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Increased Levels of the Acrolein Metabolite 3-Hydroxypropyl Mercapturic Acid in the Urine of e-Cigarette Users

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Abstract

Carcinogen and toxicant uptake by e-cigarette users have not been fully evaluated. In the study reported here, we recruited 30 e-cigarette users, 63 non-smokers, and 33 cigarette smokers who gave monthly urine samples over a period of 4-6 months. Their product use status was confirmed by measurements of exhaled CO, urinary total nicotine equivalents (TNE), cyanoethyl mercapturic acid (CEMA), and total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (total NNAL). Urinary biomarkers of exposure to the carcinogens acrolein (3-hydroxypropyl mercapturic acid, 3-HPMA), benzene (*S*-phenyl mercapturic acid, SPMA), acrylonitrile (CEMA), and a combination of crotonaldehyde, methyl vinyl ketone, and methacrolein (3-hydroxy-1-methylpropyl mercapturic acid, HMPMA) were quantified at each visit. Data from subject visits with CEMA > 27 pmol/ml were excluded from the statistical analysis of the results because of possible unreported exposures to volatile combustion products such as secondhand cigarette smoke or marijuana smoke exposure; this left 22 e-cigarette users with 4 or more monthly visits, and all 63 non-smokers. Geometric mean levels of 3-HPMA (1249 vs 679.3 pmol/ml urine) were significantly higher (P=0.003) in e-cigarette users than non-smokers, while levels of SPMA, CEMA, and HMPMA did not differ between these two groups. All analytes were significantly higher in cigarette smokers than either e-cigarette users or non-smokers. The results of this unique multi-month longitudinal study demonstrate consistent significantly higher uptake of the carcinogen acrolein in e-cigarette users vs. non-smokers presenting a warning signal regarding e-cigarette use.

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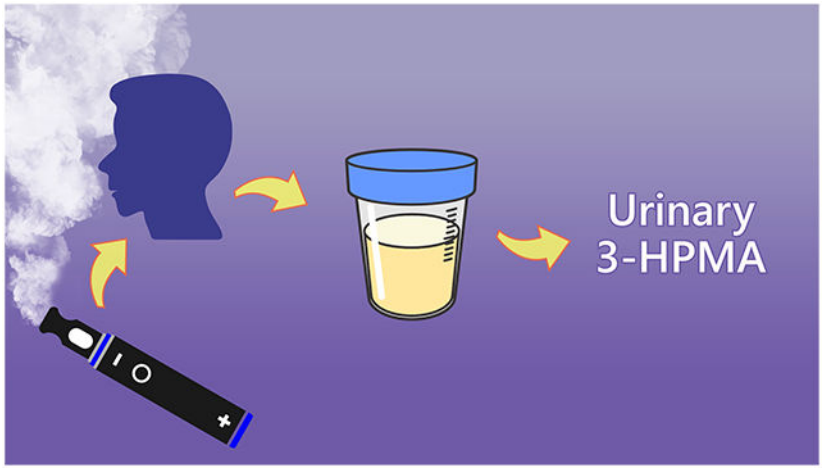
Conflict of Interest

The authors declare no competing financial interest.

Graphical Abstract



Introduction

The U.S. National Academies of Sciences, Engineering, and Medicine Consensus Study Report entitled “Public Health Consequences of E-Cigarettes” concluded that e-cigarette aerosol contains fewer toxicants and carcinogens, and lower levels of these substances, than does the smoke of tobacco cigarettes.[1] They also concluded that e-cigarettes are likely to be far less harmful than tobacco cigarettes, *but* that there is presently insufficient data to evaluate their absolute health risk.[1] Considering the growing popularity of e-cigarettes, particularly among younger people, it is essential that we explore their potential health effects.[2–5] The study reported here focuses on exposure to certain volatile carcinogens in e-cigarette users compared to non-smokers, as determined by collecting urine samples at multiple monthly visits and quantifying their mercapturic acid metabolites as biomarkers.

Multiple studies have evaluated biomarkers of carcinogen and toxicant exposure in subjects who used e-cigarettes versus conventional cigarettes or non-users of any tobacco product, as recently reviewed.[6–8] The vast majority of these studies have made biomarker measurements at a single time point, or more than one time point over a relatively short duration. While e-cigarette use consistently resulted in significantly lower exposure to carcinogens and toxicants than cigarette smoking, a few of these single time point studies demonstrated elevated levels of certain biomarkers of volatile toxicants or carcinogens such as acrolein and acrylonitrile in e-cigarette users versus non-tobacco users, as summarized in Table 1.

Another set of published reports compared biomarkers of exposure in cigarette smokers who switched to e-cigarettes, and generally showed decreases in exposure to volatile toxicants and carcinogens.[9–16] While most of these investigations were also of relatively short duration, one followed subjects who switched for up to 2 years, with multiple measurements.[17, 18] However, these studies did not include non-user comparison groups.

Overall, these biomarker results are potentially important in the evaluation of e-cigarette safety, but the measurements over a limited period of time in many studies and lack of

parallel non-user control groups in others raise questions regarding the relationship of the reported results to e-cigarette use as opposed to other exposures. Based on these studies, exposure to volatile toxicants and carcinogens due to e-cigarette use *per se* is difficult to evaluate. Therefore, we have quantified urinary biomarkers of exposure to selected volatile toxicants and carcinogens in 30 e-cigarette users, 63 non-smokers, and 33 cigarette smokers, who gave monthly urine samples over a 4-6 month period.

Materials and Methods

The participants in this study were recruited through the Tobacco Research Programs, University of Minnesota. Inclusion criteria were age >18, in stable and good physical and mental health, excellent oral health, no current infection as determined by medical history and investigator assessment, and no current use of medicinal nicotine products or any tobacco products other than cigarettes. e-Cigarette users exclusively used e-cigarettes for at least 3 months and at least 4 days per week, and had exhaled CO < 6 ppm. Smokers smoked at least 5 cigarettes per day for a minimum of 4 days per week for the past year and had exhaled CO > 8 ppm. Average urinary total nicotine equivalents (TNE, the molar sum of nicotine, cotinine, 3'-hydroxycotinine and their glucuronides plus nicotine-1'-*N*-oxide) and 2-cyanoethyl mercapturic acid (CEMA) were quantified to confirm e-cigarette use (TNE > 3 nmol/ml; CEMA < 27 pmol/ml) or non-smoking, non-user status (TNE < 0.1 nmol/ml; CEMA < 27 pmol/ml), whereas cigarette smokers had TNE > 3 nmol/ml and CEMA > 27 pmol/ml. Non-smokers smoked less than 100 cigarettes in their lifetime and had exhaled CO < 6 ppm, and never used any other tobacco product or e-cigarettes. Additional validation was provided by measurement of urinary total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (total NNAL) which was below the limit of detection at most time points in the e-cigarette users. Neither e-cigarette users nor smokers intended to quit using these products in the next 6 months. Subjects had no current use of antibiotics or anti-inflammatory agents and did not use marijuana for at least 2 days before study visits. Body mass index of all subjects was ≤40 kg/m^2 and they consumed less than 21 alcoholic drinks per week. The participants were not pregnant, nursing or planning on becoming pregnant while enrolled in the study.

Participants in this study visited the clinic monthly for 4-6 consecutive months. They came in the morning before eating breakfast or after at least a 4-h fast, and reported not ingesting alcohol, or using cigarettes or any other tobacco product or e-cigarettes immediately before the clinic visit. At each visit, participants provided a spot urine sample which was collected in a 100 mL sterile specimen cup, aliquoted, and immediately frozen at −20 °C.

TNE,[30] 3-hydroxypropyl mercapturic acid (3-HPMA),[31] hydroxymethylpropyl mercapturic acid (HMPMA),[32] *S*-phenyl mercapturic acid (SPMA),[33] CEMA,[34] and total NNAL[35] were analyzed as described previously, except that in the total NNAL assay 0.5% aqueous HCOOH was used instead of HCl in all the SPE steps, and the final elution was with 5% (v/v) NH_4OH in MeOH. The HMPMA analysis includes mercapturic acids of crotonaldehyde, methyl vinyl ketone, and methacrolein.[32] Creatinine was quantified using a colorimetric microplate assay (CRE34-K01) purchased from Eagle Bioscience.

Statistical methods

All biomarkers were analyzed in the logarithmic scale due to skewed distributions and reported as geometric means with 95% confidence intervals (CI). The limit of detection (LOD) for each biomarker is reported in Table S2, Supporting Information. Biomarkers 3-HPMA and HMPMA had no values below the LOD and were evaluated over the 6 months and compared among the three groups using a linear mixed model (SAS PROC MIXED). Biomarkers CEMA and SPMA had frequent values below the LOD for some of the study groups and were analyzed by group and month using a left-censored mixed effects model[36] (SAS PROC NLMIXED) where values below the LOD were treated as being left censored. These two mixed models can handle cases with missing time points, such that participants with less than 6 months of data were not excluded from the analysis. Using a Bonferroni adjustment for multiple comparisons, the overall group effect for each biomarker was significant at the 0.01 level and the three pairwise group comparisons within each biomarker were evaluated at the 0.017 level of significance. Since no intervention was planned for any of the study groups, no significant changes over time were expected or observed. All statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary NC).

Results

Thirty e-cigarette users (12 male), 63 non-smokers (21 male) and 33 cigarette smokers (16 male) completed 4-6 monthly visits. Mean/median ages of e-cigarette users were 32.7/28.5 (range 18-62); non-smokers were 33.0/31.0 (18-67); and cigarette smokers were 46.8/49.0 (21-67). Further demographic information is summarized in Table S1 and biomarker data validating product use are presented in Table S2, Supporting Information. We excluded from the analysis time points at which e-cigarette users' or non-users' CEMA values were >27 pmol/ml, as this would signal potential exposure to combustion products, for example from secondhand cigarette smoke or marijuana smoke or waterpipe use which could confound the analysis. The 8 e-cigarette users who were not included were subjects 1, 3, 12, 16, 21, 23, 28, and 29 (Table S2). In addition, the two time points from subject 8 and one time point from subject 26 with CEMA>27 pmol/ml were not included in the analysis. After this rigorous exclusion rule, there were 22 e-cigarette users and 63 non-smokers who finished at least 4 monthly visits.

The results summarized in Table 2 demonstrate significantly elevated levels of 3-HPMA in e-cigarette users compared to non-smokers. No significant differences were found for SPMA, CEMA, and HMPMA between e-cigarette users and non-smokers. When the data were expressed per mg creatinine instead of per ml urine, the comparative results among the 3 groups were the same. For the 18 e-cigarette users of the 22 considered in this study who were ex-smokers, the average time since stopping smoking was 45.6 months (range 1 to 108 months) which is well beyond the lifetime of these biomarkers as measured in human urine.[31] The results in Table 2 also confirm significantly higher levels of all biomarkers in cigarette smokers than in e-cigarette users or non-smokers, as previously documented in multiple studies.[25, 28, 37] There were no significant effects of gender on the results expressed

per ml of urine, and there was no relationship of any of the biomarkers to age of the subjects. Biomarker data at each time point are presented in Table S2, Supporting Information.

Discussion

This is to our knowledge the first multi-month longitudinal study to quantify urinary toxicant and carcinogen biomarkers in e-cigarette users compared to non-smokers. We quantified mercapturic acids – 3-HPMA, SPMA, and CEMA - which are specific biomarkers of exposure to acrolein, benzene, and acrylonitrile respectively; and HMPMA which is a biomarker of exposure to crotonaldehyde, methyl vinyl ketone, and methacrolein. Our findings showing higher levels of 3-HPMA in the urine of e-cigarette users compared to non-smokers are consistent with our recent report of significantly higher acrolein-DNA adducts in buccal brushings of e-cigarette users versus non-users of any tobacco product.[38] That study encompassed 3 monthly visits and 18 of the 20 subjects were the same ones who participated in the present study. Levels of DNA adducts of acrolein in buccal brushings did not significantly correlate (non-parametric, Spearman) with those of 3-HPMA in urine, possibly because DNA repair may influence adduct levels and 3-HPMA is relatively short-lived and also has endogenous sources. Our results for 3-HPMA are also consistent with the results of many studies that demonstrate the presence of acrolein in e-cigarette vapor.[39–50]

Acrolein is considered “probably carcinogenic to humans, Group 2A” by the International Agency for Research on Cancer (IARC), which concluded that “there is consistent and coherent evidence that acrolein exhibits key characteristics of carcinogens.”[51] Acrolein administered by inhalation significantly increased the incidence of malignant lymphoma in female mice and caused rare rhabdomyoma and squamous cell carcinoma of the nasal mucosa in female rats. These findings, together with a massive amount of biochemical and molecular biological data, strongly support the IARC classification.[51] The clear evidence for increased uptake of acrolein in e-cigarette users, and increased levels of acrolein-DNA adducts in buccal brushings of e-cigarette users, compared to non-users of any tobacco or nicotine product, present a warning signal regarding the potential carcinogenicity of e-cigarette use.

Several previous studies have reported increased uptake of benzene and acrylonitrile based on measurements of SPMA and CEMA in the urine of e-cigarette users versus non-users (Table 1), but all of these studies used single time points and may not have fully considered other sources of exposure to combustion products such as marijuana smoking and exposure to secondhand cigarette smoke. These issues are clearly important in the evaluation of e-cigarette safety because benzene is a known human carcinogen according to evaluations by IARC and the U.S. Government[52, 53] and acrylonitrile is considered “possibly carcinogenic to humans” by the IARC.[54] Benzene is not commonly detected as a component of e-cigarette vapor, although some reports have appeared.[46, 55] Similarly, acrylonitrile has been infrequently detected as a component of e-cigarette vapor.[46] The possible increased exposure of e-cigarette users to benzene and acrylonitrile requires further study.

HMPMA had been considered as a specific urinary biomarker of crotonaldehyde exposure until our recent study demonstrating that the LC-MS response for HMPMA could actually

be resolved into peaks corresponding to the isomeric mercapturic acids derived from crotonaldehyde, methyl vinyl ketone, and methacrolein.[32] Published data summarized in Table 1 rarely showed an increase of this mixture in e-cigarette users, consistent with the present results, and the fact that these compounds have an additional carbon atom compared to the major e-cigarette ingredients propylene glycol and glycerol. Methyl vinyl ketone, methacrolein, and crotonaldehyde are all powerful irritants and toxicants[56–58] and crotonaldehyde caused liver tumors in rats when administered in the drinking water.[59]

A clear strength of our study compared to previous reports is its longitudinal nature. Single time point studies could be misleading for the reasons noted above. Exposures could occur through inhalation of secondhand tobacco smoke, but our measurements of NNAL in e-cigarette users (Table S2, Supporting Information) provided scant evidence for that. Cooking fumes, industrial emissions, and endogenous processes are further sources of acrolein exposure[51] which could confound single time point measurements but would be less likely to be elevated over a period of months.[34]

A limitation of this study is that we did not analyze the e-cigarettes used by our subjects for acrolein, or the other volatiles which are the sources of the mercapturic acids measured here. It would be informative to carry out studies specifically linking e-cigarette vapor constituents to their urinary biomarkers under normal conditions of use. However, a more practical and important need is to decrease the unregulated use of these products.

In summary, this is the first multi-month longitudinal study to demonstrate significantly increased levels of a urinary biomarker of acrolein in e-cigarette users compared to non-smokers. While e-cigarettes are likely less carcinogenic than tobacco cigarettes, our results indicate that e-cigarette users may have increased risk relative to non-users.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Examples of toxicant and carcinogen exposure in e-cigarette users versus non-users of any tobacco or nicotine-containing product as reported in the literature.

Study	Time points	Fluid analyzed	Reported increased toxicant exposure in e-cigarette users vs. non-users
Lorkiewicz *et al.*[19, 20]	single	urine	acrylonitrile, xylene, *N,N*-dimethylformamide, glycidol
Rubinstein *et al.*[21]	single	urine	acrylonitrile, acrylamide, acrolein, crotonaldehyde, propylene oxide
Keith *et al.*[22]	single	urine	acrylonitrile, acrylamide, acrolein, xylene
Samburova *et al.*[23]	single	breath	aldehydes and methyl ethyl ketone
Fuller *et al.*[24]	single	urine	*o*-toluidine, 2-naphthylamine
Goniewicz *et al.*[25]	single	urine	acrylonitrile, lead, cadmium, pyrene
St. Helen *et al.*[26]	single	urine	acrylamide, benzene
Smith *et al.*[27]	single	urine	acrylonitrile, *N,N*-dimethylformamide
De Jesus *et al.*[28]	single	urine	acrylonitrile, acrolein
Prokopowicz *et al.*[29]	single	urine	None for various metals

Table 2.

Levels of biomarkers in 22 e-cigarette users (E, 8 male), 63 non-smokers (N, 21 male), and 33 smokers (S, 16 male) in urine samples collected monthly for 4-6 months. Among the groups, the number of monthly visits completed were: e-cigarettes - 22 participants completed 6 visits; non-smokers - 55 completed 6, 3 completed 5, 5 completed 4; smokers - 29 completed 6 and 4 completed 4. Samples from all completed visits were used in the analysis.

Biomarker	Group	Geometric mean	95% CI	Group p-value	Pairwise
3-HPMA (acrolein) pmol/ml	**N**on-smokers	679.3	554.2, 832.4		E vs S, p<0.001
	E-cig users	1249	885.7, 1762	<0.001	E vs N, p=0.003
	Smokers	5576	4210, 7384		N vs S, p<0.001
SPMA (benzene) pmol/ml	**N**on-smokers	0.12	0.09, 0.17		E vs S, p<0.001
	E-cig users	0.17	0.10, 0.30	<0.001	E vs N, p=0.250
	Smokers	10.00	6.54, 15.29		N vs S, p<0.001
CEMA (acrylonitrile) pmol/ml	**N**on-smokers	1.74	1.31, 2.32		E vs S, p<0.001
	E-cig users	2.72	1.68, 4.41	<0.001	E vs N, p=0.120
	Smokers	531.9	358.4, 789.4		N vs S, p<0.001
HMPMA (crotonaldehyde; methyl vinyl ketone; methacrolein) pmol/ml	**N**on-smokers	586.5	485.4, 708.8		E vs S, p<0.001
	E-cig users	696.3	505.5, 959.1	<0.001	E vs N, p=0.363
	Smokers	4771	3673, 6197		N vs S, p<0.001
TNE nmol/ml	**N**on-smokers	*a*			
	E-cig users	34.3			
	Smokers	58.2			

a there were only 2 TNE values above the LOD for non-smokers