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Original investigation

Comparison of Urinary Biomarkers of Exposure in Humans Using Electronic Cigarettes, Combustible Cigarettes, and Smokeless Tobacco

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Abstract

Background: Cigarette smoking is associated with an increase in cardiovascular disease risk, attributable in part to reactive volatile organic chemicals (VOCs). However, little is known about the extent of VOC exposure due to the use of other tobacco products.

Methods: We recruited 48 healthy, tobacco users in four groups: cigarette, smokeless tobacco, occasional users of first generation e-cigarette and e-cigarette menthol and 12 healthy nontobacco users. After abstaining for 48 h, tobacco users used an assigned product. Urine was collected at baseline followed by five collections over a 3-h period to measure urinary metabolites of VOCs, nicotine, and tobacco alkaloids.

Results: Urinary levels of nicotine were ≈2-fold lower in occasional e-cigarette and smokeless tobacco users than in the cigarette smokers; cotinine and 3-hydroxycotinine levels were similar in all groups. Compared with nontobacco users, e-cigarette users had higher levels of urinary metabolites of xylene, cyanide, styrene, ethylbenzene, and benzene at baseline and elevated urinary levels of metabolites of xylene, N,N-dimethylformamide, and acrylonitrile after e-cigarette use. Metabolites of acrolein, crotonaldehyde, and 1,3-butadiene were significantly higher in smokers than in users of other products or nontobacco users. VOC metabolite levels in smokeless tobacco group were comparable to those found in nonusers with the exception of xylene metabolite—2-methylhippuric acid (2MHA), which was almost three fold higher than in nontobacco users.

Conclusions: Smoking results in exposure to a range of VOCs at concentrations higher than those observed with other products, and first generation e-cigarette use is associated with elevated levels of N,N-dimethylformamide and xylene metabolites.

Implications: This study shows that occasional users of first generation e-cigarettes have lower levels of nicotine exposure than the users of combustible cigarettes. Compared with combustible cigarettes, e-cigarettes, and smokeless tobacco products deliver lower levels of most VOCs, with the exception of xylene, N,N-dimethylformamide, and acrylonitrile, whose metabolite levels were higher in the urine of e-cigarette users than nontobacco users. Absence of anatabine in the urine of e-cigarette users suggests that measuring urinary levels of this alkaloid may be useful in distinguishing between users of e-cigarettes and combustible cigarettes. However,

these results have to be validated in a larger cohortcomprised of users of e-cigarettes of multiple brands.

Introduction

The use of tobacco products is associated with an increase in the risk of several chronic diseases, such as cardiovascular disease (CVD), respiratory disease, and cancer.[1] Although in the last few decades the rates of smoking have declined significantly in the United States, the use of new tobacco products, such as electronic cigarettes (e-cigarettes), has seen an appreciable increase. According to the recent Surgeon General's report,[2] 5.7% adults (aged 25 or older) and 13.6% of young adults (18–24 years of age) used e-cigarettes on one or more occasion in the past 30 days. In addition, 5.3% of middle school students and 16.0% of high school students have also used e-cigarettes.

Although there are several reasons for the increasing acceptance of e-cigarettes, most nonsmoking young individuals and adult smokers are drawn to e-cigarettes because of their perceived safety[3,4] and the widespread belief that e-cigarettes can help smokers quit combustible cigarettes,[5,6] and deal with withdrawal symptoms.[3] It is also believed that e-cigarettes do not contain many of the harmful or potentially harmful substances (HPHCs) produced by combustible tobacco products and that they deliver nicotine at lower levels than conventional cigarettes, making them less addictive, less harmful, and easier to quit.[4,7–9]

In most e-cigarettes, a nicotine solution in propylene glycol and vegetable glycerin is heated to generate an aerosol, which when inhaled, delivers nicotine to the user. Nicotine is thus delivered without many of the combustion products present in conventional cigarettes and can reach blood levels that can match or exceed those reached after a combustible cigarette exposure.[10,11] Although in e-cigarettes, aerosolization by heating eliminates many of the combustion-derived HPHCs, polycyclic aromatic hydrocarbons (PAHs) and some volatile organic chemicals (VOCs),[12–14] recent research has shown that e-cigarette emissions contain significant levels of carbonyls including reactive aldehydes and ketones such as formaldehyde, acetaldehyde, acrolein, and acetone[15–17] albeit at much lower levels than in combustible cigarettes. The presence of these chemicals in e-cigarette aerosols is of concern because risk estimates suggest that aldehydes such as acrolein account for 80%–85% of the total noncancer risk of smoking and that they contribute 40–100 times more to such risk than any other chemical present in cigarette smoke.[18] Data from animal studies have shown that such reactive aldehydes induce significant cardiovascular and pulmonary dysfunction and injury.[19] In humans, exposure to formaldehyde can cause airway irritation and bronchial asthma,[20,21] and exposure to acrolein has been found to be associated with increased CVD risk, even in nonsmokers.[22]

The levels of VOCs in e-cigarette emissions vary with device type, use pattern, and the extent of use,[16] and it remains unclear whether the chemicals generated under standardized smoking machine conditions are also produced during normal use of e-cigarette by experienced users. Estimates of VOC exposure derived from measurements of their urinary metabolites suggest that e-cigarette users are exposed to lower levels of acrolein, benzene, crotonaldehyde, and propylene oxide than users of combustible cigarettes.[23] However, in addition to acrolein, benzene and crotonaldehyde, combustible cigarettes generate a host of other VOCs such as acetaldehyde, acrylonitrile, 1,3-butadiene, carbon disulfide, cyanide, styrene, toluene, and xylene, and it is not known whether the use of e-cigarettes results in significant exposure to these HPHCs. Although the levels of the urinary metabolites of several of these HPHCs have been reported to be reduced by switching from combustible cigarettes to e-cigarettes,[24,25] it remains unclear to what extent the use of e-cigarettes directly contributes to exposure to these VOCs. Therefore, the current study was designed to assess time-dependent changes in tobacco alkaloids (TA) and VOC metabolites, immediately after e-cigarette use so that measurements of these metabolites in the urine could provide a direct estimate of exposure attributable to e-cigarette use. To provide context and comparison, we also measured changes in urinary nicotine and VOC metabolites after the use of combustible cigarettes and smokeless tobacco (ST).

Methods

Study Design

Self-reported healthy adults of either sex (n = 48) between 21 and 55 years of age were recruited for the study. The study protocol was approved by the institutional review board at the University of Louisville, and all participants provided written informed consent. To be included in the study, participants had to be willing to refrain from smoking and tobacco products of any kind for 48 h and fast for 12 h before the scheduled visit. They had to remain at the study location for the entire study period. Criteria for exclusion included known diagnosis of thyroid disease, HIV, hepatitis, cancer, inflammatory conditions, chronic liver disease, anemia, renal replacement therapy, taking medications that may interfere with the metabolism of tobacco, testosterone or estrogen therapies, and diagnosis and active treatment of drug or alcohol abuse. Participants with BMI > 40 or weight < 100 lbs, pregnant women, prisoners, and/or other vulnerable populations were excluded from the study.

Participants were self-reported occasional tobacco and/or e-cigarette users, and were asked to abstain from tobacco, e-cigarettes, and nicotine products for 48 h before the study visit. The 48 h cessation period before the second visit was used to eliminate any residual TA and their metabolites.[26–28] Twelve healthy nontobacco users, not exposed to secondhand smoke, were recruited as control subjects. One nontobacco user was excluded due to unusually high levels of acetate and formate metabolites in the urine (over ×10 higher than other participants). We also excluded one ST user due to unusually high level of urinary cotinine at the baseline (suggesting active tobacco use) despite 48 h of reported abstinence. The participants were assigned to one of four product categories: users of (1) combustible cigarettes (self-reported occasional smokers, who have smoked at least 20 cigarettes in their lifetime and who have smoked in the last 12 months); (2) ST (have used at least one package of chewing tobacco, dry or moist snuff in their lifetime, and currently using ST once or less a week); (3) e-cigarettes (self-reported occasional users, who have used electronic cigarettes or other electronic nicotine delivery devices on at least 10 separate occasions in their lifetime and who currently use electronic cigarettes once or less per day); and (4) mentholated e-cigarettes (e-cigarette users were given an option to use mentholated e-cigarettes, e-cigarettes with other flavors, or both). Users of more than one product group at the time of the study

could participate multiple times in each product category. Specific group assignments are shown in Supplementary Table 1.

The study consisted of two visits. At Visit 1, height, weight, BMI, blood pressure were measured and summarized in Supplementary Table 2. Clean catch urine samples were collected at the end of Visit 1 (baseline for TA and VOC metabolites) and an additional sample was obtained 22–24 h after baseline in the participants' home, to ensure steady decrease in the levels of biomarkers of exposure. These "at home" urine samples were collected in a sterile urine collection cup, placed in provided insulated coolers with two-ice packs, stored in the refrigerator, and later returned to the research team at the follow up appointment 20–28 h after home urine collection (all urinary biomarkers were stable within the storage period—stability data not shown). The participants were then instructed to abstain for 48 h from tobacco, e-cigarettes, nicotine, and smoking of any kind (including marijuana and other illicit drugs). They were also asked not to eat and drink any caffeinated or alcoholic beverages or grapefruit juice 8 h prior to the second visit. During Visit 2, the home urine samples were collected and inspected for proper labeling and time of collection. At the beginning of the visit, the participants were asked to provide a urine sample and subsequently empty their bladder completely. Immediately after urine collection the participants used the tobacco product. Depending on the study group, participants were asked to smoke one Marlboro Red cigarette (nicotine 1.2 mg/cigarette), use one or two pouches (depending on participants' usage pattern) of Grizzly Premium Straight ST (~10.5 mg/g nicotine), use NJOY King e-cigarette (2.4% nicotine) or NJOY King Menthol (3.0% nicotine) e-cigarette. Both the ST and e-cigarette products were used *ad libitum* but no longer than 15 min and no less than 15 puffs (e-cigarettes). A new urine sample was obtained 20 min (±5 min) after the first collection, but after exposure to the tobacco product. Urine was collected at specific timepoints, (0 right before exposure and 20, 40, 80, 120, and 180 ± 5 min after the first urine sample). Multiple sample collections within 3 h of usage of tobacco products ensured the quantitation of VOCs with short half-lives.[27,29] All urine samples were refrigerated and processed within 1 h after the end of the study. TA, cotinine, 3-hydroxycotinine (3HC), and VOC metabolites were measured in all the specimen at all timepoints.

Sample Analyses

Urinary metabolites of 20 VOCs; free forms of TA—nicotine, anatabine, and anabasine; and free forms of nicotine metabolites—cotinine and 3HC were quantified by ultra performance liquid chromatography-mass spectrometry (UPLC-MS/MS) as described by Alwis et al.[30] with slight modifications. Urine samples were diluted with solvent A (15 mM ammonium acetate, pH 6.8), spiked with isotopically labeled internal standards (IS), and analytes were characterized and quantified by UPLC-MS/MS. Urine formate and acetate (metabolites of formaldehyde and acetaldehyde, respectively) were analyzed by gas chromatography-mass spectrometry (GC-MS) using isotopically labeled IS. Concentrations of analytes were normalized to urinary creatinine levels. Detailed description of UPLC-MS/MS and GC-MS assays are described in Supplementary Material.

Statistics and Data Analysis

Data are mean ± standard deviation (SD). The Kruskal–Wallis test was used to test for significant differences between smoking groups. The Wilcoxon Rank Sum test was used to test for significant differences between tobacco groups and non-tobacco users. Statistical significance was accepted at the $p < .05$ level. For each tobacco group, Z-scores were calculated as [Z-score = (VOC concentration – mean (VOC in NTU))/SD (VOC in NTU)] to determine, for each VOC, how many standard deviations away from the mean of NTU. In Z-Score plots, error bars are SEM. Data were analyzed using SAS, version 9.4 (SAS Institute, Inc., Cary, NC) and GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA).

Results

Subjects and Recruitment

Mean age of the participants was 34 ± 1 years, 65% were male, 92% Caucasian, 2% African American, and the remaining 6% did not disclose their ethnicity. Sixty-seven percent of participants were dual tobacco product users with 40% using both combustible cigarettes and e-cigarettes (Supplementary Table 1). For each tobacco product tested, we measured three TA, two nicotine metabolites, and metabolites of 20 VOCs in the urine. Eleven of these metabolites were below detection limit of our UPLC-MS/MS method (data not shown).

Tobacco Alkaloids and Nicotine Metabolites in the Urine of Sole Users

At Visit 1, we measured TA, cotinine, 3HC, and VOC metabolites in 12 nontobacco product users and a subset of individuals which were sole users of tobacco products—3 e-cigarette users, 4 combustible cigarette smokers, and 10 users of ST (Supplementary Table 1). As shown in Table 1, TA and nicotine metabolites were undetectable at baseline (–48 h; visit 1) in nontobacco users. Nicotine was abundant in the urine of subjects using combustible cigarettes and ST products whereas only traces of nicotine were detected in the urine of e-cigarette users (Table 1). Urine cotinine and 3HC levels in ST users was 3.2–6.7-fold higher than smokers and e-cigarette users. Appreciable amounts of anatabine and anabasine were also present in the urine of ST users but not in smokers or e-cigarette users (Table 1).

VOC Metabolites in the Urine of Sole Users

Because tobacco contains several VOCs and additional VOCs are generated by heating or burning tobacco, we measured the urinary concentration of 20 metabolites of VOCs in the urine of sole users of tobacco products and nonusers of different tobacco products (Table 1). The concentrations of VOCs in our study are similar to those reported previously.[30,31] In comparison with nontobacco users, the concentrations of metabolites derived from xylene, N,N-dimethylformamide, acrylonitrile, and crotonaldehyde were significantly higher in cigarette smokers. Similarly, the baseline urine of e-cigarette users showed higher levels of xylene, cyanide, styrene, ethylbenzene, acrolein, and benzene metabolites than nontobacco users. Levels of VOC metabolites in the urine of ST users were comparable with nontobacco users, and no statistically significant differences were observed between these groups.

Tobacco Alkaloids and Nicotine Metabolites in All the Participants After Exposure to Tobacco Products

Similar to sole users, participants using multiple tobacco products also had high levels of TA and metabolites of nicotine in the urine (Figure 1). Abstinence from the use of tobacco products for 48 h substantially depleted the levels of all of the TA and their metabolites (Figure 1). Analyses of urine at Visit 2, prior to the usage of

Table 1. Baseline Urinary Levels of Tobacco Alkaloids and Metabolites of Nicotine and VOCs at Visit 1.

Parent compound	Analyte	Average concentration (ng/mg creatinine)			
		NTU	CIG	ST	ECIG
Tobacco alkaloids					
Anabasine	ANB	0.0 (0.0)	1.6 (4.3)	4.4 (4.6)*	0.0 (0.0)
Anatabine	ANTB	0.0 (0.0)	3.6 (9.3)	8.1 (7.9)*	0.0 (0.0)
Nicotine	NIC	0.0 (0.0)	494.7 (1273.7)*	519.9 (531.0)*	17.8 (30.9)
	COT	0.1 (0.2)	69.5 (122.0)*	466.1 (390.0)*	147.3 (249.3)
	3HC	0.0 (0.0)	535.6 (1105.0) *	1713.0 (2498.9)*	498.2 (863.0)
Volatile organic compounds					
Acetaldehyde	Acetate	1480.2 (630.6)	2771.3 (1838.8)	914.0 (472.4)	4070.6 (2418.6)
Acrolein	CEMA	51.5 (26.9)	110.2 (118.5)	91.7 (53.9)	18.4 (16.1)*
	3HPMA	294.3 (344.7)	544.7 (596.5)	221.2 (139.0)	332.5 (397.4)
Acrylamide	AAMA	59.1 (57.8)	84.4 (57.3)	29.8 (9.6)	136.2 (185.3)
	GAMA	82.8 (69.3)	146.7 (218.1)	56.9 (39.8)	21.0 (36.4)
Acrylonitrile	CYMA	4.7 (15.6)	33.0 (51.2)*	1.7 (3.4)	43.5 (75.3)
Acrylonitrile, vinyl chloride, ethylene oxide	HEMA	1.1 (1.4)	1.2 (1.1)	1.3 (0.6)	0.0 (0.0)
Benzene	MU	144.0 (80.4)	186.9 (74.9)	380.2 (577.1)	317.5 (92.7)*
1-Bromopropane	BPMA	13.3 (9.2)	15.5 (10.9)	12.5 (7.9)	4.6 (7.9)
1,3-Butadiene	DHBMA	368.6 (155.9)	381.8 (150.4)	274.9 (78.9)	359.1 (8.5)
	MHBMA1	0.1 (0.2)	0.4 (0.8)	0.0 (0.0)	0.0 (0.0)
	MHBMA2	0.2 (0.4)	0.1 (0.2)	0.2 (0.4)	1.3 (2.2)
	MHBMA3	4.6 (3.7)	14.3 (19.3)	2.9 (1.0)	6.8 (11.7)
Carbon disulfide	TTCA	19.4 (19.6)	96.1 (147.9)	4.4 (7.8)	6.4 (11.0)
Crotonaldehyde	HPMMA	157.6 (48.0)	358.1 (491.0)*	111.7 (19.0)	275.9 (245.0)
Cyanide	ATCA	115.5 (77.1)	343.2 (444.5)	134.6 (213.0)	439.7 (257.8)*
N,N-Dimethylformamide	AMCC	113.9 (83.7)	237.2 (135.7)*	66.3 (27.9)	201.8 (85.1)
Ethylbenzene, styrene	PGA	205.2 (75.4)	216.6 (77.7)	201.4 (77.9)	324.5 (75.5)*
Formaldehyde	Formate	5951.7 (3108.7)	8345.0 (6095.2)	3919.9 (2514.2)	16408.6 (10155.3)
Propylene oxide	2HPMA	84.0 (133.9)	89.2 (103.4)	33.7 (16.8)	37.0 (33.6)
Styrene	PHEMA	0.4 (1.0)	0.8 (1.3)	0.0 (0.0)	0.4 (0.7)
	MA	132.0 (41.0)	187.9 (61.9)	157.7 (38.6)	197.2 (35.9)*
Toluene	BMA	6.7 (5.6)	6.6 (4.6)	3.4 (1.0)	1.5 (1.3)*
Trichloroethylene	1,2DCVMA	2.7 (4.4)	6.7 (17.8)	2.3 (3.2)	1.6 (2.7)
	2,2DCVMA	0.0 (0.0)	1.0 (2.5)	0.0 (0.0)	0.0 (0.0)
Xylene	2MHA	10.4 (7.9)	59.4 (79.1)*	51.3 (62.8)	20.8 (27.9)
	3MHA+ 4MHA	71.9 (29.6)	197.9 (207.8)*	273.7 (283.9)	316.3 (349.1)*

All the participants (nontobacco users, NTU, $n = 12$; smokers, CIG, $n = 8$; smokeless tobacco, ST, $n = 5$; and electronic cigarette, ECIG, $n = 3$) were sole users of indicated products. Urine was collected prior to tobacco cessation for 48 h. VOC metabolites in the urine were measured by LC-MS/MS. Values are mean ± *SD*. *$p < .05$ versus NTU as analyzed by the Wilcoxon Rank Sum test. 1,2DCVMA = N-Acetyl-S-(1,2-dichloroethenyl)-L-cysteine; 2,2DCVMA = N-Acetyl-S-(2,2-dichloroethenyl)-L-cysteine; 2HPMA = N-Acetyl-S-(2-hydroxypropyl)cysteine; 2MHA = 2-Methyl Hippuric Acid; 3HC = 3-hydroxycotinine; 3HPMA = N-Acetyl-S-(3-hydroxypropyl)cysteine; 3MHA+4MHA = 3-Methyl Hippuric Acid + 4-Methyl Hippuric Acid; AAMA = N-Acetyl-S-(carbamoylethyl)-L-cysteine; AMCC = N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine; ANB = Anabasine; ANTB = Anatabine; ATCA = 2-Aminothiazoline-4-carboxylic Acid; BMA = N-Acetyl-S-benzyl-L-cysteine; BPMA = N-Acetyl-S-propyl-L-cysteine; CEMA = N-Acetyl-S-(2-carboxyethyl)-L-cysteine; COT = Cotinine; CYMA = N-Acetyl-S-(2-cyanoethyl)-L-cysteine; DHBMA = N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine; GAMA = N-Acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine; HEMA = N-Acetyl-S-(2-hydroxyethyl)-L-cysteine; HPMMA = N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine; MA = Mandelic acid; MHBMA1 = N-Acetyl-S-[1-(hydroxymethyl)-2-propen-1-yl)-L-cysteine; MHBMA2 = N-Acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine; MHBMA3 = N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine; MU = trans,trans-Muconic acid; NIC = Nicotine; PGA = Phenylglyoxylic Acid; PHEMA = N-Acetyl-S-(2,5-dimethylbenzene)-L-cysteine; TTCA = 2-Thioxothiazolidine-4-carboxylic acid..

various tobacco products (0 min), showed only traces of nicotine in the participants of various groups; however, appreciable amounts of cotinine and 3HC were detected in the subjects of all study groups, which may reflect tobacco exposure over the past 2–3 days (Figure 1). Alkaloid anatabine was found in urine of 6 out of 12 participants who used a combustible cigarette and 7 out of 12 ST users (data not shown). Anatabine was not detected in urine participants exposed to e-cigarettes at Visit 2. In addition, free anabasine could not be found in the urine of any subject using multiple tobacco products (data not shown). After collection of the urine at 0 min time point, study participants used tobacco products for 15 min, and urine was obtained 5 min after the consumption of tobacco products (20 min).

Urinary elimination of nicotine occurred rapidly after exposure to all tested products (Figure 1A). The maximum concentration observed in the sampling period was reached at 20 min (5 min post-exposure) in smokers and 40 min (25 min postexposure) after using ST and e-cigarettes. Cumulative urinary nicotine levels of e-cigarette users were comparable with users of ST. However, in individuals who smoked a combustible cigarette, the accumulated levels of nicotine (collected over 180 min) were ~2 fold higher than in those who smoked e-cigarettes or used ST (Figure 1E).

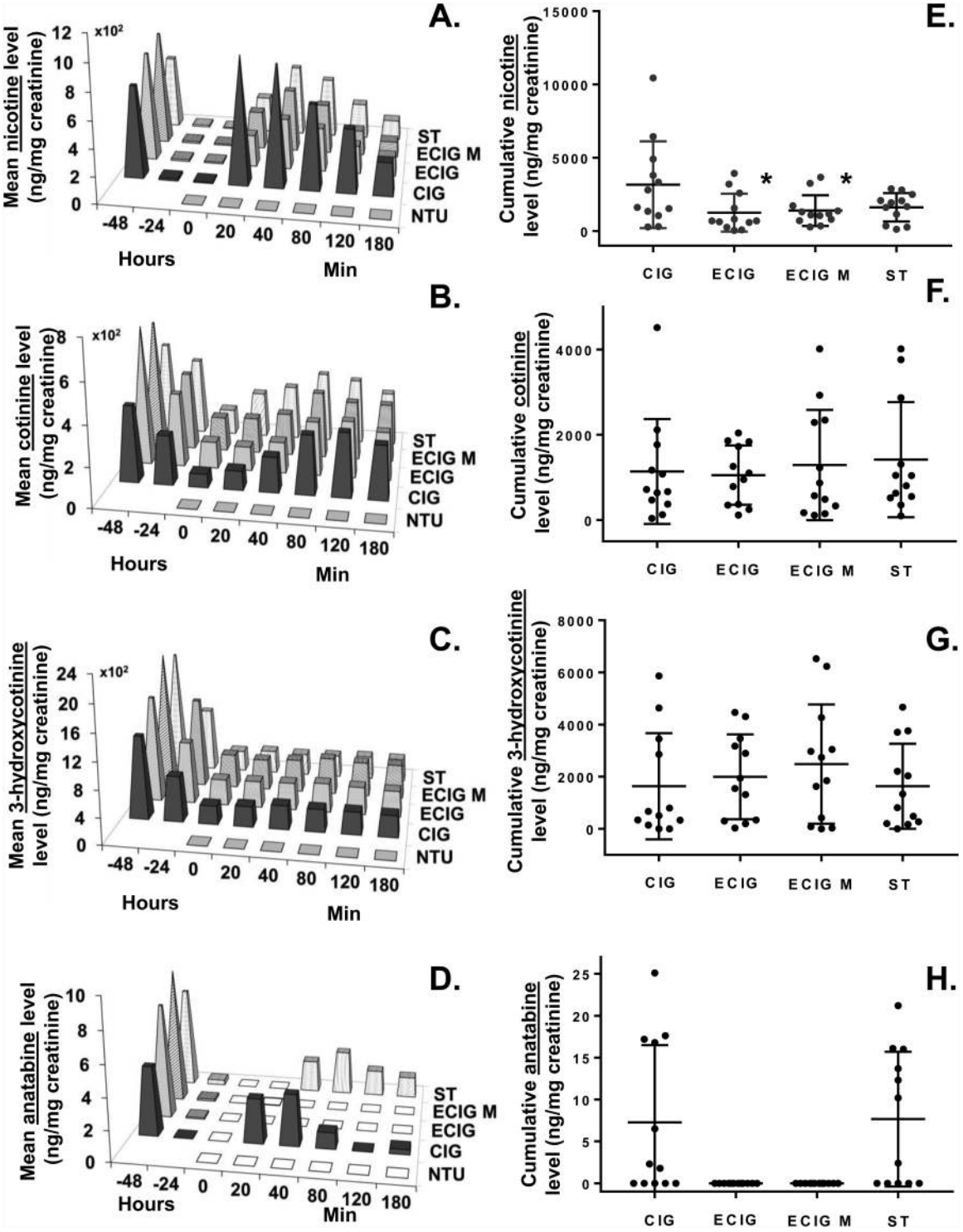

Figure 1. Urinary levels of tobacco alkaloids and nicotine metabolites in tobacco users at Visit 2. Participants abstained from the usage of any tobacco products for 48 h, and then during the visit used one of the tobacco products—one Marlboro red cigarette (CIG), one to two pouches of Grizzly premium straight smokeless tobacco (ST), NJOY king e-cigarette (ECIG), or NJOY king menthol e-cigarette (E-CIG M). Usage of all the tobacco products was restricted to a maximum of 15 min. Panels A-D show the time course of elimination of (A) nicotine, (B) cotinine, (C) 3-hydroxycotine, and (D) anatabine in the urine of participants. Each bar represents the average urinary analyte level at the indicated time point. Anatabine was detected only in the urine of six smokers (CIG) and seven smokeless tobacco (ST) users. Panels E–H illustrate cumulative urinary levels of (E) nicotine, (F) cotinine, (G) 3-hydroxycotinine, and (H) anatabine in users of tobacco products. Values are mean ± *SD*. Wilcoxon Rank Sum test was used to determine significant differences between users of various tobacco products and nontobacco users. **P* < .05 versus CIG.

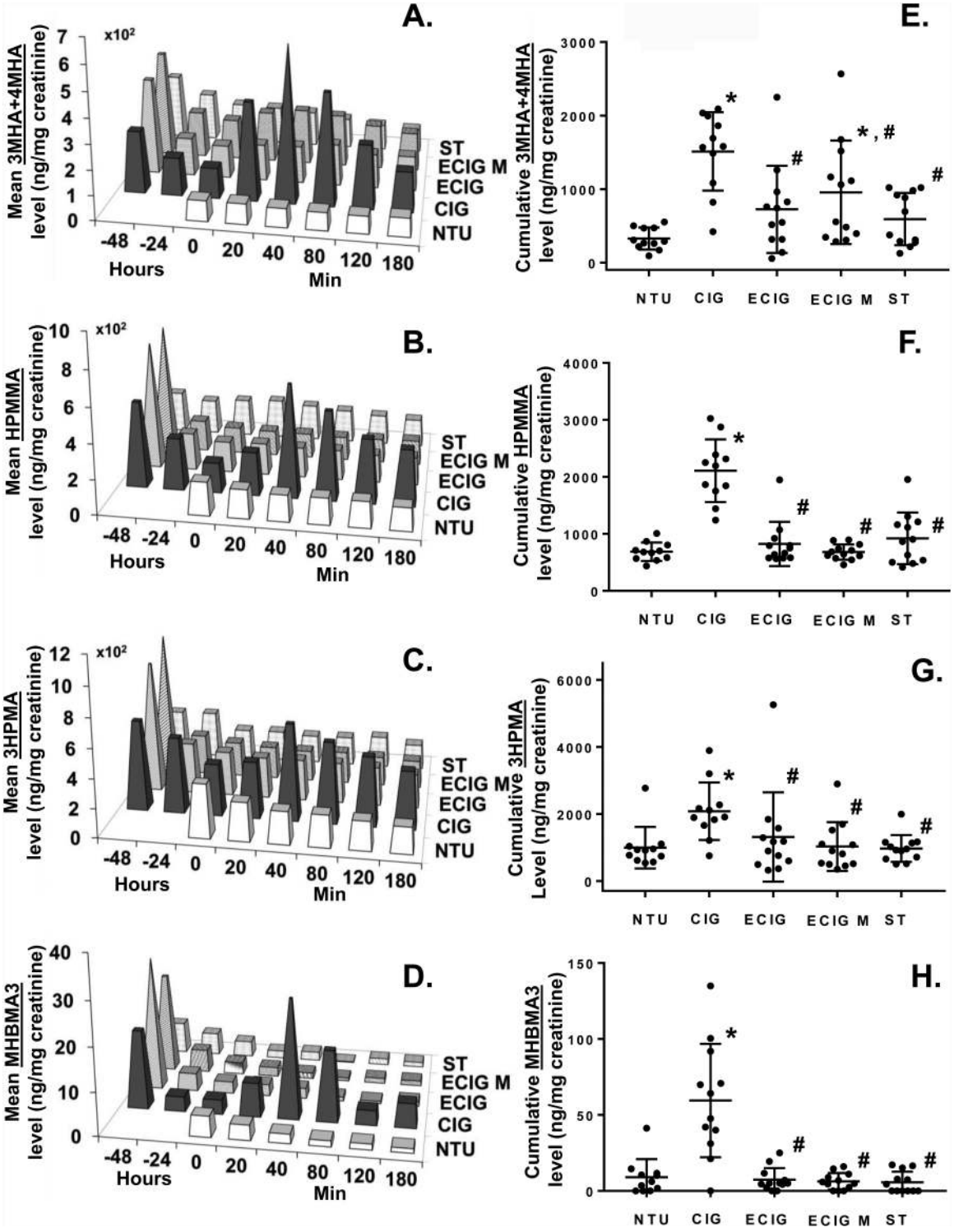

Figure 2. Urinary levels of VOC metabolites in tobacco users at Visit 2. Urine was collected as described in Figure 1. Panels A-D show the time course of the abundance of VOC metabolites in the urine of participants. Each bar represents the average urinary level of (A) 3MHA+4MHA, (B) HPMMA, (C) 3HPMA, (D) MHBMA3 at the indicated timepoint. Panels E–H illustrate cumulative urinary levels of (E) 3MHA+4MHA and (F) HPMMA, (G) 3HPMA, (H) MHBMA3 in users of various tobacco products. Values are mean ± *SD*. Wilcoxon Rank Sum test was used to determine significant differences between users of various tobacco products and non-tobacco users. *$p < .05$ versus nontobacco users (NTU) and #$P < .05$ versus smokers (CIG).

Urine cotinine levels peaked at 80 min (65 min after tobacco usage) and remained unchanged up to 180 min (Figure 1B). The cumulative amounts of cotinine recovered in the urine did not differ among users of different tobacco products (Figure 1F). Urine 3HC levels did not change upon the usage of any tobacco products for 15 min (Figure 1C). Likewise, cumulative urine 3HC levels remained constant in all the

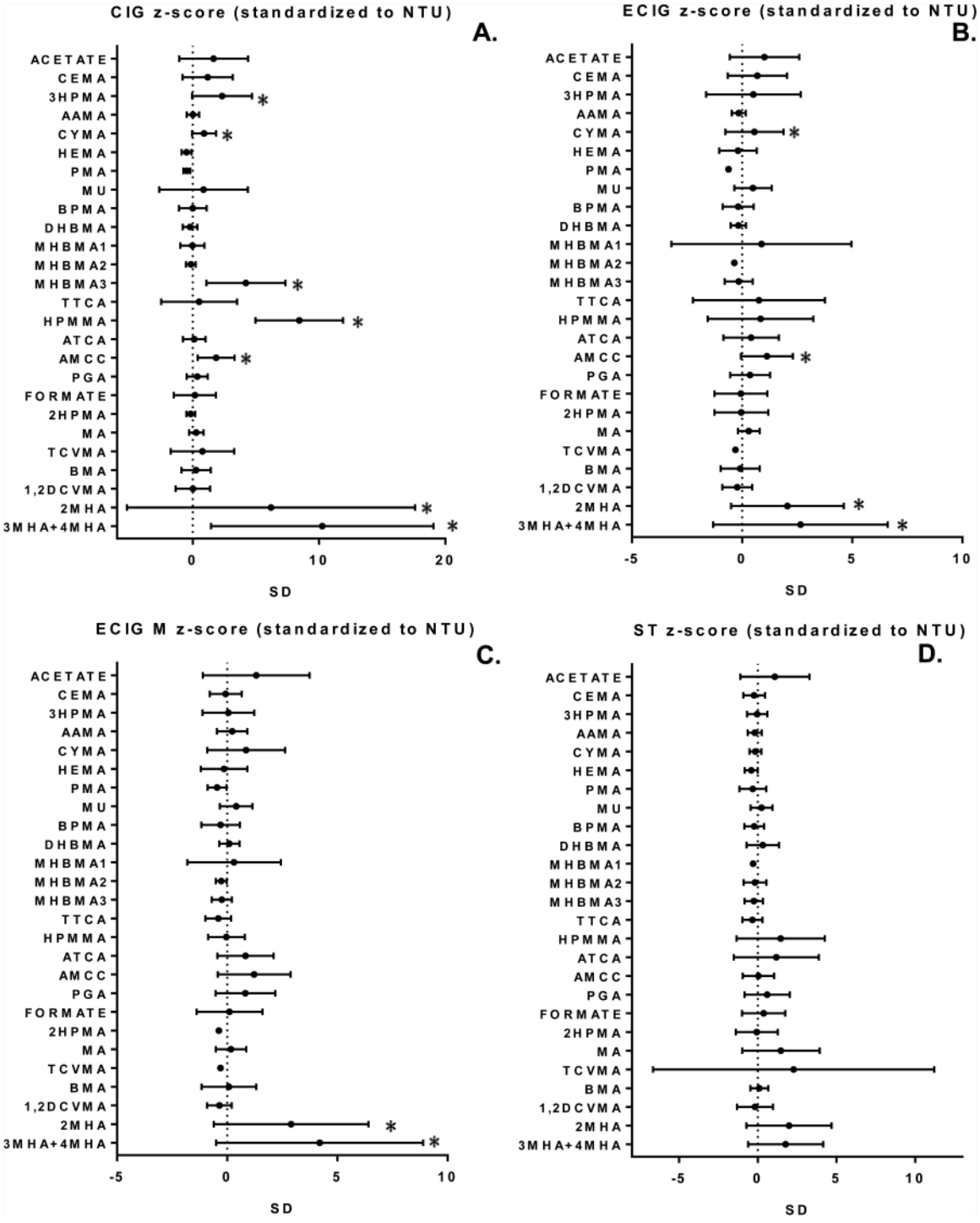

Figure 3. *Z*-score plots of cumulative urinary metabolite levels detected after the exposure to tobacco products. Urine was collected as described in Figure 1. *Z*-scores standardized to nontobacco users (NTU) were calculated for the sum of concentrations across timepoints 20–180 min for each VOC using the equation *Z*-score = (VOC concentration – mean (VOC in NTU))/*SD* (VOC in NTU). Panels A–D show *Z*-scores of smokers (CIG), e-cigarette (ECIG), mentholated e-cigarette (ECIG M), and smokeless tobacco (ST), respectively. Values are mean ± SEM. **p* < .05 versus NTU as analyzed by the Wilcoxon Rank Sum test.

study groups throughout the course of data collection for 180 min (Figure 1G). Anatabine, which was only detected in the urine of six smokers and seven ST users (Figure 1H) but not in participants exposed to any of the e-cigarettes, peaked at 40 min in smokers and 80 min in ST users (Figure 1D). Cumulative anatabine levels for 180 min in smokers were comparable with participants using ST (Figure 1H).

VOC Metabolite Levels After Usage of Various Tobacco Products: Measurement of the Urinary

VOC metabolites at Visit 2 (0 min; prior to the usage of tobacco products), after a 48-h tobacco cessation period, showed that metabolites of acrolein (3-hydroxypropylmercapturic acid [3-HPMA]), crotonaldehyde (3-hydroxy-1-methylpropylmercapturic acid

[HPMMA]), 1,3-butadiene (monohydroxy-3-butenyl mercapturic acid [MHBMA3]) (Figure 2), and acrylonitrile (N-Acetyl-S-(2-cyanoethyl)-L-cysteine [CYMA]) (data not shown) dropped to the levels comparable with non-tobacco users. Abstaining from tobacco use for 48 h also decreased the levels of metabolites of xylene 3-methylhippuric acid + 4-methylhippuric acid (3MHA+4MHA) by 40%–65%, but they were still more than 2-fold higher than the nontobacco users. Forty-eight hours of tobacco cessation did not affect the levels of other VOC metabolites (data not shown). Time course studies after the usage of tobacco products for 15 min showed that urinary metabolites of xylene 3MHA+4MHA peaked at 40 min (25 min after exposure) (Figure 2A). Similarly, the maximum concentration of HPMMA, 3-HPMA, and MHBMA3 was noted at 40 min in the urine of participants who used combustible cigarettes (Figure 2B–D). Levels of these VOC metabolites did not increase after the use of other tobacco products.

Cumulative urinary levels of the xylene metabolite 2-methylhippuric acid (2MHA) were ~7.2-fold higher in smokers (p = .087), ~2.9-fold higher (p = .033) in ST users, ~3 times higher in nonmentholated, and 3.8 times higher in mentholated e-cigarette users, whereas 3MHA+4MHA levels were ~5.7-fold higher (p = .002) in smokers and 2.9-fold higher in mentholated e-cigarette users (p = .011) than in nontobacco products users (Supplementary Table 5 and Figure 2E). Similarly, cumulative levels of CYMA—a metabolite of acrylonitrile were four times higher in smokers (p = .001) and ~2.85-fold (p = .044) and ~3.8-fold (p = .043) higher in the urine of e-cigarette and e-cigarette menthol users, respectively. Additionally, N-Acetyl-S-(N-methylcarbamoyl)-L-cysteine (AMCC)—a metabolite of N,N-dimenthylformamide was significantly higher in urine of combustible cigarette (2.6-fold, p = .002) and e-cigarette users (~2-fold and p = .022; P > .05 in mentholated e-cigarettes), whereas cumulative HPMMA, 3-HPMA, and MHBMA3 concentrations were only significantly higher in smokers than nontobacco users (p < .05). Moreover, cumulative levels of 3MHA+4MHA, HPMMA, 3-HPMA, and MHBMA3 in e-cigarette, mentholated e-cigarette, and ST users were significantly lower than the smokers (p < .05) (Figure 2E–H). Supplementary Table 5 lists cumulative levels of all VOC metabolites measured at Visit 2 after use of tobacco products (Figures 2E–H represent selected plots) and concentrations of these metabolites in urine on nontobacco users.

To analyze the product specific abundance of VOC metabolites relative to the non-tobacco users, we determined Z-scores of analytes [Z-score = (VOC concentration – mean (VOC in NTU))/SD (VOC in NTU)]. Our data show that Z-scores for the metabolites of acrolein, crotonaldehyde, xylene, acrylonitrile, 1,3-butadiene, and N,N-dimethylformamide were significantly higher in the urine of combustible cigarette users than nontobacco users (Figure 3). These findings are in agreement with previous reports.[30,32–34] We also observed that xylene metabolites were significantly elevated in the urine of e-cigarette (both nonflavored and menthol-flavored) users. Further, the Z-scores of CYMA, and AMCC were significantly higher in the urine of e-cigarette users than nontobacco users (Figure 3).

Discussion

Although the use of e-cigarettes has dramatically increased in recent years, little is known about the health effects of these devices. Few studies have been conducted to determine the rate of nicotine delivery from these devices, or to characterize and quantify VOCs generated in e-cigarettes. Because e-cigarettes aerosolize nicotine, rather than burn tobacco, certain VOCs present in the smoke of combustible cigarettes are either reduced in concentration or are not present in e-cigarette aerosols. While measurements of nicotine and VOCs in e-cigarette aerosols generated by a smoking machine suggest that e-cigarettes generate lower levels of VOCs than conventional cigarettes,[15,35] few studies have examined the relative exposure to VOCs and nicotine under conditions of normal use.

Our measurements of urinary nicotine and nicotine metabolites indicate that exposure to first generation e-cigarettes (ciga-like) used in this study results in lower levels of nicotine exposure than the use of combustible cigarettes. Previous studies have shown that e-cigarettes can deliver nicotine in the blood at levels similar to those delivered by combustible cigarettes, although time to peak concentration is delayed in e-cigarette users than in users of tobacco cigarettes.[36,37] In contrast, our measurements of urinary metabolites indicate that e-cigarettes, at least the ones used in this study, deliver less nicotine than conventional tobacco cigarettes.

Even though the cumulative levels of nicotine were higher in tobacco cigarette users, the levels of cotinine and 3HC were not different among the three groups, indicating that higher rates of nicotine delivery by combustible cigarettes are associated with lower metabolism of nicotine, such that higher levels of unmetabolized nicotine appear in the urine. The consequences of higher rates of elimination of unmetabolized nicotine remain unknown, but it appears likely that lower rates of nicotine metabolism in smokers may lead to greater receptor occupancy, and greater psychological and cardiovascular effects of nicotine in smokers than in e-cigarette and ST users, even when similar levels of nicotine are inhaled or ingested. Finally, we found that even though anatabine was present in the urine of *several* smokers and ST users, it was absent in e-cigarette users. This is expected because e-liquids contain relatively pure nicotine and most associated alkaloids are removed during the purification process. Further studies using larger cohort are required to examine whether the levels of anatabine in the urine could be used to identify e-cigarette usage and could help in distinguishing e-cigarette users from those who use ST or smoke combustible cigarettes.

Our results also show significant elevation in the urinary levels of several VOCs immediately after the use of tobacco products. At baseline, under conditions of normal tobacco product use (both at Visit 1 and Visit 2 after abstaining from tobacco for 48 h), the levels of several VOC metabolites were elevated in the urine of the users of both combustible and e-cigarette users. The high levels of VOC metabolites in the urine of e-cigarette users was surprising and may be because of dual use, exposure to secondhand tobacco smoke exposure or other sources. Although we cannot completely rule out these possibilities, the absence of anatabine in the urine of e-cigarette users suggests that the VOC metabolites in their urine are unlikely to be derived from cigarette smoking, but may be due to e-cigarette use, or nontobacco related environmental sources of VOCs. Further studies are required to identify which VOC metabolites are excreted in the urine of e-cigarette users at steady-state.

We were able to capture most of the VOC metabolites in the urine 3 h post exposure. However, we could not detect significant increases in most metabolites in the urine of e-cigarette users or those using ST products. Although the absence of VOC metabolites does not necessarily mean that e-cigarettes and ST products do not contain these VOCs or that users of these tobacco products are not exposed to VOCs, it does indicate that the levels of several VOCs in e-cigarettes and in ST may be significantly lower than those present in tobacco smoke. This notion is supported by our observations showing that smoking combustible cigarettes led to a rapid appearance of the metabolites of VOCs such as acrolein and butadiene in

the urine, suggesting that smokers are likely to be exposed to high levels of these VOCs during smoking. High levels of acrolein have been detected in tobacco smoke,[38] and measurements of urinary metabolites show that in comparison with nonsmokers, smokers have 2- to 3-fold higher levels of the acrolein metabolite 3-HPMA. In addition to cigarette smoke, acrolein is also present in several foods and is also generated endogenously during lipid peroxidation and inflammation;[39] however, a decrease in 3-HPMA levels in the urine of smokers after smoking cessation supports the view that high levels of 3-HPMA in human urine are derived from acrolein in tobacco smoke. Our observations showing a time-dependent increase in 3-HPMA levels in the urine after smoking attest to its origin from tobacco smoke.

The absence of acrolein and butadiene metabolites in the urine of e-cigarette smokers after e-cigarette use is consistent with the results of previous studies[24,25] showing a decrease in the levels of the urinary metabolites of these VOCs in smokers who switch from combustible cigarettes to e-cigarettes and indicate that the levels of these VOCs in e-cigarette aerosols may be lower than their levels in tobacco smoke. Indeed, direct measurement of e-cigarette aerosols indicate that the levels of VOCs generated by e-cigarettes are 10- to 100-fold lower than their levels in tobacco smoke.[16] In this regard, it is also significant to point out that we did not see an increase in the urinary metabolites of formaldehyde or acetaldehyde in the urine of e-cigarette smokers; but, as no increase in the levels of these metabolites was observed even in the urine of smokers, it is difficult to ascertain whether our methods were sensitive enough to detect the metabolites of these aldehydes or whether these aldehydes are metabolized at a rate that was not captured with the duration of our collection period (3 h). It is also likely, that urinary formate and acetate derived from use of tobacco are in much lower levels than those generated by exogenous and endogenous sources of formaldehyde and acetaldehyde. Therefore, the absence of several VOC metabolites in the urine of tobacco product users cannot be taken as evidence of lack of exposure.

Although we were unable to detect changes in most VOC metabolites in the urine of nonmentholated e-cigarette users, we did observe an increase in urinary metabolites of xylene—2MHA and 3MHA+4MHA, in users of e-cigarettes. Although the levels of xylene metabolites have been previously correlated with urinary cotinine and nicotine levels,[40] to our knowledge this is the first report showing that the levels of 2MHA and 3MHA+4MHA are increased in the urine of e-cigarette users. However, it remains unclear why the levels of 2MHA and 3MHA+4MHA were higher in mentholated than in nonmentholated e-cigarettes. As xylene is widely used as an extraction solvent, or synthesis precursor, and, therefore, might be used in production/isolation of flavors found in tobacco products, we speculate that this and similar aromatic compounds may be present in variable amounts in flavored products. Another likely explanation may be that xylene and other aromatic VOCs are formed through combustion, heating, or other physico-chemical processes. In fact, Pankow et al., reported measurable amounts of benzene formed during heating from glycerol, propylene glycol, and flavors, and preservatives (benzaldehyde and benzoic acid, respectively) present in e-liquids.[41] Similarly, generation of benzene and other HPHCs was observed in Camel crush cigarettes (a product that contains a capsule in the filter that, when crushed, releases a mentholated liquid), where benzene and other VOC yields have been reported to increase in the presence of menthol, especially in gas phase.[42] Further studies are required to fully assess the potential of excessive VOC production by flavors such as menthol; moreover, given the multiple adverse respiratory and cardiovascular effects of xylene,[43] additional evaluation of the health effects of flavored e-cigarettes is also warranted. Another novel finding is the increased levels of metabolites of acrylonitrile—CYMA and N,N-dimethylformamide—AMCC in the urine of e-cigarette users. Our findings are consistent with the literature, which shows that the levels of AMCC[30] and CYMA[30,44,45] are higher in the urine of smokers. However, to our knowledge, this is the first report to note the increase of these metabolites in urine after exposure to e-cigarettes. Nevertheless, the elevated levels of VOC metabolites in the urine of e-cigarette users in our study does not necessarily mean that all types and use patterns of e-cigarettes will lead to the nature or the extent of exposure described in this study. It is known that the sources of AMCC and CYMA may be dietary, occupational and/or environmental, however, due to the fact that our study participants remained in the study room for the entire duration of the study and did not eat, drink, or use tobacco (other than the study product) over the study period, it is unlikely that they were exposed to other sources of AMCC and CYMA. However, more studies, with a larger number of participants, are needed to confirm AMCC and CYMA as a possible biomarkers of e-cigarette exposure or mediators of e-cigarette toxicity.

In summary, our results show that e-cigarettes, at least the devices tested in the study, result in lower levels of nicotine delivery than combustible cigarettes and that even a single, acute exposure to combustible cigarettes results in measurable and significant increases in urinary levels of harmful VOC metabolites. These findings underscore the need for routine measurements of these biomarkers of exposure to establish a relationship between harmful constituents present in the product alone and the actual extent of exposure to these toxicants. We believe that these findings will aid in assessment of the relative toxicities and toxicity thresholds of the HPHCs present in these and emerging tobacco products.[46,47] Clearly, additional research is necessary to fully characterize exposure in users of tobacco products. In addition, apart from the 1st generation cigalikes, other e-cigarette types such as rechargeable pen-type and "mods," capable of generating variable voltages and with reported higher VOC levels in the generated aerosols,[48] need to be investigated for the presence of urinary VOC metabolites.

Limitations

Despite its many strengths, the study has some limitations. The study was designed to compare acute exposure to different tobacco products by measuring concentrations of urinary TA, cotinine, 3HC, and VOC metabolites. A 3-h postexposure observation period was chosen to balance between participants' willingness to remain confined in the study room with no food, drink or tobacco use (except the study product) and allowing time to capture metabolite elimination in the urine. Although this observation time was not sufficient to capture the entire elimination profile of the metabolites, the observed differences within the timeperiod (3 h) could be compared between products and could be attributed to product exposure. Our measurements did not provide information regarding the relative efficacy of different metabolic pathways metabolizing the same product; differences in the rate of metabolism between different individuals; or account for all possible pathways. However, our experimental protocol, which minimized confounding due to co-exposures, allowed the identification of differences between the use of different tobacco products and potential markers of exposure. Moreover, we did not

normalize or control for differences in nicotine dose across products. This prevented us from making direct comparison using VOC exposure per unit dose of nicotine, which may be different for different products. However, our measurements of VOC metabolites did provide estimates of VOC exposure, independent of nicotine dose. Further studies are required to establish quantitative exposure to VOCs based on nicotine equivalents to assess whether adjustment of nicotine dose by users of different tobacco products could alter their VOC exposure. Finally, the NJOY King e-cigarettes used in our investigation are cigalikes or first generation devices, with the smallest power output among e-cigarette products; because, at the time of the study they were one of the most popular disposable and nonrefillable devices containing fixed concentration of nicotine and same composition of e-liquids. The use of these devices ensured consistent nicotine delivery and eliminated the device dependent variability in our study, but similar investigations are needed to assess VOC exposure due to the use of other, newer, e-cigarette devices with higher power output.

Supplementary Material

Supplementary data are available at *Nicotine & Tobacco Research* online.

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Declaration of Interest

Authors do not have any conflict of interest.

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