Urine Cotinine Underestimates Exposure to the Tobacco-Derived Lung Carcinogen 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone in Passive Compared with Active Smokers

Neal Benowitz1,2, Maciej Lukasz Goniewicz1,2,4, Mark D. Eisner3, Eduardo Lazcano-Ponce5, Wioleta Zielinska-Danch4, Bartosz Koszowski4, Andrzej Sobczak4, Christopher Havel1, and Peyton Jacob III1

Abstract

Objectives: Cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are widely used biomarkers for tobacco-derived nicotine and the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), respectively. The discrepancy between cotinine levels in relation to disease risk comparing active versus passive smoking suggests a nonlinear tobacco smoke dose-response and/or that cotinine is not providing an accurate measure of exposure to the toxic constituents of secondhand tobacco smoke.

Methods: Cotinine and NNAL were measured in the urine of 373 active smokers and 228 passive smokers.

Results: Average cotinine levels were 1,155 (interquartile range, 703-2,715) for active smokers and 1.82 (0.45-7.33) ng/mg creatinine for passive smokers. Average NNAL levels were 183 (103-393) and 5.19 (2.04-11.6) pg/mg creatinine, respectively. NNAL/cotinine ratio in urine was significantly higher for passive smokers when compared with active smokers (2.85 × 103 versus 0.16 × 103, P < 0.0001).

Conclusions: Passive smoking is associated with a much higher ratio of NNAL/cotinine in the urine compared with active smoking.

Impact: Cotinine measurement leads to an underestimation of exposure to the carcinogen NNK from second-hand smoke when compared with active smoking.

Introduction

Cotinine, the major proximate metabolite of nicotine, is widely used as a biomarker of tobacco exposure (1, 2). Measurement of cotinine in blood, saliva, or urine, has been used to support epidemiologic findings of causal relationships between secondhand smoke (SHS) exposure and lung cancer, cardiovascular disease, and aggravation of chronic obstructive pulmonary disease (COPD) in adults, and asthma in children (3-6).

If one measures the concentrations of cotinine in people exposed to SHS compared with active smoking, the relative exposure with SHS exposure is typically 1% or less, comparing blood or urine cotinine levels in SHS versus active smokers (1). In contrast, the relative risk of lung cancer and cardiovascular disease with SHS exposure compared with active smoking is much higher than the relative cotinine levels. For example, the odds ratio for cigarette smoking and lung cancer is 1.25 for SHS exposure and 10 for active smoking, translating into a relative attributable risk ratio of 36 (7). The odds ratio for cardiovascular disease is about 1.3 with SHS and 2 with active smoking, translating into a relative attributable risk ratio of 3.3 (8). The discrepancy between relative cotinine levels and disease risk with SHS versus active smoking suggests that there is a nonlinear tobacco smoke dose-response and/or that cotinine is not providing an accurate measure of exposure to the toxic constituents of tobacco smoke.

Among the most important tobacco smoke toxins are tobacco-specific nitrosamines, which are derived from nicotine and other tobacco alkaloids mostly during tobacco curing (9). These compounds are known animal and human carcinogens. The most potent carcinogenic tobacco-specific nitrosamine is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK). NNK is metabolized in the human body to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is further conjugated to form NNAL N- and O-glucuronides (NNAL-Gluc). The presence of NNAL in urine is highly specific for tobacco exposure and provides a biochemical link between both active and passive smoking and lung cancer (3).
There are several reasons to suspect that cotinine levels underestimate exposure to NNK. As SHS ages, concentrations of nicotine decline faster than many other gaseous and particulate components of smoke due to the absorption of nicotine on surfaces such as walls and carpets (10). In addition, NNK levels increase as SHS ages, presumably related to the reaction of nicotine with nitric oxide in SHS (11, 12). Thus, exposure to SHS over a period of time, in between emissions from individual cigarettes, would be expected to result in lower relative intake of nicotine versus NNK as compared with that obtained by the active smoker from mainstream smoke. Additionally, the half-life of NNAL is much longer (10 d) than that of cotinine (16 h; refs. 2, 13). As a result, with intermittent exposure to SHS, cotinine levels will decline between exposures to a much greater extent than NNAL, which would further increase the ratio of NNAL/cotinine in passive versus active smokers.

The aim of our study was to examine the hypothesis that cotinine levels do not provide an accurate assessment of exposure to the tobacco smoke carcinogen NNK in passive smokers. We compared the relative concentrations of NNAL and cotinine. The subjects included 373 smokers and 228 nonsmokers with evidence of exposure to SHS. Detailed characteristics of the study groups are presented in Table 1.

Adult daily smokers were recruited in three different studies (studies A-C). The groups were selected to include both regular daily smokers and occasional smokers. Study A was a study of tobacco smoke biomarkers comparing African American and white smokers in San Francisco (n = 128). Study B assessed the effect of smoking topography on tobacco biomarkers among daily smokers recruited in the Silesia region, Poland (n = 187, Koszowski and Sobczak). Study C compared urine biomarkers in daily compared with occasional smokers in Pittsburgh (n = 58, Shiffman).

Nonsmokers exposed to SHS had participated in two previously published (studies D and F) and one unpublished (study E) studies in which urine was collected for the assessment of SHS exposure. Subjects were selected to range from very light to heavy SHS exposure. Study D was a U.S. cohort study of nonsmoking adults with COPD, who collected samples in their homes and mailed samples to the investigators (n = 72; ref. 14). Among recruited subjects, there were no current smokers, although almost half of them had a history of smoking (n = 35; refs. 1, 2). Only subjects who had biochemical evidence of SHS exposure (cotinine or NNAL levels above the limit of quantitation) were included. Study group E was a Polish cohort of nonsmoking adults with urine samples to assess secondhand exposure to tobacco smoke in home and work environments. Subjects provided morning spot samples during screening medical tests (n = 108, Zielinska-Danch). Study F included volunteers from central Mexico.

### Materials and Methods

**Study design and subjects**

A descriptive study was conducted in smoking and nonsmoking subjects from the United States, Poland, and Mexico, each of whom provided a urine sample for the measurements of concentration of NNAL and cotinine. The subjects included 373 smokers and 228 nonsmokers with evidence of exposure to SHS. Detailed characteristics of the study groups are presented in Table 1.

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### Table 1. Characteristics of the study groups (n = 601)

<table>
<thead>
<tr>
<th>Study</th>
<th>Active smokers (n = 373)</th>
<th>Nonsmokers with evidence of exposure to SHS (n = 228)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample size</td>
<td>373</td>
<td>228</td>
</tr>
<tr>
<td>Subjects with detectable cotinine levels (%)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Subjects with detectable NNAL levels (%)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Valid subjects</td>
<td>373</td>
<td>228</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>74 (58%)</td>
<td>83 (44%)</td>
</tr>
<tr>
<td>Race-ethnicity (white, non-Hispanic)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>38.2 ± 10.9</td>
<td>36.3 ± 13.8</td>
</tr>
<tr>
<td>Nationality</td>
<td>United States</td>
<td>Poland</td>
</tr>
<tr>
<td>Cigarettes per day (mean ± SD)</td>
<td>18.4 ± 8.2</td>
<td>15.0 ± 8.4</td>
</tr>
<tr>
<td>Analytical data (geometric mean, IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>1,993 (1,180-3,604)</td>
<td>928 (646-1,942)</td>
</tr>
<tr>
<td>Cotinine (ng/mg creatinine)</td>
<td>2,471 (1,662-4,393)</td>
<td>882 (542-1,914)</td>
</tr>
<tr>
<td>NNAL (pg/mL)</td>
<td>174 (76.7-367)</td>
<td>192 (119-411)</td>
</tr>
<tr>
<td>NNAL (pg/mg creatinine)</td>
<td>223 (134-366)</td>
<td>182 (102-418)</td>
</tr>
<tr>
<td>NNAL/cotinine ratio (×10^3)</td>
<td>0.09 (0.06-0.13)</td>
<td>0.21 (0.13-0.31)</td>
</tr>
</tbody>
</table>

(Continued on the following page)
who provided a urine sample after being in a discotheque in which smoking was ongoing for at least 1 hour ($n = 81$; ref. 15).

**Analytical chemistry**

Cotinine (unconjugated) was analyzed by liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry by a method similar to the one described by Bernert et al. (16). Total NNAL (after hydrolysis of NNAL glucuronide) was analyzed by liquid chromatography-tandem mass spectrometry as described by Jacob et al. (17). The limits of quantification were 0.05 ng/mL and 0.25 pg/mL for cotinine and NNAL, respectively.

**Data analysis**

Nonparametric Kruskal-Wallis ANOVA was used to compare biomarker levels and their ratios among study groups. Pearson correlations of log-transformed urine cotinine and NNAL concentrations were done. All analyses were done using STATISTICA data analysis software system v. 8 (StatSoft, Inc.).

**Results**

Geometric mean urine cotinine and NNAL concentrations and the ratio of NNAL/cotinine for each of the study groups are shown in Table 1, and comparisons between active and passive smokers in Fig. 1. The distribution of the NNAL/cotinine ratio among all subjects is shown in Fig. 2. The average ratio of NNAL/cotinine was almost 18 times higher in passive smokers (2.85; interquartile range, 1.32-6.14 $\times 10^3$) compared with active smokers (0.16; interquartile range, 0.09-0.26 $\times 10^3$; $P < 0.0001$), and there was relatively little overlap in the ratio in the active versus passive smoking groups. The two subjects with the highest NNAL/cotinine ratios (361 and 107 $\times 10^3$) were from passive smoker groups D and E, respectively; and their high ratios were due to extraordinarily high levels of urine NNAL.

Among active smokers, urine cotinine/creatinine ratios were significantly higher for group A compared with groups B and C ($P < 0.05$) and urine NNAL/creatinine ratios were significantly higher in group C compared with groups A and B ($P < 0.05$). Among smokers, the urine NNAL/cotinine ratio was significantly higher in groups B and C compared with group A ($P < 0.05$). Within group A, there was no significant difference in the NNAL/cotinine ratio comparing African American and white smokers.

Among passive smokers, group F had significantly higher urine cotinine and NNAL levels and lower NNAL/cotinine ratios compared with groups D and E (all $P < 0.05$). Among active smokers, log-transformed concentrations of cotinine and NNAL urine were highly correlated: group A, $r = 0.79$; group B, $r = 0.80$; group C, $r = 0.79$; all smokers, $r = 0.76$ (all $P < 0.05$). Among passive smokers, these biomarkers were also highly correlated: group D, $r = 0.71$; group E, $r = 0.74$; group F, $r = 0.67$; all passive smokers, $r = 0.79$ (all $P < 0.05$).

**Discussion**

The main and novel finding of our study is that the ratio of NNAL to cotinine is much higher in passive

### Table 1. Characteristics of the study groups ($n = 601$) (Cont’d)

<table>
<thead>
<tr>
<th></th>
<th>Passive smokers ($n = 228$)</th>
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<tbody>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>72 (75%)</td>
</tr>
<tr>
<td></td>
<td>54 (75%)</td>
</tr>
<tr>
<td></td>
<td>48 (67%)</td>
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<tr>
<td></td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>19 (42%)</td>
</tr>
<tr>
<td></td>
<td>42 (93%)</td>
</tr>
<tr>
<td></td>
<td>64.6 ± 5.8</td>
</tr>
<tr>
<td>United States</td>
<td>Poland</td>
</tr>
<tr>
<td></td>
<td>0.57 (0.15-2.07)</td>
</tr>
<tr>
<td></td>
<td>0.85 (0.18-4.15)</td>
</tr>
<tr>
<td></td>
<td>3.13 (0.92-8.59)</td>
</tr>
<tr>
<td></td>
<td>4.69 (1.15-10.3)</td>
</tr>
<tr>
<td></td>
<td>5.50 (2.65-11.7)</td>
</tr>
</tbody>
</table>

Abbreviations: IQR, interquartile range; N.A., data not available.
compared with active cigarette smokers. This finding supports the idea that passive smoking leads to relatively higher exposure to the carcinogen NNK in relation to nicotine compared with active smoking. Thus, the use of urine cotinine to estimate exposure to the tobacco smoke carcinogen NNK in passive smokers with the assumption of a similar relationship between nicotine and NNK as that seen in active smokers, leads to an underestimation of exposure and potential cancer risk. Our data on absolute concentrations of NNAL and cotinine in urine among active and passive smokers are similar to those reported by other researchers (1, 18); however, to the best of our knowledge, no one has published a formal comparison of ratios in active and passive smokers.

Our study focuses on NNAL, which is a highly specific biomarker of tobacco smoke exposure. We do not know if cotinine measurement also underestimates exposure to other tobacco smoke toxins based on biomarker measures in humans because the other toxins are not specific to tobacco exposure. However, studies of the concentrations of nicotine and other tobacco smoke constituents in the air immediately after smoking and then followed over time indicate that nicotine levels decline much faster than do most other smoke constituents (10). Thus, it is likely that measurement of cotinine, which reflects intake of nicotine, also underestimates exposure to other tobacco smoke toxins. Epidemiologic data comparing risks of lung cancer and cardiovascular disease in SHS exposed versus active smokers show much higher risk for SHS compared with active smokers based on relative cotinine levels (7, 8). Thus, cotinine underestimates the risk of tobacco-related diseases in passive smokers compared with active smokers.

On average for U.S. cigarettes, the mainstream/sidestream smoke ratios for nicotine and NNK are 2.31 and 0.40, respectively (19). Given these ratios and based on exposure to fresh sidestream smoke, one would expect that passive smokers would be exposed to relatively more nicotine than NNK compared with active smokers, which is opposite to what we observed. As described above, as SHS ages nicotine levels decline while NNK levels increase, which most likely explains our observations.

Our study has potential limitations with respect to generalizability. Our subjects were not representative of the general U.S. population. Our active smokers came from both the United States and Poland. Our SHS-exposed subjects were also multinational, with different levels of SHS exposure. These included heavily exposed (Mexican discotheques), moderately exposed (Polish workers), and lightly exposed (COPD subjects in the United States). The duration of exposure to SHS was also different in these three groups. The Mexican subjects were exposed for a short period of time, the Polish and COPD subjects were presumably exposed over a longer period of time. Furthermore, it is unknown if this is an effect of COPD on the absorption of nicotine or NNK.

Comparison of the urine NNAL/cotinine ratio within the three groups of smokers indicated that Polish regular smokers had a significantly higher ratio than U.S. regular smokers. These groups had similar NNAL levels but the U.S. smokers had, on average, higher cotinine levels. This difference between groups of smokers could be due to the difference in racial composition [the U.S. group included nearly half African Americans, who metabolize cotinine and possibly NNAL more slowly than whites (refs. 20, 21), and/or due to differences in NNK levels in American versus Polish cigarettes]. However, we found no difference in the NNAL/cotinine ratio comparing African American to white smokers, suggesting that racial differences in metabolism do not explain our findings. The NNK content of cigarette tobacco and the NNK yield of cigarettes by machine-testing are known to differ in different countries (22, 23). However, we were unable to locate any published data on NNK content of Polish cigarettes. Of note is that our occasional U.S. smokers had a NNAL/cotinine ratio similar to Polish regular smokers.

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**Figure 1.** Comparison of geometric means of cotinine, NNAL and NNAL/cotinine ratios among self-reported active ($n = 373$) and passive smokers ($n = 228$; bars show interquartile ranges).
Among the passive smokers, the two groups of lightly exposed smokers in the United States and Poland had similar ratios, but the heavily exposed Mexican group had a significantly higher ratio than either of the other two groups. Cigarettes from Mexico are reported to have lower NNK content and yield than U.S. cigarettes (22, 23). Despite lower NNK levels in the tobacco, the NNAL/cotinine ratios were higher in Mexican passive smokers, indicating that country differences cannot explain our results. Considering all of our groups of smokers and nonsmokers, there seems to be a pattern going from passive smokers to active smokers, such that the heavier the exposure, the lower the NNAL/cotinine ratio.

In our interpretation of the study findings, we make the assumption that urine NNAL levels quantitatively reflect systemic NNK exposure, which is expected because NNAL is a metabolite of NNK. In active smokers, urine NNAL is strongly correlated with mouth-level exposure to NNK, as assessed by measuring the latter in cigarette butts (24). The possibility of dose-dependent metabolism of NNK such that there is a saturation of metabolism at high doses with relatively less excretion of NNAL might be considered as a potential confounder. We are aware of only one study that has examined dose-dependent metabolism of NNK, a study carried out in rodents (25). This study found that urine recovery of NNAL was greater with higher compared with lower doses of NNK. If this is also the case for people, it would bias against our findings of higher NNAL/cotinine ratios in those exposed to lower compared with higher levels of tobacco smoke. Thus, the dose-dependent metabolism of NNK does not seem to explain our findings.

In conclusion, passive smoking is associated with a much higher ratio of NNAL/cotinine in the urine compared with active smoking. This finding is robust and is seen even with the inclusion of subjects from different countries and with different levels of active and passive smoke exposures. Even the most heavily exposed passive smokers had much lower ratios than occasional light active smokers, supporting the idea that the use of cotinine measurement leads to an underestimation of exposure to the carcinogen NNK from SHS when compared with active smoking.

Disclosure of Potential Conflicts of Interest

N.L. Benowitz, tobacco litigation regarding nicotine addiction.

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References

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