

## Research Article

## Effect of Differing Levels of Tobacco-Specific Nitrosamines in Cigarette Smoke on the Levels of Biomarkers in Smokers

David L. Ashley<sup>1</sup>, Richard J. O'Connor<sup>2</sup>, John T. Bernert<sup>1</sup>, Clifford H. Watson<sup>1</sup>, Gregory M. Polzin<sup>1</sup>, Ram B. Jain<sup>1</sup>, David Hammond<sup>4</sup>, Dorothy K. Hatsukami<sup>6</sup>, Gary A. Giovino<sup>3</sup>, K. Michael Cummings<sup>2</sup>, Ann McNeill<sup>7</sup>, Lion Shahab<sup>8</sup>, Bill King<sup>9</sup>, Geoffrey T. Fong<sup>5,10</sup>, Liqin Zhang<sup>1</sup>, Yang Xia<sup>1</sup>, Xizheng Yan<sup>1</sup>, and Joan M. McCraw<sup>1</sup>

## Abstract

**Background:** Smokers are exposed to significant doses of carcinogens, including tobacco-specific nitrosamines (TSNA). Previous studies have shown significant global differences in the levels of TSNA in cigarette smoke because of the variation in tobacco blending and curing practices around the world.

**Methods:** Mouth-level exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) measured in cigarette butts and urinary concentrations of its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) were examined among 126 daily smokers in four countries over a 24-hour study period.

**Results:** As mouth-level exposure of NNK increased, the urinary NNAL increased even after adjustment for other covariates ( $\beta = 0.46$ ,  $P = 0.004$ ). The relationship between mouth-level exposure to nicotine and its salivary metabolite, cotinine, was not statistically significant ( $\beta = 0.29$ ,  $P = 0.057$ ), likely because of the very limited range of differences in mouth-level nicotine exposure in this population.

**Conclusions:** We have shown a direct association between the 24-hour mouth-level exposure of NNK resulting from cigarette smoking and the concentration of its primary metabolite, NNAL, in the urine of smokers. Internal dose concentrations of urinary NNAL are significantly lower in smokers in countries that have lower TSNA levels in cigarettes such as Canada and Australia in contrast to countries that have high levels of these carcinogens in cigarettes, such as the United States.

**Impact:** Lowering the levels of NNK in the mainstream smoke of cigarettes through the use of specific tobacco types and known curing practices can significantly affect the exposure of smokers to this known carcinogen. *Cancer Epidemiol Biomarkers Prev*; 19(6); 1389–98. ©2010 AACR.

## Introduction

Tobacco smoke is a complex aerosol mixture containing many toxic and carcinogenic compounds (1). Of particular concern are the tobacco-specific nitrosamines (TSNA), which include 4-(methylnitrosamino)-

1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosornicotine (NNN), *N*-nitrosoanabasine, and *N*-nitrosoanatabine. These compounds form via nitrosation of tobacco alkaloids (nicotine, nornicotine, anabasine, and anatabine) during the curing process. Several factors contribute to TSNA formation, including use of nitrate fertilizers, NO<sub>x</sub> gas availability, microbial activity, curing temperature, humidity during curing and storage, and tobacco variety (1-4).

Given the variability of TSNA levels among commercially available brands both within and among countries (5-8), cigarette manufacturers can produce cigarettes with fewer TSNA. Although the actual degree of health benefit from lowering TSNA in cigarettes is uncertain, the WHO Study Group on Tobacco Product Regulation (TobReg; ref. 9) has recommended that TSNA levels be lowered. This recommendation is because NNN and NNK are IARC Group 1 known human carcinogens and suspected to significantly contribute to the cancer burden of smokers.

TSNA are implicated in the development of human lung adenocarcinoma and have been shown in experimental studies to produce adenocarcinomas in laboratory animals (10). NNK is metabolized to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and total (or free)

**Authors' Affiliations:** <sup>1</sup>Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia; <sup>2</sup>Department of Health Behavior, Roswell Park Cancer Institute; <sup>3</sup>Department of Health Behavior, School of Public Health and Health Professions, University at Buffalo, State University of New York, Buffalo, New York; Departments of <sup>4</sup>Health Studies and Gerontology and <sup>5</sup>Psychology, University of Waterloo, Waterloo, Ontario, Canada; <sup>6</sup>Tobacco Use Research Center and the Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota; <sup>7</sup>School of Community Health Sciences, University of Nottingham, Nottingham, United Kingdom; <sup>8</sup>Health Behaviour Research Centre, Department of Epidemiology and Public Health, University College London, London, United Kingdom; <sup>9</sup>The Cancer Council Victoria, Melbourne, Victoria, Australia; and <sup>10</sup>Ontario Institute for Cancer Research, Toronto, Ontario, Canada

Disclaimer: The findings and conclusions of this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

**Corresponding Author:** David L. Ashley, Centers for Disease Control and Prevention, 4770 Buford Highway, Mailstop F-47, Atlanta, GA 30341. Phone: 770-488-7962; Fax: 770-488-0181. E-mail: dla1@cdc.gov

doi: 10.1158/1055-9965.EPI-10-0084

©2010 American Association for Cancer Research.

NNAL and its glucuronides are established biomarkers for NNK exposure measurable in human urine (11-14). Total NNAL has been shown to correlate with urinary cotinine, although the ratio of NNAL to cotinine may decrease as cotinine levels increase (15).

Only a few studies to date have compared exposure levels of TSNA between persons smoking brands of cigarettes known to differ significantly in TSNA smoke levels. These studies have focused on potentially reduced exposure products (16-18). For example, Breland and colleagues (16) measured total NNAL levels in smokers who were switched to Advance, a brand marketed as having 80% lower nitrosamine yields based on levels determined by the International Organization for Standardization (ISO) machine smoking regimen. The researchers found that NNAL levels were reduced 51% compared with the levels found when the smokers used their usual brands—a significant reduction in NNAL, although less of a reduction than when the same individuals abstained from smoking for a week.

In another study, Hughes et al. (17) examined smoking behavior, urine cotinine, and NNAL levels in smokers who switched from their usual brand to Omni cigarettes, another purportedly low nitrosamine brand. The researchers found that, in spite of the reported lower NNK smoke emissions from Omni cigarettes, NNAL concentrations were not significantly lower in the urine of smokers who switched to Omni for 6 weeks.

Finally, Hatsukami et al. (18) examined smokers' urinary concentrations of cotinine and NNAL after smokers switched to Omni cigarettes or medicinal nicotine. They found that, for those cigarette smokers who switched to Omni cigarettes, NNAL concentrations in the urine were reduced significantly (32%).

These switching studies are informative in determining if smokers who switch to lower TSNA cigarettes will experience lower levels of these carcinogens. However, they are limited by recruitment of subjects who may not be interested in these products and, consequently, may not smoke as much as individuals who self-select to use these products or fully comply with using only the product assigned to them. When switching to a new product, smokers often adapt their smoking behavior to compensate for differences in nicotine delivery and the sensory properties of a cigarette (19). For example, smokers may change the number of cigarettes smoked per day and/or vary the amount of smoke inhaled from each cigarette (20). In these forced-switching studies, such changes can influence biomarker results.

However, the wide variability in TSNA levels across tobacco markets provides an opportunity to conduct natural experiments on the association between TSNA levels from cigarettes and biomarker concentrations of TSNA among people who smoke their accustomed cigarette brands. No study to date has actually compared biomarker levels of TSNA in persons smoking their usual brand of cigarettes, including smokers of brands that vary substantially in nitrosamine levels.

The number of cigarettes smoked per day has long served as a standard exposure measure for epidemiologic research on smoking and health (21), but it provides only a crude estimate of human exposure, particularly when assessing differences among tobacco products. Biomarkers provide an aggregate measure of exposure over hours, days, or even weeks, depending on the specific compound's clearance rate, but can be influenced by individual differences in metabolism, complicating the interpretation of results. This limitation has revived interest in complementary mouth-level indicators of smoke exposure. Mouth-level indicators include measures of smoking topography by using flow meter-type devices (20), cigarette filter staining patterns (22), or chemical assays of the filter (23, 24). A method of estimating smoke exposure by using solanesol, a naturally occurring component in tobacco deposited during smoking in the cigarette filter butt, has also been developed (25). Solanesol levels can be used to model emissions of other constituents, such as nicotine and TSNA.

In the current study, we sought to evaluate the extent to which the mouth-level exposure to NNK and nicotine assessed from filter butts related to the urinary levels of NNAL and salivary cotinine in smokers who smoked cigarette brands with different levels of TSNA. The study was conducted in four countries: the United States, where American-blend cigarettes containing burley tobacco have high levels of TSNA, and Canada, the United Kingdom, and Australia, where cigarettes typically made only from bright tobacco have substantially lower TSNA levels than cigarettes in the United States (5, 6).

## Materials and Methods

### Subjects

The subjects were 126 adult daily smokers recruited in four countries: Australia, Canada, the United Kingdom, and the United States. Subjects were recruited through advertisements in local newspapers, flyers, emails, or posters on public bulletin boards at five different sites: Waterloo, Canada; Melbourne, Australia; London, United Kingdom; and Buffalo and Minneapolis, United States. Smokers who responded to the advertisements were screened for eligibility by a brief telephone interview. Subjects were included if they were between 18 and 55 years of age, had smoked at least 10 cigarettes daily for the past year, and had been a regular smoker of one particular cigarette brand for more than 3 months. Seventeen eligible cigarette brands (between 3 and 5 brands from each country) were selected on the basis of national sales and nicotine yield to identify popular brands with a range of ventilation. The brands and associated ventilation are provided in Table 1. Smokers were excluded if they smoked an ineligible cigarette brand, had a history of lung or heart disease, or were pregnant. See Table 2 for a summary of the sample profile. The study was approved by the Institutional Review Boards at the U.S.

**Table 1.** Brands and tip ventilation for cigarettes used at each site

Study site	Brand	Ventilation (%)
Canada	Players Light	1.2
	DuMaurier	0.7
	DuMaurier Extra Light	39
United States (New York)	Marlboro	13
	Newport	0.2
	Newport Light	23
	Camel Light	30
United States (Minnesota)	Marlboro Menthol	21
	Marlboro	13
	Newport	0.2
	Marlboro Light	23
Australia	Camel Light	30
	Peter Jackson Super Mild	31
	Peter Jackson Extra Mild	22
	Peter Stuyvesant Filter	15
	Winfield Super Mild	39
United Kingdom	Winfield Extra Mild	17
	Marlboro Gold	49
	Silk Cut Purple	57
	Benson & Hedges Gold	25
	Lambert & Butler Silver	10

Centers for Disease Control and Prevention (CDC); Roswell Park Cancer Institute; the University of Waterloo, Canada; the University of Minnesota; the University at Buffalo, State University of New York; University College London; and the Cancer Council Victoria, Australia.

#### Data collection protocol

Data collection occurred during April to November 2005 at each of the five study sites. Subjects completed a brief telephone interview to ascertain eligibility and were booked for two 1-hour laboratory visits on consecutive days. The study procedure has been described elsewhere (26).

Briefly, subjects visited the laboratory on two occasions, 24 hours apart, and were instructed to abstain from smoking for at least 0.5 hour before each visit. At the subjects' first visit, researchers explained the purpose of the study; obtained written consent; and recorded basic smoking, socio-demographic, and anthropometric characteristics (i.e., height and weight). At both visits, subjects provided additional details about recent (within 24 h) smoking behavior before saliva and random urine samples were collected. At the end of each visit, subjects smoked a cigarette through a smoking topography device (data not included), and the spent cigarette butt was collected. Between visits, subjects were asked to continue smoking as usual and were provided with a container to collect all the butts from the cigarettes they smoked in the intervening 24-hour period. Subjects were reimbursed the equivalent of \$50 USD for their time.

The urine, saliva, and used cigarette butts collected from participants were frozen after each visit. On conclusion of the study, all biospecimens and cigarette butt samples were placed on dry ice and shipped to CDC for analysis.

#### Data cleanup

Data describing each subject, including demographics, smoking history, cigarette butt collection dates and times, and any deviations from the protocol, were compiled in identical spreadsheets at each site for further processing. Consistency of replies between the two questionnaire sessions was examined to assess the data for obvious outliers and compare reported information for internal consistency. The field sites were contacted for clarification of data questions, and identifiable errors were corrected. Subjects were excluded from data analysis if they failed to follow the study protocol by smoking other brands of cigarettes; failing to attend the appointments; using other nicotine products; failing to collect >75% of the butts from cigarettes smoked during the period; failing to record dates and times of smoking; smoking immediately

**Table 2.** Descriptive statistics for study subjects by site

Country	Valid subjects	Age (mean $\pm$ SE)	Sex (male/female)	Self-reported cigarettes/day (mean $\pm$ SE)	Cigarettes smoked in past 24 h (mean $\pm$ SE)	% Vent* <5	% Vent 5-20	% Vent >20
Canada	25	29.0 $\pm$ 1.9	19/6	17.3 $\pm$ 1.1	15.7 $\pm$ 0.9	84	0	16
United States (New York)	25	30.8 $\pm$ 2.1	12/13	18.3 $\pm$ 0.8	15.3 $\pm$ 0.8	24	16	60
United States (Minnesota)	28	31.2 $\pm$ 1.8	15/13	19.7 $\pm$ 1.3	17.3 $\pm$ 1.1	50	21	29
Australia	25	37.7 $\pm$ 3.9	12/13	19.6 $\pm$ 1.2	18.6 $\pm$ 1.4	0	44	56
United Kingdom	23	30.2 $\pm$ 1.4	11/12	15.6 $\pm$ 0.9	14.6 $\pm$ 0.9	0	0	100

\*Percentage of cigarettes from these subjects with tip ventilation in the range indicated.

before the visit; and returning butts that, by observing the filter, showed no sign of having been from a smoked cigarette.

Of 157 subjects who enrolled in the study, 126 (80%) fully complied with the study protocol and were included in this analysis.

### Machine smoking conditions for determining cigarette emissions

Cigarettes were conditioned at 22°C and 60% relative humidity before testing. An ASM500 16-port linear smoking machine (Cerulean) was used for machine smoking. Systematic variation of puff volume, puff frequency, and filter ventilation blocking provided a wide range of smoking conditions and resultant mainstream smoke deliveries (25). After the cigarettes were smoked, the filters were detached from the residual tobacco column and stored in cryovials (Nalgene) at -20°C until further processing.

### Nicotine and TSNA determination

The TSNA captured during machine smoking on a Cambridge filter pad (CFP) were quantified by using an Agilent 1100 liquid chromatograph (LC) and API 4000 tandem mass spectrometer (MS/MS; Sciex) as previously published (27). Sample preparation was streamlined with direct extraction of the CFPs in the LC mobile phase (28), 20 mmol/L ammonium acetate containing 5% methanol. One hour before the cigarettes were smoked, the CFPs were treated with 2 mL of 50 mmol/L ascorbic acid in methanol to reduce TSNA artifact formation (27, 29).

Mainstream smoke nicotine levels were determined by using a combined LC-MS/MS approach that incorporated nicotine in our standard TSNA method. Isotopically labeled nicotine was included in the TSNA internal standard panel for nicotine quantification. This method allowed the quantification of both nicotine and TSNA on CFPs that collected mainstream smoke, along with the analysis of solanesol on the cigarette filter from which this smoke was drawn. We compared nicotine results obtained by using LC-MS/MS and standard gas chromatography flame ionization detection method (30) and confirmed equivalency of the results.

### Solanesol analysis

Portions of the cigarette filters (1-cm length) removed from the mouth end were stripped of the wrapping paper and loaded into glass vials. The 1-cm portions were analyzed as previously described (25). Briefly, cigarette filter butts were added to geranylgeraniol, placed in 1 N methanolic potassium hydroxide, and extracted with hexane. The butts were then analyzed by using normal phase LC and single quadrupole atmospheric pressure chemical ionization mass spectrometry in selected ion monitoring mode.

### Calculation of mouth-level exposure to TSNA and nicotine

Results from analyzing solanesol in cigarette butts were used to calculate mouth-level exposure of NNN, NNK, and nicotine by using relationships established by smoking cigarettes from each brand under a wide range of conditions, as described above (25). Regression analysis was done to determine solanesol levels in butts and to determine nicotine, NNN, or NNK collected on CFPs analyzed in samples resulting from smoking the same cigarettes but under multiple smoking conditions. This analysis allowed butt levels of solanesol to be used to calculate target compound levels emitted from the mouth end of the smokers' discarded cigarette butts.

Where the relationship between solanesol and the delivered component deviated from linearity at low delivery levels, a separate correlation was determined over this range and mouth-level exposure for these study butts was recalculated. Of the butts tested, 1.6% of the NNN, 2.0% of the NNK, and 3.3% of the nicotine results were recalculated in this manner.

Some solanesol levels that were determined on submitted butts were greater than the maximum levels achievable by using the most intense machine smoking conditions. The levels of TSNA and nicotine from the smokers' cigarette butts that had solanesol levels above the highest machine-determined levels were calculated by extrapolating the solanesol-delivered component comparison curves. Of the butts tested, 1.9% of the results were determined by extrapolation.

For butts that were not collected by the subjects or for which analysis results were not reportable, a mean of all of the submitted butts for each subject was calculated and substituted for these undetermined results. Of the butts tested, 4.1% of the results were replaced by mean levels that were determined individually for each subject. This step was required so that the total mouth-level exposure of smoke constituents over the 24-hour period would not be biased for subjects for whom all results were not available.

Mouth-level exposures of the individual TSNA—NNN and NNK—and nicotine were determined for each cigarette butt submitted and summed to create 24-hour NNN, NNK, and nicotine mouth-level exposures for each subject. In the case of the cigarette butts submitted from Minneapolis, butts collected from the two cigarettes the subjects smoked during clinic visits were not obtained and submitted for analysis. Therefore, the 24-hour mouth-level exposure of constituents for subjects from Minneapolis was calculated as the submitted butts plus two times the average.

### Salivary cotinine

Saliva samples were collected by having subjects gently chew on the sterile cotton swabs from a Salivette (Sarstedt) for ~2 minutes, after which the swab was returned to the device. The intact Salivettes were stored



frozen at  $\leq -20^{\circ}\text{C}$  and shipped to the laboratories at CDC, where they were kept frozen until analysis. Samples were subsequently extracted into methylene chloride, reconstituted in water, and analyzed by LC-MS/MS by using a method described previously (31, 32). The cotinine concentration was quantified by comparing unknown relative response factors of unknowns to standards using least-squares linear regression.

### Urinary NNAL

Urinary NNAL was measured as free (i.e., nonconjugated) NNAL and also as total NNAL after hydrolysis of NNAL glucuronide. All samples were analyzed by slightly modifying a previously described method using LC-MS/MS (11). Total NNAL was measured following an overnight incubation with  $\beta$ -glucuronidase. Following the incubation, the hydrolyzed solution was made basic by adding 100  $\mu\text{L}$  of 10 N NaOH and loaded onto a prewashed ChemElute column that was eluted twice with 4 mL of methylene chloride. The pooled methylene chloride extracts were then back extracted into 3 mL of 0.1 N HCl. The aqueous HCl extract was neutralized with NaOH, and 3 mL of phosphate buffer (pH 6.4) was added. This sample was then processed through a custom molecularly imprinted polymer column (MIP Technologies). Free NNAL samples were prepared in the same manner, except the initial hydrolysis was omitted and the samples were processed directly through the ChemElute and molecularly imprinted polymer columns as described above. Analysis and quantification of NNAL were also as described previously (11), except that we used an API 5000 MS and an Xterra RP18 column (5  $\mu\text{m}$ , 3.0  $\times$  150 mm) from Waters.

### Urinary creatinine

Urinary creatinine was assayed by the Roche CREA plus procedure using a Hitachi analyzer. This enzymatic procedure is optimized to provide minimum interferences from ascorbate and ketone bodies, and it generates results that correlate well with specific high-performance LC methods.

### Statistical analysis

Data were analyzed by using SAS (SAS Institute) and SUDAAN (Research Triangle Institute). Proc MEANS and TTEST were used to compute descriptive statistics and to test for statistical differences between pairs of variables. SUDAAN Proc REGRESS was used to fit regression models for mouth-level exposures and biomarkers. Because these variables are log-normally distributed, they were log transformed before modeling and related analyses. Geometric means and their 95% confidence intervals adjusted for other variables in the model were computed for each site and gender. Because of the short half-life of salivary cotinine (18-24 h), levels for the visits were averaged to obtain more consistent measures for

each subject over the study period. Levels of total and free NNAL from only the second visit were used for comparison with mouth-level exposure of NNK because the NNAL biomarker has a half-life of approximately 40 to 45 days, and levels are not expected to change appreciably between visits (33). All reported statistical differences were based on a  $P$  value of  $<0.05$ .

## Results

Tables 1 and 2 provide summary information on brands, subjects from each site, and the filter ventilation of the cigarettes smoked. The ages of the subjects included in the study ranged from 18 to 52 years. Except for Canada, an approximately equal number of men and women participated at each individual site. The mean self-reported number of usual cigarettes smoked per day was lowest for subjects from the United Kingdom and was significantly different ( $P < 0.05$ ) from the means reported by subjects from Australia and Minnesota. The actual number of cigarettes smoked during the 24-hour study period was significantly higher ( $P < 0.05$ ) for subjects from Australia compared with subjects in New York and the United Kingdom. Across sites, the self-reported average cigarettes-per-day values were higher than the actual number of cigarettes smoked over the 24-hour period of the study, with mean discrepancies ranging from 1.0 to 3.0 cigarettes per day.

Cigarette brands from each study location reflect differences in product regulation and preferences of the subjects included in the study (Table 2). In the United Kingdom, all of the cigarette brands from the subjects tested had ventilation of  $>20\%$ . This ventilation is likely due to the European Union "10-1-10" regulation, limiting emissions to 10 mg each of tar and carbon monoxide and 1.0 mg of nicotine under machine testing by using the ISO regimen. Cigarettes from Australia also tended toward higher ventilation, with approximately half of the subjects smoking cigarette brands having ventilation of 5% to 20% and half of the subjects smoking cigarette brands with ventilation of  $>20\%$ . Cigarette brands smoked by the subjects from Canada were highly weighted toward lower ventilation, with 84% having ventilation of  $<5\%$ .

### Mouth-level exposure to nicotine and nitrosamines

Table 3 provides summary data on the mouth-level exposure of nicotine, NNN, and NNK during the 24-hour test period. The subjects from the five sites clustered in two groups based on the 24-hour mouth-level exposure to nicotine: Levels of nicotine in subjects in Australia and Canada were similar but were significantly higher than levels in subjects in New York, Minnesota, and the United Kingdom, which were not statistically different from each other based on significance testing. The 24-hour mouth-level exposure to nicotine for subjects from the United Kingdom, where all cigarettes had

**Table 3.** Smoke constituent mouth-level exposure per 24 h by site; geometric means with their 95% confidence intervals

Study site	n	Smoke nicotine (mg/24 h)	Smoke NNN (ng/24 h)	Smoke NNK (ng/24 h)
		Geometric mean (95% confidence limits)	Geometric mean (95% confidence limits)	Geometric mean (95% confidence limits)
Canada	25	24.4 (20.3-29.5)	246 (204-297)	449 (372-541)
United States (New York)	25	20.4 (17.8-23.3)	3,140 (2,720-3,630)	1,490 (1,270-1,750)
United States (Minnesota)	28	18.6 (15.9-21.8)	2,510 (2,108-2,989)	1,150 (931-1,420)
Australia	25	27.4 (23.4-32.0)	368 (303-448)	350 (289-424)
United Kingdom	23	19.0 (15.9-22.7)	1,390 (1,070-1,800)	1,010 (797-1,280)

ventilation of >20%, was not statistically different than the mouth-level exposure of subjects from Minnesota and New York, where many cigarettes have lower ventilation (Tables 2 and 3).

Also shown in Table 3 are the large differences in NNN and NNK levels among sites. The 24-hour NNN mouth-level exposure of subjects from Canada was the lowest, with smoke NNN mouth-level exposure of smokers in Australia only slightly, but statistically significantly, higher. Levels of 24-hour mouth-level NNN exposure of subjects from the United Kingdom were at an intermediate level and not significantly different from the other groups. The 24-hour NNN mouth-level exposure of subjects in both U.S. locations (New York and Minnesota) was much higher than levels in the other countries. The wide range of these mouth-level exposures is clearly evident because the geometric mean of the 24-hour mouth-level NNN exposure of subjects in New York was >12 times higher than in Canada. In all cases, the differences in the log of 24-hour mouth-level NNN exposure for subjects among all sites were statistically significant ( $P < 0.05$ ). Similarly, the 24-hour mouth-level NNK exposures were highest in New York and lowest in Australia. All of the mouth-level NNK exposures between sites were statistically different ( $P < 0.05$ ), except the 24-hour mouth-level NNK exposures of subjects in Canada versus Australia, New York versus Minnesota, and Minnesota versus the United Kingdom. Thus, we were able to obtain samples from subjects with a wide range of TSNA mouth-level exposure.

#### Relationship of urinary NNAL to mouth-level exposure

We fitted a multiple regression model to examine the relationship between 24-hour mouth-level NNK and urinary total NNAL (both natural log transformed) after adjusting for the unique contributions of covariates. These covariates included urinary creatinine, minutes since the last cigarette, cigarette ventilation, body mass index, age, study site, and gender. In this analysis, 24-hour mouth-level NNK exposure ( $\beta = 0.46$ ,  $P = 0.004$ ) was positively correlated with the log of urinary NNAL. Gender ( $\beta = 0.13$ ,  $P = 0.03$ ), age ( $\beta = 0.006$ ,  $P = 0.04$ ), and creatinine ( $\beta = 0.003$ ,  $P < 0.0001$ ) were also positive-

ly correlated with the log of urinary NNAL. Minutes since the last cigarette ( $\beta = -0.002$ ,  $P = 0.008$ ) and cigarette ventilation ( $\beta = -0.006$ ,  $P = 0.02$ ) were inversely associated with the log of urinary NNAL. Body mass index was not a statistically significant determinant of urinary NNAL. In addition, controlling for other predictors in the model, the log urinary NNAL in subjects from Canada was significantly different from subjects from the United Kingdom ( $P = 0.02$ ) and New York ( $P = 0.003$ ). The log urinary NNAL in subjects from New York was significantly different from subjects from Minnesota ( $P = 0.03$ ). The multivariate model accounted for 63% of the total variance in log urinary NNAL.

#### Relationship of salivary cotinine to mouth-level exposure

We conducted a regression analysis to examine the relationship between 24-hour mouth-level nicotine with salivary cotinine (both log transformed), controlling for the same covariates, except creatinine, as the above analysis. Surprisingly, we found that 24-hour mouth-level nicotine was not a robust predictor of salivary cotinine ( $\beta = 0.29$ ,  $P = 0.057$ ) once other factors were included in the model. Minutes since the last cigarette ( $\beta = -0.001$ ,  $P = 0.02$ ) and cigarette ventilation ( $\beta = -0.006$ ,  $P = 0.003$ ) were inversely correlated with log of salivary cotinine, whereas age ( $\beta = 0.003$ ,  $P = 0.01$ ) was positively correlated with the log of salivary cotinine. Body mass index was not significantly associated with salivary cotinine. We observed, controlling for the above factors, that cotinine levels in Australian subjects were significantly different from those in Canadian ( $P = 0.02$ ) and New York ( $P = 0.006$ ) subjects. However, the multivariate model accounted for only 30% of the total variance in salivary cotinine.

#### Discussion

This study clearly documents that smokers have lower NNAL levels in their urine when they have lower mouth-level exposure to TSNA in mainstream smoke from their usual cigarette brands. Mouth-level exposure as assessed by using spent cigarette filters is a valid predictor of downstream levels of NNAL in urine. The study design

was made possible by the substantial differences in mainstream smoke TSNA levels across countries differing substantially in tobacco blend and curing practices (5, 8, 34-38). Our finding agrees with previous switching studies that examined biomarker levels in smokers alternatively using their usual cigarette brands and cigarettes with lower TSNA levels (16-18). However, this study is the first to examine differences in TSNA exposure among smokers from different countries, in which leading cigarette brands have very greatly differing TSNA levels. This study is also the first to examine TSNA exposure by using both mouth-level exposure and urine biomarkers.

Table 4 shows the crude geometric means of biomarkers of exposure separated by study site without adjustment for covariates (other than creatinine concentration in the urinary NNAL results). The levels of salivary cotinine showed very limited variation among sites, as expected, because, on average, smokers adjust the way they smoke to obtain a similar dose of nicotine. In contrast, creatinine-corrected total NNAL levels ordered from highest to lowest in the order New York, Minnesota, the United Kingdom, Australia, and Canada. Figure 1 shows the relation between the geometric means of the 24-hour smoke intake of NNK versus the geometric means of the creatinine-normalized total urine NNAL levels for the five different sites, along with 95% confidence intervals. An overall relationship is evident: The subjects from sites with higher levels of mouth-level exposure of NNK in smoke have higher levels of NNAL in their urine.

As expected, both urinary NNAL and mouth-level exposure to NNK were much higher among smokers from the United States, where American-blend cigarettes have higher levels of TSNAs, and lower among smokers in Canada (39) and Australia, where only bright tobacco with lower levels of TSNAs predominates. Levels in the United Kingdom fell in the middle of the range of exposures. Mouth-level exposure to NNN and NNK between subjects from Minnesota and New York was moderately different, likely resulting from dissimilarity in the personal demographics of the subject populations between these two sites, their level of addiction, or the way that they consume the product (e.g., puff volume, puff frequency,

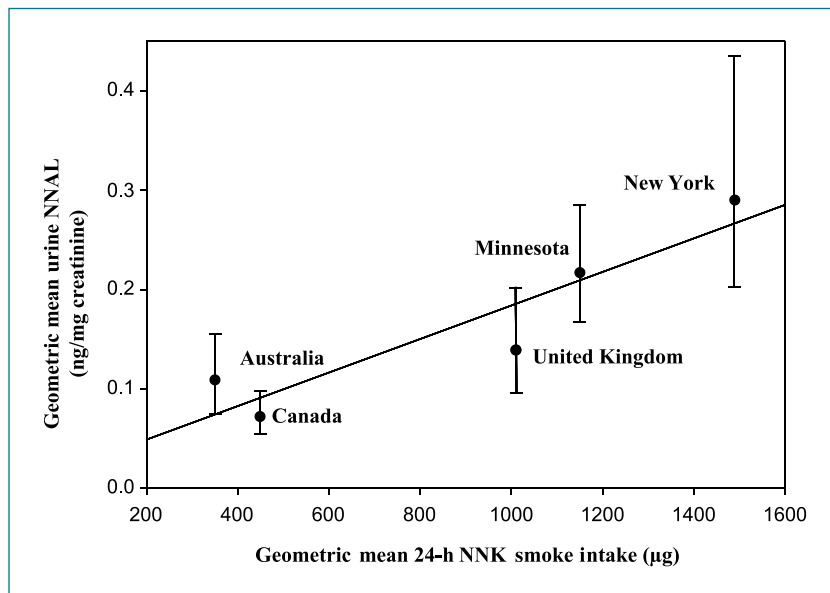
and total number of puffs per cigarette). The study was not designed to sample equivalent populations at the sites but to provide a wide range of mouth-level exposure to TSNAs. Additional analyses on differences in the products and the average and range of 24-hour mouth-level exposure are continuing.

Finding wide variation in mouth-level exposure to TSNAs reflected in biomarkers of exposure reveals that exposure to a class of potent carcinogens (i.e., TSNAs) found at high levels in cigarettes manufactured in the United States can be easily reduced in smokers by changing the curing practices and blend of tobacco used in manufacturing cigarettes. However, the selective reduction of particular classes of harmful constituents such as TSNAs does not necessarily translate into a measurably less harmful product. For example, although we found expected differences in biomarkers of TSNAs between smokers with different mouth-level exposure, we did not measure exposure to the many other toxins in tobacco smoke that could differ among cigarette brands and are known to vary by tobacco blend (40).

The range of mouth-level nicotine exposure under natural free-smoking conditions determined by using the butt solanesol method in this study (site geometric means ranged from 18.6 to 27.4 mg/24 h) was comparable with previous measures of delivered smoke nicotine. Benowitz and Jacob (41) measured nicotine intake by sampling blood and urine in smokers using their own brand, a high-delivery brand, and a low-delivery brand. While smoking their own brand, the subjects smoked an average of 35 mg nicotine over a 24-hour period. Benowitz and Jacob (42) also examined the daily intake of nicotine in 22 smokers of medium- to high-yield cigarettes who were smoking their usual brand of cigarette. In that study, the daily intake of nicotine averaged 37.6 mg (range, 10.5-78.6 mg). In both of the studies by Benowitz and Jacob, subjects were smokers who smoked at least one pack of cigarettes per day, a rate that may explain the higher daily nicotine intake in their studies compared with ours. Although our subjects smoked at least 10 cigarettes per day, they smoked fewer cigarettes and

**Table 4.** Salivary cotinine and creatinine-adjusted total NNAL levels by site

Country	n	Salivary cotinine (ng/mL)	Total NNAL—visit 2—creatinine normalized (ng/mg creatinine)
		Geometric mean (95% confidence limits)	Geometric mean (95% confidence limits)
Canada	25	268 (208-346)	0.072 (0.054-0.097)
United States (New York)	24	236 (200-279)	0.290 (0.203-0.416)
United States (Minnesota)	27	232 (174-309)	0.217 (0.167-0.283)
Australia	25	337 (289-392)	0.109 (0.075-0.158)
United Kingdom	23	213 (169-268)	0.139 (0.096-0.202)



**Figure 1.** Geometric means of 24-h mouth-level exposure of NNK (ng) versus creatinine-corrected concentrations of urinary NNAL (ng/g creatinine) for the five study sites. Error bars are 95% confidence limits.

obtained less daily total nicotine than Benowitz and Jacob's subjects. Forbes et al. (43) examined 24 subjects under normal conditions who smoked two brands of cigarettes differing in machine-smoked deliveries. They used the amount of nicotine trapped in the filter of each cigarette smoked and filter efficiency to calculate mouth-level exposure of each subject. They found that daily mouth-level nicotine exposure averaged 19.09 mg/d (range, 3.70-52.90). Thus, our results are in reasonable agreement with these previous studies.

In general, for sites with high mouth-level NNK, the levels of NNAL were higher. Thus, if the mouth-level exposure of TSNAs in smoke were decreased and all other factors (nicotine, ventilation, etc.) remained the same, a decrease should occur in the amount of TSNAs delivered to the smoker as measured in the emissions, in exposure as determined in the mouth-level exposure, and in levels of internal dose as measured by using biomarkers. When modeled, the levels of urinary NNAL were associated with variables that reflect exposure. Thus, minutes since the last cigarette and 24-hour NNK were significantly associated with NNAL. The 24-hour mouth-level exposure to NNK had a greater effect on explaining the variance in total urinary NNAL than any other variable except creatinine concentration. The effect of 24-hour mouth-level exposure was more important for urinary NNAL than it was for salivary cotinine. The increased dependence on actual delivery for NNK likely resulted because NNK levels varied much more over the range of products used than did nicotine.

Given the number of cigarettes smoked within a 24-hour period, we analyzed ~2,000 cigarette butts for solanesol, necessitating the development of a faster, higher throughput method using simpler equipment to conduct these assays (25). Although our measure of solanesol in butts reflects the amount of smoke pulled from the cigarette, it does not

account for other differences in smoking topography. Variations in subjects' depth of inhalation and length of time holding cigarette smoke in their lungs could affect exposure to tobacco TSNAs, nicotine, and other smoke constituents.

A relatively small sample size and measurement of mouth-level exposure and biomarkers of exposure over 24 hours only, dictated largely by practical considerations, limit the generalization of the results from this study. Future studies should expand the number of subjects, the length of time these subjects are monitored, and the number of cigarette specimens assessed from each subject and should include a wider range of products. This study was not designed to evaluate every possible confounder. Other variables, such as individual metabolic rates, also could be significantly associated with the relationship between delivery of smoke emissions and biomarkers of exposure and would help in assessing the variance of exposure biomarkers to the emissions from cigarette smoke.

Two recent studies (44, 45) have shown a direct association between measured levels of NNAL in smokers and the risk of lung cancer. This literature, combined with the findings in our current report, suggests that the higher levels of TSNAs in the smoke of leading U.S. cigarette varieties lead to higher mouth-level exposure to NNK and increased NNAL in smokers, which may be associated with excess lung cancer burden among U.S. smokers. However, although TSNAs have been identified as of significant concern, they are not the only harmful tobacco smoke constituents, and the extent of reductions in NNK that is necessary to see reductions in harm is unclear. Reducing TSNAs alone could adversely affect public health if it resulted in elevating other harmful constituents or if it led to public perceptions of increased product safety. Careful evaluation is critical when examining the relative risks of tobacco products, particularly when trying to juxtapose risks and health end points.



Nevertheless, this study has shown that smoking currently available cigarettes with lower TSNA emissions results in lower internal dose levels of NNAL, a biomarker of exposure to the known carcinogen NNK.

### Disclosure of Potential Conflicts of Interest

K.M. Cummings: honorarium, Pfizer; Expert testimony for plaintiffs against the tobacco industry. L. Shahab: honorarium, Pfizer.

### Acknowledgments

We thank Connie Sosnoff, LaQuasha Gaddis, Leah Henderson, and Rick Akins for assistance with the cotinine and NNAL assays and

Larry W. Hawk, Jr. and Daniel Gross for their assistance with data collection at the New York site.

### Grant Support

Data collection for this study was supported by grants P50 CA111236 (Roswell Park Transdisciplinary Tobacco Use Research Center) and P50 DA013333 (University of Minnesota Transdisciplinary Tobacco Use Research Center) from the National Cancer Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 01/19/2010; revised 03/15/2010; accepted 03/30/2010; published OnlineFirst 05/25/2010.

### References

- International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans, vol. 83. Tobacco smoke and involuntary smoking. Lyon: International Agency for Research on Cancer; 2004. Available from: <http://monographs.iarc.fr/ENG/Monographs/vol83/volume83.pdf>.
- De Roton C, Wiernik A, Wahlberg I, Vidal B. Factors influencing the formation of tobacco-specific nitrosamines in French air-cured tobaccos in trials and at the farm level. *Beitr Tabakforsch Int* 2005;21:305–20.
- Peele DM, Riddick MG, Edwards ME. Formation of tobacco-specific nitrosamines in flue cured tobacco. *Recent Adv Tobacco Sci* 2001; 27:3–12.
- Chamberlain WJ, Chortyk OT. Effects of curing and fertilization on nitrosamine formation in bright and burley tobacco. *Beitr Tabakforsch Int* 1992;15:87–92.
- Wu W, Zhang L, Jain RB, Ashley DL, Watson CH. Determination of carcinogenic tobacco-specific nitrosamines in mainstream smoke from U.S.-brand and non-U.S.-brand cigarettes from 14 countries. *Nicotine Tob Res* 2005;7:443–51.
- Ashley DL, Beeson MD, Johnson DR, et al. Tobacco-specific nitrosamines in tobacco from US brand and non-US brand cigarettes. *Nicotine Tob Res* 2003;5:323–31.
- Stepanov I, Carmella SG, Hecht SS, Duca G. Analysis of tobacco-specific nitrosamines in Moldovan cigarette tobacco. *J Agric Food Chem* 2002;50:2793–7.
- Gray N, Zaridze D, Robertson C, et al. Variation within global cigarette brands in tar, nicotine, and certain nitrosamines: analytic study. *Tob Control* 2000;9:351–2.
- Burns DM, Dybing E, Gray N, et al. Mandated lowering of toxicants in cigarette smoke: a description of the World Health Organization TobReg proposal. *Tob Control* 2008;17:132–41.
- Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem Res Toxicol* 1998;11:559–603.
- Xia Y, McGuffey JE, Bhattacharyya S, et al. Analysis of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in urine by extraction on a molecularly-imprinted polymer column and liquid chromatography/atmospheric-pressure ionization tandem mass spectrometry. *Anal Chem* 2005;77:7639–45.
- Muscat JE, Djordjevic MV, Stellman SD, Richie JP, Jr. Racial differences in exposure and glucuronidation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNK). *Cancer* 2005;103:1420–6.
- Carmella SG, Le Ka KA, Upadhyaya P, Hecht SS. Analysis of N- and O-glucuronides of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine. *Chem Res Toxicol* 2002;15:545–50.
- Carmella SG, Han S, Fristad A, Yang Y, Hecht SS. Analysis of total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine. *Cancer Epidemiol Biomarkers Prev* 2003;12:1257–61.
- Hecht S. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. *Carcinogenesis* 2002;23:907–22.
- Breland AB, Acosta MC, Eissenberg T. Tobacco specific nitrosamines and potential reduced exposure products for smokers: a preliminary evaluation of Advance. *Tob Control* 2003;12:317–21.
- Hughes JR, Hecht SS, Carmella SG, Murphy SE, Callas PW. Smoking behaviour and toxin exposure during six weeks use of a potential reduced exposure product: Omni. *Tob Control* 2004;13:175–9.
- Hatsukami DK, Lemmonds C, Zhang Y, et al. Evaluation of carcinogen exposure in people who used “reduced exposure” tobacco products. *J Natl Cancer Inst* 2004;96:844–52.
- Benowitz N. Compensatory smoking of low-yield cigarettes. In: Shopland DR, Burns DM, Benowitz NL, Amacher RH, editors. Risks associated with smoking cigarettes with low machine-measured yields of tar and nicotine. NCI Smoking and Tobacco Control Monograph No. 13. Bethesda (MD): U.S. Department of Health and Human Services, NIH, National Cancer Institute; 2001, p. 39–64.
- Hammond D, Fong GT, Cummings KM, Hyland A. Smoking topography, brand switching, and nicotine delivery: results from an *in vivo* study. *Cancer Epidemiol Biomarkers Prev* 2005;14:1370–5.
- Doll R, Hill AB. Smoking and carcinoma of the lung; preliminary report. *Br Med J* 1950;221:739–48.
- O'Connor RJ, Kozlowski LT, Hammond D, Vance TT, Stitt JP, Cummings KM. Digital image analysis of cigarette filter staining to estimate smoke exposure. *Nicotine Tob Res* 2007;9:865–71.
- St Charles FK, Krautter GR, Dixon M, Mariner DC. A comparison of nicotine dose estimates in smokers between filter analysis, salivary cotinine, and urinary excretion of nicotine metabolites. *Psychopharmacology (Berl)* 2006;189:345–54.
- Shepperd CJ, Eldridge AC, Mariner DC, McEwan M, Errington G, Dixon M. A study to estimate and correlate cigarette smoke exposure in smokers in Germany as determined by filter analysis and biomarkers of exposure. *Regul Toxicol Pharmacol* 2009;55: 97–109.
- Polzin GM, Wu W, Yan X, et al. Estimating smokers' mouth level exposure to select mainstream smoke constituents from discarded cigarette filter butts. *Nicotine Tob Res* 2009;11:868–74.
- Shahab L, Hammond D, O'Connor RJ, et al. The reliability and validity of self-reported puffing behaviour: evidence from a cross-national study. *Nicotine Tob Res* 2008;10:867–74.
- Wu W, Ashley DL, Watson CH. Simultaneous determination of five tobacco-specific nitrosamines in mainstream cigarette smoke by isotope dilution liquid chromatography/electrospray ionization tandem mass spectrometry. *Anal Chem* 2003;75:4827–32.
- Wagner KA, Finkel NH, Fossett JE, Gillman IG. Development of a quantitative method for the analysis of tobacco-specific nitrosamines in mainstream cigarette smoke using isotope dilution liquid chromatography/electrospray ionization tandem mass spectrometry. *Anal Chem* 2005;77:1001–6.
- Caldwell WS, Conner JW. Artifact formation during smoke trapping: an improved method for determination of N-nitrosamines in cigarette smoke. *J Assoc Off Anal Chem* 1990;73:783–9.
- Calafat AM, Polzin GM, Saylor J, Richter P, Ashley DL, Watson CH.

- Determination of tar, nicotine, and carbon monoxide yields in the mainstream smoke of selected international cigarettes. *Tob Control* 2004;13:45–51.
31. Bernert JT, Turner WE, Pirkle JL, et al. Development and validation of a sensitive method for the determination of serum cotinine in both smokers and nonsmokers by using liquid chromatography/atmospheric pressure ionization tandem mass spectrometry. *Clin Chem* 1997;43:2281–91.
  32. Bernert JT, McGuffey JE, Morrison MA, Pirkle JL. Comparison of serum and salivary cotinine measurements by a sensitive high-performance liquid chromatography/tandem mass spectrometry method as an indicator of exposure to tobacco smoke among smokers and non-smokers. *J Anal Toxicol* 2000;24:333–9.
  33. Hecht SS, Carmella SG, Chen M, et al. Quantitation of urinary metabolites of a tobacco-specific lung carcinogen after smoking cessation. *Cancer Res* 1999;59:590–6.
  34. King B, Borland R, Fowles J. Mainstream smoke emissions of Australian and Canadian cigarettes. *Nicotine Tob Res* 2007;9:835–44.
  35. Fischer S, Castonguay A, Kaiserman M, Spiegelhalter B, Preussmann R. Tobacco-specific nitrosamines in Canadian cigarettes. *J Cancer Res Clin Oncol* 1990;116:563–8.
  36. Fischer S, Spiegelhalter B, Preussmann R. Tobacco-specific nitrosamines in European and USA cigarettes. *Arch Geschwulstforsch* 1990;60:169–77.
  37. Fischer S, Spiegelhalter B, Eisenbarth J, Preussmann R. Investigations on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. *Carcinogenesis* 1990;11:723–30.
  38. Fisher B. Curing the TSNA problem. *Tob Reporter* 2000;August 2000:51–6.
  39. Rickert WS, Joza PJ, Sharifi M, Wu J, Lauterbach JH. Reductions in the tobacco specific nitrosamine (TSNA) content of tobaccos taken from commercial Canadian cigarettes and corresponding reductions in TSNA deliveries in mainstream smoke from such cigarettes. *Regul Toxicol Pharmacol* 2008;51:306–10.
  40. Ding YS, Zhang L, Jain RB, et al. Levels of tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons in mainstream smoke from different tobacco varieties. *Cancer Epidemiol Biomarkers Prev* 2008;17:3366–71.
  41. Benowitz NL, Jacob P III. Nicotine and carbon monoxide intake from high- and low-yield cigarettes. *Clin Pharmacol Ther* 1984;36:265–70.
  42. Benowitz NL, Jacob P III. Daily intake of nicotine during cigarette smoking. *Clin Pharmacol Ther* 1984;35:499–504.
  43. Forbes WF, Robinson JC, Hanley JA, Colburn HN. Studies on the nicotine exposure of individual smokers. I. Changes in mouth level exposure to nicotine on switching to lower nicotine cigarettes. *Int J Addict* 1986;11:933–50.
  44. Church TR, Anderson KE, Caporaso NE, et al. A prospectively measured serum biomarker for a tobacco-specific carcinogen and lung cancer in smokers. *Cancer Epidemiol Biomarkers Prev* 2009;18:260–6.
  45. Yuan JM, Koh WP, Murphy SE, et al. Urinary levels of tobacco-specific nitrosamine metabolites in relation to lung cancer development in two prospective cohorts of cigarette smokers. *Cancer Res* 2009;69:2990–5.

---

**Correction**

## Correction: Effect of Differing Levels of Tobacco-Specific Nitrosamines in Cigarette Smoke on the Levels of Biomarkers in Smokers

In this article (1), which was published in the June 2010 issue of *Cancer Epidemiology, Biomarkers & Prevention*, in Fig. 1, the x-axis is labeled with units of  $\mu\text{g}$ . The correct units, as given in the figure legend, are ng.

### Reference

1. Ashley DL, O'Connor RJ, Bernert JT, Watson CH, Polzin GM, Jain RB, Hammond D, Hatsukami DK, Giovino GA, Cummings KM, McNeill A, Shahab L, King B, Fong GT, Zhang L, Xia Y, Yan X, McCraw JM. Effect of differing levels of tobacco-specific nitrosamines in cigarette smoke on the levels of biomarkers in smokers. *Cancer Epidemiol Biomarkers Prev* 2010;19:1389–98.

---

©2010 American Association for Cancer Research.  
doi: 10.1158/1055-9965.EPI-10-0731

# Cancer Epidemiology, Biomarkers & Prevention

**AACR** American Association  
for Cancer Research

## Effect of Differing Levels of Tobacco-Specific Nitrosamines in Cigarette Smoke on the Levels of Biomarkers in Smokers

David L. Ashley, Richard J. O'Connor, John T. Bernert, et al.

*Cancer Epidemiol Biomarkers Prev* 2010;19:1389-1398. Published OnlineFirst May 25, 2010.

**Updated version** Access the most recent version of this article at:  
doi:[10.1158/1055-9965.EPI-10-0084](https://doi.org/10.1158/1055-9965.EPI-10-0084)

**Cited articles** This article cites 42 articles, 12 of which you can access for free at:  
<http://cebp.aacrjournals.org/content/19/6/1389.full#ref-list-1>

**Citing articles** This article has been cited by 8 HighWire-hosted articles. Access the articles at:  
<http://cebp.aacrjournals.org/content/19/6/1389.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cebp.aacrjournals.org/content/19/6/1389>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.