

Toxicological assessment of E-cigarette flavored E-liquids aerosols using Calu-3 cells: A 3D lung model approach

Felix Effah^{a,b,*}, John Adragna^c, David Luglio^c, Alexis Bailey^a, Tim Marczylo^b, Terry Gordon^c

^a Pharmacology Section, St George's University of London, Cranmer Terrace, SW17 0RE London, UK

^b UK Health Security Agency, Radiation, Chemical and Environmental Hazards, Chilton, Didcot, Oxfordshire OX11 0RQ, UK

^c Division of Environmental Medicine, New York University Langone Health, New York, NY, USA

ARTICLE INFO

Keywords:

E-cigarettes
In vitro lung models
Flavors
E-liquids
Calu-3

ABSTRACT

Scientific progress and ethical considerations are increasingly shifting the toxicological focus from in vivo animal models to in vitro studies utilizing physiologically relevant cell cultures. Consequently, we evaluated and validated a three-dimensional (3D) model of the human lung using Calu-3 cells cultured at an air-liquid interface (ALI) for 28 days. Assessment of seven essential genes of differentiation and transepithelial electrical resistance (TEER) measurements, in conjunction with mucin (MUC5AC) staining, validated the model. We observed a time-dependent increase in TEER, genetic markers of mucus-producing cells (*muc5ac*, *muc5b*), basal cells (*trp63*), ciliated cells (*foxj1*), and tight junctions (*tjp1*). A decrease in basal cell marker *krt5* levels was observed. Subsequently, we utilized this validated ALI-cultured Calu-3 model to investigate the adversity of the aerosols generated from three flavored electronic cigarette (EC) e-liquids: cinnamon, vanilla tobacco, and hazelnut. These aerosols were compared against traditional cigarette smoke (3R4F) to assess their relative toxicity. The aerosols generated from PG/VG vehicle control, hazelnut and cinnamon e-liquids, but not vanilla tobacco, significantly decreased TEER and increased lactate dehydrogenase (LDH) release compared to the incubator and air-only controls. Compared to 3R4F, there were no significant differences in TEER or LDH with the tested flavored EC aerosols other than vanilla tobacco. This starkly contrasted our expectations, given the common perception of e-liquids as a safer alternative to cigarettes. Our study suggests that these results depend on flavor type. Therefore, we strongly advocate for further research, increased user awareness regarding flavors in ECs, and rigorous regulatory scrutiny to protect public health.

1. Introduction

Tobacco smoking is the leading cause of preventable deaths worldwide, according to a 2023 World Health Organization report (WHO, 2023). Cigarette smoke contains around 7000 chemicals, as noted by the National Center for Chronic Disease Prevention and Health Promotion US Office on Smoking and Health (2014), and 93 of these substances, including nicotine, have been identified as potentially harmful (FDA, 2012). The addictive nature of nicotine results in tobacco dependency, and nicotine replacement therapies (NRTs) have been employed to aid in smoking cessation. Traditional NRTs including patches and gum can take a while to reach their effective concentration in the bloodstream, limiting their popularity and effectiveness. Electronic cigarettes (ECs) or electronic nicotine delivery systems (ENDS) offer the most successful smoking cessation aid in the UK (ASH, 2022b). These battery-operated

devices vaporize a solution containing nicotine, providing a faster and more efficient delivery system to the brain, akin to traditional cigarettes. The e-liquids used in EC principally contain propylene glycol (PG) and/or vegetable glycerin (VG), typically with nicotine and flavoring agents. Besides delivering nicotine, ECs provide sensory stimulation and behavioral cues similar to cigarette smoking, which can help alleviate withdrawal symptoms, reduce cravings, and decrease the likelihood of a relapse, according to a study by Wadgave and Nagesh, (2016).

While ECs are generally seen as less harmful than traditional cigarettes (McNeill et al., 2022, 2018), it is unclear how much harm EC use causes compared to non-use, especially among long-term former or never smokers. There are also concerns about EC use among young people, and the potential for progression to traditional tobacco smoking. These concerns have led to regulatory restrictions in several countries, including parts of the US, Australia, Ukraine, and Finland. Notably,

* Correspondence to: UK Health Security Agency, Radiation, Chemical and Environmental Hazards, Chilton, Didcot, Oxfordshire OX11 0RQ, UK.
E-mail address: Felix.Effah@UKHSA.gov.uk (F. Effah).

<https://doi.org/10.1016/j.tox.2023.153683>

Received 20 October 2023; Received in revised form 19 November 2023; Accepted 22 November 2023

Available online 25 November 2023

0300-483X/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

there was a 3% increase in EC use among 11–17-year-olds in the UK from 4% to 7% between 2020 and 2022 (ASH, 2022a). The flavors in ECs play a crucial role in attracting both young people, and current and former smokers to EC use. A complete ban on flavors is therefore undesirable from a harm reduction perspective (McNeill et al., 2022).

While the flavoring compounds in e-liquids are generally regarded safe for consumption and are widely used in the food industry, there are concerns about their potential toxicity when inhaled through EC use (Farsalinos et al., 2013; Khlystov and Samburova, 2016; Leigh et al., 2016; Sundar et al., 2016). Safety evidence concerning inhalation is growing but not extensive. Several studies have reported potential health risks from exposure to flavored e-liquids, such as inhibited cell growth, and increased cytotoxicity, apoptosis, oxidative stress, and mitochondrial dysfunction (Effah et al., 2022, 2023). Nonetheless, we have previously reported that these studies frequently: 1) lack appropriate controls that enable identification of hazards due specifically to flavor components, 2) have ambiguity over physiologically relevant doses of flavor/e-liquid used, and 3) have no demonstration of a dose-dependent relationship (for an extensive review see (Effah et al., 2022) and references therein). In a recent study, we adopted a high-throughput experimental approach to screening cytotoxicity in a wide range of flavored, nicotine-free e-liquids ($n = 53$) (Effah et al., 2023). In that study, we identified that out of 53 nicotine-free e-liquids, only 3 (i.e. cinnamon, hazelnut and vanilla tobacco) revealed dose-dependent toxicity independent of the PG/VG component in human bronchial epithelial cells (HBEC-3KT) in submerged cultures. Although submerged cell cultures are widely used in toxicity studies, they have several limitations. In this configuration, cells are covered entirely by e-liquids diluted in culture medium, offering a non-physiological environment for lung cells that, under normal conditions, the lung lining fluid is exposed to air on the apical side. This alteration can affect cellular behavior, morphological characteristics, and responses to e-liquids, rendering the model less predictive for in vivo situations (Barrila et al., 2010). Also, heating e-liquids during vaping may release additional toxicants such as volatile organic compounds, breakdown products, and condensation products (Effah et al., 2022) that could increase toxicity. Here, we used Calu-3 cells grown at the air-liquid interface (ALI) to assess the toxicological effects of the three flavors we identified in the previous high throughput submerged culture study (Effah et al., 2023). Although several lung cell culture models are available, none are considered perfect models for studying respiratory tract effects. Nonetheless, in vitro ALI culture models hold more potential in an inhalation toxicity assessment (Leibrock et al., 2019; Singh et al., 2021a). The Calu-3 cell line, derived from a human bronchial adenocarcinoma submucosal glands was chosen for this study because we aimed to understand the impact of flavors in e-liquid on bronchial membrane integrity and how the presence of mucus impacts flavor toxicity both in their e-liquid and aerosols forms. Calu-3 cells are akin to respiratory epithelia, they form tight junctions and secrete mucus making it a widely used in vitro 3D model in academia and industry with a robust differentiation protocol (Silva et al., 2023) for examining the pharmaco-toxicology of inhaled drugs (Ong et al., 2013). This cell line has been extensively employed to study the transport and metabolism of numerous therapeutic compounds, especially those for cystic fibrosis, due to its relatively high expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Haws et al., 1994) and environmental inhalation toxicology studies (Foster et al., 2000). A key advantage of using Calu-3 cells, as demonstrated in this study, is their ability to replicate the biological behavior of the human respiratory tract upon exposure to airborne chemicals. Thus, Calu-3 cells are apt for screening respiratory irritation and toxicity (Jeong et al., 2019; Ji et al., 2016; Pasman et al., 2021; Sakagami, 2020) because they can form tight junctions with robust barrier integrity, essential for creating a relevant lung model. Although several studies have validated Calu-3 cells grown at ALI for 21 days as an appropriate 3D lung model by measuring the transepithelial electrical resistance (TEER), the

expression of tight junction proteins, and mucus production as markers for differentiation, no studies investigated them beyond this time-frame or investigated the time course of changes that occur within the genes that encode these markers over the weeks the cells are grown at ALI. Therefore, this study had two main aims. The first aim was to evaluate Calu-3 cells beyond the typical 21 days at ALI by 1) measuring TEER using an evometer, 2) measuring the expression of genetic markers of differentiation for goblet cell (*muc5ac* and *muc5b*), the expression of ciliated cell markers (*foxj1*), and tight junction (*tjp1*), and basal markers (*trp63*, *krt5* and *itga6*) (Hewitt and Lloyd, 2021; Rock et al., 2009) using RT-qPCR and how these change over 28 days at ALI, and 3) by measuring immunofluorescence for mucin (MUC5AC) production with digital confocal microscopy after 21 days of ALI. The second aim was to assess the potential adverse effects of the three flavored e-liquids (cinnamon, hazelnut, and vanilla tobacco) previously found to induce toxicity in submerged HBEC-3KTs (Effah et al., 2023) on the ALI-validated Calu-3 cell model by assessing their effects on 1) reactive oxygen species (ROS) in submerged exposures, and 2) cytotoxicity (LDH assay) and TEER measurements following both suspension exposures and aerosol exposures. Although the manufacturer's protocol for ROS levels has been optimized for submerged exposures only, we unsuccessfully attempted to adapt the protocol for Calu-3 cells grown at ALI. Hence, ROS levels were characterized in submerged cultures only. In contrast, we strived to characterize the cytotoxic effects of flavored-e-liquid aerosols compared to 3R4F reference tobacco smoke. The selection of the 3R4F reference cigarette is grounded in its established use as a standard in toxicological studies of tobacco products. 3R4F refers to a research cigarette produced by the University of Kentucky's Tobacco Research & Development Center. It is designed to represent the average properties of the U.S. domestic cigarette, making it a suitable benchmark for comparative studies.

2. Methods

2.1. Cell culture

The human bronchial epithelial cell line Calu-3 (ATCC) was cultured in advanced Dulbecco's modified eagle medium (DMEM) with 10% foetal bovine serum (FBS, GIBCO), 1% non-essential amino acids (NEAA, GIBCO), 1% antibiotic-antimycotic (GIBCO) and 10 mM Hepes. Cells were cultured at 37 °C with 5% CO₂. 0.25% Trypsin EDTA (GIBCO) was added during passaging, and cells incubated at 37 °C with 5% CO₂ for 3–5 min. The agitation of the flask detached the cells, which could be observed using the light microscope. Phosphate buffered saline (PBS) (GIBCO) containing 5% FBS (GIBCO) was added to neutralise the trypsin EDTA before the cell suspension was centrifuged at 1000 rpm for 5 min. After discarding the supernatant, the cell pellet was resuspended in the appropriate growth medium. A small sample was added to trypan blue (Sigma-Aldrich) in a 1:1 ratio and placed into a haemocytometer for a viable cell count. Cells were seeded at 2×10^6 per T175 flask (ThermoFischer Scientific, US) for the next passage.

2.1.1. Calu-3 ALI culture

6 well PET transwells plates were used for ALI exposures (ThermoFischer Scientific, US). Calu-3 cells were seeded at passage 3 into PET transwells with 0.4 μm pores and 24 mm thickness (Corning) at 5×10^5 cells/well. The cell medium used to culture all cells during expansion was as previously described (Section 2.1). Confluence was observed using a light microscope after 48 h. All medium was removed from cells once confluence was reached. Once at an ALI, the growth medium in the basal compartment was replaced every 2–3 days whilst the transwells were incubated at 37 °C with 5% CO₂.

2.2. Characterization of Calu-3 cells as physiologically relevant 3D model

2.2.1. RNA extraction

The genetic expression of differentiation markers was assessed to validate this 3D model of the microenvironment of the human lung. Every 7 days for 28 days, 700 μ l QIAzol lysis reagent was added to Calu-3 cells in a transwell chamber for 3 min, the lysate was collected and homogenized using a 5 mL syringe, and 25 gauge needle by aspiration, repeated 10 times. The homogenate was incubated at room temperature for 5 min to promote cell dissociation of nucleoprotein complexes. Then, 140 μ l chloroform was added, vortexed, and incubated for a further 2 min at room temperature. The suspension was then centrifuged at 12,000 g, for 15 min at 4^o C. The upper colourless aqueous phase was transferred to a 2 mL Qiagen collection tube. The following automated steps were performed by the QIAcube, (Qiagen, Venlo, Netherlands). 525 μ l of 100% ethanol was added and subsequently vortexed. 700 μ l of the sample was transferred into an RNeasy mini spin column and centrifuged at 8000 g for 15 min at 20^o C. This step was repeated. Subsequently, 500 μ l RPE buffer was added to the RNeasy mini spin column and was centrifuged for 2 min at 8000 g. The RNeasy mini spin column was transferred to a new 1.5 mL collection tube, and 50 μ l RNase-free water was added and subsequently centrifuged for 1 min at 8000 g to elute the RNA. RNA purity was calculated using a nanodrop 2000/c spectrophotometer (Thermo-Scientific) from the 260/280 nm and 260/230 nm absorbance ratios.

2.2.2. Synthesis of cDNA and RT-qPCR

Primer validation and mRNA concentration optimization assays were carried out before the experiment. cDNA was synthesized from total RNA (200 ng) using the High-Capacity RNA-to-cDNA kit (Thermo Scientific, UK). Initially, serial cDNA dilutions were used to validate the primers of marker genes and genes of interest. Marker genes were used to indicate the differentiation of Calu-3 cells to specific cell types in the ALI cultures. Expression was analyzed in triplicate 10 μ l reactions containing 1 μ l cDNA, 5 μ l Fast SYBR Green Master Mix (Applied Biosystems), 3 μ l nuclease-free water and 0.5 μ l of 10 μ M forward and reverse primers of the appropriate endogenous control (*gapdh*, *hprt1*, *ywaz*, *tata*, see Fig. S1 for quality score of endogenous controls), tight junctions (*tjp1*), basal cell (*itga6*, *krt5* and *trp63*) goblet cell (*muc5ac* and *muc5b*) or ciliated cell markers (*foxj1*). All primers were purchased from Thermo-Scientific. We consulted the geNorm website (<https://genorm.cmgg.be/>), a widely accepted tool for assessing the stability of house-keeping genes. According to the geNorm analysis (supplementary Figure 2), *gapdh*, *tata*, *hprt1* and *ywaz* consistently exhibited stable expression among the screened genes in our experimental context after repeating the experiments at least twice. The $\Delta\Delta$ Ct method (Haimes and Kelley, 2014) was used in the calculations. Primers used in the in vitro study were validated before use. The primers of differentiation are seen in Table 1.

2.2.3. Transepithelial electrical resistance (TEER)

Transepithelial Electrical Resistance (TEER) barrier integrity is a measure of tight junction formation between cells (Srinivasan, et al., 2015). Basal media was removed every 2–3 days, and PBS was placed in both the apical and basal compartments, allowing TEER measurements to be recorded using an Evo meter (World Precision Instruments). Adhering to the manufacturer's protocol, the PBS was warmed to equilibrate in the incubator before measurements were recorded. Values were corrected by subtracting the measurement of the blank transwell (PBS without cells) and multiplying by the effective membrane area (4.67 cm²), resulting in measurements related to Ω cm² (Srinivasan et al., 2015). PBS was removed after TEER measurements, and ALI culture conditions were restored with ALI Medium in the basal compartment.

2.2.4. Immunofluorescence with MUC5AC and Hoechst 33342 (DAPI)

On day 21 of ALI, cells were fixed in 4% paraformaldehyde for 0.5 h.

Table 1

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

Gene	Sequence (forward and reverse)	Primer efficiency (%)
<i>gapdh</i>	F – 5' – CTGACTTCAACAGCGACACC – 3' R – 5' – TCGTTGTATACAGGAAATGA – 3'	93
<i>tjp1</i>	F – 5' – TCTATCCATGCAGAGAAGCCT – 3' R – 5' – TCCACAGCTGAAGGACTCAC – 3'	96
<i>muc5ac</i>	F – 5' – GCTGTGTACCATAGGAGCCT – 3' R – 5' – AGCGAGTACATGGAAGAGCT – 3'	96
<i>muc5b</i>	F – 5' – CCTGTCAAGTCCGCATCAAC – 3' R – 5' – CTGCTGAGTACTTGGACGCT – 3'	104
<i>itga6</i>	F – 5' – TGGGAGCCTTCATTGATGTG – 3' R – 5' – ACACAGTCACTCGAACCTGA – 3'	106
<i>trp63</i> (<i>tp63</i>)	F – 5' – ACTCCATGGATGATCTGGCA – 3' R – 5' – AAGGGGAGGAGAATTCGTGG – 3'	111
<i>foxj1</i>	F – 5' – CCTGTCGGCCATCTACAAGT – 3' R – 5' – CGGATTGAATTCGGCAGGT – 3'	107
<i>krt5</i>	F – 5' – GGAGCTCATGAACACCAAGC – 3' R – 5' – CACTGAGTCTGCATTCCTCG – 3'	75
<i>tata</i>	F – 5' – TGCTGCGGTAATCATGAGGA – 3' R – 5' – TTCTCACTCTTGGCTCCTGT – 3'	98
<i>ywaz</i>	F – 5' – CTGGTGATGACAAGAAAGGGAT – 3' R – 5' – GGATGTGTGGTTGCATTCC – 3'	103
<i>hprt1</i>	F – 5' – CCTGGCGTCGTGATTAGTGAT – 3' R – 5' – AGACGTTCAAGTCTGTCCATAA – 3'	95

The cells were permeabilized by incubating with 0.5% triton X-100 for 0.25 h. Cells were washed 3 times with PBS. Cells were blocked with 2% BSA (diluted in PBS) for 1 h at room temperature. Subsequently, cells were washed 3x with PBS. Cells were incubated with MUC5AC primary antibody (mouse monoclonal antibody, ThermoScientific, UK) at 2 μ g/mL concentration/dilution in 0.5% BSA (diluted in PBS), for 3 h at room temperature. Cells were washed 3x with PBS. Cells were incubated with a secondary antibody (rabbit polyclonal antibody, Alexa Fluor 488, ThermoScientific, UK) at 1:1000 concentration/dilution in 5% BSA (diluted in PBS), for 1 h at room temperature. The antibody solution was aspirated, and cells were counter-stained with 6 μ M Hoechst 33342 (DAPI) (ThermoScientific, UK). The cells were washed 3x with PBS, and images were taken with excitation/emission of 499/520 nm with the Z-stacking option on an ImageXpress Pico digital confocal microscope (Molecular Devices, UK) aided by CellReporterXpress software (Molecular Devices, UK).

2.3. Toxicological assessment of cinnamon, hazelnut and vanilla tobacco on Calu-3 cells

2.3.1. Cell exposure to EC e-liquid, aerosols and 3R4F Kentucky cigarette

Calu-3 cultured and validated at ALI for 21 days were used to assess the toxicity of cinnamon, vanilla tobacco and hazelnut flavored e-liquids, ALI cells were exposed to either 1% (v/v) of the e-liquid diluted with ALI maintenance media for 1.5 h (dose validation is provided in (Effah et al., 2023)) or to the aerosols of these for 0.5 h following CORESTA protocol (CORESTA, 2015). EC aerosols were generated using an in-house electronic cigarette aerosol generator (ECAG, eAerosols, Central Valley, NY) following the CORESTA protocol of a 5 s puff with a 30 s interval for 0.5 h totalling 60 puffs. The EC aerosol generator was set to puff at 12 W, and the coil resistance of the cartridges used for studies varied between 1.8 and 2.0 Ω . Cigarette smoke was generated with a smoking machine (Heinr. Borgwaldt, Germany). ALI cultures were placed in a Thermolyne incubator (37 $^{\circ}$ C), where aerosol emitted from the ECAG was diluted with HEPA-filtered air (4 L/min) and dispersed in the incubator's atmosphere, allowing the particles to impact on the apical surface. The amount of aerosol reaching the apical region of ALI cultures was characterized by measuring the mass difference (between pre-exposure and post-exposure) of a glass slide placed in an empty transwell at the same level as the cells. The masses of e-liquids deposited were: PG/VG (60/40, 1.21 mg), cinnamon (0.11 mg),

hazelnut (0.16 mg), and vanilla tobacco (0.8 mg). One entire cigarette was used to generate cigarette smoke (~12 puffs) and exposed to cells for 0.5 h.

2.3.2. Transepithelial electrical resistance (TEER)

For exposures, TEER readings were taken using an Evo meter (World Precision Instruments) before and after either the 1.5 h exposure to e-liquid in suspension culture or the 0.5 h aerosol exposure at ALI. Adhering to the manufacturer's protocol, the PBS was warmed to equilibrate in the incubator before measurements were recorded. Values were corrected by subtracting the measurement of the blank transwell (PBS without cells) and multiplying by the effective membrane area (4.67 cm²), resulting in measurements related to Ωcm² (Srinivasan et al., 2015).

2.3.3. Reactive oxygen species

In this assay, we aimed to characterize whether cinnamon, hazelnut and vanilla tobacco-flavored e-liquids stimulate reactive oxygen species (ROS) generation in Calu-3 cells in submerged cultures. Calu-3 cells were seeded at 6×10^4 cells in black-walled 96 well plates with clear bottoms (ThermoFisher Scientific, USA). Once cells reached confluence, they were treated with 1.5 mM of cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (ThermoFisher Scientific, USA) and incubated at 37 °C, 5% CO₂, and 98% humidity for 0.5 h. Subsequently, cells were washed 2x with Dulbecco's phosphate buffer saline (DPBS) and challenged with diluted e-liquids (1%, 0.5% and 0.25% diluted with DMEM/F-12, HEPES, no phenol red (Gibco, UK) or 2% hydrogen peroxide (positive control) for 3 h. Fluorescence intensity was measured using the Spectramax M2e microplate reader (Molecular Devices, San Jose, California) at 495 nm excitation and 530 nm emission wavelengths.

2.3.4. Cytotoxicity: LDH assay

After 1.5 h of hazelnut, vanilla tobacco and cinnamon flavored-e-liquid direct treatment (1%) 5 h of aerosol exposure to ALI cultures, maintenance medium was replaced, and cells were incubated at 37 °C, 5% CO₂, and 98% humidity for 24 h. Subsequently, spent media was removed from the basal region of the transwells and retained for lactate dehydrogenase (LDH) assay using the LDH cytotoxicity kit (Roche, UK) according to the manufacturer's protocol. Plate absorbance was measured at 490 nm and 680 nm using the BIO-TEK synergy HT absorbance reader (Bio-Tek, USA).

2.4. EC Aerosol size distribution characterization

As detailed here, a sampling train was used to characterise the particle size distribution of the e-cig aerosols. The exhaust from the ECAG, was attached to an inlet of an airtight, plastic exposure chamber. A Mercer-style impactor (Model SMEIMA7, CH Technologies, Westwood, NJ, USA), in-line with a Gast pump (Model 1023-1⁰¹Q-6Q608X, Benton Harbor, MI, USA), was attached to another open port of the chamber. The pump's flow rate with the impactor was set to 4.0 L/min utilizing an external valve and checked with a DryCal air flow calibrator (Mesa Laboratories, Lakewood, CO, USA). The EC cartridges were loaded with each flavor [i.e. PG/VG (60/40), cinnamon, hazelnut and vanilla tobacco]. The ECAG was set to puff every 30 s at 12 W, and the coil resistance of the cartridges used for studies varied between 1.8 and 2.0 Ω. The machine puffed into the chamber for 2 min before the Gast pump was turned on for 2 min. Each stage of the Mercer impactor was weighed before and after each sample in a humidity- and temperature-controlled weighing chamber (MTL, Minneapolis, MN, USA) with a microbalance (Model MT5, 1 µg readability; Mettler-Toledo, Columbus, OH). Each aerosol's size distribution was calculated from the mass distribution collected across each impactor stage.

2.5. Statistical analysis

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

3. Results

3.1. Validation of Calu-3 cells at ALI

The genes of differentiation that were characterized were normalized against four housekeeping genes: *gapdh*, *hprt1*, *ywaz* and *tata* (see Fig. S1 for the quality score of the housekeeping genes and Fig. S2 for the stability of the genes). The genes characterized were basal cell markers (*itga6*, *krt5* and *trp-63* Fig. 1A) mucus-production (*muc5ac*, Fig. 1B; *muc5b* Fig. 1C), a tight junction marker (*tjp1*, Fig. 1D), and cilia formation (*foxj1*, Fig. 1E). We observed a significant time-dependent increase in *trp63* (3-fold increase from day 0 to day 28), *muc5ac* (~13-fold increase from day 0 to day 28), *foxj1* (~4-fold increase from day 0 to day 28), and *tjp1* (~2.5-fold change from day 0 to day 28) gene expression, with the highest expression on day 21 or 28 at ALI. *Muc5b* increased about 400-fold from day 0 to day 7, followed by a subsequent decline to around 200-fold baseline levels from day 7 to day 14, whence levels were maintained till day 28. In contrast, we observed a decrease in *krt5* from day 0 till day 7, which remained stable till day 28. Interestingly, *itga6* mRNA levels remained constant throughout the 28 days. The presence of MUC5AC mucins was observed on day 21 at ALI (Fig. 1G).

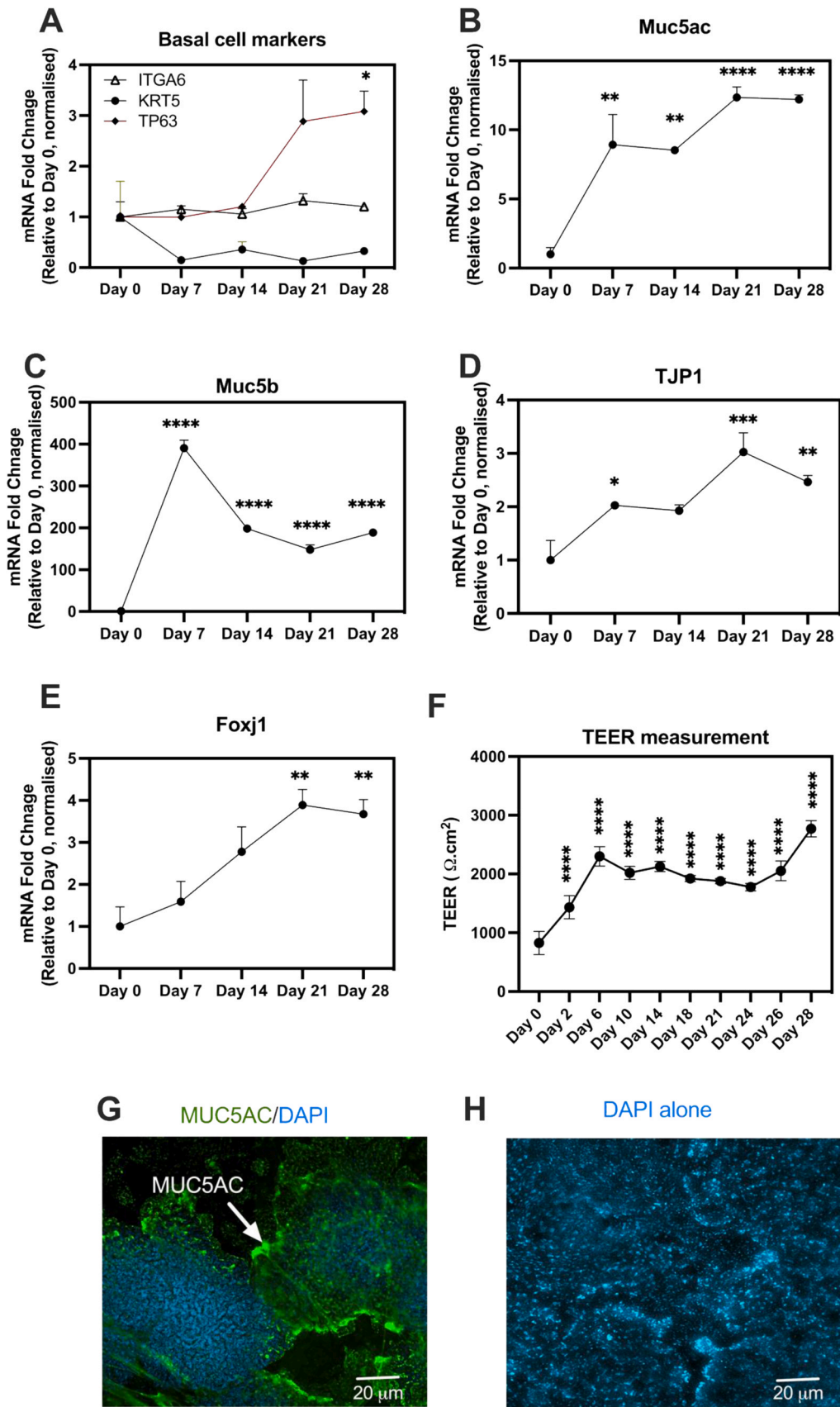
TEER measurements (Fig. 1F) increased significantly from less than 1000 Ωcm² to nearly 2500 Ωcm² over the first 6 days at ALI. Between days 6 and 24, no further change in TEER was observed until between days 24 and 28, when there was a second rapid increase in TEER, from 1800 Ωcm² to 3000 Ωcm².

3.2. LDH and TEER measurements in Calu-3 cells in suspension culture

The TEER measurements in Fig. 2A are the relative TEER value obtained from the difference between post-exposure TEER and pre-exposure TEER after ALI-cultured Calu-3 cells were exposed directly to dilutions of e-liquids for 1.5 h on the apical region. Interestingly, the relative TEER for PG/VG (*p* < 0.01), cinnamon (*p* < 0.001), and vanilla tobacco (*p* < 0.05), all at 1% (v/v) dilution (justification behind this dilution choice is provided in our most recent publication Effah et al., (2023)), were significantly reduced from the relative TEER of media-only control and had decreased compared to pre-treatment. Hazelnut, however, showed no difference compared to media-only control but was significantly higher than PG/VG control having increased following treatment. Although the 24 h LDH release in all groups was slightly higher than in the media-only control, there were no significant differences between groups (Fig. 2B).

3.3. ROS production in Calu-3 cells in submerged cultures

The ROS analysis (Fig. 3) showed no significant differences between PG/VG, vanilla tobacco, and hazelnut at 0.25%, 0.5%, and 1% vs media-only control. In contrast, cinnamon significantly and dose-dependently decreased ROS generation at 0.25% (*p* < 0.05), 0.5% (*p* < 0.001), and 1% (*p* < 0.001) vs media-only and at 0.5% (*p* < 0.05) and 1% (*p* < 0.01) vs PG/VG controls.



(caption on next page)

Fig. 1. Establishment of Calu-3 epithelium using ALI cultivation. Markers of differentiation were characterised every week for 28 days. The mRNA expression of (A) basal cell markers (*krts5*, *tp63*, and *itga6*), (B) *muc5ac*, (C) *muc5b* (D) *tjp1* (E) *foxj1* was investigated using qPCR. (F) TEER values were measured every 2–3 days with an EVOM 2 voltmeter from Day 0 (before the apical medium was aspirated). (G) Digital confocal microscopy was used to qualitatively examine the Calu-3 cells after MUC5AC (green) counterstained with DAPI (light blue) and (H) DAPI alone and revealed the expression of MUC5AC mucins on day 21. A one-way ANOVA with multiple comparisons, corrected using Bonferroni's test was used to determine statistical significance. Data are expressed as mean ± SEM (n = 3 per each day group for qPCR gene expression, n = 9 for each TEER day group). Each time point for TEER measurements was statistically significant (p < 0.0001) compared to day 0. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 vs day 0.

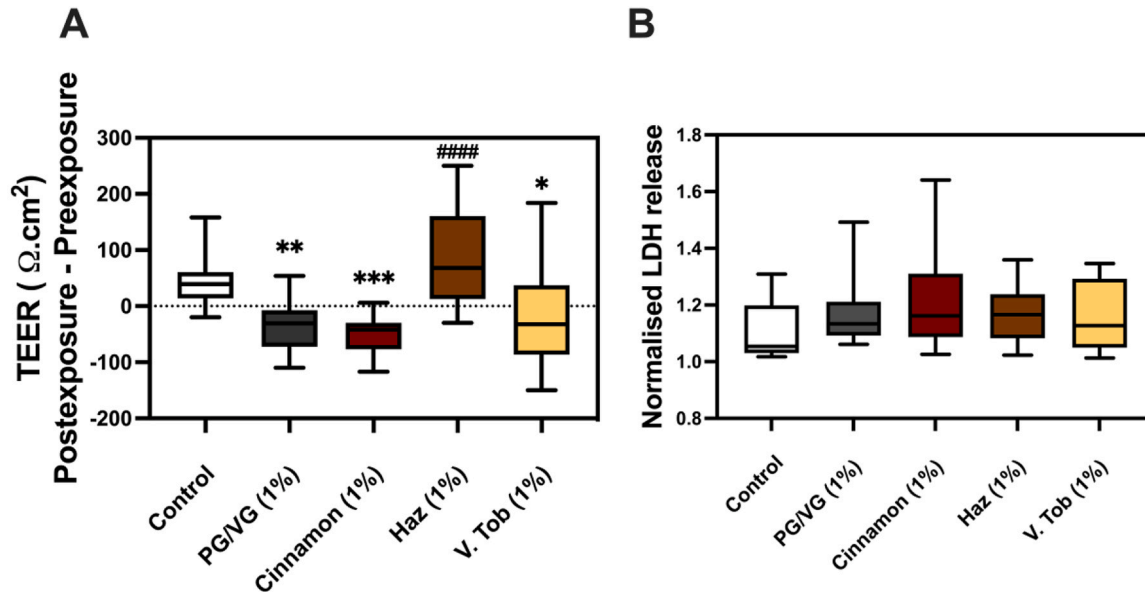


Fig. 2. Toxicological assessment of cinnamon, hazelnut, and vanilla tobacco flavored-e-liquids on Calu-3 cells. (A) Relative change in TEER and (B) LDH levels in Calu-3 cells grown at ALI and exposed for 1.5 h to diluted e-liquids. A one-way ANOVA with multiple comparisons, corrected using Tukey's test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

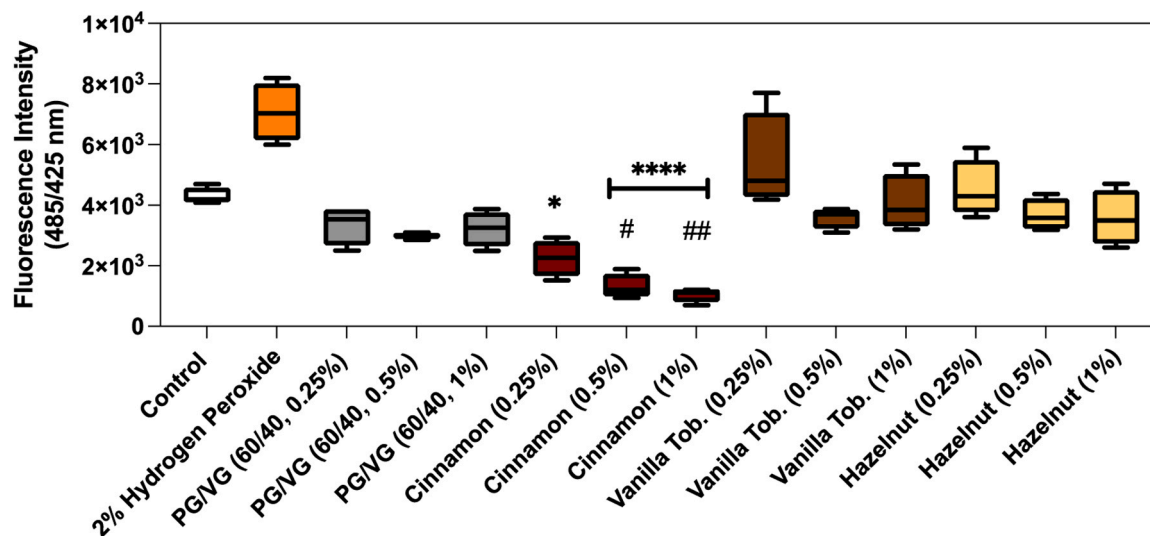


Fig. 3. ROS production in Calu-3 cells grown in monolayer exposed to diluted e-liquids for 3 h. A one-way ANOVA with multiple comparisons, corrected using Tukey's test was used to determine statistical significance. Data are presented as box and whiskers min-max (n = 6 for each group). *p < 0.05; **p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

3.4. LDH and TEER in Calu-3 ALI post-exposure to EC aerosol

This study investigated the relative toxicity of cinnamon, hazelnut, and vanilla tobacco-flavored e-cigarette aerosols on 21-day ALI-cultured

Calu-3 cells compared to the emissions from a 3R4F Kentucky reference cigarette positive control. Moreover, a PG/VG vehicle and an air-only control were included for a comprehensive assessment. As expected, we noted that the air-only control had no significant impact on the TEER

measurements (i.e., a maintained cell barrier integrity), thus underlining the appropriateness of the exposure period (Fig. 4A). TEER measurements for the vanilla tobacco-flavored aerosol exposure group were unaffected. However, the PG/VG vehicle, cinnamon, and hazelnut-flavoured aerosols each exhibited a significant decrease in TEER measurements ($p < 0.0001$) compared to the incubator and air-only controls. These results were not statistically distinguishable from the TEER reduction induced by the 3R4F Kentucky cigarette control. LDH release at 24 h hours, a marker of cellular damage and cytotoxicity, was used to ascertain further the effects of e-cig aerosol exposure. It was noted that all groups except for the vanilla tobacco exhibited a significant two-fold increase in LDH release (Fig. 4B) compared to incubator and air-only controls, thus corroborating the TEER measurements. The increase in LDH levels in the flavors and PG/VG aerosols were indistinguishable from that of reference cigarette.

3.5. Particle size distribution characterization

We hypothesized that particle size distribution might play a key role in explaining why vanilla tobacco e-liquid but not aerosol adversely affected TEER. Specifically, it was postulated that vanilla tobacco might

have a distinct particle size distribution that limits deposition when compared to the cytotoxic e-liquid aerosols (cinnamon and hazelnut). Vanilla tobacco (Fig. 4E) demonstrated a particle size distribution profile similar to that of the PG/VG (60/40) vehicle (Fig. 4C), with the highest percentage of particle size being particles with an aerodynamic size $< 0.99 \mu\text{m}$ at 74.3% and 75.9% respectively. The particle size distribution for hazelnut (Fig. 4D) and cinnamon-flavored (Fig. 4F) aerosols was larger particles with the majority of PM being $> 1 \mu\text{m}$ for hazelnut (79.9%) and cinnamon (84.6%), respectively. This suggests that flavouring in the e-liquid affects the size distribution of the generated aerosol.

4. Discussion

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

Our study aimed to evaluate an in vitro model for predicting the

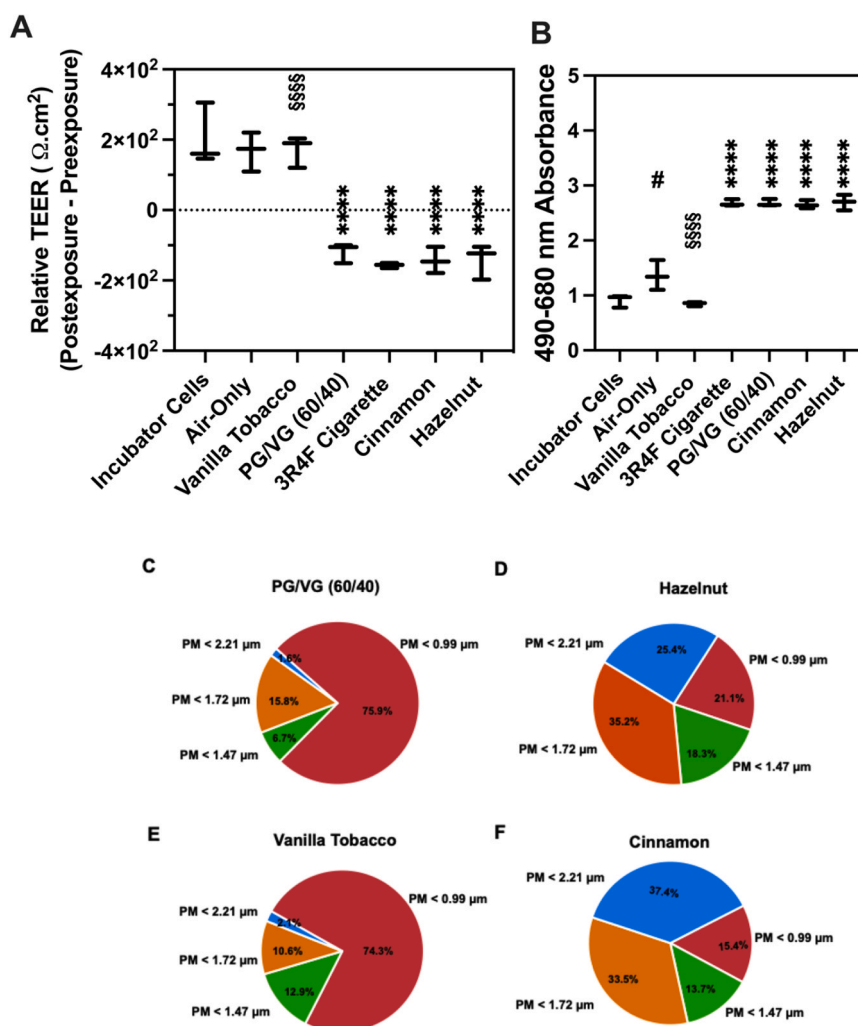


Fig. 4. Characterizing the toxic effects of cinnamon, hazelnut, and vanilla tobacco in ALI-differentiated Calu-3 to EC aerosols. (A) TEER and (B) LDH levels in Calu-3 cells grown at ALI-exposed to EC aerosols generated with the ECAG for 0.5 h. Particle size distribution of (C) PG/VG (60/40), (D) hazelnut,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

acute inhalation toxicity (Leibroek et al., 2020) of three flavored-e-liquids and their aerosols using Calu-3 epithelium. The study began with validating the cell culture model to confirm that the air-liquid interface (ALI) cultured cell layers display the expected morphological and bioelectrical characteristics. Under ALI culture conditions, mucus secretion (at day 21) and tight junction formation (as early as day 6) were confirmed. We observed a time-dependent significant increase in TEER values. The optimum window for studying bioelectric properties in several Calu-3 studies has differed; for example, Ji et al., (2016), after culturing Calu-3 cells at ALI for 22 days, observed this stable window between days 8–16. Jeong et al., (2019) only cultured the Calu-3 cells for 8 days and observed the highest TEER values around day 8, whereas Foster et al., (2000) cultured Calu-3 at ALI for 14 days and observed an increase in TEER from day 6 till 14. The TEER readings in our study were biphasic. In the first phase, it agrees with Ji et al., (2016) as we observed an increase in TEER measurement levels, which initially peaked at day 6 and then fell by about 20% until day 24. Interestingly, in the second phase, we observed a rapid increase in TEER values between day 24 and 28. Lee et al., (2021) also cultured Calu-3 cells at ALI for 28 days and observed the highest TEER at day 28, although the increment in TEER value was strictly time-dependent in their study. It is unclear why there were discrepancies among these studies. However, we assume that factors that may have influenced these results include the passage number of the cells used at ALI, culture medium, seeding density and transwell inserts.

TEER values offer insight into the physical structure and quality of the cells on the porous membrane by gauging the transcellular and paracellular resistance across the membrane, which amplifies with cell-substrate and cell-cell interaction (Chen et al., 2015). Therefore, the expression of high TEER values observed in this study may be a result of the formation of a pseudostratified structure, which, in turn, may have resulted from the time-dependent increase in the *tp1*, *muc5ac*, *muc5b* and *foxj1* genes. There was a decrease in *krt5*; whereas *itga6* gene remained constant from day 0 to day 28. The expression of *itga6* is a biomarker to measure the stemness of isolated basal cells from human airway epithelium and murine trachea (Rock et al., 2009). Basal cells are pivotal for forming and repairing the pseudostratified epithelium of the human lung following injurious insults. However, the evidence provided in this study suggests that more sub-types of basal cells are expressed within the lung epithelium recapitulated with Calu-3 cells at ALI, with each playing a distinctive role in maintaining the pseudostratified structure. For example, the *krt5*-expressing basal cells may be the ones regenerating differentiated cells, whereas the *itga6*-expressing basal cells maintain a steady state level for epithelial repair, recapitulating the physiology of the human lung. In contrast to the other basal cell markers, the expression *tp63* remained unchanged from day 0 till day 14, followed by a significant increase in expression till day 28. The tumour-suppressor gene *tp63* encodes for the transformation-related protein 63 (p63), which is pivotal for cell differentiation and development such as in basal cells where it is important in generating and maintaining pseudostratified bronchial epithelium in vitro (Arason et al., 2014). Moreover, human bronchial epithelial cells, where *tp63* gene was knocked down, failed to differentiate into goblet cells and showed non-phenotypical bronchial basal cells when grown at ALI (Arason et al., 2014). As such, the initial no-change in levels in *tp63* observed in this study might have occurred during differentiation from basal cells into more specialized bronchial epithelial cells, as demonstrated by the increased expressions of *muc5ac*, *muc5b*, *tp1*, and *foxj1*. This may also corroborate the second rapid increase in TEER values from day 24–28. Indeed, our findings agree with Felix et al., (2021) who showed an increase in TEER values in Caco-2 cells cultured at a liquid-liquid interface, followed by a decrease during differentiation and an increase after developing a full barrier. Taken together, the results from these differentiation genes and the TEER values indicate that the longer Calu-3 cells are grown at ALI, the more they differentiate into several other bronchial epithelial cell types (i.e submucosal glands,

goblet cells, ciliated cells, basal cells) to enable the recapitulation of the in vivo microenvironment of human lung epithelium. No study has reported beating cilia in Calu-3 cells grown at ALI. Although we observed a 4-fold increase in the *foxj1* gene on day 28 compared to day 0, no visible cilia were observed under light microscopy. Conversely, functional cilia have been shown in primary human bronchial epithelial cells grown at ALI for 28 days (Leung et al., 2020). This may indicate that functional cilia are not expressed in Calu-3 cells because of their submucosal gland origin, or a better visualization technique is required to view them. Moreover, the high expression of *muc5b* compared to *muc5ac* over the 28 days agrees with the literature, as Calu-3 cells derive from human bronchial submucosal glands, the main source of MUC5B (Liegeois and Fahy, 2022). In contrast, goblet cells primarily secrete MUC5AC proteins (Liegeois and Fahy, 2022). While several studies have studied the expression of ZO-1, MUC5AC, and E-cadherins in Calu-3 cells grown at ALI (Jeong et al., 2019; Ji et al., 2022; Lee et al., 2021; Pasman et al., 2021), this is the first time the time-dependent variation in marker genes for differentiation has been validated for Calu-3 cells. Moreover, this is the first time the expression of genetic markers of basal genes in differentiating Calu-3 cells has been reported that recapitulates the in vivo pattern of genetic expression. This study may help future studies investigating lung epithelium repair and other pulmonary disorders understand which basal cell markers to target. Moreover, it will help as a robust screening model to screen pulmonary toxicants and their mode of toxicological actions. Here, we used this validated model to characterize the potential toxicity of flavors in e-liquid.

Currently, more than 8000 flavored e-liquids from various brands are on the UK market (Effah et al., 2022). Although carcinogens appear to be reduced or eliminated in ECs compared to cigarette smoke (Amatngalim et al., 2016), the inhalation toxicity of EC aerosols with flavoring chemicals and flavor enhancers is poorly understood based on toxicological effects on the lungs. Several in vitro studies have shown the link between EC and e-liquids to lung tissue damage. The most frequently reported adverse effects are reduced cell count and viability, altered pro-inflammatory biomarkers, cytokine release, and increased oxidative stress in vitro (Balharry et al., 2008; Behar et al., 2018; Clapp et al., 2019; Sundar et al., 2016). These effects mentioned above are consistent across pulmonary cell types, including normal and cancer-derived cells, large and small airway epithelial cells, alveolar epithelial cells, pulmonary smooth muscle, fibroblasts, and alveolar macrophages (for a detailed review, see Effah et al., (2022b)). Although the flavoring chemicals used are generally regarded as safe (GRAS) for oral ingestion, their GRAS status post-inhalation remains elusive, and the impact of specific flavoring chemicals on airway epithelial cells remains unclear (Clapp et al., 2019; Neilson et al., 2015; Omaiye et al., 2022). In vitro assessment of inhalation toxicology has also been applied to the EC liquids (Khlystov and Samburova, 2016; Leigh et al., 2016). Nevertheless, information on flavoring-specific inhalation toxicity and the relative toxicity of flavored-e-liquids vs tobacco smoking needs further examination. In this study, we tested the acute inhalation toxicity of 3 previously identified flavored-e-liquids that demonstrated toxicity in submerged bronchioepithelial cells (Effah et al., 2023) and compared the toxicity as e-liquids and as EC aerosols to conventional cigarette emissions.

We used TEER as the main indicator of tissue stress by measuring the integrity of cellular tight junctions and, thus, epithelial permeability. LDH levels were used as a proxy to measure cytotoxicity and overall cell health. PG/VG, vanilla tobacco, and cinnamon e-liquids at 1% (v/v), but not hazelnut, significantly decreased TEER values in suspension culture. In contrast, no effect was observed on LDH release. Interestingly, with aerosol exposures, vanilla tobacco was the only flavored-e-liquid which did not impact on TEER value or LDH release, with PG/VG, hazelnut, cinnamon and 3R4F cigarette reducing TEER measurements and increasing LDH levels by 2–3 fold, respectively compared to air only exposure. Surprisingly, TEER values and LDH levels induced by aerosols from PG/VG alone did not differ from the flavoured-e-liquids. This may

suggest that 60 puffs of PG/VG alone may be enough to exacerbate the toxicological response of bronchial epithelial cells to an extent that the additive toxicity of flavors may not be significant *in vitro*. Therefore, further studies are required to establish whether the toxicity of aerosolized PG/VG is dose dependent. Nonetheless, this contrasted with our expectations, given the common perception of e-liquids as a safer alternative and the contrasting evidence on PG/VG toxicity in the literature (for an extensive review see [Effah et al., \(2022\)](#)).

EC aerosol contains fewer and significantly lower concentrations of toxic chemicals than conventional cigarettes ([Dusautoir et al., 2021](#); [Margham et al., 2016](#)). However, only a few studies have assessed the toxicity of EC flavors compared to conventional cigarettes. [Czekala et al., \(2019\)](#) have shown that aerosols from blueberry flavored-e-liquids induced less toxicity than conventional cigarettes in a 3D *in vitro* human respiratory model developed from primary cells. However, they only screened one flavor and they did not justify choosing that particular flavor. In contrast, [Glynos et al., \(2018\)](#) showed that EC aerosols, especially those containing flavor (flavor type not specified), increased bronchoalveolar lavage fluid (BALF) cellularity, Muc5ac production, as well as BALF and lung oxidative stress markers in mice at least comparably and in many cases more than did conventional cigarettes. Conversely, we observed no flavor-specific additive toxic effects in our study. Taken together, our study suggests that the potentially adverse health effects associated with these flavored e-liquid aerosols may be heavily dependent on PG/VG and not on flavor type as not all flavors exert added toxicity ([Effah et al., 2023](#)). Therefore, we strongly advocate for increased user awareness on the potential toxicity of PG/VG and rigorous regulatory scrutiny of potentially toxic flavors to protect public health.

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

To justify why the aerosolized vanilla tobacco elicited no toxic effects even though it was the flavour with the highest most deposited mass (0.8 mg) compared to cinnamon (0.11 mg), and hazelnut (0.16 mg), we hypothesized that it might have a different particle size distribution compared to cinnamon and hazelnut. Therefore, we characterized the particulate matter (PM) size produced from the EC aerosol. Hazelnut and cinnamon showed similar patterns in their particle size distribution, which reinforces the findings regarding TEER reduction and increased LDH release in the cells exposed to these e-liquid aerosols. In contrast, vanilla tobacco and PG/VG showed a similar pattern with the greatest particle size distribution below 0.99 μm . Smaller particles tend to have a larger surface area relative to their mass, which can increase their reactivity and ability to interact with biological systems ([Hewitt et al., 2020](#)); however, vanilla tobacco showed no cytotoxicity in its aerosolized form. Therefore, this suggests that factors beyond particle size distribution may contribute to the cytotoxicity of PG/VG vehicle,

cinnamon, and hazelnut. Although more studies are warranted to delineate why vanilla tobacco aerosol showed no toxicity in Calu-3 cells grown at ALI, [Rowell et al., \(2017\)](#) showed that vanilla tobacco reduced cell proliferation and decreased cell viability dose-dependently in submerged Calu-3 cells exposed to vanilla tobacco aerosol bubbled through culture medium. Therefore, differences in cell model may account for the differences in toxicological outcome in our study and that of [Rowell et al., 2017](#). In addition, we showed in our previous study that vanilla tobacco and hazelnut may elicit their toxic effect by decreasing mitochondrial membrane potential and inducing ROS production in HBEC-3KT ([Effah et al., 2023](#)). Nonetheless, hazelnut and vanilla tobacco e-liquid did not adversely affect Calu-3 cells at the doses tested compared to PG/VG controls. Interestingly, while cinnamon e-liquid showed no effect on ROS production in direct diluted e-liquid exposure to submerged HBEC-3KTs, it induced a dose-dependent decrease in ROS production compared to media-only and PG/VG vehicle controls in Calu-3 cells. We hypothesize that the differential responses in HBEC-3KT and Calu-3 cells for ROS generation may be rooted in the differential expression of antioxidative stress enzyme in cancerous (Calu3) and immortalised normal (HBEC-3KT) cells ([Perillo et al., 2020](#)). Conversely, cinnamon e-liquids have been shown to induce oxidative stress and increase ROS production in osteoblast-like MG-63 cells ([Wavreil and Heggland, 2020](#)).

The observed decrease in ROS production in Calu-3 cells exposed to diluted cinnamon e-liquids could be attributed to the presence of stilbene, a compound found in cinnamon e-liquid ([Effah et al., 2023](#)). Stilbene has antioxidant properties and can act as a ROS scavenger ([Frombaum et al., 2012](#); [Hamadouche et al., 2021](#); [Reinisalo et al., 2015](#)), leading to a dose-dependent decrease in ROS levels in this cell line. This suggests that the effects of cinnamon e-liquids on ROS production may be influenced by the specific composition of the e-liquid. Moreover, it is important to note that the antioxidant activity of stilbene compounds can be context dependent. In certain situations, this compounds may also exhibit prooxidative effects, depending on the cellular environment and concentration ([Kladna et al., 2021](#)). Therefore, further research is needed to fully understand the mechanisms underlying the effects of cinnamon e-liquids on ROS production and the potential role of stilbene in this process.

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

One important factor of Calu-3 ALI characteristics is the formation of a mucus layer, which can significant impact the deposition and dissolution of chemicals ([Silva et al., 2023](#)). Permeation profiles obtained with a PennCentury™ Dry Powder Insufflator in Calu-3 cell layers in ALI conditions revealed that mucus facilitates the dissolution and permeability of poorly soluble drugs (indomethacin), but restrains the absorption of drugs with high solubility (salbutamol) ([Cingolani et al., 2019](#)). PG is already highly soluble in water and various solvents due to its diol structure, which allows it to form hydrogen bonds with polar molecules. VG, on the other hand, is also water-soluble but has a higher viscosity and lower volatility compared to PG. The solubility of PG and VG in e-liquids is primarily determined by their interactions with other components, such as water, flavors, and nicotine. Therefore, we hypothesize that other pulmonary cells (cancerous, immortalized or primary) grown at ALI that do not present a thick mucus layer like Calu-3 cells exposed to aerosolized e-liquids in *in vitro* inhalation systems such as the CULTEX system ([Aufderheide et al., 2013](#)) may be a more robust experimental approach to screen for flavor toxicity in ECs.

Nonetheless, in the context of regulatory considerations, comparative analyses between aerosols derived not from the flavored e-liquids but the PG/VG vehicle alone and the standardized 3R4F reference cigarette smoke, can provide pivotal insights. These insights are crucial for informing policymakers and regulatory entities in the development of rigorous standards and guidelines for the manufacture and marketing

of e-cigarette products. From a public health perspective, because the base components of e-liquids, PG and VG, exhibit toxicity profiles similar to those of 3R4F cigarette smoke, the implications for harm reduction strategies associated with EC use could be negative. There is a need for a re-evaluation of the presumed reduced harm potential of EC, which may influence public health recommendations and the regulation of e-liquids constituents.

A debate ensues: can we wholly rely on in vitro models, or is there an irrefutable need for in vivo studies to bridge the knowledge gaps and offer holistic insights into the multi-dimensional toxicological landscapes? The combination of robust in vitro investigations using ALI, organ-on-chip models (Singh et al., 2021b), and bioinformatics may provide enough toxicological evidence to not rely on animal testing. In the future, scientists investigating flavor toxicity should utilize these improved physiologically relevant in vitro models with aerosol exposure. Appropriate controls, such as unflavored carrier liquids, are critical to isolate the effects of specific flavors. Testing should focus on lung cell types at realistic exposure levels. Flavored e-liquids and aerosols should be evaluated, not just individual chemicals in the solution. Reliance on older, less relevant models has propagated misconceptions that all flavor chemicals are universally safe via inhalation. We advocate for developing a machine learning algorithm meticulously trained with data encompassing flavoring chemicals proven in in vitro inhalation studies to be toxic to human lungs. The primary objective of this innovative tool would be to rigorously analyse and map the chemical composition of new e-liquids entering the market. Our proposition entails a stringent regulatory measure where if 5% or more of the chemicals in a new e-liquid align with the toxic chemical library, it should be flagged as potentially hazardous. Consequently, the MHRA would be advised to bar such products from the British market, reinforcing a proactive stance in safeguarding public health against the potential perils associated with harmful constituents in e-liquids. This approach underscores the imperative of intertwining advanced technology with regulatory oversight to elevate safety standards in the rapidly evolving EC landscape.

5. Conclusions

Flavors in EC are believed to attract long-time tobacco cigarette smokers to switch to EC use and tobacco naïve non-smokers to take up e-cigarettes; they also potentially may exert adverse effects on airways. In this study, the toxicity of PG/VG aerosols alone exert adverse effects in Calu-3 cells in ALI cultures comparable to the positive cigarette controls. Interestingly, none of the flavored-e-liquids that showed toxicity comparable to conventional cigarettes significantly differed from their PG/VG vehicle control. In contrast, while vanilla tobacco elicited adverse effects in suspension culture, it was non-toxic in its aerosol form. This suggests that the toxicity associated with aerosols of flavored-e-liquids may be due to the vehicle components and not the flavours themselves. This study suggests that the toxicity associated with aerosolized e-liquids heavily depends on the PG/VG component. As our systematic review Effah et al., (2022) identified, cancerous cells may be less sensitive to e-liquids and their aerosols. Therefore, future work needs to focus on building robust and validated lung models with primary or immortalised cells grown at ALI that are sensitive enough to predict the toxicity of aerosols and the potential additive effects of flavoring components to the toxic effects of PG/VG. This will help inform public health authorities in the UK and elsewhere about which EC flavors may need monitoring and/or restricting.

CRedit authorship contribution statement

Luglio David: Investigation, Validation, Writing – review & editing. **Adragna John:** Methodology, Validation, Writing – review & editing. **Marczylo Tim:** Conceptualization, Formal analysis, Funding acquisition, Supervision. **Bailey Alexis:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing –

review & editing. **Effah felix:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gordon Terry:** Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

All data are included in this research article.

Acknowledgements

This paper is based on independent research commissioned and funded by the National Institute for Health Research (NIHR) of the UK and NIH/NHLBI (HL139239-03), NIH/NIEHS ES000260, and NIH/NCI (P30 CA016087) grants of the U.S. Tim Marczylo and Felix Effah are part-funded by the NIHR Health Protection Research Unit (HPRU) in Health Impacts of Environmental Hazards at Imperial College London.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2023.153683.

References

- Amatngalim, G.D., Broekman, W., Daniel, N.M., Van Der Vlucht, L.E.P.M., Van Schadewijk, A., Taube, C., Hiemstra, P.S., 2016. Cigarette smoke modulates repair and innate immunity following injury to airway epithelial cells. *PLOS ONE* 11 (11), e0166255. <https://doi.org/10.1371/journal.pone.0166255>.
- Arason, A.J., Jonsdottir, H.R., Halldorsson, S., Benediktssdottir, B.E., Bergthorsson, J.T., Ingthorsson, S., et al., 2014. deltaNp63 has a role in maintaining epithelial integrity in airway epithelium. *PLoS ONE* 9 (2), e88683. <https://doi.org/10.1371/journal.pone.0088683>.
- ASH (2022a) Fears of growth in children vaping disposables backed up by new national survey. Available from: (<https://ash.org.uk/media-and-news/press-releases-media-and-news/fears-of-growth-in-children-vaping-disposables-backed-up-by-new-national-survey/>).
- ASH (2022b) Use of e-cigarettes (vapes) among adults in Great Britain. Available from: (<https://ash.org.uk/uploads/Use-of-e-cigarettes-vapes-among-adults-in-Great-Britain-2022.pdf?v=1661865959>) [Accessed].
- Aufderheide, M., Halter, B., Möhle, N., Hochrainer, D., 2013. The CULTEX RFS: a comprehensive technical approach for the in vitro exposure of airway epithelial cells to the particulate matter at the air-liquid interface. *BioMed. Res. Int.* 2013, 1–15. <https://doi.org/10.1155/2013/734137>.
- Balharay, D., Sexton, K., Bérubé, K.A., 2008. An in vitro approach to assess the toxicity of inhaled tobacco smoke components: Nicotine, cadmium, formaldehyde and urethane. *Toxicology* 244 (1), 66–76. <https://doi.org/10.1016/j.tox.2007.11.001>.
- Barrila, J., Radtke, A.L., Crabbé, A., Sarker, S.F., Herbst-Kralovetz, M.M., Ott, C.M., Nickerson, C.A., 2010. Organotypic 3D cell culture models: using the rotating wall vessel to study host–pathogen interactions. *Nat. Rev. Microbiol.* 8 (11), 791–801. <https://doi.org/10.1038/nrmicro2423>.
- Behar, R.Z., Wang, Y., Talbot, P., 2018. Comparing the cytotoxicity of electronic cigarette fluids, aerosols and solvents. *Tob. Control* 27 (3), 325–333. <https://doi.org/10.1136/tobaccocontrol-2016-053472>.
- Chen, S., Einspanier, R., Schoen, J., 2015. Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports. *Histochem. Cell Biol.* 144 (5), 509–515. <https://doi.org/10.1007/s00418-015-1351-1>.
- Cingolani, E., Alqahtani, S., Sadler, R.C., Prime, D., Stolnik, S., Bosquillon, C., 2019. In vitro investigation on the impact of airway mucus on drug dissolution and absorption at the air-epithelium interface in the lungs. *Eur. J. Pharm. Biopharm.* 141, 210–220. <https://doi.org/10.1016/j.ejpb.2019.05.022>.
- Clapp, P.W., Lavrich, K.S., van Heusden, C.A., Lazarowski, E.R., Carson, J.L., Jaspers, I., 2019. Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *Am. J. Physiol. -Lung Cell. Mol. Physiol.* 316 (3), L470–L486. <https://doi.org/10.1152/ajplung.00304.2018>.
- CORESTA (2015) E-cigarette Task Force Technical Report, 2014 Electronic Cigarette Aerosol Parameters Study.
- Czekala, L., Simms, L., Stevenson, M., Tschierske, N., Maione, A.G., Walele, T., 2019. Toxicological comparison of cigarette smoke and e-cigarette aerosol using a 3D in

- vitro human respiratory model. *Regul. Toxicol. Pharmacol.* 103, 314–324. <https://doi.org/10.1016/j.yrtph.2019.01.036>.
- Dusautoir, R., Zarcone, G., Verrielle, M., Garçon, G., Fronval, I., Beauval, N., et al., 2021. Comparison of the chemical composition of aerosols from heated tobacco products, electronic cigarettes and tobacco cigarettes and their toxic impacts on the human bronchial epithelial BEAS-2B cells. *J. Hazard. Mater.* 401, 123417. <https://doi.org/10.1016/j.jhazmat.2020.123417>.
- Effah, F., Elzein, A., Taiwo, B., Baines, D., Bailey, A., Marczylo, T., 2023. In Vitro High-Throughput Toxicological Assessment of E-Cigarette Flavors on Human Bronchial Epithelial Cells and the Potential Involvement of TRPA1 in Cinnamon Flavor-Induced Toxicity. *Toxicology*, 153617. <https://doi.org/10.1016/j.tox.2023.153617>.
- Effah, F., Taiwo, B., Baines, D., Bailey, A., Marczylo, T., 2022. Pulmonary effects of e-liquid flavors: a systematic review. *J. Toxicol. Environ. Health, Part B* 1–29. <https://doi.org/10.1080/10937404.2022.2124563>.
- Farsalinos, K.E., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Spyrou, A., Voudris, V., 2013. Impact of flavour variability on electronic cigarette use experience: an internet survey. *Int. J. Environ. Res. Public Health* 10 (12), 7272–7282. <https://doi.org/10.3390/ijerph10127272>.
- FDA (2012) *Reporting Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke Under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act*.
- Felix, K., Tobias, S., Jan, H., Nicolas, S., Michael, M., 2021. Measurements of transepithelial electrical resistance (TEER) are affected by junctional length in immature epithelial monolayers. *Histochem. Cell Biol.* <https://doi.org/10.1007/s00418-021-02026-4>.
- Foster, K.A., Avery, M.L., Yazdani, M., Audus, K.L., 2000. Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. *Int. J. Pharm.* 208 (1–2), 1–11. [https://doi.org/10.1016/S0378-5173\(00\)00452-X](https://doi.org/10.1016/S0378-5173(00)00452-X).
- Frombaum, M., Le Clanche, S., Bonnefont-Rousselot, D., Borderie, D., 2012. Antioxidant effects of resveratrol and other stilbene derivatives on oxidative stress and NO bioavailability: Potential benefits to cardiovascular diseases. *Biochimie* 94 (2), 269–276. <https://doi.org/10.1016/j.biochi.2011.11.001>.
- Glynos, C., Bibli, S.-L., Katsaounou, P., Pavlidou, A., Magkou, C., Karavana, V., et al., 2018. Comparison of the effects of e-cigarette vapor with cigarette smoke on lung function and inflammation in mice. *Am. J. Physiol. -Lung Cell. Mol. Physiol.* 315 (5), L662–L672. <https://doi.org/10.1152/ajplung.00389.2017>.
- Haimes, J. and Kelley, M. (2014) Demonstration of a $\Delta\Delta Cq$ Calculation Method to Compute Relative Gene Expression from qPCR Data. Available from: (<https://horizondiscover.com/-/media/Files/Horizon/resources/Technical-Manuals/delta-cq-solaris-technote.pdf>).
- Hamadouche, S., Ounissi, A., Baira, K., Ouddai, N., Balsamo, M., Erto, A., Benguerba, Y., 2021. Theoretical evaluation of the antioxidant activity of some stilbenes using the Density Functional Theory. *J. Mol. Struct.* 1229, 129496. <https://doi.org/10.1016/j.molstruc.2020.129496>.
- Haws, C., Finkbeiner, W.E., Widdicombe, J.H., Wine, J.J., 1994. CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated Cl⁻ conductance. *Am. J. Physiol. -Lung Cell. Mol. Physiol.* 266 (5), L502–L512. <https://doi.org/10.1152/ajplung.1994.266.5.L502>.
- Hewitt, R.E., Chappell, H.F., Powell, J.J., 2020. Small and dangerous? Potential toxicity mechanisms of common exposure particles and nanoparticles. *Curr. Opin. Toxicol.* 19, 93–98. <https://doi.org/10.1016/j.cotox.2020.01.006>.
- Hewitt, R.J., Lloyd, C.M., 2021. Regulation of immune responses by the airway epithelial cell landscape. *Nat. Rev. Immunol.* 21 (6), 347–362. <https://doi.org/10.1038/s41577-020-00477-9>.
- Jeong, M.H., Kim, H.R., Bang, I.J., Yoo, S.H., Lee, S.J., Lee, K.H., Chung, K.H., 2019. In vitro model for predicting acute inhalation toxicity by using a Calu-3 epithelium cytotoxicity assay. *J. Pharmacol. Toxicol. Methods* 98, 106576. <https://doi.org/10.1016/j.vascn.2019.04.002>.
- Ji, E.H., Sun, B., Zhao, T., Shu, S., Chang, C.H., Messadi, D., Xia, T., Zhu, Y., Hu, S., 2016. Characterization of Electronic Cigarette Aerosol and Its Induction of Oxidative Stress Response in Oral Keratinocytes. *PLOS ONE* 11 (5), e0154447. <https://doi.org/10.1371/journal.pone.0154447>.
- Ji, X., Sheng, Y., Guan, Y., Li, Y., Xu, Y., Tang, L., 2022. Evaluation of Calu-3 cell lines as an in vitro model to study the inhalation toxicity of flavoring extracts. *Toxicol. Mech. Methods* 32 (3), 171–179. <https://doi.org/10.1080/15376516.2021.1977880>.
- Khlystov, A., Samburova, V., 2016. Flavoring Compounds Dominate Toxic Aldehyde Production during E-Cigarette Vaping. *Environ. Sci. Technol.* 50 (23), 13080–13085. <https://doi.org/10.1021/acs.est.6b05145>.
- Kladna, A., Berczyński, P., Dündar, O.B., Kruk, I., Torun, B., Aboul-Enein, H.Y., 2021. A Study on Synthesis and Antioxidant Activity Comparison of Novel Stilbenebenzamide Compounds. *Med. Chem.* 17 (5), 533–544. <https://doi.org/10.2174/15734064156661911141233004>.
- Lee, D.F., Lethem, M.L., Lansley, A.B., 2021. A comparison of three mucus-secreting airway cell lines (Calu-3, SPOC1 and UNC3T) for use as biopharmaceutical models of the nose and lung. *Eur. J. Pharm. Biopharm.* 167, 159–174. <https://doi.org/10.1016/j.ejpb.2021.07.016>.
- Leibrock, L., Jungnickel, H., Tentschert, J., Katz, A., Toman, B., Petersen, E., et al., 2020. Parametric Optimization of an Air-Liquid Interface System for Flow-Through Inhalation Exposure to Nanoparticles: Assessing Dosimetry and Intracellular Uptake of CeO₂ Nanoparticles. *Nanomaterials* 10 (12), 2369. <https://doi.org/10.3390/nano10122369>.
- Leibrock, L., Wagener, S., Singh, A.V., Laux, P., Luch, A., 2019. Nanoparticle induced barrier function assessment at liquid-liquid and air-liquid interface in novel human lung epithelia cell lines. *Toxicol. Res.* 8 (6), 1016–1027. <https://doi.org/10.1039/c9tx00179d>.
- Leigh, N.J., Lawton, R.I., Hershberger, P.A., Goniewicz, M.L., 2016. Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS). *Tob. Control* 25 (Suppl 2), ii81. <https://doi.org/10.1136/tobaccocontrol-2016-053205>.
- LeMessurier, K.S., Tiwary, M., Morin, N.P., Samarasinghe, A.E., 2020. Respiratory Barrier as a Safeguard and Regulator of Defense Against Influenza A Virus and *Streptococcus pneumoniae*. *Front. Immunol.* 11, 3. <https://doi.org/10.3389/fimmu.2020.00003>.
- Leung, C., Wadsworth, S.J., Yang, S.J., Dorscheid, D.R., 2020. Structural and functional variations in human bronchial epithelial cells cultured in air-liquid interface using different growth media. *Am. J. Physiol. -Lung Cell. Mol. Physiol.* 318 (5), L1063–L1073. <https://doi.org/10.1152/ajplung.00190.2019>.
- Liegeois, M.A., Fahy, J.V., 2022. The Mucin Gene *MUC5B* Is Required for Normal Lung Function. *Am. J. Respir. Crit. Care Med.* 205 (7), 737–739. <https://doi.org/10.1164/rccm.202201-0064ED>.
- Margham, J., McAdam, K., Forster, M., Liu, C., Wright, C., Mariner, D., Proctor, C., 2016. Chemical Composition of Aerosol from an E-Cigarette: A Quantitative Comparison with Cigarette Smoke. *Chem. Res. Toxicol.* 29 (10), 1662–1678. <https://doi.org/10.1021/acs.chemrestox.6b00188>.
- McNeill, A.D., Brose, L.S., Calder, R., Bauld, L. and Robson, D.J. (2018) Evidence review of e-cigarettes and heated tobacco products 2018: A report commissioned by Public Health England. Available from: (<https://www.gov.uk/government/publications/e-cigarettes-and-heated-tobacco-products-evidence-review>) [Accessed].
- McNeill, A., Simonavicius, E., Brose, L., Taylor, E., East, K., Zuikova, E., Calder, R. and Robson, D. (2022, October) Nicotine vaping in England: an evidence update including health risks and perceptions.
- National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health (2014) *The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General*. Atlanta (GA): Centers for Disease Control and Prevention (US). Available from: (<http://www.ncbi.nlm.nih.gov/books/NBK179276/>).
- Neilson, L., Mankus, C., Thorne, D., Jackson, G., DeBay, J., Meredith, C., 2015. Development of an in vitro cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol. *Toxicol. Vitro* 29 (7), 1952–1962. <https://doi.org/10.1016/j.tiv.2015.05.018>.
- Omaie, E.E., Luo, W., McWhirter, K.J., Pankow, J.F., Talbot, P., 2022. Ethyl maltol, vanillin, corylone and other conventional confectionery-related flavour chemicals dominate in some e-cigarette liquids labelled 'tobacco' flavoured. *Tob. Control* 31 (Suppl 3), s238–s244. <https://doi.org/10.1136/tc-2022-057484>.
- Ong, H.X., Traini, D., Young, P.M., 2013. Pharmaceutical applications of the Calu-3 lung epithelia cell line. *Expert Opin. Drug Deliv.* 10 (9), 1287–1302. <https://doi.org/10.1517/17425247.2013.805743>.
- Pasman, T., Baptista, D., Van Riet, S., Truckenmüller, R.K., Hiemstra, P.S., Rottier, R.J., et al., 2021. Development of an In Vitro Airway Epithelial-Endothelial Cell Culture Model on a Flexible Porous Poly(Trimethylene Carbonate) Membrane Based on Calu-3 Airway Epithelial Cells and Lung Microvascular Endothelial Cells. *Membranes* 11 (3), 197. <https://doi.org/10.3390/membranes11030197>.
- Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., Migliaccio, A., 2020. ROS in cancer therapy: the bright side of the moon. *Exp. Mol. Med.* 52 (2), 192–203. <https://doi.org/10.1038/s12276-020-0384-2>.
- Reinisalo, M., Kärllund, A., Koskela, A., Kaarniranta, K., Karjalainen, R.O., 2015. Polyphenol Stilbenes: Molecular Mechanisms of Defense against Oxidative Stress and Aging-Related Diseases. *Oxid. Med. Cell. Longev.* 2015, 1–24. <https://doi.org/10.1155/2015/340520>.
- Rock, J.R., Rawlins, E.L., Onaitis, M.W., Hogan, B.L., 2009. Basal cells as stem cells of the mouse trachea and human conducting airways. *Dev. Biol.* 331 (2), 503. <https://doi.org/10.1016/j.ydbio.2009.05.438>.
- Rowell, T.R., Reeber, S.L., Lee, S.L., Harris, R.A., Nethery, R.C., Herring, A.H., Glish, G.L., Tarran, R., 2017. Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line. *Am. J. Physiol. -Lung Cell. Mol. Physiol.* 313 (1), L52–L66. <https://doi.org/10.1152/ajplung.00392.2016>.
- Sakagami, M., 2020. In vitro, ex vivo and in vivo methods of lung absorption for inhaled drugs. *Adv. Drug Deliv. Rev.* 161–162, 63–74. <https://doi.org/10.1016/j.addr.2020.07.025>.
- Silva, S., Bicker, J., Falcão, A., Fortuna, A., 2023. Air-liquid interface (ALI) impact on different respiratory cell cultures. *Eur. J. Pharm. Biopharm.* 184, 62–82. <https://doi.org/10.1016/j.ejpb.2023.01.013>.
- Singh, A.V., Maharjan, R.S., Kromer, C., Laux, P., Luch, A., Vats, T., Chandrasekar, V., Dakua, S.P., Park, B.-W., 2021a. Advances in Smoking Related In Vitro Inhalation Toxicology: A Perspective Case of Challenges and Opportunities from Progresses in Lung-on-Chip Technologies. *Chem. Res. Toxicol.* 34 (9), 1984–2002. <https://doi.org/10.1021/acs.chemrestox.1c00219>.
- Singh, A.V., Romeo, A., Scott, K., Wagener, S., Leibrock, L., Laux, P., et al., 2021b. Emerging Technologies for In Vitro Inhalation Toxicology. *Adv. Healthc. Mater.* 10 (18), 2100633. <https://doi.org/10.1002/adhm.202100633>.
- Srinivasan, B., Koll, A.R., Esch, M.B., Abaci, H.E., Shuler, M.L., Hickman, J.J., 2015. TEER Measurement Techniques for In Vitro Barrier Model Systems. *SLAS Technol.* 20 (2), 107–126. <https://doi.org/10.1177/2211068214561025>.
- Sundar, I.K., Javed, F., Romanos, G.E., Rahman, I., 2016. E-cigarettes and flavonoids induce inflammatory and pro-senescence responses in oral epithelial cells and periodontal fibroblasts. *Oncotarget* 7 (47), 77196–77204. <https://doi.org/10.18632/oncotarget.12857>.

- Wadgave, U., Nagesh, L., 2016. Nicotine replacement therapy: an overview. *Int. J. Health Sci.* 10 (3), 425–435.
- Wavreil, F.D.M., Hegglund, S.J., 2020. Cinnamon-flavored electronic cigarette liquids and aerosols induce oxidative stress in human osteoblast-like MG-63 cells. *Toxicol. Rep.* 7, 23–29. <https://doi.org/10.1016/j.toxrep.2019.11.019>.

WHO (2023) Tobacco. Available from: (<https://www.who.int/news-room/fact-sheets/detail/tobacco>).