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Research Article

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Menthol Smokers: Metabolomic Profiling and Smoking Behavior

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Abstract

Background: The use of menthol in cigarettes and marketing is under consideration for regulation by the FDA. However, the effects of menthol on smoking behavior and carcinogen exposure have been inconclusive. We previously reported metabolomic profiling for cigarette smokers, and novelly identified a menthol-glucuronide (MG) as the most significant metabolite directly related to smoking. Here, MG is studied in relation to smoking behavior and metabolomic profiles.

Methods: This is a cross-sectional study of 105 smokers who smoked two cigarettes in the laboratory one hour apart. Blood nicotine, MG, and exhaled carbon monoxide (CO) boosts were determined (the difference before and after smoking). Spearman correlation, χ^2, and ANCOVA adjusted for gender, race, and cotinine levels for menthol smokers assessed the relationship of MG boost, smoking behavior, and metabolic profiles. Multivariate metabolite characterization using supervised partial least squares-discriminant analysis (PLS-DA) was carried out for the classification of metabolomics profiles.

Results: MG boost was positively correlated with CO boost, nicotine boost, average puff volume, puff duration, and total smoke exposure. Classification using PLS-DA, MG was the top metabolite discriminating metabolome of menthol versus nonmenthol smokers. Among menthol smokers, 42 metabolites were significantly correlated with MG boost, which linked to cellular functions, such as of cell death, survival, and movement.

Conclusions: Plasma MG boost is a new smoking behavior biomarker that may provide novel information over self-reported use of menthol cigarettes by integrating different smoking measures for understanding smoking behavior and harm of menthol cigarettes.

Impact: These results provide insight into the biological effect of menthol smoking. *Cancer Epidemiol Biomarkers Prev; 26(1); 51–60. ©2016 AACR.*

Introduction

In 2009, the FDA was mandated by the Family Smoking Prevention and Tobacco Control Act to regulate tobacco products and ban flavor additives. However, the act exempted menthol from the ban, pending FDA review and consideration of the available science. In March 2011, the Tobacco Product Scientific Advisory Committee found adverse impact on public health by increasing the numbers of smokers with resulting premature death and avoidable morbidity and recommended that "removal of menthol cigarettes from the marketplace would benefit the public health" (1). The FDA's Center for Tobacco Products separately has done extensive literature reviews on topics regarding menthol and tobacco and concluded that the studies that examined menthol cigarette usage associated with smoking topography are inconsistent and limited (2). From its preliminary scientific evaluation (3), menthol in cigarettes was also considered by the FDA to likely have significant public health impacts. In July 2013, the FDA further issued an Advance Notice of Proposed Rulemaking (4), seeking additional information from science-based approaches to further inform decisions about regulatory action with respect to menthol in cigarettes.

Menthol cigarettes are highly popular among African American smokers, although many European Americans also smoke them (5). Used as a flavoring that provides a cooling sensory effect that reduces the harshness and irritation inherent in cigarette smoke, almost all cigarettes contain some degree of menthol (6). Menthol-labeled cigarettes are marketed with messaging such as "fresh," "clean," and "refreshing" by the tobacco industry and attract specific demographic groups, including adolescents and young adults, women, and African Americans (7–10). From a recent meta-analysis, when compared with never smokers, current menthol cigarette smokers have a statistically significant HR of 3.48 for cardiovascular mortality, whereas the ratio was 2.10 for current nonmenthol smokers (11).

Menthol cigarette usage is associated with greater addictive potential, increased initiation and dependence, and longer time to achieve cessation (3). Duner-Engstrom and colleagues reported that menthol increased salivary secretion that could facilitate the absorption of nicotine in the mouth (12). Other studies have found its effect to prolong breath-hold time (13) and to increase

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the permeability of N-nitrosonornicotine and nicotine (14). Moreover, menthol smokers showed impaired nicotine metabolism (15), had a lower detoxification rate of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; ref. 16), and retained more ultrafine particulate and fine particulate benzo(a)pyrene than nonmenthol smokers (17). As a result, menthol cigarette smokers may take in more carcinogens. Because of its sensory property and respiratory effects, menthol has been hypothesized to affect smoking topography (e.g., how someone smokes their cigarettes) by increasing the puff volumes, leading to smokers' exposure to more hazardous chemicals (18). For cancer outcomes, Sidney and colleagues (19) reported a modestly increased risk of lung cancer associated with male menthol smokers (RR = 1.45; 95% confidence interval, 1.03–2.02). Other studies had also shown an increased risk of menthol smoking with lung cancer (20), pharyngeal cancer (21), and esophageal cancer (22) among menthol smokers, although the results were not always statistically significant.

Recently, an untargeted metabolomics approach by ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS) was used with a pre–post experimental design, where dynamic changes of plasma metabolites could be examined in a cross-sectional study of smokers ($n = 105$) who smoked two cigarettes one hour apart; we reported that 31 novel metabolites were affected by cigarette smoking during this interval, uniquely including menthol-glucuronide (MG) (23). Here, the study extends our investigation by examining the associations of MG changes during smoking, termed the MG boost, as well as other smoke exposure biomarkers, smokers' puff profiles, the classification of metabolomic profiles between menthol and nonmenthol smokers, and the biological associations of MG in menthol smokers' plasma with their metabolome. Using an untargeted metabolomics approach, this study hypothesized that the MG boost would identify metabolic pathways that are changed by menthol exposure.

Materials and Methods

Study recruitment and design

A previously conducted cross-sectional study with a pre–post smoking experimental design was used to determine smoking behavior and novel blood biomarkers before and after smoking cigarettes (23). Subjects ≥18 years of age and who smoked >10 cigarettes/day for at least 5 years with a stable smoking pattern for ≥1 year were recruited through local media. Eligible subjects were excluded from the study if they had a prevalent respiratory or oral cavity disease, prior history of cancer, or had undergone general anesthesia within 6 months. Subjects who had used medications for smoking cessation (including nicotine replacement therapy) or antidepressants within 6 months or used other tobacco products in the past year were further excluded. All subjects gave informed consent, and the study protocols were approved by the Institutional Review Boards at the respective institutions.

Smoking protocol and biospecimen collection

All participants were asked to smoke two cigarettes of their usual brand in a smoking laboratory, one hour apart. Participants were first asked to smoke one of their own cigarettes as naturally as possible and to smoke their second cigarette through the mouthpiece to assess smoking topography (CReSS; Borgwaldt KC) via a transducer to measure puff volume, puff duration, interpuff interval, number of puffs, time to smoke the cigarette, and puff velocity. From these data, total smoke exposure (puff number × puff volume) was calculated. Carbon monoxide (CO) levels in the participants' expired air were determined immediately before and after each of the two cigarettes (Vitalograph Inc.). Blood was collected immediately before and 2 minutes after smoking each cigarette. This allowed for the assessment of the change of biomarker levels (nicotine and MG) due to smoking a cigarette (i.e., the "boost" measurement). Nicotine, cotinine, and trans-3′-hydroxycotinine levels in the blood samples were determined by gas chromatography (nicotine) or liquid chromatography–tandem mass spectrometry (cotinine and trans-3′-hydroxycotinine) in the laboratory of Dr. Neil Benowitz, as described previously (24, 25). The nicotine metabolic ratio (NMR) was calculated using the ratio of plasma 3′-hydroxycotinine to cotinine, as a measure of nicotine metabolic capacity. Six subjects with negative nicotine boost levels and 7 subjects with plasma cotinine levels less than 100 ng/mL were excluded to ensure that subjects had an active smoking history in the study. These 6 smokers with negative boosts likely smoked their cigarette differently, and minimally, compared with their usual smoking behavior. It is possible that the subjects with negative boost levels had minimal puff and inhalation during the boost assessment (in contrast to their normal smoking pattern), and so it is appropriate to exclude them. A total of 105 subjects were left for the analysis.

A questionnaire was administered in person by trained interviewers during the 60 minutes between cigarettes. Data were collected on demographic characteristics, smoking history, and other lifestyle factors (including alcohol use and body size) and personality traits related to smoking behavior. Subjects were also administered the Fagerstrom test for nicotine dependence (FTND).

Significant metabolites from untargeted metabolomics analysis

A total of 420 plasma samples from 105 smokers (4 samples each) and 117 quality control samples and repeats were analyzed by UHPLC-Q-TOF MS for the untargeted metabolomics profiles, using both a positive and negative ion mode (total of 1,074 assays) on an ACQUITY UPLC system (Waters) and processed by XCMS online (XCMS Public Shares Job ID: 1110757 and 1110926; ref. 26). From the previous result, novel metabolites consistently and similarly affected across two cigarettes were identified and 11 metabolites were validated by matching the retention time, mass error and isotopic pattern, and tandem mass spectrum of the parent ion from the biological sample to that of the commercially available standard metabolites and were included in this study.

Statistical analysis

All data were analyzed using SIMCA (Umetrics Inc.) and Partek Genomics Suite (Partek Inc.). t tests and χ^2 tests were performed to evaluate differences in smokers' characteristics between menthol and nonmenthol smokers. Spearman rank correlation was performed to assess correlations for smoking-related variables and metabolites. A partial least squares-discriminant analysis (PLS-DA) model was constructed for multivariate metabolite characterization. ANCOVA models adjusted for gender, race, and cotinine levels were carried out to determine the relationship of MG boost from the second cigarette among menthol smokers to their metabolomics profiles. Significant metabolites were searched

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Table 1. Study participant characteristics and differences between menthol and nonmenthol smokers

	All ($n = 105$)		Menthol ($n = 71$, 67.6%)		Nonmenthol ($n = 34$, 32.4%)		
Variable	***n* (%)**	**Mean ± SD**	***n* (%)**	**Mean ± SD**	***n* (%)**	**Mean ± SD**	***P*[a]**
Age (y)		43.1 ± 9.6		43.8 ± 9.3		41.4 ± 10.9	*0.25*
Age (y), categorical							
<44 yrs old	55 (52.4)		34 (47.9)		21 (61.8)		0.18
≥44 yrs old	50 (47.6)		37 (52.1)		13 (38.2)		
Gender							
Male	67 (63.8)		43 (60.6)		24 (70.6)		0.32
Female	38 (36.2)		28 (39.4)		10 (29.4)		
Race							
AA	63 (60.0)		58 (81.7)		5 (14.7)		<0.00001
EA	38 (36.2)		12 (16.9)		26 (76.5)		
Biracial	4 (3.8)		1 (1.4)		3 (8.8)		
Yrs smoked (y)		22.9 ± 9.9		22.5 ± 9.8		23.7 ± 10.3	*0.57*
Yrs smoked (y), categorical							
<22 yrs	52 (49.5)		39 (54.9)		13 (38.2)		0.11
≥22 yrs	53 (50.5)		32 (45.1)		21 (61.8)		
Plasma cotinine (ng/mL)		252.6 ± 103.3		247.5 ± 97.3		263.4 ± 115.6	*0.46*
Plasma cotinine, categorical							
Low (<200)	35 (33.3)		23 (32.4)		12 (35.3)		0.96
Medium (200–279)	35 (33.3)		24 (33.8)		11 (32.4)		
High (>279)	35 (33.3)		24 (33.8)		11 (32.4)		
NMR		0.38 ± 0.22		0.37 ± 0.24		0.41 ± 0.17	*0.44*
NMR, categorical							
Q1 (<0.238)	25 (23.8)		20 (28.2)		5 (14.7)		0.25
Q2 (0.238–0.34)	27 (25.7)		19 (26.8)		8 (23.5)		
Q3 (0.341–0.458)	27 (25.7)		18 (25.4)		9 (26.5)		
Q4 (>0.458)	26 (24.8)		14 (19.7)		12 (35.3)		
FTND scoring		5.0 ± 2.3		4.8 ± 2.1		5.4 ± 2.7	*0.20*
FTND scoring, categorical							
<5 points	44 (41.9)		32 (45.1)		12 (35.3)		0.31
5–6 points	33 (31.4)		23 (32.4)		10 (29.4)		
>6 points	27 (25.7)		15 (21.1)		12 (35.3)		

Abbreviations: AA, African Americans; EA, European Americans.

[a]*P* values represent differences in menthol versus nonmenthol smokers for each characteristic. Continuous variables were evaluated by two-sample *t* tests (italics), and χ^2 tests were used to investigate the differences in distributions of categorical variables from menthol to nonmenthol smokers.

against METLIN Metabolomics Database, Human Metabolome Database, and LIPID MAPS Structure Database with the mass accuracy of 10 ppm to identify putative metabolite identifications. The construction, interaction, and pathway analysis of potential biomarkers were performed by Ingenuity Pathways Analysis (IPA).

Results

Characteristics of the 105 study participants (mentholated cigarette smokers, $n = 71$ versus nonmentholated, $n = 34$) are shown in Table 1. The mean age ± standard deviation of participants was 43.1 ± 9.6 years old, with an average smoking history of 22.9 ± 9.9 years. The mean cotinine level of smokers was 252.6 ± 103.3 ng/mL, and the mean NMR was 0.38 ± 0.22. Approximately 64% were male and the mean FTND score was 5.0 ± 2.3. Aside from race, where menthol smokers were predominantly African American ($P < 0.00001$), no significant differences in participant characteristics were observed by type of cigarette smoked.

To examine the correlation of the 11 smoking-related metabolites to variables related to smoking behavior, Spearman rank correlation coefficient was computed on the metabolite boosts (i.e., the level changed) from the second cigarette to the smoking-related variables (Supplementary Table S1). As blood cotinine level is a biochemical measure of nicotine consumption, rather than self-reported use (27), plasma cotinine levels were used in the analysis to represent smokers' cigarette exposure. From the results in Supplementary Table S1, the MG boost had the most statistically significant correlations among all validated metabolites with smoking-related variables; it was positively correlated with the average puff volume ($r = 0.36$; $P = 0.0003$), CO boost ($r = 0.31$; $P = 0.001$), average puff duration ($r = 0.3$; $P = 0.003$), total smoke exposure ($r = 0.26$; $P = 0.01$), and nicotine boost ($r = 0.26$; $P = 0.04$; Supplementary Table S1; Supplementary Fig. S1). There was no correlation of the MG to cigarettes per day or time to first cigarette, probably due to the quick half-life of the MG. When adjusted with cotinine levels in the model (Supplementary Table S2), average puff volume, CO boost, number of puffs, 3-hydroxy cotinine, and NMR were significantly correlated with MG boost. However, no significant correlations were found between MG boost and average interpuff interval, maximum puff velocity, and FTND.

When smokers were stratified by menthol and nonmenthol status, the MG boost from menthol smokers was positively correlated with average puff volume ($r = 0.48$; $P = 6.22E{-}05$), average puff duration ($r = 0.33$; $P = 0.008$), total smoke exposure ($r = 0.35$; $P = 0.004$), and negatively correlated with interpuff interval ($r = -0.37$; $P = 0.002$; Table 2). On the other hand, the MG boost from nonmenthol smokers was positively correlated only with CO boost ($r = 0.47$; $P = 0.006$), and not significantly correlated with other variables. An increased correlation of MG

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Table 2. Correlation of the second MG boost to smoking-related variables

	All smokers		Menthol		Nonmenthol	
	r^a	P^a	r	P	r	P
Average puff volume (mL)	0.36	0.0003	0.48	6.22E−05	0.09	0.64
CO boost	0.31	0.001	0.22	0.06	0.47	0.006
Average puff duration (sec)	0.30	0.003	0.33	0.008	0.10	0.58
Total smoke exposure (mL)	0.26	0.01	0.35	0.004	0.14	0.46
Nicotine boost	0.26	0.04	0.32	0.05	0.28	0.19
Average interpuff interval (sec)	−0.14	0.17	−0.37	0.002	0.27	0.14
Number of puffs	0.11	0.27	0.17	0.18	0.07	0.69
Cotinine (ng/mL)	0.05	0.61	0.18	0.12	−0.15	0.40
Maximum puff velocity (mL/sec)	0.05	0.62	0.18	0.14	0.16	0.38
FTND	0.04	0.65	0.22	0.06	0.02	0.90
3HC (ng/mL)	0.04	0.69	0.23	0.06	−0.11	0.54
NMR	0.01	0.90	0.13	0.27	0.04	0.84

NOTE: Data significant at $P < 0.05$.

[a]r and P were computed on the basis of Spearman rank correlation coefficient.

boost versus FTND in menthol smokers ($r = 0.22$; $P = 0.06$) compared with nonmenthol smokers ($r = 0.02$; $P = 0.9$) was found, and an increased correlation of MG boost versus nicotine boost in menthol smokers ($r = 0.32$; $P = 0.05$) compared with nonmenthol smokers ($r = 0.28$; $P = 0.19$) was observed. Significant correlations were also found between pre- and postcigarette MG levels to the topography profiles (Supplementary Table S3).

Spearman rank correlation was further computed on the second nicotine boost and second CO boost to smoking-related variables, separately. From the results, nicotine boost was negatively correlated with NMR in all smokers ($r = -0.27$; $P = 0.03$), positively correlated with MG boost in all smokers ($r = 0.26$; $P = 0.04$), and positively correlated with FTND in menthol smokers ($r = 0.38$; $P = 0.02$, Supplementary Table S4). On the other hand, CO boost was positively correlated with MG boost in all smokers ($r = 0.31$; $P = 0.001$) and nonmenthol smokers ($r = 0.47$; $P = 0.006$), and positively correlated with average puff volume in nonmenthol smokers ($r = 0.38$; $P = 0.04$, Supplementary Table S5).

Two sample t tests were computed to assess the differences in smoking-related characteristics between self-reported menthol and nonmenthol smokers (Table 3). Menthol smokers had significantly higher MG boosts, compared with smokers of nonmenthol cigarettes that still contain small amounts of menthol ($P < 0.00002$, fold change = 11.2). The range for the MG boost in menthol smokers was −41.3 to 259.6 (Supplementary Fig. S1, red dots), with a mean of 31.2 and a median of 22.7, whereas in nonmenthol smokers, the range was −44.7 to 67.5, with a mean of −2.8 and a median of −3.7 (Supplementary Fig. S1, blue dots), respectively. However, there were no significant differences in the FTND scores, CO boost, nicotine boost, NMR, and any topography profiles between menthol versus nonmenthol smokers, including maximum puff velocity, average puff volume, average puff duration, average interpuff interval, total number of puffs, and total smoke exposure (Table 3). Specificity analysis using median MG boost values to categorize smokers with high and low plasma MG boost presents a sensitivity of 70% in identifying menthol smokers with a positive predictive value of 94.2%, and 91% in the specificity to identify nonmenthol smokers.

Metabolomics profiles of menthol and nonmenthol smokers using PLS-DA were well separated with a CV-ANOVA P value of 0.0003 (Fig. 1A) using type of cigarettes as the classifier, and MG displayed the strongest discriminatory power among all metabolites in the model (Fig. 1B) with a variable importance in projection score >2.5. To examine the biological impact of MG boost from the second cigarette to smokers' metabolomics profiles, a subset analysis was conducted using an ANCOVA model adjusted for gender, race, and cotinine levels of 71 menthol smokers in relation to the metabolomics profiles after the second cigarette. There were 653 metabolites significantly correlated with MG boost ($P < 0.05$), and 42 of them were significant after correction

Table 3. Differences in the smoking-related characteristics of participants from self-reported menthol and nonmenthol smokers in the study

	Menthol (71) Mean ± SD	Nonmenthol (34) Mean ± SD	P^a	Fold change (Menthol/nonmenthol)
MG boost	31.2 ± 42.8	−2.78 ± 18.3	2.31E−05	11.2
Maximum puff velocity (mL/sec)	47.5 ± 13.7	51.6 ± 16.1	0.20	−1.1
FTND	4.8 ± 2.1	5.4 ± 2.7	0.20	−1.1
CO boost	5.2 ± 3.1	4.4 ± 3.6	0.20	1.2
3HC (ng/mL)	90.8 ± 67.9	106.7 ± 62.7	0.25	−1.2
Nicotine boost	14.0 ± 8.6	11.9 ± 7.0	0.31	1.2
NMR	0.4 ± 0.2	0.4 ± 0.2	0.44	−1.1
Average puff volume (mL)	55.7 ± 18.5	52.7 ± 18.0	0.45	1.1
Average puff duration (sec)	1.8 ± 0.6	1.6 ± 0.8	0.46	1.1
Cotinine (ng/mL)	247.5 ± 97.3	263.4 ± 115.6	0.46	−1.1
Average interpuff interval (sec)	20.0 ± 12.7	21.0 ± 14.7	0.74	−1.0
Number of puffs	12.6 ± 7.5	12.3 ± 6.6	0.87	1.0
Total smoke exposure (mL)	691.7 ± 455.9	690.3 ± 547.8	0.99	1.0

[a]P value was computed on the basis of two-sample t tests. Data significant at $P < 0.05$.

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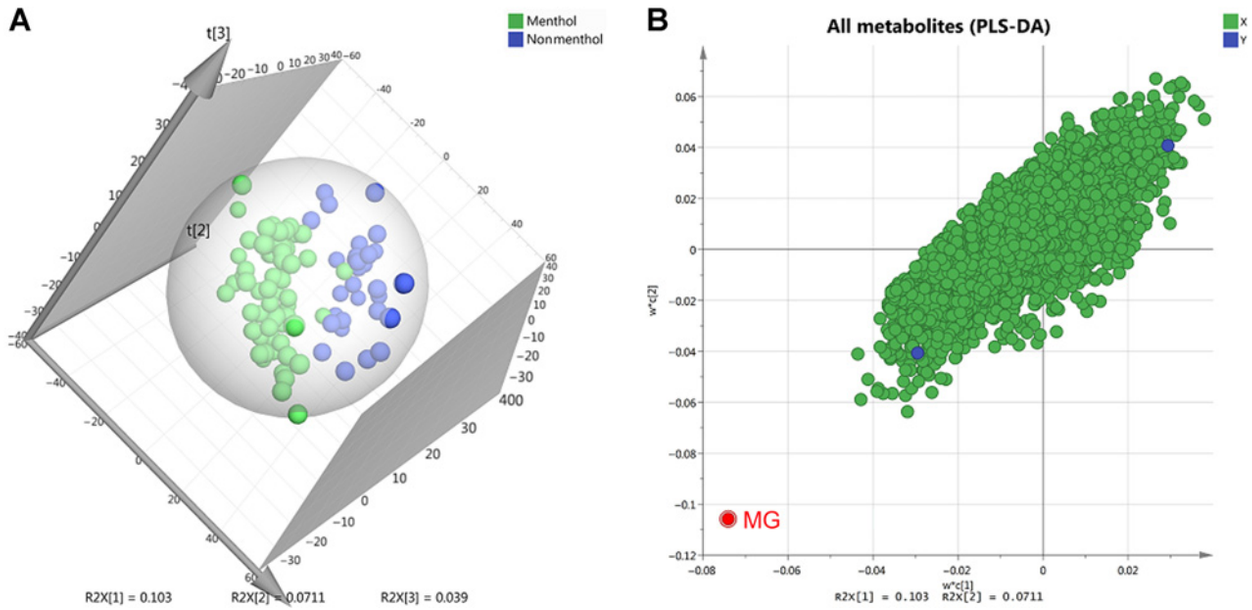

Figure 1.

PLS-DA of all menthol and nonmenthol cigarette smokers. **A,** Three-dimensional score plot between the selected principle components (PC) showed difference between menthol (green) and nonmenthol (blue) smokers in their metabolomic profiles. **B,** Loadings plot showed MG as the most influential metabolite discriminating metabolome of menthol and nonmenthol smokers with an ANOVA assessment of the cross-validatory (CV) $P = 0.0003$. The P value indicates the probability level where a model with this F value may be the result of just chance.

for multiple comparisons ($q < 0.05$). The main chemical taxonomy classes of the 42 metabolites were putatively identified as glycerophospholipids ($n = 5$), carboxylic acids and derivatives ($n = 4$), sterol lipids ($n = 2$), prenol lipids ($n = 2$), alkaloids and derivatives ($n = 1$), benzene and substituted derivatives ($n = 1$), fatty acyls ($n = 1$), indoles and derivatives ($n = 1$), organic sulfuric acids and derivatives ($n = 1$), alkaloids and derivatives ($n = 1$), and terpenoids ($n = 1$; Table 4). Among the correlated metabolites, MG, 2,4-dihydroxyacetophenone 5-sulfate, L-tryptophan, uric acid, benzyl sulfate, bilirubin, oxalosuccinic acid, PC(O-16:0/22:6), PC(22:2/16:1) were positively correlated with MG boost, and S-(PGA2)-glutathione, PC(O-12:0/O-1:0), thalicoside A, PE(P-16:0/0:0), 2-(1-ethoxyethoxy)propanoic acid, 2-deoxy-20-hydroxyecdysone 22-phosphate, N-ornithyl-L-taurine, 10-oxo-nonadecanoic acid, 20-hydroxyecdysone, isothankunic acid, and lysoPC(20:4) were negatively correlated with MG boost

Table 4. Known metabolites significantly correlated with MG boost among 71 menthol smokers

m/z	RT	Metabolites	Adducts	Category	Δppm	*q*-value	PartialCorr
331.18	3.07	MG	[M−H]−	Prenol lipids	1	0.0040	0.53
640.29	5.52	S-(PGA2)-glutathione	[M−H]−	Carboxylic acids and derivatives	1	0.0042	−0.52
231.00	1.70	2,4-Dihydroxyacetophenone 5-sulfate	[M−H]−	Benzene and substituted derivatives	6	0.0199	0.42
205.10	0.36	L-Tryptophan	[M+H]+	Indoles and derivatives	8	0.0288	0.53
167.02	0.34	Uric acid	[M−H]−	Alkaloids and derivatives	7	0.0344	0.39
187.01	1.71	Benzyl sulfate	[M−H]−	Organic sulfuric acids and derivatives	5	0.0344	0.42
462.30	5.01	PC(O-12:0/O-1:0)	[M+Na]+	Glycerophospholipids	4	0.0344	−0.54
799.48	4.65	Thalicoside A	[M+H]+	Terpenoids	7	0.0344	−0.54
460.28	4.63	PE(P-16:0/0:0)	[M+Na]+	Glycerophospholipids	6	0.0364	−0.54
185.08	4.74	2-(1-Ethoxyethoxy)propanoic acid	[M+Na]+	Carboxylic acids and derivatives	7	0.0397	−0.53
585.27	3.95	Bilirubin	[M+H]+	Tetrapyrroles and derivatives	0	0.0408	0.52
189.00	1.71	Oxalosuccinic acid	[M−H]−	Carboxylic acids and derivatives	2	0.0472	0.41
792.60	8.48	PC(O-16:0/22:6)	[M+H]+	Glycerophospholipids	8	0.0480	0.49
567.27	4.87	2-deoxy-20-hydroxyecdysone 22-phosphate	[M+H]+	Sterol lipids	0	0.0485	−0.50
240.10	4.73	N-Ornithyl-L-taurine	[M+H]+	Carboxylic acids and derivatives	3	0.0491	−0.49
313.27	4.85	10-oxo-nonadecanoic acid	[M+H]+	Fatty acyls	0	0.0491	−0.51
481.32	5.00	20-hydroxyecdysone	[M+H]+	Sterol lipids	2	0.0491	−0.52
505.35	5.03	Isothankunic acid	[M+H]+	Prenol lipids	7	0.0491	−0.51
834.60	8.73	PC(22:2/16:1)	[M+Na]+	Glycerophospholipids	1	0.0491	0.45
544.34	5.03	LysoPC(20:4)	[M+H]+	Glycerophospholipids	0	0.0498	−0.48

*q-value (corrected P value) was computed on the basis of two-way ANCOVA model adjusted for gender, race, and cotinine levels. Data significant at $q < 0.05$ after correcting for multiple testing. PartialCorr represents the correlation between the second MG boost and the metabolites.
Abbreviations: m/z, mass-to-charge ratio; RT, retention time.

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(Table 4). Twenty-two metabolites could not be mapped to current databases and their identities remain unknown.

Pathway analysis was conducted using available KEGG IDs from their putative metabolites and projected onto Ingenuity's knowledge-based networks. Considered in this analysis were direct and indirect relationships, including endogenous chemicals, focusing on interaction networks observed from all data sources. There were four KEGG IDs mapped by IPA for the analysis, namely L-tryptophan, uric acid, bilirubin, and *1*-acylglycerophosphocholine (representing 5 glycerophospholipids in the study). Network analysis was generated *de novo* based on the mapped metabolites to explore potential molecular events and mechanisms affected by MG boost. The network affected by the MG boost (Fig. 2) was associated with functions of cell death and survival, cellular movement, and hematologic system development and function, with 7 molecules involved in cancer, namely *ABCB4*,*C3*,*CASR*,*CCK*, *IDO1*, L-tryptophan, and *UMOD* (Fig. 2).

The ANCOVA model adjusted for gender, race, and cotinine levels was performed to model the association between MG boost and the metabolomics profiles of 34 nonmenthol smokers, as well as correlation between CO boost/and nicotine boost for menthol and nonmenthol smokers, separately (data not shown). However,

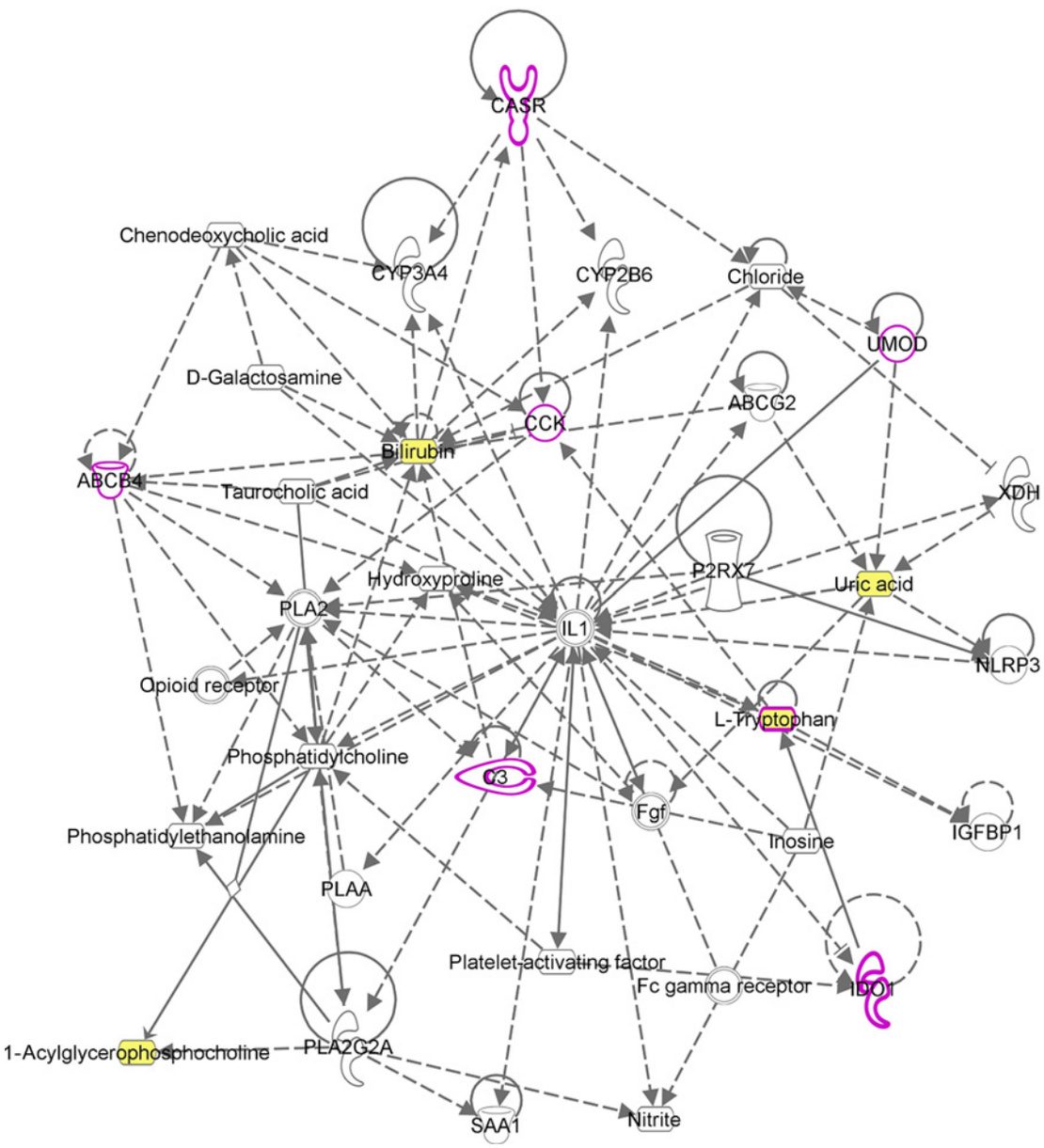

Figure 2.
Network associated with menthol boosts in menthol smokers. Yellow nodes, metabolites mapped from our study; magenta, seven molecules known as biomarkers for cancer.

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none of results reached statistical significance after correction for multiple comparisons.

Discussion

Menthol-marketed cigarettes represent more than a quarter of all cigarettes sold in the United States (28). They are known as a starter product, fostering initiation in youth (9), and menthol smokers are known to have greater nicotine dependence (10) and are less successful in quit attempts (29). The use of menthol in tobacco products is currently not regulated, but the FDA is considering the removal or regulation of menthol in tobacco and restricting its marketing (1). From our previous study using a pre–post experimental design where an individual smoked two cigarettes to assess biomarker changes with smoking, allowing for within-subject replication, MG was the most significant metabolite that rose after each cigarette smoking ($P = 6.99E{-}10$, $q = 8.90E{-}07$) independent of gender, race, and plasma cotinine levels, and baseline MG level was positively correlated with cotinine levels in all smokers ($P = 0.04$) and menthol smokers ($P = 0.008$; ref. 23). In this study, the associations of the MG boost from the second cigarette were examined, after a 1-hour standardized time interval, to various smoking-related variables, including topography profiles. The result showed that MG boost was positively associated with CO boost, nicotine boost, average puff volume, average puff duration, and total smoke exposure. Although the correlation was more significant among menthol smokers than nonmenthol smokers, some of the nonmenthol smokers did exhibit higher MG boost than menthol smokers (Supplementary Fig. S1), revealing the disadvantage of using the traditional dichotomous descriptors (menthol or nonmenthol) in the evaluation of the actual menthol delivered from cigarette smoking. The sensitivity of MG boost showed that there is a 30% probability for a menthol smoker to have low MG boost in the plasma. Here, we evaluate the impact of menthol on smoking behavior using MG level delivered in plasma rather than self-reported cigarette usage for a more accurate estimation. The correlation was also significant between the direct observation of pre- and postcigarette MG levels versus the topography profiles. This indicates the potential for the specificity without using a boost level. Thus, MG and MG boost is a marker of smoke exposure that reflects different types of exposure measurements and may provide better specificity for exposure and harm than self-reported menthol cigarette use or the other measures of exposure.

Generally, high correlation between topographic measures is expected (30). However, these measures are not interchangeable and can be affected by gender, smoking dependence, and cigarette smoked per day (31–33). Therefore, all parameters need to be recorded and evaluated separately when measuring smoking topography (31). Studies on the topography measures also indicated that they are not kept constant during the course of smoking a cigarette, where puff volume decreases and interpuff interval initially increases and then decreases (30). To date, it has been unclear whether menthol cigarette use affects exposure through topography, because prior studies are contradictory regarding its effects on puff volume (34–41), number of puffs (35–40, 42), and other topography measures (34, 35, 37). Conclusions from these studies are limited due to differences in study design, small sample sizes, differences in the nicotine and tar yield, and differences in the menthol content of the cigarettes used (2). Importantly, no studies to date assessed topography in terms of actual menthol exposure, but only self-reported use of menthol cigarettes. In contrast, in this study, the change in the MG boosts for menthol smokers was related to smoking topography.

The MG boost was positively correlated to the CO and nicotine boosts. Prior studies have not been conclusive regarding differences in nicotine and CO boosts between menthol and nonmenthol smokers, ranging from increased and decreased correlations to no effect (35–39, 42–45). From our results, no significant differences in nicotine boost and CO boost were observed between menthol and nonmenthol smokers. Correlations of nicotine boost and CO boost to the smoking-related variables were also examined, but almost none of the smoking-related variables were correlated with either nicotine or CO boost (Supplementary Tables S4 and S5). Furthermore, from the ANCOVA model adjusted for gender, race, and cotinine levels performed to model the association between CO boost and the metabolomics profiles, none of the metabolites from analyses reached significance. Thus, the MG boost provides better relationships to topography and inhalation, compared with nicotine and CO boost measurements.

Menthol is highly volatile and has a high diffusion rate into smoke (46). It is added in many synthetic forms to tobacco in ways to deliver the compound in smoke more efficiently without fluctuations in the yields (47). Mentholated cigarettes are perishable products, and thus, the levels of menthol can vary by the storage time, temperature (18), brand, product (6), and by shelf-life (48). All cigarettes contain menthol to some extent (6), but the menthol-labeled cigarettes have about 1.5- to 2-fold more menthol than nonmenthol cigarettes (6). Gelal and colleagues have reported plasma levels of the MG in nonsmokers who were administered menthol capsules, candies, and mint tea, where the metabolites have the same characteristics of the MG that we identified, and providing further evidence that we are studying the MG as a metabolite of menthol (49). However, the method requires *in vitro* treatment of the plasma samples with β-glucuronidase enzyme. Benowitz and colleagues have reported urine levels of the MG as a biomarker of exposure in menthol smokers with an improved method by liquid chromatography–mass spectrometry (50). Other studies have reported the assessment of MG in humans related menthol in the treatment of gastrointestinal disease using gas chromatography–mass spectrometry in urine and plasma (49, 51, 52). In this study, by using a robust method UPLC-Q-TOF MS, which allows better separation and resolution for the untargeted metabolomics, MG was detected simultaneously with smokers' metabolomics profiles. This provides new opportunities for human studies of menthol tobacco products. For example, e-cigarette vapors do not have most of the tobacco toxicants, but some are mentholated and so the MG might be useful for studies of e-cigarettes that need to assess exposure. The MG boost range was substantially more in the menthol smokers than the nonmenthol smokers, which is both consistent with how levels vary among these cigarettes, but also that menthol smokers are inhaling and absorbing substantially more menthol.

Some previous studies have investigated the effect of cigarette smoking on metabolomics profiles, but none have reported the presence of the MG boost. Müller and colleagues investigated the differences in the fatty acid and phospholipid species among plasma of smokers and nonsmokers by GC-TOF-MS (53) and found elevated levels of PC (glycerophosphocholines) and PE (glycerophosphoethanolamines) species in smokers

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containing monounsaturated fatty acids in smokers. KORA (Cooperative Health Research in the Region of Augsburg) study performed a targeted metabolite profiling of 198 metabolites on 283 human sera samples. They observed three long-chain acyl-alkyl-phosphatidylcholines decreased in current smokers compared with former, and nonsmokers in their first report (54), and later in the follow-up study found decreased in glycerophospholipid pathways (55). In the study reported herein, 42 metabolites were significantly correlated with MG boost ($q < 0.05$), which mapped to networks associated with functions of cell death and survival, cellular movement, hematologic system development, and function (Fig. 2). Menthol has been shown to inhibit cell proliferation and to induce cell death (56, 57). The major network affected involved 7 known molecules for cancer and its biological functions and two P450 enzymes (*CYP3A4* and *CYP2B6*; Fig. 2). Menthol was reported to be an inhibitor of *CYP3A4* (58) and *CYP2B6* (59), and both were associated with altering the nicotine *CYP2A6* metabolism (60). Nicotine metabolism is impaired among menthol smokers (15), and detoxification of the potent lung carcinogen NNAL was found lower in menthol smokers (16). The MG boost was positively correlated with NMR when controlling for cotinine (Supplementary Table S2). Furthermore, MG level in urine was highly correlated with nicotine metabolites, NNAL, and polycyclic aromatic hydrocarbon metabolites (50). Our results revealed the metabolic association of menthol on nicotine metabolism. Further studies should be conducted taking variables associated with individual's nicotine metabolic ratio into account to elucidate the impacts of menthol on the nicotine metabolism.

Among the metabolites that were correlated with the MG boost, glycerophospholipids were the most abundant compound class (Table 4). Glycerophospholipids are amphipathic lipids that serve as important constituents of cell membranes that help to maintain structural integrity and ion permeability, as well as pulmonary surfactant to reduce surface tension during expiration (37). LysoPCs, specifically, are biologically potent compounds presenting as minor phospholipids in the plasma (8%–12%) and cellular membranes ($\geq$3%; ref. 61). They are involved in inflammatory reactions (62), in the regulation of neurotransmitters (63), and as signaling molecules that transmit signals for biological responses, including cell differentiation (64), immune response (65), inflammation (66), oxidative stress (66), cell migration (67), mitogenesis (68), and apoptosis (68). Patients with atherosclerotic diseases (69) and sepsis (70) were found to have lower levels of plasma lysoPCs. LysoPCs are metabolites transiently generated by phospholipase A2 (PLA2) during the remodeling of glycerophospholipids (71, 72) and can be further hydrolyzed to glycerophosphocholine by lysophospholipase (73). From our pathway analysis, phospholipase A2 (PLA2) was involved in the major network affected (Fig. 2). It was reported that PLA2 modulated the activity of menthol receptor TRPM8 and lysophospholipids altered the sensitivity of TRPM8 (74, 75). Upregulation of lysophospholipase and decreased levels of lysoPCs have been shown upon cigarette smoke exposure in mouse model (76) and among current smokers (55), and combined activities of secreted PLA2s and eosinophil lysophospholipases were also found to cause pulmonary surfactant dysfunction (73). Consistent with our metabolomics profiling, the results from transcriptomics data of smokers in our group also showed that PLA2G15, which codes for lysophospholipase III for the hydrolysis of glycerophospholipids, was among the genes found significantly upregulated on lymphocytes of smokers treated with cigarette smoke condensate (77). Thus, circulating glycerophospholipids could play an important role in the composition and metabolism of lung surfactants, for the determination of lung dysfunction, and can be a potential biomarker for early detection of lung cancer.

There are several strengths to this study. First, the pre–post study design that assessed smokers immediately before and after smoking a cigarette allowed for an untargeted assessment of metabolite boosts. This uniquely allowed for the examination of dynamic changes on the plasma metabolome regulated by the acute effects of cigarette smoking without confounding by other exogenous exposures that would affect the metabolome (e.g., diet, medication, and lifestyle). Using subset analysis for changes in the metabolomics profiles, metabolites affected by menthol were identified among menthol smokers, which may reflect the biological impacts of menthol inhalation in blood. There are some limitations of this study, such as small sample size, limited statistical power for subset analyses, and causal relationships that cannot be established due to the cross-sectional nature of the study. Another limitation is that only one type of UPLC column and separation method was used, and although chosen to obtain the most comprehensive profile for our samples, would not identify highly polar metabolites, such as sugars and amino acids, and some smoke carcinogen metabolites. The identification of unknown metabolites is a major bottleneck in the metabolomics field. It is a complex and costly process, limited by the number of commercially available standards, with intensive effort required, and often results in a low yield of correctly characterized metabolites. Therefore, identification of the unknown metabolites was not carried out in the current article. However, spectral interpretation and structural elucidation of the unknown features are needed in the future to identify and validate the unknown features in the study.

In summary, a metabolomics assessment was applied to a pre–post experimental design in smokers to evaluate the acute effects of cigarette smoking. The identification of the plasma MG boost, which has not been previously reported, allows improvements in evaluating impacts of menthol smoke exposure and smoking behavior. This in turn may provide specificity for the assessment of exposures and harm in menthol smokers, integrating several smoking exposure parameters. The pathways identified herein may yield insights into biological effects of plasma menthol and tobacco-related pathogenesis.

Disclosure of Potential Conflicts of Interest

P.G. Shields has provided expert testimony for Tobacco Company Litigation (expert witness on behalf of the plaintiffs). No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the FDA.

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