1. **The impact of vaping on adolescent lung function and nasal epithelium gene expression.**
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**Abstract**

**Background:** Electronic cigarettes (e-cigs) are touted as safer alternatives to traditional tobacco products; however, there are no substantive data to corroborate this claim. Vaping among adolescents is a pressing public health problem and is a risk factor for negative health effects. We aimed to investigate the impact of vape exposure on lung function and nasal epithelial gene expression in adolescents. We hypothesized that vape exposure results in abnormal lung function and differential expression of inflammatory genes in the nasal epithelium of vape users in adolescence.

**Study Design and Methods:** We performed focus groups and interviews of 50 middle and high school students at high school and community-based settings in Colorado to explore youth perceptions on vaping. Participants completed focus groups and confidential surveys on their vaping use. Airflow obstruction was investigated using impulse oscillometry (IOS) and spirometry. Nasal epithelial brushing was collected for gene expression analyses. RNA-sequencing was performed using the Illumina HiSeq platform. We compared IOS measures between vaping and non-vaping groups using Mann-Whitney U tests. Differential expression testing was performed in DESeq2 and enrichment was performed using Gene Set Enrichment Analysis (GSEA). Written informed consent was obtained from participants and Institutional Review Board approval was obtained.

**Results:** Fifty-one participants were recruited. Vaping subjects were defined as those who reported vaping within the past 6 months while non-vaping control subjects did not report any vape exposure in the past 6 months. One subject did not report vape status. The mean (standard deviation) age was 15.1 (1.5) years for vaping subjects and 14.5 (1.4) years for control subjects. We found significant differences in airway resistance by vaping status. Mean airway resistance identified by the IOS R5 measure revealed that vapers (n=13) had higher values than control subjects (n=37) [p=0.016, Figure 1], an early indication of increased airflow obstruction. Of 16,860 nasal epithelial genes tested, 7,136 were significantly differentially expressed between vaping and control subjects (false discovery rate adjusted p-value FDR < 0.05), after covariate adjustment. Enrichment analyses identified overexpression of inflammatory response genes and underexpression of genes associated with ciliogenesis in the vaping group compared to controls.

**Interpretation:** Our results suggest that vaping increases airway resistance and dysresgulation of nasal epithelial genes, including genes involved in airway inflammation and ciliary function. Furthermore, dysregulation of these gene expression pathways may be a biological mechanism for the development of increased airway resistance due to vaping even during adolescence.

**Clinical Trial Registration:** N/A

1. **Background**

The increasing use of Electronic Nicotine Delivery Systems (ENDS), such as electronic cigarettes (e-cigs), is a significant and emerging public health problem [1]. ENDS represents a diverse class of products such as electronic cigarettes, vapes, vaporizers, vape pens, hookah pens, and pods [2], and exposure to ENDS aerosols depends on the user and device characteristics [2]. They may contain nicotine and can be classified as tobacco products [3]. Herein, we focus on one type of ENDS, namely e-cigs, the use of which we term vaping. E-cigs are touted as safe alternatives to traditional tobacco products, however, there is no substantive data to corroborate this assertion [4]. The solutions in e-cigs, and their resulting aerosols, contain nicotine, carcinogens, and metal particles, to which users and nonusers in close proximity can be exposed [3]. The youth and young adults are the predominant population using e-cigarettes and are at the greatest risk to the negative effects of nicotine exposure [5]. Given the attractive flavors, marketing and design, and its appeal to youth, e-cigs have the potential to reverse decades of progress achieved in nicotine and tobacco product use [6].

While traditional cigarette smoking has declined, e-cig usage (or vape use) has increased, attracting both former, current and never tobacco smokers [1]. Vaping among youth is a pressing public health issue, with prevalence of use surpassing that of tobacco cigarettes [7]. There is a reported increase in past-30-day e-cigarette use among high school students. Particularly, the National Youth Tobacco Survey reported increased from 11.3% in 2017 to 20.8% in 2018 [8]. Monitoring the Future Study also reported increased use from 11.0% in 2017 to 20.9% in 2018 [9]. A recent study on failed nicotine quit attempts, among US adolescent e-cig and traditional cigarette users, shows that levels are back to where it was 13 years ago [10]. Certainly, the contribution of e-cigs to unsuccessful nicotine quit attempts among adolescents is substantial and warrants urgent attention.

The pathophysiologic impacts of e-cig exposure on the human pulmonary system are being elucidated.Tobacco smoke is known to be highly proinflammatory and has been shown to trigger the release of inflammatory cytokines and other biological changes, including goblet cell metaplasia and neutrophil influx [11], however the impact of e-cig aerosols in the long term is not clear. The solutions found in e-cigarettes present a novel mixture of chemicals, including flavors and sweeteners designed to mask nicotine’s bitter taste [1]. Thus, while nicotine is known to adversely alter airway physiology, the effects of these novel chemical mixtures, either by itself, and/or together with nicotine have not been studied. Short-term exposure to e-cigarettes in healthy adults increases airway resistance, with some evidence demonstrating decreased lung function after exposure [12]. However, what happens in the long term during a critical development window such as adolescence, is unknown.

Given the paucity of information on the effects of vaping on the lungs in the long-term, the high-risk youth population with access to these devices, and the current epidemic, we recruited adolescents who vape to help address these knowledge gaps. By presenting evidence on biologic determinants of reduced lung function we will fill in a critical knowledge gap on the health effects of vaping in the human population. Thus, we aimed to investigate the impact of vape exposure on measures of lung function in adolescents and to compare nasal epithelial gene expression and DNA methylation in those who vape to non-vaping controls to determine the biological impact of vape exposure. The motivating hypothesis for this work is that vape exposure is associated with abnormal lung function measures and that nasal epithelial gene expression would be modified by vape exposure. To address this hypothesis, we sought to determine the impact of vape exposure on measures of lung function and its impact on nasal epithelial gene expression in adolescents who vape.

**Methods**

**Study Participants**

Study participants were enrolled in a pilot study aimed to examine vaping initiation and its respiratory effects among youth in Colorado. Adolescent participants completed focus groups on reasons for vaping initiation and confidential surveys on their vaping use. Adolescents from high schools in Pueblo, Aurora and Denver were enrolled in this pilot study. Specifically, youth aged 12 to 17 years were eligible as participants. Written informed consent was obtained from each of the participants. We used Research Electronic Data Capture (REDCap) to securely enter and store data. The Institutional Review Board at the University of Colorado Anschutz Medical Campus approved the current study.

**Vape use (or vaping) variable definition.**

Subjects who self-reported vape use in the last six months were considered to be vapers, while those who did not report vape use in the last six months were considered non-vaping control subjects.

**Impulse Oscillometry**

Impulse oscillometry (IOS) measurements were obtained from each participant. Since IOS permits passive measurement of lung mechanics, it can identify small airway obstruction and is more sensitive than spirometry for peripheral airway disease [13, 14]. Using tremoflo, a portable IOS analyzer (Thorasys INC, Montreal, Canada), forced oscillation measurements (FOT) measurements were applied in 60 second measurements in triplicate, capturing reactance (XR), resistance (R), resonance frequency (XA). Data were captured within 5–37 Hz [15]. Additionally, we performed spirometry at each visit following the American Thoracic Society/European Respiratory Society guidelines [16, 17].

**Nasal epithelium gene expression**

RNA was isolated from nasal epithelial brush specimens obtained from our study participants.RNA sequencing (RNA-seq) was conducted using the Illumina HiSeq platform at the Genomics Core at the University of Colorado. To minimize potential batch effects, all samples were submitted together for RNA-seq. We examined the quality of the sequencing reads (FastQC[18]) and removed adaptors and low quality base calls (Cutadapt [19]). Sequences were aligned to the human genome (GENCODE GRCh38) [20] and read counts overlapping each gene were reported using STAR [21]. We performed quality control at the sample and gene-level, retaining genes with an average counts per million > 0.5 and >10 counts in at least 80% of all samples for further analyses. Finally, we applied the voom [22] transformation to the counts, yielding an approximately normally distributed measure of gene expression.

**DNA methylation**

DNA was isolated and purified from participants nasal samples using the Maxwell® 16 Integrated System (Promega Corp, Madison, WI).[23] We assessed genome-wide DNA methylation using the Illumina Infinium Human Methylation 850K beadchip profiling microarray (Illumina Inc, San Diego, CA). Samples were analyzed at the Genomics Core at the University of Colorado and were processed under standardized conditions and equally distributed across chips. Illumina results were represented as average β values (methylated probe intensity over sum of methylated and unmethylated probe intensities), which were corrected for batch effect by performing parametric empirical Bayesian adjustments through ComBat function in R [24]. M-values were calculated as the log2 ratio of the intensities of methylated probe versus unmethylated probe, and used in subsequent analysis.[27]

**Covariates**

### For adjusted analyses, we include the following the covariates into the models, as these have been identified as potential confounders in the literature: 1) Recruiting center (Pueblo or ##), which encompasses a broader geographic region where the participant lives, 2) Sex 3) Age.

**Statistical Analysis**

We examined differences in demographic measures among vaping and non-vaping study participants using Fisher’s Exacttests for categorical variables and two-sample t-tests for continuous variables. Next, we conducted a series of bioinformatic analysis to evaluate associations between vape status and lung function and gene expression among study participants. For the lung function data, IOS measures were visually inspected for normality using histograms before conducting association studies with vaping status.

normalization  
To correct for unwanted technical effects, we used the between-sample normalization of Removal of Unwanted Variance using residuals (RUVr) from a first pass generalized linear model (GLM) including vape status, sex, and age [28]. Two normalization factors were included for RUVr after visual inspection of an elbow plot, relative log expression plots, and dendrograms. This analysis used edgeR to fit the first pass GLM based on recommendations for the RUVr procedure.[29]

Gene counts were then modeled using a series of negative binomial models from the R package *DESeq2* to preform Likelihood Ratio Tests (LRT) [30]. All models adjusted for sex, age, and the normalization factors. The main test of interest for each gene evaluated whether including vape status significantly increased model fit while also adjusting for recruitment center. The other resulting LRTs investigate the presence of recruitment center bias in the data. To correct for multiple testing the Benjamini and Hochberg False Discovery Rate (FDR) adjustment was used. Significance was then set at FDR < 0.05. In addition, we focused on genes with an effect cutoff of |Log2(fold change)| > 2.

**Sensitivity analysis**

To check the robustness of our results, we conducted a sensitivity analysis of only subjects from the Pueblo recruitment center (where 12/13 vapers were recruited). An LRT was fit to determine if *vape status* contributes significantly to gene expression differences with only the subjects from the Pueblo recruitment center while adjusting for age, sex, and the 2 normalization factors. FDR was used to correct for multiple testing and significance was set at FDR ≤ 0.05 and a magnitude of effect cutoff of |Log2(Fold-Change)| > 2.

**Enrichment analysis**

To identify differentially enriched biological pathways or ontologies, Gene Set Enrichment Analysis (GSEA) was conducted after obtaining differential gene expression results using the R package *fGSEA* ver. 1.23.0.[31] To compare across the pathway or ontology data base genes were mapped to Entrez (NCBI) IDs. Gene ranks were based on the direction of the FC times -log10 of the p-value from the LRT [32].

**Databases**  
We implemented GSEA for Reactome, Gene Ontology (GO), and Koyoto Encyclopedia of Genes and Genomes (KEGG). Only pathways or ontologies with 2 or more unique Entrez genes were considered for inclusion in GSEA. After the initial analysis, we determined statistically redundant pathways using the function *collapsePathways* from fGSEA.[33] Pathways or ontologies that are statistically independent from other significant pathways or ontologies are referred to as independent. All pathways were retrieved from Ensembl (ver. 106) using biomaRt ver. 2.53.2.[34, 35]

**DNA methylation analysis**

We mapped CpG sites to their respective genes using annotation for Illumina’s 850K methylation arrays[36] to generate a list of CpGs for targeted methylation analysis. Then, a subset of CpG sites was selected based upon the 7,136 genes found to be differentially expressed when vapers are compared to controls in the RNA-seq data. Finally, we repeated the FDR adjustment for multiple testing using *bacon*[37]-corrected p-values (FDR < ##). Secondly, we conducted another targeted analysis which included only the CpG sites which were annotated to the genes found in the Gene Ontologies[38] for “inflammatory response”, “immune system process”, “immune response”, and “cilium”. This search also included CpG sites annotated to the *AHRR* gene, which has been the subject of tobacco-related research.[39] We again repeated the FDR p-value adjustment for only the CpG sites associated with these genes (FDR < ##). .

* + - 1. **Results**
      2. *Descriptive statistics*
      3. A total of 51 subjects participated in the study.  Vaping subjects were characterized as adolescents who reported vaping within the past 6 months (n=13) while controls subjects were adolescents who did not have any vape exposure in the past 6 months (n=37). One subject did not report vape status and was therefore excluded from the study. The mean (standard deviation) age was 14.8 (1.4) years for vaping subjects and 14.6 (1.4) years for control subjects. We observed some demographic differences by vaping status. Most vaping subjects were recruited in Pueblo (91%) and identified as LatinX (85%). 53% of subjects were female. Spirometry measurements were missing for all but one of the vaping subjects and IOS data was available for most subjects (Table 1).
  1. **Table 1. Demographic characteristics and lung function testing results of study participants.** SD: standard deviation

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Did Not Vape in Last 6 Months (N=37)** | **Vaped in Last 6 Months (N=13)** | **Total (N=50)** |
| **Sex** |  |  |  |
| Female | 17 (45.9%) | 8 (61.5%) | 25 (50.0%) |
| Male | 20 (54.1%) | 5 (38.5%) | 25 (50.0%) |
| **Age (years)** |  |  |  |
| Mean (SD) | 14.6 (1.4) | 14.8 (1.4) | 14.6 (1.4) |
| Range | 12.0 - 17.0 | 13.0 - 17.0 | 12.0 - 17.0 |
| **Recruitment Center** |  |  |  |
| Aurora | 15 (40.5%) | 0 (0.0%) | 15 (30.0%) |
| Commerce City/Denver | 13 (35.1%) | 1 (7.7%) | 14 (28.0%) |
| Pueblo | 9 (24.3%) | 12 (92.3%) | 21 (42.0%) |
| **Ethnicity** |  |  |  |
| LatinX | 23 (62.2%) | 11 (84.6%) | 34 (68.0%) |
| Non-LatinX | 14 (37.8%) | 2 (15.4%) | 16 (32.0%) |
| **FEV1** |  |  |  |
| N of Missing | 10 | 12 | 22 |
| Mean (SD) | 2.6 (0.7) | 3.9 (NA) | 2.6 (0.7) |
| Range | 1.2 - 3.9 | 3.9 - 3.9 | 1.2 - 3.9 |
| **FEV1/FVC (%)** |  |  |  |
| N of Missing | 10 | 12 | 22 |
| Mean (SD) | 0.8 (0.1) | 0.7 (NA) | 0.8 (0.1) |
| Range | 0.5 - 1.0 | 0.7 - 0.7 | 0.5 - 1.0 |
| **R5** |  |  |  |
| N of Missing | 1 | 0 | 1 |
| Mean (SD) | 4.0 (0.9) | 5.0 (1.3) | 4.3 (1.1) |
| Range | 2.0 - 6.1 | 3.7 - 7.6 | 2.0 - 7.6 |
| **X20** |  |  |  |
| N of Missing | 4 | 2 | 6 |
| Mean (SD) | 0.1 (0.6) | 0.7 (0.9) | 0.2 (0.7) |
| Range | -1.1 - 2.4 | -1.0 - 2.3 | -1.1 - 2.4 |

***Lung function measures***

To assess the impact of vape exposure on measures of lung function, we tested the association of vape exposure with IOS measurements using linear models adjusted for age and sex. We observed significant differences in airway resistance by vaping status. Mean airway resistance (R) values calculated over a measurement period of 60 seconds at a frequency of 5 Hz (R5) revealed increased airway resistance in vaping (n=13) subjects compared to non-vaping control (n=37) subjects (p=0.026) [Fig 1], an early indication of increased airflow obstruction. Additionally, our results showed higher X20 (reactance) values in vapers compared to non-vaping controls (p=0.016), which suggests adverse effects of vaping on lung parenchyma.

1. Chart, box and whisker chart

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2. **Fig 1. Differences in impulse oscillometry (IOS)** **measures between vaping and control subjects.**

***Gene expression***

Nasal epithelial gene expression was utilized to assess the biological impact of vape exposure on the airway epithelium of the study subjects. Three participants were missing gene expression data and are excluded from subsequent analysis (N=X).

16,860 genes of the 60,651 genes were retained after removing low expressed genes. 7,136 were significantly differentially expressed between vaping subjects and control subjects after adjusting for age, sex, recruitment center, and two normalization factors (FDR<0.05) [40]. A total of 4,193 genes were underexpressed (e.g., *WNT5B*, *WNT3A, ACE2, IL7*) while 2,943 were overexpressed (e.g. *TNF*, *MUC5A, IL10, IL17C)* when vapers are compared to controls. Using a fold change cut of |log2(FC)| ≥ 2, there were 135 genes that were upregulated and 370 genes that were downregulated when comparing vapers with control subjects (Supplementary Table 1).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Table 2. The top twenty genes with the greatest fold-change (log2(FC)) when adolescents who vaped in the last 6 months are compared to adolescents who did not vape.** The fold-change is the expression in the vaping group relative to the control group (i.e., FC > 1 represents increased expression). | | | | | |
| **Ensembl ID** | **Gene Symbol** | **Log2(FC)** | **p-value** | **FDR** | **Associated function** |
| ***Negative log2 fold change*** | | | | | |
| ENSG00000147647 | *DPYS* | -3.9 | 5.8E-06 | 4.2E-05 | AGE-RAGE signaling pathway, oxidative stress |
| ENSG00000198838 | *RYR3* | -3.5 | 9.2E-04 | 3.5E-03 | 9+2 motile cilium |
| ENSG00000162782 | *TDRD5* | -3.4 | 1.1E-03 | 4.2E-03 | 9+2 motile cilium |
| ENSG00000152779 | *SLC16A12* | -3.4 | 8.7E-09 | 1.2E-07 | 9+0 motile cilium |
| ENSG00000039537 | *C6* | -3.4 | 4.6E-03 | 1.4E-02 | ADORA2B mediated anti-inflammatory cytokines production |
| ENSG00000280780 | *JAKMIP2-AS1* | -3.3 | 8.1E-12 | 2.6E-10 | Novel transcript |
| ENSG00000244067 | *GSTA2* | -3.3 | 1.7E-13 | 8.3E-12 | 9+0 motile cilium |
| ENSG00000260951 | *AC005100.1* | -3.2 | 1.5E-05 | 9.4E-05 | Novel transcript |
| ENSG00000277893 | *SRD5A2* | -3.1 | 1.4E-08 | 1.9E-07 | 9+0 motile cilium |
| ENSG00000268566 | *AC100781.1* | -3.1 | 6.5E-04 | 2.6E-03 | Novel transcript |
| ***Positive log2 fold change*** | | | | | |
| ENSG00000177257 | *DEFB4B* | 7.2 | 2.1E-03 | 7.3E-03 | ADORA2B mediated anti-inflammatory cytokines production |
| ENSG00000198692 | *EIF1AY* | 6.4 | 4.9E-03 | 1.5E-02 | 9+0 motile cilium |
| ENSG00000129824 | *RPS4Y1* | 6.4 | 4.6E-03 | 1.4E-02 | ADORA2B mediated anti-inflammatory cytokines production |
| ENSG00000012817 | *KDM5D* | 5.9 | 2.1E-03 | 7.1E-03 | 9+2 motile cilium |
| ENSG00000067048 | *DDX3Y* | 5.7 | 2.1E-04 | 9.6E-04 | 9+2 motile cilium |
| ENSG00000114374 | *USP9Y* | 4.8 | 5.8E-03 | 1.7E-02 | 9+0 motile cilium |
| ENSG00000232177 | *MTND4P24* | 4.7 | 4.2E-04 | 1.8E-03 | Novel transcript |
| ENSG00000225972 | *MTND1P23* | 4.2 | 9.3E-04 | 3.6E-03 | Novel transcript |
| ENSG00000201321 | *RNA5S9* | 3.7 | 3.8E-03 | 1.2E-02 | Novel transcript |
| ENSG00000183878 | *UTY* | 3.5 | 1.5E-04 | 7.1E-04 | 9+0 motile cilium |

***Enrichment analysis***

Pathways or ontologies were sourced from KEGG, Reactome and GO databases. Overall, enrichment analysis of the top twenty differentially expressed genes, vaping subjects tended to have dysregulated expression of pathways associated with ciliogenesis and inflammation compared to the control group (Table 2). We also observed the inflammatory response, immune system process, immune response, and cilium pathways and ontologies to be enriched (Table 4-6).

Chart

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**Figure 2 Differntial Gene Expression Results.** A volcano plot of differentially expressed genes between vaping subjects and control subjects (false discovery rate [FDR]<0.05), after adjusting for age, sex, recruitment center, and two normalization factors.

***Sensitivity analysis***

With 92% (12/13) vapers and 42% 21/50 participants recruited at the Pueblo center, we sought to understand how this demographic imbalance acts as a potential confounder or source of multicollinearity. As such we restricted analysis to participants recruited from the Pueblo center and compared it to the overall model containing all study participants to assess differences in estimates and significant gene composition between the models. The top 2,000 significant genes for the restricted and full models are identical both before and after the selected cutoff value (|Log2(Fold Change) | > 2). The variation of Log2(Fold Change) from the top 2,000 genes in the model with only subjects and the model with only Pueblo subjects were minimal (Supplementary Figure 2).

***Targeted DNA Methylation Analysis***

One CpG site, cg02123174, was significantly differentially methylated at a type-I error rate of 0.05. The site maps to *REXO1* and was less methylated in vapers compared to non-vapers. This result is consistent with RNA-Seq results where the gene was up-regulated (0.396, p = 0.02). One additional site, cg11903190 was marginally significant at an FDR-adjusted p-value of 0.06 and maps to *CERK*, which was also up-regulated in the RNA-Seq results (0.395, p = 0.00). At a type-I error rate of 0.2, there were 36 differentially methylated sites. Supplementary Table 3 gives the top 10 sorted by adjusted p-value and estimate size while Supplementary Table 3 shows boxplots of five of such CpG sites (and their associated genes).

**Discussion**

Our results suggest that adolescent vape exposure is associated with increased airflow obstruction and increased expression of inflammatory genes in the nasal epithelium of subjects who vape. Our work provides critical evidence of the negative impact of vape exposure on respiratory outcomes even in early adolescence. To the best of our knowledge, these data are the first to demonstrate that vape exposure is associated with abnormal lung function in early adolescence and shows the detrimental impact of vape exposure during a critical window of lung function development.

Our findings are consistent with existing literature in adults demonstrating that short-term exposure to e-cigs increases airway resistance and is associated with decreased measures of lung function after exposure [12]. We identified significant differences in IOS measures between vapers and non-vaping control subjects. To date, there have been few studies in humans looking at e-cig use and lung function measures and none have looked at long term exposures and their effects on lung function. A previous study on the acute effects of vape exposure on airway physiology and respiratory symptoms in COPD smokers, asthmatic smokers, "healthy" smokers and healthy never smokers after e-cig use identified an increase in airways resistance (ΔRaw) in asthmatic (p=0.034) and healthy smokers (p=0.004). Additionally, an increase in airway resistance was noted in never smokers after using e-cigs with (p<0.005) or without (p<0.001) nicotine [41]. Another study on documented e-cig exposures revealed adverse changes to respiratory metrics and markers of inflammation after a 5 minute e-cig use session. The authors observed that R5, a marker of total airway resistance, had increased post e-cig use in both healthy controls and mild asthmatics. Notably, the increase in airway resistance was significantly higher among the mild asthmatics [42].

A 2022 systematic analysis of the effects of e-cigs on lung function when compared to traditional cigarettes found that there were statistically significant increases in airflow resistance (Z5, R5 and R10), after e-cig inhalation among asthmatic smokers using data from Europe between 2018–2020 [43]. This systematic analysis used data from n= 10 to 408 participants (mean age ranging from 22.6 – 58 years) with most of the studies (16/18) assessing lung function after about 5 min to 1 month of e-cig use. The impacts of e-cig use on airway physiology have also been documented during passive vape exposures. In a study with 15 subjects with repeated measures of lung function during passive vape exposure, there were no significant changes in FEV1/FVC when exposed to one hour of passive e-cigarette smoking (indicative: 2.3% reduction in FEV1/FVC) [44]. A cross-over study also looked at 40 healthy nonsmokers (18–35 years old) exposed to e-cig emissions produced at two resistance settings, 0.5 ohm and 1.5 ohm. At the 1.5 ohm session, R5 showed a post exposure decrease trend that was not significant (0.39 pre to 0.38 kPa/L/s post exposure) [45].

While these studies are short term in nature, we believe our data which assessed lung function at about 6 months of e-cig use adds to the current state of the science on the adverse impacts on the small airways after e-cig exposure. Further studies are needed to understand the respiratory health impacts of long-term e-cig use. Additionally, these previous studies were in adult populations, some of whom had comorbidities such as asthma or chronic obstructive pulmonary disease. Our study population was in relatively healthy adolescents without previously reported respiratory disease and is the first study to our knowledge that documents the adverse impact of e-cig use on lung function.

Furthermore, we demonstrate differences in nasal epithelial gene expression in vapers compared ot non-vaping control subjects. Importantly, the top four genes (*CEACAM4, MMP25, NCF1* and *NFAM1)* that were overexpressed in vapers compared to control participants were associated with inflammatory processes or in immune function (Fig 3). For instance, *CEACAM4* is an orphan receptor of the *CEACAM* family that is associated with phagocytic function [46]. *MMP25* is a metalloprotease that regulates innate immune response through NF-κB signaling [47]. Mutations in *NCF1* is associated with chronic granulomatous disease [48] and variants of this gene are associated with an increased risk of developing autoimmune diseases [49-51] and recurrent spontaneous abortion [52]. *NFAM1* promotes pro-inflammatory cytokine production in both mouse and human monocytes and has been identified as a potential therapeutic target for treatment of autoimmune disease [53]which are characterized by abnormal inflammatory response [54].

Three of the top genes (*CAPS, DNAH7* and  *NWD1)* that were downregulated among vapers, compared to non-vaping control subjects have significant implications for respiratory health based on reports in the literature. *CAPS* encodes the calcium binding protein calcyphosine which is associated with cellular proliferation and differentiation [55]. *DNAH7* is associated with ciliary dysfunction and may be important in understanding the molecular pathogenesis of Middle East respiratory syndrome coronavirus (MERS-CoV) [56] and COVID-19 infections [57]. *NWD1* has been demonstrated to modulate androgen receptor protein levels [58] and is involved in axonal growth, with high protein levels detected in the brain of a mouse model for seizure and temporal lobe epilepsy [59]. A recent study demonstrated that the knockdown of *NWD1* inhibited dendritic growth and synaptogenesis [60]. Taken together, vaping may be associated with increased risk of inflammation and decreased ciliogenesis thereby impacting airflow obstruction.

Our results are supported by previously published investigations on the impact of e-cig use. In a cohort of ten healthy never smokers, short term e-cig use was associated with altered transcriptomes of small airway epithelial cells and alveolar macrophages among all subjects [61]. Interestingly, two genes identified in this short-term study were dysregulated in the opposite direction in our study. *NDC80* (FC: 0.56) and *PTGER3* (FC: -1.27) had differential expression in directions opposite to our current study. However, differences in e-cig products, demographics of the study population, duration of exposure as well as the location of the biological sample (brushing nasal epithelial vs brushing the 10th–12th order bronchi) limits direct comparisons with our work. Further studies are needed to understand the effects of the increasing prevalence of e-cig use. Indeed, Sayed et al compared sputum and salivafrom e-cig users and nonusers and observed reductions in markers of airway inflammation among e-cig users compared to nonusers [62]. However, plasma concentrations of certain inflammatorycytokines, chemokines, and growth factors were higher among e-cig users. Hence it is possible that changes in airway inflammatory markers may be a counter-response to general inflammation caused by chronic e-cig use [62] and additional studies are warranted.

Several members of the mucin family (eg *MUC5AC, MUC12*) were differentially expressed among vapers and nonvapers. Mucins are O-glycosylated proteins that play an essential role in forming protective mucous barriers on epithelial surfaces and have been implicated in epithelial renewal and differentiation. These glycoproteins also play a role in intracellular signaling. In our study, several signaling pathways such as ‘cytokine-mediated’, ‘cell surface receptor’, ‘signal transduction’, ‘cytokine signaling in immune system’, ‘G alpha signaling events’, ‘chemokine signaling, ‘NOD-like receptor’ and ‘B cell receptor’ signaling pathways were enriched. Another noteworthy pathway recently implicated in developmental biology is the *wnt* signaling pathway. In our study, *WNT5B, WNT3A* and *WNT4* were underexpressed in vapers compared to nonvapers. W*nt* signaling has been recently highlighted as a pathway whose dysregulation can affect lung disease development later in life [63, 64].

Pathways enriched for ciliary function involved genes such as the DNAH family. Of note, of the 12 members of the DNAH family of genes that were differentially expressed in our data set, 11 were underexpressed in vapers. *DNAH17 was the only gene that was overexpressed.* Most of the 11 DNAH genes have been detected in the lung and have been implicated in ciliary dyskinesia in airway epithelial cells [65] and DNAH17 is mostly expressed in the testis and are less abundant in the lung [66]. Since e-cig use is associated with significant inflammation in the lungs, albeit the dysregulation in the immune landscape differs from that of tobacco use, it is important for future studies to focus on e-cig use and its impact on systemic function as well [67].

An interesting pathway in our enrichment analysis was the inflammasome pathway. *IL1B* was significantly? upregulated in vapers (FC: 2.1) and this has been suggested that inflammasomes are activated, and like Lee et al, *CXCL1, CXCL2* and *NOD2* were upregulated in vapers [67]. Inflammasomes (such as nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing (NLRP)) are large protein structures primarily located in macrophages that respond to inflammatory signals [67]. Upon activation, inflammasomes cleave pro-IL1B and pro-*IL18* into *IL1B* and *IL18*, to signal the presence of xenobiotics and initiate the inflammatory response [68]. Several members of the NLRP family were differentially expressed when vapers were compared to controls (*NLRP1, NLRP3, NLRP6, NLRP12,* and *NLRP14*). All except *NLRP14* were upregulated. The dysregulation of inflammasome pathway may be an important step in immune dysregulation during chronic e-cig use. For instance, an inflammatory cytokine such as *IL1B* (upregulated in vapers) can stimulate the production of other inflammatory cytokines (eg *IL6* and *TNF*) which were also upregulated in vapers vs nonvapers in our study. Future studies may need to assess the types of e-cig devices that may stimulate different pathways due to chronic e-cig use. The study by Hickman et al highlights the importance of assessing device type. Data from third- and fourth-generation e-cig users and found an overall suppression of host defense associated with fourth-generation e-cig use [69]. This may be due to new/emerging formulations in the e-cigs such as nicotine salts and future studies need to be designed to understand the health consequences of emerging e-cigs [70].

Finally, our results suggest that DNA methylation may be one of the mechanisms by which vaping exerts its effects on the lungs. *REXO1* and *CERK* were differentially methylated when vapers are compared to controls. While not much is known about REXO1, its over expression has been associated with cell proliferation, migration, and invasion in cervical cancer.[71] However, a recent epigenome wide association study in Puerto Rican and Mexican American children and young adults (average age 12-14 years) with asthma identified REXO1 in one of two differentially methylated regions as associated with pre-FEV1/FVC.[72] Interestingly, the association signals for that study were enriched for inflammatory processes and the authors concluded that REXO1 is associated with airflow limitation in children.

*CERK* can be activated by *IL1B*, and its activity has been observed in neutrophils and the lung epithelium. [73, 74]. The gene encodes ceramide kinase and has been implicated as an important regulatory component of inflammatory response. [75]. Notably, ceramide kinase converts ceramide to cermide-1-phosphate [76], which plays a role in linking Prostaglandin E2, neurotransmitters, and airway epithelial inflammation.[75]

Together, our data provide further insights into the mechanisms involved in adverse impacts on lung function.

A pediatric pulmonary review concluded that vaping may increase risk of developing chronic lung disease [77]. The authors indicated that vaping is associated with both in vitro and in vivo airway mucociliary dysfunction, increased epithelial cell, macrophage death, as well as dysregulation of airway epithelium. Indeed, data from our current study point to these adverse respiratory impacts among adolescents who use e-cigs. Our findings demonstrate that significant changes in airway resistance that result from vape exposure may precede the development of adverse respiratory symptoms and that vape exposure negatively impacts adolescent lung function during a critical stage of their lung development. We also establish that vape exposure results in changes in gene expression in inflammatory pathways and genes involved in ciliary function in the nasal epithelium and that this may be biological mechanism that underlies the development of airflow obstruction in this at-risk population.

Limitations of our study include the small sample size, the cross-sectional nature of the study, and the lack of exposure assessment specific to e-cigarettes in this cohort. Future investigations in this at-risk adolescent population will be needed to assess the longitudinal impact of habitual vape exposure on lung function outcomes and should include a more comprehensive exposure assessment of the contents of vape smoke. Our data indicates that vaping is associated with impaired lung function in adolescents and extensive changes in nasal epithelial gene expression. While limited in sample size, our results add to the currently limited knowledge on the chronic effects of vape exposures. Given the paucity of information on the effects of vaping on the airway epithelium and the high-risk youth population with access to these devices, our work suggests that further research is needed to help characterize vaping exposure and its impact on lung function, nasal epithelial gene expression, and how vaping cessation may reverse these changes.

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**Table 4. Top 15 Gene Ontologies for differentially regulated genes when adolescents who vaped in the last 6 months are compared to adolescents who did not vape.** Pathways with low false discovery rate (FDR) values and negative Normalized Enrichment Scores (NES) represent downregulated biological processes while those with low FDR and positive NES represent upregulated biological processes.



**Table 5. Top 15 Reactome pathways for differentially regulated genes when adolescents who vaped in the last 6 months are compared to adolescents who did not vape.** Pathways with low false discovery rate (FDR) values and negative Normalized Enrichment Scores (NES) represent downregulated biological processes while those with low FDR and positive NES represent upregulated biological processes. ****

**Table 6. Top 15 KEGG pathways for differentially regulated genes when adolescents who vaped in the last 6 months are compared to adolescents who did not vape.** Pathways with low false discovery rate (FDR) values and negative Normalized Enrichment Scores (NES) represent downregulated biological processes while those with low FDR and positive NES represent upregulated biological processes.

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