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Biomarkers of chronic tobacco smoke exposure help to increase our understanding of tobacco-related cancer mechanisms and to develop preventive measures. Systemic exposure to cigarette smoke is commonly monitored by measuring nicotine and its major metabolite cotinine in urine, saliva, blood, and hair (4-12). In some studies, nicotine and cotinine in urine, plasma, and toenail cotinine correlated with cigarettes smoked per day (r = 0.24; P = 0.015 and r = 0.26; P = 0.009, respectively). Toenail cotinine correlated with plasma nicotine (r = 0.39; P < 0.001); toenail cotinine correlated with plasma cotinine (r = 0.45; P < 0.001) and plasma trans-3'-hydroxycotinine (r = 0.30; P = 0.008); and toenail NNAL correlated with urine NNAL (r = 0.53; P = 0.005). The results of this study provide essential validation data for the use of toenail biomarkers in investigations of the role of chronic tobacco smoke exposure in human cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(7):1382–6)

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Most investigations show a significant positive correlation between urinary cotinine and total NNAL (19), indicating that both of these compounds are biomarkers of tobacco toxicant exposure. Measurement of urinary cotinine and total NNAL is a common approach and has several advantages, but a disadvantage of urinary biomarkers is their transient nature (19). Thus, the distribution half-life of urinary total NNAL is 3 to 4 days (23), and cotinine has a half-life of only ~15 to 17 h (28). Recently, we developed sensitive and quantitative methods for analysis of nicotine, cotinine, and NNAL in human toenails (27). Measurement of toenail biomarkers has certain advantages, including ease of collection and storage and potential evaluation of cumulative exposure over a relatively long period due to the slow (~0.1 cm/mo) growth of toenails (28). Another important advantage of toenail nicotine, cotinine, and NNAL measurements is the seemingly indefinite stability of the collected sample caused by incorporation of these biomarkers into the keratinic matrix of the nail.

Although our previous study provided accurate and sensitive methods for analysis of nicotine, cotinine, and NNAL in human toenails, there is a need for their validation as biomarkers of human chronic exposure to tobacco smoke. The criteria for evaluation of a new biomarker include the relationship between the level of exposure and the amount of the biomarker in a biological sample; relationship between the new biomarker and one that is already proven and widely accepted; effects of gender, age, and race on the biomarker level; longitudinal intrasubject reliability; and kinetics of biomarker elimination from the biological sample after exposure is stopped. In this study, we evaluated the newly developed toenail biomarkers by investigating their relationship to demographic factors, reported exposure, plasma nicotine, cotinine, and trans-3'-hydroxycotinine, and urinary total NNAL. Structures of the biomarkers analyzed here are shown in Fig. 1.
Materials and Methods

**Caution.** NNAL is carcinogenic and mutagenic and should be handled with extreme care, using appropriate protective clothing and ventilation at all times.

**Chemicals and Enzymes.** NNAL was purchased from Toronto Research Chemicals, Inc. [Pyridine-D$_4$]NNAL was synthesized from [pyridine-D$_4$]ethyl nicotinate (Cambridge Isotope Laboratories) as described previously (29, 30). $^{13}$C$_6$NNAL was synthesized by NaBH$_4$ reduction of $^{13}$C$_6$-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Cambridge Isotope Laboratories). Nicotine, [CD$_3$]nicotine, cotinine, and [CD$_2$]cotinine were purchased from Sigma Chemical Co., and trans-3'-hydroxycotinine was purchased from Toronto Research Chemicals.

**Analyses.**

Nicotine, Cotinine, and NNAL in Toenails. Nicotine, cotinine, and NNAL in toenail clippings were analyzed as described previously (27). Because the analysis involves NaOH digestion, N-glucuronides of these compounds would be converted to their aglycones. Further experiments with β-glucuronidase indicated that NNAL-O-Gluc was not present in human toenails (27). Therefore, the values reported in this article are total nicotine, cotinine, and NNAL, but it is likely that these analytes in toenails are mainly in their nonconjugated form.

Nicotine, Cotinine, and 3'-Hydroxycotinine in Plasma. Concentrations of cotinine, and trans-3'-hydroxycotinine in plasma were determined by liquid chromatography-tandem mass spectrometry as described previously (31). Plasma nicotine concentration was measured by gas chromatography with nitrogen phosphorus detection as described previously (32). Values are free (nonconjugated) nicotine, cotinine, and 3'-hydroxycotinine.

NNAL in Urine. To measure total urinary NNAL, 10,000 units of *Helix pomatia* β-glucuronidase were added to 5 mL aliquots of urine, and the sample was incubated for 48 h at 37 °C and further worked up and analyzed as described elsewhere (33).

**Subjects.** The subjects were regular smokers who had smoked for at least 1 year and who were healthy by self-report. Users of smokeless tobacco, pipes, cigars, or nicotine medications were excluded, as were people who used medications other than vitamins, hormones, and over the counter analgesics.

Seventy-nine smokers with available toenail samples were selected from subjects being recruited in San Francisco for a larger study of the racial differences in smoking behaviors and tobacco smoking biomarkers. The subjects were recruited by newspaper advertisements, notices posted in local colleges, community centers and other public places, and via the internet (Craig’s list and our Web site). The study protocol was approved by the Institutional Review Board at the University of California San Francisco. Eligible subjects were asked to come to the General Clinical Research Center at San Francisco General Hospital Medical Center, having smoked their cigarettes in their usual way before the study visit. Written consent was obtained, and subjects were asked to complete questionnaires that included demographic and smoking history questions. A blood sample, urine sample, and toenail clippings were collected.

To improve the statistical power of the results, the sample size was increased by recruiting of another 26 active smokers from several smoking studies conducted at the Transdisciplinary Tobacco Use Research Center (Minneapolis, MN). The entrance criteria of these studies required subjects to smoke at least 10 cigarettes per day for at least 1 year. Subjects were offered the opportunity to participate in this addendum study for additional payment. All studies were approved by the University of Minnesota Research Subjects’ Protection Programs Institutional Review Board Human Subjects Committee.

**Statistical Analyses.** The distribution of each biomarker was assessed and log transformation was used if needed. In addition to the summary statistics, the analyses included the Pearson correlation, the two-sample *t* test, and multiple regression to determine predictive models for the biomarkers. All of the statistical analyses were carried out using SAS version 9.1 (SAS Institute, Inc.). *P* values <0.05 were considered statistically significant.

**Results**

There were 105 participants in the study. Their demographic characteristics and smoking and biomarker data are summarized in Table 1.

A total of 104 participants provided age information and 103 gender and race information. The mean age was 38.9 years (SD, 11.7 years); 55 (53.4%) were male and 51 (49.5%) were White. Data for number of cigarettes smoked per day (CPD) were available for 104 participants and ranged from 5 to 50 with a mean of 18 (SD, 8.4).

Data on plasma nicotine, cotinine, and trans-3'-hydroxycotinine were available for 78 subjects; data on urinary total NNAL were available for 71 subjects. Toenail samples from all

### Table 1. Characteristics of the study participants and biomarker levels in their toenails, plasma, and urine

<table>
<thead>
<tr>
<th>Characteristic/biomarker</th>
<th><em>n</em></th>
<th>Frequency</th>
<th>Mean (SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>104</td>
<td>—</td>
<td>38.9 (11.7)</td>
<td>19-68</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>103</td>
<td>55:48</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Race (White/African-American)</td>
<td>103</td>
<td>51:52</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CPD</td>
<td>104</td>
<td>—</td>
<td>18 (8.4)</td>
<td>5-50</td>
</tr>
<tr>
<td>Toenail nicotine (ng/mg)</td>
<td>105</td>
<td>—</td>
<td>5.44 (6.41)</td>
<td>0.01-33.18</td>
</tr>
<tr>
<td>Toenail cotinine (ng/mg)</td>
<td>103</td>
<td>0.67 (0.79)</td>
<td>0.01-3.87</td>
<td></td>
</tr>
<tr>
<td>Toenail NNAL (pg/mg)</td>
<td>51</td>
<td>—</td>
<td>0.18 (0.22)</td>
<td>0.01-1.10</td>
</tr>
<tr>
<td>Plasma nicotine (ng/mL)</td>
<td>78</td>
<td>—</td>
<td>9.56 (7.74)</td>
<td>0.00-35.40</td>
</tr>
<tr>
<td>Plasma cotinine (ng/mL)</td>
<td>78</td>
<td>—</td>
<td>198 (134)</td>
<td>0-580</td>
</tr>
<tr>
<td>Plasma trans-3'-hydroxycotinine (ng/mL)</td>
<td>78</td>
<td>—</td>
<td>69.3 (33.1)</td>
<td>0-251.8</td>
</tr>
<tr>
<td>Urine NNAL (ng/mL)</td>
<td>71</td>
<td>—</td>
<td>0.24 (0.26)</td>
<td>0.01-1.37</td>
</tr>
</tbody>
</table>
105 subjects were analyzed for nicotine and cotinine. Because of the limited size of a toenail sample, only 51 samples were analyzed for NNAL. Frequency histograms of toenail NNAL and toenail cotinine are shown in Fig. 2. The overall mean toenail biomarker levels were 5.44 ng nicotine/mg toenail (SD, 6.41), 0.67 ng cotinine/mg toenail (SD, 0.79), and 0.18 pg NNAL/mg toenail (SD, 0.22; Table 1). Mean levels of the other biomarkers were as follows: 9.56 ng nicotine/mL plasma (SD, 7.74), 198 ng cotinine/mL plasma (SD, 134), 69.5 ng trans-3'-hydroxycotinine/mL plasma (SD, 53.1), and 0.24 ng total NNAL/mL urine (SD, 0.26).

Toenail NNAL correlated with toenail nicotine (r = 0.68; P < 0.001) and toenail cotinine (r = 0.69; P < 0.001). There were weak but significant correlations of CPD with toenail nicotine (r = 0.24; P = 0.015) and cotinine (r = 0.26; P = 0.009) but not NNAL (r = 0.25; P = 0.076).

Relationships among the biomarkers are summarized in Table 2. Toenail nicotine and cotinine correlated with all other biomarkers. Toenail NNAL correlated with plasma cotinine and urinary total NNAL but not plasma nicotine or trans-3'-hydroxycotinine. Based on the regression analyses, the inclusion of demographic factors and reported exposure did not significantly alter the correlation between biomarkers.

Toenail biomarkers were standardized by CPD to investigate the effects of age and gender. There were no significant relationships. Toenail NNAL/CPD was higher in White than in African-American participants (P = 0.019), but there was no relationship of race to toenail nicotine/CPD or cotinine/CPD (Table 3). Controlling for age and gender did not change the effect of race on the biomarkers.

### Discussion
Measurement of nicotine, cotinine, and NNAL in human toenails is a promising new tool for investigating the role of chronic tobacco smoke exposure in human cancer. The purpose of our study was to provide validation data for these recently developed biomarkers. We investigated the relationship of toenail nicotine, cotinine, and NNAL levels to CPD as reported by the study participants. We also studied the relationship between toenail biomarkers and plasma concentrations of nicotine, cotinine, trans-3'-hydroxycotinine, and urinary total NNAL, as well as the effects of age, gender, and race on toenail biomarker levels.

The overall mean values and the ranges of toenail biomarker levels observed here are in good agreement with those obtained in our previous study (27). The correlation of toenail NNAL with toenail cotinine observed in this study (r = 0.69) was also similar to that reported previously (r = 0.77; ref. 27).

### Table 2. Relationships among biomarkers analyzed in this study

<table>
<thead>
<tr>
<th></th>
<th>Toenail nicotine</th>
<th>Toenail cotinine</th>
<th>Toenail NNAL</th>
<th>Plasma nicotine</th>
<th>Plasma cotinine</th>
<th>Plasma 3HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson correlation coefficient</td>
<td>0.43</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. samples</td>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toenail NNAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson correlation coefficient</td>
<td>0.68</td>
<td>0.69</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. samples</td>
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<td>51</td>
<td>51</td>
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<tr>
<td>Plasma nicotine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson correlation coefficient</td>
<td>0.39</td>
<td>0.26</td>
<td>0.22</td>
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<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>0.021</td>
<td>0.252</td>
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<tr>
<td>No. samples</td>
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<td>78</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma cotinine</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pearson correlation coefficient</td>
<td>0.54</td>
<td>0.45</td>
<td>0.37</td>
<td>0.86</td>
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<td></td>
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<td>0.050</td>
<td>&lt;0.001</td>
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<tr>
<td>No. samples</td>
<td>78</td>
<td>78</td>
<td>29</td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma 3HC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson correlation coefficient</td>
<td>0.39</td>
<td>0.30</td>
<td>0.24</td>
<td>0.55</td>
<td>0.67</td>
<td></td>
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<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.220</td>
<td>&lt;0.001</td>
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<tr>
<td>No. samples</td>
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<td>78</td>
<td>29</td>
<td>78</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Urine NNAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson correlation coefficient</td>
<td>0.42</td>
<td>0.43</td>
<td>0.53</td>
<td>0.35</td>
<td>0.53</td>
<td>0.40</td>
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<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.005</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No. samples</td>
<td>71</td>
<td>71</td>
<td>27</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

Abbreviation: 3HC, trans-3'-hydroxycotinine.
The major potential use of toenail biomarkers is to estimate cumulative carcinogen dose from chronic exposure to tobacco smoke. CPD was found to positively correlate with toenail nicotine ($r = 0.24$) and cotinine ($r = 0.26$); however, these correlations were lower than that reported by Al-Delaimy et al. (34) for CPD and toenail nicotine ($r = 0.85$). Several other studies that compared self-reported CPD with nicotine levels in another keratinic matrix, hair, did not produce consistent results. Some of them reported significant associations, whereas others found no association between CPD and hair nicotine (reviewed in ref. 35). In our study, correlation of CPD with toenail NNAL was not statistically significant, unlike the reported significant correlation between cigarette consumption and urinary NNAL ($r = 0.48$; $P < 0.0001$; ref. 36); however, the latter study had a much larger sample size. Considerable individual variability in toenail biomarker levels was observed at all levels of smoking, similar to that observed for urinary biomarkers (36). The CPD data were based on consumption at the time of blood and urine sampling, whereas the toenail levels reflect exposure over several months before the time of sampling. Possible lack of accuracy in self-reported CPD, the relatively small number of samples analyzed for NNAL, and the small number of low-level and high-level smokers recruited for this study limit our ability to make definitive conclusions about the relationship between CPD and toenail biomarkers.

Plasma nicotine and cotinine, and urinary total NNAL are well-established biomarkers of nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone uptake in smokers, smokeless tobacco users, and nonsmokers exposed to environmental tobacco smoke. Our results show significant positive correlations of toenail nicotine or cotinine with plasma nicotine, cotinine, and trans-3'-hydroxycotinine and of toenail NNAL with plasma cotinine and urinary total NNAL (Table 2). These findings indicate that toenail nicotine, cotinine, and NNAL promise to be valid and reliable biomarkers of human chronic exposure to nicotine and NNAL.

Age, gender, race, differences in smoking behavior, cigarette preferences, and metabolic processes may also influence toenail biomarker levels in smokers. Because in general male smokers smoke more CPD than do female smokers (37, 38) and Whites smoke more CPD than do Blacks (38, 39), we normalized toenail biomarkers by CPD before comparing them by age, gender, and race. There was no significant effect of age or gender on toenail biomarker levels, although some studies reported that male smokers have higher cotinine concentrations than female smokers (40). In addition, in our study, there was no relationship of race to toenail cotinine, although toenail NNAL was higher in White than in African-American participants (Table 3). These results disagree with earlier published data that show that Whites have lower plasma cotinine concentrations (41) and urinary cotinine and NNAL levels (42) than do Black smokers. Small sample size in this study may be responsible for this outcome.

There are several limitations to this study. As mentioned previously, toenail biomarker levels reflect exposure over several months, whereas the number of CPD and plasma and urine biomarker levels reflect exposure at one point in time. Smokers with low and high CPD were underrepresented; therefore, we could not make definitive conclusions about the relationship between the level of exposure and toenail biomarkers. In addition, only about half of all toenail samples were analyzed for NNAL, which reduced the statistical power of our analysis. Another disadvantage consists in single determinations of plasma and urinary biomarkers because significant interindividual differences in urinary biomarkers were observed in some studies (43), suggesting that a single determination of biomarker level may not be optimal.

In summary, we report here the first study that provides essential validation data for the use of toenail nicotine, cotinine, and NNAL as biomarkers in investigations of the role of chronic tobacco smoke exposure in human cancer.

Acknowledgments

We thank Dr. Delia Dempsey for medical oversight, Sandra Tinetti for assistance in the clinical study, Duan Minjiang and Lita Ramos for analyses of plasma and urine samples, Rachel Feuer and Brad Lieberman for technical assistance, Dr. Faith Allen for data management, and Bob Carlson for editorial assistance.

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Relationship of Human Toenail Nicotine, Cotinine, and 4-(MethylNitrosamino)-1-(3-Pyridyl)-1-Butanol to Levels of These Biomarkers in Plasma and Urine

Irina Stepanov, Stephen S. Hecht, Bruce Lindgren, et al.