ORIGINAL ARTICLE

Biomarkers of Airway Immune Homeostasis Differ Significantly with Generation of E-Cigarettes

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Abstract

Rationale: Numerous studies have demonstrated that e-cigarettes can impact respiratory immune homeostasis; however, the extent of these effects remains an active area of investigation, and most previous studies were conducted with model systems or subjects exposed to third-generation e-cigarettes, such as vape pens and box mods.

Objectives: Given the rise in popularity of nicotine-salt–containing pods and disposable e-cigarettes (fourth generation), we set out to better understand the respiratory effects of these newer e-cigarettes and compare their effects to early-generation devices.

Methods: We collected induced sputum samples from a cohort of nonsmokers, smokers, third-generation e-cigarette users, and fourth-generation e-cigarette users ($n = 20$–30 per group) and evaluated the cellular and fluid-phase composition for markers of inflammation, host defense, and lung injury.

Measurements and Main Results: Fourth-generation e-cigarette users had significantly more bronchial epithelial cells in the sputum, suggestive of airway injury. Concentrations of soluble intercellular adhesion molecule 1 (sICAM1) and soluble vascular cell adhesion molecule 1 (sVCAM1) were significantly lower in fourth-generation e-cigarette users in comparison with all other groups, and CRP (C-reactive protein), IFN-γ, MCP-1 (monocyte chemoattractant protein-1), MMP-2 (matrix metalloproteinase 2), uteroglobin, and VEGF (vascular endothelial growth factor) were significantly lower in fourth- versus third-generation e-cigarette users, suggestive of overall immune suppression in fourth-generation e-cigarette users. Predictive modeling also demonstrated clear separation between exposure groups, indicating that the overall mediator milieu is different between groups, particularly fourth-generation e-cigarette users.

Conclusions: Our results indicate disrupted immune homeostasis in fourth-generation e-cigarette users and demonstrate that the biological effects of fourth-generation e-cigarette use are unique compared with those associated with previous-generation e-cigarettes.

Keywords: machine learning; pod e-cigarettes; immune suppression

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This article has a related editorial.

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At a Glance Commentary

Scientific Knowledge on the Subject: Previous studies using a wide variety of models and exposure paradigms have demonstrated that e-cigarettes can alter respiratory immune homeostasis. However, most of these studies used previous-generation e-cigarette devices rather than currently popular nicotine-salt–containing e-cigarettes.

What This Study Adds to the Field: Our results reveal that users of newer, nicotine-salt–containing (fourth generation) e-cigarettes have a unique biomarker milieu indicative of immune suppression compared with the other groups in our study. These findings demonstrate the importance of considering device type in future clinical, epidemiological, and mechanistic studies on the health effects of e-cigarettes and have the potential to influence e-cigarette regulations.

Over the past decade, an increasing number of studies have demonstrated that e-cigarettes and their chemical constituents can affect the respiratory system, including respiratory immune homeostasis (1, 2), but the extent of these effects, including their magnitudes, affected cell types, and biological implications for human health, is an active area of investigation. A major challenge in the field of e-cigarette toxicology is the constant evolution of e-cigarette devices and e-liquid formulations. The most notable example of this is the shift in the e-cigarette industry that started when JUUL, a discreet, pod-based e-cigarette formulated with nicotine salts, was introduced in the United States in 2015. With JUUL's popularity (3), a new wave of similar devices entered the market, and in 2020, prefilled pods or cartridges were still the most popular e-cigarette device type among high school students in the United States (4, 5). In response to the restrictions from the FDA (U.S. Food and Drug Administration) on flavored, cartridge-based e-cigarettes (6), disposable e-cigarettes have also gained popularity, with the percentage of youth vapers using disposables increasing from 2.5% in 2019 to 26.5% in 2020 (4, 7).

This newer generation of e-cigarettes (small, low-power pod, cartridge, and disposable e-cigarettes, also referred to as fourth-generation e-cigarettes) is unique from the previous generation of e-cigarettes (vape pens and box mods, also referred to as second- and third-generation e-cigarettes, respectively) in their aerosolization parameters and nicotine formulation (8–11). E-liquid found in fourth-generation e-cigarettes typically contains nicotine salts, which results in the formation of monoprotonated nicotine and lowers the pH of the mixture and, therefore, also the resulting aerosol (9, 12, 13). The proportion of nicotine in the aerosol in protonated and freebase forms impacts the user sensory experience, with nicotine-salt–containing e-cigarettes providing a "smoother" feeling and allowing for the inhalation of higher concentrations of nicotine (5, 14). Importantly, the inhalation toxicity of organic acids commonly used in the formulation of nicotine-salt–containing e-liquids is poorly understood.

Previous studies have shown increased proteinase concentrations, enhanced neutrophil activation, and altered mucin composition in airway samples from e-cigarette users, also commonly referred to as vapers because of the misconception that e-cigarette aerosol is water vapor (15, 16). However, the samples used in these studies were collected from subjects who used third-generation e-cigarettes, as fourth-generation e-cigarettes such as JUUL had not yet gained popularity at the time of these studies. Because fourth-generation e-cigarettes are now the most popular type of e-cigarette, particularly among young never-smokers, there is a need to assess the respiratory effects of these types of e-cigarettes and determine whether these effects are unique from those observed in previous-generation e-cigarette users. This research is also critical given the recent marketing authorization of Vuse Solo, a nicotine-salt–containing fourth-generation e-cigarette, by the FDA, with other similar devices still under review (17).

In this study, we collected induced sputum samples from a cohort of nonsmokers/nonvapers, cigarette smokers, and third- and fourth-generation e-cigarette users to determine whether fourth-generation e-cigarette users exhibited unique central airway immune profiles. We evaluated cellular composition and soluble mediators associated with inflammation, host defense, and lung injury in sputum samples. We then applied a standard variable-by-variable analysis and a multivariate predictive modeling analysis to enhance our resolution to distinguish inflammatory protein concentration profiles between subject cohorts. Our results demonstrate that there are significant differences in markers of respiratory immune homeostasis between fourth-generation e-cigarette users and other groups, underscoring the importance of considering device type when assessing the inhalation toxicity of e-cigarettes. Some of the results of these studies have been previously reported in the form of abstracts (18, 19).

Methods

Study Cohort and Sample Collection

Subject recruitment. Healthy adult human nonsmokers, cigarette smokers, and e-cigarette users between 18 and 50 years old were recruited to participate in this study. Active cigarette smoking and e-cigarette use were determined as described previously (20). Subjects were classified as e-cigarette users if they were current daily users (at least 10–20 puffs per day) and did not report the use of tobacco products other than e-cigarettes in the past 3 months or a greater than 10 pack-year history of smoking cigarettes. E-cigarette users were classified as third-generation e-cigarette users if they reported using primarily vape pens, box mods, or similar devices that contain freebase nicotine. Although vape pens are typically considered second-generation e-cigarettes, we included vape pen users in the third-generation cohort to simplify the analysis. E-cigarette users were classified as fourth-generation e-cigarette users if they reported using primarily JUUL or other low-powered e-cigarettes that contain nicotine salts. Two subjects used both third- and fourth-generation e-cigarettes regularly and were excluded from the study. A flow chart showing the inclusion and exclusion of clinical study subjects by device type is available in Figure E1 in the online supplement. Exclusion criteria included current symptoms of allergic rhinitis, chronic cardiorespiratory disease, immunodeficiency, bleeding disorders, current pregnancy, and FEV_1 less than 75% predicted during the screening visit. Informed consent was obtained from all subjects, and all studies were approved by the University of North

Carolina at Chapel Hill School of Medicine Institutional Review Board (IRB #13–3454 and #17–2275).

Sample collection and processing. Induced sputum collection, processing, acquisition of fluid phase samples, and differential cell counts were performed as described previously, and fluid-phase samples were stored at −80°C until analysis (21, 22). Differential cell counts were performed on methanol-fixed, Hema 3-stained slides. Cells were counted and classified manually using light microscopy on the basis of morphology. Squamous epithelial cells were counted to determine sample quality but excluded from differential cell counts. Bronchial epithelial cells, which have a characteristic columnar shape, were included in differential cell counts. Differential cell counts were available for 89 out of 103 subjects (Figure E1). Of these slides, 90% were in an acceptable range (<80%) for percentage of squamous epithelial cells, with a mean and standard error of 30.45% ± 3.12% squamous cells across all subjects. Although our samples, on average, had higher than desired (30% vs. 20%) squamous epithelial cells, we opted to include samples with higher squamous epithelial cell percentages to allow for a higher number of samples to be included in the analysis. Notably, our primary endpoint was soluble mediator concentration and not differential cell analysis, the latter a more vulnerable endpoint to high squamous epithelial cells. To obtain serum, venous blood was allowed to clot for a minimum of 15 minutes and centrifuged at 1200 × g for 10 minutes. The serum layer was collected and stored at −80°C until analysis.

Experimental Procedures

Serum cotinine measurement. Serum cotinine, a metabolite of nicotine, was measured using a commercially available ELISA (enzyme-linked immunosorbent assay) kit (Calbiotech) to confirm smoking status. For samples below the limit of detection (5 ng/ml), a value of zero was assigned. Serum was not available for some subjects in each group. The number of subjects for which serum was available in each group is denoted in Table 1.

Induced sputum soluble mediator measurement. Soluble mediator ($n = 45$) concentrations in cell-free–induced sputum supernatants were determined using commercially available single-plex ELISAs (R&D Systems) and Mesoscale Discovery V-Plex assay kits (Table E1). Double-stranded DNA concentrations were measured using the Quant-iT Picogreen assay (Thermo Fisher).

Data Analysis

Data availability. Data input files and code used for the analysis can be found at: https://github.com/UNC-CEMALB/Biomarkers-of-airway-immune-homeostasis-differ-significantly-with-generation-of-e-cigarettes/. All analyses were conducted using R v4.1.1 using base R unless otherwise noted below (23). An overview of the analysis structure for soluble mediators is provided in Figure 1.

Normality testing. For all continuous variables, normality was tested before between-group comparative analyses using the Shapiro-Wilk test. Normally distributed data were analyzed using parametric tests, and nonnormally distributed data were analyzed using nonparametric tests when possible. Normality was also assessed through the examination of histograms and quantile–quantile plots through the *ggplot2* package (24).

Demographics. To determine which predictor covariates were significantly different between exposure groups, we performed either a Fisher's exact test (categorical variables) or a Kruskal-Wallis test with Dunn's test (continuous variables) for multiple comparisons. Predictors significantly different between groups were included in analyses as described below. Because of the small number of Black, Asian

Table 1. Study Demographics

	NS/NV ($n = 28$)	Smoker ($n = 21$)	Third gen ($n = 27$)	Fourth gen ($n = 27$)	*P* Value
Sex, *n* (%)					0.004
Male	9.00 (32.1)	8.00 (38.1)	19.0 (70.4)	19.0 (70.4)	—
Female	19.0 (67.9)	13.0 (61.9)	8.00 (29.6)	8.00 (29.6)	—
Race, *n* (%)					0.0095
White	21.0 (75.0)	10.0 (47.6)	18.0 (66.7)	23.0 (85.2)	—
Black	5.00 (17.9)	10.0 (47.6)	3.00 (11.1)	1.00 (3.7)	—
Asian/Pacific Islander	1.00 (3.6)	0 (0)	4.00 (14.8)	2.00 (7.4)	—
Mixed/other	1.00 (3.6)	1.00 (4.8)	2.00 (7.4)	1.00 (3.7)	—
Hispanic, *n* (%)					0.418
No	24.0 (85.7)	20.0 (95.2)	26.0 (96.3)	23.0 (85.2)	—
Yes	4.00 (14.3)	1.00 (4.8)	1.00 (3.7)	4.00 (14.8)	—
Age					<.001
Mean (SD)	26.5 (5.29)*	32.1 (7.42)	27.2 (7.42)*	21.6 (3.17)†	—
BMI					0.133
Mean (SD)	26.2 (5.61)	27.3 (5.98)	27.2 (5.94)	24.0 (4.04)	—
Serum cotinine, ng/ml					<0.001
Mean (SD)	0 (0)†	188 (87.5)	143 (82.2)	110 (90.5)	—
n measured	21	20	25	12	—

Definition of abbreviations: BMI = body mass index; gen = generation; NS/NV = nonsmokers/nonvapers.
For continuous variables, groups were compared using the Kruskal-Wallis test with Dunn's test for nonparametric multiple comparisons. For categorical variables, groups were compared using Fisher's Exact Test.
*$P < 0.05$ in comparison with smokers.
†At least $P < 0.001$ in comparison with all other groups.

and Pacific Islander, and “Mixed/Other” subjects in each group, these groups were collapsed into “Non-White” for further analyses.

Differences in individual sputum differential metrics and soluble mediators between exposure groups. Kruskal-Wallis test, followed by a Dunn’s test for nonparametric multiple comparisons, was used on the raw data for each cell metric and mediator value (Table E2). Data were pseudo $\log_2$ transformed, and then analysis of covariance (ANCOVA) tests were applied to control for the differences in age, sex, and race in the exposure groups (Table E3). Mediators showing significant differential expression for Device were identified using adjusted $q < 0.2$, similar to a previously published study using respiratory clinical samples with multiplex ELISA (25), with multiple test-corrected *P* values (q-values) reported in full (Table E3). For variables with significant ($q < 0.2$) overall associations with Device, Tukey’s *post hoc* test was performed on ANCOVA results for mediators with significant differences between groups associated with Device to determine significant differences between control samples and exposure groups and third- versus fourth-generation e-cigarette users.

Variable selection, hierarchical clustering, and predictive modeling to enhance the resolution of between-group differences in sputum soluble mediators. Supervised machine learning was used to determine whether these overall differences in soluble mediators were significant enough to separate subjects in each exposure group from the others. Before applying machine learning models, data were preprocessed by removing mediators that were undetected in more than 75% of subjects. For remaining mediators, values below the lower limit of detection were imputed using the square root of the lowest detected concentration for each mediator.

To design the machine learning algorithms, mediator data was used as primary predictor data. Predictors identified as significantly different between exposure groups, including age, sex, and race, were considered for inclusion. Because induced sputum cell metrics were only available for a subset of subjects in the cohort (Figure E1) and the scale and resolution of these data differed from the soluble mediator data, models were built with mediator data alone or mediator data in combination with covariates. The evaluated outcome variables were exposure group classifications: nonsmoker/non–e-cigarette user, cigarette smoker, third-generation e-cigarette user, and fourth-generation e-cigarette user. The variable selection method of best subsets regression (through the *leaps* package,

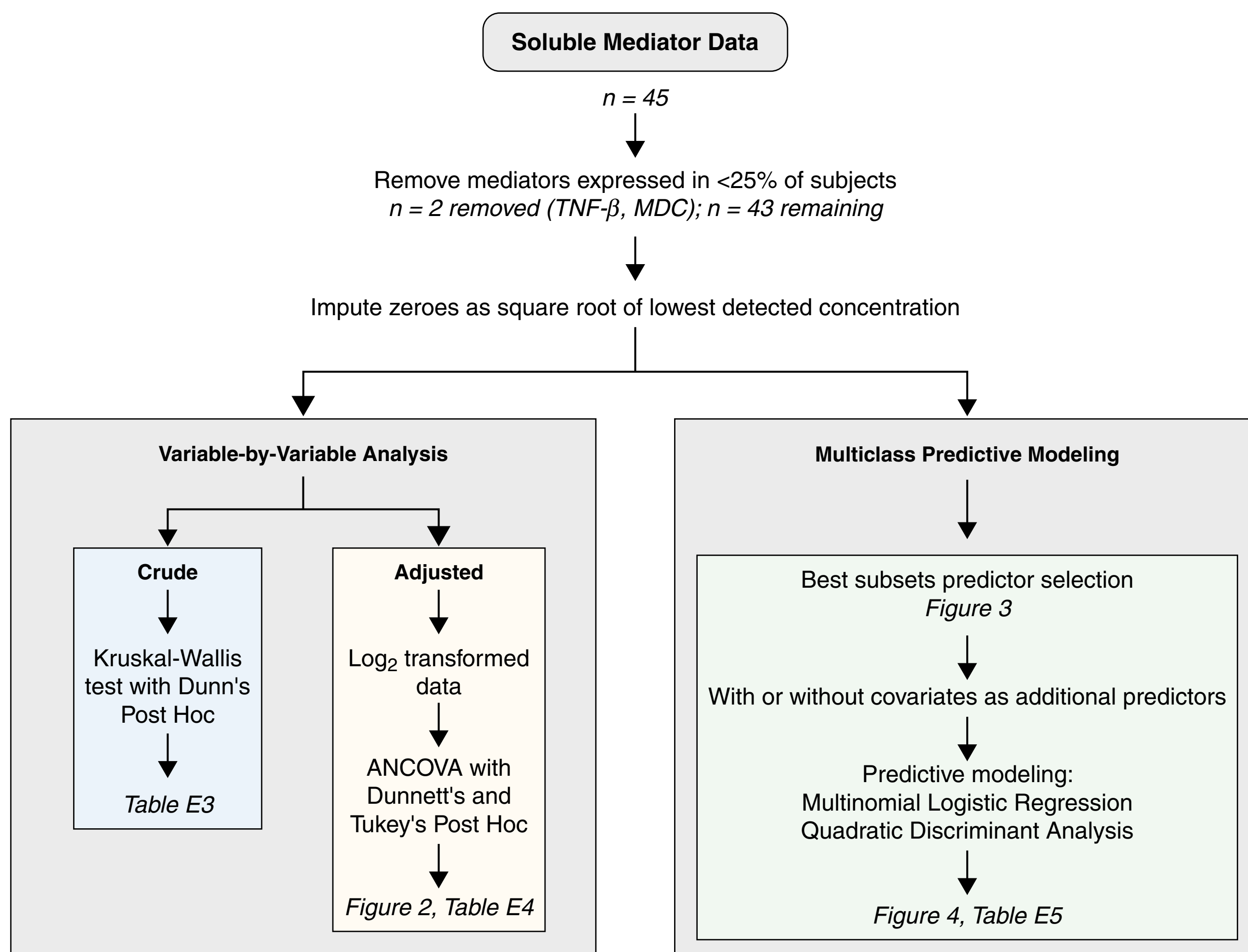

Figure 1. Flowchart depicting analysis workflow for investigating differences in soluble mediators between exposure groups. Figure created using biorender.com. ANCOVA = analysis of covariance; MDC = macrophage-derived chemokine; TNF-β = tumor necrosis factor-β.

v3.1 [26]) was also applied to address study questions and optimize the performance of the machine learning models. Best subsets regression, which tested all linear combinations of variables and prioritized which variables best explained separation between exposure group classifications (27), was the best choice to address our study's questions and optimize the performance of the algorithms. A heatmap with hierarchical clustering using the *pheatmap* package (28) was used to visualize patterns in the soluble mediators selected by best subsets regression on the basis of exposure group.

Predictive models were built using two of the most recognized classification models that allow for multiclass predictions: quadratic discriminant analysis (QDA) and multinomial logistic regression (MLR). QDA notably allows for nonlinear boundaries between classifiers, whereas MLR incorporates probability-based estimates for predictor variables on the dependent variable. Using the *caret* package (v6.0–90) (25), data were randomly split into training and test datasets (fivefold crossvalidation) before using QDA or MLR models to predict tobacco use status. Like previously published work (26, 27), model performance parameters were assessed on the basis of the resulting confusion matrix, which summarizes the classification of subjects on the basis of correct and incorrect identification of subjects as belonging to or not belonging to a specific exposure group.

Results

Subject Demographics

Demographic data are summarized in Table 1. The study cohort was comprised of 27% nonsmokers/nonvapers (NS/NV; $n = 28$), 20% smokers ($n = 21$), 26% third-generation e-cigarette users ($n = 27$), and 26% fourth-generation e-cigarette users ($n = 27$). Each exposure group contained both males and females, with a minimum of $n = 8$ per sex per group. Fourth-generation e-cigarette users were significantly younger on average than all other groups, whereas smokers were significantly older. These age differences were expected given the rise in popularity of fourth-generation e-cigarettes and the decline in popularity of cigarettes among youth (29, 30). The distribution of subjects' races was significantly different across exposure groups ($P = 0.0095$), which mirrors previous studies showing that e-cigarette users are more likely to be White (31, 32). Body mass index was not significantly different between exposure groups. As expected, smokers, third-generation e-cigarette users, and fourth-generation e-cigarette users had significantly elevated concentrations of serum cotinine, a metabolite of nicotine, in comparison with NS/NV. There were no significant differences in serum cotinine between the three tobacco user groups, indicating similar nicotine exposure at the time of sample collection. Data on previous smoking history was available for 100% of third-generation e-cigarette users and 59% ($n = 16$ out of 27) of fourth-generation e-cigarette users. Seventy percent ($n = 19$ out of 27) of third-generation e-cigarette users and 81% ($n = 13$ out of 16) of fourth-generation e-cigarette users reported previous cigarette smoking, roughly equivalent proportions. However, because of missing data for 11 subjects in the fourth-generation e-cigarette user group, previous smoking status was not included in statistical analyses.

Beyond categorizing each e-cigarette user as using third- or fourth-generation e-cigarettes, we did not collect detailed information about specific brands of devices and e-liquids used for all subjects. We collected samples for this study starting in 2014 and learned over time that subjects use a variety of devices and flavors, making it difficult to obtain reliable information on devices, e-liquids, and brands. In samples collected after 2018, we aimed to obtain more detailed device and e-liquid information, but the degree of detail in our data is not consistent across groups, and subjects do not always know/remember what they are using. Thus, these data were not included in statistical analyses.

Induced Sputum Differential Cell Counts

Induced sputum differential cell counts are summarized in Table 2. Because ANCOVA performed on sputum differential metrics

Table 2. Sputum Differential Data

	NS/NV ($n = 20$)	Smoker ($n = 19$)	Third gen ($n = 25$)	Fourth gen ($n = 25$)	Overall *P* Value
Total cells/mg	766 (190)	727 (225)	976 (135)	780 (151)	0.096
Macrophages/mg	469 (122)	204 (54.5)	**544 (89.4)***	505 (132)	**0.012**
% macrophage	67.2 (6.04)	**36.5 (5.59)**†	59.1 (5.09)	57.7 (5.12)	**0.003**
PMN/mg	259 (97.0)	505 (174)	421 (99.8)	256 (40.2)	0.102
% PMN	26.5 (5.07)	**61.3 (5.39)**†	39.8 (5.12)	38.5 (5.29)	**<0.001**
Eosinophils/mg	1.15 (0.678)	6.26 (4.01)	5.92 (3.70)	2.48 (1.16)	0.394
% eosinophil	0.230 (0.135)	0.775 (0.278)	0.456 (0.224)	0.704 (0.505)	0.564
Lymphocytes/mg	2.75 (1.73)	1.95 (1.42)	**0.04 (0.04)**‡	1.12 (0.40)	**0.018**
% lymphocyte	0.233 (0.092)	0.135 (0.084)	**0.008 (0.008)**‡	**0.095 (0.028)**§	**0.013**
Bronchial cells/mg	2.30 (0.927)	10.5 (7.69)	4.40 (1.74)	**15.5 (5.06)**‡,‖	**0.004**
% bronchial cells	0.870 (0.355)	1.33 (0.460)	0.562 (0.203)	**3.03 (1.24)**‖	**0.014**

Definition of abbreviations: gen = generation; NS/NV = nonsmokers/nonvapers; PMN = polymorphonuclear.
Data are presented as mean (standard error). Groups were compared using the Kruskal-Wallis test with Dunn's test for nonparametric multiple comparisons. Data in bold show significant differences among the different groups.
*$P < 0.01$ in comparison with smokers.
†At least $P < 0.05$ in comparison with all other groups.
‡$P < 0.05$ in comparison with NS/NV.
§$P < 0.05$ in comparison with third gen.
‖$P < 0.01$ in comparison with third gen.

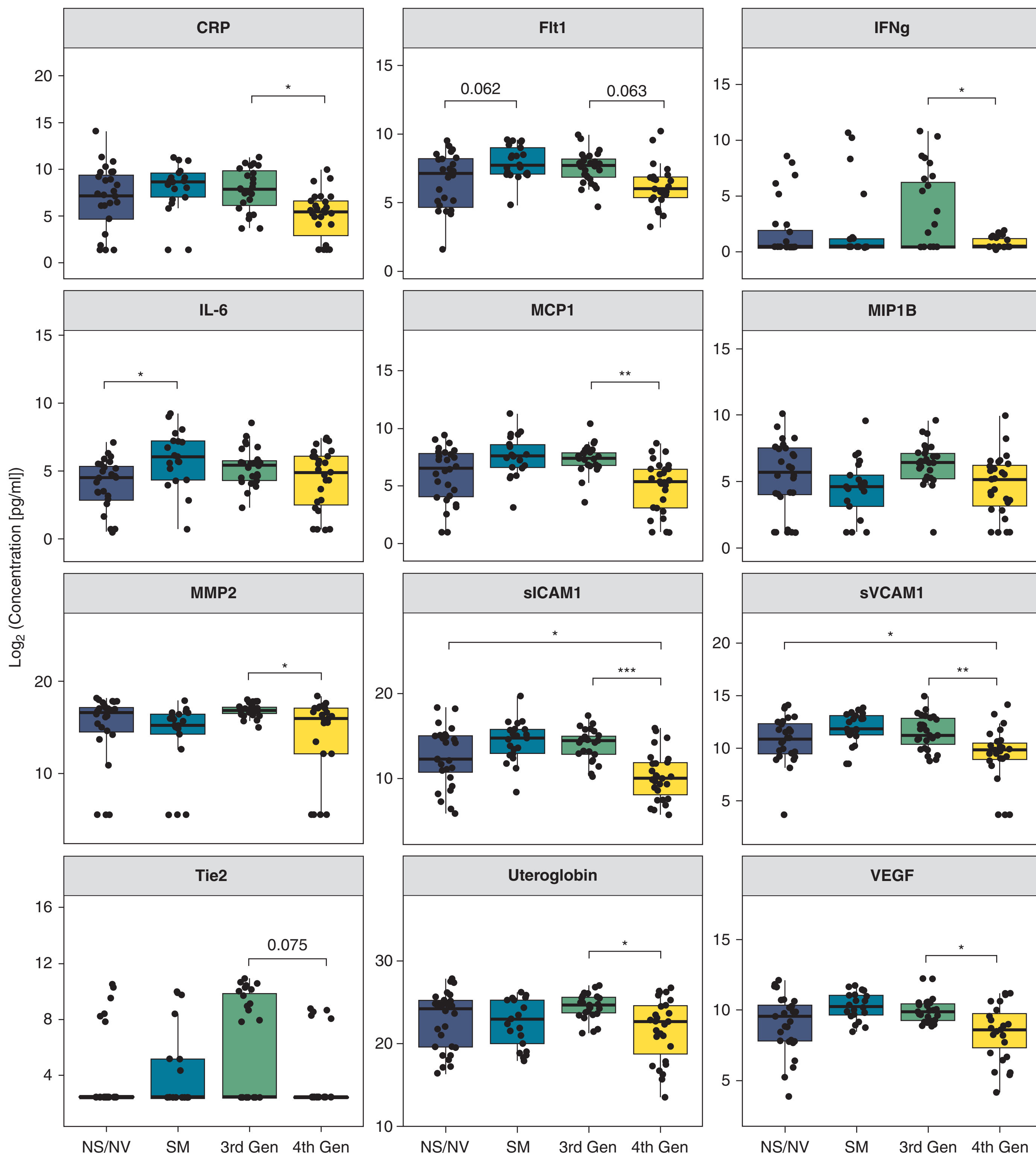

Figure 2. Soluble mediators that were significantly different between exposure groups after adjusting for age, sex, and race differences between exposure groups. Results are presented as mean ± standard error of $\log_2$ transformed mediator concentrations. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ using ANCOVA followed by Tukey's *post hoc* test. fourth Gen = fourth-generation e-cigarette users ($n = 27$), NS/NV = nonsmoker/nonvaper ($n = 28$), SM = smoker ($n = 21$), and third Gen = third-generation e-cigarette users ($n = 27$). CRP = C-reactive protein; Flt1 = Fms related receptor tyrosine kinase 1; MCP1 = monocyte chemoattractant protein-1; MIP1B = macrophage inflammatory protein 1B; MMP2 = matrix metalloproteinase-2; sICAM1 = soluble intercellular adhesion molecule 1; sVCAM1 = soluble vascular cell adhesion molecule 1; VEGF = vascular endothelial growth factor.

indicated no significant associations with sex and race and only one significant association with age (bronchial cells per mg, $P = 0.011$), Kruskal-Wallis P values with Dunn's test for multiple comparisons between exposure groups are reported for induced sputum cell differential data in Table 2. We observed that the induced sputum of smokers contained a significantly higher percentage of neutrophils in comparison with all other exposure groups, which is consistent with previous reports of smoking-associated increases in inflammatory cell recruitment to the airways (33–35). We found that third-generation e-cigarette users had significantly more macrophages per mg of sputum, fewer lymphocytes per mg of sputum, and a lower percentage of lymphocytes than smokers. Interestingly, fourth-generation e-cigarette users had significantly greater absolute cells per mg than nonsmokers/nonvapers and third-generation e-cigarette users and percent bronchial epithelial cells than third-generation e-cigarette users.

Soluble Mediators that Were Significantly Different between Exposure Groups

ANCOVA performed on soluble mediator data indicated that there were mediators that had significant associations with exposure group, age, sex, and race (Table E3). Twelve soluble mediators were significantly affected by exposure group: CRP (C-reactive protein), Flt1 (Fms related receptor tyrosine kinase 1), IFN-γ, IL-6, MCP-1 (monocyte chemoattractant protein 1), MIP-1b (macrophage inflammatory protein 1b), MMP-2 (matrix metalloproteinase 2), soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), Tie-2, uteroglobin (also known as clara cell secretory protein), and VEGF (vascular endothelial growth factor) (Figure 2), with Benjamini-Hochberg (BH) q-value < 0.2 (Table E3). sICAM-1 and sVCAM-1 showed the strongest association with exposure group (BH q-value < 0.05), followed by MCP-1 (BH q-value < 0.1) and the remaining mediators (BH q-value between 0.1 and 0.2) (Table E3). Of the 12 mediators with q-value < 0.2, a majority included significant differences between fourth-generation and third-generation e-cigarette users, and a majority were significantly decreased in fourth-generation e-cigarette users in comparison with the other exposure groups. Specifically, concentrations of CRP, IFN-γ, MCP-1, uteroglobin, MMP-2, and VEGF were significantly lower in fourth- versus third-generation e-cigarette users. We observed reduced concentrations of CRP, IFN-γ, MCP-1, and uteroglobin in fourth-generation e-cigarette users relative to nonsmokers/nonvapers, although these comparisons did not reach statistical significance. The concentrations of sICAM1 and sVCAM1 were significantly lower in fourth- versus third-generation e-cigarette users and nonsmokers/nonvapers. In addition, IL-6 concentration was significantly increased in smokers compared with nonsmokers/nonvapers, as expected (33, 36). Average concentrations stratified by exposure group with Kruskal-Wallis P values (crude/unadjusted) for all mediators are summarized in Table E2. Overall, these results indicate reduced mediator degrees in the airways in fourth-generation e-cigarette users, suggesting dysregulated immune homeostasis in the form of overall immune suppression in these subjects.

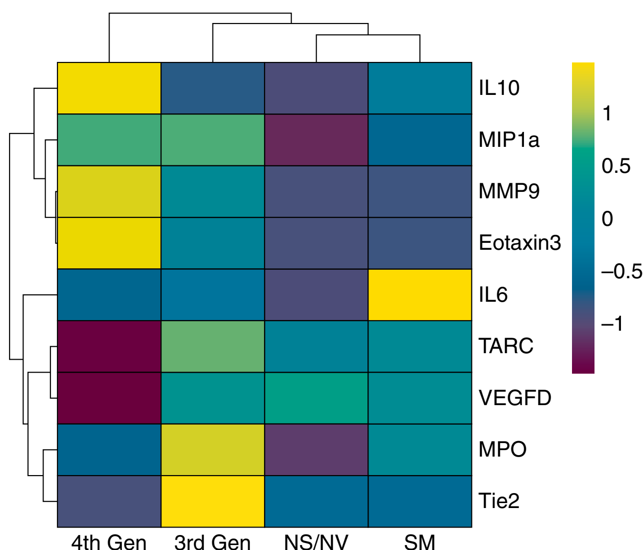

Figure 3. Heatmap showing the mean relative protein concentrations (row-scaled) of soluble mediators selected by best subsets regression by exposure group. Soluble mediator and exposure group positioning within the heatmap was determined using hierarchical clustering as part of the *pheatmap* package (28). MIP1a = macrophage inflammatory protein 1a; MMP9 = matrix metalloproteinase-9; MPO = myeloperoxidase; NS/NV = nonsmoker/nonvaper; SM = smoker; TARC = thymus and activation regulated chemokine; VEGFD = vascular endothelial growth factor D.

Variable Selection and Predictive Modeling to Evaluate the Separation between All Four Exposure Groups

In addition to the individual significant differences in induced sputum cell differential metrics and soluble mediators between exposure groups, we also examined significant overall differences in soluble mediator signatures between groups using predictive modeling. Given our cohort size and number of subjects per group, we determined that the maximum number of predictors our models would accept was nine. Best subsets regression was applied to the soluble mediator data to determine which nine variables best separated the exposure groups. The mediators that best separated the exposure groups were: eotaxin-3, IL-6, IL-10, MIP-1α, MMP-9, MPO (myeloperoxidase), TARC (thymus and activation regulated chemokine), Tie2, and VEGFD (vascular endothelial growth factor D) (Figure 3), which are associated with different functions, including immune cell recruitment, type 2 inflammation, angiogenesis, and tissue remodeling. To build models using either multinomial logistic regression or quadratic discriminant analysis, mediators were used as predictors with or without covariates. Most of these variables were different from the variables determined to be significantly different between exposure groups by ANCOVA and ANOVA, indicating novel trends not captured by traditional group comparison approaches. Notably, trends that we observed in these mediators were: decreased concentrations of TARC and VEGF and increased concentrations of IL-10, MMP9, and Eotaxin-3 in fourth-generation e-cigarette users, increased concentrations of

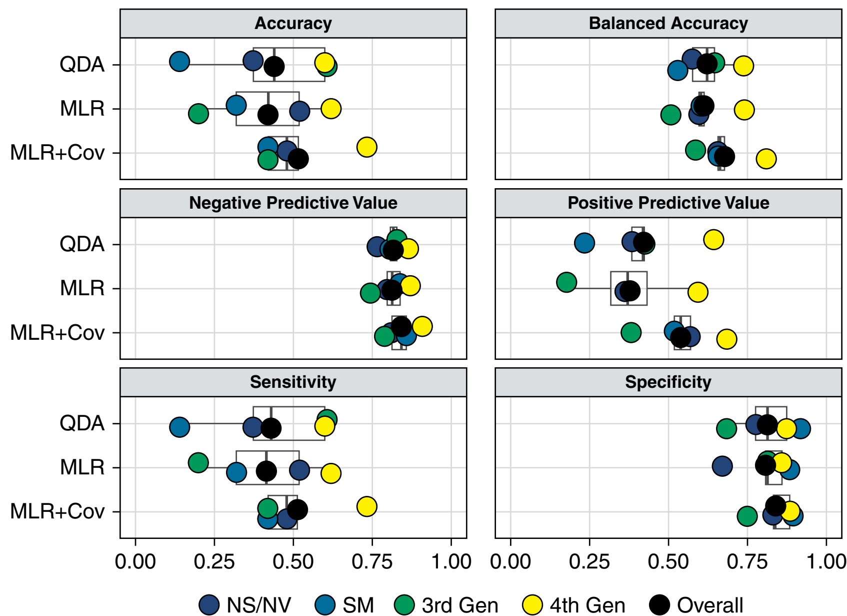

Figure 4. Performance parameters for predictive models built using soluble mediators as predictor variables for quadratic discriminant analysis (QDA) or multinomial logistic regression (MLR). MLR was also run with covariates (Cov) included as predictors (MLR + Cov). Each distinct model is shown on the *y*-axis, with performance parameter values plotted on the *x*-axis. Performance parameter values for each exposure group are represented by solid circles, and the mean values are represented by the black circle. For each performance parameter, values closer to one indicate better performance. NS/NV = nonsmoker/nonvaper; SM = smoker.

Tie2 and MPO in third-generation e-cigarette users, and increased concentrations of IL-6 in smokers (Figure 3). Hierarchical clustering of mean mediator degrees by exposure group showed that the profile of fourth-generation e-cigarette users was the most different from the other three exposure groups (Figure 3).

Overall and by class, performance metrics for each model are summarized in Figure 4 and Table E4. Performance for all models followed similar trends, without notable differences in performance between QDA and MLR. The highest values (closer to one), indicating better performance, were found in specificity and negative predictive value. The lower values (closer to zero) were found with accuracy, sensitivity, and positive predictive value. These data indicate that the models could better predict which subjects were not members of a specific group than predict which subjects were members of a specific group. Notably, for most of the algorithms, performance metrics for fourth-generation e-cigarette users were higher than for other groups, indicating higher predictivity for the classification of fourth-generation e-cigarette users on the basis of soluble mediator concentrations.

Discussion

In this study, a multifaceted approach analyzing induced sputum from healthy human nonsmokers/nonvapers, smokers, third-generation e-cigarette users, and fourth-generation e-cigarette users was employed to understand differences in biomarkers of respiratory immune homeostasis between groups. Using both variable-by-variable analyses and machine learning analyses, we demonstrated significant differences in both induced sputum cell differentials and soluble mediator milieu between exposure groups. Most notably, we observed significantly decreased protein concentrations of soluble mediators in airway samples from fourth-generation e-cigarette users, which could indicate airway immune dysfunction in these subjects. To our knowledge, this is the first study directly comparing respiratory immune biomarkers in e-cigarette users who use different generations of e-cigarette devices.

After adjusting for sex, age, and race, we found that concentrations of sICAM-1 and sVCAM-1 were significantly lower in fourth-generation e-cigarette users compared with other groups. Increased concentrations of both sICAM-1 and sVCAM-1 have been associated with acute lung injury (37, 38), but decreased concentrations of these molecules in response to a disease state or toxicant exposure, particularly in airway fluid, have not been documented. Previous studies have demonstrated that sICAM-1 can be protective during rhinovirus infection, as sICAM-1 can bind the virus, thereby reducing the amount of virus binding mICAM-1 (membrane-bound ICAM-1) and entering epithelial cells (39–41). Therefore, the significantly reduced concentrations of sICAM-1 we observed in fourth-generation e-cigarette users may indicate increased susceptibility to rhinovirus infection in fourth-generation e-cigarette users. However, it is important to note that concentrations of many other mediators were not significantly different between subjects using e-cigarettes or cigarettes and nonsmokers/nonvapers.

We also observed significantly decreased protein concentrations of CRP, IFN-γ, MCP-1, MMP-2, VEGF, and uteroglobin in fourth-generation e-cigarette users compared with third-generation e-cigarette users, although not all these comparisons reached statistical significance in comparison with nonsmokers/nonvapers. Each of these mediators is known to play an important role in respiratory host defense. On mucosal surfaces, such as in the respiratory tract, CRP acts as an antimicrobial and binds to specific residues on cell walls of bacteria that are prevalent in the respiratory tract (42, 43). As it is constitutively expressed, it is hypothesized to play a role in mediating host–microbe interactions in the respiratory tract, though more work is needed to characterize CRP in the respiratory mucosa in association with disrupted respiratory immune homeostasis. IFN-γ and MCP-1 are pleiotropic molecules that mediate the activity of both the innate and adaptive immune systems, including the priming of macrophages to respond to proinflammatory stimuli, chemotaxis, and maintenance of immune homeostasis (44–47). Uteroglobin, also known as club cell secretory protein, is regarded as antiinflammatory, and decreases in uteroglobin have been observed previously in smokers and subjects with chronic obstructive pulmonary disease (48). Therefore, decreased concentrations of these mediators may demonstrate suppressed host defense in association with fourth-generation e-cigarette use.

Our study also evaluated whether soluble mediator concentrations measured in induced sputum could be used to predict whether subjects were nonsmokers/nonvapers, smokers, third-generation e-cigarette users, or fourth-generation e-cigarette users. Our hierarchical clustering of soluble mediators revealed that fourth-generation e-cigarette users clustered differently and had the highest degree of separation from other tobacco use groups. In addition, multiclass machine learning analyses demonstrated that fourth-generation e-cigarette users were correctly classified the most frequently, indicating a unique biological response to exposure compared with the other groups. This approach also allowed us to detect shifts in the overall mediator milieu, which were not evident in individual mediator analyses. These efforts also permit the consideration of complex soluble mediator signaling interactions needed to maintain immune homeostasis. Future studies are needed to directly assess the relationship between e-cigarette use and the cellular processes highlighted in this analysis, particularly those beyond standard inflammatory pathways commonly interrogated in studies of e-cigarette effects.

We found that fourth-generation e-cigarette users had a significantly higher percentage of bronchial epithelial cells in their induced sputum than nonsmokers/nonvapers, which could indicate airway injury; however, no studies that we are aware of have established normal ranges for bronchial epithelial cells in induced sputum from healthy subjects, making it difficult to assess if the magnitude of effect observed is biologically significant. This finding is also particularly interesting in the absence of significant increases in markers of airway injury in fourth-generation e-cigarette users.

We hypothesized that the differences we observed between third- and fourth-generation e-cigarette users could be driven by the following mechanisms. First, fourth-generation e-cigarettes contain nicotine salts, which are formulated using organic acids such as benzoic acid, lactic acid, and levulinic acid (12). The effects of inhaling these organic acids are currently unknown and, therefore, could be driving some of the effects we observed. Second, fourth-generation e-cigarettes contain a higher concentration of nicotine than third-generation e-cigarettes (8), and nicotine is known to be immunosuppressive (49, 50). Although we observed similar serum cotinine concentrations across tobacco-use groups, it is possible that serum cotinine is not an accurate reflection of nicotine concentrations in the respiratory mucosal microenvironment and that the high concentration of nicotine present in the aerosol of fourth-generation e-cigarette users exerts immunosuppressive effects. Third, fourth-generation e-cigarettes aerosolize the e-liquid at lower temperatures than third-generation e-cigarettes, thereby producing fewer free radicals and carbonyls (10, 11). This phenomenon likely explains the absence of overt acute inflammation observed in fourth-generation e-cigarette users and our observation that increased concentrations of inflammatory biomarkers in third-generation e-cigarette users were more frequent than in fourth-generation e-cigarette users. Lastly, fourth-generation e-cigarette users are significantly younger, less likely to be former smokers, and more likely to be dual users with marijuana. We did not assess inhalation of cannabinoids, which may be particularly relevant given that dual use of nicotine-containing e-cigarettes and marijuana (either smoked or vaped) is prevalent in young adult users (51–54) and that cannabinoids can modulate the immune system (55). These factors may also contribute to the different patterns of soluble mediator amounts we observed in fourth- versus third-generation e-cigarette users and warrant investigation in future studies.

Although our study provides novel insight into differences in airway biomarkers of immune homeostasis between exposure groups, there are limitations to our study that are important to consider and that warrant exploration in future studies. Beyond categorizing subjects as current e-cigarette users of a specific device type, we did not collect additional information about e-cigarette use parameters, such as flavor, the number of puffs per day, power/heat settings (for third-generation e-cigarette users), previous smoking history, or length of e-cigarette use for all subjects. These variations in e-cigarette use patterns may have contributed to the high variability we observed within each of our e-cigarette groups. Future studies with larger cohort sizes and more detailed e-cigarette use questionnaires will be required to control for this variation and to more fully elucidate factors driving respiratory effects observed in e-cigarette users. In addition, larger cohort sizes would allow for more aggressive thresholding for the percentage of squamous epithelial cells in induced sputum samples and provide sufficient statistical power for applying a more stringent multiple test correction threshold, improving overall study quality and reproducibility. Because our average squamous epithelial cell percentage was slightly higher than ideal, our findings may represent both upper and lower airway inflammatory changes, and because we set a less conservative q-value of 0.2 as a threshold for reporting associations, there is potential for some of our results to be false positives.

Larger cohorts would also allow for stratification by sex and exploration of sex differences, as females were underrepresented in our vaping groups and overrepresented in our nonsmoking and smoking groups. Although we adjusted for sex in our analysis, it is still possible that the results presented in this study may be biased toward male vapers. Future studies examining sex differences in response to e-cigarette exposure are needed. Furthermore, as a crosssectional observational study, our results are primarily associative and hypothesis-generating. Prospective studies that collect and analyze samples from e-cigarette users over time will be required to more fully understand the respiratory effects associated with e-cigarette use. In addition, analyzing BAL cellular composition and soluble mediator amounts would provide complementary data to the findings presented here.

Conclusions

Taken together, our data demonstrate that there are significant differences in biomarkers of respiratory immune homeostasis in fourth-generation e-cigarette users compared with nonsmokers/nonvapers and with third-generation e-cigarette users, with an overall phenotype of suppression of immune biomarkers. Our findings highlight the importance of considering device type in studies of e-cigarette inhalation toxicity, the utility of leveraging multiple analysis approaches to understand differences between exposure groups, and the need for continued investigation of the mechanisms underlying the effects of popular e-cigarette devices, including those that have recently been authorized for use by the FDA. ■

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