Brief Communications

Electronic nicotine delivery system-induced alterations in oral health via saliva assessment

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Impact statement

The use of traditional tobacco products is a known risk factor for the development of diseases including periodontal disease. To date, the potential oral health effects related to electronic nicotine delivery systems (ENDS) use is unknown. This study collected saliva from ENDS users and never tobacco users to examine differences in the oral cavity of inflammatory cytokines and metabolites. The identification and measurement of these ENDS-related changes provide insight into disease pathways potentially associated with ENDS use. The utilization of saliva samples collected from human participates enhances the application of the findings compared to the majority of studies using cell culture and animal models. In addition, these foundational findings can inform future studies to examining specific pathways identified, interventional approaches, and application of translatable biomarkers of ENDS use.

Abstract

Use of electronic nicotine delivery systems (ENDS) is becoming increasingly prevalent. ENDS aerosols contain a variety of toxic components that may adversely impact health. Although exposure to traditional cigarette smoke is a risk factor for periodontal disease, the effects of ENDS on oral health have not been adequately examined. To evaluate potential oral health effects associated with ENDS use, a pilot study was performed with 14 current ENDS users and 16 never tobacco users. Participants completed questionnaires about their ENDS use and overall health. Saliva samples were assessed for differential biomarkers of inflammation, toxicity, and disease development. This included evaluation of specific inflammatory cytokines and the global assessment of alterations in metabolites. ENDS users were determined to have elevated saliva levels of interleukin-1β and tumor necrosis factor-α indicative of inflammation. Metabolite profiling determined 368 metabolites were differentially expressed in the saliva of ENDS users versus never tobacco users. Cotinine, the primary metabolite of nicotine, was the most significantly altered metabolite between the groups. Increased levels of prostaglandins and leukotrienes indicated that ENDS users exhibited increased arachidonic acid metabolism compared to never tobacco users. Additionally, a variety of other metabolites known to be involved in immune signaling such as gangliosides, ceramides, angiotensin, and others were also different between groups. Overall, our pilot study demonstrates differential saliva component profiles in current ENDS users, which may contribute to periodontal disease development. These alterations suggest specific pathways of oral disease induced by ENDS use and could be utilized as potential future biomarkers.

Keywords: Oral health, saliva, metabolite profiling, inflammation, smoking, electronic nicotine delivery systems, e-cigarettes, periodontal disease, biomarkers

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Introduction

The prevalence of electronic nicotine delivery systems (ENDS) use has increased in recent years, especially among middle and high school students.[1] Use of traditional tobacco products (e.g. smoking combustible cigarettes) is known to increase the risk of cancer, cardiopulmonary and metabolic diseases, and a plethora of other health consequences.[2] However, much less is known about the health effects of ENDS. ENDS are perceived to be less harmful

than conventional cigarettes due to the number and quantity of chemicals released in the aerosol.[3] However, aerosols generated by ENDS still contain toxic components such as metal nanoparticles, formaldehyde, diacetyl, acrolein, acetaldehyde, acetone, and others.[4] Inhalation of these components through cigarette smoke has been shown to have detrimental cardiovascular, pulmonary, and oral health effects.[5] The limited investigations of oral health effects of ENDS use have demonstrated altered oral microbiomes as well as increased risks of periodontal, dental, and gingival health effects.[6] These evaluations have primarily focused on the presence of clinical symptoms without in-depth examination of toxicological mechanisms of disease progression.

Metabolite profiling provides a comprehensive assessment of physiological function that is useful in the identification of toxicity concerns and establishment of biomarkers of exposure and disease. Because of our limited knowledge of oral health effects and pathways of toxicity associated with ENDS use, a broad examination of molecular alterations is warranted. Saliva is an easily assessible biological fluid in the oral cavity, which is both the site of exposure and potential diseases. Saliva has been utilized to uncover novel and useful biomarkers of initial biological events that can be used to identify potential disease risk.[7] Specifically, analysis of metabolites in saliva following smoking combustible tobacco products determined distinct metabolites that had been altered, suggesting pathways of disease.[8] Previous evaluations have used saliva from ENDS users to establish the presence of ENDS aerosol components and nicotine metabolites to determine exposure.[9] However, data are currently lacking in regards to the global assessment of alterations in endogenous metabolites due to ENDS use.

Here we present findings from a pilot study designed to examine the use of a global metabolite profiling approach to determine modifications in saliva samples between ENDS users and never tobacco users. During a single visit, participants completed questionnaires about their ENDS use and perceptions of their overall health. Saliva samples were examined via assessment of inflammatory cytokines and metabolite profiling. These initial findings represent novel data that can be built upon in future studies to elucidate biological responses and risks associated with ENDS use in the oral cavity.

Materials and methods

Participants

Recruitment of participants occurred via posted flyers on Purdue University's campus and at local shops selling ENDS products. Fourteen current ENDS users and 16 never tobacco users volunteered to participate. The sample sizes for our groups are consistent with other studies utilizing metabolite profiling to investigate oral health effects.[10,11] The Institutional Review Board at Purdue University reviewed and approved all experimental procedures, and verbal and written informed consent were obtained from all participants. The participants reported to the laboratory on a single occasion where they filled out questionnaires to understand individual demographics (age, race, alcohol use, etc.), perceived overall health, and ENDS usage (device, duration of use, flavor, previous tobacco use history, etc.). Eligible participants self-identified as healthy, between the ages of 18–40, and denied having recent dental surgery or oral disease. Individuals in the never tobacco user group indicated that they had never used ENDS or smoked tobacco products. Individuals in the ENDS group indicated that they currently used ENDS daily and either (1) only used ENDS or (2) primarily used ENDS with occasional use of conventional cigarettes. All participants were requested to refrain from eating and drinking for at least 1 h prior to their visit.

Saliva collection

Participants rinsed their mouths out with water twice prior to saliva collection. Whole saliva samples were collected into 2 mL cryovials using a saliva collection aid (Salimetrics, State College, PA) via the passive drool method. Two aliquots of saliva were stored at −80°C. One aliquot was utilized for metabolite profiling and the other for inflammatory cytokine analysis. This resulted in each aliquot only undergoing a single freeze-thaw cycle prior to analysis.

Cytokine analysis

Saliva levels of the inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) were examined by Salimetrics using electrochemiluminescent assays validated for saliva. Each sample was assessed in duplicate, producing two technical replicates per endpoint. Results are expressed as mean values ± S.E.M. with $n = 16$ for never tobacco users and $n = 14$ for ENDS users. All statistical analyses of cytokines were performed in GraphPad Prism 8 (GraphPad, San Diego, CA). Student two-tailed between-groups *t*-tests were used to determine significant differences between groups, $P < 0.05$.

Metabolite profiling assessment

Saliva samples were prepared for metabolite profiling similar to previous studies.[12] Briefly, protein removal and sample extraction was performed by adding acetonitrile to saliva. Following mixing and centrifugation, supernatants were evaporated to dryness in a vacuum concentrator. The aqueous global metabolomics experiments were performed on an Agilent HPLC 1290 system (Palo Alto, CA), using a Waters HSS T3 column and mass data (from m/z 70–1000) were collected using Agilent MassHunter Acquisition software (v. B.06). Raw data obtained from LC-MS analysis were imported into Agilent's MassHunter Profinder (v. B.06), where unique features were extracted and peak alignment was carried out based on m/z, retention time, isotopic ratio, and grouping of different adducts. Significance analysis was performed using Agilent's Mass Profiler Professional by performing an

unpaired *t*-test with Benjamini–Hochberg FDR correction. Peak annotations were performed using the METLIN (www.metlin.scripps.edu) metabolite databases (mass error < 30 ppm). Metabolites with $P < 0.05$, as determined by t-tests, were considered statistically different between never tobacco users and ENDS users. All metabolites determined to differ significantly between groups along with FDRs, calculated fold changes, and *P*-values can be found in Supplemental Table 1.

Results and discussion

Participant characteristics and self-reported health ratings are shown in Table 1. ENDS users were younger and reported higher alcohol use than never tobacco users. ENDS users also self-reported lower overall health and overall oral health compared to never tobacco users. Consistent with our findings, ENDS users were recently determined to be twice as likely to suffer from periodontal disease and three times as likely to have gingival disease than individuals that did not use ENDS or conventional cigarettes.[13,14] ENDS usage has also been demonstrated in studies to be associated with a number of oral symptoms such as dry mouth, irritation, bad breath, and others.[6] These oral symptoms related to ENDS use, however, have been shown to be reduced compared to conventional cigarette smoking.[6] Although no participants reported living with a smoker, one participant in the never tobacco group reported secondhand smoke exposure, while six participants in the ENDS user group reported secondhand smoke exposure. No participants in either group were on medications for oral health and only one participant from the ENDS user group reported an immediate family member with previous history of oral cancer.

ENDS users primarily used JUUL (10-JUUL, 2-Suorin, 1-Smok, and 1-VOOPOO). This is consistent with the other studies demonstrating that JUUL is currently the most popular ENDS on the market.[15] It is likely that ENDS users may also smoke conventional cigarettes. Nine participants stated that they only utilized ENDS, while five stated primary use of ENDS with occasional use of conventional cigarettes. The nicotine concentration in the e-liquid used was reported by 12 ENDS users as 5%, while 2 did not respond to the question. Among ENDS users, two reported having used ENDS for 1–3 months, one for 4–6 months, three for 7–12 months, six for 1–2 years, and two for over 2 years. The average self-reported frequency of ENDS use was 14.64 ± 11.25 times a day (mean $\pm$ SD) (mode 30, median 11.25). Of the 14 ENDS users, 11 reported that mint was their most utilized flavor, while 3 reported fruit.

Smoking traditional tobacco products is known to cause inflammation within the oral cavity.[16] To determine ENDS-associated inflammation, cytokines were measured in saliva samples. No differences in saliva levels of IL-6 or IL-8 were observed between never tobacco users and ENDS users (Figure 1(a) and (b)). ENDS users demonstrated elevated levels of IL-1β and TNF-α compared to never tobacco users (Figure 1(c) and (d)). This is consistent with previous findings demonstrating that ENDS aerosols cause inflammation in cell culture and animal models.[17] Increased inflammation within the oral cavity is a risk factor for the development of periodontitis. Specifically, studies have demonstrated that IL-1β and TNF-α contribute to soft tissue destruction in periodontal disease.[18] IL-1β and TNF-α contribute to increased production of matrix metalloproteinases that are considered biomarkers of periodontal disease due to their role in tissue destruction.[19,20] Further, elevations have been observed in IL-1β and TNF-α levels in the peri-implant sulcular fluid of ENDS users compared to never tobacco users which correlated with bleeding upon probing and peri-implant bone loss.[21] Alterations in inflammatory cytokine levels were not determined to correlate with duration of ENDS use or occasional use of conventional cigarettes.

To examine alterations in saliva metabolites in ENDS users, we utilized a metabolite profiling approach. Principal component analysis was employed to determine differential grouping of ENDS users compared to never tobacco users based on all metabolites assessed (Figure 2(a)). Five ENDS users were determined to group with never tobacco users. The questionnaires were examined and no similarities with regard to their characteristics (age, sex, brand usage, flavor, etc.) were observed for these five ENDS users or substantial differences compared to the other nine ENDS users. Global metabolite profiling identified 368 metabolites that were significantly different ($P < 0.05$) in the saliva between never tobacco users and ENDS users (Supplemental Table 1). Of these 368, 227 were higher and 141 were lower. A total of 90 were different by a fourfold or greater change (58 higher and 32 lower).

Table 1. Participant characteristics and self-reported health assessments.

	Never tobacco users	ENDS users	*P*-value
Total *n*	16	14	–
Males (%)	9 (56.3)	9 (64.3)	–
Females (%)	7 (43.7)	5 (35.7)	–
Age in years (Mean ± SD)	23.25 ± 3.62	20.36 ± 1.82	0.0117
Alcohol use days/week (Mean ± SD)	0.36 ± 0.56	1.11 ± 0.94	0.0112
Overall health (Mean ± SD) (Scale 1–10; with 10 being the best)	8.72 ± 0.86	7.82 ± 0.91	0.0096
Overall oral health (Mean ± SD) (Scale 1–10; with 10 being the best)	8.53 ± 1.18	7.36 ± 1.15	0.0102

Note: Self-reported information from recruited never tobacco users and current ENDS users. Questionnaires were utilized to determine sex, age, alcohol use, overall health, and overall oral health. Values represent means ± standard deviation, while *P*-values were calculated using student two-tailed *t*-tests.

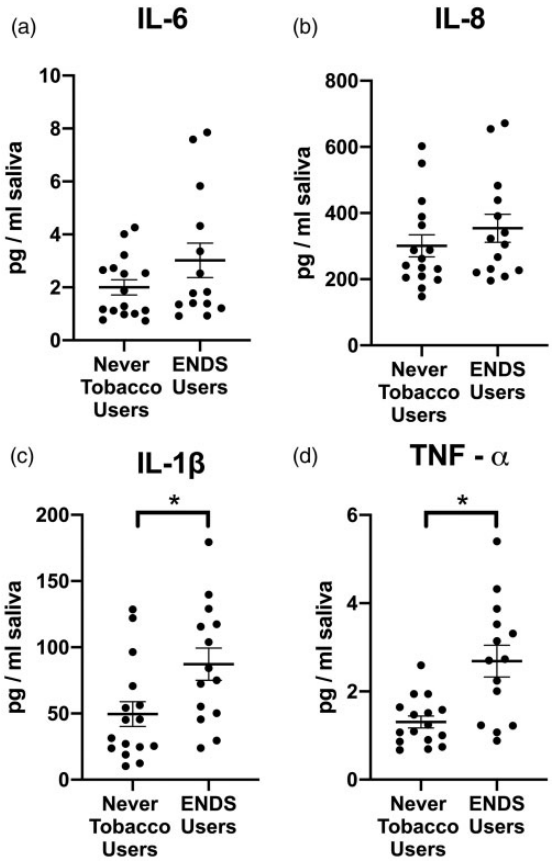

Figure 1. Assessment of inflammatory cytokine levels in saliva samples collected from never tobacco users and ENDS users including (a) interleukin-6 (IL-6), (b) interleukin-8 (IL-8), (c) interleukin-1β (IL-1β), and (d) tumor necrosis factor-α (TNF-α). Values are expressed as mean $\pm$ SEM, with an $n = 16$ for never tobacco users and an $n = 14$ for ENDS users. *Denotes significance between groups ($P < 0.05$).

Unsupervised hierarchical clustering of these metabolites for each participant demonstrated grouping of never tobacco users and ENDS users (Figure 2(b)). This demonstrates that ENDS use was associated with different metabolite profiles. The most significantly different metabolite between groups was cotinine, the primary metabolite of nicotine, which was elevated in ENDS users 120.28 fold compared to never tobacco users (Table 2).[22] Other metabolites of nicotine were also found within the saliva such as anabasine, which was elevated 72.37 fold in ENDS users (Table 2). The presence of cotinine and anabasine in saliva are indicators of ongoing nicotine exposure, which confirms participants' self-reported ENDS usage.

Metabolite profiling determined that arachidonic acid metabolism was different in ENDS users compared to never tobacco users. Specifically, significant elevations in prostaglandin G2 (3.21 fold), 16-phenoxy tetranor prostaglandin E2 (4.59 fold), and 17-phenyl trinor-13,14-dihydro prostaglandin A2 (14.79 fold) were observed in ENDS users compared to never tobacco users (Table 2). S-(PGA1)-glutathione was lower (0.41 fold), suggestive of decreased metabolism of prostaglandin A1 in ENDS users compared to never tobacco users. Additionally, ENDS users demonstrated higher amounts of leukotriene D4 methyl ester (3.80 fold) and leukotriene E4 (3.35 fold) (Table 2). Together, these differences suggest higher metabolism of arachidonic acid via cyclooxygenases and lipooxygenases leading to production of prostaglandins and leukotrienes, respectively. Elevations in these inflammatory mediators have been observed in patients with periodontitis and are involved in pathogenesis of the disease.[23,24] Further, similar to our findings, another study examining saliva metabolites found that people with periodontal disease had altered arachidonic acid metabolism compared to healthy individuals,

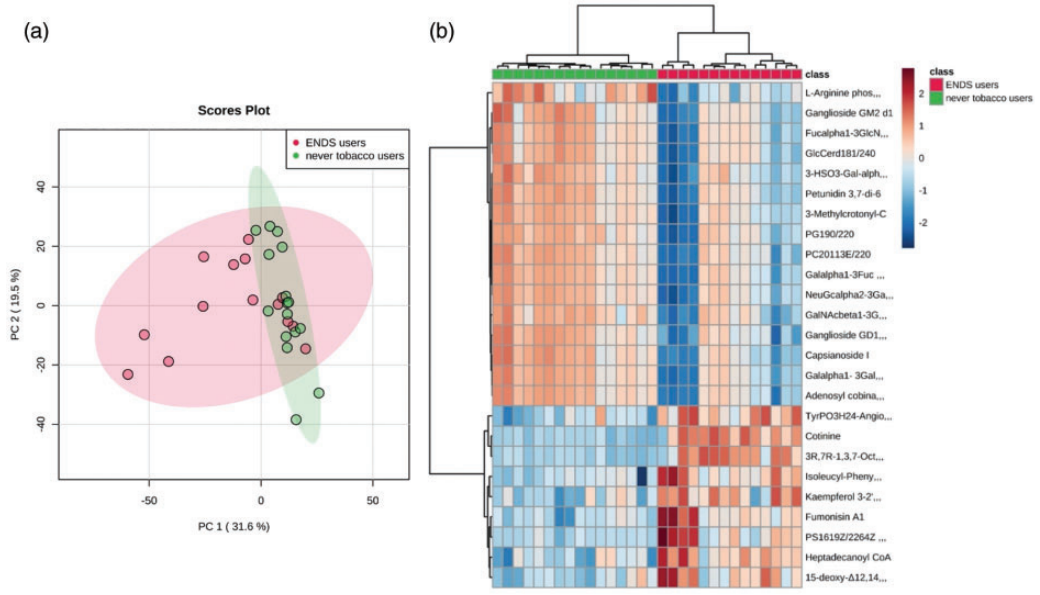

Figure 2. Alterations in metabolites were identified between never tobacco users and ENDS users. (a) Principal component analysis demonstrating grouping of study participants based on all identified metabolites. (b) Heat map of the 33 most significantly altered saliva metabolites between never tobacco users and ENDS users. The heat map was produced via unsupervised hierarchical clustering demonstrating levels of distinct metabolites within each participant's saliva sample. Red denotes increased expression of a metabolite compared to the median of all samples, whereas blue denotes decreased expression of a metabolite compared to the median.

Table 2. Comparison of saliva metabolites between current ends users and never tobacco users.

Metabolite	Fold change	*P*-value
Cotinine	120.28	7.05×10^{-10}
Anabasine	72.37	1.03×10^{-8}
Prostaglandin G2	3.20	0.02
16-phenoxy tetranor prostaglandin E2	4.59	4.31×10^{-3}
17-phenyl trinor-13,114-dihydro prostaglandin A2	14.79	0.03
S-(PGA1)-Glutathione	0.41	5.62×10^{-3}
Leukotriene D4 methyl ester	3.80	9.17×10^{-3}
Leukotriene E4	3.35	0.02
Ganglioside GM3 (d18:0/16:0)	3.12	5.94×10^{-4}
Ganglioside GM1 (18:1/9Z-18:1)	0.23	6.51×10^{-4}
Glutathionyl spermine	3.10	7.79×10^{-4}
Angiotensin II	1.71	0.04
Phosphotrosyl-angiotensin II	2.98	9.81×10^{-4}
Pentosidine	3.19	8.83×10^{-4}
Reduced coenzyme F420	3.74	3.66×10^{-4}

Note: Alterations in representative metabolites within the saliva of ENDS users and never tobacco users. Positive fold changes indicated the increased presence of the metabolite in the saliva of ENDS users compared to never tobacco users, whereas negative fold changes indicate less. *P*-values were determined by two-tailed Student's *t*-tests. Data for all metabolites determined to be significantly altered ($P < 0.05$) can be found in Supplemental Table 1.

suggesting that elevations in prostaglandins could be used as biomarkers of periodontal disease.[25] ENDS aerosol exposure increases mouse pulmonary susceptibility to bacterial infections via impairment of the immune system.[26] Our pilot study identified significant differences in 12 gangliosides (all 2 fold or more) between ENDS users and never tobacco users (Supplemental Table 1). Specifically, five gangliosides were higher and seven were lower among ENDS users. Gangliosides are a subclass of sphingolipids involved in recognition of pathogens such as bacteria and facilitate interactions with immune cells.[27] Specifically, ganglioside GM3 was elevated in our study (3.12 fold) in ENDS users and has been shown to be elevated in periapical oral lesions (Table 2).[28] Ganglioside GM1 was also lower in ENDS users (0.23 fold) compared to never tobacco users, and reductions in Ganglioside GM1 have been shown to inhibit wound repair in an airway epithelial cell model of cystic fibrosis (Table 2).[29] It is likely that these differences in gangliosides associated with ENDS usage may modulate susceptibility to bacterial infections and thereby development of periodontal disease.

Our evaluation demonstrated higher glutathionyl spermine (3.10 fold) in ENDS users, which is a product of glutathione mediated spermine metabolism (Table 2). Spermine inhibits inflammation at sites of injury and infection.[30] The removal of spermine via glutathione metabolism, as indicated by increased saliva levels of glutathionyl spermine, may contribute to enhanced inflammation as observed by analysis of cytokine levels. Other inflammatory markers such as angiotensin II (1.71 fold), phosphotrosyl-angiotensin II (2.98 fold), and others (sphingolipids, glycerophospholipids, ceramides) were elevated in ENDS users (Table 2 and Supplemental Table 1). Many of these metabolites are known to be increased in the circulation of tobacco smokers and also involved in periodontal disease development.[31,32] Further, pentosidine, a marker of advance glycation end products, was higher in the ENDS user group (3.19 fold) (Table 2). Advanced glycation end products are known to be involved in tobacco-induced inflammation and the development of periodontal disease.[33,34] Lastly, the build-up of bacteria in the oral cavity leads to the development of periodontal disease.[35] Our data demonstrate elevations in reduced coenyzme F420 (3.74 fold) in ENDS users, which is a marker of the presence of methanogenic Archaea (Table 2).[36] The presence of methanogenic archaea has been found to correlate with the severity of periodontal disease.[37]

In conclusion, the results from this pilot study demonstrate differences in components of saliva including inflammatory cytokines and metabolites in ENDS users compared to never tobacco users. The participants recruited for this study are younger than those typically diagnosed with periodontal disease.[38] Alterations observed within our pilot study demonstrate ongoing inflammatory processes. This is consistent with gingival inflammation, which is known to precede development of periodontal disease.[39] Therefore, these alterations in saliva components potentially could be utilized as indicators of oral disease risk and not as diagnostic markers. A strength of our study is that the profiling approach was able to determine a panel of molecular entities modified in the saliva of ENDS users that could be utilized in upcoming studies or for future clinical applications to classify disease risk. It is unlikely that a single entity will be specific for ENDS-induced oral disease; however, a panel of markers may be utilized to identify those at risk. This pilot study is limited by small sample size, decreasing our ability to address more nuanced questions about variations in ENDS devices, duration of ENDS use, dual use of ENDS and traditional cigarettes, and other individual characteristics that might relate to oral health. However, results suggest specific mechanisms to investigate in our upcoming expanded studies that will focus on specific immune signaling (gangliosides, arachidonic acid metabolism, etc.) associated with the development of periodontal disease, variations in ENDS products and usage, and potential differences by sociodemographic characteristics.

AUTHORS' CONTRIBUTIONS

All authors participated in interpretation and analysis of data as well as writing and editing. SA carried out the research and along with BC performed the bioinformatics. CW and JS conceptualized and supervised the study.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

Study was approved by the Purdue Institutional Review Board (Protocol: 1906022340).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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