Journal

Nicotine & Tobacco Research, 25(6)

ISSN

1462-2203

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Publication Date

2023-05-22

DOI

10.1093/ntr/ntad003

Vaping dose, device type, and e-liquid flavor are determinants of DNA damage in electronic cigarette users

Running title: *Vaping & DNA damage*

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Abbreviations

8-OH-dG, 8-hydroxydeoxyguanosine; BMI, body mass index; CO, carbon monoxide; COHb, carboxyhemoglobin; Cum e-liq; cumulative e-liquid consumed; e-cig, electronic cigarette; ELISA, enzyme-linked immunosorbent assay; *HPRT*, hypoxanthine phosphoribosyltransferase 1 gene; IRB, Institutional Review Board; LA-QPCR; long-amplicon quantitative polymerase chain reaction; PG, propylene glycol; *POLB*, DNA polymerase beta gene; ppm, parts per million; PY, pack year; ROS, reactive oxygen species ; VG, vegetable glycerin.

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Abstract

Background: Despite the widespread use of electronic cigarettes, the long-term health consequences of vaping are largely unknown. **Methods:** We investigated the DNA-damaging effects of vaping as compared to smoking in healthy adults, including *'exclusive'* vapers (never-smokers), cigarette smokers only, and non-users, matched for age, gender, and race (N=72). Following biochemical verification of vaping/smoking status, we quantified DNA damage in oral epithelial cells from the study subjects, using long-amplicon quantitative polymerase chain reaction assay. **Results:** We detected significantly increased levels of DNA damage in both vapers and smokers as compared to non-users (P = 0.005 and P = 0.020, respectively). While the mean levels of DNA damage did not differ significantly between vapers and smokers (P = 0.522), damage levels increased dose-dependently, from light users to heavy users, in both vapers and smokers as compared to non-users. Among vapers, pod users followed by mod users, and those who used sweet-, mint/menthol-, and fruit-flavored e-liquids, respectively, showed the highest levels of DNA damage. The nicotine content of e-liquid was not a predictor of DNA damage in vapers. **Conclusions:** This is the first demonstration of a dose-dependent formation of DNA damage in vapers who had never smoked cigarettes. Our data support a role for product characteristics (device type and e-liquid flavor) in induction of DNA damage in vapers, which should further be validated in future large cohorts. Given the popularity of pod and mod devices and the preferability of sweet-, mint/menthol-, and fruit-flavored e-liquids by both adult- and youth vapers, our findings may have significant implications for public health and tobacco products regulation.

Keywords: electronic cigarettes (e-cigs), DNA damage, flavor, genotoxic, nicotine, vaping.

Implications

We demonstrate a dose-dependent formation of DNA damage in oral cells from vapers who had never smoked tobacco cigarettes as well as exclusive cigarette smokers. Device type and e-liquid flavor determine the extent of DNA damage detected in vapers. Users of pod devices followed by mod users, and those who use sweet-, mint/menthol-, and fruit-flavored e-liquids, respectively, show the highest levels of DNA damage when compared to non-users. Given the popularity of pod and mod devices and the preferability of these same flavors of e-liquid by both adult- and youth vapers, our findings may have significant implications for public health and tobacco products regulation.

Introduction

Electronic cigarette (e-cig) use, otherwise known as *'vaping'*, is highly popular among adolescent never-smokers and adult smokers seeking a less-harmful alternative to tobacco cigarettes [1-3]. E-cigs are handheld battery-powered devices, which exploit 'heating' of a liquid to produce vapor for inhalation [4,5]. The liquid, also called 'e-liquid/e-juice', is a mixture of propylene glycol (PG), glycerin/vegetable glycerin (VG), flavors, and varying concentrations of nicotine, although nicotine-free e-liquid is also available [6,7]. Chemical analysis has shown that many of the same toxicants and carcinogens present in cigarette smoke are also found in e-cig vapor, albeit mostly at substantially lower levels [5-7]. E-cig vapor also contains chemicals that are not detected in cigarette smoke [8,9]. The latter compounds likely arise from mixing of the e-liquid ingredients and/or vaporization of humectants (PG/VG), flavorings, or chemicals leached from the device components [10,11]. To date, however, the long-term health consequences of vaping are largely unknown [12,13].

Many toxic and carcinogenic chemicals present in e-cig vapor or cigarette smoke exert their biological effects through induction of DNA damage leading to mutagenesis and genome instability [5,14]. DNA damage has been implicated in a wide variety of tobacco-related diseases, including cancer in multiple organ sites [15]. Quantification of DNA damage in cells and tissues of e-cig users *vs*. cigarette smokers can help determine the genotoxic potential of vaping relative to smoking. In the present study, we have compared the DNA damaging effects of vaping to smoking by measuring the level of DNA lesions in oral cells of e-cig users and cigarette smokers as compared to non-users. The study population consisted of healthy adult *'exclusive'* vapers (never-smokers), cigarette smokers only, and non-users of any tobacco products, matched for age, gender, and race. We have used the extensively validated and highly sensitive long-amplicon quantitative

polymerase chain reaction (LA-QPCR) assay [16] to quantify DNA damage in oral epithelial cells collected by brushing from our study subjects. Of significance, oral epithelium is a major target for cancer and other diseases associated with tobacco product use [17]. Moreover, we have investigated the influence of use frequency and duration (*i.e.*, vaping/smoking dose) on the induction of DNA damage in e-cig users and cigarette smokers. Among e-cig users, we have also determined the impact of product characteristics, including device type and e-liquid flavor and nicotine content, on the extent of DNA damage detected. In addition, we have biochemically verified the vaping/smoking status of the study subjects by measuring plasma cotinine, a major metabolite of nicotine, exhaled carbon monoxide (CO), and carboxyhemoglobin (COHb) levels [6].

Methods

Ethics declarations

The study was approved by the Health Sciences Institutional Review Board (IRB) of the University of Southern California (Protocol No: HS-16-00175). Written informed consent was obtained from participants prior to inclusion in the study. All research was performed in accordance with the approved IRB protocol and relevant guidelines & regulations, including the Declaration of Helsinki.

Study population

Eligible candidates for the study included healthy adults — both males and females of diverse ages, races, and ethnicities — who could read and write in English and understand and give informed consent. The catchment area for this study was the Greater Los Angeles Area. The study population consisted of 72 subjects divided equally into three groups, including Group 1: current

exclusive vapers (never-smokers); Group 2: current exclusive smokers; and Group 3: nonsmokers non-vapers (non-users). Detailed characteristics of the study population are listed in Table 1. Dual users of e-cigs and combustible cigarettes, poly users of e-cigs, cigarettes, or other tobacco products, and former smokers or vapers were excluded from the study. Criteria for classification of the study subjects as vapers, cigarette smokers, or non-users, consistent with national surveys [18], were as follows: vapers were those who reported current use of e-cigs for at least three times a week for a minimum of six months, and no use of combustible cigarettes or any other tobacco products in their lifetime. Smokers were those who reported current smoking of tobacco cigarettes at least three times per week for a minimum of one year, and no or less than five vaping sessions in their lifetime and no use of tobacco products (except for combustible cigarettes) in the past six months. Non-users were those who reported no use of any tobacco product (e-cigs or tobacco cigarettes) more than five times in their life; non-users reported smoking no or fewer than 100 cigarettes or having no or less than five vaping sessions in their lifetime (no vaping or smoking in the past six months). We note that participants in this study had equal opportunity to self-identify as former smokers or vapers, and participate in other existing studies in our laboratory. This is important in view of the fact that all of our study participants underwent stringent screening and comprehensive personal interviews complemented with biochemical verification of vaping/smoking status. Altogether, our enrollment strategy, inclusion/exclusion criteria, and verification analysis have ensured reliable and accurate classification of the study subjects in this report. We note that unlike combustible cigarettes that have been in the market for many years, e-cigs are a relatively new tobacco product [5-7]. Thus, we set the minimum use criteria for vapers and smokers to six months and one year, respectively, to allow enrollment of sufficient number of subjects into this study. More detailed information about subject recruitment and enrollment,

inclusion/exclusion criteria, and sample collection and processing are provided in refs. [17,19] and Supplementary Materials.

Quantification of DNA damage by LA-QPCR

LA-QPCR quantification of DNA damage was performed as described in ref. [16], with few modifications. Our LA-QPCR analysis interrogated a 12.2 kb region of the DNA polymerase beta *(POLB)* gene [16,20,21]. For validation purpose, we also interrogated an additional gene target, hypoxanthine phosphoribosyltransferase 1 (*HPRT*). We used the same protocol to amplify a 10.4 kb fragment encompassing exons 2–5 of the *HPRT* gene [16,20,21]. Detailed information about the LA-QPCR assay, description of the protocol, and quantification of the results are provided in Supplementary Materials.

Cotinine measurement by ELISA

Plasma cotinine was measured by a solid-phase competitive enzyme-linked-immunosorbent assay (ELISA) (Abnova Corp.) (Supplementary Materials).

CO and COHb quantification by breath monitor

Exhaled CO levels and %COHb were measured using the Bedfont Micro^{+TM} Smokerlyzer® according to the manufacturer's instructions (Bedfont Scientific Ltd.) (Supplementary Materials).

Statistical analysis

Distribution of data was evaluated by the Shapiro-Wilk test. Results are expressed as mean $\pm$ SE. Comparisons of all variables between two groups were performed by the Student's *t*-test.

Specifically, DNA damage levels between two independent groups, namely vapers and non-users, smokers and non-users, or vapers and smokers, were compared by the Student's *t*-test. To compare variables in three or more groups, we used One-Way Analysis of Variance (ANOVA) followed by post hoc Tukey HSD test. The multi-group comparison by ANOVA was used to compare damage levels among heavy vapers, light vapers, and non-users, as well as heavy smokers, light smokers, and non-users. Similarly, we used this multiple group comparison to assess DNA damage levels in vapers who used different devices or e-liquids and non-users. Relationships between different variables were examined by Pearson correlation coefficient analysis. Other statistical tests used are specified in the text. All statistical tests were two-sided. P values < 0.05 were considered statistically significant. All statistical analyses were performed using the R environment for statistical computing, available at RStudio (https://rstudio.com/), which is a free and open-source software.

Results

DNA damage quantification

As shown in Figure 1, both vapers and smokers had significantly higher levels of DNA damage in their oral cells as compared to non-users. There were 2.6- and 2.2-fold increases, respectively, in mean levels of DNA damage in the *POLB* gene in the oral cells of vapers and smokers as compared to non-users ($P = 0.005$ and $P = 0.020$, respectively). The levels of DNA damage in the *POLB* gene in vapers' oral cells were not statistically significantly different from those in smokers ($P = 0.522$) (Fig. 1A-C). To validate these results, we have used a subset of samples from which extra DNA was available (N=36 total, 12 per matched group) for LA-QPCR analysis of an independent gene target (*HPRT*). Similar to LA-QPCR results in the *POLB* gene, the levels of DNA damage in

the *HPRT* gene, as quantified by LA-QPCR, were significantly higher in both vapers and smokers as compared to non-users ($P = 0.029$ and $P = 0.033$, respectively). Furthermore, DNA damage levels in the *HPRT* gene in vapers were not significantly different from those in smokers ($P = 0.578$) (Fig. 1D-F). In addition, there was a significant correlation between DNA damage levels in the *POLB* and *HPRT* genes in the tested samples ($r = 0.647$, $P < 0.0001$). Given the confirmatory results of LA-QPCR in the *HPRT* gene, we have performed in-depth analysis of DNA damage data in the larger samples that were assayed for the *POLB* gene (N=72). This was to allow more meaningful comparison of variables among sub-groups of vapers and smokers relative to non-users. Henceforth, the following sections exclusively present the results and discussion of DNA damage data obtained by LA-QPCR in the *POLB* gene.

To examine the dose-dependency of the induced DNA damage in vapers and smokers, we have further analyzed the DNA damage data (in the *POLB* gene) using indicators of intensity and duration of e-cig- and cigarette use, expressed as cumulative e-liquid (cum e-liq) and pack year, respectively. Whereas cumulative e-liquid consumption was calculated as the total volume of e-liquid (in milliliter) vaped by a person during his/her lifetime, pack year was computed by multiplying the number of packs of cigarette a person smoked per day by the number of years he/she smoked [22]. Using the 2nd quartile as cutoff point, we have divided both the vapers and smokers in two groups, including 'light' and 'heavy' users. As illustrated in Figure 2A, levels of DNA damage increased dose-dependently, from light vapers to heavy vapers, as compared to non-users ($F = 4.571$, $P = 0.0156$ | Tukey's HSD $P = 0.0195$). A similar trend was found in smokers wherein DNA damage levels were increased from light smokers to heavy smokers when compared to non-users ($F = 4.368$, $P = 0.0185$ | Tukey's HSD $P = 0.0135$) (Fig. 2B).

To investigate the influence of device type on the extent of DNA damage detected in e-cig users, we have divided the vapers into three groups, including users of third generation devices (*'mod'*), users of 'fourth' generation devices (*'pod'* = JUUL and JUUL alike), and users of *'multiple'* generation devices. We note that except for one individual who was exclusive user of 'Cigalike' devices, all vapers in our study were users of third or fourth generation devices or users of multiple generation devices (Table 1). As shown in Table 2, users of pod-based devices had the highest levels of DNA damage in their oral cells as compared to non-users, followed by users of mod-based devices and multiple device users. There was 3.3-fold increase in mean level of DNA damage in the oral cells of pod users as compared to non-users ($F = 3.886$, $P = 0.0152$ | Tukey's HSD $P = 0.0216$). Mod users and multiple device users showed 2.6-fold and 1.6-fold increases, respectively, in mean levels of DNA damage in their oral cells as compared to non-users. One may argue that the extent and duration of use of different generation devices might modulate the levels of DNA damage in pod users *vs.* mod users *vs.* multiple device users. To account for these factors, we have adjusted the DNA damage data in vapers based on (I) 'cumulative e-liquid consumed' and (II) 'years vaped' per device(s). In both cases, the adjusted data showed a similar pattern of highest mean levels of DNA damage in pod users, followed by mod users and multiple device users (Table 2).

Furthermore, we investigated the impact of chemical composition of e-liquid on the induction of DNA damage in e-cig users. To determine the role of e-liquid flavors, we have categorized the e-liquid flavors consumed by our study subjects, and divided the vapers into five groups, including those who used e-liquid with (1) fruit flavors; (2) candy/desserts/other sweet flavors [hereinafter referred to as 'sweet' flavors]; (3) mint/menthol flavor; (4) tobacco flavor; and (5) multiple flavors. As shown in Table 2, vapers of sweet-flavored e-liquids had the highest levels

of DNA damage in their oral cells as compared to non-users ($F = 3.238$, $P = 0.0146$ | Tukey's HSD $P < 0.05$), followed by vapers of multiple e-liquid flavors, mint/menthol flavor and tobacco flavor, and fruit-flavored e-liquids. Adjusting the data for (I) 'cumulative e-liquid consumed' and (II) 'years vaped' per flavor(s), vapers of sweet-flavored e-liquids still showed the highest mean level of DNA damage, followed by vapers of mint/menthol- and fruit-flavored e-liquids (Table 2). To substantiate these results, we have further scrutinized the data, performed additional tests, and conducted statistical analyses with or without certain sub-groups. More specifically, we have demonstrated that exclusion of small sub-groups, such as "tobacco" flavor e-liquid users, from the analysis did not change the statistically significant results obtained by comparing all sub-groups to non-users ($F = 4.002$, $P = 0.0077$ | Tukey's HSD $P = 0.0112$). We have also analyzed the data using the non-parametric test of Kruskal Wallis followed by post-hoc Dunn's test, which is better equipped for smaller samples with data variability [23]. Analysis of the data by this non-parametric test yielded statistically significant results similar to those obtained by its parametric counterpart (*see*, above) [Device type: $P = 0.036$ | Dunn's $P = 0.0038$; Flavor type (tobacco group included): $P = 0.043$ | Dunn's $P = 0.0041$; Flavor type (tobacco group excluded): $P = 0.033$ | Dunn's $P = 0.0048$] (Table 2).

Moreover, we examined how the nicotine content of e-liquid may influence the induction of DNA damage in e-cig users. Specifically, we sought correlation between the cumulative amounts of nicotine in e-liquids consumed by vapers and the levels of DNA damage in their oral cells. The cumulative nicotine consumption by e-cig users (in milligram) did not correlate to the levels of DNA damage in their oral cells ($r = 0.3189$, $P = 0.1288$). Similarly, no correlation was found between indicator of *'recent'* nicotine intake, calculated as past-week nicotine consumption (mg), and DNA damage levels in vapers' oral cells ($r = -0.0457$, $P = 0.834612$).

Biochemical verification of vaping/smoking

As shown in Table 1, while both vapers and smokers had significantly higher levels of plasma cotinine than non-users ($P < 0.0001$), only smokers did show significantly increased levels of breath CO and %COHb in comparison to non-users ($P = 0.0005$ and $P = 0.0002$, respectively); vapers had similar levels of CO and %COHb to non-users (Table 1). Plasma cotinine levels in vapers and smokers were not significantly different from one another ($P = 0.607$). Of note, plasma cotinine, a primary metabolite of nicotine, is a validated marker of tobacco product use (both for smoking combustible cigarettes and vaping nicotine containing e-cigs) [19]. However, exhaled breath CO is an objective biomarker of recent exposure to combustible products, such as tobacco cigarette (but not vape) [6]. In addition, %COHb indicates the proportion of red blood cells carrying CO instead of oxygen [6]. Although the cut-off point of exhaled CO to distinguish cigarette smokers from nonsmokers varies across different studies [24], we considered the cut-off point of 7.0 ppm recommended by the manufacturer of Smokerlyzer® (Bedfont Scientific Ltd.), which was used to measure exhaled CO in our study subjects. Likewise, there is no unanimous consensus regarding the cut-off point of cotinine for distinguishing cigarette smokers from nonsmokers [25]. Nonetheless, we confirmed the self-reported exposure status of the study subjects by demonstrating that non-users had breath CO levels of 1.9 $\pm$ 0.3 ppm (all below the 3.0 ppm) and plasma cotinine of 2.6 $\pm$ 0.1 ng/ml, whereas the respective values for smokers and vapers were: [CO = 12.0 $\pm$ 1.6 ppm and cotinine = 76.7 $\pm$ 8.6 ng/ml (for smokers)] and [CO = 2.0 $\pm$ 0.3 ppm and cotinine = 84.9 $\pm$ 13.1 ng/ml (for vapers] (Table 1).

Discussion

Most adult vapers have a prior history of smoking combustible cigarettes [3,5]. Thus, many adults who are 'current' users of e-cigs, are likely to be 'former/ex-' smokers [1-3]. The existing literature on the *'potential'* health risks of vaping is often criticized by the fact that the study subjects in many reports consist of adult vapers whose 'past' smoking history is either unspecified or ambiguously defined [3,13]. This has complicated the interpretation of results as it is unclear whether the observed effects in e-cig users are solely caused by vaping or due to the persistent effects of 'past' smoking [1,3,5]. These uncertainties have fueled a highly contentious debate on the public health impact of vaping [3,26]. The design of the present study has allowed us to tease out the biological effects of 'exclusive' vaping in a thoroughly characterized population of adults.

LA-QPCR quantification of DNA damage in oral epithelial cells, a target cell type for cancer and other diseases associated with tobacco product use [17], showed significantly increased levels of polymerase-blocking lesions in both vapers and smokers as compared to non-users. The mean levels of DNA damage did not differ significantly between vapers and smokers. Importantly, DNA damage levels in both vapers and smokers increased dose-dependently, from light users to heavy users, when compared to non-users. These *in vivo* findings are novel and significant as they demonstrate, for the first time, a dose-dependent formation of DNA damage in target cells from vapers who had never smoked tobacco cigarettes. In this study, we used cumulative e-liquid consumption (Cum e-liq) and pack year (PY) [17,19] as indicators of vaping- and smoking dose, respectively. We [17,19,22] and others [27] have previously shown the utility of Cum e-liq and PY for estimation of chronic e-cig use and cigarette smoking, respectively. Soule *et al.* [28] have argued that amount of e-liquid consumed may be a useful indicator of quantity of aerosol inhaled by e-cig users, but not necessarily a precise measure of exposure to nicotine and other toxicants in the aerosol. This is consistent with our choice of Cum e-liq for estimating cumulative exposure to

complex mixture of e-cig aerosol (as a whole) but not to its individual chemical constituents. We did not use puff topography to assess exposure in the present study because e-cig users are known to puff often from the same e-cig in multiple sessions, with sessions not being consistent in total puff duration or number of puffs [28]. This is in sharp contrast with cigarette smokers who typically smoke a cigarette from start to finish in a single session. It is important to note that consensus on e-cig use intensity measures that can be used for survey research has yet to be established due to great heterogeneity in e-cig device and e-liquid characteristics and user behavior, which lead to different levels of exposure to toxicants and carcinogens by e-cig users [28].

We note that the comparable levels of DNA damage detected in vapers and smokers deserve further investigation. Given the similarities and differences in chemical composition of e-cig vapor and cigarette smoke [5-7,29], it is important to uncover the identity of DNA lesions formed in vapers and smokers. Future studies should exploit the high specificity and sensitivity of mass-spectrometry based assays [15] to characterize the type of induced DNA damage in vapers and smokers. Identifying the chemical structure of DNA lesions formed in vapers *vs*. smokers will have significant implications for tobacco products regulation.

Ganapathy *et al*. [30] have shown that *in vitro* treatment of human oral squamous cell carcinoma cells (UM-SCC-1) with e-cig aerosol condensates resulted in the formation of DNA damage in the *TP53* gene, as detected by a similar PCR-based assay. The levels of 8-hydroxy-2'-deoxyguanosine, an indicator of promutagenic oxidative DNA damage [14], were also increased significantly in treated UM-SCC-1 cells as compared to controls, as quantified by ELISA. Sundar *et al*. [31] have shown that *in vitro* exposure of human gingival epithelium progenitors pooled cells and periodontal ligament fibroblasts to e-cig aerosols at air-liquid interface caused significant DNA damage, as reflected by the increased phosphorylated γH2A.X Ser139 (a DNA damage

marker) and/or elevated comet tail lengths in the treated cells as compared to air-exposed controls. The findings were recapitulated in normal human 3D *in vitro* model of EpiGingival tissues that were similarly exposed to e-cig aerosols and afterward analyzed by immunohistochemical staining for γH2AX. Recently, Cheng *et al*. [32] have reported significantly increased levels of the major DNA adduct of acrolein, a carcinogenic aldehyde found substantially in cigarette smoke and to a lesser extent, in e-cig vapor [6,7], in buccal cells of vapers as compared to controls, using mass spectrometry-based analysis. Vapers in the Cheng *et al*'s study (*N*=20) were defined as those who used e-cigs for a minimum of three months and, at least, four days per week; albeit no information was provided on the subjects' history of smoking or other tobacco product use [32]. It is important to put into context the diverse types of assay used in the above studies, which were conducted in different settings (*in vitro* or *in vivo*). Some of the applied assays in the cited studies do not measure the exact same endpoints as those quantifiable by LA-QPCR. While detection of DNA damage in vapers and smokers in the present study is consistent with the findings reported by others in other settings, the type of assays used in some of those reports enables quantification of specific markers of DNA damage. The specific markers detected by those assays do not necessarily reflect formation of the same type of lesions detectable by LA-QPCR.

Our findings on a role for product characteristics in induction of DNA damage in vapers are novel and may have significant regulatory implications for electronic nicotine delivery systems. We observed that both e-cig device type and e-liquid flavor are determinants of DNA damage in oral epithelial cells in vapers. Users of pod devices followed by mod users had the highest levels of DNA damage in their oral cells as compared to non-users. Since entering the U.S. market around 2006-2007, e-cig devices have evolved continually and rapidly, from the first-generation 'Cigalike', to the second-generation vape pens, third-generation box mods, and the

current fourth-generation pod-based devices [5,7]. A combination of sleek and high-tech design, innovative salt-based nicotine delivery technology, large assortment of e-liquid flavors, and social media-oriented marketing has made pod-based e-cigs, such as JUUL and JUUL alike devices, widely popular among both novice and experienced vapers [5,7,33]. The small size, light weight, and easy to conceal nature of these devices together with teen-appealing e-liquid flavors have also made them a choice of preference for adolescents experimenting with tobacco products [33]. The latter is believed to have contributed to the ongoing epidemic of youth vaping in the U.S. [34]

Furthermore, we observed that users of sweet-flavored e-liquids, followed by users of mint/menthol- and fruit-flavored e-liquids had the highest levels of DNA damage in their oral cells when compared to non-users. This finding may have significant implications for public health because these e-liquid flavors, which exhibit the highest DNA-damaging potencies, are not only popular among adult vapers but also, they are the preferred flavors for youth vapers [35-37]. Common flavoring chemicals, such as ethyl maltol imparting sweet and caramel-like aromas and flavors, lactones imparting fruity and creamy flavors, piperonal, an aromatic aldehyde imparting cherry and vanilla like flavors, and benzaldehyde, a natural fruit flavorant, are known to decompose to radicals and redox active species during vaporization [38]. It has also been shown that vapor generated from JUUL pod with Cool Mint significantly increased acellular reactive oxygen species (ROS) levels when compared to control filtered air [39]. Moreover, *in vitro* experiments have confirmed that vapor produced from Classic Menthol JUUL pod caused the greatest increase in mitochondrial superoxide production in lung epithelial cells in comparison to vapors from pods with other flavors or filtered air [39]. Altogether, the higher oxidative properties of sweet-, mint/menthol-, or fruit-flavored e-liquids as compared to non-flavored e-liquids may translate to greater genotoxic potential for the former products [40]. This is reinforced by our observation that

vapers of e-liquids with such flavors exhibited the highest levels of DNA damage in their oral cells.

Moreover, we found that nicotine content of e-liquid was not a predictor of DNA damage in vapers' oral cells. This finding is in agreement with previous reports showing that *in vitro* e-cig aerosols/condensates induce genotoxic effects independently of nicotine concentrations in the e-liquid [30,41,42]. Collectively, our data suggest that flavoring components alone or in combination with other e-liquid constituents (*e.g.*, humectant or additives) can give rise to DNA reactive species, which may, in turn, cause genotoxic effects in cells and tissues of e-cig users. Potential chemicals involved in this process may include those formed during the vaporization of e-liquid flavorings and humectants, particularly reaction products of flavorant aldehydes or formaldehyde and PG/VG, such as acetals and hemiacetals, and ROS, among others [10,11,22]. Future studies should uncover the chemical structures of the herein detected DNA lesions in vapers. These follow up investigations should help inform the regulation of e-liquid ingredients that are responsible for the genotoxic effects of vaping observed in this study as well as in previous studies by others [30-32,39,41,42].

Strengths and limitations: By design, the present study accounted for relevant biological variables, specifically age, gender, and race. In addition, dietary intake data obtained from the study subjects confirmed that there was no significant difference in alcohol use or consumption of grilled/roasted/broiled foods among vapers, smokers, and non-users. Furthermore, vapers in our study were mostly young adults and likely representative of the population from which they were drawn. Young adults are known to favor pod and mod devices and prefer e-liquids with sweet-, mint/menthol- and fruit-flavors rather than tobacco-flavored e-liquids [35-37]. We demonstrated the impact of device type and e-liquid flavor on the induction of DNA damage in vapers. The small

sample size of certain subgroups in our study can be considered a limitation. Nevertheless, we have substantiated our findings by further scrutinizing the data, performing additional tests, and conducting statistical analyses with or without small sub-groups. We acknowledge that future studies with larger sample size are needed to further validate our results and allow for more detailed characterization of the ingredients of e-liquid as well as device features that may contribute to the biological effects of vaping. These follow up investigations are highly important given the diversity of e-cig devices and variation in e-liquid products [5,7,37].

The LA-QPCR assay used in this study quantifies a broad spectra of polymerase stalling/stopping DNA lesions (*e.g.*, oxidative, alkylative, and bulky lesions, and single-strand breaks) within the amplification region of the primer set designed for a specific gene [16]. The assay does not provide information on the chemical structure of the detected lesions or the distribution of DNA damage across the genome [16]. However, we and others have shown that DNA damage in reporter genes faithfully captures many aspects of lesion formation/repair in cancer-related genes, such as tumor suppressor genes or oncogenes [16,30,43,44]. In this study, the choice of *POLB* as a gene target for LA-QPCR analysis was based on previous studies by others [16,20,21] who have confirmed that *POLB* can serve as a representative gene target for DNA damage detection. In addition, the results of LA-QPCR in the *POLB* and other gene targets, such as *HPRT*, have been shown to be highly consistent and correlated [21], as reconfirmed in our study.

Of importance for this study, we stress the challenges of research in healthy volunteers with matching characteristics (*i.e.*, age, gender, and race) and strictly defined exposure, whose source materials (*e.g.*, tissues, cells, DNA/RNA) are often limited for molecular analysis. The limited source materials in these studies inevitably leads to prioritization of endpoints for quantification (*e.g.*, selection of target gene). With the same token, prioritization will be required

to detect the selected endpoint(s) in specific tissues, cells, or cellular compartments (*e.g.*, nucleolus *vs*. mitochondria). For example, while the significance and importance of damage to the nuclear genome in the pathophysiology of disease is well-established, the role of mitochondrial DNA damage in disease development is beginning to be fully appreciated [45,46] (*see*, Supplementary Materials for distinctions between nuclear and mitochondrial genomes). Given the limited source materials for molecular analysis in population-based studies [47], nuclear DNA has been extensively used as a preferred choice for direct measurement of DNA damage [15]. The focus of the present study was on nuclear DNA damage because the source materials for this study had to be shared with our ongoing genomic sequencing project, which aims to detect mutations in the nuclear genome. While there is a growing recognition of the importance of mitochondria in health *vs*. disease state [45,46,48], measuring mitochondrial DNA damage was beyond the scope of the present study and outside its prioritization scheme.

In the present study, we did not collect quantitative data on physical activity levels of the study subjects. Limited but emerging data suggest modulatory effects of exercise on DNA damage associated with lifestyle factors, such as smoking [49]. Urinary levels of 8-hydroxydeoxyguanosine (8-OH-dG) in male smokers were inversely related to physical activity of moderate/rigorous intensity. A similar trend was found in female smokers between urinary 8-OH-dG levels and total physical activity [50]. We note that investigating the effects of exercise on DNA damage in vapers *vs*. smokers is beyond the scope of this study. Lastly, while we underscore the importance of follow up studies in large populations, our power calculations showed that the present study was powered at 83% and 63%, respectively, to detect statistically significant differences in DNA damage levels between vapers and non-users, and smokers and non-users (at $\alpha = 0.05$).

Conclusions: We have demonstrated a dose-dependent formation of DNA damage in oral cells of vapers who had never smoked tobacco cigarettes as well as exclusive cigarette smokers. We have also shown that e-cig device type and e-liquid flavor determine the extent of DNA damage detected in vapers. In terms of device type, pods followed by mods, and in terms of flavor type, sweet, followed by mint/menthol- and fruit-flavored e-liquids exhibit the greatest DNA-damaging potencies in vapers. Given the popularity of pod and mod devices and the preferability of these same flavors of e-liquid by both adult- and youth vapers [33,35,36], our findings may have significant implications for public health and tobacco products regulation.

Funding

Work of the authors is supported by grants from the National Cancer Institute and National Institute of Dental and Craniofacial Research of the National Institutes of Health (1R21CA268197 and 1R01DE026043, resp.), and the University of California Tobacco-Related Disease Research Program (T31IR1839, and T32IR5144). The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit for publication.

Declaration of Interests

All the authors declare no conflict of interest.

Acknowledgements

We would like to thank Andrew Caliri and Amanda Caceres for technical support and help with laboratory assays. Special thanks to Dr. Alan Hiti and the USC-Norris Outpatient Clinical Laboratory staff for phlebotomy work. We are grateful to Drs. Bo Hang and Altaf Sarker of Lawrence Berkeley National Laboratory for sharing their expertise in LA-QPCR with our group.

Supplemental Materials

Supplemental Materials includes Supplementary Methods.

Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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Table 1. Characteristics of the study population.

		Vapers (*N*= 4)	Smokers (*N*=24)	Non-users (*N*=24)
Age *		24.3 ± 0.8	26.0 ± 0.7	25.3 ± 0.6
Gender †	Male	20 (83.3%)	20 (83.3%)	20 (83.3%)
	Female	4 (16.7%)	4 (16.7%)	4 (16.7%)
Race †	White	7 (29.2%)	7 (29.2%)	7 (29.2%)
	Hispanic	5 (20.8%)	5 (20.8%)	5 (20.8%)
	African American	2 (8.3%)	2 (8.3%)	2 (8.3%)
	Asian	8 (33.3%)	8 (33.3%)	8 (33.3%)
	Other ‡	2 (8.3%)	2 (8.3%)	2 (8.3%)
Marital status †	Single and never married	20 (83.3%)	19 (79.2%)	21 (87.5%)
	Married	2 (8.3%)	2 (8.3%)	1 (4.2%)
	Currently living with someone	2 (8.3%)	1 (4.2%)	2 (8.3%)
	Widowed	0 (0%)	0 (0%)	0 (0%)
	Separated	0 (0%)	0 (0%)	0 (0%)
	Divorced	0 (0%)	2 (8.3%)	0 (0%)
Education †	Less than high school	0 (0%)	0 (0%)	0 (0%)
	High school diploma or GED	5 (20.8%)	3 (12.5%)	0 (0%)
	Some college completed or currently enrolled in college	13 (54.2%)	6 (25.0%)	3 (12.5%)
	College degree or higher	6 (25.0%)	15 (62.5%)	21 (87.5%)
Employment status †	Full time	13 (54.2%)	15 (62.5%)	14 (58.3%)
	Part time	9 (37.5%)	7 (29.2%)	7 (29.2%)
	Retired or disability	1 (4.2%)	0 (0%)	0 (0%)
	Unemployed	1 (4.2%)	2 (8.3%)	3 (12.5%)
Pre-tax annual income †	< $15,000	1 (4.2%)	7 (29.2%)	7 (29.2%)
	≥ $15,000 - < $30,000	4 (16.7%)	4 (16.7%)	6 (25.0%)
	≥ $30,000 - < $45,000	5 (20.8%)	6 (25.0%)	1 (4.2%)
	≥ $45,000 - < $60,000	4 (16.7%)	1 (4.2%)	4 (16.7%)
	≥ $60,000 - < $75,000	1 (4.2%)	5 (20.8%)	1 (4.2%)
	≥ $75,000 - < $90,000	1 (4.2%)	0 (0%)	1 (4.2%)

	≥ $90,000 - < $105,000	1 (4.2%)	0 (0%)	2 (8.3%)
	≥ $105,000 - < $120,000	2 (8.3%)	0 (0%)	0 (0%)
	≥ $120,000	5 (20.8%)	1 (4.2%)	2 (8.3%)
BMI [*, §]		26.6 ± 1.2	26.9 ± 1.1	23.9 ± 1.0
Years smoked [*]		NA	7.3 ± 1.1	NA
Pack Year [*, ¶]		NA	3.1 ± 0.6	NA
Years vaped [*]		2.9 ± 0.4	NA	NA
Cumulative e-liquid (ml) [*, #]		5,780.1 ± 2,017.5	NA	NA
E-cig device type [†]	Cigalike	1 (4.2%)	NA	NA
	Mod	11 (45.8%)	NA	NA
	Pod	8 (33.3%)	NA	NA
	Multiple	4 (16.7%)	NA	NA
E-liquid flavor [†]	Fruit	7 (29.2%)	NA	NA
	Sweet	3 (12.5%)	NA	NA
	Mint/Menthol	5 (20.8%)	NA	NA
	Tobacco	1 (4.2%)	NA	NA
	Multiple	8 (33.3%)	NA	NA
Plasma cotinine (ng/ml) [*]		84.9 ± 13.1 [**]	76.7 ± 8.6 [**]	2.6 ± 0.1
Breath CO [(ppm) *]		2.0 ± 0.3	12.0 ± 1.6 [††]	1.9 ± 0.3
%COHb [*]		0.9 ± 0.07	2.5 ± 0.2 [‡‡]	0.9 ± 0.05
Vitamin or multi-vitamin use [‖]		7 (29.2%)	5 (20.8%)	6 (25.0%)

[*] Results are expressed as Mean ± SE.

[†] Numbers and percentages (inside brackets) are indicated.

[‡] Other = Multiracial or Native American

[§] BMI: Body Mass Index [Weight $_{(kg)}$ ÷ Height^2 $_{(m)}$]

[¶] Pack Year is calculated by multiplying the number of packs of cigarettes a person smoked per day by the number of years he/she smoked.

[#] Cumulative e-liquid is calculated as the total volume of e-liquid (in milliliter) vaped by a person during his/her lifetime.

[‖] Defined as those who used vitamin or multi-vitamin regularly (at least 3 times per week in the past year).

Plasma cotinine levels were measured using a solid phase competitive ELISA (Abnova Corp.) and exhaled breath CO levels and %COHb were quantitated by a Bedfont Micro^{+TM} Smokerlyzer® Breath CO monitor (Bedfont Scientific Ltd.) (*see*, Supplementary Materials).

[**] Statistically significant as compared to non-users, $P < 0.0001$

[††] Statistically significant as compared to non-users, $P = 0.0005$

[‡‡] Statistically significant as compared to non-users, $P = 0.0002$

GED = General Education Development or General Education Diploma; The GED or High School Equivalency Certificate shows that one has a level of knowledge equivalent to a high school graduate; CO = carbon monoxide; ppm = parts per million; COHb = carboxyhemoglobin; NA = Not applicable.

Table 2. The influence of e-cig device type and e-liquid flavors on the extent of DNA damage in vapers *vs.* non-users.

			Lesions/10 kb	Lesions/10 kb/Years vaped*	Lesions/10 kb/Cum e-liq*
Vapers	Device	Mod	0.684 ± 0.239	0.195 ± 0.059	1.80E-4 ± 6.22E-5
		Pod	0.864 ± 0.175 †	1.077 ± 0.336	3.13E-2 ± 1.70E2
		Multiple	0.423 ± 0.280	0.089 ± 0.044	1.20E-4 ± 5.10E-5
	Flavor	Fruit	0.402 ± 0.176	0.295 ± 0.187	0.001 ± 0.001
		Sweet	1.262 ± 0.148 ‡	1.513 ± 0.836	0.065 ± 0.041
		Mint/Menthol	0.692 ± 0.222	0.598 ± 0.224	0.009 ± 0.005
		Tobacco	0.670 ¶	0.164	4.6E-4
		Multiple	0.699 ± 0.320	0.193 ± 0.068	2.0E-4 ± 9.0E-5
Non-users			0.262 ± 0.049	NA	NA

Summary results of LA-QPCR in the *POLB* gene in oral cells of vapers as compared to non-users. Vapers were divided into three groups, including users of third generation devices (*'mod'*), users of fourth generation devices (*'pod'* = JUUL and JUUL alike), and users of *'multiple'* generation devices. E-liquid flavors consumed by vapers were divided into five categories, including (1) fruit; (2) sweet (*i.e.*, candy/desserts/other sweets); (3) mint/menthol; (4) tobacco; and (5) multiple.

***** To account for the extent and duration of use of different generation devices or different e-liquid flavors, data were adjusted for 'years vaped' and 'cumulative e-liquid consumed'. Cumulative e-liquid (Cum e-liq) is calculated as the total volume of e-liquid (in milliliter) vaped by a person during his/her lifetime.

† Statistically significant as compared to non-users; ANOVA: $F = 3.886$, $P = 0.0152$ | Tukey's HSD $P = 0.0216$. We have also analyzed the data using the non-parametric test of Kruskal Wallis followed by post-hoc Dunn's test, which is better equipped for smaller samples with data variability [23]. Analysis of the data by this non-parametric test yielded statistically significant results similar to those obtained by its parametric counterpart (ANOVA) as follows: $P = 0.036$ | Dunn's $P = 0.0038$.

‡ Statistically significant as compared to non-users; ANOVA: $F = 3.238$, $P = 0.0146$ | Tukey's HSD $P < 0.05$. We note that exclusion of ¶ tobacco group from the analysis did not change the statistically significant result : ANOVA: $F = 4.002$, $P = 0.0077$ | Tukey's HSD $P = 0.0112$. Furthermore, the non-parametric test of Kruskal Wallis followed by post-hoc Dunn's test yielded similar statistically significant results: Tobacco group included: $P = 0.043$ | Dunn's $P = 0.0041$ and Tobacco group excluded: $P = 0.033$ | Dunn's $P = 0.0048$.

Results are expressed as mean ± SE from duplicate samples assayed independently up to two times.

NA = Not applicable.

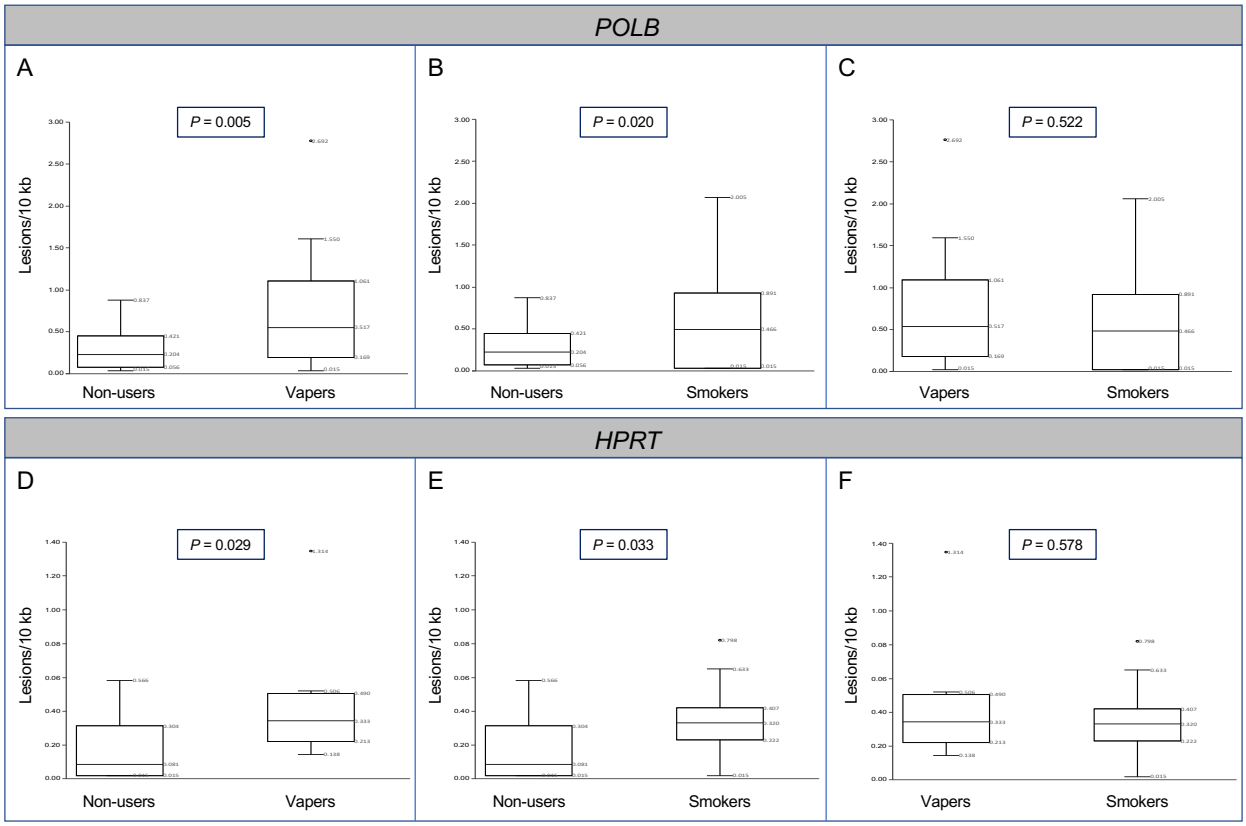

Figure 1. Quantification of DNA damage between vapers and non-users, smokers and non-users, and vapers and smokers. DNA damage levels were determined in genomic DNA of oral epithelial cells from healthy adult 'exclusive' vapers (never-smokers), cigarette smokers only, and non-users by LA-QPCR, as described in the text. Panels A-C show the LA-QPCR results in the *POLB* gene whereas Panels D-F display the respective results in the *HPRT* gene. Distribution of data within each group is shown by box and whisker plots whereby *'lower'* and *'upper'* edges of the boxes represent the 1st and 3rd quartiles, respectively, and horizontal lines within the boxes indicate the 2nd quartile. The *'lower'* and *'upper'* vertical lines extending from the boxes, also known as the "whiskers", represent the lowest and highest data points, respectively, in the set (minimum and maximum values, resp., excluding values outside the whiskers' range). The five measures of box and whisker plots are all labeled within the graphs. All samples were assayed independently up to two times and the results were averaged for each sample. DNA damage levels were compared between each two independent groups, as described in the text; *P*-values are indicated for all comparisons.

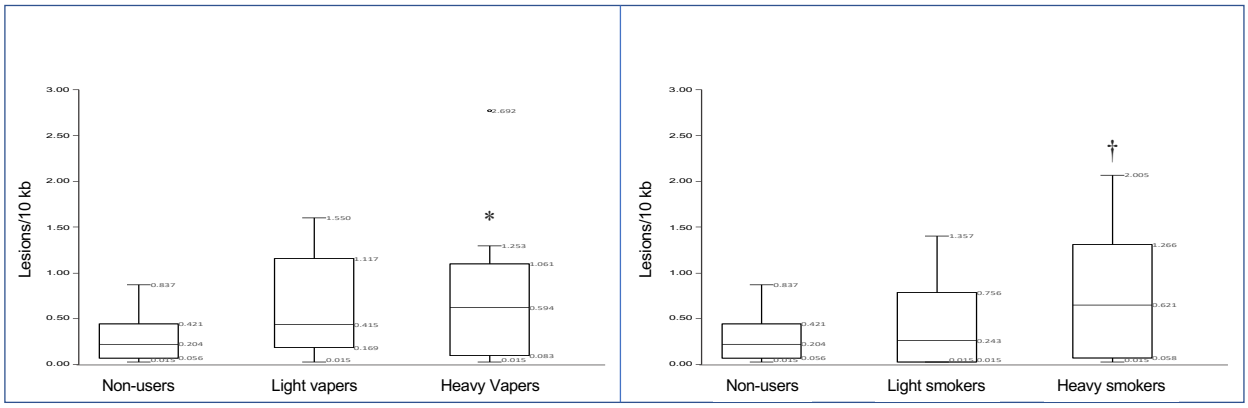

Figure 2. Examination of dose-dependent formation of DNA damage in vapers and smokers as compared to non-users. To examine the dose-dependency of DNA damage, both vapers and smokers were divided in two groups, including 'light' and 'heavy' users based on cumulative e-liquid consumption and pack year, respectively, as described in the text. Distribution of data within each group is shown by box and whisker plots, with the five indicating measures of each plot being labeled within the graphs (*see*, description in legend for Fig. 1).

[*] Statistically significant as compared to non-users: ANOVA: $F = 4.571$, $P = 0.0156$ | Tukey's HSD $P = 0.0195$

[†] Statistically significant as compared to non-users: ANOVA: $F = 4.368$, $P = 0.0185$ | Tukey's HSD $P = 0.0135$

"Supplementary Materials"

Vaping dose, device type, and e-liquid flavor are determinants of DNA damage in electronic cigarette users. *Stella Tommasi, Hannah Blumenfeld, and Ahmad Besaratinia*

Supplemental Materials include:

- Supplementary Methods (text)
- Supplementary References

"Supplementary Methods"

Subject recruitment and enrollment

The study was advertised in online forums, including Craigslist, Reddit, and myUSC (http://my.usc.edu), and on social media (Twitter, Instagram, and Facebook) [1]. Also, flyers and leaflets were used to advertise the study in local colleges, universities, and vape shops. Furthermore, an online survey was developed, validated, and subsequently employed to solicit and query potential participants. Individuals who appeared to have met the study criteria were contacted by phone to complete a screening questionnaire. Based on the information obtained during the phone screen, those who were deemed potentially eligible, were scheduled for an in-person visit to our laboratory. During the visit, an expanded version of the phone screen was administered to reconfirm eligibility and afterward, a written informed consent was obtained from all participants (*see*, below).

Personal interview

Upon reconfirmation of eligibility and informed consent, all participants were interviewed in person to provide detailed information on demographics, socio-economic status, use frequency and patterns of e-cigs, cigarettes, or other tobacco products, dietary habits (*e.g.*, grilled/roasted/broiled food consumption), lifestyle, use of recreational or illicit drugs, alcohol, and prescription- or over-the-counter medicine, specifically vitamins or multivitamins, occupational and residential history, and family history of disease.

Inclusion and exclusion criteria

Health indicators for exclusion from the study consisted of respiratory diseases (*e.g.*, asthma or chronic obstructive pulmonary disease), immune system disorders, diabetes, kidney diseases, body mass index < 18 kg/m^2 or > 40 kg/m^2, local or systemic inflammation or infection, or any medical disorder/medication that could affect subject's safety or study results. Any unstable or significant medical condition in the past 12 months, including but not limited to symptomatic heart conditions, stroke, severe angina, and hypertension was ground for exclusion. Being pregnant or having a baby in the past 12 months was also exclusionary. Other exclusion criteria included uncontrolled mental illness or substance abuse or inpatient treatment for those conditions in the past 12 months, use of recreational or illicit drugs (*e.g.*, marijuana or heroin) in the past six months, and use of any medication known to induce/inhibit CYP450 2A6 enzyme. Physical examination and health assessment of all participants were performed by highly trained staff during the personal visits and interviews.

Sampling and processing of oral epithelial cells

All subjects were required to refrain from eating, smoking, or vaping, at least, 1 h prior to visiting our laboratory. Before sampling, subjects were asked to vigorously rinse their mouths with water to remove saliva, residual food particles, and mucosal debris. An Ultra Soft Oral-B brush (SENSI.SOFT^{TM}; Cincinnati, OH) was placed in the subject's mouth, and sufficient pressure was applied to contact the surface of the inside of his/her cheeks. Rotatory motion along the face and edge of the brush was used to gently scrape the entire surface of the inside of the cheek, while avoiding bleeding. The proximal, central, and distal regions of the inside of each cheek were brushed 15 times each. Once brushing of a region was completed, the brush was swirled in a tube pre-filled with 35 ml ice-cold sterile phosphate buffer saline (PBS) to dislodge the cells from the

bristles. Cycles of brushing and washing the cells from the bristles were repeated until all regions from both cheeks were sampled. The two tubes containing the harvested cells from opposite cheeks were centrifuged at 800× *g* for 5 min at 4 °C. Pelleted cells from each tube were re-suspended in PBS, pooled into a single tube, and re-centrifuged as above. The collected cell pellet was snap frozen and kept at −80 °C until further analysis. We have confirmed that this protocol provides, on average, several million cells, the vast majority of which being intermediate and suprabasal oral epithelial cells [1]. To rule out significant contamination by other cell types, we have performed differential cell count on the collected cells and verified the overwhelming presence of oral epithelial cells in all samples. To avoid any potential bias, specimen collection and processing of samples from different groups were done in variable orders, not in batches.

Sampling and processing of peripheral blood

Peripheral blood (30 ml) was drawn from the study subjects by venipuncture. Plasma was collected by centrifugation, aliquoted into multiple microtubes (Eppendorf, Inc., San Diego, CA), snap frozen, and preserved at −80 °C until further analysis.

Quantification of DNA damage by LA-QPCR

LA-QPCR quantification of DNA damage was performed as described in ref. [2], with few modifications. Briefly, genomic DNA was isolated from snap frozen oral epithelial cells, diluted 1:10 with 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and quantified fluorimetrically using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher, Waltham, MA) per the manufacturer's instructions. Following PicoGreen quantification, equal amounts of genomic DNA (20 ng) were used, in duplicate, for long PCR amplification in 1x reaction buffer containing 2.5 U

LongAmp *Taq* DNA polymerase (New England BioLabs, Ipswich, MA), 2% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO), and 300 μM dNTPs (MilliporeSigma, Burlington, MA). The following primers (at 0.2 μM final concentration) were added to the reaction mix to amplify a 12.2 kb region of the DNA polymerase beta *(POLB)* gene: forward primer, 5'-CCTGGAGTAGGAACAAAAATTGCTG and reverse primer, 5'-CATGTCACCACTGGACTCTGCAC. Preliminary assays were carried out to determine the optimal DNA concentration and number of cycles needed to ensure the linearity of the PCR amplification. PCR products were run on agarose gel to verify the size of the long amplicons and assure that no spurious products were generated. Final PCR conditions were set as follows: 94 °C for 2 min; 94 °C for 30 sec, 58 °C for 30 sec, 65 °C for 10 min (x 30 cycles); 65 °C for 10 min; 4 °C. For background adjustment, blanks (samples containing no DNA template) were also included in the assay. After amplification, PCR products and blanks (5 μl/sample) were quantified fluorimetrically by Quant-iT PicoGreen, using dilutions of λ HindIII-cut DNA (ThermoFisher, Waltham, MA) to generate a standard curve [2].

Following the PicoGreen assay, fluorescence values expressed as relative fluorescence units (RFU) were compiled for each sample on an excel spreadsheet for data analysis. Fluorescence readings of duplicate samples from vapers, smokers and non-users were averaged, and blank values (averaged no-DNA samples) were subtracted. To minimize technical noise, fluorescence values from independent PicoGreen plates were normalized using the standards' fluorescence readings. Adjusted RFU values (blank-corrected and standard-calibrated) were then used to calculate the "relative amplification", by dividing each sample's fluorescence by the average fluorescence of all non-users' samples used as the reference. Finally, the resulting values were converted to relative lesion frequencies by applying the Poisson distribution formula:

lesions/amplicon = –ln A_e/A_c, where A_e represents the amplification of each experimental sample from vapers, smokers, and non-users, and A_c is the amplification of the non-users' samples (average) [2]. Lesion frequencies were normalized to number of lesions/10 kb [2-4], and lesion frequencies below the detection level were set to 0.015/10 kb, which is one half of the detection limit of the assay. For validation purpose, we have also interrogated an additional gene target, hypoxanthine phosphoribosyltransferase 1 (*HPRT*), by LA-QPCR using the same protocol as described above. The primer set to amplify a 10.4 kb fragment encompassing exons 2–5 of the *HPRT* gene includes: forward primer, 5′-TGG GAT TAC ACG TGT GAA CCA ACC and reverse primer, 5′-GCT CTA CCC TCT CCT CTA CCG TCC.

Quantification of plasma cotinine by ELISA

Plasma cotinine was measured by a solid phase competitive enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer (Abnova Corp., Walnut, CA). Briefly, aliquots of standard controls and samples of plasma from the study subjects (in triplicate) were loaded (10 µl each) onto a 96-microwell plate pre-coated with a polyclonal antibody raised against cotinine. After adding a cotinine horseradish peroxidase enzyme (100 µl per well), the microplate was incubated for one hour at room temperature in the dark. Unbound cotinine and cotinine enzyme-conjugate were washed off by rinsing the wells six times with distilled water (300 µl each wash). A chromogenic substrate (3,3',5,5'-Tetramethylbenzidine) was added (100 µl per well), and the plate was incubated for 30 minutes at room temperature. The reaction was terminated by adding a stop solution (100 µl per well), and absorbance was read at 450 nm using a SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, LLC., San Jose, CA). Results are expressed as nanograms (ng) of cotinine measured per milliliter of plasma [5].

Quantification of exhaled CO and COHb by breath monitor

Exhaled CO levels and %COHb were measured using the Bedfont Micro^{+TM} Smokerlyzer® according to the manufacturer's instructions (Bedfont Scientific Ltd., Harrietsham, UK). Briefly, study subjects were instructed to inhale and hold their breath for 15 seconds. Following the completion of the 15 second countdown, subjects blew slowly into the device mouthpiece aiming to empty their lungs completely. The CO levels (ppm) and equivalent %COHb were recorded by the device and displayed on the touchscreen monitor.

Prioritization of endpoints for measurement

Of importance for the present study, we should stress the challenges of research in healthy volunteers with matching characteristics (*i.e.*, age, gender, and race) and strictly defined exposure, whose source materials (*e.g.*, tissues, cells, DNA/RNA) are often limited for molecular analysis. The limited source materials in these studies inevitably leads to prioritization of endpoints for quantification (*e.g.*, selection of target gene). With the same token, prioritization will be required to detect the selected endpoint(s) in specific tissues, cells, or cellular compartments (*e.g.*, nucleolus *vs*. mitochondria). For example, while the significance and importance of damage to the nuclear genome in the pathophysiology of disease is well-established, the role of mitochondrial DNA damage in disease development is beginning to be fully appreciated [6,7]. Given the limited source materials for molecular analysis in population-based studies [8], nuclear DNA has been extensively used as a preferred choice for direct measurement of DNA damage [9]. The focus of the present study was on nuclear DNA damage because the source materials for this study had to be shared

with our ongoing genomic sequencing project, which aims to detect mutations in the nuclear genome. Other distinctions between nuclear and mitochondrial genomes are highlight below:

(1) The mitochondrial genome consists of 16,569 DNA base pairs, whereas the nuclear genome is made of 3.3 billion DNA base pairs;

(2) The mitochondrial genome contains 37 genes with few non-coding DNA sequences, whereas the nuclear genome consist of 20,000-25,000 genes, including protein-coding genes, mitochondrial genes, and thousands of non-coding genes (*e.g.*, microRNAs and long non-coding RNAs) with known regulatory functions;

(3) Mitochondrial DNA is encoded for the genetic information required by mitochondria whereas nuclear DNA is encoded for the genetic information required by the entire cell;

(4) whereas one mitochondrion contains dozens of copies of its mitochondrial genome, each cell contains numerous mitochondria (*i.e.*, hundreds to thousands). Thus, a given cell can contain several thousand copies of its mitochondrial genome, but only one copy of its nuclear genome. This leads to heterogeneous population of mitochondrial DNA within the same cell, and even within the same mitochondrion;

(5) Unlike nuclear genome, the mitochondrial genome is not enveloped and packaged into chromatin. Given the absence of many of the protective protein structures and a relatively less efficient DNA repair machinery, the mitochondrial genome has a much higher mutation rate (~100-fold higher) than the nuclear genome; and

(6) the mitochondrial mode of inheritance is strictly maternal, whereas nuclear genomes are inherited equally from both parents (reviewed in refs. [6,7,10]).

Altogether, while there is a growing recognition of the importance of mitochondria in health *vs*. disease state [7], measuring mitochondrial DNA damage was beyond the scope of the

present study and outside its prioritization scheme. Notwithstanding, the rising appreciation for elucidating the role of mitochondrial DNA damage in pathobiology warrants further investigation whereby mitochondrial DNA damage can be evaluated in vapers *vs*. smokers.

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