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Research Article

Urine Menthol as a Biomarker of Mentholated Cigarette Smoking

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Abstract

Objectives: Menthol cigarettes are smoked by 27% of U.S. smokers, and there are concerns that menthol might enhance toxicity of cigarette smoking by increasing systemic absorption of smoke toxins. We measured urine menthol concentrations in relation to biomarkers of exposure to nicotine and tobacco carcinogens.

Methods: Concentrations of menthol glucuronide (using a novel analytical method), nicotine plus metabolites (nicotine equivalents, NE), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and polycyclic aromatic hydrocarbon (PAH) metabolites were measured in the urine of 60 menthol and 67 regular cigarette smokers.

Results: Urine menthol was measurable in 82% of menthol and 54% in regular cigarette smokers. Among menthol smokers, urine menthol was highly correlated with NE, NNAL, and PAHs. In a multiple regression model NE but not menthol was significantly associated with NNAL and PAHs.

Conclusions: Urine menthol concentration is a novel biomarker of exposure in menthol cigarette smokers, and is highly correlated with exposure to nicotine and carcinogens. Menthol is not independently associated with carcinogen exposure when nicotine intake is considered. *Cancer Epidemiol Biomarkers Prev; 19(12); 3013–9.* *©2010 AACR.*

Introduction

Presently in the United States, approximately 27% of smokers smoke menthol cigarettes (1). Menthol, when inhaled, provides a cooling sensation; and mentholated cigarettes have been marketed as being associated with less irritation, with freshness, and more recently with hip culture (2, 3).

Several health concerns regarding menthol cigarettes have been raised. These include facilitation of youth initiation of smoking, more severe tobacco dependence, less successful quitting and enhancement of the direct toxicity of cigarette smoking (4–7). The latter might occur if menthol permitted deeper inhalation, longer breath hold in the lungs and/or enhanced permeation of tobacco smoke toxins.

Biomarker studies are useful in exploring the health effects of environmental exposures. Several research groups have examined the relationship between menthol cigarette smoking and exposure to biomarkers of tobacco smoke exposure, including nicotine and metabolites, carbon monoxide and tobacco-specific nitrosamines (8–10). Muscat and coworkers (8) and Heck (9) found no differences in biomarkers of nicotine and tobacco-specific nitrosamine exposure, but Clark et al. (10) found that menthol cigarette smoking was associated with higher expired carbon monoxide and serum cotinine levels than that with regular cigarette smokers. However, to the best of our knowledge no previous research has described the relationship between a biomarker of menthol exposure and exposure to tobacco smoke toxins.

We measured urine menthol concentrations in smokers of mentholated and regular cigarettes in relation to biomarkers of other tobacco smoke constituents. Menthol concentrations could be associated with other tobacco smoke constituents simply as a marker of overall smoke exposure, so we also examined the hypothesis that menthol enhances exposure to tobacco smoke carcinogens in relation to nicotine exposure.

Methods

The subjects were 127 cigarette smokers who were recruited by newspaper advertisements and notices posted in local colleges, community centers, and other public places as well as on Craigslist. Subjects were required to be 18–65 years old, to be healthy and to have

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smoked an average of 10 cigarettes per day or more for the past year or longer. Subjects had to be self-identified non-Hispanic white or African American, with 4 grandparents of the same race. Exclusions included active medical problems, pregnancy, breast-feeding, current alcohol or drug abuse, current use of smokeless tobacco, pipes, cigars and nicotine medications, and regular use of medications other than vitamins, oral contraceptives, hormone replacements, or aspirin.

Subjects were asked to come to the Clinical Research Center at San Francisco General Hospital Medical Center, where the study was explained and written consent obtained. Questionnaires were administered regarding health history, drug use history, smoking and tobacco dependence measures, including the Fagerstrom test of nicotine dependence (FTND; ref. 11). Average cigarette consumption was taken as the average number of cigarettes smoked per day in the 3 days prior to the study visit.

A questionnaire inquiring about sources of potential exposure to menthol was also administered. Subjects were asked about the use of various products containing menthol. Twelve items were included in the questionnaire: mint-flavored teas; mint-flavored coffees, mint-flavored nondairy creamers, mint-flavored liquors, mint-flavored candy or mints, mint-flavored chewing gum, mint-flavored chocolate candies, mint-flavored desserts, mint jelly, mint-flavored toothpaste, mint-flavored mouthwash, and mint-flavored medicines. Since menthol has a short half-life (~75 min; ref. 12), we scored use only in the past 24 hours. All potential sources of exposure were added to form a single composite score. The maximum score was 12. Doses of menthol from these sources were not available.

After completing the questionnaires, a blood sample was taken and urine collected. The time of smoking the last cigarette prior to blood and urine sampling was recorded. Plasma was assayed for concentrations of nicotine and cotinine. The urine samples were analyzed for concentrations of creatinine, nicotine and its 5 major metabolites, NNAL, and metabolites of several polycyclic aromatic hydrocarbons (PAHs). The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent pulmonary carcinogen, is metabolized in the body to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), also a pulmonary carcinogen. NNAL reflects NNK exposure and can be measured in the urine (13). PAHs are a class of combustion products that include benzo[*a*]pyrene and other carcinogens that are present in combustion products including tobacco smoke (13). Several PAH metabolites in urine are measured in our laboratory and are believed to reflect exposure to the carcinogenic PAHs.

Subjects were compensated financially for participation. The study was approved by the Institutional Review Board at the University of California, San Francisco.

Analytical chemistry

Plasma nicotine was measured by gas chromatography with nitrogen-phosphorous detection modified for simultaneous determination of nicotine and cotinine using a capillary column (14, 15). Urine concentrations of nicotine and its metabolites cotinine, trans-3′-hydroxycotinine (3HC) and their respective glucuronide metabolites were measured by LC-MS/MS, as described previously (16, 17). Urine concentrations of NNAL (free plus conjugated) and the PAH metabolites, 2-naphthol, 1, 2, and 3 + 4 hydroxyphenanthrenes, 1-hydroxypyrene, and 1, 2, and 3-hydroxyfluorenes, were measured by LC-MS/MS and expressed as pmol per milligram creatinine (18, 19).

Menthol is extensively metabolized such that excretion in the urine is predominantly menthol glucuronide, with no measurable unchanged menthol (9). Concentrations of menthol glucuronide were determined using a novel method developed in our laboratory. Briefly, the method involves adding a stable isotope internal standard of menthol glucuronide to the urine sample followed by separation by reverse phase HPLC and quantitation by tandem mass spectrometry. The menthol-d_4 glucuronide internal standard was prepared in this laboratory using rat liver microsomes to catalyze the formation of menthol-d_4 glucuronic acid from menthol-d_4 (9) in the presence of uridine diphosphoglucuronic acid. A standard of menthol glucuronide was obtained from Sigma-Aldrich. The mass spectrometer was operated in negative selective reaction monitoring mode (331/85 and 335/85 for d_0 and d_4, respectively) with electrospray ionization. Calibration curves in urine from a low-level exposed donor were constructed using peak height ratios of the analyte/internal standard. Peak height calibration was used because the d_4-menthol glucuronide was a mixture of isomers that were not quite baseline separated. The lower limit of quantitation (LOQ) for menthol glucuronide in urine was 1.0 μg/mL. Standard curves were linear from 1.0 to 100 μg/mL with an $r^2 > 0.99$. Over the range of 1 to 100 μg/mL, the precision ranged from 2.1% to 5.2% (coefficient of variation) and the accuracy ranged from 90.0% to 104%. In our article, urine menthol refers to menthol glucuronide, and menthol glucuronide concentration is expressed per milligram creatinine in urine.

Data analysis

Comparison of demographic and smoking history characteristics and exposure biomarkers in menthol versus regular cigarette smokers were performed by the *t* test or Wilcoxon rank-sum test. For urine biomarkers whose values were not normally distributed, including nicotine equivalents, total NNAL, total PAH metabolites and menthol, geometric mean concentrations are presented and log-transformed values were used in regression analyses.

Multivariate regression analysis was used to examine predictors of urine menthol, where independent variables included time from last cigarette, cigarettes smoked per day, menthol exposure score and age, sex, race, and BMI. Regression analysis was also used to examine the association between urine menthol and other biomarkers of exposure, in which case models included time from

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last cigarette, cigarettes per day and urine menthol, as well as age, sex, race, and BMI (model 1). Another model was examined to determine if menthol was associated with carcinogen exposure independently from nicotine exposure. In this model the cigarettes per day variable was replaced by urine nicotine equivalents (model 2). In the regressions, BMI was expressed as an ordinal variable as follows: normal (18.5 < 25), overweight (25 < 30) and obese (>30). Cigarettes per day were expressed as a categorical variable as follows: 0 < 10 CPD, 10 < 20 CPD, and ≥20 CPD. Time from last cigarette to urine collection was truncated at 4 hours and the menthol score truncated at 4 to avoid extreme values of these variables having undue influence on their estimated regression coefficients. Analyses were performed using SAS 9.2 (2008).

Urine nicotine equivalents was determined as the molar sum of nicotine, cotinine, 3HC, and their glucuronide metabolites in urine normalized for creatinine concentration. When measured at steady state, the sum of these metabolites accounts for on average 80%–90% of a daily dose of nicotine (20). We have shown that nicotine equivalents measured in this way are highly correlated with daily intake of nicotine (21). We expressed total PAHs as the molar sum of all PAH metabolites.

Results

The frequency distribution of urine menthol concentration in our study group according to cigarette type (regular vs. menthol) is shown in Figure 1. Urine menthol concentrations were below the limit of quantitation in 46.3% of regular cigarette smokers and in 18.3% of menthol cigarette smokers. The geometric mean concentrations and interquartile ranges for urine menthol as well as plasma cotinine, urine nicotine equivalents, NNAL and total PAHs in regular and menthol cigarette smokers are shown in Table 1.

Among regular cigarette smokers, there were significant simple correlations between urine menthol and menthol score and between urine menthol, urine nicotine equivalents and urine PAHs (Table 2). Multiple regression analysis found no significant predictors of urine menthol concentration in regular cigarette smokers. Of note among the regular cigarette smokers were 3 outliers with high urine menthol concentrations as seen in Figure 1. These subjects all reported consuming mint-flavored candies or breath mints (e.g., Altoids), mint-flavored gum, and/or mint-flavored toothpaste 7 days per week.

Among menthol cigarette smokers, there were significant simple correlations between urine menthol, cigarettes smoked per day, urine nicotine equivalents, plasma cotinine, urine NNAL, and urine PAH metabolites (Table 2).

Multiple regression analyses for menthol cigarette smokers are presented in Table 3. In a model of predictors of menthol in menthol cigarette smokers in which the independent variables were age, sex, race, BMI, cigarettes per day, and time from last cigarette there were no significant associations between any of the independent variable and urine menthol.

To examine the question of whether menthol influences exposure to tobacco smoke constituents, we examined models in which the independent variables were cigarettes smoked per day and urine menthol and dependent variables were urine nicotine equivalents, urine NNAL, or total PAH metabolites (model 1). Urine menthol was significantly associated with each of these

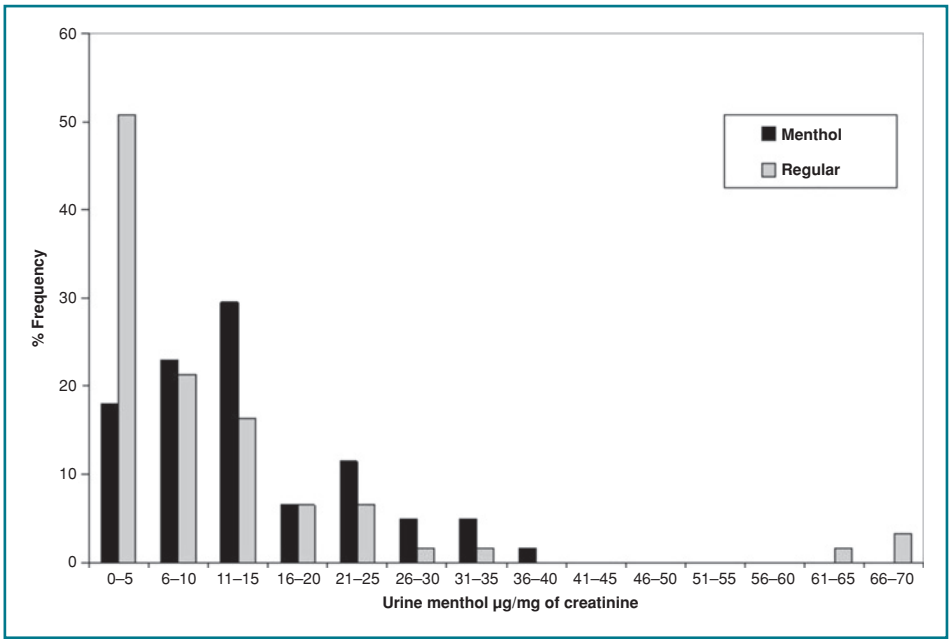

Figure 1. Frequency histogram of urine menthol concentration in smokers of menthol and regular cigarettes. Subjects whose urine concentrations were below the assay limit of quantitation are included in the 0–5 µg/mg creat bar.

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Table 1. Demographic, smoking behavior, and urine biomarker comparisons by cigarette type, mean (interquartile intervals)

Characteristic	Menthol smokers ($n = 60$)	Regular smokers ($n = 67$)	*P*
Race, % AA	70.5	29.5	<0.0001[a]
Age, y	40.8 (35–47)	35.7 (25–36)	0.007[a]
BMI	28.8 (24–32)	25.4 (22–27)	0.0007[a]
CPD (mean over 3 d)	18.8 (11–22)	17.0 (12–20)	0.28
FTC nicotine, mg	1.2 (1.1–1.3)	1.0 (0.8–1.2)	0.004[a]
FTC tar, mg	16.1 (15–18)	13.5 (11–16)	<0.0001[a]
Number of years smoked	23.7 (15–31)	18.3 (9–25)	0.006[a]
Age of smoking start, y	15.9 (13–18)	16.8 (14–18)	0.25
Time after awakening to first cigarette, min	24.8 (5–30)	27.8 (5–45)	0.30
Time from last cigarette to urine collection, min	112 (60–148)	86 (50–108)	0.02[a]
FTND score	5.6 (3–5)	3.6 (2–5)	0.22
Plasma cotinine, ng/mL	202 (125–280)	217 (124–266)	0.48
Urine nicotine equivalents,[b] pmol/mg creat	47.2 (32–79)	59.8 (42–106)	0.04[a]
Urine NNAL,[b] pmol/mg creat	0.9 (0.6–1.7)	1.2 (0.7–2.3)	0.08
Urine total PAHs,[b] pmol/mg creat	81.6 (54–117)	115.2 (79–185)	0.001[a]
Urine menthol,[b] μ/mg creat	4.8 (2.3–12.9)	2.1 (0.5–7)	0.0009[a]
Menthol exposure score	1.6 (0–2)	1.4 (0–2)	0.53

Abbreviation: AA, African Americans.

[a]Significant difference between groups by chi-square (for race), *t* test (for arithmetic mean parameters), and Wilcoxon test (for geometric mean parameters).

[b]Geometric means.

Table 2. Cross-correlations among exposure biomarkers in menthol and regular cigarette smokers

Characteristic	CPD	Menthol score	Plasma nicotine	Plasma cotinine	Urine total PAH	Urine menthol	Urine NNAL	Urine nic equiv
Menthol smoker								
CPD	**1.0**	0.08	**0.26**[b]	0.25	0.17	0.02	0.05	0.11
Menthol score		**1.0**	0.16	0.04	0.10	0.05	0.05	0.05
Plasma nicotine			**1.0**	**0.71**[a]	**0.46**[b]	**0.44**[b]	0.25	**0.54**[a]
Plasma cotinine				**1.0**	**0.41**[b]	**0.34**[b]	**0.44**[b]	**0.57**[a]
Urine total PAH					**1.0**	**0.55**[a]	**0.48**[a]	**0.80**[a]
Urine menthol						**1.0**	**0.30**[b]	**0.60**[a]
Urine NNAL							**1.0**	**0.45**[b]
Urine nic equiv								**1.0**
Regular smoker								
CPD	**1.0**	0.17	0.17	0.14	0.30[b]	0.06	**0.35**[b]	**0.25**[b]
Menthol score		**1.0**	0.01	0.07	0.09	**0.30**[b]	0.05	0.05
Plasma nicotine			**1.0**	**0.82**[a]	**0.43**[b]	0.16	**0.39**[b]	**0.63**[a]
Plasma cotinine				**1.0**	**0.34**[b]	0.15	**0.43**[b]	**0.63**[a]
Urine PAH total					**1.0**	**0.45**[b]	**0.43**[b]	**0.75**[a]
Urine menthol						**1.0**	0.15	**0.36**[b]
Urine NNAL							**1.0**	**0.67**[a]
Urine nic equiv								**1.0**

[a]Significant $P < 0.0001$.

[b]Significant $P < 0.05$.

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Table 3. Multiple regression models of predictors of urine menthol, urine nicotine equivalents, urine total NNAL, and urine total PAH metabolites in menthol cigarette smokers

Dependent variable	Model	Independent variables	Estimate	SE	*P*	R^2
Urine menthol	NA	Age	−0.0005	0.019	0.98	0.14
		Sex ($F = 1$)	0.341	0.404	0.40	
		Race ($A = 1$)	−0.573	0.430	0.16	
		BMI	0.192	0.220	0.39	
		CPD	0.412	0.253	0.11	
		TLC	−0.144	0.183	0.43	
		Menthol score	0.103	0.132	0.44	
Urine NIC Equivalents (mmol/g creat)	1	Age	0.012	0.008	0.17	0.46
		Sex	0.181	0.180	0.32	
		Race	0.012	0.181	0.95	
		BMI	−0.0308	0.098	0.003	
		CPD	0.080	0.114	0.486	
		TLC	−0.108	0.081	0.192	
		MEN	0.317	0.062	<0.001	
Urine NNAL (pmol/mg creat)	1	Age	0.027	0.009	0.003	0.33
		Sex	0.055	0.191	0.77	
		Race	0.068	0.192	0.73	
		BMI	−0.144	0.104	0.17	
		CPD	−0.096	0.121	0.43	
		TLC	−0.162	0.086	0.07	
		MEN	0.223	0.066	0.001	
	2	Age	0.013	0.005	0.01	0.58
		Sex	0.154	0.140	0.14	
		Race	−0.170	0.108	0.12	
		BMI	0.096	0.068	0.16	
		TLC	0.007	0.056	0.90	
		NEq	0.084	0.081	<0.001	
		MEN	−0.035	0.033	0.30	
Urine PAH (pmol/mg creat)	1	Age	0.025	0.007	0.004	0.47
		Sex	0.241	0.145	0.10	
		Race	−0.050	0.146	0.74	
		BMI	−0.107	0.080	0.18	
		CPD	−0.003	0.092	0.97	
		TLC	−0.003	0.066	0.21	
		MEN	0.228	0.050	<0.001	
	2	Age	0.018	0.004	<0.001	0.73
		Sex	0.169	0.097	0.089	
		Race	−0.066	0.104	0.525	
		BMI	0.064	0.062	0.308	
		TLC	0.016	0.047	0.736	
		NEq	0.558	0.081	<0.001	
		MEN	0,045	0.044	0.314	

NOTE: Models are described in the Data Analysis methods section.

Abbreviations: TLC, time from last cigarette to urine collection; NEq, urine nicotine equivalents; PAH, urine polycyclic aromatic hydrocarbon metabolites; MEN, urine menthol; NA, not applicable.

exposure measures, whereas number of cigarettes smoked per day was not. To determine whether menthol exposure influences NNK or PAH exposure independent of nicotine exposure, we examined a regression model in which both a measure of nicotine intake (urine nicotine equivalents or plasma cotinine) instead of cigarettes per day, as well as urine menthol concentration were the independent variables, and urine NNAL or PAH

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metabolites were the dependent variables (model 2). In these models, nicotine equivalents or plasma cotinine were strongly associated with urine NNAL, but urine menthol was not.

Discussion

We present novel data on urine menthol concentrations in smokers of mentholated and regular cigarettes. We also describe a novel assay for the measurement of urine menthol glucuronide. This assay is an improvement over previous methods because it measures menthol glucuronide directly without the need to treat the samples with glucuronidase enzyme with the subsequent measurement of free menthol. This eliminates any uncertainty involved with incomplete hydrolysis, and the method is faster and easier to perform as well.

The menthol levels that are presented in our article represent menthol glucuronide, which is the major form of menthol in human urine (12). On the basis of prior studies with the administration of known doses of menthol, urine excretion of menthol glucuronide accounts for 50% of a dose of menthol. Given the short half-life of menthol in the body (75 minutes), urine menthol is expected to reflect menthol exposure for only 6 to 8 hours (5–6 half-lives) prior to urine sampling. Because of the short half-life of menthol, we included time from last cigarette to time of urine collection in our regression models.

Menthol is present in numerous sources including dental hygiene products, soaps, foods, and medications (12). Menthol is present in many cigarettes, with higher concentrations in those that are characterized as menthol flavors and lower concentrations in many others (22, 23). Hopp reported average menthol concentration of tobacco in mentholated cigarettes to be between 0.1% and 0.45%; menthol concentration of nonmenthol cigarette tobacco averaged 0.003%. Celebucki et al. reported that the average menthol content of menthol cigarettes is 2.64 mg, whereas we reported previously that 1 popular brand of menthol cigarette contained 3 mg of menthol per cigarette (24, 25).

As expected, we found substantial differences in urine menthol in smokers of menthol versus regular cigarettes. In many regular cigarette smokers menthol levels were below the limit of quantitation, whereas most (80%) of menthol cigarette smokers had measurable menthol levels. We developed a menthol exposure score based on the number of sources of exposures but found no significant correlation between this score or cigarettes smoked per day and urine menthol levels in regular cigarette smokers. Perhaps, if we had more precise information on how much menthol was taken from each source, we could have better predicted menthol in regular cigarette smokers. However, there were 3 outliers among regular smokers who had very high urine menthol levels, and these smokers also had daily self-reported exposure to menthol in candies, gum, and/or toothpaste.

Among menthol cigarette smokers there were strong cross-correlations among urine menthol, urine nicotine equivalents, plasma cotinine, urine NNAL, and urine PAH metabolites. There was no significant association between cigarettes smoked per day and urine menthol. These observations are consistent with prior research showing that the number of cigarettes smoked per day is not a very good marker of smoke exposure but that biomarkers of nicotine exposure are highly correlated with exposure to other tobacco smoke constituents (26, 27).

In multiple regression analyses, urine menthol concentration was significantly associated with exposure to nicotine (urine nicotine equivalents), urine NNAL, and urine PAH metabolites. When menthol and cigarettes smoked per day were both included in the model, cigarettes smoked per day had no predictive value. Significant associations would be expected between urine menthol and other smoke constituents if menthol was simply a marker of overall tobacco smoke exposure. However, if menthol directly influences the generation, delivery or absorption of carcinogens, one would expect to see an effect of menthol independent of that of nicotine. When both nicotine equivalents or plasma cotinine and urine menthol were included in the same model as predictors of NNAL or PAHs, nicotine exposure was the stronger predictor and the menthol effect was nonsignificant. Thus, it appears that menthol exposure does not add to the predictive value of nicotine exposure in determining exposure to tobacco-specific nitrosamines or PAHs from cigarette smoking. Consistent with this finding are reports of similar levels of biomarkers of tobacco smoke exposure in smokers of menthol versus regular cigarettes with similar levels of nicotine intake (8, 9).

In summary, we find that urine menthol is measurable in the great majority of menthol cigarette smokers and that urine menthol is highly correlated with exposure to nicotine and tobacco smoke carcinogens. Menthol is not independently associated with carcinogen exposure when nicotine intake is considered. Although our study does not find that menthol in cigarettes selectively increases exposure to tobacco smoke carcinogens, our data do not exclude the possibility that menthol facilitates inhalation of cigarette smoke and increases exposure to all tobacco smoke toxins equally.

Disclosures of Potential Conflicts of Interest

N.L. Benowitz serves as a scientific advisor to several pharmaceutical companies that are developing and/or market medications to aid smoking cessation. He also has served as an expert witness in litigation against the tobacco industry. K.M. Dains, D. Dempsey, and P. Jacob, C. Havel, and M. Wilson have no potential conflicts to disclose.

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