# E-Cigarette Use Causes a Unique Innate Immune Response in the Lung, Involving Increased Neutrophilic Activation and Altered Mucin Secretion

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**At a glance commentary**

**Scientific Knowledge on the Subject**

New and emerging tobacco products (NETPs), especially e-cigarettes, have become popular and their use has increased dramatically, particularly among the younger population by attracting both former tobacco smokers and never smokers. This trend is supported by a common assumption that e-cigarette use is harmless and a safe alternative to cigarette smoking. Despite a lack of sufficient health science evidence, e-cigarettes are promoted as cigarette smoking cessation aids in some health care practices. In short, little is known about the potential adverse health effects of e-cigarette use on the lungs.

**What this study adds to the field**

To our knowledge, this is the first study utilizing human airway samples to explore the effect/harm of e–cigarette use on the airways. This study describes a unique e-cigarette-induced innate lung response that includes markers of aberrant neutrophilic response and altered mucin secretion and indicate that the effects of e-cigarettes are overlapping with yet distinct from those observed in otherwise healthy cigarette smokers. These findings challenge the concept that switching from cigarettes to e-cigarettes is a healthier alternative.

**Abstract**

**Rationale:** E-cigarettes have become increasingly popular and little is known about their potential adverse health effects.

**Objective:** To determine the effects of e-cigarette use on the airways.

**Methods:** Induced sputum samples from cigarette smokers, e-cigarette users, and non-smokers, were analyzed by quantitative proteomics, and the total and individual concentrations of mucins MUC5AC and MUC5B were determined by light scattering/refractometry and labeled mass spectrometry, respectively. Neutrophil extracellular trap (NET) formation rates were also determined for the same groups.

**Measurements and Main Results:** E-cigarette users exhibited significant increases in aldehyde-detoxification and oxidative stress related proteins associated with cigarette smoke comparing to non-smokers. The levels of innate defense proteins associated with Chronic obstructive pulmonary disease (COPD), such as elastase and matrix metalloproteinase- 9, were significantly elevated in e-cigarette users as well. E-cigarette users' sputum also uniquely exhibited significant increases in neutrophil granulocyte- and NET-related proteins, such as myeloperoxidase, azurocidin, and protein-arginine deiminase 4, despite no significant elevation in neutrophil cell counts. Peripheral neutrophils from e-cigarette users showed increased sensitivity to PMA-induced NETosis. Finally, a compositional change in the gel-forming building blocks of airway mucus, i.e., an elevated concentration of mucin MUC5AC, was observed in both cigarette smokers and e-cigarette users.

**Conclusions:** Together, our results indicate that e-cigarette use alters the profile of innate defense proteins in airway secretions, inducing both similar and unique changes relative to

cigarette smoking. These data challenge the concept that e-cigarettes are a healthier alternative to cigarettes.

**Introduction:**

The adverse health effects of long-term cigarette smoking in the lung, e.g., increased risks of cancer and COPD, have been well established (1), and public awareness regarding these risks is rising. Although this awareness has led to an ongoing trend of decreased usage of conventional tobacco products in the US, new and emerging tobacco products, and especially e-cigarettes, have been gaining popularity. These products have become popular and have recently shown dramatic increases (up to 900%) in use, particularly in the younger population attracting both former tobacco smokers and never smokers (2,3). This trend is supported by the assumption by many that e-cigarette use is harmless and a safe alternative to cigarette smoking and by the fact that e-cigarettes are promoted as cigarette smoking cessation aids in certain health care practices, despite a lack of sufficient health science evidence (4).

E-cigarettes are designed to deliver nicotine to the brain via the lungs and cardiovascular system without the combustion of tobacco, which ordinarily results in the production of thousands of toxic compounds. However, vaporization of e-liquids by current-generation e-cigarette devices can generate similar toxic compounds, such as reactive aldehydes (5). The formation of toxic aldehydes (e.g., formaldehyde and acrolein) in e-cigarette vapors has been attributed to thermal decomposition of the major vehicle components of e-cigarette e-liquids (propylene glycol and glycerol) and flavorings, which are reported to produce levels that exceed occupational safety standards (6). Several of these toxic compounds, e.g., acrolein, are already associated with the

epithelial response to cigarette smoking, specifically increasing mucin secretion (7-9).

E-cigarettes have recently been deemed to be subject to FDA regulation; however, specific aspects of their regulation are not yet in place, primarily because the potential adverse health effects and risks of e-cigarette use are still unknown. Therefore, it is of great importance to better understand how exposure to e-cigarette vapors modifies human airway biology compared with traditional cigarette smoking and to identify the potentially unique effects e-cigarette vapors might have on lung physiology.

The airway epithelial mucosal barrier is part of the innate immune system and protects the underlying epithelia when faced with microbial, physical, and chemical challenges (10), including smoking and vaping. The biophysical properties of airway mucus are primarily established by mucins MUC5B and MUC5AC and their interacting partners (11). Airway secretions from smokers and COPD patients exhibits quantitative and compositional alterations, with observable changes in mucin concentration and the ratio of MUC5B to MUC5AC, signifying these mucins' importance in the establishment of the biophysical properties of mucus (12, 13).

Neutrophils play an important role in airway maintenance due to their strong antimicrobial activity. However, when left unchecked, activated neutrophils can contribute to inflammatory lung diseases, such as COPD. Neutrophils exert their antimicrobial effects through four major mechanisms: phagocytosis, degranulation of stored mediators and enzymes, reactive oxygen generation and the release of neutrophil extracellular traps (NETs) (14). NETs are the result of a specific type of cell death called NETosis (15) and consist of chromatin filaments and specific granule proteins, including neutrophil elastase (NE) and myeloperoxidase (MPO) (16), which have been associated with the bronchial inflammation and structural damage observed in diseases such as cystic fibrosis (CF) and COPD (17, 18). Although NET formation is an antibacterial immune

response, it has also been shown to be triggered by constituents of smoke and e-cigarette vapors, such as acrolein (19).

E-cigarettes are fairly new products, and data on the effects of their long-term use remain scarce. The airway epithelium is one of the first parts of the body encountered by inhaled smoke and vapors, and the mucus secretion layer acts as the first line of defense against inhaled biological and chemical substances. Analysis of the changes in the airway secretion proteome as a result of e-cigarette vaping can identify potential biomarkers associated with adverse health effects. Using induced sputum samples from tobacco product users participating in the UNC TCORS program, we compared changes in airway mucus composition and integrity among cigarette smokers, e-cigarette users and healthy non-smokers.

**Materials and Methods:**

Additional information on the study design and the details of the methods is provided in the online data supplement.

***Subject population and sputum sample collection:*** Informed consent was obtained from all study participants, and the protocol was submitted to and approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board. Induced sputum samples from 14 current cigarette smokers, 15 current e-cigarette users and 15 never smokers were collected as previously described (20). Raw sputum expectorant was processed as described below. The smoking status of the participants was confirmed based on serum cotinine and urine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) levels.

***Label-free quantitative proteomic analysis:*** An aliquot (100 µl) of induced sputum was diluted 1:1 in the chaotropic agent GuHCl at a final concentration of 4 M, reduced, alkylated and digested by trypsin. Next, 5 µl of solubilized peptides was injected utilizing a Q-Exactive (Thermo Scientific) mass spectrometer coupled to an UltiMate 3000 (Thermo Scientific) nano HPLC system. The raw data were processed and searched against the UniProt protein database (*Homo sapiens*, January 2017) using Proteome Discoverer 1.4 (Thermo Scientific) software. Protein quantification was performed using the normalized total precursor intensity. The data were log transformed to achieve a normal distribution. Statistical significance among groups was determined by one-way ANOVA.

***Mucin concentration measurements:*** The total mucin concentrations in the sputum samples were analyzed by size-exclusion chromatography/differential refractometry (SEC-MALLS/dRI) measurements (21), and the individual concentrations of MUC5AC and MUC5B were measured using stable-isotope-labeled mass spectrometry with parallel reaction monitoring analysis, as described previously (13) .

***Isolation of peripheral blood neutrophils and quantitation of NET formation***: Neutrophils from healthy non-smokers (N=11), smokers (N=7), and e-cigarette users (N=12) were isolated from venous blood as described previously (22) . The cells were incubated at 37°C and 5% $CO_2$ for 30 min prior to challenge with 25 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich). The wells were assayed for extracellular chromatin content every hour during a 4 h challenge to assess NET formation. The amount of chromatin released each hour was quantified using a PicoGreen double-stranded DNA kit (Life Technologies).

**Results:**

To determine how the use of e-cigarettes and conventional cigarettes impacts the airway innate immune response, we collected induced sputum samples from e-cigarette users and cigarette smokers and compared them to those of non-smokers. The study participants in the non-smoker group displayed values for both nicotine exposure (cotinine) and the tobacco-specific marker NNAL at or below the detection limit (Figure E1 in the online data supplement). In the cigarette smoker group, serum cotinine and urine NNAL levels were significantly correlated with the number of cigarettes smoked per day ($p<0.05$). In the e-cigarette user group, serum cotinine levels were significantly correlated with the number of e-cigarette puffs per day ($p<0.0001$), whereas urine NNAL levels were not. Average urine NNAL levels were significantly lower in e-cigarette users compared with smokers (Table E1 in the online data supplement). Only one subject had urine NNAL levels above the cutoff point of 47 pg/ml, which distinguishes smokers from non-smokers (23), whereas the majority of e-cigarette users had urine NNAL levels comparable to those observed in non-smokers, suggesting exclusive e-cigarette use.

The average BMI and age distribution did not significantly differ among the different groups. The average number of cigarettes smoked per day in the cigarette smoker group was ~11. In the e-cigarette user category, the average number of puffs inhaled per day was ~280. Of the 15 e-cigarette users, 12 identified themselves as having previously smoked cigarettes, and three indicated no prior cigarette smoking history. In addition, five of the subjects reported occasionally smoking cigarettes (Table E2 in the online data supplement).

***Altered airway secretion proteomes in cigarette smokers and e-cigarette users:*** Using a peptide confidence interval of 95% and a minimum of 2 assigned peptides per protein, our proteomic analysis of induced sputum identified a total of approximately 1000 proteins across all cigarette users', e-cigarette users' and non-smokers' samples (Sheet E1 in the online data supplement). Our label-free quantitative analysis revealed that mucus protein composition in e-cigarette users was qualitatively and quantitatively different than in cigarette smokers and non-smokers. We detected the highest number of significant changes in protein levels in the e-cigarette user group (Fig. 1A). Compared with non-smokers, induced sputum from e-cigarette users contained approximately 81 proteins with significantly altered abundance, whereas approximately 44 proteins with altered abundance were identified in sputum from cigarette smokers. A heatmap (Fig. 1B) was used to summarize all of the proteins with significantly changed levels in cigarette smokers' and e-cigarette users' sputum, sorted by fold change in the e-cigarette group. This representation illustrates that several of the proteins showed similar abundance changes in the two user groups but, more importantly, that the overall patterns of change were relatively different between the two groups.

The cigarette smokers' sputum proteome displayed an upregulation of known markers associated with smoking (Fig. 2), such as the aldehyde-detoxifying enzyme aldehyde dehydrogenase 3A1 (ALDH3A1) (24, 25), microseminoprotein beta (MSMB) (25), nucleobindin-1 (NUCB1) (25) and anti-thrombin 3 (ANT3) (26), and an upregulation of oxidative stress response proteins, e.g., thioredoxin (TXN) (25, 27) and glutathione S-transferase (GSTP1) (28). The levels of several of these markers (ALDH3A1, TXN, GSTP1, ANT3) were also significantly elevated in the e-cigarette users (Fig. 2D-F).

Another trend that was observable in our dataset was a significant and broad elevation of the levels of mucosal defense proteins in sputum from cigarette smokers, but not e-cigarette users (Fig. 3). These proteins, which are known to be airway mucus constituents that are essential in fighting infections, are shown in Fig. 3, including deleted in malignant brain tumors 1 (DMBT1), lactotransferrin (LTF), trefoil factor 3 (TFF3) and lysozyme C (LYSC). The levels of several of these innate defense proteins, such as DMBT1 and LYSC, were significantly decreased in the e-cigarette users compared with the non-smokers.

***Elevated markers of neutrophil activation in e-cigarette users' sputum:*** Most notable among the functional protein groups with increased abundance in the sputum of e-cigarette users were secreted proteins related to the innate defense functions of leukocytes. Among these, primary neutrophil granule proteins, such as NE, proteinase 3 (PRTN3), azurocidin (AZU1) and MPO, showed significantly higher levels in e-cigarette users than in cigarette smokers or non-smokers, with slightly, but non-significantly, increased levels in the cigarette user group as well (Fig. 4). In addition, we observed significant increases in secondary neutrophil granule proteins, such as collagenase and gelatinase (also known as matrix metalloproteinase 8 (MMP8) and MMP9, respectively), in cigarette smokers' as well as e-cigarette users' sputum (Fig. 4B). After observing this pattern of neutrophil proteins, we performed a correlation analysis in which the statistical relationships between the expression profiles of all quantified proteins and the profile of NE were determined across all samples (Table E3 in the online data supplement). Proteins with a Spearman correlation coefficient above 0.6, and indeed the vast majority of proteins whose profiles were highly correlated with that of NE, were typical neutrophil proteins.

These findings of neutrophil protein enrichment in the e-cigarette group raised the question of whether the number of neutrophils was increased in the sputum samples from e-cigarette users. Interestingly, neutrophil cell counts were not significantly higher in e-cigarette users but were significantly increased in the sputum samples from cigarette smokers (Fig. 4F).

In addition, our proteomic analysis identified several proteins that have been shown to be associated with NETs (Fig. 5). In particular, sputum from e-cigarette users displayed a significant increase in calprotectin (Fig. 5A,B) a cytosolic protein in unstimulated neutrophils that is crucial for the clearance of infections when it is released as part of NETs (29). Other NET markers that were significantly increased in our analysis included coronin-1 (Fig. 5C) (30) and peptidyl arginine deiminase 4 (PAD-4) (Fig. 5D), an enzyme known to modify histones that becomes part of NETs. Notably, histone H4 was identified among the proteins with the highest correlation with NE in our analysis, providing additional evidence for increased NET formation in the airways of e-cigarette users.

***PMA-induced systemic NET formation:*** To determine whether peripheral blood neutrophils were also affected by e-cigarette use, we challenged the neutrophils isolated from cigarette smokers, e-cigarette users and non-smokers *ex vivo* with a protein kinase C (PKC) activator and the potent NET agonist PMA. Quantitation of the PMA-induced NETs formed by neutrophils isolated from the venous blood indicated that peripheral neutrophils isolated from e-cigarette users were significantly more sensitive to PMA-induced NET formation at 2 h ($p=0.01$), as assessed based on nucleic acid release over time (Fig. 6).

***Total mucin and individual MUC5AC and MUC5B concentrations in tobacco product users' sputum:*** An increased total mucin concentration and a shift in the ratio between the major gel-forming airway mucins MUC5B and MUC5AC have been shown to be correlated with cigarette

smoking and COPD progression (31). We observed that the total mucin concentrations in sputum samples were significantly increased (p=0.04) in cigarette smokers (1986 µg/ml +/- 810 SD) compared with non-smokers (1251 µg/ml +/- 964 SD) and that the total mucin concentrations in the sputum samples of the e-cigarette users, although slightly increased (1322 µg/ml +/- 663 SD), were not significantly different from those in non-smokers' samples (Fig. 6A). Analysis of the individual concentrations of MUC5B and MUC5AC (Fig. 6B, C) showed that MUC5B levels were not altered in the sputum of cigarette smokers or e-cigarette users and thus did not contribute to the observed increases in total mucin. MUC5AC concentrations, however, were significantly increased in cigarette smokers (132 pmol/ml +/- 58 SD, p=0.02) and in e-cigarette users (58 pmol/ml +/- 21 SD, p=0.05) compared with the non-smokers (15 pmol/ml +/- 6 SD). As a result, the MUC5AC/MUC5B ratio was significantly increased in cigarette smokers (0.32, p=0.02) and e-cigarette users (0.34, p=0.05) compared with non-smokers (0.11) (Fig. 6D).

**Discussion:**

In this study, using induced sputum samples from cigarette smokers, e-cigarette users, and healthy never-smokers, we aimed to determine the effects of e-cigarettes on the human airways. Given that e-cigarette use-related changes in lung secretions are mostly unknown, we first focused on the known impacts of cigarette use. Comparing our results with published data, we were able to confirm changes in the levels of numerous proteins that are established markers of cigarette smoke exposure, thus validating our quantitative approach to identifying the effects on airways. We detected upregulation of the secreted detoxifying enzyme ADH3A1, and the levels of other known *in vivo* and *in vitro* markers of cigarette smoke exposure (24, 25, 32,33) were also elevated in the e-cigarette users, suggesting that e-cigarette exposure is equally bad for the lung. Additionally, the

elevated levels of markers known to be associated with cigarette smoke and lung disease/inflammation, such as TXN and MMP9, in the sputum of both cigarette smokers and e-cigarette users indicates commonality in the impacts of these products on airway physiology, such as increased oxidative stress and activation of innate defense mechanisms. Furthermore, proteases of neutrophil and epithelial origin, such as MMP9, are inflammatory mediators known to be major contributors to chronic lung diseases (34, 35).

As shown in Figure 3, increased levels of innate defense proteins secreted by the airway epithelium were observed in cigarette smokers. Several of these proteins, such as DMBT1, TFF3, lactoferrin and LYSC, play important roles in protection against pathogens, either through direct antimicrobial activity or by acting as part of the mucosal barrier network. DMBT1 (11, 36) and trefoil factors (37) have been shown to interact with the building blocks of the mucus gel, i.e., gel-forming mucins, and can therefore potentially alter the viscoelastic properties of this important mechanical barrier (38). Interestingly, in the present study, the levels of these proteins tended to decrease in e-cigarette users; the levels of DMBT1 and LYSC, in particular, were significantly decreased in the e-cigarette users compared with the non-smokers, suggesting an altered innate immune response in the former group.

The analysis of differentially expressed proteins revealed that a group of innate defense proteins of neutrophilic origin were highly represented in the e-cigarette users (Fig.1 B, C). Most prominently, primary neutrophilic granule enzymes, such as NE, PRTN3 and MPO, showed significantly higher levels in e-cigarette users than in non-smokers (Fig. 3). Among other functions, these neutrophilic enzymes are inflammatory mediators and major contributors to the pathogenesis of chronic lung diseases, such as COPD (34). Cell counts, however, showed no

significant increase in the number of neutrophils in the sputum of e-cigarette users (Fig. 5), suggesting that the changes were not caused by a greater number of neutrophils in these subjects.

Neutrophil granulocytes possess 2 major mechanisms for releasing stored mediators and enzymes to perform their antimicrobial activity: degranulation and the release of NETs (14). To gain insight into which of these mechanisms was most likely responsible for the observed proteome changes, we investigated the abundance of marker proteins associated with these processes in our dataset. The combined observations of elevated neutrophil-derived protein levels but no increase in neutrophil cell number in the sputum of e-cigarette users suggest two potential underlying pathogeneses: e-cigarettes may cause altered activation and degranulation of these neutrophils, or e-cigarettes may cause a neutrophilic increase but induce neutrophil death at the same time.

Extensive degranulation of neutrophils is a feature of lung disorders involving inflammation, such as severe asthma and COPD (17, 39). An additional mechanism by which neutrophil granulocytes can eliminate bacteria has been described by Brinkman et al (14). Upon activation by specific mediator signals, including cytokines, lipopolysaccharide and specific complement factors (40), neutrophils were shown to form web-like extracellular structures called NETs. NETs are the product of a specific type of cell death called NETosis (15) and consist of chromatin filaments and specific globular proteins that are highly effective at trapping and killing invasive bacteria. In our proteomic analysis of sputum from tobacco product users, we were able to identify several proteins that have been shown to be associated with NETs (Fig. 4). For example, in the sputum of e-cigarette users, we identified a significant increase in calprotectin (Fig. 5A,B), a cytosolic protein in unstimulated neutrophils that is crucial for the clearance of infections when it is released as part of NETs (29). Other NET markers found to be significantly increased in our analysis include coronin-1 (Fig. 4C) (30) and PAD-4, an enzyme known to modify histones that become part of

NETs. Notably, histone H4 was identified among the proteins with the highest correlation with NE in our analysis, suggesting increased NET formation in e-cigarette users' airways. Cytokines such as IL-8 and TNF-alpha are known mediators of NET formation (41, 42) but were not elevated in the e-cigarette users (data not shown).

The observation of elevated levels of neutrophil-derived proteins, including the proteins related to NET formation, in the absence of increased neutrophil cell numbers and cytokine levels indicates that the activation state of neutrophils is altered in the airways of e-cigarette users compared with non-smokers and cigarette smokers. Indeed, the data also suggest that activation may be present systemically because peripheral blood neutrophils from e-cigarette users were more sensitive to PMA-induced NET formation. These data support the sputum proteomic data and are consistent with the findings of our previous study, which demonstrated that exposure of neutrophils to certain flavored e-liquids *ex vivo* also enhanced sensitivity to PMA-induced NET formation (22). Although the exact role of NET formation in respiratory disease is not known, aberrant activation of NET formation likely leads to the release of tissue-damaging proteases. Indeed, accumulation of NETs has been shown to be associated with inflammatory diseases, including CF and COPD (43, 44). The enhanced NET formation in peripheral blood neutrophils from e-cigarette users also suggests the potential for systemic harm beyond the lung. Given that increased NET formation is closely associated with epithelial and endothelial cell death and subsequent pathogenesis, aberrant NET formation in peripheral blood neutrophils from e-cigarette users should be examined in the context of the pathogenesis of systemic diseases, such as systemic lupus erythematosus (45), vasculitis (46), and psoriasis (45).

The secretion of elastase and other pro-inflammatory mediators by neutrophils has been observed in response to e-cigarette vapor extract exposure *in vitro* (47), indicating that e-cigarette

constituents aberrantly activate neutrophils. Despite the enhanced secretion of elastase, e-cigarette-exposed mice showed a reduced ability to clear either bacteria or an influenza virus, suggesting that innate mucosal defense systems could be impaired by e-cigarette vapors (48). The consistent elevation of innate neutrophil defense protein levels in the e-cigarette users' sputum in the present study suggests a vaping-induced increase in the inflammatory response of neutrophils in the airways that is distinct from the changes induced by cigarette smoke or more effective.

It is crucial to understand the acute and chronic effects of activated neutrophils and altered mucin secretion dynamics on the innate immune properties of airway secretions. The functional consequences of these alterations for the antibacterial and antioxidant defense mechanisms of the lung and their contributions to the pathogenesis of chronic lung diseases such as COPD remain to be elucidated.

Another key observation in this study is the marked increase in the gel-forming mucin MUC5AC in tobacco product users' sputum, which led to an altered MUC5AC/MUC5B ratio. Increased MUC5AC has previously been associated with cigarette smoke exposure (7, 49), but the current study is the first to report an increase in this mucin in response to e-cigarette use. It has been suggested that an elevated mucin concentration is an important hallmark of failed mucus transport in muco-obstructive disease and an important parameter in COPD pathogenesis (13). Additionally, the ratio of the two major gel-forming mucins, MUC5AC and MUC5B, is related to mucus pathologies, including mucus stasis (12) and mucus obstruction (50). The MUC5AC concentration or the MUC5AC/MUC5B ratio of the airway secretions can therefore serve as a biomarker of exposure to and/or the effects of tobacco smoking.

It is important to note that the majority of the subjects in the studied e-cigarette user group (12 of 15) had smoked cigarettes at some time in their tobacco product use history before becoming

predominant e-cigarette users. Therefore, the observed effects could have partly been a result of the users' smoking history. However, it has been shown that the sputum proteomes of healthy former smokers are essentially similar to those of never smokers (25) and that there is an absence of residual biomarkers of smoking in the sputum of former smokers. Future studies designed to study e-cigarette smokers who have never smoked cigarettes appears to be warranted.

To our knowledge, this the first study utilizing human airway samples to explore the effect/harm of e-cigarette use on the airways. Our study clearly demonstrates a unique e-cigarette-induced innate lung response that includes markers of an aberrant neutrophilic response. Taken together, our results indicate that the effects of e-cigarettes are both overlapping with and distinct from what is observed in otherwise healthy cigarette smokers. In conclusion, our results challenge the concept that e-cigarettes are a healthier alternative to cigarettes and reverse smoking-induced adverse health effects.



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**Figure legends:**

**Figure 1: Analysis of induced samples of tobacco product users' sputum indicates a uniquely altered airway secretome for e-cigarette users in comparison with cigarette smokers and non-smokers.** (A) Venn diagram showing the number of proteins with significantly higher levels than the means in the sputum of cigarette smokers, e-cigarette users and non-smokers. (B) Heatmap of proteins displaying significantly (ANOVA p-value ≤0.05) changed levels with respect to non-smokers based on the total precursor intensity. The protein order in the heatmap is based on descending fold change in the e-cigarette user group. (C) Interactome map showing proteins with significant increases in cigarette smokers, e-cigarette users and both with respect to non-smokers. Quantified protein hits were based on at least 2 assigned peptides per protein.

**Figure 2: Levels of proteins known to be affected by cigarette smoke exposure are also altered in e-cigarette users.** The total precursor intensity of each sample was plotted for comparison among the groups. A) Aldehyde dehydrogenase 3A1 (ALDH3A1), (B) nucleobindin-1 (NUCB1), (C) thioredoxin (TXN), (D) glutathione S-transferase (GSTP1) and (E) microseminoprotein beta (MSMB). Mean and SEM values are indicated by major and minor horizontal bars, respectively. Statistical significance was determined by one-way ANOVA, and p-values are indicated by * ≤0.05, ** ≤0.01, *** ≤0.005 and **** ≤0.001. NS: non-smokers, CS: cigarette smokers, ECU: e-cigarette users.

**Figure 3: Airway epithelial defense protein levels are significantly altered in tobacco product users.** The total precursor intensity of each sample was plotted for comparison among the groups.

(A) Deleted in malignant brain tumors 1 (DMBT1), (B) lactotransferrin (LTF), (C) trefoil factor 3 (TFF3), and (D) lysozyme C (LYSC). Mean and SEM values are indicated by major and minor horizontal bars, respectively. Statistical significance was determined by one-way ANOVA, and p-values are indicated by * ≤0.05, ** ≤0.01, *** ≤0.005. NS: non-smokers, CS: cigarette smokers, ECU: e-cigarette users.

**Figure 4: Neutrophilic granule enzyme levels are significantly increased in e-cigarette users' airways, despite no increase in neutrophil cell counts.** The total precursor intensity of each sample was plotted for comparison among the groups. (A) Neutrophil elastase, (B) myeloperoxidase, (C) proteinase 3, (D) matrix metalloproteinase 9 (MMP9) and (E) azurocidin. (F) Neutrophil cell counts in sputum samples from e-cigarette users as well as cigarette smokers and non-smokers. Mean and SEM values are indicated by major and minor horizontal bars, respectively. Statistical significance was determined by one-way ANOVA, and p-values are indicated by * ≤0.05, ** ≤0.01, *** ≤0.005. NS: non-smokers, CS: cigarette smokers, ECU: e-cigarette users.

**Figure 5: Evidence for increased NET formation in current e-cigarette users.** The levels of neutrophil extracellular trap (NET) marker proteins such as S100A8 (A) and S100A9 (B), which form a heterodimer called calprotectin; (C) coronin-1 (COR1A_HUMAN), and (D) peptidyl arginine deiminase, type IV (PADI4_HUMAN) were significantly increased. Statistical significance was determined by one-way ANOVA, and p-values are indicated by * ≤0.05, ** ≤0.01, *** ≤0.005. NS: non-smokers, CS: cigarette smokers, ECU: e-cigarette users.

**Figure 6: Isolated peripheral (blood) neutrophils from e-cigarette users are more sensitive to phorbol ester stimulation-induced NETosis.** Quantitation of PMA-induced NETs formed by neutrophils from non-smokers, cigarette smokers, and e-cigarette users. Neutrophils isolated from the venous blood of non-smokers (filled squares), cigarette smokers (filled triangles), and e-cigarette users (filled circles) were challenged with 25 nM PMA, a protein kinase C (PKC) activator and potent NET agonist, and assayed for nucleic acid release over time. Mean and SEM values are indicated by major and minor horizontal bars, respectively. Statistical significance was determined by one-way ANOVA, and the p-value is indicated by * ≤0.05. NS: non-smokers, CS: cigarette smokers, ECU: e-cigarette users.

**Figure 7: The ratio between the major airway mucins MUC5AC and MUC5B is significantly shifted toward MUC5AC in cigarette smokers and follows the same trend in e-cigarette users.** Comparison of total and individual mucin concentrations and their ratios in sputum samples from non-smokers, cigarette smokers and e-cigarette users. Individual mucin concentrations of the dominant airway mucin MUC5B (A) increased slightly but not significantly in cigarette smokers, and the concentrations of MUC5AC (B) and the MUC5AC/MUC5B ratio (D) significantly increased in both cigarette and e-cigarette users. Total mucin concentrations were increased in cigarette smokers but not in e-cigarette users (D). Mean and SEM values are indicated by major and minor horizontal bars. NS: non-smokers, CS: cigarette smokers, ECU: e-cigarette users, (*p-value <0.05).

**Figure 8: Schematic depiction of the impact of e-cigarette smoking on the airways.** Vaping uniquely alters the airway innate immune response by causing an increase in the release of neutrophil NET-associated proteins; proteins involved in maintaining the redox balance of the airways; and the ratio of the building blocks of airway mucus, namely, the mucins MUC5AC and MUC5B.

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