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REVIEW

*Electronic Cigarettes: Not All Good News?*

# Recent updates on biomarkers of exposure and systemic toxicity in e-cigarette users and EVALI

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## Abstract

Electronic nicotine delivery systems (ENDS), or e-cigarettes, are emerging tobacco products that produce aerosols by heating e-liquids, which most often consist of propylene glycol and vegetable glycerin along with various flavoring compounds, bypassing the combustion that occurs in the use of traditional tobacco cigarettes. These products have seen a drastic increase in popularity in recent years both as smoking cessation devices as well as among younger generations, due in large part to the widespread perception among consumers that e-cigs are significantly less harmful to health than traditional tobacco cigarettes. Due to the novelty of ENDS as well as their rapidly increasing use, research into biomarkers of e-cig exposure and toxicity have lagged behind their popularity, leaving important questions about their potential toxicity unanswered. Research into potential biomarkers of acute and chronic e-cig use, and e-cigarette- or vaping-associated lung injury is necessary for informing both clinical and regulatory decision-making. We aim to provide an updated review of recent research into potential circulating, genomic, transcriptomic, and epigenetic biomarkers of exposure to and toxicity of e-cigs. We additionally highlight research areas that warrant additional study to gain a better understanding of health risks associated with ENDS use, as well as to provide validation of existing data and methods for measuring and analyzing e-cig-associated biomarkers in human and animal biofluids, tissues, and cells. This review also highlights ongoing efforts within the WNY Center for Research on Flavored Tobacco for research into novel biomarkers in extracellular vesicles that may be associated with short- and long-term ENDS use.

*cigarette smoke; e-cigarettes; EVALI; exposure biomarker; pulmonary toxicity*

## INTRODUCTION

The use of electronic nicotine delivery systems (ENDS), or electronic cigarettes (e-cigs), has increased drastically in recent years, partly due to the perception among consumers that using such devices ("vaping") is less harmful to human health than smoking combustible tobacco cigarettes. ENDS are also commonly used as a tool for quitting smoking, contributing to their popularity (1). Due to the novelty of ENDS, and variability between different types of ENDS and e-cig liquids [e-liquids: commonly composed of propylene glycol (PG), vegetable glycerin (VG), nicotine, and flavoring chemicals], knowledge of their possible adverse health effects is incomplete. However, it is clear that ENDS use is associated with negative health effects including inflammation, oxidative stress, and organ toxicity (2–7). ENDS work by heating e-liquids until they are aerosolized, avoiding the combustion associated with cigarettes (5, 7). Several factors can affect toxicity of ENDS aerosols, including type/model of ENDS, battery voltage, temperature e-liquids are heated to, e-liquid constituents, and individual use patterns (8, 9). One area of research helping to elucidate the health effects of ENDS use in vivo is the identification and validation of systemic biomarkers that can be associated with exposure to and toxicity of ENDS (6). Advancing this field will allow for more accurate comparisons of the health risks associated with vaping versus smoking and will aid in clinical and regulatory decision-making.

Biomarkers of exposure are molecules that can be used to measure the amount of a particular chemical substance an organism has been exposed to, by detection of the chemical itself or its metabolites (9). Such biomarkers are necessary for the identification and assessment of e-cig use, both for clinical and regulatory reasons. Due to the novelty of e-cigs, there is a lack of validated biomarkers of exposure that allow researchers and clinicians to accurately identify and quantify e-cig use, and to differentiate e-cig use from that of other tobacco products (10). Biomarkers of exposure to ENDS that

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are being investigated and validated include cotinine and other nicotine metabolites, tobacco-specific nitrosamines (TSNAs), exhaled carbon monoxide (CO), polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), and metals (7, 9, 10).

Biomarkers of toxicity are molecules used to quantify biochemical, physiological, and other measurable effects of exposure to a specific substance (1, 9). They are widely used to quantify the biological effects that a chemical or substance has on the body, such as changes in morphology or function, and can be used to measure risk factors for disease (8). In this review, we use the term biomarkers of toxicity to include biomarkers of effect (on health/biology) and potential harm (disease risk). Biomarkers of toxicity that are currently being investigated in relation to ENDS use include proinflammatory cytokines and chemokines, reactive oxygen species (ROS), matrix metalloproteases (MMPs), markers of DNA damage and oxidation, differential gene and protein expression, and changes in DNA methylation (2, 4–8, 10). It is important to note that some biomarkers of exposure may also be considered biomarkers of toxicity if they produce notable changes on the biology of an organism following exposure (9). This review aims to summarize recent literature on potential circulating, genomic, transcriptomic, and epigenomic biomarkers of e-cig/ENDS exposure and systemic toxicity, which will be important in informing tobacco regulatory science and clinical practice.

## BIOMARKERS OF EXPOSURE

### Cotinine

Nicotine is a chemical found in tobacco as well as in many e-liquids, so nicotine and its metabolites are biomarkers that are not specific to ENDS, giving researchers the ability to compare nicotine consumption levels between users of various tobacco products. When nicotine is consumed, it is broken down into various metabolites, with the predominant one being the alkaloid cotinine. Although both nicotine and cotinine levels can be measured in various biofluids and tissues, the longer half-life of cotinine (16–18 h compared with 2 h for nicotine) and the fact that circulating cotinine levels stay more consistent throughout the day making it a more useful biomarker for nicotine exposure (1, 10). Levels of cotinine depend on the sample matrix in which it is being measured, as well as on the genetic background of the individual whose cotinine level is being analyzed (10). One recent study by Singh et al. (6) showed significant differences in the plasma cotinine levels of e-cig users ($n$ = 22, 164.70 ± 39.92 ng/mL) and nonsmokers ($n$ = 26, 3.86 ± 2.74 ng/mL). Prior study by Park and Choi (11) found significant differences in urinary cotinine levels between cigarette smokers ["smokers," geometric mean (GM): 842.5, median: 1,163.0 ng/mL, $n$ = 2,627], e-cig users (GM: 119.5, median: 309.7 ng/mL, $n$ = 44), and nonsmokers (GM: 0.8, median: 0.8 ng/mL, $n$ = 12,182).

Another recent study by Goniewicz et al. (12) using spot urine samples from 2,411 smokers, 247 e-cig users, and 1,655 never-users (never used tobacco products) found that e-cig users had significantly lower levels of all major nicotine metabolites than smokers, and the same was true for never-users compared with e-cig users. Alternatively, Rapp et al. (13) found no significant difference in mean serum cotinine levels of smokers ($n$ = 379, 205.97 ng/mL) and e-cig users ($n$ = 49, 152.96 ng/mL), indicating that ENDS expose users to nicotine levels comparable with combustible tobacco cigarettes. Bustamante et al. (14) also found similar cotinine levels in the urine of smokers (17.3 ± 10.6 nmol/mL) and e-cig users (17.5 ± 17.4 nmol/mL) ($n$ = 19/group), with nonsmokers showing only trace amounts ($n$ = 18, 0.32 ± 0.47 nmol/mL). A study by Goney et al. (15) measuring cotinine in urine also showed no significant differences between levels in e-cig users ($n$ = 32, 1,755 ± 1,848 ng/g creatinine) and smokers ($n$ = 33, 1,720 ± 1,335 ng/g creatinine), with nonsmokers' ($n$ = 33) levels being below the level of detection. Johnson et al. (16) measured salivary and urinary cotinine levels in 28 nonsmokers following secondhand exposure to e-cigs at e-cigarette conventions. Although levels varied due to sampling time and differences in event size, adjusted mean ratios for maximum cotinine levels relative to baseline in urine (ranging between 2.67 and 13.16) and saliva (ranging between 2.02 and 12.68) clearly indicate secondhand nicotine exposure from e-cigs (16). Collectively, these data show that it is possible to reliably differentiate nicotine-containing e-cig users from nonsmokers based on cotinine levels, but it is less evident when comparing cotinine exposure between e-cig users and smokers (Table 1 and Fig. 1). The differences in results among these and other studies point to the need for additional research on how cotinine levels vary between ENDS users and smokers, and whether or not cotinine can be used as a biomarker to effectively differentiate between these populations.

### Tobacco-Specific Nitrosamines

Tobacco-specific nitrosamines (TSNAs), as the name implies, are biomarkers that are recognized as being specific to tobacco products, and as such these compounds can be detected in smokers and e-cig users at varying levels. These compounds are *N*-nitroso-derivatives of pyridine-alkaloids (i.e., nicotine, nornicotine) and include *N*′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is a metabolite of NNK (9, 10). These TSNAs, primarily detected in urine, are useful biomarkers for tobacco exposure, but are considered more difficult to detect than cotinine and as such are less practical. TSNAs are widely considered to be carcinogenic, and NNAL specifically, which is the predominant and most stable TSNA in urine samples, is used as an indicator of cancer risk as well as being a tobacco-specific biomarker (10). NNN is a known tobacco-specific oral and esophageal carcinogen, which is formed endogenously in e-cig users (14).

Recently, Sakamaki-Ching et al. (4) found that NNAL levels in spot urine samples of nonsmokers ($n$ = 20, means ± SD = 2.8 ± 6.3 pg/mg creatinine) and e-cig users ($n$ = 18, 13.3 ± 18.6 pg/mg creatinine) were significantly lower than those of smokers ($n$ = 13, 105.7 ± 87.4 pg/mg creatinine), with both nonsmokers and e-cig users showing NNAL levels consistent with no tobacco use. Goniewicz et al. (12) found that never-users had significantly lower levels of urinary NNK and NNAL than e-cig users (~81% lower NNAL in never-users vs. e-cig users), and the same was true for e-cig users compared with smokers (~98% lower NNAL in e-cig users

**Table 1.** *Biomarkers of exposure in ENDS/e-cig users*

| Sample Matrix (Metabolite) | Methods | Groups (*n*/group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| Cotinine | | | | | |
| Plasma | Cotinine ELISA (Salimetrics) | Nonsmokers ($n = 26$);<br>E-cig users ($n = 22$) | Means ± SE = 3.86 ± 2.74 ng/mL<br>Means ± SE = 164.70 ± 39.92 ng/mL | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| Urine | HPLC-MS/MS (Agilent 1100 Series with API 4000) | Nonsmokers ($n = 12,182$);<br>E-cig users ($n = 44$);<br>Smokers ($n = 2,627$) | GM = 0.8, med = 0.8 ng/mL<br>GM = 119.5, med = 309.7 ng/mL<br>GM = 842.5, med = 1,163.0 ng/mL | Significantly higher levels in e-cig users vs. nonsmokers. Significantly higher levels in smokers vs. e-cig users. | (11) |
| Spot urine | Two separate isotope dilution HPLC-MS/MS analyses | Never-users ($n = 1,655$);<br>E-cig users ($n = 247$);<br>Smokers ($n = 2,411$) | GM = 4.2 E-01 ng/mg creatinine<br>GM = 124.3 ng/mg creatinine<br>GM = 1,830.9 ng/mg creatinine | Significantly higher levels in e-cig users vs. nonsmokers. Significantly higher levels in smokers vs. e-cig users. | (12) |
| Serum | Isotope dilution HPLC/APCI-MS/MS | E-cig users ($n = 49$);<br>Smokers ($n = 379$) | Mean = 152.96 ng/mL<br>Mean = 205.97 ng/mL | No significant difference between levels in smokers and e-cig users. | (13) |
| Urine | GC-MS | Nonsmokers ($n = 33$);<br>E-cig users ($n = 32$);<br>Smokers ($n = 33$) | Means ± SD = below LOD<br>Means ± SD = 1,755 ± 1,848 ng/g creatinine<br>Means ± SD = 1,720 ± 1,335 ng/g creatinine | No significant difference between levels in smokers and e-cig users.Nonsmoker levels below LOD. | (15) |
| Urine, saliva | GC-MS (Agilent 7820 and Agilent 5977 E). Results relative to BSL. | Nonsmokers with passive e-cig exposure ($n = 28$) | Adjusted MR range: 2.67–13.16,<br>Adjusted MR range: 2.02–12.68 | Secondhand e-cig exposure leads to measurable cotinine levels in urine and saliva. | (16) |
| Tobacco-specific nitrosamines (TSNAs) | | | | | |
| NNAL | | | | | |
| Spot urine | Liquid chromatography-atmospheric pressure ionization tandem mass spectrometry | Nonsmokers ($n = 20$);<br>E-cig users ($n = 20$);<br>Smokers ($n = 13$) | Means ± SD = 2.8 ± 6.3 pg/mg creatinine<br>Means ± SD = 13.3 ± 18.6 pg/mg creatinine<br>Means ± SD = 105.7 ± 87.4 pg/mg creatinine | Significantly higher levels in smokers vs. e-cig users and nonsmokers. | (4) |
| Urine | Isotope dilution HPLC/APCI-MS/MS | Never-users ($n = 1,655$);<br>E-cig users ($n = 247$);<br>Smokers ($n = 2,411$) | ~81% higher levels vs. never-users~98% higher levels vs. e-cig users | Significantly higher levels in e-cig users vs. never-users. Significantly higher levels in smokers vs. e-cig users. | (12) |
| Urine | LC/ESI-MS/MS | Nonsmokers ($n = 18$);<br>E-cig users ($n = 19$);<br>Smokers ($n = 19$) | Means ± SD = 0.04 ± 0.10 pmol/mL<br>Means ± SD = 0.07 ± 0.18 pmol/mL<br>Means ± SD = 1.28 ± 1.04 pmol/mL | Significantly higher levels in smokers vs. e-cig users and nonsmokers. | (14) |
| Urine | LC/MS/MS | No secondhand exposure ($n = 24$);<br>Secondhand e-cig aerosols ($n = 6$);<br>Secondhand cigarette smoke ($n = 25$) | 29.2% showed quantifiable NNAL<br>66.7% showed quantifiable NNAL<br>76.0% showed quantifiable NNAL | There was a significant difference in percentage of samples with quantifiable NNAL among groups. | (26) |
| Urine | Isotope dilution HPLC/APCI-MS/MS | Nonsmokers with passive e-cig exposure ($n = 28$) | N/A | Levels so low as to preclude statistical analysis. | (16) |
| NNN | | | | | |
| Saliva, urine | LC-MS/MS<br>LC-NSI-HRMS/MS (Orbitrap Fusion Tribrid) | Nonsmokers ($n = 19$);<br>E-cig users ($n = 20$);<br>Smokers ($n = 20$)<br>Nonsmokers ($n = 18$);<br>E-cig users ($n = 19$);<br>Smokers ($n = 20$) | Means ± SD = 0.25 ± 0.28 pg/mL<br>Means ± SD = 14.6 ± 23.1 pg/mL<br>Means ± SD = 94.5 ± 176 pg/mL<br>Means ± SD = 0.001 ± 0.001 pmol/mL<br>Means ± SD = 0.001 ± 0.002 pmol/mL<br>Means ± SD = 0.16 ± 0.50 pmol/mL | Significantly higher levels in smokers vs. e-cig users and nonsmokers.<br>Significantly higher levels in smokers vs. e-cig users and nonsmokers. | (14) |
| Urine | Isotope dilution HPLC/APCI-MS/MS | Nonsmokers with passive e-cig exposure ($n = 28$) | N/A | Levels so low as to preclude statistical analysis. | (16) |
| Carbon monoxide (CO) | | | | | |
| Exhaled breath | Covita Smokerlyzer | ENDS users ($n = 23$);<br>Smokers ($n = 27$) | GM = 2.21 ppm<br>GM = 16.58 ppm | Significantly higher levels in smokers vs. ENDS users. | (22) |
| Exhaled breath | | Smokers switched to e-cigs ($n = 42$);<br>Smoker controls (no switch; $n = 20$) | 13.9 ± 0.7 (BSL) to 4.2 ± 0.6 ppm (1 mo)<br>15.3 ± 1 (BSL) to 16.4 ± 0.7 ppm (1 mo) | Smokers who switched to e-cigs showed significantly lower levels vs. smokers who continued smoking. | (20) |

*Continued*

**Table 1.—** *Continued*

| Sample Matrix (Metabolite) | Methods | Groups (*n*/group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| Exhaled breath | Bedfont Micro Smokerlyzer | Nonsmokers (*n* = 44);<br>E-cig users (*n* = 39);<br>Smokers (*n* = 38) | 1.80 ± 0.11 ng/mL<br>2.18 ± 0.19 ng/mL<br>15.16 ± 1.33 ng/mL | Significantly higher levels in smokers vs. e-cig users and nonsmokers. | (23) |
| Exhaled breath | Bedfont Micro Smokerlyzer | Nonsmokers (*n* = 30);<br>E-cig users (*n* = 18);<br>Smokers (*n* = 25) | N/A | Significantly higher levels in smokers vs. e-cig users and nonsmokers. | (27) |
| Polycyclic aromatic hydrocarbons (PAHs) | | | | | |
| Naphthalene | | | | | |
| Spot urine (2-NAP) | Isotope dilution LC/MS/MS | Never-users (*n* = 1,655);<br>E-cig users (*n* = 247);<br>Smokers (*n* = 2,411) | 62% higher levels vs. e-cig users | No difference between e-cig users and never-users. Significantly higher levels in smokers vs. e-cig users and never-smokers. | (12) |
| Pyrene | | | | | |
| Spot urine (1-PYR) | Isotope dilution LC/MS/MS | Never-users (*n* = 1,655);<br>E-cig users (*n* = 247);<br>Smokers (*n* = 2,411) | ~20% higher levels vs. never-users<br>47% higher levels vs. e-cig users | Significantly higher levels in e-cig users vs. never-users. Significantly higher levels in smokers vs. e-cig users. | (12) |
| Volatile organic compounds (VOCs) | | | | | |
| Acrolein | | | | | |
| Spot urine (CEMA) | Isotope dilution UPLC-MS/MS | Never-users (*n* = 1,655);<br>E-cig users (*n* = 247);<br>Smokers (*n* = 2,411) | 60% higher levels vs. e-cig users | Significantly higher levels in smokers vs. e-cig users. | (12) |
| Urine (3-HPMA) | LC-MS/MS | Nonsmokers (adolescent *n* = 20);<br>E-cig users (adolescent *n* = 67) | 20% higher levels vs. nonsmokers | Significantly higher levels in e-cig users vs. nonsmokers. | (30) |
| Urine (CEMA) | (UPLC/ESI-MS/MS) | Nonsmokers with passive e-cig exposure (*n* = 28) | Increased up to 2.4-fold following exposure, corrected for creatinine | Levels so low as to preclude statistical analysis. | (16) |
| Urine (3-HPMA) | (UPLC/ESI-MS/MS) | Nonsmokers with passive e-cig exposure (*n* = 28) | Increased up to 3.8-fold following exposure, corrected for creatinine | Levels so low as to preclude statistical analysis. | (16) |
| Acrylamide | | | | | |
| Spot urine (CEMA) | Isotope dilution UPLC-MS/MS | Never-users (*n* = 1,655);<br>E-cig users (*n* = 247);<br>Smokers (*n* = 2,411) | 59% higher levels vs. e-cig users | Significantly higher levels in smokers vs. e-cig users. | (12) |
| Urine (AAMA) | LC-MS/MS | Nonsmokers (adolescent *n* = 20);<br>E-cig users (adolescent *n* = 67) | 30% higher levels vs. nonsmokers | Significantly higher levels in e-cig users vs. nonsmokers. | (30) |
| Acrylonitrile | | | | | |
| Spot urine (CYMA) | Isotope dilution UPLC-MS/MS | Never-users (*n* = 1,655);<br>E-cig users (*n* = 247);<br>Smokers (*n* = 2,411) | ~66% higher levels vs. never-users<br>97% higher levels vs. e-cig users | Significantly higher levels in e-cig users vs. never-users. Significantly higher levels in smokers vs. e-cig users. | (12) |
| Urine (CNEMA) | LC-MS/MS | Nonsmokers (adolescent *n* = 20);<br>E-cig users (adolescent *n* = 67) | 341% higher levels vs. nonsmokers | Significantly higher levels in e-cig users vs. nonsmokers. | (30) |
| Urine (CYMA) | Isotope dilution UPLC-MS/MS | Fruit only e-cig flavor users (*n* = ~40);<br>Other e-cig flavor users (*n* = ~65) | GM = 7.55 ng/mg creatinine<br>GM = 2.79 ng/mg creatinine | Significantly higher levels in fruit-only e-cig flavor users vs. users of other non-tobacco flavors. | (32) |
| Benzene | | | | | |
| Urine (MU) | UPLC-MS/MS | Non-tobacco product users (*n* = 12);<br>E-cig users (*n* = 3) | Means ± SD = 144.0 ± 80.4 ng/mg creatinine<br>Means ± SD = 317.5 ± 92.7 ng/mg creatinine | Significantly higher levels in e-cig users vs. non-tobacco product users. | (21) |
| Crotonaldehyde | | | | | |
| Urine (HMPMA) | LC-MS/MS | Nonsmokers (adolescent; *n* = 20);<br>E-cig users (adolescent; *n* = 67) | 20% higher levels vs. nonsmokers | Significantly higher levels in e-cig users vs. nonsmokers. | (30) |
| Cyanide | | | | | |
| Urine (ATCA) | UPLC-MS/MS | | Means ± SD = 115.5 ± 77.1 ng/mg creatinine | | (21) |

*Continued*

**Table 1.—** *Continued*

| Sample Matrix (Metabolite) | Methods | Groups (*n*/group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| | | Non-tobacco product users (*n* = 12); E-cig users (*n* = 3) | Means ± SD = 439.7 ± 257.8 ng/mg creatinine | Significantly higher levels in e-cig users vs. non-tobacco product users. | |
| Ethylbenzene, Styrene | | | | | |
| Urine (PGA) | UPLC-MS/MS | Non-tobacco product users (*n* = 12); E-cig users (*n* = 3) | Means ± SD = 205.2 ± 75.4 ng/mg creatinine<br>Means ± SD = 324.5 ± 75.5 ng/mg creatinine | Significantly higher levels in e-cig users vs. non-tobacco product users. | (21) |
| Propylene oxide | | | | | |
| Urine (2-HPMA) | LC-MS/MS | Nonsmokers (adolescent; *n* = 20);E-cig users (adolescent; *n* = 67) | 51% higher levels vs. nonsmokers | Significantly higher levels in e-cig users vs. nonsmokers. | (30) |
| Styrene | | | | | |
| Urine (MA) | UPLC-MS/MS | Non-tobacco product users (*n* = 12); E-cig users (*n* = 3) | Means ± SD = 132 ± 41 ng/mg creatinine<br>Means ± SD = 197.2 ± 35.9 ng/mg creatinine | Significantly higher levels in e-cig users vs. non-tobacco product users. | (21) |
| Xylene | | | | | |
| Urine (3MHA + 4MHA) | UPLC-MS/MS | Non-tobacco product users (*n* = 12); E-cig users (*n* = 3) | Means ± SD = 71.9 ± 29.6 ng/mg creatinine<br>Means ± SD = 316.3 ± 349.1 ng/mg creatinine | Significantly higher levels in e-cig users vs. non-tobacco product users. | (21) |
| Heavy metals | | | | | |
| Cadmium | | | | | |
| Spot urine | ICP-MS | Never-users (*n* = 1,655); E-cig users (*n* = 247);Smokers (*n* = 2,411) | 23% higher levels vs. never-users<br>30% higher levels vs. e-cig users | Significantly higher levels in e-cig users vs. never-users. Significantly higher levels in smokers vs. e-cig users. | (12) |
| Blood | ETAAS (PerkinElmer 4100ZL) | Nonsmokers (51); E-cig users (past smokers; *n* = 48); Smokers (*n* = 28) | GM = 0.31 μg/L<br>GM = 0.44 μg/L<br>GM = 1.44 μg/L | Significantly higher levels in e-cig users vs. nonsmokers. Significantly higher levels in smokers vs. nonsmokers and e-cig users. | (36) |
| Lead | | | | | |
| Spot urine | ICP-MS | Never-users (*n* = 1,655); E-cig users (*n* = 247);Smokers (*n* = 2,411) | 19% higher levels vs. never-users | Significantly higher levels in e-cig users vs. never-users. No difference between e-cig users and smokers. | (12) |
| Blood | ETAAS (PerkinElmer 4100ZL) | Nonsmokers (*n* = 51); E-cig users (past smokers; *n* = 48); Smokers (*n* = 28) | GM = 11.9 μg/L<br>GM = 14.2 μg/L<br>GM = 15.9 μg/L | Significantly higher levels in smokers vs. nonsmokers. | (36) |

AAMA, *N*-acetyl-*S*-(carbamoylethyl)-L-cysteine; ATCA, 2-aminothiazoline-4-carboxylic acid; BSL, baseline; CEMA, *N*-acetyl-*S*-(2-carboxyethyl)-L-cysteine; CNEMA, 2-cyanoethylmercapturic acid; CYMA, *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine; ELISA, enzyme-linked immunosorbent assay; ETAAS, electrothermal atomic absorption spectrometry; GC-MS, gas chromatography-mass spectrometry; GM, geometric mean; HMPMA, 3-hydroxy-1-methyl-propylmercapturic acid; HPLC/APCI-MS/MS, high performance liquid chromatography/atmospheric pressure chemical ionization-tandem mass spectrometry; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; 2-HPMA, 2-hydroxypropylmercapturic acid; 3-HPMA, 3-hydroxypropylmercapturic acid; ICP-MS, inductively coupled plasma mass spectrometry; LOD, level of detection; LC/ESI-MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; LC/MS/MS, liquid chromatography tandem mass spectrometry; LC-NSI-HRMS/MS, liquid chromatography-nanoelectrospray ionization-high resolution tandem mass spectrometry; MA, mandelic acid; med: median; 3MHA + 4MHA, 3-methyl hippuric acid + 4-methyl hippuric acid; MR, mean ratio; MU, *trans,trans*-muconic acid; N/A, details not available; 2-NAP, 2-naphthol; PGA, phenylglyoxylic acid; 1-PYR, 1-hydroxypyrene; SD, standard deviation; SE, standard error of the mean; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; UPLC/ESI-MS/MS, ultrahigh-performance liquid chromatography-electrospray ionization-tandem mass spectrometry.

vs. smokers). Bustamante et al. (14) examined salivary NNN levels among smokers (*n* = 20), e-cig users (*n* = 20), and nonsmokers (*n* = 19), finding levels in e-cig users (means ± SD; 14.6 ± 23.1 pg/mL) to be significantly lower than in smokers (94.5 ± 176 pg/mL), whereas nonsmoker levels (0.25 ± 0.28 pg/mL) were not significantly different than those of e-cig users. The same study found urinary NNN levels in smokers (*n* = 20; 0.16 ± 0.50 pmol/mL) to be significantly higher than those of e-cig users (*n* = 19; 0.001 ± 0.002 pmol/mL), with nonsmokers (*n* = 18; 0.001 ± 0.001 pmol/mL) showing similar levels to e-cig users. This study showed a similar trend in urinary NNAL, with levels in smokers (*n* = 19; 1.28 ± 1.04 pmol/mL) being significantly higher than those of e-cig users (*n* = 19; 0.07 ± 0.18 pmol/mL) and nonsmokers (*n* = 18; 0.04 ± 0.10 pmol/mL) (14). This data illustrates how TSNAs NNAL and NNN are potential biomarkers that will allow for differentiation of e-cig users and tobacco/cigarette smokers, although additional studies with large cohorts would be helpful to further investigate and validate this relationship.

NNAL has also been assessed in nonsmokers who are exposed to secondhand aerosol from e-cigs. Martinez-Sanchez et al. (26) measured urinary NNAL concentrations in nonsmokers who lived in homes with smokers (*n* = 25), e-cig users (*n* = 6), and other nonsmokers (*n* = 24, "control homes"). Measurable levels of NNAL were detected in 4 of the 6 nonsmokers living with e-cig users, though overall levels were not significantly greater than those of nonsmokers living in control homes (26). Another recent study by

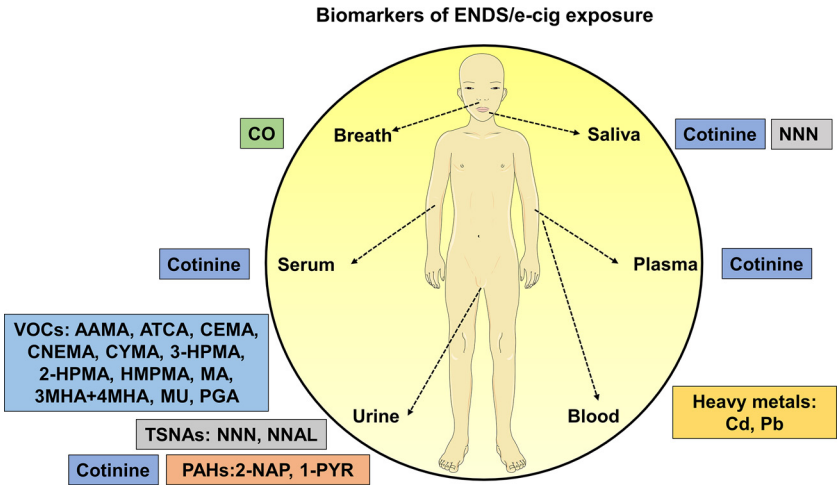


**Figure 1.** Biomarkers of electronic nicotine delivery systems (ENDS)/e-cig exposure. Biomarkers of exposure that can be detected in various biofluids including carbon monoxide (CO) in breath, cotinine in serum, plasma, saliva, and urine, tobacco-specific nitrosamines in saliva (*N*′-nitrosonornicotine, NNN) and urine (NNN; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, NNAL), volatile organic compounds in urine [e.g. *N*-acetyl-*S*-(carbamoylethyl)-L-cysteine (AAMA), 2-amino-thiazoline-4-carboxylic acid (ATCA), *N*-acetyl-*S*-(2-carboxyethyl)-L-cysteine (CEMA), 2-hydroxypropylmercapturic acid (2-HPMA), etc.], polycyclic aromatic hydrocarbons [2-naphthol (2-NAP), 1-hydroxypyrene (1-PYR)], and heavy metals in blood [cadmium (Cd) and lead (Pb)] of ENDS/e-cig users. CNEMA, 2-cyanoethylmercapturic acid; CYMA, *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine; MA, mandelic acid; 3MHA + 4MHA, 3-methyl hippuric acid + 4-methyl hippuric acid; MU, *trans*,*trans*-muconic acid; PGA, phenylglyoxylic acid. [This schematic was prepared from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.]

Johnson et al. (16) analyzed urinary levels of NNN and NNAL in nonsmokers ($n$ = 28) who attended at least one e-cigarette convention where they were exposed to secondhand e-cig aerosol and found levels of both TSNAs to be so low as to preclude statistical analysis. These results show that although TSNAs may be detected in nonsmokers exposed to secondhand e-cig aerosol, the levels may not be statistically significant (Table 1 and Fig. 1). These studies are limited in their sample sizes and thus additional experiments should be performed with larger samples sizes to better understand the relationship between secondhand e-cig aerosol exposure and urinary TSNA concentrations.

## Carbon Monoxide

Breath or exhaled CO has been established as a reliable biomarker for exposure to tobacco and has a dose-dependent correlation with exposure to tobacco products (9, 10). This relationship is therefore of interest for the potential classification of e-cig users versus smokers, though there is limited CO data available in relation to ENDS use. A recent study by Carroll et al. (22) looking at breath CO in ENDS users ($n$ = 23) and smokers ($n$ = 27) of American Indian descent found levels in ENDS users to be significantly lower than those of smokers (GM = 2.21 ppm and GM = 16.58 ppm, respectively). Ikonomidis et al. (20) measured exhaled CO concentration in 70 smokers who were assigned to use e-cigs with 12 mg/dL nicotine as part of a smoking cessation program for 1 mo and compared the values to those of 20 smoking controls. Only 60% ($n$ = 42) of participants assigned to the vaping group reported compliance (use of e-cigs only). After 1 mo, there was a significant decrease in exhaled CO levels in smokers who switched to using only e-cigs relative to baseline (decreased from 13.9 ± 0.7 to 4.2 ± 0.6 ppm), with no significant change in smoking controls (20).

Another study by Caliri et al. (23) found exhaled breath CO to be significantly elevated in smokers ($n$ = 38, 15.16 ± 1.33 ng/mL) versus nonsmokers ($n$ = 44, 1.80 ± 0.11 ng/mL), with levels in e-cig users ($n$ = 39, 2.18 ± 0.19 ng/mL) similar to those of nonsmokers. The same trend was seen in a study by Tommasi et al. (27) measuring breath CO levels in smokers ($n$ = 25), e-cig users ($n$ = 18), and nonsmokers ($n$ = 30), who found significantly elevated levels in smokers versus nonsmokers, with e-cig user levels similar to those of nonsmokers. Numeric CO concentrations were not reported for this analysis as CO concentration in this study was used to confirm smoking or vaping status and thus was not being evaluated as a potential biomarker of exposure (27). Based on these findings, exhaled CO levels in e-cig users may not be significantly higher than those of nonsmokers, but they seem to be significantly lower than those of smokers and thus may be used in conjunction with other biomarkers, such as cotinine, to differentiate ENDS users from smokers. However, due to the limited data available, more studies are needed to truly uncover the relationship between exhaled breath CO and ENDS use, particularly in diverse populations with large subject numbers (Table 1 and Fig. 1).

## Polycyclic Aromatic Hydrocarbons

PAHs are compounds that result from the incomplete combustion of organic matter, including tobacco, but knowledge of their relationship with ENDS use is incomplete (9, 10, 28). PAHs can be carcinogenic or noncarcinogenic, and some have been linked to the development of bladder cancer, including pyrene, 6-naphthalene, fluorene, and phenanthrene (12, 29). Since ENDS are noncombustible tobacco products, levels of PAHs are expected to be lower in ENDS users than tobacco smokers. Therefore, the relationship between ENDS use and PAH levels warrants investigation as using PAHs as biomarkers may allow for differentiation between smokers and e-cig users. PAHs come from many sources such as environmental pollution and food, and thus are not specific for tobacco products (9, 10, 28). The

Goniewicz et al. (12) study analyzing spot urine found levels of PAH exposure biomarkers for naphthalene [2-naphthol (2-NAP)] and pyrene [1-hydroxypyrene (1-PYR)] to be significantly lower (62% and 47%, respectively) in e-cig users than smokers, with 1-PYR also being significantly lower in never-users than e-cig users (~20%). Levels of 2-NAP were not significantly different between e-cig users and never-users (12). This study shows the potential of using PAH biomarkers to show e-cig users from both never-users and smokers (Table 1 and Fig. 1).

### Volatile Organic Compounds

VOCs are products of incomplete tobacco combustion found in emissions of various tobacco products, especially cigarettes, though they also come from atmospheric and endogenous sources (i.e., inflammation), and many are considered damaging to health (9, 10, 12). Several carcinogenic VOC biomarkers linked to bladder cancer, including acrylamide and 1,3-butadiene, have been shown to be elevated in e-cig users (29). Goniewicz et al. (12) found levels of a urinary VOC biomarker for acrylonitrile [*N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine (CYMA)] to be significantly (~66%) higher in e-cig users than never-users. The same study found urinary levels of biomarkers for acrylonitrile (CYMA), acrolein [*N*-acetyl-*S*-(2-carboxyethyl)-L-cysteine (CEMA)], and acrylamide [*N*-acetyl-*S*-(carbamoylethyl)-L-cysteine (AAMA)] to be significantly lower (by 97%, 60%, and 59%, respectively) in e-cig users than smokers (12). Another recent study by Rubinstein et al. (30) measuring levels of VOC metabolites in the urine of adolescent e-cig users ($n$ = 67) and nonsmokers ($n$ = 20) found significantly higher excretion levels of metabolites of acrylonitrile [341%; 2-cyanoethylmercapturic acid (CNEMA)], acrolein [20%; 3-hydroxypropylmercapturic acid (3-HPMA)], propylene oxide [51%; 2- hydroxypropylmercapturic acid (2-HPMA)], acrylam-ide [30%; (AAMA)], and crotonaldehyde [20%; 3-hydroxy-1-methyl-propylmercapturic acid (HMPMA)] in e-cig users relative to nonsmokers. Lorkiewicz et al. (21) compared urinary levels of VOC metabolites in e-cig users ($n$ = 3) and non-tobacco product users ($n$ = 12), and found e-cig users to have significantly elevated urinary metabolites of benzene [*trans*,*trans*-muconic acid (MU); means ± SD = 317.5 ± 92.7 vs. 144.0 ± 80.4 ng/mg creatinine], cyanide [2-aminothiazoline-4-carboxylic acid (ATCA); 439.7 ± 257.8 vs. 115.5 ± 77.1], ethylbenzene [phenylglyoxylic acid (PGA); 324.5 ± 75.5 vs. 205.2 ± 75.4), styrene [mandelic acid (MA); 197.2 ± 35.9 vs. 132 ± 41], and xylene [3-methyl hippuric acid + 4-methyl hippuric acid (3MHA + 4MHA); 316.3 ± 349.1 vs. 71.9 ± 29.6) relative to non-tobacco product users.

A recent within-subjects crossover study by St. Helen et al. (31) found significant decreases in spot urine levels of 9 VOC biomarkers in 36 participants (all "dual users" of e-cigs and cigarettes), with fold-change values ranging from 1.31 to 7.09, when using e-cigs only versus cigarettes only. Interestingly, a study by Smith et al. (32) examining data from 211 flavored e-cig users found significantly higher urinary levels of a biomarker for acrylonitrile (CYMA) in users of fruit-only flavored e-cigs ($n$ = ~40, GM = 7.55 ng/mg creatinine) relative to users of other single non-tobacco flavors such as mint, clove, or chocolate ($n$ = ~65, GM = 2.79 ng/mg creatinine). Levels of benzene [phenylmercapturic acid (PMA)] and acrolein (CEMA) showed no difference between flavor groups (32). This study shows that the relationship between certain VOCs and e-cig flavoring chemicals may be of interest in regard to exposure and toxicity differences between flavors, though further study is needed. Johnson et al. (16) found that secondhand ENDS exposure by nonsmokers attending e-cigarette conventions increased urinary acrolein biomarkers CEMA and 3-HPMA (adjusted mean ratios ranged from 1.16–2.4 to 1.28–3.82, respectively) relative to baseline levels. Taken together, these studies show that VOC biomarkers may have the potential for differentiation between vapers/e-cig users, smokers, and nonsmokers, though additional studies should be conducted to further evaluate and validate various VOCs as biomarkers of ENDS exposure (Table 1 and Fig. 1). Secondhand exposure to ENDS also appears to increase VOC metabolites, though additional studies are needed to fully understand this relationship.

VOCs also include toxic aldehydes such as formaldehyde, acrolein, and acetaldehyde that are known to negatively impact cardiovascular and pulmonary health. These harmful compounds are present in cigarette smoke and e-cig aerosols as both aldehyde and hemiacetal forms. Ogunwale et al. (19) examined the levels of acetaldehyde, acrolein, and formaldehyde in e-cig aerosols produced using various devices and e-liquid flavors, and found that these three aldehydes were detectable in 10-puff aerosol samples of all ten e-cigs tested, as well as in cigarette smoke. Detected levels of acetaldehyde and formaldehyde were higher than those of acrolein in all e-cigs tested, and e-liquids used with a newer-generation tank-type e-cig ($n$ = 6) produced higher levels of all three aldehydes than blu e-cigs ($n$ = 4), potentially due to differences in battery voltage (19). Levels of formaldehyde, acetaldehyde, and acrolein in e-liquids used with a tank-type e-cig ranged from 8.2 to 40.4 μg, 13.3 to 63.1 μg, and 1.6 to 5.8 μg, respectively, per 10-puff aerosol volume. Levels of these three aldehydes in all blu e-cig 10-puff aerosols were below 0.65 μg (19). Measured levels of all e-cig aerosols tested are lower than the corresponding values for formaldehyde (74 μg), acetaldehyde (1,240.3 μg), and acrolein (120.4 μg) produced from one tobacco cigarette (19). Researchers additionally examined aldehyde-specific hemiacetals in these e-cigs and e-liquids, as these compounds may contribute to total aldehyde levels in e-cig aerosols. Investigators found 7.1 μg of formaldehyde hemiacetal in a 10-puff aerosol from one of the e-liquids used with a newer-generation tank-type e-cig, but otherwise found no detectable hemiacetals in tested samples (19). Very different results were seen in a study by Salamanca et al. (33) that reported levels of excess formaldehyde hemiacetals to be ~14 times higher than those of formaldehyde at both 10- and 15-watt power settings. There are several factors that may have contributed to the difference in measured outcomes between these studies (e.g., type of device and coil used, resistance, and power settings, etc.). These conflicting findings reflect the need for a more detailed investigation into this topic, taking into account differences between commonly used e-cig devices, type of coils used, power settings, and the exact methodology for experiments to establish standardization of protocols and

methods of evaluation and validation in this rapidly evolving field.

### Heavy Metals

Heavy metals come from various environmental sources, including the tobacco plant, making them nonspecific biomarkers of tobacco exposure. Levels of several metals are significantly higher in tobacco users than in nonsmokers, and some such heavy metals, including cadmium (Cd) and lead (Pb), are associated with negative health effects including cardiovascular and renal damage, cancer, and neurotoxicity (9, 10, 34, 35). Cd has a very long half-life (14–23 yr) making it both particularly hazardous to health and useful as a biomarker of long-term exposure to tobacco products (9). Heavy metal exposure from ENDS is thought to come in part from the heating coils themselves as well as from soldered joints and other metallic components of these devices. Another source of potential metal exposure is the e-liquids themselves. These sources are both thought to contribute to metal levels found in e-cig vapor and ultimately in ENDS users (10, 34, 35). Goneiwicz et al. (12) analyzed urinary levels of several metals and found significantly higher GM concentrations of Cd and Pb (23% and 19%, respectively) in e-cig users relative to never-users. In the same study, levels of Cd were significantly (30%) higher in smokers than e-cig users (12). A recent study by Prokopowicz et al. (36) examined blood Cd and Pb levels in nonsmokers ($n$ = 51), e-cig users, who had switched from cigarettes (using e-cigs alone for $\geq$6 mo and previously smoked cigarettes for $\geq$2 yr, $n$ = 48), and current smokers ($n$ = 28). Researchers found significantly higher blood Cd levels in e-cig users (GM = 0.44 μg/L) versus nonsmokers (GM = 0.31 μg/L), as well as in smokers (GM = 1.44 μg/L) versus e-cig users. In addition, they found blood Pb levels to be significantly higher in smokers (GM = 15.9 μg/L) versus nonsmokers (GM = 11.9 μg/L), but no significance was detected between the other groups (36).

There are many possible reasons for variation in metal levels among e-cig users, such as variation in device type (pod- vs. tank-based designs, cig-a-likes, etc.), e-liquid composition, and device power settings such as voltage, temperature, device care, and other user-controlled parameters. Additional factors that may impact the amount of metal exposure in e-cig users are exposed to include variation in puffing characteristics among study subjects as well as the methods used by investigators during collection, processing, storage, and analysis of samples [differences in time of collection, sample matrix, storage conditions of e-liquids (i.e., temperature, exposure to light), reagents, protocols, equipment, etc.] (35). These factors should be taken into consideration when designing future studies of metal exposure in relation to human e-cig use and highlight the need for increased standardization of methods and reporting of metal exposure in e-cig research (Table 1 and Fig. 1). Due to the serious health effects associated with metal exposure, and the limited data available, metals as biomarkers of ENDS use is an area of research warranting additional attention. Studies examining the levels of metals not mentioned here, as well as comparing metal exposure in short- versus long-term ENDS use, should be conducted to gain a better understanding of metal exposure in ENDS users.

## BIOMARKERS OF SYSTEMIC TOXICITY

### Inflammatory Biomarkers

There are several proinflammatory molecules that are established biomarkers of inflammation and are also associated with cigarette smoking. Studies have shown levels of some inflammatory cytokines and chemokines to also be elevated in ENDS users, making them useful indicators of ENDS usage and allowing for comparisons between the toxicity of e-cig vapor and cigarette smoke (5–7). These molecules include cytokines (i.e., interleukins; IL-6, IL-8 [CXCL8], IL-13, IL-1β), interferon (IFN)-γ, tumor necrosis factor (TNF)-α, chemokines [i.e., monocyte chemoattractant protein (MCP)-1], and proteases [i.e., matrix metalloproteinases (MMPs)], which are known biomarkers of inflammation and are thus associated with systemic toxicity and disease, especially chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD), and cancer (2, 5–8, 17). In addition, inflammasome components such as apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) can be used to measure inflammation. Inflammasomes are protein complexes that work to clear pathogens and usually activate caspase-1 resulting in maturation of IL-1β, thereby promoting pyroptosis, a form of inflammatory cell death (37). Singh et al. (6) found significantly higher levels of IL-6, IL-8, IL-13, and MMP9 in the plasma of e-cig users relative to nonsmokers. This study also found IFN-γ and IL-1β levels to be significantly higher in the urine and saliva, respectively, of e-cig users versus nonsmokers (6). A recent study by Song et al. (7) analyzing bronchoalveolar lavage fluid (BALF) samples from never-smokers ($n$ = 42), e-cig users ($n$ = 15), and smokers ($n$ = 16) found levels of IL-1β to be significantly higher in e-cig users (means ± SD = 1.51 ± 1.48 pg/mL) versus never-smokers (0.82 ± 0.39 pg/mL), as well as in smokers (6.08 ± 5.37 pg/mL) versus e-cig users. Levels of IL-6 were found to be significantly higher in e-cig users (means ± SD = 1.56 ± 1.02 pg/mL) than never-smokers (0.99 ± 0.56 pg/mL) (7). This study also found IFN-γ levels to be significantly higher in never-smokers (means ± SD = 0.91 ± 0.27 pg/mL) versus e-cig users (0.74 ± 0.31 pg/mL), as well as in e-cig users versus smokers (0.65 ± 0.33 pg/mL), differing from the results found by Singh et al. (6). It is important to keep in mind that there could be several physical and biological factors (e.g., e-cig usage pattern/behavior, device used, amount of exposure over a period of time, age, sex) that might contribute to the observed differences in inflammatory biomarkers observed in human studies. These conflicting reports demonstrate the pressing need for additional studies to further validate and identify novel, potentially stable inflammatory biomarkers in e-cig users.

Lee et al. (38) measured the levels of inflammatory cytokines in serum samples from five nonsmokers, five cigarette smokers, and two e-cig users, all of whom were instructed to abstain from cigarettes and e-cigs products for 12 h before collection. Serum samples were taken following the abstinence period as well as 0, 1, and 3 h after smokers smoked one Marlboro cigarette and e-cig users vaped e-liquid with 16 mg/mL nicotine, each at a rate of 2 puffs/min, for 10 min or until the cigarette went out. Levels of IL-6, MCP-1, intercellular adhesion molecule 1 (ICAM-1), and macrophage colony-stimulating factor

(MCSF) were found to be significantly elevated in e-cig users and smokers 3 h after vaping or smoking, respectively, relative to prior levels (38). Another recent study by Ghosh et al. (2) found significantly elevated BALF levels of MMP2, MMP9, and neutrophil elastase in both e-cig users and smokers relative to nonsmokers ($n$ = 14/group), with no significant difference between e-cig users and smokers. MMP2 is found to be upregulated in asthmatics, and increased MMP9 is correlated with several chronic inflammatory lung diseases including asthma, COPD, and cystic fibrosis.

Prior study by Scott et al. (39) examined proinflammatory effects of 24-h exposure to nicotine-containing 0.5% e-cig vapor condensate (ECVC) on alveolar macrophages (AM) harvested from eight normal human never-smoking donors, and found significantly increased production of IL-6, CXCL8, MMP9, MCP-1, and TNFα relative to controls (untreated). Researchers also found significant increases in the production of IL-6, CXCL8, and MMP9 in AM treated with 0.5% nicotine-free ECVC (39). Higham et al. (40) analyzed inflammatory effects of e-cig vapor extract (ECVE) on neutrophils isolated from peripheral blood samples from 10 healthy never-smokers. Their findings included significantly increased MMP9, CXCL8, and NE production following a 6-h exposure to 0.003% ECVE, relative to controls. Tsai et al. (37) found significantly higher median levels of ASC in BALF of both smokers ($n$ = 16; med = 37, interquartile range (IQR) = 21–64 ng/mL) and e-cig users ($n$ = 15; med = 22, IQR = 14–35 ng/mL) versus never-smokers ($n$ = 12; med = 11, IQR = 9–15 ng/mL). Capsase-1 concentrations were significantly higher in smokers (med = 42, IQR = 27–68 pg/mL) relative to both e-cig users (med = 16, IQR = 9–35 pg/mL) and never-smokers (med = 12, IQR = 6–14 pg/mL), whereas levels of e-cig users and never-smokers were not significantly different from each other. The same trend was seen for IL-1β, with levels in smokers found to be significantly higher than those of both e-cig users and never-smokers (37). Taken together, these findings show that inflammation is associated with e-cig use, and indicate that inflammatory biomarkers including certain cytokines, chemokines, proteases, and inflammasome components can be used to identify and quantify e-cig-associated systemic toxicity (Table 2 and Fig. 2).

## Aldehydes

Potentially toxic aldehydes, most notably benzaldehyde, are commonly used to flavor certain e-cigs and e-liquids despite the potential for such compounds to cause respiratory irritation when inhaled. A recent study by Jabba et al. (41) showed that not only do flavoring compounds including benzaldehyde have harmful effects on human airway epithelial cells, but PG acetal forms of these chemicals present in e-cig liquids and aerosols may be even more harmful than their parent aldehydes. Investigators showed that for benzaldehyde and all other tested aldehydes, PG acetals reduced mitochondrial function, ATP synthesis, and maximal and spare respiratory capacities to a greater degree than the corresponding aldehyde alone in human lung epithelial cells (BEAS-2B). In addition, the benzaldehyde PG acetal was found to be more cytotoxic, in terms of both cell viability and cell growth, than benzaldehyde in BEAS-2B cells (41). Hickman and coworkers (42) showed that both benzaldehyde and the benzaldehyde PG acetal have detrimental effects on phagocytosis in human neutrophils, with the acetal resulting in more significant inhibition of neutrophil phagocytosis than the parent aldehyde ($IC_{50}$ = 4.72 ± 2.05 and 1.89 ± 0.66 mM, respectively).

Demonstrating the high prevalence of benzaldehyde among ENDS products, Omaiye et al. (49) reported that the benzaldehyde PG acetal was present in 118 out of 277 different flavored e-liquids, making it more common than benzaldehyde among the products analyzed. Another study by Kosmider et al. (50) found detectable levels of benzaldehyde in 108 out of 145 flavored e-cigs tested, with aerosols of cherry-flavored products (5.129–141.2 μg/30 puffs) containing more benzaldehyde than those of other flavors (0.025–10.27 μg/30 puffs). Interestingly, researchers found that the levels of benzaldehyde exposure following 30 puffs of many of these flavored e-cigs were higher than the amount that would be inhaled after smoking one traditional cigarette (50). Together these studies highlight the potential negative health effects of toxic aldehydes present in e-liquids and demonstrate the prevalence of these compounds in products available in the market. However, the relative lack of research in this area and the potential for significant toxicity warrants additional investigation into the safety of these common e-liquid components, especially in realistic use scenarios with human subjects. In addition, there is a need for standardized analytical protocols for the detection of aldehydes in e-cig aerosols and e-liquids so that results from different studies can be reliably compared.

## Oxidative Stress, DNA Damage, and Lipid Peroxidation Biomarkers

E-cig aerosols contain reactive oxygen species (ROS) that cause oxidative stress and can damage cells and organs leading to CVD, COPD, and cancer, making them promising biomarkers of e-cig-induced toxicity (4, 8). The prostaglandin 8-isoprostane (8-iso), a byproduct of lipid peroxidation, is one commonly measured biomarker of oxidative stress (4). The noninvasive biomarker 8-hydroxy-2′-deoxyguanosine (8-OHdG) [a.k.a. 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG)] is a well-established biomarker of oxidative DNA damage (51, 52). Club cell protein 16 (CC16) is a protein that is thought to reduce both inflammation and oxidative stress in the airways, and existing studies have found increased serum CC16 to be a result of acute lung injury (44, 53). Singh et al. (6) found significantly elevated levels of urinary 8-iso and 8-oxo-dG in e-cig users versus nonsmokers. Similarly, Sakamaki-Ching et al. (4) reported significantly higher 8-OHdG in spot urine samples of e-cig users (means ± SD: 442.8 ± 300.7 ng/mg creatinine) versus nonsmokers (221.6 ± 157.8 ng/mg creatinine). No significant difference was found between e-cig users and smokers (388 ± 235 ng/mg creatinine). This study also found higher levels of 8-iso in spot urine samples of e-cig users (750.8 ± 433 pg/mg creatinine) versus nonsmokers (411.2 ± 287.4 pg/mg creatinine), with no significant difference between e-cig users and smokers (784.2 ± 546.1 pg/mg creatinine) (4). Scott et al. (39) found that treating AM with 0.5% ECVC (with or without nicotine) significantly increased ROS production 50-fold compared with controls. Ikonomidis et al. (20) found a significant decrease in the

**Table 2.** *Biomarkers of systemic toxicity in ENDS/e-cig exposure*

| Sample Matrix (Metabolite/ Biomarker) | Methods Used for Analysis of Biomarkers | Groups (*n*/Group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| Inflammatory biomarkers: cytokines and chemokines | | | | | |
| IL-1β | | | | | |
| Saliva | ELISA | Nonsmokers ($n = 26$); E-cig users ($n = 22$). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| BALF | V-PLEX Plus Proinflammatory Combo 10 Panel (Meso Scale Discovery) | Never-smokers ($n = 42$); E-cig users ($n = 15$); Smokers ($n = 16$). | Means ± SD = 0.82 ± 0.39 pg/mL<br>Means ± SD = 1.51 ± 1.48 pg/mL<br>Means ± SD = 6.08 ± 5.37 pg/mL | Significantly higher levels in e-cig users vs. never-smokers. Significantly higher levels in smokers vs. e-cig users. | (7) |
| BALF | Bead-based ELISA (Meso Scale Discovery) | Never-smokers ($n = 12$); E-cig users ($n = 15$); Smokers ($n = 16$). | N/A | Significantly higher levels in smokers vs. both e-cig users and nonsmokers. | (7) |
| IL-6 | | | | | |
| Plasma | XL Cytokine Discovery Magnetic Luminex Panel (R&D Systems) | Nonsmokers ($n = 26$); E-cig users ($n = 22$). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| BALF | V-PLEX Plus Proinflammatory Combo 10 Panel (Meso Scale Discovery) | Never-smokers ($n = 42$); E-cig users ($n = 15$); Smokers ($n = 16$). | Means ± SD = 0.99 ± 0.56 pg/mL<br>Means ± SD = 1.56 ± 1.02 pg/mL<br>Means ± SD = 4.21 ± 4.93 pg/mL | Significantly higher levels in e-cig users vs. never-smokers. | (7) |
| Serum | Luminex (magnetic bead kit) | Nonsmokers ($n = 5$); E-cig users ($n = 2$); Smokers (n = 5). | N/A | Significantly higher levels in e-cig users and smokers 3 h after vaping or smoking, respectively, relative to prior levels. | (38) |
| Alveolar macrophages | ELISA (Biotechne) | Never-smokers ($n = 8$) | N/A | Significantly higher levels after treatment with 0.5% ECVC (with or without nicotine) vs. controls. | (39) |
| IL-8 (CXCL8) | | | | | |
| Plasma | XL Cytokine Discovery Magnetic Luminex Panel (R&D Systems) | Nonsmokers ($n = 26$); E-cig users ($n = 22$). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| Alveolar macrophages | ELISA (Biotechne) | Never-smokers ($n = 8$) | N/A | Significantly higher levels after treatment with 0.5% ECVC (with or without nicotine) vs. controls. | (39) |
| Neutrophils | ELISA (R&D Systems) | Never-smokers ($n = 10$) | N/A | Significantly higher levels after 6-h exposure to 0.003% ECVE vs. controls. | (40) |
| IL-13 | | | | | |
| Plasma | XL Cytokine Discovery Magnetic Luminex Panel (R&D Systems) | Nonsmokers ($n = 26$); E-cig users ($n = 22$). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| ICAM-1 | | | | | |
| Serum | Luminex (magnetic bead kit) | Nonsmokers ($n = 5$); E-cig users ($n = 2$); Smokers ($n = 5$). | N/A | Significantly higher levels in e-cig users and smokers 3 h after vaping or smoking relative to prior levels. | (38) |
| MCP-1 | | | | | |
| Serum | Luminex (magnetic bead kit) | Nonsmokers ($n = 5$); E-cig users ($n = 2$); Smokers ($n = 5$). | N/A | Significantly higher levels after treatment with 0.05% ECVC (with nicotine) vs. controls. | (38) |
| Alveolar macrophages | ELISA (Biotechne) | Never-smokers ($n = 8$) | N/A | Significantly higher after treatment with 0.5% ECVC vs. control. | (39) |
| MCSF | | | | | |
| Serum | Luminex (magnetic bead kit) | Nonsmokers ($n = 5$); E-cig users ($n = 2$); Smokers ($n = 5$). | N/A | Significantly higher levels in e-cig users and smokers 3 h after vaping or smoking relative to prior levels. | (38) |
| Inflammatory proteases | | | | | |
| MMP-2 | | | | | |
| BALF | Western blot normalized to albumin | Nonsmokers ($n = 14$); E-cig users ($n = 14$); Smokers ($n = 14$). | N/A | Significantly higher levels in both e-cig users and smokers vs. nonsmokers. | (2) |
| MMP-9 | | | | | |

*Continued*

**Table 2.—** *Continued*

| Sample Matrix (Metabolite/ Biomarker) | Methods Used for Analysis of Biomarkers | Groups (*n*/Group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| Plasma | Custom 9-plex Magnetic Luminex Assay (R&D Systems) | Nonsmokers (*n* = 26); E-cig users (*n* = 22). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| BALF | Western blot normalized to albumin | Nonsmokers (*n* = 14); E-cig users (*n* = 14); Smokers (*n* = 14). | N/A | Significantly higher levels in both e-cig users and smokers vs. nonsmokers. No difference between e-cig users and smokers. | (2) |
| Alveolar macrophages | ELISA (Biotechne) | Never-smokers (*n* = 8) | N/A | Significantly higher levels after treatment with 0.5% ECVC (with or without nicotine) vs. controls. | (39) |
| Neutrophils | ELISA (R&D Systems) and western blot | Never-smokers (*n* = 10) | N/A | Significantly higher levels after 6-h exposure to 0.003% ECVE vs. controls. | (40) |
| IFNγ | | | | | |
| Urine | XL Cytokine Discovery Magnetic Luminex Panel (R&D Systems) | Nonsmokers (*n* = 26); E-cig users (*n* = 22). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| BALF | V-PLEX Plus Proinflammatory Combo 10 Panel (Meso Scale Discovery) | Never-smokers (*n* = 42); E-cig users (*n* = 15); Smokers (*n* = 16). | Means ± SD = 0.91 ± 0.27 pg/mL<br>Means ± SD = 0.74 ± 0.31 pg/mL<br>Means ± SD = 0.65 ± 0.33 pg/mL | Significantly higher levels in never-smokers vs. e-cig users.<br>Significantly higher levels in e-cig users vs. smokers. | (7) |
| TNFα | | | | | |
| Alveolar macrophages | ELISA (Biotechne) | Never-smokers (*n* = 8) | N/A | Significantly higher levels after treatment with 0.5% ECVC (with nicotine) vs. controls. | (39) |
| Neutrophil elastase (NE) | | | | | |
| BALF | Western blot normalized to albumin | Nonsmokers (*n* = 14); E-cig users (*n* = 14); Smokers (n = 14). | N/A | Significantly higher levels in both e-cig users and smokers vs. nonsmokers. | (2) |
| Neutrophils | Florescence (FLUOstar omega plate reader (BMG Labtech) | Never-smokers (*n* = 10) | N/A | Significantly higher levels after 6-h exposure to 0.003% ECVE vs. controls. | (40) |
| Apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC) | | | | | |
| BALF | ELISA | Never-smokers (*n* = 12); E-cig users (*n* = 15); Smokers (*n* = 16). | Med = 11, IQR = 9–15 ng/mL<br>Med = 22, IQR = 14–35 ng/mL<br>Med = 37, IQR = 21–64 ng/mL | Significantly higher levels in both e-cig users and smokers vs. nonsmokers. | (37) |
| Caspase-1 | | | | | |
| BALF | ELISA | Never-smokers (*n* = 12); E-cig users (*n* = 15); Smokers (*n* = 16). | Med = 12, IQR = 6-14 pg/mL<br>Med = 16, IQR = 9-35 pg/mL<br>Med = 42, IQR = 27-68 pg/mL | Significantly higher levels in smokers vs. both e-cig users and nonsmokers. | (37) |
| Oxidative stress, DNA damage, and lipid peroxidation biomarkers | | | | | |
| 8-Isoprostane | | | | | |
| Urine | ELISA | Nonsmokers (*n* = 26); E-cig users (*n* = 22). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| Spot urine | ELISA (R&D) | Nonsmokers (*n* = 20); E-cig users (*n* = 20); Smokers (*n* = 13). | Means ± SD = 411.2 ± 287.4 pg/mg creatinine<br>Means ± SD = 750.8 ± 433 pg/mg creatinine<br>Means ± SD = 784.2 ± 546.1 pg/mg creatinine | Significantly higher levels in e-cig users vs. nonsmokers.No significant difference between e-cig users and smokers. | (4) |
| 8-OHdG | | | | | |
| Urine | HT 8-Oxo-dG Human ELISA (Trevigen) | Nonsmokers (*n* = 26); E-cig users (*n* = 22). | N/A | Significantly higher levels in e-cig users vs. nonsmokers. | (6) |
| Spot urine | DNA Damage (8-OHdG) ELISA Kit (Stress Marq Biosciences) | Nonsmokers (*n* = 20); E-cig users (*n* = 20); Smokers (*n* = 13). | Means ± SD = 221.6 ± 157.8 ng/mg creatinine<br>Means ± SD = 442.8 ± 300.7 ng/mg creatinine<br>Means ± SD = 388 ± 235 ng/mg creatinine | Significantly higher levels in e-cig users vs. nonsmokers.No significant difference between e-cig users and smokers. | (4) |
| ROS | | | | | |
| Alveolar macrophages | DCFDA assay (Abcam) | Never-smokers (*n* = 8) | 50-fold increase in ECVC treated AM (with or without nicotine) vs. untreated controls | Significantly higher levels after treatment with 0.5% ECVC (with or without nicotine) vs. controls. | (39) |

*Continued*

**Table 2.—** *Continued*

| Sample Matrix (Metabolite/ Biomarker) | Methods Used for Analysis of Biomarkers | Groups (*n*/Group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| MDA | | | | | |
| Plasma | Colorimetric lipid peroxidation assay (Oxford Biomedical Research) | Smokers switch to e-cigs ($n = 42$); Smokers switch to dual use ($n = 24$); Smoker controls (no switch; $n = 20$). | 1.12 ± 0.1 (prior); 1.01 ± 0.1 (1 mo) nmol/L<br>1.28 ± 0.1 (prior); 1.09 ± 0.1 (1 mo) nmol/L<br>1.12 ± 0.3 (prior); 1.15 ± 0.2 (1 mo) nmol/L | Significant decrease in smokers who switched to e-cigs or dual use (cigarettes and e-cigs) for 1 mo relative to BSL. No change in smoker controls. | (20) |
| CC16 | | | | | |
| Serum | N/A | Occasional smokers ($n = 23$); Vape session: Sham vape session: | 0.51 (BSL)–0.64 (post) μg/mg creatinine<br>0.63 (BSL)–0.49 (post) μg/mg creatinine | E-cig use significantly increased median levels relative to BSL after 30 min e-cig use vs. sham vaping. | (43) |
| Serum | Latex immunoassay using rabbit anti-CC16 antibody | Smokers ($n = 25$) Vape session (nicotine): Vape session (no nicotine): Sham vape session: | Δ: +1.2 ± 0.3 μg/L (after vaping-BSL)<br>Δ: +1.1 ± 0.3 μg/L (after vaping-BSL)<br>Δ: −0.5 ± 0.2 μg/L (after vaping-BSL) | E-cig use significantly increased means ± SE levels relative to BSL after 30 min e-cig use (with or without nicotine) vs. sham vaping. | (44) |
| EVALI-specific biomarkers: vitamin E acetate (VEA) | | | | | |
| BALF | Isotope dilution mass spectrometry | EVALI patients ($n = 51$); Nonusers ($n = 52$); E-cig users ($n = 18$); Smokers ($n = 29$) | present in 48/51; present in 0/52; present in 0/18; present in 0/29 | VEA present in most EVALI samples but not in any e-cig user, smoker or nonuser samples. | (45) |
| Transcriptomic/gene expression biomarkers | | | | | |
| Bronchial brushings | GeneChip Human Transcriptome Array 2.0 (Affymetrix) | Never-smokers ($n = 42$); E-cig users ($n = 15$); Smokers ($n = 16$). | 181 DETs unique to e-cig users | 2,452 DETs among groups (for 2,093 genes) | (7) |
| Oral cells | RNA-seq | Nonsmokers ($n = 27$); E-cig users ($n = 42$); Smokers ($n = 24$). | ~50% more DETs than e-cig users | Significant numbers of DETs in both e-cig users and smokers vs. nonsmokers. | (27) |
| SAE Alveolar Macrophages | TruSeq v2 mRNA library prep RNA-sequencing (2 × 125 bp) (Illumina HiSeq. 2500) | Never-smokers ($n = 10$) given e-cig with nicotine ($n = 7$) or without nicotine ($n = 3$). | 71 significantly altered genes vs. BSL; 65 significantly altered genes vs. BSL;<br>27 significantly altered genes vs. BSL; 61 significantly altered genes vs. BSL | For both groups, significantly altered pathways were associated with nicotine receptors and downstream p53 targets.<br>No standard pathways identified in alveolar macrophages. | (59) |
| Primary HBECs | GeneChip Human Gene 1.0 ST Array (Affymetrix) | Nonsmoker (donor; *n*); E-cig aerosol exposure; Cig smoke exposure; Air (control). | 546 genes significantly differentially expressed among the three groups;<br>493 genes differentially expressed between tobacco and menthol e-cig vapor. | Genes altered in both e-cig and smoke groups associated with cilium assembly/movement, apoptosis, xenobiotic and oxidative stress, and DNA damage pathways.Genes altered uniquely in e-cig group associated with cell division and cell cycle regulation pathways. | (60) |
| Primary NHBEs | RNA-seq<br>Results relative to $H_2O$ controls | Diacetyl (2,3-butanedione), 2,3-pentanedione. | 163 differentially expressed genes;<br>568 differentially expressed genes | Differentially expressed genes associated with cytoskeletal- and cilia-related pathways. | (24) |
| Differentiated HBECs | RNA-seq (Illumina)<br>Results relative to air controls | Nonsmokers (donors; $n = 2$);<br>E-cig vapor exposure; Cig smoke exposure; Air (control). | 57 differentially expressed genes (1 h);<br>49 differentially expressed genes (0 h) | Genes altered uniquely in e-cig group associated with the cell cycle, response to hypoxia, response to organic substances, apoptosis and acute inflammatory response. | (61) |
| Nasal epithelium biopsies | RNA-seq<br>Results relative to nonsmokers | Nonsmokers ($n = 13$); E-cig users ($n = 12$); Smokers ($n = 14$). | 358 genes significantly downregulated;<br>53 genes significantly downregulated | All 53 genes downregulated in smokers were also downregulated in e-cig users. | (62) |
| Exosomal small RNAs as circulating biomarkers | | | | | |
| Plasma-derived exosomes | RNA-seq (Illumina NextSeq. 500)<br>Results relative to nonsmokers | Non-smokers ($n = 8$); E-cig users ($n = 7$); Cigarette smokers ($n = 7$); Waterpipe smokers ($n = 7$); Dual smokers ($n = 7$). | 17 miRNAs, 7 tRNAs and 5 piRNAs differentially expressed in e-cig users | 13 miRNAs were significantly upregulated, and 4 miRNAs were significantly downregulated in e-cig users compared to nonsmokers. | (64) |

*Continued*

**Table 2.—** *Continued*

| Sample Matrix (Metabolite/ Biomarker) | Methods Used for Analysis of Biomarkers | Groups (*n*/Group) | Results | Summary of Key Findings | Citation |
|---|---|---|---|---|---|
| DNA methylation biomarkers | | | | | |
| Bronchial brushings | Infinium Methylation EPIC BeadChip (Illumina) | Never-smokers ($n = 10$); E-cig users ($n = 12$); Smokers ($n = 10$). | 14 unique CpGs differentially expressed | 451 differentially methylated CpGs (273 genes) among the three groups. | (7) |
| Peripheral blood leukocytes | DNA methylation LINE-1 kit (Active motif)<br>RNA-seq Results relative to nonsmokers | Nonsmokers ($n = 15$); E-cig users ($n = 15$); Smokers ($n = 15$). | 18% hypomethylation in LINE1 elements;<br>13% hypomethylation in LINE1 elements | No significant difference in levels of LINE-1 hypomethylation between smokers and e-cig users. | (23) |
| Proteomic biomarkers | | | | | |
| Bronchial brush biopsies | LC-MS/MS<br>Results relative to nonsmokers | Nonsmokers ($n = 8$); E-cig users ($n = 9$); Smokers ($n = 9$). | 191 differentially expressed proteins;<br>292 differentially expressed proteins | 78 proteins similarly altered in vapers and smokers.<br>131 proteins exclusively altered in vapers.<br>14 uniquely altered pathways in vapers. | (48) |
| Induced sputum | Q-Exactive mass spectrometer coupled to UltiMate 3000 nano HPLC system (Thermo Scientific). | Never-smokers ($n = 15$); E-cig users ($n = 15$); Smokers ($n = 14$). | 5 significantly upregulated proteins;<br>66 significantly upregulated proteins;<br>23 significantly upregulated proteins | 15 proteins commonly upregulated in smokers and vapers.<br>~81 proteins significantly altered in e-cig users relative to never-smokers, 44 in smokers relative to never-smokers. | (17) |
| MUC5AC | | | | | |
| Induced sputum | Stable-isotope-labeled mass spectrometry with parallel reaction monitoring analysis | Never-smokers ($n = 15$); E-cig users ($n = 15$); Smokers ($n = 14$). | Means ± SD = 15 ± 6 pmol/mL;<br>Means ± SD = 58 ± 21 pmol/mL;<br>Means ± SD = 132 ± 58 pmol/mL | Significantly higher levels in both e-cig users and smokers vs. nonsmokers. | (17) |

BALF, bronchoalveolar lavage fluid; BSL, baseline; CpGs, CpG sites; DETs, differentially expressed transcripts; ECVC, e-cigarette vapor condensate; ECVE, e-cig vapor extract; ELISA, enzyme-linked immunosorbent assay; EVALI, e-cigarette or vaping-associated lung disease; HBECs, human bronchial epithelial cells; IQR, interquartile range; LC-NSI-HRMS/MS, liquid chromatography-nanoelectrospray ionization-high resolution tandem mass spectrometry; med, median; N/A, details not available; NHBEs, normal human bronchial epithelial cells; SAE, small airway epithelium; SD, standard deviation.

levels of lipid peroxidation and oxidative stress biomarker malondialdehyde (MDA) in smokers who switched to vaping e-cigs only (1.12 ± 0.1 to 1.01 ± 0.1 nmol/L) or dual use of e-cigs and cigarettes (1.28 ± 0.1 to 1.09 ± 0.1 nmol/L) for 1 mo relative to baseline levels, whereas no significant change was seen in smoking controls.

A study by Chaumont et al. (43) exposed 23 healthy occasional smokers to 25 puffs of an e-cig and 25 puffs of sham vaping, in random order, and found a significant increase in median serum CC16/creatinine compared with baseline after vaping (0.51–0.64 μg/mg creatinine) relative to sham vaping (0.63–0.49 μg/mg creatinine). In addition, a recent within-subjects study by Chaumont et al. (44) examined serum samples from 25 young tobacco smokers who underwent one session of sham-vaping as well as one session each of vaping with and without nicotine, in a randomized order, and found significantly elevated (means ± SE compared with baseline) serum CC16 levels in subjects 30 min after sessions of vaping both with (+1.2 ± 0.3 μg/L) and without (+1.1 ± 0.3 μg/L) nicotine, relative to sham-vaping (−0.5 ± 0.2 μg/L). Starting in 2019, vaping has been implicated in potentially deadly cases of acute lung injury [e-cigarette- or vaping-associated lung injury (EVALI)], and research looking at CC16 in relation to vaping may help in illuminating mechanisms underlying recent EVALI cases (53). Together, these results clearly show increased oxidative stress in e-cig users versus nonsmokers illustrate the usefulness of these commonly employed oxidative stress biomarkers in evaluating e-cig-induced systemic toxicity (Table 2 and Fig. 2). In addition, it is important to note that in some cases vaping has been shown to cause lower levels of certain oxidative stress biomarkers than cigarette smoking.

## EVALI-Specific Biomarkers

It is worth mentioning potential toxicity biomarkers of some less prevalent vaping product additives such as vitamin E acetate (VEA), which has recently been implicated as one potential causal factor in the EVALI outbreak (18, 25, 54–56). One study by Blount et al. (45) found VEA to be present in the vast majority (48 out of 51) of EVALI patient BALF samples, almost all of which (47 out of 50) came from patients who had vaped tetrahydrocannabinol (THC) products up to 90 days before becoming ill. In addition, VEA was not found in a comparison group of BALF samples from 99 healthy nonsmokers, exclusive e-cig users and exclusive cigarette smokers, pointing to the potential of VEA as an EVALI-specific biomarker (45). High levels of VEA have also been found in nonmedical grade (counterfeit) THC-containing vaping products (25, 54–57), which many patients with EVALI have admitted to using before becoming ill (25, 47). One possible mechanism for VEA-mediated lung toxicity is that, since vitamin E is a natural component of lung surfactant, increasing levels of VEA in the lungs may alter the properties of a person's lung surfactant leading to pulmonary injury and toxicity (54, 57, 58). Toxic ketene formation upon heating of VEA is another potential mechanism for the lung injury seen in patients with EVALI warranting additional investigation (18, 54–56). Although the link between VEA and EVALI is strong, future research is necessary to fully understand this relationship and uncover the true mechanism(s) responsible for the devastating injuries seen in EVALI sufferers.

Although VEA shows potential as a causative agent in the EVALI outbreak, there are many other possible chemical

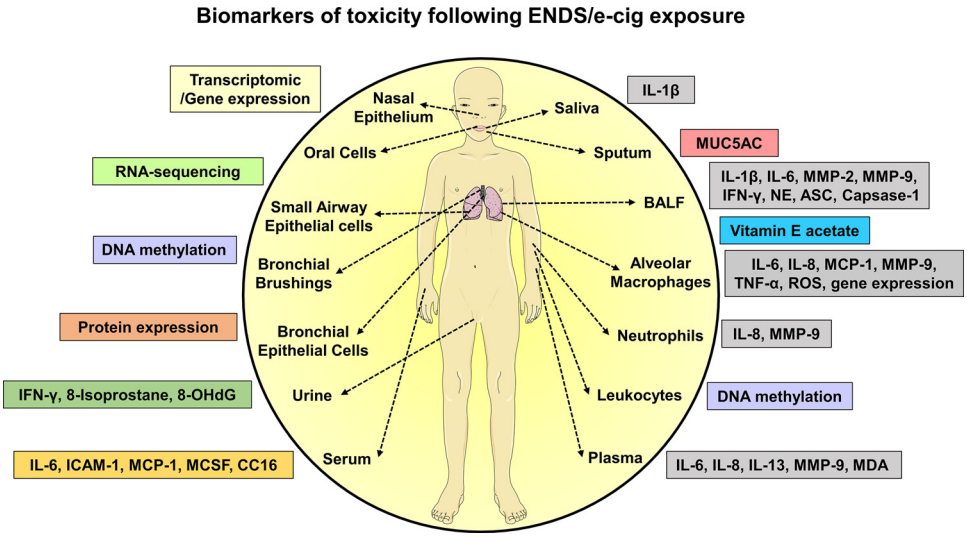


**Figure 2.** Biomarkers of toxicity following electronic nicotine delivery systems (ENDS)/e-cig exposure. Biomarkers of toxicity that can be identified in various biofluids/specimens include plasma/serum, urine, sputum, and immune inflammatory cells (e.g., alveolar macrophages, neutrophils, leukocytes, etc.). Various inflammatory markers in saliva [interleukins (IL)-1β], bronchoalveolar lavage (BAL) fluid [IL-1β, IL-6, matrix metalloprotease (MMP)-2, MMP-9, interferon (IFN)-γ, neutrophil elastase (NE), apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), Capsase-1], plasma [IL-6, IL-8, IL-13, MMP-9, malondialdehyde (MDA)], serum [IL-6, intercellular adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein (MCP)-1, macrophage colony-stimulating factor (MCSF), Club cell protein 16 (CC16)], alveolar macrophages [IL-6, IL-8, MCP-1, MMP-9, tumor necrosis factor (TNF)-α, reactive oxygen species (ROS), and altered gene expression], neutrophils (IL-8, MMP-9), leukocytes, and bronchial brushings (DNA methylation) were altered following e-cig exposure. In addition, oxidative stress, lipid peroxidation markers in urine [e.g., 8-isoprostane, 8-hydroxy-2′-deoxyguanosine (8-OHdG)], altered gene expression signatures in oral cells, nasal epithelial cells (by transcriptomics), small airway epithelial cells (SAECs), plasma-derived exosomes (by RNA-sequencing), protein expression and proteomic changes in sputum (MUC5AC) and human bronchial epithelial cells (HBECs) were reported following exposure to ENDS/e-cigs. This schematic was prepared from SMART (Servier Medical Art), licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

culprits. Muthumalage et al. (25) identified over 500 chemicals present in counterfeit THC vape cartridges ($n$ = 38) recovered from patients with EVALI. The chemicals discovered include several that, when compared to cannabidiol (CBD)-containing and medical-grade vape cartridges, were found to be unique to counterfeit THC cartridges, including decane, 2,2-dimethoxybutane, tetramethyl silicate, siloxanes, methyl and ethyl esters, as well as VEA and other acetates. This study highlights the complicated nature of EVALI by identifying the numerous potential toxicity biomarkers present in these problematic products, the interactions of which should be thoroughly studied for potential involvement in EVALI-associated lung toxicity. In addition to VEA, Blount et al. (45) measured levels of other suspected toxicants in the BALF samples of 51 patients with EVALI. Except for one sample showing the presence of coconut oil, and another sample showing detectable levels of diluent terpene limonene, the rest of the BALF samples were negative for the presence of plant oils (i.e., coconut oil), medium-chain triglyceride (MCT) oil, petroleum distillates, and diluent terpenes, serving as evidence against these compounds being causal factors in EVALI. Despite progress being made in understanding the causes and mechanisms behind EVALI, there is a need for additional in vitro and in vivo research into the roles of VEA and other chemicals identified in vaping products used by EVALI patients in the onset and progression of EVALI.

## Transcriptomic and Gene Expression Biomarkers

Examining changes in gene expression through methods including analysis of differentially expressed genes, differentially expressed transcripts (DETs) and RNA sequencing (RNA-seq) can give insight into the transcriptomic changes associated with e-cig use. Song et al. (7) analyzed bronchial brushings from never-smokers, e-cig users, and smokers, and found 2,452 DETs, corresponding to 2,093 unique genes, across the groups. Gene expression profiles of never-smokers were tightly clustered and were clearly differentiated from those of smokers. In addition, the profiles of e-cig users and smokers were relatively similar, showing that e-cig usage alters gene expression in a pattern similar to smoking (7). For 93% of these DETs, gene expression levels in e-cig users were in between those of never-smokers and smokers, showing potential for DETs as biomarkers for the differentiation of nonsmokers, e-cig users, and smokers. Interestingly, 181 of those 2,452 DETs were specific to e-cig use (i.e., significantly different from both never-smokers and smokers), with the most notable ones being MUC5B (4 transcripts), *MUC5AC*, *ZNF445*, *REEP1*, *ABHK4*, *LINC00589*, and *TMPRSS3* (7). Using ingenuity pathway analysis (IPA), the top canonical pathways for the DETs identified in this study were related to smoking and lung cancer, such as xenobiotic metabolism signaling, NRF2-mediated oxidative stress response, aryl hydrocarbon receptor (AHR) signaling, pregnane X receptor (PXR)/retinoid X receptor (RXR) activation, and LPS/IL1-mediated inhibition of RXR function (7). A recent study by Tommasi et al. (27) examined differential gene expression between e-cig users ($n$ = 42), smokers ($n$ = 24), and nonsmokers ($n$ = 27) in oral cells via RNA-sequencing. Researchers found significant numbers of DETs in both e-cig users and smokers relative to nonsmokers, with smokers showing ~50% more DETs than e-cig users (27). In e-cig users, the most affected canonical pathway was the "Wnt/$Ca^+$" pathway, whereas in smokers the "integrin signaling

pathway" was most affected. The most deregulated pathway found in common between smokers and e-cig users was the "Rho family GTPases" (27). In both e-cig users and smokers, cancer was the disease most associated with the genes identified as deregulated, indicating that potential serious health risks are associated with e-cig use.

Staudt et al. (59) analyzed gene expression via RNA-seq in small airway epithelium (SAE) and alveolar macrophages (AM) harvested from 10 healthy never-smokers before and after first-time e-cig use (10 puffs, 30 min wait, 10 more puffs) with ($n$ = 7) or without ($n$ = 3) nicotine. Following e-cig use, 71 genes were significantly altered in the SAE exposed to e-cig with nicotine group, whereas 65 genes were altered in the SAE exposed to e-cig without nicotine group, relative to baseline (59). Across both groups, significantly altered pathways were associated with nicotine receptors (genes: *KCNK15*, *PPP1R16B*, *GNB1L*) and downstream targets of p53 (upregulated genes: *EDN1, AMOTL2, LATS2, RND3*; downregulated genes: *ATAD2, GDA, MKI67, NDC80, RRM2*). In addition, e-cig use with nicotine was found to significantly alter the expression of 27 genes in AM, whereas e-cig use without nicotine significantly altered the expression of 61 genes in AM, relative to baseline. No standard pathways associated with these differentially expressed AM genes were identified (59). Moses et al. (60) examined differential gene expression in primary human bronchial epithelial cells (HBECs) isolated from a healthy 23-yr-old male donor that was exposed to either cigarette smoke, e-cig aerosol, or air controls. Researchers identified 546 genes significantly differentially expressed between the three groups. Of these 546 genes, those significantly altered in both e-cig users and smokers were linked to pathways related to cilium assembly and movement, apoptosis, xenobiotic and oxidative stress, and DNA damage (60). Genes found to be expressed more highly in response to e-cig exposure specifically were related to pathways involved in cell division and cell cycle regulation. This study similarly examined differential expression between cells exposed to menthol and tobacco flavored e-cig aerosols and found 493 genes to be differentially expressed between these conditions, indicating the importance of studying toxicity responses induced by e-cig aerosol containing flavoring chemicals. Genes differentially expressed in the menthol condition were related to cell adhesion and protein polymerization, whereas those differentially expressed in the tobacco condition were related to the cell cycle and superoxide response (60). Park et al. (24) conducted RNA-seq analysis on primary normal human bronchial epithelial (NHBE) cells exposed to commonly used e-cig flavoring chemicals diacetyl (2,3-butanedione) and 2,3-pentanedione, and found differential expression of 163 and 568 genes, respectively. The identified genes were associated with cytoskeletal- and cilia-related pathways.

Shen et al. (61) used RNA-seq to analyze differential gene expression in HBECs following 1-h exposure to air (controls), cigarette smoke, or e-cig vapor with or without nicotine (16 mg/mL). Immediately after exposure, relative to controls, cigarette smoke-exposed HBECs displayed significant differential expression of 49 genes (16 downregulated) related to several significantly enriched pathways including signal transduction, cell cycle regulation, apoptosis, response to organic substances, and response to hypoxia. E-cig vapor without nicotine resulted in significantly altered expression of six genes relative to controls, including *RPS8* and *ZNF721*, which are involved in the regulation of translation and transcription (61). In HBECs exposed to e-cig vapor with nicotine, after 1-h, there was significant differential expression of 57 genes (43 downregulated) related to pathways involved with the cell cycle, response to hypoxia, response to organic substances, apoptosis, and acute inflammatory response (61). Martin et al. (62) analyzed gene expression, via RNA-seq, in nasal epithelium biopsies from nonsmokers ($n$ = 13), e-cig users ($n$ = 12), and smokers ($n$ = 14) and found differential expression of 358 genes among the three groups. Smokers showed differential downregulation of 53 genes compared with nonsmokers, with the top five being early growth response 1 (*EGR1*), dipeptidyl-peptidase 4 (*DPP4*), chemokine (C-X-C Motif) ligand 2 (*CXCL2*), chemokine (C-X3-C Motif) receptor 1 (*CX3CR1*), and CD28 molecule (*CD82*). E-cig users showed differential downregulation of 358 genes relative to nonsmokers, including all 53 of those downregulated in cigarette smokers, with the top five genes being zinc finger and BTB domain containing 16 (*ZBTB16*), *EGR1*, polymeric immunoglobulin receptor (*PIGR*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and FK506 binding protein 5 (*FKBP5*) (62). These data illustrate unique immune-suppression effects observed in e-cig users relative to nonsmokers as well as cigarette smokers. Together, these studies indicate that there is significant deregulation of gene expression in both ENDS users and smokers, with ENDS users showing a level of deregulation lower than that of smokers (Table 2 and Fig. 2). In addition, e-cig flavorings appear to impact the type and degree of differential gene expression, though additional studies should be conducted in human subjects and primary cells from acute and chronic ENDS users to confirm and validate such findings.

## Exosomal Small RNAs as Circulating Biomarkers

Extracellular vesicles (EVs) are membrane-bound structures that contains complex biological materials such as proteins, lipids, metabolites, and nucleic acids (DNA and RNA). EVs vary in their size [exosomes (~50–150 nm) and microparticles (1,000–2,000 nm)], mode of biogenesis, and cargo depending on the physiologic state of the cells from which they are released. Recent studies have shown that EVs play an essential role in intercellular and interorganismal communications (63). Emerging reports support the idea that exosomes and EVs isolated from various biofluids, such as plasma/serum, saliva, urine, etc. can be used as novel circulating biomarkers of toxicity/injury. In a recent study, plasma-derived exosomes isolated from nonsmokers ($n$ = 8), cigarette smokers ($n$ = 7), e-cig users ($n$ = 7), waterpipe smokers ($n$ = 7), and dual smokers (cigarette and waterpipe; $n$ = 7) revealed distinct miRNA, tRNA, and piRNA signatures in specific pairwise comparisons (64). Differential expression analysis by DESeq2 showed 17 miRNAs that are differentially expressed in e-cig users versus nonsmokers. Among these, 13 miRNAs were upregulated (hsa-miR-365a-3p; hsa-miR-365b-3p; hsa-let7f-5p; hsa-miR-1299; hsa-miR-21-5p; hsa-let7i-5p; hsa-let7a-5p; hsa-miR-30a-5p; hsa-miR-193b-3p; hsa-miR-100-5p; hsa-miR-423-3p; hsa-miR-30c-5p; hsa-miR-143-3p; hsa-miR-224-5p) and four miRNAs were downregulated

(hsa-miR-362-5p; hsa-miR-29b-3p; hsa-miR-451a; hsa-miR-30e-5p) in nonsmokers compared with e-cig users (64). In addition, seven distinct tRNAs ($tRNA^{Val}$, $tRNA^{Glu}$, $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Asp}$, and $tRNA^{Cys}$) and five piRNAs (hsa-piR-016658, hsa-piR-016659, hsa-piR-017591, hsa-piR-019825, and hsa-piR-000552) were differentially expressed among e-cig users compared with nonsmokers (64). However, the exact role of the identified small RNAs in e-cig exposure-induced systemic toxicity remains unclear. These findings must be cautiously interpreted due to the very low-sample sizes used for comparisons between groups. Further validation of identified small RNA targets (miRNAs, tRNAs, and piRNAs) is needed to demonstrate their role as potential biomarkers of e-cig toxicity. Future and ongoing studies will strengthen the validity of EVs as novel circulating biomarkers using human cohorts with larger samples size both cross-sectionally and longitudinally along with complementary in vitro and in vivo models of e-cig exposure.

### DNA Methylation Biomarkers

DNA methylation, which occurs at CpG sites (CpGs) in the genome, can be analyzed in both targeted and genome-wide approaches and it alters gene expression leading to adverse health effects. As such, differential gene methylation (DGM) has potential as a biomarker for systemic toxicity associated with e-cig use (8, 65). Song et al. (7) analyzed a subset of bronchial brushings and found 451 differentially methylated CpGs, correlated to 273 unique genes, between never-users ($n$ = 10), e-cig users ($n$ = 12), and smokers ($n$ = 10), with e-cig users displaying DGM values between never-users and smokers for 97% of those CpGs (7). Of those differentially methylated CpGs, 14 were specific to e-cig users, showing potential for differentiation between e-cig- and tobacco cigarette-induced toxicity. Of these 14 CpGs, *RHBDL2*, *TTC16*, *ZNF815* and 3 intergenic CpGs were found to be significantly hypomethylated in e-cig users versus both smokers and never-smokers, whereas DGM levels for *AMZ1*, *KRT12*, *NOX5/MIR548H4* co-localized, *NRF1* and four intergenic CpGs were significantly hypermethylated in e-cig users relative to other groups (7). The top canonical pathways for DGM, similar to those for DETs discussed in the transcriptomic and gene expression biomarkers section, were xenobiotic metabolism signaling and colorectal cancer metastasis, as well as HOTAIR Regulatory Pathway and Axonal Guidance Signaling. Researchers also compared this DGM data with the aforementioned DET data from the same study, and found significant correlations related to 56 different genes. Of these 56 genes, IPA found the greatest enrichment for beta-naphthoflavone, which is involved in smoking-related mechanistic networks such as the AHR, the AHR nuclear translocator (AHRNT), and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2). These 56 genes included 51 that are related to cancer, 27 of which are related to respiratory tumors, making cancer the most represented disease in this analysis (7).

Recently, Caliri et al. (23) measured DNA methylation levels in DNA samples from peripheral blood leukocytes of nonsmokers, e-cig users, and smokers ($n$ = 15/group). This was done by measuring DNA methylation levels in LINE-1 (Long Interspersed Nucleotide Element 1), which serves as an indicator of the global level of 5-methylcytosine (5-mC) in a DNA sample (23). Researchers also measured global levels of an oxidation product of 5-mC called 5-hydroxymethycytosine (5-hmC), as well as several enzymes [DNA methyltransferases (DNMTs) and Ten-eleven translocation (TET) enzymes] involved in the methylation of DNA in isolated RNA from the same samples (23). Relative to nonsmokers, the vaping and smoking groups both displayed significant hypomethylation in LINE-1 repeat elements (by ~18% and 13%, respectively), with no significant difference between smokers and e-cig users ("vapers"). As for global DNA hydroxymethylation, quantified by 5-hmC, vapers and smokers again showed significantly lower levels compared with nonsmokers, with no significant difference between vapers and smokers. The analysis of expression levels showed no significant changes between the groups, though mRNA expression levels of DNMTs and TETs displayed nonsignificant increases in vapers and smokers relative to nonsmokers (23). Taken together, these studies clearly indicate that smokers and ENDS users have differential DNA methylation responses relative to both nonsmokers and each other, and long-term use of e-cigs may increase the risk of serious consequences such as deregulation of pathways associated with cancer and other diseases (Table 2 and Fig. 2).

### Proteomic Biomarkers

Proteomic analysis is another method used to measure the effects of e-cigs on human protein expression and may be used to identify novel biomarkers of ENDS at the molecular level. Differences in protein expression between nonsmokers, e-cig users/vapers, and smokers can provide important information regarding similarities and differences in toxicity of e-cigs and cigarettes. Ghosh et al. (48) performed proteomic analysis in bronchial brush biopsy samples from nonsmokers ($n$ = 8), e-cig users ($n$ = 9), and smokers ($n$ = 9) and found significant differences in protein expression between vapers and smokers. Researchers found 191 proteins to be significantly up- or downregulated in vapers, compared with 292 in smokers (48). Of these proteins, 78 were similarly altered in both groups, whereas 131 were exclusively altered in vapers. The mucin MUC5AC and vesicle-associated membrane protein 8 (VAMP8) were upregulated in both groups, MUC4 was upregulated in vapers only, and MUC5B was downregulated significantly in smokers only. Pathway analysis revealed 14 uniquely altered pathways in vapers, including proteins involved in cell organelle membranes, mitochondria, macromolecular complexes, and early endosomes/trafficking (48). MUC5AC and MUC5B are particularly interesting as potential markers of toxicity as they are primarily responsible for biophysical properties of the airway mucus, which serves as an important immune barrier against challenge by smoking and vaping, and are implicated in the progression of chronic inflammatory lung diseases such as COPD, asthma, and cystic fibrosis.

Reidel et al. (17) analyzed the airway secretion proteome (secretome) in induced sputum samples from never-smokers ($n$ = 15), e-cig users ($n$ = 15). and smokers ($n$ = 14). Their analysis revealed 66 proteins significantly upregulated in e-cig users, versus 23 in smokers and 5 in never-smokers, indicating differences in mucus protein composition between the three groups (17). Of these proteins, 15 were commonly

upregulated in both smokers and vapers. This study also found ~81 proteins significantly altered in e-cig users relative to never-smokers, versus 44 in smokers, indicating greater proteomic changes in e-cig users than smokers. Proteins significantly upregulated in e-cig users relative to both never-smokers and smokers include neutrophil elastase, proteinase 3, azurocidin 1, and myeloperoxidase (MPO). In addition, MMP8 and MMP9 were significantly upregulated in both e-cig users and smokers relative to never-smokers (17). These proteins are involved in inflammation and oxidative stress, giving them potential as toxicity biomarkers of ENDS use. The same study also found significantly elevated levels (means ± SD) of MUC5AC in sputum from e-cig users (58 ± 21 pmol/mL) and smokers (132 ± 58 pmol/mL) relative to nonsmokers (15 ± 6 pmol/mL), though MUC5B levels were not significantly different among the groups (17). Proteins that are uniquely dysregulated in vapers have potential as biomarkers of toxicity, though they require additional validation concerning the clinical significance of such findings (Table 2 and Fig. 2).

## CONCLUSIONS AND FUTURE DIRECTIONS

This review summarizes recent findings on a few of the most commonly tested clinical biomarkers of exposure to and systemic toxicity of ENDS use in humans. In order for these biomarkers to be properly validated for clinical and regulatory use, additional studies are needed with larger cohorts with diverse populations. In addition, the methods used to quantify these biomarkers in human biofluids (e.g., whole blood, plasma/serum, urine, exhaled breath condensate, and saliva) and primary cells (e.g., nasal epithelial cells, etc.) need to be rigorously tested and standardized using novel genomic, epigenomic, and transcriptomic approaches so they can be reliably quantified and compared across different cohorts and subject populations.

Although the focus of this review article is to provide a review of recent data from studies using human subjects, cells, and biofluids, it is also necessary to mention the role of animal research in understanding the health effects of current and emerging ENDS devices as completely and quickly as possible. An important benefit of animal research is that investigators can have full control over the exposure of animals to ENDS vapor and/or cigarette smoke, as opposed to relying on human subjects' report of their own cigarette/ENDS use (46). In addition, animal studies provide researchers with more control over their experiments compared with research using human subjects, where individual differences in puffing topography, device type and settings, e-liquid vendors, e-liquid volumes, nicotine concentrations [e.g., dosage (typical range: 0–24 mg/mL)], and several other factors serve as potential confounding variables. Preclinical e-cig exposure animal models give researchers the ability to control for such variables, allowing results to be better compared between studies. These animal models should include acute (1-day, 3-day, and 10-day), subchronic (1–3 mo), and chronic (6–12 mo) exposures, both cross-sectionally and longitudinally, conducted using similar e-cig exposure systems with well-defined standardized and optimized protocols and exposure conditions in mice (e.g., wild-type strains, susceptible transgenic strains, reporter mice). The reliability and reproducibility of animal research makes it another vital facet in the investigation of potential health hazards associated with ENDS use.

Currently, the WNY Center for Research on Flavored Tobacco (CRoFT) is focusing on investigation and validation of several existing exposure [cotinine, nicotine, TSNA (NNK, NNAL), VOCs, PAHs, flavoring chemicals and their breakdown products, etc.] and toxicity [oxidative/carbonyl stress, DNA damage, proinflammatory cytokines/chemokines, proresolving lipid mediators, global DNA methylation, and differential gene expression (inflammatory genes), etc.] biomarkers, as well as novel circulating, genomic, and epigenomic biomarkers in human and animal/mouse biofluids, cells, and tissues both cross-sectionally and longitudinally. The CRoFT also aims to identify novel potential biomarkers of ENDS use, particularly in circulating extracellular vesicles/exosomes, via small RNA-sequencing approaches. In addition, standardized experimental conditions and bioassays are currently being developed to ensure that analytical methods used for biomarker analysis are consistent, which will have wider implications in both clinical and tobacco regulatory science settings. These goals will be especially important for informing FDA regulation regarding the manufacture, marketing, and distribution of e-cigarettes/e-liquids and other emerging ENDS products (i.e., pod-based and disposable ENDS) which, as highlighted in this review, have the potential to negatively affect public health.

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## DISCLAIMERS

The updated review of literature and conclusions in this review article are solely those of the authors and do not represent the official views of the WNY Center for Research on Flavored Tobacco (CRoFT), the US Food and Drug Administration, and the US National Institutes of Health (including, NCI, NIEHS, and NHLBI).

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

S.R.M. and I.K.S. prepared figures; S.R.M. and I.K.S. drafted manuscript; S.R.M., I.R., and I.K.S. edited and revised manuscript; S.R.M., I.R., and I.K.S. approved final version of manuscript.

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