# LITTLE CIGARS EXERT MORE SEVERE EFFECTS ON AIRWAY EPITHELIA THAN CIGARETTES

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New and emerging tobacco products pose a unique threat to pulmonary health. Little cigars are a class of tobacco which differ from cigarettes in that they are wrapped in any substance containing tobacco. We set out to examine the composition and relative effects of little cigars compared to Kentucky research cigarettes, emphasizing effects on the innate defense system of the lung. The chemical profile of tar particles from these products were also analyzed to see if they differed from cigarettes. Human bronchial epithelial cultures (HBECs) grown at the air liquid interface were exposed to air and whole tobacco smoke from Kentucky cigarettes, two leading brands of little cigars (Swisher Sweets and Captain Black), and one similar brand of cigar (Cheyenne) chronically for five days. These cells were then analyzed by western blot and confocal microscopy. We also exposed C57/BL6 mice to smoke from Kentucky cigarettes and Swisher Sweets little cigars. Broncho-alveolar lavage samples were collected and measured for leukocyte infiltration. Chemical species present in the tar particles of smoke were evaluated using gas chromatography-mass spectrometry (GC-MS). Airway surface liquid height and CFTR protein levels were significantly decreased in chronic smoke-exposed cultures, although epithelial sodium channel (ENaC) levels were unaffected. This effect was more severe for little cigars compared to Kentucky cigarettes. A significant decrease in cilia abundance also suggested that innate defense was compromised, and this was significantly more prominent after little cigar exposure. Little cigar smoke exposure induced a significantly greater increase in leucocyte numbers in mice as compared to cigarette smoke exposure. Using GC-MS, we also observed increased diversity in the number of chemical species present in the tar extract of little cigars as compared to cigarettes, suggesting that little cigars expose the lung to different and potentially more harmful toxicants. Our results indicate that little cigars cause more adverse effects to airway epithelia than Kentucky cigarettes. Little cigars deliver unique chemicals to the lung which are not seen in cigarettes, as shown by smoke tar particle analysis. These effects may compromise innate defense.

# BANANA PUDDING FLAVORED E-LIQUID ALTERS CELL PROLIFERATION AND CA$^{2+}$ SIGNALING IN LUNG EPITHELIA

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E-cigarettes are popular and available in over 7,000 flavors, yet little is known about their effects on pulmonary epithelia. We purchased flavored e-liquids from the Vapor Girl (http://www.thevaporgirl.com/) to characterize their biological effects on human bronchial epithelial cultures (HBECs) and CALU3 lung epithelial cells. We screened 13 e-liquid flavors for effects on cell proliferation/viability and ability to alter cell signaling (e.g. Ca$^{2+}$). Ca$^{2+}$ signaling regulates cell division, mucus secretion, ciliary beat frequency, and Cl$^-$/fluid secretion in airway epithelia. Altered Ca$^{2+}$ signaling could change cell homeostasis, thus we measured changes in cytosolic Ca$^{2+}$ and other pathway components (i.e. STIM1 puncta, kinase phosphorylation, IP$_3$ generation).

Cells were plated on glass coverslips or in multiwell plates and exposed to e-liquids diluted into media either acutely (10 min) or over 24h. Cell proliferation/viability was measured using the MTT assay. Cytosolic Ca$^{2+}$ changes were measured using Fura-2-AM. Relative kinase phosphorylation was measured using a Phospho-Kinase Array and IP$_3$ generation was measured using a competitive ELISA. All assays were done with HBECs and/or CALU3s except STIM1 puncta visualization, which used transiently transfected STIM1-mCherry HEK293 cells.

Several flavors inhibited cell proliferation in a dose-dependent manner, including Banana Pudding (Southern Style) (BPSS). BPSS also elicited an acute cytosolic Ca$^{2+}$ signal involving both the endoplasmic reticulum (ER) and store-operated Ca$^{2+}$ entry (SOCE), formed STIM1 puncta, and altered phosphorylation of ERK1/2, which regulates STIM1 phosphorylation.

Our data showed that BPSS-flavored e-liquid altered ER/SOCE Ca$^{2+}$-signaling mechanisms in airway epithelia, which could have biological consequences to Ca$^{2+}$-mediated HBEC innate defenses. This suggested that other flavors may alter cell signaling and other important lung epithelial functions. Investigations into the effects of flavored e-cigarette aerosol on Ca$^{2+}$-dependent aspects of innate defense are ongoing.

# CIGARETTE SMOKE DISRUPTS THE FUNCTIONS OF SPLUNC1 IN REGULATING AIRWAY SURFACE LIQUID VOLUME AND ANTI-MICROBIAL ACTIVITY

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Chronic obstructive pulmonary disease is the third leading cause of death worldwide with tobacco smoke being the most common causative factor. Airway surface liquid (ASL) is a thin layer that lines the luminal side of airways which plays a major role in in innate defense facilitating the clearance of inhaled pathogens as well. SPLUNC1 is a secreted protein in the upper airway that regulates ASL height and has anti-microbial activity against respiratory pathogens. Exposure of cigarette smoke (CS) to airway epithelia is known to induce inflammation, mucus hypersecretion, and depletion of the ASL volume which together results in in bacterial infection and mucus stasis. However whether CS affects SPLUNC1 activity directly is unknown.

We investigated the role of CS on SPLUNC1 activity using an in vitro CS exposure model, ASL height measurements, mass spectrometry and antimicrobial assays. After exposing recombinant SPLUNC1 to CS we observed a reduction in the ability of SPLUNC1 to regulate ASL height (P<0.05) (N=6). Overnight dialysis of SPLUNC1 did not reverse this effect (N=6). Furthermore antimicrobial activity against Gram Negative bacteria such as *Haemophilus influenzae* and *Pseudomonas aeruginosa* was attenuated following CS exposure (P<0.05) (N=3). Further, analysis of smoked SPLUNC1 using mass spectrometry revealed oxidative modifications including tri-oxidation and crotonaldehyde, to the cysteine residues at positions 180 and 224 (N=3). Some of these oxidative modifications were also observed in endogenous SPLUNC1 from cell culture secretions. The exact mode of action derivatizing these cysteines by cigarette components warrants further investigation.

We conclude that cigarette smoke modifies SPLUNC1 resulting in its loss of ASL height regulation and attenuation of anti-microbial activity. This data expands on the severe impact of cigarette smoking on lung physiology through inactivating SPLUNC1 function. The identification of modified secreted proteins in the airway following smoke exposure may serves as biomarkers that can be used to assess the toxicity of cigarette compounds.

# EVALUATING TOXICITY AND ELECTROPHYSIOLOGICAL EFFECTS OF E-LIQUIDS

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While tobacco smoking has declined since the 1950's, e-cigarette use has increased, attracting both former smokers and non-smokers alike. E-cigarettes are able to deliver nicotine and chemical flavorings through the vaporization of a liquid propylene glycol/vegetable glycerin (PG/VG) base. The public has viewed e-cigarettes as a safer method of nicotine delivery since it does not involve the combustion of nicotine. Since little is known about the potential toxicity of e-liquids, we deigned a high-throughput assay to assess the potential harm.

As a proof of concept, we have performed a primary screen on HEK293T cells exposed to 154 e-liquids in order to categorize their potential for toxicity.  Using a multiplate reader approach, we used cell growth after exposure to e-liquids as our endpoint. Almost 60% of the e-liquids reduced cell growth at a 1% dilution. To further examine the effects of e-liquids on HEK293T cells, we ran full dose-response curves for each e-liquid. After 28 hours of exposure, cells were stained with calcein-AM and propidium iodide to quantify live and dead cells respectively. From these data, dose-response curves were generated and $IC_{50}$ values were determined (N=3).

In addition to the dose-response curves, we are beginning our tertiary screen in which we examine the electrophysiological effects of e-liquids on primary cells and more airway-relevant cell lines. Ussing chambers are being used to determine the effect that PG/VG and other e-liquids have on ion transport in the human airway. These investigations into e-liquid's toxicity and airway effects will help inform the FDA about the toxicity and underlying effects of commercially available e-liquids.

# SUSTAINED INCREASES IN CYTOSOLIC CALCIUM INDUCE CFTR INTERNALISATION

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**Introduction:** Chronic obstructive pulmonary disease (COPD), the third leading cause of death worldwide, is characterised by airflow obstruction and is primarily caused by smoking (http://www.who.int/mediacentre/factsheets/fs310/en/). Cystic fibrosis (CF) is another obstructive disease and affects approximately 80,000 people worldwide. However, patients with either COPD or CF present with similar problems, including impaired airway mucus clearance due to the production of viscous mucus, brought about by defective ion homeostasis. In particular, CFTR activity is reduced in both diseases. Recently, we have shown tobacco smoke exposure causes an increase in intracellular calcium ($[Ca^{2+}]_i$) leading to internalisation of CFTR, in both airway and non-airway cells, through an unknown mechanism (Clunes LA *et al*. 2012 *FASEB J* 26:533-45). **Aims:** To investigate the dynamics and molecular mechanism underlying the effect of an increase in $[Ca^{2+}]_i$, stimulated through thapsigargin (TG), on CFTR channel activity. **Methods:** CFTR channel activity was measured using fast whole cell patch clamp current recordings and $[Ca^{2+}]_i$ by fluorescence microscopy. **Results:** Exposure to TG caused a time-dependent decrease in forskolin-activated CFTR conductance within 10 mins of exposure, with a ~60% reduction in conductance after 20 mins. The temporal effect of TG on CFTR was mirrored by changes in $[Ca^{2+}]_i$. Furthermore, the TG-induced reduction in CFTR was eliminated when experiments were repeated in the presence of a high concentration of EGTA in the patch pipette solution, showing that the rise in $[Ca^{2+}]_i$ was temporally related to the loss of CFTR conductance. Our lab has previously shown that tobacco smoke-induced $Ca^{2+}$ release causes internalisation of CFTR into intracellular aggregates (Rasmussen JE *et al*. 2014 *JBC* 289:7671-81). Likewise, pre-incubation of cells with the endocytosis inhibitor, Dynasore, blunted the decreased CFTR activity by TG. **Conclusions:** These results show that a sustained increase in $[Ca^{2+}]_i$ decreased CFTR activity through internalisation of the channel via a dynamin-dependent, endocytic route. Additional $Ca^{2+}$ agonists and tobacco smoke components will be tested to further understand the physiological relevance and pathophysiological mechanism by which tobacco smoke-induced $Ca^{2+}$ release affects CFTR.

# ELECTRONIC CIGARETTES INDUCE INFLAMMATION AND ACTIVATE THE UNFOLDED PROTEIN RESPONSE IN HUMAN AIRWAY MACROPHAGES

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Airway macrophages (AMs) play a key role in pulmonary innate defense and serve as the primary phagocyte in the lungs. Cigarette smoke (CS) changes the morphology of AMs and impairs host defense responses. Electronic cigarettes (E-cigs) have become popular and are perceived as a healthy alternative to CS. However, the effects of E-cigs on AM morphology and function are largely unknown. CS triggers endoplasmic reticulum (ER) stress and activates the unfolded protein response (UPR) in airway epithelia, but it is not known whether it activates the UPR in human AMs. The UPR provides cellular protection and survival, but excessive ER stress can trigger apoptosis. The UPR is initiated upon activation of three ER stress transducers: 1) inositol requiring enzyme 1 (IRE1) that exists in two isoforms, α (ubiquitous) and β (expressed only in mucous cells); 2) activating transcription factor 6 (ATF6); and 3) PKR-like ER kinase/pancreatic eIF2α kinase (PERK). We have previously linked activation of IRE1α-induced mRNA splicing of X box binding protein 1 (XBP-1s) in inflammatory responses of human AMs.

In this study we evaluated whether CS or E-cigs promote inflammation associated with UPR activation in human AMs from non-smokers (NS), CS, and E-cig users. Smoking status was determined as previously described (Martin et al, 2016). AMs were obtained from bronchoalveolar lavages (BALs) according to an IRB-approved protocol. The morphology of AMs was evaluated in cytospins from the BAL fluid stained with Diff-Quik. To assess inflammation and UPR biomarkers, AMs were isolated from BAL fluid by adherence according to our method (Lubamba et al, 2015), which yields an AM purity >95%, and studied as 3 day old primary cultures. NS AMs exhibited a flattened appearance, normal nuclear morphology and presence of pseudopodia extending from the surface. In contrast, AMs from CS and E-cig users exhibited a rounder shape, evidence of chromatin damage, membrane blebs, and an increased content of particulate matter (e.g., numerous inclusion bodies in the cytoplasm). To evaluate the inflammatory status of the AMs, interleukin-6 (IL-6) mRNA was measured by RT-PCR. IL-6 mRNA levels were increased in AMs from CS and E-cig users vs. NS. Similar changes were observed for IL-6 protein secretion: AMs from CS or E-cig users displayed 5.7- and 30-fold higher levels of IL-6 secretion, respectively, vs. AMs from NS. To investigate whether these changes were linked to UPR activation, we evaluated the expression of the IRE1α-dependent biomarker XBP-1s, and the PERK pathway biomarkers ATF-4 and CHOP (transcription factors involved in defense against oxidative stress and induction of apoptosis, respectively). XBP-1s, ATF-4 and CHOP mRNA expression was increased in AMs from CS and E-cig users vs. AMs from NS (Table 1).

| Gene | NS | CS | E-cig |
|------|-----|-----|-------|
| XBP-1s | 3.0 ± 1.4 | 31.7 ± 1 | 33.4 ± 5.2 |
| ATF-4 | 0.8 ± 0.3 | 59.4 ± 21 | 32.2 ± 8.5 |
| CHOP | 0.9 ± 0.3 | 167.8 ± 55.7 | 94.2 ± 32.2 |

Table 1. Biomarkers of UPR activation are increased in AMs from CS and E-cig users. mRNA expression is shown as fold change relative to 18s mRNA (mean ± SEM; N = 4).

In conclusion, similar to CS, E-cigs induce severe morphological alterations of AMs, suggesting they have toxic effects. Up-regulation of XBP-1s likely mediates the increased inflammation observed in AMs from CS and E-cig users. Up-regulation of ATF4 may reflect a cellular response against smoking-promoted changes in the oxidative status of AMs. Finally, induction of CHOP may reflect a role for this transcription factor in AM apoptosis, which is suggested by the severe morphological changes exhibited by AMs from CS and E-cig users. Our findings raise concerns over the safety of E-cigs regarding pulmonary function. *Funded by UNC TCORS NIH (P50-HL-120100-01).*

# CIGARETTE SMOKE REDUCES AIRWAY EPITHELIAL ATP RELEASE

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Unbalanced rates of mucous secretion and hydration represent a major contributing factor to the progression of chronic lung diseases such as chronic obstructive pulmonary disease (COPD). COPD is a highly heterogeneous disease that includes chronic bronchitis (CB) and emphysema, and it is characterized by airflow obstruction that reflects defects in airway function, and/or abnormalities in the alveolar parenchyma. COPD affects 10 to 20 million people in the US and is the fourth leading cause of death in this country. COPD is largely (~90%) associated with cigarette smoking, but the disease can progress despite smoking cessation. Mucus hydration is driven by electrolyte transport activities, which in turn are regulated by airway surface liquid (ASL) concentrations of ATP and its metabolite adenosine (Ado) acting on airway epithelial purinergic receptors. Nucleotide release occurs via (a) the plasma membrane channel pannexin 1 (PANX1) and (b) exocytotic pathways driven by the vesicular nucleotide transporter VNUT expressed in secretory vesicles and mucin granules. Several ecto-enzymes metabolize ASL nucleotides, thus controlling the availability of purinergic agonists. Our previous studies with CB subjects suggest an association between defective balance of nucleotide release/metabolism rates and mucus dehydration and plugging. For example: subjects with CB exhibited defective MCC *in vivo* and increased mucus percentage solids in BAL and sputum, which were associated with increased metabolism and reduced levels of ATP and Ado in airway secretions. In the present study, we investigated the effect of cigarette smoke on airway epithelial ATP release/metabolism rates. A single, acute exposure of human bronchial epithelial (HBE) cells to cigarette smoke (CS, 13 puffs over 1h) resulted in no apparent changes in ATP release or hydrolysis rates. In contrast, exposure of HBE cells to CS for 5 consecutive days resulted in robust decrease of hypotonicity-evoked ATP release. Quantification of the entire adenyl purine pool via etheno-derivatization and HPLC analysis revealed a >6-fold increase of extracellular nucleotides in air-exposed cells subjected to a hypotonic challenge, but nucleotide release was markedly reduced in CS-exposed cells. No differences in ATP release were observed among cigarette types, i.e. Kentucky, Captain Black, and Swisher Sweets. Incubation of HBE cells with exogenous ATP (1 µM), resulted in a rapid decay of the nucleotide ($T_{1/2}$ = 1.4 min) in CS exposed cultures, relative to air. RT-PCR analysis indicated a variable increase (rather than a decrease) of the levels of expression of PANX1 and VNUT. Notably, expression levels of DUOX2, a member of the NADPH oxidase gene family reported to inhibit to PANX1 activity in HBE cells, were substantially increased after CS exposure. Collectively, these results suggest that CS inhibits the mechanisms of airway epithelial ATP release, likely, via inactivation of PANX1. Abnormal CS-associated airway epithelial ATP release may contribute to the pathogenies of CB.

# PROTEOMIC PROFILING OF SPUTUM FROM NEW AND EMERGING TOBACCO PRODUCTS USERS REVEALS BIOMARKERS OF EFFECT

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**Rationale:** Although the effect of cigarette smoking on lung health has been well documented, the impact of new and emerging tobacco products (NETPs), e.g. e-cigarettes and hookah, however are largely unknown. Therefore, the aim of this study was to assess the effects of NETPs on the airway epithelial mucosal barrier by quantitatively characterizing the secretomes of NETP users in comparison to cigarette smokers using sputum samples.

**Methods:** Induced sputum (IS) samples were collected from cigarette, e-cigarette and hookah users, as well as from non-smokers. Sputum samples were denatured in 4M GuHCl, reduced, alkylated and digested with trypsin. After glyco-peptide removal, the resulting peptide mixtures were analyzed by LC/MSMS based label-free quantitative proteomics and by bioinformatics software tools such as Scaffold and Proteome Discoverer.

**Results:** Among the three exposure groups, the highest number of significantly changing proteins was observed in the sputum of e-cigarette users followed by cigarette users. The cigarette and e-cigarette user sputum shared a significant upregulation in proteins involved in detoxification, like aldo-keto reductase and aldehyde dehydrogenase, and in oxidative stress response, indicated by glutathione s-transferase and thioredoxin increases. In addition, e-cigarette user sputum showed significantly increased levels in several neutrophil granulocyte associated proteins, exemplified by myeloperoxidase, protein disulfide isomerase, cathepsin G and neutrophil elastase.

**Conclusions:** Our data indicates that known biological responses to cigarette smoke exposure (e.g. aldehyde dehydrogenase upregulation) are also observable in response to e-cigarette use. Further, the increases in proteins related to neutrophil granulocyte activation and certain proteases in e-cigarette user sputum show an impact of vapors on the innate immune defense system of the airways. Overall, our study challenges the concept of e-cigarettes generally having a lesser impact than cigarettes on the pulmonary system and thereby the user's health.

# EFFECT OF LITTLE CIGAR EXPOSURE ON THE AIRWAY EPITHELIAL MUCUS BARRIER AND EXOSOMAL MIRNA PROFILE

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**Rationale**: Cigarette smoking is an important cause of respiratory tract morbidity and mortality worldwide. However, the health risk behind cigar/little cigar smoking is not well documented and is often considered "safer". Besides, there is an increasing concern to understand the risks associated with cigar smoking. Airway mucus is one of the first points of contact with inhaled smoke and mucus acts as the first line of defense to inhaled biological and chemical substances. Thus, the overarching aim of this study, using in vitro cell culture model to look for the effect of little-cigar smoke on airway mucosal epithelia and its innate defense system and discovering novel muco-biomarkers.

**Methods**: Cultured human primary airway epithelial (HTBE) cells were exposed to different little cigar smoking brands product: Captain-Black (LCCB), Cheyenne (CCN), Swisher-Sweets (LCSS); Kentucky Research cigarette (KCS); and air (control) for 5 days. Apical secretions were collected and processed to identify and quantitate the proteins using mass-spectrometry based proteomic analysis. Scaffold bioinformatics tools were used for the data analysis. Exosomes were purified from the secretions and subjected to proteomics and miRNA analysis using mass spectrometry and HTG-EdgeSeq technology respectively.

**Results**: Proteomic analysis identified over 1000 proteins in the HTBE secretions. The analysis of the secretions from little cigar smoked cells showed significant quantitative changes in protein expression when compared with the air group, Kentucky Research cigarette and between the different little-cigar brands. Spondin-2, BPI-fold protein-B-1(LPLUNC1), mucin-1 and 20 were significantly decreased with $p \leq 0.0001$ in the little cigar exposed group. Bioinformatic analysis of miRNA library showed that over 700 miRNAs (of 2400 total) significantly differentially expressed in little cigar exposed group while 65 miRNAs in Kentucky research cigarette group compared to the air group, for example miR-1202, miR-4257 and miR-4655-5p were differentially expressed which is significant after smoking exposure practically among little cigar smoke exposure group

**Conclusions**: Data suggests that cigar-smoke exposure changes the protein and miRNA expression in the secretions from HTBE cells. Pathway analysis indicates that proteins and miRNA related to innate defense, signaling pathways, and immune response pathways are particularly affected. Moreover, little cigars significantly altered the protein expression and miRNA profile more than Kentucky research cigarette compared to the air group. This study helps to understand the effect of little cigar-smoking on the airway epithelial barrier, and provides a more integrated view of mucus/mucin related biomarkers, thus contradicting the previous claim that little cigars are "safer."

# THE NEW AND EMERGING TOBACCO PRODUCTS IMPACT THE IMMUNE RESPONSE IN THE NASAL EPITHELIA

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## Objective

Although the effect of cigarette smoking on the airway epithelial mucosal barrier has been investigated at length, the effects of new emerging tobacco products remain unknown. The aim of this study is to identify proteomic changes in the human nasal lavage fluid (NLF) due to smoking products, specifically cigarettes, e-cigarettes, and hookahs.

## Method

NLF was collected from four different groups of subjects: non-smokers (n=11), cigarette (n=15), e-cigarette (n=12), and hookah smokers (n=15). Samples were analyzed by tandem mass spectrometry (Q-Exactive Orbitrap UPLC MS/MS System). Proteins were identified and label free quantified using different bioinformatic tools (Proteome Discoverer,Scaffold, SAS, and GraphPad).

## Results

Cigarette smoking had an impact on nasal epithelial response as evaluated by the number of proteins differentially secreted. Most of the proteins that increased were related to inflammation, wound repair, tissue remodeling and stress response (eg. granulin, kallikrein, angiogenin, and ribonuclease), while other proteins were involved with keratinocyte differentiation (fatty acid-binding protein and subrabasin). Moreover, cigarette smoking leads to a decrease of different proteins related to the immune response (Ig gamma-1 chain C region, Ig kappa chain V-I region Wes, IgG H chain, and others). Proteins such as Ig kappa chain V-I region AU and Protein IGKV1-33 decreased in e-cigarette users. Some enzymes were significantly increased in the e-cigarette group, as well in the hookah group (D-dopachrome decarboxylase, Proteasome subunit alpha type-1). Furthermore, in the hookah group S100 family proteins such as S100 –A7, -A7A, and -A8 were overexpressed, indicating activation of the immune pathway. Proteins Lipocalin-15 and BPI fold-containing family B member 4 (BPIB4) were decreased in both the hookah group and the e-cigarette group. BPIB4 may have the capacity to bind specific classes of odorants, acting as a primary defense mechanism.

## Conclusions

Our data indicate that emerging tobacco products, e-cigarette and hookah smoking, alters the proteins expression in nasal epithelia, including proteins involved in immune defense, signaling, and inflammation. Our data suggest that new and emerging tobacco products can be equally harmful to the upper airways as cigarette smoking.

# CHANGES IN LUNG GENE EXPRESSION INDUCED BY CIGARETTE SMOKE IN HEALTHY MICE AND MICE WITH CHRONICALLY INFLAMED AIRWAYS

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## INTRODUCTION
Chronic obstructive pulmonary disease (COPD) affects 210 million people; the number one risk factor for developing COPD is cigarette smoking. Males and females experience disease progression differently: females tend to display more of the chronic bronchitis phenotype and males display more the emphysematous phenotype. However, only a subset of smokers develops COPD, and the contributions of sex, tobacco smoke and chronic inflammation in the development of COPD are unclear. Changes in the lungs of mice exposed to cigarette smoke partially mimic changes induced in human lungs. Transgenic mice overexpressing the *Scnn1b* gene (BENaC) develop dehydrated airway mucus and chronic bronchitis. These studies tested the hypothesis that smoke induces sex-specific changes in mRNA profiles that can be used as biomarkers of cigarette smoke exposure and harm and as biomarkers of disease progression.

## METHODS
Sex-matched 6-week old WT and *Scnn1b*-tg mice were exposed to cigarette or sham smoke for 1 day, 5 days, or 6 months. RNA was isolated from flash-frozen lungs using the Qiagen miRNeasy RNA isolation kit. Gene expression was assessed using Affymetrix microarrays. Differentially expressed genes (DEGs) are identified at each exposure time point for males and females, between exposure types, between genotypes, and in pairwise comparisons between groups. Gene-set enrichment analyses (GSEAs) are performed to investigate pathway enrichment.

## RESULTS
To date, the chronically (6 month) exposed female gene expression results have been partially analyzed. An analysis of the differentially expressed genes with a fold change of ±1.5 and FDR <0.05 shows that the most differences were found when comparing female BENaC smoke-exposed mice to BENaC sham mice. Seven genes upregulated in the BENaC smoke to BENaC sham comparison are also upregulated in the WT smoke to WT sham comparison, indicating that they respond to cigarette smoke in a disease state-independent manner. Analysis of the top 25 pathways upregulated in the gene expression profiles of the chronically exposed mice revealed that many immune response and immune regulatory pathways were upregulated due to the BENaC genotype. Interestingly, certain xenobiotic and toxic response pathways were upregulated commonly across genotypes in response to cigarette smoke. This indicates that chronic bronchitis and smoke exposure contribute differently to the gene expression profile in the BENaC smoke exposed mice and, therefore, that biomarkers of harm and exposure can be identified independently in this model.

## CONCLUSION
Chronic bronchitis and smoke exposure each induced unique changes to the gene expression profile in murine lung tissue. The effects of smoke were different in healthy lungs compared to those with chronic bronchitis. Comparisons between male and female DEGs will be made at each time point to look for sex differences in biomarkers of harm and biomarkers of smoke exposure. Within the same sex, correlations with duration of exposure will also be analyzed to examine the expression of potential biomarkers over the course of disease progression.

# EFFECTS OF CIGARETTE AND LITTLE CIGAR SMOKE EXPOSURE IN MICE

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**INTRODUCTION:**  New and emerging tobacco products are gaining in popularity among the youth. Little cigars, which are among those products, are currently not subjected to the same FDA regulation as cigarettes, and their potential to cause lung injury is not well defined.  The composition of cigarettes and little cigars, and thus the smoke they generate, are intrinsically different.  Evaluating the effect of the same number and rate of puffs allows for a standardized comparison between the two tobacco products.  This study compares the same number of puffs given at the same rate over the same period of time in smoke exposure generated by little cigars and by cigarettes.  This study is in progress, and this abstract represents an interim analysis performed after only the 1.5 hour per day studies were completed.

**METHODS:**  Male and female C57BL/6J (wildtype) mice were exposed to smoke from Kentucky 3R4F research cigarettes, Swisher Sweets natural flavor mild strength little cigars or room air.  The mice received 1 puff of smoke (2 second duration) per minute for 1.5 hours per day for 5 days, using the SCIREQ inExpose whole body inhalation system.  Male and female mice were also exposed to cigarette smoke for 3 hours per day at the same puff rate.  Studies exposing mice to Swisher Sweets for 3 hours per day are ongoing and will be presented at the Retreat.

One hour post smoke exposure on the fifth day, the lungs were lavaged. The total protein concentration was measured and the concentration of cells and leukocyte subpopulations was determined.  ANOVAs followed by post-hoc corrections identified significant differences.

**RESULTS:**  The protein concentration in the lavage fluid was increased after 1.5 hours of cigarette smoke for both sexes when compared to sham-exposed mice.  Curiously, 1.5 hours of little cigar smoke did not cause an increase in either males or females.

The total number of cells in the lavage fluid was increased after 1.5 hour cigarette smoke exposure for males but not females, and for both sexes after 3 hours.  Little cigar smoke for 1.5 hours increased the number of cells in lavage fluid from both genders.  Cigarettes increased the number of macrophages in males after either exposure duration, and little cigars increased macrophages in both sexes.  Interestingly, 3 hours of cigarette smoke caused a significant increase in the numbers of neutrophils in both sexes, but the increase in males was greater than the increase in females (17.5% vs 6.2% respectively; $56.4 \pm 5.3 \times 10^3$ vs $18.5 \pm 3.7 \times 10^3$ neutrophils, respectively, $p < 0.001$).  The number of lymphocytes was increased in both sexes similarly after 3 hour smoke exposure.

**CONCLUSION:**  Tobacco smoke induces changes in the lungs that are very likely to impact on immune responses, and changes induced by cigarette smoke appear different than those induced by little cigar smoke.  After 1.5 hours in both males and females, cigarette smoke causes lung injury, as measured by protein concentrations, whereas little cigar smoke did not.  Macrophages increase after 1.5 hour exposure to either tobacco product.  Neutrophil recruitment is not induced by 1.5 hours of either tobacco product, but occurs after 3.0 hours of cigarette smoke.  Clear differences in neutrophil recruitment occur between sexes in response to cigarette smoke, males showing more recruitment than females. Ongoing biomarker analysis of the lavage fluid may reveal additional differences between cigarettes and little cigars and may identify interesting biomarkers that are either sex-specific or tobacco product-specific.

# EFFECT OF CHRONIC TOBACCO SMOKE EXPOSURE ON INNATE IMMUNE CELL POPULATIONS IN THE LUNG

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Rationale and Aim:  Smoking related disease in humans is associated with aberrant numbers of innate immune cells (including neutrophils and tissue macrophages) in the lung, suggesting that these cell subpopulations or their products may be useful as biomarkers.  Previous studies showed that transgenic mice that overexpress *Scnn1b* in lung epithelia mimic certain features of tobacco-related human disease more closely than other models, likely due to mucus hyperconcentration.  The aim of this study is to characterize the innate immune cell populations in the lung of wild type (WT) and Scnn1b-transgenic (Tg) mice that have been chronically exposed to cigarette smoke.  The study is in progress and this abstract presents the results of an interim analysis.

Methods:  Male and female wild type (WT) and Scnn1b-transgenic (Tg) mice were exposed to either one hour of cigarette smoke per day for 28 weeks or no tobacco smoke (sham).  Single cell digests of the lungs were prepared, and immune cell populations were identified by labeling with cell type-specific markers.  The percentages of leukocytes and innate immune cell subtypes in the lung were determined by immunophenotyping using multicolor flow cytometry.

Results:  28-week exposure to cigarette smoke resulted in decreased body weight in males.  Analysis of the innate immune cell subpopulations in the lung showed that smoke caused an increase in the Siglec F- Ly6C- CD64+ macrophage population of female Scnn1b-Tg mice.  There was a trend for this population to also increase in WT mice following smoke exposure, but the difference did not quite reach significance in this interim analysis.

| **Females** | Stained cell population | WT-Sham (n=3) | WT-Smoke (n=4) | Tg-Sham (n=3) | Tg-Smoke (n=5) |
|---|---|---|---|---|---|
| % Siglec F- Ly6C- CD64+ cells | Interstitial macrophages | $5.3 \pm 0.2$ | $8.3 \pm 0.9$† | $3.6 \pm 0.2$ | $6.6 \pm 0.7$† |

† $p<0.05$ compared with Tg-Sham

Conclusion:  Our interim analysis shows that mucus hypersecretion and smoke exposure appear to induce changes in the phenotype of some innate immune subpopulations in the mouse lung, particularly in female mice. Sex may confer differential susceptibility to the health effects of smoke exposure.  This mouse model may be useful for developing biomarkers of tobacco exposure and smoking-related injury.

# EFFECTS OF BERRY FLAVORED E-CIGARETTE LIQUIDS ON AIRWAY EPITHELIAL CELLS.

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Electronic-cigarette (e-cig) use has been gaining steady popularity especially amongst younger adults. One of the reasons it is so attractive are the hundreds of different flavored liquids that can be used in an e-cig device, prompting several studies examining the toxicological effects of various flavored e-cig liquids. Though most of the flavorings and the propylene glycol (PG) and vegetable glycerin (VG) vehicle used in e-liquids are generally recognized as safe (GRAS), this is only for oral ingestion and does not take in consideration the aerosolization, the dose, and inhalation of these flavorings. A recent survey has found that fruit flavored e-cigs are the most popular and for this reason, we have screened six  berry flavored e-cig liquids from a local company for potential toxicity using human airway bronchial cell line, Beas-2B. In addition to testing several berry flavors we also tested furaneol, a known strawberry flavoring used in cosmetics and food for potential adverse effects on airway epithelial cells. Beas-2B cells were plated in a 96 well format and treated with various berry flavored e-cig liquids at different concentrations, 1%, 0.5%, and 0.25%, and for different lengths of exposure, 24 hr and 4 hr. The cells were also challenged with different concentrations of furaneol, 1mM, 0.1mM, and 0.01mM for the same durations. We discovered that a popular flavor, 'Cotton Berry', decreased cell density as compared to the vehicle control, PG/VG at a ratio of 45/55. The decreased cell density was not caused by necrosis, as levels of lactase dehydrogenase (LDH) released by 'Cotton Berry' treated cells did not change. However, assessment of cell growth suggests that treatment with 'Cotton Berry' reduces cell proliferation as compared to the media control. In contrast, treatment with furaneol alone did not have any significant effects on cell density or LDH release as compared to the media control, suggesting that furaneol does not mediate the effects of 'Cotton Berry' on epithelial cells. Surprisingly, the vehicle control PG/VG alone also reduced cell growth as compared to the media control, suggesting that the PG/VG vehicle at 55/45 causes adverse effects in epithelial cells. Though most of the recent research on e-cigs has been focused on the role specific flavorings may have on adverse health effects, it is becoming apparent that exposure to the PG/VG vehicle of e-liquids may by itself cause adverse effects on epithelial cells. Considering the rising popularity of e-cig usage, additional efforts into understanding the potential toxicity of the PG/VG humectants contained in e-liquids should be placed.

# NOVEL APPLICATIONS FOR NON-INVASIVE SAMPLING METHOD OF THE NASAL MUCOSA

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Reliable methods for sampling the nasal mucosa provide clinical researchers with key information regarding respiratory biomarkers of exposure and disease. For quick and non-invasive sampling of the nasal mucosa, nasal lavage (NL) collection has been a widely used as a clinical tool, however, challenges in volume variability, sample dilution, and storage prevent it from being used in non-laboratory settings and for the analysis of low abundance biomarkers. In this study, we optimize and validate a described methodology using absorbent Leukosorb® paper cut to fit the nasal passage in order to extract extracellular lavage fluid (ELF) from the nasal mucosa. The ELF sampling method limits the dilution of soluble mediators, allowing for the identification of both high and low abundance soluble biomarkers, such as IL-1B, IP-10, IL-8, IL-6 and Neutrophil Elastase. Additionally, we demonstrate this method can successfully detect the presence of respiratory pathogens, such as influenza virus in the nasal mucosa. According to our findings, the efficacy of ELF collection in this method is not diminished in consecutive day sampling, and percent recovery of both recombinant IL-8 and soluble mediators are not changed despite freezing or room temperature storage conditions for 24h. Our results indicate that ELF collection utilizing Leukosorb® paper sampling is a reliable, easy-to-use, and reproducible methodology to collect concentrated amounts of soluble biomarkers from the nasal mucosa and provides researchers with a new tool to assess changes in nasal mucosal host defense status.

# FLAVORED ELECTRONIC CIGARETTE LIQUIDS IMPAIR RESPIRATORY INNATE IMMUNE CELL FUNCTION

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Innate immune cells of the respiratory tract are the first line of host defense against pathogenic and environmental insults. In the nasal cavity, these cells are primarily neutrophils (PMNs) and natural killer (NK) cells, while in the lower airways, the predominant immune cell type is the alveolar macrophage. Failure of these cells to perform their immune functions leaves the host susceptible to bacterial and viral infection, and may also contribute to impaired resolution of inflammation. While combustible tobacco cigarettes have been shown to suppress respiratory immune cell function, the effects of flavored electronic cigarette liquids (e-liquids) and individual flavoring agents on immune cell responses are unknown. To address this gap in knowledge, we assayed the effects of seven commercially available, flavored, nicotine-free e-liquids on primary human alveolar macrophages, PMNs, and NK cells. All cells were challenged with a range of e-liquid dilutions and assayed for their functional responses to pathogenic stimuli. Functional endpoints included phagocytic capacity (PMNs and macrophages), neutrophil extracellular trap formation, proinflammatory cytokine production, and cell mediated cytotoxic response (NK cells). Each e-liquid flavor was then analyzed via qualitative mass spectrometry to identify individual flavoring components. Strikingly, three cinnamaldehyde-containing e-liquids had a dose-dependent, broadly immunosuppressive effect on all cell types tested. Quantitative mass spectrometry was used to identify molar concentrations of cinnamaldehyde in each of the three e-liquids and immune cells were subsequently challenged with a broad range of cinnamaldehyde concentrations to establish dose-response relationships for changes in functional endpoints. We conclude that specific chemical flavorings commonly used in e-liquid manufacturing, such as cinnamaldehyde, have the potential to impair normal respiratory immune cell function and may thereby increase the risk of respiratory infections in e-cigarette users. These findings illustrate an immediate need for further toxicological evaluation of these products and regulation governing the use of chemical flavorings in e-liquid formulations.

# INFECTION WITH LIVE-ATTENUATED INFLUENZA VIRUS (LAIV) CAUSES ALTERED IMMUNE RESPONSES IN THE NASAL MUCOSA OF E-CIGARETTE USERS.

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Inhalation of tobacco smoke has been linked to an increased risk of viral infection, such as influenza, in humans. Inhalation of new and emerging tobacco products, such as e-cigarettes, has also recently been linked to immune suppression within the airway, specifically nasal mucosa. We propose that innate immune suppression in the airway also makes e-cigarette users more susceptible to influenza infection. To test this hypothesis, we used the controlled exposure of humans to live attenuated influenza virus (LAIV), to safely examine innate immune response to influenza infection in non-smokers, cigarette smokers, and e-cigarette users. Our observational cohort of non-smokers, cigarette smokers, and e-cigarette users were inoculated with LAIV, nasal lavage fluid (NLF), nasal biopsies, blood, and urine was collected before and after inoculation. Endpoints include inflammatory mediators from the nasal lavage fluid, gene expression changes and viral RNA from nasal biopsies, as well as markers of tobacco exposure from blood and urine. Compared to controls, LAIV-induced NLF levels of interferon gamma inducible protein (IP-10) and interleukin-6 (IL-6) were suppressed in e-cigarette users. While further examination of inflammatory markers in the NLF, associated gene expression, viral RNA, and markers of tobacco exposure are currently under analysis, these preliminary data indicate that exposure to e-cigarettes suppresses host defense responses after inoculation with LAIV.

# ETHINYL ESTRADIOL MEDIATED SEX DIFFERENCES IN THE HUMAN AIRWAY EPITHELIUM OF SMOKERS AND NON-SMOKERS.

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Sex has been identified as a risk factor for a variety of lung diseases, including early onset COPD, where 70% of the patients are women.  It is known that females are disproportionately affected, but the contribution of estradiol, the primary circulating estrogen in women, is unknown.   While inhaled pollutants like cigarette smoke contribute to an increased number of patients with COPD, it does not explain the increased incidence in women.  To investigate this question, we examined both genomic and non-genomic factors by which sex and smoking may both independently and concurrently contribute to sex-biased lung disease susceptibility.  Nasal lavage and nasal turbinate scrape biopsies were collected from smokers and non-smokers of both sexes for this study.  Samples were isolated from women during high and low circulating estrogen phases of the menstrual cycle.  After RNA isolation, sex-associated and estradiol-dependent effects on mRNA expression were evaluated using NanoString nCounter analysis.  Changes in innate immune response to infection (Influenza A) were also evaluated with concomitant exposure to philologically relevant levels of ethinyl estradiol by PCR and ELISA in immortalized human bronchial epithelial cells (Beas-2B) as well as in primary nasal epithelial cells cultured at air-liquid interface. Gene expression changes were both estrogen level dependent and smoking-status dependent.  Pro-apoptotic genes such as *bax* were upregleted in smokers and down regulated in non-smokers when estrogen was high.  Mast cell degranulation was increased, along with IL-8 secretion, however neutrophil and macrophage recruitment was suppressed in smokers, especially during the high estrogen portion of their cycle.  Independent of estrogen level, *itch* was up-regulated in smokers, preventing t-cell anergy.  Similar to our NanoString data, *in-vitro* IL-6 and IP-10 levels were more suppressed in female smokers exposed to influenza than males.  Our results indicate that the synergy of estrogen level/sex and smoking-status combines to increase inflammation in the airway while suppressing anti-inflammatory signaling and normal host-defense responses.

# BENCHMARK STUDIES OF COMBUSTIBLE TOBACCO PRODUCTS FOR COMPARISON TO EMERGING TOBACCO PRODUCTS

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*Background:* Well-differentiated primary human bronchial epithelial cell (HBEC) cultures grown at an air-liquid interface (ALI) are instrumental for studying basic and applied aspects of tobacco smoke respiratory tract toxicity. The Cell Culture and Smoke Exposure Core of the UNC TCORS project provides epithelial cell culture expertise and a reliable supply of HBEC ALI cultures. It also houses a Vitrocell® VC10 Smoking Robot System with a 24 well exposure block to enable studies of standard or new and emerging tobacco products. Our goal was to perform comprehensive "baseline" smoke dose response and time course experiments with Kentucky Reference 2R4F cigarettes as a starting point and benchmark for comparison to emerging tobacco products.

*Methods:* Smoke exposure- HBEC ALI cultures originating from 4 human lung donors were grown for 28-35 days on 6.5mm Transwell® inserts. The cultures were exposed apically to five dilutions of whole gas phase R24F cigarette smoke mixed with air, or air alone, in quadruplicate. Cells were studied after a single acute exposure and after four daily consecutive exposures. RNA was harvested at 6 and 24 hours after a single exposure and 24 hours after four exposures. Cytotoxicity was assessed by adenylate kinase release onto the apical surface and inflammatory potential was determined by measuring IL8 in the basolateral media. Four-color whole mount immunofluorescent staining was performed to detect cilia, mucin, filamentous actin, and nuclei. Paraffin sections were made for histological analysis by hematoxylin and eosin (H&E), and Alcian Blue-Periodic Acid Schiff's (AB-PAS) staining.

*Results:* The highest smoke dose was cytotoxic, leading to significant detachment of cell cultures from the Transwell membranes. Lower smoke doses were not overtly cytotoxic. Quantitative RT-PCR revealed large increases in CYP1A1 and HMOX1, and more extensive mRNA profiling is in progress. Increased IL8 was observed 24h post initial exposure, but was not increased beyond 24h. Immunofluorescent staining and histological analysis revealed a dose responsive depletion of cilia at the apical surface.

*Conclusions:* There have been many studies using liquid extract/condensate from tobacco smoke to expose transformed cell lines or poorly differentiated primary cells grown under non-differentiated, submerged conditions. Studies of ALI HBEC cultures have used proprietary commercial cells and medias. Our group is strongly committed to direct apical surface exposure of well-differentiated primary ALI HBEC cultures created using non-proprietary methods to tobacco products. These studies with whole gas-phase combustible tobacco in our model system provide a reliable benchmark for comparison to emerging tobacco products. Exposures using a third generation advanced personal vaporizer are in progress.

# THE UNC TCORS CELL CULTURE AND SMOKE EXPOSURE CORE: PRODUCTIVITY AND CAPABILITIES

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Well-differentiated primary Human Bronchial Epithelial Cell (HBEC) Air-Liquid Interface (ALI) cultures are vital for research to determine the health effects of new and emerging tobacco products. Many prior studies use proprietary, commercially produced cells, medias or ALI cultures that are available only at considerable expense. The UNC Cell Culture and Smoke Exposure Core procures human lungs and produces HBECs, media and ALI cultures using well established non-proprietary methods (Methods Mol Biol. 2013;945:109-21). Lungs originate from non-smoker donors, with no history of prior chronic lung disease, and cells are subjected to rigorous quality control measures. Depending on project needs, the Cell Culture and Smoke Exposure Core supplies passage (p) 0, or p1 cells and medias for investigator-initiated cultures, and, in some cases, already well differentiated ALI cultures. To date, we have produced $5.85 \times 10^9$ p0 HBECs; $2.99 \times 10^9$ p1 HBECs; ~70 liters of Bronchial Epithelial Growth Medium (BEGM, for cell growth on plastic) and ~300 Liters of ALI Medium (for cell growth on porous supports at an ALI), for TCORS projects. The equivalent commercial value of these resources is estimated to be greater than $5,000,000. In addition, the Core houses and manages smoke exposure capabilities, including a user-run Borgwalt LX1 smoke robot suitable for use with British American Tobacco style ALI culture exposure chambers (J Anal Toxicol. 2008 Apr;32(3):201-7). The Core maintains and operates a Vitrocell® VC10 apparatus, capable of exposing 24 HBEC ALI cultures simultaneously, employing an air control and 5 whole smoke doses in quadruplicate. Using the VC10, the Core is spearheading comprehensive "benchmark" studies of Kentucky Reference 2R4F cigarettes that will serve as a comparison for new and emerging products. These studies are reported in an accompanying abstract and poster. The VC10 has recently been adapted to accommodate third generation advanced personal vaporizers. The Core thus enables studies of new and emerging tobacco products that would otherwise be impossible, and is instrumental to meet the goals of the UNC TCORS project.

# COLLECTION AND ANALYSIS OF E-CIGARETTE AEROSOL GENERATED AT A RANGE OF COIL TEMPERATURES

Nicholas J. Wallbillich and Gary L. Glish

The variation in commercial vaporizers, particularly in the level of current applied to the atomizer coil, complicates chemical characterization of e-cigarette aerosol because first principles in aerosol chemistry dictate that temperature has a strong influence on the aerosol, including particle composition, concentration, and size. The focus of this project is two-fold: (1) to develop a sampling apparatus for reproducible collection of e-cigarette aerosol generated at constant temperatures and (2) to compare the chemical constituents of the aerosols generated at different temperatures. A Joyetech Evic vaporizer with constant temperature control on a 0.2 ohm Ni coil was used to produce the aerosol, which was collected onto 44 mm Cambridge filter pads. Four-second puffs at 30-second intervals were realized with the introduction of a 12 V ¼" solenoid valve triggered by a driver circuit. A 24 V push solenoid for depression of the vaporizer button was driven by the same circuit. Two e-liquids purchased from the local Vapor Girl, Vanilla Tobacco and Menthol Tobacco, with 12 mg/mL nicotine and no nicotine, were smoked at $120^{o}C$, $200^{o}C$, $315^{o}C$, representing the full temperature range of the device. After collection on the filter pad, sample is extracted in methanol for GC-MS analysis.

# DIRECT ANALYSIS OF NICOTINE AND NICOTINE METABOLITES BY PAPER-SPRAY MASS SPECTROMETRY

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Quantitation of nicotine and related metabolites is critical to understanding the physiological impact of emerging tobacco and e-cigarette products. Mass spectrometry provides a platform capable of high selectivity and sensitivity, but is traditionally limited by sample complexity. Analysis of biological samples by liquid or gas chromatography-mass spectrometry (LC-MS, GC-MS) requires sample preparation (e.g. liquid-liquid extractions, solid-phase extractions) that is time-consuming and prone to increasing variance through the addition of steps to the analytical workflow. For rapid analysis, direct ionization followed by mass spectrometric measurement is ideal, but most ionization techniques are not suitable for work with untreated biological samples. For example, electrospray ionization would permit immediate analysis, but the high concentration of salts would interfere with ionization of the analyte. A recently developed ionization technique, paper-spray, alleviates the problems associated with analysis of biological samples by electrospray. Samples can be spotted directly on a sharp-tipped, rigid piece of paper and analyzed immediately following application of solvent and high voltage. Paper-spray mass spectrometry (PS-MS) permits direct analysis of complex samples, as the analyte is wicked towards the paper tip via multiple porous pathways. This provides a high tolerance for salts and associated matrices. Analysis can be completed in one minute, at least an order of magnitude faster than related chromatographic techniques (GC-MS, LC-MS), and the simplification of the analytical workflow reduces experimental variance. Samples of immediate interest are lavages of the airway-surface liquid and cell media of tobacco smoke-exposed epithelial cells, as quantitation of nicotine and its metabolites will aid in the interpretation of immune response studies. Methods are currently being validated for the analysis of nicotine and cotinine in phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), and Air-Liquid Interface Medium (ALI). Methods will be expanded to include additional metabolites (nornicotine, anabasine) and additional matrices (sputum, serum, urine).

# THE REWARDING AND ANALGESIC PROPERTIES OF FLAVORED ELECTRONIC CIGARETTES

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**Purpose:** Electronic cigarettes are gaining popularity and are sold in many different flavors. Yet, little is known about whether the addition of flavorings affects the addictive potential of electronic cigarettes. The purpose of this research study is to examine the effects that different flavorings have on the rewarding properties of smoking electronic cigarettes (i.e., 'vaping') and on the ability of electronic cigarettes to produce analgesic effects (pain reducing effects), which may also contribute to their addictive potential.

**Participants**: To date, 74 medically healthy men and women aged 18 – 50 years who are habitual e-cigarette users and have a preference for one of three flavorings: tobacco, menthol, or berry have been studied. Target goal is at least 25 participants/flavor group. All participants are free of any pain condition, including frequent headaches, and free from clinical levels of depressive or anxious symptomatology. All participants reported vaping nicotine solution at least 4 days per week. Individuals who reported smoking >5 tobacco cigarettes per week were not studied (confirmed with a 5 day vaping and smoking diary).

**Methods:** Using a within-subjects, repeated measures laboratory-based protocol that controls for female menstrual cycle phase, each of the 74 participants have been tested under the following four conditions: 1) vaping an e-cigarette that contains both nicotine and their preferred flavor (+nicotine/+flavor); 2) vaping an e-cigarette that contains nicotine but no flavor (+nicotine/no flavor); 3) vaping an e-cigarette that contains no nicotine but does contain the preferred flavor (no nicotine/+flavor); and 4) vaping an e-cigarette that contains neither nicotine nor flavor (no nicotine/no flavor). Three times during each laboratory session, participants follow a paced vaping protocol (10 min). Following each of the three vaping segments, participants rate the rewarding (reinforcing) properties associated with vaping. Immediately following each rating, participants are exposed to one of three pain tests: a heat pain test, a cold pressor test, and a forearm ischemic pain test (randomizing order). Time to pain onset (pain threshold), time to pain tolerance and pain intensity and unpleasantness ratings for each pain test are measured. These pain tests were selected because sensitivity to each is mediated by different endogenous pain regulatory mechanisms (e.g. opioid, adrenergic). Subjects also provide a urine sample at the beginning of each of the four labs to test for urine cotinine (a metabolite of nicotine) and creatinine to provide an index of tobacco exposure that will be used as a control variable in analyses.

**Results:** To date, 26 berry vapers (42.3% female), 31 menthol vapers (22.6% female) and 17 tobacco vapers (29.4% female) have been studied. Preliminary results support expected gender differences in pain sensitivity (males less sensitive) to all three pain tests and expected analgesic effects of nicotine. Relative to the no nicotine/no flavor condition, comparisons of the three different flavorings (with or without the presence of nicotine) on the rewarding and analgesic effects of berry, menthol or tobacco will be presented at the poster session.