. (1993) Metabolites of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in smokers' urine, *Cancer Research*, 53 (4), pp. 721–724.

Carnesecchi, S., Deffert, C., Donati, Y., Basset, O., Hinz, B., Preynat-Seauve, O., *et al.* (2011) A Key Role for NOX4 in Epithelial Cell Death During Development of Lung Fibrosis, *Antioxidants & Redox Signaling*, 15 (3), pp. 607–619. DOI:10.1089/ars.2010.3829.

CDC (2014) *Health Effects of Secondhand Smoke*.

CDC (2023) *Current Cigarette Smoking Among Adults in the United States*. Available from: https://www.cdc.gov/tobacco/data_statistics/fact_sheets/adult_data/cig_smoking/index.htm

Cendrowicz, E., Sas, Z., Bremer, E. and Rygiel, T. P. (2021) The Role of Macrophages in Cancer Development and Therapy, *Cancers*, 13 (8), pp. 1946. DOI:10.3390/cancers13081946.

centre for public impact (2019) *Smoking ban in the United Kingdom*. Available from: https://www.centreforpublicimpact.org/case-study/smoking-ban-united-kingdom/

Cervellati, F., Muresan, X. M., Sticozzi, C., Gambari, R., Montagner, G., Forman, H. J., Torricelli, C., Maioli, E. and Valacchi, G. (2014) Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells, *Toxicology in Vitro*, 28 (5), pp. 999–1005. DOI:10.1016/j.tiv.2014.04.012.

Chapman, D. G., Casey, D. T., Ather, J. L., Aliyeva, M., Daphtary, N., Lahue, K. G., van der Velden, J. L., Janssen-Heininger, Y. M. W. and Irvin, C. G. (2019) The Effect of Flavored E-cigarettes on Murine Allergic Airways Disease, *Scientific Reports*, 9 (1), pp. 13671. DOI:10.1038/s41598-019-50223-y.

Charlton, A. (2004) Medicinal uses of tobacco in history, *JRSM*, 97 (6), pp. 292–296. DOI:10.1258/jrsm.97.6.292.

Chelombitko, M. A. (2018) Role of Reactive Oxygen Species in Inflammation: A Minireview, *Moscow University Biological Sciences Bulletin*, 73 (4), pp. 199–202. DOI:10.3103/S009639251804003X.

Chen, S., Einspanier, R. and Schoen, J. (2015) Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports, *Histochemistry and Cell Biology*, 144 (5), pp. 509–515. DOI:10.1007/s00418-015-1351-1.

Cheng, G., Li, J., Zheng, M., Zhao, Y., Zhou, J. and Li, W. (2014) NNK, a Tobacco-Specific Carcinogen, Inhibits the Expression of Lysyl Oxidase, a Tumor Suppressor, *International Journal of Environmental Research and Public Health*, 12 (1), pp. 64–82. DOI:10.3390/ijerph120100064.

Chernyavsky, A. I., Shchepotin, I. B., Galitovkiy, V. and Grando, S. A. (2015) Mechanisms of tumor-promoting activities of nicotine in lung cancer: synergistic effects of cell membrane and mitochondrial nicotinic acetylcholine receptors, *BMC Cancer*, 15 (1), pp. 152. DOI:10.1186/s12885-015-1158-4.

Chun, L. F., Moazed, F., Calfee, C. S., Matthay, M. A. and Gotts, J. E. (2017) Pulmonary toxicity of e-cigarettes, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 313 (2), pp. L193–L206. DOI:10.1152/ajplung.00071.2017.

Chung-man Ho, J., Zheng, S., Comhair, S. A., Farver, C. and Erzurum, S. C. (2001) Differential expression of manganese superoxide dismutase and catalase in lung cancer, *Cancer Research*, 61 (23), pp. 8578–8585.

Cingolani, E., Alqahtani, S., Sadler, R. C., Prime, D., Stolnik, S. and Bosquillon, C. (2019) In vitro investigation on the impact of airway mucus on drug dissolution and absorption at the air-epithelium interface in the lungs, *European Journal of*

Pharmaceutics and Biopharmaceutics, 141, pp. 210–220. DOI:10.1016/j.ejpb.2019.05.022.

Clapp, P. W., Lavrich, K. S., van Heusden, C. A., Lazarowski, E. R., Carson, J. L. and Jaspers, I. (2019a) Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 316 (3), pp. L470–L486. DOI:10.1152/ajplung.00304.2018.

Clapp, P. W., Lavrich, K. S., van Heusden, C. A., Lazarowski, E. R., Carson, J. L. and Jaspers, I. (2019b) Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 316 (3), pp. L470–L486. DOI:10.1152/ajplung.00304.2018.

Clapp, P. W., Pawlak, E. A., Lackey, J. T., Keating, J. E., Reeber, S. L., Glish, G. L. and Jaspers, I. (2017a) Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 313 (2), pp. L278–L292. DOI:10.1152/ajplung.00452.2016.

Clapp, P. W., Pawlak, E. A., Lackey, J. T., Keating, J. E., Reeber, S. L., Glish, G. L. and Jaspers, I. (2017b) Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 313 (2), pp. L278–L292. DOI:10.1152/ajplung.00452.2016.

Coppack, S. W. (2001) Pro-inflammatory cytokines and adipose tissue, *Proceedings of the Nutrition Society*, 60 (3), pp. 349–356. DOI:10.1079/PNS2001110.

CORESTA (2015) E-cigarette Task Force Technical Report, 2014 Electronic Cigarette Aerosol Parameters Study.

CORESTA (2021) *Technical Guide for Sample Handling and Sample Collection of E-Cigarettes and E-Vapour Generating Products*. Available from: https://www.coresta.org/sites/default/files/technical_documents/main/Guide-No18_Ecig-Evap-Sampling-HandlingCollection_July21.pdf

Cornelius E., M., Loretan, C. G., Jamal, A., Davis Lynn, B., Mayer, M., Alcantara, I. and Neff, L. (2023) *Tobacco Product Use Among Adults – United States, 2021*. Available from: https://www.cdc.gov/mmwr/volumes/72/wr/mm7218a1.htm

COT (2019) *Statement on the potential toxicological risks from electronic nicotine (and non-nicotine) delivery systems (E(N)NDS – e-cigarettes)*. Available from: https://cot.food.gov.uk/sites/default/files/2020-09/COT%20E%28N%29NDS%20statement%202020-04.pdf

Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., Naudon, L. and Rabot, S. (2014) Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats, *Psychoneuroendocrinology*, 42, pp. 207–217. DOI:10.1016/j.psyneuen.2014.01.014.

Cryan, J. F. and Dinan, T. G. (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour, *Nature Reviews Neuroscience*, 13 (10), pp. 701–712. DOI:10.1038/nrn3346.

Cuthbertson, L. and Britton, J. (2010) Passive smoking and children's health, *Clinical Medicine*, 10 (2), pp. 113–114. DOI:10.7861/clinmedicine.10-2-113.

Czekala, L., Simms, L., Stevenson, M., Trelles-Sticken, E., Walker, P. and Walele, T. (2019a) High Content Screening in NHBE cells shows significantly reduced biological activity of flavoured e-liquids, when compared to cigarette smoke condensate, *Toxicology in Vitro*, 58, pp. 86–96. DOI:10.1016/j.tiv.2019.03.018.

Czekala, L., Simms, L., Stevenson, M., Tschierske, N., Maione, A. G. and Walele, T. (2019b) Toxicological comparison of cigarette smoke and e-cigarette aerosol using a 3D in vitro human respiratory model, *Regulatory Toxicology and Pharmacology*, 103, pp. 314–324. DOI:10.1016/j.yrtph.2019.01.036.

Dasgupta, P. (2006) Nicotine induces cell proliferation by -arrestin-mediated activation of Src and Rb-Raf-1 pathways, *Journal of Clinical Investigation*, 116 (8), pp. 2208–2217. DOI:10.1172/JCI28164.

De Bruijne, K., Ebersviller, S., Sexton, K. G., Lake, S., Leith, D., Goodman, R., *et al.* (2009) Design and Testing of Electrostatic Aerosol *In Vitro* Exposure System (EAVES): An Alternative Exposure System for Particles, *Inhalation Toxicology*, 21 (2), pp. 91–101. DOI:10.1080/08958370802166035.

DeJarnett, N., Conklin, D. J., Riggs, D. W., Myers, J. A., O'Toole, T. E., Hamzeh, I., *et al.* (2014) Acrolein Exposure Is Associated With Increased Cardiovascular Disease Risk, *Journal of the American Heart Association*, 3 (4), pp. e000934. DOI:10.1161/JAHA.114.000934.

del Olmo, A., Calzada, J. and Nuñez, M. (2017) Benzoic acid and its derivatives as naturally occurring compounds in foods and as additives: Uses, exposure, and controversy, *Critical Reviews in Food Science and Nutrition*, 57 (14), pp. 3084–3103. DOI:10.1080/10408398.2015.1087964.

Delgado, O., Kaisani, A. A., Spinola, M., Xie, X.-J., Batten, K. G., Minna, J. D., Wright, W. E. and Shay, J. W. (2011) Multipotent Capacity of Immortalized Human Bronchial Epithelial Cells, *PLoS ONE*, 6 (7), pp. e22023. DOI:10.1371/journal.pone.0022023.

Department of Health and Social Care (2020) *Smoke-free generation: tobacco control plan for England*. Available from: https://www.gov.uk/government/publications/towards-a-smoke-free-generation-tobacco-control-plan-for-england

Dimauro, I., Mercatelli, N. and Caporossi, D. (2016) Exercise-induced ROS in heat shock proteins response, *Free Radical Biology and Medicine*, 98, pp. 46–55. DOI:10.1016/j.freeradbiomed.2016.03.028.

Diskin, B., Adam, S., Cassini, M. F., Sanchez, G., Liria, M., Aykut, B., *et al.* (2020) PD-L1 engagement on T cells promotes self-tolerance and suppression of neighboring macrophages and effector T cells in cancer, *Nature Immunology*, 21 (4), pp. 442–454. DOI:10.1038/s41590-020-0620-x.

Doll, R. and Hill, A. B. (1950) Smoking and Carcinoma of the Lung, *BMJ*, 2 (4682), pp. 739–748. DOI:10.1136/bmj.2.4682.739.

Doll, R., Peto, R., Boreham, J. and Sutherland, I. (2005) Mortality from cancer in relation to smoking: 50 years observations on British doctors, *British Journal of Cancer*, 92 (3), pp. 426–429. DOI:10.1038/sj.bjc.6602359.

Donaldson, G. C., Seemungal, T. A. R., Patel, I. S., Bhowmik, A., Wilkinson, T. M. A., Hurst, J. R., MacCallum, P. K. and Wedzicha, J. A. (2005) Airway and Systemic Inflammation and Decline in Lung Function in Patients With COPD, *Chest*, 128 (4), pp. 1995–2004. DOI:10.1378/chest.128.4.1995.

Duell, A. K., Pankow, J. F. and Peyton, D. H. (2019) Nicotine in tobacco product aerosols: 'It's déjà vu all over again', *Tobacco Control*, pp. tobaccocontrol-2019-055275. DOI:10.1136/tobaccocontrol-2019-055275.

Dusautoir, R., Zarcone, G., Verriele, M., Garçon, G., Fronval, I., Beauval, N., *et al.* (2021) Comparison of the chemical composition of aerosols from heated tobacco products, electronic cigarettes and tobacco cigarettes and their toxic impacts on the human bronchial epithelial BEAS-2B cells, *Journal of Hazardous Materials*, 401, pp. 123417. DOI:10.1016/j.jhazmat.2020.123417.

Effah, F., Elzein, A., Taiwo, B., Baines, D., Bailey, A. and Marczylo, T. (2023) In Vitro High-Throughput Toxicological Assessment of E-Cigarette Flavors on Human Bronchial Epithelial Cells and the Potential Involvement of TRPA1 in Cinnamon Flavor-Induced Toxicity, *Toxicology*, pp. 153617. DOI:10.1016/j.tox.2023.153617.

Effah, F., Taiwo, B., Baines, D., Bailey, A. and Marczylo, T. (2022) Pulmonary effects of e-liquid flavors: a systematic review, *Journal of Toxicology and Environmental Health, Part B*, pp. 1–29. DOI:10.1080/10937404.2022.2124563.

El-Zayadi, A.-R. (2006) Heavy smoking and liver, *World Journal of Gastroenterology*, 12 (38), pp. 6098–6101. DOI:10.3748/wjg.v12.i38.6098.

Etter, J.-F., Zäther, E. and Svensson, S. (2013) Analysis of refill liquids for electronic cigarettes: E-liquids, *Addiction*, 108 (9), pp. 1671–1679. DOI:10.1111/add.12235.

European Medicines Agency (2023) *Use of herbal medicinal products containing estragole - Scientific guideline*. Available from: https://www.ema.europa.eu/en/use-herbal-medicinal-products-containing-estragole-scientific-guideline

Evans, M. J., Van Winkle, L. S., Fanucchi, M. V. and Plopper, C. G. (2001) CELLULAR AND MOLECULAR CHARACTERISTICS OF BASAL CELLS IN AIRWAY EPITHELIUM, *Experimental Lung Research*, 27 (5), pp. 401–415. DOI:10.1080/019021401300317125.

Fahy, J. V. and Dickey, B. F. (2010) Airway mucus function and dysfunction, *The New England Journal of Medicine*, 363 (23), pp. 2233–2247. DOI:10.1056/NEJMra0910061.

Farsalinos, K. E., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A. and Voudris, V. (2013a) Impact of flavour variability on electronic cigarette use experience: an internet survey, *International Journal of Environmental Research and Public Health*, 10 (12), pp. 7272–7282. DOI:10.3390/ijerph10127272.

Farsalinos, K., Gillman, G., Poulas, K. and Voudris, V. (2015) Tobacco-Specific Nitrosamines in Electronic Cigarettes: Comparison between Liquid and Aerosol Levels, *International Journal of Environmental Research and Public Health*, 12 (8), pp. 9046–9053. DOI:10.3390/ijerph120809046.

Farsalinos, K., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A. and Voudris, V. (2013b) Impact of Flavour Variability on Electronic Cigarette Use Experience: An Internet Survey, *International Journal of Environmental Research and Public Health*, 10 (12), pp. 7272–7282. DOI:10.3390/ijerph10127272.

FDA (2012) *Reporting Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke Under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act*.

Fedan, J. S., Dowdy, J. A., Fedan, K. B. and Hubbs, A. F. (2006) Popcorn worker's lung: In vitro exposure to diacetyl, an ingredient in microwave popcorn butter flavoring, increases reactivity to methacholine☆, *Toxicology and Applied Pharmacology*, 215 (1), pp. 17–22. DOI:10.1016/j.taap.2006.02.001.

Felix, K., Tobias, S., Jan, H., Nicolas, S. and Michael, M. (2021) Measurements of transepithelial electrical resistance (TEER) are affected by junctional length in immature epithelial monolayers, *Histochemistry and Cell Biology*. DOI:10.1007/s00418-021-02026-4.

Feron, V. J., Til, H. P., de Vrijer, F., Woutersen, R. A., Cassee, F. R. and van Bladeren, P. J. (1991) Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment, *Mutation Research/Genetic Toxicology*, 259 (3–4), pp. 363–385. DOI:10.1016/0165-1218(91)90128-9.

Fetterman, J. L., Weisbrod, R. M., Feng, B., Bastin, R., Tuttle, S. T., Holbrook, M., *et al.* (2018) Flavorings in Tobacco Products Induce Endothelial Cell Dysfunction, *Arteriosclerosis, Thrombosis, and Vascular Biology*, 38 (7), pp. 1607–1615. DOI:10.1161/ATVBAHA.118.311156.

Fiegel, J. (2003) [No title found], *Pharmaceutical Research*, 20 (5), pp. 788–796. DOI:10.1023/A:1023441804464.

Foronjy, R. and D'Armiento, J. (2006) The effect of cigarette smoke–derived oxidants on the inflammatory response of the lung, *Clinical and Applied Immunology Reviews*, 6 (1), pp. 53–72. DOI:10.1016/j.cair.2006.04.002.

Foster, K. A., Avery, M. L., Yazdanian, M. and Audus, K. L. (2000) Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery, *International Journal of Pharmaceutics*, 208 (1–2), pp. 1–11. DOI:10.1016/S0378-5173(00)00452-X.

Freeman, R. and Perks, D. (1990) Carboxyhaemoglobin, *Anaesthesia*, 45 (1), pp. 57–57. DOI:10.1111/j.1365-2044.1990.tb14509.x.

Fröhlich, E., Mercuri, A., Wu, S. and Salar-Behzadi, S. (2016) Measurements of Deposition, Lung Surface Area and Lung Fluid for Simulation of Inhaled Compounds, *Frontiers in Pharmacology*, 7. DOI:10.3389/fphar.2016.00181.

Frombaum, M., Le Clanche, S., Bonnefont-Rousselot, D. and Borderie, D. (2012) Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and NO bioavailability: Potential benefits to cardiovascular diseases, *Biochimie*, 94 (2), pp. 269–276. DOI:10.1016/j.biochi.2011.11.001.

Frost-Pineda, K., Liang, Q., Liu, J., Rimmer, L., Jin, Y., Feng, S., *et al.* (2011) Biomarkers of Potential Harm Among Adult Smokers and Nonsmokers in the Total Exposure Study, *Nicotine & Tobacco Research*, 13 (3), pp. 182–193. DOI:10.1093/ntr/ntq235.

Funabashi, T., Sano, A., Mitsushima, D. and Kimura, F. (2005) Nicotine Inhibits Pulsatile Luteinizing Hormone Secretion in Human Males But Not in Human Females, and Tolerance to This Nicotine Effect Is Lost within One Week of Quitting Smoking, *The Journal of Clinical Endocrinology & Metabolism*, 90 (7), pp. 3908–3913. DOI:10.1210/jc.2005-0041.

Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., *et al.* (2019) Chronic inflammation in the etiology of disease across the life span, *Nature Medicine*, 25 (12), pp. 1822–1832. DOI:10.1038/s41591-019-0675-0.

Fuxe, K., Andersson, K., Eneroth, P., Härfstrand, A. and Agnati, L. F. (1989) Neuroendocrine actions of nicotine and of exposure to cigarette smoke: Medical implications, *Psychoneuroendocrinology*, 14 (1–2), pp. 19–41. DOI:10.1016/0306-4530(89)90054-1.

Gallucci, G., Tartarone, A., Lerose, R., Lalinga, A. V. and Capobianco, A. M. (2020) Cardiovascular risk of smoking and benefits of smoking cessation, *Journal of Thoracic Disease*, 12 (7), pp. 3866–3876. DOI:10.21037/jtd.2020.02.47.

Ganguly, K., Nordström, A., Thimraj, T. A., Rahman, M., Ramström, M., Sompa, S. I., *et al.* (2020) Addressing the challenges of E-cigarette safety profiling by assessment of pulmonary toxicological response in bronchial and alveolar mucosa models, *Scientific Reports*, 10 (1), pp. 20460. DOI:10.1038/s41598-020-77452-w.

Gerloff, J., Sundar, I. K., Freter, R., Sekera, E. R., Friedman, A. E., Robinson, R., Pagano, T. and Rahman, I. (2017) Inflammatory Response and Barrier Dysfunction by Different e-Cigarette Flavoring Chemicals Identified by Gas Chromatography–Mass Spectrometry in e-Liquids and e-Vapors on Human Lung Epithelial Cells and

Fibroblasts, *Applied In Vitro Toxicology*, 3 (1), pp. 28–40. DOI:10.1089/aivt.2016.0030.

Gizurarson, S. (2015) The Effect of Cilia and the Mucociliary Clearance on Successful Drug Delivery, *Biological & Pharmaceutical Bulletin*, 38 (4), pp. 497–506. DOI:10.1248/bpb.b14-00398.

Glynos, C., Bibli, S.-I., Katsaounou, P., Pavlidou, A., Magkou, C., Karavana, V., *et al.* (2018) Comparison of the effects of e-cigarette vapor with cigarette smoke on lung function and inflammation in mice, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 315 (5), pp. L662–L672. DOI:10.1152/ajplung.00389.2017.

Gonçalves, V. S. S., Matias, A. A., Poejo, J., Serra, A. T. and Duarte, C. M. M. (2016) Application of RPMI 2650 as a cell model to evaluate solid formulations for intranasal delivery of drugs, *International Journal of Pharmaceutics*, 515 (1–2), pp. 1–10. DOI:10.1016/j.ijpharm.2016.09.086.

Goniewicz, M. L., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., *et al.* (2014a) Levels of selected carcinogens and toxicants in vapour from electronic cigarettes, *Tobacco Control*, 23 (2), pp. 133–139. DOI:10.1136/tobaccocontrol-2012-050859.

Goniewicz, M. L., Knysak, J., Gawron, M., Kosmider, L., Sobczak, A., Kurek, J., *et al.* (2014b) Levels of selected carcinogens and toxicants in vapour from electronic cigarettes, *Tobacco Control*, 23 (2), pp. 133. DOI:10.1136/tobaccocontrol-2012-050859.

Goniewicz, M. L., Smith, D. M., Edwards, K. C., Blount, B. C., Caldwell, K. L., Feng, J., *et al.* (2018) Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes, *JAMA Network Open*, 1 (8), pp. e185937. DOI:10.1001/jamanetworkopen.2018.5937.

Gorsline, J., Gupta, S. K., Dye, D. and Rolf, C. N. (1993) Steady-State Pharmacokinetics and Dose Relationship of Nicotine Delivered from Nicoderm® (Nicotine Transdermal System), *The Journal of Clinical Pharmacology*, 33 (2), pp. 161–168. DOI:10.1002/j.1552-4604.1993.tb03938.x.

Gourlay, S. G. and Benowitz, N. L. (1997) Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine*, *Clinical Pharmacology & Therapeutics*, 62 (4), pp. 453–463. DOI:10.1016/S0009-9236(97)90124-7.

Guénette, J., Breznan, D. and Thomson, E. M. (2022) Establishing an air-liquid interface exposure system for exposure of lung cells to gases, *Inhalation Toxicology*, 34 (3–4), pp. 80–89. DOI:10.1080/08958378.2022.2039332.

Haimes, J. and Kelley, M. (2014) *Demonstration of a ΔΔCq Calculation Method to Compute Relative Gene Expression from qPCR Data*. Available from: https://horizondiscovery.com/-/media/Files/Horizon/resources/Technical-Manuals/delta-cq-solaris-technote.pdf

Hajek, P., Phillips-Waller, A., Przulj, D., Pesola, F., Myers Smith, K., Bisal, N., *et al.* (2019) A Randomized Trial of E-Cigarettes versus Nicotine-Replacement Therapy, *New England Journal of Medicine*, 380 (7), pp. 629–637. DOI:10.1056/NEJMoa1808779.

Hamadouche, S., Ounissi, A., Baira, K., Ouddai, N., Balsamo, M., Erto, A. and Benguerba, Y. (2021) Theoretical evaluation of the antioxidant activity of some stilbenes using the Density Functional Theory, *Journal of Molecular Structure*, 1229, pp. 129496. DOI:10.1016/j.molstruc.2020.129496.

Hamidi, H. and Ivaska, J. (2018) Every step of the way: integrins in cancer progression and metastasis, *Nature Reviews Cancer*, 18 (9), pp. 533–548. DOI:10.1038/s41568-018-0038-z.

Havermans, A., Mallock, N., Zervas, E., Caillé-Garnier, S., Mansuy, T., Michel, C., *et al.* (2022) Review of industry reports on EU priority tobacco additives part A: Main outcomes and conclusions, *Tobacco Prevention & Cessation*, 8 (July), pp. 1–18. DOI:10.18332/tpc/151529.

Haws, C., Finkbeiner, W. E., Widdicombe, J. H. and Wine, J. J. (1994) CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated Cl- conductance, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 266 (5), pp. L502–L512. DOI:10.1152/ajplung.1994.266.5.L502.

He, S.-H., Zheng, J. and Duan, M.-K. (2004) Induction of mucin secretion from human bronchial tissue and epithelial cells by rhinovirus and lipopolysaccharide, *Acta Pharmacologica Sinica*, 25 (9), pp. 1176–1181.

Hecht, S. S. (2003) Tobacco carcinogens, their biomarkers and tobacco-induced cancer, *Nature Reviews Cancer*, 3 (10), pp. 733–744. DOI:10.1038/nrc1190.

Herrero, A. and Barja, G. (1999) 8-oxo-deoxyguanosine levels in heart and brain mitochondrial and nuclear DNA of two mammals and three birds in relation to their

different rates of aging, *Aging Clinical and Experimental Research*, 11 (5), pp. 294–300. DOI:10.1007/BF03339803.

Hewitt, R. E., Chappell, H. F. and Powell, J. J. (2020) Small and dangerous? Potential toxicity mechanisms of common exposure particles and nanoparticles, *Current Opinion in Toxicology*, 19, pp. 93–98. DOI:10.1016/j.cotox.2020.01.006.

Hewitt, R. J. and Lloyd, C. M. (2021) Regulation of immune responses by the airway epithelial cell landscape, *Nature Reviews Immunology*, 21 (6), pp. 347–362. DOI:10.1038/s41577-020-00477-9.

Hiemstra, P. S. and Bals, R. (2016) Basic science of electronic cigarettes: assessment in cell culture and in vivo models, *Respiratory Research*, 17 (1), pp. 127. DOI:10.1186/s12931-016-0447-z.

Ho, E., Karimi Galougahi, K., Liu, C.-C., Bhindi, R. and Figtree, G. A. (2013) Biological markers of oxidative stress: Applications to cardiovascular research and practice, *Redox Biology*, 1 (1), pp. 483–491. DOI:10.1016/j.redox.2013.07.006.

Ho, T. N. T., Abraham, N. and Lewis, R. J. (2020) Structure-Function of Neuronal Nicotinic Acetylcholine Receptor Inhibitors Derived From Natural Toxins, *Frontiers in Neuroscience*, 14, pp. 609005. DOI:10.3389/fnins.2020.609005.

Hoi, J. K., Lieder, B., Liebisch, B., Czech, C., Hans, J., Ley, J. P. and Somoza, V. (2020) TRPA1 Agonist Cinnamaldehyde Decreases Adipogenesis in 3T3-L1 Cells More Potently than the Non-agonist Structural Analog Cinnamyl Isobutyrate, *ACS Omega*, 5 (51), pp. 33305–33313. DOI:10.1021/acsomega.0c05083.

Hu, H., Tian, M., Ding, C. and Yu, S. (2019) The C/EBP Homologous Protein (CHOP) Transcription Factor Functions in Endoplasmic Reticulum Stress-Induced Apoptosis and Microbial Infection, *Frontiers in Immunology*, 9, pp. 3083. DOI:10.3389/fimmu.2018.03083.

Hu, T., Zhou, R., Zhao, Y. and Wu, G. (2016) Integrin α6/Akt/Erk signaling is essential for human breast cancer resistance to radiotherapy, *Scientific Reports*, 6 (1), pp. 33376. DOI:10.1038/srep33376.

Huang, C. and Shi, G. (2019) Smoking and microbiome in oral, airway, gut and some systemic diseases, *Journal of Translational Medicine*, 17 (1), pp. 225. DOI:10.1186/s12967-019-1971-7.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2006) Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, 88, pp. 1–478.

Improgo, Ma. R., Tapper, A. R. and Gardner, P. D. (2011) Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer, *Biochemical Pharmacology*, 82 (8), pp. 1015–1021. DOI:10.1016/j.bcp.2011.05.020.

Innocentini, G. C. P., Hodgkinson, A. and Radulescu, O. (2018) Time Dependent Stochastic mRNA and Protein Synthesis in Piecewise-Deterministic Models of Gene Networks, *Frontiers in Physics*, 6, pp. 46. DOI:10.3389/fphy.2018.00046.

International Agency for Research on Cancer (IARC) (2004) *Tobacco Smoke and Involuntary Smoking*. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 83. Available from: https://publications.iarc.fr/Book-And-Report-

Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Tobacco-Smoke-And-Involuntary-Smoking-2004

Jain, R. B. (2019) Concentrations of cadmium, lead, and mercury in blood among US cigarettes, cigars, electronic cigarettes, and dual cigarette-e-cigarette users, *Environmental Pollution*, 251, pp. 970–974. DOI:10.1016/j.envpol.2019.05.041.

Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B. and Beeregowda, K. N. (2014) Toxicity, mechanism and health effects of some heavy metals, *Interdisciplinary Toxicology*, 7 (2), pp. 60–72. DOI:10.2478/intox-2014-0009.

Jeong, M. H., Kim, H. R., Bang, I. J., Yoo, S. H., Lee, S. J., Lee, K. H. and Chung, K. H. (2019) In vitro model for predicting acute inhalation toxicity by using a Calu-3 epithelium cytotoxicity assay, *Journal of Pharmacological and Toxicological Methods*, 98, pp. 106576. DOI:10.1016/j.vascn.2019.04.002.

Ji, E. H., Sun, B., Zhao, T., Shu, S., Chang, C. H., Messadi, D., Xia, T., Zhu, Y. and Hu, S. (2016) Characterization of Electronic Cigarette Aerosol and Its Induction of Oxidative Stress Response in Oral Keratinocytes, *PLOS ONE*, 11 (5), pp. e0154447. DOI:10.1371/journal.pone.0154447.

Ji, X., Sheng, Y., Guan, Y., Li, Y., Xu, Y. and Tang, L. (2022a) Evaluation of Calu-3 cell lines as an *in vitro* model to study the inhalation toxicity of flavoring extracts, *Toxicology Mechanisms and Methods*, 32 (3), pp. 171–179. DOI:10.1080/15376516.2021.1977880.

Ji, X., Sheng, Y., Guan, Y., Li, Y., Xu, Y. and Tang, L. (2022b) Evaluation of Calu-3 cell lines as an *in vitro* model to study the inhalation toxicity of flavoring extracts,

Toxicology Mechanisms and Methods, 32 (3), pp. 171–179. DOI:10.1080/15376516.2021.1977880.

Jiang, H., Gao, X., Gao, Y. and Liu, Y. (2023) Current Knowledge and Challenges of Particle Size Measurements of Mainstream E-Cigarette Aerosols and Their Implication on Respiratory Dosimetry, *Journal of Respiration*, 3 (1), pp. 7–28. DOI:10.3390/jor3010003.

Johansen, M. E., Reilly, C. A. and Yost, G. S. (2006) TRPV1 Antagonists Elevate Cell Surface Populations of Receptor Protein and Exacerbate TRPV1-Mediated Toxicities in Human Lung Epithelial Cells, *Toxicological Sciences*, 89 (1), pp. 278–286. DOI:10.1093/toxsci/kfi292.

Jones, D. P. (2008) Radical-free biology of oxidative stress, *American Journal of Physiology-Cell Physiology*, 295 (4), pp. C849–C868. DOI:10.1152/ajpcell.00283.2008.

Juarez Facio, A. T., Yon, J., Corbière, C., Rogez-Florent, T., Castilla, C., Lavanant, H., *et al.* (2022) Toxicological impact of organic ultrafine particles (UFPs) in human bronchial epithelial BEAS-2B cells at air-liquid interface, *Toxicology in Vitro*, 78, pp. 105258. DOI:10.1016/j.tiv.2021.105258.

Kaisani, A., Delgado, O., Fasciani, G., Kim, S. B., Wright, W. E., Minna, J. D. and Shay, J. W. (2014) Branching morphogenesis of immortalized human bronchial epithelial cells in three-dimensional culture, *Differentiation*, 87 (3–4), pp. 119–126. DOI:10.1016/j.diff.2014.02.003.

Kalabus, J. L., Cheng, Q., Jamil, R. G., Schuetz, E. G. and Blanco, J. G. (2012) Induction of carbonyl reductase 1 (CBR1) expression in human lung tissues and lung cancer cells by the cigarette smoke constituent benzo[a]pyrene, *Toxicology Letters*, 211 (3), pp. 266–273. DOI:10.1016/j.toxlet.2012.04.006.

Karadogan, D., Onal, O., Sahin, D. and Kanbay, Y. (2018) Factors associated with current smoking in COPD patients:A cross-sectional study from the Eastern Black Sea region ofTurkey, *Tobacco Induced Diseases*, 16 (May). DOI:10.18332/tid/90665.

Kärkelä, T., Tapper, U. and Kajolinna, T. (2022) Comparison of 3R4F cigarette smoke and IQOS heated tobacco product aerosol emissions, *Environmental Science and Pollution Research*, 29 (18), pp. 27051–27069. DOI:10.1007/s11356-021-18032-x.

Kavvalakis, M. P., Stivaktakis, P. D., Tzatzarakis, M. N., Kouretas, D., Liesivuori, J., Alegakis, A. K., Vynias, D. and Tsatsakis, A. M. (2015) Multicomponent analysis of replacement liquids of electronic cigarettes using chromatographic techniques, *Journal of Analytical Toxicology*, 39 (4), pp. 262–269. DOI:10.1093/jat/bkv002.

Kerasioti, E., Veskoukis, A. S., Skaperda, Z., Zacharias, A., Poulas, K., Lazopoulos, G. and Kouretas, D. (2020) The flavoring and not the nicotine content is a decisive factor for the effects of refill liquids of electronic cigarette on the redox status of endothelial cells, *Toxicology Reports*, 7, pp. 1095–1102. DOI:10.1016/j.toxrep.2020.08.029.

Khalil, A. A., Jameson, M. J., Broaddus, W. C., Lin, P. S. and Chung, T. D. (2013) Nicotine enhances proliferation, migration, and radioresistance of human malignant glioma cells through EGFR activation, *Brain Tumor Pathology*, 30 (2), pp. 73–83. DOI:10.1007/s10014-012-0101-5.

Khlystov, A. and Samburova, V. (2016) Flavoring Compounds Dominate Toxic Aldehyde Production during E-Cigarette Vaping, *Environmental Science & Technology*, 50 (23), pp. 13080–13085. DOI:10.1021/acs.est.6b05145.

Kim, H.-J. and Shin, H.-S. (2013) Determination of tobacco-specific nitrosamines in replacement liquids of electronic cigarettes by liquid chromatography–tandem mass spectrometry, *Journal of Chromatography A*, 1291, pp. 48–55. DOI:10.1016/j.chroma.2013.03.035.

Kim, S. A., Smith, S., Beauchamp, C., Song, Y., Chiang, M., Giuseppetti, A., *et al.* (2018) Cariogenic potential of sweet flavors in electronic-cigarette liquids, *PLOS ONE*, 13 (9), pp. e0203717. DOI:10.1371/journal.pone.0203717.

Kimura, Y. and Okuda, H. (2010) Effects of Naturally Occurring Stilbene Glucosides from Medicinal Plants and Wine, on Tumour Growth and Lung Metastasis in Lewis Lung Carcinoma-Bearing Mice, *Journal of Pharmacy and Pharmacology*, 52 (10), pp. 1287–1295. DOI:10.1211/0022357001777270.

Kılıç Süloğlu, A., Koçkaya, E. A. and Selmanoğlu, G. (2022) Toxicity of benzyl benzoate as a food additive and pharmaceutical agent, *Toxicology and Industrial Health*, 38 (4), pp. 221–233. DOI:10.1177/07482337221086133.

Kładna, A., Berczyński, P., Dündar, O. B., Kruk, I., Torun, B. and Aboul-Enein, H. Y. (2021) A Study on Synthesis and Antioxidant Activity Comparison of Novel Stilbenebenzamide Compounds, *Medicinal Chemistry*, 17 (5), pp. 533–544. DOI:10.2174/1573406415666191114123004.

Knezevich, A., Muzic, J., Hatsukami, D. K., Hecht, S. S. and Stepanov, I. (2013) Nornicotine Nitrosation in Saliva and Its Relation to Endogenous Synthesis of N'-Nitrosonornicotine in Humans, *Nicotine & Tobacco Research*, 15 (2), pp. 591–595. DOI:10.1093/ntr/nts172.

Kroemer, G., Galluzzi, L. and Brenner, C. (2007) Mitochondrial Membrane Permeabilization in Cell Death, *Physiological Reviews*, 87 (1), pp. 99–163. DOI:10.1152/physrev.00013.2006.

Krüsemann, E. J. Z., Boesveldt, S., de Graaf, K. and Talhout, R. (2019) An E-Liquid Flavor Wheel: A Shared Vocabulary Based on Systematically Reviewing E-Liquid Flavor Classifications in Literature, *Nicotine & Tobacco Research*, 21 (10), pp. 1310–1319. DOI:10.1093/ntr/nty101.

Ladel, S., Schlossbauer, P., Flamm, J., Luksch, H., Mizaikoff, B. and Schindowski, K. (2019) Improved In Vitro Model for Intranasal Mucosal Drug Delivery: Primary Olfactory and Respiratory Epithelial Cells Compared with the Permanent Nasal Cell Line RPMI 2650, *Pharmaceutics*, 11 (8), pp. 367. DOI:10.3390/pharmaceutics11080367.

Lalo, H., Leclerc, L., Sorin, J. and Pourchez, J. (2020) Aerosol droplet-size distribution and airborne nicotine portioning in particle and gas phases emitted by electronic cigarettes, *Scientific Reports*, 10 (1), pp. 21707. DOI:10.1038/s41598-020-78749-6.

Lamb, T., Muthumalage, T. and Rahman, I. (2020) Pod-based menthol and tobacco flavored e-cigarettes cause mitochondrial dysfunction in lung epithelial cells, *Toxicology Letters*, 333, pp. 303–311. DOI:10.1016/j.toxlet.2020.08.003.

Laugesen Murray (2008) *Safety Report on the Ruyan® e-cigarette Cartridge andInhaled Aerosol*. Available from: http://www.healthnz.co.nz/RuyanCartridgeReport30-Oct-08.pdf

Lechasseur, A., Altmejd, S., Turgeon, N., Buonanno, G., Morawska, L., Brunet, D., Duchaine, C. and Morissette, M. C. (2019) Variations in coil temperature/power and e-liquid constituents change size and lung deposition of particles emitted by an electronic cigarette, *Physiological Reports*, 7 (10), pp. e14093. DOI:10.14814/phy2.14093.

Lee, D. F., Lethem, M. I. and Lansley, A. B. (2021) A comparison of three mucus-secreting airway cell lines (Calu-3, SPOC1 and UNCN3T) for use as biopharmaceutical models of the nose and lung, *European Journal of Pharmaceutics and Biopharmaceutics*, 167, pp. 159–174. DOI:10.1016/j.ejpb.2021.07.016.

Lee, H.-J., Guo, H.-Y., Lee, S.-K., Jeon, B.-H., Jun, C.-D., Lee, S.-K., Park, M.-H. and Kim, E.-C. (2005) Effects of nicotine on proliferation, cell cycle, and differentiation in immortalized and malignant oral keratinocytes, *Journal of Oral Pathology and Medicine*, 34 (7), pp. 436–443. DOI:10.1111/j.1600-0714.2005.00342.x.

Leigh, N. J., Lawton, R. I., Hershberger, P. A. and Goniewicz, M. L. (2016a) Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS), *Tobacco Control*, 25 (Suppl 2), pp. ii81. DOI:10.1136/tobaccocontrol-2016-053205.

Leigh, N. J., Lawton, R. I., Hershberger, P. A. and Goniewicz, M. L. (2016b) Flavourings significantly affect inhalation toxicity of aerosol generated from

electronic nicotine delivery systems (ENDS), *Tobacco Control*, 25 (Suppl 2), pp. ii81. DOI:10.1136/tobaccocontrol-2016-053205.

Leigh, N. J., Lawton, R. I., Hershberger, P. A. and Goniewicz, M. L. (2016c) Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS), *Tobacco Control*, 25 (Suppl 2), pp. ii81–ii87. DOI:10.1136/tobaccocontrol-2016-053205.

Leigh, N. J., Lawton, R. I., Hershberger, P. A. and Goniewicz, M. L. (2016d) Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS), *Tobacco Control*, 25 (Suppl 2), pp. ii81–ii87. DOI:10.1136/tobaccocontrol-2016-053205.

Lemasters, J. J., Qian, T., Bradham, C. A., Brenner, D. A., Cascio, W. E., Trost, L. C., Nishimura, Y., Nieminen, A.-L. and Herman, B. (1999) [No title found], *Journal of Bioenergetics and Biomembranes*, 31 (4), pp. 305–319. DOI:10.1023/A:1005419617371.

Lemasters, J. J., Qian, T., He, L., Kim, J.-S., Elmore, S. P., Cascio, W. E. and Brenner, D. A. (2002) Role of Mitochondrial Inner Membrane Permeabilization in Necrotic Cell Death, Apoptosis, and Autophagy, *Antioxidants & Redox Signaling*, 4 (5), pp. 769–781. DOI:10.1089/152308602760598918.

LeMessurier, K. S., Tiwary, M., Morin, N. P. and Samarasinghe, A. E. (2020) Respiratory Barrier as a Safeguard and Regulator of Defense Against Influenza A Virus and Streptococcus pneumoniae, *Frontiers in Immunology*, 11, pp. 3. DOI:10.3389/fimmu.2020.00003.

Leopold, P. L., O'Mahony, M. J., Lian, X. J., Tilley, A. E., Harvey, B.-G. and Crystal, R. G. (2009) Smoking Is Associated with Shortened Airway Cilia, *PLoS ONE*, 4 (12), pp. e8157. DOI:10.1371/journal.pone.0008157.

Lerner, C. A., Sundar, I. K., Yao, H., Gerloff, J., Ossip, D. J., McIntosh, S., Robinson, R. and Rahman, I. (2015) Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung, *PLOS ONE*, 10 (2), pp. e0116732. DOI:10.1371/journal.pone.0116732.

Leslie Fulcher, M., Gabriel, S., Burns, K. A., Yankaskas, J. R. and Randell, S. H. (2004) Well-Differentiated Human Airway Epithelial Cell Cultures, in: *Human Cell Culture Protocols*. New Jersey: Humana Press,107, pp. 183–206.

Leslie, L. J., Vasanthi Bathrinarayanan, P., Jackson, P., Mabiala Ma Muanda, J. A., Pallett, R., Stillman, C. J. P. and Marshall, L. J. (2017) A comparative study of electronic cigarette vapor extracts on airway-related cell lines *in vitro*, *Inhalation Toxicology*, 29 (3), pp. 126–136. DOI:10.1080/08958378.2017.1318193.

Leung, C., Wadsworth, S. J., Yang, S. J. and Dorscheid, D. R. (2020) Structural and functional variations in human bronchial epithelial cells cultured in air-liquid interface using different growth media, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 318 (5), pp. L1063–L1073. DOI:10.1152/ajplung.00190.2019.

Lewis, D. I. (2019) Animal experimentation: implementation and application of the 3Rs, *Emerging Topics in Life Sciences*, 3 (6), pp. 675–679. DOI:10.1042/ETLS20190061.

Li, H., Liu, B., Lian, L., Zhou, J., Xiang, S., Zhai, Y., *et al.* (2021) High dose expression of heme oxigenase-1 induces retinal degeneration through ER stress-related DDIT3, *Molecular Neurodegeneration*, 16 (1), pp. 16. DOI:10.1186/s13024-021-00437-4.

Li, J.-M. (2003) Role of mitochondrial dysfunction in hydrogen peroxide-induced apoptosis of intestinal epithelial cells, *World Journal of Gastroenterology*, 9 (3), pp. 562. DOI:10.3748/wjg.v9.i3.562.

Liao, Q., Du, R., Ma, R., Liu, X., Zhang, Y., Zhang, Z., *et al.* (2022) Association between exposure to a mixture of benzene, toluene, ethylbenzene, xylene, and styrene (BTEXS) and small airways function: A cross-sectional study, *Environmental Research*, 212, pp. 113488. DOI:10.1016/j.envres.2022.113488.

Liegeois, M. A. and Fahy, J. V. (2022) The Mucin Gene *MUC5B* Is Required for Normal Lung Function, *American Journal of Respiratory and Critical Care Medicine*, 205 (7), pp. 737–739. DOI:10.1164/rccm.202201-0064ED.

Lin, A.-H., Liu, M.-H., Ko, H.-K., Perng, D.-W., Lee, T.-S. and Kou, Y. R. (2018) Menthol Cigarette Smoke Induces More Severe Lung Inflammation Than Non-menthol Cigarette Smoke Does in Mice With Subchronic Exposure – Role of TRPM8, *Frontiers in Physiology*, 9, pp. 1817. DOI:10.3389/fphys.2018.01817.

Lucas, J. H., Muthumalage, T., Wang, Q., Friedman, M. R., Friedman, A. E. and Rahman, I. (2020) E-Liquid Containing a Mixture of Coconut, Vanilla, and Cookie Flavors Causes Cellular Senescence and Dysregulated Repair in Pulmonary Fibroblasts: Implications on Premature Aging, *Frontiers in Physiology*, 11, pp. 924. DOI:10.3389/fphys.2020.00924.

Lykkesfeldt, J., Viscovich, M. and Poulsen, H. E. (2004) Plasma malondialdehyde is induced by smoking: a study with balanced antioxidant profiles, *British Journal of Nutrition*, 92 (2), pp. 203–206. DOI:10.1079/BJN20041191.

MacArthur Clark, J. (2018) The 3Rs in research: a contemporary approach to replacement, reduction and refinement, *British Journal of Nutrition*, 120 (s1), pp. S1–S7. DOI:10.1017/S0007114517002227.

Madison, M. C., Landers, C. T., Gu, B.-H., Chang, C.-Y., Tung, H.-Y., You, R., *et al.* (2019) Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine, *Journal of Clinical Investigation*, 129 (10), pp. 4290–4304. DOI:10.1172/JCI128531.

Magnani, F. and Mattevi, A. (2019) Structure and mechanisms of ROS generation by NADPH oxidases, *Current Opinion in Structural Biology*, 59, pp. 91–97. DOI:10.1016/j.sbi.2019.03.001.

Mantey, J. A., Rekhadevi, P. V., Diggs, D. L. and Ramesh, A. (2014) Metabolism of benzo(a)pyrene by subcellular fractions of gastrointestinal (GI) tract and liver in Apc Min mouse model of colon cancer, *Tumor Biology*, 35 (5), pp. 4929–4935. DOI:10.1007/s13277-014-1647-0.

Marczylo, T. (2020) How bad are e-cigarettes? What can we learn from animal exposure models?, *The Journal of Physiology*, 598 (22), pp. 5073–5089. DOI:10.1113/JP278366.

Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D. and Proctor, C. (2016) Chemical Composition of Aerosol from an E-Cigarette: A Quantitative

Comparison with Cigarette Smoke, *Chemical Research in Toxicology*, 29 (10), pp. 1662–1678. DOI:10.1021/acs.chemrestox.6b00188.

Martin, E. M., Clapp, P. W., Rebuli, M. E., Pawlak, E. A., Glista-Baker, E., Benowitz, N. L., Fry, R. C. and Jaspers, I. (2016) E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 311 (1), pp. L135–L144. DOI:10.1152/ajplung.00170.2016.

Maslanik, T., Tannura, K., Mahaffey, L., Loughridge, A. B., Benninson, L., Ursell, L., Greenwood, B. N., Knight, R. and Fleshner, M. (2012) Commensal Bacteria and MAMPs Are Necessary for Stress-Induced Increases in IL-1β and IL-18 but Not IL-6, IL-10 or MCP-1, *PLoS ONE*, 7 (12), pp. e50636. DOI:10.1371/journal.pone.0050636.

McDonough, P. and Moffatt, R. J. (1999) Smoking-Induced Elevations in Blood Carboxyhaemoglobin Levels: Effect on Maximal Oxygen Uptake, *Sports Medicine*, 27 (5), pp. 275–283. DOI:10.2165/00007256-199927050-00001.

McEvoy, C. T. and Spindel, E. R. (2017) Pulmonary Effects of Maternal Smoking on the Fetus and Child: Effects on Lung Development, Respiratory Morbidities, and Life Long Lung Health, *Paediatric Respiratory Reviews*, 21, pp. 27–33. DOI:10.1016/j.prrv.2016.08.005.

McGraw, M. D., Kim, S.-Y., Reed, C., Hernady, E., Rahman, I., Mariani, T. J. and Finkelstein, J. N. (2020) Airway basal cell injury after acute diacetyl (2,3-butanedione) vapor exposure, *Toxicology Letters*, 325, pp. 25–33. DOI:10.1016/j.toxlet.2020.02.012.

McNeill, A. D., Brose, L. S., Calder, R., Bauld, L. and Robson, D. J. (2018) Evidence review of e-cigarettes and heated tobacco products 2018 : A report commissioned by Public Health England. Available from: https://www.gov.uk/government/publications/e-cigarettes-and-heated-tobacco-products-evidence-review [Accessed

McNeill, A., Simonavičius, E., Brose, L., Taylor, E., East, K., Zuikova, E., Calder, R. and Robson, D. (2022, October) Nicotine vaping in England: an evidence update including health risks and perceptions.

Medrano-Padial, C., Puerto, M., Moreno, F. J., Richard, T., Cantos-Villar, E. and Pichardo, S. (2019) In Vitro Toxicity Assessment of Stilbene Extract for Its Potential Use as Antioxidant in the Wine Industry, *Antioxidants*, 8 (10), pp. 467. DOI:10.3390/antiox8100467.

Melendez-Colon, V. J., Luch, A., Seidel, A. and Baird, W. M. (1999) Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites, *Carcinogenesis*, 20 (10), pp. 1885–1891. DOI:10.1093/carcin/20.10.1885.

Mercier, C., Hodin, S., He, Z., Perek, N. and Delavenne, X. (2018) Pharmacological Characterization of the RPMI 2650 Model as a Relevant Tool for Assessing the Permeability of Intranasal Drugs, *Molecular Pharmaceutics*, 15 (6), pp. 2246–2256. DOI:10.1021/acs.molpharmaceut.8b00087.

Méry, B., Guy, J.-B., Vallard, A., Espenel, S., Ardail, D., Rodriguez-Lafrasse, C., Rancoule, C. and Magné, N. (2017) In Vitro Cell Death Determination for Drug

Discovery: A Landscape Review of Real Issues, *Journal of Cell Death*, 10, pp. 117967071769125. DOI:10.1177/1179670717691251.

MHRA (2015) *The Standardised Packaging of Tobacco Products Regulations 2015*. Available from: https://www.legislation.gov.uk/ukdsi/2015/9780111129876

Micera, A., Balzamino, B. O., Zazzo, A. D., Biamonte, F., Sica, G. and Bonini, S. (2016) Toll-Like Receptors and Tissue Remodeling: The Pro/Cons Recent Findings: TLRs AND TISSUE REMODELING, *Journal of Cellular Physiology*, 231 (3), pp. 531–544. DOI:10.1002/jcp.25124.

Milnerowicz, H., Ściskalska, M. and Dul, M. (2015) Pro-inflammatory effects of metals in persons and animals exposed to tobacco smoke, *Journal of Trace Elements in Medicine and Biology*, 29, pp. 1–10. DOI:10.1016/j.jtemb.2014.04.008.

Mishra, A., Chaturvedi, P., Datta, S., Sinukumar, S., Joshi, P. and Garg, A. (2015) Harmful effects of nicotine, *Indian Journal of Medical and Paediatric Oncology*, 36 (01), pp. 24–31. DOI:10.4103/0971-5851.151771.

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P. and Malik, A. B. (2014) Reactive Oxygen Species in Inflammation and Tissue Injury, *Antioxidants & Redox Signaling*, 20 (7), pp. 1126–1167. DOI:10.1089/ars.2012.5149.

Moazed, F. and Calfee, C. S. (2017) The Canary in the Coal Mine Is Coughing: Electronic Cigarettes and Respiratory Symptoms in Adolescents, *American Journal of Respiratory and Critical Care Medicine*, 195 (8), pp. 974–976. DOI:10.1164/rccm.201611-2259ED.

Molavian, H. R., Goldman, A., Phipps, C. J., Kohandel, M., Wouters, B. G., Sengupta, S. and Sivaloganathan, S. (2016) Drug-induced reactive oxygen species (ROS) rely on cell membrane properties to exert anticancer effects, *Scientific Reports*, 6 (1), pp. 27439. DOI:10.1038/srep27439.

Morsch, A. L. B. C., Wisniewski, E., Luciano, T. F., Comin, V. H., Silveira, G. D. B., Marques, S. D. O., Thirupathi, A., Silveira Lock, P. C. and De Souza, C. T. (2019) Cigarette smoke exposure induces ROS-mediated autophagy by regulating sestrin, AMPK, and mTOR level in mice, *Redox Report*, 24 (1), pp. 27–33. DOI:10.1080/13510002.2019.1601448.

Munis, A. M., Hyde, S. C. and Gill, D. R. (2021) A human surfactant B deficiency air-liquid interface cell culture model suitable for gene therapy applications, *Molecular Therapy - Methods & Clinical Development*, 20, pp. 237–246. DOI:10.1016/j.omtm.2020.11.013.

Munnia, A., Giese, R. W., Polvani, S., Galli, A., Cellai, F. and Peluso, M. E. M. (2017) Bulky DNA Adducts, Tobacco Smoking, Genetic Susceptibility, and Lung Cancer Risk, in: *Advances in Clinical Chemistry*. Elsevier,81, pp. 231–277.

Murphy, S. E. (2021) Biochemistry of nicotine metabolism and its relevance to lung cancer, *Journal of Biological Chemistry*, 296, pp. 100722. DOI:10.1016/j.jbc.2021.100722.

Murphy, S. E., Isaac, I. S., Ding, X. and McIntee, E. J. (2000) Specificity of cytochrome P450 2A3-catalyzed alpha-hydroxylation of N'-nitrosonornicotine enantiomers, *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, 28 (11), pp. 1263–1266.

Muthumalage, T., Lamb, T., Friedman, M. R. and Rahman, I. (2019) E-cigarette flavored pods induce inflammation, epithelial barrier dysfunction, and DNA damage in lung epithelial cells and monocytes, *Scientific Reports*, 9 (1), pp. 19035. DOI:10.1038/s41598-019-51643-6.

Muthumalage, T., Prinz, M., Ansah, K. O., Gerloff, J., Sundar, I. K. and Rahman, I. (2018) Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine, *Frontiers in Physiology*, 8, pp. 1130. DOI:10.3389/fphys.2017.01130.

Neczypor, E. W., Saldaña, T. A., Mears, M. J., Aslaner, D. M., Escobar, Y.-N. H., Gorr, M. W. and Wold, L. E. (2022) e-Cigarette Aerosol Reduces Left Ventricular Function in Adolescent Mice, *Circulation*, 145 (11), pp. 868–870. DOI:10.1161/CIRCULATIONAHA.121.057613.

Neilson, L., Mankus, C., Thorne, D., Jackson, G., DeBay, J. and Meredith, C. (2015) Development of an in vitro cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol, *Toxicology in Vitro*, 29 (7), pp. 1952–1962. DOI:10.1016/j.tiv.2015.05.018.

Neurath, M. F. and Finotto, S. (2011) IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer, *Cytokine & Growth Factor Reviews*, 22 (2), pp. 83–89. DOI:10.1016/j.cytogfr.2011.02.003.

Nguyen, H. M.-H., Torres, J. A., Agrawal, S. and Agrawal, A. (2020a) Nicotine Impairs the Response of Lung Epithelial Cells to IL-22, *Mediators of Inflammation*, 2020, pp. 1–9. DOI:10.1155/2020/6705428.

Nguyen, N. D., Memon, T. A., Burrell, K. L., Almestica-Roberts, M., Rapp, E., Sun, L., *et al.* (2020b) Transient Receptor Potential Ankyrin-1 and Vanilloid-3 Differentially Regulate Endoplasmic Reticulum Stress and Cytotoxicity in Human Lung Epithelial Cells After Pneumotoxic Wood Smoke Particle Exposure, *Molecular Pharmacology*, 98 (5), pp. 586–597. DOI:10.1124/molpharm.120.000047.

NHS (2019) *Statistics on Smoking, England - 2019*. Available from: https://digital.nhs.uk/data-and-information/publications/statistical/statistics-on-smoking/statistics-on-smoking-england-2019/part-1-smoking-related-ill-health-and-mortality

NHS (2020) *Statistics on Smoking, England 2020*. Available from: https://digital.nhs.uk/data-and-information/publications/statistical/statistics-on-smoking/statistics-on-smoking-england-2020

NICE (2018) *Stop smoking interventions and services*.

NICE (2023a) *Smoking cessation*. Available from: https://bnf.nice.org.uk/treatment-summaries/smoking-cessation/#:~:text=Nicotine%20replacement%20therapy%20%28NRT%29%2C%20varenicline%2C%20and%20bupropion%20hydrochloride%2C,cessation%20and%20should%20be%20offered%20alongside%20behavioural%20support.

NICE (2023b) *Tobacco: preventing uptake, promoting quitting and treating dependence*. Available from: https://www.nice.org.uk/guidance/ng209/resources/impact-on-nhs-workforce-and-resources-11188073773

NICE (2023c) *What is the prevalence of smoking?* Available from: https://cks.nice.org.uk/topics/smoking-cessation/background-information/prevalence/#:~:text=Smoking%20prevalence%20in%20the%20UK%20has%20fallen%20from,reduction%20of%20more%20than%206.9%20percent%20since%202011.

Noël, A., Hansen, S., Zaman, A., Perveen, Z., Pinkston, R., Hossain, E., Xiao, R. and Penn, A. (2020a) In utero exposures to electronic-cigarette aerosols impair the *Wnt* signaling during mouse lung development, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 318 (4), pp. L705–L722. DOI:10.1152/ajplung.00408.2019.

Noël, A., Hossain, E., Perveen, Z., Zaman, H. and Penn, A. L. (2020b) Sub-ohm vaping increases the levels of carbonyls, is cytotoxic, and alters gene expression in human bronchial epithelial cells exposed at the air–liquid interface, *Respiratory Research*, 21 (1), pp. 305. DOI:10.1186/s12931-020-01571-1.

Novak, I. (2003) ATP as a Signaling Molecule: the Exocrine Focus, *Physiology*, 18 (1), pp. 12–17. DOI:10.1152/nips.01409.2002.

O'Brien, K. A. F., Smith, L. L. and Cohen, G. M. (1987) Inability of a human lung tumour cell line (A549) to detect chemically induced organ-specific toxicity to the lung, *Toxicology in Vitro*, 1 (2), pp. 85–90. DOI:10.1016/0887-2333(87)90005-1.

O'Byrne, P. M. and Postma, D. S. (1999) The Many Faces of Airway Inflammation: Asthma and Chronic Obstructive Pulmonary Disease, *American Journal of Respiratory and Critical Care Medicine*, 159 (supplement_2), pp. S1–S63. DOI:10.1164/ajrccm.159.supplement_2.mfa-1.

OECD Health statistics (2020) *Health at a Glance: Europe 2020 : State of Health in the EU Cycle*.

O'Farrell, H. E., Brown, R., Brown, Z., Milijevic, B., Ristovski, Z. D., Bowman, R. V., Fong, K. M., Vaughan, A. and Yang, I. A. (2021) E-cigarettes induce toxicity comparable to tobacco cigarettes in airway epithelium from patients with COPD, *Toxicology in Vitro*, 75, pp. 105204. DOI:10.1016/j.tiv.2021.105204.

Ogunwale, M. A., Li, M., Ramakrishnam Raju, M. V., Chen, Y., Nantz, M. H., Conklin, D. J. and Fu, X.-A. (2017) Aldehyde Detection in Electronic Cigarette Aerosols, *ACS Omega*, 2 (3), pp. 1207–1214. DOI:10.1021/acsomega.6b00489.

Okuda, K., Chen, G., Subramani, D. B., Wolf, M., Gilmore, R. C., Kato, T., *et al.* (2019) Localization of Secretory Mucins MUC5AC and MUC5B in Normal/Healthy Human Airways, *American Journal of Respiratory and Critical Care Medicine*, 199 (6), pp. 715–727. DOI:10.1164/rccm.201804-0734OC.

Olivieri, M., Murgia, N., Carsin, A., Heinrich, J., Benke, G., Bono, R., *et al.* (2019) Effects of smoking bans on passive smoking exposure at work and at home. The European Community respiratory health survey, *Indoor Air*, pp. ina.12556. DOI:10.1111/ina.12556.

Omaiye, E. E., Luo, W., McWhirter, K. J., Pankow, J. F. and Talbot, P. (2022) Ethyl maltol, vanillin, corylone and other conventional confectionery-related flavour chemicals dominate in some e-cigarette liquids labelled 'tobacco' flavoured, *Tobacco Control*, 31 (Suppl 3), pp. s238–s244. DOI:10.1136/tc-2022-057484.

Omaiye, E. E., McWhirter, K. J., Luo, W., Tierney, P. A., Pankow, J. F. and Talbot, P. (2019a) High concentrations of flavor chemicals are present in electronic cigarette refill fluids, *Scientific Reports*, 9 (1), pp. 2468. DOI:10.1038/s41598-019-39550-2.

Omaiye, E. E., McWhirter, K. J., Luo, W., Tierney, P. A., Pankow, J. F. and Talbot, P. (2019b) High concentrations of flavor chemicals are present in electronic cigarette refill fluids, *Scientific Reports*, 9 (1), pp. 2468. DOI:10.1038/s41598-019-39550-2.

O'Neill, S. T. and Parrott, A. C. (1992) Stress and arousal in sedative and stimulant cigarette smokers, *Psychopharmacology*, 107 (2–3), pp. 442–446. DOI:10.1007/BF02245173.

Ong, H. X., Traini, D. and Young, P. M. (2013) Pharmaceutical applications of the Calu-3 lung epithelia cell line, *Expert Opinion on Drug Delivery*, 10 (9), pp. 1287–1302. DOI:10.1517/17425247.2013.805743.

Ooi, B. G., Dutta, D., Kazipeta, K. and Chong, N. S. (2019) Influence of the E-Cigarette Emission Profile by the Ratio of Glycerol to Propylene Glycol in E-Liquid Composition, *ACS Omega*, 4 (8), pp. 13338–13348. DOI:10.1021/acsomega.9b01504.

Otterbein, L. E., Hedblom, A., Harris, C., Csizmadia, E., Gallo, D. and Wegiel, B. (2011) Heme oxygenase-1 and carbon monoxide modulate DNA repair through ataxia-telangiectasia mutated (ATM) protein, *Proceedings of the National Academy of Sciences*, 108 (35), pp. 14491–14496. DOI:10.1073/pnas.1102295108.

Paradowska-Gorycka, A. (2015) U1-RNP and Toll-like receptors in the pathogenesis of mixed connective tissue diseasePart II. Endosomal TLRs and their biological

significance in the pathogenesis of mixed connective tissue disease, *Reumatologia*, 53 (3), pp. 143–151. DOI:10.5114/reum.2015.53136.

Park, H.-R., O'Sullivan, M., Vallarino, J., Shumyatcher, M., Himes, B. E., Park, J.-A., Christiani, D. C., Allen, J. and Lu, Q. (2019) Transcriptomic response of primary human airway epithelial cells to flavoring chemicals in electronic cigarettes, *Scientific Reports*, 9 (1), pp. 1400. DOI:10.1038/s41598-018-37913-9.

Parrott, A. C. (1993) Cigarette smoking: Effects upon self-rated stress and arousal over the day, *Addictive Behaviors*, 18 (4), pp. 389–395. DOI:10.1016/0306-4603(93)90055-E.

Parrott, A. C. (1994) Individual differences in stress and arousal during cigarette smoking, *Psychopharmacology*, 115 (3), pp. 389–396. DOI:10.1007/BF02245082.

Pasman, T., Baptista, D., Van Riet, S., Truckenmüller, R. K., Hiemstra, P. S., Rottier, R. J., *et al.* (2021) Development of an In Vitro Airway Epithelial–Endothelial Cell Culture Model on a Flexible Porous Poly(Trimethylene Carbonate) Membrane Based on Calu-3 Airway Epithelial Cells and Lung Microvascular Endothelial Cells, *Membranes*, 11 (3), pp. 197. DOI:10.3390/membranes11030197.

Patel, J., Pal, D., Vangala, V., Gandhi, M. and Mitra, A. K. (2002) [No title found], *Pharmaceutical Research*, 19 (11), pp. 1696–1703. DOI:10.1023/A:1020761514471.

Pearsall, D. M. (1992) The origins of plant cultivation in South America. In The origins of agriculture: An international perspective, (Washington, DC: Smithsonian Institution Press.), pp. 173–204.

Pereira, E. J., Sim, L., Driver, H. S., Parker, C. M. and Fitzpatrick, M. F. (2013) The effect of inhaled menthol on upper airway resistance in humans: A randomized controlled crossover study, *Canadian Respiratory Journal*, 20 (1), pp. e1–e4. DOI:10.1155/2013/383019.

Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G. and Migliaccio, A. (2020) ROS in cancer therapy: the bright side of the moon, *Experimental & Molecular Medicine*, 52 (2), pp. 192–203. DOI:10.1038/s12276-020-0384-2.

Peters-Hall, J. R., Coquelin, M. L., Torres, M. J., LaRanger, R., Alabi, B. R., Sho, S., Calva-Moreno, J. F., Thomas, P. J. and Shay, J. W. (2018) Long-term culture and cloning of primary human bronchial basal cells that maintain multipotent differentiation capacity and CFTR channel function, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 315 (2), pp. L313–L327. DOI:10.1152/ajplung.00355.2017.

Pezzuto, A., Citarella, F., Croghan, I. and Tonini, G. (2019) The effects of cigarette smoking extracts on cell cycle and tumor spread: novel evidence, *Future Science OA*, 5 (5), pp. FSO394. DOI:10.2144/fsoa-2019-0017.

Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Research*, 29 (9), pp. 45e–445. DOI:10.1093/nar/29.9.e45.

Phillips, D. H. (2002) Smoking-related DNA and protein adducts in human tissues, *Carcinogenesis*, 23 (12), pp. 1979–2004. DOI:10.1093/carcin/23.12.1979.

Phillips, D. H. and Venitt, S. (2012) DNA and protein adducts in human tissues resulting from exposure to tobacco smoke, *International Journal of Cancer*, 131 (12), pp. 2733–2753. DOI:10.1002/ijc.27827.

Pigeolet, E., Corbisier, P., Houbion, A., Lambert, D., Michiels, C., Raes, M., Zachary, M.-D. and Remacle, J. (1990) Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals, *Mechanisms of Ageing and Development*, 51 (3), pp. 283–297. DOI:10.1016/0047-6374(90)90078-T.

Ping Wu, J. (2019) Nicotine is Insufficient as a Carcinogen, It's Functions as a Tumor Promoter on Purpose, *American Journal of Biomedical Science & Research*, 5 (1), pp. 47–48. DOI:10.34297/AJBSR.2019.05.000872.

Pinkston, R., Zaman, H., Hossain, E., Penn, A. L. and Noël, A. (2020) Cell-specific toxicity of short-term JUUL aerosol exposure to human bronchial epithelial cells and murine macrophages exposed at the air–liquid interface, *Respiratory Research*, 21 (1), pp. 269. DOI:10.1186/s12931-020-01539-1.

Poburko, D., Santo-Domingo, J. and Demaurex, N. (2011) Dynamic Regulation of the Mitochondrial Proton Gradient during Cytosolic Calcium Elevations, *Journal of Biological Chemistry*, 286 (13), pp. 11672–11684. DOI:10.1074/jbc.M110.159962.

Popa, C., Netea, M. G., van Riel, P. L. C. M., van der Meer, J. W. M. and Stalenhoef, A. F. H. (2007) The role of TNF-α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk, *Journal of Lipid Research*, 48 (4), pp. 751–762. DOI:10.1194/jlr.R600021-JLR200.

Popko, K., Gorska, E., Stelmaszczyk-Emmel, A., Plywaczewski, R., Stoklosa, A., Gorecka, D., Pyrzak, B. and Demkow, U. (2010) Proinflammatory cytokines IL-6 and TNF-α and the development of inflammation in obese subjects, *European Journal of Medical Research*, 15 (S2), pp. 120. DOI:10.1186/2047-783X-15-S2-120.

Prakash, Y. S., Pabelick, C. M. and Sieck, G. C. (2017) Mitochondrial Dysfunction in Airway Disease, *Chest*, 152 (3), pp. 618–626. DOI:10.1016/j.chest.2017.03.020.

Pray, L. (2008) *Genes, Smoking, and Lung Cancer*. Available from: https://www.nature.com/scitable/topicpage/genes-smoking-and-lung-cancer-804/

Rafferty, S. M. (2002) Identification of Nicotine by Gas Chromatography/Mass Spectroscopy Analysis of Smoking Pipe Residue, *Journal of Archaeological Science*, 29 (8), pp. 897–907. DOI:10.1006/jasc.2001.0747.

Ramirez, R. D., Sheridan, S., Girard, L., Sato, M., Kim, Y., Pollack, J., *et al.* (2004) Immortalization of Human Bronchial Epithelial Cells in the Absence of Viral Oncoproteins, *Cancer Research*, 64 (24), pp. 9027–9034. DOI:10.1158/0008-5472.CAN-04-3703.

Raub, J. A., Mathieu-Nolf, M., Hampson, N. B. and Thom, S. R. (2000) Carbon monoxide poisoning — a public health perspective, *Toxicology*, 145 (1), pp. 1–14. DOI:10.1016/S0300-483X(99)00217-6.

Rawlins, E. L., Okubo, T., Xue, Y., Brass, D. M., Auten, R. L., Hasegawa, H., Wang, F. and Hogan, B. L. M. (2009) The Role of Scgb1a1+ Clara Cells in the Long-Term Maintenance and Repair of Lung Airway, but Not Alveolar, Epithelium, *Cell Stem Cell*, 4 (6), pp. 525–534. DOI:10.1016/j.stem.2009.04.002.

Reddel, R. R., Salghetti, S. E., Willey, J. C., Ohnuki, Y., Ke, Y., Gerwin, B. I., Lechner, J. F. and Harris, C. C. (1993) Development of tumorigenicity in simian virus 40-immortalized human bronchial epithelial cell lines, *Cancer Research*, 53 (5), pp. 985–991.

Redza-Dutordoir, M. and Averill-Bates, D. A. (2016) Activation of apoptosis signalling pathways by reactive oxygen species, *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1863 (12), pp. 2977–2992. DOI:10.1016/j.bbamcr.2016.09.012.

Reinisalo, M., Kårlund, A., Koskela, A., Kaarniranta, K. and Karjalainen, R. O. (2015) Polyphenol Stilbenes: Molecular Mechanisms of Defence against Oxidative Stress and Aging-Related Diseases, *Oxidative Medicine and Cellular Longevity*, 2015, pp. 1–24. DOI:10.1155/2015/340520.

Rock, J. R., Rawlins, E. L., Onaitis, M. W. and Hogan, B. L. (2009) Basal cells as stem cells of the mouse trachea and human conducting airways, *Developmental Biology*, 331 (2), pp. 503. DOI:10.1016/j.ydbio.2009.05.438.

Roe, F. J. C. and Wood, D. (1992) Review : Acetaldehyde and Formaldehyde: Is There a Cancer Risk for Man?, *Indoor Environment*, 1 (1), pp. 8–15. DOI:10.1177/1420326X9200100103.

Roos, D. (2016) Chronic granulomatous disease, *British Medical Bulletin*, 118 (1), pp. 50–63. DOI:10.1093/bmb/ldw009.

Rose, C. S. (2017) Early detection, clinical diagnosis, and management of lung disease from exposure to diacetyl, *Toxicology*, 388, pp. 9–14. DOI:10.1016/j.tox.2017.03.019.

Rowell, T. R., Keating, J. E., Zorn, B. T., Glish, G. L., Shears, S. B. and Tarran, R. (2020) Flavored e-liquids increase cytoplasmic Ca $^{2+}$ levels in airway epithelia, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 318 (2), pp. L226–L241. DOI:10.1152/ajplung.00123.2019.

Rowell, T. R., Reeber, S. L., Lee, S. L., Harris, R. A., Nethery, R. C., Herring, A. H., Glish, G. L. and Tarran, R. (2017) Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 313 (1), pp. L52–L66. DOI:10.1152/ajplung.00392.2016.

Russell, C., McKeganey, N., Dickson, T. and Nides, M. (2018) Changing patterns of first e-cigarette flavor used and current flavors used by 20,836 adult frequent e-cigarette users in the USA, *Harm Reduction Journal*, 15 (1), pp. 33. DOI:10.1186/s12954-018-0238-6.

Russell, M. A. H. (1980) Nicotine intake and its regulation, *Journal of Psychosomatic Research*, 24 (5), pp. 253–264. DOI:10.1016/0022-3999(80)90015-X.

Russo, P., Nastrucci, C., Alzetta, G. and Szalai, C. (2011) Tobacco Habit: Historical, Cultural, Neurobiological, and Genetic Features of People's Relationship with an Addictive Drug, *Perspectives in Biology and Medicine*, 54 (4), pp. 557–577. DOI:10.1353/pbm.2011.0047.

Sabnis, A. S., Shadid, M., Yost, G. S. and Reilly, C. A. (2008) Human Lung Epithelial Cells Express a Functional Cold-Sensing TRPM8 Variant, *American Journal of Respiratory Cell and Molecular Biology*, 39 (4), pp. 466–474. DOI:10.1165/rcmb.2007-0440OC.

Sakagami, M. (2020) In vitro, ex vivo and in vivo methods of lung absorption for inhaled drugs, *Advanced Drug Delivery Reviews*, 161–162, pp. 63–74. DOI:10.1016/j.addr.2020.07.025.

Salib, R. J., Lau, L. C. and Howarth, P. H. (2005) The novel use of the human nasal epithelial cell line RPMI 2650 as an in vitro model to study the influence of allergens and cytokines on transforming growth factor-beta gene expression and protein release, *Clinical <html_ent Glyph="@amp;" Ascii="&"/> Experimental Allergy*, 35 (6), pp. 811–819. DOI:10.1111/j.1365-2222.2005.02258.x.

Sassano, M. F., Davis, E. S., Keating, J. E., Zorn, B. T., Kochar, T. K., Wolfgang, M. C., Glish, G. L. and Tarran, R. (2018) Evaluation of e-liquid toxicity using an open-source high-throughput screening assay, *PLOS Biology*, 16 (3), pp. e2003904. DOI:10.1371/journal.pbio.2003904.

Schick, S. F., Blount, B. C., Jacob, P., Saliba, N. A., Bernert, J. T., El Hellani, A., *et al.* (2017) Biomarkers of exposure to new and emerging tobacco delivery products, *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 313 (3), pp. L425–L452. DOI:10.1152/ajplung.00343.2016.

Schober, W., Szendrei, K., Matzen, W., Osiander-Fuchs, H., Heitmann, D., Schettgen, T., Jörres, R. A. and Fromme, H. (2014) Use of electronic cigarettes (e-cigarettes) impairs indoor air quality and increases FeNO levels of e-cigarette consumers, *International Journal of Hygiene and Environmental Health*, 217 (6), pp. 628–637. DOI:10.1016/j.ijheh.2013.11.003.

Schweitzer, R. J., Wills, T. A. and Behner, J. D. (2017) E-cigarette Use and Indicators of Cardiovascular Disease Risk, *Current Epidemiology Reports*, 4 (3), pp. 248–257. DOI:10.1007/s40471-017-0118-8.

Sciacchitano, S., Lavra, L., Morgante, A., Ulivieri, A., Magi, F., De Francesco, G., Bellotti, C., Salehi, L. and Ricci, A. (2018) Galectin-3: One Molecule for an Alphabet of Diseases, from A to Z, *International Journal of Molecular Sciences*, 19 (2), pp. 379. DOI:10.3390/ijms19020379.

Scott, R. S., Frame, S. R., Ross, P. E., Loveless, S. E. and Kennedy, G. L. (2005) Inhalation Toxicity of 1,3-Propanediol in the Rat, *Inhalation Toxicology*, 17 (9), pp. 487–493. DOI:10.1080/08958370590964485.

Seriani, R., Paula, C. P. de, Cunha, A. F. da, Oliveira, M. A. de, Krempel, P. G., Frias, D. P., Negri, E. M., Mauad, T. and Macchione, M. (2021) Expression patterns of peroxiredoxin genes in bronchial epithelial cells exposed to diesel exhaust particles, *Experimental and Molecular Pathology*, 120, pp. 104641. DOI:10.1016/j.yexmp.2021.104641.

Shahab, L., Goniewicz, M. L., Blount, B. C., Brown, J., McNeill, A., Alwis, K. U., Feng, J., Wang, L. and West, R. (2017) Nicotine, Carcinogen, and Toxin Exposure in Long-Term E-Cigarette and Nicotine Replacement Therapy Users: A Cross-sectional Study, *Annals of Internal Medicine*, 166 (6), pp. 390. DOI:10.7326/M16-1107.

Shahsavar, A., Gajhede, M., Kastrup, J. S. and Balle, T. (2016) Structural Studies of Nicotinic Acetylcholine Receptors: Using Acetylcholine-Binding Protein as a Structural Surrogate, *Basic & Clinical Pharmacology & Toxicology*, 118 (6), pp. 399–407. DOI:10.1111/bcpt.12528.

She, Y. X., Yu, Q. Y. and Tang, X. X. (2021) Role of interleukins in the pathogenesis of pulmonary fibrosis, *Cell Death Discovery*, 7 (1), pp. 52. DOI:10.1038/s41420-021-00437-9.

Shen, B. Q., Finkbeiner, W. E., Wine, J. J., Mrsny, R. J. and Widdicombe, J. H. (1994) Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 266 (5), pp. L493–L501. DOI:10.1152/ajplung.1994.266.5.L493.

Sherwood, C. L. and Boitano, S. (2016) Airway epithelial cell exposure to distinct e-cigarette liquid flavorings reveals toxicity thresholds and activation of CFTR by the chocolate flavoring 2,5-dimethypyrazine, *Respiratory Research*, 17 (1), pp. 57. DOI:10.1186/s12931-016-0369-9.

Shimoji, M., Shimizu, S., Sato, K., Suda, K., Kobayashi, Y., Tomizawa, K., Takemoto, T. and Mitsudomi, T. (2016) Clinical and pathologic features of lung cancer expressing programmed cell death ligand 1 (PD-L1), *Lung Cancer*, 98, pp. 69–75. DOI:10.1016/j.lungcan.2016.04.021.

Silva, C. P. and Kamens, H. M. (2021) Cigarette smoke-induced alterations in blood: A review of research on DNA methylation and gene expression., *Experimental and Clinical Psychopharmacology*, 29 (1), pp. 116–135. DOI:10.1037/pha0000382.

Silva, S., Bicker, J., Falcão, A. and Fortuna, A. (2023) Air-liquid interface (ALI) impact on different respiratory cell cultures, *European Journal of Pharmaceutics and Biopharmaceutics*, 184, pp. 62–82. DOI:10.1016/j.ejpb.2023.01.013.

Simonavicius, E., McNeill, A., Shahab, L. and Brose, L. S. (2019) Heat-not-burn tobacco products: a systematic literature review, *Tobacco Control*, 28 (5), pp. 582–594. DOI:10.1136/tobaccocontrol-2018-054419.

Skjesol, A., Yurchenko, M., Bösl, K., Gravastrand, C., Nilsen, K. E., Grøvdal, L. M., *et al.* (2019) The TLR4 adaptor TRAM controls the phagocytosis of Gram-negative bacteria by interacting with the Rab11-family interacting protein 2, *PLOS Pathogens*, 15 (3), pp. e1007684. DOI:10.1371/journal.ppat.1007684.

Son, Y., Mishin, V., Laskin, J. D., Mainelis, G., Wackowski, O. A., Delnevo, C., *et al.* (2019) Hydroxyl Radicals in E-Cigarette Vapor and E-Vapor Oxidative Potentials under Different Vaping Patterns, *Chemical Research in Toxicology*, 32 (6), pp. 1087–1095. DOI:10.1021/acs.chemrestox.8b00400.

Sopori, M. (2002) Effects of cigarette smoke on the immune system, *Nature Reviews Immunology*, 2 (5), pp. 372–377. DOI:10.1038/nri803.

Soriano, J. B., Polverino, F. and Cosio, B. G. (2018) What is early COPD and why is it important?, *European Respiratory Journal*, 52 (6), pp. 1801448. DOI:10.1183/13993003.01448-2018.

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L. and Hickman, J. J. (2015) TEER Measurement Techniques for In Vitro Barrier Model Systems, *SLAS Technology*, 20 (2), pp. 107–126. DOI:10.1177/2211068214561025.

Stahl, A. B. (1994) Change and Continuity in the Banda Area, Ghana: The Direct Historical Approach, *Journal of Field Archaeology*, 21 (2), pp. 181–203. DOI:10.1179/009346994791547670.

Stead, L. F., Koilpillai, P., Fanshawe, T. R. and Lancaster, T. (2016) Combined pharmacotherapy and behavioural interventions for smoking cessation, *Cochrane Database of Systematic Reviews*, 2016 (3). DOI:10.1002/14651858.CD008286.pub3.

Stefaniak, A. B., Ranpara, A. C., Virji, M. A. and LeBouf, R. F. (2022) Influence of E-Liquid Humectants, Nicotine, and Flavorings on Aerosol Particle Size Distribution and Implications for Modeling Respiratory Deposition, *Frontiers in Public Health*, 10, pp. 782068. DOI:10.3389/fpubh.2022.782068.

Steinberg, M. B. (2009) Triple-Combination Pharmacotherapy for Medically Ill Smokers: A Randomized Trial, *Annals of Internal Medicine*, 150 (7), pp. 447. DOI:10.7326/0003-4819-150-7-200904070-00004.

Stepanov, I., Carmella, S. G., Briggs, A., Hertsgaard, L., Lindgren, B., Hatsukami, D. and Hecht, S. S. (2009) Presence of the Carcinogen *N'*-Nitrosonornicotine in the Urine of Some Users of Oral Nicotine Replacement Therapy Products, *Cancer Research*, 69 (21), pp. 8236–8240. DOI:10.1158/0008-5472.CAN-09-1084.

Stewart, C. E., Torr, E. E., Mohd Jamili, N. H., Bosquillon, C. and Sayers, I. (2012) Evaluation of Differentiated Human Bronchial Epithelial Cell Culture Systems for Asthma Research, *Journal of Allergy*, 2012, pp. 1–11. DOI:10.1155/2012/943982.

Strzelak, A., Ratajczak, A., Adamiec, A. and Feleszko, W. (2018) Tobacco Smoke Induces and Alters Immune Responses in the Lung Triggering Inflammation, Allergy, Asthma and Other Lung Diseases: A Mechanistic Review, *International Journal of Environmental Research and Public Health*, 15 (5), pp. 1033. DOI:10.3390/ijerph15051033.

Sun, Y., Oberley, L. W., Elwell, J. H. and Sierra-Rivera, E. (1989) Antioxidant enzyme activities in normal and transformed mouse liver cells, *International Journal of Cancer*, 44 (6), pp. 1028–1033. DOI:10.1002/ijc.2910440615.

Sundar, I. K., Javed, F., Romanos, G. E. and Rahman, I. (2016a) E-cigarettes and flavorings induce inflammatory and pro-senescence responses in oral epithelial cells and periodontal fibroblasts, *Oncotarget*, 7 (47), pp. 77196–77204. DOI:10.18632/oncotarget.12857.

Sundar, I. K., Javed, F., Romanos, G. E. and Rahman, I. (2016b) E-cigarettes and flavorings induce inflammatory and pro-senescence responses in oral epithelial cells and periodontal fibroblasts, *Oncotarget*, 7 (47), pp. 77196–77204. DOI:10.18632/oncotarget.12857.

Sundar, I. K., Maremanda, K. P. and Rahman, I. (2019) Mitochondrial dysfunction is associated with Miro1 reduction in lung epithelial cells by cigarette smoke, *Toxicology Letters*, 317, pp. 92–101. DOI:10.1016/j.toxlet.2019.09.022.

Szafran, B. N., Pinkston, R., Perveen, Z., Ross, M. K., Morgan, T., Paulsen, D. B., Penn, A. L., Kaplan, B. L. F. and Noël, A. (2020) Electronic-Cigarette Vehicles and Flavoring Affect Lung Function and Immune Responses in a Murine Model, *International Journal of Molecular Sciences*, 21 (17), pp. 6022. DOI:10.3390/ijms21176022.

Talavera, K., Startek, J. B., Alvarez-Collazo, J., Boonen, B., Alpizar, Y. A., Sanchez, A., Naert, R. and Nilius, B. (2020) Mammalian Transient Receptor Potential TRPA1 Channels: From Structure to Disease, *Physiological Reviews*, 100 (2), pp. 725–803. DOI:10.1152/physrev.00005.2019.

Talih, S., Balhas, Z., Eissenberg, T., Salman, R., Karaoghlanian, N., El Hellani, A., Baalbaki, R., Saliba, N. and Shihadeh, A. (2015) Effects of User Puff Topography, Device Voltage, and Liquid Nicotine Concentration on Electronic Cigarette Nicotine Yield: Measurements and Model Predictions, *Nicotine & Tobacco Research*, 17 (2), pp. 150–157. DOI:10.1093/ntr/ntu174.

Tamura, Y., Iwasaki, Y., Narukawa, M. and Watanabe, T. (2012) Ingestion of Cinnamaldehyde, a TRPA1 Agonist, Reduces Visceral Fats in Mice Fed a High-Fat and High-Sucrose Diet, *Journal of Nutritional Science and Vitaminology*, 58 (1), pp. 9–13. DOI:10.3177/jnsv.58.9.

Tantucci, C. and Modina, D. (2012) Lung function decline in COPD, *International Journal of Chronic Obstructive Pulmonary Disease*, pp. 95. DOI:10.2147/COPD.S27480.

Tierney, P. A., Karpinski, C. D., Brown, J. E., Luo, W. and Pankow, J. F. (2016) Flavour chemicals in electronic cigarette fluids, *Tobacco Control*, 25 (e1), pp. e10–e15. DOI:10.1136/tobaccocontrol-2014-052175.

Tiwari, R. K., Sharma, V., Pandey, R. K. and Shukla, S. S. (2020) Nicotine Addiction: Neurobiology and Mechanism, *Journal of Pharmacopuncture*, 23 (1), pp. 1–7. DOI:10.3831/KPI.2020.23.001.

Tokunaga, R., Zhang, W., Naseem, M., Puccini, A., Berger, M. D., Soni, S., McSkane, M., Baba, H. and Lenz, H.-J. (2018) CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation – A target for novel cancer therapy, *Cancer Treatment Reviews*, 63, pp. 40–47. DOI:10.1016/j.ctrv.2017.11.007.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J. and Jemal, A. (2015) Global cancer statistics, 2012: Global Cancer Statistics, 2012, *CA: A Cancer Journal for Clinicians*, 65 (2), pp. 87–108. DOI:10.3322/caac.21262.

Tsoutsoulopoulos, A., Gohlsch, K., Möhle, N., Breit, A., Hoffmann, S., Krischenowski, O., *et al.* (2019) Validation of the CULTEX® Radial Flow System for the assessment of the acute inhalation toxicity of airborne particles, *Toxicology in Vitro*, 58, pp. 245–255. DOI:10.1016/j.tiv.2019.03.020.

Tsujimoto, Y., Nakagawa, T. and Shimizu, S. (2006) Mitochondrial membrane permeability transition and cell death, *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1757 (9–10), pp. 1297–1300. DOI:10.1016/j.bbabio.2006.03.017.

Tweed, J. O., Hsia, S. H., Lutfy, K. and Friedman, T. C. (2012) The endocrine effects of nicotine and cigarette smoke, *Trends in Endocrinology & Metabolism*, 23 (7), pp. 334–342. DOI:10.1016/j.tem.2012.03.006.

Uguna, C. N. and Snape, C. E. (2022) Should IQOS Emissions Be Considered as Smoke and Harmful to Health? A Review of the Chemical Evidence, *ACS Omega*, 7 (26), pp. 22111–22124. DOI:10.1021/acsomega.2c01527.

U.S. Food and Drug Administration (2009) *Summary of results: Laboratory analysis of electronic cigarettes conducted by FDA*. Available from: http://www.fda.gov/NewsEvents/PublicHealthFocus/ucm173146.htm

Valavanidis, A., Vlachogianni, T. and Fiotakis, K. (2009) Tobacco Smoke: Involvement of Reactive Oxygen Species and Stable Free Radicals in Mechanisms of Oxidative Damage, Carcinogenesis and Synergistic Effects with Other Respirable

Particles, *International Journal of Environmental Research and Public Health*, 6 (2), pp. 445–462. DOI:10.3390/ijerph6020445.

van Rooy, F. G. B. G. J., Rooyackers, J. M., Prokop, M., Houba, R., Smit, L. A. M. and Heederik, D. J. J. (2007) Bronchiolitis Obliterans Syndrome in Chemical Workers Producing Diacetyl for Food Flavorings, *American Journal of Respiratory and Critical Care Medicine*, 176 (5), pp. 498–504. DOI:10.1164/rccm.200611-1620OC.

Vermot, A., Petit-Härtlein, I., Smith, S. M. E. and Fieschi, F. (2021) NADPH Oxidases (NOX): An Overview from Discovery, Molecular Mechanisms to Physiology and Pathology, *Antioxidants (Basel, Switzerland)*, 10 (6), pp. 890. DOI:10.3390/antiox10060890.

Vestbo, J., Hurd, S. S., Agustí, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., *et al.* (2013) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary, *American Journal of Respiratory and Critical Care Medicine*, 187 (4), pp. 347–365. DOI:10.1164/rccm.201204-0596PP.

Viana, F. (2016) TRPA1 channels: molecular sentinels of cellular stress and tissue damage: TRPA1 channels and cellular stress, *The Journal of Physiology*, 594 (15), pp. 4151–4169. DOI:10.1113/JP270935.

Villablanca, A. C. (1998) Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro, *Journal of Applied Physiology*, 84 (6), pp. 2089–2098. DOI:10.1152/jappl.1998.84.6.2089.

Vitale, A. and Ricceri, L. (2022) The principle of the 3Rs between aspiration and reality, *Frontiers in Physiology*, 13, pp. 914939. DOI:10.3389/fphys.2022.914939.

Volckens, J., Dailey, L., Walters, G. and Devlin, R. B. (2009) Direct Particle-to-Cell Deposition of Coarse Ambient Particulate Matter Increases the Production of Inflammatory Mediators from Cultured Human Airway Epithelial Cells, *Environmental Science & Technology*, 43 (12), pp. 4595–4599. DOI:10.1021/es900698a.

Wadgave, U. and Nagesh, L. (2016) Nicotine Replacement Therapy: An Overview, *International Journal of Health Sciences*, 10 (3), pp. 425–435.

Wagner, C. E., Wheeler, K. M. and Ribbeck, K. (2018) Mucins and Their Role in Shaping the Functions of Mucus Barriers, *Annual Review of Cell and Developmental Biology*, 34 (1), pp. 189–215. DOI:10.1146/annurev-cellbio-100617-062818.

Waldum, H. L., Nilsen, O. G., Nilsen, T., Rørvik, H., Syversen, U., Sandvik, A. K., Haugen, O. A., Torp, S. H. and Brenna, E. (1996) Long-term effects of inhaled nicotine, *Life Sciences*, 58 (16), pp. 1339–1346. DOI:10.1016/0024-3205(96)00100-2.

Wang, G.-Z., Cheng, X., Li, X.-C., Liu, Y.-Q., Wang, X.-Q., Shi, X., *et al.* (2015) Tobacco smoke induces production of chemokine CCL20 to promote lung cancer, *Cancer Letters*, 363 (1), pp. 60–70. DOI:10.1016/j.canlet.2015.04.005.

Wang, J., Yang, H., Shi, H., Zhou, J., Bai, R., Zhang, M. and Jin, T. (2017) Nitrate and Nitrite Promote Formation of Tobacco-Specific Nitrosamines via Nitrogen Oxides

Intermediates during Postcured Storage under Warm Temperature, *Journal of Chemistry*, 2017, pp. 1–11. DOI:10.1155/2017/6135215.

Wang, M., Zhang, Y., Xu, M., Zhang, H., Chen, Y., Chung, K. F., Adcock, I. M. and Li, F. (2019) Roles of TRPA1 and TRPV1 in cigarette smoke -induced airway epithelial cell injury model, *Free Radical Biology and Medicine*, 134, pp. 229–238. DOI:10.1016/j.freeradbiomed.2019.01.004.

Wang, Q., Sundar, I. K., Li, D., Lucas, J. H., Muthumalage, T., McDonough, S. R. and Rahman, I. (2020) E-cigarette-induced pulmonary inflammation and dysregulated repair are mediated by nAChR α7 receptor: role of nAChR α7 in SARS-CoV-2 Covid-19 ACE2 receptor regulation, *Respiratory Research*, 21 (1), pp. 154. DOI:10.1186/s12931-020-01396-y.

Warden, H., Richardson, H., Richardson, L., Siemiatycki, J. and Ho, V. (2018) Associations between occupational exposure to benzene, toluene and xylene and risk of lung cancer in Montréal, *Occupational and Environmental Medicine*, 75 (10), pp. 696–702. DOI:10.1136/oemed-2017-104987.

Wavreil, F. D. M. and Heggland, S. J. (2020) Cinnamon-flavored electronic cigarette liquids and aerosols induce oxidative stress in human osteoblast-like MG-63 cells, *Toxicology Reports*, 7, pp. 23–29. DOI:10.1016/j.toxrep.2019.11.019.

Ween, M. P., Hamon, R., Macowan, M. G., Thredgold, L., Reynolds, P. N. and Hodge, S. J. (2020a) Effects of E-cigarette E-liquid components on bronchial epithelial cells: Demonstration of dysfunctional efferocytosis, *Respirology*, 25 (6), pp. 620–628. DOI:10.1111/resp.13696.

Ween, M. P., Moshensky, A., Thredgold, L. L., Bastian, N. A., Hamon, R., Badiei, A., *et al.* (2020b) E-cigarettes and health risks: more to the flavour than just the name, *American Journal of Physiology-Lung Cellular and Molecular Physiology*, pp. ajplung.00370.2020. DOI:10.1152/ajplung.00370.2020.

Weimann, A., Belling, D. and Poulsen, H. E. (2001) Measurement of 8-oxo-2′-deoxyguanosine and 8-oxo-2′-deoxyadenosine in DNA and human urine by high performance liquid chromatography-electrospray tandem mass spectrometry, *Free Radical Biology and Medicine*, 30 (7), pp. 757–764. DOI:10.1016/S0891-5849(01)00462-2.

Wen, J., Fu, J.-H., Zhang, W. and Guo, M. (2011) Lung carcinoma signaling pathways activated by smoking, *Chinese Journal of Cancer*, 30 (8), pp. 551–558. DOI:10.5732/cjc.011.10059.

Werley, M. S., Kirkpatrick, D. J., Oldham, M. J., Jerome, A. M., Langston, T. B., Lilly, P. D., Smith, D. C. and Mckinney, W. J. (2016) Toxicological assessment of a prototype e-cigaret device and three flavor formulations: a 90-day inhalation study in rats, *Inhalation Toxicology*, 28 (1), pp. 22–38. DOI:10.3109/08958378.2015.1130758.

Weydert, C. J. and Cullen, J. J. (2010) Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue, *Nature Protocols*, 5 (1), pp. 51–66. DOI:10.1038/nprot.2009.197.

WHO (2022a) *Cancer*. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer

WHO (2022b) Tobacco. Available from: https://www.who.int/news-room/fact-sheets/detail/tobacco [Accessed

WHO (2022c) *Tobacco: E-cigarettes*. Available from: https://www.who.int/news-room/questions-and-answers/item/tobacco-e-cigarettes

WHO (2023) *Lead poisoning*.

Wickham, R. J. (2020) The Biological Impact of Menthol on Tobacco Dependence, *Nicotine & Tobacco Research*, 22 (10), pp. 1676–1684. DOI:10.1093/ntr/ntz239.

Winton, Wan, Cannell, Gruenert, J. Thompson, Garrod, Stewart, and Robinson (1998) Cell lines of pulmonary and non-pulmonary origin as tools to study the effects of house dust mite proteinases on the regulation of epithelial permeability: Dust mite proteinases and epithelial permeability, *Clinical & Experimental Allergy*, 28 (10), pp. 1273–1285. DOI:10.1046/j.1365-2222.1998.00354.x.

Wong, H. L., Murphy, S. E. and Hecht, S. S. (2005) Cytochrome P450 2A-Catalyzed Metabolic Activation of Structurally Similar Carcinogenic Nitrosamines: *N'* -Nitrosonornicotine Enantiomers, *N* -Nitrosopiperidine, and *N*- Nitrosopyrrolidine, *Chemical Research in Toxicology*, 18 (1), pp. 61–69. DOI:10.1021/tx0497696.

Woodall, M., Jacob, J., Kalsi, K. K., Schroeder, V., Davis, E., Kenyon, B., *et al.* (2020) E-cigarette constituents propylene glycol and vegetable glycerin decrease glucose uptake and its metabolism in airway epithelial cells in vitro, *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 319 (6), pp. L957–L967. DOI:10.1152/ajplung.00123.2020.

Wu, J., Wang, Y., Liu, G., Jia, Y., Yang, J., Shi, J., Dong, J., Wei, J. and Liu, X. (2018) Characterization of air-liquid interface culture of A549 alveolar epithelial cells, *Brazilian Journal of Medical and Biological Research*, 51 (2), pp. e6950. DOI:10.1590/1414-431x20176950.

Wu, S.-W., Fowler, D. K., Shaffer, F. J., Lindberg, J. E. M. and Peters, J. H. (2017) Ethyl Vanillin Activates TRPA1, *The Journal of Pharmacology and Experimental Therapeutics*, 362 (3), pp. 368–377. DOI:10.1124/jpet.116.239384.

Wu, S.-Y., Fu, T., Jiang, Y.-Z. and Shao, Z.-M. (2020) Natural killer cells in cancer biology and therapy, *Molecular Cancer*, 19 (1), pp. 120. DOI:10.1186/s12943-020-01238-x.

Xia, B., Blount, B. C., Guillot, T., Brosius, C., Li, Y., Van Bemmel, D. M., *et al.* (2021) Tobacco-Specific Nitrosamines (NNAL, NNN, NAT, and NAB) Exposures in the US Population Assessment of Tobacco and Health (PATH) Study Wave 1 (2013–2014), *Nicotine & Tobacco Research*, 23 (3), pp. 573–583. DOI:10.1093/ntr/ntaa110.

Yach, D. (2005) *Tobacco: science, policy and public health/ Tobacco smoke and involuntary smoking (IARC Monographs, Volume 83)*. ulletin of the World Health Organization. 2005;83:398-9.

Yang, S., Jin, H. and Zhao, Z. (2019) Epidermal growth factor treatment has protective effects on the integrity of the blood-brain barrier against cerebral ischemia injury in bEnd3 cells, *Experimental and Therapeutic Medicine*. DOI:10.3892/etm.2019.7186.

Yang, Y., Liu, L., Naik, I., Braunstein, Z., Zhong, J. and Ren, B. (2017) Transcription Factor C/EBP Homologous Protein in Health and Diseases, *Frontiers in Immunology*, 8, pp. 1612. DOI:10.3389/fimmu.2017.01612.

Yeo, C. D., Kim, I. K., Ban, W. H., Kang, H. S., Kim, J. W., Kim, S. J., Park, J. Y. and Lee, S. H. (2019) Chronic nicotine exposure affects programmed death-ligand 1 expression and sensitivity to epidermal growth factor receptor-tyrosine kinase inhibitor in lung cancer, *Translational Cancer Research*, 8 (S4), pp. S378–S388. DOI:10.21037/tcr.2019.05.02.

Yi, M., Tan, Y., Wang, L., Cai, J., Li, X., Zeng, Z., *et al.* (2020) TP63 links chromatin remodeling and enhancer reprogramming to epidermal differentiation and squamous cell carcinoma development, *Cellular and Molecular Life Sciences*, 77 (21), pp. 4325–4346. DOI:10.1007/s00018-020-03539-2.

Youn, H. S., Lee, J. K., Choi, Y. J., Saitoh, S. I., Miyake, K., Hwang, D. H. and Lee, J. Y. (2008) Cinnamaldehyde suppresses toll-like receptor 4 activation mediated through the inhibition of receptor oligomerization, *Biochemical Pharmacology*, 75 (2), pp. 494–502. DOI:10.1016/j.bcp.2007.08.033.

Young, H. W. J., Williams, O. W., Chandra, D., Bellinghausen, L. K., Pérez, G., Suárez, A., *et al.* (2007) Central Role of Muc5ac Expression in Mucous Metaplasia and Its Regulation by Conserved 5′ Elements, *American Journal of Respiratory Cell and Molecular Biology*, 37 (3), pp. 273–290. DOI:10.1165/rcmb.2005-0460OC.

Younus, H. (2018) Therapeutic potentials of superoxide dismutase, *International Journal of Health Sciences*, 12 (3), pp. 88–93.

Yuan, J.-M., Knezevich, A. D., Wang, R., Gao, Y.-T., Hecht, S. S. and Stepanov, I. (2011) Urinary levels of the tobacco-specific carcinogen N'-nitrosonornicotine and its glucuronide are strongly associated with esophageal cancer risk in smokers, *Carcinogenesis*, 32 (9), pp. 1366–1371. DOI:10.1093/carcin/bgr125.

Zahedi, A., Phandthong, R., Chaili, A., Remark, G. and Talbot, P. (2018) Epithelial-to-mesenchymal transition of A549 lung cancer cells exposed to electronic cigarettes, *Lung Cancer*, 122, pp. 224–233. DOI:10.1016/j.lungcan.2018.06.010.

Zhang, D., Lei, J., Ma, J., Chen, X., Sheng, L., Jiang, Z., *et al.* (2016) β2-Adrenogenic signaling regulates NNK-induced pancreatic cancer progression via upregulation of HIF-1α, *Oncotarget*, 7 (14), pp. 17760–17772. DOI:10.18632/oncotarget.5677.

Zhang, R., Zhang, L., Li, P., Pang, K., Liu, H. and Tian, L. (2023) Epithelial Barrier in the Nasal Mucosa, Related Risk Factors and Diseases, *International Archives of Allergy and Immunology*, 184 (5), pp. 481–501. DOI:10.1159/000528969.

Zhang, Y. and Wong, H. S. (2021) Are mitochondria the main contributor of reactive oxygen species in cells?, *Journal of Experimental Biology*, 224 (5), pp. jeb221606. DOI:10.1242/jeb.221606.

Zhou, C., Gao, Y., Ding, P., Wu, T. and Ji, G. (2023a) The role of CXCL family members in different diseases, *Cell Death Discovery*, 9 (1), pp. 212. DOI:10.1038/s41420-023-01524-9.

Zhou, X., Gao, S., Yue, M., Zhu, S., Liu, Q. and Zhao, X.-E. (2023b) Recent advances in analytical methods of oxidative stress biomarkers induced by environmental

pollutant exposure, *TrAC Trends in Analytical Chemistry*, pp. 116978. DOI:10.1016/j.trac.2023.116978.

Zhu, S.-H., Sun, J. Y., Bonnevie, E., Cummins, S. E., Gamst, A., Yin, L. and Lee, M. (2014) Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation, *Tobacco Control*, 23 (suppl 3), pp. iii3–iii9. DOI:10.1136/tobaccocontrol-2014-051670.

Ziebarth, D. (1997) N-nitrosation of medicinal drugs catalysed by bacteria from human saliva and gastro-intestinal tract, including Helicobacter pylori, *Carcinogenesis*, 18 (2), pp. 383–389. DOI:10.1093/carcin/18.2.383.

Zorov, D. B., Juhaszova, M. and Sollott, S. J. (2014) Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release, *Physiological Reviews*, 94 (3), pp. 909–950. DOI:10.1152/physrev.00026.2013.

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., *et al.* (2018) Mitochondrial membrane potential, *Analytical Biochemistry*, 552, pp. 50–59. DOI:10.1016/j.ab.2017.07.009.