Articles in PresS. Am J Physiol Lung Cell Mol Physiol (September 25, 2015). doi:10.1152/ajplung.00272.2015

Will Chronic E-Cigarette Use Cause Lung Disease?

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Running title: E-Cigarettes and the Lung

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September 9th, 2015

Abstract

Chronic tobacco smoking is a major cause of preventable morbidity and mortality world-wide. In the lung, tobacco smoking increases the risk of lung cancer, and also causes chronic obstructive pulmonary disease (COPD), which encompasses both emphysema and chronic bronchitis. E-cigarettes (E-Cigs), or electronic nicotine delivery systems, were developed over a decade ago and are designed to deliver nicotine without combusting tobacco. Whilst tobacco smoking has declined since the 1950s, E-Cig usage has increased, attracting both former tobacco smokers and never smokers. E-Cig liquids (e-liquids) contain nicotine in a glycerol/propylene glycol vehicle with flavorings, which are vaporized and inhaled. To date, neither E-Cig devices, nor e-liquids, are regulated by the Food and Drug Administration (FDA). The FDA has proposed a deeming rule, which aims to initiate legislation to regulate E-Cigs, but the timeline to take effect is uncertain. Proponents of E-Cigs say that they are safe and should not be regulated. Opposition is varied with some opponents proposing that E-Cig usage will introduce a new generation to nicotine addiction, reversing the decline seen with tobacco smoking or that E-Cigs generally may not be safe and will trigger diseases like tobacco. In this review, we shall discuss what is known about the effects of E-Cigs on the mammalian lung and isolated lung cells *in vitro*. We hope that collating this data will help illustrate gaps in the knowledge of this burgeoning field, directing researchers toward answering whether or not E-Cigs are capable of causing disease.

Overview

Electronic cigarettes or e-cigarettes (E-Cigs), also known as electronic nicotine delivery systems (ENDS), were designed to deliver aerosolized nicotine in a minimal liquid vehicle that was thought to be relatively safe compared to tobacco. It has been proposed by E-Cig manufacturers that since these products do not burn tobacco, that they will not expose the lung to the same toxic chemicals as regular smoked tobacco and so, will not cause the lung disease that is frequently associated with chronic tobacco inhalation including lung cancer and chronic obstructive pulmonary disease (COPD). E-Cig users are a fast-growing subset of nicotine users who are described as "vapers" rather than smokers, since E-Cigs heat and generate aerosols, but do not burn e-liquids. There is considerable controversy regarding the disease risk and toxicity of E-Cigs (112, 116, 127). However, since E-Cigs do not currently fall under the auspices of the Food and Drug Administration (FDA), they have not undergone the typical toxicological evaluation, followed by human clinical trials that are required of other inhaled products (e.g. inhaled therapeutic agents) and as such, no safety data exists from either humans or animals. Because of this, it is hard to predict whether these products will be benign when chronically inhaled, possibly over a lifetime, or whether they will induce tobacco-like disease or other types of lung disease such as bronchiolitis obliterans, a disease that has been caused by the inhalation of the buttery-tasting flavor, diacetyl (83). The clinical evaluation of biomarkers of harm (e.g. inflammatory and cytotoxic markers) is required to inform the FDA and for ensuring safety and proper regulation. However, these studies are only just beginning in what can at best be described as "investigator-initiated trials", rather than formal clinical trials. A further confounder is that many E-Cig users have switched after chronically smoking tobacco products, making it difficult to differentiate between the previous effects of tobacco vs. the effects of the E-Cigs (40). To date, there are currently 1273 E-Cig articles on Pubmed, of which 135 are reviews and only 85 include the terms "e-cigarette" and "lung". In contrast, "tobacco" and "lung" yields 9769 hits, indicating the lack of maturity of this field. In this review, we shall list and evaluate what is known about the effects of E-Cig exposure on lungs/airways *in vivo* and *in vitro*.

The Problem Explained

Nicotine is a highly addictive compound that, through nicotinic acetylcholine receptors (nAChR), exerts potent effects on the brain including the saturation and desensitization of nAChRs ($\alpha_4\beta_2$ subtype) leading to significant changes in the brain's physiology such as activation of the reward/pleasure regions of the cortex and reduced anxiety (17, 50, 128). Inhaling tobacco is a relatively simple and efficient way of delivering nicotine into the bloodstream. Nicotine is a weak base which can be absorbed across the lung in its unionized form into the bloodstream (18). The effects of nicotine on the brain are complex and only just beginning to be understood. Importantly, its effects on the adolescent brain are markedly different compared to the adult brain and can affect neural development. For example, exposure to nicotine in adolescent rats led to an increased sensitivity to nicotine in these rats as adults, even if a smoking cessation period was introduced, suggesting that vaping by adolescents may have serious consequences later in life (41).

Nicotine craving causes a huge drive to continue smoking and smokers maintain fairly constant plasma nicotine levels during waking hours, despite the significant risks: Lung cancer (including both small cell and non-small cell) accounts for 27% of all diagnosed cancers and is the deadliest form of cancer, killing ~150,000 people in the USA per year (27, 132). It is thought that ~85% of all lung cancer is caused by smoking and secondhand smoke exposure increases the chance of lung cancer by ~25% (27). COPD is also caused primarily by tobacco exposure in Western countries and kills a similar amount of people as lung cancer (~140,000 per year) (12, 60). COPD is also the third leading cause of death in the USA and worldwide (157), though tobacco exposure is a primary risk factor of COPD in first world countries, smoke from biomass fuels is also a risk factor for COPD in second and third world countries (49). COPD often occurs with other co-morbidities and presentation with COPD is a major risk factor for the development of lung cancer (73, 106). The fact that tobacco exposure is a key factor in the development of both of these diseases was first denied by the tobacco industry and later accepted, following the Tobacco Master Settlement Agreement in 1998 (52, 79).

In the 1950s, approximately 50% of the adult population were regular tobacco smokers in the USA (30). Following the Surgeon General's 1954 report linking tobacco smoke with disease, a series of public health campaigns led to (i) increased public awareness about the dangers of smoking, (ii) bans on tobacco advertising, (iii) age limits for purchasing tobacco, (iv) bans on tobacco products that allegedly target minors (e.g. flavored cigarettes), (v) increased taxation on tobacco and (vi) restrictions on where tobacco products can be smoked (e.g. bans on smoking in public places) (76, 119). Due to these pressures, tobacco companies began developing "safe cigarettes". In the 1980s, "low tar" cigarettes were developed, which were purportedly safer than regular cigarettes because they exposed users to less of the tar phase from cigarettes. This premise was based on flawed science: In part, the reduced tar output was generated by putting holes at the base of the cigarette, which served to reduce airflow through the cigarette. However, these cigarettes were not safer than regular cigarettes. In fact, users learned to compensate by either covering up the holes with their fingers or taking larger puffs, thus negating the "low tar effects" (67, 117). Furthermore, the gas phase of cigarette smoke is also highly toxic and was not addressed in "low tar cigarettes" (111, 125). Additional types of "safe cigarette" have been developed including "heat not burn" types (e.g. the "Eclipse") which heats a rod in the middle of the cigarette to give off tobacco smoke without burning it. This style of cigarette was not successful commercially (6) and there is no evidence that they are actually safer (71).

Despite these failed attempts at 'safe cigarettes', the Institute of Medicine issued a report in 2001 that outlined the feasibility of focusing efforts on harm reduction tobacco products, which were referred to as "Potential to Reduced Exposure Products" or "PREPs" as they wanted to avoid implying "that any product currently known is *safe*" (141). At that time, there was no conclusive data that the current products on the market were reducing the individual users' exposure to harmful tobacco substances. However, the committee did conclude that there was potential merit in "harm reduction" as a part of the national tobacco program that "emphasizes abstinence oriented prevention and treatment" (141). Thus when E-Cigs were brought to market, they appeared to be likely candidate PREPs as they neither contain

tobacco, nor is the vapor produced by combustion. E-Cigs were introduced into European markets in 2006 and in the USA in 2007 (126). The first generation of E-Cigs were dubbed "cigalikes" due to their resemblance to conventional cigarettes and they came in both rechargeable/refillable and disposable formats (164) (126). Subsequently, as their popularity grew, second and third generation E-Cigs have been developed which, though they have ceased to resemble cigarettes, have significantly improved their ability to deliver nicotine through the lung and into the bloodstream (164). However, the health effects of long-term inhalation of E-Cig aerosols are currently unknown.

What are/what is in E-Cigs?

Cigarette smoke is a complex and highly reactive mixture that includes metals (e.g. Cr, Cd, Hg), aldehydes (e.g. 4-ABP, acrolein, formaldehyde), carbon monoxide, free radicals and of course, nicotine (57, 145). Adverse effects can be caused by adduct formation, especially of aldehydes, with DNA or proteins (114) or due to excessive oxidative stress (81). Aldehydes and heavy metals have been shown to have a number of cytotoxic effects on epithelia including adduct formation to DNA (32, 140, 154). Additionally, tobacco smoke, as well as aldehydes, cadmium and oxidative stress also affect plasma membrane proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR)(25, 33, 70, 137), which is required for fluid secretion in the lung (35, 60). In contrast, e-liquids (the flavored liquids which are heated to form the E-Cig vapor) are thought to be much simpler and ostensibly contain nicotine (~6-18 mg/ml) in a liquid vehicle (typically propylene glycol and/or glycerin), along with sweeteners and flavorings (65).

To date, over 400 different brands of E-Cigs have been produced (164). Unlike disposable E-Cigs, second and third generation E-Cigs contain a refillable tank, to which the e-liquid is added, a battery-powered atomizer which generates the aerosol from the e-liquid, and a mouthpiece which collects and delivers the aerosol. This function is usually controlled by a microchip, which may activate an LED at the tip of the E-Cig during inhalation for aesthetics. The amount of aerosol that is generated is directly proportional to the power of the battery, which has led some users to modify their E-Cig to increase

battery power in order to get a greater nicotine "hit". This is not without risks however, as there is a chance of battery explosion, which can lead to injury (23). Though the number of fires and explosions from E-Cig devices has increased since inception, interestingly, many of these instances occurred while the device was being charged and are still considered rare (https://www.usfa.fema.gov/downloads/pdf/publications/electronic_cigarettes.pdf). An additional, behavioral modification that has developed amongst E-Cig users is "dripping", which entails dripping e-liquid directly on the atomizer (i.e. the heating element) and inhaling the resultant vapor, which is supposed to give the largest amount of nicotine delivery possible with current E-Cig devices (146). Parameters of E-Cig emission such as aerosol size, mass output, and chemical composition vary by device and e-liquid types and are predicted to impact the user's exposure to the E-Cig aerosol. For example, aerosol size strongly affects how much of an aerosol is delivered to different regions of the lung and how much is retained in the oral cavity (16, 82).

There are currently over 7,000 different e-liquids that are commercially available (164). Since these e-liquids are not FDA regulated, the vendors do not have to list their e-liquid ingredients, nor to have performed any safety testing before they reach the market, nor generate these products under Good Manufacturing Practice-type conditions. For example, the reported amount of nicotine has been found to vary by up to 20% from what is reported on the e-liquid label and has even been found in purportedly nicotine-free e-liquids (38, 39, 63). It is likely that many of the compounds used in e-liquids fall within the FDA's Generally Regarded As Safe (GRAS) list [http://www.accessdata.fda.gov/scripts/fdcc/?set=SCOGS]. The typical vehicle for e-liquids contains a mix of propylene glycol and glycerol, which are both GRAS products (23). To date, most GRAS testing/toxicology has been performed following oral ingestion rather than following aerosolization to the lung. For example, diacetyl is sometimes added to foods as a buttery flavor and is on the GRAS list, based on its oral toxicology (135). Due to the potential for adverse health effects from inhaling other chemicals, the Flavor and Extract Manufacturers' Association of the United States (FEMA) issued a

statement warning that additives on the GRAS list apply to food only and should not be characterized as safe for use in E-Cig products without further testing (https://www.femaflavor.org/safety-assessment-and-regulatory-authority-use-flavors-focus-e-cigarettes). Propylene glycol is used as a common e-liquid vehicle component in part because of its perceived low toxicity. However, both ocular and upper respiratory irritation were reported in non-asthmatic adults following a short, controlled occupational exposure (158). Furthermore, diacetyl inhalation is known to cause bronchiolitis obliterans or "popcorn workers lung" (83). Bronchiolitis obliterans caused by the inhalation of diacetyl can cause a range of symptoms from mild, reversible respiratory impairment to a more severe non-reversible lung obstruction from extensive scarring in the small airways (11). With no current consensus on E-Cig user topography, it is possible that even non-asthmatic users who vape frequently could experience, at the very least, respiratory irritation. Although to date, no long-term data is available regarding chronic propylene glycol or flavorant exposure in humans.

Do E-liquids Undergo Thermal Decomposition (Pyrolysis) when Vaped?

E-Cig aerosols are typically generated at temperatures of 100-250°C, which is predicted to cause pyrolysis of the e-liquid vehicle (162) and may also induce breakdown of other e-liquid constituents. Recently, formaldehyde has been detected in E-Cig emissions (77). However, this data has been disputed (55). Part of the problem lies in deciding which temperature the e-liquid is heated to during the experiment vs. what occurs during actual vaping. For example, Jensen et al. found significant amounts of formaldehyde (~380 μg per 10 puffs) in the emission from a tank-style E-Cig device when the battery voltage was set at 5.0V, with no formaldehyde being detected when a lower voltage (3.3V) was used (77). Since the power consumption/electrical resistance of the coil was not quoted by Jensen et al., it will be hard to see how this observation transfers to other E-Cig devices. That is, the power generated by the heating coil cannot be determined purely by the quoted voltage since it also depends on the current, and the temperature reached by the e-liquid is dependent on the power output of the heating element. Thus, for reproducibility, it may be useful for researchers to quote the power output of their E-Cig device in

addition to the puff profile used. Farasalinos et al., have reported that E-Cig users do not use this higher voltage setting and they also proposed that E-Cigs only produce formaldehyde in "dry puff" conditions (55), where a dry puff refers to the scenario where there is little liquid on the atomizer coil and temperatures get higher than would be seen with sufficient liquid, leading to the potential for increased pyrolysis. However, acrolein and other carbonyls have also been found by other investigators both in neat e-liquids and in E-Cig aerosols that were generated by unmodified E-Cig devices (133), suggesting that the occurrence/production of these compounds may be more common than originally suspected. Interestingly, neat glycerin does not pyrolyze at 900°C. However, when diluted, significant amounts of acrolein were produced following pyrolysis of glycerol (28). Similarly, these aldehydes are known to be released from vegetable oil (of which glycerol is a major component) when it is heated during cooking, even to 180°C, which is close to temperatures reported for E-Cigs (130°C to 350°C) (146). For example, the acrid smell that occurs when oil is burnt on a stove is from acrolein (13, 29). Similarly, the chemical decomposition of sugars also causes the release of aldehydes including acrolein (144).

It has been proposed that E-Cig users tend to avoid the bitter taste that is associated with release of aldehydes during overheating/dry puffing and that in actual E-Cig users, aldehyde exposure never actually happens (55). However, during the aforementioned practice of "dripping", where the e-liquids are placed directly on the coil, it is possible that significant pyrolysis occurs. Certainly, cigarettes can produce a harsh taste that is concomitant with the production of significant amounts of acrolein, formaldehyde and other aldehydes, along with many other toxicants (144). However, this relatively unpleasant taste is soon overcome in new smokers due to the power of the nicotine drive (136) and due to cross-desensitization of Transient receptor potential ankyrin subtype 1 (TRPA1) channels in sensory neurons (19). Therefore, it is also possible that E-Cig users will "learn" to overcome any unpleasant taste due to increased aldehyde production if the nicotine drive is great enough. It is also worth pointing out at this point that many flavors are themselves aldehydes including anisaldehyde (sweet), cinnamaldehyde (cinnamon) and isovaleraldehyde (nutty). The effects of these flavors on pulmonary surfaces are not known. However,

their potential inclusion in e-liquids may increase overall aldehyde exposure to the lung. Indeed, cinnamaldehyde is present in some e-liquids (14) and activates TRPA1 (108), suggesting that they may exert effects on the lung. Similarly, activation of this ion channel in sensory neurons in the airways of rodents by unsaturated aldehydes has previously been shown to trigger neurogenic inflammation (7) and to inhibit the CFTR ion channel (4), suggesting that a higher aldehyde burden may indeed be toxic to the lung. However, the degree of adverse effects will likely depend on dose-ranging and whether aldehydes are actually generated in sufficient quantities during real vaping conditions to trigger these responses.

In addition to aldehydes, Lerner et al., also found that E-Cig aerosols generated from 2 separate devices produced oxidants and reactive oxygen species (OX/ROS) (94). Since the amount of OX/ROS changes with time as smoke matures, these data suggest that freshly-produced E-Cig aerosols may be more potent than "aged" E-Cig aerosols, which has important implications for studying these aerosols. Indeed, with regular cigarette smoke, different biological effects are seen with freshly-produced vs. aged smoke, with aged smoke often being less biologically potent, which has previously been attributed to the decline in OX/ROS over time (74). Furthermore, since OX/ROS are highly reactive, they may also react with other components in the E-Cig aerosol, further changing its chemical composition. Indeed, Sussan et al., demonstrated that E-Cigs contain 10^{11} free radicles per puff, which is about 100 times less than is seen in regular cigarettes (142), but still likely to exert significant biological effects (45).

E-Cig Topography

When generating cigarette smoke through a smoke machine, there are several international standards. These standards are important since the rate and duration that air passes through a cigarette affects the burn temperature and the relative amount of chemicals that are subsequently produced (68, 99), and this is likely true for E-Cigs. Also, having tobacco be smoked in a reproducible fashion facilitates cross-laboratory data comparisons. Smoking profiles are designed to mimic the inhalation topography seen in actual smokers. For example, the Federal Trade Commission/International Standard Organization (FTC/ISO) protocol calls for a 2 s/35 ml puff every 60 s and this is likely the most common

puff profile used in the laboratory. However, it has been suggested that this profile underestimates how much people actually inhale and a second profile, called "Canadian Intense", which uses a 2 s/55 ml puff every 30 s has also been adopted and it has recently be recommended that experiments be repeated with both profiles to study smoke generation over the range of exposures (68, 99). Similarly, for E-Cigs, knowing users puff topography characteristics will be important for setting smoke machine parameters in the laboratory and for studying appropriate E-Cig emissions. To date, no consensus exists on how to set E-Cig parameters and nothing comparable to the Canadian Intense profile has been developed. However, Farasalinos et al., found that E-Cig users took puffs of 4.2 s every 23 s, although they didn't record the puff volume (53), while Lee et al. found the average puff duration to be 3.1 s (93). In contrast, Behar et al. found that the average puff duration was 2.75 s every 17 s, with a inhalation volume of 56 mls (15). These authors studied several different types of E-Cig and found that parameters varied only slightly with the type of E-Cig used. It may be that the users puff harder/more frequently on E-Cig devices that are less efficient at delivering nicotine in order to maintain sufficient plasma nicotine levels. Indeed, data suggested that users were able to maintain constant nicotine uptake, despite switching brands (15). Importantly, until a greater consensus is reached, these data suggest that a modified Canadian Intense profile may be a suitable parameter for studying E-Cig aerosol generation.

Will Nicotine and Chemical Constituents in E-liquids/E-Cigs Alter Airway Physiology?

Nicotine is a highly addictive substance that is a major component of both cigarette smoke and E-Cig aerosols that can cause physiological changes to users through nAChRs expressed throughout the body (36). Traditionally, nAChRs were primarily studied as part of the acetylcholine neurotransmitter signaling system in the central and peripheral nervous system. However, nAChR expression has been characterized in the airways as well (36, 96, 101, 165). These ligand gated ion channels are permeable to both Na^+ and divalent cations and are physiologically stimulated by acetylcholine. nAChRs contain 5 subunits, of which different subtypes exist (e.g. α, β, γ, δ) (3, 107). For example, in the brain, $(\alpha4)_3$, $(\beta2)_2$ is the most common type of nAChR, while in the lung, $(\alpha7)_5$ and $\alpha3$, $\alpha5$, $\beta4$ are common (147). Lee et al.

found that inhaled nicotine from cigarette smoke caused airway irritation and a cough reflex via nAChRs expressed in pulmonary afferent neurons (89).

Interestingly, nAChRs regulate cell proliferation as well as inhibit apoptosis (48). For instance, Maouche et al. found that α7 nAChRs were enriched in basal lung epithelia and that during development, α7 regulated basal cell proliferation (98), which is important for the maintenance of epithelial cell turnover and differentiation. It is well established that smoking is linked to lung cancer and a hallmark of lung cancer is uncontrolled cell proliferation. West et al. reported that both nicotine and its metabolite (NNK) stimulated Akt signal transduction downstream of nAChR activation, which altered cell proliferation and apoptosis in bronchial epithelia (156). Specifically, α3, α5, and β4 were identified as candidate genes for a potential role in lung cancer from genomic wide association studies (26, 75, 130, 139). Additionally, Lam et al. found different nAChR subunit gene expression profiles between nonsmokers and smokers with non-small cell lung cancer (86). In the same study, human bronchial epithelial cell lines (HBEC) were exposed to nicotine and expression was compared before and after removal of nicotine. Interestingly, exposing HBECs briefly upregulated nAChR α1, α5, and α7 expression at 72 h that returned to baseline levels after removal of nicotine. While all classes of nAChRs are capable of desensitization through chronic agonist exposure, there are definite immediate effects of nicotine on nAChRs in a subunit-dependent manner. Though it is currently unknown whether chronic exposure of nAChR to nicotine via E-Cigs can cause lung cancer, the role of nAChR α7 in contributing to non-small cell lung cancer by altering cell proliferation and apoptotic resistance has been reported (86, 113).

Many inflammatory cells contribute to COPD pathogenesis including, but not limited to dendritic cells, T and B lymphocytes, monocytes, macrophages and neutrophils (12, 72). Of note, monocytes, macrophages, and neutrophils, which are impacted by inhaling cigarette smoke in the lungs, also express nAChRs. The effects of non-cholinergic signaling in airway inflammatory cells has been described (64). Nicotine suppressed inflammation in human monocytes and in mouse macrophages (100, 160).

Neutrophil influx occurs in COPD and indeed neutrophils present in smokers have upregulated nAChR expression and display a reduced ability to undergo apoptosis (9, 37). Likely, these neutrophils are more sensitive to inhaled nicotine and have extended life spans, which may serve to prolong inflammation in the lungs. Taken together, these data indicate that nicotine has a pro-inflammatory effect on neutrophils. However, nicotine also has an anti-inflammatory effect on monocytes/macrophages, which may be negated in the case of cigarette smoke due to the inhalation of other pro-inflammatory products such as the tar phase. This dualism has curious implications for the chronic inhalation of nicotine from E-Cig aerosols as many of the cigarette tobacco and tar byproducts that contribute to inflammation are not present in E-Cig aerosols. It is possible that the anti-inflammatory effects of nicotine, in the absence of pro-inflammatory constituents, could suppress the user's immune system. Certainly, it is reasonable to assume that high nicotine exposure from E-Cigs will be a major pharmacological player following E-Cig exposure in any organ where nAChR are expressed. Thus E-Cig use may affect inflammation in the airways that could alter a user's susceptibility to infection and/or increase the risk of developing COPD or lung cancer.

Despite nicotine's known addictive and airway irritant properties, it is also known to be bitter-tasting. Due to this, E-Cigs and their e-liquids present a novel mix of chemical constituents that not only contain nicotine but also flavors, sweeteners and other chemicals, many of which have not been studied in the lung. Many of these chemicals are present to mask the bitter nicotine taste. Thus, while nicotine has been shown to alter many aspects of airway physiology, the potential exists for sweet and bitter flavored constituents from e-liquids to stimulate taste receptor signaling pathways that could alter airway physiology with chronic use. To date however, there is no current literature on the effects of E-Cigs and chronic vaping in pulmonary physiology of nAChRs or taste receptors. Moreover, the ability to taste bitter substances may to contribute to smoking behavior and nicotine addiction (24, 51, 97). Bitter taste receptors (T2Rs) are ligand activated G protein-coupled receptors (GPCRs) that use intracellular Ca^{2+} as a downstream signaling molecule. There are approximately 30 T2Rs expressed in humans. T2Rs are most

abundant in the tongue and T2R polymorphisms (e.g. T2R38) that impair the ability to taste bitter compounds have been correlated with populations that are more nicotine dependent and/or heavy smokers (80, 97). Furthermore, when tongue tissue was compared for T2R mRNA expression in smokers versus non-smokers, overall T2R gene expression was reduced in the smoking compared to the non-smoking group (8). It is unknown whether the reduction of T2R gene expression was genetic or was suppressed by a component of cigarette smoke. Yet a correlation between T2R expression and age was present in the non-smoker group and absent in the smoker group, suggesting that starting smoking earlier in life could suppress T2R gene expression and contribute to nicotine addiction.

Many T2Rs have been identified in the upper and lower airway epithelia as well as airway smooth muscle cells (34, 44, 134, 150). Interestingly, T2R38 polymorphisms have also been linked to increased susceptibility of upper respiratory infections (92). Though an endogenous ligand is still unknown, known bitter agonists activate these T2Rs and increase intracellular Ca^{2+}, stimulating ciliary beat frequency. Thus, they play a role in detecting noxious inhalants and expelling them from the airways due to increased rates of mucociliary clearance. Nasal mucosa have been reported to express both sweet receptors (T1Rs) as well as T2Rs in special non-ciliated epithelial cells called solitary chemosensory cells (SCCs) (90). SCCs in the nasal epithelium harbor these receptors along with known components of taste receptor signaling pathway and trigeminal nerve innervation. Tizzano et al. characterized the presence of SCCs with T2Rs and the T2Rs' ability to detect known bitter agonists and acyl-homoserine lactones (150), which are intercellular chemical signaling compounds secreted by Gram negative bacteria, providing more evidence for T2R roles in innate immunity. Furthermore, Lee et al. found that T1Rs and T2Rs in nasal epithelium converge to arbitrate innate immunity (91). That is, when T1Rs are activated (e.g. hyperglycemia, chronic rhinosinusitis), they can block the antimicrobial effects of T2Rs, causing persistent airway infections. Together, these data suggest that taste reception in the airways is important to innate immunity.

Non-ciliated SCCs are found throughout the lower airways, though T1Rs are not detected there (104, 105). Interestingly, Dehkordi et al. reported that intrapulmonary epithelial SCCs coexpress T2R38, its T2R signaling components, and many nAChR subunits in the same cells (42). Though it is unknown whether these two signaling pathways directly interact, it is possible that the coexpression of multiple chemosensation receptor types may increase the repertoire and sensitivity of airway cells to inhaled irritants, specifically nicotine. In this case, nicotine might be sensed by either receptor type and an interaction might exist between downstream in the signal transduction pathway. For example, triggering Ca^{2+} influx from the activation of one nAChR subunit can attenuate the response of a second subunit through desensitization of the stimuli or prolong increases in intracellular Ca^{2+} (59).

The Effects of E-Cig Aerosols and E-liquids on Cultured Cells from the Lung

Tobacco smoke is highly pro-inflammatory and has been shown to trigger the release of inflammatory cytokines from endothelia, epithelia and leukocytes (88) (20, 66). These cytokines can then trigger additional changes including goblet cell metaplasia and neutrophil influx (85). Inflammation may be beneficial in the short term, especially when resolving infection. However, chronic inflammation can act as a precursor to cancer, and continued influx of neutrophils, with the subsequent increase in free elastase levels, can lead to cell damage and denudation of the epithelia (22, 143). Tobacco exposure is also associated with cellular cytotoxicity including increased apoptosis, autophagosome formation, membrane permeability and mitochondrial damage (46, 78, 87, 129). Furthermore, micronuclei form when chromosomes or parts of chromosomes are excluded from daughter nuclei following cell division (56). As such, micronuclei formation is associated with a high risk of cancer and is a common assay that is used to screen for genotoxic substances and increased micronuclei formation has been observed in cigarette smokers (149) (43). Tobacco smoke has also been shown to alter gene expression and DNA methylation in both the whole lung and in airway epithelia (69, 115, 153), macrophages (47) and endothelia (161). Many of these assays have been established as outcome measures for tobacco smoke exposure and they should be useful for probing the effects of E-Cig exposure.

To date, many cell types have exposed to e-liquids and/or E-Cig aerosols. These cell types include lung epithelial cell lines (H292, A549), lung fibroblasts, human primary trachea-bronchial cells and HaCaT keratinocytes (31, 94, 159). Whilst e-liquids are aerosolized, a common early approach has been to add e-liquids directly to cells at various dilutions. Although this protocol would not pick up any additional effects of pyrolysis due to heating the e-liquid, it is a useful first step to determine whether e-liquids themselves have inherent toxicity. Wu et al, exposed non-differentiated tracheobronchial cultures to a tobacco-flavored e-liquid that contained either 18 mg/ml of nicotine (which equates to 111 mM) or was nicotine free for 24 - 48 h over the range (*v/v*) 0.01 - 0.3% [InnoVapor LLC, Boise, ID] and found that exposures in this range did not increase lactate dehydrogenase (LDL) levels, suggesting that they were not cytotoxic (159). However, the upper levels of dosing caused significant increases in IL-6 and IL-8 levels, as well as increasing rhinovirus infection and rhinovirus-induced IL-6 secretion and decreasing mRNA levels of SPLUNC1, an innate defense molecule (148, 159). While increases in IL-6 secretions have been detected after rhinovirus infections (122), the implication of this observation in the context of E-Cig and/or tobacco exposure is not fully understood and needs additional testing. However, increased IL-6 responses to viral infection have been detected in COPD patients, suggesting that this may be a relevant assay for E-Cig exposure (131). Lerner (CA) et al. found that e-liquids altered HFL-1 cell morphology (94). Bahl et al. tested the effects of e-liquids, directly added to murine pulmonary fibroblasts, human embryonic stem cells and murine neural stem cells (10). While effects of e-liquids were typically seen with $\geq$ 0.1% (*v/v*) addition, in general, the stem cells were more sensitive than the fibroblasts, suggesting that some cell types in the lung may be more vulnerable to E-Cigs than others. Furthermore, of the 36 e-liquids tested, ~15 showed cytotoxicity, with cinnamon flavors being especially toxic. Of interest, the authors found significant variability in cytotoxicity from batch to batch, even for one flavor from one vendor, which suggests that poor quality control may exist in some cases.

In addition to directly studying the effects of e-liquids, they can be heated/aerosolized and then studied. Cervellati et al., exposed A549 (lung epithelial) and HaCaT (keratinocytes) cells to whole

cigarette smoke or E-Cig vapor from three combinations of E-Cigs (nicotine; nicotine + flavor; no flavor, no nicotine)(31). After 50 min of smoke or aerosol exposure, cultures were then left for 24 h and LDH release and cell viability were studied. No information was given, regarding whether these cells were polarized or not. However, they found that under these conditions, E-Cigs with nicotine and/or flavor induced similar cytotoxicity (increased LDH release and decreased cell viability) as standard cigarettes, while nicotine and flavor-free E-Cigs did not have any effect. E-Cig aerosols (generated using a 4 s/35 ml pulse) also caused an increase in IL-6 and IL-8 secretion, which in the case of 1 flavor (Cinnamon roll), was greater than the IL-8 secretion seen with cigarette smoke extract addition (94).

The effects of E-Cig exposure have also been studied on the lung's microvasculature. For example, Schweitzer et al., found that E-Cigs decreased the electrical resistance of endothelial cells derived from mice, rats and humans, as well as exerting significant effects on cell viability and MTT production that were associated with changes in cell signaling (activation of p38 MAPK). Interestingly, these changes were similar to those observed after exposure to cigarette smoke extract (133). They also detected increased phosphorylation of myosin light chain (MLC) and Rho Kinase following E-Cig exposure which may have been due to activation of sphingolipids (133). Changes in the permeability in the lung's microvasculature may induce edema and/or increase the number of leukocytes that can enter the lung, thus increasing inflammation, as described elsewhere (84).

The Effects of E-Cigs on the Murine Lung

While little is known about the effects of E-Cigs on humans, some studies have been performed in mice. E-Cig exposure has been shown to elicit neuro-pharmacological effects including upregulation of nAChR in different areas of the brain, and also caused signs of addiction and increased serum and cotinine levels, suggesting that comparable, systemic nicotine levels can be obtained with E-Cig exposure as are seen with tobacco exposure (118) (2). Lerner CA et al., exposed mice to E-Cig aerosols for 5 h/day for 3 days and sacrificed the mice 1 day later. They found that several cytokines were increased in the broncho-alveolar lavage of these mice, including IL-1α, IL-6, IL-13 and MCP-1 (94). Of note, MCP-1

recruits macrophages to the lung, IL-6 is a pro-inflammatory cytokine and IL-13 induces cellular remodeling and goblet cell hyperplasia. Schweitzer et al. found that an E-Cig exposure regimen, which was equivalent in dose to exposure to smoke from 2 cigarettes, caused a significant increase in 8-Oxo-2'-deoxyguanosine (8-oxo-dG) in both plasma and BAL (133). 8-oxo-dG is a marker of systemic oxidative stress and is indicative of DNA damage (109). They also detected increased nitrotyrosine levels in plasma. Nitrotyrosine can be formed following exposure to reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide, and is also a marker of cell stress/damage (124). A 2-week exposure to E-Cigs smoked under relatively standard conditions (2 s 35 ml puff) caused a significant increase in the number of macrophages in murine lungs and actually decreased IL-6 (133). The difference in IL-6 levels observed between the 2 experiments are most likely due to differences in smoking (vaping) regimens since one was acute and the other was chronic (94, 133). Mouse strain differences and/or differences in E-Cig device/e-liquid may also have been factors.

After infection with *Streptococcus pneumonia*, E-Cig-exposed mice were less able to clear this infection, suggesting that innate defense was impaired (142). They also found that H1N1 influenza virus infection also was poorly cleared. Whilst this data will need to be repeated by other groups, it is the first report that E-Cig exposure leads to increased susceptibility to infection, which has important implications for the safety of E-Cig users. Interestingly, increased susceptibility to pathogens is a hallmark of tobacco exposure and is seen following both viral and bacterial infections (58, 110, 123). In addition to these aerosol exposures, a 50 fold-diluted E-Cig liquid has been shown to increase IL-4, IL-5 and IL-13 in allergen-sensitized mice when tracheally instilled (95), again suggesting that e-liquids can still have adverse effects even before they are vaporized.

The adverse effects of tobacco on both pre-natal and post-natal development have been well described and include low birth weight, increased incidence of sudden infant death syndrome and of developing lung disease later in life (e.g. asthma) (1, 62). Whilst most published studies to date have focused on adult mice, it has been demonstrated that E-Cig exposure also adversely effects neonatal mice

and leads to impaired development, including decreased weight gain and reduced cell proliferation in the lungs, suggesting that secondhand vaping may also potentially be a cause for concern and could adversely affect lung development (103).

What is Known about the Effects of E-Cig Exposure in Humans?

Currently, many adult E-Cig users are former smokers and have a significant history of tobacco usage prior to using E-Cigs and/or continue to be mixed tobacco/ E-Cig users (61, 120). This will make studying the chronic effects of E-Cigs difficult since the airways/lung retain a significant memory of smoking history/exposure even after smoking cessation. For example, Jaspers et al., found significant evidence of DNA methylation in the nasal epithelia of ex-smokers (121). Thus, for any observed effects on E-Cig smokers, the previous and/or current tobacco smoking history must be taken into account. That said, the largest, and fastest growing population of E-Cig users who have never smoked tobacco is adolescents. For example, in North Carolina, 15% of high school students have vaped E-Cigs, and 60% thought that E-Cigs were safe. In contrast, amongst the same group, 24% had smoked cigarettes (5). This trend is reflected nationally (102).

It has been shown that short term (e.g. 5 min) E-Cig inhalation leads to comparable plasma cotinine levels as regular tobacco smoking and exerts rapid physiological effects on the cardiovascular system, including elevated heart rate (151). These data indicate that modern E-Cigs are delivering significant amounts of nicotine to the bloodstream. Whilst to date, no studies have been performed to look at the adverse effects of E-Cigs in pulmonary health (e.g. inflammation, etc.), Vardavas et al. looked at the effects of 5 min E-Cig exposure on pulmonary function using standard spirometry (152). Interestingly, they found that a 5 min E-Cig exposure caused a significant increase in peripheral airway resistance, which is indicative of changes to the small airways. The authors noted that these changes were relatively small and likely not great enough to be of immediate clinical significance. However, the changes were observed after only 5 minutes of exposure, and they speculated that chronic exposure may lead to greater changes in resistance. They also found that this 5 min exposure caused a significant decrease in exhaled

NO levels. NO has a number of functions in the lung, and changes in NO levels can affect ciliary beating, transcription, inflammation, ion transport and airway smooth muscle tone (21). NO is altered in many diseases including asthma (increased), cystic fibrosis (decreased), primary ciliary dyskinesia (decreased) and COPD (may be suppressed or mildy increased) (163). Thus, it is possible that E-cig exposure could cause different lung disease to COPD.

Conclusions and Future Directions

There is a long history of deceptive marketing tactics used by tobacco industry regarding the 'safety' of cigarettes (52). Thus, it is interesting to speculate whether the same will hold true for the nascent E-Cig industry. Certainly, users want to believe that E-Cig products are safe, but unfortunately, no definitive data currently exists to prove or disprove this hypothesis. Studying E-Cig exposure is very much like trying to hit a moving target, but one where researchers are not completely sure what the target looks like, since E-Cig devices, the way that they are used, as well as the types/flavors of e-liquids available are constantly changing. However, some facts have been established: (i) Current E-Cig devices deliver nicotine at comparable levels to cigarettes, and certainly at levels high enough to evoke physiological responses in humans and rodents (54, 103, 138), (ii) nicotine is highly addictive and, along with its metabolites, can cause cancer and affect neuronal development in adolescents irrespective of its source (50, 155), (iii) e-liquids have been shown to contain potentially toxic aldehydes and ROS (146) and (iv) some type of a biological response (e.g. change in cytokine levels) has been observed in the vast majority of murine *in vivo* and *in vitro* studies following E-Cig vapor/e-liquid exposure (94, 133, 152). Whilst it seems certain that E-Cig aerosols contain toxicants, it is fair to say that they likely contain less types of toxicants than cigarette smoke (i.e. E-Cig aerosols likely have hundreds of chemicals in them while tobacco smoke has thousands of chemicals). The remaining question is then one of dose ranging. That is, are the toxicants in E-Cigs present in sufficiently high concentrations to elicit lung disease over a similar time frame as tobacco smoking?

Given the paucity of information that is available regarding the effects, not only of E-Cigs, but also of many of the chemical constituents of e-liquids, on the lungs, we propose that E-Cigs/e-liquids be regulated in a similar fashion as any inhaled therapeutic agent. That is, thorough inhalation toxicology and safety based, Phase-1 style clinical trials be performed on all commercially available E-Cig products. Though this would be an undeniably expensive undertaking, the estimated value of the E-Cig market is in the billion dollar range, indicating that tobacco and E-Cig companies could likely foot this bill.

Acknowledgments

We thank our colleagues at UNC and elsewhere for stimulating discussion regarding studying the effects of E-Cigs exposure.

Funding

This work was funded by NIH P50 HL120100. Research reported in this publication was in part supported by NIH and the FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Food and Drug Administration.

Conflicts of Interests

None

Figure Legend

Figure 1: Current knowledge of the effects of E-Cig and e-liquid exposures on pulmonary cell types. Included in the table is a short list of the current *in vitro* and *in vivo* study outcomes for lung-related cell types that are depicted in the cartoon and labeled appropriately.

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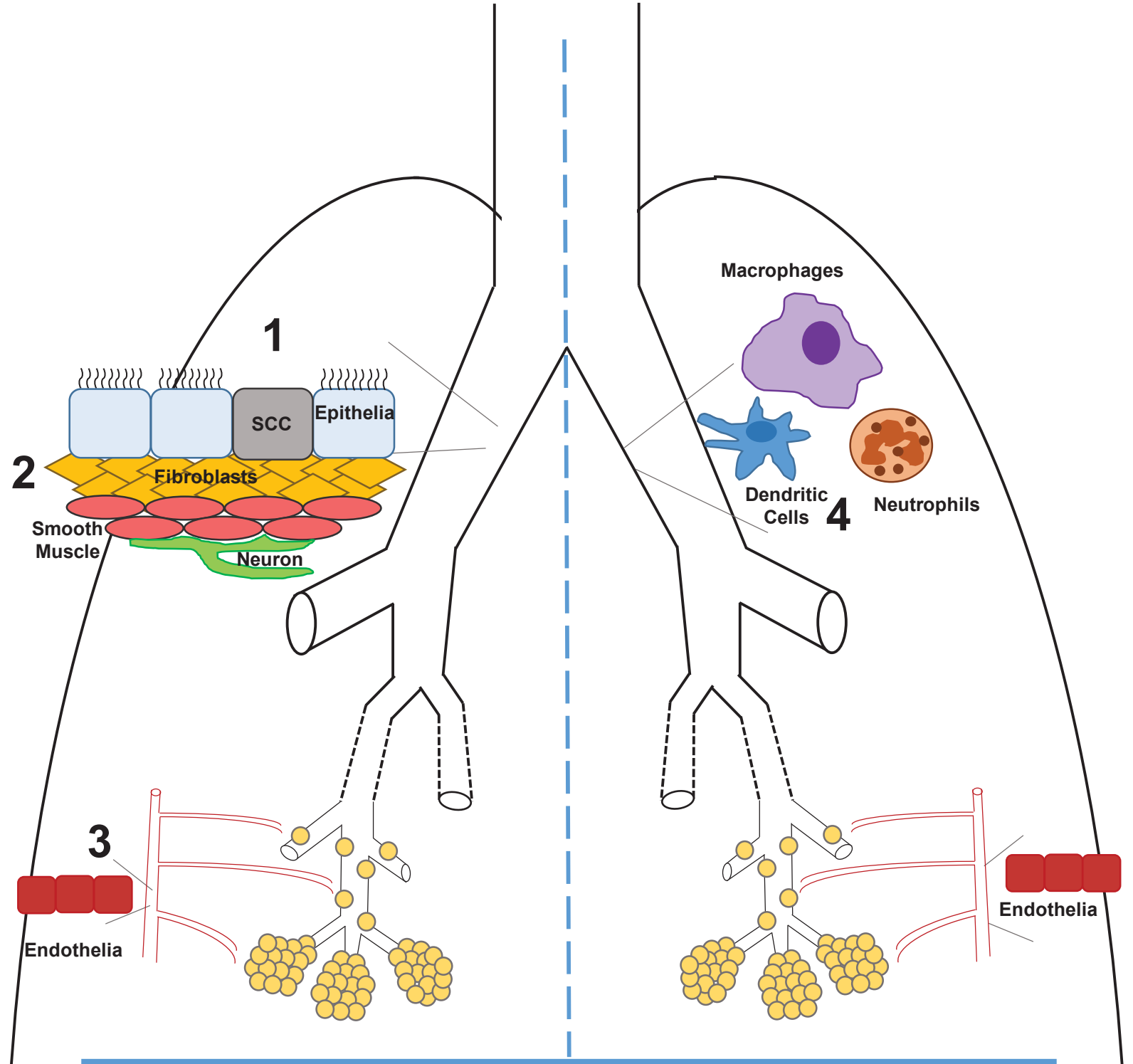

Current Data for the Effects of E-cigarettes/E-liquids on the Lung

Tissue/Cell Type	*Effects*
(1) Epithelia	↑ Cytotoxicity[31], ↓Cell Viability[31], ↑ Inflammation[94,159], ↑ Infection[159]
(2) Fibroblasts	↑ Cytotoxicity[10,94], ↓ Cell Viability[10,94], Altered Morphology[94]
(3) Endothelia	↓ Cell Viability[133], ↓ Electrical Resistance[133]
(4) Inflammatory Cells (BALF)	↑ Macrophages[142], ↑ Cytokine Secretion[94,95], ↑ Infection[142]