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Urinary Tobacco Smoke Constituent Biomarkers for Assessing Risk of Lung Cancer

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Abstract

Tobacco constituent biomarkers are metabolites of specific compounds present in tobacco or tobacco smoke. Highly reliable analytical methods, based mainly on mass spectrometry, have been developed for quantitation of these biomarkers in both urine and blood specimens. There is substantial inter-individual variation in smoking-related lung cancer risk that is determined in part by individual variability in the uptake and metabolism of tobacco smoke carcinogens. Thus, by incorporating these biomarkers in epidemiological studies we can potentially obtain a more valid and precise measure of *in vivo* carcinogen dose than by using self-reported smoking history, ultimately improving the estimation of smoking-related lung cancer risk. Indeed, we have demonstrated this by using a prospective study design comparing biomarker levels in urine samples collected from smokers many years prior to their development of cancer, versus those in their smoking counterparts without a cancer diagnosis. The following urinary metabolites were associated with lung cancer risk, independent of smoking intensity and duration: cotinine plus its glucuronide, a biomarker of nicotine uptake; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL), a biomarker of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); and *r*-1-,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT), a biomarker of polycyclic aromatic hydrocarbons (PAH). These results provide several possible new directions for using tobacco smoke constituent biomarkers in lung cancer prevention, including improved lung cancer risk assessment, intermediate outcome determination in prevention trials and regulation of tobacco products.

1. Introduction

The tobacco epidemic is responsible for 12% of all deaths worldwide among adults over 30 years of age and is considered the world's leading cause of preventable premature death (1). While tobacco consumption has decreased in the U.S. since 2000 (2), worldwide tobacco consumption has increased by 75% over the past 30 years to 7.4 million metric tons (3). It is

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estimated that there are approximately 1.4 billion smokers worldwide (4). The highly addictive nature of nicotine combined with effective exposure of susceptible target tissues to carcinogens in tobacco smoke distinguish tobacco products from all other commodities as the single greatest cause of cancer-related deaths.

Lung cancer is one of the most commonly diagnosed cancers, and the leading cause of cancer death in the U.S. and worldwide (5, 6). Cigarette smoking is the most important causal factor for lung cancer; an estimated 90% of all lung cancer deaths are attributable to cigarette smoking (7). An estimated 11% of female smokers and 24% of male smokers may die from lung cancer over their lifetime, assuming no competing cause of death (8). Cigarette smoke contains 73 established animal carcinogens, 16 of which are rated as carcinogenic to humans (9). The inter-individual variation in smoking-related lung cancer risk may be determined in part by variability in the uptake and metabolism of tobacco smoke carcinogens, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N'*-nitrosonornicotine (NNN) and polycyclic aromatic hydrocarbons (PAH) (10). While there is no doubt that exposure to these compounds is a major cause of smoking-related lung cancer, we have little ability to predict who among the more than one billion smokers in the world will actually get lung cancer.

This review will discuss the possible future application of tobacco smoke carcinogen and toxicant biomarkers in lung cancer prevention, including improved lung cancer risk assessment, intermediate outcome determination in prevention trials and regulation of tobacco products.

2. Biomarkers Associated with Tobacco Smoking

Our group has developed and applied laboratory methods to quantify urinary tobacco constituents and their metabolites in humans. The quantified constituents include metabolites of nicotine, a highly addictive, non-carcinogenic compound, as well as established carcinogens, such as tobacco-specific nitrosamines, PAH, and volatile organic compounds (VOC). Below are brief descriptions of the specific biomarkers illustrated in Figure 1.

2.1 Nicotine and its metabolites

Nicotine's stimulant effect is a major contributing factor to the dependence-forming properties of tobacco smoking. However, nicotine and its major metabolites are non-carcinogenic. Nicotine is extensively metabolized, primarily in the liver, and its major proximate metabolite is cotinine: on average, 75% of nicotine is converted to cotinine, primarily by the liver enzyme cytochrome P450 2A6 (CYP2A6) (11). Cotinine and cotinine-*N*-glucuronide are metabolites of nicotine; their sum is "total cotinine". Total cotinine is a reliable measure of uptake of nicotine, and widely used as a biomarker of exposure to active and passive tobacco smoking. Cotinine is further metabolized to *trans*-3'-hydroxycotinine (3-HC) by the same liver enzyme CYP2A6. The *in vivo* half-life ($t_{1/2}$) of cotinine is longer (16 hours) than that of nicotine (2 hours) (12). Urinary cotinine concentrations average 4- to 5-fold higher than those in plasma (13), making urine a more sensitive medium for the detection of low-level exposure to tobacco smoke and use of other nicotine-containing

products. Alternatively, urinary total nicotine equivalent, i.e., the sum of total nicotine, total cotinine and total 3-HC, represents the uptake and metabolism of total nicotine, and presumably a better biomarker for the consumption of cigarettes and other nicotine-containing products.

2.2 Tobacco-specific nitrosamines

The tobacco-specific nitrosamines comprise one of the major groups of carcinogenic chemicals in tobacco and cigarette smoke. The formation of tobacco-specific nitrosamines occurs primarily during tobacco curing. Among the seven tobacco-specific nitrosamines found in tobacco, NNK and NNN are considered the most carcinogenic (10). Both NNK and NNN are classified as human carcinogens (Group I) by the International Agency for Research on Cancer (IARC) (14).

NNK is a strong lung carcinogen capable of inducing lung tumors in rodents independent of its route of administration (15). In the rat, the lowest total dose of NNK shown to induce lung tumors was 1.8 mg/kg, with a significant dose-response trend (16). This lowest total dose in rats is close to the estimated average uptake of 1.1 mg/kg of NNK for a heavy smoker with 40 years of smoking (15). NNK itself is not detectable in human urine because of its rapid and extensive metabolism to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNAL) and other products (15, 17).

NNAL is a major metabolite of NNK. Like NNK, NNAL is an established powerful lung carcinogen in rats and mice, although somewhat less carcinogenic than NNK. Urinary total NNAL, i.e., the sum of free NNAL and its glucuronides, is a well-established biomarker of NNK uptake (10). Thus, NNAL detection in urine signifies exposure to, and uptake of, the lung carcinogen NNK. Given that NNK is found only in tobacco products, a major advantage of the total NNAL biomarker is its specificity to tobacco smoke exposure. Another advantage of urinary total NNAL over other tobacco constituents for epidemiologic studies is its relatively long half-life (i.e., 10 days to 3 weeks) (10).

Similar to NNK, NNN is produced during curing, aging, and burning of tobacco. Human exposure to NNN can be measured via quantitation of unchanged NNN (also called free NNN) and its detoxification product NNN-pyridine-*N*-glucuronide (NNN-*N*-Gluc) in urine (18). The sum of free NNN and NNN-*N*-Gluc in urine is referred to as total NNN.

Virtually all unburned commercial tobacco products contain NNN and NNK, and they always occur together (14). NNN is also present in smoke from cigars and cigarettes, in the saliva of people who chew betel quid with tobacco, and in the saliva of oral-snuff users (19). There is great variation in levels of NNN and NNK in mainstream smoke of cigarettes. This is mainly due to differences in tobacco types used, agricultural practices, curing methods, and manufacturing processes (14). Levels of NNN range from 20 to 58,000 ng per cigarette and NNK from 19 to 10,745 ng per cigarette in tobacco from commercial cigarettes sold in different parts of the world. In mainstream smoke, the ranges of NNN and NNK were reported from 4 to 2830 ng per cigarette and 3 to 1749 ng per cigarette, respectively (14).

A collaborative study between the World Health Organization and the U.S. Centers for Disease Control and Prevention found that the levels of NNK plus NNN in the mainstream smoke of Marlboro cigarettes purchased in 10 different countries were significantly higher than local top-selling brands of cigarettes from the same countries (20). For example, the median levels of NNK plus NNN in Marlboro cigarettes in the U.S. were very similar to the Marlboro-brand cigarettes sold in China (i.e., 216.3 and 263.1 ng/cigarette, respectively), whereas considerably lower median levels were reported in the local popular brands of cigarettes in China (Hongtashan: 5.8 ng/cigarette) and India (Gold Flake: 12.4 ng/cigarette) (20). These data suggest that differences in cancer risk among smokers, after taking into account the number of cigarettes consumed, may be due in part to differences in NNK and NNN uptake among smokers consuming different brands of cigarettes.

2.3 Polycyclic aromatic hydrocarbons (PAH)

PAH as a group include hundreds of chemicals that commonly occur as mixtures in the environment. PAH are present in cigarette smoke as well as in the general environment resulting from incomplete combustion of organic matter. Extensive investigations have demonstrated that PAH-enriched cigarette smoke condensate fractions are carcinogenic to mouse skin and rat lung (21, 22). Fourteen individual PAH compounds, including the widely studied PAH benzo[*a*]pyrene (BaP), have been rated as having sufficient evidence for carcinogenicity in laboratory animals, and BaP is considered carcinogenic to humans (23, 24).

We have developed and validated several biomarkers for PAH uptake and metabolism (25-27). *r*-1,*t*-2,3,*c*-4-Tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT) is a metabolite of phenanthrene, the simplest PAH with a bay region, a feature that is closely associated with the carcinogenicity. 1-Hydroxypyrene is a metabolite of the noncarcinogenic pyrene that is always a component of PAH mixtures. We applied these biomarkers in smokers and nonsmokers in epidemiological studies (28-30).

2.4 Volatile Organic Compounds

Besides tobacco-specific nitrosamines and PAH, tobacco smoke contains a myriad of volatile organic compounds that are toxic and some may be carcinogenic to humans. Levels in mainstream smoke of the routinely quantified volatile organic compounds acrolein, benzene, 1,3-butadiene, and crotonaldehyde are 100 to 1000 times greater than those of typical PAH and NNK (28). Urinary mercapturic acids are well-established and validated biomarkers of uptake of these compounds (shown in Figure 1), and all are found at higher levels in the urine of smokers than in non-smokers (28, 31). Benzene, 1,3-butadiene, and ethylene oxide are considered carcinogenic to humans by the IARC, based mainly on occupational studies and mechanistic data related to hematopoietic malignancies (32, 33). Acrolein is a highly toxic but marginally carcinogenic compound while crotonaldehyde is a relatively weak hepatocarcinogen (34, 35).

3. Biomarkers of tobacco smoke constituents in relation to risk of lung cancer

Only an estimated 11% to 24% of lifelong smokers may die from lung cancer over their lifetimes (8). This variability in susceptibility to lung cancer among smokers is due in part to the fact that not all smokers of a given frequency of cigarette use are exposed to the same levels of tobacco constituents. Sources of variability of the *in vivo* dose of tobacco smoke carcinogens include preference for certain brands of cigarettes that contain different levels of carcinogens, as well as differences in smoking behaviors, such as intensity of smoking, number of puffs per cigarette, and depth of inhalation. A biomarker approach would more directly and closely measure the internal dose to tobacco smoke constituents, therefore potentially providing a better estimate of the risk of developing lung cancer.

Utilizing the Shanghai Cohort Study, we conducted a series of nested case-control studies to examine the association between urinary biomarkers of cigarette smoke constituents and the risk of developing lung cancer among current smokers. The Shanghai Cohort Study enrolled 18,244 men between January 1, 1986 and September 30, 1989 (36, 37). At enrollment all subjects were between 45 and 64 years old and lived in one of four geographically defined communities in the city of Shanghai, China. In-person interviews were conducted and a urine sample was obtained from each subject upon enrollment. Cases of lung cancer were identified annually by in-person re-interviews of all surviving cohort members and routine review of reports from the Shanghai Cancer Registry and the Shanghai Municipal Vital Statistics Office. For the nested case-control study, we included lung cancer patients who smoked cigarettes at enrollment (i.e., at the time of urine collection). For each case, we randomly selected one control subject from all cohort members who were current smokers at enrollment, without a history of cancer, and alive at the time of cancer diagnosis of the index case. Controls were matched to the index case by age at enrollment (± 2 years), year and month of urine collection (± 1 month), and the same neighborhood of residence at recruitment. Urine samples were retrieved from the biorepository of the cohort study and analyzed for the following tobacco smoke carcinogen and toxicant biomarkers: total cotinine, total NNAL, total NNN, PheT, and mercapturic acid metabolites of acrolein, benzene, 1,3-butadiene, crotonaldehyde, and ethylene oxide. In this paper, we review of findings from the Shanghai Cohort Study and other prospective studies for associations between urinary metabolites of nicotine, tobacco-specific nitrosamines, PAH and volatile organic compounds and lung cancer risk. Table 1 summarizes the main findings on these biomarkers in relation to lung cancer from the Shanghai Cohort Study and other selected studies with a similar design.

3.1 Nicotine Metabolites

Several epidemiologic studies, mainly using a cross-sectional or retrospective study design, have been carried out to examine the association between plasma or urinary cotinine, a major metabolite of nicotine, and risk of lung cancer. Among the earliest reported findings based on prospective epidemiologic data was from a nested case-control study of 92 lung cancer cases and 305 controls from over 26,000 women aged 40-64 living in The Netherlands (38). The study reported an odds ratio of lung cancer for the highest tertile level

of urinary total nicotine metabolites to be 18.8 (95% CI: 8.2, 42.9), compared with those with negative measurement of urinary nicotine and its pyridyl-containing metabolites. A trend was observed with increasing lung cancer risk among smokers by level of urinary total nicotine metabolites. Similar findings have since been replicated in a few prospective epidemiological studies (39, 40).

Based on the Shanghai Cohort Study, we conducted a nested case-control study of lung cancer among men who were self-reported current smokers and whose urinary cotinine levels were 35 ng/ml or higher. Our initial report among 155 lung cancer cases and 152 controls showed that current smokers with the highest tertile of urinary total cotinine (2615 ng/mg creatinine) had an odds ratio of 3.76 (95% confidence interval = 1.75 – 8.06) relative to smokers with the lowest tertile of total cotinine (35 – 1196 ng/mg creatinine) after adjustment for number of cigarettes per day, number of years of smoking, and urinary total NNAL (P for trend = 0.002) (39). We expanded this initial study to include 476 lung cancer cases and 476 controls from the same Shanghai Cohort Study and reported a similar association between urinary total cotinine and lung cancer risk; odds ratios [95% confidence intervals (CI)] for the 2nd and 3rd tertile of total cotinine were 2.18 (1.43-3.32) and 3.52 (2.30-5.41), respectively, compared to the lowest tertile (P for trend <0.001), also with adjustment for smoking intensity (cigarettes per day) and duration (number of years of smoking), and urinary total NNAL, in addition to urinary PheT (30).

In addition to the Shanghai cohort study, two nested case-control studies of lung cancer were also conducted among current smokers. Using the Singapore Chinese Health Study dataset, Yuan *et al.* conducted a nested case-control study of lung cancer (91 cases and 93 controls) and reported a two-fold increased risk of lung cancer for the highest relative to the lowest tertile of urinary total cotinine among current smokers, and the elevated risk diminished slightly after adjustment for urinary total NNAL (39). In contrast, a positive association was not observed between serum cotinine level and lung cancer risk in a nested case-control study of lung cancer among current smokers (100 cases and 100 matched controls) that was conducted by Church *et al.* utilizing the dataset of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) conducted and did not find a positive association (41).

The following two nested case-control studies were conducted among both nonsmokers and smokers. Based on the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, an ongoing prospective study of over 520,000 men and women between 1992 and 2000 from 10 European countries, Timofeeva *et al.* conducted a nested case-control study of lung cancer including current and former smokers as well as never smokers (894 cases and 1,805 matched controls). Lung cancer risk increased monotonically with increasing serum cotinine levels; odds ratio of lung cancer was 12.4 (95% CI: 7.1, 21.9) for subjects in the highest decile (serum cotinine >1,800 nmol/L or >316.8 ng/mL) relative to nonsmokers (serum cotinine <75 nmol/L or <13.2 ng/mL) after adjustment for number of cigarettes per day (42). In a similar analysis involving 1,741 lung cancer cases and the same number of matched controls of smokers and nonsmokers in Norway, Boffetta *et al.* reported a statistically significant positive association between serum cotinine levels and lung cancer risk in a dose-dependent manner. Compared with nonsmokers (serum cotinine <5 ng/mL or

<29 nmol/L), current smokers in the highest level of serum cotinine (>378.8 ng/mL or >2,149 nmol/L) had an odds ratio of 55.1 (95% CI: 35.7, 85.0) of lung cancer (40). Among those with smoking information in the analytic sample, there were 16% never, 17% former and 67% current smokers. Compared with individuals who had serum cotinine levels 5 ng/mL, which included never smokers, smokers with 24.8-114.7 ng/mL of serum cotinine (i.e., the third highest group of cotinine) had an odds ratio of 2.80 (95% CI: 1.55, 5.05) and smokers with >378.8 ng/mL of serum cotinine (i.e., the eighth or highest group of cotinine levels in the study) had an odds ratio of 55.1 (95% CI: 35.7, 85.0) for lung cancer. These results were not adjusted for smoking history (e.g., status, intensity, duration) or other biomarkers of tobacco carcinogens. It is worth pointing out that while there is no definitive cutpoint for cotinine to separate smokers from nonsmokers (43) the most widely used cutpoint is 14 ng/mL in serum and 50 ng/mL in urine (44).

A stronger association between cotinine levels and lung cancer risk observed in the studies by Timofeeva *et al.* and Boffetta *et al.* was due to the inclusion of never smokers and former smokers who supposedly had very low or undetectable levels of cotinine in their body fluids given a relatively short half-life of cotinine. On the other hand, the studies by Yuan *et al.* (30, 39) and Church, *et al.* (41) included only current smokers with certain elevated levels of cotinine in urine or serum, which resulted in a narrow range of cotinine and a flattened dose-response relationship for cotinine and lung cancer risk. In addition, the adjustment for smoking intensity and duration and additional biomarkers of tobacco carcinogens (e.g., NNAL and PheT) could further attenuate the association between cotinine and lung cancer risk. Nonetheless, a statistically significant association between urinary total cotinine and lung cancer risk even after adjustment for these tobacco carcinogen biomarkers (i.e., NNAL and PheT) in the Shanghai Cohort Study (30) suggests that cotinine, while not carcinogenic, is acting as a surrogate for other important compounds in cigarette smoke that have yet to be characterized in terms of lung cancer risk in humans.

3.2 Total NNAL and total NNN

A limited number of studies have examined levels of total NNAL and risk of lung cancer in smokers. Using the nested case-control study of lung cancer within the Shanghai Cohort Study described above, we found that smokers who developed lung cancer had statistically significantly higher levels of total NNAL in baseline urine samples than smokers who did not develop lung cancer ($P < 0.001$). The risk of lung cancer doubled for smokers with the highest tertile of urinary total NNAL relative to the lowest tertile after adjustment for smoking intensity and duration and urinary total cotinine (odds ratio = 2.04; 95% CI = 1.02, 4.05; P for trend = 0.04) (39). In the follow-up study with a larger sample size (476 cases and 476 controls) within the same Shanghai Cohort Study, we found a similar relative risk for lung cancer associated with elevated levels of urinary total NNAL (odds ratio = 1.93; 95% CI: 1.28, 2.90) after adjustment for smoking intensity and duration, and urinary total cotinine and PheT (30). We also conducted a case-control study nested within the Singapore Chinese Health Study and found a similar result; the multivariate-adjusted odds ratio for lung cancer was 2.64 (95% CI: 1.10, 6.34) for the highest tertile versus the lowest tertile of total NNAL (39).

In a U.S.-based case-control study nested within the PLCO, total NNAL was associated with a 1.57 times increase in lung cancer risk per standard deviation, or 40 fmol/mL ($P=0.018$) (41). In this study, total NNAL, PheT and total cotinine were measured in serum collected prior to lung cancer diagnosis among 100 cases and 100 matched controls; all of them were current smokers consuming 10 or more cigarettes/day. The statistically significant association for NNAL remained after adjustment for number of years of smoking, serum cotinine and PheT.

Like NNK, NNN is produced by the nitrosation of alkaloids specific to tobacco, therefore, NNN and NNK always occur together (14). Experimental studies have demonstrated that NNN is a potent carcinogen to the oral cavity and esophagus, but a relatively weak carcinogen to the lung of rats (15, 45). Utilizing the Shanghai cohort study database, we observed no association between urinary total NNN and lung cancer risk; the odds ratios (95% CIs) for the 2nd and 3rd tertiles of total NNN were 0.82 (0.36-1.88) and 1.02 (0.39-2.89), respectively (P for trend = 0.959), after adjustment for smoking intensity and duration, and urinary total cotinine (46). In the same cohort, we have previously observed and reported a strong, dose-dependent relationship between urinary NNN and esophageal cancer risk in current smokers (47). The odds ratios (95% CIs) of esophageal cancer for the 2nd and 3rd tertiles of urinary total NNN were 3.99 (1.25-12.7) and 17.0 (3.99-72.8), respectively, compared to the 1st tertile after adjustment for smoking intensity and duration, and urinary total cotinine and total NNAL (P for trend <0.001). These results are strikingly coherent with the findings of studies in F-344 rats demonstrating that NNK is a carcinogen selective for the lung while NNN affects the esophagus and oral cavity.

3.3 PheT

As described above in the nested case-control study of lung cancer in current smokers within the Shanghai Cohort Study, we quantified urinary levels of PheT in 476 lung cancer cases and the same number of matched controls to examine if PheT levels are associated with risk of lung cancer (30). Compared with the lowest quintile, odds ratios (95% CIs) for lung cancer in the 2nd, 3rd, 4th, and 5th quintiles of urinary PheT were 1.70 (1.00-2.88), 1.07 (0.62-1.84), 1.48 (0.86-2.53) and 2.34 (1.33-4.11), respectively (P for trend = 0.023) after adjustment for number of cigarettes smoked per day, number of years of smoking, and urinary total cotinine and total NNAL. In the same cohort, we also conducted a nested case-control of lung cancer in lifelong nonsmokers and found a statistically significant positive association between urinary biomarkers of PAH, including PheT, and lung cancer risk in a dose-dependent manner (29). These results also demonstrate a role of PAH in the development of lung cancer independent of tobacco carcinogens.

Results from the case-control study nested within the PLCO, however, do not support the role of PAH in the development of lung cancer in smokers (41). Mean serum PheT levels were higher in cases than controls, but the difference was not statistically significant ($P=0.204$). Results from the multivariable logistic regression model also indicated a small, but statistically non-significant association after adjustment for smoking duration, serum total cotinine and total NNAL (odds ratio=1.23; 95% CI: 0.88, 1.72, per one standard deviation increase in PheT). The small size of the study (100 case-control pairs), as well as

the general occurrence of PAH in the diet and the environment (as opposed to tobacco-specific compounds such as nicotine, NNK, and NNN), may have contributed to the null results (41).

3.4 Volatile Organic Compounds

We quantified urinary mercapturic acid metabolites of acrolein, benzene, 1,3-butadiene, crotonaldehyde, and ethylene oxide in addition to urinary biomarkers of PAH, NNK, and nicotine in 343 lung cancer cases and 392 matched controls within the Shanghai Cohort Study described above (48). Compared with the lowest quartiles, highest quartiles of all measured mercapturic acids were associated with statistically significantly approximately two-fold increased risk for lung cancer (all P 's for trend <0.01) after adjustment for smoking intensity and duration. These positive associations were completely explained by urinary total cotinine in addition to smoking intensity and duration. Therefore, mercapturic acid metabolites of these volatile organic compounds are not independent risk predictors of lung cancer among male smokers in Shanghai, China.

3.5 Multivariable Model of Lung Cancer Risk Among Smokers

We examined the joint effect of three urinary biomarkers – total cotinine, total NNAL, and PheT on risk of lung cancer in current smokers. For example, smokers in the highest tertiles of urinary total NNAL and total cotinine exhibited an 8.5-fold (95% CI: 3.7, 19.5) increased risk for lung cancer relative to smokers with comparable smoking intensity and duration but possessing the lowest tertiles of urinary total NNAL and total cotinine (39). When we simultaneously examined the number of cigarettes per day, number of years of smoking, urinary total cotinine, PheT, and total NNAL in current smokers, all five variables were statistically significantly associated with an increased risk of lung cancer (all P for trend <0.05) (30). Odds ratios (95% CIs) for lung cancer were 1.93 (1.27-2.93) for number of packs of cigarettes per day (20 cigarettes per pack) and 1.94 (1.52-2.47) for every 10 years of smoking (30). Among the three urinary biomarkers, the multivariable-adjusted odds ratios (95% CIs) for lung cancer for one unit in natural logarithmic value (an equivalent of 2.7-fold increment) of total cotinine, total NNAL, and PheT was 1.64 (1.34-2.01), 1.28 (1.02-1.59), and 1.41 (1.02-1.93), respectively (30). In summary, these three biomarkers were positively associated with lung cancer risk among current smokers, independent of smoking history.

4. Discussion

The worldwide burden of lung cancer is expected to continue growing given that worldwide consumption of tobacco continues to increase and cigarette smoking will remain the single most important causal factor for lung cancer. However, only one quarter of deaths among male smokers are attributed to lung cancers over their lifetime (8). Interview-based assessment of smoking intensity and duration has limitation in capturing inter-individual variability in uptake and metabolism of tobacco carcinogens. In this review, we have demonstrated that appropriately chosen biomarkers would have potential to improve the assessment of lung cancer risk over a model based solely on self-reported history of smoking habits for current smokers.

A biomarker approach to evaluate the heterogeneity of lung cancer risk among smokers has advantages. The measurement of the biomarker is objective and provides a direct link between the parent compound and lung cancer risk. These biomarkers, when validated for their direct relation with lung cancer, could be used as intermediate markers to assess the effectiveness of cancer prevention strategies among smokers. Important among these could be the identification of susceptible smokers at a young age, when preventive interventions are more likely to be effective.

The strengths and limitations of a specific biomarker are also dependent on their characteristics of being tobacco-specific (e.g., without significant non-tobacco exposure sources), having a relatively long half-life (e.g., representative of long-term exposure), and/or being a carcinogen or a metabolite of an established carcinogen (17). Among the four biomarkers for which our data support being independent risk factors for cancer among smokers, total cotinine, total NNAL, and total NNN are excellent measures of nicotine, NNK, and NNN uptake, respectively. Given their specific source of tobacco, total cotinine and total NNAL have been used to evaluate environmental tobacco smoke exposure in nonsmokers (43, 49, 50). On the other hand, the PAH biomarker, PheT, could be derived from multiple sources. PAH are ubiquitous environmental contaminants formed in all processes involving incomplete combustion of organic matter (24, 51). The ubiquity of PAH in the environment and their lack of tobacco specificity could contribute to the relatively weak association between PheT and lung cancer risk in the PLCO study (41).

Of the four tobacco constituents, NNAL and its parent compound NNK as well as NNN are carcinogenic (15). PheT is a metabolite of the non-carcinogenic PAH phenanthrene, but the metabolism of phenanthrene to PheT closely parallels that of BaP, a strong PAH carcinogen capable of inducing tumors of the lung and other tissues in rodents, and rated as carcinogenic to humans (24). Cotinine is the major metabolite of nicotine, a non-carcinogenic constituent of tobacco smoke. However, each dose of nicotine delivered from a cigarette is accompanied by at least 70 established carcinogens. Thus, nicotine and its metabolites could be a good surrogate biomarker for the uptake of other carcinogens in tobacco smoke for which biomarkers have yet to be developed.

A biomarker with a longer half-life has advantages over shorter-lived biomarkers in terms of being able to capture exposures that occurred in the more distant past. NNAL has the longest half-life ($t_{1/2}$ =10 days to 3 weeks) of the four biomarkers. The positive association between total NNAL and lung cancer risk among the three study populations (30, 39, 41) support that notion. Although cotinine is a well-established biomarker for nicotine due to its high and frequent uptake, a well-known limitation is the inability to distinguish the source of nicotine from non-tobacco (e.g., nicotine patch) versus tobacco products. Furthermore, the half-life of cotinine is short compared to that of NNAL.

In summary, no single biomarker of tobacco smoke constituents stands out as the best for stratifying smokers by risk of lung cancer. Our finding that urinary cotinine was positively associated with lung cancer risk among smokers, after adjustment for urinary NNAL and PheT, as well as smoking duration and intensity, supports the hypothesis that other compounds in tobacco smoke are also likely to play a role in the development of lung cancer

in smokers. Additional research is required to develop and validate biomarkers that could be used to predict the risk of lung cancer for smokers. An effective set of non-invasive, predictive markers of lung cancer risk could allow the identification of the relatively small fraction of smokers at very high risk for lung cancer. This would help to target lung cancer screening and smoking cessation efforts to smokers at extremely high risk. The current recommendations for lung cancer screening are for annual low-dose computed tomography among smokers 55 to 79 years of age and with 30 or more pack-years of smoking (52). While these screening efforts will play an important role in identifying lung tumors at their early stage when effective treatment options are available, the high false-positive detection rate hinders the application of this screening method to large populations of smokers (53). A metabolite panel in addition to smoking history information may be able to reduce the false positive rate associated with the use of CT scanning alone to identify lung tumors among asymptomatic smokers.

A demonstrated utility of tobacco-constituent biomarkers that has not been fully explored is their use as intermediate outcomes in chemoprevention trials among smokers. Our group and others have contributed to the body of laboratory evidence that implicates NNK in lung carcinogenesis in humans and 2-phenethyl isothiocyanate (PEITC) as a chemopreventive agent against NNK-induced lung carcinogenesis (51, 54, 55). To elucidate the potentially causal relationship between dietary isothiocyanates (ITC) and lung cancer protection in humans, Hecht *et al.* assessed the changes in urinary total NNAL in 11 cigarette smokers following quantified consumption of watercress, a rich source of PEITC. Similar to results in the rodent experiments (54), the consumption of watercress significantly enhanced urinary excretion of total NNAL, indicating the activation of a possible NNK detoxification pathway (56). Therefore, the identified biomarkers of lung cancer risk could be further developed as intermediate markers for the evaluation of cancer prevention strategies that involve the detoxification of NNK in smokers.

Using a biomarker-based approach to measure metabolites in human urine that reflect the *in vivo* dose to tobacco smoke constituents and evaluate the relationship between metabolite levels and lung cancer risk has important implications in public health and in setting policy that limits the health and in setting policy that limits the contents and levels of specific compounds in cigarettes, including nicotine and tobacco carcinogens (e.g., tobacco-specific nitrosamines) (9, 57). The technology is certainly available to reduce these carcinogens in tobacco products by selecting certain types of tobacco leaves and by altering the agricultural, curing and storage processes. For example, it has been shown that levels of NNN and NNK in selected popular brands of smokeless tobacco that were more recently introduced into the market is about 6- and 8-fold lower than the popular traditional brands, respectively (58). Cigarettes containing lower amounts of nicotine (i.e. 0.05 mg per cigarette) are associated with reduced carcinogen exposure and reduced nicotine dependence (59). In addition to establishing regulation of cigarettes that set maximum allowable toxicant levels, additional studies are warranted to demonstrate the reduction of lung cancer risk in smokers who consume cigarettes or other tobacco products with reduced carcinogens and toxicants.

A relatively new product, the electronic cigarette (e-cigarette), is growing in popularity, particularly among youths. The National Youth Tobacco Survey by the Centers for Disease Control and Prevention found that the prevalence of e-cigarette use in high school students in the U.S. doubled in the recent 2 to 3 years from 4.7% in 2011 to 10% in 2012 ($P<0.05$) (60). Currently e-cigarettes are unregulated, although the FDA is being urged to propose regulations that will address the advertising, ingredients and sale to minors of e-cigarettes. E-cigarettes deliver nicotine to the user in the form of a vapor. Nicotine, although not carcinogenic per se, often contains nornicotine as an impurity, and nornicotine is easily converted to NNN endogenously by reaction with salivary nitrite. Previous studies have shown large increase of urinary total NNN in individuals after they used oral nicotine replacement therapy products such as nicotine gum or lozenge (61, 62). The rise of e-cigarette use in the U.S., particularly among youths, and the potential for endogenous formation of NNN from nicotine present in e-cigarette vapor suggest a need for future research in which the values of urinary biomarker of tobacco-specific nitrosamines and nicotine metabolites could be used to quantify carcinogen exposure and possibly cancer risk associated with e-cigarette use.

In summary, the data reviewed here demonstrate that several tobacco smoke toxicant and carcinogen biomarkers – total cotinine, total NNAL, PheT, and total NNN – are not only biomarkers of exposure but also are biomarkers of cancer risk. Based on comparison of smokers who eventually developed lung cancer versus their healthy counterparts, total cotinine, total NNAL, and PheT were risk biomarkers for lung cancer while total NNN was a risk biomarker for esophageal cancer. These results provide several possible new directions by which tobacco smoke biomarkers could be used for lung cancer prevention. With future research devoted to identifying additional objective biomarkers of tobacco constituents, we hope to improve lung cancer risk assessment. Tobacco constituent biomarkers are as intermediate endpoints in lung cancer prevention trials among smokers. Given the long latent period for cancer development, using changes in biomarker levels, for example, between intervention and control groups could provide results more quickly and at less cost than waiting for cancer outcomes. Finally, the use of these and newly discovered tobacco smoke constituent biomarkers for the purpose of establishing targets for regulation of tobacco products is another direction that deserves emphasis in future research.

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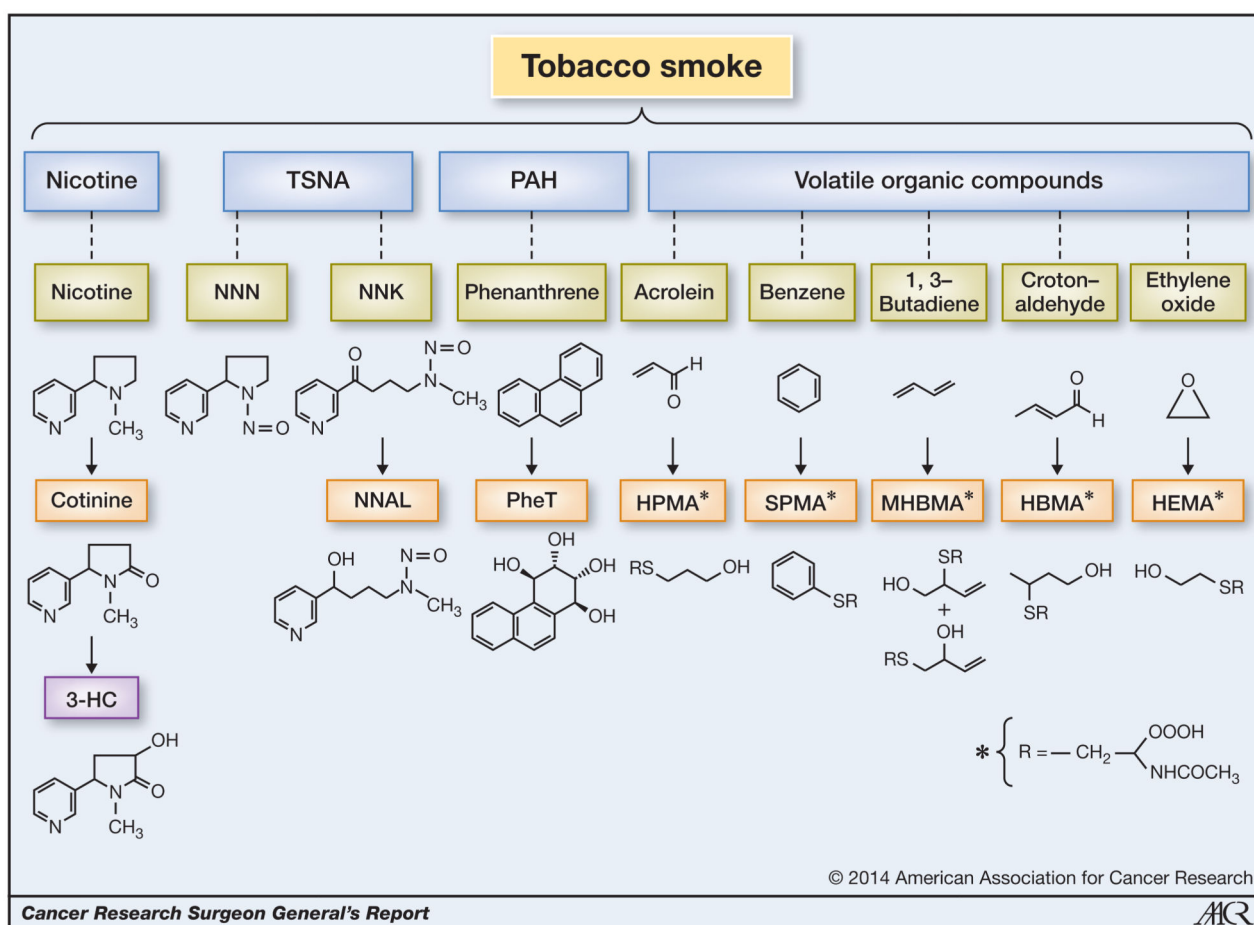


Figure 1. Overview of tobacco smoke constituents and their metabolites as biomarkers of toxicant and carcinogen exposure in smokers

Abbreviations: HBMA, 4-hydroxybut-2-yl mercapturic acid; 3-HC, trans-3'-hydroxycotinine; HEMA, 2-hydroxyethyl mercapturic acid; HPMA, 3-hydroxypropyl mercapturic acid; MHBMA, monohydroxybutyl mercapturic acid; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosornicotine; PAH, polycyclic aromatic hydrocarbons; PheT, *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene; SPMA, *S*-phenyl mercapturic acid; TSNA, tobacco-specific nitrosamines.

Table 1
Summary of findings from our prospective cohort studies for urinary tobacco constituent biomarkers and lung cancer risk among current smokers

Tobacco constituent	Metabolite/Biomarker	Study population	OR (95% CI) for highest versus lowest biomarker level	Comments	Reference
Nicotine	Total cotinine	Men, Shanghai (476 cases and 476 controls)	3.52 (2.30-5.41)	Specimen type: urine. OR was adjusted for age, date of specimen collection, neighborhood of residence, number of cigarettes per day, number of years of smoking, and urinary total NNAL and PheT.	Yuan, 2011 (30)
		Men and women, Singapore (91 cases and 93 controls)	1.65 (0.69, 3.97)	Specimen type: urine. OR was adjusted for age, sex, date of specimen collection, dialect group, number of cigarettes per day, number of years of smoking, and urinary total NNAL.	Yuan, 2009 (39)
		Men and women, U.S. (100 cases and 100 controls)	0.85 (0.59, 1.23)	Specimen type: serum. OR is associated with a unit standard deviation increase adjusted for sex, age, number of years of smoking, and urinary NNAL and PheT.	Church, 2009 (41)
TSNA	Total NNN	Men, Shanghai (93 cases and 93 controls)	1.02 (0.39, 2.89)	Specimen type: urine. OR was adjusted for number of cigarettes per day, number of years of smoking, and urinary total cotinine and PheT.	Stepanov, 2013 (46)
		Men, Shanghai (476 cases and 476 controls)	1.93 (1.28, 2.90)	Specimen type: urine. OR was adjusted for number of cigarettes per day, number of years of smoking, and urinary total cotinine and PheT.	Yuan, 2011 (30)
		Men and women, Singapore (91 cases and 93 controls)	2.64 (1.10, 6.34)	Specimen type: urine. OR was adjusted for age, sex, date of specimen collection, dialect group, number of cigarettes per day, number of years of smoking, and urinary total cotinine.	Yuan, 2009 (39)
PAH	PheT	Men and women, U.S. (100 cases and 100 controls)	1.57 (1.08, 2.28)	Specimen type: serum. OR is associated with a unit standard deviation increase adjusted for sex, age, number of years of smoking, and urinary cotinine and PheT.	Church, 2009 (41)
		Men, Shanghai (476 cases and 476 controls)	2.34 (1.33, 4.11)	Specimen type: urine. OR was adjusted for number of cigarettes per day, number of years of smoking, and urinary total cotinine and total NNAL.	Yuan, 2011 (30)
		Men and women, U.S. (100 cases and 100 controls)	1.23 (0.88, 1.72)	Specimen type: serum. OR is associated with a unit standard deviation increase adjusted for sex, age, number of years of smoking, and urinary cotinine and total NNAL.	Church, 2009 (41)
VOC	HPMA	Men, Shanghai (343 cases and 392 controls) *	1.06 (0.62, 1.80)	Specimen type: urine. OR was adjusted for number of cigarettes per day, number of years of smoking, and urinary cotinine *	Yuan, 2012 (48) *
	SPMA		1.20 (0.74, 1.96)		
	MHBMA		1.08 (0.66, 1.75)		
	HBMA		0.97 (0.56, 1.66)		
	HEMA		1.12 (0.66, 1.89)		

Abbreviations: CI, confidence interval; HBMA, 4-hydroxybut-2-yl mercapturic acid; 3-HC, trans-3'-hydroxycotinine; HEMA, 2-hydroxyethyl mercapturic acid; HPMA, 3-hydroxypropyl mercapturic acid; MHBMA, monohydroxybutyl mercapturic acid; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosomocotine; OR, odds ratio; PAH, polycyclic aromatic hydrocarbons; PheT, *r*-1,*r*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene; SPMA, *S*-phenyl mercapturic acid; TSN_A, tobacco-specific nitrosamines; VOC, volatile organic compounds.

* For all volatile organic compound metabolite results presented.