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# A Vaper Paradox: The Relationship Between Electronic Cigarette Uses and SARS-CoV-2 Infection

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## Dedication

This dissertation is dedicated to my father, Phandpong, and my uncle, Johnny. Now that I am a PhD doctor, they can finally listen to me.

I want to dedicate this dissertation to my mother, Sureporn, for believing in me and supporting me.

Lastly, I want to dedicate this dissertation to my wife, Aerialle, for putting up with me by being patience and understanding. I thank you for your love and compassion…*Always*.

ABSTRACT OF THE DISSERTATION

A Vaper Paradox: Evaluation of the Relationship
Between Electronic Cigarette Uses and SARS-CoV-2 Infection

by

Rattapol Phandthong

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
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Dr. Prue Talbot, Chairperson

The relationship between electronic cigarette (EC) use and SARS-CoV-2 infection is poorly understood and contradictory with claims that vaping both increases and decreases the likelihood of contracting COVID-19. The objective of this dissertation was to determine how EC fluids and aerosols affect SARS-CoV-2 infection of human cells and tissues. Responses of human bronchial epithelial cells to EC fluids and aerosols and their individual constituents were evaluated using submerged cultures, air liquid interface (ALI) exposure in a cloud chamber, and ALI exposure in a Cultex® system, which produces authentic heated EC aerosols. Both monolayers of BEAS-2B cells and 3D organotypic cultures of EpiAirway tissues were studied. Results were generally in agreement across the three exposure platforms and the two cell models. EC aerosols made from fluids with nicotine, BLU™ EC aerosols, and pure nicotine increased ACE2 (the viral receptor) and TMPRSS2 activity, a protease essential for viral entry. In contrast to BLU™ EC, aerosols from JUUL™ “Virginia Tobacco” decreased TMPRSS2 activity. Using SARS-CoV-2 viral pseudoparticles, we demonstrated that exposure to EC fluids or aerosols with nicotine or to pure nicotine increased infection dose dependently. PG/VG also increased infection but

only when aerosols were made in the Cultex® exposure system, suggesting reaction products contributed to increased infection. Aerosols produced at both low (8 watt) and high (20 watt) powers increased nicotine-enhanced infection. EC fluid ingredients and aerosol nicotine dosimetry modulated the response of human bronchial epithelial cells to infection. Specifically, inclusion of benzoic acid reduced EC fluid pH, which reduced TMPRSS2 activity, providing protection against the enhanced infection produced by PG/VG and nicotine. This protection lasted at least 48 hours. Finally, JUUL™ “Virginia Tobacco” aerosols, which contain benzoic acid, protected against pseudoparticle infection, while BLU™ EC aerosols, which lack benzoic acid, did not. Our investigation demonstrates that the effect of ECs on SARS-CoV-2 infection are complex, and infection can either be increased or not affected depending on the ingredients and the pH of EC fluids, TMPRSS2 activity, and nicotine concentration.

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## Introduction

### Introduction to Electronic Cigarettes (ECs)

ECs are tobacco products that generate aerosol, which the EC user inhales through a mouthpiece (Trtchourian et al., 2010; 2011; NAS, 2018). EC models vary greatly in design, chemical constituents, and operating power, which makes evaluation of health effects challenging. Common EC components are a battery, heating coil/atomizer, tank or cartridge containing e-liquid, and a mouthpiece.

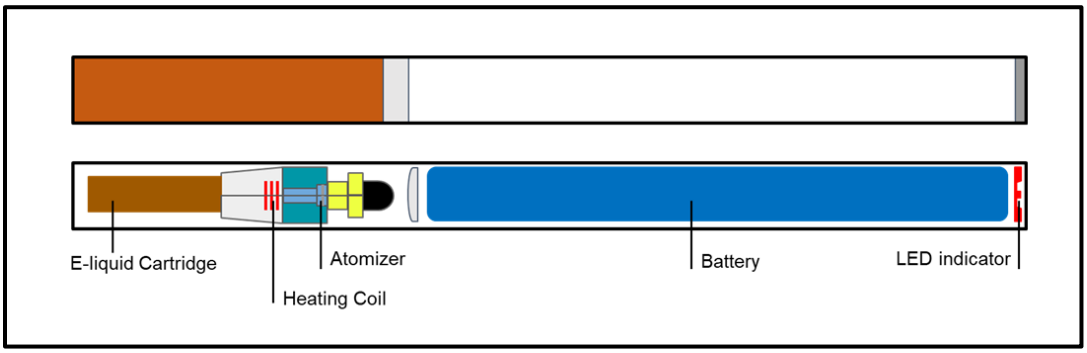


**Figure I.1. Diagram of an EC and its internal components.**

E-liquid, also called e-juice or vape juice, is a mixture of chemicals that are heated to produce aerosol. E-liquid consists of solvents, usually propylene glycol (PG) and vegetable glycerin (VG), flavor chemicals, and varying concentrations of nicotine, which can range from 0 to 60 mg/mL (Trtchourian et al. 2011; Davis et al., 2015; NAS 2018; Omaiye et al., 2021; 2022). The ratio of the two solvents varies from one manufacture to another, but often is 50PG/50G or 30PG/70G (Talih et al., 2018; Peace et al., 2016). Flavor chemicals are added to the e-liquids to flavor the aerosol and make ECs more appealing to users. Both solvents and flavor chemicals are food grade additives that are generally regarded as safe (GRAS) when ingested, but their effects on the respiratory system when inhaled are largely unknown. In fact, the Flavor Extract and Manufacturers Association

(FEMA) has explicitly stated that their GRAS designation applies only to ingested chemicals and not to those inhaled in EC aerosols (FEMA; 2018). Nicotine is a highly addictive chemical extracted from tobacco plants. The health effects of nicotine in tobacco cigarettes are well-document, and include nicotine dependency, cardiovascular disease, emphysema, Gastro Esophageal Reflux Disorder (GERD), and cancer (Le Foll et al., 2022; Kaijser et al., 1985; Chu et al., 2013; Beck et al., 1986; Mishra et al., 2015). While nicotine addiction has been documented in EC users (Snow et al., 2020; National Institute on Drug Abuse, 2020), other health consequences of inhaling nicotine from ECs are poorly understood. Each component in e-liquid will be discussed in detail in later sections.

*Construction of ECs*

The common components of ECs are a battery, atomizer, and cartridge/tank that stores e-liquid. During vaping, the battery activates an indicator, usually an LED light and heats the filament in the atomizer. Depending on the EC model, the battery may be non-rechargeable (disposable device), rechargeable, or replaceable (Williams et al., 2019; Brown et al., 2014). Batteries used to power ECs are usually nickel or lithium-based, with lithium being more common because they are smaller and can store a large amount of energy.

Depending on the product, e-liquids are either stored in a plastic or glass bottle (refill fluids), plastic cartridge, plastic pod, or a glass/plastic tank. The tank model can hold the largest volume of e-liquid and can be manually refilled. The cartridge and pod models are usually sold for one time use, after which they are discarded. As EC manufacturers continue to evolve their products, the cartridge designs are being replaced by pod models (Voos et al., 2019; Huang et al., 2019).

*Electronic cigarette generations*

ECs have evolved since their introduction in about 2006. Currently, ECs are classified into four different generations: cig-a-like (first generation), clearomizer (second generation), mods/tanks (third generation), and the pods (fourth generation). The early generation ECs were designed to resemble tobacco cigarettes. The second and third generation ECs are highly customizable by the user. Possible modifications include the use of different types of battery, atomizer, heating coils, wicking materials, and reservoirs size.

*First-generation: cig-a-like*

The first-generation ECs were designed to look and feel like traditional cigarettes, and therefore are often called cig-a-likes (Breland et al., 2016). The cig-a-like model has three different variations: 1-piece disposable, 2-piece cartomizer style (cartomizer with battery), and 3-piece style (cartridge, atomizer and battery).

The 1-piece disposable cig-a-like is a single unit containing a battery, atomizer and cartridge filled with e-liquid. The cig-a-like cannot be recharged or refilled and was designed to be discarded after the battery runs out of power or the e-liquid is depleted, generally after 200-800 puffs (Williams et al., 2019).

The 2-piece cartomizer is the most common cig-a-like on the market. Unlike the 1-piece model, it is composed of two parts: a battery and a cartomizer. The battery is rechargeable enabling reuse of the device. The cartomizer is prefilled and can be disconnected from the battery. Once the e-liquid is depleted, the cartomizer is discarded and replaced with a new one.

The 3-piece cartridge model is made up of a battery, atomizer, and a pre-filled cartridge. Like the 2-piece model, the battery is rechargeable, however, unlike the other models, the atomizing unit can be separated. The customization of this model influenced the preferred designs of later generations of ECs.

*Second-generation: Clearomizer*

The second-generation ECs are larger, and no longer resemble traditional cigarettes. The shape of clearomizer ECs is determined by the design of the rechargeable battery, which are often cylindrical, but may have other shapes such as rectangular. This generation of EC can be recognized by its clear atomizer unit or clearomizer, which contains an atomizer and a transparent reservoir that holds large volume of e-liquid and can be refilled. The clearomizers are sometimes called tank style ECs. The clearomizer units contain a preassembled wick and filament that can be replaced by the user. The wicking materials draw e-liquid in the tank to the coil, and upon activation, the coil surrounding the wick heats up and produces aerosol.

*Third generation: Mods*

The third generation ECs are commonly known as “mods”, because this generation offers the highest degree of modification. The mod ECs in many ways are similar to the second generation, but they extend the modification capabilities. Generally, the mod ECs are larger than other generations because they can hold larger and multiple batteries that can be removed and externally charged. Beyond the mechanical modification, the mod ECs have a display screen that shows device settings, such as voltage and power, and permits the user to customize the voltage and the wattage to generate varying volumes of aerosol (Etter et al., 2016).

*Fourth generation*: *Pods*

The pod ECs use low powered batteries and operate similarly to the first-generation cartomizer ECs. Unlike the first-generation ECs, the pods may contain very high concentrations of nicotine (60 mg/mL) (Pankow et al., 2017; Omaiye et al., 2019). Additionally, acids are added to the e-liquid to protonate nicotine to make the aerosol less harsh. Various acids have been used for this purpose, but benzoic acid is the one most often used (Harvanko et al., 2019). Since its introduction by JUUL™, the pod style ECs has become very popular (Huang et al., 2019). Due to its small size, it is concealable and predominately popular with high school age children.

## Effects of ECs on the Public Health

*Smoking cessation and harm-reduction*

Major campaigns and restrictions on indoor smoking have raised awareness of the diseases caused by tobacco use and helped to reduce cigarette smoking (Institute of Medicine, 1994). ECs are sometimes viewed as a safer alternative than tobacco cigarettes because their aerosols contain fewer chemicals (U.S. Department of Health and Human Services; USDHHS, 2014). Some EC manufacturers suggest their products are effective smoking cessation tools by associating their products with key words such as ““quit””, “smoke-free”, “switch”, and alternative” (Ramamurthi et al., 2016), but after several lawsuits, EC companies stopped advertising ECs for smoking cessation. Recent meta-analyses showed mixed and limited evidence supporting the idea that ECs are effective aids for smoking cessation (Hartmann-Boyce et al., 2020; US Preventive Service Task Force, 2021; Wang et al., 2021a).

The Hartmann-Boyce group at the University of Oxford, United Kingdom (UK), analyzed randomized clinical trial (RCT) data of adult who smoked and quit for longer than 6 months, then compared the quit rate of ECs with nicotine against NRT, nicotine free EC, and nicotine support and counselling (Hartmann-Boyce et al., 2020). These comparisons demonstrated low to moderate evidence that smokers benefit from using EC nicotine and had quit smoking for more than 6 months. The study concluded with moderate certainty that overall smokers benefit by using ECs with nicotine.

In contrast, the US Preventive Service Task Force (USPSTF; 2021) identified five RCTs (random clinical trials) on ECs as smoking cessation devices and find inconsistent results. These data were obtained from participants who either wanted to stop smoking or were attending a stop smoking service. The control group was using either NRT (nicotine replacement therapy) or nicotine-free ECs, while the other group used ECs with nicotine. Two of the studies concluded that those who used ECs with nicotine had a significantly higher chance of quitting that lasted longer than 6 months, the first reported 3% higher and the second reported 10% higher. However, the other 3 trials reported no significant difference between the two groups. In addition to this analysis, Wang et al. (2021a) conducted a meta-analysis study that analyzed observational trials and RCT data and found mixed results. The meta-analysis results of the observational data showed that EC product use was not significantly associated with quitting smoking or smoking cessation (odd risk; OR = 0.95, 95% confidence intervals; CI 0.77-1.16). While the analysis of RCT showed that EC use was more associated with quitting than conventional therapy (odd ratio (OR) = 1.55, 95% (CI) 1.17-2.06).

However, due to some data imprecision, the limited number of trials, and the low number of data points, the evidence is not sufficient to definitively conclude that ECs are effective tools for smoking cessation and further studies are needed.

*EC use among youth*

The rapid increased use of EC among youth in middle school and high school students is a pressing public health concern (Wang et al., 2018; Cullen et al., 2019; Lee et al., 2020b). The Surgeon General raised the issue of EC use among young adult and declared that the EC addiction is an epidemic among youth (CDC, 2021b ). Most ECs contained nicotine, which is particularly dangerous to children as nicotine exposure can impact learning and attention, harming a developing brain (Office of the Surgeon General, 2016).

ECs were introduced in the US market in 2007, and since then ECs have been the most preferred tobacco product among youth (U.S. Department of Health and Human Services, 2014). In 2015, JUUL™ introduced the 4$^{th}$ generation EC, which has a replaceable pod that rapidly became popular among youth and dominated EC sales (Huang et al., 2019) The pod-based design became popular among middle and high school students because these ECs contain appealing flavors, have sleek designs allowing better concealment, and efficiently deliver nicotine (Barrington-Trismis et al., 2018; Omaiye et al., 2019; Goniewicz et al., 2019).

A survey by Cullen and colleagues (2019) estimated that in 2019 EC use among high school student had increase by 7%, from 20.8% in 2018 (Wang et al., 2018) to 27.5% in 2019 (Cullen et al., 2019), Based on this estimation, more than 4.1 million high school students have used ECs (Cullen et al.,2019). In 2020, EC use decreased among high

school students from 27.5% to 19.6%, perhaps because the pandemic limited their access to ECs. Despite the encouraging decrease in EC use among youth, about 1 in 5 high school students have used ECs.

## EC Constituents/Chemicals

### *Nicotine*

Nicotine is an alkaloid that is extracted from tobacco leaves and the cause of tobacco addiction (Benowitz et al., 2009). While not all ECs contain nicotine, most do, and its concentration varies among the EC brands and refill fluids (Davis et al., 2015). Goniewicz et al. (2015) measured nicotine concentrations in 91 e-liquids purchased in US, South Korea, and Poland and found that nicotine concentration in the e-liquid samples ranged between 0 to 36.6 mg/mL. The concentrations of nicotine in 9 e-liquid samples deviated from the labeled by more than 20% and nicotine-free e-liquid contained traces of nicotine.

Davis et al. (2015) used high performance liquid chromatography to measure nicotine concentrations in 71 EC refill fluids, and 54 of the 71 refill fluids contained nicotine. 60% (35/54) of the refill fluids had inaccurate labeling of the nicotine concentration, which deviated from the label by more than 10%. Etter et al. (2017) assessed 11 popular e-liquids from different companies in the US, UK, France, and Switzerland. Nicotine concentrations on the label of the e-liquids were 16 to 48 mg/mL, while measured concentrations ranged from 15.5 to 52.0 mg/mL. In contrast to the study by Goniewicz et al., Davis's and Etter's group found that the measured nicotine concentration deviated from the label by about 10%.

Some ECs labeled 0 mg/mL of nicotine had significant quantities of nicotine when analyzed by gas chromatography/mass spectrometry (GC/MS) (Omaiye et al. (2017). These samples were subsequently shown to be counterfeit ECs products. Some EC users choose nicotine free products to avoid addiction. Clearly mislabeling of refill fluids opens the possibility of inadvertent addiction to nicotine.

These studies show that nicotine concentrations vary among EC brands and e-liquids. Significant inaccuracies have been found between nicotine content on the label and nicotine measured in the e-liquid, which could lead to accidental exposure to nicotine and unwanted health effects including addiction.

*Solvents*

ECs contain solvents such as, propylene glycol (PG) and vegetable glycerin (VG), which are mixed with flavor chemicals and nicotine. Upon activation of an EC, the atomizer heats up the coil and aerosolizes the solvent to produce aerosols.

PG and VG are the most common solvents in e-liquids, while ethylene glycol has been infrequently used (Hahn et al., 2014; Hutzler et al., 2014). PG is a clear, colorless fluid with relatively low viscosity. It is used as a humectant to maintain moisture in cigarettes, cosmetics, food, and pharmaceutical products (USDHHS, 2015). PG is generally regarded as safe (GRAS) for ingestion by the Flavor and Extract Manufacturers Association (FEMA). PG is used in oral liquid medicine and in food. While PG is considered safe for ingestion, it is not recognized by FEMA as safe for inhalation. Like PG, VG is GRAS by FEMA when ingested and has been widely used in food, cosmetics, and pharmaceutical (USDHHS, 2020). In e-liquids, PG and VG are mixed at various ratios; often e-liquids contain 50%-70% of PG with the remainder being VG. The PG:VG ratio

affects the emission profile of EC aerosols. Aerosols produced from e-liquid with high PG produced more “throat hits”, while high VG aerosols produced smoother inhalation (Jahnson, 2016).

When PG and VG are aerosolized by heating, they produce reaction products, such as acetaldehyde, formaldehyde and acrolein, that can be toxic (Ooi et al., 2019; Son et al., 2019; Kienhuis et al., 2015). For example, short-term exposure to PG/VG aerosol inhibited glucose uptake in human primary lung epithelial cells, reduced mitochondrial ATP synthesis, and increased transepithelial solute permeability (Woodall et al., 2020). *In vivo* exposure of PG/VG aerosols increased cellular oxidation of proteins and elevated MUC5AC in the bronchoalveolar lavage fluid of mice (Glynos et al., 2018).

*Flavor chemicals*

While there are more than 10,000 flavor chemicals available to EC manufacturers (Burdock, 2009), the data on flavor chemical inhalation are limited. Although many of the flavor chemicals are considered GRAS when used for ingestion, they have generally not been evaluated for inhalation safety (FEMA, 2018). A number of studies have demonstrated that flavor chemicals in ECs can have adverse effects on respiratory cells (Bahl et al., 2012; Muthumalage et al., 2017; Hua et al., 2020; Omaiye et al., 2020; 2022). Additionally, when heated, flavor chemicals degrade and produce reaction products, such as aldehydes (Tierney et al., 2016), free radicals (Muthumalage et al., 2017) and benzene, which is a carcinogen (Pankow et al., 2017).

*Organic acids in 4$^{th}$ generation ECs*

Organic acids are common ingredients in 4$^{th}$ generation e-liquids. Their purpose is to protonate free-base nicotine and produce nicotine salt, which is less harsh and

attractive to novice users (Pankow et al., 2001). Harvanko et al. (2019) identified six acids that are commonly used in the poplar EC brands: lactic, benzoic, levulinic, salicylic, malic, and tartaric acid.

In 2015, JUUL™ incorporated benzoic acid into their e-liquid and subsequently other manufacturers (Vuse Alto, MyBlu) offered similar products. Often, the products with acids contain high concentrations of nicotine (up to 60 mg/mL), The addition of acids reduces the harshness of nicotine and provides a more pleasant sensation for the user (Duell et al., 2020). While these acids are used in food products and are generally safe when ingested, there is little information on their safety when inhaled. For example, benzoic acid has been used as a preservative for food and beverages, but when heated in an EC, the acid can breakdown and produce cancer-causing benzene (Pankow et al., 2017).

*Reaction products and metals*

During storage, e-liquid ingredients such as PG, VG and flavor chemicals can interacts at room temperature and form a new chemical like aldehyde propylene glycol acetal. (Erythropel et al., 2019b; Klager et al., 2017). Upon EC activation, e-liquid is heated to produce aerosol, which can form reaction products, such as formaldehyde and acetone, and volatile organic chemicals, such as acrolein and acetaldehyde (Goniewicz et al., 2014; Lim et al., 2017). Although these reaction products are not added intentionally and are usually presence at low concentrations, they can accumulate and may have adverse effect health (Erythropel et al., 2019a). These reaction products include formaldehyde, a known human carcinogen and diacetyl, which causes bronchiolitis

obliterans (Behar et al., 2016; Swenberg et al., 2012; Uchiyama et al., 2020; van Rooy et al., 2006).

Metals such as zinc, copper, lead, nickel, and chromium have been reported in EC aerosols and EC fluids (Aherrera et al., 2017; Goniewicz et al., 2014; Hess et al., 2017; Olmedo et al., 2018; Williams et al., 2013; 2017; 2019). These metals likely originate from the joints, wire, and metallic coil in the atomizer. (Olmedo et al., 2018; Williams et al., 2017). Some EC devices can vary their power, which can increase metals emitted into the aerosols. Metal concentrations in some EC aerosols exceeded current occupational or environmental standards (Olmedo et al., 2018). Additionally, during storage when the metal-coil comes in contact with the e-liquid, metals can leech into the e-liquid. Hess et al., (2017) found cadmium, chromium, lead, and nickel in e-liquids touching unused cartomizer coils. Among the metals, Zn transfers efficiently into aerosol and is commonly present in different EC brands and in relatively high concentrations compared to other EC metals (Olmedo et al., 2018; Williams et al., 2013; 2017; 2019). Urine samples from EC users had elevated levels of Zn, which were correlated with oxidative damage to DNA (Sakamaki-Ching et al., 2019), supporting the conclusion that metal in EC aerosols can produce harm.

## Effect of ECs on the Respiratory System

### *Inflammation and injury in the respiratory system caused by EC ose*

Due to the delicate nature of lung tissue, mild irritation and inflammation can be damaging and in some cases life threatening (Moldoveanu et al., 2008). This is a concern because major ingredients in EC aerosols, such as PG, VG, and flavor chemicals, can cause irritation in the lung. The bronchial lining in healthy EC users was brittle, easily torn,

and abnormally red (Ghosh et al., 2018); and in cases of electronic cigarettes and vaping product use-associated lung injury (EVALI), EC users presented with serious damage to the lung and required hospitalization (Carter et al., 2017).

Additionally, bronchoalveolar lavage fluid and brushings from EC users had more immune cells, oxidative stress response biomarkers, and mucus protein than the non-users (Stadut et al., 2018; Gosh et al., 2018; Song et al., 2019; Singh et al., 2019). Significant elevation of IL-2, IL-1β, IL-6, and IFN-γ were observed in the airway of EC users, and an increase of these specific biomarkers has been associated with expression of lung oncogenesis and chronic obstruction pulmonary disease in tobacco smokers (Ridker et al., 2017; Reynolds et al., 2018; DeCotiis et al., 2016). Increased MUC5AC and MUC5B were observed in the bronchial epithelia of the EC users, suggesting a decline in lung function and airway clearance (Reidel et al., 2017; Ghosh et al., 2018; Song et al., 2019).

*EC and Vaping Product Use-Associated Lung Injury (EVALI)*

A case report showed that EC aerosol was associated with lipoid pneumonia, which led to respiratory failure (Viswam et al., 2018). EC use may also be associated with eosinophilic pneumonitis, which is a rare disease caused by accumulation of white blood cells in the lungs (Thota et al., 2014). Alveolar hemorrhage has also been linked to EC use (Villeneuve et al., 2020). It is possible that EC aerosol causes the alveolar space to be brittle, as observed during bronchoscopy of EC users (Agustin et al., 2018; Ghosh et al; 2018). These plus additional case reports (Hua et al 2016; 2020) and EVALI (Schier et al., 2019; Gordon et al., 2020; Diaz et al., 2020; Werner et al., 2020) have linked EC use

to multiple life-threaten pulmonary illnesses. EVALI alone has caused 2,807 hospitalizations and 68 deaths as of 2021 (CDC, 2021a).

The symptoms of EVALI include fever, vomiting, respiratory distress, abdominal pain and difficulty breathing, which can require intensive care and ventilation assistance (Agustin et al., 2018; Kalininskiy et al., 2019). Patients who are diagnosed with EVALI often have high concentrations of leukocytes in their bronchoalveolar lavage fluid (BAL) (Kalininskiy et al., 2019). Alveolar macrophages from BAL contained numerous lipid vacuoles, which were associated with lipoid pneumonia in the EVALI patients (Guerrini et al., 2020). The x-rays or CT scans of EVALI patient's chest showed high opacity, ground-glass infiltrates, and sub-pleural sparing, which indicate severe inflammation and obstruction in the alveoli (Agustin et al., 2018; Kalininskiy et al., 2019; Kligerman et al., 2021).

EVALI cases were associated with the use of EC products containing tetrahydrocannabinol and vitamin E-acetate (Butt et al., 2019), although there could be additional factors effecting EVALI. Following inhalation, vitamin E acetate is postulated to incorporate in the phospholipids of the alveolar surfactant and reduce its function. This alteration in the physiochemical properties of the alveolar surfactant increased surface tension inside the alveoli, which decreased lung compliance and reduced the surface area for gas exchange, making the breathing difficult (Blount et al., 2019; DiPasquale et al., 2020; Lee et al., 2020a).

*The effect of EC use on the immune response*

It is well documented that the use of cigarettes impairs the immune system and is a risk factor for respiratory viral infection (Gotts et al., 2019; Jiang et al., 2020). Recent

reports suggest EC use also compromises the immune system. Nasal scrapes from EC users showed elevation of immunosuppression genes (Martin et al., 2016). Staudt et al. (2018) showed that the expression of alveolar macrophage genes was altered in EC users. For example, cinnamaldehyde, a flavor chemical used in ECs, impaired the efficiency of macrophage phagocytosis ( Clapp et al., 2017). Additionally, EC use increased neutrophil granulocyte-related and neutrophil extracellular trap–related protein, and upregulated neutrophil activation and degranulation genes (Reidel et al., 2018; Pozuelos et al., 2022).

The host-defense responses of EC user to infection were evaluated in a clinical study using attenuated influenza virus (Rebuli et al., 2020). Compared to nonsmokers or cigarette smokers, EC users were not more susceptible to infection,

EC use altered immune-gene expression and decreased the release of cytokines critical for antiviral responses, which in turn delayed the production of IgA antibody specific to influenza virus. *NT5E*, which encodes CD73, cell surface protein necessary for an innate anti-viral immune response, was highly down regulated in EC users. The decrease in the CD73 protein delayed activation of the innate anti-viral immune response and subsequently delay adaptive immune responses, which produce virus neutralizing antibody.

In vivo studies with mice concluded that EC use reduced anti-viral immune protection. Mice subjected to whole body exposure to EC aerosol, then infected with influenza A (H1N1) had significantly elevated viral concentrations in their lavage fluid, delayed recovery time, and increased mortality when compared to unexposed mice. (Sussan et al., 2015). Similarly, Madison et al. (2019) showed that mice exposed to EC

aerosol then exposed to influenza A (H3N2) exhibited an increase in mortality and weight loss, and impaired lung lipid metabolism that led to impaired macrophage function and worsening illness.

Another *in vitro* study found that e-liquid reduced anti-viral immunity and increased susceptibility to viral infection (Wu et al., 2014). Primary human airway cells exposed to e-liquid had an altered airway defense against human rhinovirus infection, and after infection, aerosol exposed cells exhibited a higher concentration of virus than the control groups (Wu et al., 2014). These studies support the conclusion that EC aerosol exposure impaired host anti-viral defense leading to an increase in viral infection and altered inflammatory cytokines.

*Relationship between EC use and SARS-CoV-2 infection*

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes COVID-19, was first identified in December 2019 and has rapidly spread causing a worldwide pandemic (Li et al., 2020; Morens and Fauci et al., 2020). SARS-CoV-2 is an enveloped, positive-stranded RNA virus from the Betacoronavirus genus and shares 79% genomic similarities with SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) (Hu et al., 2020), which establishes infection by membrane fusion with the host cells (Belouzard et al., 2012). SARS-CoV-2 is comprised of a nucleocapsid, membrane, envelope, and spike (S) protein, which is responsible for viral attachment to the host cells receptor, angiotensin-converting enzyme 2 (ACE2; Cai et al., 2020; Hoffman et al., 2020).

SARS-CoV-2 spike protein is a homotrimer, and each subunit of the trimer is comprised of S1 and S2 subunits, which are modified by furin a transmembrane protease

serine 2, prior to infecting cells. The first cleavage occurs during viral biosynthesis in the Golgi apparatus of the infected cells that produce virus (Shang et al., 2020; Hoffman et al., 2020). Furin in the Golgi apparatus recognizes the multibasic site, Arg-Arg-Ala-Arg, and cleaves the S1 and S2 subunits, which remain non-covalently associated (Peacock et al., 2021). This cleavage by furin activates the S reactive binding domain (RBD), by changing 1 of the 3 RBDs from the inactive 'down' conformation into the active 'up' conformation, which facilitates viral attachment to the host cell's ACE2 receptors. This allows the spike protein to bind to ACE2 ~20 times more efficiently than SARS-CoV making SARS-CoV-2 highly contagious (Wrap et al., 2020; Yuan et al., 2020). The second cleavage is brought about by TMPRSS2 or cathepsin-L, which recognize an internal site on the S2 subunit. During infection, the 'up' RDB of the spike binds to ACE2, then S2 is either cleaved at the cell surface by TMPRSS2 or by cathepsin L in an endosomal vesicle after the virus is internalized by endocytosis (Jackson., 2022; Huang et al., 2006). However, SARS-CoV-2 infection is more dependent on TMPRSS2 spike modification than cathepsin-L in HEK293T cells expressing human ACE2 (Ou et al., 2021).

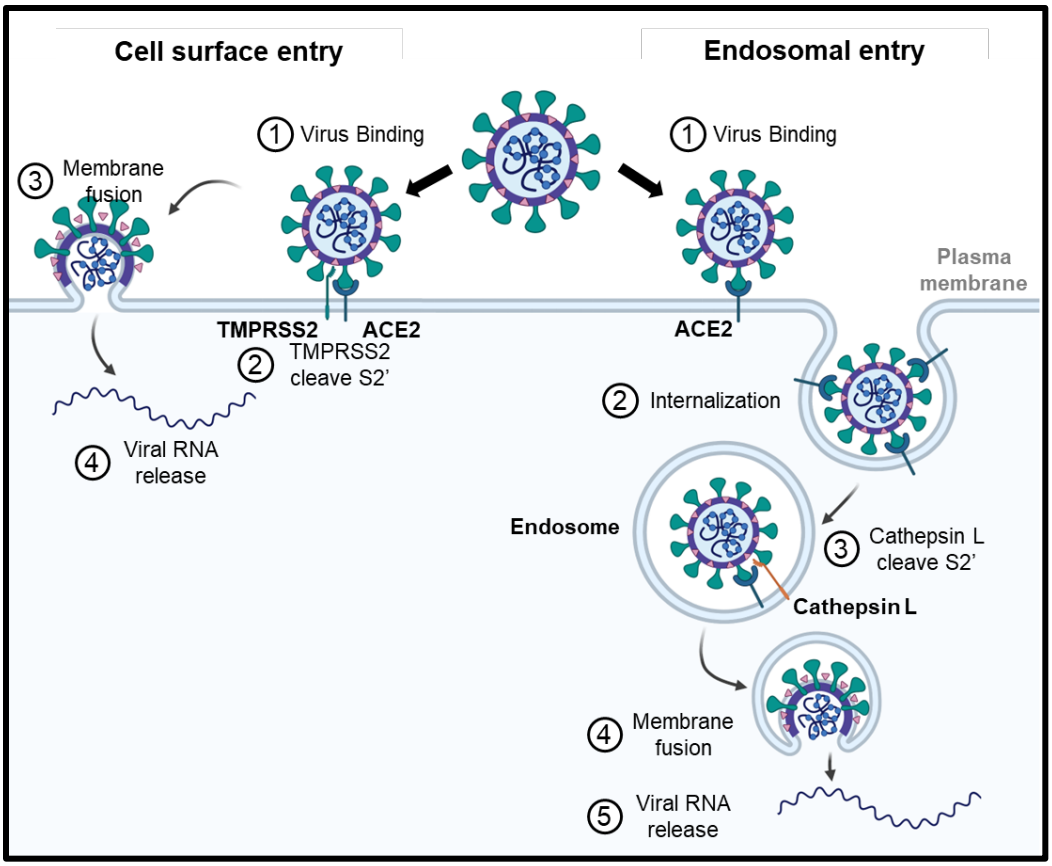


**Figure I.2. Two mechanisms of SARS-CoV-2 entry into cells**

Since the COVID-19 outbreak began in 2019, SARS-CoV-2 has infected an estimated 111 million people worldwide and resulted in the death of 1 million people in the United States (World Health Organization, 2020; 2022). The scientific community and the government have implemented multiple strategies to control the spread of COVID-19 (Honein et al., 2020; Riva et al., 2020), including development of novel mRNA vaccine technologies (Polack et al., 2020).

At the beginning of the pandemic, one strategy was to find prophylactic medicines to protect the public against SARS-CoV-2 infection. Nicotine and tobacco products were speculated to help prevent infection, as hospital observational studies reported a lower prevalence of infection in tobacco users than in the general population (Table 1). It has

been questioned whether these data, collected during times when hospital were severely stressed, are accurate and complete (van Westen-Lagerweij et al., 2021; Usman et al., 2021).

**Table I.1: Early Hospital Observational Studies of Smokers and SARS-CoV-2 Infection.**

| Studies | Country | Sample Size N | Active Smokers N (%) |
|---|---|---|---|
| Zhang et al., 2020 | China | 140 | 7 (5%) |
| Guan et al., 2020 | China | 1085 | 21 (1.9%) |
| Huang et al., 2020 | China | 41 | 3 (7.3%) |
| Yang et al., 2020 | China | 52 | 2 (3.8%) |
| Richardson et al., 2020 | USA | 3567 | 558 (15.6%) |
| Goyal et al., 2020 | USA | 393 | 20 (5.1%) |
| Cummings et al.,2020a | USA | 257 | 33 (12.8%) |
| Miyara et al., 2020;2022 | France | 482 | 34 (7%) |

These observations led to the hypothesis that tobacco smoke and nicotine prevent SARS-CoV-2 infection. Hypothetical reports (Changeux et al., 2020; Farsalinos et al., 2020) speculated that nicotine protects against SAR-CoV-2 infection by: (1) maintaining an imbalanced hyperinflammatory response induced by heightened levels of circulating cytokines during an active SAR-CoV-2 infection, and (2) possibly blocking cellular α7 nicotinic acetylcholine receptor (α7nAChR) interactions with viral spike protein. Spike protein was shown *in silico* to contain a nAChR binding motif similar to nicotinic-neurotoxin agents (Lagoumintzis et al., 2021). As a cholinergic agonist, nicotine may reduce pro-inflammatory cytokines during infection and prevent the infection from worsening. As a result, nicotine-product users may be protected from developing severe symptoms that require intensive care.

However, the nicotine hypothesis was never proven experimentally. In fact, the early hospital studies were criticized for several methodological issues, particularly that

hospital data were cross-sectional studies, which are the weakest form of observational studies in epidemiology (Van Western-Lagerweij et al., 2021). The nicotine hypothesis contradicts current knowledge about smoking and tobacco use, which shows that smoking causes several lung diseases (Balbi et al., 2010), reduces lung function (Tantisuwat et al., 2014), increases viral and bacterial infection (Arcavi et al., 2008), and impairs the immune system (Qiu et al., 2017; Strzelak et al., 2018). Thus much knowledge regarding the effects of smoking on disease contradicts the idea that nicotine or smoking reduces the risk of contracting SARS-CoV-2 and decreases the likelihood of becoming severely infected.

In contrast to the previous observational studies (Table 1), recent work supports the idea that smoking increases the risk of SARS-CoV-2 infection and the risk of COVID-19 related hospitalization. Jackson et al., (2021) reported that current smokers are 1.8 times more likely to be confirmed with COVID-19 relative to non-smokers, and speculated that hand-to-mouth action and social smoking increase the likelihood of contracting the COVID-19. Hopkins et al., (2020) analyzed data from the “Zoe COVID-19 symptom study app”, which has a collection of 2.4 million reports, and found that current smokers were likely to report the three classic covid symptoms (cough, fever, breathlessness).

Other observations support the conclusion that smoking increases the risk of SAR-CoV-2 infection. Experimental data involving human subjects found that ACE2 expression is upregulated in the lungs of smokers compared to non-smokers (Zhang et al., 2020b, Leung et al., 2020, Cai et al., 2020, Brake et al., 2020, Smith et al., 2020, Muus et al., 2021), which may facilitate infection of the host cells. Cai et al., (2020) point out that ACE2 receptors are mostly expressed in the goblet cells, and extended use of tobacco led to goblet cell hyperplasia, which increased the abundance of ACE2 positive cells that can be infected by the virus. Smith et al (2020) showed that expression of ACE2 in smokers is

30-55% higher than in the lungs of non-smokers and former smokers. Additionally, smokers who used more than 80 packs/year exhibited a two-fold higher ACE2 expression than those who smoked less than 20 packs/year. Muus et al., (2021) performed a single-cell meta-analysis, which assessed cell-type-expressing ACE2, TMPRSS2, and cathepsin L, and found a strong correlation between ACE2 expression and smoking status in different cell types. ACE2 levels were higher in former and current smokers in the basal and secretory cells. Additionally, bronchial brushings showed upregulation of ACE2 and TMPRSS2 in the current smokers. Increases in ACE2 and TMPRSS2 in the lung suggest that SARS-CoV-2 would bind and penetrate the respiratory cells of smokers more efficiently than in non-smokers (Rodrigues et al., 2021; Zamorano Cuervo et al., 2020).

A growing body of scientific evidence support the conclusion that nicotine and tobacco cigarettes do not provide protection against SARS-CoV-2 infection, and worsen COVID-19 progression. Russo et al. (2020) found that 0.1 μM of nicotine induced ACE2 expression in human bronchial epithelial cells by interaction with the α7-nAChR (α-7 nicotinic acetylcholine receptor). Tobacco smoke can also activate the aryl hydrocarbon receptor (AhR), which can transcriptionally upregulate the expression of ACE2 (Lv et al., 2021). An in vitro study using airway stem cells found that short-term exposure to cigarette smoke increased SARS-CoV-2 infection and reduced the innate immune and airway stem cells repair response, leading to an increase in disease severity (Purkayastha et al., 2020). A meta-analysis of 19 studies concluded that smoking is a risk factor for worsening the progression of COVID-19, and smokers have a higher (1.91 times) likelihood of severe COVID than non-smokers (Patanavanich and Glantz 2020). Clift et al (2022) performed an observational meta-analysis and mendelian randomization on a United Kingdom cohort. The meta-analysis found a strong association between smoking and increased risk

for COVID-19, while the mendelian randomization provided genetic evidence that a lifetime of smoking led to a higher risk for hospitalization and death due to severe COVID-19 (Clift et al., 2022). Furthermore, a clinical trial study using nicotine patches on severe COVID-19 patient did not find that nicotine treatment provide protection against SARS-CoV-2 infection or alleviate infection (Labro et al., 2022). Data supporting the idea that smoking can increase susceptibility to SARS-CoV-2 and increase the likelihood of severe COVID-19 is in agreement with studies on other viruses showing that viral infection is more likely and more severe in smokers (Arcavi et al., 2004; Jiang et al., 2020).

Some smokers have acknowledged that they are at a higher risk for ARS-CoV-2 infection and are motivated to quit (Kowitt et al., 2020), but those who have high nicotine dependence and are unable to quit nicotine have switched from smoking to other nicotine replacement therapies including EC (Klemperer et al., 2020). In spite of an increase in EC use, especially among adolescents (Singh et al., 2016; King et al., 2020), very little work has been done on the relationship between EC use and SAR-CoV-2 infection. Like tobacco cigarette smoke, EC aerosols with high concentrations of nicotine could increase ACE2 expression in the respiratory epithelium leading to increased SAR-CoV-2 infection. In addition to nicotine, EC aerosols contain PG, VG, and flavor chemicals, which could also contribute to SARS-CoV-2 infection and severe COVID-19.

Currently, the data on EC use and SARS-CoV-2 are limited and contradictory. Hospital observational data found that EC use is not a risk factor of SARS-CoV-2 infection nor a cause for severe COVID-19 (Jose et al., 2021; Burnett-Hartman et al., 2022). Jose et al. (2021) performed logistic regression models to assess the correlations between EC use and SAR-CoV-2 susceptibility with data from 69,264 patients, which contained smokers and EC users. This study concluded that EC users were not likely to be

diagnosed with COVID-19 (OR 0.93; CI 0.69 -1.25; $p$=0.63) and smokers had a decreased risk of infection (OR 0.43; CI 0.35 - 0.53; $p$<0.001). Burnett-Hartmann et al. (2022) performed a multivariable regression model on data from 126,475 individuals and did not find association between EC use and SARS-CoV-2 infection. The analyses determined the risk of SARS-CoV-2 infection for both former and current EC users. Both regression models failed to show an association between SARS-CoV-2 infection and former EC users (hazard ratio (HR) = 0.99; CI 0.83-1.18) or current EC users (HR = 1.089; CI 0.76-1.52). These clinical observational studies support the conclusion that EC use is not a risk factor for SARS-CoV-2 infection, although EC use had been shown to impair the immune response in humans and increase the severity of viral infection severity in mice (Rebuli et al., 2020; Sussan et al., 2015; Madison et al. 2019). It is noteworthy that in both studies, the confidence intervals are wide for correlations between current EC users and SARS-CoV-2 infection, suggesting the data are noisy due to some unlaying factors.

In contrast, *in vivo* studies with mice found that whole body exposure to EC aerosols and nicotine aerosols elevated ACE2 expression, which increased the likelihood of SARS-CoV-2 binding to cells and presumably caused more infections (Wang et al., 2020; Naidu et al., 2021; Masso-Silva et al., 2021). Sub-chronic exposure of mice to EC aerosol emitted from 25 mg/mL nicotine increased ACE2 levels, particularly in female mice, which had ~2-fold increase compared to the control (Wang et al., 2020). Similar studies observe similar results that is EC aerosol or nicotine exposures increased ACE2 in mice, but showed that male mice had higher responses to the exposures and ACE2 increased more than female mice (Naidu et al., 2021 and Lallai et al., 2021). Furthermore, nicotine in the EC aerosol activated α7-nAChR in mice causing elevation of ACE2. These data support the prior observation that nicotine in cigarette smokers increased ACE-2 via

α7-nAChR activation (Russo et al., 2020). Flavor chemicals in authentic mint JUUL™ pods also increased ACE2 expression in mice (Masso-Silva et al., 2021). These *in vivo* studies reach the conclusion that EC aerosols with nicotine or without nicotine increased ACE2 mRNA expression and ACE2 abundance in the lung of mice.

While there are limited human data on EC use and SARS-CoV-2 infection, *in vitro* studies using human bronchial epithelial cells (BEAS-2B) and primary respiratory cells showed that EC products increased ACE2 levels and ACE2 activity, which increased SARS-CoV-2 infection. Submerged treatment of BEAS-2B cells with Watermelon-flavored refill fluid increased ACE2 transcripts in BEAS-2B cells (McAliden et al., 2021). JUUL™ "Virginia Tobacco" in submerged cultures also increased ACE2 enzymatic activity in the primary respiratory cells, and this correlated with increased cell susceptibility to SARS-CoV-2 pseudoparticle infection (Ghosh et al., 2022).

Despite differences in experimental models, exposure protocols, and nicotine concentrations, data from these *in vitro* studies of human cells and the *in vivo* mouse studies complement each other and suggest that nicotine induced an increase in ACE2 in mice that can be extrapolated to human respiratory cells.

Although the relationship between tobacco product use and SARS-CoV-2 infection has been controversial, a growing body of evidence supports the idea that tobacco smoke, EC aerosols, and nicotine increase ACE2, which could exacerbate SARS-Cov-2 susceptibility and infection. Additional experiments using laboratory-controlled conditions are critical to understand the relationship between EC use and entry of the SARS-CoV-2 virus into cells of the human respiratory system.

The purpose of this dissertation was to test the hypothesis that EC aerosols increase the likelihood of SARs-CoV-2 infection in human bronchial epithelial cells. In vitro

exposures were done at the air liquid interface (ALI) using both monolayer cultures of BEAS-2B cells and 3D organotypic cultures of bronchiolar epithelium. The latter contain basal cells, ciliated cells and mucous-secreting cells, and represents the best model currently available for this type of study. ACE2 levels and TMPRSS2 activity were measured. Infection of cells was quantified using SARs-CoV-2 viral pseudoparticles that carried a green fluorescent marker. Both authentic EC aerosols and aerosols made from fluids containing individual EC chemicals or mixtures were tested to isolate an effect to a particular chemical or mixture. Our data will show that the factors that affect SARs-CoV-2 infection are complex and depend on the composition of the e-liquid. Our data help resolve the controversy in the literature regarding tobacco product use and COVID-19 and define a better strategy for studies on ECs and COVID-19 that should improve the quality of future data.

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# CHAPTER 1

## New Insights into How JUUL™ Electronic Cigarette Aerosols and Aerosol Constituents Affect SARS-CoV-2 Infection of Human Bronchial Epithelial Cells

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**Abstract**

The relationship between the use of tobacco products and SARS-CoV-2 infection is poorly understood and controversial. Most studies have been done with tobacco cigarettes, while few have examined the effect of electronic cigarettes (ECs) on SARS-CoV-2 infection. We tested the hypothesis that EC fluids and aerosols with high concentrations of nicotine promote SARS-COV-2 infection by increasing viral entry into human respiratory epithelial cells. Responses of BEAS-2B cells to authentic JUUL™ aerosols or their individual constituents (propylene glycol (PG)/vegetable glycerin (VG) and nicotine) were compared using three exposure platforms: submerged culture, air-liquid-interface (ALI) exposure in a cloud chamber, and ALI exposure in a Cultex® system, which produces authentic heated EC aerosols. SARS-CoV-2 infection machinery was assessed using immunohistochemistry and Western blotting. Specifically, the levels of the SARS-CoV-2 receptor ACE2 (angiotensin converting enzyme 2) and a spike modifying enzyme, TMPRSS2 (transmembrane serine protease 2), were evaluated. Following each exposure, lentivirus pseudoparticles with spike protein and a green-fluorescent reporter were used to test viral penetration and the susceptibility of BEAS-2B cells to infection. Nicotine, EC fluids, and authentic JUUL™ aerosols increased both ACE2 levels and TMPRSS2 activity, which in turn increased viral particle entry into cells. While most data were in good agreement across the three exposure platforms, cells were more responsive to treatments when exposed at the ALI in the Cultex® system, even though the exposures were brief and intermittent. In the Cultex® system, PG/VG, PG/VG/nicotine, and JUUL™ aerosols significantly increased infection above clean air controls. However, both the PG/VG and JUUL™ treatments were significantly lower than nicotine/PG/VG. PG/VG increased infection only in the Cultex® system, which produces heated aerosol. Our data

are consistent with the conclusion that authentic JUUL™ aerosols or their individual constituents (nicotine or PG/VG) increase SARS-CoV-2 infection. The strong effect produced by nicotine was modulated in authentic JUUL™ aerosols, demonstrating the importance of studying mixtures and aerosols from actual EC products. These data support the idea that vaping increases the likelihood of contracting COVID-19.

## Introduction

COVID-19 (corona virus disease-2019), a serious respiratory illness caused by the SARS-CoV-2 virus (severe acute respiratory syndrome coronavirus 2), has resulted in the death of over 1,000,000 people in the United States and an estimated 15,000,000 people worldwide (World Health Organization, 2020; 2022). Smoking can lead to lung diseases, including cancer and increases in viral infection (Arcavi et al., 2004; USDHHS, 2014), and while there has been intense interest in the relationship between tobacco product/nicotine use and COVID-19 infection, progression, and severity, this relationship is currently poorly understood and sometimes contradictory. Patient-derived data have sometimes led to the conclusion than smoking protects against COVID-19 (Simons et al., 2021; Changeux et al., 2020; Farsalinos et al., 20200, while most studies, including several recent meta-analyses, have concluded that smoking is a risk factor for the progression of COVID-19 (Patanavanich et al., 2020, Jackson et al., 2020; Hopkinson et al., 2020) and that patients with a smoking history have a higher probability of developing more severe symptoms of COVID-19 (Shastri et al., 2021).

Several studies have addressed the effect of smoking on COVID-19 experimentally using *in vitro* and animal models. SARS-CoV-2 viral fusion with the host cell is aided by TMPRSS2 (transmembrane serine protease 2), an enzyme that modifies the viral spike protein, enabling it to fuse with the host cell (Hoffmann et al., 2020). The ACE2 receptor (angiotensin converting enzyme 2) for SARS-CoV-2 spike protein is elevated in biopsies from the respiratory tract of human smokers compared to non-smokers, supporting the idea that smokers are more susceptible to COVID-19 (Smith et al 2020; Zhang et al., 2020; Leung et al., 2020; Cai et al., 2020a; Brake et al, 2020). Smith et al (2020) also found that smokers had elevated cathepsin B, an enzyme involved in

spike processing following infection. In a single cell meta-analysis across various tissues, smoking was correlated with increased levels of ACE2 and TMPRSS2, which may contribute to COVID-19 pathogenesis (Muss et al., 2021). TMPRRS2 expression was differentially regulated in different respiratory cell types of smokers (Muss et al., 2021). Studies that directly examined viral entry into human cells concluded that cigarette smoking increases SARS-CoV-2 infection (Purkayastha et al., 2020; Ghosh et al., 2022). One group further showed that cigarette smoking inhibited the airway basal cell repair processes and reduced the response of the innate immune system by suppressing interferon β-1 (Purkayastha et al., 2020), both factors that may contribute to the severity of the disease.

Electronic cigarettes (ECs) are nicotine delivery devices that have gained popularity in the last decade (Trtchourian et al., 2011; NAS, 2018; Becker et al., 2021). ECs heat e-liquids to produce aerosols that contain numerous chemicals, some of which are distinct from those in tobacco smoke (Hua et al., 2019; Omaiye et al., 2020; USDHHS, 2010). The relationship between EC use and COVID-19 is not well understood. EC aerosols can have multiple adverse effects on the respiratory system (Pisigner et al., 2017; Gotts et al., 2019; NAS, 2018; Hua 2016; Hua et al.,2020). These include negative effects on lung physiology and the immune response, which may make fighting and recovery from a COVID-19 infection more difficult.

Several in vivo studies with mice and in vitro studies with human cells have investigated the effects of vaping and SARS-CoV-2 infection. Elevated ACE2 activities were found in the bronchial-lavage fluid from EC users and smokers (Ghosh et al., 2022). Full body exposures to “Mint” but not “Mango” aerosols generated from JUUL™ pods increased ACE2 levels in mouse lung (Masso-Silva et al., 2020 Three additional lung

studies found elevated ACE2 levels following treatment with aerosols containing a mixture of PG (propylene glycol)/VG (vegetable glycerol) and nicotine (Wang et al., 2020; Lallai et al., 2021; Naidu et al., 2021). While these studies reported gender differences, they were not in agreement with respect to the gender affected.

The effects of EC use and ACE2 levels has been studied in vitro with cultured human cells from the respiratory system. BEAS-2B cells treated with e-liquids in submerged culture showed that PG/VG elevated ACE2 mRNA expression, and the effect was dependent on other chemicals in the e-liquid mixture (McAliden et al., 2021). JUUL™ “Virginia Tobacco” e-liquid increased infection in primary tracheobronchial and small airway epithelium in submerged culture (Ghosh et al., 2022); however, e-liquid rather than authentic aerosol was tested.

The relationship between vaping authentic EC aerosols and acquisition of COVID-19 is poorly understood. To add clarity to this topic, we tested the hypothesis that EC aerosols increase SARS-CoV-2 infection by increasing levels of ACE2 in human bronchial epithelium, increasing levels and activities of TMPRRS2, and increasing viral infection. We performed controlled laboratory experiments with human cells that were exposed to known concentrations of both JUUL™ “Virginia Tobacco” EC fluids and aerosols, nicotine, PG and VG, and then examined discrete endpoints relevant to infection. We chose JUUL™ “Virginia Tobacco” because this product is currently marketed, popular, and has few flavor chemicals (Omaiye et al., 2019), making its fluid and aerosol a relatively simple mixture for testing. Moreover, tobacco flavored products are likely to remain marketable in the future as they are not subject to the FDA’s enforcement policy that proposes to remove flavored EC products from the market (Food and Drug Administration (FDA), 2020). We tested both JUUL™ “Virginia Tobacco” fluid and aerosols equivalent to those

inhaled during vaping. Testing was done across three *in vitro* exposure platforms: submerged cultures, cloud chamber ALI exposure, and Cultex® ALI exposure. Submerged culture has historical importance and is still frequently used (Bahl et al., 2012; Behar et al.,2012; 2018; Omaiye et al., 2022; Smart et al., 2020). The cloud chamber enables individual chemicals to be studied without using heat to create the aerosol, so that endpoint effects can be attributed to a particular chemical(s) without interference from other chemicals, solvents, or reaction products (Nair et al., 2020). The Cultex® system was used to vape JUUL™ ECs by heating their fluid to create authentic JUUL™ aerosols, which are equivalent to those an EC user inhales. Cultex®-generated aerosols contain the chemicals normally present in the fluid (Omaiye et al., 2020) plus any reaction products or metals added when fluid is heated in an EC atomizer (Kosmider et al 2014; Uchiyana et al 2020, Talih et al 2019; Omeda et al., 2018; Williams et al., 2013; Williams et al 2017; Williams et al., 2019a). Infection of cells was evaluated using SARS-CoV-2 pseudoparticles with a green-fluorescent reporter protein. Data were compared across exposure platforms and across exposure groups (JUUL™ fluids, JUUL™ aerosols, and individual chemicals).

## Materials and Methods

### BEAS-2B, HEK 293, and HEK 293T $^{ACE2}$ Cell Culture and Maintenance.

BEAS-2B cells (ATCC, Manassas, VA, USA) were cultured in Bronchial Epithelial Basal Medium (BEBM; Lonza, Walkersville, MD, USA) supplemented with the Bronchial Epithelial Growth Bullet Kit (BEGM; Lonza, Walkersville, MD, USA) without the gentamycin antibiotic. Cells were cultured in Nunc T-25 tissue culture flasks (Fisher Scientific, Tustin, CA, USA), pre-coated overnight with collagen type I, bovine serum albumin, and fibronectin. Cells were passaged at 80% confluency. Cell cultures were washed with Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS-; Lonza, Walkersville, MD, USA) and detached with 0.25% trypsin with 0.53 mM EDTA (ATCC, Manassas, VA, USA) and poly-vinyl-pyrrolidone (Sigma-Aldrich, St. Louis, MO, USA). Cells were seeded at 3,000 cells/cm$^2$ in a pre-coated T-25 flask, and culture medium was replaced every other day.

For in vitro submerged treatments, cells were seeded at a density of 9,000/cells/cm$^2$ in pre-coated 6-well and 12-well plates and allowed to attach overnight prior to treatments. For ALI culture, cells were seeded at a density of 12,000 cells/cm$^2$ in pre-coated 12-well Transwell® inserts with a pore size of 0.4 µm (Corning, Inc., Corning, NY, USA) and allowed to form a monolayer. Once a monolayer formed, the medium on the apical side of the transwell was removed, and the monolayer was acclimated to air for 24 hrs prior to an exposure. Medium in the basal-lateral side of the transwell was replaced every other day. Cells were incubated at 37$^o$C, 5% $CO_2$, and 95% relative humidity.

HEK 293T cells (ATCC, Manassas, VA, USA), and HEK 293T cells over expressing ACE2 (HEK 293T$^{ACE2}$, BEI resource, Manassas, VA, USA; NR-52511) were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS;

ATCC Manassas, VA, USA). At 80-90% confluency, cells were washed and detached as described above. Cells were seeded at 3,000 cells/$cm^2$ and incubated in a 37$^oC$, 5% $CO_2$, 95% relative humidity incubator.

**Submerged Treatments**

Refill fluids contained PG (Thermofisher, Tustin CA, USA) and VG (Thermofisher, Tustin CA, USA). At 80% confluency, BEAS-2B cells were treated for 24 hours with nicotine, PG/VG, PV/VG with nicotine, or JUUL™ fluids. All treatments were diluted with BEGM culture medium to reach working concentrations. Liquid (-)- nicotine (Sigma-Aldrich, St. Louis, MO, USA) was diluted to reach final concentrations of 0.03 mg/mL or 0.3 mg/mL. PG/VG (30/70 ratio) was diluted to reach a final concentration of 0.5% in volume. Stock solutions of PG/VG with nicotine were made and then diluted with culture medium to 0.5% PG/VG with either 0.03 mg/mL nicotine or 0.3 mg/mL nicotine. JUUL™ "Virginia Tobacco" was diluted to 0.5%, which contains PG/VG at 0.5% concentration and nicotine at 0.3 mg/mL.

**Nicotine Aerosol Exposure at the ALI in the VITROCELL® Cloud Chamber**

Monolayers of BEAS-2B cells cultured on 12 well Transwell® inserts were placed into a VITROCELL® cloud chamber (VITROCELL® Walkirch, Germany) for an ALI exposure of various chemical aerosols that were generated without heating. Prior to exposures, pre-warmed culture medium was dispensed into wells of the exposure chamber and allowed to equilibrate to 37 ºC. Stock nicotine was diluted with PBS- to make exposure solutions with final concentrations of either 0.03 mg/mL or 0.3 mg/mL nicotine. For each exposure, 200 µL of exposure solution was added into a VITROCELL® nebulizer

to generate a uniform aerosol with a flow rate of 200 µL/min without using heat. Control cells were exposed to PBS- aerosols.

BEAS-2B cells cultured on 12-well inserts were exposed to 1 puff of PBS- and nicotine (0.03 or 0.3 mg/mL) aerosol during a 1.5-minute aerosol generation period followed by a 3-minute aerosol deposition period. After the exposure, the cells were returned to the incubator to recover for 24 hrs.

**EC Aerosol Exposure at the ALI in the Cultex® RFS Compact Exposure System**

A Cultex® RFS compact exposure module (Cultex® Laboratories GmbH, Hannover, Germany) was used to expose cells to humified sterile air (clean air control) or EC aerosols generated from EC devices. Prior to each exposure, cells were placed into the exposure chambers, which contained culture medium maintained at 37ºC.

The Cultex® exposure system consisted of a sampling module and an aerosol guiding module. The sampling module contained a custom designed EC smoking robot (RTI International, North Carolina, USA) that draws filtered air from the biosafety cabinet or EC aerosol from the EC device into a 200 mL syringe. After collecting a 55 mL sample of either filtered air or EC aerosols, the Cultex® system then dispensed the sample into the aerosol guiding module, where it was combined with humified zero air (1 L/min). This mixture step diluted the sample and generated a uniform flow before the exposure mixtures were directly distributed onto each biological sample. Exposure mixtures were allowed to settle onto cells for 5 seconds, then vented out of the exposure chamber at a flow rate of 5 mL/min, generated by a mass flow controller (Boekhorst, Bethlehem, PA, USA), and finally dispensed into a waste container.

Each exposure consisted of 55 mL of filtered air or EC aerosol generated using a 4 second puff duration and a 30 second puff interval. BEAS-2B cells were exposed to 10 puffs of either clean air or EC aerosol and allowed to recover for 24 hrs prior to analyses.

**Quantification of Deposited Nicotine in the ALI Exposure Systems**

To quantify the nicotine deposited in the ALI exposure systems, exposures were repeated as described in the previous protocol sections using isopropyl alcohol (IPA; Fisher Scientific, Fair Lawn, NJ, USA) in place of the biological samples. Ten mL of IPA were added to each exposure well in both the cloud chamber and Cultex® system. After the exposures, the IPA was collected and shipped overnight on ice to Portland State University where it was analyzed using gas chromatography-mass spectrometry (GC-MS). Chemical analysis was performed with an Agilent 5975 GC/MS system (Agilent, Santa Clara, CA, USA) using an internal standard-based calibration procedure and method previously described (Omaiye et al., 2019; Brown et al., 2014).

**Immunocytochemistry of BEAS-2B cells.**

BEAS-2B cells were seeded in 8-well chamber slides (Ibidi, Gräfelfing, Germany). At 80% confluency, cells were treated with nicotine or EC fluids for 24 hrs in an incubator. Cells were then fixed in 4% paraformaldehyde for 15 mins at room temperature and washed several times with DPBS+. Samples were permeabilized with 0.1% Triton X and blocked using donkey serum (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature, followed by an overnight incubation in primary antibody. After several washes with PBS-T (DPBS+0.1% Tween), the samples were incubated at room temperature in the dark for 2 hrs with appropriate secondary antibodies. Samples were

washed several times and mounted using Vectashield with DAPI (Vectashield, San Francisco, CA, USA). Fluorescent cells were imaged with a Nikon Eclipse Ti inverted microscope (Nikon Instrument, Melville, NY, USA) using a 60X objective, and images were captured using a high-resolution Andor Zyla VSC-04941 camera (Andor, Belfast, UK). Antibodies used were anti-ACE2 (1:100; R&D System, Minneapolis MN, USA), anti-TMPRSS2 (1:200; Santa Cruz, Dallas, TX, USA), Secondary antibodies were Alexa fluor-488 or Alexa fluor-594 (Thermofisher, Tustin CA, USA).

**Lysate Preparation for Western Blot and Proteolytic Assay.**

After submerged treatments or exposures at the ALI, RIPA buffer with or without PMSF protease inhibitors (ChemCruz Biochemical, Dallas, TX, USA) was used to lyse cells. Lysates for Western blots were prepared using RIPA buffer with protease inhibitors, while lysates for proteolytic assays used RIPA buffer without protease inhibitors. The cell lysates were vortexed every 10 mins for 30 mins, pipetted through 23-gauge needles several times, then centrifuged at 3,000 x g for 5 mins at 4$^{o}$C. The lysate protein was quantified using the Pierce BCA assay kit (Thermo Scientific, Waltham, MA. USA). Each Western blot used 30 µg of protein, and each proteolytic assay used 5 µg of protein.

**Western Blotting.**

Following lysate preparation, denaturing buffer (β-mercaptoethanol and Laemeli buffer, 1:10) was added to each Western blot lysate at a 1:4 ratio. The buffer/lysate mixtures were heated at 95°C for 2 mins, then loaded onto an SDS gel (BioRad, Carlsbad, CA, USA) for electrophoretic separation of proteins (120V for 1-2 hrs), and afterwards transferred to a BioRad PVDF membrane at 200mA overnight at 4$^{o}$C. Following transfer,

the membrane was cut horizontally, either below or above the expected location of the protein of interest based on its molecular weight (kDa). The membranes were then blocked with 5% milk in TBS-T (TBS with 1%Tween-100) buffer for 2 hrs. and incubated overnight at 4$^{o}$C with antibodies against ACE2 (1:400; R&D systems, Minneapolis, MN, USA), TMPRSS2 (1:1000; Santa Cruz, Dallas, TX, USA ), and GADPH (1:2000; Cell Signaling Technology, Danvers, MA, USA). Next, the membranes were washed for 30 mins in TBS-T, then incubated in an HRP-conjugated secondary antibody (1:1000; Santa Cruz, Dallas, TX, USA or Cell Signaling Technology, Danvers, MA, USA) for 2 hrs. at room temperature. Finally, the membranes were developed using BioRad Clarity$^{TM}$ Western ECL Substrate reagent (BioRad, Carlsbad, CA, USA) in a BioRad ChemiDoc$^{TM}$ Imaging System (BioRad, Carlsbad, CA, USA), which determined the optimal exposure time for each protein (ACE2 = 120 ms; TMPRSS2 = 24 ms; GADPH = 4 ms).

**TMPRSS2 Proteolytic Assay.**

The TMPRSS2 enzyme assay was performed using a modification of a previously published protocol (Shrimp et al., 2020). The fluorogenic substrate, Boc-Gln-Ala-Arg-AMC · HCl, (Bachem, Torrance, CA. USA) was dissolved in DMSO and diluted in reaction buffer (50 mM Tris pH 8, 150 mM NaCl) to a final concentration of 10 μM. The fluorogenic substrate was added to each well of a 96-well plate and the fluorescence intensity was measured at 340/440 nm over a 1 hr period at 37 ˚C using a Bio-Tek Synergy HTX (Agilent, Santa Clara, CA, USA) plate reader.

**Spike Viral Pseudoparticle Production.**

Pseudoparticle production was performed as outlined in Crawford et al., 2020. In brief, HEK293T cells were plated with antibiotic-free medium at a density of 7 x $10^{6/}$T75 flask and transfected with Lipofectamine3000 (Thermo Fisher Sci, Waltham, MA. USA) using a total of 15 µg of BEI lentiviral plasmids (NR-52520: pHAGE2-CMV-ZsGreen-W, 7.5 µg; NR-52517: HDM-Hgpm2, 1.65 µg; NR-52519: pRC-CMV Rev1b, 1.65 µg; NR-52518: HDM-tat1b, 1.65 µg; NR-52314: pHDM-SARS-CoV-2 Spike, 2.55 µg), 60 µL of Lipofectamine, and 20 µL of P3000 reagent following the manufacturer's protocol. After overnight incubation, fresh medium supplemented with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. Fluorescence microscopy was used to visually inspect transfection efficiency and the expression of ZsGreen**.** Wild-type HEK293T cells, which were not transfected by the lentiviral plasmids, were used as a transfection control. The cell culture medium from transfected and wild-type HEK293T cells was collected 48 hours after transfection, centrifuged, and the resulting supernatant was filtered using a 0.45 µm syringe filter. The filtered supernatant was mixed with 5X PEG (Abcam, Cambridge, UK) and precipitated overnight at 4˚C. The lentivirus was collected by centrifugation and resuspension of the pellet in Viral Re-suspension Solution (Abcam, Cambridge, UK). The virus aliquots were stored at -80°C. Prior to infection experiments, the transduction efficiency of each viral batch was determined by infecting $293T^{ACE2}$ cells and quantifying the number of infected cells with flow cytometry. The medium from transfection control cells were processed the same, and produced a mock transfection solution, which did not contain virus and was used for mock infection.

**Viral Pseudoparticle Infection.**

For each viral pseudoparticle infection experiment, a 0.3 multiplicity of infection (MOI) was used to infect BEAS-2B cells. Viral pseudoparticles were delivered as a mixture with the appropriate fresh culture medium

In the submerged culture infection experiments, cells were treated for 24 hours, then the treatment was replaced with pseudoparticle medium. In the ALI culture infection experiments, following the recovery period, 100 µL of pseudoparticle medium was added directly onto the apical side of the Transwell®.

In all infection experiments, cells or tissues were incubated with viral pseudoparticles for 24 hrs. Subsequently, the pseudoparticles were removed and the cells or tissues were allowed to incubate another 24 hrs to amplify the expression of the green fluorescence reporter protein in the infected cells. Cells were harvested and analyzed with flow cytometry to determine the number of infected cells.

Prior to flow cytometry, fluorescence microscopy was used to validate the expression of ZsGreen signal. All samples were pipetted several times and passed through a 35 µm filter of a Falcon™ Round-Bottom 5 mL polystyrene test tube (Fisher Scientific, Tustin, CA, USA) to generate single cell suspensions. Forward-Scatter-Height (FSC-H) and Forward-Scatter-Area (FSC-A) were used to generate a gate to select single cells for each sample. A gate to exclude small debris was created using Side-Scatter-Area (SSC-A) and FSC-A. Non-fluorescent mock infected cells, which were incubated in viral re-suspension solution without viral pseudoparticles, were used to produce a gate to quantify the fluorescence signal of infected cells. Final results were represented as percent of fluorescent infected cells in each sample.

**Statistical Analysis**

In all cases, three independent experiments were performed using different passages of BEAS-2B cells. Statistical analyses were done using Minitab Statistics Software (Minitab, State College, PA, USA). Data were first checked for normality of distribution and homogeneity of variances. When data did not satisfy the assumptions of analysis of variance (ANOVA), they were subjected to a Box-Cox transformation, and again checked to verify that data satisfied the ANOVA model. Statistical analyses were done using one-way ANOVA. When significance was found ($p < 0.05$), data were further analyzed using Tukey's multiple comparison posthoc test to isolate significant differences to specific groups. Means were considered significantly different for $p < 0.05$. Data were plotted using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA)

## Results

### Effect of JUUL™ Fluids, Aerosols, and EC Chemicals (Nicotine and PG/VG) on ACE2 in BEAS-2B Cells During Submerged Culture and ALI Exposures.

The effects of JUUL™ "Virginia Tobacco" fluids and aerosols and individual components in JUUL™ products (specifically nicotine and PG/VG) on ACE2 levels were examined in BEAS-2B cells using submerged and ALI exposures (**Figure 1**). BEAS-2B cells were treated in submerged culture for 24 hours with 0.5% of 5% nicotine JUUL™ "Virginia Tobacco" fluid, which contained 0.3 mg/mL of nicotine after dilution, nicotine (0.03 mg/mL or 0.3 mg/mL), 0.5% PG/VG (ratio = 30/70), or 0.5% PG/VG with nicotine (0.03 mg/mL or 0.3 mg/mL) (Figure 1A). Micrographs of treated BEAS-2B cells labeled with ACE2 antibodies showed increased expression of ACE2 in those groups that contained 0.3 mg/mL of nicotine (**Figure 1B**). Increased expression was confirmed using Western blots (**Figures 1 C-D**). Nicotine increased ACE2 dose dependently, and the increase was significant in those groups that contained 0.3 mg/mL of nicotine. ACE2 also increased in the JUUL™ "Virginia Tobacco" treated group, but it was not significantly different than the control, even though JUUL™ has 0.3 mg/mL of nicotine. Overall, nicotine treatment for 24 hours in submerged cultures increased the expression of ACE2 dose dependently. PG/VG alone increased ACE2, but the effect was not significant.

Similar experiments were performed in which BEAS-2B cells were exposed to aerosols at the ALI in a VITROCELL® cloud chamber or Culte® exposure system (**Figures 1 G-K**). In the cloud chamber, BEAS-2B cells were exposed to either 1 puff of aerosol generated from solutions of PBS or nicotine (0.03 mg/mL or 0.3 mg/mL) in PBS, then allowed to recover for 24 hours before Western blotting **(Figures 1G-H)**. ACE2 increased in both nicotine-treated groups, and was close to significance ($p = 0.08$) in the

0.3 mg/mL treated cells (**Figure 1H**). There was no significant difference between the incubator and PBS controls, indicating that incubation in the cloud chamber did not affect the cells.

In the Cultex® system, BEAS-2B cells were exposed to either 10 puffs of humidified, filtered clean air or 10 puffs of aerosols made using an authentic JUUL™ "Virginia Tobacco" EC. In addition, the JUUL™ EC battery was used with refillable third-party pods containing lab made refill fluids to determine which components in JUUL™ fluid affected ACE2 levels. The fillable pods contained PG/VG, PG/VG with 6 mg/mL of nicotine, or PG/VG with 60 mg/mL of nicotine. There was no significant difference between the incubator and clean air controls. PG/VG and authentic JUUL™ "Virginia Tobacco" aerosols elevated ACE2 expression, but not significantly (**Figure 1J and K**). Aerosols from **l**ab made fluids containing PG/VG and nicotine increased ACE2 expression significantly compared to the clean air control.

In both submerged and ALI exposures, nicotine and EC aerosols with nicotine resulted in a concentration-dependent elevation of ACE2 levels in BEAS-2B cells.

The exposures in the cloud chamber and Cultex® experiments were compared by measuring nicotine deposited in the transwell culture medium after exposure. When aerosolized in the cloud chamber, solutions containing 0.3 mg/mL of nicotine deposited 1,328 ng/mL of nicotine into the fluid in each insert (**Figure 1F)**. In the Culte® system, 1,863 ng/mL of nicotine were deposited into each insert from aerosols generated using PG/VG with 60 mg/mL of nicotine. JUUL™ aerosols deposited a similar concentration of nicotine into the fluid in each insert (1,543 ng/mL) (**Figure 1I**). These results demonstrate that similar amounts of nicotine were deposited in each insert in the two ALI exposure systems.

**The Effect of JUUL™ Fluids, PG/VG, and Nicotine on the Concentrations and Activities of TMPRSS2 in BEAS-2B Cells During Submerged Exposure.**

Submerged treatments were done to determine the effects of PG/VG, nicotine, and JUUL™ "Virginia Tobacco" fluid on TMPRSS2 levels and enzymatic activity (**Figure 2**). TMPRSS2 is critical for infection, as it modifies SARS-CoV-2 spike protein after binding to ACE2 and facilitates virus and host membrane fusion.

Similar exposure experiments were done to determine if TMPRSS2 is affected by JUUL™ "Virginia Tobacco" fluid, PG/VG, or nicotine. Treated BEAS-2B cells labeled with TMPRSS2 antibodies showed increased expression of TMPRSS2 in those groups exposed to 0.3 mg/mL of nicotine (**Figure 2A**). In Western blots, TMPRSS2 expression was not significantly affected by JUUL™ fluid or PG/VG alone (**Figure 2 B, C**). However, groups treated with lab-made fluids containing nicotine had elevated levels of TMPRSS2, an effect that was dose dependent (**Figure 2B-C**). TMPRSS2 activity, as measured by cleavage of a specific fluorescent substrate, increased ($p = 0.07$) when BEAS-2B cells were exposed to authentic JUUL™ "Virginia Tobacco" EC fluid. TMPPRS2 activity was dose dependently increased by nicotine and was significantly greater than the control in groups having 0.3 mg/mL of nicotine. TMPRSS2 activity was not significantly affected by PG/VG alone (**Figures 2D, 2E**).

**The Effect of Authentic JUUL™ Aerosols, PG/VG, and Nicotine Aerosols on TMPRSS2 Concentration and Activity in BEAS-2B Cells Following ALI Exposures.**

Similar experiments were performed with BEAS-2B cells exposed at the ALI to aerosols generated in a cloud chamber or Cultex® exposure system. TMPRSS2 levels were evaluated in Western blots, and its activity was analyzed using specific fluorogenic substrates.

In the cloud chamber, BEAS-2B cells were exposed to 1 puff of aerosol generated from solutions of either PBS or nicotine (0.03 mg/mL or 0.3 mg/mL) in PBS, then allowed to recover for 24 hours before Western blotting and analysis of proteolytic activities **(Figures 3A-D)**. Nicotine exposures did not significantly affect TMPRSS2 levels **(Figures 3A, 3B)**. However, the enzymatic activity was significantly increased in 0.3 mg/mL of nicotine (**Figure 3C, 3D**).

In the Cultex® system, BEAS-2B cells were exposed to 10 puffs of aerosol containing JUUL™ "Virginia Tobacco" aerosol, PG/VG only, or PG/VG with nicotine **(Figure 3E-3H)**. TMPRSS2 levels did not differ significantly in any of the treatments when compared to the clean air control group (**3E, 3F**). However, TMPRSS2 activity decreased significantly in the PG/VG group, increased significantly in the two groups containing PG/VG with nicotine, but did not change significantly in the authentic JUUL™ aerosol group, which was generated from fluid containing 60 mg/L of nicotine. **(Figure 3G, 3H)**.

These results demonstrate that aerosols containing nicotine plus PG/VG can elevate TMPRSS2 activity in BEAS-2B cells, which may facilitate infection by enabling more rapid cleavage of the viral spike protein after ACE2 binds to host cells.

**JUUL™ Fluids and Aerosols and Nicotine Increased Viral Pseudoparticle Infection of BEAS-2B Cells in Submerged Cultures and ALI Exposures**

Experiments were done to determine how authentic JUUL™ fluids and aerosols, and their components (nicotine and PG/VG) affect infection of BEAS-2B cells. SARS-CoV-2 viral pseudoparticles were constructed using lentivirus as the infecting agent. The pseudoparticles contain SARS-CoV-2 spike protein in their envelope and a reporter plasmid that encodes ZsGreen, which is used to identify infected cells.

293T cells, genetically modified by stable transfection to overexpress ACE2 ($293T^{ace2}$), were used to test viral pseudoparticle infectivity, determine pseudoparticle density, and optimize the expression of ZsGreen. $293T^{ace2}$ cells were infected using an MOI of 0.3 **(Figure 4A)**. These data were used to optimize infection of BEAS-2B cells (**Figure 4B-4H).**

After 24 hours of submerged treatment, viral pseudoparticles were added to the cell cultures for 24 hours, then fluorescence was examined using microscopy and flow cytometry (**Figure 4B**). Infection increased significantly in cells treated with authentic JUUL™ "Virginia Tobacco" fluid (**Figure 4 C, E**). Infection increased slightly, but not significantly, in PV/VG alone. Nicotine significantly increased infection dose dependently, and infection was slightly higher when nicotine was combined with PG/VG, rather than used alone **(Figure 4C, E)**.

Similar results were obtained with BEAS-2B cells exposed to nicotine-containing aerosols at the ALI in a cloud chamber (**Figure 4G**). Infection was significantly elevated in the group exposed to 0.03 mg/mL of nicotine and was close to significance ($p = 0.06$) in the group exposed to the lower concentration of nicotine (**Figure 4G**). ALI exposure to various aerosols in the Cultex® system significantly increased infections in all treatment

groups with the effect being strongest in the groups that had nicotine **(Figure 4H)**. The increase in infection was significant in both the JUUL™ aerosols and the high nicotine plus PG/VG group; however, the increase in the JUUL™ group was significantly lower than in the nicotine PG/VG group. These increases in infection are likely due to higher ACE2 expression (**Figure 1**) and elevated TMPRSS2 activities **(Figure 2-3**) in the nicotine-treated BEAS-2B cells.

## Discussion

Our results, summarized in Figure 5, show the effects of exposure protocol (submerged vs ALI), heated vs unheated aerosols, and authentic JUUL™ aerosol versus individual aerosol chemicals on the machinery involved in SARS-CoV-2 infection. These results support the conclusion that JUUL™ "Virginia Tobacco" e-liquids and aerosols can increase ACE2 levels, TMPRSS2 activity, and infection of BEAS-2B bronchial epithelial cells by SARS-COV-2 pseudoparticles. By examining different exposure protocols (submerged vs ALI) and chemical variables (fluids, aerosols, PG/VG, and nicotine), we show that the effects of ECs on viral infection depend on both the context in which exposures occur (submerged vs ALI) and the chemical formulations of the aerosols *per se*. In submerged cultures and cloud chamber exposures, increases in ACE2 levels and infection can be attributed mainly to nicotine. In the Cultex® system, these increases were associated with both nicotine and PG/VG, perhaps due to formation of reaction products (e.g., aldehydes, ketones and alcohols) from the solvents during heating (Bitzer et al., 2018, Erythropel et al., 2019, Jensen et al., 2017; Uchiyama et al., 2020). Metals (e.g., zinc, copper, iron, lead, nickel, tin) are also added to aerosols during heating (Olmedo et al., 2018; Williams et al., 2017, 2019a), and these could also influence the outcome. While

both the levels and activity of TMPRSS2 increased in submerged cultures, only activity increased in ALI exposures. Increases in TMPRSS2 activity were associated mainly with nicotine, while PG/VG produced a small but significant decrease in the Cultex® system. While JUUL™ aerosols increased infection significantly, this increase was not as great as in the group containing only PG/VG/nicotine in concentrations equivalent to those in JUUL™, showing both that JUUL™ e-liquid can modulate the effects of nicotine and the importance of testing authentic EC aerosols, such as JUUL™, which decreased the effectiveness of nicotine. For most endpoints, both submerged and ALI exposures lead to similar conclusions, but the ALI provides data on an exposure similar to that an EC user would receive, and the Cultex® exposure is the best protocol for evaluating the human response.

**Table 1.1: Nicotine concentrations across the in vitro platforms compared to. nicotine concentrations in alveolar lining fluid of human smokers[1]**

| Platform | Concentration in submerged culture fluid and aerosolized fluid | Concentration in culture fluid and insert fluid | Concentration in alveolar lining fluid of smokers after 1 cigarette[1] |
|---|---|---|---|
| Submerged Culture | 30 and 300 µg/mL | 30 and 300 µg/mL | 1 -10 µg/mL |
| Cloud Chamber ALI Exposure | 30 and 300 µg/mL | 1.3 µg/mL. | 1 -10 µg/mL |
| Cultex® ALI Exposure | 6 and 60 mg/mL | 1.5 µg/mL Authentic JUUL™<br>1.8 µg/mL Lab-made Nicotine | 1 -10 µg/mL |

[1] Ranges are taken from references in the Eiserich et al., 1995; Clunes et al., 2008.

Table 1 summarizes the nicotine concentrations used in our three exposure platforms (fluid concentrations in submerged exposures and concentrations measured in insert culture media after ALI exposures) and compares them to estimated nicotine concentrations in human alveolar lining fluid after smoking a cigarette (Eiserich et al., 1995; Hoshino et al., 2001). We adjusted exposures in the two ALI platforms to produce similar final concentrations of nicotine in the insert fluids after exposure. As seen in Table 1, the nicotine concentrations in both the Cultex® and cloud chamber inserts were similar and were within the range reported in the alveolar lining fluid of smokers after smoking one cigarette (Eiserich et al., 1995; Clunes et al., 2008.), supporting the conclusion that our ALI exposures were reasonable and representative of those received in vivo by humans during vaping. A much higher starting concentration, which is similar to concentrations found in actual EC products, was used in the Cultex® system as the aerosol is diluted before entering the exposure chamber to ensure uniform distribution. In contrast, undiluted aerosol can be used in the cloud chamber, where it disperses uniformly and hence a lower starting concentration and puff number was used. These data demonstrate that chemical exposures were similar between the cloud chamber and Cultex® system and that exposures in the ALI systems, based on nicotine concentration, were within the range a tobacco product user would have in their alveolar lining fluid.

The effective concentrations and length of exposure to nicotine differed across exposure platforms. Nicotine increased SARS-CoV-2 pseudoparticle infection of BEAS-2B cells in all exposure systems; however, in submerged culture, exposures were continuous over 24 hours, while in the ALI systems, exposures were intermittent and short in duration, suggesting cells are more sensitive when exposed at the ALI. This is in agreement with other studies showing that zinc nanoparticle exposure of A549 cells was

more likely to produce an effect in ALI exposures than in submerged cultures (Lenz et al., 2012) and that gaseous exposure to aldehydes at the ALI caused significantly higher levels of IL-8 secretion than submerged culture exposures (Dwivedi et al., 2017). The apparent increase in cell sensitivity at the ALI may come about because nicotine or other chemicals in the EC aerosols were not diluted by the medium and interact directly with the surface of cells in the absence of culture medium. Because the effects of nicotine on infection are concentration dependent, results of both the ALI and submerged exposures are likely to vary with EC products, which are available in a broad range of nicotine concentrations (Omaiye et al., 2020; Davis et al., 2015). Our in vitro models support the conclusion that cells are more responsive to nicotine at the ALI than when submerged and that nicotine dose dependently increases ACE2 levels, TMPRSS2 activity, and SARS-CoV-2 pseudoparticle infection.

One of our goals was to examine the effects of individual chemicals in JUUL™ aerosols on ACE2, TMPRSS2, and infection. JUUL™ “Virginia Tobacco” was chosen because its concentrations of nicotine and PG/VG are known, and it has relatively few flavor chemicals, which are used at low concentrations (Omaiye et al., 2019). Our experiments compared authentic JUUL™ “Virginia Tobacco” aerosols to aerosols made with fluids containing PG/VG, nicotine, and/or PG/VG/nicotine mixtures using chemical concentrations that replicated those in JUUL™ “Virginia Tobacco” fluid. JUUL™ “Virginia Tobacco” aerosol increased the levels of ACE2 in BEAS-2B cells across all platforms, in agreement with and extending prior work showing that submerged treatment of BEAS-2B cells with Watermelon-flavored refill fluid increased ACE2 transcripts in BEAS-2B cells (McAliden et al., 2021). JUUL™ fluids also increased ACE2 enzymatic activity in vitro (Ghosh et al 2022), although ACE2 serves as a spike protein receptor and its activity is

not required for infection (Jackson et al., 2022). Our data isolated these increases to nicotine and PG/VG in the Cultex® system. Our data further showed that: (1) nicotine increased ACE2 levels dose dependently, (2) the nicotine concentration in JUUL™ "Virginia Tobacco" was sufficient to significantly elevate ACE2, and (3) the concentrations of nicotine reaching insert media in both the cloud chamber and Cultex® system were within the range reported in smokers alveolar lining fluid (Eiserich et al., 1995; Clunes et al., 2008). Based on our data and other publications (Russo et al., 2020; Maggi et al., 2021; Lallai et al 2021), there is a consensus that nicotine increases the ACE2 receptor in human bronchial epithelial cells.

Most prior SARS-CoV-2 studies have focused on ACE2 expression (Wang et al., 2020, McAliden et al., 2021; Gosh et al., 2021; Naidu et al., 2021; Masso-Silva et al 2021), and did not examine the effects of EC chemicals on TMPRSS2 concentration and activity. Increased TMPRSS2 activity would promote spike cleavage at the S2' site, internally to the S2 subunit, and fully activate the viral fusion process (Hoffman et al., 2020, Shang et al., 2020). Our data show that PG/VG, nicotine, and JUUL™ aerosols had different effects on TMPRSS2 activity. In most cases, TMPRSS2 activities were significantly increased by nicotine. An interesting exception was the lack of effect of JUUL™ "Virginia Tobacco" aerosols on TMPRSS2 activity, even though JUUL™ aerosol had a nicotine concentration similar to that in the group with 60 mg/mL of pure nicotine in PG/VG. This again shows the importance of examining authentic EC aerosols as the effects of nicotine may be modulated in complex aerosols containing mixtures of chemicals.

In summary, our data are consistent with the conclusion that JUUL™ "Virginia Tobacco" aerosols modulate the infection machinery in BEAS-2B cells in a manner that increases infection by SARS-CoV-2 viral pseudoparticles. Nicotine in PG/VG was more

effective than nicotine in JUUL™ aerosol, showing that chemicals in authentic EC aerosols can reduce nicotine's effect. In ALI exposures, increases in infectivity were generally correlated with nicotine and/or PG/VG induced increases in ACE2 concentrations and increases in TMPRRS2 activity. Results were generally similar across the three exposure platforms, but exposures in the ALI systems were brief and intermittent and involved lower concentrations of nicotine than in submerged cultures, which received 24 hours of continuous exposure. Given the highly variable nature of EC devices and their fluids (Cheng et al., 2014), it is probable that infection by SARS-CoV-2 is influenced by the product, user topography, and the e-liquid used for aerosol generation. Our data identify chemicals in EC liquids and aerosols that affect SARS-CoV-2 infection, show that EC aerosols can modulate infection, and identify nicotine and PG/VG as chemicals that can increase SARS-CoV-2 infection of human bronchial epithelium. Given the number of factors that can influence infection, it is not surprising that prior studies on tobacco products, mainly conventional cigarettes, have sometimes differed in their conclusions regarding the relationship between smoking/vaping and COVID-19 (Simons et al., 2020; Changeux et al., 2020; Vardavas et al., 2020; Farsalino et al., 2020b, Zhang et al., 2020 , Leung et al., 2020, Cai et al., 2020, Brake et al., 2020, Smith et al., 2020, Muus et al., 2021). EC products are highly variable and have nicotine concentrations ranging from 0 to 60 mg/mL (Trtchourian et al. 2011; NAS 2018; Omaiye et al 2021; Davis et al., 2015), operate at different powers, have different atomizer designs (Williams et al., 2019b), and have varying chemical mixtures in their fluids, all of which can affect SARS-CoV-2 infection and severity differently. Based on our BEAS-2B data, it is probable that some EC products deliver sufficient nicotine to increase infection, while those that are nicotine-free or have low concentrations of nicotine may not influence SARS-CoV-2 infection.

Our data support the conclusion that understanding the relationship between EC use and COVID-19 will be challenging due to the many variables related to vaping. Variations in EC design, power, user topography, and e-liquid ingredients are some of the factors that can influence SAR-CoV-2 infection. Nevertheless, our data can be valuable in improving the safety of EC. For example, since nicotine increased infection dose dependently, capping the allowable concentration of nicotine in e-liquids could reduce the likelihood of SARS-CoV-2 infection in EC users.

**Limitations of the Study**

We examined the effect of EC aerosols on BEAS- 2B cells. In the future, it will be important to extend these studies to a 3D organotypic model of the respiratory epithelium that closely resembles *in-vivo* exposure in humans. Our study is limited to one SARS-CoV-2 variant and could in the future be extended to new variants as they arise. Our work included JUUL™ “Virginia Tobacco”, which operates with a relatively low voltage and power. Variations in power can affect formation of reaction products, which may in turn alter infectability of cells. Finally, our exposures were acute and could be extended to chronic exposure in the future.

## Author Contributions

Project administration and funding acquisition, P.T.; Conceptualization, R.P. and P.T.; Investigation R.P.; Sample preparation, data collection, and data processing were done by R.P., M.W., A.S., and T.M.; Data Interpreted by R.P. and P.T.; Writing – original draft by R.P. and P.T.; Writing- Review & Editing, all authors.


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## Declaration of Interest

The authors have no competing interests to declare.

## Figures and Legends

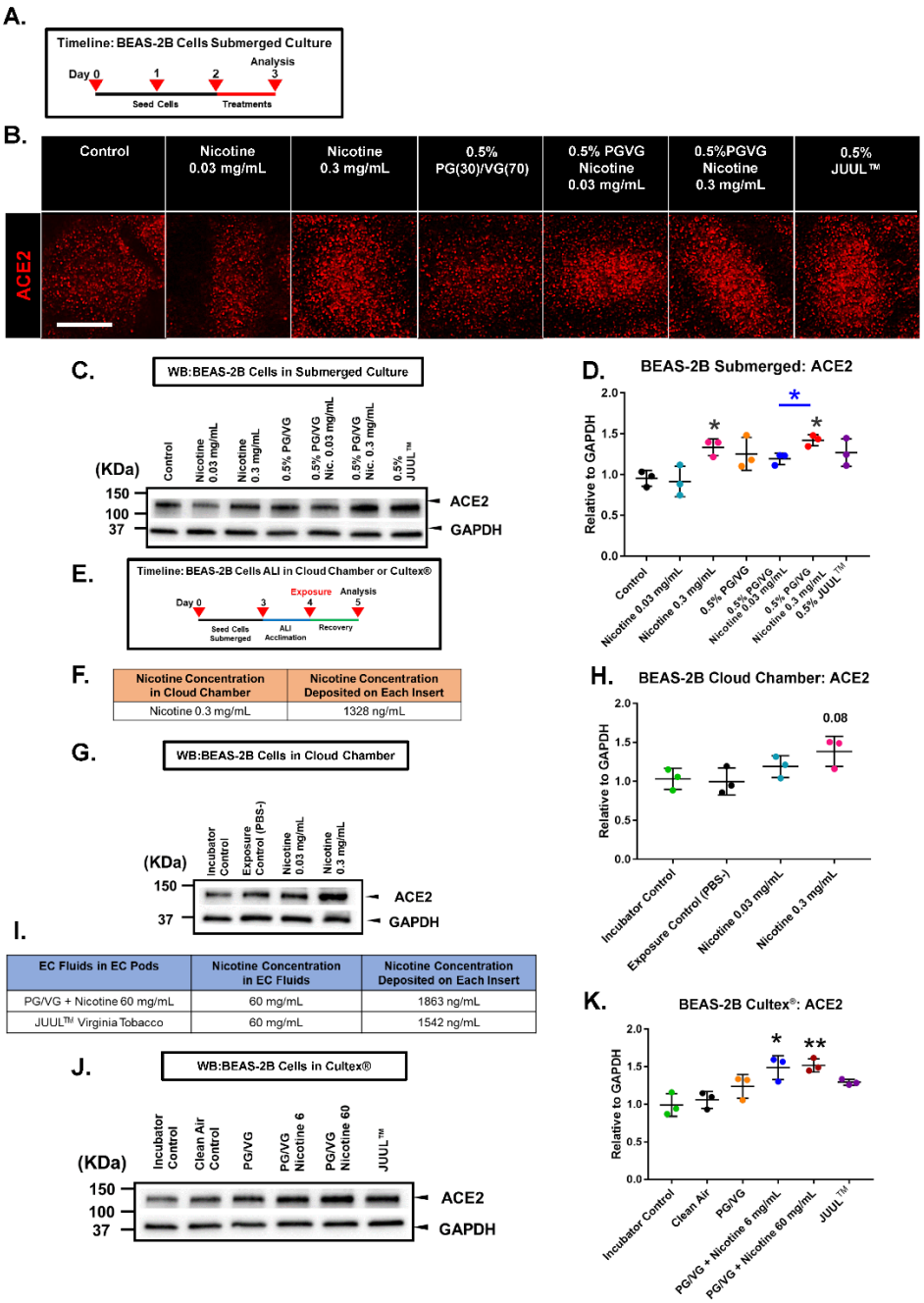


**Figure 1.1. Nicotine, EC Fluid, and EC Aerosols Increased ACE2 Expression in BEAS-2B Cells During Exposure in Submerged Culture and at the Air-liquid Interface.**

**(A)** Timeline showing experimental treatment used with BEAS-2B cells in submerged culture.
**(B)** BEAS-2B cells labeled with ACE2 antibody. Micrographs show a concentration dependent increase of ACE2 following treatment with nicotine in submerged culture. Scale bar = 50 μm.
**(C-D)** Western blots showing the effect of JUUL™ "Virginia Tobacco" e-liquid, nicotine, and PG/VG on ACE2 in BEAS-2B cells during submerged culture. High nicotine concentrations significantly increased ACE2.
**(E)** Timeline showing experimental treatment used with BEAS-2B cells exposed at the ALI in a VITROCELL® cloud chamber or the Cultex® exposure system.
**(F)** Concentration of nicotine deposited in each well following aerosolization of a solution containing 0.3 mg/mL of nicotine in a VITROCELL® cloud chamber.
**(G-H)** Western blot data showing a concentration-dependent increase in ACE2 in BEAS-2B cells following ALI exposure to 1 puff of nicotine-containing aerosol in a VITROCELL® cloud chamber.
**(I)** Concentration of nicotine deposited in each well following exposure to aerosol generated from PG/VG with nicotine 60 mg/mL or JUUL™ "Virginia Tobacco" in a Cultex® exposure system.
**(J-K)** Western blot data showing an increase of ACE2 in BEAS-2B cells following ALI exposure to 10 puffs of nicotine-containing EC aerosols in a Cultex® system. The aerosols were generated from PG/VG, PG/VG with nicotine, and a JUUL™ "Virginia Tobacco" EC.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, data were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. $*p < 0.05$, $**p < 0.01$. Black asterisks: significant difference from the control. Blue asterisks: significant difference from 0.5% PG/VG + Nicotine 0.03 mg/mL

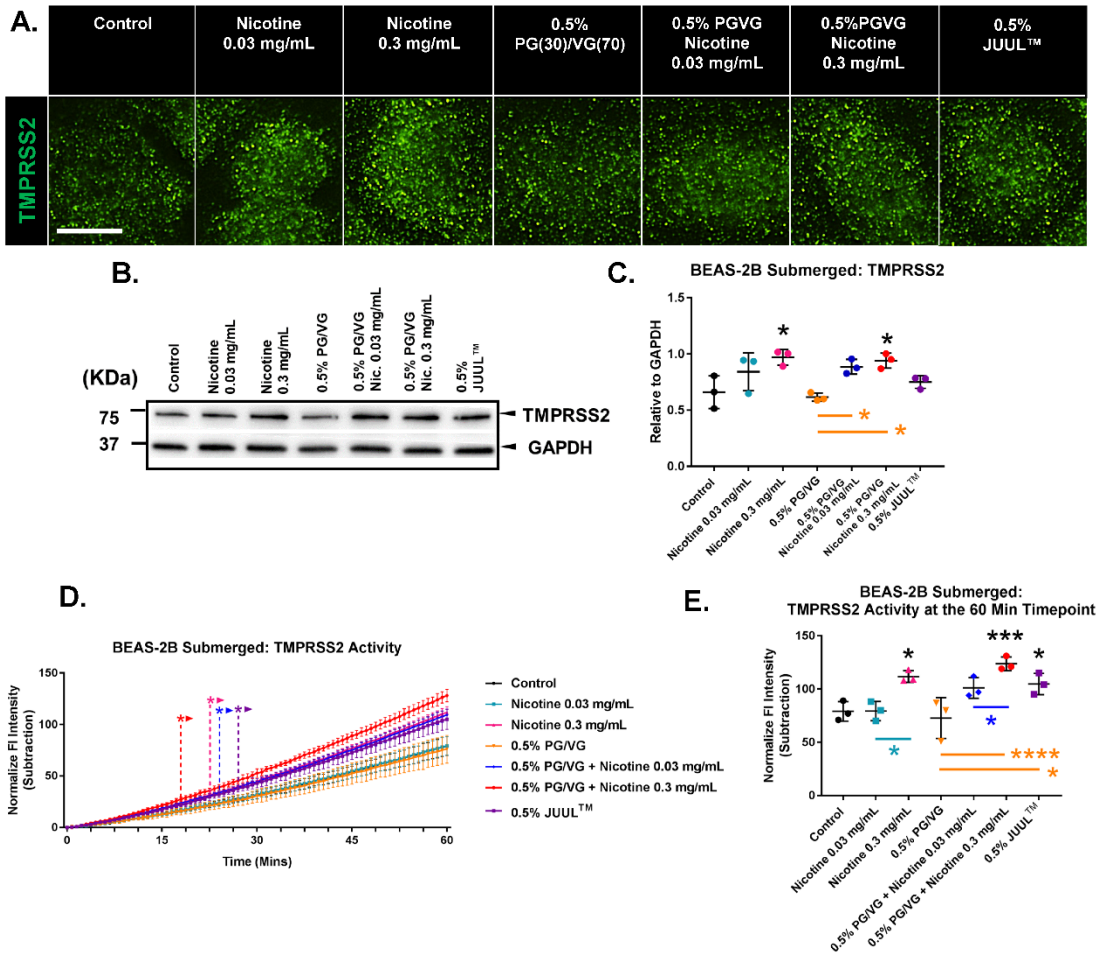


**Figure 1.2. Effect of JUUL™ E-liquid, Nicotine, and PG/VG on TMPRSS2 Levels and Activity in BEAS-2B Cells Exposed in Submerged Culture.**

**(A.)** BEAS-2B cells labeled with TMPRSS2 antibody. Micrographs show TMPRSS2 labeling increased in the presence of nicotine in submerged culture. Scale bar = 50 µm. **(B.- C.)** Western blot data showing the effect of JUUL™ "Virginia Tobacco" e-liquid, nicotine, and PG/VG on TMPRSS2 concentration in BEAS-2B cells during submerged culture.
**(D.- E.)** TMPRSS2 activity was increased by JUUL™ e-liquid and by nicotine following 24 hours of treatment in submerged cultures. (**D**.) Activity over 60 minutes: 0.3 mg/mL of nicotine significantly increased activity between 22 and 60 minutes (*p* ranged from 0.05 to 0.0001), the 0.5% PG/VG + nicotine 0.03 mg/mL was significantly increased between 24 and 60 minutes (*p* values ranged from 0.05 to 0.0001), the 0.5% PG/VG + nicotine 0.3 mg/mL was significantly increase between 18 and 60 minutes (*p* values ranged from 0.05 to 0.0001), and the JUUL™ group was significantly increase between 27 and 60 minutes (*p* values ranged from 0.05 to 0.0001). (**E**) Activity at the 60-minute timepoint.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, **C** and **E** were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. Black asterisks: significantly different than the control. Light blue asterisks: significantly different than the nicotine 0.03 mg/mL group. Orange asterisks: significantly different than the 0.5% PG/VG group. Dark blue asterisks: significantly different than the 0.5% PG/VG + nicotine 0.03 mg/mL group. **D** was analyzed using a two-way ANOVA followed by Dunnett's posthoc test to compare means against the control. For all figures: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

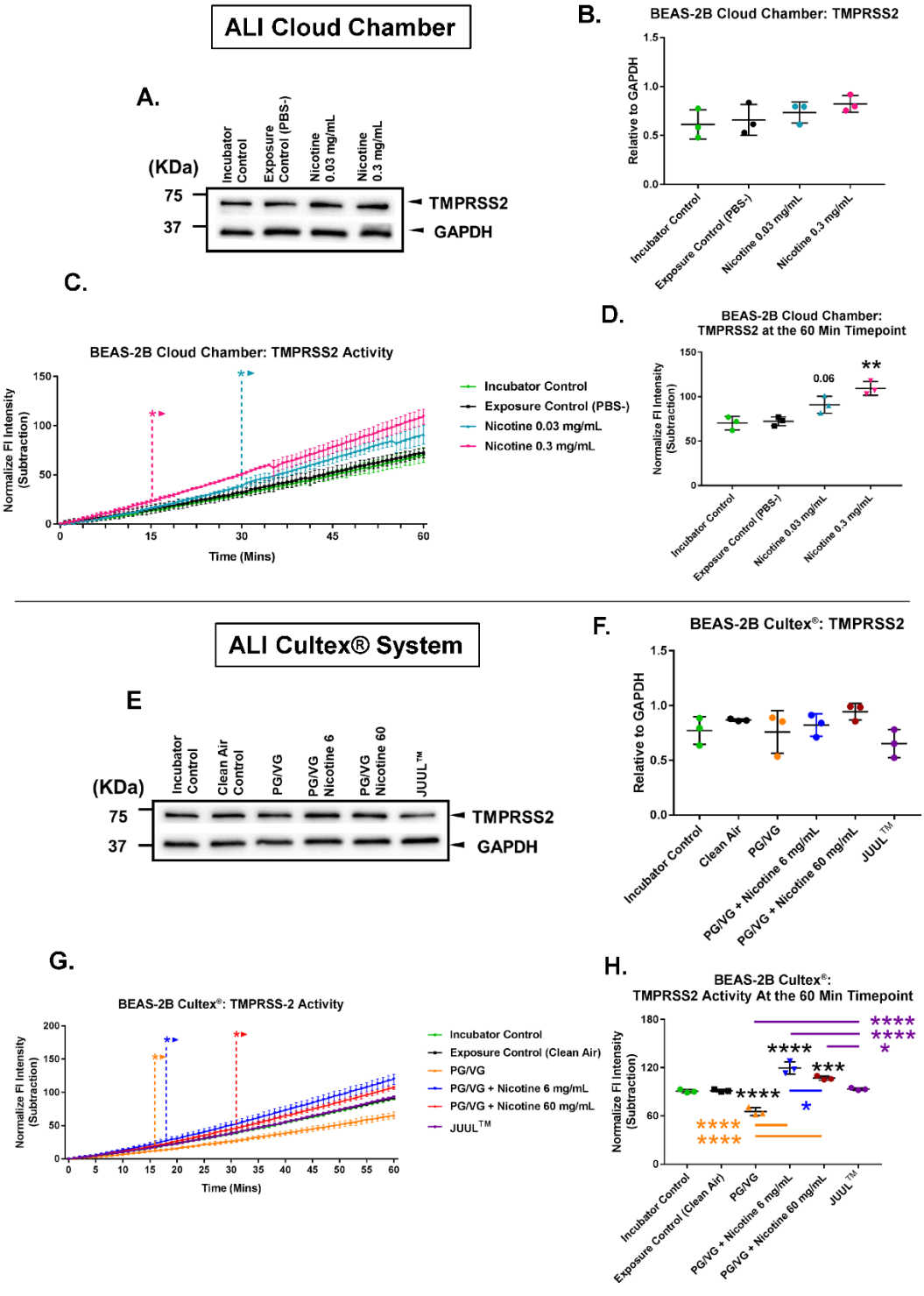


**Figure 1.3. The Effect of EC Aerosols on TMPRSS2 Activity in BEAS-2B Cells Exposed at the Air-liquid Interface.**

**(A - B)** Western blot showing the effect of 1 puff of pure nicotine aerosol on TMPRSS2 levels in BEAS-2B cells when exposure occurred at the ALI in a VITROCELL® cloud chamber.
**(C- D)** TMPRSS2 activity was increased by nicotine following exposure at the ALI in a VITROCELL® cloud chamber. (C.) Activity over 60 minutes: 0.03 mg/mL of nicotine significantly increased activity between 30 and 60 minutes (*p* values ranged from 0.05 to 0.0001), and the nicotine 0.03 mg/mL group was significantly increased between 15 and 60 minutes (*p* values ranged from 0.05 to 0.0001). (D.) Activity at the 60-minute timepoint.
**(E - F)** Western blot showing the effect of 10 puffs of PG/VG, PG/VG with nicotine, and a JUUL™ "Virginia Tobacco" EC aerosol on TMPRSS2 concentration in BEAS-2B cells when exposure occurred at the ALI in the Cultex® system.
**(G - H)** TMPRSS2 activity was increased by nicotine following exposure at the ALI in the Cultex® system, while PG/VG decreased TMPRSS2. JUUL™ aerosols also increased TMPRSS2 activity (**G**.) Activity over 60 minutes: the PG/VG group significantly decreased between 16 and 60 minutes (*p* ranged from 0.05 to 0.0001), the PG/VG + nicotine 6 mg/mL group was significantly increased between 18 and 60 minutes (*p* values ranged from 0.05 to 0.0001), and the PG/VG + nicotine 60 mg/mL was significantly increased between 31 and 60 minutes (*p* values ranged from 0.05 to 0.0001), (**H.**) Activity at the 60-minute timepoint.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, **B, D, F** and **H** were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. Black asterisks: significantly different from the control. Orange asterisks: significantly different than the PG/VG group. Dark blue: asterisks significantly different than the PG/VG + nicotine 60 mg/mL group. Purple asterisks: significantly different than the JUUL™ group. **C** and **G** were analyzed using a two-way ANOVA followed by Dunnett's posthoc test to compare means to the exposure control. For all figures: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

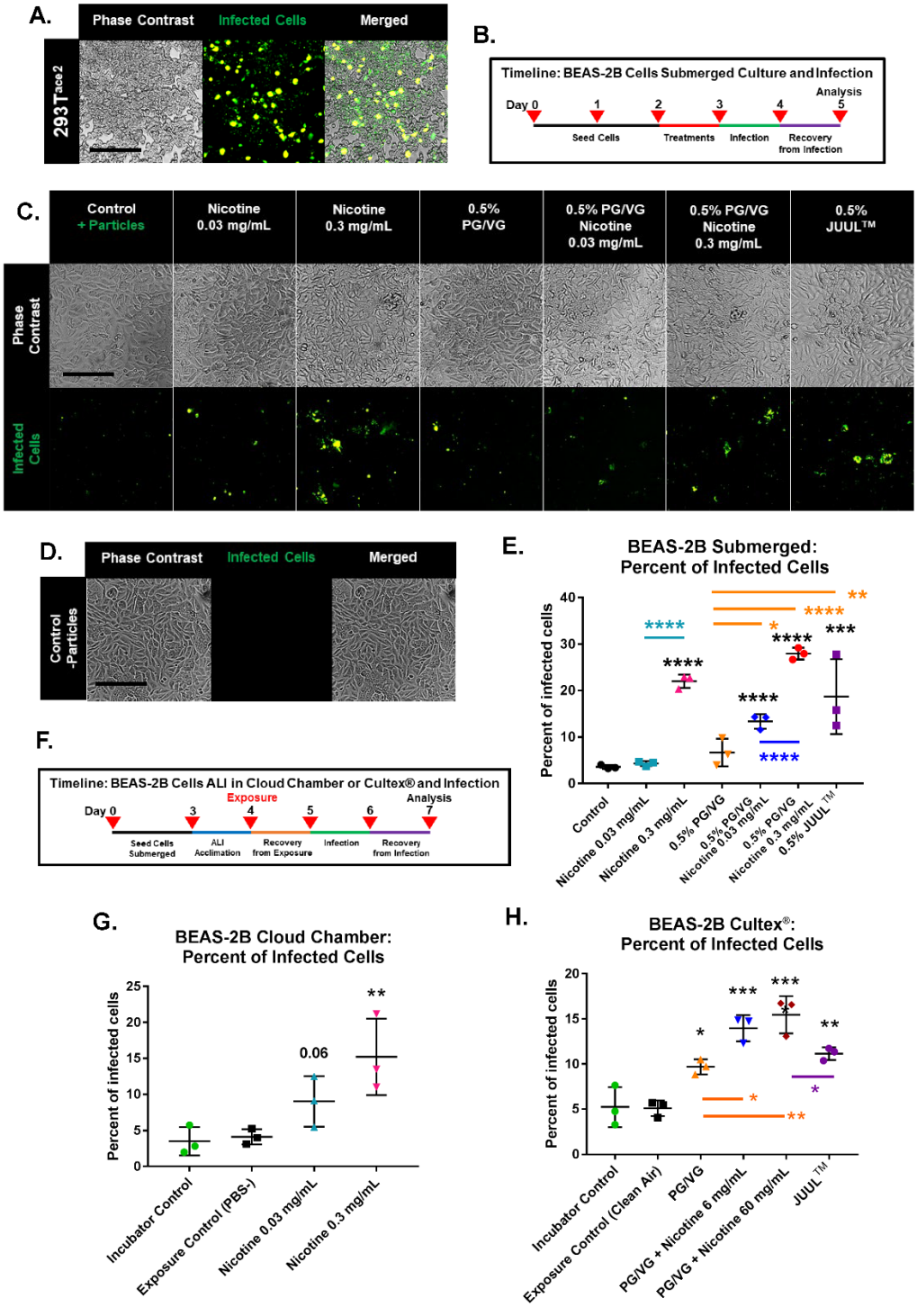


**Figure 1.4. JUUL™ Aerosols and Nicotine, Increased Infection of BEAS-2B Cells by SARS-CoV-2 Pseudoparticles After Submerged Treatment or ALI Exposure to Aerosols.**

**(A)** Phase contrast, fluorescent, and merged micrographs showing 293T$^{ace2}$ cells that were infected by SARS-CoV-2 viral pseudoparticles. Infected cells express the green fluorescence reporter protein. Scale bar = 250 μm.
**(B)** Timeline showing experimental treatments used with BEAS-2B cells in submerged culture followed by infection with viral pseudoparticles.
**(C- E)** Micrographs and flow cytometry data showing infection of BEAS-2B cells treated for 24-hrs with EC chemicals or JUUL™ "Virginia Tobacco" fluid then infected for 24 hrs with viral pseudoparticles. (C-D) Phase contrast and fluorescent micrographs showing (C) BEAS-2B cells infected with viral pseudoparticles and (D) uninfected control cells. (E) Flow cytometry data showing highest infection in cells treated with PG/VG with 0.3 mg/mL of nicotine. Scale bars in C and D = 250 μm.
**(F)** Timeline showing experimental treatment used with BEAS-2B cells exposed at the ALI in a VITROCELL® cloud chamber or a Cultex® exposure system and then infected with viral pseudoparticles for 24 hours.
**(G)** Flow cytometry data showing the percent of infected BEAS-2B cells after exposure to 1 puff of PBS (control), 0.03 mg/mL nicotine, or 0.3 mg/mL nicotine, followed by 24 hrs of exposure to viral pseudoparticles. Exposure was at the ALI in a VITROCELL® Cloud Chamber. Infection was significantly higher in the group exposed to 0.3 mg/mL of nicotine.
**(H)** Flow cytometry data showing the percent of infected BEAS-2B cells after exposure to 10 puffs of clean air or EC aerosols, followed by 24 hrs of exposure to viral pseudoparticles. Exposure was at the ALI in a Cultex® system. All treatment groups increased infection significantly.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, data were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. Black asterisks: significantly different than the control group in **E** or the exposure control group in **G and H**. Light blue asterisks: significantly different than the nicotine 0.03 mg/mL group. Orange asterisks: significant different than the 0.5% PG/VG group in **E** or the PG/VG group in **G, H**. Dark blue asterisks: significantly different than the 0.5% PG/VG + nicotine 0.03 mg/mL group in **E**. Purple asterisks: significantly different than the JUUL™ group in **H**. For all figures: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

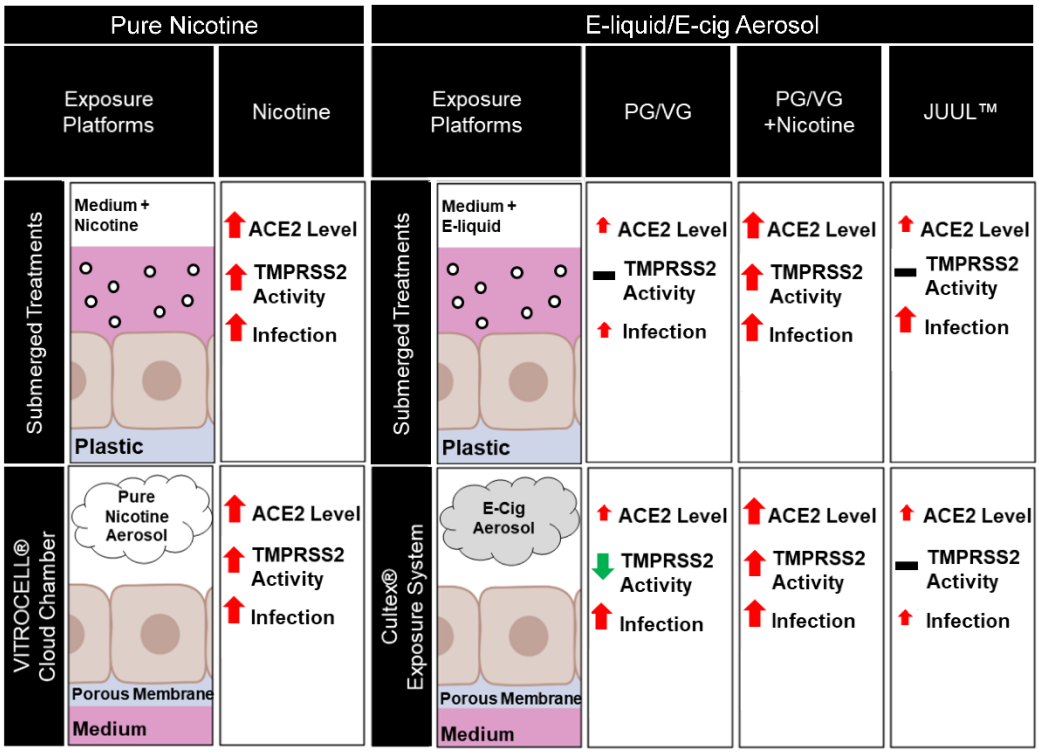


**Figure 1.5: The Relationship Between JUUL™ E-liquid, Nicotine, and PG/VG Exposure and ACE2, TMPRSS2, and Viral Pseudoparticle Infection.**

Diagram summarizing our major findings in BEAS-2B cells following exposure to authentic JUUL™, mixture (PG/VG plus nicotine), and individual chemicals (nicotine and PG/VG solvent) in three different exposure models: submerged treatment (top), air-liquid interface (ALI) exposure in the cloud chamber (bottom left), and ALI exposure of authentic EC aerosols in a Cultex® system (bottom right). ACE2 levels increased with all treatments in each exposure platform. TMPRSS2 activity varied with the platform and treatment. Most treatments increased TMPRSS2 activity, three treatments had no effect (PG/VG in submerged and PG/VG and JUUL™ in the Cultex® exposures), and one treatment decreased activity (PG/VG in the Cultex®). Infection was increased in all treatments/exposures. Size of the arrow indicates relative change from the control

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# CHAPTER 2

## Does Vaping Increase the Likelihood of SARS-CoV-2 Infection? Paradoxically Yes and No

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**Abstract**

Data on the relationship between electronic cigarette (EC) use and SARS-CoV-2 infection are limited and contradictory. Some evidence indicates that EC aerosols or nicotine increase ACE2 receptors, which can bind SARS-CoV-2 virus and may thereby increase infection susceptibility. Our objectives were to determine if authentic EC aerosols increased SARS-CoV-2 infection of human bronchial epithelial cells and to identify those chemicals that produced the observed effects. A 3D organotypic model (EpiAirway™) in conjunction with air liquid interface (ALI) exposure was used to test the effects of aerosols produced from JUUL™ "Virginia Tobacco" and BLU™ ECs, or individual chemicals (nicotine, propylene glycol (PG)/vegetable glycerol (VG), and benzoic acid) on infection using SARS-CoV-2 viral pseudoparticles. Exposure of EpiAirway™ to JUUL™ and BLU™ EC aerosols increased ACE2, while aerosols from mixtures of nicotine and PG/VG or PG/VG alone increased both ACE2 levels and TMPRSS2 activity (an enzyme that cleaves spike protein to enable viral-host cell fusion). SARS-CoV-2 pseudoparticle infection of EpiAirway increased with aerosols produced from PG/VG alone, PG/VG plus nicotine, or BLU™ ECs. JUUL™ EC aerosols did not increase infection above clean air controls. The reduced infection in JUUL™ aerosol treated groups was attributed to benzoic acid, which reduced the infection enhancement produced by aerosols with PG/VG or PG/VG plus nicotine. The protection from elevated infection that benzoic acid provided continued at least 48 hours after aerosol exposure. TMPRSS2 activity was significantly correlated with e-liquid pH, which in turn was significantly correlated with infection, with lower pH blocking PG/VG and nicotine-induced elevation of infection. While ACE2 levels increased in EpiAirway tissues exposed to EC aerosols, infection depended on the ingredients of the

e-liquids. PG/VG and nicotine enhanced infection, an effect that was mitigated by benzoic acid.

## Introduction

Electronic cigarettes (ECs) are nicotine delivery devices that aerosolize e-liquids, which usually contain nicotine, the solvents propylene glycol (PG) and vegetable glycerin (VG), and flavor chemicals (Trtchounian et al., 2011; Omaiye et al., 2019;). While sometimes promoted as less harmful than tobacco cigarettes, ECs are not harm free (Pisigner et al., 2017; Sakamaki-Ching et al 2020; Kalininsky et al., 2019; Diaz et al., 2020; Hua et al., 2016; 2020; Gio et al., 2022; Gotts et al., 2021; Becker et al., 2021). Adverse effects reported for EC users include impaired pulmonary immune defenses against bacterial and viral infections (Sussan et al., 2015) and a weakened innate immune response in the lung compared to non-users (Reidel et al., 2018). Despite their popularity, especially among adolescents and young adults (Singh et al.,2016; Goldenson et al., 2017; Vogel et al 2020), little work has been done on the relationship between EC use and users' susceptibility to infection by the SARS-CoV-2 virus which causes Coronavirus Disease 2019 (COVID-19). The entry of SARS-CoV-2 into host cells depends on the viral spike protein, cellular angiotensin converting enzyme (ACE2), and cellular transmembrane protease, serine 2 (TMPRSS2), which cleaves an internal site of the spike S'2 subunit preparatory to fusion.

In a survey of adolescents and young adults, EC users were five times more likely to be diagnosed with COVID-19 than non-users (Gaiha et al., 2020), and a correlative study found a positive association between EC use and the number of SAR-CoV-2 cases (Li et al., 2020). Whole body exposure of mice to EC aerosol with or without nicotine elevated ACE2 concentration in the lung (Wang et al., 2020; Naidu et al 2021; Masso-Silva et al.,2021; Lallai et al., 2021), suggesting an increased likelihood of infection. Although human data are limited, Ghosh et al., (2022) showed that EC aerosol increased

ACE2 activity and soluble ACE2 levels in EC users' bronchoalveolar lavage fluid; these changes were correlated with infection of human bronchial epithelial cells by SARS-CoV-2 pseudoparticles following exposure to JUUL™ "Virginia Tobacco" e-liquid in submerged cultures (Ghosh et al., 2022). Additionally, e-liquid with and without nicotine increased ACE2 expression in BEAS-2B cells after submerged treatment (McAliden et al., 2020), an effect linked to nicotine concentration in air-liquid interface (ALI) exposures of BEAS-2B cells (Phandthong et al., 2022). Using BEAS-2B cell monolayer culture, we further showed that submerged treatments with nicotine and e-liquid with nicotine as well as ALI exposure to EC aerosol with nicotine increased ACE2 levels, TMPRSS2 activity, and SARS-CoV-2 pseudoparticle infection (Phandthong et al., 2022). While preliminary, these studies suggest that EC use may increase the likelihood of contracting COVID-19. In contrast, several hospital observational studies have concluded that EC use is not associated with an increased risk for COVID-19 (Jose et al., 2021; Burnett-Hartman et al., 2022). Because data on EC use and SARS-CoV-2 susceptibility are limited and contradictory, our objective was to test the hypothesis that using ECs is a risk factor for SAR-CoV-2 infection.

The current study is the first to examine SARS-CoV-2 infection using 3D organotypic cultures of human bronchial epithelium (EpiAirway) exposed at the ALI. EpiAirway tissues, which contain ciliated, basal, and mucus producing cells, are the best in vitro model currently available for examining how EC use affects SAR-CoV-2 infection. To elucidate the effect of vaping on COVID-19 and to identify the specific chemicals in EC liquids that affect SARS-CoV-2 infection, authentic JUUL™ or BLU™ EC aerosols and aerosols made with specific e-liquid ingredients (nicotine and PG/VG or mixtures thereof) were tested using ALI exposures of 3D organotypic cultures of human bronchial epithelium (EpiAirway). Infection was evaluated using SARS-CoV-2 viral pseudoparticles containing

spike protein and a green fluorescent reporter. The three endpoints examined for each exposure treatment included: (1) ACE2 levels in EpiAirway tissues; (2) TMPRSS2 protease activity; and (3) SARS-CoV-2 viral pseudoparticle infection. Our data will show that the relationship between vaping and SARS-CoV-2 infection is complex and highly dependent on the ingredients of the e-liquid used to create the aerosol. These data will provide EC users with options that may reduce their risk of contracting COVID-19, affect future research studies, and help improve the design of clinical trials involving the use of tobacco products and SARs-CoV-2 infection.

## Materials and Methods

### HEK 293T $^{ACE2}$ Cell Culture and Maintenance.

HEK 293T cells (ATCC, Manassas, VA, USA), and HEK 293T cells, which over expressed ACE2 (293T$^{ACE2}$, BEI resource, Manassas, VA, USA; NR-52511) were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS; ATCC, Manassas, VA, USA). At 80-90% confluency, cells were washed and detached as described above. Cells were seeded at 3,000 cells/cm$^2$ and incubated in a 37ºC, 5% $CO_2$, 95% relative humidity incubator

### EpiAirway™ Tissue Maintenance.

EpiAirway™ primary cell tissues cultured at the ALI have beating ciliated cells, mucus producing goblet cells, and keratin-5-positive basal cells. Upon arrival, EpiAirway™ inserts from MatTek Corporation (Ashland, MA, USA) were transferred from the agarose shipping plate to a 12-well culture plate pre-filled with AIR-100-ASY medium in basal-lateral compartments. These tissues were acclimated and stored in an incubator with 37ºC, 5% $CO_2$, and 95% relative humidity for 24 hours prior to any experiments. Basal lateral medium was replaced every day. Prior to aerosol exposure, the apical side of the EpiAirway™ was washed with Dulbecco's phosphate buffered saline with calcium and magnesium (DPBS+; Lonza, Walkersville, MD, USA).

**Reagents and Procedure for Compounding Lab-Made EC Refill fluids.**

Refill fluids contained propylene glycol (PG; Thermofisher, Tustin CA, USA) and vegetable glycerin (VG; Thermofisher, Tustin CA, USA) made at a 30 PG/70 VG ratio. Liquid (-)- nicotine (Sigma-Aldrich, St. Louis, MO, USA) was added to PG/VG to obtain either 6 or 60 mg/mL of nicotine. In some refill fluids, benzoic acid was dissolved in heated VG and allowed to cool at room temperature; PG was then added to make a 30 PG/70VG ratio containing 40 mg/mL of benzoic acid Sigma-Aldrich (St. Louis, MO, USA). To replicate JUUL™ "Virginia Tobacco" refill fluid, the VG with benzoic acid solution was mixed with PG containing 2,3,5 TMP, ethyl maltol, corylone, and ethyl lactate (Sigma-Aldrich, St. Louis, MO, USA) to give the following final concentrations: 0.027 mg/mL 2,3,5 TMP, 0.033 mg/mL ethyl maltol, 0.036 mg/mL corylone, 0.125 mg/mL ethyl lactate, 40 mg/mL benzoic acid, and 60 mg/mL nicotine in a 30 PG/70 VG solvent, as reported in previous publications (Pankow et al., 2017; Omaiye et al., 2019).

**Source of EC Refill fluids, EC Devices, and Pods.**

BLU™ ECs, JUUL™ ECs, Drag S VOOPOO ECs, VOOPOO "PnP" pods and coils, JUUL™ "Virginia Tobacco" pods, and blank JUUL™ compatible pods (OVNS JC01 pods with 1.5Ω ohm ceramic wick) were purchased online from third party vendors, at a local gas station, and at grocery stores. JUUL™ "Virginia Tobacco" refill fluids were extracted from pods by removing the cover cap, rubber stopper, and directly collecting the refill fluids.

**Nicotine aerosol exposure at the ALI in the VITROCELL® cloud chamber**

Monolayers of EpiAirway™ tissues cultured on 12 well Transwell® inserts were placed into a VITROCELL® cloud chamber (VITROCELL®, Walkirch, Germany) for an ALI exposure to various chemical aerosols that were generated without heating. Prior to exposures, pre-warmed culture medium was added into wells in the exposure chamber and allowed to equilibrate to 37 ºC. Stock nicotine was diluted with PBS- to make exposure solutions with final concentrations either 0.03 mg/mL or 0.3 mg/mL nicotine. Benzoic acid flakes were dissolved in warm water and diluted with PBS- to make an exposure solution with a final concentration of 0.2 mg/mL benzoic acid. For each exposure, 200 µL of exposure solution were added into a VITROCELL® nebulizer to generate a uniform aerosol with a flow rate of 200 µL/min without using heat. Control cells were exposed to PBS- aerosols.

EpiAirway™ tissues were exposed to 5 puffs of PBS-, nicotine (0.3 mg/mL), or benzoic acid (0.2 mg/mL) aerosol. This protocol is similar to the 1 puff protocol, except for the inclusion of a 1 min venting step between each puff. The aerosols were allowed to settle onto cells until the chamber becomes visually clear, which takes 3 mins. After the exposure, the cells were returned to the incubator to recover for 24 hrs.

**EC aerosol exposure at the ALI in the Cultex® RFS compact exposure system**

A Cultex® RFS compact exposure module (Cultex® Laboratories GmbH, Hannover, Germany) was used to expose cells or tissue cultures to humified sterile air (clean air control) or EC aerosols generated from EC devices. Prior to each exposure, cells or tissues samples were placed into the exposure chambers, which contained culture medium and were regulated at a temperature at 37 ºC.

The Cultex® exposure system consisted of a sampling module and an aerosol guiding module. The sampling module used a custom designed EC smoking robot (RTI International, NC, USA) that draws filtered air in the biosafety cabinet or EC aerosol from the EC device into a 200 mL syringe. After collecting 55 mL of either filtered air or aerosols, the Cultex® system then dispense the sampled air or aerosols into the aerosol guiding module, which mixes either the filtered air or EC aerosols with humified zero air (1 L/min). This mixture diluted the aerosol and generated a uniform flow before aerosols were directly distributed onto each tissue sample. Exposure mixtures were allowed to settle onto cells for 5 second and then vented out the exposure chamber at a flow rate of 5 mL/min, generated by a mass flow controller (Boekhorst, Bethlehem, PA, USA), and finally dispensed into a waste container.

Each exposure consisted of 55 mL of filtered air or EC aerosol. Puffs lasted 4 seconds and were spaced 30 seconds apart. EpiAirway™ 3D tissues were exposed to 50 puffs of air or EC aerosols/day for 3 days. Between each exposure day, tissues were returned to the incubator and after the last exposure, tissues were allowed to recover in the incubator for 24 hrs prior to analyses.

**Immunofluorescence of EpiAirway™ Tissues.**

After EC aerosol exposures and recovery, EpiAirway™ tissues were fixed in 4% paraformaldehyde (PFA) at 4°C for 20 hrs, then the apical and basal surfaces were washed with DPBS several times. Fixed tissues were stored in cold DPBS solution and shipped to MatTek for histology. The samples were returned to us as unstained histology sample slides, which were rehydrated through a graded series of ethanol washes, until reaching pure nano water. Antigen retrieval was performed using a 10 mM citrate buffer

(pH 6.0) in a 95 ºC water bath for 20 mins. Permeabilization, blocking, and primary and secondary antibody incubation steps were the same as for immunocytochemistry. Samples slides were mounted using Vectashield with DAPI (Vectashield, San Francisco, CA, USA) and glass cover slips. Fluorescent cells were imaged with a Nikon Eclipse Ti inverted microscope (Nikon Instrument, Melville, NY, USA) using a 60X objective, and images were captured using a high-resolution Andor Zyla VSC-04941 camera (Andor, Belfast, UK). Antibodies used were anti-ACE2 (1:200; R&D system, Minneapolis, MN, USA), anti-TMPRSS2 (1:200; Santa Cruz, Dallas, TX, USA), anti-acetylate α-tubulin conjugated to Alexa fluor-594 (1:200; Santa Cruz, Dallas, TX, USA ), anti-MUC5AC conjugated to Alexa fluor-594 (1:100; Santa Cruz, Dallas, TX, USA ), and anti-keratin 5 (1:200, Biolegend, San Diego, CA, USA). Secondary antibodies were Alexa fluor-488 or Alexa fluor-594 (Thermofisher, Tustin CA, USA).

**Lysate Preparation for Western Blot and Proteolytic Assay.**

After exposure at the ALI, RIPA buffer with or without PMSF protease inhibitor (ChemCruz Biochemical, Dallas, TX, USA) was used to lyse cells and tissues. The lysates prepared for Western blot use RIPA buffer with protease inhibitor, while lysates prepared for proteolytic assay used RIPA buffer without a protease inhibitor. The cell lysates were vortexed every 10 mins for 30 mins, pipetted through 23-gauge needles several times, then centrifuged at 3,000 x g for 5 mins at 4$^{o}$C. The lysate protein was quantified using the Pierce BCA assay kit (Thermo Scientific, Waltham, MA, USA). Each Western blot used 20 µg of protein, and each proteolytic assay used 5 µg of protein.

**Western Blotting.**

Following the lysate preparation step, denaturing buffer (β-mercaptoethanol and Laemeli buffer, 1:10) was added to each Western blot lysate at a 1:4 ratio. The buffer/lysate mixtures were heated at 95°C for 2 mins, then loaded onto an SDS gel (BioRad, Carlbad, CA, USA) for electrophoretic separation of proteins (120V for 1-2 hrs), and then transferred onto a PVDF membrane (BioRad; 200mA overnight at 4$^{o}$C). After transfer, the membrane was cut horizontally, either under or above the expected location of the protein of interest based on its molecular weight (kDa). Membranes were blocked with 5% milk in TBS-T (TBS with 1%Tween-100) buffer for 2 hrs. and incubated overnight at 4$^{o}$C with antibodies against anti-ACE2 (1:400; R&D systems, Minneapolis, MN, USA), anti-TMPRSS2 (1:1000; Santa Cruz, Dallas, TX, USA), anti-GADPH (1:2000; Cell Signaling Technology, Danvers, MA, USA). Membranes were washed for 30 mins in TBS-T, then incubated in an HRP conjugated secondary antibody (1:1000; Santa Cruz, Dallas, TX, USA or Cell Signaling Technology, Danvers, MA, USA) for 2 hrs. at room temperature. Membranes were developed using BioRad Clarity$^{TM}$ Western ECL Substrate reagent (BioRad, Carlbad, CA, USA) in a BioRad ChemiDoc$^{TM}$ Imaging Systems (BioRad, Carlbad, CA, USA), which determined the optimal exposure time for each protein (exposure times were ACE2 27ms; TMPRSS2 24ms; GADPH 4ms).

**TMPRSS2 Proteolytic Assay.**

The TMPRSS2 enzyme assay was performed using a previously published protocol, with some modification (Shrimp et al., 2020). The fluorogenic substrate, Boc-Gln-Ala-Arg-AMC · HCl (Bachem, Torrance, CA. USA) was dissolved in DMSO and diluted in reaction buffer (50 mM Tris pH 8, 150 mM NaCl) to a final concentration of 10 μM. The fluorogenic substrate was added to each well of a 96-well plate, and fluorescence intensity was measured at 340/440 nm over a 1 hr period at 37 ˚C using a BioTek Synergy HTX (Agilent, Santa Clara, CA, USA).

**Spike Viral Pseudoparticle Production.**

Pseudoparticle production was performed as outlined in Crawford et al., 2020. In brief, HEK293T cells were plated with antibiotic-free medium at a density of 7 x $10^{6/}$T75 flask and transfected with Lipofectamine3000 (Thermo Fisher Sci, Waltham, MA. USA) using a total of 15 μg of BEI lentiviral plasmids (NR-52520: pHAGE2-CMV-ZsGreen-W, 7.5 μg; NR-52517: HDM-Hgpm2, 1.65 μg; NR-52519: pRC-CMV Rev1b, 1.65 μg; NR-52518: HDM-tat1b, 1.65 μg; NR-52314: pHDM-SARS-CoV-2 Spike, 2.55 μg), 60 μL of Lipofectamine and 20 μL of P3000 reagent following the manufacturer's protocol. After overnight incubation, fresh medium supplemented with 1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA) was added to the cells. Fluorescence microscopy was used to visually inspect transfection efficiency and the expression of the ZsGreen**.** The cell culture medium was collected 48 hours after transfection, centrifuged, and the supernatant was filtered with a 0.45 μm syringe filter. The filtered supernatant was mixed with 5X Polyethlyene glycol (Abcam, Cambridge, UK) and precipitated overnight at 4˚C. The lentivirus was collected by centrifugation and resuspension of the pellet in Viral Re-

suspension Solution (Abcam, Cambridge, UK). The virus aliquots were stored at -80°C. Prior to infection experiments, the transduction efficiency of each viral batch was determined by infecting $293T^{ACE2}$ cells and quantifying the number of infected cells with flow cytometry.

**Viral Pseudoparticle Infection.**

For each viral pseudoparticle infection experiment, 0.3 multiplicity of infection (MOI ) was used to infect 3D EpiAirway™ tissues. Viral pseudoparticles were delivered as a mixture with the appropriate fresh culture medium

For ALI culture, after the recovery period, 100 µL of pseudoparticle medium was added directly onto the apical side of the Transwell®.

In ALI culture, viral pseudoparticles were incubated with cells for 24 hrs, then the pseudoparticle mixture was removed. Cells or tissues were allowed to incubate another 24 hrs to amplify the expression of the green fluorescence reporter protein in the infected cells. Cells were harvested and analyzed with flow cytometry to determine the number of infected cells.

Prior to flow cytometry, fluorescence microscopy was used to validate the expression of ZsGreen signal. All samples were pipetted several times to generate single cell suspensions and passed through a 35 µm filter of a Falcon™ Round-Bottom 5 mL polystyrene test tube (Fisher Scientific, Tustin, CA, USA). Forward-Scatter-Height (FSC-H) and Forward-Scatter-Area (FSC-A) were used to generate a gate to select single cell for each sample. Side-Scatter-Area (SSC-A) and FSC-A were used to generate a gate to exclude small debris. Non-fluorescent mock infected cells were used to generate a gate

to quantify fluorescence signal of infected cells. Final results were represented in percent of fluorescent infected cells in each sample.

**Measuring pH of lab made, BLU™ and JUUL™ "Virginia Tobacco" e-liquid**

The pH meter (Thermofisher, Tustin CA, USA) was calibrated with solutions at pH 4, 7, and 10 before measuring the pH of each diluted e-liquid sample. Lab made, BLU™ and JUUL™ e-liquid were diluted at a 1:10 ratio in ultrapure water, then the pH of the diluted sample was measured. For each session of measurement, the pH electrode is fully submerged in the sample. Between each sample, the pH electrode was rinsed with ultra-pure water and patted dry before measuring new samples.

**Correlation plots**

ACE2 concentration, TMPRSS2 activity, and infection data were normalized to their respective control groups. The normalized data were then used to calculate correlations between each group in Minitab statistic software (Minitab, State College, PA, USA). Pairwise Pearson correlation was used to test correlation and significance between each group, and GraphPad Prism 7 (GraphPad, San Diego, CA, USA) was used to plot the linear regression graphs.

**Statistical Analysis**

In all experiments, three independent EpiAirway tissues were used. Statistical analyses of all data were done with Minitab Statistics Software (Minitab, State College, PA. USA). If data satisfied the assumption of ANOVA (homogeneity of variances and normal distribution), they were analyzed using a one-way ANOVA. If the assumptions were not meet, data were subjected to a Box-Cox transformation, and retested to verify they satisfied the assumptions of ANOVA. When the ANOVA detected significance (p , 0.05), Tukey's postdoc test was used to compare group means. In the kinetic assays of TMPRSS2 activity, two-way ANOVAs were used to study the effects of treatment and time. Means were considered significantly different for $p < 0.05$. Data were plotted using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA)

## Results

### EC Aerosol Exposures at the ALI in the Cultex® System Increased ACE2 in the 3D EpiAirway tissues.

The effects of authentic JUUL™ "Virginia Tobacco" aerosols, PG/VG aerosols, and PG/VG + nicotine aerosols were evaluated using MatTek EpiAirway™ tissues, which are 3D organotypic tissues that include ciliated, mucus-producing, and basal cells (**Figure 1A)**. A 3-day EC aerosol exposure protocol was used to replicate an acute exposure that an EC user could receive. EpiAirway was exposed to 50 puffs/day to be within the range (30-200 puffs day) reported for EC users (**Behar et al., 2015**).

The timeline in Figure 1B shows the experimental design and the sequence of EC aerosol exposure over 3 days. Tissues were exposed to 50 puffs of clean air or 50 puffs of aerosol generated from a JUUL™ EC. The EC aerosols were produced using authentic JUUL™ "Virginia Tobacco" pods and refillable pods containing either PG/VG only, PG/VG with 6 mg/mL of nicotine, or PG/VG with 60 mg/mL of nicotine. After the last exposure, tissues recovered in the incubator for 24 hours. They were then fixed for immunofluorescence microscopy **(Figure 1C),** lysed for Western blot analysis **(Figure 1D-1E),** or assayed for TMPRSS2 activity (**Figure 1 F-G**).

Following 24 hours of recovery, ACE2 receptors increased in all exposure groups except the clean air and incubator controls, which were similar to each other, showing that incubation in the Cultex® exposure system did not affect ACE2 levels **(Figure 1C)**. In Western blots, controls were not significantly different, but ACE2 levels increased significantly in all treatment groups compared to the clean air control **(Figure 1D-4E).** These results indicate that acute exposure of EpiAirway to authentic JUUL™ aerosols, PG/VG, or PG/VG + nicotine can increase ACE2 levels, the SARS-CoV-2 point of entry.

**ALI Exposure of EpiAirway Tissues to EC Aerosols with Nicotine Altered Proteolytic Activity of TMPRSS2**

Following the exposure session, the activity of the spike-cleaving protease, TMPRSS2, was analyzed using a specific fluorogenic substrate **(Figure 1F-G)**. TMPRSS2 activity in EpiAirway tissues varied among treatment groups**.** Tissues exposed to PG/VG aerosols showed a small, but significant decrease in TMPRSS2 activity when compared to the clean air control. EC aerosols containing PG/VG plus 6 or 60 mg/mL of nicotine increased TMPRSS2 activity significantly. In contrast, TMPRSS2 activity in the JUUL™ exposed group was significantly lower than tissue exposed to PG/VG with nicotine, even though JUUL™ fluid contained PG/VG and 60 mg/mL of nicotine. The TMPRSS2 data show the importance of both testing mixtures, including authentic EC aerosols, and measuring enzymatic activity.

**Nicotine and PG/VG Increased Infection of EpiAirway Tissues by Viral Pseudoparticles**

Because EC aerosols increased ACE2 expression in EpiAirway tissues **(Figure 1C-1E)**, the following experiments were done to test the hypothesis that EC aerosols increase viral pseudoparticle infection. **Figure 2A** shows the experimental timeline for short-term 3-day exposures of EpiAirway™ tissues. Cells were exposed to 50 puffs of air or EC aerosols/day for 3 days. Tissues were returned to the incubator and allowed to recover for 24 hrs after each exposure. After recovery, tissues were infected with SARS-CoV-2 viral pseudoparticles for 24 hours then allowed to incubate for another 24 hours to amplify the expression of the green reporter protein. Prior to flow cytometry assays, mock-

infected and virus-infected cells were harvested, and fluorescence microscopy was used to verify that the infected cells were expressing ZsGreen **(Figure 2B)**.

ALI exposure to aerosols containing PG/VG or PG/VG plus nicotine significantly increased viral pseudoparticle infection of EpiAirway tissues **(Figure 2C)**. In contrast, JUUL™ "Virginia Tobacco" aerosols caused a slight but significant decrease in infection. In addition, tissues exposed to JUUL™ aerosols had significantly fewer infected cells than the PG/VG or PG/VG with 60 mg/mL nicotine groups. These data indicate that authentic JUUL™ pod aerosols negated the effect of PG/VG or PG/VG with 60 mg/mL nicotine on infection.

**Benzoic Acid in EC Aerosols Decreased Infection of EpiAirway Tissues by Viral Pseudoparticles**

Unlike PG/VG or PG/VG with nicotine, JUUL™ "Virginia Tobacco" fluid contains 40 mg/mL of benzoic acid and several flavoring chemicals, which have been identified and quantified previously (Pankow et al., 2017; Omaiye et al., 2019). The data in **Figure 2C** suggest that other chemicals in JUUL™ aerosols nullified the effect of PG/VG and nicotine on viral pseudoparticle infection. To identify the chemical(s) that reduced infectivity, EpiAirway tissues were exposed to EC aerosols generated from: PG/VG only, PG/VG with flavor chemicals (2,3,5 TMP, ethyl maltol, corylone and ethyl lactate = PG/VG+FLV), PG/VG with 40 mg/mL of benzoic acid (PG/VG+BA), PG/VG with flavor chemicals and benzoic acid (PG/VG+FLV+BA), PG/VG with flavor chemicals, benzoic acid and 60 mg/mL nicotine (PG/VG+FLV+BA+NIC), and JUUL™ "Virginia Tobacco" (**Figure 2D**). Flavor chemicals were used at the concentrations found in JUUL™ "Virginia Tobacco" (Omaiye et al., 2019). Viral pseudoparticle infection was increased only in tissues exposed to

PG/VG and PG/VG+FLV. All aerosols containing benzoic acid significantly reduced infection (**Figure 2D**).

**Aerosols made with Pure Benzoic Acid did not alter Viral Infection of the Primary Bronchial Epithelial Cells in the ALI Culture**

The results showed that aerosols containing benzoic acid mitigated the effect of nicotine and PG/VG on viral pseudoparticle infection. We next tested pure aerosolized benzoic acid in the cloud chamber to determine if it alone affects EpiAirway infection during exposure to viral pseudoparticles. Nicotine-containing aerosol increased viral pseudoparticle infection in EpiAirway (positive control); however, benzoic acid alone did not significantly alter infection compared to the clean air control (**Figure 2E)**.

**The Protective Effect of Benzoic Acid-containing Aerosols lasted at least 48 hours after ALI EC Exposure of EpiAirway in the Cultex®.**

In our earlier experiments (**Figure 2C-2E**), EpiAirway tissues were allowed to recover for 24-hrs before inoculation with viral pseudoparticles. To determine if benzoic acid in EC aerosol can provide protection against infection for a longer period of time, the recovery period was extended to 48 hours, as shown in the experimental timeline in **Figure 2F**.

Consistent with the previous result, PG/VG aerosol increased infection in the 24 and 48 hour recovery groups compared to the clean air controls. However, aerosols containing benzoic acid significantly reduced PG/VG-induced infection in both the 24- and 48-hour recovery groups (**Figure 2G)**. These results show that benzoic acid containing

aerosols continue to protect EpiAirway tissues from infection at least 48 hours after the exposure had stopped.

**BLU™ Disposable EC Aerosol Increased Viral Pseudoparticle Infection, while JUUL™ Aerosol with Benzoic Acid did not.**

BLU™ “Classic Tobacco” is a popular first-generation EC brand that has been marketed for many years and is still available. BLU™ disposable EC fluid contains 24 mg/mL nicotine and lacks benzoic acid, unlike JUUL™ EC fluid. Consistent with the previous infection results (**Figure 2C, 2D)**, EpiAirway tissues exposed to BLU™ aerosols showed an increase in viral infection (**Figure 2H)**. These data further support the idea that benzoic acid in EC aerosols reduces viral susceptibility and further show that EC aerosols that do not have benzoic acid increase infectivity.

**Comparison of a High and Low Power EC on Viral Pseudoparticle Infection**

JUUL™ ECs produce aerosols at relatively low power (8W-LP) (Talih et al., 2019), in contrast to third generation products that have variable and often higher powers (Williams et al., 2019). VOOPOO, which is a popular high-power EC (20W-HP), produces a larger aerosol volume/puff that often increases aerosol complexity (Son et al., 2019; Ogunwale et al., 2017; Zhao et al., 2019). PG/VG aerosols produced from both the low and high-power device increased viral pseudoparticle infection relative to the clean air control. The aerosols made with the high-power device produced a slightly higher percent of infection with a lower p-value than the low power device (but the groups were not significantly different). All groups containing benzoic acid were equivalent to or slightly lower than the clean air control (**Figure 2I**). Consistent with previous data (**Figure 2C, 2D,**

**2E**), EC aerosols with benzoic acid protected tissue from viral pseudoparticle infection. This protection was slightly less effective, but not significant, in the benzoic acid aerosol generated in the high-power device.

**EC Aerosols with Benzoic Acid Reduced TMPRSS2 Activity and Prevented the Increase in Infection Caused by PG/VG and Nicotine.**

We next examined the effects of benzoic acid on ACE2 levels and TMPRSS2 activity. Consistent with previous data, all EC aerosols tested increased ACE2 levels in the EpiAirway tissues compared to the clean air control (**Figure 3A, 3B**), although the increase in the BLU™ aerosol group was not significant ($p$=0.14). The inclusion of benzoic acid did not prevent this increase (**Figure 3A**), and benzoic acid by itself did not cause the increase when tested in the cloud chamber **(Figure 3C**).

TMPRSS2 activity assays revealed that EpiAirway tissues exposed to aerosols containing PG/VG or PG/VG + benzoic acid had significantly lower activity than the clean air control group (**Figure 3D, 3E**). In contrast, tissues exposed to BLU™ aerosol, which contained nicotine, but not benzoic acid, had a significant increase in TMPRSS2 activity, similar to the results in figure 1F and G. Exposure to pure benzoic acid in the cloud chamber decreased TMPRSS2 activity, but not significantly (**Figure 3F-3G)**, while nicotine alone increased its activity.

Overall, tissues exposed to aerosols containing benzoic acid showed a decrease in the activity of TMPRRS2, a protease essential for viral pseudoparticle infection.

**pH of Test Solutions and Correlations between pH, TMPRSS2 Activity, ACE2 Levels, and Infection**

To further investigate the relationship between pH and viral infection, the pH values of lab made and commercial e-liquids were measured (**Figure 4A).** Solutions with nicotine, but not benzoic acid, usually had a high pH ranging from 8.02 in BLU™ to 9.83 in lab made PG/VG + 60 mg/mL nicotine (**Figure 4A** green boxes). Addition of benzoic acid neutralized the solutions and brought the pH down to 6.08 in lab made liquids or 5.62 in authentic JUUL™ "Virginia Tobacco" e-liquid (**Figure 4A** white and pink boxes). Solutions with benzoic acid but without nicotine had lower pH values ranging from 2.87 - 3.87 (**Figure 4A** red boxes).

Linear regression analyses were performed on pH, ACE2 levels, TMPRSS2 activity, and infection to determine which factors affected infection (**Figure 4B-4G)**. There were weak positive correlations for ACE2 levels and pH ($r^2$=0.34; **Figure 4B**), and for ACE2 levels and infection ($r^2$=0.26; **Figure 4C**), but neither were significant ($p > 0.05$). TMPRSS2 activity showed a strong and significant positive correlation to pH ($r^2$=0.83, p=0.01; **Figure 4D**). The correlation between TMPRSS2 activity and infection was also strong, but not significant ($r^2$=0.66), however, when an outlier PG/VG point was removed, the correlation was both stronger and significant ($r^2$=0.75, p=0.02; **Figure 4E)**. There was a significant and strong positive correlation between pH and infection ($r^2$=0.65, p=0.04; **Figure 4F**). The correlation table summarizes the results of linear regression analysis on pH, ACE2 concentration, TMPRSS2 activity, and infection **(Figure 4G)**.

To determine the pH optimum for TMPRSS2, TMPRSS2 activity in clean air exposed tissue lysate was tested with several buffers at different pH values (5,7,8,9). Optimal activity was observed between 7-8 (**Figure 4H**).

Taken together, these data demonstrated that ACE2 concentration does not always correlate with infectibility and that factors, such as pH, influence TMPRSS2 activity, which can alter infectivity.

## Discussion

Our results, which are summarized in Figure 5, provide evidence that PG/VG and nicotine-containing aerosols dose-dependently increased infection of EpiAirway 3D organotypic tissues by SARS-CoV-2 viral pseudoparticles and that benzoic acid in JUUL™ aerosols mitigates the effect of PG/VG or nicotine. The decrease in infection by benzoic acid was strongly correlated with reduced TMPRRS2 activity, which is required for viral fusion to host cells after binding to the ACE2 receptor (Hoffman et al., 2020; Jackson et al., 2022). The protection from PG/VG- and nicotine-enhanced-infection that benzoic acid provided remained at least 48 hours after exposure stopped. BLU™ EC aerosols, which contain nicotine, but not benzoic acid, increased pseudoparticle infection, showing that infection of EpiAirway varies with EC brand and e-liquid content. Infection of EpiAirway tissues occurred when both low- and high-power ECs were used to create aerosols, showing that even the low powered JUUL™ battery when used with a third-party pod was sufficient to induce PG/VG or nicotine enhanced infection. TMPRSS2 activity was highly correlated with the pH of the aerosolized e-liquid, and infection was highly correlated with pH, with low pH values producing low levels of infection. These data are consistent with the conclusion that the relationship between EC use and SARS-CoV-2 infection depends on the e-liquid ingredients, which can either increase or protect against infection by SARS-CoV-2 pseudoparticles.

We designed our Cultex® exposure protocol to simulate an acute EC exposure over 3 days. EC exposures vary among users and can range from 1 to 1,265 puffs/day with reported averages of 140 and 200 puffs/day (Behar et al., 2015; Dautzenberg et al., 2015). We chose 50 puffs/day as this is within the range that an EC user receives, although it is below several reported averages. The Cultex® system generates authentic

EC aerosols, which include any chemicals present in the e-liquid plus reaction products and metals that are produced during heating of the e-liquid (Bitzer et al., 2018; Erythropel et al., 2019; Jensen et al., 2017; Uchiyama et al., 2020; Williams et al., 2017). The cloud chamber enabled further study of the effects observed in the Cultex® through isolation of the chemicals. The combination of these two ALI exposure systems provides an innovative technology for understanding how aerosols affect the human respiratory system, and when used in conjunction with 3D EpiAirway models represent a powerful approach for assessing the effect of viruses on the respiratory system, a problem that cannot readily be addressed experimentally in human subjects.

In our previous study, we used monolayers of BEAS-2B cells in submerged cultures and ALI exposures (Phandthong et al., 2022). Treatments that increased ACE2 (nicotine, nicotine plus PG/VG, and authentic JUUL™ aerosol) also increased viral pseudoparticle infection; however, the increase in the JUUL™ group was significantly less than in the nicotine only group, even though both groups were exposed to a similar concentration of nicotine (Phandthong et al., 2022). It is likely that benzoic acid in JUUL™ aerosol suppressed nicotine-enhanced infection in our prior study, but the effect was not as great as in the current study. This difference is likely due to variations in the exposures. In the BEAS-2B study, cells in the Cultex® were exposed only once to 10 puffs of JUUL™ aerosol. In the current study, exposures were 50 puffs/day over a 3-day span. The latter exposure protocol was sufficient to completely prevent nicotine enhanced infection of EpiAirway, while 10 puffs on one day only partially reduced the effect of nicotine on infection of BEAS-2B cells. Comparison of these studies shows that user topography could affect the outcome of experiments and the health effects that EC users experience.

A critical result in this study was the finding that authentic JUUL™ aerosols and mixtures containing benzoic acid kept SARS-CoV-2 pseudoparticle infection at or below clean air control levels. Although benzoic acid has been used for many years as a food preservative (National Center for Biotechnology Information, 2022) and recently became a common additive in 4$^{th}$ generation EC products (Cunningham et al., 2020), its effect on the respiratory epithelium is not well characterized. Our data showed that EpiAirway treated with EC aerosols containing benzoic acid caused infection results similar to the control group, while aerosols from BLU™, which does not have benzoic acid, elevated infection. Benzoic acid by itself did not affect infection (cloud chamber), but when combined with EC chemicals that normally increase infection, such as nicotine or PV/VG, benzoic acid held infection to control levels. These data clearly show that infection by SARS-CoV-2 is strongly influenced by the ingredients in e-liquids and inclusion of benzoic acid can reverse the increases in infection induced by nicotine and PG/VG.

The suppression of PG/VG or nicotine-enhanced infection by benzoic acid was correlated with EC-liquid pH and with TMPRSS2 activity. These data support the conclusion that benzoic acid lowers the pH at the ALI to a level that reduces TMPRSS2 activity, which in turn reduces infection. This conclusion is supported by our data showing that TMPRSS2 had optimal activity between pH 7-8, well above that in benzoic acid-containing solutions. Our optimum is in reasonable agreement with Shrimp et al (2020) who reported the pH optimum of isolated TMPRSS2 protein to be between 8-9. The differences in range for pH optima may be due to the use of cell lysates vs. isolated TMPRSS2 enzyme. In either case, TMPRSS2 has optimal activity at 7 or above, and our infection data show that aerosols produced from e-liquids with higher pH (i.e., those containing nicotine without benzoic acid or those made from BLU™ EC) in most cases

had higher levels of viral pseudoparticle infection. Other types of viruses may also be susceptible to the low pH levels in benzoic acid containing e-liquids. Influenza A type virus undergoes an irreversible loss of activity at pH levels between 4.6 and 6.0 when tested in vitro (Scholtissek et al.,1985).

The low pH aerosols containing benzoic acid may also affect binding of the virus to its ACE2 receptor. The spike protein has four receptor binding domains (RBDs) that are cryptic at low pH and become exposed and available for binding as pH is elevated (Jackson 2022; Zhou et al., 2020). Additionally, pH 7.5-9 was found the be an optimum pH for binding of ACE-2 and spike (Xie et al., 2022). Low pH aerosols likely reduce the exposure of the cryptic RBDs and therefore also reduce the ability of the SARS-CoV-2 pseudoparticles to bind to ACE2. The pH of exhaled aerosol may also inactivate both influenza A and SARS-CoV-2 viruses in indoor environments (Luo et al., 2022), further suggesting that the low pH of JUUL™ and other 4$^{th}$ generation aerosols may be directly damaging to SARS-CoV-2 virus. This in combination with reduced TMPRSS2 activity are three mechanisms that likely reduced viral infection in JUUL™ and other aerosols containing benzoic acid.

The suppression of nicotine- or PG/VG-induced infection by benzoic acid was sustained 48 hours after ALI exposure, suggesting that benzoic acid may have additional roles in reducing SARS-CoV-2 infection. While pH may be lowered during inhalation of benzoic acid-containing aerosols during vaping, it likely returns to normal after vaping has stopped, and this probably does not take 48 hours to occur. Our study focused on the infection step of COVID-19. It is possible that benzoic acid also affects later stages in pathogenesis. *In silico* models have shown that benzoic acid and its derivatives can

interact with proteases involved in SARS-CoV-2 replication and reduced their functions (Stefaniu et al., 2020).

In contrast to nicotine-containing aerosols, aerosols produced from PG/VG alone had a low pH (5), yet increased infection. This may be due to PG's ability to stabilize protein interactions and to increase transepithelial permeability. PG can stabilize small molecules and proteins and extend their reactivity for a long period (Rocchitta et al., 2018; Qarawi et al., 2017). Prior to SARS-CoV-2 entry, spike protein is cleaved by furin at the boundary of the S1 and S2 subunits. This modification increases instability of spike and can cause the S1 subunit to be shed from spike, leading to a premature change to post-fusion conformation of spike, which would make it non-functional (Jackson et al., 2022). Exposure to PG/VG aerosol may help stabilize the tenuous association between the S1-S2 subunits, increasing the chance of infection. In support of this idea, the SARS-CoV-2 D614G strain acquired a mutation that stabilized the S1-S2 association and increased its infectability (Zhang et al 2020, Li et al., 2021). PG/VG e-liquid treatments also rapidly decrease transepithelial electrical resistance, weaken cellular tight junctions, and increased transepithelial permeability (Woodall et al., 2020). Such weakening of cellular-tight junctions could allow the virus to penetrate deeper into the tissue, causing more infection.

Our finding that nicotine increased ACE2 agrees with prior studies (Wang et al., 2020; Naidu et al 2021; Masso-Silva et al.,2021; Lallai et al., 2021; McAliden et al., 2020; Phandthong et al., 2022). To the best of our knowledge, our data are the first to show that PG/VG aerosol can also increase ACE2, an important finding since these solvents are the dominant chemicals in most EC liquids (Omaiye et al., 2019; Omaiye et al., 2022; NAS, 2018). We also showed that both nicotine and PG/VG increase ACE2 when present in

authentic JUUL™ aerosols made using the Cultex® system. The parallel exposures in a cloud chamber and Cultex® thus enabled authentic aerosol to be evaluated and the chemicals responsible for ACE2 elevation to be identified (cloud chamber). Elevation of ACE2 by nicotine is generally considered an indicator that viral infection would increase (Wan et al., 2020; Gheware et al., 2022). While our data in part support this conclusion (e.g., PG/VG elevated both ACE2 and pseudoparticle infection), increases in ACE2 were not always correlated with increased infection. For authentic JUUL™ aerosols and mixtures containing benzoic acid, ACE2 was usually significantly elevated, but infection was at or below clean air control levels, indicating an inverse relationship between ACE2 and infection. Thus, caution should be used in interpreting ACE2 levels, as they do not always correlate with infection.

Increases in PG/VG-induced infection of EpiAirway occurred with both the VOOPOO (20 watt) and JUUL™ (8 watt) ECs. While the VOOPOO's infection mean was higher than that of the JUUL™ with the third-party pod, there was not a significant difference between the group means, showing that even low powered 4$^{th}$ generation ECs can produce the PG/VG-induced increase in infection as effectively as the higher powered VOOPOO. In both cases, benzoic acid prevented the PG/VG-induced increases in infection.

Most work on the relationship between tobacco product use and COVID-19 has been done with cigarette smokers and tobacco cigarettes. Several recent meta-analyses found that cigarette smokers have a higher susceptibility to COVID-19 infection than non-smokers, and following infection, patients with smoking histories were more likely to develop severe COVID-19 symptoms leading to hospitalization and death (Dorjee et al., 2020; Patanavanich et al., 2020; Li et al., 2021; Muus et al., 2021). Nevertheless, the

relationship between smoking and COVID-19 remains unresolved and some investigators have proposed that nicotine, from tobacco smoke, protects against SARS-CoV-2 infection (Changeux et al., 2020; Farsalinos et al., 2020).

Similarly, there is some discrepancy in the EC literature regarding the influence of EC use to SARS-CoV-2 susceptibility (Gaiha et al., 2020; Li et al., 2020; Wang et al., 2020; Ghosh et al., 2022; Jose et al., 2021; Burnett-Hartman et al., 2022). Our study clearly shows that the EC brand and the presence of benzoic acid in e-liquids mitigates the enhanced infection caused by nicotine. Because prior EC studies with humans have not segregated EC users based on whether they used acid containing ECs, the data are likely noisy, which may account for some of the contradictory reports regarding the correlation between EC use and SARS-CoV-2 infection. In two clinical studies (Jose et al 2021; Burnett-Hartman et al., 2022), all EC user data were collected into a single group without regard to the EC products used. Based on our data, it would be interesting, if possible, to reanalyze these clinical data and separate the EC users into subgroups based on their EC products (acid containing or not). Perhaps, another conclusion could be extracted from new analysis and could explain some conflicting information surrounding EC use and SARS-CoV-2 susceptibility.

In summary, our data address the question: “Does EC use increase or decrease the likelihood of SARS-CoV-2 infection?”. The answer is EC use can do either depending on the ingredients in the e-liquid that is aerosolized. EC aerosols, with or without nicotine and with or without benzoic acid, increased ACE2 in the EpiAirway tissues, and this could facilitate infection by SARS-CoV-2. However, TMPRSS2 activity, which is seldom monitored, was decreased at the acidic pH levels of EC aerosols containing benzoic acid and this held infection to clean air control levels. Many 4$^{th}$ generation EC products use

benzoic or other acids that lower aerosol pH, and these are also likely to reduce SARS-CoV-2 infection. Given the diversity of e-liquids that have been sold in the past and currently, it is not surprising that prior studies have drawn different conclusions regarding the relationship between smoking/vaping and COVID-19 (Gaiha et al., 2020; Li et al., 2020; Wang et al., 2020; Ghosh et al., 2022; Jose et al., 2021; Burnett-Hartman et al., 2022). Overall, these data demonstrate that individual ingredients (nicotine, PG/VG, and benzoic acid) can influence SARS-CoV-2 infection of human bronchial epithelial tissues and that benzoic acid, if present, will override the increased infection produced by nicotine and PG/VG. Future studies involving organotypic cultures, animals, or humans should separately evaluate groups with and without e-liquids containing benzoic or other acids. If low and high pH e-liquids are not studied separately, the data will likely be unclear and significant effects of vaping will be missed. We do not advocate acid containing EC products as a prophylactic for avoiding COVID-19; however, if already vaping, then using an acid containing product may help reduce viral infection. It is important to be aware that the long-term effects of inhaling benzoic or other acids in the context of EC aerosol is not known and may itself have adverse health consequences.

**Limitations of the Study**

We examined one 4th generation product (JUUL™ “Virginia Tobacco”). It will be important to determine if aerosols produced from other 4th generation ECs that have benzoic and other acids can offset the effects of nicotine and PG/VG. The EpiAirway model provides an *in-vitro* analysis technology that closely resembles *in-vivo* exposure in humans. Because standard Mat-Tek EpiAirway uses primary epithelium from a single donor, it would be informative to test 3D cultures from other human donors. The effect of acid pH on infection lasted at least 48 hours. In the future, other acids and longer intervals after exposure could be tested. Finally, during heating of e-liquids, metals can be added to the aerosols and reaction products can form from the solvents and flavor chemicals (Son et al., 2019; Ogunwale et al., 2017; Zhao et al., 2019: Williams et al., 2019). It is not yet known how these chemicals affect SARS-CoV-2 infection.

## Author Contributions

Project administration and funding acquisition, P.T.; Conceptualization, R.P. and P.T.; Investigation R.P.; Sample preparation, data collection, and data processing were done by R.P., M.W., A.S., and T.M.; Data Interpreted by R.P. and P.T.; Writing – original draft by R.P. and P.T.; Writing- Review & Editing, all authors.


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## Declaration of Interest

The authors have no competing interests to declare.

## Figures and Legends

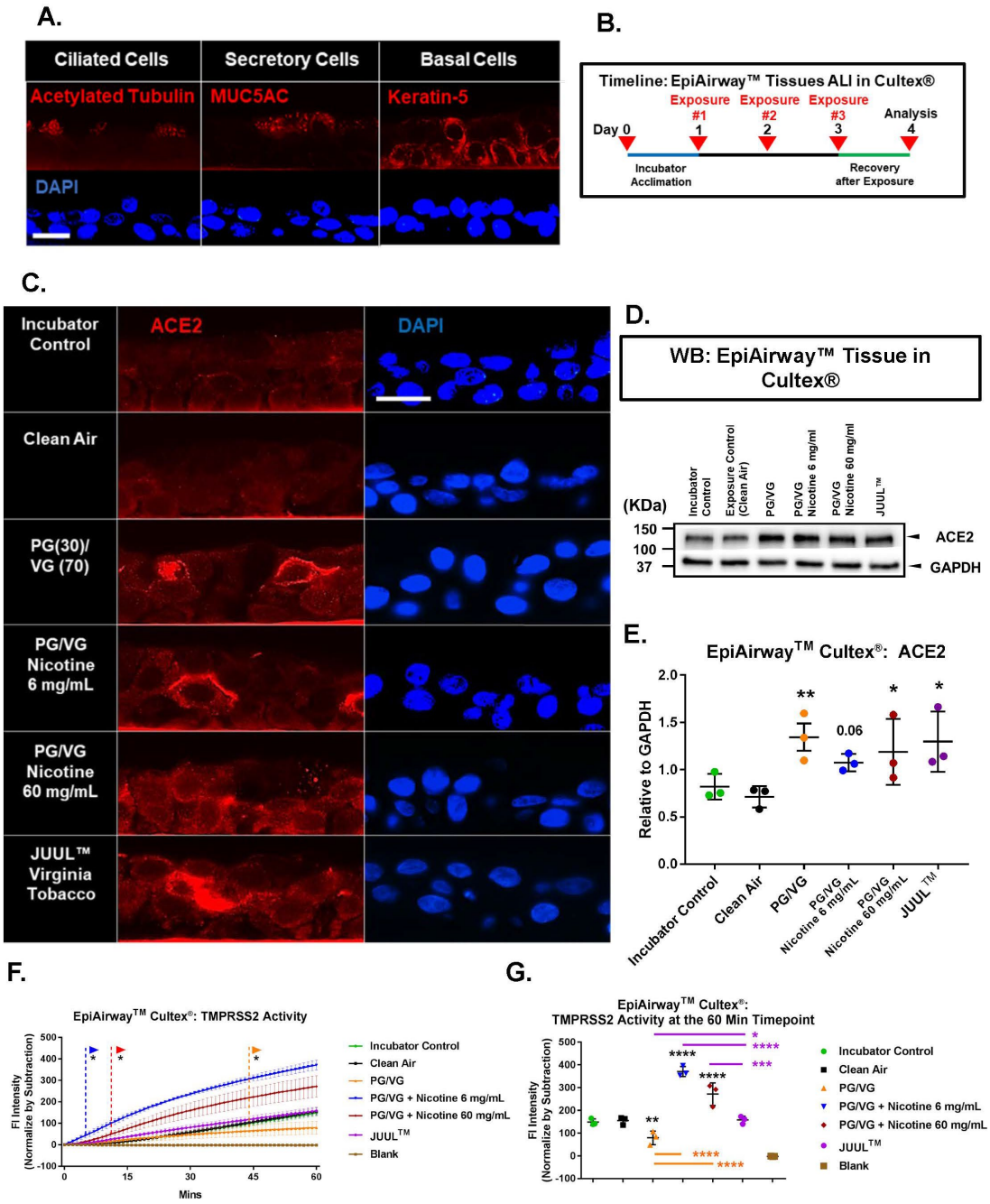


**Figure 2.1. PG/VG, Nicotine, and JUUL™ EC Aerosols Affect ACE2 and TMPRSS2, in EpiAirway™ Tissue Following ALI Exposure in a Cultex® System.**

**(A)** Micrographs showing cell types in EpiAirway™ tissues. Ciliated cells are labeled with acetylated tubulin. Secretory cells are labeled with MUC5AC. Basal cells are keratin-5 positive. Cell nuclei are shown by DAPI (blue) labeling. Scale bar = 50 μm
**(B)** Timeline showing experimental design used with EpiAirway™ tissue at the ALI in a Cultex® exposure system.
**(C)** Immunofluorescence labeling of EpiAirway™ tissues exposed to 50 puffs/day of EC aerosols for 3 days. Exposure was at the ALI in a Cultex® system. The aerosols were generated from PG/VG, PG/VG with 6 or 60 mg/mL of nicotine, and a JUUL™ "Virginia Tobacco" EC. Cells are labeled with ACE2 antibody (red) and DAPI (blue). Treatments induced an increase of ACE2. Scale bar = 50 μm
**(D, E)** Western blot showing effects on ACE2 of ALI exposure of EpiAirway™ tissue to EC aerosols in a Cultex® system. The aerosol exposures significantly increased ACE2.
**(F, G)** ALI exposure of EpiAirway™ in the Cultex® system produced different effects on TMPRSS2 activity. (**F)** Activity over 60 minutes: PG/VG significantly decreased activity between 43 and 60 minutes (*p* ranged from 0.05 to 0.0001), the PG/VG + nicotine 6 mg/mL significantly increased activity between 5 and 60 minutes (*p* values ranged from 0.05 to 0.0001), the PG/VG + nicotine 60 mg/mL significantly increased activity between 11 and 60 minutes (*p* values ranged from 0.05 to 0.0001). (**G)** Activity at the 60-minute timepoint. JUUL™ aerosol did not affect TMPRSS2 activity.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, **E** and **G** were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. Orange asterisks: significantly different than the PG/VG group in **G**. Purple asterisks: significantly different than the JUUL™ group in **G**. **F** was analyzed using a two-way ANOVA followed by Dunnett's posthoc test to compare means to the exposure control. For all figures: Black asterisks: significantly different from the clean air control. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

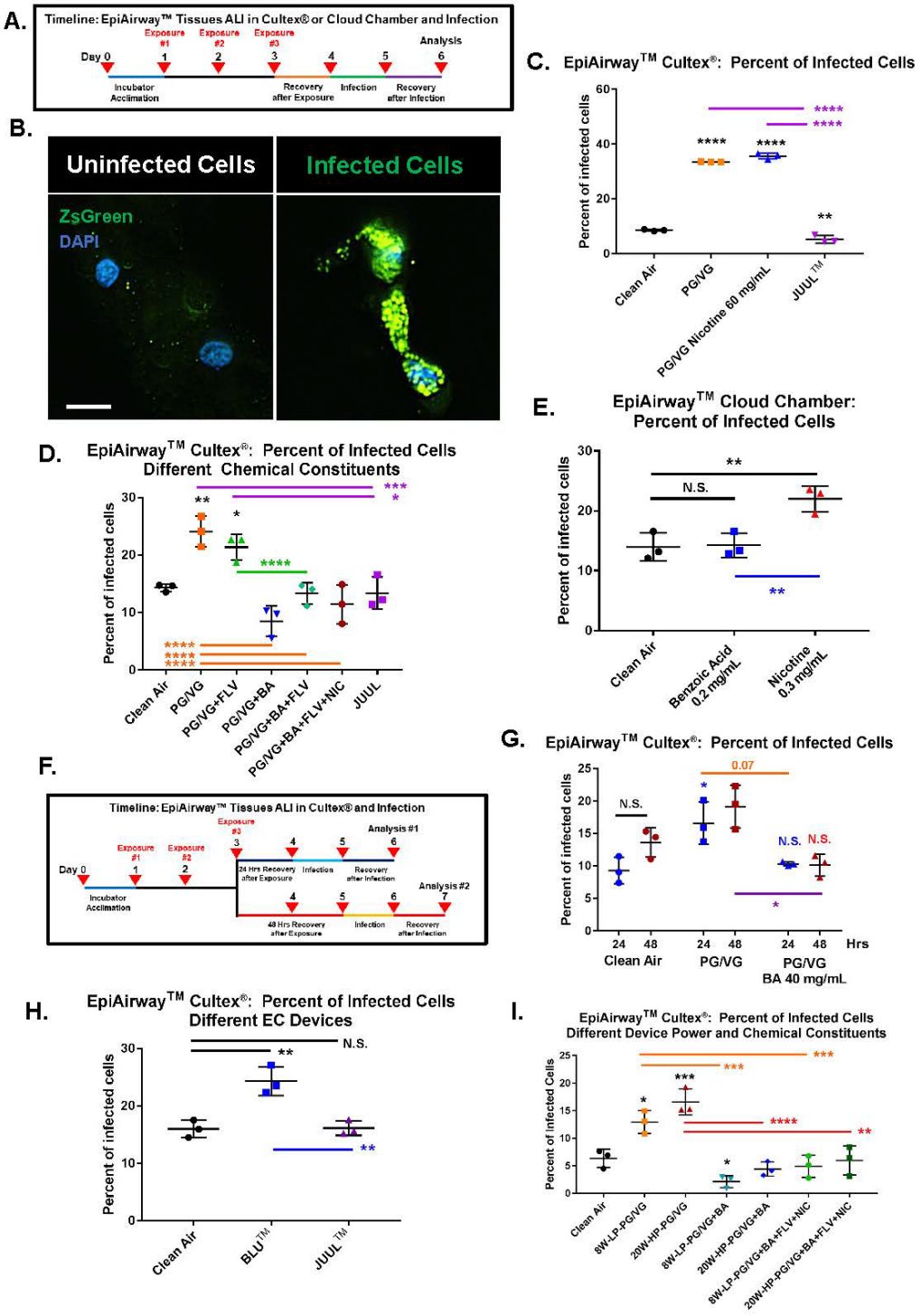


**Figure 2.2. EC Aerosols Increased EpiAirway™ Tissue Susceptibility to Viral Pseudoparticle Infection Except When Exposed to EC Aerosols with Benzoic Acid**

**(A)** Timeline showing experimental design used with EpiAirway™ tissue exposed at the ALI to aerosols in a Cultex® exposure system or Cloud Chamber, then infected with viral pseudoparticles. Purple asterisks: significantly different than the JUUL™ group
**(B)** Micrographs showing EpiAirway™ cells infected with viral pseudoparticles. Infected cells expressed ZsGreen. Scale bar = 50 μm.
**(C)** Flow cytometry data showing the percent of infected cells in the EpiAirway™ tissue after exposure to EC aerosols, followed by 24 hrs of exposure to pseudoparticles. PG/VG and PG/VG + nicotine 60 mg/mL increased infection, but JUUL™ "Virginia Tobacco" EC fluid did not.
**(D)** Effect of chemical constituents in aerosols on infection of EpiAirway tissues. Exposures were done in a Cultex® system using JUUL™ compatible pods filled with different mixtures. Infection did not increase in treatments containing benzoic acid. Orange asterisks: significantly different than the PG/VG group. Green asterisks: significantly different than the PG/VG+FLV group. Purple asterisks: significantly different than the JUUL™ group.
**(E)** Benzoic acid reduced nicotine enhanced infection of EpiAirway™ tissues. Exposures were done in a VITROCELL® cloud chamber using aerosols produced without heating. Blue asterisks: significantly different than the benzoic acid 0.2 mg/mL group.
**(F.)** Experimental design used with EpiAirway™ tissue exposed at the ALI to aerosol with either a 24 hrs (blue and cyan) or 48 hrs (red and orange) recovery period, followed by viral pseudoparticle exposure at the ALI in a Cultex® exposure system.
**(G.)** Flow cytometry data showing that protection against infection lasted at least 48 hrs after exposure to benzoic acid. Blue asterisk and labels show comparison among the 24 hours post-exposure group. Red labels show comparisons among for the 48 hours groups. Orange numbers show comparison against 24 hours PG/VG group. Purple asterisks: significantly different than the 48 hours PG/VG group.
**(H)** Flow cytometry data showing that JUUL™ "Virginia Tobacco" aerosol imitated enhanced infection, but aerosols Classic Tobacco BLU™ ECs did not and increase the infection. Blue asterisks: significantly different than the BLU™ group.

**(I)** Effect of power and chemical constituents in aerosols on infection of EpiAirway™ tissues. Exposures were done in a Cultex® system using a low power JUUL™ device that generated aerosol using 8 watts of power and a high power VOOPOO EC device that use 20 watts of power. Orange asterisks: significantly different than the 8W-LP-PG/VG group. Red asterisks: significantly different than the 20W-HP-PG/VG group.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, data were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. For all figures: Black asterisks and labels show comparison against the exposure control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. N.S. = not significant.

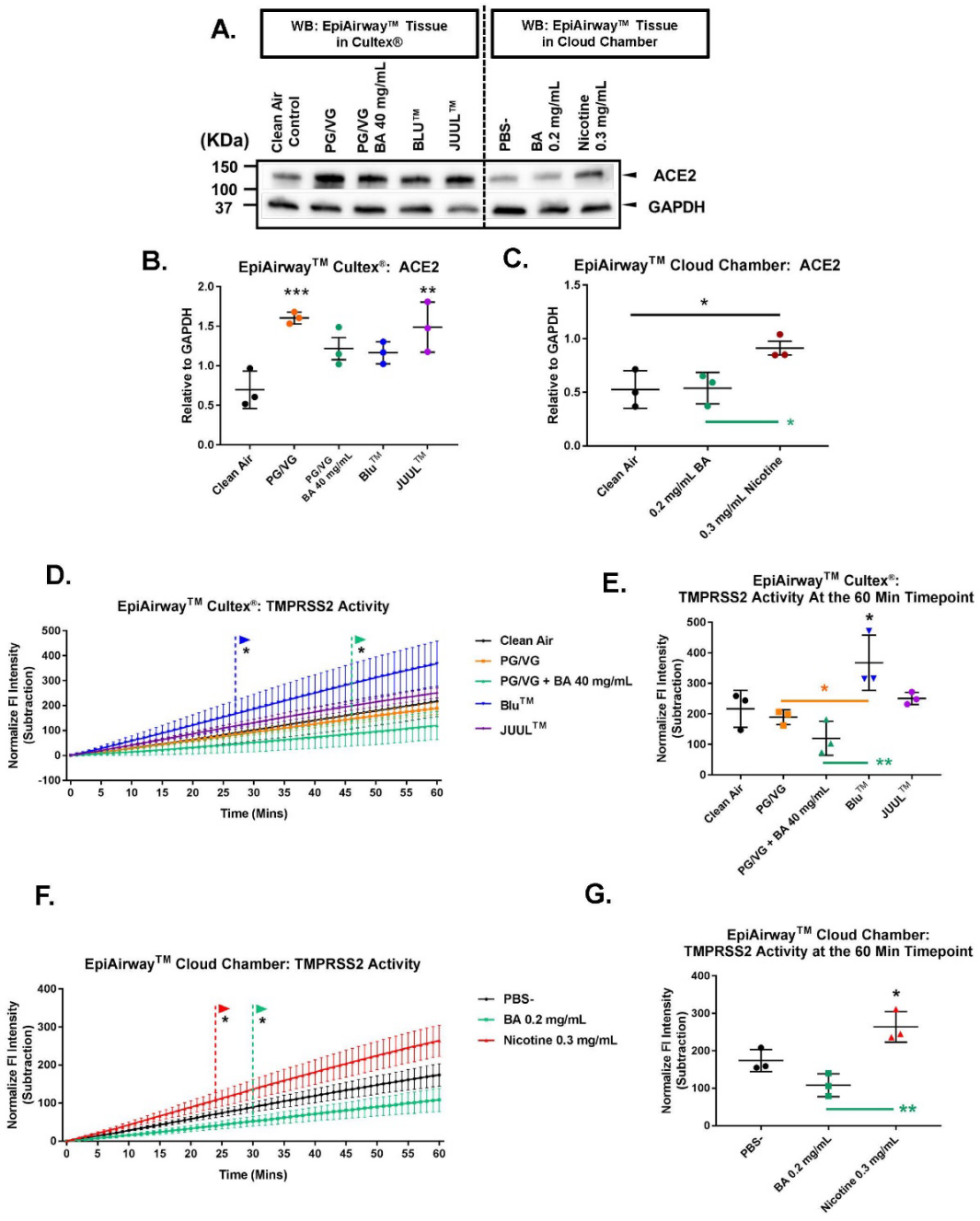


**Figure 2.3. Benzoic Acid in EC Aerosol Nullified PG/VG or Nicotine Enhanced Infection in the EpiAirway™ Tissue by Decreasing TMPRSS2.**

**(A-C)** Western blots showing the effects on ACE2 levels on EpiAirway™ tissues exposed at the ALI in the Cultex® system (A, B) or Cloud Chamber (A, C). EC aerosols were generated from JUUL™ EC using compatible pods filled with PG/VG, PG/VG + 40 mg/mL benzoic acid, and JUUL™ "Virginia Tobacco" EC fluid, or Classic Tobacco BLU™ EC. Aerosols in the Cloud Chamber were generated from benzoic acid 0.2 mg/mL solution or nicotine 0.3 mg/mL. Teal asterisks: significantly difference than the benzoic acid 0.2 mg/mL group.
(**D-E**) TMPRSS2 activity assays showing effects of EC aerosols on the EpiAirway™ tissues in the Cultex® system. **(D)** Activity over 60 minutes: PG/VG + 40 mg/mL benzoic acid significantly decreased activity between 46 and 60 minutes (*p* ranged from 0.05 to 0.0001), the BLU™ significantly increased activity between 27 and 60 minutes (*p* values ranged from 0.05 to 0.0001), the PG/VG + nicotine 60 mg/mL was significantly increase activity between 11 and 60 minutes (*p* values ranged from 0.05 to 0.0001). (**E)** Activity at the 60-minute timepoint. JUUL™ aerosol and PG/VG only did not changed TMPRSS2 activity.
Orange asterisks: significantly difference than the PG/VG group. Teal asterisks: significantly difference than the PG/VG + 40 mg/mL benzoic acid. Purple asterisks: significantly difference than the JUUL™ group.
(**F-G**) TMPRSS2 activity assays showing pure benzoic acid aerosol decreased TMPRSS2 activity, while nicotine significantly increased TMPRSS2 activities. **(F)** Activity over 60 minutes: the 0.2 mg/mL benzoic acid significantly decrease activity between 30 and 60 minutes (*p* ranged from 0.05 to 0.0001), the 0.3 mg/mL nicotine significantly increased activity between 5 and 60 minutes (*p* values ranged from 0.05 to 0.0001). (**G)** Activity at the 60-minute timepoint.

Data are plotted as the mean ± standard deviation of three independent experiments. Following a Box-Cox transformation, **B, C, E** and **G** were analyzed using a one-way ANOVA followed by Tukey's posthoc test to compare means between groups. **D** and **F** were analyzed using a two-way ANOVA followed by Dunnett's posthoc test to compare means to the exposure control. For all figures: Black asterisks: significantly difference than the exposure control. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

A.

| Treatments | pH |
|---|---|
| Nicotine 0.3 mg/mL | 9.41 |
| Benzoic acid (BA) 0.2 mg/mL | 3.87 |
| PG/VG | 5.05 |
| PG/VG Nicotine 6mg/mL | 9.09 |
| PG/VG Nicotine 60mg/mL | 9.83 |
| PG/VG + BA 40mg/mL | 2.90 |
| PG/VG + BA 40 mg/mL + FLV | 2.87 |
| PG/VG + FLV | 5.01 |
| PG/VG+FLV+NIC 60mg/ml+BA 40mg/ml | 6.08 |
| JUUL | 5.62 |
| BLU | 8.02 |

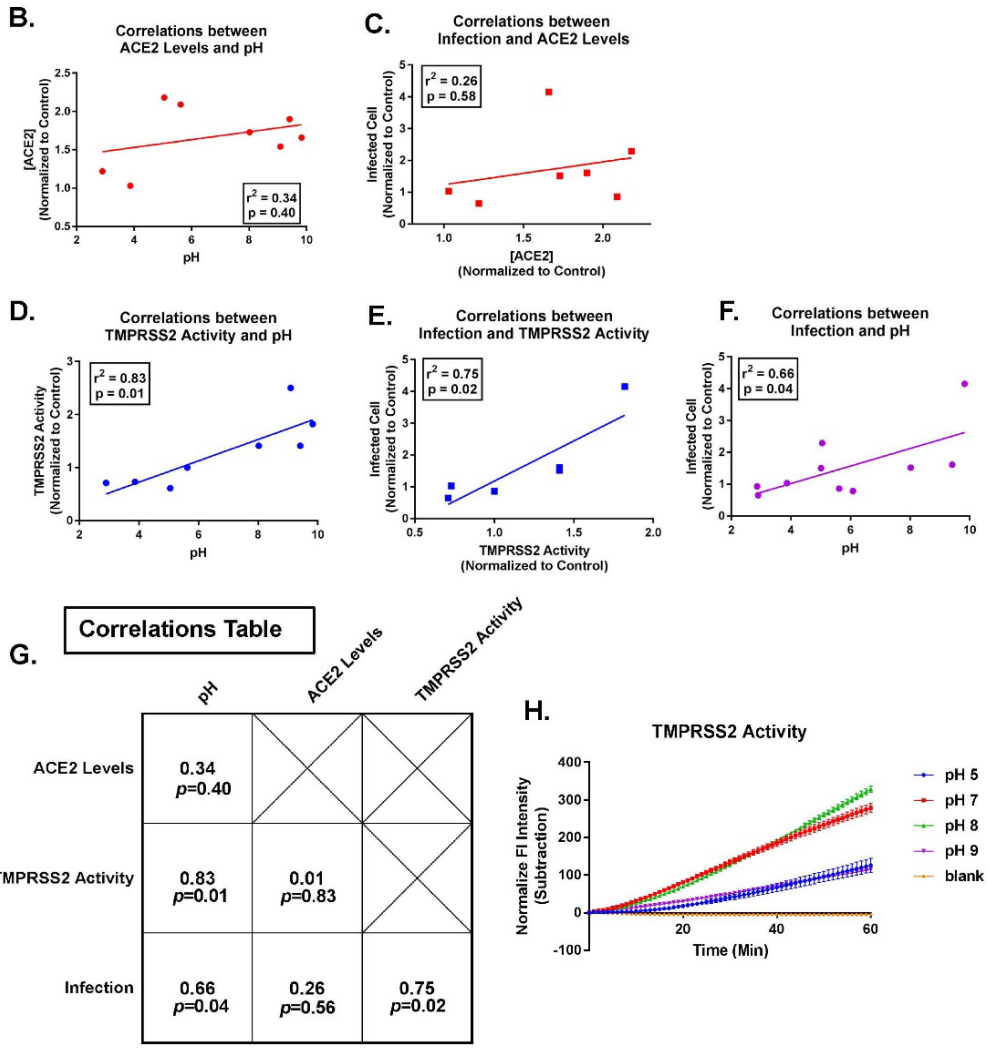


**Figure 2.4: The correlation between e-liquid pH, ACE2, TMPRSS2 activity, and Infection.**

**(A)** The table shows the pH of different treatment solutions and e-liquids before aerosolized exposure in cloud chamber or Cultex® system.
**(B-F)** Linear regression analysis comparing: **(B)** ACE2 concentration and pH, (**C)** infection and ACE2 concentration, **(D)** TMPRSS2 activity and pH, **(E)** infection and TMPRSS2 activity, and **(F)** infection and pH. Pairwise-Pearson correlation was used to test correlation and significance for each group.
**(G)** The correlation table summarizes each relationship of e-liquid, ACE2, TMPRSS2, and infection. $R^2$ values show for each interaction. The color gradient indicates the strength of each interaction from gray ($r^2$=0 to red ($r^2$=1).
**(H)** Graphs show TMPRSS2 activity of cell lysates in buffers with different pHs over 60 minutes.

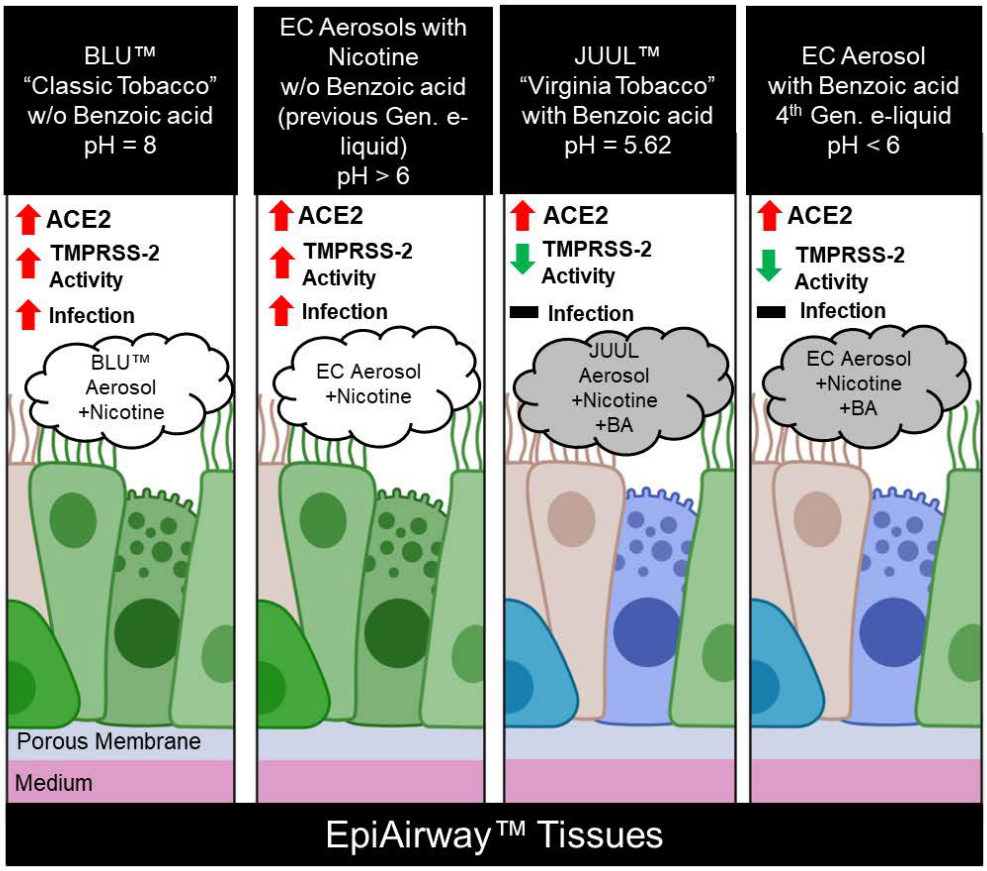


**Figure 2.5: The Effects of Nicotine, Benzoic Acid and pH on ACE2 Levels, TMPRSS2 Activity, and SARS-CoV-2 Pseudoparticle Infection.**
Summary of the responses of EpiAirway tissues to aerosols produced from authentic BLU™ "Classic Tobacco", authentic JUUL™ "Virginia Tobacco", mixtures (PG/VG plus nicotine, PG/VG plus nicotine and benzoic acid), and individual chemicals (PG/VG, nicotine, benzoic acid). All EC aerosols increased ACE2 levels in the EpiAirway™ tissue. However, the infectivity of the EpiAirway™ tissues by the SARS-C0V-2 pseudoparticles was not directly correlated with ACE2 levels. The pHs of the e-liquid modulated TMPRSS2 activity and both were critical factors that affected the infectivity of the SARS-C0V-2 viral pseudoparticles. E-liquids with nicotine, but not benzoic acid, such as BLU™ "Classic Tobacco", had pHs >6, which elevated TMPRSS2 activity and enhanced infection. E-liquids with nicotine and benzoic acid, such as JUUL™ "Virginia Tobacco", had lower pHs ranging from 5.62 – 6, which reduced TMPRSS2 activity and mitigated nicotine-enhanced infection. Surprisingly, PG/VG only aerosol with pH of 5 increased infection were involved (not shown in this figure).

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## Conclusions

The purpose of this dissertation was to answer the question: “Does EC use increase infection of human respiratory cells by the SARS-CoV-2 virus? This question was addressed by exposing human BEAS-2B cells and 3D organotypic bronchial epithelial tissues to authentic EC aerosols (JUUL™ and BLU™) or their individual constituents using three exposure platforms (submerged culture, air liquid interface (ALI) exposure in a cloud chamber, and ALI exposure in a Cultex® system), followed by inoculation with SARS-CoV-2 pseudoparticles to test infection. Our methods allowed manipulation of exposures at the ALI, chemicals in aerosols, their concentrations, and exposure methods. While data agreed well across our exposure platforms, there were some differences between platforms. For example, PG/VG only increased infection of cells/tissues that were exposed in the Cultex® system, perhaps due to formation of reaction products from the solvents and/or release of metals during heating. These data clearly show the importance of using ALI exposures and heat-aerosols generated from an actual EC. Our data show that the effect of EC use on infection is complex and depends on the chemicals (and their concentrations) in the EC liquids and, in the case of PG/VG, on the method of aerosol generation (heated vs non-heated). The most salient results were: (1) EC aerosols, with or without nicotine and with or without benzoic acid, increased ACE2 levels, thereby increasing the likelihood of SARS-CoV-2 binding to cells; (2) EC aerosols with nicotine, but without benzoic acid, increased TMPRSS-2 protease activity, which would facilitate infection by enabling more rapid modification of the spike protein after ACE2 binds to the host cells; (3) nicotine in EC aerosols increased infection by viral pseudoparticles dose-dependently; and (4) benzoic acid in JUUL™ EC nullified the enhanced infection produced by nicotine and PG/VG, and suppression lasted at least 48 hours. The influence of benzoic

acid on SARS-CoV-2 infection is important and not previously reported. Our data clearly show that the answer to our original question -"Does EC use increase infection of human respiratory cells by the SARS-CoV-2 virus?"- is paradoxically both "yes" and "no", depending on many factors, the most important being the chemical constituents and pH of the e-liquid used to generate aerosols. TMPRSS2 activity was decreased at the acidic pH levels of EC aerosols containing benzoic acid and this held infection to clean air control levels. Our findings provide explanations for the contradictory data in the EC literature regarding the influence of EC use to SARS-CoV-2 susceptibility. Unless EC users were separated based on their EC brand ($4^{th}$ generation vs generations 1-3), data on SARS-CoV-2 would likely be noisy and may lead to the conclusion there is no effect. Given the variety of EC liquids and their formulations, prior studies may have come to different conclusions on the relationship between EC use and SARS-CoV-2 susceptibility. It would be interesting to reanalyze prior data and separate the data into subgroups based on EC products (acid containing or not). Perhaps, another conclusion could be drawn from new analysis and could explain some conflicting information surrounding EC use and SARS-CoV-2 infection. Future studies investigating the relationship between EC use and SARS-CoV-2 infection should consider benzoic or other acids as major factors that can alter infection results and should separately evaluate the effect of e-liquids with and without acid. If this is not done, data will likely be noisy and significance will be lost. We do not advocate the use of acid containing EC products as a prophylactic for avoiding SARS-CoV-2 infection; however, if already vaping, then using an acid containing product may help prevent enhanced viral infection produced by PG/VG and nicotine. It is important to note that EC use is not harm-free, and the long-term effects of inhaling acid in EC aerosol are not known and it may have adverse health consequences.