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Original investigation

Biomarkers of Exposure for Dual Use of Electronic Cigarettes and Combustible Cigarettes: Nicotelline, NNAL, and Total Nicotine Equivalents

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Abstract

Introduction: Dual use of electronic cigarettes (e-cigarettes) and combustible cigarettes is a major public health issue. It is generally accepted that exclusive e-cigarette use is less harmful than exclusive combustible cigarette use, but most e-cigarette users continue to smoke combustible cigarettes as well. To what extent the use of e-cigarettes reduces harm in people who continue to smoke combustible cigarettes has been debated. The aim of this study was to explore the utility of biomarkers as measures of dual use.

Methods: In two human studies of participants who used e-cigarettes only or both combustible cigarettes and e-cigarettes, we measured urine concentrations of the metabolites of nicotine (total nicotine equivalents) as well as two biomarkers of tobacco exposure: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a tobacco-specific carcinogen metabolite, and nicotelline, a tobacco alkaloid not found in significant concentrations in e-cigarette products.

Results: The presence of nicotine metabolites indicates either e-cigarette or combustible cigarette use. Nicotelline (half-life of 2–3 hours) indicates recent combustible cigarette use and NNAL (half-life of 10 days or more), indicates combustible cigarette use occurring within several weeks prior to sample collection.

Conclusions: Nicotelline and NNAL are useful biomarkers for combustible tobacco use in users e-cigarettes. The application of these biomarkers provides a tool to help assess whether, or to what extent, dual use of e-cigarettes and combustible cigarettes reduces harm compared to sole use of combustible cigarettes. These biomarkers can also verify exclusive use of e-cigarettes over short (24 hour) or long (several week) time periods.

Implications: To what extent dual use of e-cigarettes and combustible cigarettes reduce harm compared to smoking combustible cigarettes only is of considerable public health interest. We show that the levels of the minor tobacco alkaloid nicotelline and the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are extremely low in electronic cigarette fluids. The urine biomarkers nicotelline and the NNK metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are indicative of cigarette smoking and can be used to assess recent and past smoking in dual users.

Introduction

Switching from tobacco cigarettes to electronic cigarettes (e-cigarettes) as a strategy for harm reduction is controversial. Although most public health professionals agree that sole e-cigarette use would be less harmful than tobacco cigarette use, most smokers who switch to e-cigarettes continue to use combustible cigarettes as well.[1] For this reason, from a public health standpoint, the benefits versus risks of e-cigarettes for harm reduction are uncertain. Some have argued that e-cigarettes sustain nicotine dependence, most e-cigarette users continue to use tobacco cigarettes, and on a population basis it would be better public health policy to recommend total cessation from all types of nicotine delivery products and overcome nicotine dependence. Others maintain that any significant reduction in cigarette smoking can reduce harm. The Aim of our study was to explore the utility of biomarkers as measures of dual use that could be used as a tool to investigate this public health issue.

There is considerable interest in the physiological, toxicological, and subjective effects of e-cigarettes compared to combustible cigarettes, as well as that of dual use of both products. A major issue is how to verify sole use of e-cigarettes. Verifying sole use of one product might be challenging, as self-reports are not always accurate, especially if there is a monetary incentive for study completion, or if a study participant has been urged to quit smoking by his or her health care provider.[2–4] Even if self-reports were accurate, quantification of the extent of dual use from self-report may be difficult to achieve.

Verifying self-reports of tobacco use, or lack thereof, and the extent of use (heavy vs. light smoking) can be accomplished by the use of biomarkers of tobacco smoke constituents.[2] The most widely applicable biomarkers of tobacco exposure are nicotine and its metabolites, but these are not applicable for discriminating cigarette smoking from the use of e-cigarettes containing nicotine. Product-selective biomarkers for tobacco cigarettes and for e-cigarettes are needed. Most desirable would be constituents of e-cigarette aerosols, or metabolites of these substances, that are present in biofluids of e-cigarette users but are not present in biofluids of tobacco smokers. However, at this time, we are unaware of any useful biomarkers that are specific to e-cigarette use.[5]

An alternative approach is to use substances found exclusively in conventional tobacco products as biomarkers to detect and quantify tobacco use, and to distinguish tobacco use from e-cigarette use. In this regard, we considered the tobacco-specific nitrosamine metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)[6,7] and the minor tobacco alkaloids anabasine, anatabine, and nicotelline[8,9] as candidates in this approach. We carried out three studies to evaluate this approach.

The first was a study to verify that an electronic nicotine delivery device would not expose the user to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the metabolic precursor of NNAL, or to the minor tobacco alkaloids since these substances are present in tobacco products but not in pure nicotine. However, if the product contained tobacco-derived nicotine, as e-cigarette products generally do, that had not been sufficiently purified, it might contain minor alkaloids and/or NNK, and the specificity of the biomarker for conventional tobacco products would be compromised. For this reason, we analyzed 70 different e-liquids for NNK, anabasine, anatabine, and nicotelline, and compared their concentrations to those found in cigarette smoke to evaluate specificity. The second study was an inpatient study of e-cigarette use, to demonstrate that with enforced cigarette abstinence, the two tobacco-specific biomarkers nicotelline and NNAL would decrease, or if the biologic half-life were short enough compared to the period of forced abstinence, the biomarker concentration would be undetectable. The third study was an outpatient study in which e-cigarette users or dual users of e-cigarettes and combustible cigarettes were instructed to only use e-cigarettes during a 3- to 4-day period without supervision. This study serves as an example of how these biomarkers can be used to interpret real-life data in an uncontrolled setting.

Materials and Methods

Study Procedures

Study 1 was the chemical analysis of 70 e-cigarette fluids to measure concentrations NNK, nicotelline, anabasine, and anatabine, to determine whether e-cigarettes might deliver significant amounts of NNK, the metabolic precursor of NNAL, nicotelline, or other minor tobacco alkaloids. The e-liquids were chosen to represent the most common brands that participants were using and were popular at that time. They were purchased from local retailers or online, and were stored at room temperature until analyzed, as described below. The products included non-refillable "cig-a-likes," and refill liquids for tank models or re-buildable atomizers.

Previously unpublished data from two clinical studies are presented. Study 2 (Inpatient Study) was an inpatient hospital research ward study on the pharmacology of e-cigarettes, in which urine specimens for biomarker measurements were also collected. The details of this study have been previously described.[10,11] Study 3 (Outpatient Study) involved a subset of participants who used e-cigarettes exclusively or in addition to combusted cigarettes, from a larger set of participants who used a variety of tobacco products. Urine specimens for tobacco biomarkers of exposure were collected at enrollment and at the conclusion of the study period.

Study 2 participants, 13 exclusive e-cigarette users by self-report, were healthy based on a limited physical exam and not currently trying to quit e-cigarette use. They included 6 females, 7 males, 9 Caucasians, 2 Asians, 1 African American, and 1 mixed-race, who came to the Clinical Research Center at the Zuckerberg San Francisco General Hospital for a 1-day pharmacokinetic study.[10] Participants were required to have >30 ng/mL saliva cotinine to indicate significant nicotine intake.

Participants were admitted to the hospital the evening before study activities were conducted. Use of their usual brand of e-cigarette was allowed until 10 PM, at which time all e-cigarettes, including e-liquids, and any other tobacco products were removed by the nurse and stored in a locked cabinet. Participants provided a urine sample at admission. Participants were awakened at 7:00 AM and an intravenous (IV) line for blood sampling was placed in the forearm at 8:00 AM, followed by a light breakfast. Baseline blood and urine were collected. At approximately 9:30 AM, the participants were asked to use their usual brand of e-cigarette, which was supplied by the study, and told to take 1 puff every 30 seconds for a total of 15 puffs. Blood was collected over the next 3 hours at several time points and urine was collected after 4 hours.

Study 3 participants were experienced users of nicotine-containing e-cigarettes, were healthy, based on a limited physical exam, and not currently trying to quit e-cigarette use. The 40 participants included 24 men and 15 women, one did not disclose sex; 23 Caucasian, 5 Asian, 4 Mixed, 4 Hispanic, 2 Black, and 2 Native

Hawaiian/Pacific Islanders. Participants were required to have >50 ng/mL saliva cotinine to indicate significant nicotine intake. The Study 3 participants were exclusive e-cigarette users or dual users of <5 tobacco cigarettes per day, who used e-cigarettes daily for at least 3 months or more, and agreed to abstain from tobacco cigarette use over the duration of the study.

On the first study day, the participants came to the outpatient clinic, provided a urine sample, were given a 5-day supply of their usual e-cigarette product to last for the duration of the study, and were instructed to only use the e-cigarette product given to them as part of the study. Users of cigarette-like e-cigarettes (cig-a-likes, first-generation products) were provided either a starter kit with several replacement cartridges or disposable cig-a-likes, depending on what they usually used. Users of 2nd and 3rd generation tank e-cigarettes and re-buildable atomizers (RBA) were given their usual e-liquid. After 3 to 5 days, to allow sufficient use and nicotine intake reaching steady state, the subjects returned to the clinic and provided a second urine sample.

All urine samples from both clinical studies were analyzed for concentrations of nicotelline, NNAL, and total nicotine equivalents (TNE). Both clinical studies were approved by the Institutional Review Board at the University of California San Francisco.

Analytical Chemistry

The methods used to measure biomarker concentrations have been previously described in publications. Nicotelline and NNAL concentrations were measured by liquid chromatography—tandem mass spectrometry (LC-MS/MS) as described by Jacob et al.[6,8] TNE was comprised of the molar sum of concentrations of nicotine, cotinine, trans-3′hydroxycotinine, nornicotine, norcotinine, nicotine N-oxide and cotinine N-oxide, and their respective glucuronide conjugates, which were measured by LC-MS/MS as described for cotinine and trans-3′hydroxycotinine,[12] with minor modification for the additional analytes.[13,14] Analysis of e-liquids for nicotelline, NNK, anabasine, and anatabine were carried out by LC-MS/MS by the method described in Whitehead et al.,[15] with modifications to include the analytes anabasine and anatabine, and utilizing a simper extraction procedure (sample prep) that is suitable for this less complex sample matrix. The modifications of sample prep were carried out by diluting 10 mg of e-liquid with 1 mL of .01 M hydrochloric acid, adding 1 mL of 50% aqueous tripotassium phosphate, and extracting with methylene chloride as described in Jacob et al.[12] for cotinine and trans-3′hydroxycotinine. Liquid chromatography (LC) was modified by using a 3 × 150 mm Waters X-Bridge BEH C18 column with a pH 9 ammonium formate in methanol/water mobile phase. Concentrations of nicotine in e-liquids were determined as previously described.[16]

Data Analysis

Within-subject changes were analyzed by paired *t*-test. All analyses were carried out using SAS v. 9.4 (SAS Institute, Inc., Cary, NC). Statistical tests were considered significant at $\alpha < 0.05$.

Results

Nicotine and Minor Alkaloid Concentrations in E-liquids

Anabasine and anatabine were found in measurable concentrations in the majority of e-liquids, and averaged about 20% (normalized to nicotine) of those found in the smoke of a reference cigarette (Supplementary Tables S1 and S2). Of the 70 e-liquids, 42 (60%) had measurable amounts of anabasine, and for those with measurable amounts the mean was .68 µg/mg of nicotine (range undetectable—2.80), 65 (93%) had measurable amounts of anatabine, mean of 1.07 µg/mg of nicotine (range undetectable—8.89), and 5 had undetectable levels of both. For comparison, the smoke of a reference cigarette (Supplementary Table S1) had 2.24 µg anabasine/mg nicotine, and 5.89 µg anatabine/mg nicotine. Of the 70 e-liquids, 36 (51%) had measurable amounts of NNK, and for those with measurable amounts the mean was .00049 µg/mg of nicotine (range undetectable—0.0032), and 6 (9%) had measurable amounts of nicotelline, mean .0019 µg/mg of nicotine (range undetectable—0.0043). For comparison, the smoke of a reference cigarette (Supplementary Table S1) had .20 µg NNK/mg nicotine, and 1.57 µg nicotelline/mg nicotine.

Inpatient Study

Nine of 13 participants self-reported exclusive e-cigarette use, which was initially confirmed by their low-expired CO at screening (range 1–4 ppm). Two participants used first generation e-cigarettes, 8 used second generation tank devices, and 3 used re-buildable atomizers (RBAs). Biomarker data from the inpatient study were of particular value for this article because abstention from conventional tobacco products was enforced. Biomarker data were available for 11 subjects, and are presented in Table 1 and Supplementary Tables S3 and S4. Of the 9 self-reported exclusive e-cigarette users, 7 were confirmed by nicotelline concentrations below the limit of quantitation (BLQ) on admission. As expected, nicotelline concentrations were BLQ for all participants on the study day, as sufficient time had elapsed for its concentrations to fall below the LOQ. The half-life is of nicotelline is 2–3 hours,[8] and subjects had no access to cigarettes. NNAL concentrations and TNE were essentially the same post-vaping as compared to admission, as expected because NNAL has a half-life of >10 days.[17,18]

Outpatient Study

Mean concentrations of the 3 biomarkers measured at enrollment and after 3–5 days during which participants were asked not to smoke cigarettes were compared. The data are presented in Table 1 and in Supplementary Tables S5 and S6. On average, nicotelline concentrations were about 50% lower at follow-up than at enrollment ($p = .03$). NNAL concentrations had dropped slightly, but the change was not statistically significant. TNE levels were not significantly different at follow-up and at enrollment. Some participants appeared to be solely e-cigarette users, based on the observations that nicotelline and NNAL were not measurable both at enrollment and at follow-up, or in the case of NNAL, low enough to be derived from secondhand smoke exposure ($n = 15$, Supplementary Table S5).[19] All of those appearing to be sole e-cigarette users by biomarker levels, also self-reported exclusive e-cigarette use. Others appeared to be dual users, with significant concentrations of nicotelline and/or NNAL present at enrollment ($n = 25$). Of those appearing to be dual users by biomarker levels, $n = 25$, 15 (60%) were dual users by self-report and 10 (40%) were exclusive e-cigarette users by self-report. Participants who were compliant with the study protocol requiring abstention from smoking had nicotelline concentrations below the detection limit at follow-up ($n = 27$).

Discussion

Identifying biomarkers of exposure to e-cigarettes, and biomarkers to determine the extent of dual use of tobacco and e-cigarettes are

Table 1. Concentrations of Three Biomarkers in Urine of E-cigarette Users

	Inpatient study (n = 11)			Outpatient study (n = 40)		
Biomarker	Admission	Post-vaping	p	Enrollment	Follow-up	p
Nicotelline (pmol/mg Creatinine)[a]						
Mean	0.17	0	.04	0.65	0.22	.03
Range	0–0.77	0		0–6.7	0–4.4	
SD	0.29	0		1.6	0.74	
NNAL (pmol/mg Creatinine)[a]						
Mean	0.44	0.34	NS	0.27	0.24	NS
Range	0–3.9	0–2.8		0–1.4	0–1.3	
SD	1.2	0.83		0.38	0.32	
TNE (nmol/mg Creatinine)						
Mean	63	56	NS	45	44	NS
Range	8.4–53	19–184		0.80–190	0.57–141	
SD	41	46		38	34	

NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; TNE = total nicotine equivalents. p-values are for differences between admission /enrollment and post-vaping/follow-up.

[a]If below the limit of quantitation (BLQ) zero was used.

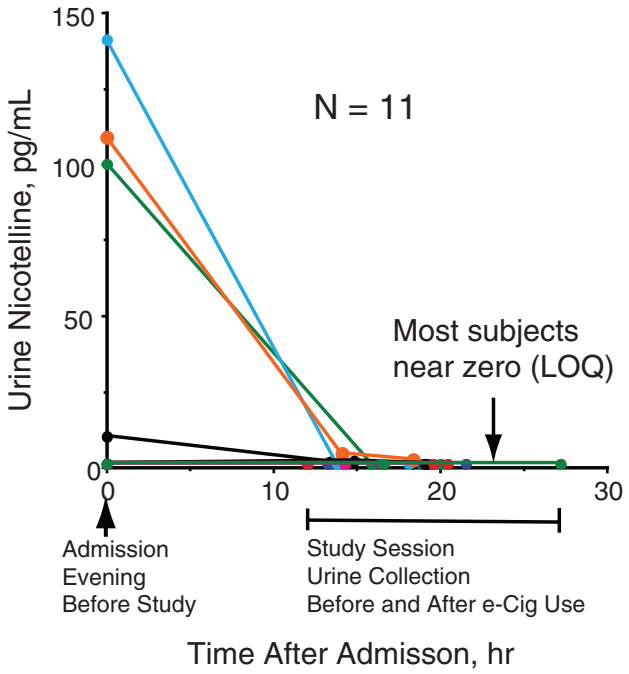

Figure 1. Nicotelline concentrations in urine of 11 e-cigarette users and dual users at admission to an inpatient study, during and after a vaping cession on a research ward. Each line represents an individual subject.

topics of considerable interest.[5] In prior research, we have determined that nicotelline is a biomarker of exposure to tobacco smoke-derived particulate matter.[8] NNAL, a metabolite of the potent lung carcinogen NNK, a tobacco-specific nitrosamine, has been extensively used as a biomarker of tobacco exposure.[6,7] Our studies described here support the use of nicotelline and NNAL to detect use of combusted tobacco products in e-cigarette users.

To determine the specificity of nicotelline and NNAL for tobacco use as compared to e-cigarette use, we analyzed 70 different e-liquids (marketed in the United States in 2015) for nicotelline and NNK, the metabolic precursor of the biomarker NNAL. Specificity would be verified if concentrations in e-liquids were negligible compared to the concentrations in tobacco smoke. Since nicotine intake by e-cigarette users is fairly similar to nicotine intake by cigarette smokers (observed in this study and in published studies), we normalized the nicotelline and NNK concentrations to nicotine. Thus, comparison of the nicotelline/nicotine ratios and NNK/nicotine ratios in e-liquids to those in cigarette smoke would be a measure of specificity. We found that mean nicotelline/nicotine ratios were about 10 000 times lower in e-liquids compared to smoke from a reference cigarette, and NNK/nicotine concentration ratios were about 1000 times lower (Supplementary Table S1). Even the highest concentration ratios in individual e-liquids were lower than in cigarette smoke by about 400-fold and 60-fold, respectively, verifying the high degree of specificity of these two biomarkers for conventional tobacco products. Thus, although various tobacco-derived substances such as minor tobacco alkaloids[20] and tobacco-specific nitrosamines, including NNK,[21] can be detected in some e-liquids and their aerosols, nicotelline and NNK concentrations are too low to result in significant concentrations of nicotelline and NNAL in urine of exclusive e-cigarette users.

We also measured concentrations of the minor tobacco alkaloids anabasine and anatabine in e-liquids and smoke from reference cigarettes, because these two alkaloids have been used as biomarkers for tobacco use in people using nicotine medications (eg, gum or patches)[9] and likewise might be considered for detection of tobacco use in e-cigarette users. In a recently published study, anabasine and anatabline were not detected in the urine of 11 e-cigarette users.[22] However, in contrast to nicotelline and NNK, we found measurable and sometimes high concentrations of anabasine and anatabine in many e-liquid products, on average about 20% of those found in cigarette smoke, normalized to nicotine, and in some products comparable to or higher than those found in cigarette smoke (Supplementary Tables S1 and S2; Supplementary Information). Essentially all nicotine of commerce is extracted from tobacco. Pharmaceutical grade nicotine used in smoking cessation aids is highly purified, but it appears that many e-liquids are relatively impure in comparison. We suspect that NNK and nicotelline are efficiently removed by simple distillation due to their high boiling points, but anabasine and anatabine are more volatile and not removed without the use of an efficient fractional distillation. Therefore, anabasine and anatabine may not have sufficient selectivity to be generally useful for detection of tobacco use in e-cigarette users. Anabasine and anatabine can be

recommended as biomarkers to distinguish cigarette smoking from e-cigarette use only if the e-liquids are confirmed to have very low levels or no anabasine and antabine.

In studies of dual use of e-cigarettes and tobacco cigarettes, nicotelline and NNAL have complementary attributes. Nicotelline has a short half-life, 2–3 hours.[8] In contrast, NNAL has a long half-life, >10 days.[17,18] If the goal of a study were to examine the acute effects of varying degrees of dual use of e-cigarettes and combustible cigarettes, NNAL would not be useful. Due to its long half-life, it would take weeks or months to achieve steady-state levels, and short-term changes in use patterns would be under-estimated or undetected. However, with a half-life of 2–3 hours, nicotelline can detect changes in the extent of dual use occurring over a short time frame. On the other hand, if the goal of a study were to examine the effects of exclusive e-cigarette use, in subjects who had not used tobacco for at least a few months, for example, a smoking cessation study, a highly sensitive NNAL analytical method[6] could confirm long-term, exclusive e-cigarette use. Urine TNE, the biomarker that is considered to be the best measure of daily nicotine intake,[5] may correlate with the extent of nicotine dependence and can be used as an overall measure of nicotine-containing product use.

Figures 1 and 2 illustrate the utility of these three biomarkers. Figure 1, prepared with data from the inpatient study, shows that if abstention from tobacco is enforced, nicotelline concentrations are undetectable or near the lower limit of quantitation (LLOQ) within 24 hours. Figure 2 was prepared with data from the outpatient study. Subject 1 was a long-term pure e-cigarette user. Both nicotelline and NNAL concentrations were undetectable at enrollment and at follow-up. Subject 2 was a dual user of e-cigarettes and combustible cigarettes. Nicotelline concentrations were undetectable at enrollment and at follow-up, indicating that the subject was not using tobacco on the day of enrollment or at follow-up. Significant NNAL was present at enrollment, declining during the study, indicating that the subject had used tobacco in the past. Subject 3 was a dual user who was compliant with the study protocol. Nicotelline concentrations were significant at enrollment, indicating recent tobacco

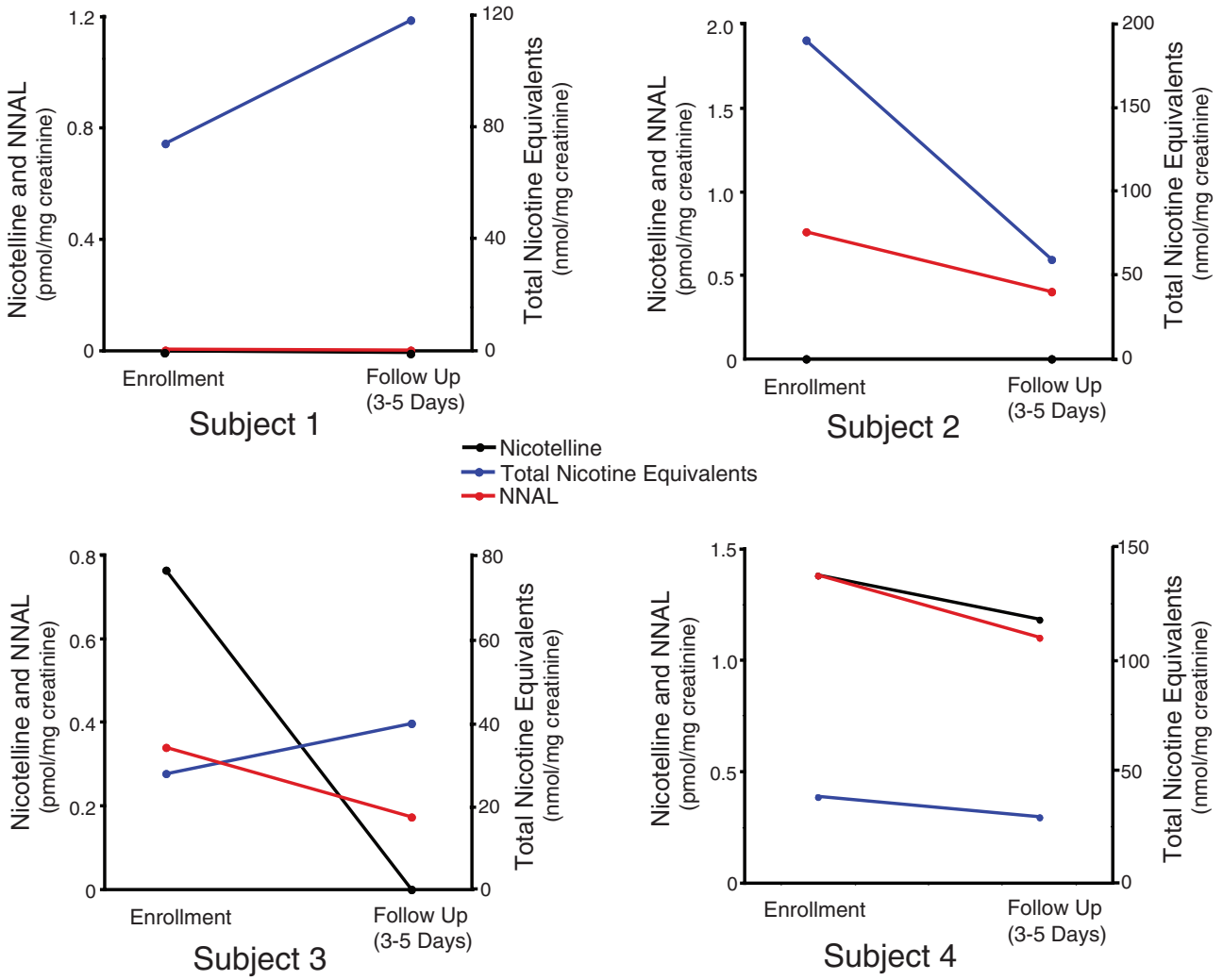

Figure 2. Nicotelline, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and total nicotine equivalents (TNE) in urine of e-cigarette users in the outpatient study. Subject 1 was a pure e-cigarette user because nicotelline and NNAL concentrations are below the limit of quantitation (BLQ) at enrollment and at follow-up, but TNE concentrations are substantial. Subject 2 was a dual user who had not used tobacco recently at the time of enrollment, as nicotelline concentrations were BLQ, but NNAL concentrations were significant. Subject 3 was a dual user who had used tobacco recently at enrollment, with substantial concentrations of nicotelline and NNAL at enrollment. Subjects 1, 2, and 3 were compliant with the study protocol, as nicotelline concentrations were BLQ at follow-up. Subject 4 was noncompliant, because nicotelline concentrations were substantial at follow-up.

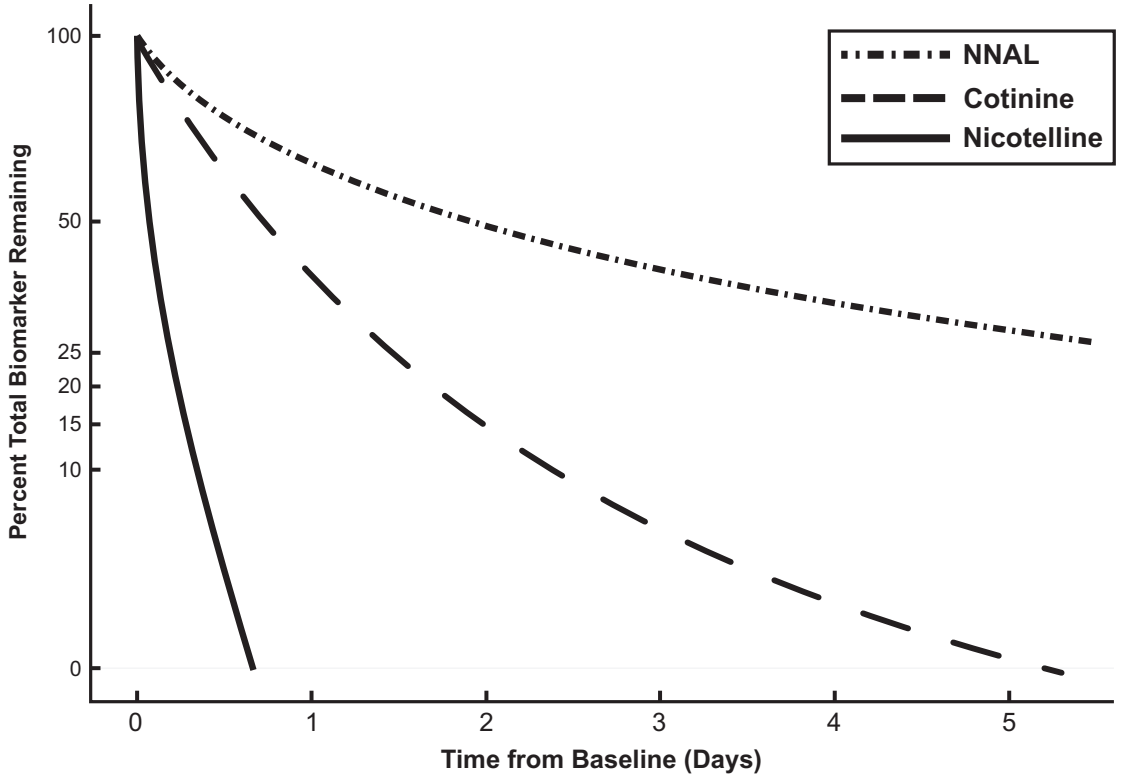

Figure 3. Elimination of nicotelline, cotinine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) over the course of 5 days, from a hypothetical person with average half-lives for the biomarkers.

use, but undetectable at follow-up. As expected, NNAL concentrations declined during the course of the study but were still significant at follow-up. Subject 4 was not compliant. At enrollment and at follow-up, nicotelline concentrations were significant. All subjects had substantial levels of TNE, which increased in some and decreased in other participants during the course of the outpatient study. This enabled us to confirm the use of nicotine-containing products, and gave us an idea as to the relative extent of use of e-cigarettes or combustible cigarettes, illustrated by whether TNE went up or down during the course of the study.

We should point out a limitation in Study 2 and in the use of NNAL and nicotelline in general. If subjects reported dual use, biomarkers cannot confirm that the subjects were using e-cigarettes for which there are no specific biomarkers. Another limitation in Study 2 was the small sample size, and the subjects were limited to one geographical area (San Francisco Bay Area), and therefore, the results for biomarker analyses may not be generalizable to the general population of e-cigarette users. However, based on our data on nicotelline and NNK in e-liquids compared to cigarette smoke, the utility of the biomarkers per se should be independent of the population studied.

In summary, we demonstrate that in studies of dual use of e-cigarettes and tobacco cigarettes, nicotelline can be used to detect and provide a measure of recent combusted tobacco use, and NNAL can be used in the same manner for tobacco use occurring over a long period of time. This is illustrated in Figure 3, which shows the elimination of nicotelline, NNAL, and cotinine (a surrogate for TNE) following cessation of tobacco use, in a hypothetical person with average half-lives for the three biomarkers. An example application of such biomarkers of exposure would be in studies of tobacco-derived toxicant intake or biomarkers of biological effects, and therefore potential health effects, in dual users of e-cigarettes and combustible cigarettes. Future studies will develop models to estimate the extent of dual use using biomarker excretion data.

Supplementary Material

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

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Declaration of Interests

NLB is a consultant to Pfizer and Achieve Life Sciences, companies that market medications to aid smoking cessation and has served as a paid expert witness in litigation against tobacco companies. The other authors have no conflicts to disclose.

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