Addiction Neuroscience 6 (2023) 100077

Contents lists available at ScienceDirect

Addiction Neuroscience

journal homepage: www.elsevier.com/locate/addicn

Review

Biomarkers of Electronic Nicotine Delivery Systems (ENDS) use

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ARTICLE INFO

Keywords:
Electronic Nicotine Delivery Systems
ENDS
E-cigarettes
Vaping
Biomarkers
Exposure

ABSTRACT

This perspective summarizes available evidence on biomarkers of exposure in electronic nicotine delivery system (ENDS) users to aid the overall assessment of the health consequences of using ENDS. Identification of novel biomarkers of exposure specific to ENDS use remains challenging because chemicals emitted from ENDS devices have many familiar sources. The biomarker levels of many tobacco-related toxicants measured in biological samples collected from ENDS users did not differ significantly from non-users, except for nicotine metabolites and a small number of biomarkers of exposure to volatile organic compounds and tobacco-specific tobacco nitrosamines. Several studies have shown that while exposed to nicotine, long-term exclusive ENDS users showed significantly lower levels of toxicant biomarkers than cigarette smokers. Studies have also shown that concurrent users of ENDS and combustible cigarettes ('dual users') are not reducing overall exposure to harmful toxicants compared to exclusive cigarette smokers. Because of an absence of validated ENDS-specific biomarkers, we recommend combining several biomarkers to differentiate tobacco product user groups in population-based studies and monitor ENDS compliance in randomized controlled trials. Using a panel of biomarkers would provide a better understanding of health effects related to ENDS use.

Abbreviations

ENDS	electronic nicotine delivery system
TSNAs	tobacco-specific nitrosamines
PAHs	polyaromatic hydrocarbons
VOCs	volatile organic compounds
HPHCs	harmful and potentially harmful constituents
TNE	total nicotine equivalents
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N'*-nitrosonornicotine
3HPMA	N-Acetyl-S- (3-hydroxypropyl)-L-cysteine
2CyEMA	N-Acetyl-S-(2-cyanoethyl)-L-cysteine
4HBeMA	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine
PATH	Population Assessment of Tobacco and Health study
NAB	*N'*-nitrosoanabasine
NAT	*N'*-nitrosoanatabine
NTR	nicotine replacement therapy
CPD	cigarettes per day
HTP	heated tobacco product
OTP	oral tobacco product

1. Introduction

Electronic Nicotine Delivery Systems (ENDS, also called electronic cigarettes, e-cigarettes, vaping devices, or vape pens) emerged over a decade ago and have significantly changed the tobacco product landscape. ENDS are engineered to heat a liquid nicotine solution so that the aerosol generated (colloquially called 'vapor') can be inhaled by the user. Standard features of ENDS devices include a heating element that heats a glycerin and propylene glycol-based nicotine solution ('e-liquid') that also contains various flavorings (fruit, candy, menthol, tobacco, beverage-themed, and more). Many laboratory-based studies on ENDS have focused on measuring nicotine and potentially harmful chemicals that these products may produce. Chemicals identified in aerosols emitted from ENDS include nicotine, metals, volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), and aldehydes [1–3]. Overall, concentrations of toxicants identified in ENDS aerosols are lower than those found in cigarette smoke [3–5]. It should also be noted that the levels of toxicants in ENDS aerosols can vary significantly, depending in large part on the device parameters (e.g., vaporization temperature), type of solvent (e.g., propylene glycol and glycerin), and user behaviors (e.g., individual puff topography and frequency of use) [6,7].

Biomarkers help identify and assess potential health risks of using novel tobacco products, like ENDS [8]. They can be measured in almost every biological material, although the most used biological samples for biomarker testing are blood (serum or plasma) and urine [9]. They are commonly used for informing clinicians, public health communities, and regulatory agencies when decision-making about health risks cannot be delayed, awaiting long-term epidemiology studies' results. Accurate and

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https://doi.org/10.1016/j.addicn.2023.100077
Received 24 December 2022; Received in revised form 21 February 2023; Accepted 22 February 2023

precise measurements of exposure to tobacco product constituents are essential for confirming the use of single or multiple tobacco products, quantifying exposure to nicotine and toxicants delivered from tobacco products, and assessing the potential biological effects of tobacco products on users. In this perspective paper, we focus on biomarkers of exposure. A biomarker of exposure is a constituent of a tobacco product or its metabolite that can be measured in a biological sample. It is sometimes considered a measure of internal dose. For this article, we defined biomarker as any measurable substance, such as a metabolite of an ENDS constituent, that can be directly related to the uptake or effects of this product's ingredients. This perspective does not focus on biomarkers of potential health effects.

This review summarizes available evidence on biomarkers of exposure in ENDS users to aid the overall assessment of the consequences of using ENDS. We discuss the strengths and limitations of currently available biomarkers of ENDS use. We recommend applying a panel of selected biomarkers in observational and experimental studies with ENDS users.

1.1. Biomarkers as early indicators of health risk

In studies focusing on understanding the health effects of tobacco use, biomarkers can demonstrate internal exposure to toxic chemicals present and emitted from specific products [10,11]. For example, biomarkers of exposure are used to characterize tobacco users' exposure to harmful and potentially harmful constituents (HPHC), including nicotine, TSNAs, PAHs, VOCs, and toxic metals [12]. Although clinical outcomes such as respiratory and cardiovascular diseases and cancer are definitive endpoints in assessing the potential health risks of novel tobacco products, they can take decades to develop and thus are not always practical to determine quickly. As clinical outcomes are hard to assess when new tobacco products like ENDS are introduced to the market, biomarkers of exposure document exposure to harmful chemicals that could potentially lead to potential early indications of long-term health effects. Thus, biomarkers of exposure can play an essential role in characterizing the potential health risks of ENDS.

1.2. Biomarkers as objective indicators of dose

For exposure assessment in longitudinal studies or clinical trials, self-reported use of ENDS products can be helpful. Still, this approach has significant limitations due to self-reporting bias and the need for validated survey tools that precisely measure product use patterns (e.g., the number of puffs taken or frequency of vaping during a day). Studies on the acute and chronic use of ENDS will require quantifiable biomarkers of exposure that can provide an objective dose indicator. Biomarkers previously used in human studies with ENDS include nicotine metabolites, metabolites of TSNAs, PAHs, VOCs, and metals [8]. An important limitation of those biomarkers is the lack of specificity to ENDS products.

Some biomarkers that were developed and validated to measure exposure to nicotine and toxicants from combustible cigarettes could also be used for measuring exposure to ENDS. Nicotine exposure is commonly assessed using cotinine alone or total nicotine equivalents (TNE), calculated most often as the molar sum of urinary nicotine, cotinine, trans-3′-hydroxycotinine, and their glucuronides [13]. These urinary biomarkers represent approximately 73%–96% of the nicotine dose and offer an excellent measurement tool for nicotine uptake [13]. Since some ENDS do not contain nicotine, users of nicotine-free products are expected to test negative for nicotine biomarkers (if they do not use any tobacco products).

Many biomarkers used for exposure assessment to combustible tobacco products are metabolites of chemicals present in tobacco plant filler or generated during tobacco combustion. For example, NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol), a metabolite of NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone plus its glucuronides) is a commonly studied biomarker that provides a measure of exposure to cancer-causing tobacco-specific nitrosamines (TSNA) [14]. 1-Hydroxypyrene and 2-hydroxyfluorene are polyaromatic hydrocarbons (PAH) metabolites; 3HPMA (N-Acetyl-S-(3-hydroxypropyl)-L-cysteine), a metabolite of acrolein, 2CyEMA (N-Acetyl-S-(2-cyanoethyl)-L-cysteine), a metabolite of acrylonitrile, and 4HBeMA (N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine), a metabolite of 1,3-butadiene are often analyzed as biomarkers of exposure to volatile organic compounds (VOCs) generated during tobacco combustion [15,16]. Since most of those biomarkers are specific to combustible tobacco products, they are unsuitable for ENDS exposure assessment. However, they still can be important indicators of exposure to combustible cigarettes in dual users.

1.3. Use of biomarkers to verify tobacco use status

Detection of biomarkers in various biological samples, like serum, urine, and saliva, has been used in observational studies and clinical trials to distinguish smokers from nonsmokers [17]. Numerous cotinine cut-points specific for each type of biological sample have been proposed for biochemical validation of tobacco use status [18]. Those cut-points are primarily used for validating abstinence in smoking cessation trials with ENDS to assess smoking cessation and compliance with ENDS. They are also critical for validating self-reported use of tobacco products in observational studies of the health effects of ENDS use or national surveillance surveys. Additionally, biomarkers would be essential to assess the patterns of dual use of ENDS and combustible cigarettes. However, biomarkers specific to ENDS have yet to be developed. The primary reason for the lack of specificity of ENDS biomarkers is that the major constituents of ENDS are nicotine, propylene glycol, glycerol, and flavoring chemicals, all of which are found in other tobacco products and have familiar dietary and environmental sources [19].

2. Biomarkers in ENDS users

2.1. Levels of biomarkers in ENDS users compared to non-user

Compared with cigarette smokers, toxicant exposure is significantly reduced in exclusive ENDS users [20–23]. However, ENDS still emit low quantities of several cardiovascular and respiratory toxicants, including particulates, metals, and carbonyl compounds like formaldehyde, acetaldehyde, acrolein, and benzaldehyde [20–23]. Safety concerns have emerged regarding toxicant levels in ENDS, considering studies consistently showing the emission of thermal degradation byproducts of nicotine solvents during product use [24–26].

Identifying biomarkers specific to ENDS remains challenging because most chemicals identified in ENDS liquids and emissions are ubiquitous in other tobacco products and consumer products, including food, cosmetics, and medications. Levels of most toxicant biomarkers measured in biological samples collected from ENDS users are statistically the same as in samples from non-users, except for a small number of biomarkers of exposure to VOCs and TSNAs [20–23]. Examples of biomarkers that have been found elevated in exclusive ENDS users compared to non-users of any tobacco products are listed in Table 1. Those elevated levels of selected biomarkers suggest a potential increase in health risk associated with ENDS use compared to not using tobacco products, indicating an increased ***absolute risk*** related to ENDS use.

2.2. Levels of biomarkers in ENDS users compared to tobacco cigarette smokers

Several cross-sectional studies have shown that exclusive ENDS users showed significantly lower levels of toxicant exposure biomarkers while exposed to nicotine than cigarette smokers [20–23]. Compared to users of combustible cigarettes, ENDS users have lower biomarkers associated with several respiratory, developmental, reproductive, and carcinogenic

Table 1
Examples of biomarkers that have been shown to be elevated in ENDS users compared to non-users ($p<0.05$) [21].

Biomarker (*sample type*)	Biomarker source and relevance	Difference between ENDS users vs. non-users (*geometric means; 95%CI*)
Cotinine (*urine*)	Cotinine is a major metabolite of **nicotine** which is a causal factor in dependence to tobacco products, addiction, and withdrawal disorders.	124.3 (95%CI 68.9–224.4) vs. 0.42 (95%CI 0.36–0.49) ng/mg creatinine
TNE (*urine*)	Total nicotine equivalent (TNE) is a molar **sum of nicotine metabolites** indicating cumulative exposure to nicotine from tobacco products.	2.0 (95%CI 1.1–3.5) vs. 0.006 (95%CI0.005–0.007) nmol/mg creatinine
NNAL (*urine*)	NNAL is a primary metabolite of tobacco-specific nitrosamine **4-(methylnitrosamino)−1-(3-pyridyl)−1-butanone (NNK)**, a potent lung carcinogen.	4.887 (95%CI 3.817–6.257) vs. 0.921 (95%CI 0.819–1.035) pg/mg creatinine
2CyEMA (*urine*)	2CyEMA (*N*-Acetyl-*S*-(2-cyanoethyl)-L-cysteine) is a major metabolite of **acrylonitrile** that is classified by IARC as Group 2B carcinogen.	3.959 (95%CI 3.002–5.219) vs. 1.315 (95%CI 1.230–1.406) ng/mg creatinine
1-Hydroxypyrene (*urine*)	1-Hydroxypyrene is a metabolite of **pyrene** and accepted biomarker of carcinogenic polycyclic aromatic hydrocarbons (PAHs).	0.161 (95%CI 0.143–0.181) vs. 0.128 (95%CI 0.121–0.136) ng/mg creatinine
Lead (*urine*)	Exposure to lead has been associated with brain damage, mental retardation, behavioral problems, developmental delays.	0.432 (95%CI 0.382–0.488) vs. 0.351 (95%CI 0.330–0.373)
Cadmium (*urine*)	Cadmium is an IARC Group 1 known human carcinogen with adverse effects on kidney and bone.	0.193 (95%CI 0.165–0.225) vs. 0.149 (95%CI 0.140–0.159) ng/mg creatinine

toxicants. Since PAHs and VOCs are primarily byproducts of incomplete combustion of organic materials, smokers of combustible tobacco products have significantly higher levels of all PAHs and VOCs than users of non-combustible nicotine-containing products, including ENDS users [27,28]. Examples of biomarkers significantly reduced in exclusive ENDS users compared to users of combustible tobacco cigarettes are listed in Table 2.

Several studies have prospectively assessed changes in biomarker levels after cigarette smokers were assigned to use ENDS only [29–34]. All studies consistently observed decreases in biomarkers of exposure to TSNAs and VOCs after smokers became exclusive ENDS users. Analysis of Population Assessment of Tobacco and Health (PATH) study data revealed that smokers who completely switched to ENDS experienced significant reductions in nicotine metabolites and most TSNAs, PAHs, and VOCs [35]. This national cohort study provides strong evidence of the potential harm reduction associated with transitioning from exclusive cigarette use to exclusive ENDS use (indicating a lower ***relative risk*** of ENDS compared to combustible cigarettes). Although this may suggest plausible effects on reducing the disease risk among smokers transitioning from combustible cigarettes to ENDS, confirmatory data from extensive epidemiological studies are not yet available.

2.3. Levels of biomarkers in ENDS users compared to users of other alternative tobacco products

Since 2018, several studies (primarily from industry) have compared exposure from ENDS to other alternative tobacco products, including heated tobacco products (HTPs) and oral nicotine products (OTPs). Generally, those studies have compared exposure from ENDS, HTPs, and OTPs to cigarettes or abstinence. However, exceedingly few studies have directly compared exposures from HTPs to ENDS. Scherer et al. [36] investigated the exposure to NNK, NNN, *N*'-nitrosoanabasine (NAB), and N'-nitrosoanatabine (NAT) as well as to the minor tobacco alkaloids anabasine and anatabine by measuring biomarkers in regular users of combustible cigarettes, ENDS, HTPs, OTPs, and nicotine replacement therapy products (NRTs) in a clinical study comprising a period of 74 h under confinement. Users of combustible cigarettes exhibited the highest levels for almost all biomarkers measured. Levels of biomarkers observed in ENDS users were lower than in the users of HTPs. In a large cross-sectional study from South Korea [37], researchers compared exposure biomarker levels by tobacco product type, including ENDS, HTPs, and combustible cigarettes. They found that ENDS-exclusive users had biomarker levels lower than HTP users and smokers but higher than

Table 2
Examples of biomarkers that have been shown to be lower in ENDS users compared to combustible cigarette smokers ($p<0.05$) [21].

Biomarker (*sample type*)	Biomarker source and relevance	Difference between ENDS users vs. tobacco cigarette smokers
Cotinine (*urine*)	Cotinine is a major metabolite of **nicotine** which is a causal factor in dependence to tobacco products, addiction, and withdrawal disorders.	124.3 (95%CI 68.9–224.4) vs.1830.9 (95%CI 1577.4–2125.1) ng/mg creatinine
TNE (*urine*)	Total nicotine equivalent (TNE) is a molar **sum of nicotine metabolites** indicating cumulative exposure to nicotine from tobacco products.	2.000 (95%CI 1.100–3.500) vs. 27.90 (95%CI 23.80–32.70) nmol/mg creatinine
NNAL (*urine*)	NNAL is a primary metabolite of tobacco-specific nitrosamine **4-(methylnitrosamino)−1-(3-pyridyl)−1-butanone (NNK)**, a potent lung carcinogen.	4.887 (95%CI 3.817–6.257) vs. 203.5 (95%CI 181.7–227.9) pg/mg creatinine
2-Napthol (*urine*)	**2-Napthol** is a biomarker of exposure to **naphthalene**, a possible human carcinogen (IARC Group 2B) that has been linked to respiratory tract lesions.	5.287 (95%CI 4.693–5.956) vs. 13.91 (95%CI 13.21–14.65) ng/mg creatinine
1-Hydroxyprene (*urine*)	1-Hydroxypyrene is a metabolite of **pyrene** and accepted biomarker of carcinogenic polycyclic aromatic hydrocarbons (PAHs).	0.161 (95%CI 0.143–0.181) vs. 0.303 (95%CI 0.287–0.321) ng/mg creatinine
3HPMA (*urine*)	3HPMA (*N*-Acetyl-*S*-(2-carboxyethyl)-L-cysteine) is a major metabolite of **acrolein** which may cause eye, nasal, and respiratory tract irritations	108.0 (95%CI 95.93–121.6) vs. 271.5 (95%CI 255.1–289.0) ng/mg creatinine
2CyEMA (*urine*)	2CyEMA (*N*-Acetyl-*S*-(2-cyanoethyl)-L-cysteine) is a major metabolite of **acrylonitrile** which is classified by IARC as Group 2B carcinogen.	3.959 (95%CI 3.002–5.219) vs. 123.9 (95%CI 109.9–139.7) ng/mg creatinine
1CaHEMA (*urine*)	1CaHEMA (*N*-Acetyl-*S*-(2-carbamoylethyl)-L-cysteine) is a major metabolite of **acrylamide** which is a probable contributor to cancer risk (IARC Group 2A carcinogen) and can be neurotoxic.	56.05 (95%CI 51.07–61.50) vs. 136.4 (95%CI 129.3–143.8) ng/mg creatinine
Cadmium (*urine)*	**Cadmium** is an IARC Group 1 known human carcinogen with adverse effects on kidney and bone.	0.193 (95%CI 0.165–0.225) vs. 0.277 (95%CI 0.259–0.297) ng/mg creatinine

nonsmokers. In conclusion, biomarkers of exposure to tobacco-related toxicants in users of ENDS appear to be lower than in users of HTPs, and other alternative tobacco products.

2.4. Levels of biomarkers in concurrent users of ENDS and combustible tobacco cigarettes ('dual users')

Additional concerns surround users of both cigarettes and ENDS ('dual users'), who overall appear to maintain or even increase their nicotine intake while significant reduction in biomarkers levels compared to cigarette smokers have not been observed. Although most dual users report that their primary reason for using ENDS is to reduce smoking tobacco cigarettes [38], in aggregate, studies have shown that dual users are not reducing exposure to harmful toxicants compared to exclusive cigarette smokers because of continued smoking [20–29]. However, distinctive patterns of dual use may result in different levels of exposure to nicotine and toxicants. A study by Smith et al. [39] has shown that cigarette smoking frequency (daily vs. non-daily) is a primary driver of toxicant exposure among dual users. Dual users who smoked daily had significantly higher biomarker concentrations than those who did not smoke daily. Notably, the frequency of ENDS use had a negligible effect on toxicant exposure among dual users. The number of cigarettes smoked per day (CPD) by dual users also affects exposure to toxicants in dual users. In a small international study [40], daily dual users who smoked fewer than five cigarettes per day (CPD) exhibited lower exposure to nicotine and toxicants than daily dual users who smoked 10 CPD or more. Taken as a whole, the findings presented above suggest that most dual users mirror exposure profiles of exclusive cigarette smokers with similar smoking frequency.

Three studies of smokers measured changes in biomarker levels up to 4 weeks after participants became dual users [29,32,34]. Those studies observed no change in biomarkers of exposure when smokers became dual users. However, in one study, dual users who reduced their daily cigarette consumption by at least half experienced a significant decrease in VOC exposure [34]. Analysis of PATH study data from Waves 1 (2013–2014) and 2 (2014–2015) [35] revealed that levels of NNAL significantly decreased in smokers who initiated dual use. At the same time, no significant reductions in nicotine metabolites or most PAHs and VOCs were observed. A stratified analysis of the data from the PATH study confirmed that changes in biomarkers while switching from exclusive smoking to dual use were strongly correlated with a reduction in CPD. Specifically, urinary concentrations of biomarkers of exposure to nicotine, TSNAs, PAHs, and VOCs decreased significantly if smokers reduced their CPD by at least 50%. In summary, biomarker studies indicate that smokers who initiate using ENDS are more likely to experience a reduction in toxicant exposure if they reduce their cigarette smoking by at least half. But those smokers who do not change their smoking pattern after initiating ENDS use will not experience any meaningful reduction in toxicant exposure.

2.5. Factors that may potentially affect biomarkers of exposure in ENDS users

While measuring biomarker levels is helpful for exposure assessments resulting from the current use of tobacco products, interpreting biomarker data will require careful consideration of potential factors that may affect biomarker levels. Those sources of variation include frequency and intensity of product use, product characteristics and design, differences in toxicant metabolism (incl. biomarker half-life), co-use of other substances, environmental and dietary sources of exposure, and inconsistency in laboratory methods.

Studies suggested significant interactions between device characteristics, incl. nicotine concentration in ENDS liquid and its flavor, and toxicant exposure levels in ENDS users [23,41–44]. For example, one study observed that nicotine exposure from tank ENDS devices with high power was similar to smoking, while using low-power devices resulted in low nicotine exposure [41]. Rostron et al. [42] compared nicotine exposure among users of open (rechargeable and refillable) and closed (not rechargeable or with prefilled cartridges) ENDS devices in the US. Urinary concentrations of the total nicotine equivalence (TNE) were higher among open-system users than closed-system users. Still, levels were similar when users were stratified by frequency of use. Country-level differences in ENDS devices may explain differences in biomarker levels among ENDS product users in different countries [23]. For example, ENDS users in Poland, who primarily use refillable tank devices, showed higher biomarkers of exposure to nicotine and several toxicants than ENDS users in the US and UK. Dawkins et al. [43] reported that using ENDS devices with a lower nicotine concentration resulted in compensatory behavior (e.g., ENDS users took more puffs, and puffs were longer). That compensatory behavior led to increased exposure to formaldehyde measured with urinary formate [43]. Smith et al. [44] observed elevated concentrations of urinary biomarkers of exposure to acrylonitrile among those ENDS users who used ENDS flavored other than fruit or menthol.

When selecting biomarkers for ENDS research, one needs to be aware of the lack of sensitivity for some biomarkers (e.g., arsenic). Many biomarkers may come from other sources, such as diet, environmental pollution, or the use of other substances (e.g., cannabis). Mainly, smoking cannabis appears to be an essential confounding in assessments of exposures to tobacco-related toxicants among ENDS users. A study by Smith et al. [45] found ENDS users who reported concurrently smoking cannabis products had higher urinary concentrations of biomarkers of exposure to many combustion byproducts than exclusive ENDS users. This finding suggests potential cumulative and elevated toxicant exposures among co-users of ENDS and cannabis. This has important implications since many tobacco users also smoke cannabis.

Further, several toxicant biomarkers measured in studies cited above have exposure sources other than tobacco products. For example, acrylamide, consistently reported in ENDS emissions, is also present in foods cooked at high temperatures [46]. Likewise, lead and cadmium accumulate in the body over the years of cigarette smoking, and they can be detected in the urine over many years after quitting smoking [47]. Therefore, elevated urinary lead and cadmium concentrations among ENDS users may be attributed, at least partially, to previous cigarette smoking than exclusively from current ENDS use.

3. Recommendations

3.1. Development and validation of biomarkers specific to ENDS use

Measurement of nicotine and toxicant doses delivered from ENDS devices could be accomplished by measuring biomarkers of exposure for ENDS constituents in biological samples collected from ENDS users. However, a significant challenge is that the chemicals emitted from ENDS devices have many familiar sources and need to be more specific for ENDS use. Thus, there is a need to identify novel biomarkers specific to ENDS use. This would facilitate exposure assessment in observational studies and allow compliance monitoring in clinical trials. Burkhardt et al. [48] have suggested measuring propylene glycol in urine or plasma as a biomarker specific to ENDS. They developed a sensitive LC-MS/MS method for quantifying propylene glycol and glycerol, two main constituents of ENDS solutions. Both chemicals were analyzed in plasma and urine samples from a clinical study comparing five nicotine product user groups, users of combustible cigarettes, ENDS users, HTP users, OTP users, NRT products, and a control group of non-users. Results have shown significantly elevated propylene glycol levels in urine and plasma in ENDS users compared to users of other tobacco products and non-users. Notably, propylene glycol in plasma and urine of ENDS users significantly correlated with nicotine in plasma and total nicotine equivalents in urine. Hiler et al. [49] successfully used urinary propylene glycol in their human laboratory study to distinguish ENDS users from non-users and verify short-term abstinence from ENDS. Al-

though propylene glycol appears to be a promising biomarker, concerns have been raised about its specificity to ENDS due to its widespread occurrence in many commonly used consumer products [19]. This promising potential biomarker requires further verification under real-life conditions.

Efforts have also been made to evaluate whether potential thermal degradation byproducts of heated solvents used in ENDS (and their respective metabolites) can serve as biomarkers specific to ENDS use. For example, Lorkiewicz et al. [50] have shown that exposure to ENDS has increased metabolites of acrolein and glycidol in the urine of exposed animals and ENDS users. Acrolein and glycidol are two thermal degradation byproducts of vegetable glycerin, another solvent commonly used in ENDS. A human experiment showed elevated levels of urinary 2,3-dihydroxypropylmercapturic acid (23HPMA), a metabolite of glycidol, but not of urinary 3-hydroxypropylmercapturic acid (3HPMA), a metabolite of acrolein in ENDS users following brief use of the product. Importantly, authors reported that urinary 23HPMA increased in ENDS users but not in users of combustible cigarettes. The authors concluded that urinary 23HPMA might be a relatively specific biomarker of ENDS use, but they also emphasized that further validation is required.

Stable-isotope tracers are often measured in biological samples collected in clinical studies to understand novel therapeutics' pharmacokinetics. Landmesser et al. [51] conducted a proof-of-concept study showing that the application of isotope-labeled ENDS ingredients (nicotine, propylene glycol, and glycerin) allowed the accurate quantification of the dose of nicotine and solvents delivered from ENDS. By labeling the ENDS ingredients, researchers could isolate exposure specific to ENDS from other sources. Although the proposed approach can be applied in randomized controlled studies with in-clinic settings, its application in population-based studies would be minimal.

3.2. Use of tobacco-specific biomarkers to verify tobacco abstinence among ENDS users or smoking reduction among dual users

Since nicotine in most ENDS products has been purified adequately so that TSNAs and minor tobacco alkaloid concentrations are low compared to the amounts present in tobacco, biomarkers of exposure to TSNAs and minor alkaloids could be used to identify concurrent smoking and co-use of other tobacco-containing products in people using ENDS. Exclusive ENDS users showed much lower TSNA levels than other tobacco users, incl. combustible cigarette smokers and oral tobacco users [52]. Such a significant reduction in exposure to TSNAs is consistent with lower levels of TSNAs in ENDS products compared with cigarettes and oral tobacco products [3]. Significantly, TSNA concentrations are associated with tobacco use frequency and intensity and may be used to assess exposure to tobacco smoke in dual users [29–34]. TSNA can also be measured prospectively in clinical trials evaluating the potential reduction in smoking frequency or intensity among smokers who initiate ENDS use [53–56]. Another advantage of NNAL is that it has a much longer elimination half-life (16–18 days) [57] and thus can be used to monitor the intermittent use of tobacco-containing products [52]. Minor tobacco alkaloids that include anabasine, anatabine, anatalline, and nicotelline, may also be used as biomarkers of concurrent use of conventional tobacco product use in ENDS users [58,59]. Those biomarkers have been previously used in people who participated in smoking cessation clinical trials with nicotine replacement therapies (NRT) [60–62]. Another promising biomarker specific to combustible tobacco products is nicotelline which does not naturally occur in the tobacco plant and is primarily formed from anatalline, another minor tobacco alkaloid, by pyrolysis and oxidation in burning tobacco [63]. Since both nicotelline and anatalline have not been found in ENDS products, they should be more selective for conventional tobacco products. They can be used to confirm smoking abstinence in clinical trials with ENDS. When selecting an appropriate biomarker for a clinical trial, one must consider its half-life. Since anatabine, anabasine, and anatalline have half-lives ranging from 10 to 16 h [58,59], they may be helpful in studies of the effects of short-term transitioning from combustible cigarettes to ENDS. NNAL has a half-life of more than 10 days [57] and hence could be used in long-term trials to detect tobacco use (incl. smoking combustible cigarettes) occurring over several weeks.

3.3. Use of biomarkers specific to combustible products to verify smoking abstinence among ENDS users

Combustible tobacco products, incl. conventional cigarettes, large and little cigars, cigarillos, and water pipes, generate smoke that users inhale during use. Numerous chemicals are generated during the combustion process and inhaled by users, such as carbon monoxide (CO), VOCs, and PAHs. Those chemicals or their metabolites can be detected in various biological samples collected from users of combustible tobacco products. Exhaled CO is a practical and validated marker for identifying individuals who have recently smoked a combustible tobacco product. Exhaled CO can be measured conveniently in clinical and research settings using commercially available portable CO monitors. A commonly used cut point for verification of self-reported abstinence in smoking cessation trials has been <10 ppm. However, new data suggest that the cut point for exhaled CO should be lowered to 4 ppm [64].

At present, a biological specimen that tests positive for nicotine metabolites and at the same time tests negative for biomarkers of combustion products suggests either the use of ENDS, non-combustible oral nicotine products (e.g., snus, nicotine pouches) or a nicotine replacement therapy (NRT) product, such as nicotine patch or gum. Several cross-sectional studies consistently showed that users of combustible tobacco products showed significantly higher concentrations of most VOC biomarkers than ENDS users [20–23]. Moreover, ENDS users have VOC Moreover, ENDS users have VOC exposure like never users for most VOCs, but not for 2CyEMA (a metabolite of acrylonitrile) [20–23]. Those findings strongly suggest that selected VOC biomarkers are appropriate for monitoring the use of combustible tobacco products and may allow for verification of smoking status in clinical trials with ENDS products. Specifically, the biomarker 2CyEMA (a significant metabolite of acrylonitrile) was shown to effectively discriminate combustible and non-combustible tobacco product users (incl. ENDS users) [65,66]. exposure like never users for most VOCs [20–23]. Those findings strongly suggest that selected VOC biomarkers are appropriate for monitoring the use of combustible tobacco products and may allow for verification of smoking status in clinical trials with ENDS products. Specifically, the biomarker 2CyEMA (a significant metabolite of acrylonitrile) was shown to effectively discriminate combustible and non-combustible tobacco product users (incl. ENDS users) [65,66]. A potential limitation of using VOC metabolites as indicators of smoking reduction in dual users is their relatively short physiological half-time (few hours) [67]. VOCs may not be sensitive enough to detect the non-daily use of combustible products in clinical trials with ENDS products.

3.4. Use of a panel of biomarkers instead of a single biomarker

Using more than one biomarker may be beneficial to verify self-reported data on smoking and vaping status to urinary biomarkers (Fig. 1). For example, cotinine measurement may be supplemented with the measurement of tobacco-specific biomarkers, like NNAL. A positive cotinine result indicates either combusted tobacco or ENDS use. Because NNAL is a tobacco-specific biomarker, it could distinguish between tobacco smokers and exclusive ENDS users [68,69]. Testing for cotinine could also be supplemented with measuring VOC biomarkers, like 2CyEMA. Because significantly elevated levels of 2CyEMA are observed in users of combustible tobacco products, it can also be used to distinguish between conventional cigarette smokers and exclusive ENDS users [65,66]. In addition to reporting absolute concentrations of two biomarkers, biomarker ratios (e.g., 2CyEMA/cotinine) may offer an additional way of distinguishing ENDS users from users of other tobacco products and nonusers.

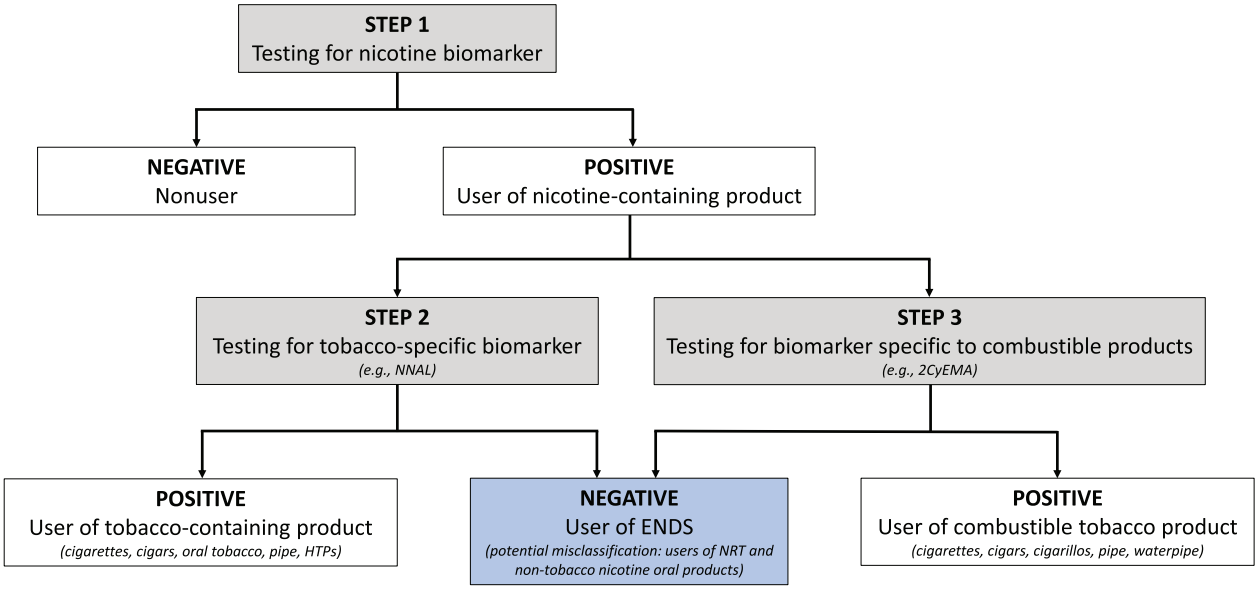

Fig. 1. Use of a panel of biomarkers specific to nicotine products (Step 1), specific to tobacco-containing products (Step 2), and specific to combustible tobacco products (Step 3) to verify ENDS use in human studies.

The potential strength of the approaches described above is the use of existing and previously validated biomarkers. However, analyzing two or more biomarkers will usually require the application of two or more analytical assays leading to increased cost of the study and longer time needed to complete all analyses. An optimal solution would be a new analytical method that allows for the simultaneous determination of multiple biomarkers in a single run. Jacobs et al. [70] have developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to simultaneously determine numerous biomarkers with a wide range of biological half-lives, ranging from 2 to 3 h for nicotelline, to more than 10 days for NNAL. The new method has high sensitivity (low limits of quantitation), facilitating low-level exposure assessment. Although such complex analytical assays allow for analyzing multiple biomarkers in a single run, they also provide many challenges. They often require expensive, state-of-the-art analytical instruments with high sensitivity. Maintaining a high accuracy, precision, sensitivity, and selectivity for all biomarkers measured in a single assay may not be feasible. Finally, the need for highly-qualified personnel to run such complex assays and the overall cost may be significant barriers for small-scale research laboratories.

A combination of several biomarkers may lead to a more precise separation of tobacco product user groups, providing a better understanding of exposure patterns and related health effects. A panel of biomarkers may also assess relative exposure to two tobacco products among dual users. The ratio of cotinine to NNAL in urine has been suggested as a valuable biomarker of dual use of combustible tobacco products and ENDS, as it is expected to be significantly lower in combustible tobacco product users than in ENDS users [71]. The advantages of the new method developed by Jacob et al. [70] and described above (highly sensitive measurement of biomarkers with variable half-time) should also make it especially suitable in studies of dual use of ENDS and conventional tobacco products.

3.5. Use of cannabis-specific biomarkers to identify study participants who use cannabis

Screening subjects in ENDS studies for biomarkers of cannabis use can improve the interpretation of results from nonspecific biomarker tests. Urinary 11-nor-Δ9-tetrahydrocannabinol carboxylic acid (THCA) is a major metabolite of THC and a well-established biomarker of cannabis use [72]. It is usually measured in urine samples using commercially available test kits. However, more sensitive chromatography methods have been previously developed and published. A commonly used cutpoint that indicates regular use of cannabis is 50 ng/ml [72]. Due to the relatively long urinary elimination half-life of THCA (approximately 30 h after seven days and 44–60 h after twelve days post cannabis use) [72], it is challenging to determine whether a positive THCA result in regular cannabis user reflects a recent episode of drug use or continued elimination of the residual drug.

3.6. Collecting data on other potential sources of exposure

Although smoking tobacco products is an essential source of exposure to VOCs, PAHs, exhaled CO, and metals, there are numerous other sources of exposure, including dietary (e.g., smoked and grilled food), occupational (e.g., fumes) and environmental (e.g., vehicle exhaust) exposures. Thus, the metabolites of those toxicants are not specific to the use of tobacco products. To measure nonspecific biomarkers of exposure to tobacco products, collecting questionnaire data on dietary, occupational, and environmental exposure is recommended. Standard protocols for collecting self-reported non-tobacco exposure data are provided in the PhenX Toolkit (consensus measures for **Phen**otypes and e**X**posures) [73]. They included recommended protocols and tools used across large national health surveys (including the National Health and Nutrition Examination Survey (NHANES), the National Health Interview Survey (NHIS), and the Population Assessment of Tobacco and Health (PATH) Study) facilitating cross-study analysis.

4. Recommendations for future research on ENDS biomarkers

- The absence of unique biomarkers for ENDS products is an urgent problem. To support research on ENDS, it is essential to identify product-specific biomarkers. There is a need for rapid, sensitive, accurate, and affordable laboratory-based analytical assays. There is also a need for affordable and sensitive test kits to measure ENDS use '*on-site*' in human studies.
- Studies are needed to validate biomarkers of exposure in serum, urine, and saliva and establish cut-points (across various biological

matrices) that can be used for biochemical validation of ENDS use status.

- Studies are needed to establish cut-points (across various biological matrices) for biochemical validation of smoking abstinence among ENDS users. Primary applications of these cut-points include validating abstinence in smoking cessation trials with ENDS and validating self-reported use of combustible products for inclusion in observational studies or population-based surveillance systems.
- Due to changes in the nicotine formulations (e.g., use of nicotine salts, synthetic nicotine) and power outputs used in the newer generation of ENDS products, it is essential to consider how these changes may affect toxicant exposure among ENDS users. As the market continues to evolve with newer-generation ENDS products and an increasing assortment of ENDS flavors, there is a need for continued surveillance of toxicant exposure among ENDS users.
- Although the measurement of expired CO is an established biomarker of smoking that has been used in numerous smoking cessation trials, it only verifies short-time abstinence. A rapid and affordable test that also detects infrequent or occasional smoking in ENDS users would be of great value to researchers who plan to evaluate the smoking cessation efficacy of ENDS in clinical trials.

5. Summary

Biomarkers indicate exposure to nicotine and toxicants from ENDS use and inform the overall risk-benefit of ENDS use. Overall, the concentrations of several tobacco exposure biomarkers, including metabolites of nicotine, TSNAs, VOCs, and metals (e.g., cadmium and lead), are elevated in exclusive ENDS users compared to non-users but significantly lower than in current smokers. Cigarette smokers who switch entirely to ENDS show reductions in biomarkers of exposure to numerous tobacco-related toxicants. There is also some evidence to suggest that ENDS users are exposed to fewer harmful substances overall, and in lower concentrations, than users of other alternative tobacco products like oral or heated tobacco products. ENDS product characteristics and use patterns may be associated with elevated levels of biomarkers, particularly potential thermal degradation byproducts like acrolein, acrylamide, and acrylonitrile. To verify self-reported data on smoking and vaping status to urinary biomarkers, using more than one biomarker may be beneficial. Currently, the optimum way to identify the use of nicotine-containing ENDS is to confirm the presence of nicotine metabolites and the absence of other biomarkers for combustible tobacco products or specific to tobacco-containing products. Studies are needed to validate biomarkers of exposure in serum, urine, and saliva and establish cut-points (across various biological matrices) that can be used for biochemical validation of ENDS use status.

Funding

This work was supported by the National Cancer Institute at the National Institutes of Health and the Food and Drug Administration Center for Tobacco Products (grant number U54 CA228110). The content is solely the responsibility of the author and does not necessarily represent the official views of the NIH or FDA.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: *MLG received research grant from Pfizer and served as a member of scientific advisory board to Johnson&Johnson.*

Data availability

This review discussed results that have been previously published. All applicable references are provided.

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