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Research Article

Cancer Prevention Research

Evidence Supporting Product Standards for Carcinogens in Smokeless Tobacco Products

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Abstract

Smokeless tobacco products sold in the United States vary significantly in yields of nicotine and tobacco-specific nitrosamines (TSNA). With the passage of the Family Smoking Prevention and Tobacco Control Act, the Food and Drug Administration now has the authority to establish product standards. However, limited data exist determining the relative roles of pattern of smokeless tobacco use versus constituent levels in the smokeless tobacco product in exposure of users to carcinogens. In this study, smokeless tobacco users of brands varying in nicotine and TSNA content were recruited from three different regions in the U.S. Participants underwent two assessment sessions. During these sessions, demographic and smokeless tobacco use history information along with urine samples to assess biomarkers of exposure and effect were collected. During the time between data collection, smokeless tobacco users recorded the amount and duration of smokeless tobacco use on a daily basis using their diary cards. Results showed that independent of pattern of smokeless tobacco use and nicotine yields, levels of TSNA in smokeless tobacco products played a significant role in carcinogen exposure levels. Product standards for reducing levels of TSNA in smokeless tobacco products are necessary to decrease exposure to these toxicants and potentially to reduce risk for cancer. *Cancer Prev Res; 8(1); 20–26. ©2014 AACR.*

Introduction

Smokeless tobacco products sold in the United States and across the world vary in levels of nicotine and harmful and potentially harmful constituents (1–3). Two important harmful constituents in smokeless tobacco products are the tobacco-specific nitrosamines (TSNA), *N*′-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; 4–6). These constituents are classified by the International Agency for Research on Cancer as human carcinogens and are considered to be causative agents for cancers of the oral cavity, esophagus, and pancreas in smokeless tobacco users (7–11).

Among the 39 top-selling U.S. moist snuff brands, the levels of NNN range from 2.2. to 42.6 μg/g wet weight of product and those of NNK from 0.4 to 9.9 μg/g (2). Newer brands of smokeless tobacco products, referred to as snus (the Swedish name for smokeless tobacco) that are marketed towards cigarette smokers as a substitute for smoking, appeared on the U.S. market in about 2001, with major tobacco companies introducing snus in 2006 and 2007. These products contain lower levels of TSNA (e.g., NNN ranging from 0.9 to 3.3 μg/g dry weight and NNK from 0.1 to 0.3 μg/g dry weight) than some of the most popular conventional smokeless tobacco products (1). Variations of TSNA levels are even more dramatic in globally available smokeless tobacco products. For example, the NNN and NNK content of some toombak smokeless tobacco samples found in Sudan reached as high as 368 μg/g and 516 μg/g wet weight, respectively (3). The NNN and NNK content in some of the smokeless tobacco products in India ranged from 0.09 to 76.9 μg/g and 0.08 to 28.4 μg/g wet weight, respectively (12). This wide variation in TSNA levels may account in part for differences in disease risks attributed to smokeless tobacco products across these countries (13).

The carcinogenic effects of NNK and NNN and the relatively high levels of these constituents found in smokeless tobacco products have led to recommendations by the World Health Organization (WHO) Tobacco Regulatory Study Group to set limits for these constituents in tobacco products (14). To date, few studies have examined the impact of NNK and NNN levels in the tobacco product to actual exposure levels in smokeless tobacco users. One factor that might have a substantial effect on exposure levels over and above constituent levels in the tobacco product is the pattern of product use, which may be moderated by nicotine yields in the smokeless tobacco products. Nicotine can be a highly addictive agent that can determine the extent of tobacco use (15, 16) and like TSNA levels, varies tremendously across brands sold in the United States (2, 17, 18) and globally (3).

The overall goals of this study were to determine the effects of varying levels of nicotine and TSNA in smokeless tobacco products, patterns of use, and demographic and tobacco history on extent of exposure to these carcinogens.

Materials and Methods

Subject recruitment

Adult participants 18 years and older were recruited from Minneapolis/St. Paul, MN, Eugene, OR and Morgantown, WV. The study was conducted after approval from the institutional review board at each of the sites and in accordance with an assurance filed

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with and approved by the U.S. Department of Health and Human Services. At each site, we recruited smokeless tobacco users of each brand listed in Table 1. These brands were selected because they vary in nicotine, NNK, and NNN content. The recruitment advertisements stated that daily smokeless tobacco users were needed for a study that examined the effects of smokeless tobacco use. Interested participants were asked to call the research clinic number. During this telephone call, potential participants were informed that the study determines the extent of exposure to toxicants and the levels of these exposures across different brands of smokeless tobacco, and would involve two visits. Study procedures were explained, a brief phone screening was conducted, and interested and potentially eligible participants were asked to attend an orientation meeting. Inclusion criteria were the following: (i) using a consistent and daily amount of smokeless tobacco for the at least the past six months, (ii) in good physical health (no unstable medical condition); and (iii) stable, good mental health (e.g., no recent unstable or untreated psychiatric diagnosis, including substance abuse). Subjects could not be currently using other tobacco or nicotine products. Female participants could not be pregnant or nursing.

Clinic visits

Participants attended an orientation meeting to be further informed about the study, to obtain written informed consent, and to determine eligibility. Once determined to be eligible, the study procedures for visit 1 were initiated.

Participants were asked to complete questionnaires on demographic and tobacco use history (e.g., age, income, duration of smokeless tobacco use, age of regular use), the Severson Smokeless Tobacco Dependency Scale (19), psychiatric (PRIME MD; ref. 20), and medical and alcohol use history (Michigan Alcohol Screening Test; ref. 21). A second visit was scheduled approximately one week apart.

At each of the two clinic visits, vital signs and alveolar carbon monoxide measurements were obtained and use of any other tobacco or nicotine products assessed. Carbon monoxide levels needed to be <8 parts per million. Participants were asked to provide three samples (usual size dips) of their smokeless tobacco product, one sample on the first visit and two on the second visit. Each of these dips was weighed to obtain an estimate of the median weight per dip. Between the two visits, participants kept a daily diary to monitor their smokeless tobacco intake (time of dip onset and time when dip was expectorated).

Before the second clinic visit, the first morning urine void was collected for biomarkers of exposure assessment. All deidentified urinary biosamples were sent to the University of Minnesota (Minneapolis, MN). Urine samples were analyzed for total nicotine equivalents (TNE), the sum of total nicotine, total cotinine, and

Table 1. Median values of constituent yields (per gram wet weight) in smokeless tobacco brands

Product	Total nicotine, mg/g	Free nicotine, mg/g	NNN, μg/g	NNK, μg/g	NNN+NNK, μg/g
Copenhagen					
Copenhagen snuff	11.79	2.42	2.66	0.74	3.42
Copenhagen long cut	11.27	3.51	2.05	0.83	2.88
Copenhagen long cut straight	10.40	2.56	1.62	0.46	2.07
Copenhagen long cut wintergreen	11.23	5.67	0.98	0.28	1.23
Skoal					
Skoal long cut straight	12.52	3.10	2.19	0.82	2.88
Skoal fine cut original	14.17	2.79	1.48	0.47	1.95
Skoal long cut wintergreen[a]	11.23	2.70	1.80	0.77	2.57
Skoal long cut wintergreen[b]	13.00	2.51	1.07	0.21	1.28
Skoal bandits wintergreen	12.68	3.07	2.86	1.00	3.89
Skoal long cut mint	12.48	1.29	1.04	0.22	1.25
Skoal wintergreen pouches	13.02	3.89	1.92	0.80	2.72
Skoal mint pouches	11.88	5.19	1.67	0.44	2.11
Skoal snus	17.19	0.98	1.41	0.25	1.65
Kodiak					
Kodiak wintergreen	10.85	4.87	2.42	0.52	2.97
Kodiak wintergreen moist snuff pouches	13.03	5.88	2.32	1.07	3.40
Grizzly					
Grizzly long cut straight	12.49	4.15	3.02	0.82	3.84
Grizzly straight pouches	14.92	0.48	2.77	1.11	3.89
Grizzly fine cut natural	16.27	4.41	11.11	3.44	14.55
Grizzly long cut mint	13.07	7.58	3.24	0.47	3.72
Grizzly mint pouches	13.08	6.99	4.65	1.19	5.84
Grizzly long cut wintergreen	12.33	5.09	2.64	0.39	3.04
Grizzly wintergreen pouches	11.32	6.25	3.03	0.64	3.67
Red seal					
Red seal fine cut natural	10.96	1.67	2.00	0.62	2.61
Marlboro					
Marlboro snus peppermint	16.01	0.91	0.63	0.19	0.82
Marlboro snus original	12.30	0.64	0.53	0.19	0.72
Marlboro snus mint	11.89	0.57	0.48	0.16	0.64
Marlboro snus rich	18.16	0.99	0.63	0.15	0.78
Camel					
Camel snus robust	8.77	2.27	1.22	0.48	1.71
Camel snus mellow	9.57	2.55	1.29	0.46	1.74
Camel snus frost	9.63	2.73	1.32	0.47	1.79
Camel snus winterchill	8.91	2.54	1.22	0.44	1.66

[a]Sample was collected in May 2011.
[b]Samples were collected in December 2011.

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total 3′-hydroxycotinine (22, 23), which account for ≥80% of the nicotine dose (24), NNK metabolites, total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; 25), and NNN metabolites, total *N*′-nitrosonornicotine (NNN; 26, 27). For each analyte, total refers to the analyte plus its glucuronide conjugate(s).

Participants were paid $50 per visit.

Constituent analysis of smokeless tobacco brands

Constituent analyses of the products listed in Table 1 were conducted according to our validated procedures. For NNN and NNK analysis, tobacco samples were extracted and purified as described (28), and further analyzed by liquid chromatography/tandem mass spectrometry (18). Total nicotine was analyzed by gas chromatography/mass spectrometry (28). The amount of free, or unprotonated, nicotine was calculated using the Henderson–Hasselbalch equation (28). Free nicotine is the pH-dependent biologically available fraction of the total amount of nicotine present in a smokeless tobacco product. This form of nicotine can readily reach bloodstream by easily passing through cellular membranes, and products with higher free nicotine content have been suggested to be more addictive (29, 30). Therefore, free nicotine was the constituent that was included in our analysis.

Statistical analysis

The baseline characteristics, including demographics and tobacco use history were summarized for the three sites combined. The frequency and percent were reported for categorical items and the mean, SD, median, and range described the continuous variables. The median value from multiple assays of the constituent levels (NNK, NNN, free nicotine, NNK/mg free nicotine, NNN/mg free nicotine) of each tobacco brand was used to represent the amount of toxicant the participant was exposed to from that product. Both NNK and NNN per mg free nicotine were examined to determine the impact of these TSNA when accounting for levels of nicotine. Data on patterns of use was extracted from the daily diaries and the following smokeless tobacco topography measures were calculated for each subject: mean dips per day, mean duration per dip, mean daily dip duration (number of dips × duration per dip), and median dip weight. Other covariates included self-reported demographic (age, income) and tobacco history (age of first smokeless tobacco use, duration of daily smokeless tobacco use, tins per week, smokeless tobacco dependence, history of smoking) variables.

The nonparametric Spearman correlation coefficient and the Wilcoxon rank sum test assessed the relationship and statistical significance of each covariate to the level of three urinary biomarkers (TNE, total NNAL, and total NNN) adjusted for the amount of urinary creatinine. All variables significant with a $P < 0.1$ were included in a multiple regression analysis to determine which factors in combination were predictors of the biomarkers. Following this analysis, a stepwise regression approach was used to find the most parsimonious final model that contained only covariates with a $P < 0.05$.

Regression results for urinary TNE are reported as the estimated coefficient for each covariate and its corresponding SE and *P* value. Urinary NNAL and NNN biomarkers were analyzed in the logarithmic scale due to highly skewed distributions. For recording the results, the coefficients are exponentiated to represent the ratio of the biomarker due to a one unit increase in the covariates plus the 95% confidence interval (CI). The interaction terms between the constituents and significant pattern of use variables in the final models were tested. None were significant at the 5% level.

Results

A total of 391 subjects were recruited with sample data collected or analyzed on 359 subjects ($N = 112$ Minnesota, 208 Oregon, and 39 West Virginia). Of the participants enrolled, six had CO > 8 ppm; four were current marijuana users and/or four reported being exposed to second-hand smoke. Only one participant may have been a smoker. When data was analyzed excluding these participants, the results were similar to the inclusion of all participants; therefore, we present the data that includes all participants.

Table 2 shows demographic, tobacco use history, and baseline biomarker level information.

Table 3 shows the univariate analysis of the relationship between specific tobacco history, patterns of smokeless tobacco use, and constituent levels in the smokeless tobacco product with urinary TNE, total NNAL, and total NNN. The results show that age of first using smokeless tobacco had a significant but modest negative correlation with various biomarkers of exposure (with the exception of total NNN): the younger the age of beginning to use smokeless tobacco, the greater the exposure levels. Number of years of daily smokeless tobacco use, level of dependence, and smokeless tobacco topography measures with the exception of median dip weight were significantly positively correlated with biomarkers of exposure. When the effects of constituent levels on the respective biomarkers were examined, no significant correlation was observed between free nicotine levels in the smokeless tobacco products and biomarkers of nicotine exposure. Significant correlations, however, were observed between NNK and NNK/mg of free nicotine in the product with total NNAL, and NNN and NNN/mg free nicotine with total NNN.

Table 2. Subject demographics and tobacco use history items ($N = 349$–359)

Characteristics	Number of subjects (%)	
Site, $N = 359$		
Oregon	208 (57.9)	
Minnesota	112 (31.2)	
West Virginia	39 (10.9)	
Gender, $N = 357$		
Male	345 (96.6)	
Race, $N = 357$		
White, non-Hispanic	331 (92.7)	
Other	26 (7.3)	
Annual personal income, $N = 356$		
<$15K	130 (36.5)	
15K–30K	54 (15.2)	
>30K	172 (48.3)	
Smoked at least 100 cigarettes, $N = 350$		
Yes	181 (51.7)	
No	169 (48.3)	
	Mean (SD)	**Median (min/max)**
Age, y	37.1 (12.6)	37 (18/89)
Age when first used snuff	17.7 (8.5)	16 (4/55)
Number of years using snuff daily	13.9 (11.9)	10.5 (0/69)
Dependence score	8.1 (3.7)	8.0 (0/17)
Median dip weight (g)	2.3 (1.5)	1.9 (0.3/8.8)
Tins per week	3.6 (2.2)	3 (0.5/14)
Mean dips per day	7.0 (3.5)	6 (1/25)
Mean duration per dip (minutes)	69.3 (42.0)	56.9 (4.2/229.2)
Mean daily dip duration (hours)	6.8 (3.9)	6.5 (0.4/17.4)
TNE, nmol/mg creatinine	72.8 (70.9)	54.6 (0.1/510.9)
Total NNAL, pmol/mg creatinine	3.29 (3.77)	2.23 (0.04/29.91)
Total NNN, pmol/mg creatinine	0.284 (3.374)	0.059 (0.003/63.068)

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Table 3. Univariate analysis: correlations[a] (*P* value) with urinary biomarkers ($N = 344–358$)

	TNE nmol/mg creatinine	Total NNAL pmol/mg creatinine	Total NNN pmol/mg creatinine
Age when first used snuff	−0.18 (0.001)	−0.18 (0.001)	−0.09 (0.088)
Number of years using snuff daily	0.50 (<0.001)	0.52 (<0.001)	0.42 (<0.001)
Dependence score	0.22 (<0.001)	0.16 (0.003)	0.13 (0.012)
Median dip weight (g)	−0.001 (0.980)	0.06 (0.297)	0.02 (0.686)
Tins per week	0.36 (<0.001)	0.36 (<0.001)	0.31 (<0.001)
Mean dips per day	0.45 (<0.001)	0.41 (<0.001)	0.34 (<0.001)
Mean duration per dip (minutes)	0.40 (<0.001)	0.36 (<0.001)	0.32 (<0.001)
Mean daily dip duration (hours)	0.67 (<0.001)	0.61 (<0.001)	0.53 (<0.001)
Free nicotine mg/g wet weight	−0.10 (0.069)	−0.002 (0.956)	−0.03 (0.522)
NNK μg/g wet weight		0.23 (<0.001)	
NNK/Free nicotine		0.22 (<0.001)	
NNN μg/g wet weight			0.25 (<0.001)
NNN/free nicotine			0.28 (<0.001)

[a]The nonparametric Spearman correlation coefficient.

The relationships between income and past history of smoking to these biomarkers were also examined. Significant effects were observed between levels of income (<$15,000 $US vs. ≥$15,000 $US) with urinary TNE and total NNAL (data not shown, both *P* values <0.003), with lower levels observed among the low income smokeless tobacco users. A similar trend was observed for urinary total NNN, but the results were not statistically significant ($P = 0.098$). Having smoked at least 100 cigarettes in a lifetime was associated with higher levels of urinary total NNN ($P = 0.01$).

Table 4 shows the multiple regression analysis including the covariates that were significant at the $P < 0.1$ level in the univariate

Table 4. Multiple regression analysis for biomarkers (adjusted for creatinine)

Covariates	Coefficent (SE)	*P*
Dependent variable = TNE nmol/mg creatinine ($N = 343$) $R^2 = 0.32$		
Intercept	1.9 (20.0)	0.925
Annual income ≥ 15K	−5.1 (7.1)	0.470
Number of years using snuff daily	1.3 (0.3)	<0.001
Age when first used snuff	0.04 (0.42)	0.937
Dependence score	−0.5 (1.0)	0.608
Tins per week	5.0 (1.7)	0.004
Mean dips per day	−0.2 (2.2)	0.945
Mean duration per dip (minutes)	−0.01 (0.20)	0.958
Mean daily dip duration (hours)	7.6 (2.4)	0.002
Constituent yield: Free nicotine mg/g wet weight	−1.9 (2.2)	0.402

Covariates	exp(coeff) (95% CI)	*P*
Dependent variable = total NNAL pmol/mg creatinine[a] ($N = 342$) $R^2 = 0.50$		
Annual income ≥ 15K	0.97 (0.81–1.16)	0.724
Number of years using snuff daily	1.02 (1.01–1.03)	<0.001
Age when first used snuff	1.00 (0.99–1.01)	0.520
Dependence score	1.00 (0.98–1.03)	0.939
Tins per week	1.13 (1.08–1.18)	<0.001
Mean dips per day	1.05 (0.99–1.11)	0.089
Mean duration per dip (minutes)	1.01 (1.00–1.01)	0.045
Mean daily dip duration (hours)	1.07 (1.01–1.14)	0.027
Constituent yield: NNK μg/g wet weight	1.38 (1.16–1.64)	<0.001
Constituent yield: NNK/free nicotine	0.84 (0.50–1.39)	0.497

Covariates	exp(coeff) (95% CI)	*P*
Dependent variable = total NNN pmol/mg creatinine[a] ($N = 326$) $R^2 = 0.43$		
Annual income ≥ 15K	0.85 (0.69–1.06)	0.156
Number of years using snuff daily	1.02 (1.01–1.03)	<0.001
Age when first used snuff	1.01 (1.00–1.02)	0.180
Dependence score	1.01 (0.98–1.04)	0.473
Smoked >100 cigarettes in lifetime	1.21 (0.99–1.49)	0.067
Tins per week	1.12 (1.06–1.18)	<0.001
Mean dips per day	1.04 (0.97–1.11)	0.301
Mean duration per dip (minutes)	1.01 (1.00–1.01)	0.067
Mean daily dip duration (hours)	1.07 (1.00–1.15)	0.058
Constituent yield: NNN μg/g wet weight	1.12 (1.05–1.20)	0.001
Constituent yield: NNN/free nicotine	0.94 (0.76–1.18)	0.614

NOTE: The model for each biomarker includes all the covariates from the univariate analysis that were significant with $P < 0.1$. For total NNN/creatinine, one subject with NNN >60 was excluded from the analysis.

[a]Analyzed in the logarithmic scale.

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Table 5. Multiple regression analysis for biomarkers (adjusted for creatinine)

Covariates	Coefficent (SE)	P
Dependent variable = TNE nmol/mg creatinine ($N = 351$) $R^2 = 0.32$		
Intercept	−10.6 (7.8)	0.175
Number of years using snuff daily	1.3 (0.3)	<0.001
Tins per week	4.8 (1.5)	0.002
Mean daily dip duration (hours)	7.2 (0.9)	<0.001
Covariates	**exp(coeff) (95% CI)**	**P**
Dependent variable = total NNAL pmol/mg creatininea ($N = 345$) $R^2 = 0.49$		
Number of years using snuff daily	1.02 (1.01–1.03)	<0.001
Tins per week	1.13 (1.09–1.18)	<0.001
Mean daily dip duration (hours)	1.13 (1.10–1.16)	<0.001
Constituent yield: NNK μg/g wet wt	1.32 (1.16–1.51)	<0.001
Covariates	**exp(coeff) (95% CI)**	**P**
Dependent variable = total NNN pmol/mg creatininea ($N = 337$) $R^2 = 0.40$		
Number of years using snuff daily	1.02 (1.01–1.03)	0.001
Tins per week	1.10 (1.05–1.15)	<0.001
Mean daily dip duration (hours)	1.14 (1.11–1.17)	<0.001
Constituent yield: NNN μg/g wet wt	1.12 (1.06–1.17)	<0.001

NOTE: The model contains only the covariates that were significant in the final regression (Table 4) with a p-value < 0.05. For total NNN/creatinine, one subject with NNN >60 was excluded from the analysis.

[a]Analyzed in the logarithmic scale.

analysis. For TNE, only number of years of daily smokeless tobacco use, tins per week and mean daily dip duration were significant (all P values < 0.004). For total NNAL, these same variables were significant plus mean duration per dip and NNK constituent yield ($P < 0.001$ to 0.045). The significant variables for NNN in the full regression model were number of years using smokeless tobacco daily, tins per week, and NNN constituent content (all P values < 0.05). Using a stepwise approach to produce regression equations that included only covariates that are significant in combination with a $P < 0.05$ (Table 5), the final models for both TNE and TSNA biomarkers contained number of years using smokeless tobacco daily, tins per week, and mean daily dip duration. Total NNAL and NNN, but not TNE, also included the corresponding constituent yield. All covariates in these final models were positively correlated with the biomarkers and were associated with increased exposure. For every one unit (μg/g wet wt) increase of NNK and NNN in the tobacco products, the estimated increase of the corresponding nitrosamine biomarkers was 32% for total NNAL and 12% for total NNN. The regression results show that these significant variables explain from 30 to 50% of the variance in the exposure biomarkers.

Discussion

Most prior smokeless tobacco studies have examined the relationship between patterns of use and biomarkers of exposure, not taking into account constituent yields in tobacco products. These studies have shown that patterns of smokeless tobacco use affect exposure levels. In one recent study of 54 male smokeless tobacco users, a regression analysis showed that daily dip duration was significantly associated with exposure biomarkers (total nicotine and/or cotinine and total NNAL; ref. 31). Age, tins per week, and dipping duration (time of first dip to time of last dip) were also significantly related to urinary total nicotine levels. In one small study ($N = 13$), a significant relationship was observed between tins per week (the only variable assessed) and urinary total NNAL (32). Other studies have also shown a relationship of cotinine levels to both frequency and durational measures (33–36) and weight of dip (36). Similar to the results from prior studies, this study found that duration and amount of smokeless tobacco use played an important role in levels of exposure to constituents. However, this study is the first to primarily focus on the contribution of constituent levels in smokeless tobacco products to exposure biomarkers. This study found that TSNA constituent levels, but not nicotine, play an important role in the extent of biomarker exposure, even when taking into account smokeless tobacco pattern of use. Therefore, a product standard for reducing these TSNA, specifically NNK and NNN (the most potent carcinogens), would likely lead to a decrease in exposure.

Consistent with this conclusion is a prior study in which we examined the effects of switching smokeless tobacco users of conventional U.S. smokeless tobacco product to Swedish snus of similar nicotine levels but lower NNK levels than popular U.S. brands of smokeless tobacco. In this study, a significant reduction in urinary total NNAL (approximately 50%) was observed (37), providing further support to reduce NNK and NNN levels in U.S. smokeless tobacco products. In a recent analysis, NNK content of popular U.S. smokeless tobacco brands ranged from 1.10 to 1.64 μg/g of wet weight compared with 0.46 μg/g of wet weight for a popular Swedish snus brand; for NNN, the values for U.S. brands ranged from 3.76 to 6.86 compared with 1.66 for Swedish snus (1).

Reducing yields of NNN and NNK may be associated with lower risk for cancer. The lack of association between snus use in Scandinavian countries to oral cancer might be attributable to the lower TSNA smokeless tobacco products sold in Sweden, whereas other countries have shown an increased risk of oral cancer among smokeless tobacco users, likely due to higher levels of NNK and NNN levels in the smokeless tobacco products (13). Furthermore, epidemiologic studies in smokers have shown dose-response effects of cancer risk with TSNA biomarkers of exposure; higher levels of total NNAL were associated with greater lung cancer risk (38–40) and higher levels of NNN exposure were associated with increased risk of esophageal cancer (26). Although these studies were conducted among smokers, studies in rats have shown that NNN is a powerful oral cavity carcinogen (10) and a combination of NNK and NNN exposure have produced oral tumors in rats (41) and possibly in human smokeless tobacco users (5, 42–45). Kresty and colleagues (46) observed a strong association between leukoplakia and increasing levels of total NNAL, indicating that higher levels of exposure are potentially associated with

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precancerous oral lesions. Collectively, these findings indicate that larger doses of carcinogens will result in greater cancer risk.

Even if substantial evidence of dose-response effects were not available in smokeless tobacco users, it is prudent to reduce levels of known carcinogens particularly in view of the relative ease by which TSNA can be reduced. For example, the Swedish government and one of the largest manufacturing companies for Swedish snus have established standards for their products (47). Through the selection of specific tobacco leaves, and implementing standards for hygiene, methods of curing, pasteurization, and requirements for storage, the limit for the level of NNN plus NNK is below 1.0 mg/kg (50% water content). A 2013 content analysis of Swedish Match moist snus products revealed levels lower than this limit (0.47, 95% CI, 0.46–0.48; ref. 48). Limits have also been established for other constituents such as nitrite, benzo[*a*]pyrene (BaP), *N*-nitrosodimethylamine and metals. Furthermore, the WHO Tobacco Regulatory Study Group has recommended reducing NNN plus NNK levels to 2 μg/g dry weight in smokeless tobacco along with a reduction in BaP to 5 ng/g dry weight, using similar manufacturing practices imposed in Sweden (14). On the basis of our results, reducing levels of TSNA could occur irrespective of the levels of nicotine in the product. Free nicotine levels did not contribute significantly to NNK and NNN exposure levels. Implementation of these product standards is possible through the Family Smoking Prevention and Tobacco Control Act (FSPTCA), which gives the U.S. Food and Drug Administration the authority to establish product standards and globally, through Article 9 (regulation of tobacco product contents) of the WHO Framework Convention on Tobacco Control.

As noted previously, the results from this study, consistent with other studies, also showed the length of time a user keeps smokeless tobacco in his/her mouth and the amount of smokeless tobacco use are strongly associated with levels of urinary TNE, total NNAL, and total NNN. In addition, this study showed that how long a person has been using smokeless tobacco daily is a significant contributor to exposure levels. A prior study also demonstrated that the duration (in years) of smokeless tobacco use was positively associated with total NNAL and cotinine levels ($N = 212$; ref. 49). These findings would suggest that quitting use of smokeless tobacco earlier and reducing duration and amount of use would be associated with less overall toxicant exposures. This decrease has been demonstrated in studies where reduction in amount and frequency of use was associated with significant reductions in total NNAL and cotinine levels (50, 51).

The relationship between amount of use and exposure levels may also account for the association between lower income levels and lower exposure biomarkers. That is, individuals with lower incomes tended to use lower amounts of smokeless tobacco. In a *post hoc* analysis, smokeless tobacco users with income <$15,000 used less tins per week (mean = 3.2 vs. 3.8, $P = 0.006$) and fewer dips per day (mean 5.7 vs. 6.8, $P = 003$) than smokeless tobacco users with income ≥$15,000.

This study is not without limitations. One of the limitations of this study is that the sample was comprised predominantly or white males; thus, the extent to which these findings can be generalized to other populations is unknown. For example, prior studies have shown that estradiol level influences the activity of various metabolic enzymes associated with the activation of NNK to carcinogenic forms (52). Therefore, women might have a different profile of carcinogen exposure than men depending on their hormonal levels.

In conclusion, there are unnecessarily high levels of tobacco-specific carcinogens found in smokeless tobacco products. Exposure to these carcinogens is related to constituent levels in the product itself and independent of pattern of use. Therefore, to protect public health, we recommend that under the U.S. FSPTCA and Articles 9 of FCTC, regulations are issued to reduce levels of the potent carcinogens, NNK and NNN in smokeless tobacco products, to the lowest levels possible.

Disclosure of Potential Conflicts of Interest

D.K. Hatsukami is a consultant for Campaign for Tobacco Free Kids. No potential conflicts of interest were disclosed by the other authors.

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