Lung mitochondrial DNA copy number, inflammatory biomarkers, gene transcription and gene methylation in vapers and smokers

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Summary

Background Mitochondrial DNA copy number (mtCN) maintains cellular function and homeostasis, and is linked to nuclear DNA methylation and gene expression. Increased mtCN in the blood is associated with smoking and respiratory disease, but has received little attention for target organ effects for smoking or electronic cigarette (EC) use.

Methods Bronchoscopy biospecimens from healthy EC users, smokers (SM), and never-smokers (NS) were assessed for associations of mtCN with mtDNA point mutations, immune responses, nuclear DNA methylation and gene expression using linear regression. Ingenuity pathway analysis was used for enriched pathways. GEO and TCGA respiratory disease datasets were used to explore the involvement of mtCN-associated signatures.

Findings mtCN was higher in SM than NS, but EC was not statistically different from either. Overall there was a negative association of mtCN with a point mutation in the D-loop but no difference within groups. Positive associations of mtCN with IL-2 and IL-4 were found in EC only. mtCN was significantly associated with 71,487 CpGs and 321 transcripts. 263 CpGs were correlated with nearby transcripts for genes enriched in the immune system. EC-specific mtCN-associated-CpGs and genes were differentially expressed in respiratory diseases compared to controls, including genes involved in cellular movement, inflammation, metabolism, and airway hyperresponsiveness.

Interpretation Smoking may elicit a lung toxic effect through mtCN. While the impact of EC is less clear, EC-specific associations of mtCN with nuclear biomarkers suggest exposure may not be harmless. Further research is needed to understand the role of smoking and EC-related mtCN on lung disease risks.

Funding The National Cancer Institute, the National Heart, Lung, and Blood Institute, the Food and Drug Administration Center for Tobacco Products, the National Center For Advancing Translational Sciences, and Pelotonia Intramural Research Funds.

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Keywords: Mitochondria copy numbers; Smokers; Vaping; DNA methylation; Gene expression; Inflammation
Introduction

Mitochondria play a central role in cellular energy production, control of metabolic stress, and maintenance of cellular homeostasis, while also inducing reactive oxygen species (ROS) involved in multiple diseases.1,4–10 The number of mitochondria and mitochondrial DNA (mtDNA) in each cell varies depending on the function of the cells.5 Alteration of mtDNA is thought to be due to the generation of ROS, oxidative damage, and inflammation.5,11 Unlike the fixed copy number of the nuclear genome, mtDNA copy number (mtCN) can fluctuate. While mitochondria carry their own small genome (16,569 bp) containing 37 genes, they require nuclear genes for their function.5 Several in vitro studies show the bi-directional communication between the two genomes to maintain cellular function and homeostasis.6–9 In humans, increased or decreased mtCN have been implicated in mtDNA damage and dysfunction in a tissue- or disease-specific manner.10–12 Clinically, altered mtCN is an emerging biomarker of respiratory diseases and is found in lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and lung cancer.1,4,6–13

Mitochondria are frequently a target of toxicants and may represent the biological burden of exposure.14 Cigarette smoke and electronic cigarette (EC) use are both positively associated with mtCN and mitochondrial gene expression,15–19 which is thought in part due to toxicant-induced oxidative stress.20–22

Studying mtCN in a target organ is particularly critical for assessing potential disease risk. Recent studies, including ours, indicate that EC use has toxic effects in human lungs, notably in different inflammatory responses from never-smokers, but are more similar to never-smokers than smokers.23–25 We examined levels of mtCN in the lung epithelium of EC users, smokers, and never-smokers and extended its association with lung inflammation, nuclear DNA methylation, and gene expression. Further, we explored the potential involvement of mtCN-associated nuclear genes identified from this study in lung diseases.

Methods

Study participants

For between smoking group comparisons, we had 26 SM, 15 EC, and 43 NS. The initial power was computed for the primary aim of identifying differences in mtCN between the smoking groups. Because there are three pairwise comparisons, for power we considered \( p = 0.05/3 = 0.017 \). Powering on the two smallest groups, with 15 EC and 26 SM, a two-sided, two-sample t-test achieves 96% power to detect a 1.15-fold difference between the two groups, considering \( p = 0.017 \) and a coefficient of variation = 0.1 (estimated from preliminary data for the current study); therefore, we had adequate power to test our primary hypothesis that mtCN differs by smoking group. Recruitment and study procedures were reported previously.26–28 Briefly, 84 participants, aged 21–30 years, who completed the informed consent form and were willing to undergo bronchoscopy were recruited from Columbus, OH between 2015 and 2019. All participants were considered healthy. Exclusion criteria included: significant medical illness such as cancer and COPD, autoimmune disorders, BMI > 40 and other illnesses or procedures that could affect lung inflammation. Smokers (SM; \( n = 26 \)) were defined as daily smokers of \( >10 \) cigarettes per day for \( >6 \) months and additionally had not used an electronic
cigarette for at least 1 year. Electronic cigarette users (EC; n = 15) used nicotine-containing EC daily for at least 1 year and had not smoked a cigarette for >6 months, a majority of EC were former smokers (n = 11). Never-smokers (NS; n = 43) had smoked <100 cigarettes in their lifetime, as defined by the Centers for Disease Control and also had not smoked a cigarette or used an EC for at least one year prior to enrolment. Table 1 shows the characteristics of participants in this study, including results for biomarkers of nicotine and EC exposure. At bronchoscopy, both bronchoalveolar lavage (BAL) and bronchial brushing samples were collected.

Immune cells and cytokines

The methods and results were previously reported. BAL fluid samples underwent differential cell counting using Diff-Quik stained cytopsins and light microscopy. For the immune cytokine analyses, cell-free BAL samples were assayed with a Meso Scale Discovery Sector Imager 2400A (Meso Scale Discovery, Rockville, MD) alongside a V-PLEX Plus Proinflam Combo 10 panel. This includes pro-and anti-immune cytokines such as IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. Mitochondrial DNA copy numbers in lung epithelium

Genomic DNA was extracted using AllPrep DNA/RNA mini Kit (Qiagen) and was quantified with NanoDrop and Qubit. Relative mtCN (mtDNA to nuclear DNA [nDNA]) was measured using a fluorescence-based quantitative real-time polymerase chain reaction (qPCR). The assays (ThermoFisher, Waltham, MA) included four mitochondrial genes, mtND1 (mitochondrially encoded NADH dehydrogenase 1, Hs02596873_s1-FAM), mtND2 (Hs02596874_g1-FAM), mtCO1 (mitochondrially encoded cytochrome c oxidase 1, Hs02596864_g1-FAM), and mtCO2 (Hs02596865_g1-FAM), and nuclear PKM (pyruvate kinase, muscle, Hs00761782_s1-VIC). 5 ng of gDNA was used in the singleplex reaction mix. The TaqMan Fast Advanced Master Mix was used to perform the qPCR experiment and each TaqMan assay was ran at 1X final concentration. The Fast protocol consist in 20 s 95 °C incubation, to activate the AmpliTaqTM Fast DNA Polymerase, and 40 Cycles amplification, 1 second at 95 °C (denaturation) followed by 20 s at 60 °C (annealing/extension). All samples were randomized for groups into a total of 7 plates, and multiplex reactions were performed in duplicates for each sample.

### Table 1: Characteristics of study participants.

|                     | NS (n=43) | EC (n=15) | SM (n=26) | P          |
|---------------------|-----------|-----------|-----------|------------|
| **P**               | SM v NS   | NS v EC   | EC v SM   |            |
| **Age, years, median (IQR)** | 25 (23–27) | 27 (25–29) | 26 (25–27) | 0.22       |
| **Gender**          |           |           |           |            |
| Females, N (%)      | 25 (58%)  | 5 (33%)   | 8 (31%)   | 0.05       |
| **Race**            |           |           |           |            |
| White, N (%)        | 31 (72%)  | 12 (80%)  | 22 (85%)  | 0.38       |
| Non-White, N (%)    | 12 (28%)  | 3 (20%)   | 4 (15%)   | 0.74       |
| **Smoking**         |           |           |           |            |
| Former, N (%)       | -         | 11 (73%)  | -         | 0.068*     |
| Current, N (%)      | -         | -         | 26 (100%) |            |
| Never, N (%)        | 43 (100%) | 4 (27%)   | -         | 0.064*     |
| Years of smoking, median (IQR) | - | 6.5 (4.3–9.3)* | 9.5 (6–10.5) | - | - | 0.068* |
| Pack Years, median (IQR) | - | 3.6 (0.8–5.8)* | 8.1 (3.9–10)    | - | - | 0.017*     |
| Cigarettes per day, median (IQR) | - | 13.5 (2.8–20)* | 20 (11.5–20) | - | - | 0.064*     |
| Days since last cigarettes, median (IQR) | - | 660(278.3–1147)* | - | - | - |
| **Electronic cigarette (e-cig) use** |           |           |           |            |
| Years of e-cig use, median (IQR) | - | 3 (2–3) | - | - |
| Puffs per day, median (IQR) | - | 100 (30–200) | - | - |
| E-liquid (ml) per day, median (IQR) | - | 8.3 (5–10) | - | - |
| Nicotine (mg/ml), median (IQR) | - | 6 (3–12) | - | - |
| **Urinary Biomarkers** |           |           |           |            |
| Cotinine+3-hydroxycotinine (nmol/mg creatinine), median (IQR) | 0.003 (0.001–0.006) | 152 (2.7–39.6) | 19.5 (6.6–43.5) | 1.78E–07 | 4.08E–06 | 0.64 |
| Propylene glycol (mg/mL), median (IQR) | 1.9 (0.8–5.0) | 25.9 (5.0–52.6) | 6.6 (2.7–20.8) | 0.005 | 0.0003 | 0.08 |

Table 1: Characteristics of study participants.

EC Electronic cigarette users, SM Smokers, NS Never-Smokers.
* former smoking EC.
Fisher’s exact test and Mann–Whitney for categorical variables and continuous variables, respectively.
To minimize the batch effect, mtDNA and nDNA assays were analysed on the same plate. mtCN was quantified using the delta CT (ΔCT) of mtDNA and nuclear DNA (ΔCT = CT [mtDNA] − CT [PKM]) in the same well and an exponent of 2 (2−ΔCT). The no-template control was included to monitor contamination. The correlation for mtCN from 5% blinded repeat samples confirmed the high quality of data (Spearman correlation $P < 0.001, r = 1$). No batch effects were found.

**Genome-wide DNA methylation and gene expression in lung epithelium**

Previously reported genome-wide DNA methylation and gene expression data were utilized for 67 and 70 participants of the 84 in this study. DNA methylation was determined using the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA), and raw data was imported into the Partek Genomics Suite™ 6.6 (St. Louis, MO) for Subset-quantile Within Array Normalization and logit-transformation of β-values to convert to M-values for normality. Any probes with detection $P > 0.05$ were filtered out before further analysis. As a reference genome, we used GRCh37/hg19 (Human Genome version 19). We excluded probes in the X and Y chromosomes to avoid gender bias, SNP-associated to avoid any potential effect of SNPs, and probes with off-target sequences.

For transcriptome analysis, we used the GeneChip Human Transcriptome Array 2.0 (Affymetrix Inc, Santa Clara, CA). The CEL files were imported into Partek for log$_2$ transformation and quantile normalization.

**Urinary biomarkers of exposure**

Trans-3-hydroxycotinine (3HC) and cotinine (COT) were analysed as previously reported by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Urine creatinine was used to normalize 3HC and COT levels, and then molar sums were calculated for the nicotine equivalent combination using 3HC + COT as 2 nicotine metabolites (2NE). Propylene Glycol (PG) was measured with deuterated 6(±)-1,2-propanediol-d8 [CDN, D-1656] as an internal standard and then analysed by LC-MS/MS (Agilent 1290 Infinity II UPLC system). The coupled Agilent 6495 Triple Quadrupole in addition to an electrospray ion (ESI) source was used to separate and quantify the eluted compound.

**mtDNA mutations**

In 68 out of the 84 available samples, mtDNA somatic mutation analysis was performed by LC Sciences (Hangzhou, China) using VariantPro Mitochondrial Panel. Briefly, mtDNA-targeted libraries were prepared using the VariantPro™ Mitochondrion Panel Library Preparation Kit (LC Sciences). The PCR product was purified by Agen-court AMPure XP beads (Beckman Coulter Genomics, UK), and diluted to 20 pmol/L, then directly sequenced on the Illumina Hiseq X Ten platform based on the 150-bp paired-end reads. Raw reads were processed following the Genome Analysis Toolkit best practices guideline. Reads were trimmed using Trim Galore and cutadapt to remove adapters and bases. The trimmed reads were then aligned to the human mtDNA relative to the revised Cambridge Reference Sequence (rCRS) GeneBank accession NG012920.1) using Burrows-Wheeler Alignment.

**Statistics**

For between smoking group comparisons, we had 26 SM, 15 EC, and 43 NS. The initial power for this study was computed for the primary aim of identifying differences in mtCN between the smoking groups. Participant characteristics (age, gender, race), smoking history indicators (years of smoking, pack-years and cigarettes per day), and urinary biomarkers of exposure (2NE, PG) were tested for associations with smoking status using Wilcoxon tests for continuous variables and Fisher’s exact tests for categorical variables. Smoking status (independent variable) was tested for associations with participant characteristics, smoking history indicators, and urinary biomarkers of exposure (dependent variables). In separate linear regression models, mtCN (dependent variable) was tested for associations with age, urinary biomarkers, and smoking history indicators. Associations of mtCN with race and gender were performed using t-tests. T-tests were also used to determine associations of mtCN with smoking status for normally distributed mtCN measures (i.e., ND1, ND2, and CO2). Wilcoxon rank sum tests were used to determine significant differences in mtCN by smoking status for the mtCN measure non-normally distributed (i.e. COI). Normality was determined using the Shapiro-Wilk W test.

Four mitochondrial DNA copy number measures were recorded per participant. The average of these measures was calculated per participant and used as the main indicator of mtCN content since all four measurements were highly correlated with each other (Pearson FDR < 0.0001, $r = 0.82–0.99$). Average mtCN was log$_{10}$ transformed to achieve a normal distribution before statistical analyses. mtCN associations with nuclear biomarkers, including immune cells, immune cytokines, gene methylation, and gene expression, were assessed through linear regression. The model for the associations was mtCN = nuclear biomarkers + smoking status + (biomarkers x smoking status). In order to further investigate any group-specific associations, significant biomarkers or biomarkers x smoking status were followed up by within-group tests. A False Discovery Rate (FDR) of 0.1 was considered significant.

Spearman correlations were used for pairs of mtCN-associated DNA methylation (mtCN-CpGs) and matched mtCN-associated gene expression (mtCN-transcripts, within 1500 bps upstream or downstream of the corresponding CpG) in 67 samples with both assays. Spearman correlation FDR < 0.1 was considered statistically significant.
For mtDNA mutations, Fisher’s exact test, Kruskal-Wallis test, and Wilcoxon rank test were used to analyse the association between numbers of mtDNA mutations and smoking status. T-tests and ANOVA were used to determine associations of mtCN with mtDNA mutations at each position (reference vs. mutation). Spearman correlations were used to investigate a relationship between the number of mutations and mtCN, for all participants and within smoking group. Fisher’s exact test was used to determine associations of smoking status with each position (reference vs. mutation) across all groups and pairwise. Only positions with mutations occurring with >10% of participants were considered in the analysis. Raw \( P < 0.05 \) was considered significant.

**Ingenuity pathway analysis (IPA) analysis**

IPA was performed for the correlated genes between mtCN-CpGs and mtCN-transcripts. The probe IDs (Affymetrix) were identified to corresponding genes in the IPA and were sorted in the IPA Ingenuity Knowledge Base.

**Publicly available datasets**

To explore the potential involvement of EC-specific mtCN-associated methylation and gene expression (significant associations among only EC) in respiratory disease, we used the GEO2R web tool from GEO (Gene Expression Omnibus). Using this resource, we obtained differential expression between cases and controls, an FDR < 0.1 was considered significant. We utilized GEO: GSE43024 (42 cases and 28 controls)\(^{63}\) and GSE6142 (128 cases and 27 controls, as available)\(^{64}\) for asthmatics, with asthmatic participants classified as “cases” and healthy non-asthmatic participants as “controls”. GEO: GSE11906 (20 cases and 44 controls)\(^{65}\) and GSE11784 (7 cases and 34 controls, as available)\(^{66}\) were utilized for COPD, using COPD participants as “cases” and healthy smokers as “controls”. It should be noted that in the datasets of COPD, 15 cases and 38 controls were found to be overlapping between the two sets. Thus, overlapping cases and controls were removed from GSE11784 before statistical analyses. For lung cancer, we utilized The Cancer Genome Atlas (TCGA) lung adenocarcinoma (PanCancer Atlas, \( n = 566 \) tumour and paired normal tissue) and lung squamous cell carcinoma (PanCancer Atlas, \( n = 487 \) tumour and paired normal tissue) using cBioPortal Cancer Genomics.\(^{67,68}\) A list of EC-specific mtCN-associated methylation and gene expression was inquired for gene expression as z-scores calculated relative to matched adjacent normal tissues in adenocarcinoma and lung squamous cell carcinoma datasets. The z-score \( \geq 2 \) or \( \leq -2 \) in any queried genes was considered altered expression.\(^{49}\) The percent altered samples threshold was set to greater than or equal to 90%.

**Figure 1. Correlation plot between mtCN measures and associations of mtCN with IL-2, IL-4, and the percentage of neutrophils.** (a) Pearson correlations visualizing the correlation coefficient (\( r \)) between mitochondrial DNA copy number (mtCN) measures. Scale bar represents the range of the correlation coefficients (\( r \)) displayed with the range from -1 (blue) to 1 (red). mtCN measures, mtND1, mtND2, and mtCO2 were log10 transformed for Pearson correlations to achieve normal distribution. (b-d) Dot plots of associations between mtCN (log10 transformed, y-axis) and inflammatory responses (x-axis) of (b) IL-2, (c) IL-4, and (d) the percentage of neutrophils measured in BAL by never-smokers (NS, blue), electronic cigarette users (EC, green), and smokers (SM, red). Each dot represents each individual. Open green circles represent never-smoking EC (smoked <100 cigarettes in their lifetime). The line displayed for each group reflects the linear regression line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Role of funders
The content is solely the responsibility of the authors. Funders had no role in study design, data collection, data analyses, interpretation, or writing of report.

Ethics
The study protocol was approved by the Ohio State University Institutional Review Board (clinical trials.gov ID: NCT02596685). Informed consent was provided from all participants.

Results

mtCN among never-smokers, electronic cigarette users, and smokers
The four mtCN genes assayed from lung epithelial cells, were significantly and strongly correlated with each other (FDR < 0.1, r = 0.82–0.99, Figure 1a). All four genes, mtND1, mtND2, mtCO1, and mtCO2, were significantly higher in SM compared to NS (FDR < 0.1 for all) (Table 2). EC mtCN was between NS and SM, but not statistically different from either group. The mean of the four mtCN genes was significantly different between SM (mean 103.6) and NS (mean 89.6) (FDR = 0.03, Table 2). There were no significant associations of mtCN with age, gender, or race. Within a group analysis, tobacco history, including cigarettes per day, PG as a marker of EC intake, and 2NE were not associated with mtCN.

In mtDNA somatic mutation analysis, there was an overall significantly negative association between mtCN and a point mutation in the D-loop at nucleotide position T16519C (P = 0.025) (Supplementary Figure 1), but no association was found in the within-group analysis. Separately, there were five point mutations in NADH dehydrogenase 4 (ND4, position A11467G), NADH dehydrogenase 5 (ND5, position C12705T and G12372A), tRNA leucine 2 (TL2, position A12308G), and the D-loop (position C16223T) found to have significantly high frequencies in SM compared to NS (Supplementary Table 1). One point mutation in the D-loop (position T310C, upstream of 12S rRNA) had a significantly higher frequency in EC compared to SM, but no difference was found compared to NS (Supplementary Table 1).

Associations between mtCN and inflammatory cells and cytokines
Associations of mtCN with BAL immune cells and immune cytokines are shown in Table 3. In the smoking group-adjusted analysis, IL-2 and the percentage of neutrophils were overall significantly associated with increased and decreased mtCN, respectively (FDR = 0.03 and FDR = 0.06, respectively, Table 3). Within-group analysis by smoking status showed that IL-2 and IL-4 had significant associations in only EC (FDR=0.065 for both), not in SM (FDR = 0.75 and 0.87, respectively) or NS (FDR = 0.92 and 0.87, respectively) (Figure 1b-c). These associations are significantly different by smoking group (FDRInteraction = 0.06 for both, Table 3). The percentage of neutrophils was significantly negatively associated with mtCN in only SM (FDR = 0.065), but this association was not significantly different across the groups (FDRInteraction =0 . 4 3 , Figure 1d).

Association of mtCN with genome-wide DNA methylation
About 10% of the CpGs analysed (n = 71,487 CpGs, 13,846 unique genes) were overall significantly associated with log10 transformed mean mtCN after adjusting for smoking group (mtCN-CpGs, FDR < 0.1). The similarities and differences of mtCN-CpGs and their corresponding genes between the smoking groups are shown in Figure 2a from the within-group analysis. Among 71,487 mtCN-CpGs, associations were mostly driven by NS (14,032 CpGs including 6132 genes) and SM (13,299 CpGs including 5580 genes). There were 3,929 mtCN-CpGs (2299 genes) found to be EC-specific (Figure 2a). Between groups, the majority of the

### Table 2: Mitochondrial DNA copy numbers (MtCN) in bronchial epithelium of never-smokers, electronic cigarette users, and smokers.

| Mitochondrial genes | Never-smokers (NS, N=43) Mean (SD)a | E-cig users (EC, N=15) Mean (SD)b | Smokers (SM, n=26) Mean (SD)b | SM v NS P FDR | SM v EC P FDR | NS v EC P FDR |
|---------------------|-------------------------------------|-----------------------------------|-------------------------------|--------------|-------------|-------------|
| mtND1               | 76.7 (17.1)                         | 89.3 (32.9)                       | 90.2 (24.0)                   | 0.02         | 0.03        | 0.79        |
| mtND2               | 77.7 (16.0)                         | 90.7 (30.6)                       | 90.8 (23.3)                   | 0.02         | 0.03        | 0.87        |
| mtCO1               | 73.3 (14.4)                         | 84.8 (29.1)                       | 82.2 (26.3)                   | 0.07         | 0.07        | 0.92        |
| mtCO2               | 130.7 (26.0)                        | 149.5 (47.3)                      | 151.3 (38.3)                  | 0.02         | 0.03        | 0.80        |
| Mean mtCN           | 89.6 (18.2)                         | 103.6 (34.6)                      | 103.6 (26.2)                  | 0.02         | 0.03        | 0.86        |

The mean copy number of four mitochondrial genes.

a Log10 transformed data used for t-tests.

Non-transformed data was used for Wilcoxon test.

*Significant P values after correction for multiple testing by adjusted FDR at the 0.1 level are bolded.
mtCN-CpGs were overlapped between SM and NS (n = 29,930), followed by 1756 between EC and SM, and 1645 between EC and NS (Figure 2a). There are 1153 mtCN-CpGs (712 genes) to be significantly associated with mtCN in all three groups (FDR < 0.1, Figure 2a, Supplementary Table 2), including ATPase families such as ATP6V0E2, ATP8A2, ATP8B1, and ATP10B (Figure 2b). The top canonical pathways include inflammation and cytoskeleton such as leukocyte extravasation signalling, Fcγ receptor-mediated phagocytosis in macrophages and monocytes, actin cytoskeleton signalling, paxillin signalling, and ErbB signalling (Figure 2c).

Association of mtCN with genome-wide expression
Smoking group-adjusted association analysis showed 321 transcripts (180 unique genes) to be significantly associated with mtCN in all three groups (FDR < 0.1, Figure 2a, Supplementary Table 3). Separately, 4 transcripts (CLPB, FAM104A, PPP1R32, and MIR2114) had significantly different patterns of associations with mtCN by smoking group (Interaction FDR < 0.1) (Figure 2e). Of these mtCN-transcripts, we found ten genes to be EC-specific (CLPB, COLCA1, HLA-DRB1, LINCO1184, LOC103798653, MIR2114, RPL13A, SNU13, TCRG1, TRIM9), eight genes to be SM specific (COBL, GIMAP7, IFI16, MAN2A1, MIR3198-1, MX2, PTPN22, and SAMD9), and four genes to be NS specific (IFITM4P, LOC101929759, PBOV1, and TRGV4) (Figure 2d). 147 transcripts (45%, 147/325) are significantly associated with mtCN in all three groups (FDR < 0.1) (Figure 2d). These mtCN-transcripts corresponded to genes enriched mainly in immune systems by IPA (Figure 2f).

Correlation between mtCN associated methylation and gene expression
Of 71,487 mtCN-CpGs and 321 mtCN-transcripts, there were 300 CpG-transcript pairs (matched genes within +/- 1.5Kb from methylation sites). Among them, 263 pairs are significantly correlated (89 positives and 174 negative correlations) (FDR < 0.1) Supplementary Table 4). Ingenuity pathway analysis of those significantly correlated genes revealed a significant enrichment of genes involved in antigen presentation, protein synthesis, cell death/survival, cellular compromise, and cellular function and maintenance (Figure 3a). The top enriched networks were antimicrobial response, cancer, immune response (Figure 3b), antigen presentation, cell death and survival, protein synthesis (Figure 3c), antimicrobial response, connective tissue disorders, and immune response (Figure 3d).

EC-specific mtCN associated methylation and gene expression in pulmonary diseases
Given that the mean mtCN was significantly associated with IL-2 and IL-4 in EC only, we explored the potential involvement of EC-specific mtCN-associated signatures in respiratory diseases. Separately, IPA identified 624 respiratory disease-related corresponding genes to a subset of EC-specific mtCN-CpGs (Supplementary Table 5). The model for the associations was mtCN → inflammatory markers + smoking status + (inflammatory markers x smoking status). Significant P-values after correction for multiple testing by adjusted FDR at the 0.1 level are bolded.

### Table 3: Association of mtCN with inflammatory markers (cytokines and immune cells).

The model for the associations was mtCN → inflammatory markers + smoking status + (inflammatory markers x smoking status). Significant P-values after correction for multiple testing by adjusted FDR at the 0.1 level are bolded.
We utilized lung expression data from publicly available GEO for asthma (GSE4302 and GSE63142) and COPD (GSE11906 and GSE11784), and TCGA Pan-Cancer Atlas for lung adenocarcinoma and squamous.

Among 2299 EC-specific mtCN-CpGs genes in healthy lungs from this study (Figure 2a), 149 and 289 genes were significantly differentially expressed between cases and healthy controls in GSE4302 and GSE63142 for asthma datasets, respectively (FDR < 0.1, Figure 4a). Of them, 35 EC-specific mtCN-CpGs genes were identified in both datasets and 29 had the same direction of expression between cases and controls (Figure 4a, Supplementary Table 6). These genes included those involved in cellular movement, inflammatory response, respiratory diseases, and metabolisms such as ALOX15, CYP2J2, IRS2, MUC5B, and NTRK2 (Figure 4c). From the COPD datasets, 75 EC-specific mtCN-CpGs genes were found to be significantly differentially expressed in cases compared to controls in only GSE11906 (Figure 4b, Supplementary Table 6). Among these, some genes are known to play a role in airway hyperresponsiveness and pulmonary hypertension, including AHRR, CD35, CXCL14, F2RL1, and MUC3B (Figure 4d). Of the 2299 EC-specific mtCN-CpGs genes, 2219 unique genes were available for analysis in cBioPortal and of the 10 EC-specific mtCN-transcripts genes, 9 unique genes were available for analysis in cBioPortal. In addition, 58 and 182 EC-specific mtCN-CpGs genes had altered expression in 90% of lung adenocarcinoma and squamous cell carcinoma tissues compared to adjacent normal tissues, regardless of high or low expression, respectively (Supplementary Table 8), with 37 genes altered in both subtypes (Figure 4e-f).

Figure 2. Associations of mtCN with (a-c) nuclear DNA methylation and (d-f) gene expression. (a) Venn diagram indicating mtCN associated CpGs (mtCN-CpGs) by smoking groups. A summary table of mtCN-CpGs and corresponding unique genes is shown. (b) Example dot plots of mtCN-CpGs in all three smoking groups. Each dot represents individual NS (blue), EC (green), and SM (red). Open green circles represent never-smoking EC (smoked < 100 cigarettes in their lifetime). The line displayed reflects the linear regression line. * represents the gene had multiple CpGs associated with it, the most significant is displayed. (c) The top 5 canonical pathways for 1,153 CpGs genes to be significantly associated with mtCN in all three groups. (d) Venn diagram indicating mtCN associated transcripts (mtCN-transcripts) by smoking groups. A summary table of mtCN-transcripts and corresponding unique genes is shown. (e) Example dot plots of mtCN-transcripts that have different patterns of association between mtCN and transcripts by smoking group (FDRInteraction < 0.1). Each line represents the linear regression line for NS (blue), EC (green), and SM (red). (f) The top 5 canonical pathways for 147 transcripts to be significantly associated with mtCN in all three groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Here, we found that mtCN was significantly higher in SM compared to NS, and EC mtCN was numerically intermediate between SM and NS. These associations remained after adjusting for age, gender, and race (Supplementary Table 10). mtCN was significantly associated with a number of nuclear DNA methylation CpGs (mtCN-CpGs) and gene expression (mtCN-transcripts). IPA showed highly enriched genes involved in inflammation for mtCN associated signatures. There

**Figure 3.** The functional annotation and top networks of significantly correlated genes between mtCN-CpGs and mtCN-transcripts. (a) The top five molecular and cellular functions (y-axis) are reported with the number of genes (x-axis). (b-d) The top three networks of correlated genes are shown. Genes encoding for red molecules are positively correlated, while green ones are negatively correlated between gene expression and DNA methylation. The brightness of colour is related to the correlation coefficient ($r$), and the darker the colour represents a stronger correlation. The nodal relationships indicated in solid or dashed lines indicate direct or indirect interactions, respectively, from Ingenuity Pathways Analysis. Different shapes represent the various functional classes of proteins. A detailed explanation of molecule shapes can be found [here](https://qiagen.secure.force.com/KnowledgeBase/articles/Basic_Technical_Q_A/Legend). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Discussion**

Here, we found that mtCN was significantly higher in SM compared to NS, and EC mtCN was numerically intermediate between SM and NS. These associations remained after adjusting for age, gender, and race (Supplementary Table 10). mtCN was significantly associated with a number of nuclear DNA methylation CpGs (mtCN-CpGs) and gene expression (mtCN-transcripts). IPA showed highly enriched genes involved in inflammation for mtCN associated signatures. There
were significant EC-specific positive associations between mtCN and lung disease-associated cytokines such as IL-2 and IL-4, but not for SM and NS. EC-specific mtCN-CpGs and transcripts genes were found to be differentially expressed in respiratory diseases such as asthma, COPD, and lung cancers, including genes involved in cellular movement, inflammatory response, metabolisms, and airway hyperresponsiveness.

The association of mtCN with cigarette smoking is reported largely in blood. The effects of cigarette smoking on mtDNA are thought to be due to inflammation, generation of ROS, and oxidative damage. Both increased and decreased mtCN are reported in relation to smoking, but in the lung we found only increased mtCN. Increased mtCN likely reflects increased mtDNA expression to compensate for the deleterious effect of mtDNA mutations, while decreased mtCN may be associated with mitochondrial dysfunction. Here, we identified lung mtCN associated point mutation at a position C16519T in the D-loop, indicating a possible impact of this mutation site on mtCN, and we did not find a group-specific association. Also, in a separate analysis, while there was no significant differences between the number of mtCN mutations and smoking status (Supplementary Table 11), we identified five somatic mutations that showed significantly different frequencies of the mutations between groups. Of them, a higher frequency of a somatic mutation in the D-loop at C16223T in the saliva of SM (4%) compared to NS (10%) was also reported in our previous study, which is opposite to our finding in the lungs of SM (92%) and NS (41%). This discrepancy may indicate a tissue-specific somatic mutation in mitochondrial DNA. When it comes to target organ effects, the association of smoking with mtCN in our study is consistent with previous studies in the normal region of the resected lung tissue from patients with various pulmonary diseases. Separately, our previous study using buccal cells showed higher mtCN in SM compared to NS in a dose-response manner. A recent study of various cancers showed increased mtCN in lung cancers while others had decreased, indicating tissue- and disease-specific manner of mtCN alteration.

We observed intermediate levels of mtCN for EC between SM and NS, and although not statistically different from SM and NS, EC may be less harmful than cigarette smoking but not completely safe compared to...
Deficiency of lipid pumps is associated with a poor prognosis of lung cancer.

This inverse relationship is consistent with previous findings. Additionally, we found a significant correlation between neutrophils (%) and mtCN among only SM. Therefore, further study is needed to investigate the clinical relevance of our findings.

Among a number of EC-specific mtCN-CpG genes to be significantly differentially expressed in asthma datasets, \textit{ALOX15} is highly expressed in M2 macrophages, and higher expression is found in relation to Th2 cytokines. \textit{CYP2J2} is a member of the cytochrome P450 superfamily of enzymes which has been thought to play an important role in pulmonary physiology, and genetic variation of this gene is associated with asthma susceptibility.

Given that only EC had significant associations of mtCN with IL-2 and IL-4, we further explored EC-specific mtCN associated signatures in respiratory diseases. Using GEO datasets for asthma and COPD, some EC-specific signatures from our study were observed to be differentially expressed between cases and controls. Among a number of EC-specific mtCN-CpG genes to be significantly differentially expressed in asthma datasets, \textit{ALOX15} is highly expressed in M2 macrophages, and higher expression is found in relation to Th2 cytokines. \textit{CYP2J2} is a member of the cytochrome P450 superfamily of enzymes which has been thought to play an important role in pulmonary physiology, and genetic variation of this gene is associated with asthma susceptibility.

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age range allowed us to minimize any age-related effects on lung physiology (although it limits generalizability to older users). Also, we investigated the biological implication of altered mtCN in association with mtDNA mutations, nuclear biomarkers, including immune responses, genome-wide DNA methylation, and gene expression. Further, we confirmed a subset of EC-specific mtCN associated signatures to be differentially expressed in lung diseases compared to controls in publicly available datasets. Thus, we revealed a broad view of the altered mtCN effects in possible underlying mechanisms that may contribute to lung disease susceptibility.

It is important to note that there are some limitations to be considered in interpreting the study findings. This study had a small sample size. While there was sufficient power to identify mtCN associated immune responses, methylation, and gene expression, a larger study can explore potential confounders. We could not explore seasonality, air pollution, occupation, and other potential confounders, but believe the confounding would be minimal compared to the larger exposures of smoking and vaping. There was a wide range of EC devices, flavours, and nicotine concentrations used by participants and with a small sample size, specific product design could not be assessed. However, given that primary EC carriers are mostly PG and VG, our findings may broadly apply to EC on the market. Additional larger studies are warranted, especially to determine the associations of mtCN with urinary exposure biomarkers and EC use variables (e.g., years of vaping, puffs per day, or nicotine concentration). Additionally, many of the EC were former smokers, which may lead to more similarities with SM than NS. We were also unable to determine the number of mitochondria copies per mitochondrion. Due to the nature of our cross-sectional design, it is impossible to assess the causal relationship of mtCN with nuclear biomarkers. Thus, there may be unknown confounders in the relationship.

In conclusion, smoking may elicit a lung toxic effect through mtCN. mtCN was significantly associated with inflammation and nuclear DNA methylation and gene expression in the lungs of healthy individuals. While a toxic effect in mtCN by EC use is less clear, EC-specific associations of mtCN with nuclear biomarkers were found. These findings support further research on the role of mtCN in lung disease related to smoking and EC use.

Contributors
MAS and PGS made significant contributions to the conception or design of this study. KMM and JPM analysed data. KMM and MAS interpreted findings. KMM wrote the first draft. KMM and MAS had full access to all data included in this study and MAS had final responsibility for the decision to submit for publication. All authors read, revised, and approved the manuscript.

Data sharing statement
The data used to support this manuscript and all supplementary materials is reported in its entirety. Patient-level data cannot be reported because of privacy and ethical issues. Readers may request to access this data for non-commercial use by reaching out to corresponding authors. Requests must explain the intended purposes for the data.

Declaration of interests
All authors declare that they have no potential conflicts of interest.

Acknowledgements
Research reported in this publication was supported by funding from the National Cancer Institute of the National Institutes of Health (NIH) (P30 CA016058), the Food and Drug Administration Center for Tobacco Products (CTP) (P0CA180908), the National Heart, Lung, and Blood Institute (R21HL147401), the National Center For Advancing Translational Sciences (UL1TR001070), and from Pelotonia Intramural Research Funds and the Prevent Cancer Foundation. We acknowledge the support of the Genomics Shared Resource who performed the mitochondria copy number assays of this study. We also acknowledge the support of the Recruitment, Intervention and Survey Shared Resource (RISSR). We thank the study participants, the staff, and nurses of the OSU Clinical Research Center, and Mrs. Sahar Kamel for assisting in recruiting participants. We thank Ms. Jiayung Zhu for her assistance in processing and managing our mitochondrial DNA mutation data.

Supplementary materials
Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104301.

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