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

David L. Ashley1, Richard J. O’Connor2, John T. Bernert1, Clifford H. Watson1, Gregory M. Polzin1, Ram B. Jain1, David Hammond3, Dorothy K. Hatsukami6, Gary A. Giovino3, K. Michael Cummings2, Ann McNeill7, Lion Shahab8, Bill King9, Geoffrey T. Fong5,10, Liqin Zhang1, Yang Xia1, Xizheng Yan1, and Joan M. McCraw1

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 TSNAs 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 (β = 0.46, P = 0.004). The relationship between mouth-level exposure to nicotine and its salivary metabolite, cotinine, was not statistically significant (β = 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-butano (-N NA), N-nitrosornicotine (NNN), N-nitrosodentamine, and N-nitrosanatabine. 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, NOx 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 TSNAs. Although the actual degree of health benefit from lowering TSNAs 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.

TSNAs 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-(methylamino)-1-(3-pyridyl)-1-butanol (NNAL), and total (or free)
smokers who switched to Omni for 6 weeks. Researchers found that, in spite of the reported lower NNK smoke emissions from Omni cigarettes, NNAL concentrations in the urine were found that, for those cigarette smokers who 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 filler (23, 24). A method of estimating smoke exposure by using solanesol, a naturally occurring component in tobacco deposited during smoking in the cigarette filter butts, 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.
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 after a laboratory visit; or providing saliva samples containing nicotine or cotinine concentrations below the limits of detection.

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

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

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

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

*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 fre-
quency, 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 TSNAs 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 pre-
viously published (27). Sample preparation was stream-
lined 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 in-
corporated nicotine in our standard TSNA method. Iso-
topically labeled nicotine was included in the TSNA
internal standard panel for nicotine quantification.
This method allowed the quantification of both nicotine
and TSNAs 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 de-
tection 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 quadruple atmo-
spheric pressure chemical ionization mass spectrometry
in selected ion monitoring mode.

**Calculation of mouth-level exposure to
TSNAs 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 collect-
ed on CFPs analyzed in samples resulting from smoking
the same cigarettes but under multiple smoking
conditions. This analysis allowed butt levels of solane-
sol 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 de-
ivered component deviated from linearity at low deliv-
er 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 submit-
ted butts were greater than the maximum levels achiev-
able by using the most intense machine smoking
conditions. The levels of TSNAs 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 com-
parison 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 TSNAs—
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
were averaged to obtain more consistent measures for half-life of salivary cotinine (18–24 h), levels for the visits computed for each site and gender. Because of the short intervals adjusted for other variables in the model were analyses. Geometric means and their 95% confidence in- sion models for mouth-level exposures and biomarkers. Because these variables are log-normally distributed, SUDAAN (Research Triangle Institute) was used to fit regression.

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 sub- jects 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

Frozen at ≤−20°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., nonconju- gated) 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 μL 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 μm, 3.0 × 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 interfer- ences 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 in- tervals 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.

Mouth-Level TSNA Exposure and Biomarkers
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 NNK 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 (β = 0.46, P = 0.004) was positively correlated with the log of urinary NNAL. Gender (β = 0.13, P = 0.03), age (β = 0.006, P = 0.04), and creatinine (β = 0.003, P < 0.0001) were also positively correlated with the log of urinary NNAL. Minutes since the last cigarette (β = −0.002, P = 0.008) and cigarette ventilation (β = −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 (β = 0.29, P = 0.057) once other factors were included in the model. Minutes since the last cigarette (β = −0.001, P = 0.02) and cigarette ventilation (β = −0.006, P = 0.003) were inversely correlated with log of salivary cotinine, whereas age (β = 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 TSNAs 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 cigarette-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 TSNAAs, and lower among smokers in Canada (39) and Australia, where only bright tobacco cigarettes 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>
<thead>
<tr>
<th>Country</th>
<th>n</th>
<th>Salivary cotinine (ng/mL) Total NNAL—visit 2—creatinine normalized (ng/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Geometric mean (95% confidence limits)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Geometric mean (95% confidence limits)</td>
</tr>
<tr>
<td>Canada</td>
<td>25</td>
<td>268 (208-346)</td>
</tr>
<tr>
<td>United States</td>
<td>24</td>
<td>236 (200-279)</td>
</tr>
<tr>
<td>(New York)</td>
<td></td>
<td>0.072 (0.054-0.097)</td>
</tr>
<tr>
<td>United States</td>
<td>27</td>
<td>232 (174-309)</td>
</tr>
<tr>
<td>(Minnesota)</td>
<td></td>
<td>0.217 (0.167-0.283)</td>
</tr>
<tr>
<td>Australia</td>
<td>25</td>
<td>337 (289-392)</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>23</td>
<td>213 (169-268)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.109 (0.075-0.158)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.139 (0.096-0.202)</td>
</tr>
</tbody>
</table>
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.

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


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