Biomarkers of exposure to new and emerging tobacco delivery products

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Schick SF, Blount BC, Jacob P 3rd, Saliba NA, Bernert JT, El Hellani A, Jatlow P, Pappas RS, Wang L, Foulds J, Ghosh A, Hecht SS, Gomez JC, Martin JR, Mesaros C, Srivastava S, St. Helen G, Tarran R, Lorkiewicz PK, Blair IA, Kimmel HL, Doerschuk CM, Benowitz NL, Bhatnagar A. Biomarkers of exposure to new and emerging tobacco delivery products. *Am J Physiol Lung Cell Mol Physiol* 313: L425–L452, 2017. First published May 18, 2017; doi:10.1152/ajplung.00343.2016.—Accurate and reliable measurements of exposure to tobacco products are essential for identifying and confirming patterns of tobacco product use and for assessing their potential biological effects in both human populations and experimental systems. Due to the introduction of new tobacco-derived products and the development of novel ways to modify and use conventional tobacco products, precise and specific assessments of exposure to tobacco are now more important than ever. Biomarkers that were developed and validated to measure exposure to cigarettes are being evaluated to assess their use for measuring exposure to these new products. Here, we review current methods for measuring exposure to new and emerging tobacco products, such as electronic cigarettes, little cigars, water pipes, and cigarillos. Rigorously validated biomarkers specific to these new products have not yet been identified. Here, we discuss the strengths and limitations of current approaches, including whether they provide reliable exposure estimates for new and emerging products. We provide specific guidance for choosing practical and economical biomarkers for different study designs and experimental conditions. Our goal is to help both new and experienced investigators measure exposure to tobacco products accurately and avoid common experimental errors. With the identification of the capacity gaps in biomarker research on new and emerging tobacco products, we hope to provide researchers, policymakers, and funding agencies with a clear action plan for conducting and promoting research on the patterns of use and health effects of these products.

exposure; NNAL; tobacco; biomarker; cotinine

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WE DEFINE NEW and emerging tobacco and nicotine delivery products as products that have been introduced to the United States market in the past 15 years, products that have become significantly more popular in the past 15 years, or products that are being modified and used in new ways. We limit our focus to products currently used by >1% of the U.S. population, based on nationally representative survey data. The products that currently meet these criteria are electronic cigarettes (e-cigarettes), little cigars, water pipes, and cigarillos.

NEW AND EMERGING PRODUCTS

For researchers who are new to tobacco and nicotine delivery product exposure biomarkers or new to the use of biomarkers to study e-cigarettes, water pipe, cigars, little cigars, and cigarillos, this paper offers guidance on choosing biomarkers that support specific study goals and are financially and practically feasible. For physiologists, this paper describes the challenges presented by new and emerging tobacco and nicotine delivery products and potential solutions to these problems. For pulmonary physiologists, this paper offers a discussion of biomarkers in samples collected in the respiratory tract. For agencies and policymakers who fund research on tobacco and nicotine delivery products, this paper outlines an action plan for promoting research on the use and health effects of these products.

Biomarkers of Exposure

The use of tobacco products results in the uptake of nicotine and a wide range of other chemicals. These chemicals and their metabolites, measured in bodily fluids and tissues, constitute biomarkers of exposure. Biomarkers of exposure to tobacco and nicotine delivery products are limited to the chemicals taken up during product use or during exposure to product emissions. Thousands of chemicals are present in tobacco smoke, and hundreds have been identified in e-cigarette aerosols and liquids. Although some biomarkers of exposure to tobacco and nicotine delivery products are metabolites of known toxicants or carcinogens, e.g., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), in this paper we focus on biomarkers of exposure, not on biomarkers of potential health effects.

No Validated Biomarkers Specific for E-Cigarettes and Other New Products

The lack of validated biomarkers for e-cigarettes is an urgent public health problem. The market for e-cigarettes has expanded rapidly since they came to market in 2007. In 2015, estimated U.S. sales totaled $3.5 billion (179, 267). To measure the health effects of e-cigarettes, researchers need biomarkers for exposure to both e-cigarettes that contain nicotine and those that do not. However, no validated biomarkers specific to nicotine-containing or nicotine-free e-cigarettes are currently available. At present, a biological specimen that tests positive for nicotine metabolites and negative for metabolites of combustion products and tobacco-specific nitrosamine (TSNA) metabolites suggests either the use of e-cigarettes with nicotine or of a nicotine replacement therapy (NRT) product, such as nicotine gum. Questionnaire data can be used to provide more accurate answers (67), but the known biomarkers of tobacco and nicotine exposure cannot. A second gap is the current lack of validated biomarkers that differentiate among the use of various combustible products (e.g., cigars, little cigars, cigarillos, water pipes, and cigarettes), as shown in Fig. 1. To support research on new products, it is essential to identify product-specific biomarkers and to develop sensitive, accurate, and affordable assays. Currently, few laboratories perform assays that can differentiate cigarette use from the use of other nicotine-containing products, and the existing assays are expensive.

BIOMARKERS OF EXPOSURE TO TOBACCO AND NICOTINE

Overview

There were three major categories of tobacco products on the market in 2016: combustion, heat delivery, and smokeless products (Table 1). Combustion products, which generate smoke enriched with nicotine and other chemicals (248), include cigarettes, cigars, little cigars, cigarillos, and water pipes. Many of the chemicals present in tobacco products and/or generated by combustion are taken up by the body in appreciable quantities. Therefore, exposure can be assessed by measuring these chemicals or their metabolites in biological specimens (261a) from various compartments of the body.

Heat delivery products, such as e-cigarettes, heat a solution of humectants, flavors, and tobacco extract to generate a nicotine-containing aerosol (45). Although the chemical composition of e-cigarette aerosols is simpler than that generated by combustible tobacco systems (58), they often contain a large number of flavorings and other additives. Furthermore, specific biomarkers of exposure to e-cigarettes and their constituents have not been identified.

Smokeless tobacco products—chewing tobacco, snuff and snus, and NRT products, such as gums, patches, lozenges, and

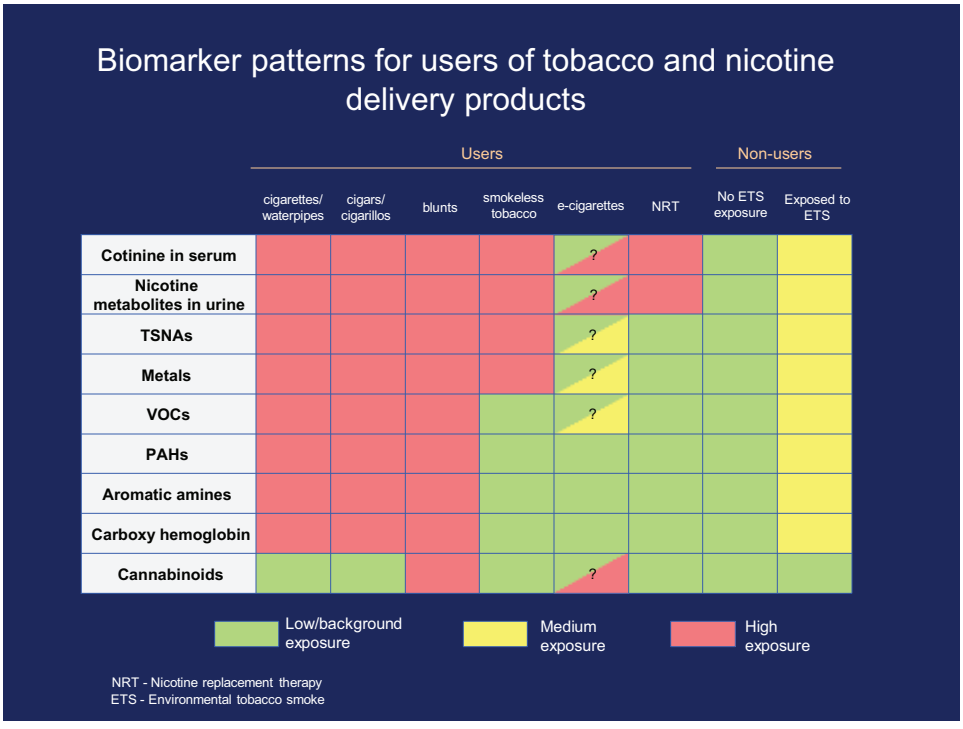

Fig. 1. Biomarker patterns for users of tobacco and nicotine.

Table 1. *Categories of tobacco and nicotine delivery products*

Tobacco Combustion	Heat Delivery	Smokeless
Cigarettes	E-cigarettes	Lozenges
Cigars	Heated, tobacco-based, noncombusting cigarettes	Gums
Cigarillos		Smokeless tobacco
Little cigars		Chewing tobacco
Water pipes		Snuff
		Snus

sprays—do not require combustion or heating to deliver nicotine. To date, only nicotine, nicotine metabolites, and TSNAs have been used to assess exposure to smokeless tobacco products (44, 106, 115, 152, 187, 241, 243, 261). Nicotine and nicotine metabolites are also the only known exposure biomarkers for nicotine replacement products. In this paper, we discuss biomarkers of exposure to tobacco and nicotine in the three defined categories and highlight the limitations in identifying selective markers of exposure to specific tobacco products.

What Makes a Good Biomarker?

Exposure to a chemical or group of chemicals can be quantified by measuring the chemical or its metabolites in the body or excreta. A good biomarker has four key traits: *1*) a clear dose-response relationship with exposure: the concentration of the biomarker chemical increases with an increase in exposure and decreases upon cessation of exposure with a known time course, *2*) qualitative and quantitative identification over a wide range of concentrations so that both low and high levels of exposure are accurately estimated, *3*) detection in readily collected biospecimens (e.g., saliva, urine, and blood), and *4*) stability upon storage for prospective analyses.

Biomarkers of exposure, such as nicotine or nicotine metabolites (including cotinine) and TSNA metabolites (e.g., NNAL), are specific to the use of tobacco and nicotine delivery products (of all the categories discussed above). Dietary or environmental exposures contribute little to the body burden of these chemicals. Nevertheless, other less-specific biomarkers of tobacco product exposure, such as carbon monoxide (CO), volatile organic chemicals (VOCs), and polycyclic aromatic hydrocarbons (PAHs), can provide additional information relevant to a more comprehensive exposure assessment and can help relate exposure to injury. Because some chemicals or their metabolites accumulate in tissues, their measurement can provide exposure estimates that integrate the duration and extent of exposure, as well as the rate of chemical or metabolite clearance. Therefore, in selecting a biomarker to quantify exposure, it is important to consider the source, persistence, and pharmacokinetics of the biomarker, dose of exposure, as well as duration between exposure and measurement.

Biomarkers of Nicotine

Nicotine was one of the first biomarkers to be used for assessing exposure to cigarette smoke (26, 215). However, its short half-life ($t_{1/2}$; ~2 h) and variable rate of metabolism led to the use of cotinine and other nicotine metabolites as biomarkers of nicotine exposure (28, 29). Cotinine is the major metabolite of nicotine, and its longer $t_{1/2}$ (16–18 h) makes it a good biomarker for nicotine uptake in various biological fluids and tissues (47, 138, 159, 198, 228, 264). Nicotine and its metabolites are discussed in detail in *Nicotine in Blood* through *Over-the-Counter Cotinine Tests*.

Biomarkers of Tobacco Use Other Than Nicotine

Tobacco and nicotine delivery product chemicals originate from the tobacco production process, the product manufacturing process, chemical reactions during product storage, or combustion or pyrolysis during product use. Table 2 summarizes the major categories of these chemicals and the corresponding biomarkers commonly used to assess tobacco and nicotine delivery product exposure (102, 173). These include exhaled CO, VOCs, PAHs, and TSNAs [mainly 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolite, NNAL]. NNAL is a biomarker for the use of combustible and smokeless tobacco products. Because e-cigarettes do not attain true combustion temperatures, they do not emit CO or as many different VOCs and PAHs in measureable quantities (97, 104, 145, 177). Thus exhaled CO and metabolites of PAHs are not associated with the use of e-cigarettes.

Biological Cut-Point Values

Biological cut-point values differentiate tobacco and nicotine delivery product users from nonusers (e.g., smokers from nonsmokers) and can be used to identify product-use patterns. Specific cut-point values that separate populations with different tobacco product exposures are established by obtaining biomarker data from a large number of users and nonusers who

Table 2. *Biomarkers of tobacco and nicotine delivery product use and exposure*

Smoke Constituent	Example/Biomarker	Measured in	References
Nicotine	Cotinine	Blood Urine Saliva Respiratory fluids	(22, 27, 28, 38)
Tobacco-specific nitrosamines	NNK/NNAL	Urine	(41, 49, 53, 62, 98, 118, 144)
Volatile organic compounds (VOCs)	1,3-Butadiene/*N*-acetyl-*S*-(4-hydroxy-2-buten-1-yl)-L-cysteine (MHBMA-3)	Urine	(151, 223)
Polycyclic aromatic hydrocarbons (PAHs)	Pyrene/1-hydroxypyrene	Urine	(87, 221)
Metals	Cadmium	Blood Urine	(158, 175)
CO	Exhaled CO carboxyhemoglobin	Blood Breath	(31, 63, 134, 153, 208, 218, 220)

are classified using a separate method (e.g., self-report) to arrive statistically at an optimum cutoff value (90). Both the sample matrix (e.g., blood vs. urine) and metabolic variations among individuals influence these values. For example, the cutoff value of cotinine in urine is much higher than that of cotinine in serum, because cotinine concentration is four to six times higher in the urine than in blood (14, 274). In addition, optimum cutoffs may vary slightly, based on the subjects' genetic backgrounds (269). For example, the optimal serum cotinine level to separate adult smokers from nonsmokers is 6 ng/ml for non-Hispanic blacks, but 5 and 1 ng/ml for non-Hispanic Whites and Mexican Americans, respectively (21). Moreover, in recent years, biological cut-point values have decreased as people tend to smoke less and are less likely to be exposed to secondhand smoke (SHS) because of indoor smoking bans (14, 123, 137, 139, 229).

It is relatively simple to separate nonsmokers from typical daily smokers using any of the aforementioned markers, as plots of such data typically result in two distinct distribution curves. However, it is not always possible to arrive at a clear distinction between the two groups. For example, biomarker values for heavy SHS exposure overlap with those from occasional smokers for some biomarkers (14, 121, 124). Hence, to distinguish SHS exposure from intermittent smoking, biomarker measurement should be supplemented with data from questionnaires. In case of mismatches, a positive biomarker value may be more reliable than self-reported data.

PLANNING FOR BIOMARKER ANALYSIS

Biomarkers of tobacco and nicotine exposure are present in trace concentration in chemically complex biological fluids. The first step in selecting a method for biomarker measurement is to determine the lowest concentration of the chemical or metabolite that must be quantified to address the primary study aims. This lower limit is defined by biological cut-point values separating active tobacco product users from nonusers and by biomarker patterns separating users of one product from users of other products (Fig. 1, and see Table 5). Biomarkers of tobacco exposure have been measured in almost every biological material, including hair and nails, although the vast majority of information has come from measurements in blood (serum or plasma) and urine. The matrices of choice are discussed in conjunction with each biomarker. The measurement of biomarkers of exposure in animals is useful and often critical for understanding human disease, because these models allow experiments and samples that are not feasible in human studies.

LOD and LLOQ

The limit of detection (LOD) and lower limit of quantitation (LLOQ) define limits of identification and quantification, respectively. The LOD is the lowest concentration of a chemical that can be detected over the background noise in a given biological sample. LOD is technology and assay dependent: assays with elaborate sample preparation procedures that remove interfering compounds often have a lower LOD than those with simpler sample preparation. However, it is easier to detect a chemical in a specimen than to quantify it. The coefficient of variation (CV; reproducibility) at the LOD may be much larger than that at the LLOQ, and thus data may not be acceptably precise at the LOD.

The LLOQ is the concentration at and above which the assay is sufficiently reliable and reproducible for measuring the quantity of a chemical in a given specimen. For example, the acceptable LLOQ for precise cotinine measurement following SHS exposure is lower than that for precise cotinine measurement following cigarette smoking. In general, the smaller the CV, the better the assay can discriminate among experimental groups or conditions. Whereas multiple methods exist for defining LOD and LLOQ (182, 252), the guidelines typically used for tobacco studies involving biomarkers are those developed for drug clinical trials (225, 266). These guidelines also describe criteria for method development, validation, and quality control and for compound quantification (e.g., setting the LLOQ at CV $<$ 20%). Commonly available assays and the LLOQ for each are listed (see Table 6).

Nicotine in Blood

Blood nicotine concentration—whether measured in whole blood, serum, or plasma—is a key determinant of the pharmacologic effects of tobacco products. The time course of nicotine in the body and resultant pharmacologic effects are highly dependent on dose, as well as the route and rate of dosing. Smoking a cigarette, for example, delivers nicotine rapidly to the pulmonary venous circulation, from which it moves quickly to the left ventricle of the heart and to the systemic arterial circulation and brain. Venous blood concentration after smoking a single cigarette ranges from 5 to 30 ng/ml, depending on how the cigarette is smoked. The mean nicotine boost in a large study of smokers was 10.9 ng/ml (195). Nicotine concentrations in arterial blood after smoking a cigarette can be quite high (up to 100 ng/ml) but usually range between 20 and 60 ng/ml (101, 119, 166, 213). For most purposes, a single blood sample within 1 or 2 min of smoking a cigarette will give an acceptable estimate of the peak nicotine concentration. However, to capture the true "peak," multiple blood samples must be collected during and immediately after use.

Blood nicotine levels typically peak at the end of smoking a cigarette and decline rapidly over the next 20 min due to tissue distribution. The initial $t_{1/2}$ of nicotine decline, during which the drug distributes into tissue, averages ~8 min. Peak venous blood levels of nicotine are similar among cigarette smokers, cigar smokers, snuff users, and chewing tobacco users, although the rate of rise of nicotine is faster among cigarette smokers (20). Pipe smokers, particularly those who have previously smoked cigarettes, may have blood and urine levels of nicotine and cotinine as high as cigarette smokers (174, 273). Cigar and pipe smokers who have previously smoked cigarettes may inhale more deeply and achieve higher blood levels of nicotine than primary cigar or pipe smokers (258). Waterpipe users attain blood nicotine levels that are, on average, lower than those seen with cigarette smoking, but their total nicotine exposure can be higher, because the duration of use is much longer (128, 235). E-cigarette use yields variable blood nicotine levels, dependent on the type of device used, the power output of the device, the nicotine content of the e-liquid, and the user's puffing behavior (249). Cigarette-like e-cigarettes usually generate much lower blood nicotine levels, whereas tank or modifiable devices generate peak levels as high as seen with cigarette smoking (237, 263).

The elimination $t_{1/2}$ of nicotine in blood is determined by a combination of clearance rate and redistribution of nicotine out-of-body tissues. Based on an average $t_{1/2}$ of ~2 h, one would predict a progressive rise in nicotine blood and tissue levels over 6–8 h (3 to 4 $t_{1/2}$) of regular smoking and persistence of significant levels for 6–8 h after cessation of smoking. Studies of nicotine blood levels in regular cigarette smokers confirm these predictions (32). Peak and trough levels follow each cigarette, but as the day progresses, trough levels rise, and the influence of peak levels becomes less important. Thus regular smoking is a multidosing situation, where nicotine concentrations rise during waking hours and decline during sleep, but because the nicotine $t_{1/2}$ is 2 h, levels persist at significant levels for 24 h each day. Light, intermittent smoking results in less nicotine accumulation in the body over the day, and oscillations in nicotine blood levels are more prominent. Plasma nicotine $t_{1/2}$ in rodents is generally shorter than in humans: 45 min in the rat and 6 to 7 min in the mouse. This means that studies in rodents require higher daily doses of nicotine to achieve blood nicotine concentrations similar to those seen in smokers (172). Perhaps the best measure of the nicotine-related pharmacologic effect is the area under the blood nicotine concentration time curve, which reflects the time-weighted exposure of body tissues to nicotine. This measure has been used to study the pharmacology of cigarettes, smokeless tobacco, and water pipes (26, 30, 128). The plasma nicotine concentration curves for various tobacco products are presented in Fig. 2.

Analytical methods. Generally, GC-based methods are most suitable for measuring blood nicotine concentrations and are reasonably economical. For the utmost sensitivity that may be required for studies of occasional tobacco users or users of products delivering low nicotine levels, GC-tandem MS (GC-MS/MS) is the method of choice (235).

Cotinine in Blood, Saliva, and Urine

Cotinine is the major proximate metabolite of nicotine and is the most widely used biomarker of nicotine exposure. On average, 75–80% of nicotine is converted to cotinine, primar-

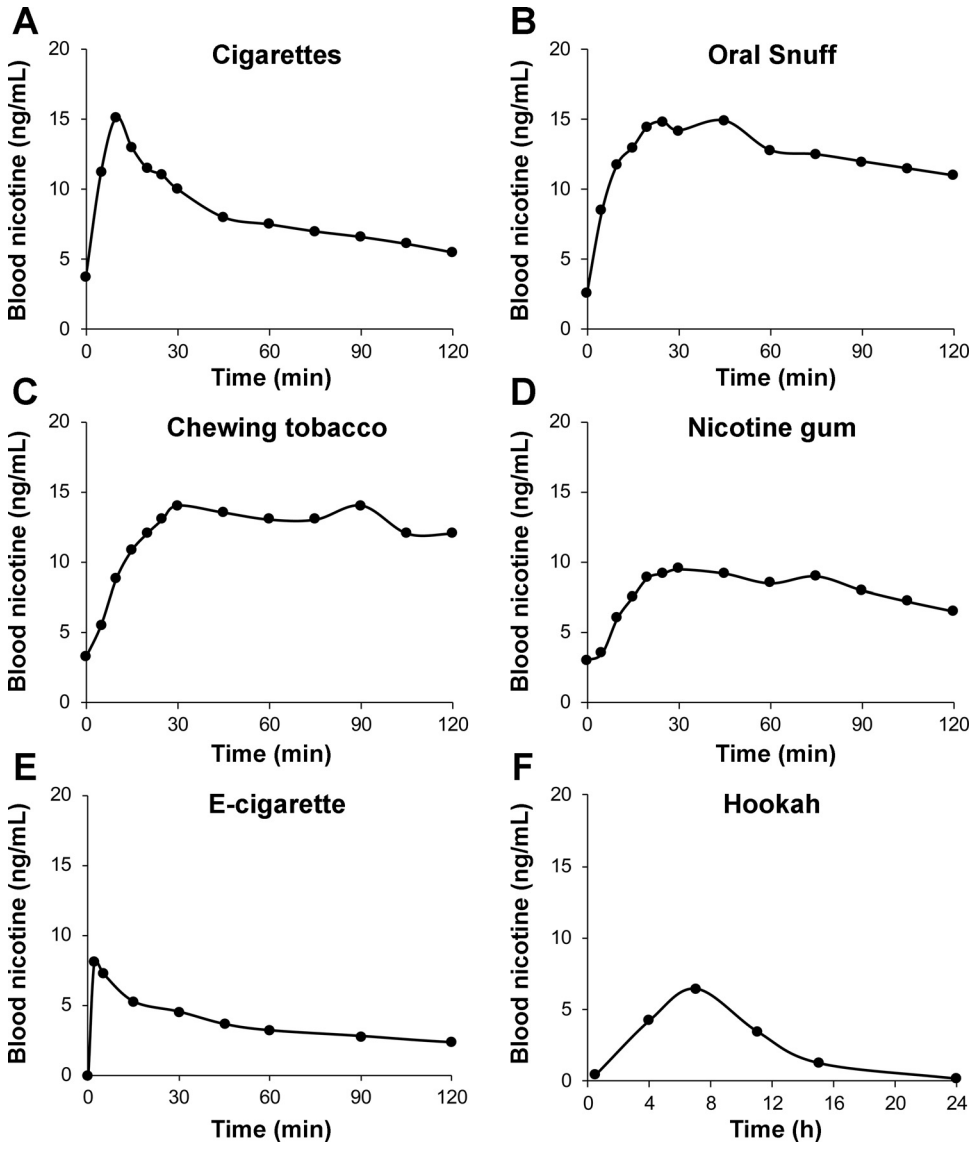

Fig. 2. Average blood nicotine concentration comparison. *A*: average blood nicotine concentrations in 10 subjects during and after cigarette smoking for 9 min (34); *B*: oral snuff (2.5 g) (34); *C*: chewing tobacco (average 7.9 g) (34); and *D*: nicotine gum (2, 2-mg pieces) (34). *E*: average plasma nicotine concentrations, corrected for baseline level, in 14 experienced e-cigarette users after 15 puffs from their usual brand of e-cigarette (237). *F*: average plasma nicotine concentration for hookah (water pipe) users over 24 h after 2+ sessions of hookah use between 900 and 1800 (128).

ily by the liver enzyme cytochrome *P*-450 family 2 subfamily A member 6 (CYP2A6) (126). Cotinine can be measured in whole blood, serum, plasma, saliva, and urine. Because its $t_{1/2}$ (16–18 h) is longer than that of nicotine (2 h), cotinine concentrations fluctuate much less than nicotine concentrations throughout the day, making it the most practical biomarker for measuring nicotine exposure (27, 126). Cotinine concentrations in blood and saliva are highly correlated, with saliva concentrations averaging 15–20% higher than plasma (239). Urine cotinine concentrations, on average, are four to six times higher than blood or saliva levels, making urine a more sensitive matrix to detect low-dose exposure (22). Whereas the $t_{1/2}$ of 16 h makes cotinine a more stable biomarker than nicotine, cotinine levels still reflect a relatively short-term exposure to tobacco, over the past 3 to 4 days.

Cotinine blood concentrations average ~150–250 ng/ml in daily cigarette smokers. Due to its longer $t_{1/2}$ than nicotine, cotinine levels rise gradually during the day, peaking at the end of smoking, and persisting at high concentrations overnight. The daily variation in blood cotinine levels throughout the day in regular smokers is ~30%. Blood cotinine concentrations are similar in smokers and regular smokeless tobacco users (3). Urine cotinine levels are generally lower in exclusive pipe and cigar smokers compared with cigarette smokers (92, 210). To date, the limited available data indicate that plasma and saliva cotinine levels in regular e-cigarette users are similar to those of smokers. However, because most e-cigarette users also smoke cigarettes, it is difficult to disentangle the contributions of each (82, 177, 265).

The mathematical relationship between nicotine intake and steady-state cotinine blood levels, based on steady-state exposure conditions, can be expressed as follows: $D_{nic} = CL_{COT} \times C_{COT}/f$, where D_{nic} is the intake (dose) of nicotine, CL_{COT} is the clearance of cotinine, C_{COT} is the steady-state blood concentration of cotinine, and f is the fraction of nicotine converted to cotinine (27). With the rearrangement of the equation, $D_{nic} = (CL_{COT}/f) \times C_{COT} = K \times C_{COT,}$ where K is a constant that converts a given blood level of cotinine to nicotine intake. On average, $K = 0.08$ mg·24 h^{-1}·ng^{-1}·ml^{-1} (range 0.05–1.1, CV = 21.9%) (27). Thus a cotinine level of 200 ng/ml in blood corresponds, on average, to a nicotine intake of 16 mg/day. The K value is an average based on a small group of healthy volunteer smokers and is expected to vary among smokers and to be influenced by genetic and environmental factors that influence nicotine and cotinine metabolism and, therefore, to vary in smokers of different racial groups. Results from specific populations may vary.

Whereas cotinine functions well as a marker of nicotine intake, individual variation in metabolism makes it an imperfect biomarker of exposure. The pathway from nicotine to cotinine is affected by genetic variation in the liver enzyme CYP2A6; race; sex; use of certain medications, including estrogen-containing hormones (e.g., oral contraceptives); alcohol use; pregnancy; and existing liver or kidney disease (126). Certain CYP2A6 gene variants slow cotinine formation and removal, although unequally, generally resulting in higher cotinine levels for a given daily nicotine intake (286). Because African Americans and Asians have, on average, lower CYP2A6 activity, they tend to have higher cotinine levels than whites for the same daily nicotine dose. However, in rare cases with extremely low CYP2A6 activity, little cotinine is generated, so levels of this biomarker are lower than expected for a given daily nicotine dose (35). Cotinine levels are also higher in African Americans because of lower rates of conversion to cotinine-*N*-glucuronide by uridine diphosphate-glucuronosyltransferase 2B10 (UGT2B10) (185). The same is true for men compared with women, whose higher estrogen levels induce higher CYP2A6 activity (33). For these reasons, the most accurate biomarker of daily nicotine intake is urine total nicotine equivalents (TNEs; see *Total Nicotine Metabolites in Urine*).

Analytical methods. GC (72, 130) and HPLC (105, 254) are appropriate methods for quantifying cotinine concentrations in specimens from daily tobacco users. Liquid chromatography (LC)-MS/MS is the best method for quantifying cotinine concentrations in samples from nondaily users and those exposed to SHS (39, 132).

Total Nicotine Metabolites in Urine

Assessment of daily nicotine intake in tobacco product users is important, as daily nicotine intake is related to nicotine/tobacco dependence. Urine TNE is defined as the molar sum of nicotine and all of its known metabolites in urine. It is considered the "gold standard" biomarker of daily nicotine intake. TNE levels are independent of factors that affect the rate and pattern of nicotine metabolism, such as genetics, sex, diet, and medication use. Cotinine is the proximate metabolite of nicotine and primarily a product of CYP2A6-mediated nicotine metabolism (126). Cotinine is metabolized further by CYP2A6 to *trans*-3′-hydroxycotinine (3-HC) and then primarily through the action of UGT enzymes (UGT2B10) to cotinine glucuronide (56, 126). Nicotine is metabolized, to a lesser extent, to its glucuronide by UGT2B10 and to nicotine *N*′-oxide by flavin-containing monooxygenase 3 (56, 126, 143). Given the high prevalence of polymorphisms in genes that encode the major nicotine metabolizing enzymes and the influence of factors, such as sex hormones and diet, on the rate of various nicotine metabolic pathways, a single nicotine metabolite cannot comprehensively assess daily nicotine intake. For example, African Americans usually have slower CYP2A6 activity and/or slower UGT2B10 activity and thus have higher cotinine levels for a given nicotine exposure compared with those with normal enzymatic activity (33, 286).

The combination of metabolites included in the term TNE may vary among studies. Most commonly, TNE is based on the six main metabolites: nicotine, cotinine, 3-HC, cotinine-*N*-glucuronide, nicotine-*N*-glucuronide, and 3-HC-O-glucuronide. Nornicotine, norcotinine, nicotine 1′-*N*-oxide, cotinine *N*-oxide, 4-hydroxy-4-(3-pyridyl)butanoic acid ("hydroxy-acid"), and other glucuronide metabolites that are present in low abundance can be measured but are rarely included in TNE determinations. When measured at steady state, these compounds account for ~80–90% of a daily nicotine dose (29, 86, 126). Defined this way, TNE is highly correlated with daily nicotine intake, as validated by administration of labeled nicotine in steady-state conditions (23).

Analytical methods. The method of choice is LC-MS/MS. It provides high sensitivity and specificity and can measure multiple metabolites in one analytical run. Administration of stable, isotope-labeled nicotine to human study participants has

been used to correlate TNE with daily nicotine intake in multiple studies (131a, 132).

Biomarkers of Exposure in the Airways

The airways, directly exposed to the ambient atmosphere, are the body's first point of contact with inhaled tobacco products. Theoretical models and studies of airway casts predict that most inhaled tobacco smoke deposits in the central airways (209). Smoke and other aerosol deposition is influenced by breathing patterns and the ways particles change in the airways and move as a cloud (12, 122, 164, 170, 171, 184, 199). For example, low-tar tobacco products prompt more intense smoking and variability among smokers; in the high humidity of the airways, smoke particles enlarge and coagulate, which changes their deposition pattern (209).

Exposure biomarkers have been studied less in the airways and lung than in blood and urine. Because tobacco components deposit directly into the airways, sampling from airway surfaces provides a reliable and local assessment of smoke exposure. The upper airways include the nasal cavity, pharynx, and larynx, and the lower airways include the trachea, bronchi, and bronchioles. All airway surfaces are lined with a thin film (~7 μm in depth) of airway surface liquid (ASL), which is approximately isotonic with plasma, has a pH between 7 and 7.4, and contains ~1,000 proteins, many of which are involved in innate immune defense (65, 250, 251, 279). Nicotine is inhaled into the lung and then converted to cotinine by cytochrome *P*-450 (25). Most nicotine metabolism by *P*-450 enzymes occurs in the liver (25). Although cytochrome *P*-450s are expressed in airway epithelia, these enzymes are not present in the ASL (146, 200), suggesting that any cotinine in the ASL is likely to be from the underlying airway epithelia or from the liver via the bloodstream. The reason to test ASL for exposure biomarkers is to estimate the local concentration in airway tissues.

The three main specimen options for ASL assessment are nasal lavage fluid (NLF), sputum, and bronchoalveolar lavage (BAL) fluid. These first two are noninvasive, and samples can be easily obtained in the field. BAL requires sedation and bronchoscopy to obtain but is considered the gold standard and perhaps the specimen most representative of the deep lung. However, all three specimen types are reliable for assessing environmental tobacco exposure. Solid-phase microextraction of volatile and semivolatile compounds using extraction fiber is a common method for sample extraction and for estimation of nicotine and cotinine in sputum (83).

Nasal lavage fluid. NLF collection is a noninvasive way to procure specimens for tobacco product exposure assessment. Typically, subjects simply expel NLF into a specimen cup after spraying a saline solution into both nostrils. After centrifugation to remove cells and other solids, cotinine levels in NLF, measured by a competitive immunoassay, provide a specific and sensitive measurement of smoking with a cutoff of exposure of 1 ng cotinine/ml NLF (188). To normalize samples and to facilitate comparisons among subjects, the total protein concentration can be measured using standard bicinchoninic acid assays. However, whereas nasal cotinine can be a useful marker of active smoking, SHS exposure does not increase cotinine in NLF when quantified using the competitive immunoassay technology (188).

Sputum samples. Induced sputum samples represent ASL from the large/central airways in the lung. The relatively easy sample collection method involves inhalation of hypertonic saline mist, followed by coughing to expel the sputum. Rinsing of the mouth minimizes sample contamination with salivary secretions (64). Carefully collected sputum samples are a reliable tool to identify nicotine and cotinine levels after smoking and could possibly be used to assess SHS exposure (64). Sputum samples are first treated with 0.1% DTT to break down the mucins and then filtered and centrifuged. The resulting supernatant can be used to measure nicotine and cotinine by HPLC-MS/MS. Sputum samples collected immediately following smoke exposure have been shown to have greater nicotine and cotinine levels than predicted from serum and plasma. Cotinine levels (6.5 ± 1.1 μM) were lower than nicotine (33.6 ± 5.5 μM). In contrast, nicotine and cotinine plasma levels were in the nanomolar and micromolar ranges, respectively (64, 131). Similar data have been generated in vitro using well-differentiated human bronchial epithelial cultures, followed by ASL lavage and subsequent MS analysis (64). A strong correlation was observed between different tobacco smoke dilutions and nicotine level, as well as cotinine level of ASL in vitro. In all dilutions, the nicotine level detected was in the micromolar range, although consistent with the lack of expression of cytochrome *P*-450 enzymes in the ASL, cotinine concentration was much lower than nicotine concentration; the ratio of nicotine follows: cotinine was ~6:1 in sputum and ~100:1 in ASL in vitro (64). Furthermore, whereas Clunes et al. (64) observed ~33 μM nicotine in sputum, they observed varying nicotine levels in vitro (from ~3,001 μM), suggesting that this technique can be used to adjust dosimetry for in vitro experiments.

BAL samples. BAL samples are collected by squirting saline solution into the lung and aspirating the solution from lung surface fluid and cellular components. Although evaluation of BAL fluid for tobacco components and metabolites may prove a critical tool for analyzing pathogenesis of tobacco smoke-induced pulmonary diseases and evidence of exposure, most BAL fluid analysis is focused on proteomics indicating the exposure effects. The only study, to date, relating smoke-induced effects identified aluminum silicate crystals in "black macrophages" in BAL samples from cigarette smokers (167).

Breath biomarkers. The breath is a well-validated indicator of concentrations of volatile chemicals in the respiratory tract. Benzene, 2,5-dimethylfuran, toluene, and xylenes have been measured in breath samples of smokers and nonsmokers. The use of each of these chemicals as a smoking biomarker, however, varies. The VOC, 2,5-dimethylfuran is invariably detected in smokers, regardless of use patterns, but not in nonsmokers, suggesting that it is a useful biomarker of combustible tobacco product exposure (6). Elevations in acrolein levels in smokers are matched by elevations of lipid oxidation products, including malondialdehyde and hydroxynonenal, which suggests that the acrolein may derive partially from lipid oxidation rather than cigarette smoke (11); the measurable presence of microgram quantities of acrolein in the smoke from each cigarette indicates that some of the acrolein exposure biomarkers measured in smokers also likely arise directly from smoke acrolein (78, 197). Development of similar noninvasive techniques to assess exposure to e-cigarettes and other novel tobacco products would facilitate large population studies.

Over-the-Counter Cotinine Tests

Although sometimes referred to as "nicotine" or "smoking" tests, the analyte measured by over-the-counter (OTC) test kits is cotinine. These kits require no instrumentation and are the fastest and least expensive cotinine assays available. With the exception of NicCheck I (Mossman Associate, Milford, MA), which is an older, colorimetric device, all currently available OTC cotinine test kits use some form of lateral diffusion immunoanalysis on disposable strips containing cotinine antibodies. These strips are designed to give a simple, qualitative smoker/nonsmoker response, based on a cutoff level. Most kits are meant for use with urine samples and according to the vendors, have a lower cutoff for a positive result of 200 ng/ml. As described earlier, most smokers and other active tobacco users typically have urine cotinine concentrations significantly higher than 200 ng/ml, so these strips, when positive, would indicate a likely tobacco user. These strips, however, are not sensitive enough to detect exposure to SHS and may not detect nondaily smoking.

Similar kits are available for use with saliva and have been reported to have greater sensitivity. These include the 1-Step Cotinine Rapid Saliva Test (20 ng/ml cutoff; Alere, Waltham, MA), the iScreen OFD Test (30 ng/ml cutoff; Alere), and the "Second Hand Smoke" NicoTest (10 ng/ml cutoff; USHealthTests, Albany NY). Since cotinine concentrations in saliva are much lower than in urine, saliva tests must be more sensitive to discriminate between tobacco users and nonusers. Some of these kits include supplies and devices for sample collection, whereas others do not. In general, with the assumption that these OTC kits perform accurately at indicated cutoff values, a positive result indicates an active tobacco user, whereas a negative result indicates a likely nonsmoker, infrequent smoker, or a user of a low-nicotine e-cigarette.

Although all of the kits described here use standard, lateral diffusion immunoassays, specific information about the antibodies or other test-strip components has not been published. With the exception of the NicAlert strips (Nymox Pharmaceutical, Hasbrouck Heights, NJ) described below, most of these kits, although marketed under various brand names, appear to be made by one manufacturer: Gemc Technology in Shenzhou, China. These tests are usually described by the manufacturer as providing preliminary results, requiring a more specific method, such as GC-MS, for confirmation.

Nymox Pharmaceutical's NicAlert and TobacAlert tests are similar in concept but use multiple band ("reland," or release ligand) regions that vary in their affinity for the analyte and use colloidal gold particles coated with cotinine conjugate for visual detection. This approach produces a more complex result pattern on the strip, which is visually evaluated by the user based on the lowest colored band. The manufacturer states that this approach provides for a semiquantitative assay. The lowest band cutoff value is 10 ng/ml, with progressively higher cutoffs assigned to higher bands, enabling the use of these strips with urine or saliva.

Cotinine test strips can provide a simple, inexpensive, and noninstrumental approach to assessing an individual's current tobacco exposure and potentially distinguishing between regular tobacco users and nonusers. Although these devices lack the sensitivity necessary to address low-level exposures reliably, including SHS and nondaily tobacco use, and generally provide results with greater variability than standard laboratory assays, they can be helpful for certain applications. One important advantage is the ability to provide nearly immediate feedback to a subject, as is also the case with breath CO measurements.

TSNA METABOLITES IN URINE

The TSNAs include the potent lung carcinogen NNK and the oral cavity and esophageal carcinogen *N*′-nitrosonornicotine (NNN) and are—as indicated by their common name—regarded as completely specific to tobacco. Consequently, these compounds and their metabolites are among the most important biomarkers for monitoring tobacco exposure and evaluating tobacco and nicotine delivery products (109, 110, 118). Urine is the preferred biospecimen, and the primary biomarker—considered as specific as nicotine or cotinine for tobacco exposure—is NNAL, a metabolite of NNK and itself a carcinogen. A key benefit of NNAL assays is the compound's estimated terminal $t_{1/2}$ of 10–18 days (112), which is longer than other tobacco biomarkers. The main disadvantage is that the urinary concentration of NNAL is many times lower than that of cotinine, so the assay is more technically challenging and expensive to perform. Measurements of NNAL typically require extensive sample prep, with analysis by LC-MS/MS, and fewer laboratories can reliably measure NNAL than cotinine or nicotine.

Figure 3 shows the formation of the nitrosamines NNN and NNK from nicotine. Whereas nicotine is converted to cotinine in the body, the formation of NNK and NNN from nicotine occurs mainly within tobacco itself, partly during plant development but predominantly by nitrosation of nicotine during

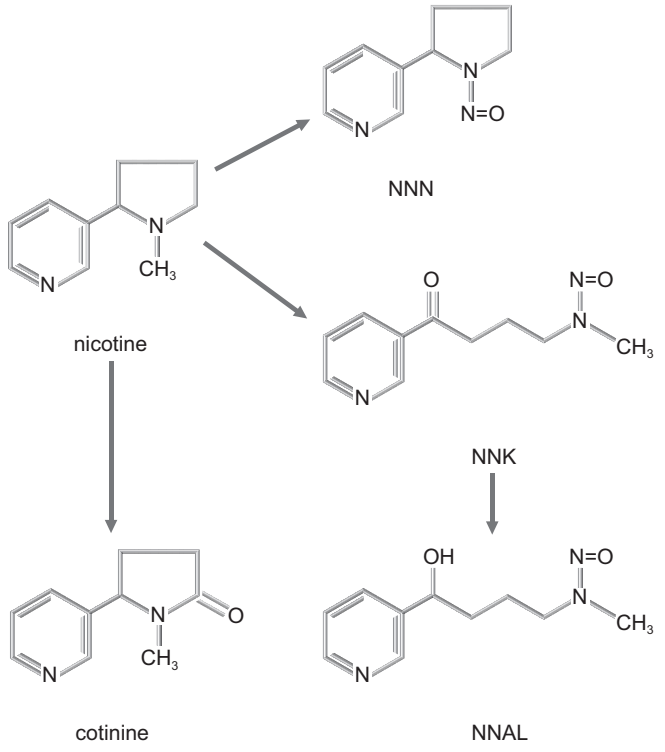

Fig. 3. Tobacco-specific nitrosamine formation from nicotine, a process that occurs mainly during the curing and processing of tobacco [modified from Kotandeniya et al. (150)].

tobacco leaf processing and curing (88, 89). (NNN and NNK are also formed from nornicotine and pseudo-oxy-nicotine during curing.) Additional TSNAs can be formed from related tobacco alkaloids, but NNN and NNK are believed to be the primary carcinogenic forms in humans. There is strong evidence that the TSNAs are carcinogenic in both experimental animals and humans and that NNAL is not only a biomarker of tobacco exposure but also an indicator of cancer risk (117, 244).

NNAL, the NNK metabolite, is the most stable and abundant TSNA metabolite in urine samples, occurring both free and as a glucuronide. NNN is also present in urine, but its concentration is lower than that of NNAL, making it more difficult to detect and quantify. Measurements made after hydrolyzing the glucuronides of NNAL provide "total" NNAL values, which are the values most commonly used. Whereas NNAL is usually measured in urine, where its concentration is highest, it has also been measured in blood and in toenail clippings (a potentially longer term storage site) (242).

TSNAs are released into the air when tobacco is burned, and nonsmokers are also exposed (46, 114). There is evidence that nicotine can continue to form NNK in the environment (224, 231). Nonsmokers exposed to SHS may have a higher NNAL-to-cotinine ratio in their urine (96). NNAL concentrations in urine of both smokers and nonsmokers have been reported in several large studies, including a multiethnic cohort study (193) and in all U.S. National Health and Nutrition Examination (NHANES) surveys since 2007 (37a, 277, 280). The total NNAL geometric mean for nontobacco users in the 2011–2012 NHANES survey of the U.S. population was 1.19 pg/mg creatinine (95% confidence interval 1.09, 1.29), whereas it was 216 pg/mg creatinine (95% confidence interval 182, 257) among cigarette smokers (277). Although creatinine concentrations vary with age, sex, and muscle mass, they average 0.5–1.5 mg/dl in healthy adult humans and 0.1–0.5 mg/dl in healthy adult mice. Mean values were lower in pipe and cigar smokers and much higher in oral tobacco users, although the sample sizes for the latter two groups were smaller. In a multinational study of 631 smokers and nonsmokers, the cut point for total NNAL between smokers and nonsmokers was 47.3 pg/ml (96). It is likely that occasional nonsmokers, with heavy SHS exposure, may exceed this cut point, but most nonsmokers fall well below this limit (96).

Since TSNAs are found in the tobacco leaf and also form during combustion, they are also delivered via other tobacco products, such as chewing tobacco, cigars, and pipes. Relatively high TSNA exposure has been reported among some smokeless tobacco users (115) and may contribute to the increased oral cancer risk associated with the use of smokeless tobacco products. Purified nicotine, such as that found in nicotine patches, gums, and lozenges, should contain no TSNA. However, NNN has been found in the urine of users of some oral NRT products, possibly through endogenous formation by nitrosation of nornicotine (241). Additionally, studies have shown that some e-cigarettes and e-cigarette liquids provide low-level nitrosamine exposure through their aerosols (99, 147). However, urinary NNAL concentrations decrease in smokers who switch to e-cigarettes (97, 203), and urinary NNAL concentrations in sole users of e-cigarettes are 1–10% of the concentrations seen in cigarette smokers (113, 226, 268) (e.g., 1.47 pg/mg creatinine vs. 53.4 pg/mg) (226).

Concentrations of TSNA in tobacco can be reduced by selecting specific types of tobacco and by modifying the curing and manufacturing process. Lower TSNA deliveries have been reported for Swedish Snus (a smokeless tobacco product that uses tobacco with relatively low TSNA content), the Omni "reduced carcinogen" cigarette, and a medicinal nicotine patch (107). The authors noted, however, that the patch delivered significantly lower TSNA than any of the other recreational products studied, and only trace levels of NNK have been detected in a typical nicotine patch (243). The specificity of NNAL to tobacco and its role as a human carcinogen make it a fundamental marker for any evaluation of a new tobacco or nicotine delivery product.

VOC METABOLITES IN URINE

VOCs are a diverse group of chemicals that are abundant in tobacco product emissions and in the atmosphere, even where no one is smoking (73, 74, 78, 94, 260). Many VOCs are formed by incomplete combustion of organic materials, and tobacco is not the only source of exposure. VOCs are also present in foods and beverages. In addition to exogenous sources, VOCs, such as acrolein, are generated by endogenous processes, such as inflammation and lipid peroxidation. Acrolein is a product of the reactions catalyzed by myeloperoxidase (10), and it is also generated as a result of lipid peroxidation reactions (259). Given that smoking increases both oxidative stress and inflammation, the measured levels of acrolein exposure biomarkers likely reflect a combination of inhaled acrolein from tobacco smoke (197) and endogenous inflammatory responses and lipid peroxidation. Hence, measurements of VOC metabolites in the urine provide a somewhat nonspecific estimate of exposure to tobacco products. Nevertheless, the levels of many VOCs and VOC metabolites are elevated in smokers' urine compared with nonsmokers (48, 77, 168, 238). Concentrations of VOCs, such as acrolein and crotonaldehyde, are up to two orders of magnitude higher in cigarette smoke than in ambient air (78). Therefore, many VOC metabolites are found at background levels in all urine samples, and cigarette smoking increases these exposure biomarkers above that background.

Several VOCs in tobacco smoke, including acrolein, benzene, and 1,3-butadiene, are high-priority chemicals on the U.S. Food and Drug Administration's list of harmful and potentially harmful tobacco product constituents (261b). Acrolein can cause cardiovascular and lung damage. Benzene is a human carcinogen (International Agency for Research on Cancer, Class 1A) known to cause leukemia. 1, 3-Butadiene is also a human carcinogen. Acrolein forms during heating of glycerol (glycerin) or glycerol-derived fats (e.g., triglycerides), making it of particular interest for e-cigarettes, which commonly use glycerol ("vegetable glycerin") in their e-liquids (240). Benzene exposure from hookah use may be higher than from cigarette smoking, possibly due to the burning charcoal generally placed on top of the moist fruit–tobacco mixture (128).

A number of harmful VOCs can be measured directly in human blood, urine, and breath (13, 43, 100). Furthermore, many toxic VOCs are metabolized to forms, such as mercapturic acids, that are useful biomarkers of exposure (8). Some mercapturic acid biomarkers that are useful in tobacco studies are listed in Table 3. The acrylonitrile metabolites 2-cyanoeth-

Table 3. *Volatile organic compounds and their biomarkers*

VOC	Biomarker	Abbreviation
Acrolein	3-Hydroxypropylmercapturic acid	3-HPMA
Acrylamide	2-Carbamoylethylmercapturic acid (acrylamide mercapturic acid)	AAMA
Acrylonitrile	2-Cyanoethylmercapturic acid	CNEMA, CYMA
Benzene	Phenylmercapturic acid	PMA
1,3-Butadiene	*N*-Acetyl-*S*-(4-hydroxy-2-buten-1-yl)-L-cysteine	MHBMA-3
Crotonaldehyde	3-Hydroxy-1-methyl-L-propylmercapturic acid	HMPMA
N,*N*-Dimethylformamide	*N*-Acetyl-*S*-(*N*-methylcarbamoyl)-L-cysteine	AMCC
Ethylbenzene	Phenylglyoxylic acid	PGA
Ethylene, ethylene oxide	2-Hydroxyethylmercapturic acid	HEMA
Methylating agents	Methylmercapturic acid	MMA
Propylene, propylene oxide	2-Hydroxypropylmercapturic acid	2-HPMA
Styrene	*N*-Acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteine + *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteine + mandelic acid	PHEMA, MA
Xylene	*N*-Acetyl-*S*-(2,4-dimethylphenyl)-L-cysteine, methylhippuric acids	DPMA, 2MHA, 2MPHA, 4MPHA

ylmercapturic acid and/or *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine are highly selective biomarkers of smoke exposure that effectively assess toxic acrylonitrile exposure and serve as a surrogate measure for smoke exposure (128, 135, 180, 219, 222, 223). Nevertheless, it is important to remember that several VOCs, such as acrolein and crotonaldehyde, and their metabolites are highly reactive and readily form covalent adducts with cell constituents, such as proteins, DNA, lipids, and carbohydrates, and therefore, are retained in tissues for extended periods. Consequently, the absence of urinary metabolites of VOCs, especially at low levels of exposure, cannot be taken to indicate absence of exposure. Conversely, because VOCs, such as acrolein, can also be generated by inflammation (10) and oxidative stress (259), the presence of tissue acrolein–protein adducts in smokers may not be entirely attributable to exposure from tobacco smoke.

Analytical Methods

Characterization and quantitation of VOC mercapturates usually require chromatographic separation—both GC and LC have been used—followed by MS analyses. GC-MS is especially useful for the detection and quantitation of low molecular weight VOCs and VOC metabolites, such as short-chain carboxylic acids, some mercapturic acid conjugates, phenols, and alcohols. These analytes are usually detected after extraction and derivatization (79, 142, 148, 155, 214, 219, 256, 262). The development of ultrahigh performance LC and highly sensitive MS detectors with ultrahigh scan speeds can eliminate the need for sample derivatization, enhance sensitivity, and decrease assay time ($<$10 min). These advances have led to the development of a new generation of multimetabolite, high-throughput assays, such as the one used by the Centers for Disease Control and Prevention for detection of 28 VOC metabolites (8).

With advances in MS technology and especially the advent of high-accuracy and high-resolution MS time-of-flight and Orbitrap mass analyzers, the monitoring and identification of tens or even hundreds of compounds in one chromatographic run are becoming a possibility. These new methods have enabled the discovery of new mercapturates (140, 270, 271). New assays are required to characterize and quantify signature metabolites of emerging tobacco products, such as e-cigarettes, cigarillos, and hookahs, as well as the flavoring reagents used in these products.

PAH METABOLITES IN URINE

PAH formation results from the incomplete combustion of organic compounds, including tobacco, during smoking. The lower molecular weight PAHs, comprising two or three aromatic rings, occur mostly in the gas phase of tobacco smoke and appear noncarcinogenic, except naphthalene. However, a number of the higher molecular weight PAHs and their alkyl derivatives, which occur mainly in the particulate matter of tobacco smoke, are strong carcinogens and are considered to be major factors in the development of lung cancer (13), due to their conversion to reactive metabolites that form DNA adducts. Exposure to both mainstream and sidestream tobacco smoke is associated with increased risk of lung cancer, cardiovascular disease, and chronic obstructive pulmonary disease. Because tobacco is often dried and cured using fire and smoke, smokeless tobacco products also contain PAHs. The quantitative pattern of PAH exposure can differ for different tobacco products, such as cigarettes vs. water pipes, as discussed later. Yet, PAHs are not specific to tobacco, and exposures also come from air pollution, food, and the workplace. Adducts of PAHs with DNA or proteins have been measured as carcinogen exposure biomarkers. Yet these PAH adduct biomarkers give only nonspecific information about the source of carcinogens and are difficult to measure, often yielding low or negative numbers (111).

PAH biomarkers of tobacco smoke exposure that are commonly measured include 1-hydroxypyrene (1-HOP); 1-, 2-, and 3-fluorenols; 1- and 2-naphthols; monohydroxyphenanthrenes; and 1,2,3,4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (Fig. 4) (284, 285). Tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (284) and hydroxylated metabolites of methylnaphthalenes (MeNs) (160) have also been reported in smokers, but the potential toxicity and health effects of MeNs are not well studied (160). PAH metabolites are found in biofluids, mainly in the conjugated (glucuronide and sulfate) forms, and deconjugation is typically performed before quantitative analysis. Table 4 shows levels of various PAH metabolites measured in U.S. smokers and nonsmokers.

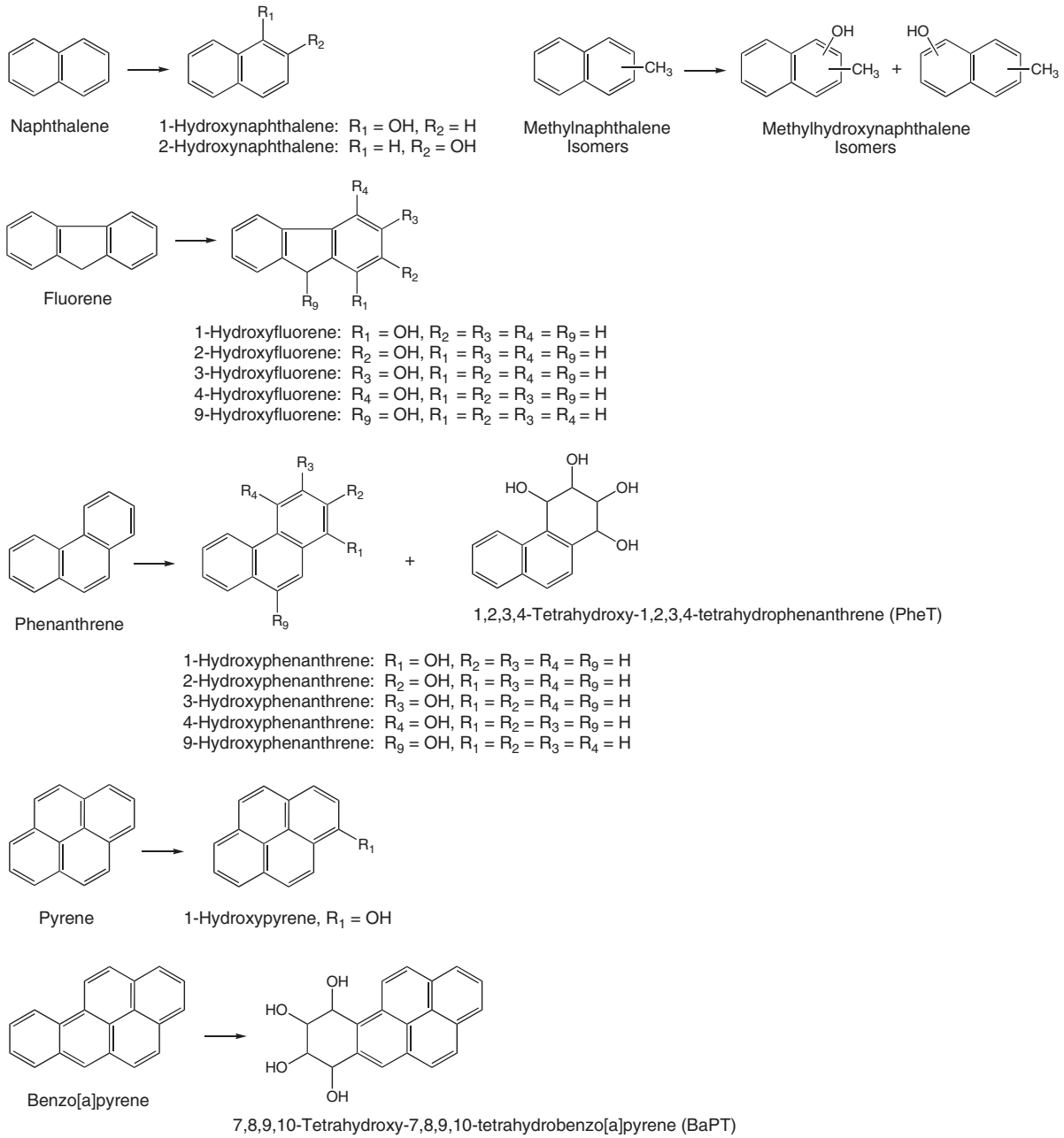

Fig. 4. Main PAH metabolites measured as biomarkers in urine of tobacco smokers.

1-HOP has been most commonly used as a PAH biomarker of tobacco smoke exposure. It is a four-member aromatic ring compound found predominantly in the particulate phase and is thought to be the best surrogate biomarker for the potent carcinogen, benzo[a]pyrene, a PAH, present in extremely low levels and whose metabolite biomarkers are difficult to measure in urine. Several studies have shown increased concentrations of 1-HOP in smokers; after smoking cessation, levels of urinary 1-HOP were reduced by 50% (50). 1,2,3,4-Tetrahydroxy-1,2,3,4-tetrahydrophenanthrene is also increased in smokers' urine compared with nonsmokers (282). In one study, levels of urinary MeNs were elevated 37-fold in smokers, on average (160), but larger cohort studies are needed to confirm this finding.

2-Naphthol and hydroxylated fluorenes appear more specifically related to smoking exposure and nicotine intake than 1-HOP, having correlation coefficients of 0.66 and 0.71, respectively, with urine nicotine equivalents, and are the best PAH measures for daily smoke exposure (236). Naphthalene metabolites in smokers are present in the highest concentration, in the >10-ng/ml range and may be 5–10 times higher than levels in nonsmokers. Levels of fluorenols are lower but were found to have the highest probability of predicting smokers from nonsmokers in both the United States and Poland (236). However, fluorene is not known to be carcinogenic and is not metabolized to diol epoxides, which are often highly potent proximate carcinogens derived from higher molecular weight PAHs. Fluorene's predictive value for smoke exposure does

Table 4. *PAH metabolites in urine from smokers and nonsmokers (pg/ml)*

Biomarker	Smokers		Nonsmokers	
	Least-Squares Geometric Means	95%Confidence Interval	Least-Squares Geometric Means	95%Confidence Interval
1-Hydroxypyrene (1-HOP)	104	91–119	40	35–46
1-Hydroxynaphthalene (1-NAP)	6,293	5,570–7,111	1,523	1,367–1,697
2- Hydroxynaphthalene (2-NAP)	8,597	7,325–10,090	1,682	1,466–1,931
2-Hydroxyfluorene (2-FLUO)	990	871–1,125	236	211–265
3-Hydroxyfluorene (3-FLUO)	592	516–679	90	81–100
9-Hydroxyfluorene (9-FLUO)	342	310–377	200	174–231
1-Hydroxyphenanthrene (1-PHEN)	193	170–219	132	117–150
2-Hydroxyphenanthrene (2-PHEN)	88	75–103	48	38–57
3-Hydroxyphenanthrene (3-PHEN)	194	167–224	91	81–102
4-Hydroxyphenanthrene (4-PHEN)	53	40–70	39	28–54
9-Hydroxyphenanthrene (9-PHEN)	88	77–102	26	23–30

not, therefore, predict cancer risk, unless it is determined that these lower molecular weight PAHs are produced in the same ratio during smoking, as the higher molecular weight carcinogenic PAHs.

Because PAH metabolites are not specific for tobacco smoke exposure, their main use is in distinguishing the use of combusted vs. noncombusted tobacco products and in characterizing different types of combusted tobacco products. For example, pyrene metabolite levels are higher and naphthalene and fluorene metabolite levels are lower in water-pipe users than in cigarette smokers, presumably reflecting the contribution of the burning charcoal in the water pipe (128). Similarly, different PAH profiles were observed between cigarette smokers in the United States and in China, presumably reflecting differences in both tobacco blends and environmental exposures (24).

There are several limitations on the use of PAH metabolite measurement to assess tobacco smoke exposure. First, the environmental sources of PAH exposure may vary across regions and countries, resulting in regional and national differences between nonsmokers' and smokers' test values. Additionally, the ratio of lower to higher molecular weight PAHs varies with region and type of tobacco-filler composition (236). Second, there are no clear-cut points for PAH metabolites that can differentiate between smokers and nonsmokers and are generalizable across regions. For example, optimal cut points were found to be at least two times higher for Polish than for U.S. samples, because Polish subjects were exposed to higher background levels of PAHs (236). Regional, normal ranges are therefore necessary to establish baseline background exposure. Third, since PAH metabolites have short $t_{1/2}$ (4–10 h), metabolite levels in a spot urine sample reflect the time interval between the subject's last smoking episode and urine collection (236). Fourth, concentrations of urinary PAH biomarkers are generally low in picograms/milliliters.

Because hydroxylated PAH metabolites are excreted mainly as conjugates, PAH exposure is generally quantified after de-conjugation with β-glucuronidase. Because of the complexity of the urine matrix and the low concentrations of PAH metabolites present, extractive clean-up procedures are generally necessary. Mass spectrometric methods with stable isotope-labeled internal standards provide the greatest sensitivity, precision, and specificity and are most commonly used. An exception is 1-HOP, for which HPLC with fluorescence detection has provided satisfactory results (52, 59).

Currently, the most commonly used instrumental methods are GC-MS (51, 103, 116, 212) and LC-MS/MS (129, 186, 205, 281). Derivatization is necessary for GC-MS methods, and generally, trimethylsilyl derivatives have been used (51, 103, 116, 212). High specificity and sensitivity in GC-MS analysis have been achieved by using an accurate mass/high-resolution mass spectrometer (212). Derivatization has been used in LC-MS/MS analysis to increase the sensitivity and specificity (129). Specific urinary biomarkers of tobacco exposure, such as TNEs and NNAL, can be correlated with the nonspecific PAH metabolite biomarkers and used as a measure of tobacco exposure.

METALS

Tobacco plants readily absorb metal ions and compounds from the soil. The amount of metals absorbed is influenced by the concentrations of metals in the soil and in soil amendments, such as phosphate fertilizers, animal waste, or sewage sludge (2, 15, 183). Cadmium is a toxic and carcinogenic metal that is absorbed from the soil by tobacco plants and is, therefore, present at high concentrations in mainstream tobacco smoke (192, 261a). Cadmium levels are higher in the blood and urine of current smokers than in former smokers or nonsmokers (1, 108, 176, 196, 207). During combustion, cadmium is reduced to the neutral form and is transported through the cigarette rod in the gas phase before condensation (16, 191). Pulmonary elimination of cadmium after inhaling tobacco smoke is slow. The biological $t_{1/2}$ of cadmium is 14–23 yr (91, 189, 247). Its slow elimination from the lungs after inhalation exposure from combustible tobacco products is consistent with exposure to cadmium in the poorly soluble, neutral form. It is measured in blood and urine using inductively coupled plasma MS (108, 178, 207, 230). Toxicokinetic studies suggest that cadmium concentration in blood reflects recent exposure (1, 136), whereas its concentration in urine reflects chronic exposure (1, 211). However, cadmium is a nonspecific biomarker of tobacco exposure, because cadmium in human specimens may also reflect dietary and occupational exposures. Cadmium levels increase with age, and the average urinary cadmium concentration for nonsmokers in the 1988–2004 NHANES was 0.21 ng/ml (253). For smokers, it was 0.40 ng/ml, and values above 5 ng/ml were associated with the occupational history of working with metal.

With few exceptions, exposure to toxic metals from noncombustible-inhaled tobacco products, such as e-cigarettes, is likely to be lower than for combustible products. In e-cigarettes, the aerosolization temperature is lower than combustion

Fig. 5. Light microscope image of the nichrome heating element coiled around the vitreous fiber wick of an e-cigarette before (*left*) and after (*right*) use. Note the evidence of thermally decomposed organic substances and fragmented vitreous fibers in the vicinity of the heating element.

temperature. These temperatures are not high enough to cause evaporation of metals from the heating element. However, any metal in the e-liquid may be sputtered and entrained in the heated aerosol as the heating element boils the liquid from the wick. Moreover, the tobacco extract used as a source of nicotine in e-juices is not the complete tobacco matrix. Temperatures between 200 and 300°C are sufficient to aerosolize propylene glycol or glycerol—the most common e-cigarette solvents. However, the temperatures of the heating element are sufficient to cause thermal decomposition of some extract and solvent constituents, which may, in turn, contribute to e-cigarette users' exposure (Fig. 5) (81). E-cigarettes components—including exposed wires, wire coatings, solder joints, electrical connectors, heating element material, and vitreous fiber wick material—constitute the second major source of inorganic toxicants to which e-cigarette users may be exposed. Figure 5 shows an example of an e-cigarette's nichrome heating element wrapped around a vitreous fiber wick. Thermal decomposition of some substances and possibly heat-induced breakage of the wick fibers are apparent in the vicinity of the heating element after use.

Figure 6 shows corrosion on a brass electrical connector from an e-cigarette. The corrosion is reflected in the copper- and zinc-containing particulate in e-liquid that was trapped with polytetrafluoroethylene filters (Fig. 7). This source might account for the elevated levels of copper and zinc in the aerosol produced by some e-cigarettes (278). Similarly, a tin solder joint could undergo corrosion (Fig. 8), leading potentially to elevated levels of tin in some e-cigarette liquids (278).

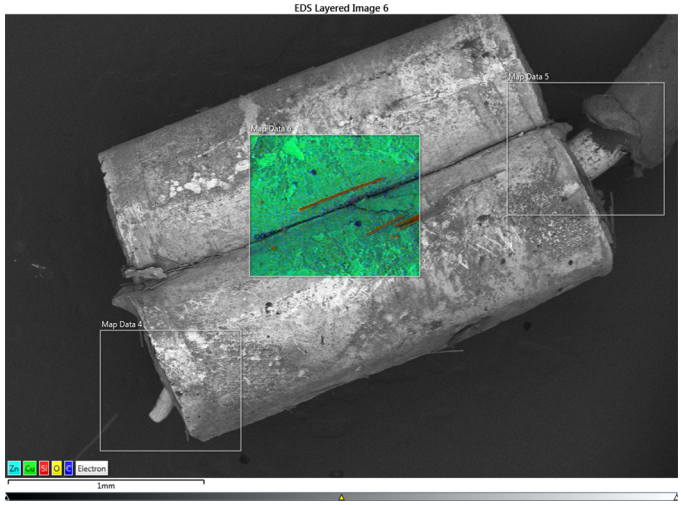

Fig. 6. Zinc and copper corrosion has apparently occurred on the surface of this brass electrical connector from an e-cigarette. [Image obtained using scanning electron microscopy–energy dispersive spectroscopy (EDS).] The orange-colored fibers are fragments of the vitreous wick fibers composed of silicate.

EXHALED CO

CO is formed by incomplete combustion of organic materials and is prevalent in the environment at low concentrations due to its presence in motor vehicle exhaust. However, high CO levels are generated during tobacco combustion, making exhaled CO a useful and validated marker for identifying individuals who have recently used a combustible tobacco product. Exhaled CO can be measured easily and reliably by asking study participants to blow into a portable CO monitor after holding their breath for 15 s. The concentration of exhaled CO [measured in parts per million (ppm)] correlates well ($r > 0.95$) with the concentration of carboxyhemoglobin in blood (percent hemoglobin saturation), and so measurement of exhaled CO has become a standard method for assessing recent smoking (25a, 272).

Although CO is produced in the human body (largely via heme-oxygenase-1) and therefore, can affect both blood carboxyhemoglobin and exhaled CO (227), the contribution of biologically generated CO to exhaled CO is small (in the range of 1–5 ppm) relative to the increase in exhaled CO directly attributable to active smoking (typically in the range of 7–60 ppm). For example, Cheng et al. (57) reported on health correlates of exhaled CO in the Framingham community sample. In that group, although approximately one-third included smokers, 78% of those with an exhaled CO $>$ 5 ppm were self-reported tobacco smokers, whereas only 10% of them were never smokers (of whom some could have been misreporting their smoking). Studies that have confirmed abstinence from smoking using cotinine have found that $<$3% of recent ex-smokers have an exhaled CO $>$ 4 ppm. Based on these data, some investigators have recommended that the cutoff for exhaled CO should be lowered to 4 ppm to confirm smoking abstinence (70), although values of 6 ppm continue to be used (see below).

Cut Points

Smoking cessation. The $t_{1/2}$ of exhaled CO varies from 2 h in someone actively exercising (e.g., jogging) to 8 h in someone sleeping, with the average of 4 h (25a). The initial CO cut point for verification of self-reported abstinence in a smoking cessation study was 8–10 ppm, and CO $<$ 10 ppm has been

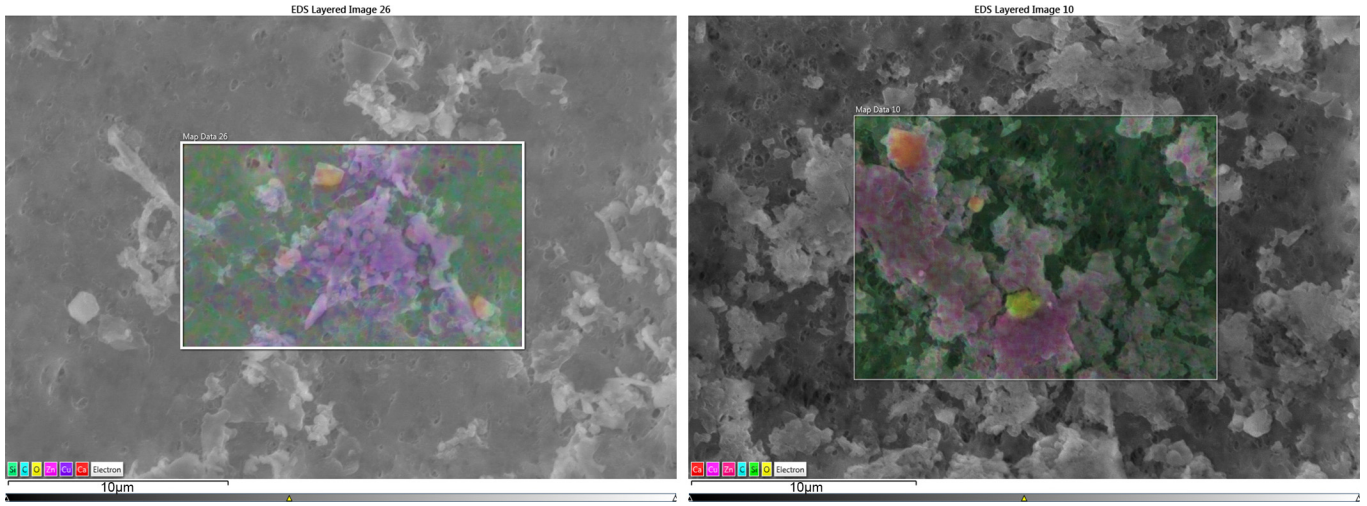

Fig. 7. Piles of fine and nano-sized particles obtained by filtration of the liquid from an e-cigarette before (*left*) and after (*right*) use. The violet and magenta colors represent copper and zinc in the particulate. The orange and yellow–green particles are calcium silicate and silica particles.

widely used in smoking cessation clinical trials (typically to verify self-reported smoking abstinence for the past 7 days). However, ambient indoor CO levels have decreased as smoking prevalence and SHS exposure have fallen over the past two decades. Many studies now suggest that CO $<$ 6 ppm (169) may be an optimal cut point to verify self-reported smoking cessation for at least 1 wk (Table 5), and this value is now being used in clinical trials (17, 216).

Because it is readily measured, exhaled CO is frequently used in tobacco research to confirm smoking status. Both 8 h "overnight abstinence" and 12 h abstinence are common in studies that do not require zero blood nicotine concentrations at baseline. If zero blood nicotine is necessary, then 16–24 h abstinence may be necessary, as the average nicotine $t_{1/2}$ can reach 4 h in slow metabolizers. Without real-time blood nicotine measurement, however, it is difficult to decide which exhaled CO cut point to use to verify abstinence compliance. For example, many compliant volunteers will likely have exhaled CO $>$ 6 or $>$ 10 ppm after overnight abstinence, partly due to the longer $t_{1/2}$ of CO during sleep. If an afternoon baseline CO measurement (with normal smoking) is available, then a verification cut point at least 50, 60, or 70% lower for overnight (8 h) and 12 and 24 h abstinence is reasonable. If no baseline smoking CO measure is available, then absolute cut points of $<$16, $<$12, and $<$10 ppm are reasonable to verify overnight (8 h) and 12 and 24 h of smoking abstinence. Although exhaled CO does not offer "perfect" validation of short periods of smoking abstinence, these guidelines may be practical for laboratory studies. If saliva or urinary nicotine is also measured, then researchers can exclude subjects with unacceptably high nicotine concentrations.

Fig. 8. A tin solder connection on a battery of an e-cigarette appears to have undergone some corrosion.

Exhaled CO As An Estimate of Smoke Inhalation

A measurement of exhaled CO is generally regarded as a valid and reliable estimate of recent smoke inhalation. However, the measure increases by 1–8 ppm with every cigarette smoked and then immediately starts falling, with an average $t_{1/2}$ of 4 h. Thus the time of day and time since last smoke are highly relevant, with afternoon or evening measurement preferable to early morning. Moreover, in a repeated-measures study, it is preferable to repeat CO measurements at the same time of day and at the time of measurement to record how many cigarettes a subject has smoked that day and the time since the last smoke. With regular smoking, afternoon CO measurements correlate well with intake of nicotine and other tobacco toxicants (141). As research participants may be exposed to a range of tobacco/nicotine products, as well as nontobacco sources of inhaled CO (e.g., smoked marijuana) (181, 206), it may be advisable to exclude recent users of products, other than the product of interest, to enable greater confidence in the source of measured CO (and nicotine).

CONFOUNDING EXPOSURES FOR NONSPECIFIC BIOMARKERS

Metabolites of VOCs, PAHs, exhaled CO, and metal are not specific to the use of and exposure to tobacco products. Although the smoking of tobacco products causes characteristic increases in the concentrations of these biomarkers, diet and

Table 5. *Validated biological cut-point values separating smokers and nonsmokers*

Biomarker	Specimen	Concentration
Carbon monoxide	Breath	6 parts/million (169)
Cotinine	Urine	31 ng/ml (96)
Cotinine	Saliva/blood	3 ng/ml (21)
Cotinine	Nasal lavage fluid	1 ng/ml (188)
NNAL	Urine	47.3 pg/ml (96)
NNAL-to-cotinine ratio	Urine	0.74×10^{-3} (96)

occupational and recreational exposure to smoke, vehicle exhaust, and welding fumes can also cause increases in these biomarkers (7, 18, 135, 162, 202, 217, 275, 276). With the measurement of nonspecific biomarkers of exposure to tobacco and nicotine delivery products, it is very helpful to collect questionnaire data on recreational, environmental, and occupational exposure to smoke, exhaust, dust, and metal fumes.

The use of cannabis (marijuana) is an important potential, confounding exposure for VOCs, PAHs, CO, and metals. Cannabis can be smoked, heated, and aerosolized; used as a concentrate in an e-cigarette; or consumed orally. The prevalence of cannabis use is higher among smokers than among nonsmokers (204, 206, 245). The compounds in cannabis smoke are similar to those in tobacco smoke, except for differences in the concentrations of nicotine, TSNAs, and cannabinoids (181). The smoking of cannabis may cause increases in nonspecific biomarkers of combustion aerosol exposure, including CO and metabolites of VOCs and PAHs. The aerosolizing of cannabis and the use of cannabis extracts in e-cigarettes may also cause increases in metabolites of VOCs and PAHs. The transfer of metals during the use of cannabis products has not been studied sufficiently. The screening of subjects in tobacco and nicotine delivery studies for biomarkers of exposure to cannabis can improve the interpretation of results from these nonspecific biomarker tests, reduce the prevalence of anomalous findings, and improve data quality.

There are two federal cut-point concentrations for cannabis metabolites: one for the initial immunoassay test (50 ng/ml) and a second for confirmation of a positive initial test, usually by GC- or LC-MS (15 ng/ml) (75a). These concentrations do not correlate with intoxication. The initial 50-ng/ml test may be performed using an OTC test or by submitting a specimen to an accredited testing laboratory. The OTC tests are usually less expensive and offer low levels of false-positive and -negative results (71).

Tetrahydrocannabinol (THC), the primary psychoactive compound in cannabis, is lipophilic, so the $t_{1/2}$ for elimination of the primary metabolite of THC (delta-9-THC-9-carboxylic acid) can be long. People who smoke cannabis more than once a day, every day, can test positive at the initial federal cutoff (50 ng/ml) for over 24 days after cessation (165). In infrequent users, a single use will not be detectable after 3–4 days (54). Likewise, even very heavy secondhand exposure to cannabis smoke is unlikely to result in a positive drug test after 12 h (66).

When a study participant smokes both tobacco and cannabis, analysis of cotinine and NNAL will yield data that can quantify tobacco use. Where spectrophotometric analysis of THC metabolites is available, it is also possible to quantify cannabis use. However, in dual users, it is not possible to apportion the sources of CO, VOCs, and PAHs. In individuals who use e-cigarettes with nicotine and cannabis regularly, the expected pattern would be cotinine above 30 ng/ml in urine and THC metabolite levels between 20 and 60 ng/ml.

POTENTIAL BIOMARKERS OF E-CIGARETTE USE

E-cigarettes are electrically powered devices that heat and aerosolize a flavored liquid to produce an inhalable aerosol without combustion (42, 45). Most e-cigarettes contain nico-

Fig. 9. Example of e-cigarette (ECIG) system and parts.

tine. First-generation e-cigarettes (cig-alikes) are frequently disposable and resemble combustible cigarettes but generally deliver less nicotine (42). Second-generation e-cigarettes (e.g., "tank-style," "vape pens," "e-Gos") are refillable, have easily assembled components, are usually cigar sized, and are more likely rechargeable than disposable (163). The nicotine delivery is more similar or in some cases, higher than a combustible cigarette (80, 85, 237). Third-generation devices (e.g., "mods," "rebuildables," or "advanced personal vaporizers") come in a large array of customizable formats that generally include stronger batteries, variable voltage, low amperage coils, and refillable tanks for e-liquid (Fig. 9) (75, 249). These combinations affect toxicant emissions in the e-cigarette aerosol (93, 95, 149, 283).

E-cigarette solution, known as e-liquid, can be a mixture of propylene glycol and glycerol (typically, 75:25) or glycerol alone, water, tobacco–nicotine extract, and flavorings. Flavorings can include menthol, sugars, esters, and pyrazines (161). More than 7,760 flavored e-liquids are now available (255, 287). E-liquid can also be contaminated with alkaloids other than nicotine, carbonyls, VOCs, PAHs, TSNAs, and metals (58, 127).

E-liquid is a starting point for the composition of e-cigarette aerosol, but the aerosol is the source of human exposure and thus, potential biomarkers. The physical composition of the aerosol can be altered by many factors: the temperature of the metal coil, rate of e-liquid flow through the heated coil, chemical composition of the coil, the coil connection to the power source, the wicking material transporting e-liquid, and the hot aerosol contacts.

It is challenging to identify exposure biomarkers specific to e-cigarette use because many e-liquid components are also found in common foods and personal care products. For example, the flavorings used in e-liquids are also used in many foods. Similarly, propylene glycol and glycerol are in many baked goods, beverages, sauces, soaps, lotions, and toothpaste. The body metabolizes propylene glycol to D- and L-lactic acid. Whereas L-lactate is a normal, endogenous metabolic product, production of D-lactate is characteristic of propylene glycol exposure, absent other causes of D-lactate acidosis (61). The extent to which plasma D-lactate is increased following frequent e-cigarette use is unknown and worthy of further study. Glycerol is an endogenous constituent in the synthesis and catabolism of triglycerides. Nicotine and its metabolites are present in the body fluids of consumers of all tobacco products, including nicotine-containing e-cigarettes.

During e-cigarette use, propylene glycol and glycerol can react by dehydration and oxidation pathways to yield methyl glyoxal (201, 232, 246); glyceraldehyde (60); propylene oxide (154); glycidol (190); dioxolanes (69, 76, 125); and oxalic, lactic, and pyruvic acids (156, 257), as shown in Figs. 10 and 11. In addition, the heating of e-liquids creates low molecular weight carbonyls (formaldehyde, acrolein, acetaldehyde) and reactive oxygen species (19, 99, 120, 233). However, all of these chemicals are also found in cigarette smoke (248). As discussed earlier, at this time, the best way to identify use of nicotine-containing e-cigarettes is to confirm the presence of cotinine and the absence of other biomarkers for the use of combustible tobacco products. It is also necessary to exclude the use of NRT.

In comparison with cigarette smoke, e-cigarette aerosols contain undetectable or low concentrations of TSNAs (84, 147). The levels of TSNAs, such as NNAL, are expected to be lower in e-cigarette users than in smokers or oral tobacco users. Likewise, levels of PAH metabolites and some VOC metabolites should be lower in e-cigarette users than in smokers. Some less-than-specific constituents of e-cigarettes and/or their metabolites—including nicotine and menthol (as menthol glucuronide)—may still be suitable as biomarkers of e-cigarette use in well-characterized subjects whose prior exposure to non-e-cigarette nicotine sources is known and can be controlled and baseline concentrations established.

Flavor Biomarkers

Flavored noncigarette tobacco products are widely available, and they may have particular appeal to youth (9, 68). Among e-cigarette users who responded to an online survey, fruit flavors are most preferred, and the most prevalent reason for e-cigarette is the belief that they might be less harmful than cigarettes (36). These results suggest that at least some users are attracted to the various e-cigarette flavorings available and have an unsubstantiated perception of the relative safety of these products. Health effects of these flavorant additives are not well understood. E-cigarette flavor compounds include eucalyptol, camphor, menthol, methyl salicylate, pulegone, ethyl salicylate, cinnamaldehyde, eugenol, diphenyl ether, and coumarin (161). Except for the menthol biomarker, menthol glucuronide, biomarkers for potentially toxic flavorants—including cinnamaldehyde and cherry flavorants—have not been well studied. Because flavors are often created from a mixture of synthetic compounds, their exact compositions can vary, even among flavors with the same name. For example, different strawberry-flavored products, including cigarettes, e-cigarette liquid, snus, and hookah tobacco, were found to contain different flavoring compounds. These compounds included esters and aldehydes; the terpenes linalool; α-terpineol; nerolidol and limonene; as well as the lactones γ-decalactone, γ-dodecalactone, and γ-undecalactone (194). Several studies using in vitro assays indicate that these flavorants may cause oxidative and inflammatory responses in lung cells (human) and tissues (rodents) (157). Together, these studies indicate that flavored tobacco product users are exposed to potentially harmful chemicals; however, we do not currently have the data to link these inhaled exposures to health effects. Although we now have considerable data on e-liquid constituents and aerosols, the derivation of useful and specific exposure biomarkers from these data remains a challenge because of the ubiquity of propylene glycol, glycerol, and flavorants in consumer products.

TOBACCO EXPOSURE BIOMARKER RESEARCH AND CAPACITY GAPS

Expanding Access to Biomarkers of Exposure

Biomarkers of exposure to tobacco and nicotine delivery products are critical to establish links between tobacco product use and subsequent health effects. These biomarkers allow us to quantify exposure, ascertain dose-response relationships, and in some cases, identify sources of exposure to tobacco product toxicants. Accurate and sensitive biomarkers are essential for characterizing exposure patterns and adverse health

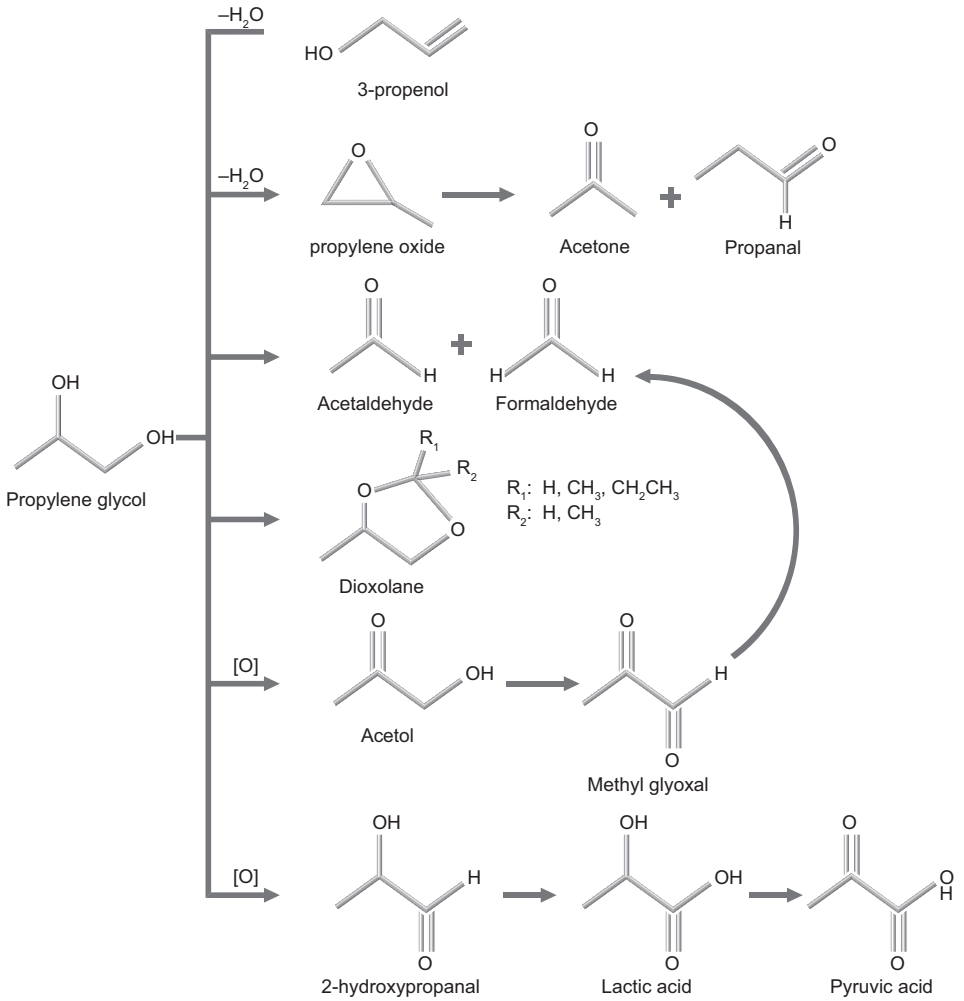

Fig. 10. Degradation pathways and products of propylene glycol.

effects of new and emerging tobacco and nicotine delivery products.

Few laboratories can currently perform the most sophisticated and sensitive assays of all of the biomarkers of exposure to tobacco and nicotine delivery products. Although assays for metabolites of nicotine, NNK, VOCs, and PAHs are necessary to confirm nicotine-containing e-cigarette use and to differentiate between active product use and SHS exposure, this suite of assays is costly and time consuming, especially for large studies and sample sets. Limited laboratory capacity impedes research at a time when the market for tobacco and nicotine delivery products is undergoing rapid change, and information is needed to inform policymaking and regulations. In addition to expanding access to existing assays, new biomarker assays are needed to assess population harm attributable to new and emerging tobacco and nicotine delivery products.

The development of biomarker assays proceeds through distinct stages. First is the identification of new candidate biomarkers. Next is assay development: the painstaking process of finding the optimal way to quantify a biomarker. This effort may include synthesis of the molecule of interest as an internal standard, experimentation with different types of chromatographic techniques, development of methods for preliminary sample purification and/or derivatization, and development of well-characterized control and test specimens to demonstrate specificity and reproducibility.

Once identified, the biomarker must be validated. The process of validation should address the following questions: how well does the candidate biomarker correlate with tobacco and nicotine delivery product use and exposure? If the biomarker does correlate well, is it the best biomarker for exposure to a specific product type or group of toxins, or are there better candidates? Can the assay be improved? Does the assay generate reliable and valid results in samples from different populations? Can cut-point values be determined to distinguish among populations with different use and exposure patterns? As a scientific consensus develops around a particular detection method, the details are disseminated to other research laboratories, which then test the same samples using the same or similar assays and compare findings—so-called interlaboratory testing (37). Concurrence of findings suggests that the assay is valid and reproducible; discordant findings require additional research and investigation. Moreover, interlaboratory validation assures that unavoidable, minor differences in equipment and methods across laboratories do not affect the quality of the results (37).

Currently, we are in the discovery and identification stage for biomarkers of e-cigarettes, little cigars, cigarillos, and

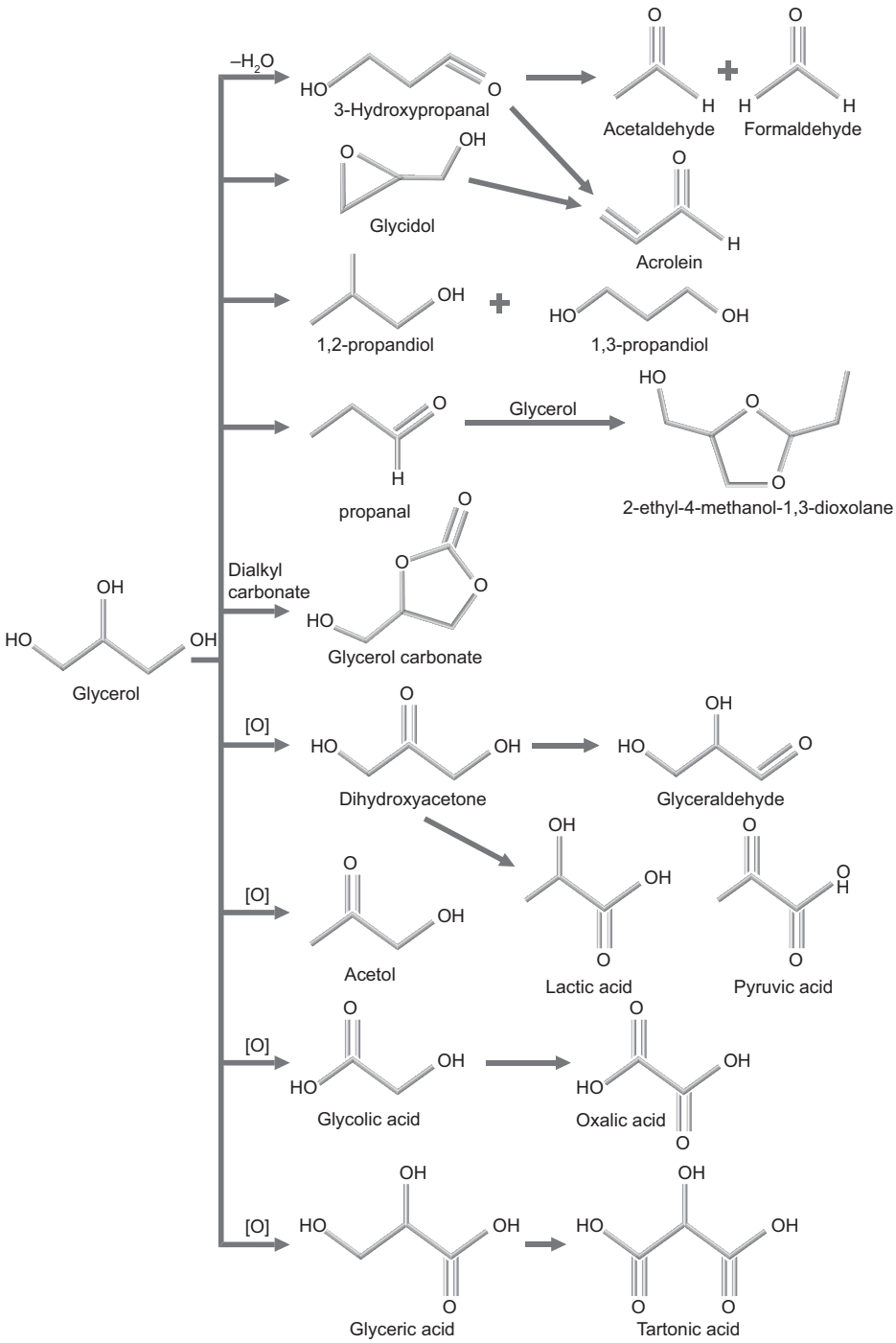

Fig. 11. Degradation pathways and products of glycerol.

water pipes. Validated biomarkers of use and exposure to these tobacco products are urgently needed. Cut-point values distinguishing between active cigarette use and SHS exposure have been identified for serum cotinine (21) and for NNAL (96) (see Table 5). The high-sensitivity LC-MS/MS assay for urinary cotinine is in interlaboratory testing, and the high-sensitivity LC-MS/MS assay for blood cotinine has completed interlaboratory testing and been published (37). The candidate biomarker, NNAL, appears to be the optimal biomarker for TSNA exposure, and many laboratories are using the assay validated for distinguishing between active smoking and SHS exposure to test samples from populations with different product use and exposure patterns. Several laboratories are in the early stages of validating biomarkers of VOC and PAH exposure (debating the best biomarkers, refining methods, and comparing results from different sample sets).

The measurement of nicotine exposure in smokers can be costly (Table 6). Laboratories with limited throughput may be able to analyze these samples at a lower cost but often have longer turnaround times, slowing study progress. Studies with large sample sets routinely face long turnaround times. In addition, researchers often need to measure several biomarkers

Table 6. *Assay sensitivity, cost, and availability*

Biomarker	Specimen	Method	LLOQ	Cost Per Sample, $	Availability
Exhaled CO	Breath	Portable instrument	0.5 ppm	200–3,000 for instrument[a]	Commercial
Cotinine	Urine	OTC kit	NA (200 ng/ml[b])	0.50–5.00	Commercial
Nicotine + metabolites	Urine	GC-MS	2 ng/ml	120–200	Commercial
Nicotine + metabolites	Blood	GC-MS	2 ng/ml	180–300	Commercial
Nicotine and cotinine	Urine, saliva, blood	GC-NPD	1 and 10 ng/ml, respectively	50–100	Academic/govt.[c]
Nicotine	Urine, saliva, blood	GC-MS/MS	0.2 ng/ml	75–200 (for nic + cot)	Academic/govt.[c]
Cotinine	Urine, saliva, blood	GC-MS/MS	2 ng/ml		Academic/govt.[c]
Cotinine	Urine	LC-MS/MS	0.05 ng/ml	100–200 (for cot + 3-OH cot)	Academic/govt.[c]
3-OH cotinine	Urine	LC-MS/MS	0.1 ng/ml		Academic/govt.[c]
Total nicotine equivalents	Urine	LC-MS/MS	Not applicable	180–300	Academic/govt.[c]
NNAL	Urine	LC-MS/MS	0.25 pg/ml	180–300	Academic/govt.[c]
Menthol glucuronide	Urine, plasma	LC-MS/MS	Plasma: 4 ng/ml, urine: 500 ng/ml	40–100	Academic/govt.[c]
VOC metabolite panel	Urine	See Table 3	See Table 3	180–300	Academic/govt.[c]
PAH metabolite panel	Urine	See Table 4	See Table 4	180–300	Academic/govt.[c]

Costs and LLOQs are constantly changing. This table was accurate at the time of publication. Please contact the laboratories when you are creating your budget and sample collection plans. The minimum sample volume for most chromatographic assays is 1 ml. NNAL and PAH metabolite assays require 3 ml. However, it is best practice to provide enough volume to allow for losses in pipetting and for repeat testing. Thus the optimal sample volumes are 2.1 ml for most tests and 6.2 ml for NNAL and PAH tests. NPD, nitrogen phosphorous detector; nic, nicotine; cot, cotinine; 3-OH, 3-hydroxycotinine. [a]Cost per test depends on frequency of use after instrument acquisition. [b]As this is a qualitative test, there is no LLOQ [not applicable (NA)]. A positive result is at or above 200 ng/ml. [c]Investigators must contact the laboratory in advance to ascertain availability, cost, and turnaround time for tests at academic and government laboratories.

to create a detailed profile reflecting exposure to new, emerging, and traditional tobacco products. For example, to ascertain whether a study participant uses nicotine-containing e-cigarettes or nicotine-replacement products, but does not use combustible tobacco products, researchers must measure cotinine, NNAL, and/or VOC or PAH metabolites in a single sample. This multivariate analysis can be cost prohibitive but is necessary for an accurate exposure and outcome profile.

The equipment needed for these biomarker analyses is highly sensitive and expensive. Additionally, it must often be housed in a controlled environment and connected to uninterrupted and consistent electrical power. High-quality GC-MS/MS, LC-MS/MS, and ultrahigh performance LC-MS instruments cost hundreds of thousands of dollars and need expensive technical support to install, calibrate, and maintain. Newer instruments offer greater sensitivity and throughput than older systems, but few laboratories have the resources required to acquire and maintain a critical core of instrumentation and staff.

The expansion of access to tobacco and nicotine delivery system biomarker tests requires additional work across the entire continuum of biomarker development. For example, research is needed to investigate potential biomarkers of e-cigarette use, including flavors and the pyrolysis and metabolism products of propylene glycol and glycerol (all typical e-liquid constituents). Laboratories now validating assays for biomarkers, such as VOC and PAH metabolites, need access to more specimens to identify specific biomarkers for combustible products. Interlaboratory NNAL validation testing is also a high priority to distinguish between light and infrequent smoking and SHS exposure—an important distinction as product use patterns change.

Expanded biomarker testing will also expand exposure data sets that improve our ability to link biomarker measurements with the use of specific tobacco products and subsequent health effects, which is critical to inform public health policymaking. To further this effort, we recommend five strategies to assess better exposure to new and emerging tobacco and nicotine delivery products.

Leverage advanced biomarker research. Advanced approaches will yield significant improvements in test sensitivity, selectivity, and throughput—three crucial quality metrics. Advanced instrumentation and innovative sample preparation can improve sensitivity and selectivity. Increased automation in sample prep and data evaluation can drive down costs while increasing throughput, making large population studies feasible to characterize population harm.

Build capacity for established biomarker methods. We must build capacity for established biomarker methods by disseminating information about validated assays to more laboratories, including commercial and hospital/clinical laboratories. This strategy requires additional training and regular interlaboratory testing. With the increase in the number of facilities able to perform biomarker assays, we can increase access and enhance collaboration among researchers toward the common goal of assay improvement.

Educate researchers and policymakers. We must educate researchers and policymakers about the importance of the individual and quantitative exposure data provided by biomonitoring. The generation of such data requires additional resources but is crucial to attribute health effects accurately in populations that are using new tobacco and nicotine delivery products and potentially using more than one product or subject to other recreational or occupational exposures . Increased biomonitoring will help researchers to characterize better exposure and subsequent health effects caused by new and emerging tobacco and nicotine delivery products.

Establish biospecimen repositories and data archives. Establishment of biospecimen repositories and data archives is necessary to support assay development and validation. Large numbers of well-characterized samples are needed to establish biomarker cut-point values and to support optimization of assay protocols. The proposed biospecimen repositories could include samples from well-characterized small studies and from large existing studies, such as NHANES and the Population Assessment of Tobacco and Health study.

Develop and validate OTC kits. OTC kits need to be developed and validated so that that they can detect more reliably

cotinine at the current biological cut-point values, distinguishing smokers from nonsmokers based on both urine and saliva specimens. Such tests might be useful in clinical settings to screen patients for tobacco exposure or to assess response to smoking cessation therapy. Furthermore, they could be administered in research settings to assess rapidly eligibility for experimental studies by determining smoking status. As noted in *Over-the-Counter Cotinine Tests*, there are commercial products that claim levels of sensitivity that meet these needs, but they are not yet validated by use and may need further development to be effective. Affordable, effective OTC kits will vastly increase the pace of research on new and emerging tobacco products.

Biomarkers of exposure to tobacco and nicotine delivery products are critical tools for identifying and quantifying the health effects of tobacco products. The absence of unique biomarkers for new and emerging tobacco products, especially e-cigarettes, is an urgent problem. The implementation of these five strategies will dramatically improve the characterization of harmful and addictive exposures related to tobacco and nicotine delivery products, as well as characterize the resulting health effects of these products on individuals and populations.

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DISCLAIMERS

The findings and conclusions in this report are solely those of the authors and do not represent the official position of the Centers for Disease Control and Prevention, the U.S. Food and Drug Administration, and the U.S. National Institutes of Health (including NCI, NIDA, NIEHS, and NHLBI).

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N. L. Benowitz is a consultant to pharmaceutical companies that market smoking-cessation medications and has been a paid expert witness in litigation against tobacco companies. J. Foulds does paid consulting for pharmaceutical companies making smoking cessation medicines (e.g., Pfizer) and has received a research grant from Pfizer, but there is no conflict between that work and the content of this biomarkers paper.

AUTHOR CONTRIBUTIONS

R.S.P. conceived and designed research; J.T.B. and R.S.P. performed experiments; J.T.B. and R.S.P. analyzed data; J.T.B. and R.S.P. interpreted results of experiments. S.F.S., B.C.B., N.A.S., A.E.H., R.S.P., C.M., G.S.H., and C.M.D. prepared figures; S.F.S., B.C.B., P. Jacob, N.A.S., J.T.B., A.E.H., P. Jatlow, R.S.P., L.W., J.F., A.G., S.S.H., J.C.G., J.R.M., C.M., S.S., G.S.H., R.T., P.K.L., I.A.B., H.L.K., C.M.D., and N.L.B. drafted manuscript; S.F.S., B.C.B., P. Jacob, L.W., S.S.H., R.T., H.L.K., N.L.B., and A.B. edited and revised manuscript; S.F.S. and A.B. approved final version of manuscript.

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