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**Evaluating the biological effects of electronic cigarettes using a novel in-house designed aerosol delivery system and an *in-vitro* co-culture model of the human airways**

Pranav Vasanthi Bathrinarayanan  
Doctor of Philosophy

Aston University

June 2018

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# Aston University

## **Evaluating the biological effects of electronic cigarettes using a novel in-house designed aerosol delivery system and an *in-vitro* co-culture model of the human airways**

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### Summary

Although electronic cigarettes (ECs) have been widely promoted as safer alternatives to tobacco cigarettes, limited scientific data is currently available on their possible health effects. The current study aims to investigate the potential effects of ECs using a novel in-house designed cigarette/EC aerosol delivery system and physiologically relevant 2D and 3D *in-vitro* human airway models. Submerged cultures of BEAS 2B and CALU 3 bronchial epithelial cells were used to investigate the effects of nicotine and its oxidative metabolite cotinine. Results demonstrated that neither nicotine nor cotinine had any significant impact on the bronchial epithelial cell viability or IL-6/IL-8 pro-inflammatory mediators' production. Further, treatment of submerged cultures of a number of airways related cell types to extracts of commercially available ECs of different nicotine strength, flavourings and brands showed that while the differing nicotine strengths had no impact on the cell viability, flavourings significantly influenced cell viability, with strawberry and cherry flavoured ECs demonstrating the highest cytotoxicity. Moreover, same flavours from different brands produced different effects on cells. Finally, a co-culture human airways model consisting of CALU 3 bronchial epithelial cells and MRC-5 pulmonary fibroblasts cultured at air-liquid-interface were treated to whole cigarette smoke (WCS) and EC vapour (ECV) at different exposure times (7 m, 1 h, 2 h, 3 h, 4.5 h and 6 h) as per the ISO:3308 standard smoking regime using an in-house designed bespoke, automated aerosol delivery system. Results demonstrated that while WCS caused a significant reduction in cell viability post 7 min exposure, ECV produced cytotoxic effects only at exposure times  $\geq 3$  h. A significant increase in oxidative stress and IL-6/IL-8 production was observed post 3 h ECV treatment, both of which are hallmark characteristics of airway inflammatory conditions like chronic obstructive pulmonary disorder. Overall, results from the current study suggest that ECs have the potential to cause substantial airways damage, and as yet, cannot be regarded as safe alternatives to tobacco cigarettes. Importantly, a standardised testing method is urgently required in order to elucidate on the long-term health effects of ECs.

Key words: E-cigarettes, co-culture, human airways, CALU 3, MRC-5

## Dedication

**Dedicated to all the eminent scientists of the past, both the heard and the unheard, on whose sturdy shoulders of original thought, hard-work, sacrifice, dedication and rational thinking, I stand upon today. At the face of the unknown and at the depth of mystery, you dared to smile. Thank you, great people.**

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## List of abbreviations

ASH	Action on smoking and health
ATSDR	Agency for Toxic Substances and Disease Registry
ALI	Air-liquid interface
Bax	Bcl-2-associated X protein
HbCO	Blood carboxyhaemoglobin
BAT	British American Tobacco
BALF	Bronchoalveolar lavage fluid
CTB <sup>®</sup>	Cell Titre Blue <sup>®</sup>
CDC	Centre for Disease Control and Prevention
CoA	Certificate of Analysis'
COPD	Chronic obstructive pulmonary disease
CSC	Cigarette smoke condensate
CSE	Cigarette smoke extract
CBC	Complete blood count
CORESTA	Cooperation centre for scientific research relative to tobacco
CFTR	Cystic fibrosis transmembrane conductance regulator
DAQ	Data Acquisition system
DNA	Deoxyribonucleic acid
DMSO	Dimethyl-sulfoxide
DMEM-F12	Dulbecco's Modified Eagle Medium Ham's F-12
DPBS	Dulbecco's Phosphate-Buffered Saline
ECs	E-cigarettes
ENDS	Electronic nicotine delivery systems
ECC	EC condensate
ECE	EC extract

ECV	EC vapours
EFS	Electronic flow sensor
ELISA	Enzyme Linked Immuno Sorbent Assay
EMEM	Essential Medium Eagle: Earl's balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
ECVAM	European Centre for the Validation of Alternative Methods
ECM	Extra Cellular Matrix
FEMA	Flavour Extracts Manufacturers Association
F.I.	Fluorescence intensity
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GRAS	Generally regarded as safe
HCI	Health Canadian Intense
HESCs	Human embryonic stem cells
HPF	Human pulmonary fibroblast
Hcl	Hydrochloric acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
IL-6	Interleukin-6
IL-8	Interleukin-8
LED	Light emitting diode
MMP	Matrix metalloproteinase
MCP-1	Monocyte chemoattractant protein-1
NHS	National Health Service
NACHRs	Nicotine acetylcholine receptors
NRTs	Nicotine replacement therapies
NHBE	Normal human bronchial epithelial cells
PMA	Phorbol 12-Myristate 13-Acetate

PAH	Polycyclic aromatic hydrocarbons
PSU	Power supply unit
PDS	Product Design Specifications
PG	Propylene glycol
PHE	Public Health England
ROS	Reactive oxygen species
RA	Retinoic Acid
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
SCW	Serial connection wire
SW	Snapwells
TSNA	Tobacco-specific nitrosamines
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TW	Transwells
USP	United States Pharmacopeia
UT	Untreated control
VG	Vegetable glycerine
VOC	Volatile organic compounds
WCS	Whole cigarette smoke
WHO	World Health Organisation
ZO	Zonula occludens protein

## List of units

L	Litre
mL	millilitre
$\mu$ L	microlitre
M	molar
mM	millimolar
$\mu$ M	micromolar
s	seconds
min	minutes
h	hour
g	grams
mg	milligrams
$\mu$ g	micrograms
pg	picograms
V	volts
m	metre
cm	centimetre
mm	millimetre
$^{\circ}$ C	degree celcius
U/mL	units/millilitre
v/v	volume/volume

# 1 Structure of the thesis

This thesis reports the various methodologies and findings of the current study pertaining to different aspects of EC research, comparing them with the existing literature and providing future directions for research in this area. This thesis has 8 main chapters excluding the current chapter, references (Chapter 10) and appendices (Chapter 11). Chapter 1, the current chapter introduces the reader to the different chapters of the thesis with the intention of providing an overview of the current study.

Chapter 2 is the literature review chapter which consists of a comprehensive review of several aspects of E-cigarettes (ECs) such as usage, safety, challenges associated with EC research, existing studies on EC toxicity etc. Additionally, the chapter also reviews the different models, ranging from animal models to various *in-vitro* models that have been employed in past cigarette/EC studies. The chapter ends with a description of different aims and objectives of the current study.

Chapter 3, the materials and methods chapter describes the different cell types, reagents, assays, detection kits and cell-culturing procedures that were employed to perform the different experiments of the project.

Chapter 4 is the design chapter of the thesis which describes the concept, design, construction and validation of an in-house built bespoke smoking machine which was used to deliver cigarette smoke and EC vapour to the cells in a controlled fashion. Additionally, the chapter reviews a number of existing commercial smoking machines, compares the cost of in-house built smoking machine with the commercial machines and elaborates on the various advantages of the in-house built smoking machine compared to the commercial smoking machines.

Although the design, construction and validation of the in-house built smoking machine can be viewed as a result in itself, Chapter 5 is the first result chapter pertaining to cell exposure experiments. Chapter 5 experiments investigate the effects of chief individual constituents of ECs namely nicotine, its oxidative metabolite called cotinine and EC base liquid which is referred to as E-vehicle throughout the thesis.

Chapter 6 is the second results chapter which focuses on assessing the influence of different variables of ECs such as nicotine content, flavourings and brand on the cytotoxicity mediated. ECs from 5 different brands with different nicotine strengths and flavours were tested on a variety of airways related cell types.

Chapter 7, the third and final results chapter investigates the effect of exposure of a co-culture human airways model to aerosols of cigarette smoke and EC vapour using the in-house built smoking machine described in Chapter 4. The experimental design and methodology of this chapter can be viewed as a human physiologically relevant methodology where a 3D differentiated multi-cellular model of human airways were exposed to cigarette smoke or EC aerosols in a fashion that mimicked the human smoking behaviour as closely as possible. Each results chapter (Chapter 5, 6 and 7) has its own introduction, aims and objectives, materials and methods, results, discussion and conclusion sections.

Chapter 8 is the overall discussion chapter which draws the results from the individual chapters and comprehensively discusses the various aspects of EC research from an overall perspective. The chapter ends with overall conclusions which provides a succinct report of the significant findings of the current study. Chapter 9 is the future works chapter which provides input on the potential experiments and design improvements that could be implemented to the aerosol delivery system based on the current study in order to further expand the existing knowledge on EC toxicity and possible health effects.

Chapter 10 provides a complete list of all references that were used throughout the thesis. Chapter 11 includes supplementary data from additional experiments, published journal papers, posters presented at different conferences during the PhD program.

Wherever appropriate, the author has acknowledged the work contributed by different people towards the completion of this study.

## 2 Literature review and background research

### 2.1 Traditional cigarette smoking

Cigarette smoke is the single greatest cause of preventable death globally. The World Health Organisation (WHO) states that cigarette smoke causes approximately 6 million deaths world-wide annually and by 2030, the number is estimated to increase to 8 million (WHO., 2011). As stated by International Agency for Research on Cancer (IARC), both mainstream and side-stream cigarette smoke consist of a complex mixture of more than 5000 chemicals including at least 50 carcinogens such as arsenic, cadmium, tobacco-specific nitrosamines (TSNA) and benzopyrenes (IARC, 2004). Cigarette smoking is a major risk factor for a number of diseases including chronic obstructive pulmonary disease (COPD), atherosclerosis, hypertension, Crohn's disease and cancers of the lung, mouth, larynx, trachea, pancreas, bladder etc. (Doll and Peto, 1976, Sherman, 1991, Sachs, 1986).

#### 2.1.1 Cigarette smoking statistics of the UK

Although the smoking rates in the UK has halved since 1974, cigarette smoking is still one of the major causes of deaths in UK (Wald and Nicolaides-Bouman, 1991). Current statistics estimate that there are approximately 10 million smokers in the UK i.e. one-sixth of the total population of UK. 22% of the adult men and 17% of the adult women are smokers (ASH., 2015). Recent data also reveals that two-third of the smokers start before the age of 18 putting adolescents at high risk of getting addicted to cigarette smoking (ASH., 2015).

#### 2.1.2 Deaths in UK due to active and passive cigarette smoking

The National Health Service (NHS) reported that between 2015-2016, 474,000 hospital admissions, including 79,000 deaths were associated with smoking; 23 % and 47 % of which were respiratory illnesses and cancer admissions respectively (NHS Digital, 2017). Also, it is estimated that every year around 12,000 deaths are associated with passive smoking and around 165,000 new cases of young children develop respiratory disorders due to passive smoking (CRUK, 2014). Treatment of smoking associated diseases significantly impacts the economics of the health care system. Action on smoking and health (ASH), a charitable organisation focused on reducing the harm caused by tobacco and related products, states that cigarette smoking alone costs the NHS £2 billion annually in treating smoking related diseases (ASH., 2017a).

## 2.2 An introduction to Electronic cigarettes (EC)

Electronic nicotine delivery systems (ENDS) or ECs were first introduced in China in the year 2003 as an alternative to tobacco cigarettes (Dockrell et al., 2013). By the year 2008, ECs had penetrated the US and European markets and by 2013 many multinational tobacco companies started producing ECs (Grana et al., 2014). Since then popularity of ECs have risen steadily across the globe. ECs are battery operated devices that heat a nicotine containing solution called EC liquid sufficiently high temperatures and deliver the resulting aerosols to the user (Hajek, 2014). The EC liquid generally consists of a propylene glycol (PG) and vegetable glycerine (VG) based solution containing nicotine, proprietary flavouring additives and in some cases, distilled water. Since the PG/VG mixture make up the bulk of EC liquid, they are collectively referred to as 'base liquid' or 'E-vehicle'. While nicotine-free EC are also available in the market, it is used by only a small fraction of EC users (Hajek, 2014). In the current study, unless specifically mentioned, the word 'EC' refers to nicotine containing ECs. ECs are widely marketed via television, internet and print advertisements as a less-harmful or 'safer' alternative to tobacco cigarettes or as useful aids to help quit smoking (Grana and Ling, 2014). The act of puffing an EC is generally referred to as 'vaping' and the person vaping is generally referred to as a 'vaper' as opposed to a 'smoker'.

### 2.2.1 Basic components of an EC

ECs, although varied in types, generally consist of three main parts as shown in Figure 2.1:

1. **A cartridge or tank containing EC liquid:** ECs, depending upon the type, consist of either a cartridge which contains the EC liquid or a tank (reservoir) into which the EC liquid can be filled.
2. **A rechargeable battery:** The battery provides the power required for EC operation and hence forms the bulk of an EC. Most ECs (apart from single use disposable ECs) consist of a rechargeable lithium-ion battery intended for multiple usage.
3. **An atomizer:** The atomizer consists two components namely a heating coil and a wicking material. While the wicking material is that part of the atomizer that draws/soaks the EC liquid, the heating coil vapourises the EC liquid into aerosols which is subsequently inhaled by the user.

Since a 1<sup>st</sup> generation EC was used in the current study, the same has been used for demonstration purpose as depicted in Figure 2.1. The working of an EC is relatively simple. When the user inhales or 'puffs' an EC, a sensor inside the EC detects air-flow and activates the atomizer (Orr, 2014). The EC liquid is then heated by the atomizer at

sufficiently high temperatures to produce vapours of the EC liquid which is then delivered to the user through a mouth piece. Most ECs also have a light emitting diode (LED) connected to a sensor placed near the atomizer device which glows when the heating element is activated (i.e. when the user puffs), thus indicating EC usage (Breland et al., 2014).



**Figure 2.1. Components of a first generation EC.**

All ECs consist of three main parts namely (i) a rechargeable battery which provides the power for EC operation (ii) a tank or a cartridge which contains the EC liquid and (iii) an atomizer which heats the EC liquid at high temperatures to produce vapours which is then delivered to the user. (Image obtained from [www.biomedicalcentral.com](http://www.biomedicalcentral.com))

### 2.2.2 Different types of EC

There are different types of ECs available in the market as described in Figure 2.2. The most well-known and also the first type of ECs to be introduced in the market are called the 1<sup>st</sup> generation ECs. This EC type looks similar to traditional tobacco cigarettes and hence are referred to as 'cig-alikes' (Etter, 2016). The 'cig-alike' ECs may either be single-use disposable ones or multiple use ones, in which case a rechargeable lithium-ion battery is included. 'Cig-alikes' are generally used by beginners due to its easy design, low cost and low-complexity. The 2<sup>nd</sup> generation ECs include E-pens or 'vape-pens'. Apart from the 1<sup>st</sup> generation cig-alikes, all other advanced ECs consist of a refillable transparent tank that can be filled with the EC liquid of desired choice (Hitchman et al., 2015). Also, starting from the 2<sup>nd</sup> generation, all ECs allow the user to manually activate the heating element for the desired amount of time by pressing a button. In these advanced ECs, the atomizer/tank combination is sometimes collectively referred to as 'clearomizers' (Goniewicz et al., 2014a). The 2<sup>nd</sup> generation ECs are generally used by starters and intermediate vapers.



**Figure 2.2. Different types of ECs available in the market.**

(Images obtained from EC companies as well as EC forums such as Go2vape.com and smoketastic.com.)

3<sup>rd</sup> generation and 4<sup>th</sup> ECs include Box mods and Advanced personal vapourisers (APVs) respectively. The battery pack usually has a box-like appearance (hence called box mods) which consists of one or two rechargeable lithium-ion batteries for extended use (Munoz et al., 2014). Box mods and APVs are customisable ECs where the user can set different parameters such as voltage, wattage and heating coil resistance in order to produce aerosols of desired strength (Clapp and Jaspers, 2017). Box mods and APVs are often regarded as the range of ECs that produce the best vaping experience (Etter, 2016). Hence, the 3<sup>rd</sup> and 4<sup>th</sup> generation ECs are amongst the most expensive ECs in the market and are used only by advanced/experienced users.

### 2.2.3 EC usage in the UK

There has been a consistent rise in the use of ECs amongst the adults and adolescents of UK in recent times. As shown in Table 2.1, according to a 2017 survey, there are approximately 2.9 million EC users in the UK (6 % of the adult population), 1.5 million amongst whom are ex-smokers and 1.3 million are dual users of tobacco cigarettes and ECs (ASH, 2017).

**Table 2.1. Statistics of EC usage in UK.**

Table demonstrating the consistent rise in EC usage in the UK. Data obtained from ASH.org (ASH, 2017a)

	2012	2013	2014	2015	2016	2017
Number of EC users	700,000	1.3 million	2.1 million	2.6 million	2.8 million	2.9 million
% of adult population	1.7 %	2.7 %	4.2 %	5.4 %	5.7 %	5.8 %
Year on year rate of change		+86 %	+62 %	+24 %	+8 %	+4 %

The main reason of current smokers to use ECs is to reduce their tobacco smoking frequency while the main reason stated by ex-smokers is to help them quit smoking completely (ASH, 2017). Some other reasons given by smokers for using ECs include saving money compared to tobacco cigarettes, to vape in smoke-prohibited zones, to avoid putting non-smokers at risk etc. The perception of ECs as a safe alternative to tobacco cigarettes has greatly changed in recent years amongst public and smokers. Compared to 2013, when 21 % of the adult population identified ECs as a 'lot less harmful' alternative to tobacco cigarettes, only 13 % of the adult population in 2017 perceive ECs in the same manner. Moreover, 26 % of the adult population in 2017 perceive ECs to be equally or more harmful than tobacco cigarettes compared to a mere 7 % of adult population who identified EC in a similar manner in 2013 (ASH, 2017).

#### 2.2.4 Use of ECs amongst youth and adolescents

A rising concern associated with ECs is its increased use amongst the young population, especially amongst non-smoking youth and adolescents. The Centre for Disease Control and Prevention (CDC) in 2013 reported that in the US, the number of 11-18 year olds using ECs increased by more than two folds in one year i.e. from 3.3 % in 2011 to 6.8 % in 2012 (CDC, 2013). Further, the CDC also reported that 61 % of the middle school students and 80 % of the high school students were dual users of tobacco cigarette and ECs (CDC,

2013). Although highly debatable, there have been emerging concerns on the ability of ECs to act as a gateway to tobacco cigarettes or to act as new tools of nicotine dependence (Mantey et al., 2016). ECs are particularly appealing to the youth and adolescents due to several reasons such as curiosity, social status (use of ECs by celebrities) availability of a wide-array of attractive flavours and even peer-influences (Moore et al., 2015, Kong et al., 2015). In the UK, the prevalence of ECs amongst the younger population is low compared to other countries with no strong evidence for the conversion of experimental EC users into regular EC users (ASH., 2016, Bauld et al., 2017, Moore et al., 2015).

#### 2.2.5 Safety aspects of EC usage

Despite being more than a decade since the introduction of ECs, the health effects of ECs have not been conclusively established. Although studies investigating long-term chronic effects of ECs are still lacking, a number of EC studies have reported on the production of carcinogenic aldehydes (Goniewicz et al., 2014b), metal particles (Williams et al., 2013) and other toxic chemicals (Allen et al., 2016) which could have possible adverse health effects. However, other studies dispute that the levels of many of the hazardous chemicals emitted by ECs are significantly low compared to tobacco cigarettes and hence their ability to cause adverse effects is quite limited (Farsalinos et al., 2015b, Farsalinos et al., 2015c). More studies are hence required to conclusively establish the harmful or beneficial effects of ECs.

#### 2.2.6 Challenges associated with EC research

##### 2.2.6.1 *Inconsistencies in labelling EC constituents*

It has been reported by studies that there can exist disparities between nicotine levels stated on the pack and the actual nicotine content in the EC cartridge (Cobb et al., 2010, Trehy et al., 2011). There have also been reports of some nicotine free ECs to contain substantial amounts of nicotine (Kubica et al., 2013, Cheah et al., 2014). Since nicotine is a potent psycho-stimulator and can have several physiological effects, these discrepancies in the concentrations of nicotine in EC liquid can have significant health effects and have raised concerns. In fact in 2014, the Food and Drug Administration (FDA) conducted a laboratory analysis of EC samples from two leading EC brands (FDA., 2014). The samples included ECs of 18 different flavours with or without nicotine. Some of the findings of the FDA included:

- a. All the cartridges which were labelled as 'nicotine free' contained detectable levels of nicotine (except one cartridge where no disparity existed).

- b. Three different EC cartridges with the same label were tested and each sample delivered noticeably different amounts of nicotine with each puff. Such a discrepancy in nicotine concentration in EC aerosols have been reported by other studies as well. McCauly et al (2012) analysed the nicotine concentration in the aerosols of 'high-strength' ECs from 4 different brands and reported variable levels of nicotine in each EC aerosol (McAuley et al., 2012).
- c. Most of the samples contained the carcinogenic TSNAs namely anabasine and myosmine. Conclusively, the FDA suggested that the ECs cannot be treated as a harmless product (FDA., 2014).

Another challenging factor is the varying proportion of the carrier solutions namely PG and VG used in the EC liquid production. Most brands either do not state the ratio of PG:VG used in their product or vaguely mention the carrier concentrations such as 'nicotine in >70% PG' or 'nicotine in ~50 % PG'. Most 'medium' strength EC contain an E-vehicle consisting of 70% PG and 30% VG respectively (Vapourlites™). Never-the-less, this ratio varies across the different brands and types of ECs giving rise to more complexities (Uryupin, 2013).

#### *2.2.6.2 Lack of a standardised test method*

ECs unlike tobacco cigarettes are not just a single product but a class of products with several variables including different nicotine strengths, flavours, PG/VG ratio, battery strength, coil resistance, atomiser type, wicking materials employed etc. Perhaps the most important drawback impeding EC research is the lack of a standardised test method. Different studies employ different EC types (with different nicotine strength, PG/VG ratio and flavourings) and test them on different cell models at different test regimes and experimental designs. Such an approach can give rise to confounding and often contradicting results on ECs, thus making it difficult to reach a consensus on their possible health effects. For instance, the aerosols produced by different EC types can vary substantially both qualitatively and quantitatively. The higher generation ECs can produce more aerosols and for a longer period of time due to their superior operational settings. Hence the research output obtained from employing a 1<sup>st</sup> generation EC would possibly be different from that obtained using a 3<sup>rd</sup> or 4<sup>th</sup> generation EC. Similarly, the method of EC delivery can also significantly influence the output of a study. For instance, studies evaluating EC effects by exposing cells to aerosols of EC would obtain results different from studies that exposed cells directly to EC liquids or EC extracts. Hence, one of the chief requirements in EC research is the development of a standardised methodology to assess their possible health effects.

## 2.3 The human respiratory system

The respiratory tract can be divided into three main regions as shown in Figure 2.3 namely:

1. **Upper respiratory tract:** This region consists of the oral and nasopharyngeal cavity. The upper-respiratory tract acts as an entry-way for air and other inhaled foreign substances. The mucus lining of the nasopharyngeal cavity and the cells equipped with hair-like cilia of this cavity trap most of the inhaled foreign particles, thus restricting its entry into the respiratory tract (Suzumoto et al., 2006).
2. **Lower respiratory tract:** This region consisting of the larynx, trachea, primary bronchus and subsequent branching are also called the conducting airways since their chief aim is to conduct inhaled air into the deeper regions of the lung (West, 2011). Apart from conducting air, the several specialised cells of the conduction airways chiefly mucus producing cells and ciliated cells further trap and eliminate any foreign particles that escaped the upper respiratory tract.
3. **Distal respiratory tract:** This region consists of the terminal bronchioles (respiratory bronchioles) and alveoli which are the functional respiratory units. The vast alveoli network are covered by a thick network of blood capillaries (sites called gas-blood barrier) where the gas exchange takes place.



**Figure 2.3. Pictorial representation of the human respiratory tract.**

The respiratory tract can be divided into three distinct segments. The first segment called the upper respiratory tract consists of the oral and nasopharyngeal cavity through which the inhaled air is warmed and moisturised before entering into subsequent parts of the respiratory tract. The second segment called the lower respiratory tract consists of the larynx, trachea, primary bronchus and its divisions (secondary bronchus, tertiary bronchus and bronchioles) which conducts the inhaled air into deeper regions of the lungs. The final segment called the distal respiratory tract or the pulmonary respiratory tract consists of the terminal bronchioles called the respiratory bronchioles and alveoli units where the gas exchange between oxygen and carbon-dioxide takes place. Image obtained from (BeruBe et al., 2009).

### 2.3.1 Airway epithelium: prominent cells of the human airways

The airway epithelial cells are the first line of cells to come in contact with inhaled environmental particles and pathogens, thus acting as a physical barrier between the external environment and internal milieu (Jeffery, 1983, Tam et al., 2011). At least eight distinct airway epithelial cells exist in the respiratory epithelium distributed at different regions of the airways (Spina, 1998). The current study chiefly focuses on the conducting zone of the airways involving tracheobronchial epithelium since it is the major line of cells that come in contact with environmental agents including cigarette smoke or EC aerosols (Li et al., 2010a, Martinez-Garcia et al., 2008, Bodas et al., 2016). The tracheobronchial epithelium is pseudostratified and consists of three chief airway epithelial cell types namely columnar ciliated cells, basal cells and mucus producing goblet cells (Velden and Versnel, 1998).

1. **Columnar ciliated epithelial cells:** Columnar ciliated epithelial cells are the most prevalent airway cells accounting to about 50 % of the total airway epithelial cells (Knight and Holgate, 2003). These ciliated cells perform a complex process called mucociliary clearance which through co-ordinated activity of cilia transports mucus upwards towards the upper respiratory tract and subsequently out of the airways (Ayers and Jeffery, 1988).
2. **Basal epithelial cells:** Basal cells are ubiquitous throughout the airways although their number decreases with decreasing airway size (Evans and Plopper, 1988). Basal cells act as progenitor to other epithelial cells and also provide structural support by helping in the attachment of other epithelial cells to the basement membrane (Evans et al., 1990). Besides, they produce several important cytokines and enzymes to aid efficient airways functions (Boers et al., 1998).
3. **Secretory epithelial cells:** Secretory cells such as goblet cells, brush cells, serous cells are chiefly responsible for producing mucus on the apical surface of the airways to trap foreign particles and eliminate them via mucociliary clearance (Howarth et al., 1994). Goblet cells are the predominant secretory cells and consist of acidic mucin granules that secrete appropriate amount of mucus relative to the environmental stimulus (Lumsden et al., 1984).

Apart from the epithelial cells, a number of other cells types such as endothelial cells, airways smooth muscle cells, pulmonary fibroblasts also play an important roles in maintaining normal airways functions. Pulmonary fibroblasts which are lined in close proximity to airway epithelial cells are chiefly responsible for Extra Cellular Matrix (ECM) protein deposition in the airways, thus providing structural support to the airway architecture (Knight, 2001). The interaction between airway epithelial cells and sub-epithelium fibroblasts have several important consequences such as robust tissue differentiation during lung development, modulation of inflammatory process by secreting several important mediators and growth factors and repair of damaged airway tissues (Minoo and King, 1994). Pulmonary fibroblasts are also involved in ECM remodelling by maintaining a balance between the secretion of pro-collagen and anti-collagen deposition factors (McAnulty et al., 1997).

## 2.4 Different respiratory models used in cigarette/EC inhalation studies

Several *in-vivo* and *in-vitro* models of human airways have been employed to investigate the toxicological effects of cigarettes/ECs.

### 2.4.1 Animal models

Animal models can be beneficial in analysing whole body response post exposure to different cigarette smoke or EC aerosols. (Martorana et al., 2006). Animal studies can provide useful information about the possible effects of cigarette smoke/EC aerosols on other non-targeted organs apart from airways. They can also be useful in long-term studies where investigations span several months (Martorana et al., 2006). Rodents, especially mice are the most widely used animal models for cigarette smoke/EC studies due to low cost, increased availability, easy handling and high-throughput experimentation compared to non-human primates.

Testing cigarette smoke/EC effects using mice models involve complex exposure systems that expose mice to cigarette smoke/EC aerosols in a controlled fashion. Smoke exposure systems for rodents can be of two types: nose-only (or head-only) exposure system and whole body exposure system (Pauluhn and Mohr, 2000). As shown in Figure 2.4, nose-only exposure systems consist of mice confined in a containment tube such that the animals' nose is pointed towards a 'delivery tube' or 'inhalation point' of the exposure system which subsequently delivers the cigarette smoke/EC aerosols directly into the animal nose (Mainelis et al., 2013). In comparison, whole body exposure systems consists of mice placed in an exposure chamber where cigarette smoke/EC aerosols are delivered into the chamber (Pauluhn, 2003). The mice are not constrained in this method and are free to move about within the chamber.



**Figure 2.4. Nose-only and whole-body rodent exposure system.**

Nose-only exposure system image obtained from (Chen and Lippmann, 2015) and whole-body exposure system image obtained from (Sussan et al., 2015)

**2.4.1.1 Advantages of mice models**

Mice models are the most commonly employed *in-vivo* model for cigarette smoke/EC toxicological studies owing to several advantages they possess over other animal models such as:

1. The rapid breeding rates of mice enables long-term investigation relatively easy especially in multi-generational studies cigarette smoke/EC studies. (Snider et al., 1986)
2. Compared to primates, the small size of mice enables easy cigarette/EC exposure procedure. (Polverino et al., 2015)
3. Extensively studied model. Compared to other species, the anatomy, physiology, immune-system and genetics of mice have been thoroughly researched and findings have been corroborated. (Paigen, 1995, Brusselle et al., 2006)
4. Low cost compared to other animal models. (Paigen, 1995)

#### 2.4.1.2 Drawbacks of employing mice models in cigarette/EC studies

Although mice models can be useful in whole body exposure studies, long-term exposure analysis, such models do come with inherent limitations. These limitations include:

1. Inter-species, intra-species and inter-laboratory variability
2. Variability in exposure systems
3. Poor translation into human medicine
4. Ethical concerns

With respect to inter-species variability, mice are obligatory nose breathers which have significantly different airway anatomy and physiology compared to humans (Marcelino et al., 2014). For instance, the tracheal epithelium in humans is pseudostratified with large number of ciliated and goblet cells (Wright et al., 2008). In contrast, the majority of the tracheal epithelial cells in mice are non-ciliated with few goblet cells (Pack et al., 1980, Plopper et al., 1980). This could potentially lead to differential mucus production and mucociliary clearance in response to cigarette/EC aerosol between the two species. Moreover, mice have less extensive airways branching and lack respiratory bronchioles as opposed to humans who have several generations of membranous and respiratory bronchioles which are the sites of cigarette smoke induced small airways remodelling and emphysema respectively (Pinkerton et al., 1993, Wright et al., 2008). This results in differential deposition patterns of cigarette/EC aerosols along the airways in mice and humans leading to differential effects (Hecht, 2005, Leberl et al., 2013).

With respect to intra-species variability, several strains of mice such as C57Bl/6, DBA/2, Balb/C, A/J, ICR, FVB are available for inhalation studies (Leberl et al., 2013). Each strain has specific characteristics and is used according to the experimental aims. With respect to EC studies performed till date, 4 studies have employed C57Bl/6 strain (McGrath-Morrow et al., 2015, Sussan et al., 2015, Lerner et al., 2015, Lauterstein et al., 2016), 2 studies have employed Balb/C strain (Ponzoni et al., 2015, Lim and Kim, 2014) and one study has employed A/J strain (Garcia-Arcos et al., 2016). Each study reported different findings and no specific reasons were given by the authors for choosing a particular strain for their studies. The employment of different strains can render comparability between studies difficult and this is one of the drawbacks of using animal models (Bartalesi et al., 2005). Moreover, even employing the same strains under the same experimental conditions may give different results in different laboratories (Wright and Churg, 2008).

With respect to exposure system variability, nose-only and head-only exposure systems involve excessive animal handling that place extreme restraints on mice often resulting in stress, anxiety that can lead to unexpected physiological effects (Coggins, 1998, Leberl et al., 2013). Also, forced inhalation of cigarette smoke/EC aerosols in rodents leads to significant alterations in their breathing patterns such as development of avoidance reactions which is starkly different from active inhalation of cigarette smoke/EC aerosols by human (Hecht, 2005). Whole body exposure can lead to other complications such as absorption of a substantial portion of the cigarette smoke/EC aerosols by other routes such as skin (Coggins, 1998) or ingestion (food placed in exposure chamber can get contaminated with cigarette smoke/EC aerosol particles) (Pauluhn, 2003). Rodents while cleaning their fur may ingest nicotine, tar and other particular substances, thus rendering it difficult to assess overall dose inhaled (Wright and Churg, 2002, Pauluhn et al., 1996). With respect to ethical considerations, experimentation with rodents draw ethical concerns and public criticisms under several grounds such as pain perception, consciousness and sentience (Ferdowsian and Beck, 2011).

#### 2.4.2 *In-vitro* models of human airways

*In-vitro* modelling involves isolating cells from specific tissues of an organism and culturing the cells under specialised conditions that mimic the *in-vivo* environment as closely as possible (Brandenberger et al., 2010). Compared to animal models, *in-vitro* models have the advantages of increased human physiological relevance, flexibility, low-cost, repeatability, controlled experimental conditions, more opportunities for intervention and assessing a particular cell type in isolation (Karp et al., 2002). The cells used for *in-vitro* modelling can be of three types namely primary cells, transformed cell lines and tumour-derived cells. Primary cells are cells derived directly from an organism and hence they closely resemble the morphological, biochemical and genetic characteristics of the *in-vivo* cells (Lechner et al., 1982). However, primary cells have a short life-span i.e. they can be used only upto limited number of passages (upto ~P 5 to P 10) after which they start losing their properties. Thus performing large number of experiments especially long-term experiments with primary cells can be difficult and expensive. Additionally, procuring primary cells from healthy volunteers or patients may involve laborious ethical approval processes and there also exists the problem of donor variability. Cell lines on the other hand are isolated from an organism and immortalized via viral transformation (Maqsood et al., 2013). This allows the cell lines to grow indefinitely and hence can be used upto several passages. Compared to primary cells, cell lines are ready available, cost-effective, easy-to-use and provide a homogeneous population of cells over an extended period of time (Kaur

and Dufour, 2012). More importantly, cell lines do not have the problem of donor variability, thus making them a valuable biological tool. Not all cell lines are virally transformed to achieve immortality. Tumour-derived cell lines are naturally immortal and hence can be used indefinitely for *in-vitro* experiments (van Staveren et al., 2009). A comparison of the various advantages and disadvantages of *in-vivo* and *in-vitro* models of human airways is tabulated in Table 2.2.

**Table 2.2. Comparison between *in-vivo* and *in-vitro* airways models used in inhalation studies**

<i>In-vivo</i> rodent models		<i>In-vitro</i> models	
Pros	Cons	Pros	Cons
Ability to analyse whole body effects	Lack of physiological relevance and poor translation into human medicine	Increased physiological relevance and relatively good translation into human medicine	Short life-span. Mostly suitable for short-term studies
Ability to perform prolonged exposure studies (several months)	Inter-species and intra-species variability.	Ability to perform high-throughput experiments in order to obtain large amounts of data relatively quickly	Difficult to culture multiple (more than two) cell types together. More suitable for single-cell investigation studies
	High cost compared to <i>in-vitro</i> models. Ethical concerns.	Economical, flexible, ability to analyse cell types in isolation	Possibilities of contamination

### 2.4.2.1 Common cell lines used in the different *in-vitro* models of human airways

Depending upon the cell type employed, different *in-vitro* models represent different regions of the human airways such as tracheobronchial region or distal alveolar region. Hence a number of transformed and tumour derived bronchial epithelial cell lines have been employed in many cigarette/EC studies. Some of the widely employed transformed and tumour derived cell lines of airways have been described in Table 2.3.

**Table 2.3. Description of popular cell lines used in inhalation studies of cigarette/EC aerosols.**

Region of the airways	Cell line	Description	Properties	Studies
Proximal conducting zone	BEAS 2B	Transformed bronchial epithelial cell line. Immortalised by transfecting with SV-40 adenovirus 12	Secretes cytokines, expresses anti-oxidants and can differentiate into squamous phenotype. Does not produce mucus or cilia and lacks tight junction formation. (Park et al., 2015)	(Laan et al., 2004, Antherieu et al., 2017, Veljkovic et al., 2011)
Proximal conducting zone	CALU 3	Immortal bronchial epithelial cell line derived from human adenocarcinoma of the bronchus	Forms commendable tight junctions and secretes mucus. There can be inter-laboratory variation in tight junction values. (Grainger et al., 2006)	(Olivera et al., 2007, Clunes et al., 2012, Rowell et al., 2017)

Proximal conducting zone	16HBE14o-	Transformed bronchial epithelial cell line. Immortalised by transfecting with SV-40 adenovirus large T antigen	Secretes cytokines, can differentiate into multi-layered tissue, may/may not produce cilia. Can form tight junctions but does not produce mucus. (Forbes et al., 2003)	(Gangl et al., 2009, Shaykhiev et al., 2011, Sherwood and Boitano, 2016)
Proximal conducting zone	NCI-H292	Immortal bronchial epithelial cell line derived from human muco-epidermoid carcinoma of bronchus	Secretes cytokines, produces mucus and cilia and forms tight junctions. Lacks metabolic, activation especially CYP2A6/2A13 activity. (Carney et al., 1985)	(Lee et al., 2006, Thorne et al., 2009, Taylor et al., 2016)
Distal respiratory zone	A549	Immortal alveolar epithelial cell line derived from human alveolar type II carcinoma	Expresses mucin granules. Does not form tight junctions. (Williams, 1984)	(Witherden et al., 2004, Ito et al., 2001, Hwang et al., 2016)

The method of culturing cells can have a significant impact on its physiological and morphological properties. Two types of culturing methods exist in producing *in-vitro* models of human airways:

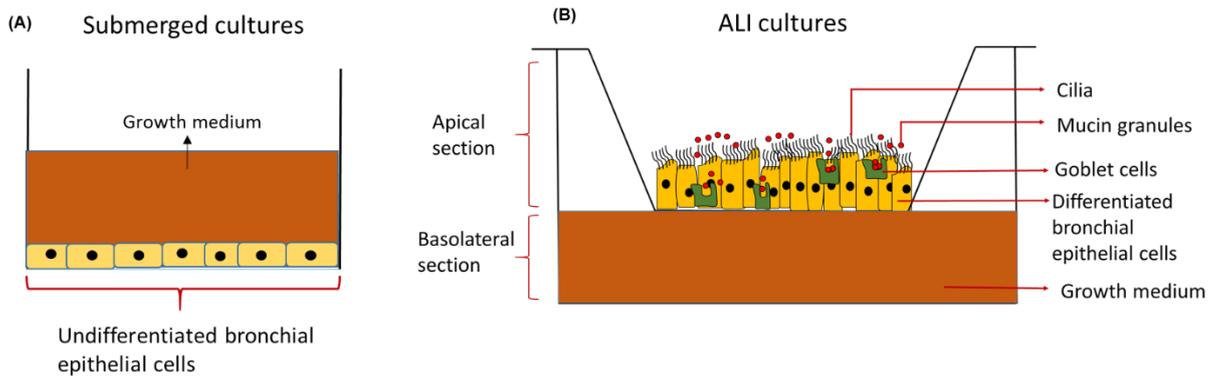
1. Submerged culturing (two dimensional cell cultures)
2. Air-liquid interface (ALI) culturing (three dimensional cell cultures)

#### 2.4.3 *In-vitro* submerged culturing

Submerged cell culture is the simplest and fastest method of culturing cells. In this culturing method, cells are grown by submerging them under appropriate growth media at specialised conditions (37°C, 5 % CO<sub>2</sub>) such that the cells grow, proliferate and expand in number to form two dimensional monolayers as shown in Figure 2.5. Using tissue culture flasks, both primary cells and cell lines are maintained and expanded under submerged conditions (Randell et al., 2011). In a given tissue culture flask, only one particular cell type can be grown and hence they are often called submerged monocultures. Submerged monocultures have several advantages such as easy maintenance, high cell yields, ability to produce high-throughput data and good reproducibility of results (Krimmer and Oliver, 2011). Hence, submerged cultures are immensely useful in obtaining preliminary high-throughput data relatively quickly. Never-the-less, in order to closely mimic the physiological, morphological and biochemical characteristics of *in-vivo* human airways environment, a more advanced culturing method called ALI culturing is preferred.

#### 2.4.4 *In-vitro* ALI culturing

The ciliated airway epithelial cells of the conducting airways face the lumen on one side and the sub-epithelial tissue on the other side. The production of such a physiologically relevant model of human airways *in-vitro* has been made possible by ALI culturing (Whitcutt et al., 1988). ALI cultures involve growing cells on permeable membrane supports called Transwells™ (TW) or Snapwells™ (SW) inserts which are commercially available. These membrane supports have an apical section where the cells are added and a basolateral section where the appropriate growth medium is added as shown Figure 2.5. Initially the cells are added to the apical surface of the membrane supports and grown under submerged conditions for 2 - 4 days during which the cells grow and proliferate to form a monolayer of undifferentiated cells.



**Figure 2.5. Diagrammatic representations of human bronchial epithelial cells grown under (A) submerged conditions and (B) ALI conditions.**

(A) The human bronchial epithelial cells grown in submerged conditions were unable to differentiate into the airway epithelial cell morphology and hence do not accurately mimic the *in-vivo* airway physiology. (B) The bronchial epithelial cells are seeded in the apical section of the TW/SW while the basolateral section is supplied with growth medium. Cells cultured under ALI conditions for 2-3 weeks undergo robust pseudostratified differentiation exhibiting features of *in-vivo* airway epithelium such as cilia formation, appearance of mucus producing goblet cell and barrier formation. The ALI cultures display characteristics closely resembling that of *in vivo* human airway epithelium and hence are considered good *in-vitro* models for human airway studies.

An ALI condition is established when the growth medium from the apical section is removed such that the cells are apically exposed to air and basolaterally exposed to growth medium. When cultured under ALI conditions for 2 - 3 weeks, the monolayer of cells stop proliferating and undergo pseudo-stratified differentiation to produce columnar ciliated epithelial cells, mucus-producing goblet cells and basal cells (Wu et al., 1997, Yankaskas et al., 1985). It is believed that this polarized differentiation occurs due to the increased availability of oxygen relative to submerged cultures (Lopez-Souza et al., 2004, Ghio et al., 2013). ALI culturing also leads to barrier formation between cells as evidenced by good Transepithelial electrical resistance (TEER) values (measured using Volt-Ohm meter) and expression of tight junction proteins namely Zonula occludens protein (ZO-1) (Fu et al., 2009, Relova et al., 2005), thus mimicking the *in-vivo* human airway epithelium as closely as possible. The major factors impacting the differentiation of the cells at ALI include:

1. **ECM substrate:** The ECM of the airways involves a mesh of nano-sized fibres in which the airway cells grow, differentiate and proliferate in 3D (Dunsmore and Rannels, 1996). The ECM substrate in which the cells grow is an important factor since the interaction between the cells and the ECM substrate has a significant influence on mucociliary differentiation and cilia formation (Gray et al., 1996, Harrington et al., 2014). Thus in ALI airways models, the membrane supports are generally coated with an ECM protein such as collagen such that the cells grow,

proliferate and expand in an *in-vivo* like environment (Gruenert et al., 1995, Yankaskas et al., 1985).

2. **Growth medium:** For cells cultured at ALI, growth medium with high Retinoic Acid (RA) content is preferred. Studies have found that growth medium with high RA promotes robust mucociliary differentiation in the cells whereas growth medium with low RA leads to flattened squamous epithelial phenotype. Dulbecco's Modified Eagle Medium Ham's F-12 (DMEM/F-12) and Essential Medium Eagle: Earl's balanced salt solution (EMEM) are good growth medium for the bronchial epithelial cell lines (van Wetering et al., 2007).

#### 2.4.4.1 ALI mono and co-cultures

As of yet, there are no standardised models of human airways and hence different cell lines are employed for different purposes. For instance, ALI monocultures of CALU 3 are widely employed in ion-transport and cystic fibrosis studies since it is the only human airway cell line to express the cystic fibrosis transmembrane conductance regulator (CFTR) (Florea et al., 2003, LeSimple et al., 2013). Similarly, monocultures of BEAS 2B and A549 have been employed as *in-vitro* models of respiratory epithelium in many past studies investigating several aspects such as drug transport, air-borne particles or cigarette smoke cytotoxicity (Forbes et al., 2003, Zhang et al., 2017b, Bitterle et al., 2006).

Since human airways consists of several other cell types apart from epithelial cells, there exists a constant cross-signalling between the different cell types in order to maintain tissue homeostasis (Knight and Holgate, 2003). One of the advantages of ALI culturing is that unlike submerged cultures which permits growth of only one cell type, ALI models can involve simultaneous growth of more than one cell type on the same permeable membrane. Such models involving two cell types are called ALI co-cultures. These co-culture models have potential to investigate the inter-play between two cell types in response to environmental challenges such as cigarette smoke or EC aerosols.

Compared to monocultures, very few studies have employed co-culture ALI models for cigarette smoke/EC studies. The co-culture models employed in past studies often involve culturing airway epithelial cells with fibroblasts since the interaction between these two cell types have been shown to play many important roles including airway epithelium differentiation, wound healing and inflammation etc. (Knight, 2001). In one study, Ishikawa et al (2016) employed an ALI co-culture model of bronchial epithelium consisting of normal human bronchial epithelial cells seeded on top of a collagen matrix embedded with human

foetal lung fibroblasts to study the effects of cigarette smoke exposure on cellular differentiation and inflammatory mediators' production (Ishikawa and Ito, 2017). In another study, an ALI co-culture model consisting of A549 alveolar epithelial cells seeded on top of a collagen gel containing either normal lung fibroblasts or cancerous fibroblasts was employed to investigate the tumour promoting influence of cancer associated fibroblasts on the alveolar epithelial cells (Horie et al., 2012). Studies have also employed *ex-vivo* tissue constructs involving multiple cell types to study cigarette smoke induced effects. Iskander et al (2015) employed an organotypic co-culture model called 'MucilAir-HF' consisting of primary bronchial epithelial cells and primary lung fibroblasts isolated from a healthy 63 year old non-smoker to study the difference in cigarette smoke induced effects on the co-culture model (Iskandar et al., 2015). This study reported that the commercially available, ready-to-use MucilAir model differentiates into ciliated cells, basal cells and mucus producing goblet cells, thus resembling *in-vivo* airway epithelium quite closely.

In the current study a co-culture human airway model consisting of CALU 3 bronchial epithelial cells and HPF human pulmonary fibroblasts were used to assess the effects of cigarette smoke and EC aerosols. This HPF-CALU 3 co-culture model has been exhaustively characterised by a previous research student from the authors' research group (Bielemeier, 2012). The HPF-CALU 3 co-culture model was shown to stain strongly for cytokeratins' CK 5 and CK 8 which are basal cell and differentiated columnar epithelial cell markers respectively via immunohistochemistry. Additionally, the model also stained positively for IB10 and vimentin which are fibroblast cell markers, thus indicating the presence of fibroblasts below the epithelial cells. Further immunohistochemistry analysis also showed strong staining for ZO-1 tight junction complex proteins, thus indicating formation of barrier between epithelial cells. The co-culture model also exhibited commendable barrier integrity after 14 days of co-culturing (reaching a TEER value of  $291.96 \pm 14.90 \Omega \times \text{cm}^2$  on the 14<sup>th</sup> day). Mucus Dot-blot analysis revealed positive staining for MUC5AC mucin secretion in supernatants collected from the apical compartment of the permeable membranes. Further, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis demonstrated apical microvilli and cilia formation in the differentiated columnar epithelial cells (Bielemeier, 2012).

## 2.5 Exposure methods employed in *in-vitro* cigarette/EC studies

As previously mentioned, cigarette smoke and EC aerosols are a complex mixture of multitude of chemicals that are distributed between the gaseous and particulate phase (Benowitz and Jacob, 2000). Traditional cigarette/EC testing method involves exposing

submerged monocultures to either extracts or condensates of cigarette smoke/EC aerosols. Cigarette smoke extract (CSE) or EC extract (ECE) is prepared by smoking (i.e. drawing) cigarette smoke/EC aerosols through appropriate growth medium or saline and adding the resulting mixture onto the submerged cells (Blue and Janoff, 1978). Cigarette smoke condensate (CSC) or EC condensate (ECC) is prepared by capturing the particulate particles of cigarette smoke or EC aerosols on Cambridge filter pads and eluting the particles in an organic solvent such as Dimethylsulfoxide (DMSO) (Andreoli et al., 2003). This eluted mixture is then added onto the submerged cells. Examples of experimental methodologies used in past studies to obtain CSE and CSC have been described in Figure 2.6.



**Figure 2.6. Examples of set-ups used to produce (A) Extracts and (B) Condensates of cigarette/ECs.**

The set-up on the left (A) used in a study by (Bernhard et al., 2004) to produce cigarette smoke extracts (CSE) consists of a lit-cigarette (1) connected to sterile tubes (2) which in-turn are connected to a sterile wash-bottle (3) and a 1 litre sterile tank (6). When the fluid pump (7) is switched on, it removes appropriate amount of water from the tank which causes vacuum to be applied in the wash-bottle. This in-turn causes cigarette smoke to be pulled into the wash-bottle through a Pasteur pipette (4) into the growth medium to form CSE (5). In the set-up on the right (B) used in a study by (Semlali et al., 2014) to produce cigarette smoke condensates (CSC), the same principle is applied wherein a pump-mediated vacuum application causes cigarette smoke to be pulled into a flask containing saline solution. This solution is then filtered using a 0.22  $\mu\text{m}$  filter to obtain the particulate particles of cigarette smoke. The particles are then eluted in an organic solvent like DMSO to obtain the CSC solution.

While the extracts collect only the water-soluble vapour-phase components of cigarette smoke or EC aerosols, condensates collect only the particulate particles of cigarette smoke or EC aerosols (Fukano et al., 2004). However, during real-time smoking/vaping, when cigarette smoke or EC aerosol is inhaled, the cells of the respiratory system interact with whole cigarette smoke (WCS) or whole EC vapours (ECV) which includes constituents of both the vapour and particulate phase. Hence, in order to expose the 3 D ALI cultures airway cells to WCS/ECV in fashion mimicking human smoking/vaping behaviour, aerosol delivery systems referred to as smoking machines have been developed (Thorne and Adamson, 2013). These smoking machines enable exposure of physiologically relevant ALI cultures of human airway cells to both vapour and particulate phases of WCS/ECV, thus increasing the physiological relevance of cigarette/EC studies (a detailed description of smoking machine principle, working and different types is explained in Chapter 4 of this study). Thus, the exposure method varies according to the type of cultures employed in the study. While submerged cultures are exposed to extracts or condensates of cigarette/EC, ALI cultures are mostly exposed to WCS/ECV (although ALI cultures can also be exposed to extracts or condensates of cigarette/EC although the physiological relevance of such an exposure would be low)

## 2.6 Review of existing EC studies

The existing studies performed on ECs can be divided into the following sections:

1. Analytical studies investigating the different chemicals found in EC liquids and aerosols
2. Acute EC studies performed on humans
3. EC studies performed using *in-vivo* rodent models
4. EC studies performed using *in-vitro* airways models

### 2.6.1 Chemical evaluation of EC liquid and aerosols

The composition of tobacco cigarette smoke and the nature of its chemical constituents have been extensively characterised by many previous studies (Stedman, 1968, Bonnet and Neukomm, 1957, Talhout et al., 2011). Since ECs are a different class of nicotine delivery systems compared to tobacco cigarettes, a number of analytical studies have evaluated the chemical composition of EC liquids and generated aerosols in order to identify the presence of potentially toxic chemicals or impurities that may be present in them.

### *2.6.1.1 Detection of metal particles in EC liquid and aerosols*

In a study by Williams et al (2013), EC aerosols from a popular brand was found to contain silicate beads and 22 different metal particles including nickel, lead and chromium which are included in the FDA list of 'harmful and potentially harmful chemicals' (FDA., 2012). The study also reported that the concentration of 9 metal particles in EC aerosols were equal to or higher than that found in tobacco cigarettes. This study additionally reported that many of the metal particles may have originated from poor solder joints, improper heating filaments and wicking materials construction, thus calling for improved quality control in EC manufacturing (Williams et al., 2013). In another study, Hess et al (2017) evaluated 48 EC liquids from across five different EC brands for the presence of cadmium, chromium, lead, nickel and manganese (Hess et al., 2017). According to the study, all five metals were detected in all the samples tested although with inter-brand variance. The presence of metal particles in EC liquids and aerosols have been reported by two other studies (Lerner et al., 2015, Goniewicz et al., 2014b). As stated by the Agency for Toxic Substances and Disease Registry (ATSDR), nickel and chromium are established carcinogens, the inhalation of which can cause chronic bronchitis, irritation effects, reduced lung function, nasal/sinus cancer and pulmonary fibrosis (ATSDR., 2005, Chervona et al., 2012, Wu et al., 2016). The inhalation of silica, copper, magnesium and zinc metal particles can lead to several respiratory effects such as airway irritation, shortness of breath, coughing, chest pain etc. (Chiba and Masironi, 1992). Cadmium inhalation has been reported to cause acute respiratory distress syndromes (Barbee and Prince, 1999). These trace metals have also been shown to produce adverse cardiovascular and neurotoxicity effects (Andrade et al., 2017, Mulware, 2013), thus raising concerns over their detection in ECs.

### *2.6.1.2 Detection of aldehydes in EC liquid and aerosols*

As stated earlier, EC liquid consists of three chief components namely PG, VG and nicotine apart from flavouring substances. When the user puffs an EC, the EC liquid is heated at temperatures that can reach as high as 300°C - 350°C (Talih et al., 2015). At these temperatures, EC liquid constituents undergo several chemical modifications that may lead to the production of potentially harmful chemicals. PG and VG can undergo pyrolysis to form low molecular weight carbonyl compounds such as formaldehyde, acetaldehyde and acrolein (Clapp and Jaspers, 2017). Goniewicz et al (2014) analysed the liquid and aerosols of ECs from 12 different brands and reported the presence of formaldehyde and acetaldehyde in EC liquid as well as aerosols of all 12 samples, while acrolein was found only in EC aerosols (in 11 out of 12 samples) possibly due to pyrolysis of VG (Goniewicz et al., 2014b). The study also reported the presence of two volatile organic compounds (VOCs)

namely toluene and m,p-xylene and three toxic metal particles namely nickel, lead, cadmium in EC aerosols. The study concluded that ECs can produce potentially toxic chemicals although the levels were 9 - 450 times lower than that detected in conventional cigarettes (Goniewicz et al., 2014b). Lauterbach and Laugesen (2012) reported a similar result where acetaldehyde, formaldehyde, TSNAs were detected in EC aerosols but the levels were 90 % less than that found in tobacco smoke (Lauterbach J.H. , 2012). However, other studies have detected high levels of aldehydes in EC aerosols, sometimes at levels comparable to that found in cigarette smoke. Kosmider et al (2014) reported the detection of formaldehyde and acetaldehyde in 8 out of the 13 EC aerosol samples tested. The emittance level of these chemicals was found to be proportional to the operational voltage of the EC device, with high-voltage ECs producing highest formaldehyde levels, some even comparable to that found in tobacco smoke (Kosmider et al., 2014). Other recent studies additionally substantiate the ability of ECs to produce harmful aldehydes at toxic levels (Jensen et al., 2015, Salamanca et al., 2017). The production of high levels of aldehydes in ECs is a subject of dispute as a number of studies argue that such high level of aldehydes can be produced only during 'dry puff' conditions i.e. when insufficient EC liquid is delivered to the atomizer at the selected power settings (Bates and Farsalinos, 2015, Farsalinos et al., 2015d). Studies debate that 'dry-puffs' are unrealistic vaping conditions and that such conditions are generally avoided by experienced EC users (Bates and Farsalinos, 2015). Irrespective of the aldehydes levels compared to that found in tobacco smoke, the presence of these toxins even at modest levels have raised a lot of concern amongst public. Formaldehyde and acetaldehyde are classified as Group 1 and Group 2B human carcinogens respectively (IARC, 2004, Park and Taniguchi, 2008). Studies have shown acrolein to cause endothelial dysfunction, platelets activation and various adverse cardiovascular diseases (Sithu et al., 2010, Srivastava et al., 2011). In fact, 92 % of all the non-cancer cardiopulmonary diseases that arise from smoking is attributed to acrolein, formaldehyde and acetaldehyde (Hausmann, 2012).

#### *2.6.1.3 Detection of other chemicals in EC liquid and aerosols*

Goniewicz et al (2014) reported that trace amounts of carcinogenic TSNAs compounds were detected in EC aerosols which correlates with another study that detected TSNAs in EC cartridges (Laugesen, 2008). Other studies have also detected TSNAs in EC liquid and aerosols (Tayyarah and Long, 2014, Farsalinos et al., 2015a, Trehy et al., 2011). Besides, many analytical studies have shown the presence of other harmful chemicals like polycyclic aromatic hydrocarbons (PAHs) (Pellegrino et al., 2012, Tayyarah and Long, 2014), phenolics (Tayyarah and Long, 2014), tobacco alkaloids (Beauval et al., 2016, Lisko et al.,

2015) etc. Studies have also detected the presence of EC specific harmful chemicals that are not generally analysed in cigarette smoke studies such as ethylene glycol, diethylene glycol, methylglyoxal (Uchiyama et al., 2013), acetyl and diacetyl propionyl. The detection of these chemicals and metal particles have raised questions on the long-term usage of ECs.

#### *2.6.1.4 Toxic flavouring compounds found in ECs*

As per 2014, there were about 7700 different EC flavours available in the market (Barrington-Trimis et al., 2014). Many of these EC flavours have alluring names such as Café Latte, oatmeal cookie, cotton candy, tutti-frutti etc. in order to appeal to the youth. Some of the common flavouring chemicals used in EC liquids include diacetyl, 2,3-pentanedione,  $\alpha$ -diketone, cinnamaldehyde, acetoin, maltol, vanillin, menthol etc. (Allen et al., 2016). Although most of these flavouring chemicals have a “Generally regarded as safe” (GRAS) approval for ingestion, the effect of heating these compounds at high temperatures and inhaling them is not well-understood (Tierney et al., 2016). Diacetyl (2,3-butanedione) for instance, is an FDA approved food additive used to give foods a characteristic creamy/buttery flavour. However, inhalation of diacetyl aerosols has been reported to cause severe irreversible lung damage called bronchiolitis obliterans which is popularly known as ‘pop-corn’ lung disease (Kanwal et al., 2006, Kreiss et al., 2002). 2,3-pentanedione and  $\alpha$ -diketone are other flavouring substances, chemically and structurally similar to diacetyl, that have been employed by some EC companies instead of diacetyl. A number of studies have evaluated EC liquids and aerosols for the presence of these potentially harmful flavouring compounds. Allen et al (2016) investigated the presence of three flavouring compounds namely diacetyl, 2,3-pentanedione and acetoin in 51 flavoured ECs and reported that at least one chemical was detected in 47 of the 51 samples, in some samples even above permissible levels (Allen et al., 2016). In a 2015 study, Farsalinos et al (2015) investigated samples from 156 EC liquids across 36 different brands for the presence of diacetyl and  $\alpha$ -diketone. The study reported detection of diacetyl and  $\alpha$ -diketone in 74.2% samples, with many of them detected at levels well above define safety limits (Farsalinos et al., 2015b). The presence of these potentially harmful chemicals in the EC liquid flavourings have raised concerns on the possible adverse health implications, especially amongst adolescents.

## 2.6.2 Human studies investigating the acute physiological effects of ECs

A number of studies have investigated the acute effects of EC vaping in humans. These studies have produced mixed results with some studies reporting short-term detrimental effects while other studies reporting little to no effects of EC vaping.

### 2.6.2.1 *EC studies reporting significant physiological effects*

Vardavas et al (2012) reported that after using a Nobacco 'black line' EC of 11 mg/mL nicotine concentration for 5 min, the user experienced increased total airway resistance, total respiratory impedance and peripheral airway resistance, thus suggesting that short term vaping can produce acute physiological effects similar to that produced by cigarette smoking (Vardavas et al., 2012). Vlachopoulos et al (2016) investigated the cardiovascular effects of vaping on 24 smokers (all free from any cardiovascular risk factors) after 5 min of tobacco cigarette smoking, 5 min EC vaping, 30 min EC vaping and 60 min normal breathing (control) (Vlachopoulos et al., 2016). The study reported that there was a significant increase in heart rate and systolic blood pressure post 30 min EC vaping, similar to that observed after 5 min tobacco smoking. The study concluded that long-term EC vaping may have detrimental cardiovascular effects as both aortic stiffness and increased blood pressure play a key role in many cardiovascular diseases progression (Vlachopoulos et al., 2016). Reidel et al (2017) analysed the sputum samples of cigarette smokers, EC users and non-smokers for oxidative stress related proteins and mucin protein concentrations (Reidel et al., 2018). The study reported an increased production of aldehyde detoxification proteins, oxidative stress proteins, innate defence proteins such as matrix metalloproteinase 9 (MMP-9), elastase and MUC5AC mucins in EC users' sputum and the levels were comparable to that found in cigarette smokers' sputum.

### 2.6.2.2 *EC studies reporting little to no physiological effects*

Farsalinos et al (2013) assessed coronary blood circulation and blood carboxyhaemoglobin (HbCO) levels in 60 healthy volunteers (30 smokers and 30 ex-smokers currently using ECs) post smoking 2 cigarettes (0.7 mg nicotine) or vaping an EC (9 mg/mL nicotine) for 15 min (Farsalinos et al., 2013a). The study reported that while cigarette smoking significantly increased the HbCO levels in both groups, no elevation in HbCO levels was observed in either groups post EC vaping. In a similar fashion, cigarette smoking significantly increased the coronary blood flow rate in both groups while EC vaping had no effect on either groups (Farsalinos et al., 2013a). Flouris et al (2012) analysed the complete blood count (CBC) markers in 15 smokers and 15 non-smokers post 30 min smoking and vaping and reported

that while active and passive tobacco smoke caused a significant increase in the number of leukocytes, lymphocytes and granulocytes, neither active nor passive EC aerosols had any significant effects on the CBC (Flouris et al., 2012). Another study by Flouris et al (2013) concluded that short-term EC vaping did not produce any significant decrease in lung-function compared to tobacco cigarettes (Flouris et al., 2013). Van Staden et al (2013) reported that post switching from tobacco cigarettes to ECs for 2 weeks, there was a significant decrease in the arterial and venous HbCO levels, increase in the oxygen saturation levels and decrease in serum cotinine levels in heavy smokers (van Staden et al., 2013). The study concluded that EC may provide a healthier and acceptable alternative to tobacco cigarettes. Thus there exists discrepancies amongst current human studies pertaining to EC acute effects.

### 2.6.3 EC studies using *in-vivo* rodent models

Although it is impossible to accurately replicate the vaping behaviour of humans using animal models, there has never-the-less been a steady rise in the number of animal studies evaluating the physiological effects of ECs using mice. McGrath-Morrow et al (2015) reported that post exposing neonatal mice to aerosols of a propylene glycol based EC (1.8 % nicotine content) for 10 post-natal days, there was a significant decrease in total body weight, elevated plasma cotinine levels, diminished alveolar cell proliferation and impaired post-natal lung growth compared to the air-exposed control mice (McGrath-Morrow et al., 2015). Sussan et al (2015) reported that when ECV (1.8 % nicotine content) exposed mice were intra-nasally infected with bacteria *Streptococcus pneumonia*, they exhibited significantly impaired pulmonary bacterial clearance ability compared to the air-exposed control mice, thus suggesting the influence of ECV on the immune system (Sussan et al., 2015). A recent study by Lee et al (2018) reported that mice treated to ECV (10 mg/mL nicotine content) for 3 h a day, 5 days a week, for 12 weeks exhibited DNA damage in lungs, bladder, heart and the DNA repairing proteins of lungs were significantly reduced (Lee et al., 2018). The study concluded that EC aerosols can act as a carcinogen to different organs and could also cause potential heart problems. Garcia-Arcos et al (2016) reported that mice exposed ECV (unflavoured ECs with nicotine) for 1 h daily for 4 months demonstrated increased airway hyper-responsiveness, mucin production, distal airspace enlargement and increased production of several pro-inflammatory cytokines including interleukins (IL) IL-1 $\beta$ , IL-6, MCP-1 (monocyte chemoattractant protein-1), CXCL 10, CXCL 2 and proteases such as MMP-9, MMP-12, thus demonstrating typical COPD pathophysiological features (Garcia-Arcos et al., 2016).

## 2.6.4 EC studies employing *in-vitro* models

This section has further been divided into the following sub-sections:

1. Studies employing submerged cultures of non-human i.e. animal derived cells
2. Studies employing submerged cultures of human non-airway cell types
3. Studies employing submerged cultures of human airway epithelial cells
4. Studies employing ALI mono and co-cultures of human airway cell types

### 2.6.4.1 *EC in-vitro studies employing submerged cultures of animal derived cell lines*

A number of EC studies have employed non-human cell lines to assess EC cytotoxicity *in-vitro*. Romagna et al (2013) employed 3T3 murine fibroblasts to study the effects of extracts of 21 ECs and reported that only one EC was cytotoxic (Romagna et al., 2013). Farsalinos et al (2013) investigated the cytotoxicity of extracts of 20 ECs on H9c2 murine cardiomyoblasts and reported that only 3 ECs produced cytotoxic effects on the H9c2 cells (Farsalinos et al., 2013b). Schweitzer et al (2015) used rat lung endothelial cells to demonstrate that exposure to EC liquid and extracts had a detrimental impact on barrier formation irrespective of the nicotine content (Schweitzer et al., 2015). Bahl et al (2012) employed mouse neural stem cells to demonstrate the cytotoxic effect induced by EC liquid exposure (Bahl et al., 2012). Sherwood et al (2016) employed mouse tracheal epithelial cells to prove the cytotoxic effects of 2,5-dimethylpyrazine, the chocolate flavouring used in ECs (Sherwood and Boitano, 2016). Although animal derived cell lines can form stable cultures and can provide useful information similar to human cell lines, the translation of data from these studies to human conditions is unreliable.

### 2.6.4.2 *EC in-vitro studies employing submerged cultures of human non-airway cell types*

Numerous *in-vitro* studies have investigated EC cytotoxic effects on a broad range of cell types apart from airway epithelial cells. Although not directly elucidating the effect of ECs on the respiratory system, these studies are however useful in understanding the wider implications of ECs on other cell types. Schweitzer et al (2015) treated human lung endothelial cells to both nicotine containing and nicotine-free EC liquid/vapour condensates and observed that irrespective of the nicotine content, EC liquid/vapour condensates demonstrated prominent cytotoxic effects by triggering a dose-dependent barrier dysfunction in the endothelial cells (Schweitzer et al., 2015). Sancilio et al (2016) employed human gingival fibroblasts to assess the effects of EC liquid exposure and reported that both nicotine containing and nicotine-free EC liquids produced increased reactive oxygen species (ROS) production, Bcl-2-associated X protein (Bax) pro-apoptotic gene expression

ultimately leading to apoptosis 48 h post exposure (Sancilio et al., 2016). Some studies have also investigated the effect of EC exposure on inflammatory immune cells. In one such study, kupffer cells (liver-resident macrophages) were treated to ECE post which a significant increase in pro-inflammatory mediators' production and oxidative stress was observed (Rubenstein et al., 2015). The study concluded that exposure to ECE can instigate kupffer cells to initiate a systemic inflammatory response (Rubenstein et al., 2015). Another study also reported on the increased pro-inflammatory activity of neutrophils caused by ECE exposure, thus suggesting the ability of ECs to produce adverse inflammatory reactions similar to those found in COPD patients (Higham et al., 2016). One particular study has also analysed the effect of EC liquid exposure on human embryonic stem cells (HESCs) (Bahl et al., 2012). This particular study by Bahl et al (2012) reported that EC liquid exposure proved cytotoxic to HESCs but the cytotoxicity was not due to nicotine but rather the concentration of substances used in EC liquid flavourings (Bahl et al., 2012).

#### *2.6.4.3 EC in-vitro studies employing submerged cultures of airway epithelial cells*

Studies employing airway epithelial cells to investigate potential EC cytotoxic effects offer more physiological relevance since they are the first and principal cells to interact with any inhaled toxicants. The airway epithelial cells employed in these studies can range from immortalised cell lines to primary cells to tumour cell lines. One such study treated immortalized bronchial epithelial cell line BEAS 2B to ECE and reported a significant accumulation of poly-ubiquitinated proteins, a decrease in proteasomal activities, aggresome formation, and an impaired autophagy ultimately leading to an increase in cellular oxidative stress, senescence or apoptosis (Shivalingappa et al., 2015). Wu et al (2014) employed primary human airway epithelial cells obtained from bronchial brushings of healthy non-smokers to study the effects of EC liquid exposure. The study reported that EC liquid exposure caused a significant increase in IL-6 pro-inflammatory cytokine, promoted human rhinovirus infection and inhibited expression of anti-microbial defense proteins in the cells (Wu et al., 2014). Rowell et al (2017) employed submerged cultures of CALU 3 bronchial epithelial cells to analyse the effects of a number of flavoured EC liquids (Rowell et al., 2017). The team reported that a number of flavoured EC liquids caused a dose-dependent decrease in cell viability post 24 h exposure, with four flavours namely Banana Pudding (Southern Style), Kola, Hot Cinnamon Candies, and Menthol Tobacco demonstrating highest cytotoxicity. Husari et al (2016) treated A549 adenocarcinoma alveolar epithelial cells to EC total particulate matter extract post which a significant decrease in cell viability was observed although the concentration required to induce this cytotoxic effect was 32 times lower than CSE (Husari et al., 2016). Interestingly, majority of

the *in-vitro* studies employing human airway epithelial cells have reported on the detrimental effects exhibited by ECE or EC liquids.

#### 2.6.4.4 *EC in-vitro studies employing ALI mono and co-cultures of human airways*

Compared to EC studies employing submerged monocultures, very few studies have employed ALI cultures to assess the possible effects of WCS/ECV. Possible reasons for this could be that studies employing ALI cultures are expensive (costs associated with TWs/SWs permeable supports, collagen etc.), time-consuming (ALI cultures require a minimum of 2-3 weeks to undergo mucociliary differentiation while co-culture models take longer), necessitates a smoking machine (to expose cells to WCS/ECV as opposed to extracts or condensates of cigarette/ECs), post-exposure analysis are difficult to perform relative submerged cultures (ALI cultures are more sensitive to disturbances and hence disrupting the differentiated cells during the different post-exposure analysis may accidentally damage the cells). Never-the-less, a few studies employing ALI cultures have added valuable information on ECs induced cytotoxic effects. One of the earliest studies to employ differentiated ALI monocultures to study EC cytotoxicity was performed by Neilson et al (2015). This study which was a collaboration work between British American Tobacco (BAT) and MatTek Corporation, a company that produces a diverse range of *in-vitro* models, employed differentiated tracheobronchial epithelial *ex-vivo* tissues called EpiAirway™ to assess EC cytotoxicity and reported that upon exposing the EpiAirway™ tissues to ECV, no cytotoxicity was observed although a significant decrease in TEER values were observed (Neilson et al., 2015). Some studies have interestingly cultured cells on permeable TW/SW membranes under submerged conditions and air-lifted the cells only a day prior to WCS/ECV exposure. Azzopardi et al (2016) employed an airway cell model where NCI-H292 bronchial epithelial cells were cultured on 12 mm TW inserts under submerged conditions up until the day of exposure (Azzopardi et al., 2016). Just prior exposing the cells to WCS or ECV, the apical medium was removed from the TWs, thus air-lifting the cells although the NCI-H292 cultures remained undifferentiated. Two other studies by Leigh et al (2016) and Lerner et al (2015) treated undifferentiated cultures of NCI-H292 bronchial epithelial cells to ECV at ALI and reported increased cytotoxicity and IL-6/IL-8 pro-inflammatory cytokine production respectively (Leigh et al., 2016, Lerner et al., 2015).

Scheffler et al (2015) performed two studies employing ALI cultures of primary NHBE bronchial epithelial cells obtained from two different volunteers as the cell model. In the first study, undifferentiated NHBE cells were exposed to WCS/ECV and the results were analysed (Scheffler et al., 2015b). In a follow-up study by the same team, Scheffler et al (2015) employed three different cell types to evaluate EC cytotoxicity namely NHBE, A549

and a third cell type called CL-1548 which was obtained by immortalising NHBE primary cells using lentiviral constructs (Scheffler et al., 2015a). All three cell types were cultured under submerged conditions on TWs and undifferentiated cultures were subsequently exposed to WCS/ECV. In this study however, the team additionally investigated the differentiation capacity of NHBE and CL-1548 cells by culturing them on collagen coated TW inserts under ALI conditions for 21 days and reported that both cell types underwent pseudostratified differentiation producing ciliated, basal, goblet cells. Further, the study reported that the differentiation capacity of CL-1548 cells were comparable to that of its parent NHBE cells, thus emphasising the point that immortalized cell lines can produce as much airways-relevant features as primary cells and hence can act as a useful and economical alternative to primary cells (Scheffler et al., 2015a). Exposure of differentiated cultures of bronchial epithelium to WCS/ECV is a more physiologically relevant *in-vitro* airway model since it involves production of several distinct airway epithelial cell features and also various cell types (basal, goblet, ciliated columnar etc.) as opposed to undifferentiated bronchial epithelium which mostly consists of basal epithelial cells (Scheffler et al., 2015b).

Only one study thus far has employed a co-culture model to study EC cytotoxicity. In this study by Bengalli et al (2017), an alveolar-blood barrier was reconstructed by co-culturing NCI-H441 alveolar adenocarcinoma epithelial cells with human pulmonary microvascular endothelial cells on permeable TW inserts (Bengalli et al., 2017). The team however induced differentiation and barrier formation not by establishing ALI but by chemical induction. From day 3 of cultivation, the co-cultures were treated with an array of chemicals including 1  $\mu$ M Dexamethasone, 25  $\mu$ g/mL copper oxide (CuO) and titanium dioxide (TiO<sub>2</sub>) suspensions such that the co-culture models exhibited good TEER values. The study reported that exposure of this alveolar co-culture model to ECV had a differential effect depending upon the flavour of the ECs (Bengalli et al., 2017). Although successfully employed to study ECV effects, a major limitation of this co-culture model is that it does not represent the tracheobronchial epithelium region which are the primary line of epithelial cells exposed to cigarette smoke/ECV *in-vivo*. The co-culture model of the current study consisting of bronchial epithelial cells and pulmonary fibroblasts fulfils that purpose where differentiated bronchial epithelial cells are exposed to WCS/ECV.

## 2.7 Aims of the current study

The overall aim of the current study was to design novel cigarette/EC delivery systems and develop physiologically relevant *in-vitro* methodologies to perform a comprehensive analysis of potential EC effects.

### **The Engineering/design objectives of the current study were as follow:**

- To design and construct a bespoke 'bubbling' system in order to collect extracts of cigarette smoke, EC aerosols and E-vehicle.
- To construct a novel, automated aerosol delivery system that would deliver cigarette smoke/EC aerosols to the co-culture human airway model in a fashion that would mimic the human smoking/vaping behaviour as closely as possible.

### **The experimental objectives of the current study were as follow:**

- To analyse the effects of individual EC constituents' chiefly nicotine, its oxidative metabolite cotinine and E-vehicle (PG/VG base-liquid) on submerged bronchial epithelial cells.
- To assess the effects of extracts of tobacco cigarette and a variety of ECs from different commercial brands with varying nicotine strengths and flavours on submerged cultures of a number of airways cell types.
- To produce a 3D *in-vitro* co-culture model of the human airways consisting of differentiated human bronchial epithelial cells and pulmonary fibroblasts
- To evaluate the effects of aerosols of WCS and ECV on the co-culture human airways model cell viability, pro-inflammatory mediator production, oxidative stress and apoptosis at different exposure times ranging from 1 h – 6 h.
- To compare and discuss the results between the different methodologies of the current study and also the existing literature.

### 3 Materials and methods

#### 3.1 Cell types

Several transformed and cancer-derived cell lines were used in the current study according to the different aims of the individual experiments. Two human bronchial epithelial cell lines namely BEAS 2B and CALU 3 were extensively used throughout the study. BEAS 2B are transformed bronchial epithelial cell line (transformed via adenovirus 12-SV40 virus hybrid) originally isolated from bronchial epithelium of healthy humans (Lechner et al., 1982). CALU 3 are lung adenocarcinoma cell line derived from the metastatic site of a cancer patient. MRC-5 are human pulmonary fibroblast (HPF) cell line derived from normal lung tissues of humans. Four subsidiary cell lines (i.e. used in Chapter 6 experiments only) namely IB3-1, C38, J774, THP-1 cells were used in this project. IB3-1 are transformed bronchial epithelial cell line derived from a cystic fibrosis patient containing a F508 mutation allele (Andersson et al., 2008). C38 cell line are transformed bronchial epithelial cells with the corrected cystic fibrosis mutation of IB3-1 (Andersson et al., 2008). J774 are transformed macrophage cell line derived from mouse reticulum sarcoma (Lam et al., 2009) and THP-1 are human monocytes derived from a patient with acute monocytic leukemia (Tsuchiya et al., 1980). All the cell lines were purchased from American Type Culture Collection (ATCC, Middlesex, UK).

**Table 3.1. Description of the different cell lines used in the current project.**

Cell line	Description	Type	Reference
1. BEAS 2B	Normal tissue derived human bronchial epithelial cells	Transformed (via adenovirus 12-SV40 virus hybrid)	(Lechner et al., 1982)
2. CALU 3	Lung adenocarcinoma derived human bronchial epithelial cells	Cancer-derived	(Grainger et al., 2006)
3. MRC-5 (HPF)	Normal lung tissue derived human pulmonary fibroblasts	Transformed	(Yamamoto et al., 1990)

4. IB3-1	Human bronchial epithelial cell with cystic fibrosis phenotype	Transformed	(Andersson et al., 2008)
5. C38	Human bronchial epithelial cells with corrected cystic fibrosis phenotype	Transformed (via adenovirus 12-SV40 virus hybrid)	(Andersson et al., 2008)
6. THP-1	Acute monocytic leukemia derived human monocytes/macrophage	Cancer-derived	(Tsuchiya et al., 1980)
7. J774	Reticulum sarcoma derived mouse macrophages	Cancer-derived	(Lam et al., 2009)

## 3.2 Cell culture

### 3.2.1 Maintenance of cell lines

Specific growth medium were used for different cell lines in order to promote optimum growth and proliferation. Bronchial epithelial cell lines namely BEAS 2B, CALU 3, IB3-1 and C38 were cultured using DMEM - F12 growth medium consisting of 1:1 ratio of Dulbecco's Modified Eagle's Medium and Ham's F12 with 2.5 mM L-glutamine (Lonza BioWhittaker®, Verviers, Belgium) supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin (Hyclone®, Thermo Scientific, Utah, USA) and 10 % (v/v) foetal bovine serum (FBS) (Gibco®, Life Technologies Corporation, New York, USA). HPF cells were cultured in EMEM (Eagle's minimum essential medium) (Lonza BioWhittaker®, Verviers, Belgium) supplemented with 2 mM L-Glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 10 % (v/v) FBS. J774 and THP-1 cells were cultured using RPMI-1640 medium (Roswell Park Memorial Institute medium) supplemented with 2 mM L-Glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 10 % (v/v) FBS. THP-1 cell culturing involved a two-step process. First, in order to induce a macrophage-like differentiation,  $2 \times 10^5$  cells/mL THP-1 cells were pre-cultured for 72 h with 250 nM Phorbol 12-Myristate 13-Acetate (PMA) (Takashiba et al., 1999, Daigneault et al., 2010). After 72 h, the differentiation process was enhanced by removing the PMA and culturing the THP-1 cells for a further 5 days. Macrophage phenotype was confirmed by morphological changes in the THP-1 cells such as enhanced adherence to

the culture flask and increase in the cytoplasmic volume (microscopic observation). All cell lines were cultured in Nunc™ T-75 or T-25 tissue culture flasks (75 cm<sup>2</sup> or 25 cm<sup>2</sup> growth area respectively) (Thermo Fisher Scientific, Loughborough, UK) and maintained in a humidified incubator at 37°C and 5 % CO<sub>2</sub>. Culture medium was changed three times per week and cell cultures were examined microscopically daily in order to monitor any changes in viability (reduction in adherence to the flask) or morphology that would indicate bacterial/fungal infection (turbidity of culture medium were indicators of infection).

### 3.2.2 Cell number determination

Since each experiment, depending upon its nature, required different cell seeding densities, it was imperative to determine accurate cell number. In order to obtain maximum number of cells from each flask, flasks (T-25 or T-75) which were at least 80 – 90 % confluent were used for experiments. The existing culture medium in the flask was discarded and the cells were rinsed with 8 mL (or 3 mL for a T-25 flask) of Dulbecco's Phosphate-Buffered Saline (DPBS) (Fisher scientific, Loughborough, UK) in order to remove any remnant serum proteins of the growth medium that would inhibit the action of a cell-dissociating agent like trypsin. After aspirating and discarding the DPBS wash, 5mL (or 3mL for a T-25 flask) of 0.05 % trypsin-EDTA enzyme (Gibco®, Life Technologies Corporation, New York, USA) was added to the flask and incubated at 37°C for 5 - 10 min. Once the cells dissociated from the flask under the enzymatic action of trypsin, 3 mL (or 1 mL for a T-25 flask) of growth medium was added to the flask to neutralize the action of trypsin enzyme. The cells, along with the medium, were then transferred into a centrifuge tube (Thermo Scientific, Utah, USA) and centrifuged at 1000 x g for 5 min. Post centrifugation, while the cells formed a pellet at the bottom of the centrifuge tube, the culture supernatant was discarded. The cell pellet was re-suspended in appropriate quantity of growth medium and pipetted up and down carefully in order to homogenously distribute the cells in the growth medium. For cell counting, appropriate amount of this homogenous cell suspension was mixed with 0.20 % Trypan Blue (Gibco®, Life Technologies Corporation, New York, USA) in a microfuge tube in a 1:1 ratio. Using a pipette, 10 µL of this mixture was loaded onto a Neubauer Haemocytometer cell counting chamber (Hawksley, Sussex, UK) in both the upper and lower compartments. The number of cells in the centre quadrant of both the compartments was counted and averaged. By convention, this cell count was multiplied by 10<sup>4</sup> to obtain the cell density in terms of cells/mL. For example, if the cell count after averaging was 200, then the cell density would 200 x 10<sup>4</sup> cells/mL. Using this cell count, the cell suspension was diluted with growth medium appropriately in order to obtain the required cell density. If not used for experiments, the cells were seeded back into more T-25 or T-75 flasks for

expansion. Each time the cells were trypsinised, the passage number was increased by 1 and the same number was labelled on the flasks' exterior surface. This labelling also ensured that cells of two different passage number were not mixed or used together.

### 3.2.3 Cryo-preservation of cells

Stocks of all cell types were routinely frozen down and stored in liquid nitrogen containers for long-term future use. In order to freeze cells down, the cells were first trypsinised and centrifuged at 1000 x g for 5 min as explained in section 3.2.2. After centrifugation, the culture supernatant was discarded and the cell pellet was re-suspended in appropriate amount of freezing medium. Different cell types required different freezing medium ingredients. For BEAS 2B, C38 and IB3-1, the freezing medium consisted of 70 % growth medium, 20 % FBS and 10 % DMSO (Sigma-Aldrich, St. Louis, USA). For all other cell types, the freezing medium consisted of 95 % growth medium and 5 % DMSO. The amount of freezing medium added to the cells depends upon the number of flasks trypsinised. For example, for cells from a confluent T-75 flask, the cell pellet post centrifugation was suspended in 3 mL of appropriate freezing medium and subsequently transferred into 3 cryo-vials, each containing 1 mL of cells in freezing medium. The cryo-vials were then labelled with the cell line name, passage number, initials of the user, date of freezing and subsequently transferred into an Mr.Frosty™ freezing container (Fisher scientific, Loughborough, UK) for 24 h at -80°C. Mr. Frosty™ was used to freeze the cells down gradually at a rate of 1°C/min which was critical for efficient cryo-preservation of cells. After 24 h, the cryo-vials were finally transferred from Mr. Frosty™ into liquid nitrogen container and maintained at -180°C for at least a year.

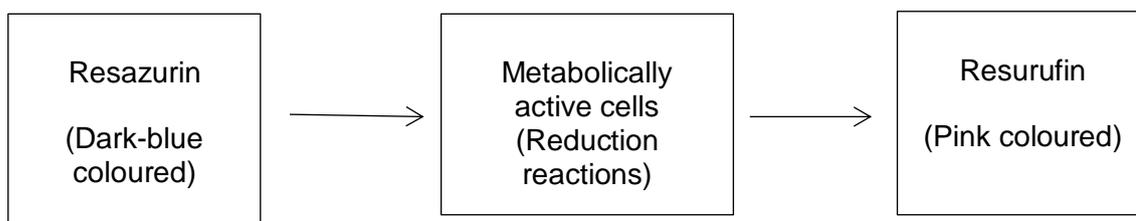
## 3.3 Cell viability assays

Depending upon the nature of the experiments including the number of samples to be analysed, time and cost, two different cell viability assays were employed.

### 3.3.1 Cell Titre Blue® cell viability assay

Cell Titre Blue® (CTB) (Promega Ltd, Southampton, UK) is a fluorometric assay used to determine the viability of cells. CTB consists of a buffered solution that contains an indicator dye called resazurin. As illustrated in Figure 3.1, the assay is based on the principle that when resazurin (a dark-blue coloured cell permeable compound) enters a metabolically active cell, it is reduced into a fluorescent end product called resorufin (pink coloured compound) (Raz et al., 1997) which can then be quantified using a spectrophotometer.

During experiments, cells were incubated at 37°C for 2 h with appropriate quantity of CTB reagent (the amount of CTB reagent added was equivalent to 20 % of the total volume of cells being analysed). After 2 h, the fluorescence intensity (F.I.) was measured using a spectrophotometer (Spectramax Gemini XS, Molecular devices, Berkshire, UK) at 560 nm excitation wavelength and 590 nm emission wavelength. The amount of F.I. was directly proportional to the cell viability of the samples.

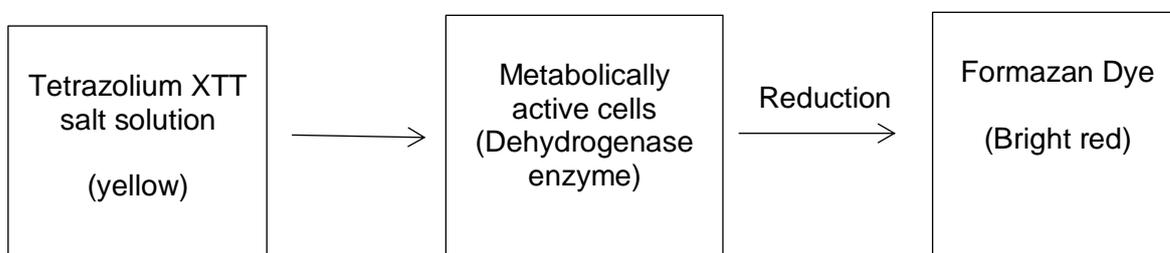


**Figure 3.1. Principle of CTB cell viability assay.**

CTB's active ingredient called Resazurin which is a dark-blue coloured compound is reduced by metabolically active cells to a pink coloured end-product called Resurufin. The fluorescence intensity (F.I.) of the resulting resurufin compound (measured using a spectrophotometer) is directly proportional to the viability of the cell sample.

### 3.3.2 XTT cell viability assay

XTT assay consists of two components namely tetrazolium XTT salt (Sigma-Aldrich, Dorset, UK) and Menadoine (Sigma-Aldrich, Dorset, UK). XTT assay is based on the principle that the dehydrogenase enzyme present in metabolically active cells reduces the yellow coloured tetrazolium XTT salt into a calorimetric red coloured end product called formazan dye as described in Figure 3.2. The amount of formazan is directly proportional to the viability of the cells (Roehm et al., 1991). Contrary to CTB, which is a fluorescence based assay, XTT is an absorbance based assay.



**Figure 3.2. Principle of XTT cell viability assay.**

XTT's active yellow coloured ingredient namely tetrazolium salt is reduced by dehydrogenase enzyme present in metabolically active cells to a bright red coloured end-product called formazan dye. The optical density of the resulting formazan dye (measured using a spectrophotometer) is directly proportional to the viability of the cell sample.

XTT salt was dissolved in 1 X DPBS to obtain a concentration of 1 mg/mL which was subsequently mixed with 1 mM menadione (diluted in acetone) at a ratio of 12.5 : 1 to produce the XTT working solution. During experiments, cells were incubated at 37°C for 2 h with appropriate quantity of XTT working solution (the amount of XTT working solution added was equivalent to 25 % of the total volume of cell samples being analysed). After 2 h, the absorbance (optical density) was measured at 450 nm using a spectrophotometer (MultiSkan, ThermoScientific, USA). The measured absorbance was directly proportional to the viability of the cells.

### 3.4 IL-6 and IL-8 detection via ELISA (Enzyme Linked Immuno Sorbent Assay)

In the current study, secretion of two chief pro-inflammatory mediators namely IL-6 and IL-8 were investigated.

#### 3.4.1 Harvesting cell culture supernatants for ELISA

24 h post exposure to different treatment conditions, the cell culture supernatants were collected using pipettes in microfuge tubes and centrifuged at 1000 x g for 5 min in order to remove any cells that might be present in the supernatants. After centrifugation the supernatants were either used immediately or transferred into fresh microfuge tubes and stored at -80°C for use at a later date.

#### 3.4.2 e-Bioscience ELISA kit

Commercially available Ready-Set-Go!<sup>®</sup> ELISA kits from e-Bioscience Ltd. (San Diego, USA) were used to quantify the amount of IL-6 and IL-8 pro-inflammatory mediators present in the collected cell culture supernatants. The kit included all the necessary reagents such as:

1. **IL-6/IL-8 capture antibody:** captures the IL-6/IL-8 present in the samples.
2. **IL-6/IL-8 detection antibody:** binds to the captured IL-6/IL-8
3. **Avidin-HRP enzyme:** binds to the detection antibody
4. **Substrate:** Binds to the Avidin-HRP enzyme
5. **IL-6/IL-8 standards:** Used to produce a standard curve to quantify the IL6/IL-8 in pg/mL
6. **ELISA diluent:** Used to dilute the different reagents and also to block wells
7. **Coating buffer:** Used to dilute the capture antibody

All the reagents including the IL-6 and IL-8 standards were prepared as per the Certificate of Analysis' (CoA) document. Additionally, the following reagents which were not provided in the kit were prepared in the lab:

1. **Wash buffer:** Used to wash away excess or unbound antibodies post incubation time. Wash buffer was prepared by adding 90 g Sodium chloride and 5 mL Tween-20 detergent to 10 L distilled water. The resulting buffer solution was aliquoted in 0.5 L squeezer bottles for use.
2. **Stop solution:** Used to end the ELISA process. In the current study, 1 M Hydrochloric acid (HCl) was used as the stop solution.

### 3.4.3 ELISA experimental procedure

All assays were performed according to the manufacturer's instructions. Only specific high-affinity protein binding 96 well plates (NUCN Maxisorp™) were used for all ELISA experiments. The first step involved coating all wells of the 96 well plate with IL-6/IL-8 capture antibody. 100 µL of IL-6/IL-8 capture antibody (diluted in coating buffer as per CoA) was added to each well of the 96 well plate and incubated overnight at 4°C during which the capture antibody adhered to the plate. The following day, after discarding the existing capture antibody supernatant, the wells were washed with wash buffer for 3 times. Post washing, 200 µL/well of ELISA diluent was added to each well and incubated at room temperature for 1 h during which the ELISA diluent blocked the spaces in the well not occupied by the capture anti-bodies, thus restricting non-specific binding and background noise. After 1 h, the ELISA diluent was discarded and washed once with wash buffer. After this, 100 µL of the IL-6/IL-8 top standard concentration (IL-6: 200 pg/mL ; IL-8: 250 pg/mL) was added to the respective plates in triplicates. The top standard concentration was then serially half-diluted until the IL-6/IL-8 bottom standard concentration (IL-6: 1.56 pg/mL ; IL-8: 1.95 pg/mL) was reached. After this, 100 µL of samples (cell culture supernatants) were added to appropriate wells in triplicates. Two wells consisting of ELISA diluent only acted as blank. The plate was incubated at room temperature for 2 h. After 2 h, the wells were aspirated with wash buffer for 3 - 5 times. After washing, 100 µL of IL-6/IL-8 detection antibody was added to each well of the plate and incubated for 1 h at room temperature. After 1 h, the plates were washed with wash buffer for 3 - 5 times in order to remove any excess/unbound detection antibodies. After washing, 100 µL/well of Avidin-HRP enzyme solution was added and incubated at room temperature for 30 min. After 30 min, post washing the plate for 5 - 7 times to remove excess/unbound Avidin-HRP, 100 µL of substrate solution was added to each well and the plate was incubated at room temperature for 15 min. Depending upon the amount of IL-6/IL-8 detected, the standards and the

samples developed deep-blue (high IL-6/IL-8 concentration) to pale-blue colour (low IL-6/IL-8 concentration) at this stage. The reaction was stopped by adding 50  $\mu\text{L}$  of 1 M HCl to each well. Absorbance of each well was measured using a spectrophotometer (Multiskan, ThermoScientific, USA) at 450 nm wavelength. Using the absorbance data, a standard curve was generated in MS Excel software. Using the values from the standard curve, the absolute amounts of IL-6 and IL-8 (in  $\text{pg/mL}$ ) were then obtained.

### 3.5 Oxidative stress analysis

The cellular oxidative stress was analysed using a kit called ROS-Glo™ hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) assay (Promega Ltd, Southampton, UK). The kit consisted of three key components namely  $\text{H}_2\text{O}_2$  substrate solution, ROS-Glo™ Detection reagent and D-cysteine solution. The ROS-Glo™ assay works under the principle that when the substrate solution is added to the cells, it reacts with the  $\text{H}_2\text{O}_2$  present within the cell in order to get converted to a precursor of luciferin. Upon subsequent addition of D-cysteine, the luciferin precursor is converted to luciferin. Finally, when the ROS-Glo™ detection reagent containing recombinant luciferase enzyme is added, it reacts with the converted luciferin to produce a luminescent signal. The amount of luminescence (measured using a luminometer) is directly proportional to the amount of  $\text{H}_2\text{O}_2$  within the cells. All reagents were prepared according to the manufacturer's instruction.

#### 3.5.1 ROS-Glo™ assay experimental procedure

24 h post exposure to different treatment conditions, the cells were incubated for 3 h with 300  $\mu\text{L}$  of 25  $\mu\text{M}$   $\text{H}_2\text{O}_2$  substrate diluted in growth medium. After 3 h, the solution was transferred in 100  $\mu\text{L}$  triplicates into a white-walled 96 well plate. 100  $\mu\text{L}$  of detection solution was then added to each sample (1:1 ratio) and incubated at room temperature for 20 min after which the luminescence was measured using a luminometer. The amount of luminescence was directly proportional to the amount of  $\text{H}_2\text{O}_2$  produced within the cells.

### 3.6 Investigating apoptosis

Caspases are a family of protease enzyme that play a key role in mediating a controlled biological process called apoptosis which means programmed cell death. Several different caspases are involved in apoptosis although caspase 3 and 7 are the final set of caspases that are activated before apoptosis ensues (Elmore, 2007). Hence, these two caspases were investigated in the current study to explore the mechanism of cell death post exposure

to different treatment conditions. For this purpose, a kit called Caspase-Glo® 3/7 assay was purchased from Promega Ltd (Southampton, UK).

### 3.6.1 Caspase-Glo® 3/7 assay kit

The kit consisted of two chief components namely, lyophilised Caspase-Glo® 3/7 substrate and Caspase-Glo® 3/7 buffer solution. The assay works under the principle that upon addition of the substrate to the cells, the caspases 3 and 7 within the cells cleave the substrate which in-turn releases a luciferase substrate called aminoluciferin. Subsequently, aminoluciferin reacts with recombinant UltraGlo™ luciferase enzyme present in the detection reagent to produce a luminescent signal. The luminescence is directly proportional to the caspase 3/7 activity within the cells.

### 3.6.2 Caspase-Glo® 3/7 assay experimental procedure

Prior to addition to cells, the lyophilised Caspase-Glo® 3/7 substrate and the Caspase-Glo® 3/7 buffer solution were allowed to equilibrate to room temperature. The entire buffer solution was then added into the bottle containing the substrate and mixed together. This formed the caspase 3/7 detection reagent which was now ready to be used with cells. 24 h post exposure to different treatment conditions, caspase 3/7 detection reagent (mixed with growth medium in a 1:1 ratio) was added to cells and incubated at 37°C for 1 h. After 1 h, the solution was transferred in 100 µL triplicates into a white-walled 96 well plate and the luminescence of the samples was measured using a luminometer.

### 3.7 HPF-CALU 3 *in-vitro* co-culture model of human airways

The co-culture human airways model used in this study consisted of CALU 3 human bronchial epithelial cells and HPF cells co-cultured together on Costar® 12 mm SW inserts (Corning, New York, USA). Each SW insert consisted of a culture cup made of a porous polycarbonate membrane (0.4 µm pore size,  $1 \times 10^8$  pores/cm<sup>2</sup>) on which the cells were cultured as shown in Figure 3.3 (A). The culture cup was supported by a detachable holder which held the culture cup as shown in Figure 3.3 (A). The SW inserts with cells were placed in a 6 well plate supplied with growth medium. Once the cells were grown to confluence on the culture cup, it could be detached from the cup holder as shown in Figure 3.3 (A). The top of SW membrane on which the cells were cultured was referred to as the apical compartment and the bottom of the SW membrane where growth medium was supplied was referred to as the basolateral compartment as shown in Figure 3.3 (B).



**Figure 3.3. Pictorial representation of the SWs used produce the co-culture human airways model in the current study.**

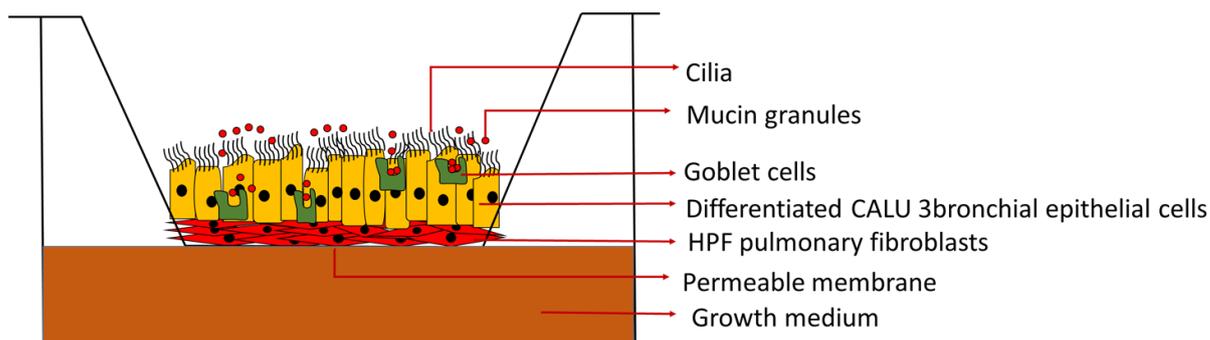
**(A) Two components of a typical SW.** As shown in the figure, the SW inserts consisted of two parts namely a culture cup and a detachable cup holder. While the culture cup consisted of a porous polycarbonate membrane (0.4 µm pore size) on which the cells were seeded and grown at ALI, the cup holder held the SWs on plate wells. After culturing the cells on the SW membrane, the culture cup could be detached from the holder and used for further investigations. **(B) Diagrammatic representation of the apical and basolateral compartments of a SW.**

### 3.7.1 Collagen coating of SW membrane

Prior to seeding cells, in order to increase the physiological relevance of the co-culture model, the SW membranes were pre-coated with human placental type IV collagen (Sigma, St. Louis, USA). Human placental type IV collagen powder was dissolved in appropriate amount of 3 % sterile filtered 0.5 M acetic acid to give a stock collagen concentration of 1 mg/mL. The stock was subsequently aliquoted in several microfuge tubes and stored at -20°C. On the day of coating, depending on the number of SW to be coated, sufficient number of frozen aliquots of collagen were thawed and used. Collagen was added to each SW at a concentration of 10 µg/cm<sup>2</sup>. The SW's used in the current project had a surface area of 11.2 cm<sup>2</sup> and hence 112 µg of collagen (112 µL of aliquoted collagen) was added to each SW membrane and incubated at room temperature for at least 2 h. This allowed sufficient time for the collagen to form a uniform feeding layer on the SW membrane. After 2 h, the excess collagen was removed and any remnant collagen on the SW membrane was neutralised by washing with 500 µL of growth medium 2 - 3 times. The collagen coated SW's were then used immediately to culture cells or stored at 4°C for up to a month.

### 3.7.2 HPF-CALU 3 co-culture model production on SW inserts

First, 300 µL of HPF cells were seeded on top of the collagen coated SW membrane (apical compartment) at a cell density of 3 x 10<sup>5</sup> cells/SW and the basolateral compartment was supplied with 2 mL of EMEM growth medium. After 4 days of submerged culturing in order to allow optimum proliferation of HPF cells, the apical medium was removed and 300 µL CALU 3 cells were added on top of the HPF cells at a cell density of 5 x 10<sup>5</sup> cells/SW and cultured with 2 mL of DMEM-F12 medium for a further 4 days under submerged conditions. After 4 days, the cells were established at ALI by aspirating the apical medium and refreshing the basolateral section with 2 mL DMEM- F12 medium, thus exposing the cells apically to air and basolaterally to growth medium. The cells were co-cultured at ALI for at least 11 - 14 days with the basolateral medium being refreshed every 2 - 3 days. After 14 days of ALI culturing, typical characteristics of pseudo-stratified epithelium such as microvilli expression, goblet cells production and inter-cellular tight junctions formation were exhibited (Bielemeier, 2012) as diagrammatically illustrated in Figure 3.4.



**Figure 3.4. Diagrammatic representation of the HPF-CALU 3 co-culture human airways model.**

11-14 days post ALI establishment, the CALU 3 bronchial epithelial cells differentiated in a pseudostratified fashion exhibiting cilia formation and mucus producing goblet cells production (Bielemeier, 2012). The cells also formed tight junction evidenced by minimal seepage of basolateral medium into the apical compartment.

### 3.8 Overall experimental design used in the project

For a comprehensive evaluation of EC *in-vitro*, a three-step experimental approach was employed in the current study. The three-step experimental design involved (1) high-throughput screening of chief EC constituents (nicotine, cotinine and E-vehicle) using submerged monocultures of bronchial epithelial cells cultures followed by (2) evaluation of different aspects of commercially available ECs (nicotine strength, flavours, brands) using submerged monocultures of several airways related cell types followed by (3) investigation of EC aerosols using physiologically relevant HPF-CALU 3 co-culture models. Such an experimental design can be viewed as a progressive experimental design with each methodology inspiring/informing the next method and moving towards a more physiologically relevant method than the prior. Additionally, such three-step experimental approaches have also been proposed by recent EC framework studies (Iskandar et al., 2016) which adds further confidence to the different methodologies employed in the current study.

Hence, according to the aims of the individual experiments of the current study, different methodologies were designed and employed based on two chief variables:

1. **Culture method:** Either submerged monocultures or ALI co-cultures
2. **Exposure method:** Direct exposure or extracts exposure or aerosol exposure

Based on these two chief variables, three different methodologies were employed in this study:

1. Direct exposure of submerged monocultures to individual constituents of cigarette/EC (Chapter 5 experiments)
2. Exposure of submerged monocultures to extracts of cigarette/EC (Chapter 6 experiments)
3. Exposure of ALI co-cultures to aerosols of cigarette/EC (Chapter 7 experiments)

## 4 Design, construction and validation of the in-house built smoking machine

### 4.1 Introduction

Just as different cell culture models used in cigarette/EC studies have a profound implication on the outcome of the study, the method of delivering cigarette smoke/EC aerosols to cells also has a major impact on the physiological relevance of the study. The chemicals of cigarette smoke or EC aerosols are dispersed between the vapour and particulate phases, with some chemicals present in both phases (Borgerding and Klus, 2005). During real-time smoking/vaping, the various cells of the *in-vivo* human airways environment interact with both the gaseous and particulate phase of cigarette/EC aerosols. As stated earlier in Chapter 2, sub-section 2.5, one of the ways to physiologically simulate this interaction between the human airway cells and cigarette/EC aerosols *in-vitro* is by exposing ALI cultured human airways cells to cigarette smoke/EC aerosols in their native form involving both gaseous and particulate phases (Aufderheide et al., 2003). For such an exposure, an aerosol delivery system capable of generating and delivering WCS/ECV over relevant cell culture systems in a controlled manner, closely simulating the human smoking/vaping behaviour is required (Ritter et al., 2004, Neilson et al., 2015). A typical aerosol delivery system consist of two chief components namely: (1) A smoking machine which is the system that generates WCS or ECV from a cigarette or EC respectively, ultimately delivering the generated aerosols to the cells in a controlled fashion and (2) A cell exposure module which is the system that houses relevant cell culture systems (mostly ALI systems) and exposes them to WCS/ECV (Aufderheide et al., 2003). An aerosol delivery system makes certain that the cells are exposed to WCS/ECV aerosols in as quick time as possible such that the aerosols do not undergo major chemical changes during the delivery process.

A number of smoking machines and exposure modules are available from commercial companies such as Vitrocell®, Borgwaldt, CULTEX® etc. These commercial systems were primarily produced for chemical characterisation of cigarette smoke constituents and hence the majority of the commercial smoking systems are designed for tobacco cigarette analysis, though in recent years there has been an increase in the production of EC specific systems as well (Fukano et al., 2004). The commercial smoking machines are sophisticated systems that provide options for manipulation with several factors such as the aerosol dilution, delivery rate, multiple cigarette puffing, multiple sample collection etc. Nevertheless, the commercial smoking systems are highly priced and most often require technical

experience to operate (Thorne and Adamson, 2013). Apart from commercial smoking machines, a number of bespoke smoking systems have also been developed by individual research groups (Thorne and Adamson, 2013). Although less sophisticated than commercial smoking machines, bespoke smoking systems are often less complex, easy-to-setup and cost-effective options compared to the commercial smoking machines. Also, bespoke smoking machines have the distinct advantage of modifying the system according to the particular requirements of a study.

In this context, in the current study, in order to investigate the effects of aerosols of EC and tobacco cigarette, a bespoke, automated aerosol delivery system was constructed to deliver WCS/ECV to the co-culture model of human airways in a controlled fashion. Throughout the study, the aerosol delivery system has been referred to as the 'smoking machine'. Using several electrical, mechanical and computing components, the current smoking machine was developed to be a user-friendly, cost-effective, automated and a flexible system that would mimic the human smoking/vaping conditions as closely as possible.

## 4.2 Aims and objectives

The main aim of this part of the study was to construct a bespoke, automated smoking machine that would generate and deliver WCS/ECV to the co-culture human airways model (as detailed in the Chapter 3, sub-section 3.7 ) in a controlled fashion in order to mimic the human smoking behaviour as closely as possible.

The specific objectives in order to accomplish this aim are as follow:

1. Review of literature of existing commercial and bespoke smoking machines
2. Develop a set of user requirements for the smoking machine
3. Develop product design specifications for the smoking machine
4. Develop operational design of the smoking machine
5. Construct the first prototype of the smoking machine for review
6. Construct the final working model of the smoking machine
7. Validate the smoking machine by verifying the ISO flow rate parameters
8. Compare and contrast the current smoking machine to the existing commercial models

### 4.3 Smoking machine types

Smoking machines can be broadly classified into two types:

1. **Rotatory systems:** Rotary machines consist of a rotor with several cigarette ports arranged across the circumference of the rotor. Most rotary machines have a central suction pump and as the rotor rotates at a specified timing, each cigarette port is puffed individually, thus collecting a cumulative sample from several cigarettes per run (Klus Hubert, 2016). This smoking machine type is more suitable for collection of chemicals present in very small quantities in the test aerosol. Figure 4.1 depicts one such rotary smoking machine called RM20D produced by Borgwaldt KC GmbH company (Hamburg, Germany)



**Figure 4.1. The Borgwaldt RM20D rotary smoking machine.**

As shown in the above figure, the cigarette ports of the Borgwaldt RM20D smoking machine are arranged around a rotor that puffs cigarettes one at a time. (Image source: Granat company website: [http://granat-e.ru/borgwaldt\\_rm20d.html](http://granat-e.ru/borgwaldt_rm20d.html) )

2. **Linear systems:** Linear machines consist of several cigarette ports arranged linearly. Unlike rotary systems, each cigarette port in linear machines has its own separate suction device such that samples are collected individually per port (Klus Hubert, 2016). This smoking machine type is more suitable for the collection of chemicals present in larger quantities in the test aerosol (Tindall). Figure 4.2 depicts one such linear smoking machine namely SM450 produced by a company called Cerulean (Milton Keynes, UK).



**Figure 4.2. The Cerulean SM450 20-channel linear smoking machine.**

As shown in the above figure, the cigarette ports of the Cerulean SM450 smoking machine are arranged in a linear fashion. (Image source: Visiontek company: <http://visionteknik.com/cerulean/smoking-machines/sm450-20-channel/>)

#### 4.4 Smoking regimes

Smoking regimes describe the standard conditions under which smoking machines operate in a controlled fashion. A typical human smoking behaviour consists of two phases: (i) a puff or drag phase, during which a cigarette is puffed and the resulting smoke inhaled and (ii) normal breathing phase, during which air from the ambient environment is inhaled between two consecutive puffs. For a smoking machine to fulfil these two conditions, it requires essential information such as puff volume, puff duration, inter-puff interval, puff number, ventilation blocking etc. Smoking regimes provide these essential parameters. There are several smoking regimes for tobacco cigarettes. Amongst them, the two most widely employed regimes are:

1. International Organization for Standardization (ISO) 3308: 2012 regime (ISO 3308, 2012)
2. Health Canadian Intense (HCI) regime (ISO/TR 19478-2, 2015)

#### 4.4.1 ISO 3308:2012 smoking regime

According to the ISO regime, the puff phase consists of a 35 mL puff for a duration of 2 s (or 1.050 L/min) drawn every 58 s without modifying the cigarette (ISO 3308, 2012). The normal breathing phase consists of air (ambient air) being delivered at a rate of 0.150 L/min for 58 s (ISO 3308, 2012).

#### 4.4.2 Health Canadian Intense (HCI) regime

HCI regime, also called the ISO/TR 19478-2:2015 standard, is a modified, more intense version of the ISO 3308 smoking regime. All parameters of the HCI regime are identical to ISO 3308 standards except the puff volume and inter-puff interval. The HCI regime consists of a 55 mL puff for a duration of 2 s occurring every 30 s instead of 60 s as in the case of ISO 3308 regime. Additionally, cigarettes used in HCI regime are required to have 100 % blockage of filter ventilation holes (ISO/TR 19478-2, 2015).

**Table 4.1. Comparison of smoking regime parameters between ISO 3308 and HCI smoking regime.**

<b>Regime</b>	<b>Puff volume</b>	<b>Puff duration</b>	<b>Puff interval</b>	<b>Filter blocking</b>
ISO 3308:2012	35 mL	2 s	60 s	0 %
HCI	55 mL	2 s	30 s	100 %

## 4.5 Review of commercially available smoking machines

Although a number of companies manufacture and sell smoking machines commercially, some companies are more established than others and their products have been well-characterised by studies. Popular smoking machine companies include, but are not limited to, Borgwaldt, Burghart, Vitrocell® and CH technologies Ltd. Similarly, some of the popular companies specialised in manufacturing cell exposure modules include CULTEX®, BAT Ltd., and Vitrocell®. For the purpose of this study, the workings and limitations of the four most widely-employed commercial smoking machines have been described below.

### 4.5.1 CSM-SCSM smoking machine

The CSM-SCSM smoking machine, manufactured by CH technologies (New Jersey, USA), is capable of analysing cigarettes, ECs, cigars and even pharmaceuticals. Apart from ISO and HCl regimes, it can also operate under customised regimes. The CSM-SCSM can collect mainstream as well as side-stream smoke and has an air-dilution system to produce different concentrations of aerosols. One of the limitations of the CSM-SCSM is that it can puff only one cigarette at a time and hence is applicable only for small scale applications (CH Technologies). Additionally, this smoking machine does not come with a cell-exposure module, thus mandating purchase of a separate cell-exposure module which increases the overall cost involved.

### 4.5.2 Burghart MSB-01 smoking machine

The Burghart MSB-01 smoking machine manufactured by Burghart Tabaktechnik (Wedel, Germany) has a distinct advantage over the other commercial smoking machines in that it comes with a dedicated multi-well exposure plate specifically designed for *in-vitro* experimentation (Scian et al., 2009a). Cells cultured on the exposure plate (96 well plate) can be directly exposed to WCS generated from MSB-01 smoking machine, thus eliminating the requirement for an independent exposure system. One major limitation of MSB-01 system is its inability to test cells cultured at ALI. The smoking machine's design permits exposure of WCS aerosols on cells grown under submerged cultures only, there-by lacking physiological relevance of the *in-vivo* smoke-airways interaction (Thorne and Adamson, 2013).

#### 4.5.3 Borgwaldt RM20S smoking machine

The Borgwaldt RM20S manufactured by Borgwaldt KC GmbH (Hamburg, Germany) is a rotary smoking machine capable of puffing up to 8 cigarettes simultaneously. This smoking machine has the added flexibility of analysing cigarette smoke (of one cigarette type) at eight different concentrations or cigarette smoke from 8 different types of cigarette at a single concentration (Kaur et al., 2010). A syringe system is used to draw cigarette smoke and a subsequent dilution system delivers the cigarette smoke at preferred concentrations (Kaur et al., 2010). One of the limitations of RM20S smoking machine is that it is frequently paired with the BAT cell exposure chamber (Curbridge Engineering, Southampton, UK), thus limiting comparisons with studies employing other exposure systems (Thorne and Adamson, 2013). Besides, one particular study which characterised the RM20S smoking machine reported that there was considerable loss of mainstream smoke (~48%) within the system before reaching the cell chamber (Adamson et al., 2011).

#### 4.5.4 Vitrocell® smoking machines

A variety of smoking machines namely VC1, VC 1/7, VC 10®, VC 10®-S, each with varying features/specifications, are produced by Vitrocell® Ltd (Waldkirch, Germany). For the purpose of this study, only VC 10® is described (VITROCELL®systems) since this model has been used in many past studies (Sailland et al., 2017, Adamson et al., 2017, Thorne et al., 2018b). VC 10® is a rotary smoking machine consisting of a rotating cigarette carousel. A piston/syringe system is used to puff a cigarette/EC and the resulting smoke/vapour is diluted with air delivered via a flow-dilution chamber (Adamson et al., 2013, VITROCELL®systems). The diluted WCS/ECV is then delivered to the Vitrocell® cell exposure system where individual permeable inserts (SWs/TWs) carrying ALI cell cultures are treated to different dilutions of WCS/ECV. One limitation of Vitrocell® smoking machines is that, similar to the RM20S smoking machine, Vitrocell® systems are most compatible with CULTEX exposure systems, thus limiting the flexibility to use this smoking machine with other exposure systems (Thorne and Adamson, 2013). The characteristics of the four different commercial smoking machines described above have been tabulated and compared in Table 4.2 below.

**Table 4.2. Comparison of characteristics of different commercially available smoking machine.**

While the author of the current study reviewed each of the smoking machines individually by contacting relevant companies and obtaining data-sheets, some of the technical specifications of each smoking machine was obtained from a study by Thorne and Adamson (2013), permission for which had been granted by the author of the study.

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## 4.6 Review of *in-vitro* cell exposure studies performed using smoking machines

Studies employing smoking machines can be broadly classified into:

1. **Chemical evaluation studies:** Involves employing smoking machines for the assessment of quantity/quality of the various chemicals found in aerosols of WCS or ECV.
2. **Cell exposure studies:** Involves employing smoking machines to deliver WCS or ECV to cultured cells and analyse post-exposure WCS/ECV cytotoxicity.

Since chemical analysis of WCS/ECV was outside the scope of the current study, the current sub-chapter focuses on the various *in-vitro* cell exposure studies that employed smoking machines for WCS/ECV delivery to cells. Both commercial as well as bespoke smoking machines have been employed in a number of past *in-vitro* cell-exposure studies.

### 4.6.1 Studies employing commercial smoking machines

The CSM-SCSM smoking machine has been used in several toxicological studies. Lerner et al (2015) employed CSM-SCSM smoking machine to investigate the effects of ECV generated by the CSM-SCSM on ALI cultured H292 human lung epithelial cells (Lerner et al., 2015). ECV was delivered to the cells at an intense customised regime consisting of a 4 s puff every 30 s for different time durations such as 5 min, 10 min and 15 min. Lei et al (2017) employed the CSM-SCSM smoking machine under a similar intense smoking regime as Lerner et al to study the effects of nicotine and ECV on myofibroblasts differentiation (Lei et al., 2017).

Very few studies have been performed using the Burghart MSB-01 smoking machine. Scian et al (2009) performed an exhaustive characterisation of the Burghart MSB-01 machine including smoke loss measurement, particle analysis, dilution reproducibility and also tested WCS cytotoxicity on BEAS 2B bronchial epithelial cells (Scian et al., 2009a). The study reported that the intra-day and inter-day variability in WCS particulate matter depositions produced by MSB-01 was less than 5 % and 13 % respectively. Moreover, the study also reported that the intra-day and inter-day variability of EC<sub>50</sub> (dose required to produce half-maximal cytotoxic effect) of WCS on BEAS 2B cells were 13 % and 20 % respectively, thus exhibiting good reproducibility of results (Scian et al., 2009a). However, the same team in a separate study, reported on the excessive loss of WCS and also the changes in chemical composition of WCS that occurs in the MSB-01 smoking system prior to reaching the exposure chamber (Scian et al., 2009b).

The Borgwaldt RM20S smoking machine has been used in several *in-vitro* cytotoxicity studies. Phillips et al (2005) investigated the acute effects of WCS produced using the RM20S smoking machine on ALI cultured NCI-H292 bronchial epithelial cells (Phillips et al., 2005). Cigarettes were smoked at ISO 3308 regime for 30 min and post-exposure analysis was performed 20 h after exposure (Phillips et al., 2005). In a 2007 follow-up study by the same research group, Maunders et al (2007) used RM20S smoking machine and BAT exposure module to investigate the genetic changes in NCI-H292 bronchial epithelial cells in response to acute WCS exposure (Maunders et al., 2007). Leigh et al (2016) employed Borgwaldt LX-1 smoking machine model to report on the effect of flavouring substances in EC aerosol induced cytotoxicity in NCI-H292 bronchial epithelial cells (Leigh et al., 2016).

Different models of Vitrocell® smoking systems have been employed in a number of past *in-vitro* studies, especially to analyse EC cytotoxicity. Neilson et al (2015) employed a Vitrocell® VC1 smoking machine to investigate the acute effects of tobacco as well as ECs on 3D EpiAirway™ human bronchial epithelial tissues (Neilson et al., 2015). While cells were exposed to WCS at ISO 3308 regime, ECs were delivered to cells at a customised intense regime of 80 mL puff over 3 s every 30 s. Misra et al (2014) employed a Vitrocell® VC10 smoking machine to generate aerosols of cigarette/EC in order to compare the *in-vitro* cytotoxicity profiles of EC pad collected aerosols and tobacco cigarette wet particulate matter (Misra et al., 2014). ECV and WCS were both generated at HCI regime and collected on filter pads post which the eluted condensates were treated to A549 carcinoma cells and cytotoxicity analysed post 24 h. (Misra et al., 2014).

#### 4.6.2 Studies employing bespoke smoking machines

As stated in the introduction of this chapter, although bespoke smoking systems may not be comparable to the level of sophistication of commercial smoking machines, such bespoke systems however offer several advantages such as low-cost, easy maintenance and less complexity. A number of studies have designed and constructed bespoke smoking systems to suit the specific requirements of their studies. In one such study, a bespoke smoke delivery system was employed to investigate the effects of WCS on the innate immune response of healthy and COPD patients to *Moraxella catarrhalis* infection (Zhang et al., 2011). The smoking apparatus consisted of a 1000 mL flask which housed the ALI cultured bronchial epithelial cells on permeable supports. Using a mini pump, standard reference cigarettes were puffed and WCS was drawn into the flask for 10 min (Zhang et al., 2011). In another study, St-Laurent et al (2009) employed a bespoke smoking system consisting of a 60 mL syringe and an impinge apparatus which was used to draw cigarette smoke from standard reference cigarettes at HCI smoking regime. A customised exposure

chamber was used to expose WCS to ALI cultured bronchial epithelial cells (St-Laurent et al., 2009).

In another study, Gualerzi et al (2012) employed a simple, yet unique design for generating WCS. As shown in Figure 4.3, the smoking system of this study consisted of a T-shaped instrument (like a T-shaped tube) to which small pneumatic pipes were plugged at each end. At one end of the T-tube, a regular cigarette was connected while a syringe was connected at the opposite end (Gualerzi et al., 2012). A sterile flask containing partially submerged oral mucosa explants from healthy non-smoking volunteers was connected to the perpendicular end of the T-tube. The T-tube had a 'central tap' at the connection point which served to regulate the flow of generated cigarette smoke. The cigarette smoke was drawn by the pulling the syringe and when the central tap was 'switched', the cigarette smoke was directed into the flask. During the time between two puffs, the flask was dismantled from the set-up, thus allowing 'exhalation' of cigarette smoke and entry of ambient air (Gualerzi et al., 2012).



**Figure 4.3. The bespoke smoking system employed by Gualerzi et al (2012) in their study.**

The set-up consisted of a T-shaped instrument connected to a reference cigarette, syringe and a flask containing the oral mucosa explants partially submerged in PBS. A 'central tap' modulated the flow of cigarette smoke into the flask from the syringe. (B) Schematic illustration of the smoking process using the T-shaped instrument. (Image reproduced from: Gualerzi et al, 2012)

In this respect, several economical, functional bespoke smoking systems have been constructed and employed in many cell exposure studies. The smoking machine used in the current project was one such set-up which was designed to suit the requirements of the current study.

## 4.7 Design/build phase of the smoking machine

A number of user-requirements and product design specifications were first conceived using which the current smoking machine was subsequently designed.

### 4.7.1 User requirements of the smoking machine

The smoking machine of the current study was conceived to:

1. Closely mimic the human smoking conditions i.e. the different parameters of the smoking regimes including the puff volume, puff duration and puff frequency
2. Mimic the physiological/biological conditions as closely as possible i.e. deliver WCS/ECV to ALI co-cultures
3. Deliver WCS/ECV to the ALI co-culture in the shortest route possible in order to prevent major chemical changes in the WCS/ECV during the delivery time
4. Keep cigarettes alight during the time between two consecutive puffs
5. Be completely automatic except replacement of cigarette/ EC's
6. Be flexible i.e. ability to operate under different smoke regimes (ISO or HCl or custom)
7. Have minimal leakage of WCS/ECV before reaching the cells
8. Be compatible with the cell exposure module and the cell manifolds blocks
9. Be user-friendly i.e. easy connection and disconnection of different components
10. Experience minimal wear and tear issues i.e. minimal moving parts which would need regular replacement
11. Have good visibility of each component
12. Be as sterile/clean as possible in order to prevent possible contamination of cells
13. Prevent contamination between tests involving different products e.g. Cigarettes vs. EC
14. Have good reproducibility of flow rates
15. Be low cost with easy to obtain parts

#### 4.7.2 Product Design Specifications (PDS) of the smoking machine

Based on the user-requirements, the following PDS were chosen:

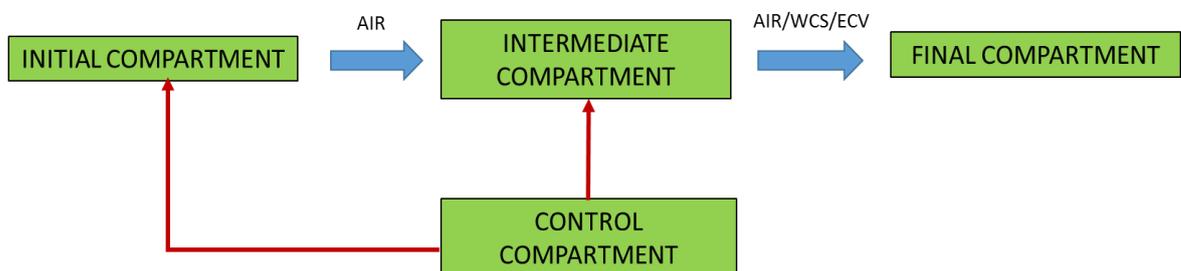
1. The smoking machine must have the capacity to be operated under standard ISO 3308 smoking regime in order to mimic the human smoking pattern as close as possible. The puff phase of the ISO 3308 standard consists of a 35 mL puff drawn over 2 s every 60 s i.e. at a flow rate of 1.050 L/min during puff phase. During the normal breathing phase i.e. intermediate time between two puffs, air from ambient atmosphere would be delivered at a rate of 0.150 L/min to the cells.
2. For tobacco cigarettes, in order to keep the cigarette alight during the time between two consecutive puffs, an air-flow of 0.150 L/min would be delivered to the cigarettes. This phase was called 'cigarette in ash-tray' phase. (This air-flow will not be required for ECs since no burning is involved)
3. In order to minimise WCS/ECV losses, push-fit pneumatic tubes and associated adapters would be used to construct smoking machine.
4. In order to deliver WCS/ECV to the cells in the shortest route possible, the smoking machine would be designed such that the pneumatic tube delivering WCS/ECV to the exposure chamber will be no longer than 15 cm.
5. In order to facilitate independent and automatic operation, the smoking machine would be computer controlled.
6. The smoking machine would run as per a specific set of computer codes. If a different smoke regime was to be employed, the codes would be altered accordingly.
7. In order to facilitate good visibility of components, the smoking machine would be aesthetically designed such that there would be no concealed components.
8. Components that come in direct contact with WCS/ECV would be water-washable in order to keep them clean/sterile.
9. The smoking machine would be designed to have separate components for each treatment condition i.e. separate components for air, WCS and ECV.
10. The smoking machine would have the capacity to incorporate an electronic flow sensor to confirm reproducibility of flow rates in order to validate the smoking machine.
11. The maximum cost of the smoking machine will be kept as low as possible without compromising the functionality of the smoking machine.

## 4.8 Design phase

### 4.8.1 Designing the different smoking machine compartments

Considering the user-requirements and PDS, the current smoking machine was designed to have four distinct compartments as shown in Figure 4.4:

1. **The initial compartment:** The components of this compartment would pump air from the ambient atmosphere into the smoking machine system.
2. **The intermediate compartment:** This compartment consisted of a cigarette chamber in which the cigarette or EC would be housed and puffed.
3. **The final compartment:** The components of this compartment would house the SWs containing the co-culture human airway cells which would be exposed to WCS/ECV.
4. **The control compartment:** The components of this compartment would automate the smoking machine and regulate the smoking machine flow rates and timings as per the desired regime.



**Figure 4.4. Over-view of the construction design of the smoking machine.**

The smoking machine was designed such that the components of initial compartment pumped air into the intermediate compartment which housed the cigarette or EC and was subsequently puffed. The aerosols were then delivered to the components of the final compartment consisting of the co-culture model while the control compartment components modulated the flow rates and timings of the smoking machine.

#### 4.8.2 Components used to construct the smoking machine

The smoking machine consisted of several inter-connected mechanical, pneumatic and electrical components. While a detailed description of each component would be explained in subsequent relevant sections, a brief introduction of the different components and their functions is as follow:

1. **12 V diaphragm gas pump (x 2):** Two 12 V diaphragm gas pumps (Gardner Denver Thomas, Munich, Germany) namely P 1 and P 2 were used to pump ambient air into the smoking machine system.
2. **3/2 ON/OFF solenoid valves (x 4):** Four 3/2 solenoid valves (M&M international, Italy) namely V 1, V 2, V 3 and V 4 were used to direct the pumped air into the appropriate subsequent components of smoking machine.
3. **Analogue flow meters (x 3):** Three analogue flow meters (Key Instruments, Pennsylvania, USA) namely AF 1, AF 2 and AF 3 were used to manually adjust and visually verify the air/WCS/ECV flow rates. While AF 1 and AF 2 were of 0.05 - 0.5 L/min range, AF 3 was of 0.1 - 1.2 L/min range.
4. **A customised cigarette chamber:** A bespoke cigarette chamber consisting of a cylindrical glass tube and an aluminium cigarette holder was used to house and subsequently puff cigarette/EC in order to generate WCS/ECV.
5. **Cell manifold Perspex blocks (x 3):** Three cell manifold blocks made of Perspex (Harvard Apparatus, Massachusetts, USA) were used to house the SWs which contained the co-culture human airways model.
6. **Gas diffusion system:** A horizontal gas diffusion system called NAVICYTE (Massachusetts, USA) was used to simultaneously and uniformly deliver air/WCS/ECV to the cell manifold Perspex blocks.
7. **Arduino microcontroller:** An Arduino (Torino, Italy) is a micro-controller device that is used to operate/automate different electro-mechanical devices. In the current study, an Arduino 2560 was used to operate the smoking machine under standard ISO 3308 conditions. The Arduino program regulated the timings and the flow rates of the smoking machine during operation.

8. **Pneumatic tubes and pneumatic Y-adapters:** Hard push-fit pneumatic tubes with 6 mm outer diameter and 4 mm inner diameter and associated pneumatic push-fit Y-adapters were used to connect the different components of smoking machine.

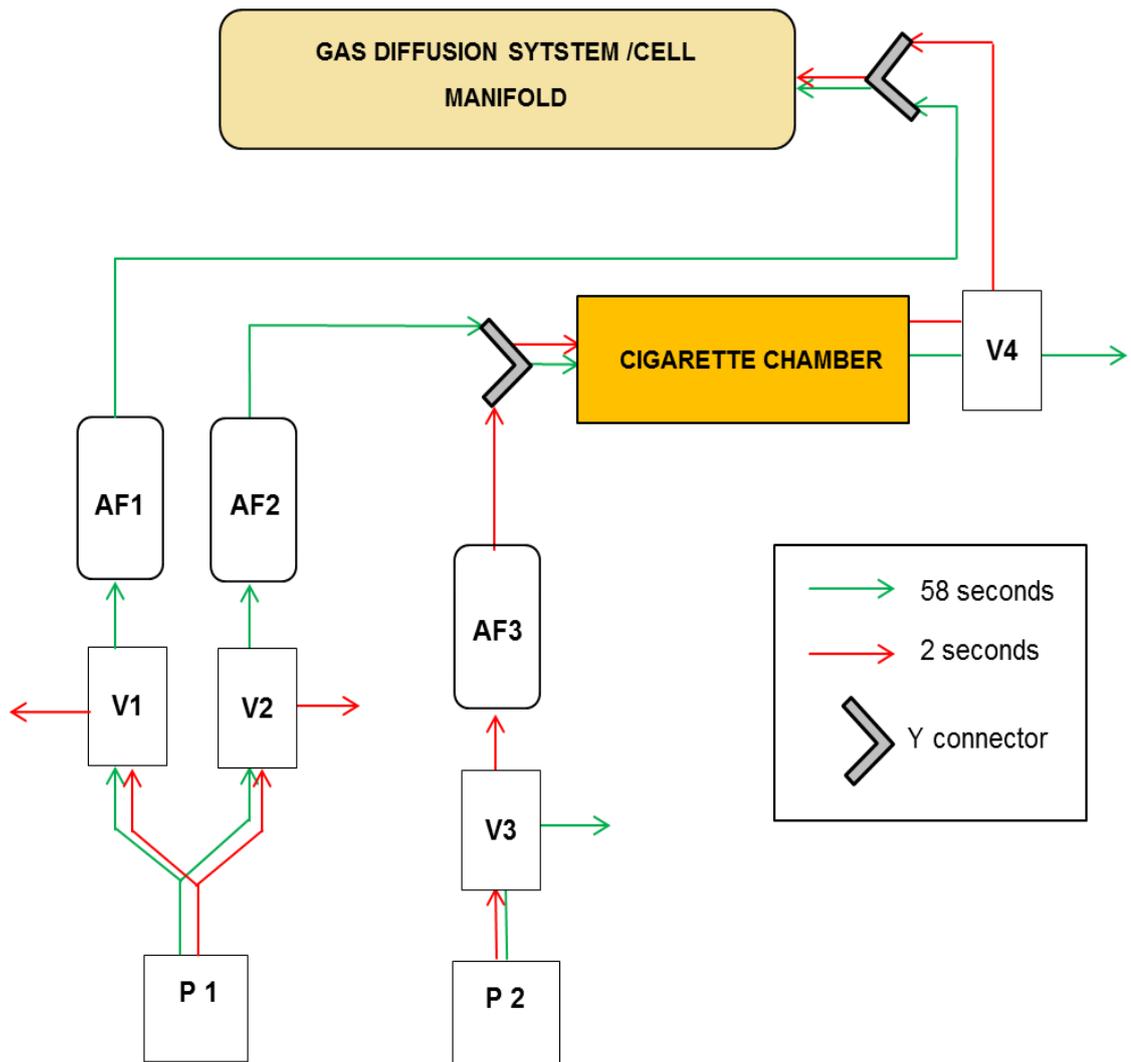
The different components used in the construction of the smoking machine belonged to specific compartments of the smoking machine design as illustrated in Table 4.3.

**Table 4.3. Components associated to different compartments of the smoking machine design**

Initial compartment	Intermediate compartment	Final compartment	Control compartment
Diaphragm pumps P1, P 2	Cigarette chamber	Cell manifold Perspex blocks	Arduino and associated modules
Solenoid valves V 1, V 2 and V 3	Solenoid valve V 4	Gas diffusion system	
Analogue flow meters AF 1, AF 2 and AF 3			

#### 4.8.3 Designing the air-flow system (initial compartment)

As described earlier in the PDS sub-chapter (section 4.7.2), the current smoking machine was designed to simulate the three phases of the human smoking cycle i.e. the puff phase, the normal air breathing phase and the 'cigarette in ash-tray' phase. Figure 4.5 represents the pneumatic flow diagram illustrating the direction of flow of air/WCS/ECV occurring during a typical one minute cycle of the smoking machine operation. As illustrated in Figure 4.5, diaphragm pump P 1 was connected to solenoid valves V 1 and V 2 via a pneumatic Y-adapter. V 1 and V 2 were in-turn connected to analogue flow meters AF 1, AF 2. Similarly, diaphragm pump P 2 was connected to solenoid valve V 3 which in-turn was connected to analogue flow meter AF 3.



**Figure 4.5. Pneumatic flow design illustrating the air/WCS/ECV flow in the smoking machine during a one minute cycle.**

In the following flow diagram, the green arrow represents the direction of flow occurring during the 58 s phase of the smoking cycle and the red arrow represents the direction of flow occurring during the 2 s puff phase. This alternating process of 58 s normal breathing phase and 2 s puff phase was continuously implemented until the power supply was turned off. At common points where two inlets lead to the same outlet, such as the point before cigarette chamber (common point for tubes arising from AF 2 and AF 3) or the point before the gas diffusion system (common point for tubes arising from AF 1 and V 4), a pneumatic push-fit Y-connector was used to connect two push-fit pneumatic tubes to a common outlet tube.

The 3/2 'normally open' solenoid valves used in the study had one inlet and two outlets, one of which was vent. Depending upon the voltage applied, each solenoid valve switched between the two outlets i.e. the valve switched to the outlet port when a voltage was applied (referred to as 'ON' condition) or vented out in the absence of voltage (referred to as 'OFF' condition). This switching of the solenoid valves was critical in the current smoking machine design since it was this switching process that determined the direction of flow of air/WCS/ECV in the system.

Under the Arduino program (which is described in detail in the later section 4.8.6) used to operate the smoking machine under ISO 3308 regime, for 58 s during the air-breathing phase, the air from pump P 1 splits into two streams. One of the stream passed into solenoid valve V 1 (and subsequently AF 1) and the other stream passed into V 2 (and subsequently AF 2). The stream of air from V 1 and AF 1 entered the gas diffusion system at a flow rate of 0.150 L/min, thus delivering air to the cells for 58 s. The other stream of air from V 2 and AF 2 entered the cigarette chamber at a similar flow rate of 0.150 L/min and vented via V 4 in order to keep the cigarette alight during the 58 s. During this entire time of 58 s, the air from pump P 2 vented out via V 3 (controlled via Arduino program). Alternatively, during the 2 s puff phase, the air from pump P 2 passed into V 3 (and subsequently AF 3) which then entered the cigarette chamber at a flow rate of 1.050 L/min and puffed the cigarette or EC housed in the cigarette chamber. The generated WCS/ECV then exited the cigarette chamber via solenoid valve V 4 and entered the gas diffusion system and subsequently into Perspex cell manifolds. During this 2 s time, the air from pump P 1 vented out via V 1 and V 2. In Figure 4.5, the direction of flow of air/WCS occurring during the 58 s phase is depicted by green lines whereas the direction of flow of air/WCS/ECV occurring during the 2 s phase is depicted by red lines.

The analogue flow meters used in the current study served two chief purposes. Firstly, it could be used to manually adjust the flow rate of the air flowing through the smoking machine. Using the flow meter dial, the flow rate could be adjusted manually such that AF 1 and AF 2 (connected to V 1 and V 2) invariably delivered air at 0.150 L/min corresponding to the air breathing phase and AF 3 (connected to V 3) invariably delivered air at 1.050 L/min into the cigarette chamber corresponding to the puff phase. Secondly, the analogue flow meters also served as a way to visually verify the flow rates during the different phases of smoking machine operation.

The smoking machine was designed such that each individual component of the machine was specifically associated with one of the three phases of the human smoking cycle. The association between the gas pumps, solenoid valves, flow meters and the different phases

is tabulated in Table 4.4. As described in Table 4.4, the diaphragm pump P 1 was associated with the air breathing phase and the ‘cigarette in ash-tray’ phase while the diaphragm pump P 2 was associated with the puff phase. The solenoid valves V 1 and V 2 were associated with the air breathing phase and the ‘cigarette in ash-tray’ phase respectively while the valves V 3 and V 4 were associated with the puff phase. Similarly, the analogue flow meters AF 1 and AF 2 were associated with the air breathing phase and ‘cigarette in ash-tray’ phase respectively while the analogue flow meter AF 3 was associated with the puff phase.

**Table 4.4. Connection, timings and association of the different components of the smoking machine with the different phases of the smoking cycle.**

The rows highlighted in green represents the components involved in the 58 s phase and the rows highlighted in red represents the components involved in the 2 s phase of the smoking cycle.

Time ‘ON’	Pump connection	Solenoid valve connection	Flow meter connection	Function/ flow rate
58 s	P 1	V 1	AF 1	Normal breathing (0.150 L/min)
58 s	P 1	V 2	AF 2	‘Cigarette in tray’ (0.150 L/min)
2 s	P 2	V 3/ V 4	AF 3	Cigarette/ECV (1.050 L/min)

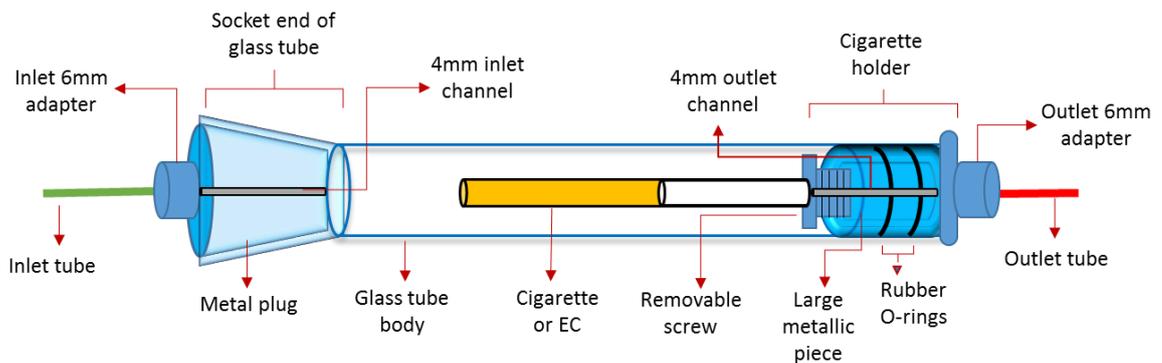
#### 4.8.4 Designing the cigarette chamber (intermediate compartment)

The cigarette chamber consisted of a three parts:

- a. A cylindrical, transparent custom-made glass tube
- b. A customised aluminium cigarette holder which was used to hold the cigarette/EC
- c. A aluminium metal plug which was used to close the glass tube in order constitute a closed-system

#### 4.8.4.1 Customised cigarette chamber glass tube

A SIMAX ground joint-socket glass tube with customised dimensions purchased from Scientific glass Ltd. (Staffordshire, UK) was used as cigarette/EC chamber in which the cigarette/EC would be puffed. The length and diameter of the glass tube was chosen such that it would accommodate all the ECs of different lengths used in the current study. The longest EC used in the current study was 100 mm in length and hence a glass tube with the following shank dimensions was chosen: 180 mm length, 34 mm outer diameter and 30 mm inner diameter. The socket end on the other side of the glass tube had a length of 32 mm and size B 29. An aluminium cigarette holder was fitted at the shank end and of the glass tube and the aluminium metal plug was fixed at the socket end as shown in Figure 4.6. When all three components were connected, the cigarette chamber formed a sealed system with an inlet and an outlet tube.



**Figure 4.6. Diagrammatic illustration of the bespoke cigarette chamber.**

The cigarette chamber consisted of three parts namely: a customised ground joint socket glass tube, an aluminium metal plug fixed at the socket end of the glass tube and an aluminium cigarette holder secured at the shaft end of the glass tube. As shown in the above figure, the metal plug at the socket end had an inlet 6 mm push-fit adapter which delivered the air from diaphragm pumps P 1 or P 2 (during different phases) into the cigarette chamber via a 4 mm inlet pipe which ran along the length of the metal plug. The cigarette holder consisted of two parts namely a threaded removable screw and a larger metallic piece (referred as main piece), both of which were made of aluminium. While the removable screw secured the cigarette/EC in position, the main piece had a threaded socket into which held the removable screw was fixed. The cigarette holder was then inserted at the shaft end of the glass tube which was firmly held in position with the help of two rubber O-rings fixed to the main piece. While each EC, depending upon its diameter had a dedicated removable screw, the main piece remained constant for all ECs. Once puffed, the WCS/ECV exited the cigarette chamber via a 4 mm outlet channel in the cigarette holder into a 6 mm outlet adapter. The outlet tube subsequently delivered the WCS/ECV to either the gas diffusion system or vented out depending on the Arduino program supplied to the smoking machine

#### 4.8.4.2 Customised aluminium metal plug

The bespoke metal plug made of aluminium (John Keatleys, Birmingham, UK) was designed (performed by engineering technician Bill) and manufactured in-house in order to close the socket end of the glass tube such that the cigarette chamber formed a closed system in which the cigarette/EC could be puffed. It had an outer diameter of 30 mm such that it firmly secured into the socket of the ground joint glass tube. The metal plug also had a threaded port into which a 6 mm push-fit adapter was fixed as shown in Figure 4.7. A pneumatic tube connected to this push-fit adapter served as the inlet tube of the cigarette chamber which delivered air from the diaphragm pump P1 or P2 into the cigarette chamber via a 4 mm inlet channel that ran along the length of the metal plug as shown in Figure 4.6.



**Figure 4.7. Different parts of the (A) aluminium metal plug and (B) the cigarette holder.**

#### 4.8.4.3 Customised aluminium cigarette holder

The bespoke cigarette holder made of aluminium (John Keatleys, Birmingham, UK) was designed in association with Shaun McClelland who was a summer project student mentored by the author of this study. It consisted a removable threaded screw which held the cigarette/EC and a main piece which in-turn held the removable screw in its socket. In essence, the term 'cigarette holder' was a collective term given to the combination of the removable screw and main piece. The reason for incorporating a removable screw in the cigarette holder design was to accommodate the variable diameters of different ECs used in the current study. Hence each EC, according to its diameter had a dedicated removable screw while the main piece remained constant for all ECs.

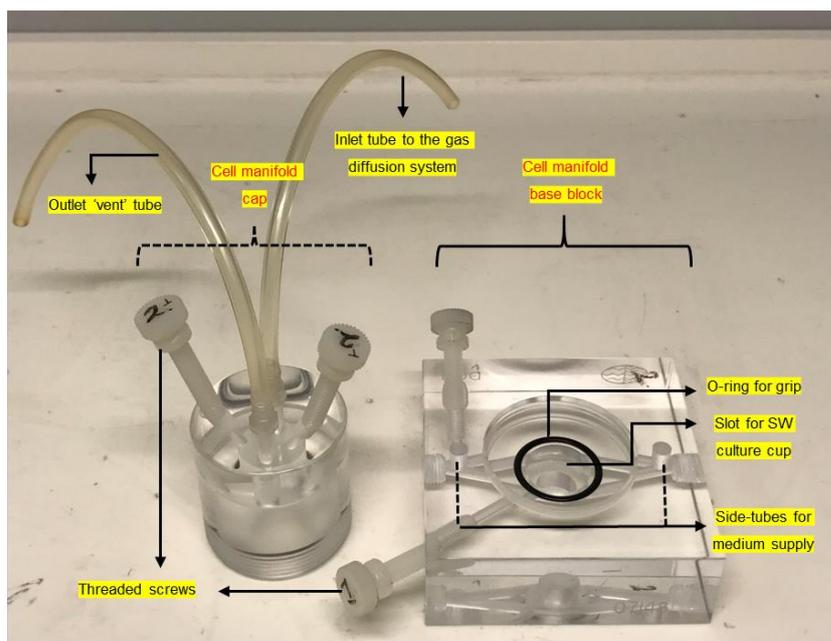
The main piece had two rubber O-rings (outer diameter = 30 mm, inner diameter = 22 mm, cross-section = 4 mm) fixed along its length at 10 mm apart as shown in Figure 4.6. The O-

rings served to firmly hold the cigarette holder inside the glass tube. Additionally, a smaller O-ring (outer diameter = 14 mm, inner diameter = 10 mm, cross-section = 2 mm) was attached at the bottom of the threaded socket of the main piece. This smaller O-ring secured the removable screw firmly into the socket. Similar to the metal plug at the socket end, the cigarette holder also had a 6 mm push-fit adapter and 4 mm outlet channel that ran along the length of the cigarette holder as shown in Figure 4.7 and Figure 4.6 respectively. The tube attached to this 6 mm adapter acted as the outlet tube and would deliver air/WCS/ECV either to the gas diffusion system or vent, depending upon the Arduino program supplied.

#### 4.8.5 Integrating the cell exposure system (final compartment)

##### 4.8.5.1 *Cell manifold Perspex blocks*

The Perspex cell manifold blocks were purchased from Harvard apparatus (Massachusetts, USA). These blocks housed the SWs containing the co-culture human airway cells which would be exposed to air/WCS/ECV. As depicted in Figure 4.8, each cell manifold consisted of a base block and a cap. The base block consisted of a circular slot (fixed with a corresponding size O-ring for extra grip) into which the SW culture cup was inserted. The base block also had two side tubes through which growth medium was supplied. Additionally, the base block had two threaded plastic screws which prevented medium leakage from the block. The cell manifold cap had slots for two Tygon<sup>®</sup> custom tubings, one each for inlet and outlet tubes respectively. The cell manifold cap also had two threaded plastic screws which served to prevent leakage of gas (air/WCS/ECV) from the cell manifold. Once the SW culture cups were fixed and medium supplied in the base block such that the base of the SW membrane touches the medium, the cell manifold cap was secured on top of the base block. During exposure, three cell manifold blocks were placed on the gas diffusion systems' base platform and the inlet tube of each block was connected to gas ports of the gas diffusion system.



**Figure 4.8. Picture of a cell manifold Perspex block used to expose the co-culture human airways cells to air/WCS/ECV in the current study.**

As shown in the above figure, the base block of the cell manifolds consisted of a circular slot for the SW culture cup to be inserted. Once the SW culture cup was inserted into the base block, the cap was secured on top of the base block. The cell manifold cap consisted of two custom tubes (inlet and vent) which would be connected to the gas diffusion system ports. Threaded screws served to prevent leakage of medium from the base block and loss of gas from the cell manifold respectively.

#### 4.8.5.2 Horizontal gas diffusion system

In order to deliver air/WCS/ECV simultaneously and uniformly to the three cell manifold Perspex blocks, a horizontal gas diffusion system called NAVICYTE from Harvard apparatus (Massachusetts, USA) was employed. The NAVICYTE gas diffusion system consisted of a flat platform as shown in Figure 4.9 on which the cell manifold Perspex blocks would be placed. The inlet tubes from each of the three cell manifold caps was connected to three gas diffusion ports (indicated by green arrows in Figure 4.9). All other gas ports were tightly wrapped with Parafilm tapes (Heathrow Scientific, Nottingham, UK) in order to avoid loss of air/WCS/ECV through the unused ports (indicated by red arrows in Figure 4.9). The gas diffusion system also had screws to tightly secure the cell manifold blocks on the platform during experiments.



***Figure 4.9. The NAVICYTE gas diffusion system used in the current study to deliver air/WCS/ECV simultaneously to the individual cell manifold Perspex blocks.***

The cell manifold blocks were secured firmly to the base platform by tightening the screws provided at two corners of the base block. Post connecting the inlet tubes of the three cell manifold blocks to their respective ports (indicated by the green arrows in the above figure), the unused gas ports were tightly sealed with Parafilm paper (indicated by red arrows in the above figure), thus preventing loss of air/WCS/ECV from the unused ports. The final tube from the smoking machine carrying air/WCS/ECV from the cigarette chamber can also be seen in the above figure.

#### 4.8.6 Designing the control compartment

For the smoking machine to operate under standard ISO 3308 conditions, it required a control system that regulated the timings and flow of air/WCS/ECV during the two phases of the smoking cycle. An Arduino 2560 microcontroller (Arduino, Torino, Italy) was used to fulfil this role.

##### 4.8.6.1 An introduction to Arduino

An Arduino micro-controller board is an open source electronic platform used to control different electro-magnetic devices. The board consists of several components etched on it including an integrated circuit chip that acts as the central processing unit, several analogue and digital pins, a quartz crystal clock timer and a USB port by means of which it can be connected to a computer or laptop (Badamasi, 2014). Depending on the purpose of use, there are a diverse range of Arduino boards available in the market although the core components are generally the same for most Arduino boards. Figure 4.10 describes the core components of an Arduino UNO.



**Figure 4.10. Diagrammatic description of different components of an Arduino UNO micro-controller board.**

An Arduino UNO board has a 8 bit Atmel® micro-processor, 14 digital input/output (I/O) pins, 6 analogue inputs, 7 power pins, a 16 megahertz quartz crystal oscillator which acts as the internal clock, an USB jack and a DC power jack. The operating voltage of Arduino UNO (and most other Arduino boards including Arduino 2560 used in the current study) is between 1.8 - 5.5 V. Most Arduinos also have a 'Reset' button which resets the Arduino to the last uploaded program. (Image source: RoboMart.com, Information source: Arduino UNO data-sheet from Arduino website)

#### 4.8.6.2 Overview of Arduino programming

The latest version of the Arduino software namely Arduino IDE 1.8.3 was used in the current project. A typical Arduino program consists of two parts namely

1. Void setup (): The set-up is where the variables used in the program were declared as inputs or outputs. It is the first function to be executed in a program and it runs only once. An example of set-up function is described below where pin 13 of the Arduino board was described as an output using the pinMode:

```
Void setup () {  
    pinMode (13, OUTPUT);  
}
```

2. Void loop (): The loop is the core part of a program. It consists of codes that would be executed continuously. Continuing with the example mentioned in the set-up function, the loop was written in such a way that an LED connected to pin 13 (Anode of the LED into pin 13, cathode into GND) would glow continuously if the pin 13 was defined as HIGH using the 'digitalWrite' function. Hence, the loop function would be written as follow:

```
Void loop () {  
    digitalWrite (13, HIGH);  
}
```

The digitalWrite function commands the Arduino to deliver either 0 V (LOW) or 5 V (HIGH) to pin 13. The LED glows continuously because the Arduino is continuously delivering 5 V to pin 13. For instance, in order to make the same LED blink i.e. turn ON/OFF alternatively, the loop function would be written as follow:

```
Void loop () {  
  
    digitalWrite (13, HIGH);           // turns LED ON  
  
    delay (1000);                       // pauses for a second  
  
    digitalWrite (13, LOW);           //turns LED OFF  
  
    delay (1000);                       //pauses for a second  
  
}
```

Every statement of an Arduino program needs to end with a semi-colon which confirms that a particular statement is complete. Without a semi-colon at the end of each statement, the compiler cannot compile the program and an error message would appear. The timings in an Arduino program are mentioned in milliseconds as shown in the above example. Also, in an Arduino program, anything written after a double slash '/' is not considered as a part of the actual program. '/' are generally used to add comments to the program such as the ones shown in the example above.

#### 4.8.7 Problem solving: increasing Arduino operational flexibility

In the current smoking machine, the Arduino 2560 was mainly used to control the timings of the four solenoid valves V 1, V 2, V 3 and V 4. But an Arduino 2560 board can only provide a maximum output voltage of 5 V. Hence, devices that have an operating voltage higher than 5 V are generally controlled by Arduino using a supporting device such as an Arduino shield or a relay switch module that increases the operational flexibility of Arduino. Since the 3/2 solenoid valves had an operating voltage of 12 V, an 8 channel relay module was used in association with Arduino to control the 4 solenoid valves.

#### 4.8.7.1 8 channel relay module

A picture of a typical 8 channel relay module is shown in Figure 4.11. As described in Figure 4.11, the relay module has 8 input pins namely IN 1 - IN 8 (one for each channel) apart from a GND and Vcc pin, thus capable of controlling 8 different devices simultaneously. Each channel had a switch and three terminals namely 'common', 'normally open' (or simply 'open') and 'normally closed' (or simply 'closed') which are common denotations used in electrical switches. The switch connects or disconnects the circuit depending upon the presence or absence of voltage. Each channel had a dedicated LED which indicated the channel that is under operation. Only 4 out of the 8 input pins were used in the current study, one pin per solenoid valve V 1, V 2, V 3 and V 4.

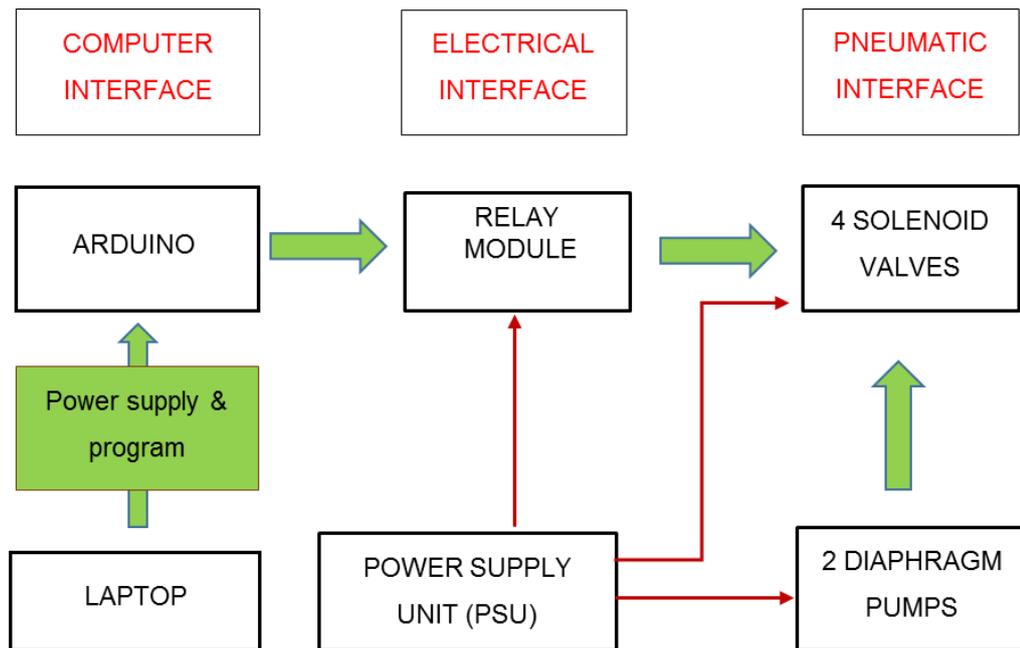


**Figure 4.11. An 8 channel relay module used to control the 4 solenoid valves through the Arduino.**

The relay module had an operating voltage of 12 V and consisted of 8 input pins namely IN 1 - IN 8, one for each channel, apart from a GND and Vcc pin. Each channel had a dedicated LED, a switch and three connection terminals namely 'common', 'open' and 'closed'. The switch activates or deactivates the electrical circuit depending upon the presence or absence of voltage. A glowing LED indicates a channel that is activated.

#### 4.8.7.2 Controlling the four solenoid valves through Arduino-relay module complex

The regulation of 4 solenoid valves by the Arduino and relay module complex played a central role in the working of the smoking machine. It was the solenoid valve 'ON/OFF' switching mechanism that determined the direction of flow of air/WCS/ECV in the system. Figure 4.12 represents an over-view block diagram describing the association between the power supply unit (PSU), four solenoid valves, two diaphragm pumps, 8 channel relay module and Arduino.



**Figure 4.12. A block diagram depicting the association between the Arduino, Relay module, the four solenoid valves and the two diaphragm pumps.**

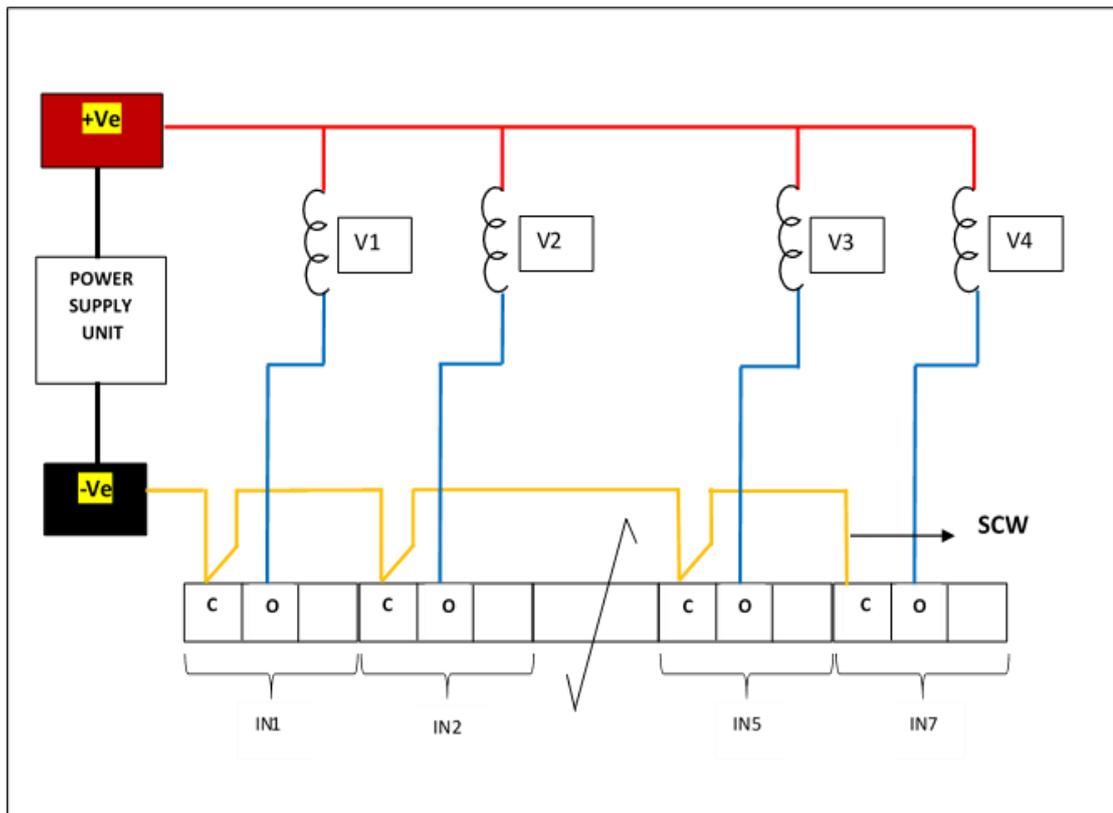
The four solenoid valves V 1, V 2, V 3 and V 4 were connected to the relay module and the relay module was in-turn connected to the Arduino, thus forming an intermediate between the four solenoid valves and the Arduino. The program to control the four solenoid valves was uploaded onto Arduino through a laptop which also provided the 5 V power to operate Arduino. Depending upon the Arduino program, the solenoid valves switched ON (open) or OFF (vent), thus regulating the direction of flow of air pumped into the system by the 2 diaphragm pumps. The PSU supplied power to the 2 diaphragm pumps, solenoid valves and the relay module

#### 4.8.7.3 Electrical connection between the Arduino, Relay module and the solenoid valves

The GND and Vcc pins of the relay module were connected to the GND and 5 V pins of the Arduino board respectively. Similarly, input pins IN 1, IN 2, IN 5, and IN 7 of the relay board were connected to digital input pins 31, 33, 39, 41 on the Arduino board respectively using male-female jumper wires. After this, the 4 solenoid valves were connected to the relay module in the following manner:

1. The positive terminal of all 4 solenoid valves were connected directly to the positive terminal of the PSU
2. The negative terminal of each of the four solenoid valves were connected in series to the 'open' terminal (denoted by 'O' in Figure 4.13) of their respective channel on the relay board i.e. negative terminals of valve V 1, V 2, V 3 and V 4 were connected to the open terminal of IN 1, IN 2, IN 5 and IN 7 channels of the relay module respectively.

- In order to complete the solenoid valve electrical circuit, an additional wire was used to connect the negative terminals on the relay board serially. This additional wire, called the 'serial connection wire' (SCW) (denoted by the yellow line in the Figure 4.13) was connected to the 'common' terminal (denoted by 'C' in Figure 4.13) of each of the 4 channels on relay board and ultimately connected to the GND of PSU.



**Figure 4.13. Schematic representation of the 4 solenoid valves connection to the 8 channel relay module.**

In the above schematic, the four solenoid valves V 1, V 2, V 3 and V 4 are represented as coils (electrical denotation). The 'common' and 'open' terminals of the relay module are denoted by 'c' and 'o' respectively in the above schematic. While the positive terminal of each solenoid valve (red coloured line) was directly connected to the positive of the PSU (red box), the negative terminal of each solenoid valve (blue coloured line) was connected to the open terminal of their respective channels on the relay board i.e. V 1 was connected to the open terminal of IN 1, V 2 to IN 2, V 3 to IN 5 and V 4 to IN 7. An additional wire called the serial connection wire SCW (yellow coloured line) was connected to the common terminal of each channel, thus serially connecting the negative terminals of the four solenoid valves. The SCW was ultimately connected to the GND of the PSU, thus completing the circuit pertaining to the 4 solenoid valves

Table 4.5 represents the connection between the Arduino, relay module and the four solenoid valves V 1, V 2, V 3 and V 4.

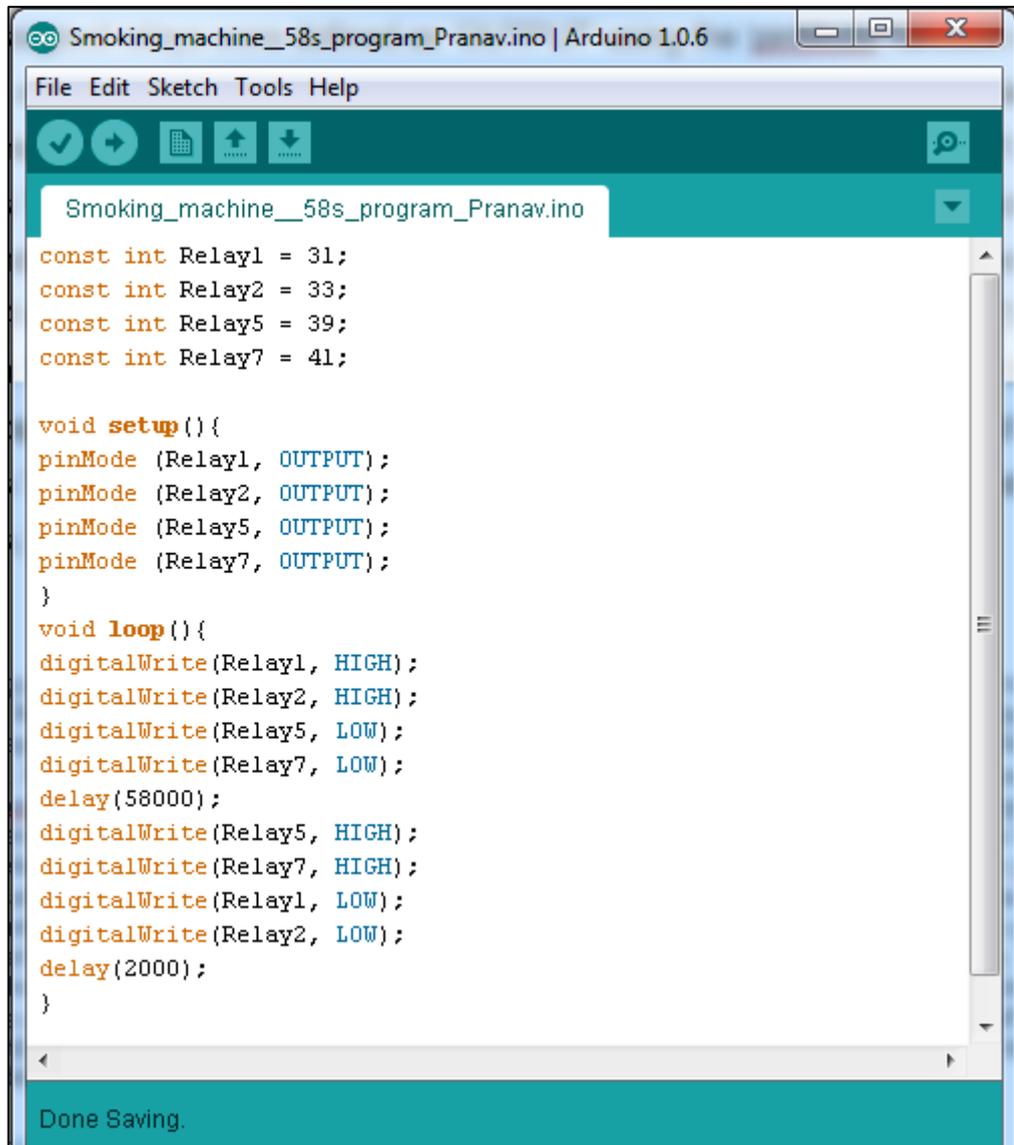
**Table 4.5. Table depicting the pin connection between the Arduino and 8 channel relay module and the subsequent connection of the negative terminals of the four solenoid valves to their respective channels on relay board.**

ARDUINO (digital pins)	RELAY MODULE (input pins)	SOLENOID VALVE (negative terminals)
GND	GND	NA
5 V	Vcc	NA
31	IN 1	V1
33	IN 2	V2
39	IN 5	V3
41	IN 7	V4

#### 4.8.7.4 Arduino program used to operate the smoking machine under ISO 3308 regime

A screen-shot of the Arduino program used to control the four solenoid valves is depicted in Figure 4.14. In the Arduino program, the four channels IN 1, IN 2, IN 5, and IN 7 were defined as Relay1, Relay2, Relay5 and Relay7 respectively as shown in Figure 4.14. Using 'const int' function, Relay1, Relay2, Relay5 and Relay7 were then assigned to pins 31, 33, 39 and 41 of the Arduino board respectively. In the void set-up section, the assigned constants (Relay1, Relay2, Relay5 and Relay7) were defined as 'OUTPUT' using the 'pinMode' function since they were used to control the 4 solenoid valves. In the void loop section, in order to simulate the ISO 3308 smoking regime, Relay1 and Relay2, which were connected to valves V 1 and V 2, were defined as 'HIGH' (equivalent to 'ON') for 58 s using 'digitalWrite' function. Hence solenoid valves V 1 and V 2 remained 'open' for 58 s. At the same time, Relay5 and Relay7 which were connected to solenoid valves V 3 and V 4 were

defined as 'LOW' (equivalent to 'OFF') for 58 s. Hence, valves V 3 and V 4 were venting for 58 s, thus executing the air-breathing phase of the smoking cycle. In the second part of the loop, Relay5 and Relay7 were defined as 'HIGH' for 2 s and Relay1 and Relay2 were defined as 'LOW' for 2 s. Hence solenoid valves V 3 and V 4 were 'open' for 2 s and V 1 and V 2 were venting for 2 s, thus executing the puff phase of the smoking cycle.



```
Smoking_machine__58s_program_Pranav.ino
const int Relay1 = 31;
const int Relay2 = 33;
const int Relay5 = 39;
const int Relay7 = 41;

void setup(){
  pinMode (Relay1, OUTPUT);
  pinMode (Relay2, OUTPUT);
  pinMode (Relay5, OUTPUT);
  pinMode (Relay7, OUTPUT);
}

void loop(){
  digitalWrite(Relay1, HIGH);
  digitalWrite(Relay2, HIGH);
  digitalWrite(Relay5, LOW);
  digitalWrite(Relay7, LOW);
  delay(58000);
  digitalWrite(Relay5, HIGH);
  digitalWrite(Relay7, HIGH);
  digitalWrite(Relay1, LOW);
  digitalWrite(Relay2, LOW);
  delay(2000);
}
```

Done Saving.

**Figure 4.14. Computer screen-shot of the Arduino program used to control the four solenoid valves through the relay module.**

The four channels of the relay module namely IN 1, IN 2, IN 3 and IN 4 were defined as 'Relay1', 'Relay2', 'Relay5' and 'Relay7' respectively. Using the 'const int' function, the four channels were assigned to their respective pins on the Arduino board. The four channels were then defined as OUTPUT in the set-up section using the 'pinMode' function. In the loop section, the four Relays were defined 'HIGH' or 'LOW' for appropriate periods of time such that the ISO 3308 smoking regime was executed. The loop was executed indefinitely until the Arduino was plugged off the power supply.

This alternating process of the four solenoid valves as illustrated in Table 4.6 was continuously executed until the power supply was switched off.

**Table 4.6. Command assigned to the four relay channels in the Arduino program and the corresponding action performed by the four solenoid valves.**

	Relays in Arduino program	Arduino Command	Associated solenoid vales	Action
Normal breathing phase (58s)	Relay 1, Relay 2	HIGH	V1, V2	ON
	Relay 5, Relay 7	LOW	V3, V4	OFF/VENT
Puff phase (2s)	Relay 1, Relay 2	LOW	V1, V2	OFF/VENT
	Relay 5, Relay 7	HIGH	V3, V4	ON

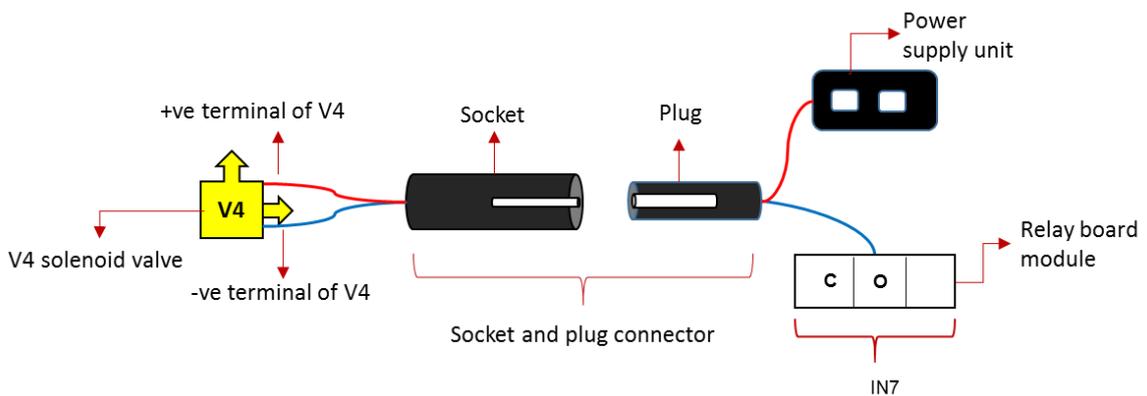
#### 4.8.8 Problem solving: designing a contamination control

The smoking machine was designed such while that the components of the initial, final and control compartments remained constant throughout all experiments, the components of intermediate compartment namely the cigarette chamber and V4 solenoid valve, were alterable depending on the condition tested. The two components of the intermediate compartment were removable and re-attachable to the initial and final compartments of the smoking machine for each condition tested i.e. air/WCS/ECV. The main reason to have three separate sets of intermediate compartment was to avoid any cross-contamination that could potentially occur by using the same components to test cigarette, EC and air. Hence, three sets of cylindrical glass tube, aluminium cigarette holder and metal plug, solenoid valve V 4 and the connecting pneumatic push-fit tubes were assigned for each condition: 'air only' or 'WCS only' or 'ECV only'.

##### 4.8.8.1 Plug and socket adapter design for the V 4 valve

Since there were three sets of V 4 valve, one each for air, WCS and ECV respectively, the positive terminal and negative terminal of V 4 valve had to be unwired from the relay module and PSU respectively each time the valve had to be changed. Subsequently, the terminals of the new V 4 valve had to be re-wired to the relay module and the PSU. This process of changing V 4 valve three times during a typical experiment consisting of testing air, WCS and ECV was time-consuming. In order to rectify this situation, a 2.1 mm in-line socket and plug power adapter was installed to connect the V 4 valve to the relay board. As illustrated

in Figure 4.15, the positive and negative terminal originating from each V 4 valve was soldered to the socket of the adapter (the soldering was performed by one of the lab technicians namely David Palmer). On the other end, two additional wires were soldered to the plug of the adapter, one of which served as the positive terminal and the other as negative terminal. While the positive terminal of the plug was connected to the PSU, the negative terminal was connected to the open terminal of the IN 7 channel on relay module. This socket and plug adapter design was applied to all three V 4 solenoid valves. Hence, the positive and negative terminals of the plug remained permanently connected to the PSU and IN 7 channel of the relay module respectively. When a V 4 valve was required to be replaced, the socket of the existing V 4 valve was disconnected from the plug and subsequently the socket of the new valve was connected to the plug, thus completing the circuit.



**Figure 4.15. Diagrammatic representation of the in-line socket and plug power adapter design used for the V 4 solenoid valve connection.**

A 2.1 mm in-line socket and plug power connector was used to alleviate the time-consuming process of changing the three V 4 solenoid valves during experiments and also improve the design flexibility of the smoking machine.

## 4.9 Implementation of the smoking machine: Building a practical model

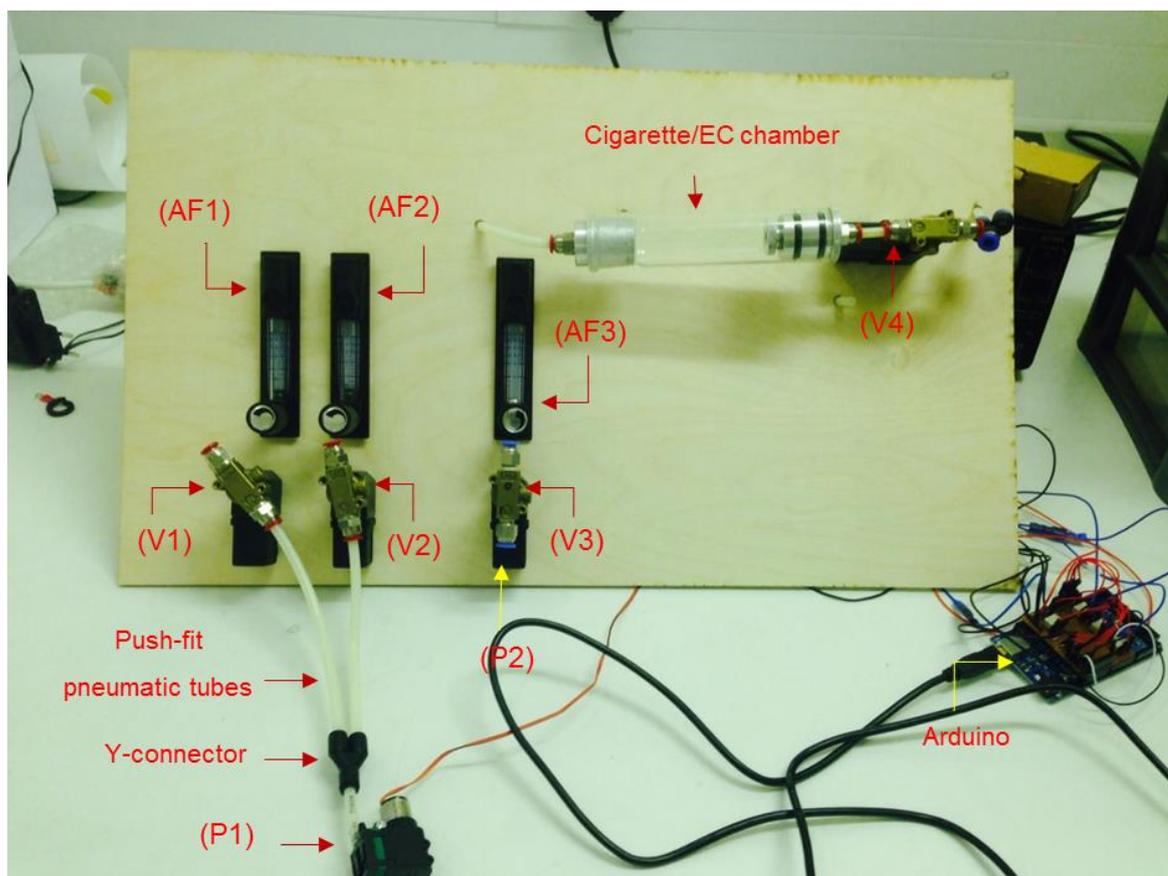
Once the individual compartments of the smoking machine were designed, the next important task was to assemble the different components in a flexible and user-friendly model that would permit maximal functionality of the smoking machine. As described in the user requirements and PDS (section 4.7.1 and 4.7.2), the smoking machine was designed such that:

1. Each component of the smoking machine was visible to the operator
2. The connections between the different compartments were easily achievable
3. The process of detaching and re-attaching the intermediate phase components with the rest of the system was easily achievable

### 4.9.1 Wooden board model

Prior to reaching its final form, an initial wooden board prototype of the smoking machine was developed. This model was developed in association with Mr. Shaun McClelland, a summer project student mentored by the author of this study. Although no experiments were performed using this model, it never-the-less, served as a useful initial prototype to trial different aspects of the smoking machine design.

The wooden board model consisted of a rectangular wooden board into which the different components of the smoking machines were fixed as shown in Figure 4.16. The dimensions of all the relevant smoking machine components namely diaphragm pumps, solenoid valves and analogue flow meters were measured and designed in SOLIDWORKS™ software (SolidWorks Corporation, Massachusetts, US). The dimensions of the different components were then subsequently laser-cut (this process was carried out by the Design Workshop technician namely James Duggan) on a rectangular wooden board. The components were then fixed onto the wooden board in their appropriate positions. Since this initial model was developed in the first year of the current doctoral study, some components (like the diaphragm pump P 2 and relay module) had not been yet installed at the time the picture displayed in Figure 4.16 was taken.



**Figure 4.16. Picture of the preliminary wooden board model of the smoking machine.**

The wooden board model was the first prototype of the smoking machine based on which the final working model was developed. The wooden board model consisted of a large single rectangular wooden board on which the accurate dimensions of the different components of the smoking machines were indented using laser-cutting. The components were then fixed into the wooden board and the pneumatic tube connections between the components were made at the backside of the wooden board in this model, thus permitting limited visibility to all the components of the system. Since the above picture was taken during the first year of the current doctoral study, some components such as diaphragm pump P 2 and relay module (indicated by the yellow arrow) is missing in the above picture.

#### 4.9.2 Drawbacks of the wooden board model

Although useful as an initial prototype, the wooden board model had some major drawbacks associated to it. Firstly, the wooden board model had all the components fixed to one rectangular wooden block. This rendered limited flexibility to the system such as difficulty in inserting or removing cigarette/EC from the glass tube. Moreover, since the initial and the intermediate compartments could not be separated under this model, the entire system had to be transported inside the fume cupboard as whole which was difficult. For instance, if some error occurred in the initial compartment of the smoking machine, it was not possible to detach only the initial compartment from the rest of the system. Either the entire board had to be transported together or the individual components were to be removed for testing. Another marked disadvantage was that under this model, the gas diffusion system and the

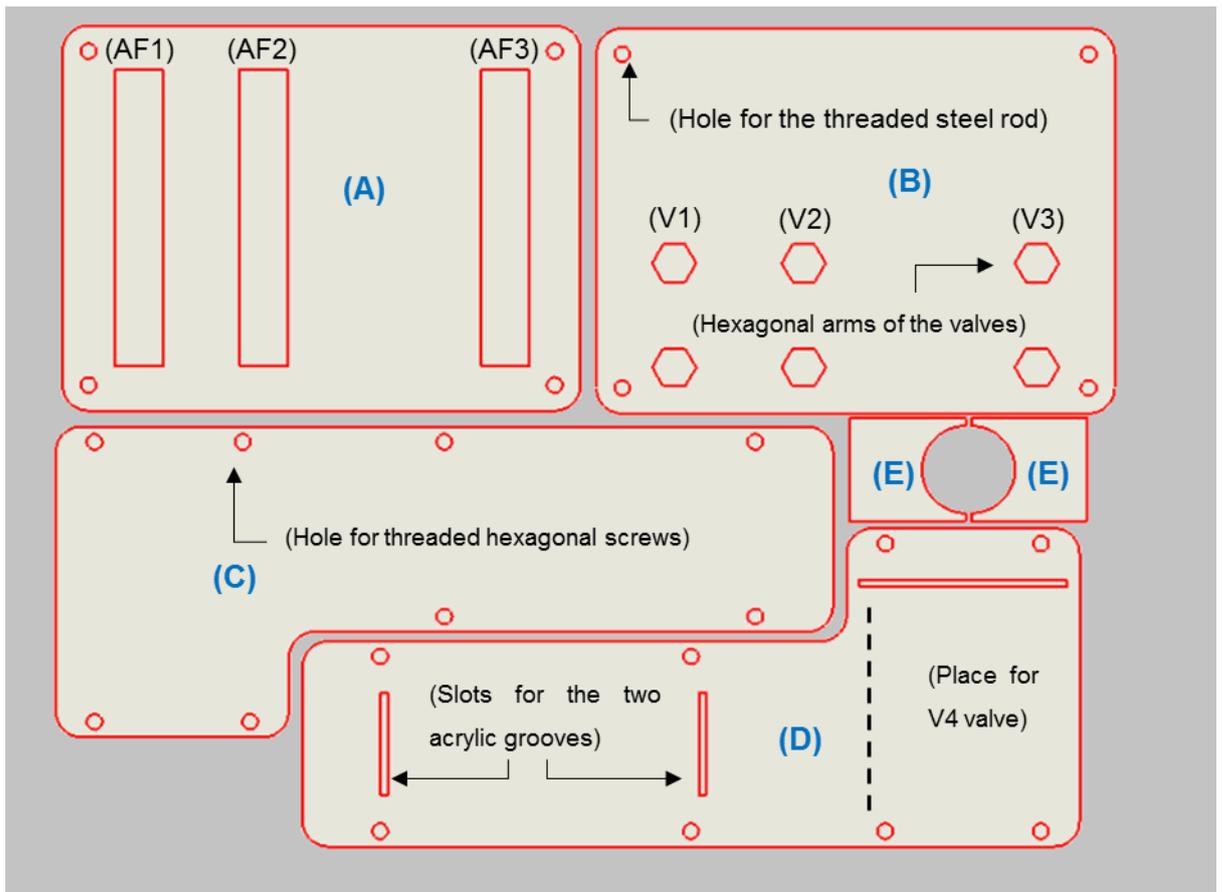
Perspex cell manifold blocks had to be placed at the same height as the cigarette chamber (which was placed at an elevated level) in order to deliver the air/WCS/ECV in the shortest route possible. This would require attachment of an additional plank to the wooden board which would further compromise the flexibility of the system and also increase the overall weight of the system. Another disadvantage was that in the wooden board design, the V 4 valve was fixed firmly into its slot on the wooden board. Since there were 3 sets of this valve, removing and re-attaching them into the slot between experiments was difficult. Lastly, one of the practical drawbacks of this model was that the overall size of the wooden board was quite space-consuming leaving very little room within the fume cupboard. This rendered practical problems such as not being able to view the pneumatic tubing that were at the backside of the wooden board. Considering these drawback, an improvised, more robust, flexible and user-friendly model using acrylic boards was designed which constituted the final working model of the smoking machine.

#### 4.9.3 Double acrylic board model

Considering the different user-requirements and PDS, a double acrylic board model of the smoking machine was developed in association with Mr. Anikeme Udofia, who was a Product Design Development summer placement student mentored by the author of this study. The rationale behind this model was that since all mechanical components (except the V 4 valve) of the smoking machine including the 2 diaphragm pumps (P 1 and P2), 3 solenoid vales (V 1, V 2 and V 3) and 3 analogue flow meters (AF 1, AF 2 and AF 3) were located in the initial compartment, this compartment formed the bulk of the smoking machine. Hence, a model consisting of two rectangular acrylic boards, was chosen to accommodate the components of the initial compartment.

##### 4.9.3.1 *Designing the double acrylic board model*

The dimensions of solenoid valves and analogue flow meters were measured using Vernier callipers. The measured dimensions were then designed in SOLIDWORKS™ software as shown in Figure 4.17. Figure 4.17 (A) represents the acrylic board on which the three analogue flow meters AF 1, AF 2 and AF 3 would be fixed. Figure 4.17 (B) represents the acrylic board on which the three solenoid valves namely V 1, V 2 and V 3 would be fixed. Post designing the component's dimensions on SOLIDWORKS™ (performed by Anikeme Udofia), laser-cutting was used to cut out the measured components' dimensions on the two acrylic board. The two acrylic boards were held in position using 4 threaded steel rods (one at each corner) and fastened using 16 hexagonal nuts (2 at each corner on either side of the 2 boards) as described in Figure 4.17 (A) and (B).



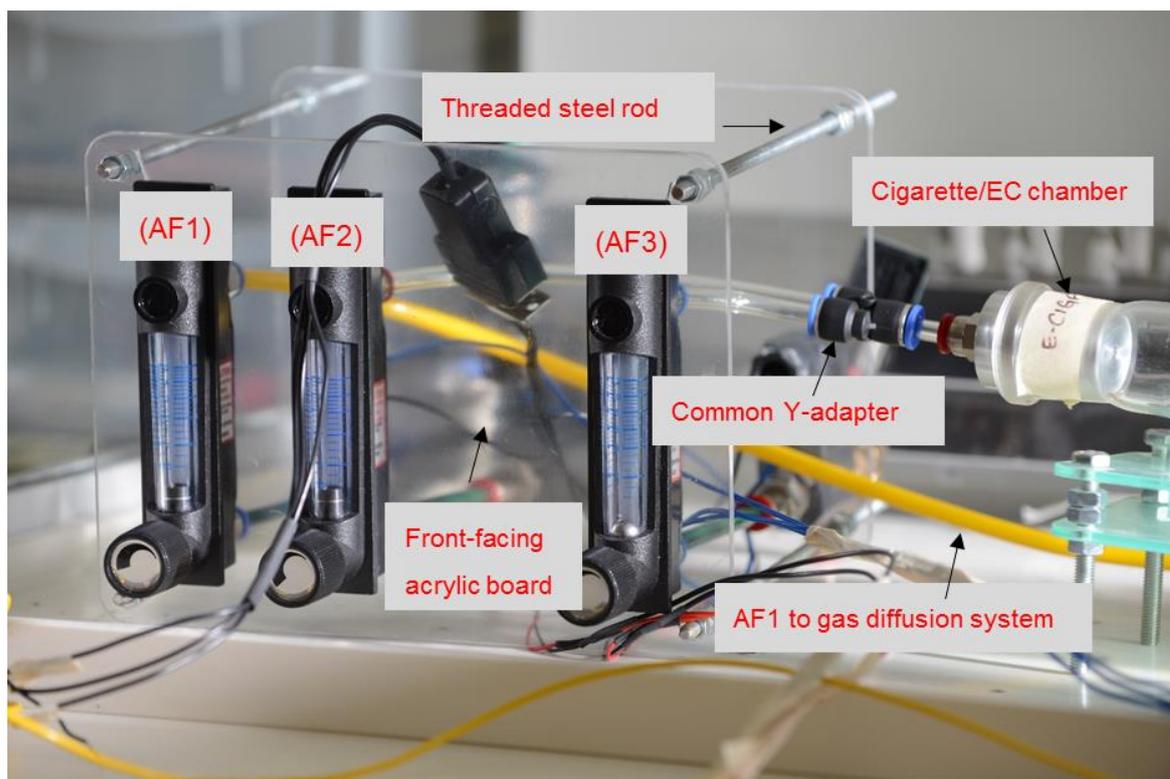
**Figure 4.17. A screenshot of SOLIDWORKS™ drawing used to laser cut the two rectangular and L-shaped acrylic boards.**

In the above diagram, (A) and (B) represent the two rectangular acrylic boards that was used to accommodate the components of the initial compartment of the smoking machine. (A) represented the rectangular acrylic board that would accommodate the three analogue flow metres AF 1, AF 2 and AF 3 and (B) represented the rectangular acrylic board that would accommodate the three solenoid valves V 1, V 2 and V 3. The dimensions of the analogue flow meters and solenoid valves were measured using Vernier callipers and the measured dimensions were designed in SOLIDWORKS™ software and subsequently laser-cut on the acrylic boards. The two boards were tightly held in position with the help of a threaded stainless steel rod and two hexagonal nuts at each corner of the board. Figure (C) and (D) represent the two L-shaped acrylic board that was used to support the cigarette chamber glass tube and the V 4 solenoid valve of the intermediate compartment. In addition, one of the L-shaped board had slots for two acrylic grooves (represented by Figure (E)) that held the cigarette chamber glass tube in position as shown in diagram (D). The L-shaped acrylic boards were also held in position using 8 threaded hexagonal screws and nuts, as indicated in the diagram (C).

For the components of the intermediate compartment, similar to the initial compartment, a double acrylic board design was developed to accommodate the cigarette chamber glass tube and V 4 solenoid valve. The only difference was that instead of a rectangular acrylic board, a customised L-shaped double acrylic board model was used. Figure 4.17 (C) and (D) represented the two L-shaped acrylic boards which accommodated the cigarette chamber glass tube and V 4 solenoid valve. The two L-shaped acrylic boards were stacked above each other separated by a small distance and fixed together using hexagonal threaded screws and nuts at eight different points as shown in Figure 4.17 (C) and (D). Figure 4.17 (E) represented the acrylic grooves which held the cigarette chamber glass tube in position on the L-shaped acrylic board. The acrylic grooves were designed according to the curvature of the cigarette chamber glass tube such that the glass tube sat on the grooves comfortably.

#### *4.9.3.2 Fixing the smoking machine components into the double acrylic board set-up*

Once the dimensions of the different components were laser-cut on the rectangular acrylic boards, they were then fixed into their respective positions on the acrylic boards. Figure 4.18 represents a real-time picture of the initial compartment components of the smoking machine. As represented in Figure 4.18, the three analogue flow meters (AF 1, AF 2, and AF 3) and the solenoid valves (V 1, V 2 and V 3) were fixed into the two rectangular acrylic boards. Since the analogue flow meters were used to manually adjust and visually verify the flow rates, they were fixed to the front facing acrylic board. The solenoid valves were fixed to the backward facing board (not visible in Figure 4.18). Once fixed, the diaphragm pumps were connected to the solenoid valves using push-fit pneumatic tubes. While P 1 was connected to V 1 and V 2 through a push-fit Y-adaptor, P 2 was connected to V 3 directly (not visible in Figure 4.18). Subsequently, each solenoid valve was connected to its respective analogue flow meters using pneumatic push-fit tubes i.e. V 1, V 2, V 3 to AF 1, AF 2 and AF 3 respectively.

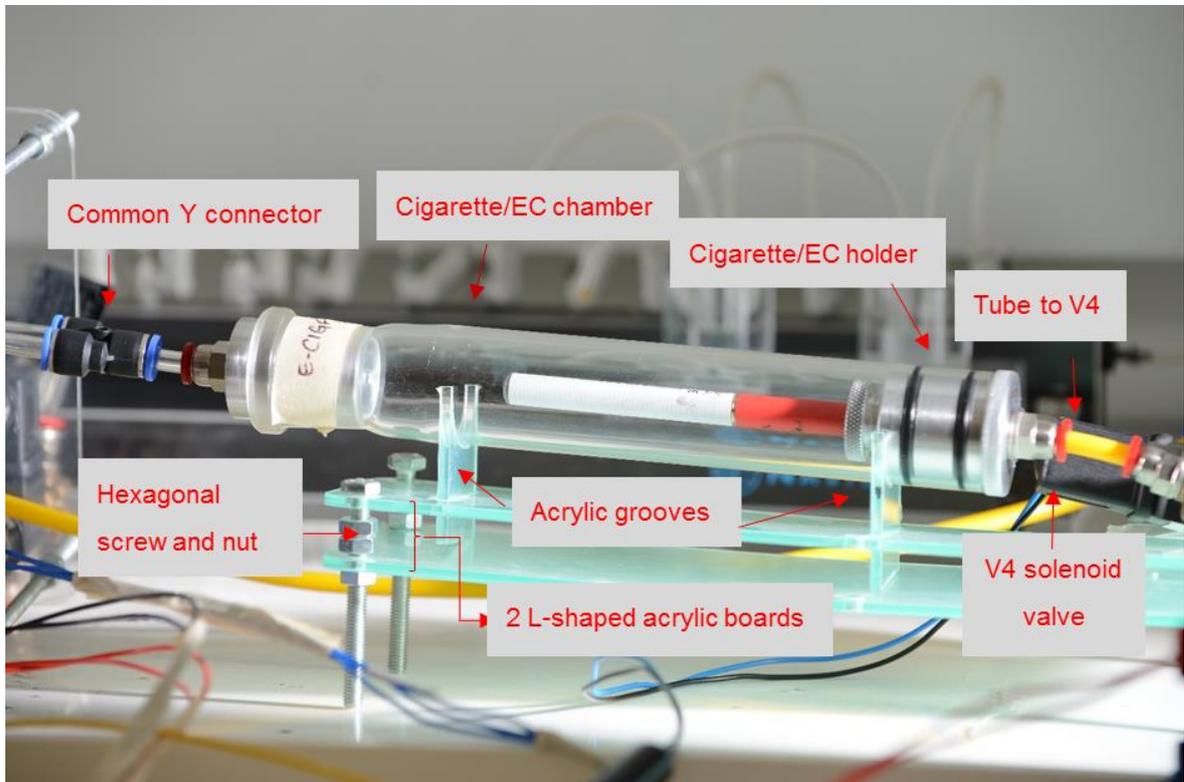


**Figure 4.18. Rectangular double acrylic board design to accommodate the initial compartment components of the smoking machine.**

Starting from behind, the three solenoid valves V 1, V 2 and V 3 were fixed to the second (or backward facing) acrylic board (not visible in the above picture). The three analogue flow meters AF 1, AF 2 and AF 3 were fixed to the first (or front-facing) acrylic board such that the flow meters were visible to the operator and hence could be used to manually adjust the flow rates. Once the components were fixed into their respective boards, the two diaphragm pumps P 1 and P 2 were connected to their respective solenoid valves (P 1 was connected to V 1/V 2 and P 2 was connected to V 3). The solenoid valves were in-turn connected to their respective analogue flow meters using pneumatic push-fit tubes (V 1, V 2, V 3 to AF 1, AF 2 and AF 3 respectively). The tubes from AF 2 and AF 3 were connected to the cigarette chamber glass tube via a push-fit pneumatic Y-adaptor as shown in the above figure.

Similar to the components of the initial compartment, the components of the intermediate compartment namely the cigarette chamber glass tube and V 4 valve were placed on the L-shaped acrylic boards as shown in Figure 4.19. Once placed on the acrylic grooves, the cigarette chamber glass tube was connected to the solenoid valve V 4. The placement of this valve V 4 on the L-shaped acrylic board was horizontal, as opposed to the vertical placement of the other three valves. Also a significant advantage of the L-shaped acrylic board model was that the V 4 valve, unlike the other three valves, was not fixed to the L-shaped acrylic board but rather placed on top of the board since it would be frequently replaced with each of the three test conditions. Hence, such a design would not just accommodate V 4 valve horizontally but also enable easy removal and replacement of V 4 valves according to the different treatment conditions. Post placement, the pneumatic tubes arising from AF 2 and AF 3 were connected to the cigarette chamber glass tube via a push-

fit common pneumatic Y-connector. On the other end, the outlet tube from cigarette holder was connected to the solenoid valve V 4 as shown in Figure 4.19, which was ultimately connected to the gas diffusion system via another push-fit pneumatic Y-adapter.

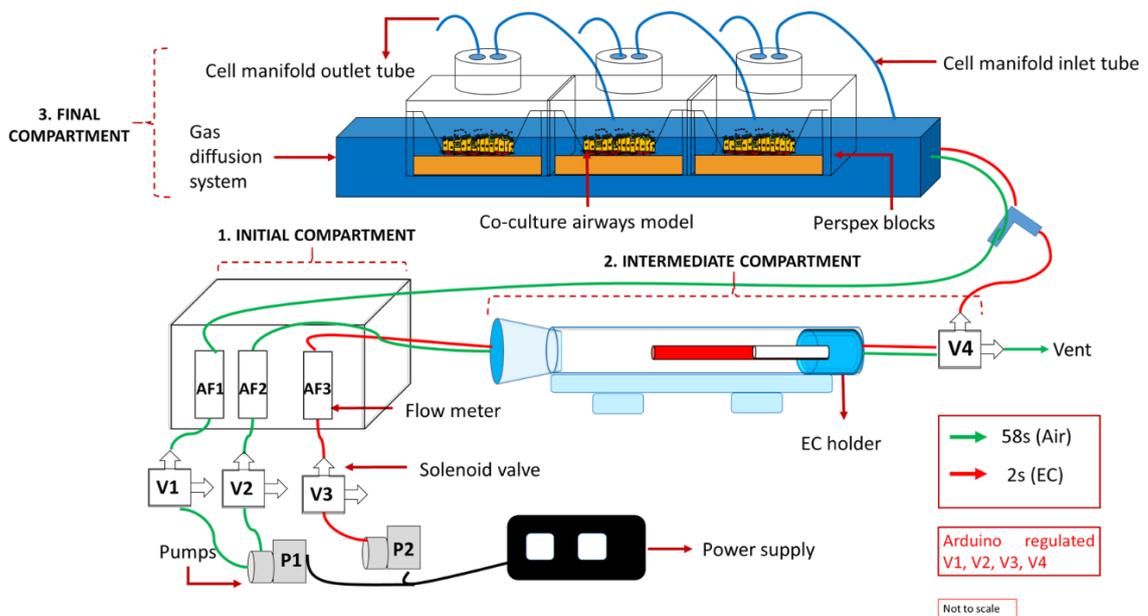


**Figure 4.19. L-shaped double acrylic board design to accommodate the intermediate compartment components of the smoking machine.**

(Note: Figure 4.19 can be viewed as a continuation of Figure 4.18). The cigarette chamber glass tube was placed on top of the acrylic grooves (described in Figure 4.17 (E)) on the top L-shaped acrylic board. The glass tube was then connected to the pneumatic tubes arising from AF 1 and AF 2 flow meters via a push-fit Y-adapter as shown in the above figure. The pneumatic tube arising from the cigarette holder end of the glass tube was connected to the solenoid valve V 4 (short yellow tube indicated in the above figure). The 'L-shape' of the acrylic board was designed to accommodate solenoid valve V 4 horizontally. V 4 was ultimately connected to the gas diffusion system via another push-fit pneumatic Y-adapter (not visible in the picture). Thus, the components of the initial, intermediate and final compartments of the smoking machine were now connected which formed the final working model of the smoking machine.

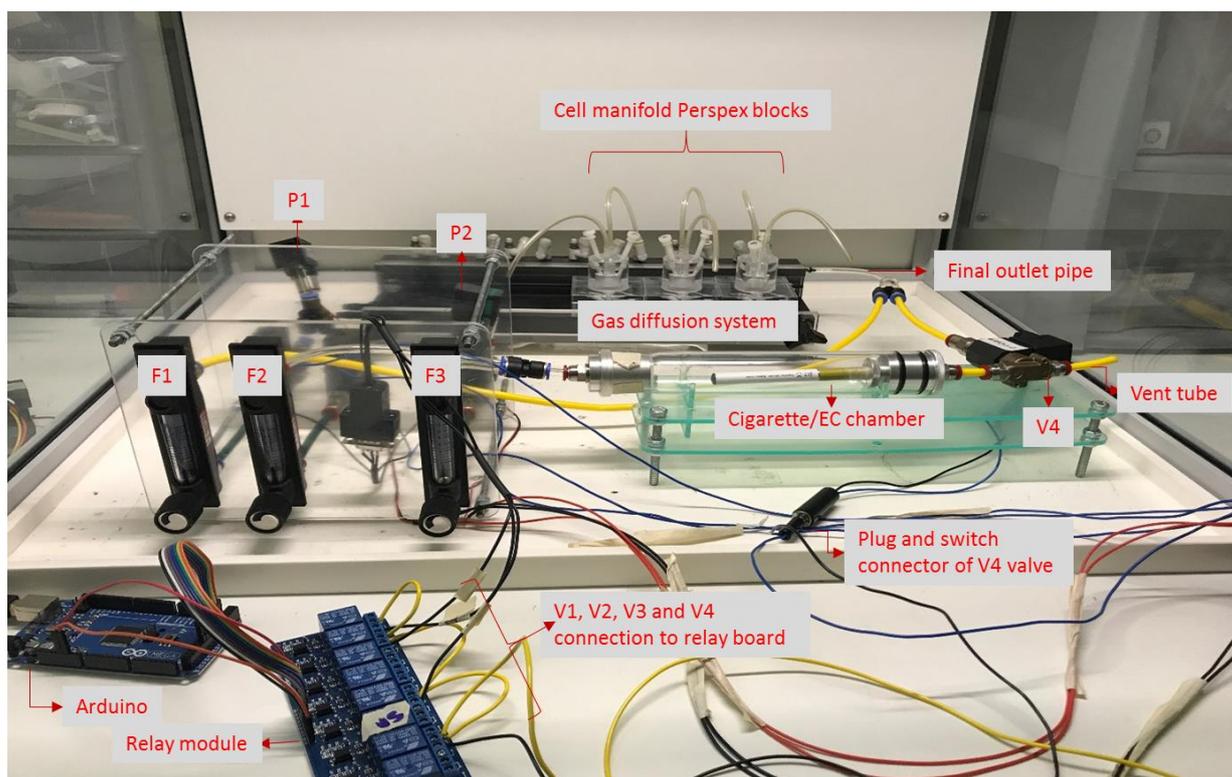
## 4.10 Final working model of the smoking machine

Once all the components were fixed into their respective positions on their respective board, the appropriate components were connected to the PSU. The positive and negative terminals of the diaphragm pumps were connected directly to the PSU. For the 4 solenoid valves, while the positive terminals were connected directly to the PSU, the negative terminals were connected to the relay module and subsequently to the Arduino as described previously in section 4.8.7.3. Thus all the components of the smoking machine were connected to their respective subsequent components to form a system extending from the diaphragm pumps to the cell manifold Perspex blocks. This model formed the final working model of the smoking machine which was placed inside a fume cupboard (Airone FC 750 model, Safelab systems Ltd. Somerset, UK). Figure 4.20 is a diagrammatic depiction of the final model of the smoking machine used to expose the co-culture human airways model to air/WCS/ECV. Figure 4.21 is a real-time picture of the final model of the smoking machine. The two pictures depicted via Figure 4.20 and Figure 4.21 can be used for comparison purposes.



**Figure 4.20. Diagrammatic depiction of the final working model of the in-house designed smoking machine.**

The components of the initial and intermediate compartments were connected in their respective positions on their respective acrylic boards as described in Figure 4.18 and 4.19. Cigarette/EC was inserted into the cigarette holder and the outlet tube from the cigarette chamber glass tube was connected to the V 4 solenoid valve. The outlet tube from V 4 valve was connected to the gas diffusion system via a pneumatic push-fit Y-adapter. Thus, the smoking machine was connected to the final compartment consisting of the gas diffusion system and the Perspex cell manifold blocks as shown in the above picture. Once the Arduino program was uploaded and PSU switched on, the WCS/ECV was delivered to the co-culture model as per the ISO regime continuously. (This figure can be compared with Figure 4.21 which is the real-time look of the smoking machine final model)



**Figure 4.21. Real-time picture of the final working model of the smoking machine.**

The above picture depicts a real-time holistic view of the final working model of smoking machine used for experimentation (refer figure 4.20 for comparison). Specifically, the connection between the Arduino, 8 channel relay module and the 4 solenoid valves can be clearly observed in the above figure. Also, the horizontal placement of the V 4 solenoid valve (right extreme in the above figure) on the L-shaped acrylic board can also be observed in the above figure. The connection of V 4 solenoid valve to the final output pipe of the smoking machine and its subsequent connection to the gas diffusion system can be noticed in the above figure. Additionally, the plug and socket design for V4 valves (described earlier in sub-section 4.8.8) can be seen in the above figure. Lastly, the connection of the Perspex cell manifold inlet tubes to the gas ports of the diffusion system can be also be noticed in the above diagram.

## 4.11 Measurement and validation of the smoking machine

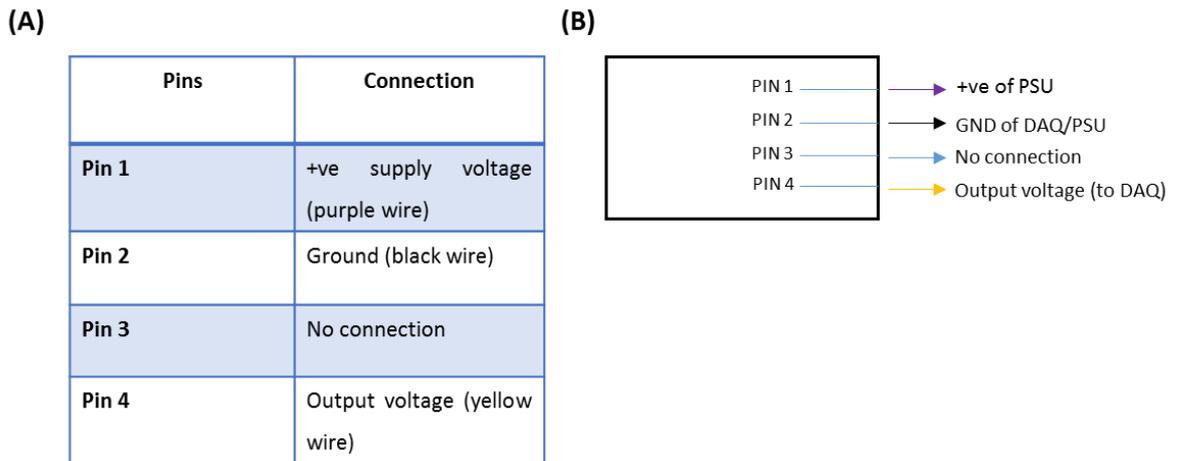
Before performing experiments with the smoking machine, it was important to ascertain that the flow rates of air/WCS/ECV entering the gas diffusion system and subsequently into the cell manifold blocks complied with the expected ISO 3308 parameters. Such a validation step would also ensure that the smoking machine was free from any major leaks or loose connections that would potentially affect the delivery process.

### 4.11.1 Validation of the smoking machine using an electronic flow sensor (EFS)

An EFS namely AWM 5000 series micro-bridge mass air-flow sensor was used to measure the smoking machine flow rates electronically (Honeywell International, Minneapolis, USA). The EFS worked on the principle of heat transfer. When in contact with a mass of air, heat transfer occurred across the sensor's surface and the output voltage varies proportional to the mass of air (Honeywell). As per the EFS data-sheet, the media flowing through the EFS must be free from condensing moisture and particulate contaminants in order to prevent damage to the sensor. Tobacco cigarettes contain ultrafine particles and ECV contains water vapour, both of which could potentially damage the EFS sensor. Hence the EFS was used to verify air flow rates only. Never-the-less, the WCS and ECV flow rates were verified using analogue flow meters. (An initial attempt made to measure ECV flow rates using EFS resulted in damage of the sensor. The test results have been included in the Appendix section 11.1, Figure 11.1)

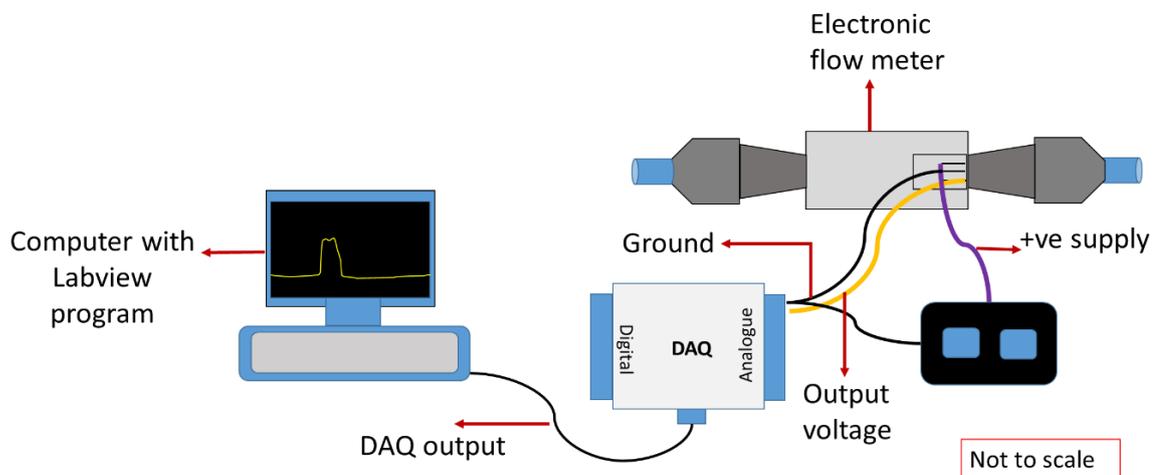
### 4.11.2 Setting up the EFS

The EFS had a 4-pin latch connector which connected the EFS to PSU and external connections. Figure 4.22 describes the connection of the four pins of EFS. Using 3 interface wires soldered on the latch connector (performed by technician David Parmer), pin 1 was connected to the positive terminal of PSU which provided the operating voltage. Pin 2 was connected to the GND of a Data Acquisition system (DAQ) USB 6009 (National instruments, Berkshire, UK) which in-turn was connected to the GND of the PSU using another interface wire. Pin 3 had no connection while pin 4 was connected to the analogue input ('A01') port of DAQ which received the output signal (voltage) from the EFS. The DAQ was in-turn connected to a computer installed with LabVIEW program (National instruments, Berkshire, UK). LabVIEW is a software programme used to acquire, process and display the data obtained by DAQ. Figure 4.23 illustrates the EFS connection to the DAQ and subsequently to a computer containing LabVIEW software.



**Figure 4.22. (A) Table representing the connection of each pin of the EFS latch connector to its respective terminals. (B) Diagrammatic representation of the pin connection to its respective terminals.**

Pin 1 of the EFS was connected to the positive terminal of PSU. Pin 2 was connected to the GND of DAQ which in-turn was connected to the GND of PSU. No connection was made from Pin 3 while Pin 4 was connected to an analogue input port (A01) of DAQ which received the output voltage from the EFS.

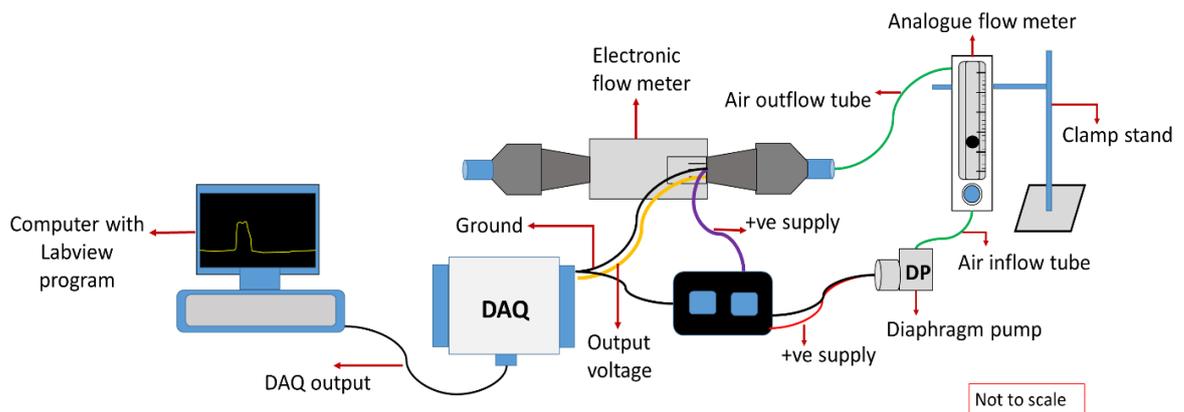


**Figure 4.23. Diagrammatic representation of EFS and its connection to DAQ to measure flow rates electronically.**

The 4 pins of the EFS was connected to DAQ and PSU as described in Figure 4.22. The DAQ was in-turn connected to a computer containing LabVIEW program through an output USB cable. The DAQ received the output voltage from the EFS and the obtained data was processed and displayed in LabVIEW software.

### 4.11.3 Calibrating the EFS

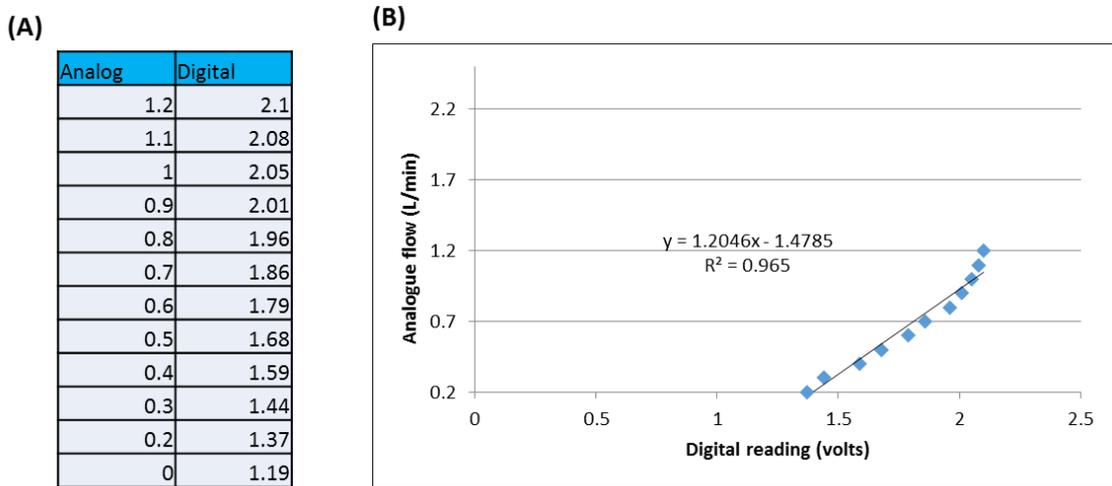
Before using the EFS to measure flow rates from the smoking machine, it was necessary to calibrate the EFS using an analogue flow meter. The slope and the y-intercept values obtained from such a calibration-plot was then be fed into LabVIEW program to produce a customised scale. Figure 4.24 illustrates the set-up used to calibrate the EFS. A 12 V diaphragm pump was connected to an analogue flow meter through a pneumatic tube (referred as the in-flow tube) while another pneumatic tube (referred as the out-flow tube) was connected to the EFS. The EFS was connected to the DAQ and in-turn to a computer with LabVIEW as described previously in Figure 4.23. Data acquisition parameters were defined such that samples were acquired continuously at a rate of 100 Hz for 30 s. When the PSU was switched on and pump activated, the flow rate in the analogue flow meter was adjusted to 0 L/min and the corresponding digital reading on LabVIEW (in volts) was recorded. The flow rate was then increased linearly in 0.100 L/min increments and the corresponding LabVIEW digital reading was recorded at each increment, until the final analogue flow rate of 1.2 L/min was reached.



**Figure 4.24. EFS calibration using analogue flow meter.**

An analogue flow meter was connected to the EFS at one end and a 12 V diaphragm pump was connected to the analogue flow meter at the other end. The EFS was connected to the DAQ which in-turn was connected to a computer installed with LabVIEW as described in Figure 4.23. When the PSU was switched on, the analogue flow rate was adjusted from 0 L/min to 1.2 L/min in increments of 0.100 L/min and the corresponding EFS digital reading on LabVIEW was noted. The analogue and the digital readings were plotted against each other and the slope and y-intercept values were obtained (described in the next figure).

Figure 4.25 (A) and (B) represent the digital values obtained during the calibration process and the corresponding plot obtained respectively. As shown in Figure 4.25, the LabVIEW digital readings were plotted on the x-axis against their corresponding analogue flow rates which were plotted on the y-axis. The slope and y-intercept values of the plot was obtained. These values were then fed into the LabVIEW software in order to produce a new customised scale. The ‘custom scaling’ section of the LabVIEW software is explained in detail in the next sub-section 4.11.4.



**Figure 4.25. (A) Table depicting the different analogue flow rates and its corresponding EFS digital readings observed in LabVIEW. (B) Graph obtained by the plotting Digital Vs Analogue values.**

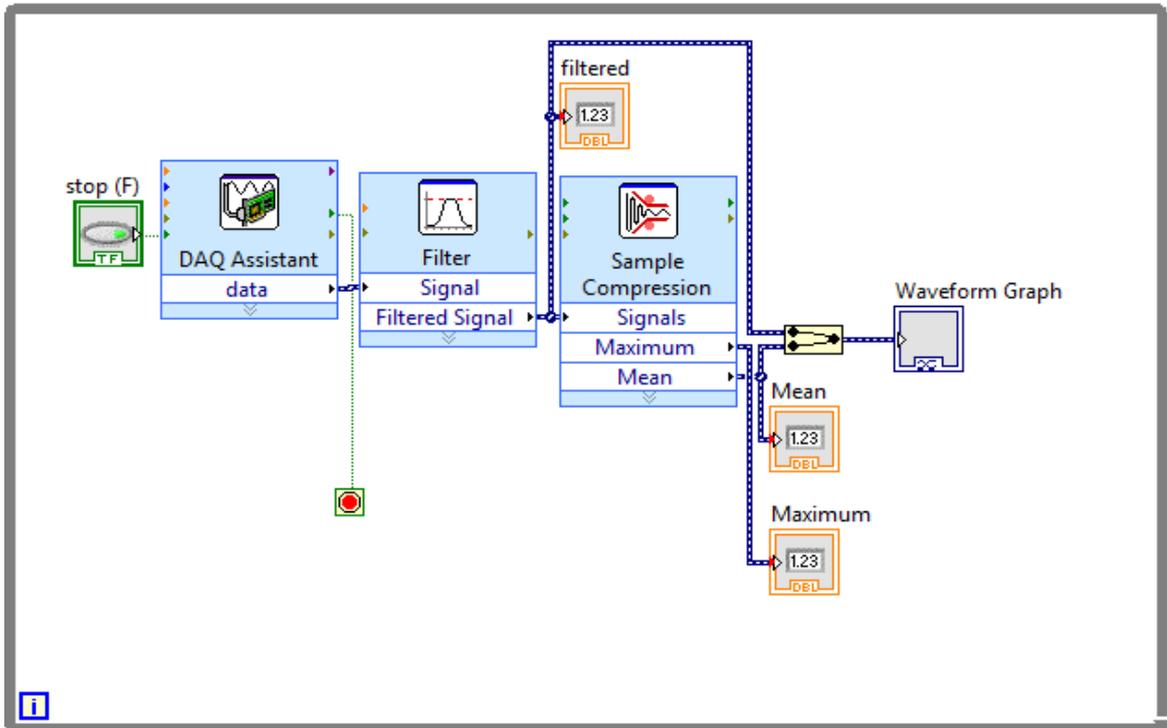
The analogue flow rate reading were plotted on the Y-axis and the observed digital readings were plotted on the X-axis. A trend-line was added onto the graph and the slope and the y-intercept values were obtained. The obtained slope and y-intercept values were then used to produce a custom scale in LabVIEW signal graph which is explained in further sections.

#### 4.11.4 Setting up LabVIEW

##### 4.11.4.1 Overview of LabVIEW block diagram

The LabVIEW software allows users to construct programs via virtual block diagrams representing the instrument to be analysed via LabVIEW. The LabVIEW block diagram used to acquire data from the EFS (via DAQ) is described in Figure 4.26. The block diagram had three main blocks namely the ‘DAQ assistant’ block, the ‘Filter’ block and ‘Sample compression block’. The bulk of data acquisition settings were made in the ‘DAQ assistant’ block. Once the different setting of the ‘DAQ assistant’ block were completed, an appropriate filter was selected from the ‘Filter’ block. Filters, in general, remove unwanted frequencies continuously as the data is being acquired. In the current program, a low-pass filter was employed which passes low frequencies but attenuates unusually high

frequencies. The 'Filter' block was followed by the 'Sample compression' block which compressed the data and obtained the 'mean' and 'maximum' values. The filtered mean and maximum values were continuously displayed in their respective boxes in the front panel of LabVIEW during data acquisition process. A 'stop' button in the block diagram stopped the data acquisition process as shown in Figure 4.26.

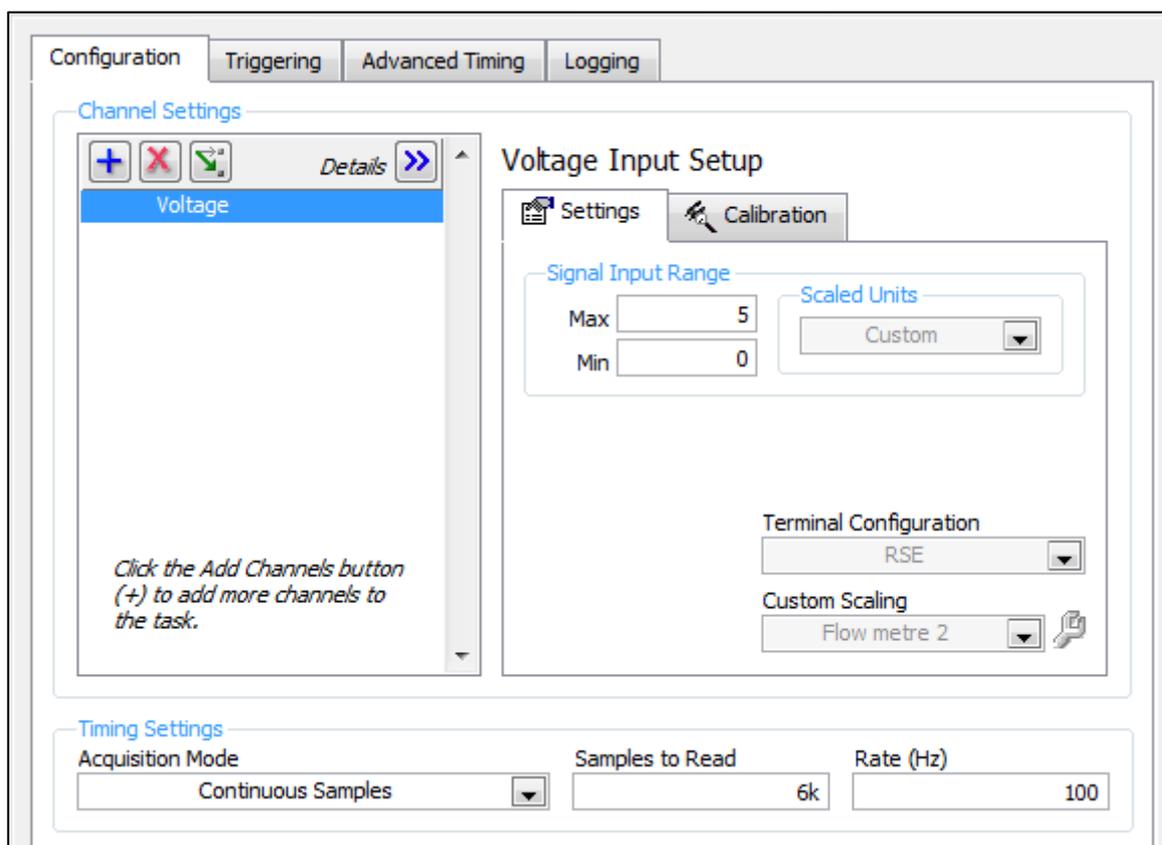


**Figure 4.26. LabVIEW block diagram used to acquire data from the DAQ.**

A virtual block diagram was designed to obtain, process and graphically display data from DAQ which received the EFS output signal. The block diagram consisted of three major blocks namely the 'DAQ assistant' block, 'Filter' block and the 'Sample compression' block. Of the three blocks, the DAQ block contained the data acquisition settings. The DAQ block was followed by the 'filter block' which filtered the obtained data continuously using a low-pass filter. This filtered data was combined with the mean and maximum data valves obtained from the 'sample compression' block to form the final waveform graph. The DAQ block was preceded by a 'stop' button that was used to stop the data acquisition process by DAQ.

#### 4.11.4.2 Customizing the data acquisition parameters

The 'DAQ assistant' block provided options for customising several data acquisition parameters such as sample number, sample acquisition rate (in Hz), data acquisition type (continuous or 'N' number of samples), terminal configuration (RSE or differential) and signal input range i.e. minimum and maximum voltage expected from the EFS. A screenshot of dialogue box depicting the different parameters employed in the current study is shown in Figure 4.27. As per the EFS's data-sheet, maximum output voltage capacity of the EFS was 5 V and hence the input signal range was assigned between 0 V to 5 V. Samples were read at a rate of 100 Hz (100 samples/s) for required period of time (E.g. for 60 s as shown in Figure 4.27) such that data from both the normal breathing phase and the puff phase of the smoking cycle were acquired. Data samples were acquired continuously under RSE terminal configuration.

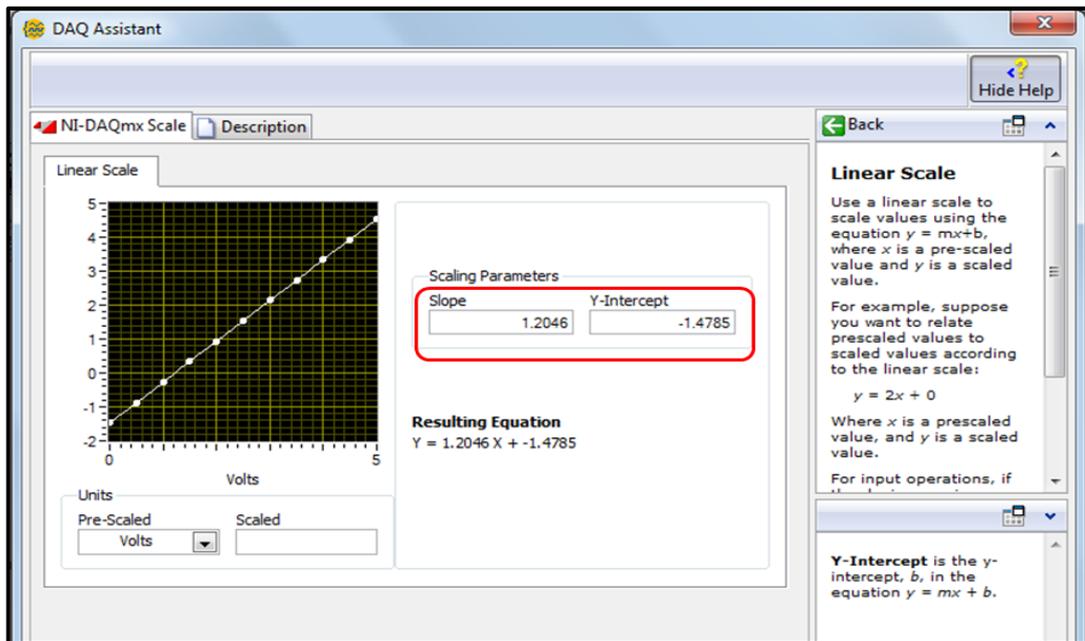


**Figure 4.27. Screen-shot of the dialogue box which contained the essential data acquisition settings.**

Data from the DAQ which receives signal from the EFS, were read at a rate of 100 Hz for required period of time. For instance, if the data is continuously read for 60 s, the program would acquire 6000 samples which would include data from both, the 'normal breathing' phase as well as the 'puff' phase of a smoking cycle. Based on the EFS calibration slope and y-intercept values, a custom scale called 'Flow metre 2' was created as displayed in the above figure.

#### 4.11.4.3 Custom scale based on EFS calibration

The custom scaling section ('spanner' icon in Figure 4.27) was used to enter the slope and y-intercept values obtained from EFS calibration process (refer Figure 4.25) in order to produce a customised scale for flow rate measurements on LabVIEW. This custom-scaling dialogue box is depicted in Figure 4.28.



**Figure 4.28. Screen-shot of the dialogue box which was used to produce a custom scale.**

As described earlier in Figures 4.24 and 4.25, the EFS was calibrated using analogue flow meter and the obtained values were plotted against each other in order to obtain slope and the y-intercept. These values were then entered in the designated space in the 'custom scaling dialogue box' (high-lightened using a red rounded-rectangle in the above figure) in order to obtain the new linear equation and scale which was used to measure the air flow rates via EFS.

## 4.12 Validating the smoking machine via flow rate measurements

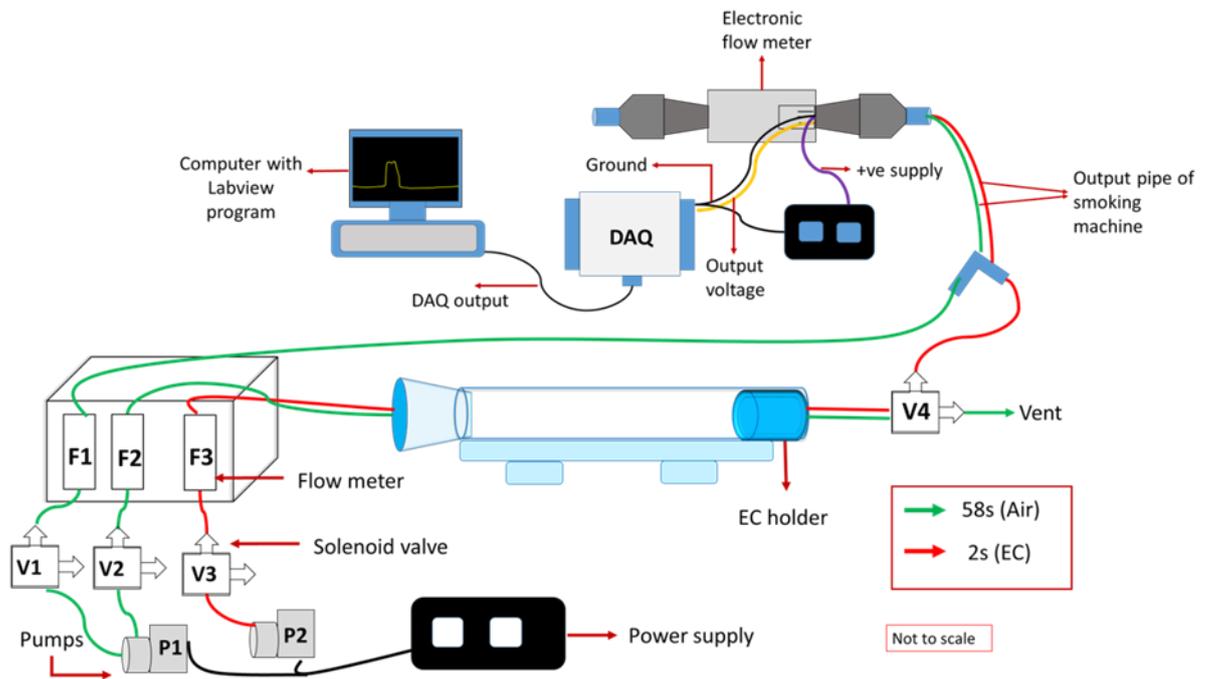
Thus, prior to using EFS to measure air-flow rates during smoking machine operation, three important steps were performed in LabVIEW software:

1. An appropriate virtual block diagram consisting of DAQ assistant' block, the 'Filter' block and 'Sample compression block' was constructed (refer Figure 4.26)
2. The appropriate data acquisition parameters were chosen (refer Figure 4.27)
3. A customised graph scale based on the EFS calibration slope and y-intercept values was produced (refer Figure 4.28)

Once these steps were completed, LabVIEW software was now ready to measure the air flow rates using EFS during smoking machine operation in order to validate the smoking machine.

### 4.12.1 Measurement of air flow rates using EFS

The validation process set-up is diagrammatically illustrated in Figure 4.29. The final output pipe from the smoking machine (the pipe that delivers air/WCS/ECV into the gas diffusion system) was connected to the EFS. Since theoretically after this point, there could be no further loss of air/WCS/ECV, measuring the flow rate at this point would represent the volume of air/WCS/ECV that was ultimately delivered to the cell manifold Perspex blocks. Since only air flow rates were to be measured, the cigarette chamber was kept empty as shown in the Figure 4.29. The EFS was connected to the DAQ in a manner as shown earlier in Figure 4.22. With the LabVIEW set-up complete, the PSU was switched on which started the smoking machine operation. The 'start' button in LabVIEW was then pressed which started the data acquisition process. For validation process, the Arduino was programmed to operate the smoking machine under standard ISO 3308 conditions with the only exception being the inter-puff timings. Instead of a 58 s inter-puff time, the Arduino was programmed to deliver a puff after every 28 s such that the data was continuously obtained for 30 s at a rate of 100 Hz. Hence, the DAQ block diagram and data acquisition parameters were identical to that described in Figure 4.26 and Figure 4.27 respectively, except that 3000 samples were read instead of 6000 as shown in Figure 4.27. After 30 s, the LabVIEW front panel displays the data obtained in the waveform graph format.

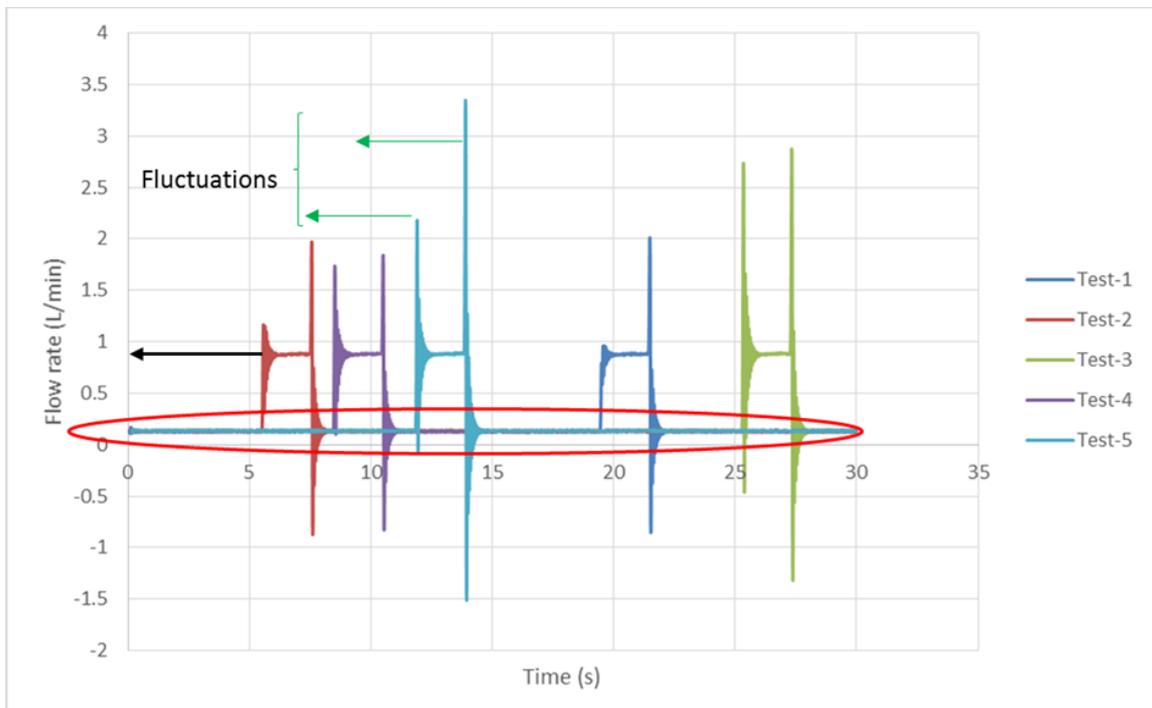


**Figure 4.29. Validation of the smoking machine air flow rates using the EFS.**

The appropriate data acquisition and custom scale parameters as described in Figure 4.27 and Figure 4.28 respectively were fed in the 'DAQ assistant' block, with the only difference being that samples were recorded for 30 s at a rate of 100 Hz (3000 samples in total). The PSU was switched on and when the smoking machine operation started, the LabVIEW data acquisition was also started. After 30 s, the LabVIEW generated the recorded data in the waveform graph. The raw data was exported into MS excel where data analysis was performed.

The data from LabVIEW was then exported into MS Excel where the raw filtered data were obtained. An x-y scatter graph was plotted with time (s) on the x-axis and Flow (L/min) on the y-axis. The validation process was repeated 5 times such that the 2 s puff phase occurred at different time points within the 30 s cycle. Similar to the first test, the LabVIEW results were exported into MS Excel and raw data from the 5 tests were plotted on a single x-y scatter graph as depicted in Figure 4.30. During smoking machine operation under ISO 3308 regime, the air-flow through the EFS produced a square-wave profile in all 5 tests. As it can be observed from Figure 4.30, air flowed at a rate of  $\sim 0.150$  L/min during the 28 s normal breathing phase in all 5 tests (the raw values ranged between 0.150 L/min - 0.165 L/min). The 2 s puff phase was marked by a square wave which peaked at  $\sim 1$  L/min in all 5 tests (the raw values ranged between 0.950 L/min - 1.1 L/min), thus corroborating the adherence of the air-flow rates to ISO 3308 standard. The square wave observed in the data signifies the difference in puffing pattern between ECs and tobacco cigarettes. A tobacco cigarette which burns continuously during smoking would generate a smooth bell-shaped wave since there would be a gradual increase in the flow rate during the puff phase and a gradual decrease in flow-rate post the puff phase to reach the baseline. On contrary,

ECs are activated only during the puff-time after which the power supply to heating coil stops and hence there is no further vapour produced, thus demonstrating a square wave profile. Since the EFS was highly sensitive to changes in flow rates, the instant at which the flow rate changed from 0.150 L/min to 1 L/min and vice versa, some fluctuations in the signal occurred producing extremely high or low flow rates at that particular instant (~0.01 second). It is speculated that these aberrant fluctuations could be due to potential pressure build-up within the EFS with each testing. Another possible reason could also be weak filtering of the data obtained from the EFS by the LabVIEW program. A low-pass filter was employed in the current study and hence the effect of other filters of LabVIEW on the EFS data needs further investigation. These fluctuations were however short lived and the flow rates returned back to its normal rates.



**Figure 4.30. X-Y scatter graph depicting the air flow rates measured using EFS from 5 individual validation tests.**

The smoking machine validation process using EFS was repeated 5 times for a 30 s time period each time. Each test result is represented by a different colour in the above graph. As depicted in the above graph, the data produced a square-wave profile in all 5 tests. The air flow rate during the 2 s phase was uniform in all 5 tests at ~0.150 L/min and hence appeared superimposed on each other (indicated by red circle). Each validation test was repeated in such a manner that in each test the 2 s puff phase occurred at different time points within the 30 s range. In all the 5 tests, the flow rate during puff phase was ~1 L/min (indicated by the black arrow), thus validating the smoking machine's functionality by adhering to ISO 3308 smoking parameters. Due to the extremely sensitive nature of the EFS, at the instant of change from normal breathing phase to puff phase and vice versa, some fluctuations in flow rate was observed at that particular instant of about 0.01 s (indicated by the green arrow). Never-the-less, these fluctuations were extremely short-lived (0.01s) and returned to the expected flow rate the next instant.

#### 4.12.2 Measurement of ECV and WCS flow rates using Analogue flow metre

As stated earlier in section 4.11.1, the EFS could not be used to measure the flow rates of WCS or ECV due to the presence of particulate contaminants and condensing moisture in them. Although, it was possible to employ the EFS to measure the air flow rate prior to entering the cigarette chamber, such a measurement was not of particular interest in the current study since this inlet flow rate was visually verified by analogue flow meter. Additionally, since it is the WCS/ECV flow rate at the final outlet pipe post leaving the cigarette chamber that the cells would be ultimately exposed to, measurement of this flow rate was the main objective pertaining to the validation process. Hence, an analogue flow meter was used to visually verify WCS and ECV flow rates in the smoking machine. The steps involved in WCS/ECV validation were similar to that of air-flow validation via EFS. The only difference was that the final output pipe from the smoking machine was connected to an analogue flow meter instead of EFS. The smoking machine was operated under standard ISO 3308 conditions and the flow rates were visually verified. Table 4.7 represents the air/WCS/ECV flow rate readings observed using analogue flow meters during the two phases of the smoke cycle. Although verified using EFS, the air flow rates were additionally verified using analogue flow meter as well. As shown in Table 4.7, the air flow rates observed using analogue flow meter were in agreement with the EFS results, thus further corroborating the smoking machine functionality and reproducibility.

**Table 4.7. Table depicting the observed analogue flow meter measurements of air/WCS/ECV.**

An analogue flow meter was connected to the final output pipe of smoking machine and the flow rates of air/WCS/ECV was recorded during both phases of the smoke cycle during a 1 min period.

Medium flowing through the smoking machine	2 s puff phase		58 s normal breathing phase	
	EXPECTED FLOW RATE	OBSERVED FLOW RATE	EXPECTED FLOW RATE	OBSERVED FLOW RATE
AIR	1 L/min	0.950 L/min	0.150 L/min	0.150 L/min
WCS	1 L/min	~0.900 L/min	0.150 L/min	0.150 L/min
ECV	1 L/min	~0.900 L/min	0.150 L/min	0.150 L/min

#### 4.13 Discussion of design aspect of the smoking machine

The main aim of this part of the project was to conceive, design, construct and validate a bespoke, automated smoking machine that would generate and deliver WCS/ECV to the co-culture human airways model according to the ISO 3308 smoking regime, in a fashion mimicking the human smoking pattern as closely as possible. Such a smoking system offered specific advantages over evaluating cigarette/EC cytotoxicity using traditional methods such as treating submerged cultures to extracts or condensates of cigarette smoke/ECV. In real-time, the actual impact of cigarette smoke/ECV depends on the multitudes of chemicals produced during interaction between the particulate phase and vapour phase (Fukano et al., 2004). Hence, to understand the underlying mechanism of tobacco smoke or EC induced biological effects, a smoking system that delivered both phases of WCS or ECV to the cells is required. Although a number of commercial smoking machines are employed in many cigarette/EC studies, very few bespoke smoking systems have been designed and constructed for both WCS and ECV delivery. In fact, most bespoke systems described in the sub-section 4.6.2, were uniquely designed for WCS delivery only. Till date, only one study has reported of a bespoke system for EC aerosol generation and characterisation (Havel et al., 2017). In this respect, the current study reports a novel bespoke, automated and economical smoking machine for WCS/ECV delivery at ISO 3308 smoking regime.

The smoking machine of the current study was constructed using several easily-available and widely employed electro-mechanical components including diaphragm pumps, 3/2 solenoid valves and analogue flow meters, thus enabling a relatively easy construction of the system compared to commercial systems. A cigarette chamber consisting of a customised cigarette holder made of aluminium and a ground joint glass tube was employed to house the WCS/ECV. An Arduino 2560 micro-computer along with an 8 channel relay module was used to control the four solenoid valves V 1, V 2, V 3 and V 4. The Arduino was programmed such that when V 1 and V 2 were OPEN for 58 s, V 3 and V 4 vented for the same time, thus mimicking the normal air breathing phase of the ISO 3308 regime. Alternatively, when V 3 and V 4 were OPEN for 2 s, V 1 and V 2 vented during this time, thus mimicking the puff phase of the ISO regime. Thus, the co-culture cells were continuously exposed to air/WCS/ECV aerosols as per the ISO 3308 regime until the PSU was switched off.

#### 4.13.1 Justification of constructing a smoking machine

A number of commercial smoking machines which have been employed in many toxicological studies are available in the market. The decision to construct a bespoke automated smoking machine to generate and deliver WCS/ECV instead of purchasing a commercial smoking system was made based on a number of factors. The advantages of constructing an in-house smoking system were as follow:

1. **Potential breathing machine:** One of the long-term aims of the research group was to develop a “breathing system” i.e. a system capable of delivering aerosolized therapies including inhalable drugs to the relevant human airway cells. In this respect, the current smoking machine was constructed such that it would act as a good foundation for the intended breathing system concept. This potential of further developing the current smoking machine into a breathing machine is discussed in Chapter 10 (Future works).
2. **Lab-space:** Most of the commercial smoking machines, especially the rotary type machines, are generally cumbersome and space consuming. The cell culture lab MB 186D, where the lab-built smoking machine was housed inside a fume cupboard (~70 cm<sup>2</sup>), could not have accommodated most of the commercial smoking machines. Most commercial machines would have required an appropriate sized fume cupboard ( $\geq 1$  m<sup>2</sup>) to be installed in MB186D. Practically, this was not feasible with the available time and resources.
3. **Service and maintenance:** Most of the commercial smoking machines are dependent on their company for services. Although this might be an easy process (as stated in many of the commercial companies’ websites), there would incur an additional expenditure of time and energy in waiting for the service to finish and for experiments to resume. Additionally, maintenance of large smoking machines might have led to unexpected additional expenditures such as replacing a malfunctioning component, purchasing an extra feature etc.
4. **Cost:** Most of the commercially available smoking machines were expensive and had a cost associated with them that could not have been purchased with the available funding of the current project. To confirm the prices, the author contacted relevant companies requesting a quote of their products. Table 4.8 describes the costs of different commercial smoking machines.

**Table 4.8. Table depicting the prices of some of the popular commercially available smoking machines.**

Prices were obtained by contacting the manufacturers via email for a quote of their product. All prices stated are in accordance with the quote obtained at the time of contacting the manufacturers. (Current prices might vary)

	<b>Product</b>	<b>Manufacturer</b>	<b>Price</b>
1.	Single Cigarette smoking machine CSM-SCSM	CH technologies	<b>£11,226</b>
2.	Vitrocell® Manual smoking machine VC1	Vitrocell® systems	<b>£36,200</b>
3.	Human puff profile single cigarette smoking machine	CH technologies	<b>£16,000</b>
4.	E-cigarette aerosol generator (ECAG)	CH technologies	<b>£6363</b>

As it can be observed from Table 4.8, the cheapest smoking machine available in the market was priced > £6000 (and which was only compatible with ECs). Although the in-house smoking machine cannot be directly compared to a commercial smoking system in terms of features and sophistication, the current smoking machine was never-the-less a fully functional system built at a cost-effective price. Table 4.9 describes the total cost of construction of the lab-built smoking machine used in the current study.

**Table 4.9. Cost breakdown of the in-house built smoking machine of the current study.**

All prices stated are in accordance with the quote obtained from the manufacturers of individual components at the time of purchasing the components. (Current prices might vary. Some components were bought in bulk and hence price per unit was difficult to assess. The total cost involved of those components have been stated instead)

Component	Company	Price per unit	Cost in GBP (incl. of VAT)
Diaphragm pumps (x2)	GD Thomas	110.10	220.20
3/2 Solenoid valves (x4)	M&M International	75.84	303.36
Analogue flow meters (0.05-0.5 L/min range) (x2)	Key instruments	52.45	104.9
Analogue flow meters (0.1-1.2 L/min range) (x1)	Key instruments	65.94	65.94
Cigarette holders (Aluminium bars)	John Keatley Ltd.	NA	~40
Glass tubes (x 3)	Dixon Glass Ltd.	9.30	27.90
Acrylic board	In-house	NA	~10
Pneumatic tubes/connectors	RS components	NA	~50
Arduino ATMEGA 3560 (x1)	Arduino	30	30
8 Channel Arduino relay module (x1)	NA	5.91	5.91
Jumper wires	Maplin	NA	10
<b>Total cost</b>			<b>858.21</b>

The cell exposure apparatus namely the gas diffusion system and cell manifold Perspex blocks were purchased separately from Harvard apparatus (Massachusetts, USA). Although perceived as an integral compartment (final compartment) of the smoking machine, technically the term ‘smoking machine’ referred only to the system that generated and delivered air/WCS/ECV. Hence the costs associated with purchasing the gas diffusion system and the cell manifold Perspex blocks have not been included towards the total cost of constructing the smoking machine and have been separately described in Table 4.10.

**Table 4.10. Cost of the cell exposure system components used in the current study.**

<b>Component</b>	<b>Company</b>	<b>Price per unit</b>	<b>Cost in GBP (incl. of VAT)</b>
Gas diffusion system/ Perspex cell manifolds	Harvard apparatus		~2000
Cell manifold inlet/outlet Tygon® soft tubing	Saint-Gobain Ltd.		15.91

Never-the-less, even including the cell exposure apparatus, the overall cost involved with the smoking machine was still < £3000 which was quite economical compared to the commercial smoking machine.

#### 4.13.2 Choosing a double acrylic board model for the smoking machine

Based on the user requirements and PDS described in section 4.7.1 and 4.7.2, a double acrylic board model of the smoking machine was constructed in order to make the smoking machine operation user-friendly and increase its flexibility. This design consisted of two rectangular acrylic board which held the different components of the smoking machine initial compartment including the two diaphragm pumps P 1/ P 2, 3 solenoid valves V 1, V 2, V 3 and 3 analogue flow meters AF 1, AF 2 and AF 3. Additionally, two L-shaped acrylic board were used to accommodate the intermediate compartment of the smoking machine including the cigarette chamber and solenoid valve V 4. Moreover, in order to prevent cross-contamination that could possibly occur from using the same components to deliver air/WCS/ECV, three separate sets of intermediate compartment components was constructed for the smoking machine. Compared to the double acrylic board model, a

number of drawbacks associated with the wooden board model as described in section 4.9.2 which led to the construction of an enhanced version of the smoking machine. Table 4.11 compares the two models of the smoking machine.

**Table 4.11. Comparison of properties of the two models of the smoking machine.**

<b>Properties</b>	<b>Wooden board model</b>	<b>Double acrylic board model</b>
Flexibility	Poor	Good
Transportability	Difficult	Easy
Overall size and weight	Big and relatively heavy	Small and compact
Visibility of components	Poor	Good
Ease of connections between the components	Difficult and time-consuming	Easy and quick
Ease of removing and re-attaching glass tube and V4 valve	Difficult and time-consuming	Easy and quick
Cost of the model	Low-cost	Moderate cost (compared to the wooden board)

#### 4.14 Discussion of validation process of the smoking machine

Before the smoking machine was employed to performed experiments, a validation step was performed. Such a validation step involved measuring the flow rates of air/WCS/ECV during smoking machine operation and verifying its adherence to the ISO 3308 smoking regime flow rates. In the current study, the air flow rates were verified using an EFS. Such a validation process of a bespoke smoking system using EFS has also been performed in one past study by Havel et al (2017), thus further adding confidence to the employed methodology of the current study. As described in the validation section 4.12, the LabVIEW results showed that during smoking machine operation for 30 s, the air-flow produced a square wave profile wherein the flow-rates ranged between 0.150 L/min - 0.165 L/min for 28 s and 0.950 L/min - 1.1 L/min during the 2 s puff phase, thus adhering to the ISO smoking regime flow rates. WCS/ECV flow rates were verified using an analogue flow meter since the condensing matter present in WCS/ECV damaged the EFS sensor which could subsequently produce false data (when verified against manual observation of analogue flow meter). ECV flow rate measurement using EFS (data depicted in Appendix, Figure 11.1) showed that, although the first repeat (represented as 'E-cig\_1' in Figure 11.1) provided the expected flow rate (0.150 L/min) during the 28 s phase, the subsequent repeats demonstrated a gradual increase in the air flow rates. It is speculated that the ECV condensed matter during the first repeat (E-cig\_1) temporarily accumulates on the EFS sensor which produces a default false voltage (since EFS, as stated earlier, works on the principle of heat transfer). This false voltage then gets added on to the voltage produced by the ECV condensing matter of the subsequent repeats which maybe the reason for the gradual increase in the air flow rates. In this scenario, further investigation of WCS/ECV flow rates by employing vapour-resistant flow sensors is required. Never-the-less, analogue flow meter results showed that WCS/ECV flow rates followed ~0.150 L/min during the 28 s normal breathing phase and ~900 L/min during the 2 s puff phase, thus adhering to the ISO 3308 smoking regimes.

It can be observed from the analogue flow meter readings (Table 4.7) that there was small difference between the expected and measured flow rates of air/WCS/ECV during the 2 s puff phase. A possible explanation to this discrepancy may be the inevitable loss of gas during operation. Although, the smoking machine was constructed to be an air-tight system, the air pumped into the system passed through a number of components before reaching the final output pipe where the flow rates were measured. Starting from the diaphragm pumps to the solenoid valves (V 1, V 2 and V 3) to the analogue flow meters (AF 1, AF 2 and AF 3) to a pneumatic Y-adaptor to the cigarette chamber glass tube to the V 4 solenoid

valve to another pneumatic Y-adapter to the final output pipe, the air/WCS/ECV passed through at least 8 different components before reaching the gas diffusion system. Hence, it is possible for some minor unpreventable leakages to exist amongst the components. Additionally, although push-fit tubes and rubber O-rings were used wherever necessary, the efficiency of these components may not be 100 % and hence there could be some minor loss of gas at some of these points. Such a loss of medium through smoking systems is not uncommon as similar loss of cigarette smoke in commercial smoking machines have been previously reported in studies (Scian et al., 2009a, Scian et al., 2009b).

#### 4.15 Conclusions

The main aim of the current chapter was to design, construct and validate a bespoke, automated smoking machine in order to deliver aerosols of cigarette smoke or ECV to the co-culture model of human airways in a controlled fashion as per the ISO 3308 smoking regime. Thus, using a specific set of user-requirements and PDS, such a bespoke smoking machine was designed, constructed using commercially available electro-mechanical components and validated using EFS. This smoking machine was automated (using Arduino micro-controller), flexible (compared to a preliminary wooden board model), economical (compared to the commercial smoking machines), user-friendly and had good visibility of different components, thus permitting easy accessibility of different components. Validation of the smoking machine was performed by verifying the flow rates of air/WCS/ECV during the smoking machine operation using EFS (for air) and analogue flow meter (for WCS/ECV). The validation process showed that the smoking machine adhered to the expected ISO 3308 smoking regime parameters with minimal loss of air/WCS/ECV during operation. Such a validation thus adds confidence to the results to be obtained from the cell-exposure experiments performed using the smoking machine (Chapter 7 experiments).

## 5 Investigating the impact of individual EC constituents on submerged bronchial epithelial cells

### 5.1 Introduction

Nicotine is a natural alkaloid present in tobacco leaves that is chiefly responsible for tobacco addiction. During smoking, the aerosolised nicotine is readily absorbed by the respiratory tract, buccal mucosa, skin after which it enters the blood circulation and reaches the brain in as little time as 10 - 20 s (Benowitz et al., 2016, Martinez-Garcia et al., 2008). Since tobacco cigarettes deliver many other carcinogenic compounds apart from nicotine, alternative nicotine delivery systems have been introduced. ECs, in recent years have been promoted as one such alternative nicotine delivery system (Bullen et al., 2013). Although highly popular, the health effects of ECs remain unclear and many aspects of ECs, including the potential effects of its individual constituents on human airways have been sparsely investigated. As per the three-step experimental design described chapter 3, sub-section 3.8, this chapter of the current study forms the first step of EC evaluation wherein individual EC constituents namely nicotine, its oxidative metabolite cotinine and E-vehicle base liquid are assessed in their liquid form using submerged BEAS 2B and CALU 3 cultures, prior to evaluating ECs in their extracts form and finally evaluating EC aerosols using complex differentiated ALI co-culture models.

Although the pharmacological and addictive effects of nicotine are well-documented (Benowitz, 2009, Dani and De Biasi, 2001), the toxicity of nicotine per se is still not well-understood and remains debatable. Past studies have shown nicotine to produce marked cardiovascular effects such as increased heart rate, blood pressure, constriction of coronary blood vessels (Tseng et al., 1993, Benowitz, 2003), impairment of immune system (Singh et al., 2000), oxidative stress effects (Barr et al., 2007) and abnormal enzymatic activity (Yildiz et al., 1999). Nicotine, although not carcinogenic, has been reported to aid lung cancer progression via promoting tumour angiogenesis or protecting tumour cells from the effects of chemotherapy (Heeschen et al., 2001, Tsurutani et al., 2005). However contradiction arises when other studies emphasise the fact that nicotine replacement therapies (NRTs) thus far have not caused any significant detrimental health effects and have not demonstrated any cancer-promoting activities (Murray et al., 2009, Moore et al., 2009, Robles et al., 2008).

Cytotoxicity studies of nicotine have predominantly focused on neuronal cell types, possibly since neurons are the chief cells influenced by nicotine. Apart from neuronal cells, airway epithelial cells also express nicotine acetylcholine receptors (NACHRs) and are capable of metabolising nicotine (Hukkanen et al., 2005, Martinez-Garcia et al., 2008). Very few past *in-vitro* studies have analysed the effect of nicotine on bronchial epithelial cells. Ginskey et al (2011) reported that BEAS 2 B bronchial epithelial cells demonstrated significant DNA damage post 1 h exposure to 1 mM nicotine (Ginzkey et al., 2012). The DNA damage however was inhibited when the cells were co-incubated with an anti-oxidant, thus demonstrating that the nicotine exposure induced DNA damage was oxidative stress-dependent. Another study showed that treatment of BEAS 2B cells to 5 mM nicotine for 6 h caused a significant impact on apoptosis and senescence which demonstrates the ability of nicotine to significantly influence cell fate (Bodas et al., 2016). Hence, while studies investigating neuronal cells have reported on the protective effects of nicotine (Akaike et al., 1994, Copeland et al., 2007, Barreto et al., 2015), other studies investigating airway epithelial cells have reported on the cytotoxic effects of nicotine. This demonstrates the variability of nicotine effects depending upon the cell types tested, nicotine dose range employed and the exposure time (Martinez-Garcia et al., 2008). Post absorption, nearly 80 - 90 % of the nicotine from cigarette smoke is metabolised into cotinine by enzymes called CYP2A oxydases (Urakawa et al., 1994, Hukkanen et al., 2005). Since cotinine has a half-life 10 times longer than that of nicotine and is highly expressed in the blood, saliva and urine of tobacco smokers as well as ECs users (Etter and Bullen, 2011), cotinine is regarded as a good indicator of nicotine metabolism studies (Schroff et al., 2000). However, data on the cytotoxicity of cotinine is limited. Similar to nicotine, cotinine has been shown to demonstrate cell-proliferative, tumour promoting (Nakada et al., 2012, Zevin et al., 2000) and neuro-protective activities (Riveles et al., 2008). One *in-vitro* study employed MRC-5 cells to compare cytotoxicity of nicotine and cotinine and reported that cotinine was less cytotoxic than nicotine (Vlasceanu, 2018). However, the effect of cotinine on bronchial epithelial cells has not been reported to date and hence the current study aims to fill this gap.

The consistency and efficiency of nicotine vapourisation varies according to the different ratios of PG/VG employed in EC liquid (Goniewicz et al., 2013). Whilst there are several customised EC liquids available in the market such as EC liquids without VG (i.e. 100 % PG based solution) or without PG (i.e. 100 % VG based solution), for the purpose of the current study, an E-vehicle fluid consisting of 70 % PG : 30 % VG ratio has been used since this is the most commonly employed E-vehicle ration (Vapourlites™). PG is a viscous, colourless fluid that is widely used as a food additive (Lessmann et al., 2005) and in several

other applications such as in pharmaceutical preparations (Wieslander et al., 2001). VG is a sweet-tasting compound that is also widely used in the cosmetic, paint, tobacco, food, pharmaceutical industry (Lechasseur et al., 2017). VG is mainly added to EC liquids to produce a plume or mist of aerosol when heated at sufficiently high temperatures (McCauley et al., 2012, Clapp and Jaspers, 2017). Both PG and VG are GRAS certified chemicals approved by the FDA (FDA., 1973). However, their toxicity have chiefly been assessed for irritation and ingestion effects while the inhalation effects of PG or VG on the respiratory system remain largely unknown. While the skin or digestive tract may not be affected by PG and VG, the lungs may respond to PG and VG differently and may produce unexpected health consequences (Lechasseur et al., 2017, Clapp and Jaspers, 2017). In fact, it has been reported that thermal degradation of PG or VG at high temperatures can lead to the production of formaldehyde, acetaldehyde and acrolein which are established mutagenic/carcinogenic agents (Jamshidi et al., Clapp and Jaspers, 2017). In this context, addition of PG/VG as a carrier vehicle in ECs, under the pretext that both these chemicals are FDA approved GRAS compounds have raised concerns amongst public as well as health professionals. The current study thus aims to investigate the possible biological effects of E-vehicle on human airway cells.

## 5.2 Aims

The main aim of this chapter was to evaluate the effects of nicotine; its oxidative metabolite cotinine; and an E-vehicle fluid consisting of 70 % PG : 30 % VG on the cell viability and IL-6/IL-8 pro-inflammatory mediators' production of BEAS 2B and CALU 3 bronchial epithelial cells.

The specific objectives were as follow:

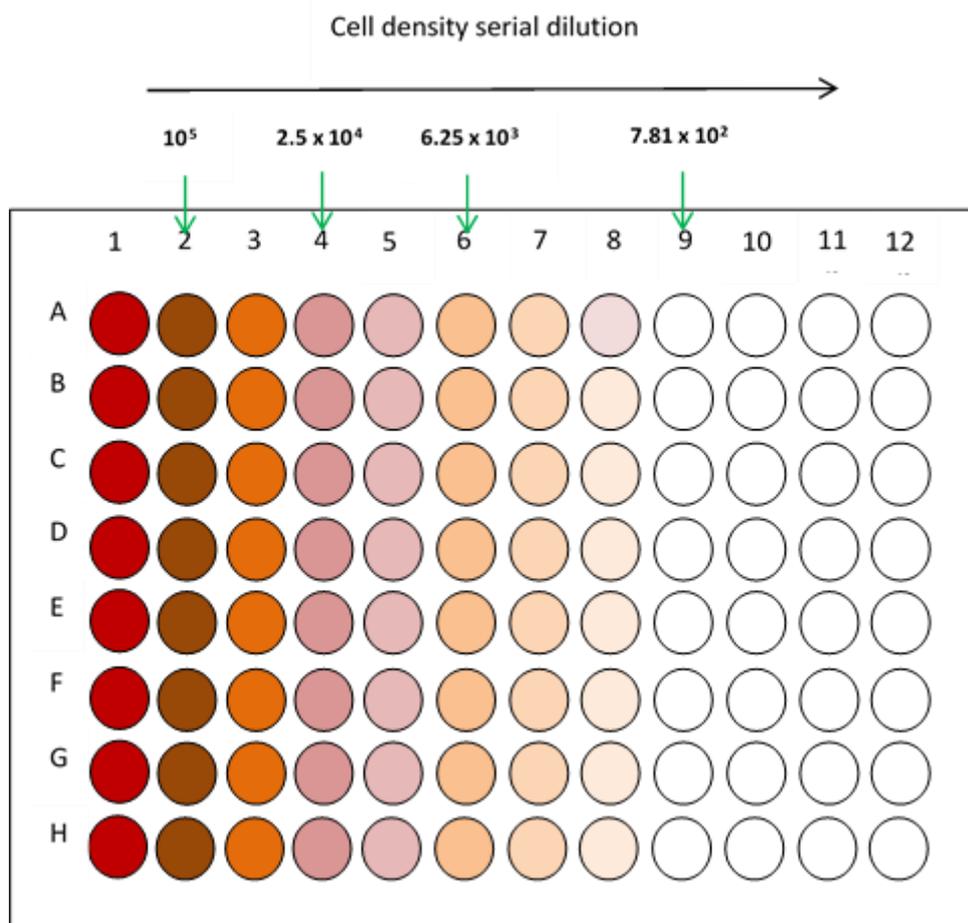
1. To perform a preliminary cell density optimisation assay in order to decipher an appropriate cell seeding density for BEAS 2B and CALU 3 cells to perform the subsequent nicotine/cotinine/E-vehicle submerged culture experiments.
2. To investigate the 4 h and 24 h exposure effects of varying concentrations of nicotine (1.17  $\mu$ M – 75  $\mu$ M) and cotinine (1.56  $\mu$ M – 100  $\mu$ M) on the cell viability and IL-6/IL-8 pro-inflammatory mediators' production of BEAS 2B and CALU 3 bronchial epithelial cells.
3. To analyse the 24 h exposure effects of varying concentrations (1.56 % - 100 %) of E-vehicle fluid on BEAS 2B and CALU 3 bronchial epithelial cell viability.

## 5.3 Materials and methods

### 5.3.1 Preliminary cell viability experiments

The main objective of this set of experiments was to seed BEAS 2B and CALU 3 cells at different cell densities in a 96 well plate in order to determine the optimum cell density for the subsequent nicotine/cotinine/E-vehicle submerged culture experiments. This optimum cell density (i.e. number of cells added per well of a 96 well plate) would be constant for all experiments carried out in a 96 well plate throughout the study.

The experimental procedure was identical for both BEAS 2B and CALU 3 cells. The experiments were performed on a transparent flat bottomed 96 well plate (Greiner bio-one, Frickenhausen, Germany). The highest cell density was decided to be  $10^5$  cells/well for both the cell types and a serial dilution was carried out in the 96 well plate in the following manner. Appropriate number of culture flasks were trypsinised (as described in section 3.2.2) and a cell suspension of  $10^6$  cells/mL was obtained. 200  $\mu$ L of this suspension was added to each well of the second column of the 96 well plate as shown in Figure 5.1. Using a multi-channel pipette (Discovery Comfort, HTL lab solutions Warsaw, Poland), 100  $\mu$ L of the cell suspension (equal to  $10^5$  cells) from the second column was mixed carefully with 100  $\mu$ L of growth medium in the next column. In this manner, the pipetting process was continued until a cell density of  $7.81 \times 10^2$  cells/well was reached in the 9<sup>th</sup> column as shown in Figure 5.1. The plate was then transferred into a standard, humidified cell culture incubator for 24 h. After the incubation period, 20  $\mu$ L of CTB was added to each well containing cells and incubated for 2 h at 37° C after which the fluorescence intensity (F.I.) was measured using a spectrophotometer as previously described in section 3.3.1. The F.I. values were plotted against their respective cell densities and the optimum cell density was determined by choosing the average cell density of the three points with the highest F.I. values. Such an optimum seeding density would provide a strong signal (F.I.) in response to an increase or decrease in cell number (viability) since it ranges between the highest and the third highest cell density.



**Figure 5.1. Pictorial representation of the serial dilution process of cell optimisation experiments performed in a 96 well plate.**

As shown in the above figure, 200  $\mu\text{L}$  of  $10^6$  cells/mL cell suspension was added to each well of the second column of a flat-bottomed transparent 96 well plate which would give  $2 \times 10^5$  cells in each well of the second column. In order to perform serial dilution, 100  $\mu\text{L}$  of growth medium was added to each well of the subsequent columns 3-9. Then, 100  $\mu\text{L}$  of the top cell density (which would equal  $10^5$  cells/mL) was added to the 100  $\mu\text{L}$  growth medium in the subsequent column to obtain a cell density of  $5 \times 10^4$  in each well of the third column. The serial dilution was continued until the lowest cell density of  $7.81 \times 10^2$  was obtained in each well of the 9<sup>th</sup> column of the 96 well plate.

### 5.3.2 Nicotine preparation

0.003738 g of Nicotine ditartate (molecular weight = 492.94) obtained from TOCRIS bioscience (Bristol, UK) was diluted in 100  $\mu\text{L}$  DMSO (Sigma Aldrich, Dorset, UK) to achieve a stock concentration of 75 mM. The stock was aliquoted into sterile centrifuge tubes and stored at  $-20^\circ\text{C}$ . On the day of experiments, appropriate number of centrifuge tubes were thawed and a 1:1000 dilution was performed with growth medium to achieve the top working nicotine concentration of 75  $\mu\text{M}$ . The top concentration was then serially diluted until the bottom nicotine concentration of 1.17  $\mu\text{M}$  was obtained.

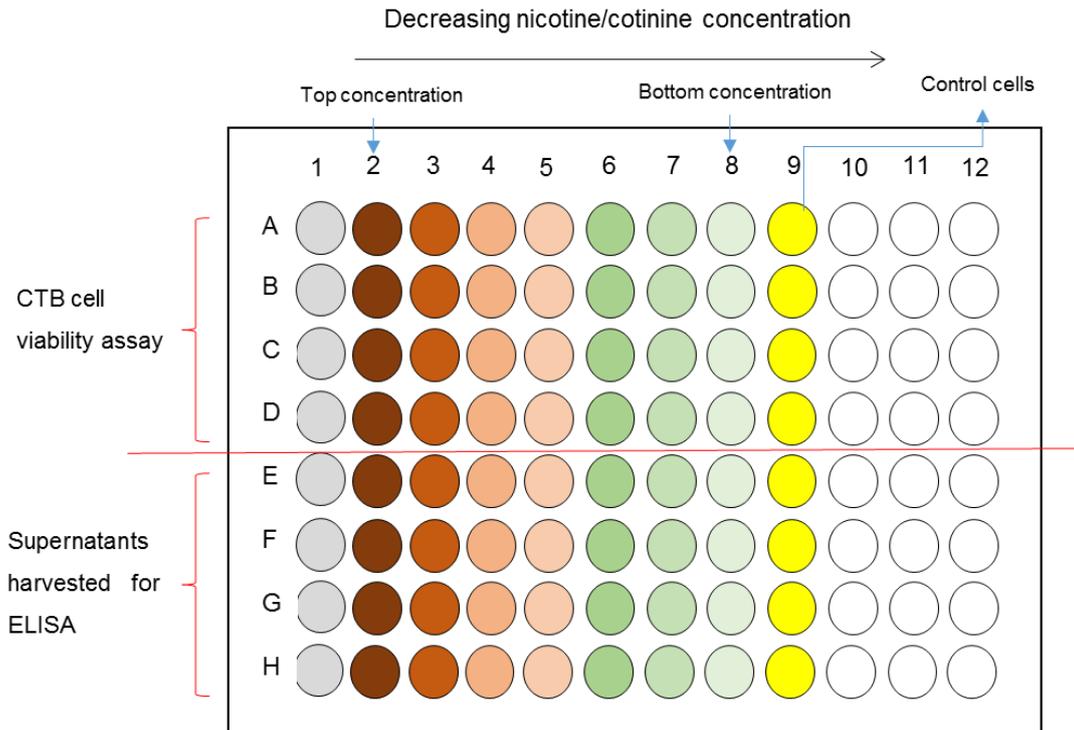
### 5.3.3 Cotinine preparation

0.00178 g of Cotinine (Molecular weight = 178.02) obtained from TOCRIS bioscience (Bristol, UK) was diluted in 100  $\mu$ L of DMSO to get the stock cotinine concentration of 100 mM. Similar to nicotine, cotinine stock was also aliquoted in sterile centrifuge tubes and stored at  $-20^{\circ}\text{C}$ . On the day of experiment, appropriate number of centrifuge tubes were thawed and a 1:1000 dilution was performed with growth medium to obtain the top working cotinine concentration of 100  $\mu$ M. The top concentration was then serially diluted until the bottom cotinine concentration of 1.56  $\mu$ M was obtained.

### 5.3.4 Exposure of submerged bronchial epithelial cells to nicotine and cotinine

The plate-layout of nicotine/cotinine exposure experiments is depicted in Figure 5.2. For cell seeding, appropriate number of culture flasks were trypsinised and a cell suspension of  $10^5$  cells/mL was obtained. 100  $\mu$ L of this cell suspension was seeded in each well of a 96 well plate (equalling to  $5 \times 10^4$  cells/well) and incubated at  $37^{\circ}\text{C}$  overnight to allow the cells to adhere to the plate. The following day, the existing medium was removed and the cells were treated to 100  $\mu$ L of increasing nicotine (1.17  $\mu$ M to 75  $\mu$ M) or cotinine (1.56  $\mu$ M to 100  $\mu$ M) concentrations. As shown in Figure 5.2, the top nicotine (75  $\mu$ M) or cotinine (100  $\mu$ M) concentration was added to the cells in the second column of the plate (A2- H2). The top concentration was then serially diluted until the bottom nicotine (1.17  $\mu$ M) or cotinine (1.56  $\mu$ M) concentration was added to cells in the 8th column of the plate (A8 - H8). Cells treated with growth medium alone acted as the untreated control cells (9<sup>th</sup> column). Cells were then incubated at  $37^{\circ}\text{C}$ , 5 %  $\text{CO}_2$  either for either 4 h or 24 h. After 4 h or 24 h exposure time, cell viability was analysed using CTB assay as previously described in section 3.3.1. A condition was deemed cytotoxic if, according to the ISO UNI EN ISO 10993-5 standard, it caused a reduction in cell viability to less than 70 % of the untreated control cells.

In order to assess the pro-inflammatory mediator release in response to different concentrations of nicotine/cotinine, supernatants ( $\sim 100$   $\mu$ L) from each well of the bottom four rows of the 96 well plate (as shown in Figure 5.2) were pooled together in a centrifuge tube and centrifuged at  $1000 \times g$  for 5 min to clear cells (if any). The supernatants were then used immediately or stored at  $-20^{\circ}\text{C}$  for ELISA analysis which was performed as per the procedure described in section 3.4 (sub-sections 3.4.1 - 3.4.3).



**Figure 5.2. Plate lay-out of nicotine and cotinine exposure experiments.**

100  $\mu$ L of BEAS 2B or CALU 3 cells were seeded in each well of a 96 well plate (columns 2-9) at a density of  $5 \times 10^4$  cells/well and incubated at 37°C overnight in order allow the cells to adhere to the plate. The following day, the existing medium was removed from the wells and 100  $\mu$ L of different concentrations of nicotine (1.17  $\mu$ M to 75  $\mu$ M) or cotinine (1.56  $\mu$ M to 100  $\mu$ M) was added to the appropriate wells in columns 2-8. The 9<sup>th</sup> column (A9 - H9) consisted of control cells which were the untreated cells. The cells were then incubated for 4 h or 24 h after which the cell viability and ELISA analysis was performed. As shown in the above figure, the supernatants from the bottom four rows (E-H) were harvested, centrifuged at 1000 x g for 5 min to remove any existing cells and stored at -20°C for pro-inflammatory mediator release investigation via ELISA. Cell viability was analysed in the top four rows (A-D) using CTB as previously described in section 3.3.1.

### 5.3.5 Exposure of submerged bronchial epithelial cells to E-vehicle fluid

The main aim of this set of experiments was to investigate the impact of E-vehicle fluid exposure on BEAS 2B and CALU 3 bronchial epithelial cell viability. From review of literature and EC online stores, it was observed that 70 % PG: 30 % VG was the most commonly employed E-vehicle ratio in most first generation commercially available ECs and thus this ratio of E-vehicle was chosen for investigation in the current study. PG and VG were purchased from Sigma Aldrich (Dorset, UK). An E-vehicle consisting of 70 % PG and 30 % VG (e.g. 700  $\mu$ L of PG and 300  $\mu$ L of VG in order to get 1 mL of E-vehicle fluid) was prepared in-house in the lab which constituted the top E-vehicle working concentration. Similar to nicotine/cotinine experiments, the highest E-vehicle concentration was serially diluted with growth medium until the lowest E-vehicle concentration of 1.56 % was attained. The experimental procedure including the BEAS 2B/CALU 3 cell seeding density and incubation

times were similar to that of the nicotine/cotinine exposure experiments. Briefly, 100  $\mu$ L of BEAS 2B or CALU 3 cells were added to appropriate wells of a 96 well-plate (Figure 5.2) at a density of  $5 \times 10^4$  cells/well and incubated overnight. The following day, after discarding the existing medium, cells were treated with 100  $\mu$ L of different E-vehicle concentrations (from 100 % to 1.56 %) and incubated for 24 h after which the CTB cell viability analysis was performed.

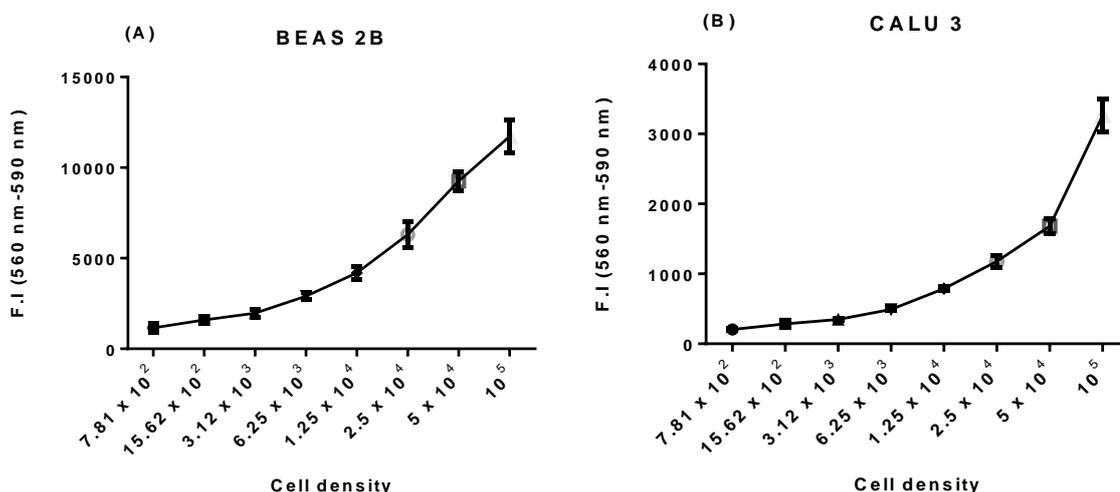
### 5.3.6 Statistics

All statistical analysis were performed in GraphPad<sup>®</sup>, V7 (GraphPad Software Inc., La Jolla, CA, USA). Data was analysed using either one-way ANOVA followed by either Dunnet or Tukey's post-hoc test. In all experiments, statistical difference higher than 0.1 was considered as non-significant (indicated by 'ns' in figure legends).

## 5.4 Results

### 5.4.1 Determination of BEAS 2B and CALU 3 optimum cell seeding density

Figure 5.3 (A) and (B) represent the results of the preliminary cell viability assay (section 5.3.1) which was performed to determine the optimum BEAS 2B and CALU 3 cell seeding density respectively for subsequent nicotine/cotinine/E-vehicle submerged culture experiments. As it can be observed from Figure 5.3 (A), there was a linear increase in the F.I. values as the BEAS 2B cell density increased whereas for CALU 3 cells, the trend seemed linear as well although an exponential increase in F.I. was observed at the highest cell density as shown in Figure 5.3 (B). For both cell types,  $10^5$  cells/well resulted in the highest F.I. value ( $11785.25 \pm 865.82$  F.I. unit for BEAS 2B;  $3261.57 \pm 223.01$  F.I. units for CALU 3). However, choosing the top cell density as the optimum seeding density would not be able to produce a strong signal in response to cell proliferation and hence a cell density that can potentially produce a sharp signal in response to both increase and decrease in cell viability is required. Considering the average cell density between the top three points,  $5 \times 10^4$  cells/well was chosen as the optimum cell seeding density for both BEAS 2B and CALU 3 cells. This cell density produced a F.I. of  $9320.19 \pm 542.78$  F.I. units for BEAS 2B and  $1682.44 \pm 105.75$  F.I. units for CALU 3 cells, thus capable of producing a marked change in F.I. in response to an increase or decrease in cell viability during experiments.

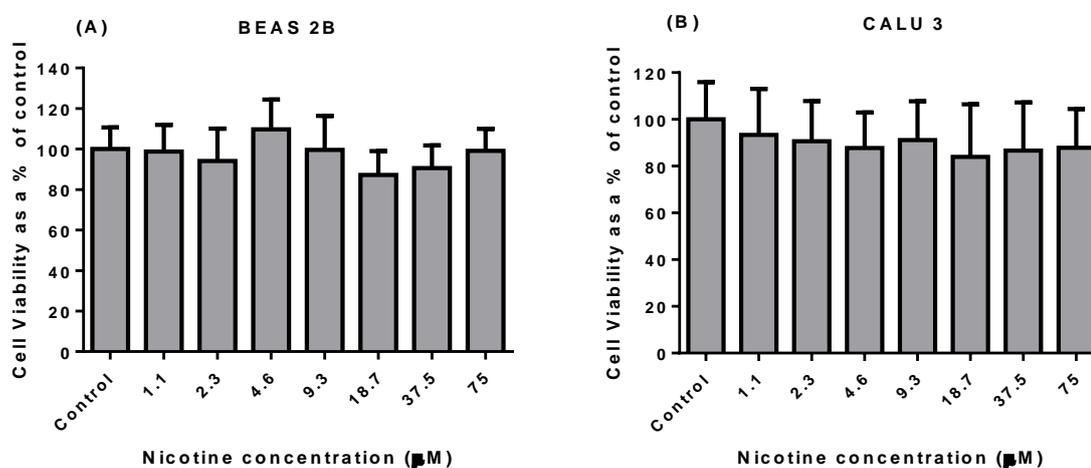


**Figure 5.3. Determination of optimum cell density of (A) BEAS 2B and (B) CALU 3 for submerged culture experiments in a 96 well plate.**

While a linear increase in F.I. values was observed in both BEAS 2B cells, a slightly exponential trend towards the highest cell density was observed in CALU 3 cells. Considering a cell density which would give a strong F.I. signal in response to an increase or decrease in cell viability,  $5 \times 10^4$  cells/well which was the average of the top three cell densities was chosen as the optimum cell density for both BEAS 2B and CALU 3 cells. Each data point represents Mean  $\pm$  SD of three individual experiments with triplicate samples per experiment.

#### 5.4.2 Influence of 4 h treatment of different concentrations of nicotine on submerged bronchial epithelial cells

As described in the methodology,  $5 \times 10^4$  cells/well of BEAS 2B and CALU 3 bronchial epithelial cells were exposed to different concentrations of nicotine ranging from 1.1  $\mu\text{M}$  to 75  $\mu\text{M}$  for 4 h after which the CTB cell viability analysis was performed as previously explained in sub-section 5.3.4. As shown in Figure 5.4 (A) and (B), nicotine did not have any significant influence on the cell viability of BEAS 2B or CALU 3 respectively at any of the different tested concentrations, compared to the untreated control.

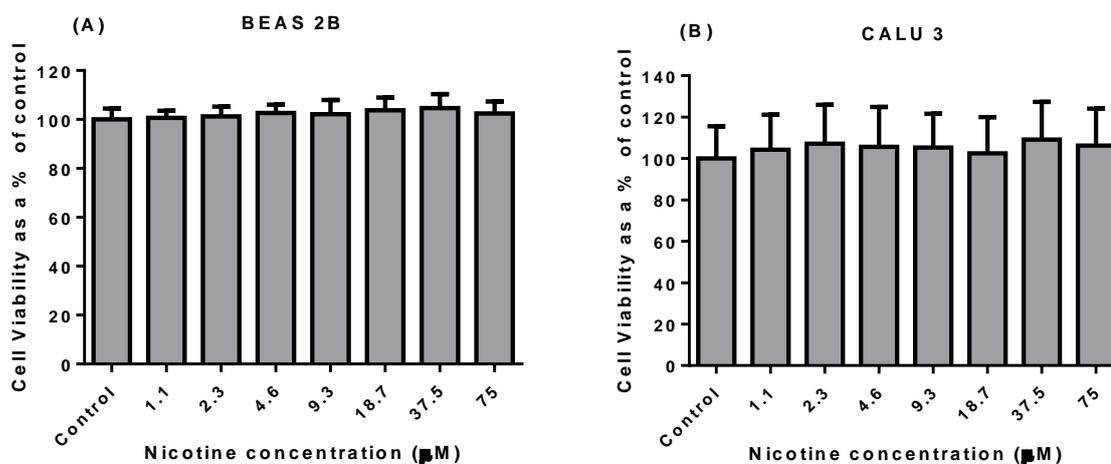


**Figure 5.4. Influence of 4 h nicotine treatment on the viability of (A) BEAS 2B and (B) CALU 3 submerged bronchial epithelial cells.**

$5 \times 10^4$  cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to varying concentrations of nicotine for 4 h post which CTB cell viability analysis was performed as per the procedure described in sub-section 5.3.4. As it can be observed from the above figures, 4 h nicotine treatment did not significantly influence the cell viability at any of the tested concentration for either of the cell types. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of three individual experiments, with triplicate samples per experiment.

### 5.4.3 Influence of 24 h treatment of different concentrations of nicotine on submerged bronchial epithelial cells

Upon treating BEAS 2B and CALU 3 bronchial epithelial cells to varying concentrations of nicotine for 24 h, CTB cell viability analysis as shown in Figure 5.5 (A) and (B), showed that, even longer nicotine exposure did not significantly influence the cell viability. The viability of nicotine treated cells was comparable to that of the untreated control cells in both BEAS 2B (Figure 5.5 A) and CALU 3 (Figure 5.5 B) cells.

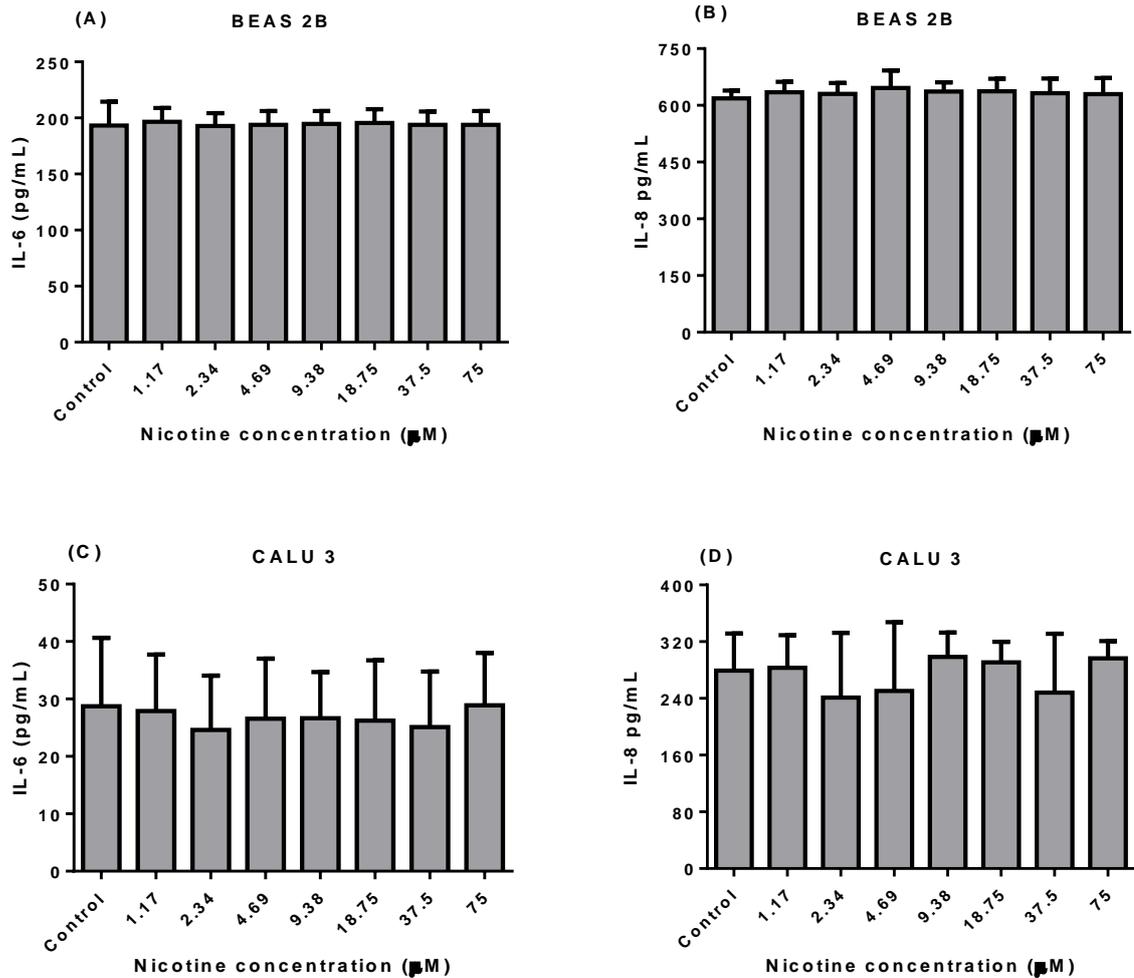


**Figure 5.5. Influence of 24 h nicotine treatment on the viability of (A) BEAS 2B and (B) CALU 3 submerged bronchial epithelial cells.**

$5 \times 10^4$  cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to varying concentrations of nicotine for 24 h post which CTB cell viability analysis was performed as per the procedure described in subsection 5.3.4. As shown in the above figures, 24 h nicotine treatment did not significantly influence the cell viability at any of the tested concentrations in both the cell types. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of three individual experiments, with triplicate samples per experiment.

#### 5.4.3.1 *Influence of 24 h nicotine treatment on submerged bronchial epithelial cell IL-6 and IL-8 pro-inflammatory mediators' production*

After treating BEAS 2B and CALU 3 bronchial epithelial cells to varying concentrations of nicotine for 24 h, the cell culture supernatants were analysed via ELISA for IL-6 and IL-8 levels as described in sub-section 5.3.4. Figure 5.6 (A) and (B) illustrates the IL-6 and IL-8 release, respectively, by BEAS 2B cells, 24 h post exposure to different nicotine concentrations. It can be observed from Figure 5.6 (A) and (B) that nicotine did not have any significant influence on IL-6 or IL-8 production at any of the tested concentrations. The IL-6/IL-8 produced by nicotine treated cells (IL-6 levels ranged between  $193.10 \pm 11.31$  pg/mL to  $196.99 \pm 12.27$  pg/mL ; IL-8 levels ranged between  $630.37 \pm 27.28$  pg/mL to  $645.75 \pm 44.55$  pg/mL) were comparable to that of the control cells (IL-6:  $193.17 \pm 21.33$  pg/mL ; IL-8:  $618.52 \pm 19.64$  pg/mL). CALU 3 cells exposed to different nicotine concentrations demonstrated a similar response to that of BEAS 2B. Figure 5.6 (C) and (D) describes the IL-6 and IL-8 levels respectively produced by CALU 3 cells 24 h post exposure to different concentrations of nicotine. As it can be observed from Figure 5.6 (C) and (D), nicotine did not exert any significant influence over CALU 3 IL-6 and IL-8 production respectively. Similar to BEAS 2B, the levels (IL-6 levels ranged between  $28.88 \pm 8.61$  pg/mL to  $24.60 \pm 8.91$  pg/mL ; IL-8 levels ranged between  $241.19 \pm 86.13$  pg/mL to  $298.34 \pm 32.63$  pg/mL) were comparable to that of the untreated control cells (IL-6:  $28.71 \pm 11.26$  pg/mL ; IL-8:  $279.25 \pm 49.21$  pg/mL)

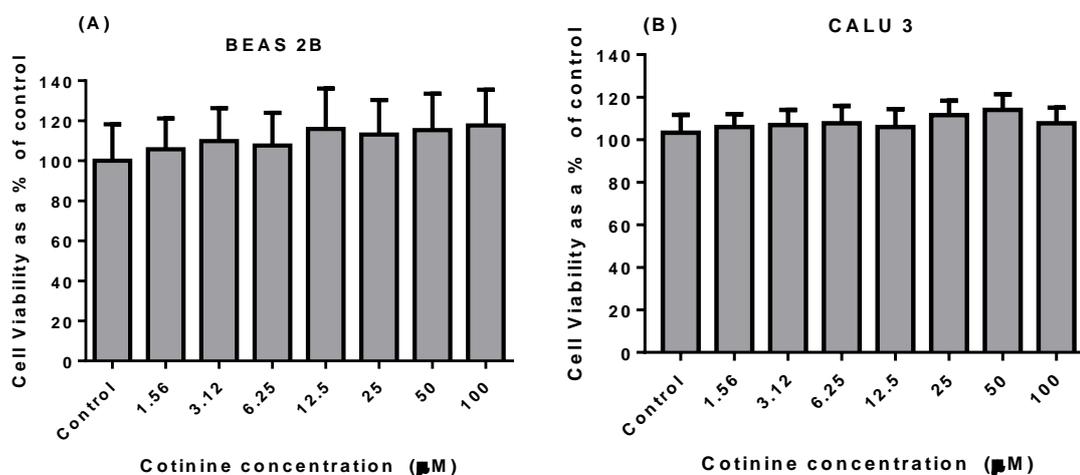


**Figure 5.6. Effect of 24 h nicotine treatment on bronchial epithelial cell pro-inflammatory mediators' production. (A) BEAS 2B IL-6 (B) BEAS 2B IL-8 production (C) CALU 3 IL-6 and (D) CALU 3 IL-8 production.**

5 x 10<sup>4</sup> cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to varying concentrations of nicotine for 24 h post which the cell culture supernatants were collected as described in sub-section 5.3.4 and ELISA analysis was performed as per the procedure explained in section 3.4.1-3.4.3. As seen from the above figure, nicotine did not have any significant effect on the release of IL-6 or IL-8 by either cell type at any of the different tested concentrations. The levels of IL-6 and IL-8 produced by the treated cells were comparable to that of the untreated control cells. Each bar represents Mean ± S.D. of three individual experiments, with triplicate samples per experiment.

#### 5.4.4 Influence of 4 h treatment of different concentrations of cotinine on submerged bronchial epithelial cells

Using a similar methodology to the nicotine exposure experiments,  $5 \times 10^4$  cells/well of BEAS 2B and CALU 3 bronchial epithelial cells were exposed to different concentrations of cotinine ranging from 1.56  $\mu\text{M}$  to 100  $\mu\text{M}$  for 4 h, after which CTB cell viability analysis was performed. As shown in Figure 5.7, cotinine did not have any significant impact on the cell viability of BEAS 2B (Figure 5.7A) or CALU 3 (Figure 5.7B) at any of the tested concentrations.

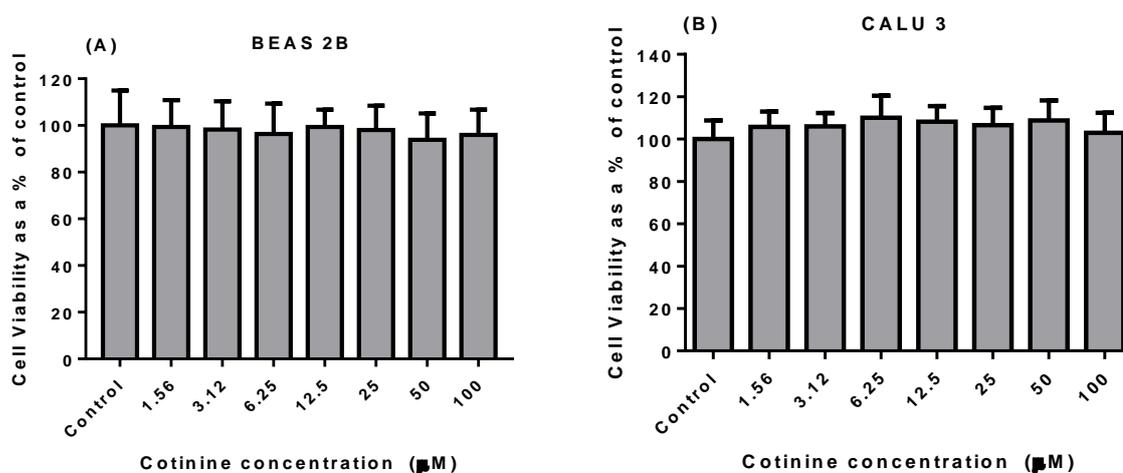


**Figure 5.7. Influence of 4 h cotinine treatment on the viability of (A) BEAS 2B and (B) CALU 3 submerged bronchial epithelial cells.**

$5 \times 10^4$  cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to varying concentrations of cotinine for 24 h post which CTB cell viability analysis was performed as per the procedure described in subsection 5.3.4. As shown in the above figures, 4 h cotinine treatment did not significantly influence the cell viability at any of the tested concentrations in both the cell types. The viability of cotinine treated cells were comparable to that of the control cells for both cell types. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of three individual experiments, with triplicate samples per experiment.

#### 5.4.5 Influence of 24 h treatment of different concentrations of cotinine on submerged bronchial epithelial cells

Similar to 4 h cotinine exposure, BEAS 2B and CALU 3 cells were treated to varying concentrations of cotinine for 24 h. Figure 5.8 (A) and (B) describes the CTB cell viability results for BEAS 2B and CALU 3 respectively, performed 24 h post exposure to different cotinine concentrations. As shown in Figure 5.8, none of the cotinine concentrations tested had a significant impact on BEAS 2B or CALU 3 cell viability.

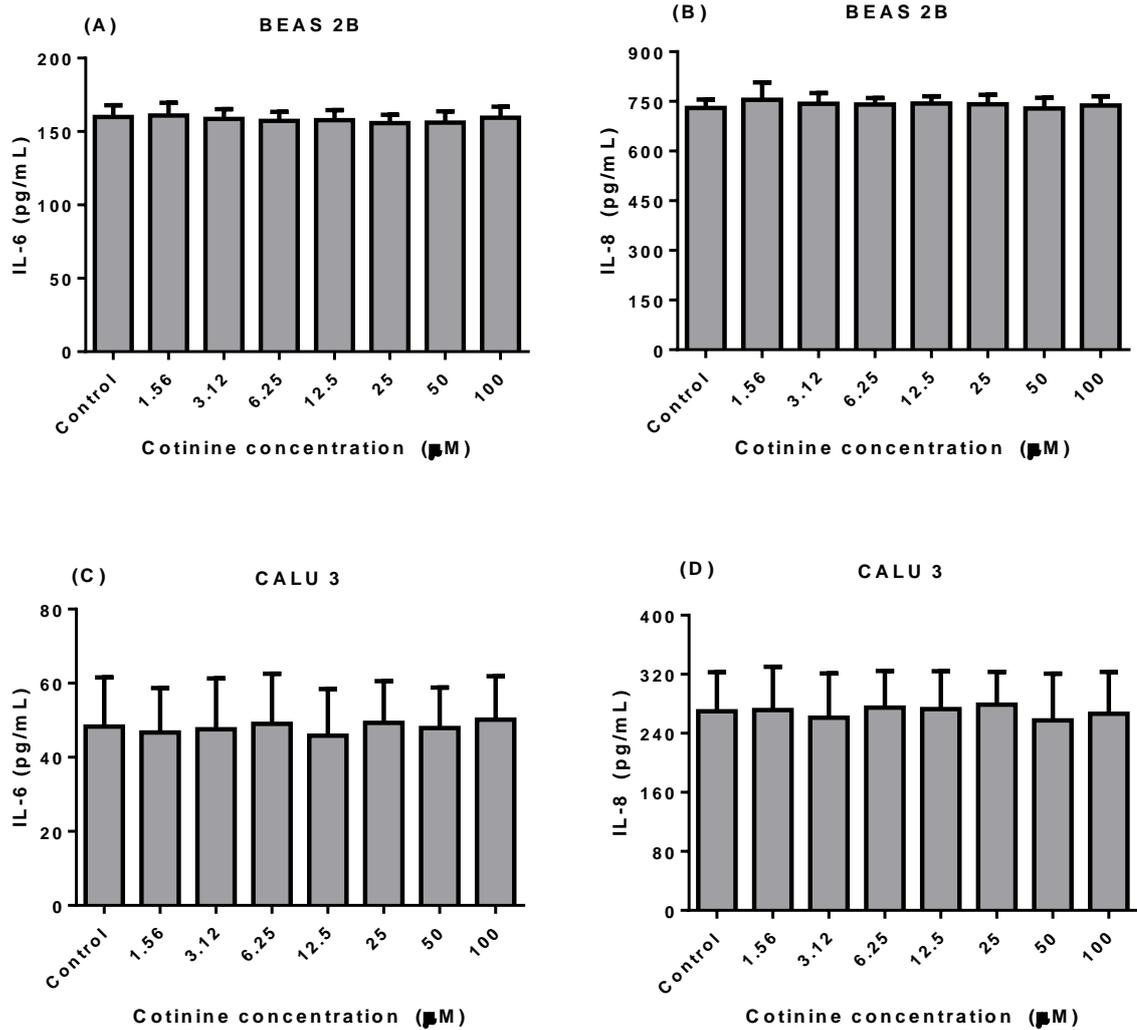


**Figure 5.8. Influence of 24 h cotinine treatment on the viability of (A) BEAS 2B and (B) CALU 3 submerged bronchial epithelial cells.**

$5 \times 10^4$  cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to varying concentrations of cotinine for 24 h post which CTB cell viability analysis was performed as per the procedure described in subsection 5.3.4. As shown in the above figure, 24 h cotinine treatment did not significantly influence the cell viability at any of the tested concentration in both the cell types. Similar to 4 h cotinine exposure results, the viability of cotinine treated cells were comparable to that of the control cell for both cell types. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of three individual experiments, with triplicate samples per experiment.

#### 5.4.5.1 *Influence of 24 h cotinine treatment on submerged bronchial epithelial cells IL-6 and IL-8 pro-inflammatory mediators' production*

Following treatment of BEAS 2B and CALU 3 bronchial epithelial cells to varying concentrations of cotinine for 24 h, the cell culture supernatants were harvested and analysed via ELISA for IL-6 and IL-8 production as described in subsection 5.3.4. Figure 5.9 (A) and (B) illustrates the IL-6 and IL-8 levels, respectively produced by BEAS 2B cells 24 h post exposure to different cotinine concentrations. It can be observed from Figure 5.9 (A) and (B) that cotinine did not influence the IL-6 or IL-8 production at any of the tested concentrations. For cotinine treated BEAS 2B cells, IL-6 levels ranged between  $160.94 \pm 8.16$  pg/mL to  $155.68 \pm 5.48$  pg/mL and IL-8 levels ranged between  $754.60 \pm 49.90$  pg/mL to  $728.44 \pm 30.64$  pg/mL; and this was comparable to that of the untreated control (IL-6:  $159.96 \pm 7.58$  pg/mL ; IL-8:  $729.81 \pm 24.48$  pg/mL), indicating an absence of any significant inflammatory response to cotinine. CALU 3 cells exposed to cotinine demonstrated a similar result to that of BEAS 2B. Figure 5.9 (C) and (D) describes the IL-6 and IL-8 levels respectively produced by CALU 3 cells 24 h post exposure to different concentrations of cotinine. As it can be observed from Figure 5.9 (C) and (D), cotinine did not exert any significant influence over CALU 3 IL-6/IL-8 production. The IL-6/IL-8 levels produced by cotinine-treated CALU 3 cells (IL-6 levels ranged between  $50.15 \pm 11.04$  pg/mL to  $45.80 \pm 11.87$  pg/mL ; IL-8 levels ranged between  $278.84 \pm 41.74$  pg/mL to  $257.39 \pm 59.70$  pg/mL) were comparable to that of the untreated control cells (IL-6:  $48.30 \pm 12.52$  pg/mL ; IL-8:  $269.85 \pm 49.96$  pg/mL)

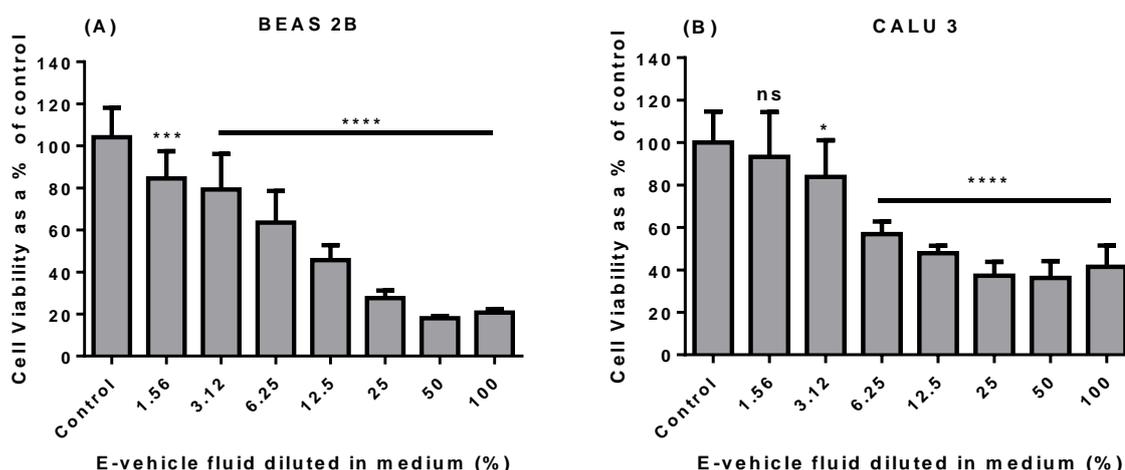


**Figure 5.9. Effect of 24 h cotinine treatment on bronchial epithelial cell pro-inflammatory mediators' production. (A) BEAS 2B IL-6 (B) BEAS 2B IL-8 production (C) CALU 3 IL-6 and (D) CALU 3 IL-8 production.**

$5 \times 10^4$  cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to varying concentrations of cotinine for 24 h post which the cell culture supernatants were collected as described in sub-section 5.3.4 and ELISA analysis was performed as per the procedure explained in section 3.4.1-3.4.3. As seen from the above figure, cotinine did not have any significant effect on the IL-6 or IL-8 production by either cell type at any of the different tested concentrations. The levels of IL-6 and IL-8 produced by the treated cells were comparable to that of the untreated control cells. Each bar represents Mean  $\pm$  S.D. of three individual experiments, with triplicate samples per experiment.

#### 5.4.6 Influence of E-vehicle fluid on submerged BEAS 2B and CALU 3 bronchial epithelial cells

As described in the methodology sub-section 5.3.5, BEAS 2B and CALU 3 bronchial epithelial cells ( $5 \times 10^4$  cells/well of a 96 well plate) were exposed to different concentrations of 70 PG : 30 VG E-vehicle fluid. Figure 5.10 (A) and (B) depicts the CTB cell viability results of BEAS 2B and CALU 3 respectively, 24 h post exposure to different E-vehicle concentrations. As it can be observed from Figure 5.10 (A), all the tested E-vehicle concentrations caused a significant decrease in the BEAS 2B cell viability compared to the untreated control cells. A dose-dependent decrease in cell viability was observed between the concentrations points of 50 % and 3.12 %. CALU 3 cells produced a similar result to that of BEAS 2B, although a dose-dependent trend was not observed in CALU 3 cells. Never-the-less, all the tested E-vehicle concentrations, except the 1.56 %, produced a significant decrease in CALU 3 cell viability compared to control cells.



**Figure 5.10. Influence of 24 h E-vehicle treatment on the viability of (A) BEAS 2B and (B) CALU 3 submerged bronchial epithelial cells.**

$5 \times 10^4$  cells/well of BEAS 2B or CALU 3 bronchial epithelial cells were treated to different E-vehicle fluid concentrations ranging from 1.56 % to 100 % for 24 h, post which cell viability was analysed using CTB assay as described in sub-section 5.3.5. As seen from the above figures, all the tested E-vehicle concentrations had a significant impact on the BEAS 2B cell viability compared to the untreated control cells. CALU 3 cells too demonstrated a similar response wherein, except the 1.56 % concentration, all the other tested E-vehicle concentrations caused a significant reduction of cell viability compared to the control cells. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of three individual experiments, with triplicate samples per experiment. (ns = not significant, \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ )

## 5.5 Discussion

Despite increasing popularity amongst adults and adolescents world-wide, research data on acute and chronic health effects of ECs are currently limited (Knorst et al., 2014). Besides, data on their ability to act as alternatives to tobacco cigarettes or as smoking cessation tools lacks scientific consensus (Grana et al., 2014). Under these circumstances, the promotion of ECs as smoking cessation tools or effective alternatives to tobacco cigarettes, especially by regulatory bodies like Public Health England (PHE), could be highly misleading to the public (Flint and Jones, 2018). Currently, more research is required on the biological impact of ECs on the human airways which are the predominant organ that interact with aerosols of ECs. Prior to investigating the effect of aerosols of ECs using complex 3D ALI cell culture models, exploring the impact of the chief components of EC liquid such as nicotine and E-vehicle base liquid using submerged cultures would provide useful information on the possible effects mediated by these individual EC constituents. In this context, the main aim of this chapter of the study was to assess the effects of direct exposure of the chief individual constituents of ECs namely nicotine, its oxidative metabolite cotinine and E-vehicle on BEAS 2B and CALU 3 bronchial epithelial cells.

### 5.5.1 Nicotine and cotinine do not influence bronchial epithelial cell viability

The nicotine/cotinine levels in the plasma of smokers vary depending upon the smoking habit of individuals. It has been noted that the nicotine plasma levels in heavy smokers is  $\sim 10 \mu\text{M}$  ( $2 \mu\text{g/mL}$ ) (Hukkanen et al., 2005, Teneggi et al., 2002). However, the term “heavy-smoking” is subjective and hence the plasma nicotine levels can vary widely with individuals. Hence, in the current study, a nicotine concentration range around  $10 \mu\text{M}$ , i.e.  $1.1$  to  $75 \mu\text{M}$ , was tested; in agreement with a number of previous studies which investigated nicotine effects *in-vitro* around similar dose-range (Li et al., 2010a, Ginzkey et al., 2014). Since cotinine concentrations are generally found to be higher than nicotine in the plasma of smokers (Langone et al., 1973, Etter and Bullen, 2011), in the current study, cotinine was tested at concentrations higher than that of top nicotine concentration i.e. up to  $100 \mu\text{M}$ .

As described in Figure 5.4 - 5.5, compared to the untreated cells, none of the tested nicotine concentrations significantly impacted the cell viability of BEAS 2B or CALU 3 at either exposure time (4 h and 24 h). This result agrees with a number of past studies where nicotine did not induce any cytotoxic effect and in fact exhibited a cell-protective effect. Utsumi et al (2004) reported that nicotine ( $1 - 100 \mu\text{M}$ ) protected PC 12 rat pheochromocytoma from apoptosis induced by tunicamycin (Utsumi et al., 2004) while Kihara et al (1997) reported on the protective effects of nicotine which inhibited  $\beta$ -amyloid

toxicity in neuronal cells (Kihara et al., 1998). However, a direct comparison of these studies with the current study is difficult due to different cell types being assessed. As previously stated in the introduction of this chapter, there is a dearth of studies investigating the cytotoxic effects of nicotine on human airways cells and hence comparability is currently limited. One possible reason for the observed null effect of nicotine on cell viability could be that the tested nicotine concentrations were in the sub-toxic range and that higher nicotine concentrations could have possibly impacted BEAS 2B and CALU 3 cell viability. For instance, Ramage et al (2006) treated A549 lung epithelial cells to high concentrations of nicotine (10 mM) for 4, 24 and 48 h and reported that nicotine treatment lead to a significant increase in cell death of A549 cells (Ramage et al., 2006). Other studies employing high concentrations of nicotine (millimolar range) also reported significant decrease in cell viability (Bodas et al., 2016, Patil et al., 2009). Further, in line with the speculated dose-dependent effects of nicotine, Guan et al (2003) reported an interesting correlation between nicotine concentration and cell response (Guan, 2003). The study reported that nicotine can act as a cytotoxic agent or non-cytotoxic agent depending on the concentration of nicotine the cells are exposed to. Higher dosages of nicotine (1 - 10 mM) produced oxidative stress leading to cytotoxicity whereas at lower doses (1 – 100  $\mu$ M) nicotine behaved more like an anti-oxidant and had a protective effect (Guan, 2003). In another study that reported a similar dual-effect of nicotine, An et al (2014) reported that low nicotine concentrations (10  $\mu$ M – 1 mM) had no significant impact on cell viability/proliferation while higher concentrations of nicotine (10 mM) inhibited cell proliferation and decreased cell viability post 4 h incubation (An et al., 2014). Never-the-less, the dose-dependent effects of nicotine must be interpreted with caution. The cytotoxic effect observed at high nicotine concentrations is not a uniform trend and is cell-type dependent as there have been reports of micro-molar concentrations of nicotine exhibiting cytotoxicity (Crowley-Weber et al., 2003, Kondeva-Burdina et al., 2010). It can thus be ascertained that the biological effects of nicotine depends on a number of factors including the cell type employed, duration of nicotine exposure and the concentration range of nicotine tested (Buisson and Bertrand, 2001, Sopori and Kozak, 1998b).

Cotinine, the main metabolite of nicotine is often used as the primary exposure marker in smoking studies (Hatsukami et al., 2006). As observed from Figure 5.7 – 5.8, the different doses of cotinine (1.56  $\mu$ M to 100  $\mu$ M) had no significant influence BEAS 2B and CALU 3 cell viability. Very few studies have investigated the cytotoxic effects of cotinine *in-vitro* and thus far no study has reported on the effects of cotinine on bronchial epithelial cells. Although a direct comparison is not possible, past studies employing neuronal cell-types to assess cotinine effects have reported on the protective effects of cotinine similar to nicotine

(Buccafusco and Terry, 2003, Riveles et al., 2008). Never-the-less, airway cells may not respond to cotinine in a similar fashion as neuronal cells and hence more studies are required to determine the precise effects of cotinine on airway cells. It is interesting to note that past human studies have reported that cotinine has minimal physiological effects. Benowitz et al (1983) showed that infusion of cotinine at blood concentrations found in heavy smokers had no impact on several cardiovascular parameters such as heart rate, blood pressure, skin temperature, all three of which were sensitive to nicotine administration (Benowitz et al., 1983). It is also interesting to note that cotinine by itself has limited pharmacologic effects and does not influence smoking cessation (Hatsukami et al., 1997). Few studies have measured cotinine levels post EC usage. Studies which measured plasma or saliva cotinine levels post EC vaping reported that the EC usage can generate cotinine levels comparable to that of tobacco cigarettes although the levels may vary with individuals depending upon several factors such as nicotine concentration in EC liquids, EC brand, EC power setting, user experience, prior level of tobacco dependence, volume of puffs, breathing characteristics etc. (Etter and Bullen, 2011, Flouris et al., 2013, Etter, 2014). Since past studies have suggested cotinine to be less cytotoxic than nicotine, and since nicotine delivery via ECs is not as rapid as tobacco cigarettes, it is reasonable to speculate that cotinine may not demonstrate any significant cytotoxic effects on the airway cells *in-vivo* post vaping. Future studies are never-the-less required to establish the toxicity profile of cotinine on bronchial epithelial cells.

#### 5.5.2 Nicotine and cotinine treatment does not influence bronchial epithelial IL-6/IL-8 production

Some of the common cytokines/chemokines released by airway epithelial cells include IL-8 (acts as neutrophil chemoattractant), IL-6 (facilitates acute phase reactions), eotaxin (acts as eosinophil chemoattractant), IL-1 $\beta$  (involved in injury, infection and inflammation) (Devalia et al., 1993, Khair et al., 1995, Abdelaziz et al., 1995, Adachi et al., 1997). In the current study, the secretion of two chief pro-inflammatory mediators namely IL-6 cytokine and IL-8 chemokine was investigated owing to their crucial involvement in several airway inflammatory conditions such as COPD. (Kent et al., 2008, Sopori and Kozak, 1998a). As shown in Figure 5.6 and 5.9, neither nicotine nor cotinine had any significant impact on the IL-6 and IL-8 pro-inflammatory mediators' production in both BEAS 2B and CALU 3 bronchial epithelial cells. This result was found to be in agreement with previous reports. Li et al (2006) reported that nicotine (at concentrations up to 100  $\mu$ M) did not significantly influence the production of IL-6, IL-8 and TNF- $\alpha$  pro-inflammatory mediators' in human bronchial epithelial cells (Li et al., 2010a). In fact, the study reported that nicotine produced

an anti-inflammatory effect, attenuating the production of pro-inflammatory cytokines/chemokines (Li et al., 2010a). Although an anti-inflammatory effect of nicotine was not observed in the current study, it is an interesting aspect of nicotine that has been highlighted by many past studies. Further, it has also been reported that the anti-inflammatory activity of nicotine may play a key role in the low incidence of asthma amongst smokers (Mishra et al., 2008).

There exists several discrepancies in the existing literature pertaining to the inflammatory effects of nicotine and hence comparisons need to be drawn with caution. Similar to nicotine's dose-dependent influence on cell viability, the inflammatory activity of nicotine depends on several other factors such as nicotine dose range, exposure time, cell model employed (cultured cells Vs animal studies) and study design (Lau et al., 2006). Studies have shown nicotine to produce little to no effect on inflammatory mediators' production at low concentrations (micro-molar range) while higher concentrations (milli-molar range) either have a significant pro-inflammatory (Lau et al., 2006, Almasri et al., 2007) or anti-inflammatory effect (Ouyang et al., 2000, Zhou et al., 2012). In a study highlighting this dual-effect, An et al (2014), reported that lower concentrations of nicotine (10  $\mu$ M – 1 mM) had no significant impact on the production of MCP-1 and IL-8 pro-inflammatory mediators in endothelial cells while higher nicotine concentrations (10 mM) demonstrated an anti-inflammatory effect by suppressing expression of MCP-1 and IL-8 (An et al., 2014). Although recent EC studies have reported on the increased production of pro-inflammatory mediators' in response to EC liquids/aerosols (Leigh et al., 2016, Lerner et al., 2015, Schweitzer et al., 2015), the contribution of nicotine to these observed effects have not been established. Further studies are thus required to ascertain the impact of different doses of nicotine on the inflammatory mediators' production by bronchial epithelial cells at different exposure times.

### 5.5.3 E-vehicle fluid exposure leads to a significant decrease in the bronchial epithelial cell viability

PG and VG are both FDA approved compounds widely employed to fabricate E-vehicle base liquids in ECs (Boulay et al., 2017). One of the aims of this chapter was to analyse the exposure effects of E-vehicle fluid in isolation i.e. without nicotine or flavourings on bronchial epithelial cells BEAS 2B and CALU 3. As shown in Figure 5.10, exposure of submerged BEAS 2B or CALU 3 bronchial epithelial cells to E-vehicle fluid for 24 h caused a significant decrease in cell viability at all dilutions. While most EC studies have focused on the effect of nicotine strength or flavourings (which will be discussed in detail in Chapter 6), very few

studies have investigated the biological effects of E-vehicle in isolation. Only two previous studies have analysed the effect of direct exposure of E-vehicle fluid on airway epithelial cells (Gonzalez-Suarez Ignacio, 2017, Rowell et al., 2017). In the study by Ignacio et al, 5 different E-vehicle ratio (0 % PG : 100 % VG, 30 % PG : 70 % VG, 50 % PG : 50 % VG, 70 % PG : 30 % VG, 100 % PG : 0 % VG) were added to NHBE cells for 24 h post which the cell viability was analysed. The study reported that all E-vehicle ratios exhibited a dose-dependent effect on NHBE cells. In the study by Rowell et al (2017), only one E-vehicle concentration of 70 % PG : 30 % VG, similar to that employed in the current study, was tested in its liquid form on submerged CALU 3 cells for 24 h (Rowell et al., 2017). E-vehicle liquid results from the Rowell et al study showed that post 24 h exposure, a significant decrease in cell viability was observed compared to control cells. It is interesting to note that the results of both these studies is in agreement with the current study wherein an E-vehicle liquid consisting of 70 % PG : 30 % VG caused a significant decrease in the viability of bronchial epithelial cells and demonstrating cytotoxicity at concentrations greater than 6.25 %.

While more studies are required to further determine the toxicity profiles of PG and VG on human airways cells, a number of chemical characterisation studies have reported on the detection of carcinogenic compounds in EC aerosols as a result of thermal degradation of PG/VG (Wang et al., 2017, Sleiman et al., 2016). Further, human studies not directly related to ECs have reported on the various detrimental impact of PG/VG inhalation such as exacerbating airway allergic disorders (Choi et al., 2010), acute ocular irritation, increased cough, upper airway irritation (Wieslander et al., 2001), reduction in lung function (Varughese et al., 2005) and development of exogenous lipid pneumonia (McCauley et al., 2012). Although these studies did not investigate into the airway epithelial cell damage mechanisms post exposure to PG or VG, they never-the-less suggest that inhalation of PG or VG aerosols can cause unexpected acute or chronic damage to the airways and thus requires further investigation.

## 5.6 Conclusions

Overall, it can be concluded that under the performed experimental conditions, neither nicotine nor cotinine at any of the tested concentrations exert any cytotoxic or pro-inflammatory effects on BEAS 2B and CALU 3 bronchial epithelial cells. On contrary, E-vehicle fluid exposure caused a significant decrease in BEAS 2B and CALU 3 cell viability, thus exhibiting a cytotoxic effect. Whilst the fluid exposure is unlikely to occur in EC users during real-time vaping, this type of delivery method has several advantages such as high-throughput screening ability due to reduced time and cost, increased repeatability between studies since only PG and VG were used to formulate the E-vehicle and finally, it acts as a good preliminary EC exposure model prior to employing more physiologically relevant EC exposure methods such as extracts and aerosols which would be investigated in the subsequent chapters.

## 6 Investigating the impact of commercially available EC extracts on various airways related cell types

### 6.1 Introduction

ECs unlike tobacco cigarettes are a class of rapidly evolving products with many variables associated with it (Hajek et al., 2014). Comparability and repeatability of studies becomes difficult since the contents and the efficiency of vapourising differ between ECs of different brands, nicotine strength, flavours and EC designs, thus rendering comprehensive analysis of potential health effects of EC very challenging (Thorne et al., 2018a). Hence, there is a requirement for studies to evaluate the influence of the different variables on the cytotoxicity mediated by ECs *in-vitro*. In this chapter of the current study, the influence of three chief variables namely nicotine content, flavourings and brands were investigated.

The impact of flavourings and nicotine content in mediating EC effects has been studied *in vitro*. Bahl et al. exposed various cell types, including embryonic stem cells and human pulmonary fibroblasts, to EC refill fluids from three different manufacturers and found no correlation between cytotoxicity and nicotine content although flavourings had a significant effect on the observed cytotoxicity (Bahl et al., 2012). This association of cytotoxicity with the EC flavourings has been verified with other studies as well (Clapp et al., 2017, Lerner et al., 2015, Muthumalage et al., 2018). Studies have also shown that ECs from a particular brand can be cytotoxic to one cell type but not to others which highlights the cell-type specific effects of ECs (Muthumalage et al., 2018, Yu et al., 2016). Further, identically-labelled ECs from the same brand can have differential composition and may produce differential cytotoxic effects (Bahl et al., 2012).

Since EC fluid exposure does not represent the *in-vivo* vaping process precisely, a more physiologically relevant delivery method was employed in the current chapter experiments where EC extracts were delivered to the cells. Extracts as stated previously in Chapter 2, sub-section 2.5 captures most of the vapour-phase components of cigarette smoke or EC aerosols which includes toxicants such as PAHs', aldehydes and acrolein. Thus, extracts provide a relatively easy, quick method of screening toxicity of a large number of ECs. The data from this set of experiments would then be used to evaluate the most cytotoxic ECs using more physiologically relevant ALI co-culture models.

Studies of EC vapour extract (ECE) on human airways cells are limited. Past *in-vitro* studies evaluating the cytotoxicity of large number of ECEs (~ 20 ECs) have been performed using non-human cell lines including rat cardiomyoblasts (Farsalinos et al., 2013b) and mouse

fibroblasts (Romagna et al., 2013). Pertaining to ECE studies employing human cells, data thus far predominantly includes the effects of ECE on immune cells such as kupffer cells (Rubenstein et al., 2015) or neutrophils (Rubenstein et al., 2015) or other immune cells (Clapp et al., 2017) as previously described in literature review section 2.6.4.2. Thus, more data is required on the effect of ECEs on airways epithelial cells. In this scenario, this is the first study to comprehensively analyse the cytotoxicity of 15 commercially available ECs from 5 different brands with different nicotine strengths and flavours on multiple airways related cell types namely 4 bronchial epithelial cell types (BEAS 2B, IB3-1, C38 and CALU 3), 2 immune cell types (J774 macrophages and THP-1 monocytes) and one fibroblast cell line (HPF). These cells were selected in order to test the effects of ECE on the most relevant cell types likely to be affected by vaping; namely the bronchial epithelial cells lining the upper respiratory tract, the underlying fibroblast cells, and the macrophages, defensive cells that patrol the airways to maintain sterility and minimise damage. The majority of the experiments of this chapter were performed by two placement students mentored by the author namely Arnold Mabilia and Pamela Jackson, though many other experiments and statistical analyses were performed by the author. The experiments of this chapter lead to the production of a group journal paper titled “A comparative study of electronic cigarette vapour extracts on airway-related cell lines *in vitro*” which was published in Inhalation Toxicology journal (refer Appendix section 11.5)

## 6.2 Aims

The main aim of this chapter was to study the effects of extracts of a variety of commercially available ECs from 5 different brands with different nicotine strengths and flavours on submerged cultures of human bronchial epithelial cells, macrophages and fibroblasts *in vitro*.

The specific objectives of this chapter includes:

1. To investigate the influence of CSE and E-vehicle extracts (EVE) on the cell viability
2. To investigate the influence of ECEs with identical flavours but different nicotine content on the cell viability (nicotine variable effect)
3. To investigate the influence of ECEs with identical nicotine content but different flavours on the cell viability (flavouring variable effect)
4. To investigate the influence of same-flavoured ECEs from different brands on cell viability (Brand variable effect)

## 6.3 Materials and Methods

### 6.3.1 Cigarette and ECs selection

A number of branded ECs were purchased from consumer websites. Marlboro Reds (Philip Morris International, New York, USA) were employed as standard tobacco cigarettes for validation purposes. The anonymised EC brands are listed in Table 6.1, along with the information provided by the manufacturer on nicotine content and other additives.

**Table 6.1. List of cigarette and ECs used in the current study along with the manufacturer's information provided on the packaging of the cigarette and five different EC brands (anonymised as A to E).**

Sample (Brand)	Flavour	Nicotine content	Other additives / Information on packaging
Cigarette	N/A	0.8 mg	10 mg tar; 10 mg carbon monoxide
A (EC)	Unflavoured	2.4 % / mL	Water, polyethylene glycol, glycerine & flavours
B (EC)	Dark Cherry	12.5 mg 3.5 % v/v	Dark cherry regular flavour
	Tobacco	12.5 mg 3.5 % v/v	Tobacco flavour
	Crisp Mint	12.5 mg 3.5 % v/v	Crisp mint flavour regular
C (EC)	Tobacco	1.8 %	Propylene glycol, traces of nuts
	Menthol	1.8 %	Propylene glycol, traces of nuts

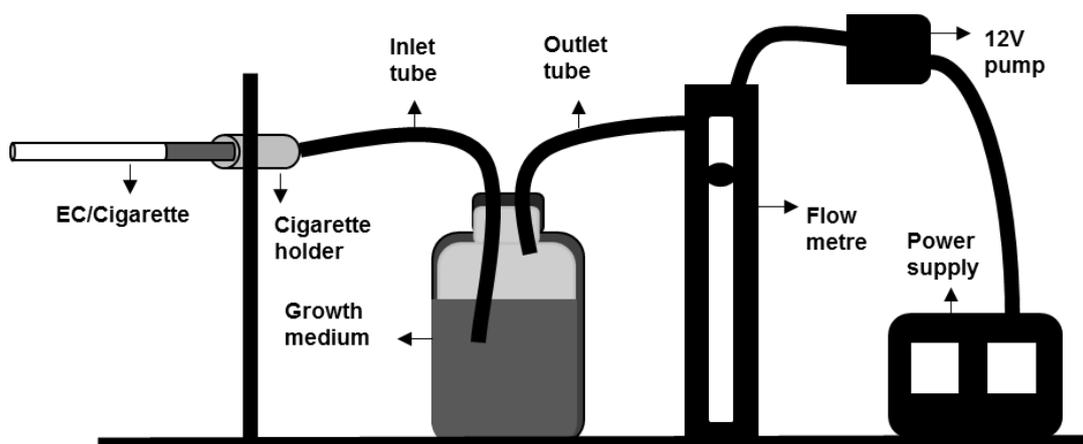
D (EC)	Tobacco	6 mg 0.6 %	Classic Tobacco flavour, propylene glycol
	Tobacco	12 mg 1.2 %	Classic Tobacco flavour, propylene glycol
	Tobacco	18 mg 1.8 %	Classic Tobacco flavour, propylene glycol
E (EC)	Apple	16 mg 1.6 % / mL	Water, propylene glycol, glycerine and flavours. Traces of nuts
	Coffee	16 mg 1.6 % / mL	Water, propylene glycol, glycerine and flavours. Traces of nuts
	Vanilla	16 mg 1.6 % / mL	Water, propylene glycol, glycerine and flavours. Traces of nuts
	Cherry	16 mg 1.6 % / mL	Water, propylene glycol, glycerine and flavours. Traces of nuts
	Strawberry	16 mg 1.6 % / mL	Water, propylene glycol, glycerine and flavours. Traces of nuts
	Tobacco	16 mg 1.6 % / mL	Water, propylene glycol, glycerine and flavours. Traces of nuts

### 6.3.2 Cell Culture

Seven cell lines in total were used in the current chapter experiments to investigate the toxicity of EC. Four human derived bronchial epithelial cell lines namely BEAS 2B, IB3-1, C38 and CALU 3, two macrophage cell lines, namely J774 (a mouse macrophage cell line) and THP-1 (a monocyte-derived macrophage) and a human derived fibroblast cell line namely HPF. The maintenance and culturing of these cell lines have been previously described in Chapter 2, sub-section 3.2.1 – 3.2.3.

### 6.3.3 Extract Collection

A bespoke set-up was designed in-house for CSE, EVE and ECE collection based on standard methodologies (Carp and Janoff, 1978). The technique allows the water-soluble components of the ECs and cigarette to be applied to the cell cultures. The water-insoluble components of cigarettes/ECs (e.g. tar, particulate particles) were not captured in this methodology and hence have not been assessed. In brief, a lit cigarette or activated EC was attached to the inflow tube of the apparatus as illustrated in Figure 6.1. A PSU was used to activate a 12 V diaphragm pump (Gardner Denver Ltd., Germany) and draw the cigarette smoke/EC aerosol through the inflow tube and into the small sterile glass bottle, containing 10 mL of appropriate sterile cell culture medium. One 35 mL puff was drawn over a time period of 2 s at a flow rate of 1.050 L/min, followed by a pause of 28 s before repeating the process (ISO, 1991). Flow rates were monitored and regulated via an analogue flow meter (Key Instruments, Trevoise, PA, USA). The time taken to smoke the whole cigarette completely was approximately 7 min which involved 14 puffs. ECE was obtained in a similar process involving the same puff rate, puff duration and total number of puffs as CSE. Individual sets of tubes and bottles were used for cigarettes or ECs to minimise the chance of cross contamination. All bottles and tubes were thoroughly washed and autoclaved between uses. The CSE and ECE were collected (100 % concentration) and were serially diluted in growth medium to obtain 50 %, 25 % and 12.5 % dilutions whereas EVE was analysed only at 100 % concentration. All extracts were collected and applied to the cells as soon as possible in order to minimise the possible chemical changes that might occur in the collected extracts.



**Figure 6.1. Diagrammatic illustration of the experimental set-up designed in-house to produce extracts of cigarette, EC and E-vehicle.**

CSE/ECE/EVE were collected by bubbling the smoke/vapour from the cigarette or EC through 10 mL of growth medium contained in a sterile glass bottle. The sterile glass bottle had an inlet tube submerged in appropriate growth medium to collect the resulting smoke/vapour and an outlet tube attached to an analogue flow meter. A diaphragm pump attached to the PSU was used to apply a vacuum for 2 s every 28 s, drawing air through the cigarette/EC and thus pulling smoke/EC vapour at a rate of 1.050 L/min into the medium. The collected CSE/ECE/EVE was transferred into a new sterile bottle and diluted appropriately with growth medium prior to addition to cells.

#### 6.3.4 Exposure of cell lines to CSE, EVE or ECE

For experiments involving bronchial epithelial cells and J774 cells, 1 mL of  $3 \times 10^5$  cells/mL were seeded into each well of a 24 well plate and incubated overnight at 5 % CO<sub>2</sub>, 37°C. Pertaining to other cell types, HPF cells were plated at a seeding density of  $1 \times 10^5$  cells/mL/well while THP-1 cells were seeded at a density of  $2 \times 10^5$  cells/mL/well. After 24 h, after the cells had formed a confluent monolayer, the existing medium was aspirated from each well and immediately replaced with CSE, EVE or ECE as appropriate. 1 mL of each dilution of CSE or ECE (100 %, 50 %, 25 % and 12.5 %) was added into the appropriate wells. Control samples consisted of untreated cells submerged in untreated medium. Plates were incubated at 37°C and 5 % CO<sub>2</sub> for 24 h.

#### 6.3.5 Cell viability analysis

Cell viability was analysed using XTT since this method was more appropriate and economical compared to CTB for this set of experiments. The preparation of XTT has been previously described in Chapter 2, sub-section 3.2.2. 24 h post exposure to CSE/EVE/ECE, 250 µL of XTT working solution was added to each well containing the extracts or untreated medium and incubated at 37°C for 2 h. After this time, 100 µL of the supernatants from each

well was transferred into a 96 well microplate in quadruplicates and the absorbance read at 450 nm using a spectrophotometer (MultiSkan, ThermoScientific, USA). The same procedure was followed for all replicates of each experimental condition.

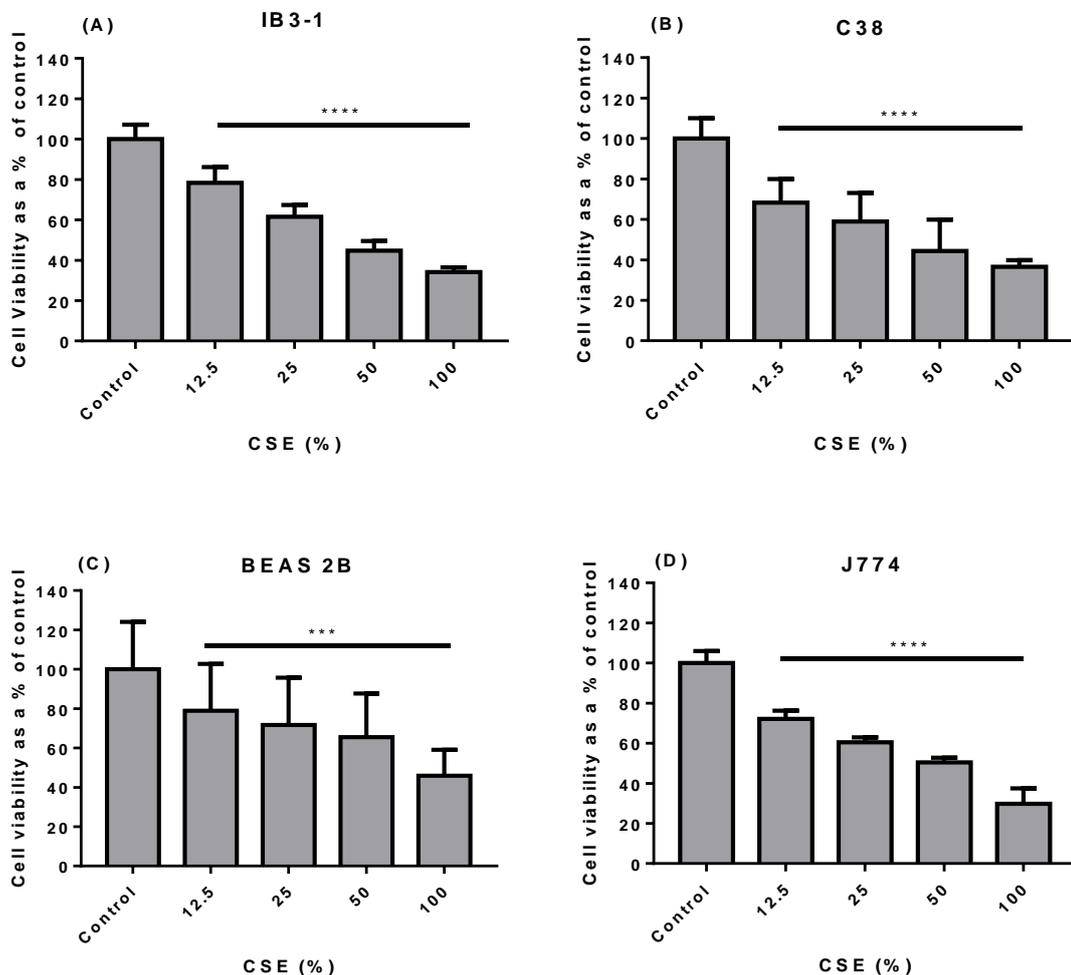
#### 6.3.6 Statistical analysis

All statistical analysis were performed using GraphPad®, V7 (GraphPad Software Inc., La Jolla, CA, USA). All experiments were repeated at least four times, with four replicate wells of each dilution tested in each repeat. Data was analysed using either One-way or Two-way ANOVA was performed, followed by either a Dunnett's or Tukey-Kramer post-hoc test. In all experiments, statistical difference higher than 0.1 was considered as non-significant (indicated by 'ns' in figure legends).

## 6.4 Results

### 6.4.1 Influence of CSE on cell viability

CSE was obtained using established techniques as explained in sub-section 6.3.3. As shown in Figure 6.2 (A) – (D), 24 h post exposure to different concentrations of CSE, the XTT cell viability results demonstrated that there was a dose-dependent decrease in cell viability such that viability was significantly less than control for all cell types and for all four concentrations of CSE tested. Cytotoxicity (< 70 % decrease in cell viability compared to untreated cells) was demonstrated in the C38 cell line at all concentrations of the CSE (Figure 6.2 B), and at concentrations of 25 % and higher for the other cell types tested.

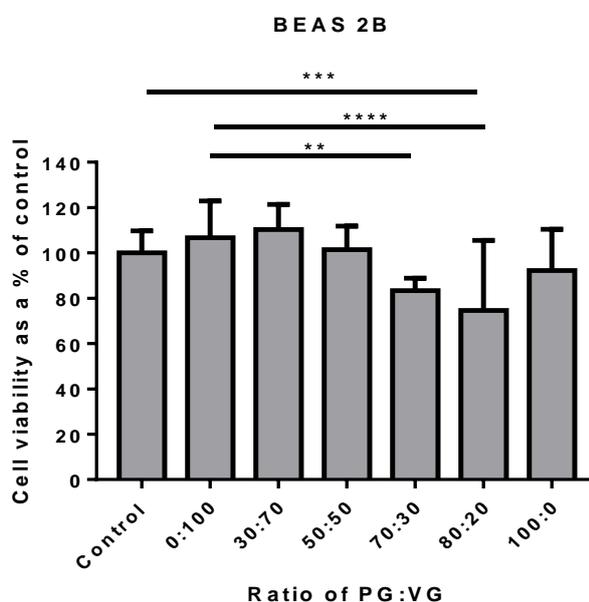


**Figure 6.2. Effect of CSE on (A) IB3-1 (B) C38 (C) BEAS 2B and (D) J774 cell viability.**

$3 \times 10^5$  cells/mL of all four cell types were treated to the different dilutions of CSE for 24 h as described in sub-section 6.3.4, post which XTT cell viability analysis was performed as described in sub-section 6.3.5. As seen from the above figures, CSE caused a statistically significant decrease in cell viability ( $p < 0.0001$ ) compared to the control in all four cell types. Further, statistically significant differences were observed between each serial dilution in for all four cell lines. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate samples per experiment. (\*\*\*) =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ )

#### 6.4.2 Influence of EVE on cell viability

In order to ascertain the effects of the bulk constituents of ECs, the effect of E-vehicle in which the EC nicotine and flavours are delivered was investigated. The combination of PG and VG was investigated at several ratios. Extracts from these ratios (only 100 % concentrations were tested) was added to submerged BEAS 2B cells for 24 h while the cells treated to medium only acted as the control cells. As shown in Figure 6.3, there was a significant decrease in cell viability compared to control only at the 80:20 (PG: VG) ratio although the viability of cells was not linearly related to the ratio of PG: VG i.e. increase or decrease in PG or VG had no significant influence on cell viability. The 70:30 ratio was significantly different from 0:100, 30:70, 50:50 but not the control, 80:20 and 100:0, as shown in Figure 6.3.



**Figure 6.3. Effect of different ratios of EVE on BEAS 2B bronchial epithelial cells.**

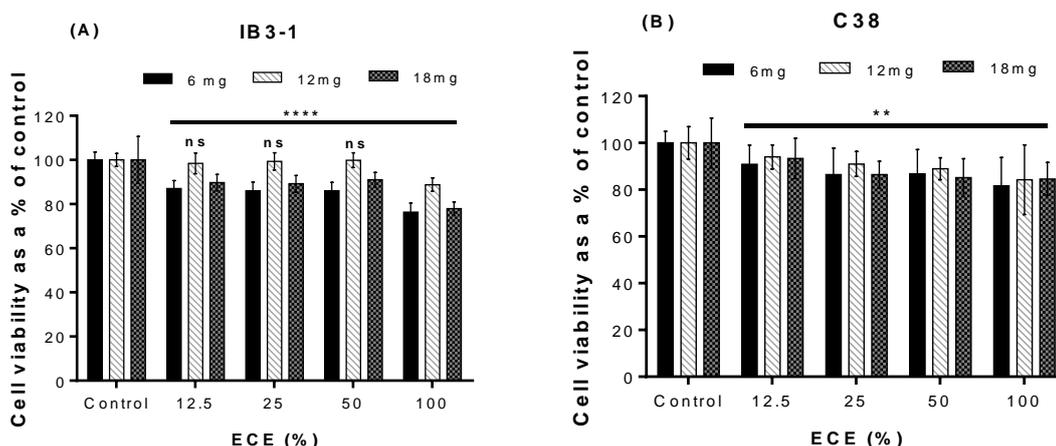
$3 \times 10^5$  cells/mL of BEAS 2B cells were treated to the different ratios of EVE for 24 h as described in sub-section 6.3.4, post which XTT cell viability analysis was performed as described in sub-section 6.3.5. As seen from the above figure, compared to the control, only the 80:20 ratio caused a significant reduction in cell viability although there was a big variability in this data-set. All other ratios were non-significant with respect to the control and other ratios and hence there was no ratio dependent effect. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate samples per experiment (\*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ )

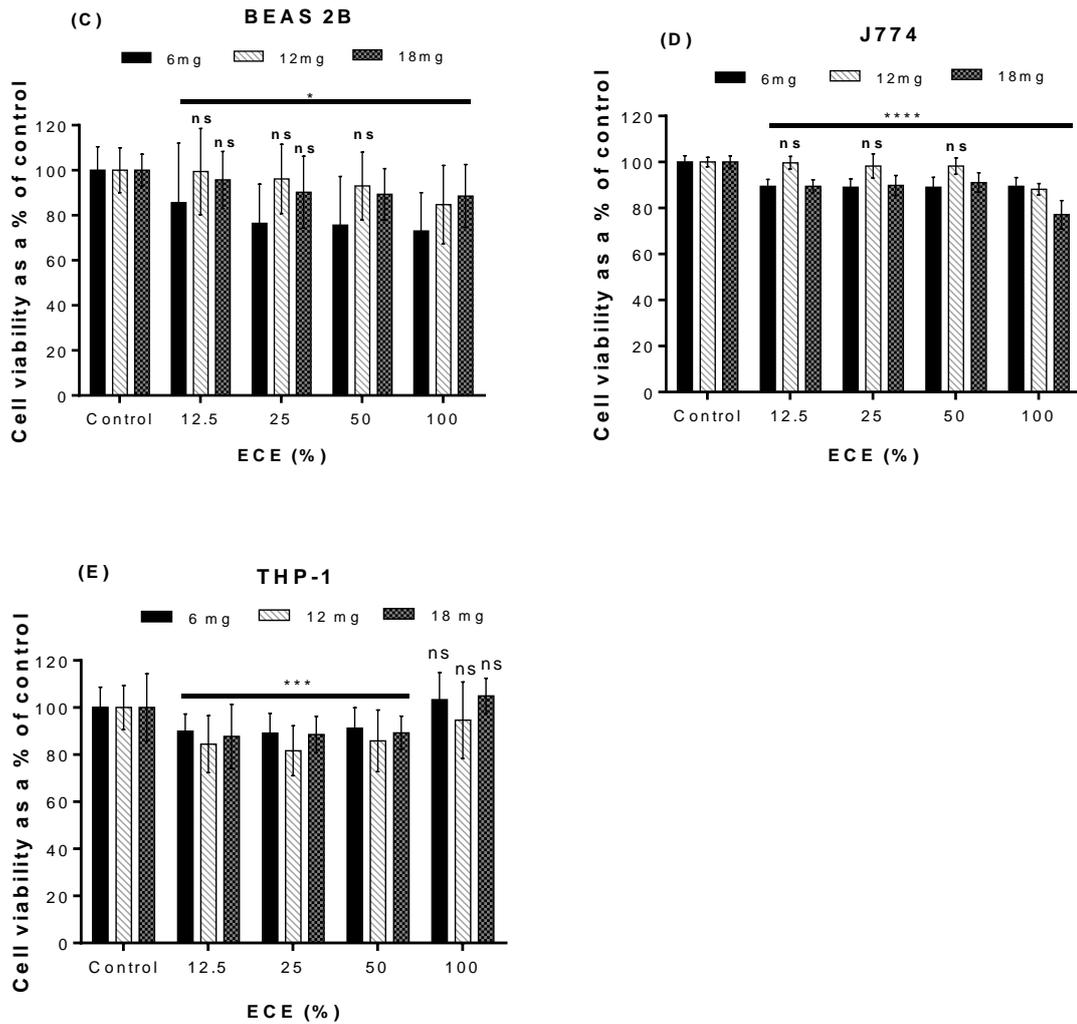
### 6.4.3 Influence of ECE on cell viability

Several ECs were purchased and the ECE was obtained in the same manner as CSE/EVE as described earlier in 6.3.3. Cells were exposed to 1 mL of ECE for 24 h post which viability was measured and cytotoxicity was defined as per the ISO standard UNI EN ISO 10993-5. (Besides the EC data being represented graphically in the below Figures (6.4 – 6.8), a comprehensive table depicting the XTT cell viability data values of all the 15 ECs tested in this chapter is represented in Appendix 11.2, Table 11.1.)

#### 6.4.3.1 The effect of varying nicotine strength

In order to assess the effects of EC nicotine concentration on cell viability, results were compared across three epithelial cell lines (IB3-1, C38 and BEAS 2B) and macrophage/monocyte cell lines (J774 and THP-1) following 24 h exposure to ECE of Brand D ECs. This brand contained three ‘Tobacco’ flavoured ECs with nicotine concentrations of 6, 12 and 18 mg respectively as stated on the packaging. The results as shown in Figure 6.4 (A) – (E) showed that there were significant decreases in the viability compared to the control, and this was true for all five cell lines. However, there was no concentration-dependent relationship between increasing nicotine content and decreasing cell viability, suggesting that the drop in viability was not simply due to nicotine content. None of the ECE tested here were cytotoxic.



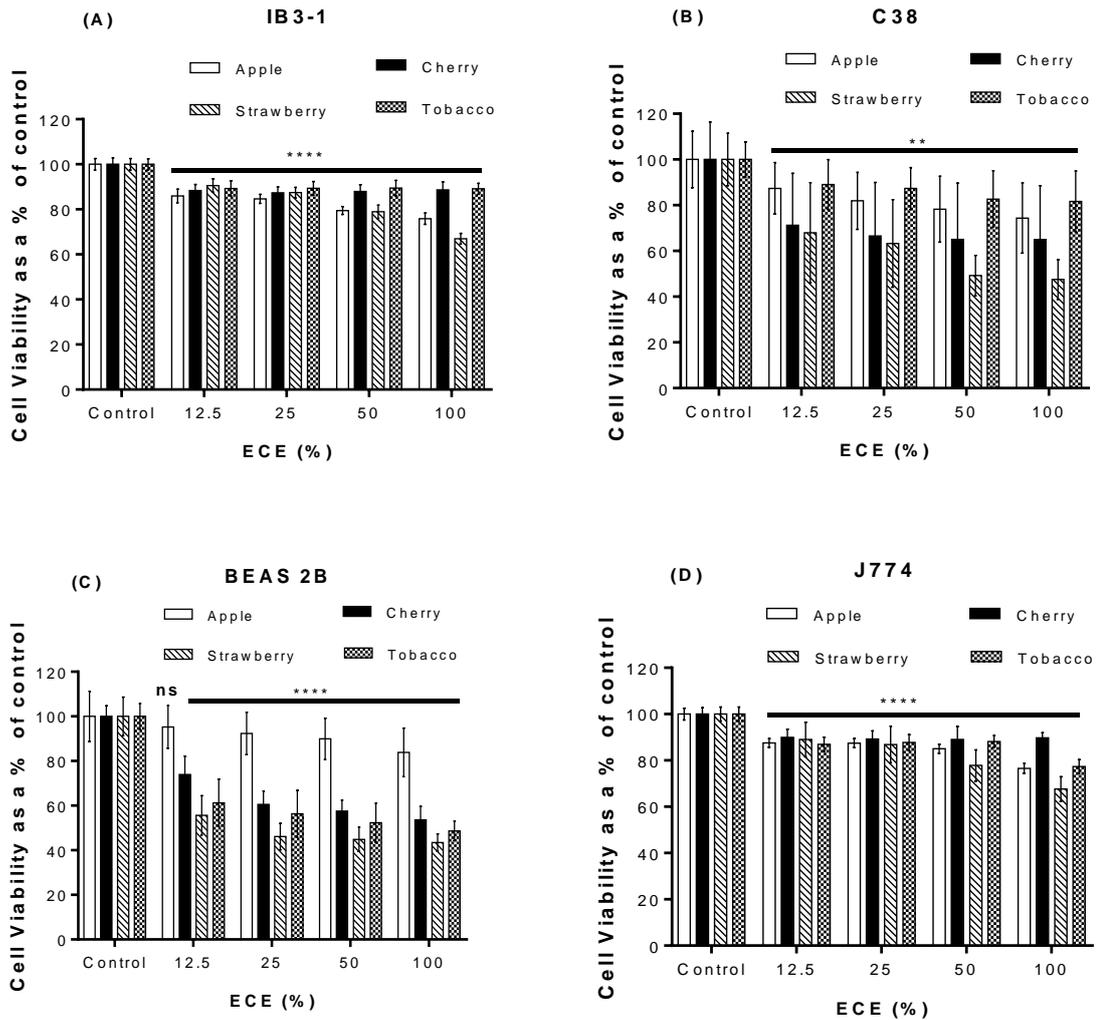


**Figure 6.4. Effect of varying nicotine concentration on (A) IB3-1 (B) C38 (C) BEAS 2B and (D) J774 cell viability.**

Appropriate number of different cell types were treated to the different dilutions of ECE for 24 h as described in sub-section 6.3.4, post which XTT cell viability analysis was performed as described in sub-section 6.3.5. As seen from the above figures, the extracts of all three ECs with 6, 12 and 18 mg nicotine content respectively caused a significant decrease in cell viability compared to the control in all 5 tested cell types. Never-the-less, there was no significant nicotine concentration-dependent effects in all 5 tested cell types, thus suggesting that higher nicotine content does not necessarily correlate to higher cytotoxicity. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate wells per experiment (ns = not significant, \* =  $p < 0.1$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ )

#### 6.4.3.2 *The effect of different flavourings*

In order to assess the influence of different flavours on cell viability, four ECs from the same brand (Brand E) was chosen. These four ECs had the same nicotine content (16 mg) but different flavours namely apple, cherry, strawberry and tobacco, thus eliminating the variables of nicotine content and brand. As shown in Figure 6.5 (A) – (D), upon treating submerged cultures of IB3-1, C38, BEAS 2B and J774 to extracts of the four flavoured ECs from Brand E, a significant decrease in cell viability was observed in all four cell types across all four ECE concentrations (except 12.5 % apple ECE in BEAS 2B) compared to the control cells. Strawberry flavour ECE at 100 % concentration demonstrated cytotoxicity in all four cell types and the cytotoxicity ranged from  $66.9 \pm 2.8$  % control (IB3-1 cells) to  $43.6 \pm 3.6$  % control (BEAS 2B cells). In C38 and BEAS 2B cells as observed from Figure 6.5 (B) and 6.5 (C) respectively, strawberry flavoured ECE proved cytotoxic at all concentrations. Cherry flavour demonstrated cytotoxicity in C38 and BEAS 2B as observed from Figure 6.5 (B) and 6.5 (C) respectively at dilutions of 25 % and higher. In the case of BEAS 2B, tobacco flavoured ECE also proved cytotoxic at all dilutions tested (Figure 6.5 (C)).

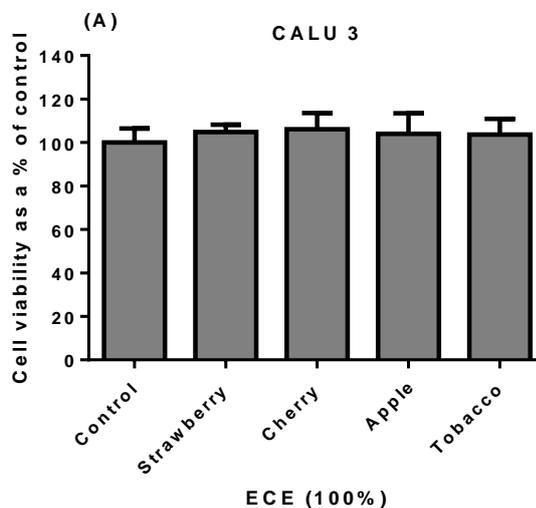


**Figure 6.5. Effect of different flavours on (A) IB3-1 (B) C38 (C) BEAS-2B and (D) J774 cell viability.**

$3 \times 10^5$  cells/mL of all four cell types were treated to the different dilutions of ECE for 24 h as described in sub-section 6.3.4, post which XTT cell viability analysis was performed as described in sub-section 6.3.5. As seen from the above figures, all the four EC flavours caused a significant decrease in cell viability compared to the control in all four cell types. Cytotoxicity was demonstrated by strawberry flavoured ECE at 100 % concentration in all four cell types. Especially in C38 and BEAS 2B cells, while strawberry flavour demonstrated cytotoxicity at all four dilutions, cherry flavour demonstrated cytotoxicity at 25 % dilutions and above. BEAS 2B was found to be the most susceptible cell line with strawberry, cherry and tobacco flavour demonstrating cytotoxicity. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate wells per experiment (ns = not significant, \* =  $p < 0.1$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ )

Further, the statistical differences between the different flavours at each ECE concentrations across the four cell types were analysed using two-way ANOVA. This analysis between the different flavours has been described in Appendix 11.3 (Table 11.2).

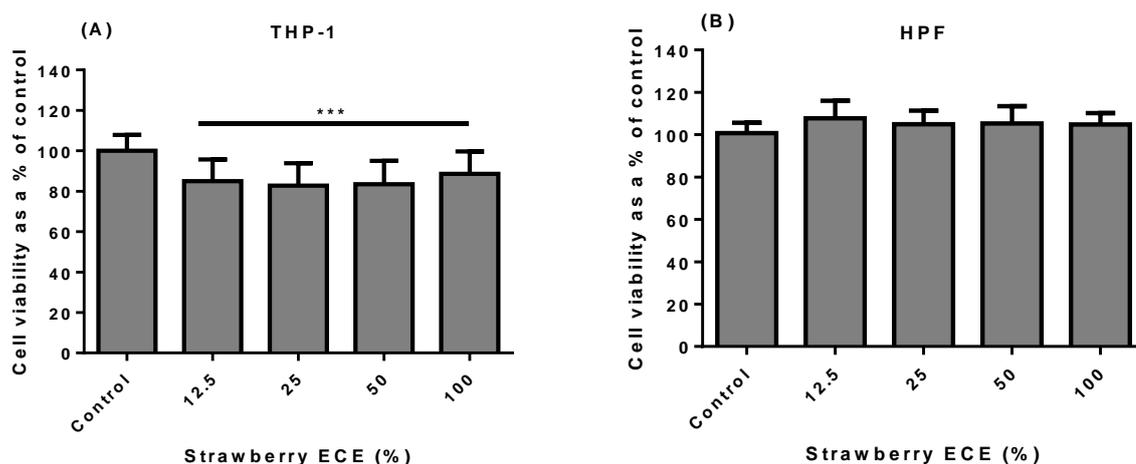
In addition, CALU 3 cells were exposed to the flavoured ECEs at 100 % dilution only. As shown in Figure 6.6, there was no significant impact of any of the flavours tested on the CALU 3 cell viability.



**Figure 6.6. Effect of different flavours on CALU 3 cell viability.**

$3 \times 10^5$  cells/mL of CALU 3 cells were treated to 100 % dilution of different flavour ECEs for 24 h post which the XTT cell viability analysis was performed as described in sub-section 6.3.5. As shown in the above figure, none of the tested flavours proved cytotoxic to CALU 3 cells. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate wells per experiment.

Since the strawberry flavour proved most cytotoxic of all the flavours tested, this EC was again tested on two additional cell lines namely THP-1 and HPF. The results as shown in Figure 6.7 (A) and (B) represent the XTT cell viability data of THP-1 and HPF cells respectively post 24 h exposure to ECE. As shown in Figure 6.7 (A), although a significant decrease in THP-1 cell viability was observed at all four concentrations compared to the control cells, the effect was not concentration-dependent. Figure 6.7 (B) shows that none of the tested strawberry ECE concentrations had any significant influence on the HPF cell viability. Further, no cytotoxicity was demonstrated at any concentration on either cell line as the lowest cell viability recorded was 83 % and 104 % of controls, for THP-1 and HPF respectively, across the varying concentrations of strawberry ECE.

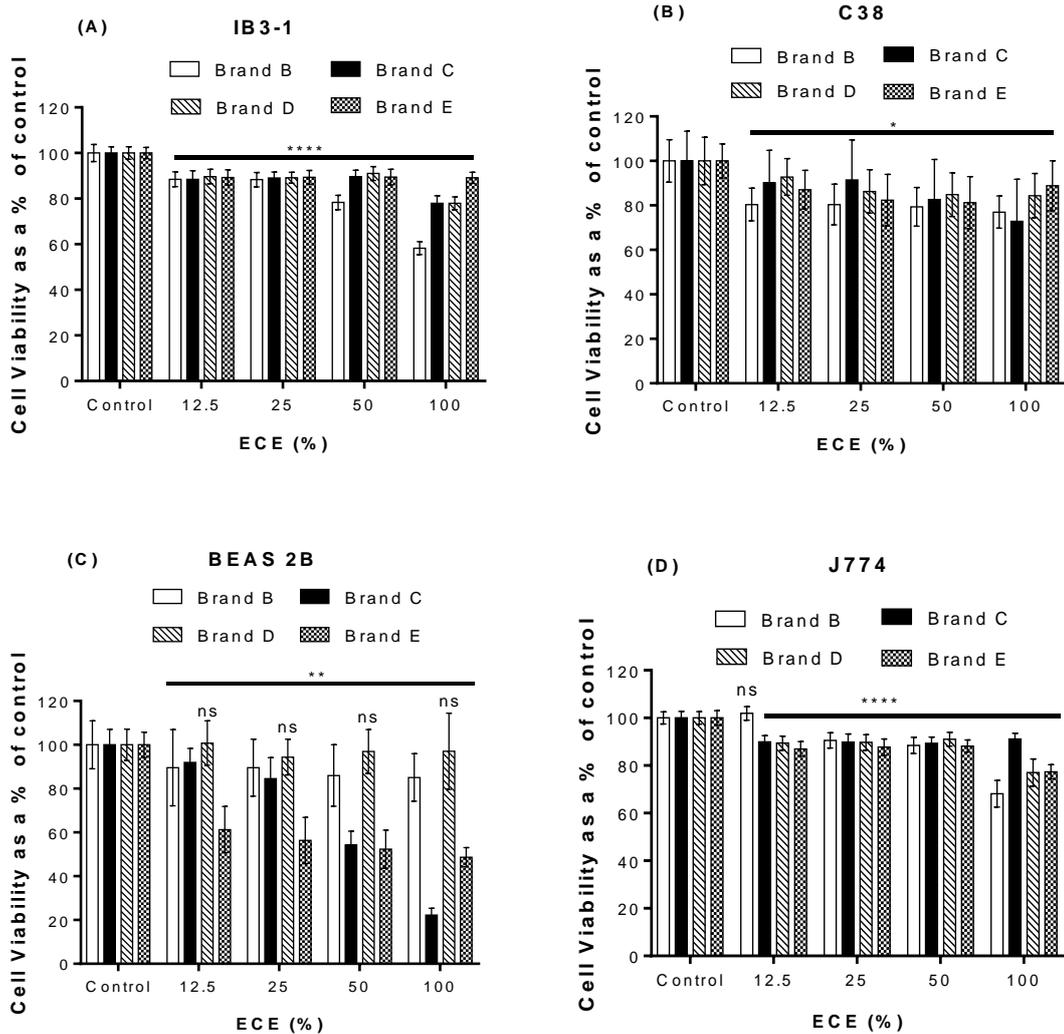


**Figure 6.7. Effect of strawberry flavoured ECE on (A) THP-1 and (B) HPF cell viability.**

Since strawberry flavoured EC was found to be the most cytotoxic of all flavours, its effect was tested on two additional cell types namely THP-1 and HPF. As seen from the above figure, none of the tested concentrations of strawberry ECE demonstrated cytotoxicity in both the cell types although a significant reduction in cell viability compared to the control was observed in THP-1 cells. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate wells per experiment. (\*\*\*) =  $p < 0.001$ )

#### 6.4.3.3 The effect of the same flavourings across different brands

In order to analyse brand variability, the effect of 4 tobacco flavoured ECs from Brand B, C, D and E were compared. The nicotine content of these ECs from different brands varied between 12.5 and 18 mg/mL. However, as no correlation had been found between nicotine content and cytotoxicity (Figure 6.4), this was deemed as an acceptable variable. As shown in Figure 6.8 (A) – (D), all four ECEs caused a significant reduction in cell viability compared to the control in all four cell types (except Brand D ECE in BEAS 2B), although the cytotoxic effects were variable and cell-type dependant. The tobacco flavoured EC from Brand B demonstrated cytotoxicity in IB3-1 and J774 at 100 % concentration only. In BEAS 2B cells, while the tobacco flavoured EC from Brand C demonstrated cytotoxicity at 50 % and 100 % concentration only, Brand E demonstrated cytotoxicity at all concentrations. The tobacco flavoured EC from Brand D did not demonstrate cytotoxicity in any cell type although it did cause a significant decrease in cell viability compared to control. Similar to flavourings result (Figure 6.5), BEAS 2B was the most sensitive cell type, with viability falling to  $22.4 \pm 3.7$  % of control after exposure to 100 % ECE from Brand C, and  $48.8 \pm 5.3$  % of control following exposure to 100 % ECE from Brand E.



**Figure 6.8. Effect of identical-flavoured ECs from different brands on (A) IB3-1 (B) C38 (C) BEAS-2B and (D) J774 cell viability.**

$3 \times 10^5$  cells/mL of all four cell types were treated to the different dilutions of ECE for 24 h as described in sub-section 6.3.4, post which XTT cell viability analysis was performed as described in sub-section 6.3.5. As seen from the above figures, except Brand D ECE in BEAS 2B, all four Brand ECs caused a significant decrease in cell viability compared to the control in all four cell types at different concentrations. Pertaining to cytotoxicity, different brands demonstrated cell-type dependent cytotoxicity at different concentrations. BEAS 2B exhibited most sensitivity wherein Brand E ECs caused cytotoxicity at all four concentrations while Brand C ECs demonstrated cytotoxicity at 50 % and 100 % concentrations. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  S.D. of four individual experiments, with quadruplicate wells per experiment. (ns = not significant, \* =  $p < 0.1$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ )

## 6.5 Discussion

The current study aimed to analyse the effects of CSE and ECE on four cell lines; namely, three human bronchial epithelial cell lines (BEAS 2B, IB3-1 and C 38), and one macrophage cell line (J774). Using these four different cell lines allowed comparison of the potentially damaging effects of CSE and ECE. Based on the information gleaned from the four cell types, further studies were then performed using additional cell types, fibroblasts (HPF), bronchial epithelial cell (CALU 3) and monocytes (THP-1). This is the first study on the influence of CSE and ECE on cytotoxicity for these airway related cell types. Using cigarette smoke or vapour extract on submerged cells to examine viability and cytotoxicity is an established methodology. Whilst not as physiologically relevant as work involving ALI cell culture models such as the studies by Neilson et al (2015) or Leigh et al (2016), the methodology used has allowed testing a large range of ECs from different brands; fifteen in total, at four concentrations, across four major and 3 subsidiary cell lines, performed in quadruplicates. Such a screening process enabled identification of ECs with the potential to cause most cytotoxicity under these conditions.

### 6.5.1 CSE has a cytotoxic effect on cell viability

The use of CSE to investigate the effects of cigarettes on cultured cells has been extensively reported previously (Bernhard et al., 2004, Carp and Janoff, 1978). In the current study, all four concentrations of CSE caused a statistically significant decrease in viability compared to the control and to each other in all four cell types. The results from the current study are in accordance with a number of past studies which reported on the cytotoxic effects exhibited by CSE even at low concentrations. Yoon et al. showed that there was a significant decrease in the cell viability of human bronchial smooth muscle cell lines with increasing concentrations of CSE up to 30 % concentration (Yoon et al., 2011). In a study with alveolar type II airway epithelial cells, CSE at 5 % concentration, caused a reduction in viability, down to 19 % of the control after 72 h (Hoshino et al., 2001). Since there are as yet no standard techniques for generating ECE or for exposing cells to ECE, the data from Figure 6.2 demonstrates the responsiveness of the cell model employed and in the methodology used for the generation of extracts, thus providing further confidence in the ability to use the data from these models/methods to analyse of the effects of ECE.

### 6.5.2 EVE has no cytotoxic effect on the cell viability

EVE with varying ratios of PG: VG was obtained using an EC which contained E-vehicle only. Figure 6.3 shows that 80 PG: 20 VG was the only E-vehicle ratio that demonstrated some statistically significant decreases in cell viability compared to control though there was a wide variability in this data. Importantly, no concentration-dependent relationship was found between increasing PG or VG and cell viability and no E-vehicle ratio was found to be cytotoxic. This result agrees with previous work by Farsalinos et al (2013) which reported on the non-cytotoxic effects of the base liquid of ECs when applied as an extract to myocardial cells (Farsalinos et al., 2013b). However, apart from this study, there is little in the literature which assesses the effects of the extracts from PG and VG, or indeed the combination of the two as seen in ECs and hence need further investigation. On the other hand, *in-vitro* studies employing ALI models to assess the effects of PG/VG aerosols instead of extracts have been performed in the past. As of yet only three such studies exist (Scheffler et al., 2015b, Scheffler et al., 2015a, Leigh et al., 2016). All three studies reported that E-vehicle aerosols without nicotine or flavourings can have significant damaging effects of its own and hence cannot be treated as completely safe.

An important aspect to consider in the study design of such experiments is the choice of E-vehicle materials employed. Some past studies have investigated the effect of E-vehicle using commercially available, nicotine-free EC liquids. For instance, Yu et al (2016) employed nicotine free, tobacco flavoured ECs from two brands, both with 70% PG: 30% VG E-vehicle solution (Yu et al., 2016). In such studies employing commercial nicotine-free, flavoured ECs, although the contribution of nicotine can be ruled out, it cannot be ascertained whether or not the flavouring additives had any contribution towards the observed cytotoxicity. Such a drawback is eliminated in the current study where only USP-grade (United States Pharmacopeia-grade) PG and VG were added to formulate the E-vehicle and hence the observed cytotoxicity can be attributed directly to the E-vehicle.

### 6.5.3 Nicotine content of ECs does not influence cell viability

As observed in Figure 6.4, increasing nicotine content in ECs did not linearly effect the cell viability of different cell types. These results are consistent with previous work performed on EC cytotoxicity. Farsalinos et al (2013) concluded that nicotine concentration had no effect on myocardial cell survival when comparing different ECEs (Farsalinos et al., 2013b), and Bahl et al. reported that cells did not survive better in samples without nicotine when subjected to direct e-liquid exposure (Bahl et al., 2012). Cervellati et al. however, showed that nicotine did have an effect on the viability and inflammatory response of cells exposed

to EC vapour, though this was not using ECE (Cervellati et al., 2014). The mechanisms of actions of nicotine in terms of protection versus cytotoxicity are not clearly understood as previously discussed in chapter 5. Nevertheless, it is interesting to note that previous works on nicotine have focused on its anti-apoptotic properties (Argentin and Cicchetti, 2006, Argentin and Cicchetti, 2004, Laytragoon-Lewin et al., 2011). Hence, though the mechanism by which the cells remain unaffected by increasing nicotine doses in the current study is unclear, the fact that nicotine may not have any major influence on the cell viability has been reported by many past studies, and the current study findings support this.

#### 6.5.4 EC flavourings significantly influence cell viability

As shown in Figure 6.5, the different flavourings caused a varied outcome in cell viability across the four cell lines, with BEAS 2B appearing the most susceptible, whereas CALU 3 showed no cytotoxic response to any of the four flavours tested (Figure 6.6). Moreover, fruit flavoured ECs especially strawberry and cherry flavours demonstrated highest cytotoxicity. As previously stated in Chapter 2, sub-section 2.6.1.4, although the flavourings used in EC liquid fabrication are often common food additives, the effects of these additives once heated and aerosolised, in conjunction with the other constituents is still largely unknown. Additional variability may arise from the type and amount of flavourings included in the EC which vary between manufacturers, with the potential to affect the cytotoxicity (Tierney et al., 2016). Moreover, even the method of production of natural flavourings such as ‘tobacco’ or ‘coffee’ and ‘cinnamon’ are different among the various manufacturers (Konstantinos Farsalinos, 2018). Bahl et al (2012) showed that the concentration of the flavouring chemicals varied even between same-flavoured EC liquids purchased from the same manufacturer (Bahl et al., 2012) and cinnamon flavourings have been particularly noted for its cytotoxicity in the literature (Behar et al., 2014). Hence, the flavouring constituents of ECs cannot be assumed to be completely non-toxic or safe. For example, the presence of formaldehyde-releasing agents has been found in EC vapour (Jensen et al., 2015) and the main ingredient of cinnamon flavour, cinnamaldehyde, is converted to benzaldehyde at high temperatures (Friedman et al., 2000). The results of the current study support that the flavourings used in ECs affect the potential cytotoxicity of the EC vapour.

#### 6.5.5 Different brands have variable influence on cell viability

Analysis of the different flavoured ECEs indicated that there are significant differences in the cytotoxic effects within one brand, and thus there is a concern that flavour choice may influence the health effects of EC. In order to assess brand variability, the effects of one flavour (tobacco) across different brands (Brand B, C, D and E) were examined. As shown

in Figure 6.8, these data indicate that there are significant differences in the contents of EC, shown by the variation in effects of ECs across each cell line, despite similar packaging labelling (e.g. all brands tested here simply declared 'Tobacco' flavourings). This suggests that there is a requirement for more rigorous regulation of EC contents and further testing of the different EC products. Previous research has shown that the brand variability is not just unique to ECs but also cigarettes. For instance, Pickett et al (2010) showed that identical doses of cigarette smoke condensates obtained from 10 different brands of cigarette had differential genotoxic effects on human bronchial epithelial cells, with a large number of genetic alterations unique to one specific brands of cigarette (Pickett et al., 2010). Brand variability exists possibly due to differences in the flavourings fabrication process or tobacco curing procedures which can vary from company to company (Farsalinos et al., 2015b). If ECs are to be used as a safe alternative to tobacco or nicotine replacement products for smoking cessation (ASH 2015), then the consumer needs to know the precise contents, and the smoking cessation clinics and advisors need to know the likely health effects, since the data from the current study indicate that not all EC are the same.

#### 6.5.6 Different cell types demonstrate variable responses to ECE

In this study, a total of seven different cell types was employed for various experiments. Four of these were bronchial epithelial cell lines, two were macrophages and one was a fibroblast cell line. Different cell types exhibited differential susceptibility to ECE and this may be related to the cell type specific biology. There was a difference in response between the epithelial cells to the fibroblasts, the epithelial cells to the macrophages and the fibroblasts to the macrophages. However, there were also difference in response within the different epithelial cell lines (BEAS 2B, IB3-1, C38 and CALU 3) and different macrophage cell lines (THP-1 and J774). The BEAS 2B cell line appeared to be the most sensitive of all the cells tested in this study. PMA treated THP-1 cells have been previously found to have a resistance to apoptotic stimuli which is comparable to primary human macrophage cell lines (Daigneault et al., 2010). These were less susceptible to cytotoxicity than the mouse macrophage J774. These data suggest that the cell types and kind (e.g. primary vs cell lines) chosen to explore the cytotoxicity of ECs can significantly influence the findings of studies in cytotoxicity testing, thereby influence overall EC safety data. Thus there is still a need to standardise a testing protocol for examination of the potential health effects of ECE using an *in vitro* model of human airways.

## 6.6 Conclusions

A number of conclusions can be drawn from the results of this chapter. Firstly, CSE has a dose-dependent detrimental impact on the cell viability across all four cell types tested. This proved as a good validation of the cell model and methodology employed in this study. The extract obtained from the base liquid alone, composed of PG and VG, showed no cytotoxicity across the different ratios tested. Nicotine concentration in ECs has little or no dose-dependent influence on the cell viability across all cell types. In contrast to nicotine, different EC flavourings had a significant influence over the observed cytotoxicity, with fruit flavours especially strawberry and cherry demonstrating greater cytotoxicity. Also, cytotoxicity varied widely across different brands, with identical descriptions of products, including flavour, resulting in significantly different effects on cell viability. And finally, the choice of cell line employed in the testing of ECs can produce significant differences in the outcome of the study. Overall, the experiments of this chapter permitted screening of the cytotoxic effects of a wide-variety of ECs using extracts on submerged cell culture. One limitation of this chapter was that the extracts deliver only the water soluble components of cigarette/EC. The next chapter would hence analyse the effect of aerosols, which would deliver both vapour and particulate phases of the strawberry flavoured EC from Brand E, which demonstrated highest cytotoxicity in this set of experiments using more physiologically relevant co-culture ALI models of human airways.

## 7 Inhalation exposure of co-culture human airways model to aerosols of ECs

### 7.1 Introduction

Unlike tobacco cigarettes that have widely-accepted standards such as ISO 3308 (ISO 3308, 2012) or HCI regime (ISO/TR 19478-2, 2015), there are currently no standardised testing methods for toxicological evaluation of ECs. Issues with current EC testing methodologies include variability in both, the 'vaping regime' employed and the cell model and delivery system used. As a result, different EC studies employ different puff regimes such as HCI regime (Misra et al., 2014), Cooperation centre for scientific research relative to tobacco (CORESTA) regime (Haswell et al., 2017) or customised puff regimes (Lerner et al., 2015) to generate and deliver EC aerosols to the cells. This lack of a consistent puffing regime for EC evaluation renders comparability between studies difficult.

A number of early studies have evaluated EC cytotoxicity by treating submerged mono-cultures of various cell types to EC extract (Farsalinos et al., 2013b, Bahl et al., 2012, Yu et al., 2016, Leslie et al., 2017). Although extract exposure studies provide valuable information on EC cytotoxicity, they do not represent the *in-vivo* EC exposure process accurately. Recent advancements in *in-vitro* methodologies have however made it possible to deliver WCS or ECV directly over cells cultured at ALI as described previously in Chapter 4, sub-section 4.1. Despite these advancements, there is a paucity of studies investigating EC cytotoxicity using these physiologically relevant ALI cell culture models. As previously described in Chapter 2, sub-section 2.6.4.4, only a few studies have employed differentiated airway epithelial cell cultures (Neilson et al., 2015, Scheffler et al., 2015a, Bishop et al., 2018), while the other studies have employed undifferentiated cultures of airway epithelial cells. Using differentiated cultures of airway epithelial cells to assess WCS/ECV cytotoxicity is a more physiologically relevant cell model as they produce many of the characteristic morphological and biochemical features of the *in-vivo* airway cellular environment.

Since ECV is a water soluble aerosol, much of its deposition is expected to happen at the level of conducting zone of the airways which predominantly consist of ciliated columnar epithelial cells and mucus producing goblet cells (Scheffler et al., 2015a, Zhang et al., 2012). In the current study, an ALI co-culture model of human airways consisting of CALU 3 bronchial epithelial cells and HPF cells was used to study the effects of ECV. Although many previous *in-vitro* models of human airway epithelium exist, this is the first study to employ a differentiated bronchial epithelium model with underlying pulmonary fibroblasts to

study EC cytotoxicity. CALU 3 cells, although derived from a tumour site, have been shown to exhibit many characteristics of the *in-vivo* bronchial epithelium such as tight barrier formation, goblet cell production and apical microvilli and cilia formation when cultured at ALI (Foster et al., 2000, Wan et al., 2000, Grainger et al., 2006). In fact, CALU 3 cells have been shown to develop permeability properties comparable to that of *in-vivo* airway epithelium (Mathia et al., 2002, Grainger et al., 2006). Sub-epithelial pulmonary fibroblasts have been shown to play a key role in the epithelial cell differentiation, cilia formation and cytokine production (Sacco et al., 2004, Costea et al., 2003), thus increasing the physiological relevance of the co-culture model employed in the current study. As stated earlier, co-culturing HPF and CALU 3 at ALI lead to the formation of a polarized pseudo-stratified epithelial layer with barrier formation, expression of tight junction protein ZO-1, cilia and microvilli production and secretion of MUC5AC protein apically (Bielemeier, 2012). Subsequently, the EC aerosols were delivered to the co-culture models using the in-house built smoking machine as previously described in Chapter 4, sub-section 4.10. Similar to Chapter 6, the experiments of this chapter lead to production of a journal paper titled “An investigation into E-cigarette cytotoxicity *in-vitro* using a novel 3D differentiated co-culture model of human airways” in Toxicology In-vitro journal (refer Appendix section 11.5.)

## 7.2 Aims

The main aim of this chapter was to assess the cytotoxicity of aerosols of the strawberry flavoured EC from Brand E which exhibited highest cytotoxicity in the previous set of experiments described in Chapter 6.

The specific objectives of this chapter includes:

1. To investigate the effect of WCS acute exposure (7 min) on the co-culture model cell viability.
2. To investigate the effect of single block (1 h, 2 h and 3 h) ECV exposure on the co-culture model cell viability, IL-6/IL-8 pro-inflammatory mediators' production, oxidative stress and caspase 3/7 activity.
3. To investigate the effect of double block (4.5 h and 6 h) ECV exposure on the co-culture model cell viability and IL-6/IL-8 pro-inflammatory mediators' production.

## 7.3 Materials and methods

### 7.3.1 Cigarette and EC selection

A strawberry flavoured EC from Brand E was chosen for investigation in this part of the study since the extracts of this particular EC was found demonstrate most cytotoxicity of the several ECs tested (refer Chapter 6 results section, Figure 6.5). The tobacco cigarettes used in this study were Marlboro red cigarettes (Philip Morris International, New York, USA) with 0.8 mg nicotine, 10 mg tar and 10 mg carbon monoxide.

### 7.3.2 Co-culture human airways model

Two human derived cell lines namely CALU 3 and HPF cells were used to produce the co-culture human airways model employed in this study. Refer Chapter 3, sub-section 3.7.1 and 3.7.2 for a detailed description of the process.

### 7.3.3 Smoking machine and cell-exposure apparatus

An in-house constructed smoking machine was used to deliver air/WCS/ECV to the co-culture human airways model. The development, construction and validation of the in-house built smoking machine has been described in detail previously in Chapter 4. The final working model of the smoking machine used to deliver air/WCS/ECV to the co-culture model has been previously described in section 4.10 and illustrated in Figures 4.20 and Figure 4.21. The smoking machine was operated under the standard ISO 3308 regime. Although EC puff volume and duration has been reported to be longer than conventional cigarettes by some studies (Farsalinos et al., 2013b, Hua et al., 2013), due to the absence of a standardised puff regime for ECs, ISO 3308:2012 regime was employed for both cigarettes and ECs. Hence for both cigarettes/ECs, one 35 mL puff was drawn over 2 s at a flow rate of 1.050 L/min every 58 s. During the time between two puffs, air from ambient atmosphere was delivered to the cells at a rate of 0.150 L/min. A NAVICYTE horizontal Ussing chamber and associated cell manifold Perspex blocks was used to expose the co-culture human airways model to air/WCS/ECV as described in detail previously in Chapter 4, sub-section 4.8.5, figures 4.8 and 4.9.

### 7.3.4 Experimental design

Each experiment consisted of three independent SWs being exposed to the different experimental conditions. Cells exposed to air acted as the working control while the cells maintained in the incubator at 37°C, 5 % CO<sub>2</sub> acted as the untreated control (UT). Cells were exposed to WCS for 7 min (equivalent of one tobacco cigarette) at ISO 3308 smoking regime and incubated for 24 h at 37°C after which the post-exposure cellular analysis was performed. Cells were exposed to ECV either in a single or double block exposure regime. Single block exposure consisted of cells exposed to 1 h, 2 h or 3 h of ECV at ISO 3308 smoking regime followed by 24 h of incubation at 37°C after which the post-exposure analysis was performed. Double block exposure consisted of cells exposed to two blocks of ECV. The first block consisted of a 3 h ECV exposure followed by 24 h of incubation at 37°C. After 24 h, the cells were then treated to the second block of either 1.5 h or 3 h of ECV, thus equating to a total ECV exposure time of either 4.5 h or 6 h. Post exposure analysis was performed 24 h after the second exposure. The EC exposure time range of 1 h – 6 h was decided based on the prolonged vaping habits of EC users (Farsalinos et al., 2015c, Etter and Bullen, 2014) and also the time range employed in previous studies relevant to this research (Neilson et al., 2015, Scheffler et al., 2015b).

### 7.3.5 Cell viability analysis

Cell viability was analysed using a standard XTT assay, the preparation of which is described in the Chapter 3, sub-section 3.3.2. After exposure to different treatment conditions and 24 h of incubation, 400 µL of growth medium was added to the surface of the SW's to which 100 µL of XTT solution was added. The cells were then incubated for 2 h at 37°C, 5 % CO<sub>2</sub>. After 2 h, 100 µL of supernatant from each SW was transferred in quadruplicates to a 96 well microplate and the absorbance was read at 450 nm using a spectrophotometer (MultiSkan, ThermoScientific, USA). The same procedure was followed for all replicates of each treatment condition. As per the ISO standard UNI EN ISO 10993-5, any treatment condition that lead to a reduction in cell viability to below 70 % of the control was deemed cytotoxic.

### 7.3.6 IL-6/IL-8 release analysis

24 h post exposure to different treatment conditions, the basolateral medium (cell-culture supernatants) was collected in microfuge tubes and cleared of any cells by centrifuging at 1000 x g for 5 min. The supernatants were then analysed for IL-6 and IL-8 pro-inflammatory mediators via commercially available e-Bioscience ELISA kits (San Diego, USA). All assays

were performed according to the manufacturer's instructions, including the assay protocol and preparation of reagents. A detailed description of the ELISA process is described in Chapter 3, sub-section 3.4.2 and 3.4.3.

### 7.3.7 Oxidative stress analysis

ROS-Glo™ H<sub>2</sub>O<sub>2</sub> luminescence assay (Promega, Southampton, UK) was used to analyse oxidative stress in cells 24 h post exposure to different treatment conditions. This particular kit was chosen for oxidative stress analysis since it has previously been employed to assess oxidative stress in an ALI cell-culture system post exposure to WCS/ECV (Scheffler et al., 2015b). All assays were performed according to the manufacturer's instructions, including the positive control which involved cells incubated with 50 µM menadione (diluted in growth medium) for 2 h at 37°C. The final luminescence of samples were measured using a luminometer (Orion II, Titertek-Berthold, Germany). This process has been described in detail in Chapter 3, sub-section 3.5.

### 7.3.8 Caspase 3/7 activity analysis

24 h post exposure to different treatment conditions, the caspase 3/7 activity was assessed using a Caspase-Glo® 3/7 luminescence assay kit (Promega, Southampton, UK). All assays were performed according to the manufacturer's instructions. The positive control involved cells treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> (diluted in growth medium) for 2 h at 37°C. At this concentration range, H<sub>2</sub>O<sub>2</sub> has previously been shown to induce apoptosis (Xiang et al., 2016, Li et al., 2010b). The final luminescence of samples were measured using a luminometer (Orion II, Titertek-Berthold, Germany). This process has been described in detail in Chapter 3, sub-section 3.6.1 and 3.6.2.

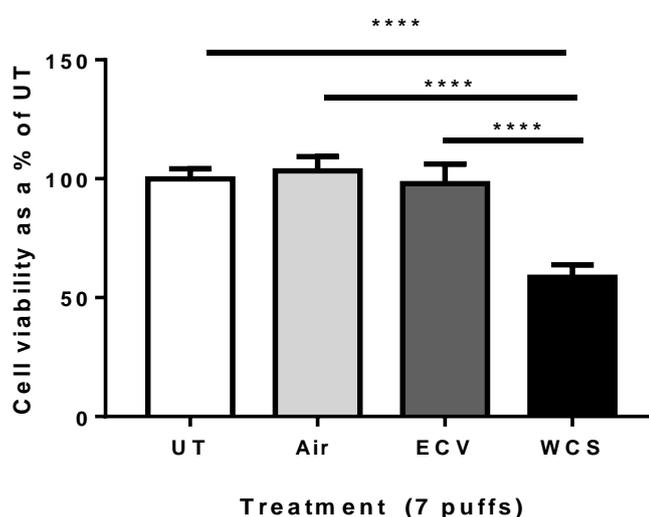
### 7.3.9 Statistics

All statistical analysis were performed in GraphPad®, V7 (GraphPad Software Inc., La Jolla, CA, USA). Data was analysed using either one-way ANOVA followed by Tukey's post-hoc test or two-way ANOVA followed by Sidak's post-hoc test. In all experiments, statistical difference higher than 0.1 was considered as non-significant (indicated by 'ns' in figure legends).

## 7.4 Results

### 7.4.1 Effect of acute exposure of WCS on the co-culture model cell viability

In order to gauge the employed methodology including the aerosol delivery system operation and the co-culture model sensitivity, the cells were treated with WCS. Upon exposing the co-culture model to 7 puffs of WCS, it was observed that WCS had a cytotoxic effect, reducing the cell viability to  $58.55 \pm 5.03$  % UT ( $p < 0.0001$ ) as shown in Figure 7.1. This reduction in cell viability caused by WCS was significant compared to cells treated to 7 puffs of ECV ( $97.92 \pm 7.89$ % UT,  $p < 0.0001$ ) as well as air-treated cells ( $103.25 \pm 5.78$ % UT,  $p < 0.0001$ ), neither of which caused any significant reduction in cell viability after 7 puffs.

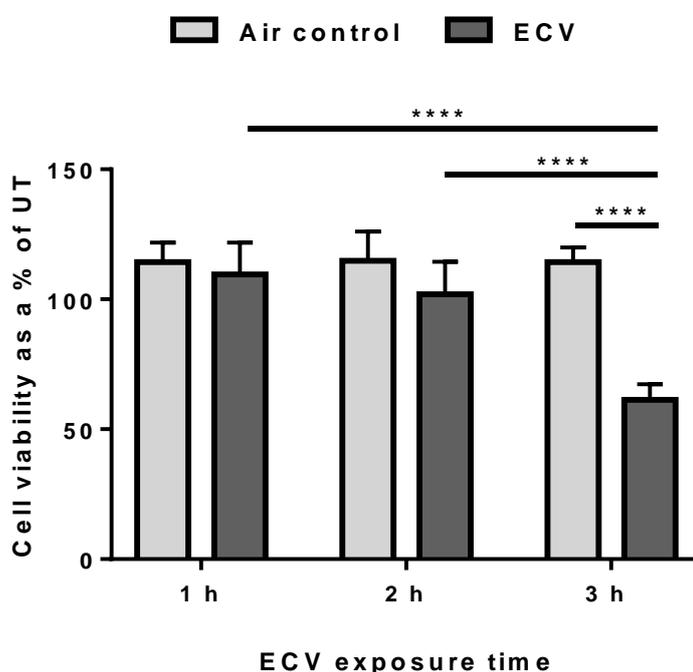


**Figure 7.1. Influence of WCS exposure on the co-culture model cell viability after 7 puffs exposure.**

The co-culture model was exposed to WCS as described in sub-section 7.3.4, post which the XTT cell viability analysis was performed as described in sub-section 7.3.5. As seen from the above figure, 7 puffs of WCS had a cytotoxic effect on the co-culture model cell viability compared to the UT and the air-treated cells. This reduction in cell viability was also significant compared to the ECV treated cells which did not influence the cell viability after 7 puffs. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  SD of 3 independent SWs. (\*\*\*\* =  $p < 0.0001$ )

#### 7.4.2 Effect of single block ECV exposure on the co-culture model cell viability

The co-culture model was exposed to ECV at different single block exposure times. As shown in Figure 7.2, while 1 h ( $109.52 \pm 11.80$  % UT) and 2 h ( $101.98 \pm 11.93$  % UT) ECV exposure had no significant impact on the cell viability, 3 h ECV exposure caused a significant drop in the cell viability ( $61.31 \pm 5.75$  %,  $p < 0.0001$ ) compared to that of the UT ( $100 \pm 3.96$  %). This drop in cell viability was significant compared to 3 h air-treated cells ( $114.31 \pm 5.36$  % UT,  $p < 0.0001$ ) as well as 1 h ( $p < 0.0001$ ) and 2 h ( $p < 0.0001$ ) ECV treated cells. A small but significant increase in cell viability ( $\sim 3 - 14$  %,  $p < 0.001$ ) of air-treated cells was observed at all three single block exposure times compared to the UT.

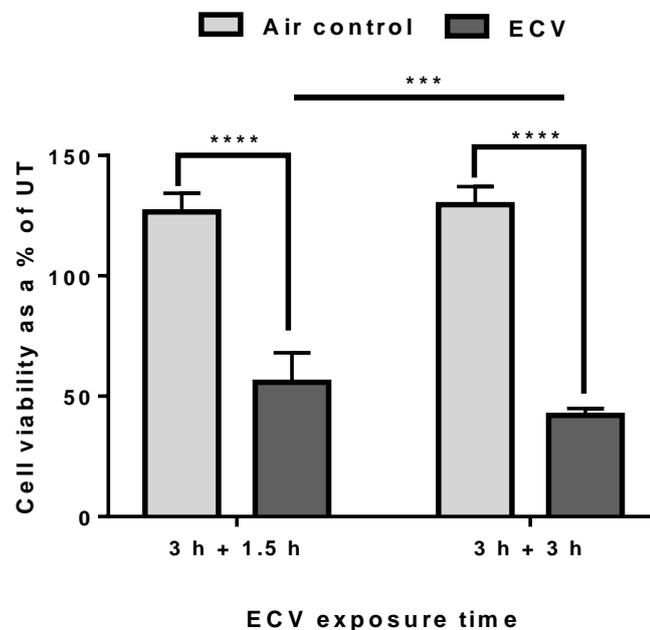


**Figure 7.2. Influence of single block ECV exposure on the co-culture model cell viability.**

The co-culture model was exposed to the different ECV single block exposure times as described in sub-section 7.3.4, post which the XTT cell viability analysis was performed as described in sub-section 7.3.5. As observed from the above figure, while 1 h and 2 h ECV exposure did not impact the cell viability, a significant reduction in cell viability was observed at the 3 h ECV exposure time compared to both the UT and 3 h air-treated cells. This decrease in cell viability was also significant compared to the 1 h and 2 h ECV treated cells. While air exposure did not impact the cell viability at any exposure time, a marginal increase (3 -14 %) in cell viability was observed at all three single block exposure times compared to the UT. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  SD of 3 independent SWs. (\*\*\*\* =  $p < 0.0001$ )

### 7.4.3 Effect of double block ECV exposure on the co-culture model cell viability

Upon exposing the co-culture model to two blocks of ECV exposure, it was observed that at both the double block exposure times, there was a significant decrease in cell viability compared to that of the UT ( $100 \pm 3.96\%$ ) and the respective air-treated cells as shown in Figure 7.3. The cell viability was reduced to  $55.81 \pm 11.68\%$  UT ( $p < 0.0001$ ) and  $42.10 \pm 2.69\%$  UT ( $p < 0.0001$ ) at the 4.5 h and the 6 h ECV exposure times respectively. Moreover, the cell viability observed at the 6 h exposure time was significantly lower than the cell viability observed at the 4.5 h exposure time ( $p < 0.001$ ). Similar to single block exposures, although air treatment did not impact the cell viability at either of the double block exposure times, there was a significant increase in cell viability ( $\sim 30\%$ ,  $p < 0.0001$ ) compared to the UT.

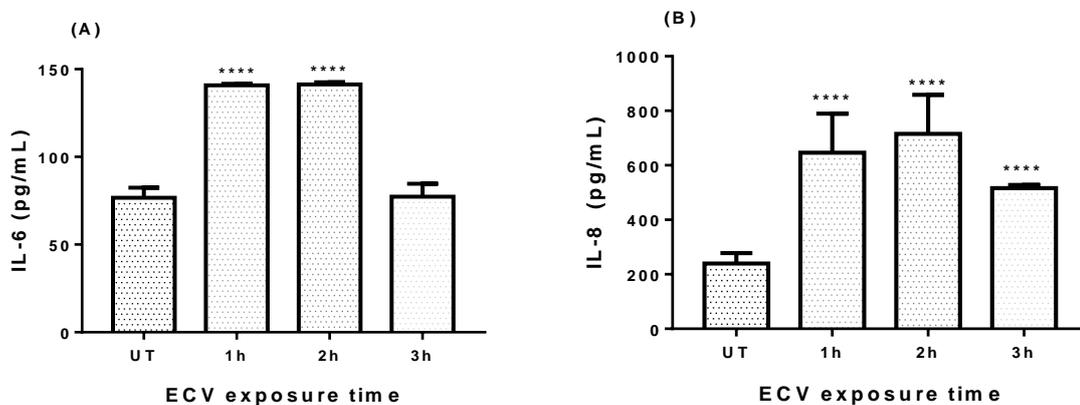


**Figure 7.3. Influence of double block ECV exposure on the co-culture model cell viability.**

The co-culture model was exposed to the different ECV double block exposure times as described in sub-section 7.3.4, post which the XTT cell viability analysis was performed as described in sub-section 7.3.5. As observed from the above figure, double block ECV exposure caused a significant reduction in cell viability at both 4.5 h and 6 h exposure times compared to the UT and air-treated cells. Moreover, the reduction in cell viability caused by 6 h ECV exposure was significantly lower than that caused by 4.5 h ECV exposure. While air treatment did not induce any cytotoxic effect at either of the double block exposure times, a significant increase ( $\sim 30\%$ ) in cell viability was observed compared to the UT. Cell viability is expressed as a percentage of the control (untreated cells). Each bar represents Mean  $\pm$  SD of 3 independent SW's. (\*\*\*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.001$ )

#### 7.4.4 Effect of single block ECV exposure on the co-culture model IL-6 and IL-8 production

Analysis of cell-culture supernatants 24 h post single block ECV exposures demonstrated elevated production of both IL-6 and IL-8 inflammatory mediators' compared to the UT. As shown in Figure 7.4 (A) and (B), at the 1 h and 2 h ECV exposure times, a significant increase in the levels of IL-6 (1 h :  $140.8 \pm 0.7$  pg/mL,  $p < 0.001$  ; 2 h :  $141.3 \pm 1.1$  pg/mL,  $p < 0.001$ ) and IL-8 (1 h :  $646.2 \pm 133$  pg/mL,  $p < 0.0001$  ; 2 h :  $715.8 \pm 134$  pg/mL,  $p < 0.0001$ ) was observed compared to the UT (IL-6 :  $76.7 \pm 5.4$  pg/mL ; IL-8 :  $239.2 \pm 36.1$  pg/mL). At the 3 h exposure time too, IL-8 levels ( $516.6 \pm 10.5$  pg/mL,  $p < 0.0001$ ) were significantly higher compared to the UT while the IL-6 levels ( $77.3 \pm 6.9$  pg/mL) were comparable to that of the UT levels, possibly due to the reduction in cell viability ( $61.31 \pm 5.75$  % UT) observed at this exposure time.

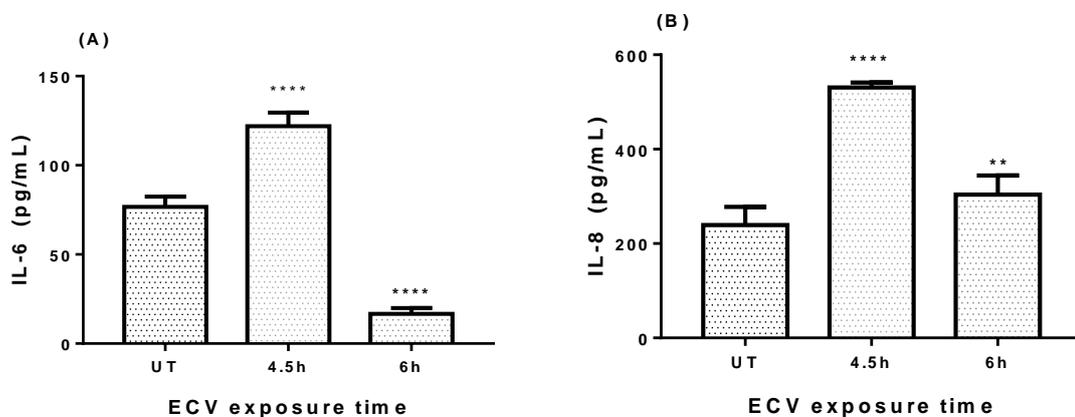


**Figure 7.4. Influence of single block ECV exposure on the co-culture model (A) IL-6 and (B) IL-8 production.**

The co-culture model was exposed to the different ECV single block exposure times as described in sub-section 7.3.4, post which the IL-6/IL-8 analysis was performed as described in sub-section 7.3.6. As observed from the above figure, compared to the UT, IL-6 and IL-8 levels at the 1 h and 2 h ECV exposure times were significantly higher despite observing a cell viability comparable to that of the UT. At the 3 h exposure time, although the cell viability was ~60 % UT, there was a significant increase in the IL-8 chemokine levels while the IL-6 cytokine levels were comparable to that of the UT. Air treatment did not have any significant influence on IL-6/IL-8 production at any of the tested time-points (data not shown). Each bar represents Mean  $\pm$  SD of 3 independent SW's. (\*\*\*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.001$ )

#### 7.4.5 Effect of double block ECV exposure on the co-culture model IL-6 and IL-8 production

It can be observed from Figure 7.5 (A) and (B) that both the double block ECV exposure times had a significant impact on IL-6 and IL-8 production. At the 4.5 h exposure time, a significant elevation in IL-6 ( $121.9 \pm 7.1$  pg/mL,  $p < 0.0001$ ) and IL-8 ( $530.9 \pm 9.7$  pg/mL,  $p < 0.0001$ ) levels was observed compared to UT levels despite the cell viability at this exposure time being recorded as less than 60 % of UT ( $55.81 \pm 11.68$  % UT). Similarly, at the 6 h exposure time, although the cell viability was  $42.10 \pm 2.69$  % UT, the IL-8 levels ( $303.8 \pm 37.9$  pg/mL,  $p < 0.01$ ) were significantly higher compared to the UT while the IL-6 levels ( $16.6 \pm 3$  pg/mL,  $p < 0.0001$ ) were significantly reduced compared to the UT.

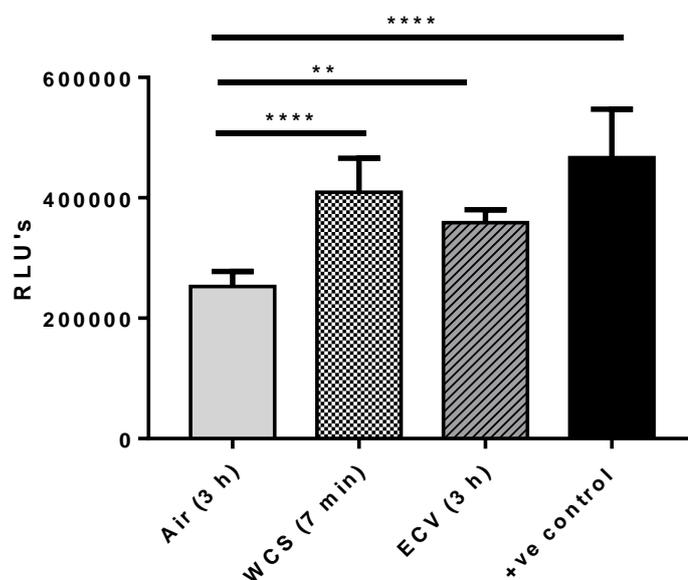


**Figure 7.5. Influence of double block ECV exposure on the co-culture model (A) IL-6 and (B) IL-8 production.**

The co-culture model was exposed to the different ECV double block exposure times as described in sub-section 7.3.4, post which the IL-6/IL-8 analysis was performed as described in sub-section 7.3.6. As observed from the above figure, at the 4.5 h exposure time, where the cell viability was ~50 % UT, there was a sharp increase in the levels of both IL-6 and IL-8 pro-inflammatory mediators. At the 6 h exposure time, although the IL-8 level was significantly higher compared to the UT, there was a significant drop in the level of IL-6. Each bar represents Mean  $\pm$  SD of independent SW's. (\*\*\*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.001$ , \*\* =  $p < 0.01$ )

#### 7.4.6 Effect of ECV exposure on the co-culture model oxidative stress levels

Since ECV demonstrated a significant decrease in cell viability at the 3 h single block exposure time, the co-culture model oxidative stress levels and caspase 3/7 activity were further analysed at this exposure time. Analysis of cellular oxidative stress 24 h post-exposure to different treatment conditions showed that both WCS and ECV exposure caused a significant increase in H<sub>2</sub>O<sub>2</sub> levels compared to the air-treated cells (3 h) as shown in Figure 7.6. While WCS caused a ~1.6 fold ( $p < 0.0001$ ) increase in H<sub>2</sub>O<sub>2</sub> levels, ECV caused a ~1.4 fold ( $p < 0.01$ ) increase in H<sub>2</sub>O<sub>2</sub> levels compared to the air-treated cells. It must be noted that cells were exposed to different doses of WCS and ECV. The cellular H<sub>2</sub>O<sub>2</sub> levels induced by 7 puffs of WCS was higher (~1.1 fold) than that produced by 180 puffs of ECV. Positive control cells (50  $\mu$ M menadione treatment) produced the highest H<sub>2</sub>O<sub>2</sub> levels, thus providing confidence in the assay data.

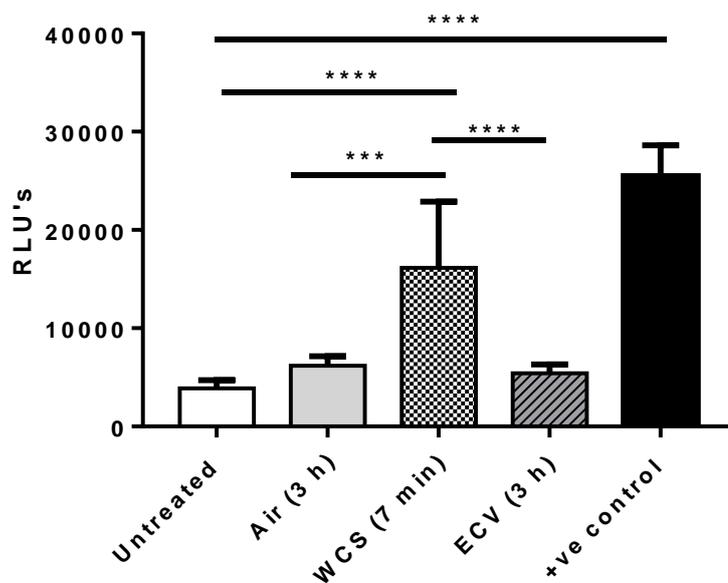


**Figure 7.6. Influence of single block 3 h ECV exposure on the co-culture model oxidative stress levels.**

The co-culture model was exposed to WCS/ECV as described in sub-section 7.3.4, post which the oxidative stress analysis was performed as described in sub-section 7.3.7. As observed from the above figure, both WCS (7 min) exposure and ECV (3 h) exposure caused a significant increase in cellular H<sub>2</sub>O<sub>2</sub> levels compared to the air-treated cells (3 h). Interestingly, 7 puffs of WCS exposure incited higher H<sub>2</sub>O<sub>2</sub> levels than 180 puffs of ECV exposure. Each bar represents Mean  $\pm$  SD of 3 independent SW's. (\*\*\*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.001$ , \*\* =  $p < 0.01$ )

#### 7.4.7 Investigating apoptosis: effect of ECV exposure on the co-culture model caspase 3/7 activity

24 h post exposure to different treatment conditions, the co-culture model was analysed for the expression of pro-apoptotic mediators' namely caspase 3 and 7. It can be observed from Figure 7.7 that 3 h ECV exposure did not have any significant impact on caspase 3/7 activity whereas 7 min WCS exposure caused a significant increase ( $p < 0.0001$ ) in caspase 3/7 activity compared to the UT cells. This increase in caspase 3/7 activity caused by WCS was also significant compared to that caused by 3 h ECV ( $p < 0.0001$ ) and 3 h air treated cells ( $p < 0.001$ ). While 3 h air-treatment did not have any significant influence on caspase 3/7 activity compared to the UT, positive control cells (0.5 mM  $H_2O_2$  treatment) produced a significant increase ( $p < 0.001$ ) in caspase 3/7 activity, thus providing confidence in the assay data.



**Figure 7.7. Influence of single block 3 h ECV exposure on the co-culture model caspase 3/7 activity.**

The co-culture model was exposed to WCS/ECV as described in sub-section 7.3.4, post which the caspase 3/7 activity analysis was performed as described in sub-section 7.3.8. As observed from the above figure, while 7 min WCS exposure caused significant increase in the expression of caspase 3/7, neither 3 h ECV nor 3 h air treatment influenced the caspase 3/7 activity compared to the UT cells. Positive control cells (0.5 mM  $H_2O_2$  treated cells) produced a significant increase in caspase 3/7 activity compared to the UT, thus validating the assay methodology. Each bar represents Mean  $\pm$  SD of 3 independent SW's. (\*\*\*\* =  $p < 0.0001$ , \*\*\* =  $p < 0.001$ )

## 7.5 Discussion

Although use of ECs has increased rapidly worldwide, there is currently no overall consensus on the possible detrimental EC health effects. Due to the lack of a standardised testing method to evaluate EC cytotoxicity, there are inconsistencies in the EC data reported so far (Hiemstra and Bals, 2016). Crucial factors such as the cell model employed and the method of EC delivery determines the physiological significance of any individual EC study. Since human airways consist of several other cell types apart from epithelial cells, there exists a constant cross-signalling between the different cell types in order to maintain tissue homeostasis (Knight and Holgate, 2003). In this respect, the present chapter aimed to investigate the effects of ECV using a physiologically relevant ALI co-culture model of human airways consisting of CALU 3 bronchial epithelial cells and MRC-5 human pulmonary fibroblasts. To the authors' knowledge, this is the first study to report a co-culture model of differentiated bronchial epithelium with human pulmonary fibroblasts to investigate EC aerosol cytotoxicity. Our results demonstrate that extended ECV exposure ( $\geq 3$  h) has a significant impact on the cell viability, IL-6/IL-8 production and oxidative stress but not caspase 3/7 activity.

### 7.5.1 Acute WCS exposure has a cytotoxic impact on the co-culture model cell viability

Exposure of HPF-CALU 3 co-culture models to cigarette smoke provided a quick, relevant method of demonstrating the sensitivity of the co-culture model to inhalable toxicants such as WCS. The current study found that 7 min acute WCS exposure had a significant cytotoxic impact on the co-culture model cell viability as shown in Figure 7.1, while air and ECV had no significant impact at this exposure time. This result correlates with a number of previous studies employing ALI monocultures of bronchial epithelial cells that reported on similar acute cytotoxic effects of WCS although the time taken to exhibit cytotoxicity varied across studies (Aufderheide et al., 2001, Li et al., 2014, Scheffler et al., 2015a). For instance, in the study by Scheffler et al (2015), 60 puffs of WCS (6 puffs from 10 reference cigarettes each) was required to produce a cytotoxic effect ( $< 70$  % cell viability compared to the control) on bronchial epithelial cells whereas in the current study, a cytotoxic effect was observed after 7 puffs of WCS. Possible reasons for this could be differences in the study designs including the type of smoking machine used, type of smoking regime employed, type of cigarette used, dilution rates of WCS and flow rate of WCS delivery. While undiluted WCS was delivered at ISO 3308 regime using a bespoke set-up in the current study, Scheffler et al half-diluted WCS, generated at a customised intense smoking regime, with synthetic air at 1 L/min (which would decrease the potency of WCS) using a commercial

CULTEX<sup>®</sup> RFS smoking machine. Also, Scheffler et al delivered WCS to the cells at a rate of 5 mL/min/insert while in the current study WCS was delivered at a rate of 350 mL/min/insert. Moreover, while a commercial tobacco cigarette (Marlboro) was used in the current study, a reference cigarette (K3R4F) was used in the study by Scheffler et al. Lastly, while Scheffler et al employed undifferentiated cultures of primary bronchial epithelial cells in their study, a differentiated co-culture model of bronchial epithelium using cell lines was employed in the current study. All of the above mentioned factors may have potentially contributed towards the observed difference in WCS result between the two studies. Nevertheless, this WCS result demonstrates that the co-culture model employed in the current study responded to WCS in a fashion similar to that of the ALI monocultures of past studies although offering more physiological relevance due to the presence of underlying fibroblasts (Costea et al., 2003, BeruBe et al., 2009). The WCS result reported here therefore provides confidence in our methodology including the aerosol delivery system and responsiveness of the co-culture models to WCS aerosols.

An interesting observation was that although a 7 min air-exposure did not produce any significant impact on the co-culture model cell viability ( $103.25 \pm 5.78\%$  UT, Figure 7.1), longer exposures ranging from 1 h to 6 h produced a small but significant increase in cell viability compared to the untreated incubator control cells (Figures 7.2 and 7.3). This suggests that the continuous exposure of cells to a steady flow rate of air for prolonged periods of time could have a protective effect on the cells, possibly via accumulation of moisture over the apical surface of the cells (Azzopardi et al., 2015). Another possible explanation could be that the continuous exposure of cells to air stimulates the various intracellular signalling pathways associated with cell viability (e.g. MAPK, ERK1/2) although further investigations are required to precisely ascertain this observed effect.

#### 7.5.2 ECV induces a cytotoxic effect only at prolonged exposure times ( $\geq 3$ h)

On a behavioural level, ECs are generally used for extended periods of time compared to tobacco cigarettes which are used rather more acutely. Previous studies and EC forums (like [www.vaping.com](http://www.vaping.com)) have suggested that the number of puffs an 'average' or 'moderate' EC user inhales can vary between 150 - 600 puffs/day (Farsalinos et al., 2015c, Scheffler et al., 2015b, Etter and Bullen, 2014) although this number fluctuates largely with individual EC users, nicotine strength and device type and hence as of yet, a consensus on the vaping topography of an 'average' EC user is still lacking. In the current study, we employed an EC exposure time range of 1 h - 6 h (equating to 60 – 360 puffs) which we found to be relevant to the vaping habits of majority of 'average' EC users (E-cigarette forum webpage). When the co-culture model was exposed to ECV, an exposure-time dependant decrease in cell

viability was observed from the 2 h ECV exposure time onwards i.e. cell viability at 6 h < 4.5 h < 3 h < 2 h as shown in Figure 7.2 and 7.3, thus demonstrating a clear cytotoxic effect with increasing number of puffs. Very few studies employing an ALI cell culture system have investigated EC aerosol cytotoxicity across a broad range of exposure times such as the present study. Most previous studies reported either exposure of cells to a fixed number of EC puffs or analysed cells across short range of different exposure times (such as 0 – 20 min). Only one previous study has analysed ECV cytotoxicity across a range of exposure times comparable to that of the present study (Neilson et al., 2015). In this study, Neilson et al (2015) reported that ECV had no cytotoxic effect at any of the exposure times ranging from 1 h to 6 h even though ECV was delivered to tracheobronchial epithelial tissues at a highly intense puff regime (80 mL puff drawn over 3s, every 30s). Other studies have however shown EC aerosols to produce significant cytotoxic effects similar to that observed in the current study (Scheffler et al., 2015b, Leigh et al., 2016). Such disparities amongst EC studies clearly demonstrates the requirement of a standardised testing method to analyse EC cytotoxicity *in-vitro*. Such a standardised method must indicate the different vaping topography parameters such puff-volume, puff-duration, inter-puff interval, air-dilution ratio etc. such that comparability between studies is enhanced.

### 7.5.3 ECV exposure leads to increased oxidative stress but lesser than that caused by WCS

In line with the cell viability data, ECV exposure caused a significant increase in oxidative stress levels in the co-culture model compared to air-treated cells. It was never-the-less interesting to note that 7 puffs of WCS exposure produced markedly higher oxidative stress levels than that produced by 180 puffs of ECV as shown in Figure 7.6, which clearly demonstrates the intense cytotoxic profile of tobacco smoke in comparison to ECV. Whilst the oxidative stress inducing effects of WCS is well established (Nowak et al., 1996, Dekhuijzen et al., 1996, Tanni et al., 2012), a number of recent studies have reported on similar oxidative stress inducing effects of ECs (Putzhammer et al., 2016, Anderson et al., 2016). Interestingly, one previous study that employed ALI model of bronchial epithelial cells reported a result similar to that of the current study wherein ECV exposure lead to increased oxidative stress but lesser than that caused by WCS exposure (Scheffler et al., 2015b). In correlation with the *in-vitro* studies, *in-vivo* mice studies (Lerner et al., 2015, Schweitzer et al., 2015) and human studies (Carnevale et al., 2016, Ikonomidis et al., 2018) have also shown EC aerosols to produce significant oxidative stress effects. Since it is well-established from past cigarette smoke studies that increased oxidative burden plays a key role in the pathogenesis of smoking induced COPD (MacNee, 2005), the oxidative stress-

inducing effects of EC aerosols, even if less potent than cigarette smoke, needs to be treated with high significance and further in-depth investigation is required in this area.

#### 7.5.4 ECV exposure causes increase in IL-6/IL-8 pro-inflammatory mediators' production

Increased oxidative stress can have several biological implications and one of the significant consequences is the amplified expression of pro-inflammatory genes such as IL-6, IL-8 and TNF- $\alpha$  (Rahman, 2003, Yang et al., 2006) which correlates with the increased IL-6/IL-8 levels observed in the current study. ECV exposure caused elevated levels of IL-6 and IL-8 at most exposure times (except IL-6 levels at 6 h due to decreased cell viability) as seen from Figure 7.4 and 7.5. Especially at the 3 h, 4.5 h and 6 h exposure times, IL-8 levels were significantly elevated compared to UT, thus indicating a sustained inflammatory response post exposure to EC aerosols. A number of previous studies have reported similar findings on ECV-induced elevated IL-6 and IL-8 production (Leigh et al., 2016, Lerner et al., 2015, Cervellati et al., 2014). Elevated IL-6 and IL-8 levels have major implications in mucus hyper-secretion, immune cells infiltration and airway fibrosis (Hogg et al., 2004, Tetley, 2005). Synergistically, elevated pro-inflammatory cytokine/chemokine production and increased ROS levels have been demonstrated to play a key role in COPD progression (Morrison et al., 1999, Rahman and Adcock, 2006, Yao et al., 2014), thus suggesting that long-term exposure to aerosols from certain ECs may lead to substantial airways damage. Although the cell-type specific origin of the pro-inflammatory mediators was not been investigated in the current study, previous studies have demonstrated that the cytokines secreted by sub-epithelium fibroblasts have a marked influence on bronchial epithelial cells proliferation (Skibinski et al., 2007) especially during wound healing and recovery (Iskandar et al., 2015). In this respect, employing a co-culture model such as ours would be beneficial in understanding the influence of fibroblasts on the bronchial epithelial cell response to inhaled aerosols such as WCS or ECV.

#### 7.5.5 ECV induced cell death occurs in a caspase-independent fashion

In order to investigate into the mechanism of cell death, caspase 3 and 7, the executing caspases of the intrinsic apoptotic pathway was analysed. As shown in Figure 7.8, while 7 min WCS caused a significant increase in caspase 3/7 activity compared to the UT, 3 h ECV exposure did not significantly alter caspase 3/7 activity despite reduced cell viability observed at this exposure time. One possible explanation for the observed reduction in cell viability may be that ECV induced cell death via a caspase-independent pathway. Apoptosis can occur via granzyme A/B mediated mitochondrial damage resulting in upsurge of intracellular ROS (Heibein et al., 1999, Beresford et al., 1999, Beresford et al., 2001). This

explanation correlates well with the observed elevated H<sub>2</sub>O<sub>2</sub> levels in ECV exposed cells, thus suggesting that increased ROS levels may be a chief contributing factor towards the observed cytotoxicity in ECV exposed cells. Alternatively, it could also be possible that the cells underwent necrosis as a consequence of the physico-chemical stress (Festjens et al., 2006) that was placed on them during ECV exposure. Anderson et al (2016) reported that post exposure of vascular endothelial cells to EC aerosols, a significant proportion of both apoptotic and necrotic endothelial cells were observed and that subsequent treatment with anti-oxidants prevented EC induced necrosis but not apoptosis (Anderson et al., 2016). This suggests a possibility that different constituents of ECV such as nicotine, base-humectants or flavouring compounds may impact the cells via different death pathways. Further analysis of cellular surface markers and genes of apoptosis/necrosis is required to elucidate the precise mechanisms of cell death that are involved in these exposure systems. To the authors' knowledge, this is the first study to investigate apoptosis in an ALI cell system post exposure to EC aerosols. Two previous studies have reported on increased Bax gene expression (Sancilio et al., 2016) and Annexin V/PI (Yu et al., 2016) respectively in cells post exposure to EC liquid/extracts. Our result does not agree with these studies, possibly due to the difference in study design as the previous studies involved the exposure of submerged cultures to either EC liquid or extracts, both of which have limited physiological significance compared to ALI culture exposure to EC aerosols.

#### 7.5.6 Cause of ECV cytotoxicity

It is as yet unclear what the precise factors that mediate EC cytotoxicity are. Previous studies have implicated different agents such as ROS (Lerner et al., 2015), particulate metals (Williams et al., 2013) or copper nanoparticles (Lerner et al., 2016) emitted by ECs that could chiefly be responsible for the cytotoxic effects of ECs. Alternatively, it could also be possible that the low-molecular weight carbonyl compounds such as formaldehyde, acetaldehyde, and acrolein, possibly produced via pyrolysis of glycerine (Goniewicz et al., 2014b, Salamanca et al., 2017) are responsible for mediating EC cytotoxicity. In recent years, there has been an upsurge in the number of studies, including the current study (Leslie et al., 2017) that have found flavouring substances used in EC liquids as a chief cause of cytotoxicity (Clapp et al., 2017, Bahl et al., 2012, Leigh et al., 2016, Allen et al., 2016). With more than 7700 different EC flavours currently available in the market (Tierney et al., 2016), this area of EC requires further investigation in order to regulate the flavouring substances more rigorously such that EC users are prevented from inhaling toxic flavouring additives (Allen et al., 2016).

## 7.6 Conclusion

The current chapter employed a novel physiologically relevant ALI co-culture model of differentiated bronchial epithelium with underlying pulmonary fibroblasts to evaluate EC cytotoxicity *in-vitro*. Results indicate that EC aerosols can have cytotoxic, pro-inflammatory and oxidative-stress inducing effects at prolonged exposure times ( $\geq 3$  h) and hence cannot be deemed completely harmless. Further research is required to ascertain the source, nature of the cytotoxic agents and precise mechanisms that mediate EC cytotoxicity. This would ultimately help EC users to assess their vaping habits and also make an informed decision about the EC type and flavours they choose.

## 8 Overall discussion

Despite establishing strong associations between cigarette smoking and multiple airway diseases including COPD and lung cancer, tobacco smoking continues to remain the leading preventable cause of deaths globally, responsible for more than 5 million deaths annually (Jha et al., 2013). Several smoking cessation strategies have been introduced in order to reduce mortality and to decrease the costs associated with treating tobacco related illnesses (Alberg, 2008). Although proven to be effective, common smoking cessation strategies such as NRTs (nicotine gums, patches etc.) or medications like bupropion and varenicline (Rigotti and Clair, 2013) do not cater to the sensory and behavioural aspects of tobacco smoking (Bullen et al., 2013). Under these circumstances, ECs were introduced in the year 2003 as 'safer' alternatives to tobacco cigarettes and as a potential smoking cessation tool (McRobbie et al., 2014). Since their introduction, there has been a tremendous increase in the number of EC users worldwide. In the UK, for the first time, there are more number of ex-smokers using ECs compared to current smokers (ASH., 2017b). More alarmingly, ECs are the most popular tobacco product amongst high-school students and youngsters (Singh et al., 2016).

Although introduced in 2003, research on ECs has been limited; substantially increasing only in recent years. Many aspects of ECs such as the long-term health implications and smoking cessation efficacy of ECs still remains unclear. ECs are widely perceived as a less-harmful alternative to tobacco cigarettes since there is no burning of tobacco leaves involved in ECs. However, this does not guarantee the absence of any potentially harmful chemicals in EC liquids and aerosols. In fact, there is growing evidence demonstrating that ECs contain or emit several toxicants including carbonyl compounds, volatile organic compounds, and metal particles (Williams et al., 2013, Salamanca et al., 2017) which are established carcinogenic/mutagenic compounds associated with the onset of several airway diseases. Further, due to the lack of a standardised protocol, the test methodologies used for EC evaluation vary hugely between different studies which limits comparability between studies. Of special importance is the model employed to assess EC biological effects. The relevance of EC data obtained from animal models is questionable due to the various physiological, genetic and behavioural differences between rodents and humans as described earlier in sub-chapter 2.4.1.2. Hence, there is a requirement for more human relevant research on ECs.

In this context, the main aim of the current study was to design and employ human physiologically relevant test methodologies to investigate the effects of ECs *in-vitro*.

Submerged monocultures of two chief bronchial epithelial cells BEAS 2B and CALU 3 and other airways related cell types (IB3-1, C38, J774, THP-1 and HPF) were used to assess the different aspects related to ECs such as the influence of nicotine, E-vehicle and flavourings and brand variability using either direct exposure method or extracts exposure (Chapter 5 and Chapter 6 experiments). Based on the results from these two set of experiments, in Chapter 7 experiments, a HPF-CALU 3 co-culture model of human tracheobronchial epithelium was employed to study the cytotoxicity of WCS and ECV at different exposure times ranging from 1 h – 6h under single block and double block regimes delivered via a bespoke, automated smoking machine that was designed and constructed in-house. This three-step approach i.e. from EC fluids to extracts to aerosols and from submerged monocultures to ALI co-cultures enabled an in-depth investigation into various aspects of EC.

### 8.1 The role of nicotine in ECs in mediating cytotoxicity is limited

Nicotine is a highly addictive substance and mediates its addictive effects by binding to NACHRs receptors, which are specific ion channels highly-expressed in neurons of the central nervous system and also in other cell types like bronchial epithelial cells, endothelial cells, smooth muscle cells etc. (Grando et al., 1995, Wessler et al., 1999). This interaction leads to the rapid production of a number of neuro-transmitters including dopamine, the release of which is associated with the pleasure cycle, ultimately leading to tobacco addiction (Benowitz, 2009, Hukkanen et al., 2005). While the mechanisms of neurological effects of nicotine is well-understood (Dani and De Biasi, 2001), the role of nicotine in exacerbating airway diseases lacks consensus amongst scientists.

In chapter 5 of the current study (sub-sections 5.4.2 and 5.4.3), when different physiologically relevant concentrations of nicotine ranging from 1.1  $\mu\text{M}$  to 75  $\mu\text{M}$  were treated to submerged BEAS 2B and CALU 3 bronchial epithelial cells for 4 h or 24 h, none of the nicotine and cotinine concentrations had a significant impact on the cell viability or IL-6/IL-8 production (Figure 5.4 - 5.9). The current study is one of the few studies that have investigated the cytotoxic effects of nicotine on human bronchial epithelial cell types namely BEAS 2B and CALU 3. When comparing the results of the current study to existing literature, it was evident that the cytotoxic effect of nicotine depends upon several factors such as the dose ranged tested, exposure time and cell type analysed. While majority of the past studies that investigated the effect of nicotine on neuronal cell types have reported on its cell-protective effects (Kihara et al., 1997, Utsumi et al., 2004), other studies that tested the effect of nicotine on human airway cells have reported on the variable effects of nicotine

depending upon the dose-range tested i.e. micro-molar doses produced an anti-apoptotic effect (West et al., 2003) while milli-molar doses produced a cytotoxic effect (Ramage et al., 2006). It is also important to consider that the rate of nicotine delivery hugely varies between ECs and tobacco cigarettes due to differences in heating process, vapourisation temperatures and puffing topography (Farsalinos et al., 2014). Studies have shown that compared to tobacco cigarettes, ECs deliver smaller quantities of nicotine at a slower rate and that the rate of delivery depends upon the type of EC used (Eissenberg, 2010, Vansickel et al., 2010) and the experience of the EC user (Schroeder and Hoffman, 2014). In this context, it is reasonable to speculate that the amount of nicotine delivered via ECs in an average EC user may not reach cytotoxic levels and hence any significant physiological effect of nicotine at this dose-range would not be anticipated. Never-the-less, usage of high-powered ECs (3<sup>rd</sup> and 4<sup>th</sup> generation) by experienced vapers for prolonged periods of time may deliver high nicotine doses (Etter and Bullen, 2014, Etter and Bullen, 2011), in which case, the effect of nicotine on airway cells remains unknown needs further investigation.

The precise influence of nicotine on the fate of different human airway cells can have significant physiological implications. For instance, although NRTs to date have not shown to cause an increased risk of cancer, past studies have reported on nicotine's ability of its own, irrespective of other tobacco smoke constituents to promote tumour, angiogenesis and gastrointestinal cancer (Schaal and Chellappan, 2014, Jensen et al., 2012, Grando, 2014). In similar lines, the inflammatory effects of nicotine is another aspect where a clear consensus has not been reached. While the current study found that nicotine/cotinine had no influence on the IL-6/IL-8 production in BEAS 2B and CALU 3 at any of the tested concentrations (Figure 5.6 and 5.9), past studies have shown nicotine to produce pro-inflammatory (Vassallo et al., 2008, Wang et al., 2012) and anti-inflammatory effects (van Westerloo et al., 2005, Tracey, 2007). While some past EC studies have shown an increase in pro-inflammatory mediators' production post EC exposure (Wu et al., 2014, Schweitzer et al., 2015, Lerner et al., 2016), it has not been established whether this effect was due to nicotine or other components of EC liquid/aerosols. As reported by Schweitzer et al (2015) and Yu et al (2016), nicotine-free ECs were found to cause barrier dysfunction and cytotoxicity comparable to the nicotine containing ECs (Yu et al., 2016, Schweitzer et al., 2015), thus further suggesting that nicotine may not be the chief contributing agent towards the observed cytotoxicity. These studies also support the observation from Chapter 6 experiments (Figure 6.4) where the effect of ECs with different nicotine strengths was investigated. It was observed from these experiments that the increasing nicotine content of ECs (6, 12 and 18 mg nicotine strength respectively) had minimal impact on the cell viability on four cell type namely IB3-1, C38, BEAS 2B and J774. Overall, since the

pharmacokinetics of nicotine delivery via ECs including the absorption time and the time taken to reach peak nicotine levels vary considerably compared to tobacco cigarettes (Farsalinos et al., 2014, Etter and Bullen, 2011), the biological effects of nicotine delivered via ECs on the different mechanisms such as cell proliferation and inflammatory mediators' production by human airway cells is currently unexplored and requires further investigation.

## 8.2 E-vehicle effects depend upon the methodology employed

PG and VG, the commonly used chemicals to produce E-vehicle, are both FDA approved food additives and hence they contribute to a great extent to the widely held perception of ECs as safe devices (Lechasseur et al., 2017). However, the influence of these compounds on human airways have been sparsely evaluated. In the current study, the effect of E-vehicle was investigated via two different methods, namely E-vehicle fluid exposure and E-vehicle extract exposure. The results of the first method as observed from Figure 5.10 showed that E-vehicle fluid consisting of 70 % PG and 30 % VG, induced a significant decrease in cell viability in both BEAS 2B and CALU 3 cells. In contrast, the results of the second method, as illustrated in Figure 6.3, showed that the extracts of E-vehicle did not produce any significant impact on the cell viability, with only one out of the six tested ratios of PG:VG (80:20) producing a significant decrease in cell viability. This illustrates the variability in study outcomes produced by employing different delivery methods.

While assessing the cytotoxicity of E-vehicle fluid, it is important to employ an E-vehicle fluid prepared using pure USP-grade PG and VG solutions such that the influence of nicotine, flavourings and added substances are eliminated. As stated earlier in Chapter 5 (sub-section 5.5.3), only one past study has investigated the cytotoxicity of an in-house prepared E-vehicle consisting of pure PG/VG solution (Gonzalez-Suarez Ignacio, 2017). The study observed a dose-dependent decrease in cell viability at all E-vehicle concentrations. An interesting comparison could be drawn between the current study and the study by Ignacio et al. All the 5 E-vehicle fluid ratios employed by Ignacio et al (0 % PG : 100 % VG, 30 % PG : 70 % VG, 50 % PG : 50 % VG, 70 % PG : 30 % VG, 100 % PG : 0 % VG) were investigated in the current study though not in their fluid form but rather in their extract form (Figure 6.3). In such a comparison, the observation reported by Ignacio et al that increasing PG concentration resulted in increased cytotoxicity does not correlate with the results of the current study. In the current study, increasing PG levels in E-vehicle did not have any dose-dependent effect as reflected by the fact that 100 % PG E-vehicle did not produce any significant decrease in cell viability compared to the other ratios of E-vehicle and the untreated control cells. This comparison further demonstrates the significant

influence of employing different E-vehicle delivery methods (extract vs fluid) to assess its cytotoxicity.

It is also interesting to note that *in-vivo* animal studies investigating inhalation of PG/VG aerosols have reported mixed results. Past rodent studies have shown inhalation of PG/VG mixture aerosols to cause alterations in circadian cycle associated genes (Lechasseur et al., 2017), diminish thymus and spleen growth, increased bronchoalveolar lavage fluid (BALF) lactate dehydrogenase and inflammatory cells (Werley et al., 2016) while other studies have shown PG/VG aerosols to produce no significant effects in rodents (Phillips et al., 2017). Such a contradiction is also evident in past human studies. In a randomised cross-over human clinical study, no significant difference was observed in the lung functions of normal and asthmatic volunteers who inhaled nicotine containing and nicotine and flavour free EC aerosols (i.e. only 70 % PG: 30 % VG E-vehicle) for 1 h (Boulay et al., 2017). However other studies have shown long-term inhalation of VG based EC can cause lipid pneumonia (McCauley et al., 2012) or inhalation of PG based theatrical fog can significantly influence the lung function (Varughese et al., 2005). Hence, there is a requirement for further investigation into the effects of PG/VG aerosols delivered via ECs using human relevant methods.

### 8.3 Flavouring additives need to be regulated with caution

One of the reasons for the global increase in EC usage is the multitude of different flavours available (ASH., 2017b). As per a 2014 study, there were 466 EC brands which cumulatively offered more than 7764 EC flavours at the time of the study (Zhu et al., 2014). The availability of a wide-variety of flavoured ECs is especially attractive amongst young populations, as never-smokers indulge in ECs in order to try different flavours (Bunnell et al., 2015). In the current study, the significant effect of flavourings was evident since different flavours exhibited different levels of cytotoxicity with fruit flavoured ECs especially strawberry and cherry flavours demonstrating the most cytotoxic effects (Figure 6.5). This result correlates with many previous studies that observed high cytotoxicity in strawberry and cherry flavoured ECs (Leigh et al., 2016, Kosmider et al., 2016, Clapp et al., 2017). In fact, Leigh et al (2016) reported an alarming finding where the cytotoxicity produced by strawberry flavoured ECs on bronchial epithelial cells were comparable to that produced by tobacco cigarettes. These studies, including the current study are timely research since fruit flavours are currently the most popular EC flavourings (out-competing tobacco flavour) according to recent surveys (ASH., 2017b, Farsalinos et al., 2013c).

The Flavour Extracts Manufacturers Association (FEMA) have listed 1037 flavouring additives identified as potential respiratory hazards due to their adverse irritant properties (FEMA., 2014). It is alarming to note that many common EC flavourings including diacetyl, acetoin, 2,3-pentanedione (buttery flavours), benzaldehyde (cherry or almond flavours), cinnamaldehyde (cinnamon flavour), butyraldehyde (chocolate flavour) are present in this list. In fact, FEMA has urged EC manufactures to not mislead EC users by suggesting that the flavourings added to EC liquids have a FEMA GRAS™ certification (FEMA., 2014). This is an important and timely announcement by FEMA since there have been many past incidents where inhalation of ingestion-safe flavouring agents have been shown to cause adverse respiratory conditions in humans such as acute onset bronchiolitis obliterans ('pop-corn lung' disease) caused by chronic inhalation of a food-safe flavouring compound namely diacetyl (Kreiss et al., 2002, van Rooy et al., 2007).

Aldehydes such as acetaldehyde, formaldehyde, acrolein, crotonaldehyde which are FDA identified airway toxicants have been shown to be present in high quantities in EC liquids (Hutzler et al., 2014, Han et al., 2016) as well as aerosols (Klager et al., 2017, Flora et al., 2016). Although the most likely route for the production of formaldehyde and acetaldehyde is the pyrolysis of PG and VG, one past study (Khlystov and Samburova, 2016) demonstrated that unflavoured ECs produced minimal amounts of aldehydes compared to flavoured ECs, thus suggesting that thermal decomposition of flavouring chemicals could be another possible route for the formation of aldehydes in EC aerosols. Benzaldehyde is a low-molecular weight aldehyde, commonly used to produce cherry-flavoured and other fruit flavoured EC liquids. Kosmider et al (2015) reported that the benzaldehyde levels emitted from 30 EC puffs were greater than that emitted from one conventional cigarette, with cherry flavoured ECs emitting the highest levels of benzaldehyde (Kosmider et al., 2016). This could be one possible explanation for the observed high cytotoxicity demonstrated by cherry flavoured EC extracts from Brand E in the current study (Figure 6.5). Further, there have been reports of contamination of unexpected flavouring compounds in EC liquids. Behar et al (2016) reported that cinnamaldehyde was found in non-cinnamon flavoured ECs, including tobacco and fruit flavoured ECs (Behar et al., 2016). Similarly, diacetyl which is used to produce 'buttery' or 'creamy' flavours, was detected in menthol flavoured ECs (Allen et al., 2016). These studies shed light on the lack of regulation of flavouring substances in EC liquid fabrication. Stringent regulatory measures are required to eliminate such discrepancies since inhalation of unexpected chemicals can possibly have severe health implications in the EC users.

It is not clear how the different EC flavouring substances mediate the observed cytotoxicity. One possible mechanism that has been suggested by past studies is that the different compounds produced by flavouring chemicals may induce oxidative stress which would trigger the Nf- $\kappa$ B pathway leading to the production of pro-inflammatory genes (Lerner et al., 2016, Rahman, 2003). This hypothesis fits well with the observation of current study wherein exposure of ALI co-culture models to strawberry flavoured EC aerosols for  $\geq 3$  h lead to a significant increase in oxidative stress and also elevated levels of IL-6/IL-8 pro-inflammatory mediators, ultimately leading to reduced cell viability (Figure 7.3 – 7.7). IL-8 specifically is a neutrophil chemo-attractant and activator which are known to play an important role in exacerbating many inflammatory conditions such as COPD (Mukaida, 2003), pulmonary fibrosis (Kinnula et al., 2005). Further, Gerloff et al (2017) reported on the pro-inflammatory effects of several ECs flavourings on a number of cell types including BEAS 2B, 16HBE and H292 bronchial epithelial cells and HFL-1 human lung fibroblasts (Gerloff et al., 2017). The influence on the various flavouring chemicals were found to be cell type dependant with BEAS 2B being the most sensitive cell type. This observation correlates well with the current study where BEAS 2B was found to be the most sensitive cell type in response to various flavoured EC extracts (Figure 6.6 and 6.7). The possible and reported detrimental effects of EC flavourings has led to scrutiny of the EC industry since the very point of introducing ECs was to deliver nicotine in a relatively safer way compared to tobacco cigarette as opposed to creating a possibility of introducing a spectrum of unexpected diseases in EC users.

#### 8.4 Significance of in-house built smoking machine in the current study

The different constituents of the vapour and particulate phases of WCS/ECV may contribute in different ways towards airways injury and hence it becomes important to deliver components of both phases over the cells in order to investigate their biological impact accurately. Hence most cigarette/EC studies employing ALI cell culture models mandate a smoking machine that can deliver WCS/ECV over the apical surface of the ALI cultures. Though several smoking machines are commercially available from different companies, many of these smoking machines are expensive, involve complex parts and cannot be streamlined for specific purposes of an individual study. Moreover, the majority of the commercial smoking machines are designed primarily for tobacco cigarette research and since EC working is distinctly different from tobacco cigarettes, many of these machines are either not compatible or require modifications to test ECs which results in additional costs. In this respect, the bespoke, automated smoking machine designed in the current study using commercially available electro-mechanical components provides an easy, convenient

and economical (refer sub-chapter 4.13.1) system for delivering smoke/EC aerosols to the co-culture human airways model in a controlled manner mimicking the human smoking behaviour as closely as possible. Very few past studies have employed bespoke smoking systems for evaluation of EC aerosols as the majority of the past EC studies have adapted commercially available cigarette smoking machines for their research which has been previously discussed in section 4.6.1 and 4.6.2.

To the authors' knowledge, only one study thus far has reported on a bespoke 'vaping' system chiefly intended for the EC evaluation. This bespoke set-up developed by Havel et al (2017) consisted of a vacuum pump (1 – 4 L/min capacity), a solenoid relay which was used to activate the EC and a three-way solenoid valve, similar to the current study which controlled the direction of flow of EC aerosols (Havel et al., 2017). Instead of an Arduino micro-controller which was used to control the switching of solenoid valves in the current study (refer sub-section 4.8.7.2), a puff-controller device was used to synchronise the solenoid relay device and the solenoid valve. During working, a 15 V pulse of 4 s was delivered by the puff-controller device to the solenoid valve which activated the EC and simultaneously a vacuum of 1.2 L/min (approximately similar to the current study) was applied. The EC aerosols were then directed by the solenoid valves into three gas dispersion bottles containing different chemicals to capture nicotine and aldehydes present in EC aerosols. Interestingly, this smoking set-up by Havel et al, was validated in a similar manner to that of the smoking machine of the current study using a flow sensor which produced a square-wave profile. This adds further credibility to the validation process of the current smoking machine and provides confidence in the functioning of the current smoking machine. Never-the-less, the smoking machine by Havel et al was used chiefly for chemical characterisation of EC aerosols and not for cell-exposure purpose. In this context, the current study is the first study to employ a novel, in-house designed, validated smoking machine to deliver WCS/ECV to ALI cultured cells in order to investigate their cytotoxicity. Apart from mechanical validation of the smoking machine working, using the in-house built smoking machine to treat the co-culture model to WCS, as emphasised earlier in sub-section 7.5.1, produced a significant cytotoxic effect. This result, comparable to that observed in past studies that performed similar delivery of WCS using commercial smoking machines to ALI airways model, provides further verification of the functionality of the smoking machine as a robust delivery system of air/WCS/ECV. Further improvements to the operational flexibility of the current smoking machine in order to accommodate all models of ECs (2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> generation) and also to deliver aerosolised therapies is currently under consideration which is further discussed in the Chapter 9, sub-section 9.1.

## 8.5 Choosing a physiologically relevant cell model and delivery method: vital criteria's of EC research

As explained earlier in the Chapter 2, sub-section 2.3.1, different types of airway epithelial cells exist in different regions of the respiratory tract. There currently exists no validated *in-vitro* models of human airways and hence depending upon the aims of each individual study, appropriate airway epithelial cells are employed. While some studies model the alveolar epithelium by culturing A549 epithelial cell line (Holownia et al., 2018, Zhang et al., 2017a), a vast majority of cigarette smoke/EC studies including the most recent ones have focused on the tracheobronchial region of the airways (Aufderheide and Emura, 2017, Aufderheide et al., 2003, BeruBe et al., 2009, Scheffler et al., 2015b). One possible advantage of using bronchial epithelial cells for EC studies is that most EC constituents including PG, VG and nicotine are highly water-soluble compounds and hence its deposition is expected to occur at the conducting zone of the airways which is the tracheobronchial zone (Zhang et al., 2012). Also, due to the presence of mucus lining in the tracheobronchial epithelium, the water-soluble compounds of the EC aerosols adhere predominantly in this region with only low-solubility compounds reaching the alveolar zone (Zhang et al., 2013). Thus, this study employed a range of bronchial epithelial cells (BEAS 2B, CALU 3, IB3-1, C38) to assess EC nicotine, flavour and brand variability effects and also employed ALI co-culture models using CALU 3 bronchial epithelial cells and HPF cells to assess the effects of EC aerosols.

Past studies have demonstrated ALI cultures of bronchial epithelial cells to produce ciliated columnar epithelial cells with robust tight junction formations and production of mucus producing goblet cells (Ehrhardt et al., 2002, Bitterle et al., 2006, Grainger et al., 2006) which correlates with the current study where the HPF-CALU 3 co-culture model has been shown to produce all the above stated physiologically relevant features of the human airways (Bielemeier, 2012). Although many bronchial epithelial cell types exist, CALU 3 bronchial epithelial cells were chosen for this study since they have been extensively characterised by many past studies and have been shown to exhibit permeability properties comparable to that of the *in-vivo* bronchial epithelial cells (Mathia et al., 2002, Grainger et al., 2006), thus adding more confidence in the cell model of the current study.

Employing different cell models can give rise to different results. For instance, in the current study, when submerged cultures of CALU 3 bronchial epithelial cells and HPF cells were treated to extracts of strawberry flavoured ECs, there was no significant decrease in cell viability compared to the control cells as seen from Figure 6.6 and Figure 6.7 (B)

respectively. The message interpreted from these results alone would be that strawberry flavoured ECs were not cytotoxic to bronchial epithelial cells or fibroblasts. However, when both these cells were co-cultured together at ALI and exposed to aerosols of strawberry flavoured ECs, the co-cultures produced significant reduction in cell viability, increased IL-6/IL-8 production and oxidative stress as seen from Figure 7.3 – Figure 7.7, thus producing an entirely different result when the culturing conditions and EC delivery methods were altered. Many past studies have observed similar disparity in results produced by using different cell culture models. In one such study, Lenz et al (2013) observed that the ALI cultures of A549 alveolar epithelial cells were significantly more sensitive to zinc oxide nanoparticles demonstrating increased cytokine profiles compared to submerged cultures of A549 cells (Lenz et al., 2013). This study agreed with the observed increased co-culture model sensitivity of the current study compared to submerged cultures. In another study by Bengalli et al (2017) however, a co-culture model consisting of A549 alveolar epithelial cells and lung endothelial cells was found to be more resilient to the effects of flavoured ECs compared to submerged monocultures of A549 cells, thus suggesting that co-culture models are less sensitive to cytotoxic effects of EC aerosols (Bengalli et al., 2017). This result contradicted the observations of the current study where co-culture models were more sensitive to EC effects than submerged cultures. This difference in results could possibly be due to difference in ECs used, cell types used and study design variability including EC puff regime and exposure method.

Differences in results can also arise between ALI monoculture and co-culture models. Iskandar et al (2015) reported that ALI monocultures of differentiated bronchial epithelial cells were more susceptible to cigarette smoke induced injury compared to a co-culture model consisting of bronchial epithelial cells and fibroblasts (Iskandar et al., 2015). These differences demonstrate the difficulty involved in developing validated *in-vitro* models of human airways. In the absence of such a validated *in-vitro* model of human airways for testing toxicity of inhalable agents such as ECV, it is important to choose physiologically relevant cell types, culturing method and aerosol delivery system, such as those employed in the current study, in order to decipher the precise effects of ECV on the human airways. While it may not be feasible to produce an *in-vitro* model that recapitulates the entire human airways from oral cavity to the lungs, the different regions of human airways can be effectively modelled on an *in-vitro* platform and data can be compared. For instance, the study by Bengalli et al (2017) would provide information on the potential effects of EC aerosols on the alveolar-blood barrier of the airways whereas the findings of the current study would provide information on the potential effects of EC aerosols on the tracheobronchial region of the airways. Hence, more studies employing multicellular ALI co-

cultures, modelling different regions of the airways are required for in-depth analysis of EC health effects.

## 8.6 An urgent requirement: standardised testing method to evaluate ECs

ECs are a relatively new phenomenon compared to tobacco cigarettes and conclusive data on many aspects of ECs including overall toxicological effects, efficacy of harm reduction, long-term safety are still lacking (Orr, 2014). One of the reasons for this is the lack of a standardised testing method to evaluate ECs (Hiemstra and Bals, 2016). Although several internationally recognised standard smoking regimes exist for tobacco cigarettes, such a standard regime is still absent for ECs. As a result, comparability between studies becomes difficult when different research groups employ ECs from different brands with different power settings and at different puff regimes. Reproducibility between studies is hindered if the authors of studies do not communicate the type, brand, flavour and power settings of ECs they employed in their study. On the other hand, one plausible reason for authors to not disclose the ECs employed in their studies is to avoid facing possible legal charges from the concerned EC companies.

A number of other reasons hinder the establishment of a standardised vaping regime. Firstly, ECs, unlike tobacco cigarettes are a class of products which come in a variety of type (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> generation), each with unique power settings, atomizer type and wicking materials which ultimately influences the vaping topography achieved. Gillman et al (2016) analysed the aerosols from five EC devices, each with different power settings and reported that the amount of aerosols produced per puff from different devices ranged between 1.5 – 28 mg/watt, a 17 fold difference and also exhibiting a 750 fold difference in the total aldehyde yield between different devices (Gillman et al., 2016). It can thus be gleaned that different atomizer designs and power settings of different ECs can significantly influence the quantity and quality of yields obtained from the different EC types. In this scenario, it can be increasingly difficult to establish a standard vaping regime for each of the individual EC types.

Secondly, the puffing topography of ECs are diverse varying with each individual users. Some users like to inhale low to moderate amount of aerosols while some other prefer to vape heavily in order to experience what is referred to as 'throat-hit'. Few studies that have evaluated EC puffing topography have reported highly variable results. Farsalinos et al (2013) reported that an average EC user inhales a  $4.2 \pm 0.7$  s puff every 20 – 30 s compared to tobacco cigarette users who took  $2.3 \pm 0.5$  s (Farsalinos et al., 2013d). Lee et al (2015) reported that the average puff duration of EC users was  $3.1 \pm 0.3$  s (Lee et al., 2015) while

Behar et al (2015) reported that the average EC puff time was 2.75 s every 17 s (Behar et al., 2015) and Hua et al (2014) reported an average EC puff duration of 4.3 s (Hua et al., 2013). The discrepancies amongst these studies is possibly due to the difference in EC devices, study designs and participant cohort differences. Although there seems to be an agreement amongst many EC studies that EC puffs are significantly longer and more frequent than tobacco cigarettes, a consensus on the EC puffing parameters has not been reached yet. In 2015, in an attempt to introduce a standard vaping regime for ECs, the CORESTA committee recommended an EC vaping regime involving a 55 s puff taken over 3 s puffed every 30 s. However, the CORESTA recommended regime has been employed only in a few chemical analysis EC studies till date (Gillman et al., 2016, Tayyarah and Long, 2014). To the authors' knowledge, no cell exposure EC study has employed CORESTA regime and many studies post 2015, continue to use ISO 3308/HCI or customised regimes for individual studies. Hence further studies are required to investigate the EC vaping topography that best simulates the vaping experience of an average EC user and ultimately reach a consensus on a standard vaping regime.

On similar lines, attempts to produce validated *in-vitro* models of the respiratory system is urgently required. The respiratory models used for evaluating ECs is highly variable amongst EC studies ranging from rodent models to *in-vitro* submerged cultures or *in-vitro* ALI cultures using animal cells, human primary cells or human derived cell lines. For robust evaluation of EC toxicity, there is a need to move away from animal models of the past and obtain more data from physiologically relevant *in-vitro* methods (Benam et al., 2016). It is anticipated that a standardised EC test method would also suggest one or more validated, physiologically relevant *in-vitro* human airways model such that data from different *in-vitro* models can be compared to the *in-vivo* data, thereby providing an opportunity to choose the *in-vitro* model that best resembles the *in-vivo* data. As an example, such validated *in-vitro* models of skin have been approved in the past by regulatory bodies such as European Centre for the Validation of Alternative Methods (ECVAM) which are now being widely used instead of animal tests to evaluate several topical irritants (ECVAM, 2009). In a similar manner, development of physiologically relevant validated *in-vitro* models of human airways would eventually accelerate EC research by producing more human relevant data, thus informing the public in a faster and more efficient way on the potential health effects of ECs. The co-culture model used in the current study is a step forward in this direction although more work needs to be performed before it could be submitted to regulatory bodies for validation. It is also anticipated that such a standardised EC test method would provide a list of different standardised biochemical assays to evaluate the different relevant end-points. The current study assessed four end-points namely cell viability via CTB, pro-

inflammatory mediator production via ELISA, oxidative stress and caspase 3/7 release via luminescence assays, all four of which are widely-employed standard assays which enables easy repeatability and enhances comparability between studies.

## 9 Overall conclusions of the current study

The current study aimed to evaluate EC potential effects using different physiologically relevant *in-vitro* methodologies including novel, in-house designed cigarette/EC delivery systems and *in-vitro* human airway models ranging from submerged monocultures to ALI co-cultures consisting of critical cells of the airways such as bronchial epithelial cells and pulmonary fibroblasts. Two chief engineering/design objectives were accomplished in the current study. Firstly, a bespoke 'bubbling' system was designed in-house in order to collect extracts of cigarette, EC and E-vehicle. Secondly, a bespoke, automated and economical in-house designed smoking machine was constructed, validated and employed to deliver WCS/ECV to the co-culture human airways models at ISO 3308 smoking regime. Additionally, pertaining to the EC experiments, a number of conclusions can be drawn from the current study.

Nicotine and cotinine do not produce any significant cytotoxic or inflammatory effects under the tested experimental conditions. Further, the varying nicotine content of ECs has minimal impact on the cytotoxicity mediated by the extracts of different ECs. This demonstrates that nicotine per se may not be chiefly responsible for any cytotoxic or inflammatory effects of ECs observed in the current study.

The effect of E-vehicle carrier depends on the delivery method employed since E-vehicle fluid caused a significant decrease in the cell viability of bronchial epithelial cells whereas E-vehicle extracts did not produce any significant influence on the bronchial epithelial cell viability. Many past studies have reported on the detrimental impacts of E-vehicle and hence further research on the potential effects of aerosols of E-vehicle is required.

EC flavourings play a significant role in the cytotoxicity demonstrated by the extracts of a variety of ECs, with fruit flavoured ECs such as strawberry and cherry flavours demonstrating highest cytotoxicity. Further, same-flavoured ECs across different brands varied in their cytotoxicity. In this context, EC flavourings needs to be effectively screened and appropriately regulated in order to inform the public the exact constituents of the ECs.

Aerosols of strawberry flavoured EC caused a significant decrease in the HPF-CALU 3 co-culture model cell viability, elevated IL-6 and IL-8 pro-inflammatory mediators' production and also caused increased oxidative stress post  $\geq$  3h exposure time. Interestingly, the observed reduction in cell viability was caspase 3/7 independent, thus suggesting execution of alternative cell death pathways and hence this requires further investigation. Overall, this

result clearly demonstrates the potential of ECs to produce significant physiological effects during prolonged vaping.

Lastly, there is a requirement for a standardised testing method to evaluate EC cytotoxicity *in-vitro* in order to render consistencies and repeatability amongst EC studies. The current study emphasises the importance of using physiologically relevant *in-vitro* models, both simple submerged cultures as well as complex ALI co-cultures to screen the cytotoxicity of a wide-variety of ECs efficiently and produce human relevant data. The employment of such physiologically relevant models could greatly accelerate EC safety assessment, ultimately helping the public to make a more informed decision about EC usage.

### 9.1 Future works

In the current study, when co-culture models were exposed to ECV, although a decrease in cell viability was observed post  $\geq 3$ h exposure time, there was no significant expression of caspase 3 and 7 apoptotic mediators', thus mandating further investigation into cell-death mechanisms. One of the important future works would be to investigate the cell-surface receptors via different dyes (such as propidium iodide) to differentiate between early apoptotic, late apoptotic and necrotic cells. A further in-depth analysis would involve analysing the expression levels of specific apoptotic genes such as Bcl-2, Bax via PCR.

A preliminary test investigating the cytotoxicity of cherry ECV at the longest exposure time only (6 h, double block exposure) demonstrated a significant decrease in cell viability compared to the control cells although cherry flavour was not as cytotoxic as strawberry at this exposure time (Refer Appendix 11.4, Figure 11.2). Hence, apart from the strawberry flavour ECs of Brand E that were analysed in detail in the current study, other EC flavours that demonstrated high cytotoxicity in extract form such as cherry and tobacco flavours need to be further investigated in their aerosol form using the HPF-CALU 3 co-culture models at the different exposure times. Further, chemical evaluation of EC and E-vehicle aerosols is required in order to investigate if, as reported by many past studies, the EC liquids and aerosols contain harmful aldehydes and metal particles. This would require analysis of EC liquids and aerosols via chromatographic and spectroscopy methods such as HPLC or Inductively-coupled Plasma Mass Spectrometry (ICP-MS).

With respect to the smoking machine, one of the limitations of the current smoking system was that its design permits only 1<sup>st</sup> generation ECs. Hence, further work focused on expanding the flexibility of the system to accommodate all ECs type including the tank systems needs to be performed. Additionally, with respect to experimental design of the

current study, the EC aerosols were tested at ISO 3308 standards. It would be interesting to investigate the effects produced by ECs at HCl regime or CORESTA recommended regime such that they could be compared with the results of the current study. Lastly, it is intended to convert the current smoking machine into a breathing machine which could be used not only to deliver cigarette smoke/EC aerosols but also for testing aerosolised drugs. This conversion would potentially involve changes in the design of many aspects of the current smoking machine depending upon the nature of the drug aerosol to be delivered.

One of the overall aims of the current project is to significantly reduce and possibly replace the use of animal models in inhalation toxicity studies with that of the *in-vitro* co-culture model of the current study. As a part of this aim, current research is focused on improving the physiological relevance of the co-culture model in a number of different ways such that its resemblance to the *in-vivo* airways environment is further enhanced. Firstly, an automated perfusion system could be introduced which would pump growth medium into the co-culture system continuously, thus providing the cells with nutrients as well as eliminating cellular waste product continuously. Such a perfusion system would also increase the physiological relevance of the model by providing cells with the optimum shear stress equivalent to that experienced by cells in the *in-vivo* airways micro-environment. Secondly, an automated sampling system could be implemented in the current system wherein samples of cell culture supernatants could be withdrawn instantaneously for evaluation of different biomarkers. Thirdly, recent advances in microfluidics and microfabrication technologies have given rise to a novel branch of *in-vitro* modelling called 'organ-on-a-chip' technologies. These chips consist of co-culture models cultured in microfluidic chambers which are expanded and relaxed using computer-controlled vacuum system. Such an alternating expansion and relaxation action provided by the chip mimics the mechanical breathing action of the *in-vivo* human lungs, thus increasing the physiological, mechanical as well as the biochemical properties of the lung. Such a 'lung-on-a-chip' model along with the perfusion system and automated sampling system would provide a robust *in-vitro* platform which best represents the *in-vivo* human airways conditions and hence could prove to be a suitable alternative to animal models.

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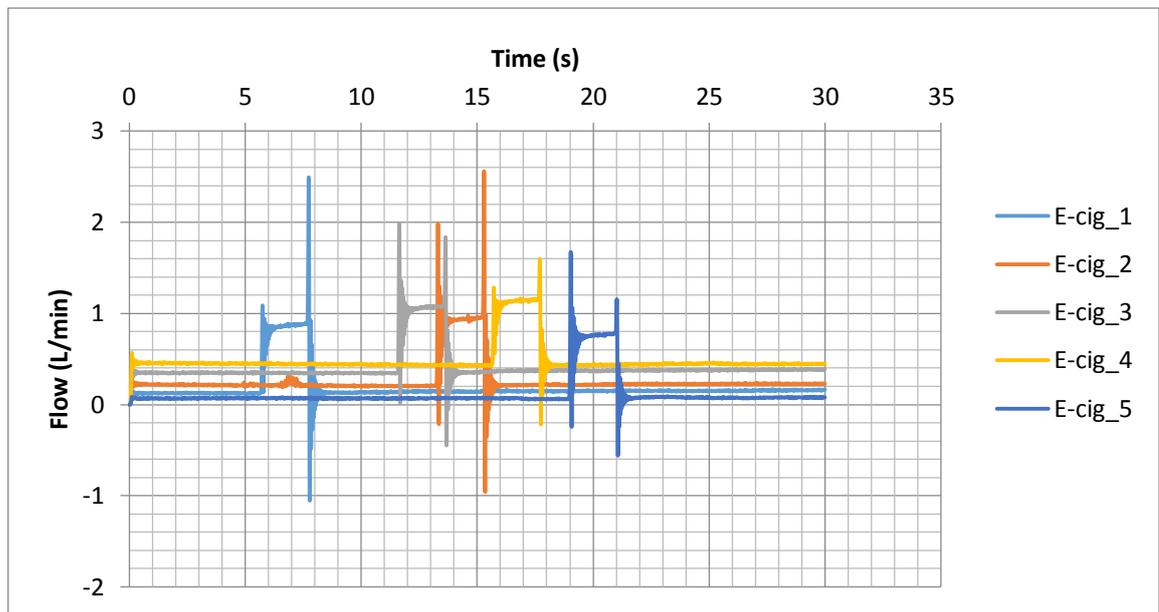
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## 11 Appendices

### 11.1 ECV flow rate measurement using EFS resulting in damage of EFS



**Figure 11.1.** X-Y scatter graph depicting the ECV flow rates measured using EFS from 5 individual validation tests.

Passage of condensing ECV through the EFS resulted in damage to the sensor. As it can be observed from the above graph, the expected base-line air-flow rates (0.150 L/min) during the 28 s cycle varied highly with some repeats even recording > 0.500 L/min. There were also substantial discrepancies in the expected ECV peak flow rate (1.050 L/min) in each test. Measurement of the same ECV flow rates using analogue flow meters demonstrated the expected flow-rates as previously described in Table 4.7.

## 11.2 XTT cell viability data set of all the 15 ECs tested in Chapter 6

**Table 11.1. Viability data as compared to control for the four main cell lines following exposure to varying concentrations (%) of ECE.**

Mean values are shown with standard deviations in brackets. Shaded boxes show cytotoxicity, i.e. viability which fell below 70% of control.

Sample (type)	Cell line	C38					BEAS-2B					IB3-1					J774					
		12.5	25	50	100	12.5	25	50	100	12.5	25	50	100	12.5	25	50	100	12.5	25	50	100	
A (EC)	Nicotine Content	95.5 (5.9)	91.0 (5.4)	90.6 (4.7)	88.5 (4.6)	89.6 (7.5)	85.7 (7.6)	85.4 (7.6)	84.8 (7.5)	89.7 (4.2)	89.6 (3.9)	88.5 (4.1)	79.2 (3.7)	99.7 (4.5)	99.4 (5.9)	98.9 (2.7)	99.4 (4.4)	99.7 (4.5)	99.4 (5.9)	98.9 (2.7)	99.4 (4.4)	98.9 (3.5)
	2.4 % / mL																					
Dark Cherry	12.5 mg	98.6 (5.4)	93.9 (5.4)	89.2 (3.7)	89.5 (4.4)	94.3 (7.3)	92.1 (7.6)	86.1 (7.2)	81.5 (7.6)	90.6 (7.1)	90.9 (7.0)	92.3 (6.5)	83.6 (6.3)	97.6 (3.6)	97.2 (4.7)	96.9 (2.9)	97.6 (3.6)	97.6 (4.7)	97.2 (4.7)	96.9 (2.9)	97.2 (2.9)	
	3.5 % v/v																					
B (EC)	12.5 mg	80.9 (5.0)	80.6 (8.7)	79.8 (8.6)	77.3 (7.3)	91.8 (9.3)	92.2 (9.8)	88.1 (7.6)	88.1 (9.6)	88.6 (4.3)	88.4 (4.1)	78.4 (4.0)	58.4 (3.9)	102.0 (4.4)	90.6 (4.4)	88.5 (5.6)	102.0 (4.4)	90.6 (4.4)	90.6 (4.4)	88.5 (5.6)	88.5 (5.6)	
	3.5 % v/v																					
Crisp Mint	12.5 mg	95.5 (3.6)	94.6 (5.7)	93.1 (3.7)	93.1 (4.7)	92.3 (4.2)	90.8 (4.1)	89.9 (4.6)	82.3 (2.7)	88.3 (4.0)	87.4 (4.2)	89.3 (3.7)	79.1 (4.4)	89.6 (5.1)	91.2 (5.8)	90.1 (4.8)	89.6 (4.2)	89.6 (5.1)	91.2 (5.8)	90.1 (4.8)	90.1 (4.8)	
	3.5 % v/v																					
Tobacco	1.8 %	90.3 (5.2)	92.3 (6.7)	83.4 (6.9)	72.9 (6.4)	92.1 (5.5)	85.1 (5.8)	54.8 (8.9)	22.4 (3.7)	88.5 (4.8)	89.1 (4.1)	89.7 (3.4)	78.0 (4.2)	90.0 (3.8)	89.9 (4.0)	89.4 (3.8)	90.0 (3.8)	90.0 (3.8)	89.9 (4.0)	89.4 (3.8)	89.4 (3.8)	
	1.8 %																					
Menthol	1.8 %	89.4 (8.9)	89.8 (6.9)	87.9 (7.5)	83.2 (5.4)	89.6 (5.7)	89.3 (5.1)	87.7 (6.2)	83.9 (5.5)	89.5 (3.8)	87.1 (9.9)	88.2 (2.6)	89.5 (3.0)	91.4 (4.4)	90.2 (4.2)	88.7 (3.9)	88.2 (4.4)	90.2 (4.2)	90.2 (4.2)	88.7 (3.9)	89.1 (5.3)	
	1.8 %																					
Tobacco	6 mg	88.8 (5.2)	84.6 (5.3)	84.9 (4.4)	79.9 (4.1)	85.6 (5.9)	76.4 (5.7)	75.6 (6.9)	73.1 (4.3)	87.0 (3.8)	86.1 (2.9)	86.1 (4.1)	76.4 (3.8)	89.4 (3.1)	89.1 (4.4)	89.4 (3.9)	86.1 (3.8)	89.4 (3.1)	89.1 (4.4)	89.4 (3.9)	89.4 (3.9)	
	0.6 %																					
Tobacco	12 mg	94.1 (5.1)	90.9 (5.3)	88.9 (4.6)	84.2 (6.8)	99.4 (7.4)	96.2 (7.7)	93.1 (7.3)	84.7 (7.3)	98.4 (4.7)	99.3 (3.9)	99.9 (3.4)	88.8 (3.0)	99.7 (2.8)	98.3 (5.3)	98.2 (2.5)	98.4 (3.0)	99.7 (2.8)	98.3 (5.3)	98.2 (2.5)	98.2 (2.5)	
	1.2 %																					
Tobacco	18 mg	93.3 (5.6)	86.4 (5.7)	85.2 (5.1)	84.6 (5.1)	95.7 (2.9)	90.3 (4.2)	89.3 (3.7)	88.6 (4.2)	89.7 (3.7)	89.2 (3.8)	91.1 (3.3)	77.9 (3.0)	89.5 (2.9)	89.8 (4.4)	91.1 (6.3)	89.5 (2.9)	89.5 (2.9)	89.8 (4.4)	91.1 (6.3)	91.1 (6.3)	
	1.8 %																					
Apple	16 mg	87.4 (4.5)	81.9 (4.6)	78.2 (4.6)	74.4 (5.6)	95.5 (7.4)	92.4 (5.6)	90.2 (8.6)	83.8 (7.6)	86.0 (3.5)	84.7 (2.9)	79.5 (3.0)	75.8 (3.0)	87.6 (3.6)	85.1 (2.8)	76.6 (2.8)	86.0 (3.5)	87.6 (3.6)	85.1 (2.8)	76.6 (2.8)	76.6 (2.8)	
	1.6 % / mL																					
Coffee	16 mg	89.3 (5.4)	87.9 (4.8)	87.3 (4.0)	86.7 (4.1)	92.0 (5.1)	91.5 (5.5)	91.9 (3.9)	87.1 (3.7)	90.1 (4.5)	89.9 (3.9)	89.4 (4.1)	79.8 (3.1)	93.1 (4.3)	90.8 (3.8)	79.9 (3.8)	89.9 (3.9)	89.4 (4.1)	90.8 (3.8)	90.9 (3.8)	90.9 (3.8)	
	1.6 % / mL																					
Vanilla	16 mg	87.9 (6.9)	82.8 (6.1)	82.2 (4.4)	79.4 (5.1)	93.2 (3.9)	89.3 (3.8)	88.4 (4.1)	85.6 (5.4)	90.8 (3.6)	90.5 (4.1)	89.8 (4.4)	78.8 (3.4)	90.5 (4.9)	88.5 (6.1)	79.6 (3.1)	89.8 (4.1)	89.8 (4.1)	89.0 (3.9)	88.5 (6.1)	88.5 (6.1)	
	1.6 % / mL																					
Cherry	16 mg	71.2 (2.8)	66.5 (3.5)	65.1 (4.6)	64.9 (3.6)	74.0 (4.7)	60.6 (4.1)	57.6 (5.5)	53.7 (4.2)	88.4 (4.2)	87.4 (3.5)	88.0 (4.4)	88.7 (4.3)	90.0 (4.0)	89.3 (6.1)	89.8 (3.5)	88.4 (4.2)	87.4 (3.5)	89.3 (6.1)	89.1 (3.5)	89.8 (3.5)	
	1.6 % / mL																					
Strawberry	16 mg	67.9 (4.9)	63.3 (4.2)	49.2 (3.8)	47.5 (4.7)	56.2 (3.8)	46.3 (3.7)	45.1 (4.3)	43.6 (3.6)	90.6 (3.9)	87.5 (3.3)	78.9 (4.0)	66.9 (2.8)	90.7 (4.4)	88.3 (4.2)	68.9 (4.0)	87.5 (3.3)	87.5 (3.3)	88.3 (4.2)	79.3 (4.2)	68.9 (4.0)	
	1.6 % / mL																					
Tobacco	16 mg	89.1 (2.8)	87.3 (2.8)	82.7 (2.1)	81.7 (3.2)	61.3 (3.1)	56.5 (3.7)	52.5 (3.8)	48.8 (5.3)	89.4 (4.0)	89.4 (3.9)	89.5 (4.3)	89.2 (3.5)	87.1 (4.3)	87.8 (4.2)	77.5 (4.5)	89.4 (4.0)	89.4 (3.9)	87.8 (4.2)	88.3 (4.2)	77.5 (4.5)	
	1.6 % / mL																					

### 11.3 Two-way ANOVA comparison between the different EC flavours

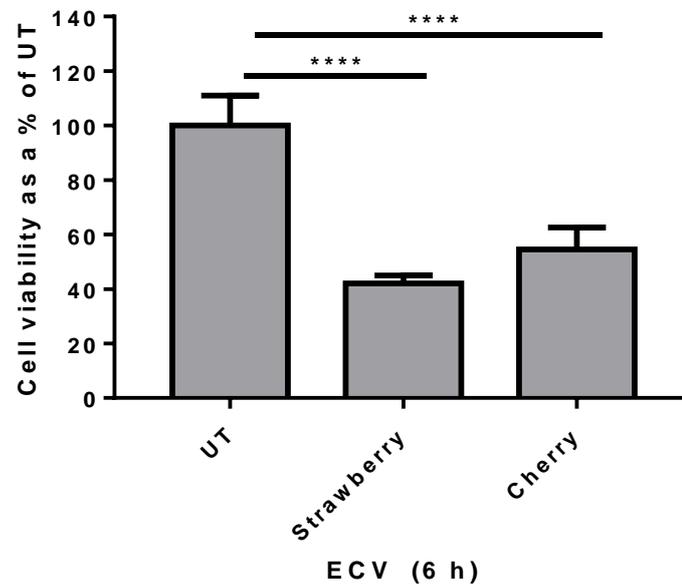
Two-way ANOVA was performed in order to analyse the difference between the effects of different flavours. Since strawberry was found to demonstrate the highest cytotoxicity, the effect of other flavours were compared to that of strawberry across all concentrations. In the BEAS 2B cell line, the effect of strawberry flavour was significantly different from the effect of apple, cherry and tobacco at all concentrations. In IB3-1, C38 and J774, at 100 % ECE concentration, the effect of strawberry flavour was statistically significantly different compared to other flavours. At other ECE concentrations, this statistic varied. The results of this statistical analysis can be seen in Table 11.2.

**Table 11.2. Statistical analysis of EC flavourings in the four main cell lines employed.**

All values relate to the Two-way ANOVA p-value result of each flavour compared to the strawberry viability data following a Dunnet's post hoc test.

Cell line	Comparison flavour	ECE concentration (%)			
		12.5	25	50	100
IB3-1	Apple	0.0001	0.01	ns	0.0001
	Cherry	0.05	ns	0.0001	0.0001
	Tobacco	ns	ns	0.0001	0.0001
C38	Apple	0.0001	0.0001	0.0001	0.0001
	Cherry	ns	ns	0.0001	0.0001
	Tobacco	0.0001	0.0001	0.0001	0.0001
BEAS-2B	Apple	0.0001	0.0001	0.0001	0.0001
	Cherry	0.0001	0.0001	0.0001	0.0001
	Tobacco	0.01	0.0001	0.0001	0.01
J774	Apple	0.01	ns	0.0001	0.0001
	Cherry	ns	ns	0.0001	0.0001
	Tobacco	0.001	ns	0.0001	0.0001

## 11.4 Preliminary investigation of cherry flavour ECV



**Figure 11.2.** Effect of cherry flavour ECV exposure on the co-culture model cell viability.

In chapter 6 experiments, besides strawberry flavour, extracts of cherry flavoured ECs from Brand E also demonstrated significant cytotoxicity (Figure 6.5) and hence this flavour was further analysed using the HPF-CALU 3 co-culture models in their aerosol form although only at one exposure time (6 h, double block). As shown in Figure 11.2, cherry ECV caused a significant decrease in cell viability compared to the UT although not as much as that caused by strawberry flavour ECV.

## 11.5 Journal publications

1. **Pranav Vasanthi Bathrinarayanan.**, James EP Brown, Lindsay J Marshall, Laura J Leslie. “An investigation into E-cigarette cytotoxicity *in-vitro* using a novel 3D differentiated co-culture model of human airways”. 2018. *Toxicology In-vitro*, 52, 255-264.
2. Laura J Leslie., **Pranav Vasanthi Bathrinarayanan.**, Pamela Jackson., Justin A Mabilia Ma Muanda., Ross Pallett., Christopher JP Stillman., Lindsay J Marshall. “A comparative study of electronic cigarette vapour extracts on airway-related cell lines *in vitro*”. 2017. *Inhalation Toxicology*, 29 (3), 126-136.

### **Published conference abstract:**

1. **Pranav Vasanthi Bathrinarayanan.**, James EP Brown, Lindsay J Marshall, Laura J Leslie. “An investigation into the effects of E-cigarette aerosols using a physiologically relevant *in-vitro* model”. *Toxicology Letters*, 280 (Supple), S266 (2017). DOI: <https://doi.org/10.1016/j.toxlet.2017.07.941>

## 11.6 Conference proceedings

1. Presented a poster titled “An investigation into the effects of E-cigarette aerosols using a physiologically relevant *in-vitro* model” at the 53rd Congress of European Societies of Toxicology (EUROTOX 2017), Bratislava, Slovakia; 09/2017
2. Presented a poster titled “Towards an *in-vitro* multi-cellular human airways model to study E-cigarette effects” at Dr Hadwen Trust Animal Replacement Science conference, Charles Darwin House, London; 12/2016
3. Delivered an oral talk titled “A comparative study of two *in-vitro* methodologies used to assess E-cigarette cytotoxicity” at *In-vitro* Toxicology Society conference, Glasgow; 11/2016
4. Presented a poster titled “Evaluating E-cigarette cytotoxicity *in-vitro*” at *In-vitro* Toxicology Society conference, Birmingham; 11/2015
5. Presented a poster titled “Using an *in-vitro* human airways model to study the toxic effects of components of E-cigarettes” at Dr Hadwen Trust Animal Replacement Science conference, Charles Darwin House, London; 11/2014.