# Effects of Menthol on Tobacco Smoke Exposure, Nicotine Dependence, and NNAL Glucuronidation

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## Abstract

**Menthol is a controversial cigarette additive because its physiologic or pharmacologic effects may possibly increase the risk for cancer and its targeted market is the Black community. In a community-based cross-sectional study on 525 Black and White volunteers, we compared levels of urinary and plasma cotinine, plasma thiocyanate, urinary 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanol (NNAL), and its detoxified form (NNAL-Gluc) between menthol and nonmenthol smokers. In regression models that adjusted for daily cigarette intake, no significant differences were observed in the concentration of these biomarkers by menthol status in both races. There was no significant association between high Fagerstrom nicotine dependence scores and the use of menthol cigarettes (odds ratio, 1.1; 95% confidence interval, 0.6-2.0), but an increased risk was observed with smoking a cigarette soon (≤30 minutes) after waking (odds ratio, 2.1; 95% confidence interval, 1.0-3.8). The ratio of NNAL-Gluc to NNAL, a possible indicator of lung cancer risk, was significantly lower in menthol versus nonmenthol smokers. The NNAL-Gluc/NNAL ratio was 34% lower in Whites ($P < 0.01$) and 22% lower in Blacks. In subsequent human liver microsome studies, menthol inhibited the rate of NNAL-*O*-glucuronidation and NNAL-*N*-glucuronidation. Collectively, these results show that menthol does not affect biological exposure to tobacco smoke constituents but indicates that menthol might inhibit the detoxification of the potent lung carcinogen NNAL.** (Cancer Epidemiol Biomarkers Prev 2009;18(1):35–41)

## Introduction

Numerous flavoring agents are added to tobacco during the manufacturing process. Most cigarette additives are approved for use in foods but their possible toxic effects in a complex mixture of chemical constituents in tobacco smoke is difficult to assess (1-4). Menthol in tobacco smoke may theoretically increase cancer risk by cooling the upper airways, causing a depression in respiratory activity and subsequent increase in exposure to tobacco smoke toxins (5), but menthol cigarettes have not been shown to increase lung and esophageal cancer risk versus nonmenthol cigarettes (6-11). Menthol and other additives mask the harshness of tobacco smoke, which has been implicated in the marketing of cigarettes to youth and increased nicotine delivery in young smokers (12). Menthol increases the *in vitro* absorption of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (13) and inhibits the glucuronidation of nicotine, which may lead to a decrease in nicotine dependence (14, 15). Conflicting data have been reported on whether menthol smokers are less likely to quit smoking than smokers of nonmentholated cigarettes (16-21).

The House of Representatives 1108: Family Smoking Prevention and Tobacco Control Act is being considered by the US Congress. The bill would provide the Food and Drug Administration with some authority to regulate tobacco products, including the banning of most flavor additives. Menthol is currently exempted from this bill because it is currently written, although the Centers for Disease Control and others have identified menthol as an important issue in health research (22, 23). Although there have been substantial investigations on the chemical and pharmacologic properties of menthol, the health risks to humans is still debatable because the epidemiologic studies were not designed to detect a small effect size that would be expected in comparing the risk for cancer from one cigarette brand or formulation to another. As an alternative or complimentary approach to estimating the hazards associated with menthol smoking, the use of exposure and intermediate biomarkers can be used to address several important questions on this issue, including (*a*) ''Does menthol increase biological exposure to tobacco smoke constituents and carcinogens?,'' (*b*) ''Is menthol associated with behavioral measures of nicotine dependence?, '' and (*c*) ''Does menthol affect the detoxification of the potent tobacco-specific lung carcinogen 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanol (NNAL)?''

## Materials and Methods

**Human Subjects—Study Recruitment.** The research methods and procedures, including inclusion-exclusion criteria, blood collection procedures, blood processing protocols, and analytic methods for cotinine, NNAL and

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its detoxified form (NNAL-Gluc), and thiocyanate (SCN) have been described in detail elsewhere (24). In brief, a community-based cross-sectional study on cigarette smoke metabolism was conducted to determine whether racial differences in tobacco smoke exposure and metabolism may help explain the higher Surveillance, Epidemiology and End Results rates of lung cancer in Blacks than in Whites. The study was conducted in lower Westchester County, NY, including the cities or towns of Mt. Vernon, Yonkers, and White Plains. Subjects were recruited using fliers, newspaper advertisements, Web-based announcements, word of mouth, and recommendations from community and church leaders. Three community locations were established to meet subjects, including a church, Young Men's Christian Association, and the laboratories of the American Health Foundation in Valhalla. Subjects were healthy volunteers who smoked at least five cigarettes per day and were not taking antidepressants or other medications thought to inhibit liver enzyme function. Subjects were asked to fast and abstain from smoking from 12:00 A.M. the previous night. Urine collection was scheduled at 9:00 A.M. the following day. Trained interviewers administered a structured questionnaire that contained detailed items on cigarette smoking history, including the brand of cigarette, the number of cigarettes smoked per day, the number of minutes after waking up that the first cigarette is smoked, and other questions that comprise the six-item Fagerstrom Test for Nicotine Dependence (FTND; ref. 25). All subjects signed a consent form approved by the American Health Foundation institutional review board and received remuneration. Smoking cessation literature and referrals to smoke cessation programs were also offered to participants. The current analysis is based on 525 smokers with a cigarette brand that could be classified according to FTC menthol status and either a plasma or urinary cotinine determination. Among these subjects, we administered the FTND for 278 subjects. There were 456 subjects with a plasma and urinary cotinine determination. Urinary NNAL levels were determined for 147 subjects. Based on our findings of menthol and total NNAL glucuronidation, we subsequently conducted *in vitro* studies using human liver microsomes to determine the effects of menthol on the rates of NNAL-*N*-glucuronidation and NNAL-*O*-glucuronidation.

***In vitro* Methods.** NNAL was purchased from Toronto Research Labs, and menthol and alamethicin from Sigma-Aldrich. All other chemicals were purchased from Fisher Scientific unless specified otherwise. The three normal human liver tissue specimens used for these studies have been described previously (26). Liver microsomes were prepared through differential centrifugation as previously described (27) and stored (10-20 mg protein/mL) at −80°C. Microsomal protein concentrations were measured using the bicinchoninic acid assay.

Glucuronidation assays were done similarly as previously described (28). Human liver microsomes (25 μg protein) were initially incubated with alamethicin (50 μg/mg protein) for 15 min in an ice bath. A serial dilution of menthol was made in 60% DMSO, and the final concentration of DMSO in reaction was 1.5%. Incubations (20 μL) with 0.5 mmol/L of NNAL were subsequently done at 37°C in 50 mmol/L Tris buffer (pH 7.5), 10 mmol/L $MgCl_2$, 4 mmol/L uridine 5′-diphosphoglucuronic acid, and different concentration menthol for 2 h. Reactions were terminated by the addition of 100 μL of cold acetonitrile. Protein was then removed by centrifugation at 13,000 × *g* for 10 min at 4°C. The NNAL-*O*-gluc and NNAL-*N*-gluc formed by human liver microsomes toward NNAL were determined by liquid chromatography–mass spectrometry/mass spectrometry multiple reaction monitoring (LC-MS/MS MRM) analysis.

Samples (10 μL) were analyzed for NNAL-*O*-gluc, NNAL-*N*-gluc, and NNAL by ultra performance liquid chromatography coupled to a TQD (Waters) tandem mass spectrometer with an electrospray interface. The ultra performance liquid chromatography system (Waters) consisting of a binary gradient pump, an autosampler (4°C), and a column oven (35°C) were used for separation. An Acquity ultra performance liquid chromatography hydrophilic interaction chromatography column (100 × 2.1 mm i.d.) with 1.7-μm particles (Waters) was used for separation. A 0.2-μm prefilter was installed before the column. Eluents were (*a*) 5 mmol/L $NH_4AC$ in 50% acetonitrile (pH 6.7) and (*b*) 5 mmol/L $NH_4AC$ in 90% acetonitrile (pH 6.7). The flow rate was 0.5 mL/min. Sample volumes of 10 μL were injected. Gradient conditions were as follows: 0 to 2 min, 20% *a*; 2 to 3 min, linear gradient to 100% *a*; and 3 to 5 min, 100% *b*.

The mass spectrometer was operated in the multiple reaction monitoring mode for quantitative analyses. Argon was used as the collision gas. Nitrogen was used as desolvation gas and cone gas. During the run, the system was in positive electrospray ionization mode. The source temperature was 140°C. The curtain gas was 20 L/h. The desolvation gas flow was 760 L/h, and desolvation temperature was 450°C. Collision gas flow rate is 0.1 mL/min. The capillary voltage was 0.64 kV. The mass transitions for NNAL-*O*-gluc and NNAL-*N*-gluc was *m/z* 386→210, with cone voltage of 25 V and collision energy of 15 eV. The mass transitions for NNAL was *m/z* 210→93, with cone voltage of 25 V and collision energy of 20 eV. As show in Fig. 1, retention times for NNAL-*O*-gluc, NNAL-*N*-gluc, and NNAL were 1.03, 2.09, and 0.73 min, respectively. The peak areas from each of these peaks were obtained, and ratios of the NNAL-*O*-gluc and NNAL-*N*-gluc to NNAL were calculated.

For the estimation of $IC_{50}$ values, glucuronidation assays were done at a fixed concentration of NNAL (0.5 mmol/L), which is close to the $K_M$ for glucuronidation of NNAL by human liver microsomes (0.85 mmol/L; ref. 28). The concentrations of menthol ranged from 0 to 2.5 mmol/L. The $IC_{50}$ was calculated by Prism Version 4.01 software (GraphPad Software) using log(inhibitor) versus response-variable slope.

**Statistical Analyses.** The statistical analysis was conducted using SAS software (version 9.13). Univariate statistics included means, SDs, and frequency distributions. There were no significant differences in urinary creatinine concentrations between menthol and nonmenthol smokers. Bivariate analysis included correlations, parametric *t* tests, and $\chi^2$ tests. The validity of self-reported smoking amount was previously described

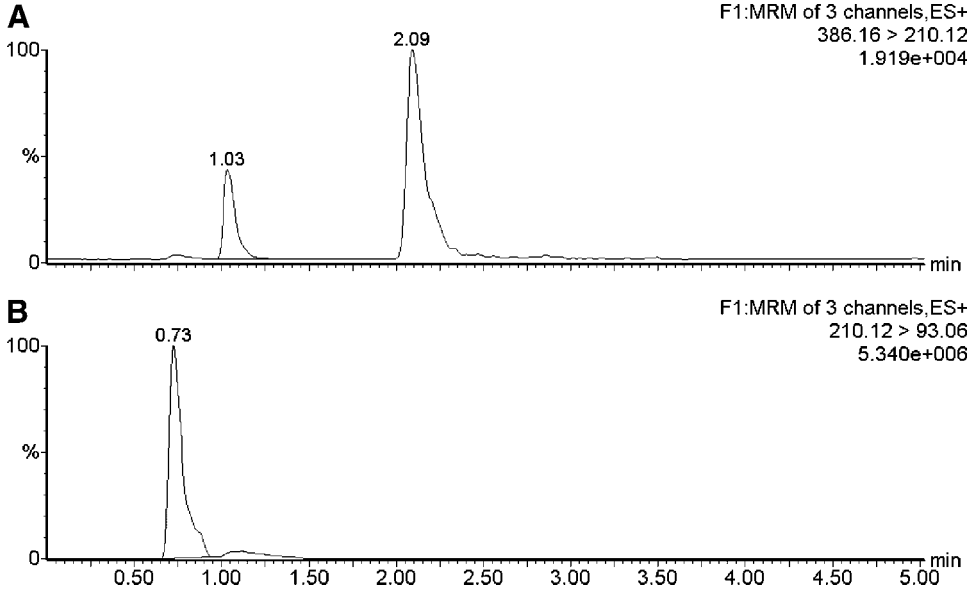


**Figure 1.** Determination of NNAL, NNAL-*O*-glucuronide, and NNAL -*N*-glucuronide in human liver microsome reacted with NNAL using LC-MS/MS MRM. The representative LC-MS/MS MRM ion chromatogram for the transitions *m/z* 386→210 for NNAL-*O*-glucuronide (1.03 min) and NNAL-*N*-glucuronide (2.09 min; **A**) and *m/z* 210 →93 (0.73) for NNAL (**B**).

(*r*, 0.95 in Blacks and 0.83 in Whites; ref. 29). Multiple least-square regression models for biomarkers were fitted where the independent effects of menthol were determined after adjusting for cigarettes smoked per day, age, and sex. Separate models were fit for Blacks and Whites. The relationship between cigarettes smoked per day and biomarker level was nonlinear, and for most models, quadratic and cubic terms were fitted. For each biomarker, we modeled the effects of cigarettes smoked per day using all subjects that had a measurement, regardless if they did not have measurements for other biomarkers. The Fagerstrom Index scores were grouped into three categories for descriptive purposes (0-3 points, low; 4-5 points, medium; and ≥6 points, high) and two categories (0-6 points, low/moderate dependence; 7-10 points, high dependence) in odds ratio calculations. The response categories for a component of the Fagerstrom Index, the time to first cigarette after waking up, are ≤5, 6-30, 31-60, and ≥ 60 min. These were then collapsed into two categories (≤30 versus >30 min) for odds ratio calculations. Smokers with low glucuronidation capacity are considered at increased risk for lung cancer, and we therefore tested the third aim by comparing NNAL-Gluc/NNAL ratios between smokers of mentholated and nonmentholated cigarettes.

## Results

**Subject Characteristics.** Table 1 shows the smoking characteristics of the subjects by race and sex, including mean years smoked, cigarettes per day, percent menthol smokers, FTND scores, and the time between waking up and the first cigarette smoked. There were no significant differences in the years of smoking between Black and White subjects. About 86% of Black subjects smoked menthol cigarettes, whereas 28% of Whites smoked menthol ($P < 0.01$). Whites smoked significantly more menthol and nonmenthol cigarettes per day, respectively, than Blacks ($P < 0.01$). There were no significant differences in FTND categories between Blacks and Whites, whereas more Whites reported smoking their first cigarette ≥30 minutes after waking up ($P = 0.05$).

**Table 1. Smoking characteristics by race and sex**

| | Black men ($n$ = 124) | Black women ($n$ = 113) | White men ($n$ = 134) | White women ($n$ = 154) |
|---|---|---|---|---|
| Years smoked | 18.6 ± 9.8 | 18.0 ± 8.9 | 16.4 ± 10.5 | 16.0 ± 10.3 |
| Menthol (%)* | 111 (90) | 93 (82) | 33 (25) | 47 (31) |
| Cigarettes per day* | 16.0 ± 7.9 | 16.1 ± 9.0 | 22.5 ± 12.2 | 19.0 ± 8.4 |
| Nonmenthol* | 18.8 ± 9.0 | 13.5 ± 6.1 | 23.7 ± 13.3 | 19.7 ± 9.6 |
| Menthol* | 14.9 ± 8.2 | 13.7 ± 7.0 | 21.9 ± 9.0 | 19.8 ± 7.5 |
| FTND | | | | |
| Low | 16 (27) | 19 (38) | 22 (28) | 36 (40) |
| Medium | 21 (36) | 11 (22) | 20 (26) | 29 (32) |
| High | 22 (37) | 20 (40) | 36 (46) | 26 (29) |
| Time since first cigarette (min) † | | | | |
| <5 | 25 (43) | 21 (43) | 31 (40) | 30 (33) |
| 6-30 | 23 (40) | 15 (29) | 23 (29) | 31 (34) |
| 31-60 | 5 (9) | 4 (8) | 16 (21) | 19 (21) |
| >60 | 5 (9) | 10 (20) | 8 (10) | 12 (13) |

*$P < 0.01$ for differences between Black men and women combined versus White men and women combined.
†$P = 0.05$ for differences between Black men and women combined versus White men and women combined.

**Table 2. Differences in mean levels of cotinine, SCN, and total NNAL levels by cigarette type**

| Metabolite | Blacks | | | | *P* | Whites | | | | *P* | All subjects | | | | *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | Menthol | *n* | Nonmenthol | | *n* | Menthol | *n* | Nonmenthol | | *n* | Menthol | *n* | Nonmenthol | |
| Plasma cotinine (ng/mL) | 186 | 389 ± 261 | 31 | 348 ± 261 | 0.09 | 69 | 323 ± 220 | 195 | 328 ± 248 | 0.56 | 255 | 371 ± 252 | 226 | 330 ± 249 | 0.49 |
| Urinary cotinine (μg) | 194 | 4,418 ± 4,229 | 30 | 4,206 ± 3,899 | 0.19 | 76 | 3,807 ± 3,908 | 200 | 4,191 ± 4,629 | 0.51 | 270 | 4,246 ± 4,143 | 230 | 4,193 ± 4,532 | 0.97 |
| SCN (μm/L) | 168 | 162 ± 48 | 93 | 155 ± 49 | 0.33 | 59 | 176 ± 47 | 165 | 169 ± 50 | 0.97 | 227 | 166 ± 48 | 193 | 167 ± 50 | 0.34 |
| NNAL+NNAL-Gluc (pmol/mg creatinine) | 50 | 3.0 ± 2.3 | 11 | 3.2 ± 1.4 | 0.43 | 17 | 2.7 ± 1.3 | 69 | 3.2 ± 2.3 | 0.44 | 67 | 2.9 ± 2.1 | 80 | 3.2 ± 2.2 | 0.23 |

NOTE: *P* values adjusted for age, body mass index, cigarettes per day, race, and sex (all subject models).

**Menthol and Smoking Exposure.** Table 2 compares the mean concentrations of cigarette smoke metabolites between smokers of mentholated cigarettes and nonmentholated cigarettes.

There were no significant differences in the mean concentrations of all cigarette smoke metabolites between menthol and nonmenthol smokers in Blacks and Whites, after adjustment for sex and other factors. In all models, except for SCN in Whites, sex was not a significant predictor of biomarker levels. White women had significantly higher adjusted SCN levels than White men.

**Menthol and Nicotine Dependence.** The association between high FTND scores and menthol was 1.1 [95% confidence interval (95% CI), 0.6-2.1; Table 3]. In multivariate analysis, younger age was the only significant predictor of high nicotine dependence scores. The association between smoking within the first 30 minutes after waking up and menthol was 2.1 (95% CI, 0.96-3.8). Race was not a significant predictor in these models. Menthol was not a significant predictor of daily cigarette amount or heavy smoking (odds ratio, 0.8; 95% CI, 0.5-1.4) after adjusting for race, sex, and other factors. In race-specific models, the inverse association of menthol with heavy smoking was slightly greater in Blacks (odds ratio, 0.702; 95% CI, 0.144-3.426) than in Whites (odds ratio, 0.855; 95% CI, 0.459-1.591).

The FTND and the time to first cigarette after waking were moderately correlated with levels of plasma cotinine ($r = 0.28$, $P < 0.01$; $r = 0.26$, $P < 0.01$), urinary cotinine-creatinine ($r = 0.27$, $P < 0.01$; $r = 0.26$, $P < 0.01$), and total NNAL ($r = 0.21$, $P = 0.08$; $r = 0.15$, $P = 0.23$).

When subjects were asked about their reasons for not quitting, the responses were similar between menthol and nonmenthol smokers. Similar percentages reported that it was a nervous habit, they enjoyed smoking too much, the craving was too great, and fear of weight gain (data not shown). The only difference in reported reason for continued smoking was that more menthol smokers "never thought about quitting" than nonmenthol smokers (17% versus 3.6%).

**Menthol and NNAL Glucuronidation.** Table 4 compares the race-specific differences in the ratio of NNAL-Gluc/NNAL levels between smokers of mentholated and nonmentholated cigarettes. The mean substrate ratio was 34% lower in Whites ($P < 0.01$) and 22% lower in Blacks. The difference in Blacks did not reach statistical significance. For all subjects combined, the difference was statistically significant after controlling for race and other factors.

**Menthol and NNAL Glucuronidation *In vitro*.** The inhibitory effects of menthol on NNAL-*O*-glucuronidation

**Table 3. Association between nicotine dependence and menthol cigarettes**

| Measure | Nonmenthol | Menthol | OR | 95% CI |
|---|---|---|---|---|
| Low or medium FTND | 96 (46) | 96 (45) | 1 | |
| High FTND | 78 (53) | 78 (55) | 1.1 | 0.6-2.1 |
| TTF > 30′ | 26 (34%) | 26 (21%) | 1 | |
| TTF = 30′ | 98 (66%) | 98 (79%) | 2.1 | 0.96-3.8 |

NOTE: Adjusted for age, race, sex and education.
Abbreviation: OR, odds ratio; TTF, time to first cigarette.

**Table 4. NNAL-G/NNAL ratio levels by race and menthol status**

| | Black | | White* | | All subjects* | |
|---|---|---|---|---|---|---|
| | Menthol ($n$ = 50) | Nonmenthol ($n$ = 11) | Menthol ($n$ = 17) | Nonmenthol ($n$ = 69) | Menthol ($n$ = 67) | Nonmenthol ($n$ = 80) |
| NNAL-G/NNAL | 3.1 ± 1.7 | 4.0 ± 2.1 | 2.5 ± 1.1 | 3.8 ± 2.6 | 3.0 ± 1.6 | 3.8 ± 2.5 |

*$P$ < 0.05. Adjusted for age, race (all subjects model), sex, education, and cigarettes per day.

and NNAL-*N*-glucuronidation were assessed in three human liver microsomes. Figure 1 has representative LC-MS/MS MRM chromatograms for determination of NNAL-*O*-glucuronide and NNAL-*N*-glucuronide and NNAL in human liver microsome reacted with NNAL. NNAL-*O*-glucuronide and NNAL-*N*-glucuronide were detected by transition *m/z* 386→210 with retention times of 1.03 and 2.09 minutes, respectively (Fig. 1A). NNAL was detected by transition *m/z* 210 →93 at 0.73 minute (Fig. 1B). Menthol inhibits NNAL-*O*- and NNAL-N-glucuronidation with IC50 values of 0.41 F 0.10 mmol/L and 0.26 F 0.04 mmol/L, respectively. Representative NNAL glucuronidation inhibition profiles in human liver microsome from one subject are presented in Fig. 2. Figure 2A and B shows NNAL-*O*-glucuronide and NNAL-*N*-glucuronide formation in human liver microsome are decreased with increasing concentration of menthol.

## Discussion

The pooled odds ratio from four case-control studies of lung cancer associated with menthol versus nonmenthol cigarettes was 0.93 (95% CI, 0.82-1.05; ref. 4). Menthol was not associated with an increased risk for other cancers (30, 31). Menthol is not pyrolized during tobacco combustion (http://tobaccodocuments.org/product_design/504331475-1477.html) and does not induce tumors in animals via i.p. injection (32) or drinking water supplementation (24). Menthol may not be a direct carcinogen or cocarcinogen, but it causes several pharmacologic and physiologic effects that may affect cancer risk (33). Despite the lack of increased risk in the case-control studies, the effect size and statistical power to detect differences of one type of cigarette relative to another is likely to be small. However, even a small relative effect of menthol could cause substantial numbers of cancers because millions of Americans smoke menthol cigarettes.

Consequently, biomarker and mechanistic studies are seemingly important tools for indirectly assessing possible risks. One of the limitations of studying the effects of menthol has been separating its effects from race. Most Blacks who smoke prefer menthol, and studies on smoking topography or cotinine levels may have controlled for race but did not examine the race-specific effects of menthol (34-36). The current study examined the race-specific levels of several tobacco smoke biomarkers between menthol and nonmenthol smokers. We found no significant differences in mean cotinine and SCN levels.

A limitation of the study is that it included subjects who smoked at least five cigarettes per day. The mean cotinine levels were higher than that reported for National Health and Nutrition Examination Survey data (37), wherein about 25% of smokers reported smoking seven or fewer cigarettes daily. The current study therefore does not address possible differences in biomarker levels by menthol status among very light smokers. We observed no significant differences in the urinary levels of the lung carcinogen NNAL between menthol and nonmenthol smokers in race-specific analyses, although the number of Blacks who smoked nonmenthol cigarettes was relatively small and the differences in NNAL by menthol status was not significant. Assuming the same effect size and variances that were observed, the study would have required about six times the number of nonmenthol Black smokers to detect significant differences. However, if the effect size is similar to that what was observed in Whites, much smaller numbers would be needed.

Menthol has been studied in relation to increased addiction to nicotine. In a 2-week randomized pharmacokinetic cross-over study on 14 smokers who switched from menthol to nonmenthol or nonmenthol to menthol, menthol was on average unrelated to blood levels of unlabelled nicotine (14). The effect of menthol on behavioral measures of nicotine dependence, such as the FTND, was negligible in adolescent smokers (38).

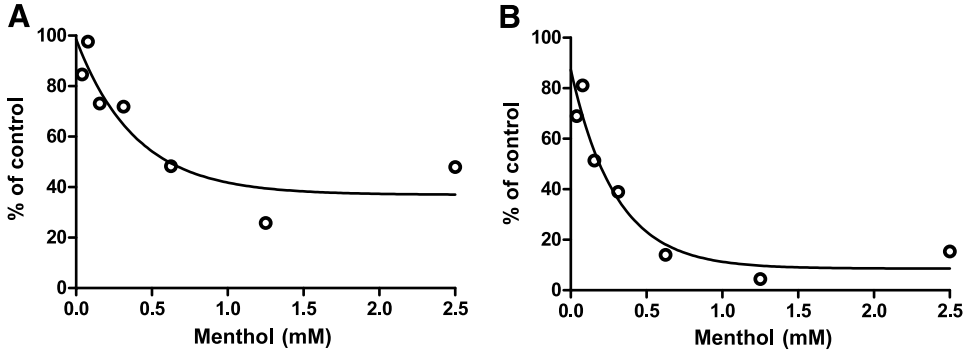


**Figure 2.** Inhibition of NNAL glucuronidation by menthol in human liver microsome NNAL-*O*-glucuronidation (**A**) and NNAL-*N*-glucuronidation (**B**). NNAL (0.5 mmol/L) was incubated with human liver microsomes (1.25 mg/mL) for 2 h with varying concentrations of menthol. Data from one of the three liver microsomes tested.

The current study showed similar findings in adult smokers. It has been suggested that the amount of time between waking up and smoking the first cigarette is possibly a better measure of nicotine dependence than smoking amount or the FTND because it was shown to be a better predictor of plasma cotinine concentrations than daily smoking amount in adults (39). However, in our data, the correlations of cotinine (plasma and urinary) were very similar between cigarettes per day, time since waking, and FTND. We did observe that menthol smokers were more likely to smoke cigarettes within 30 minutes after waking up than nonmenthol smokers, which is similar to findings in the adolescent smoker study but differs from the Community Intervention Trial for Smoking Cessation, which found a longer time to first cigarette after waking for menthol smokers (16). The literature is inconsistent in this area and more studies might be helpful to understand this relationship. Menthol may inhibit quitting (19, 22), although other large scale studies show similar quit rates between menthol and nonmenthol smokers (17).

Another limitation is the interpretation of these analyses is that menthol content varies by cigarette brand, and younger smokers have been reported to smoke menthol brands with lower menthol content than older menthol smokers (12). In the present study, cigarettes were classified as menthol or nonmenthol, and therefore, the magnitude of the effects (or lack thereof) associated with menthol reported here may not necessarily be generalizeable to all smokers.

We previously reported no racial differences in the ratio of NNAL-Gluc/NNAL levels in men, although in a subgroup of nine women who had a very high NNAL-Gluc/NNAL ratio ($\geq$6), eight were White (29). We examined the cigarette brands of these women and found that only one subject was a menthol smoker, so it is possible that the racial differences in NNAL glucuronidation in women could be attributed to menthol.

In the current study, menthol smokers had lower urinary ratios of NNAL-Gluc/NNAL than nonmenthol smokers, and menthol inhibited the glucuronidation of NNAL-*N*-Gluc and NNAL-*O*-Gluc formation *in vitro*. These effects might be mediated by UGT2B10, which is the major enzyme involved in the *N*-glucuronidation of NNAL (40), and by UGT2B17 and UGT2B7, which are the major enzymes involved in NNAL-*O*-glucuronidation (41, 42). Although UGT2B7 genotype was not associated with human liver microsomal glucuronidation activity against menthol (43), *in vitro* studies have shown that UGT2B7 is active against menthol and could be potentially inhibited by this substrate (44, 45). The potential exists that menthol could therefore act as a substrate and inhibitor of other UDP-glucuronosyltransferases as well. Studies by Benowitz et al. (14) have shown that menthol significantly inhibits nicotine-glucuronide formation *in vivo*. Because UGT2B10 is the major enzyme responsible for the *N*-glucuronidation of nicotine and NNAL, it is likely that UGT2B10 may be the target for the inhibitory effects of menthol on nicotine and NNAL glucuronidation activity *in vivo*. Studies examining the interaction between menthol and NNAL or nicotine glucuronidation in cells specifically overexpressing UDP-glucuronosyltransferases 2B7, 2B10, and 2B17 are currently planned. The major limitation of *in vitro* studies that attempt to identify the underlying mechanisms in epidemiologic data is that it is difficult to mimic *in vivo* conditions experimentally. In particular, the human liver microsomes are not equivalent to endoplasmic reticulum in intact cells and do not interact similarly with other cellular components and vascular supply and nutrients. The levels of exposure in the *in vitro* model may not reflect *in vivo* exposure. Despite these limitations, cellular pathways are often activated similarly in *in vitro* and *in vivo* systems, and human liver microsomes are commonly used to screen for drug interactions in pharmacologic research (46).

In summary, these data indicate that menthol is not associated with a higher exposure to tobacco smoke carcinogens, but the findings on nicotine dependence are inconclusive. Menthol may not be more hazardous than other cigarette formulations for most smokers, although it cannot be ruled out at this time that some menthol smokers are possibly at increased risk for lung cancer because of selective inhibition of UDP-glucuronosyltransferase enzymes.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

1. Paschke T, Scherer G, Heller WD. Effects of ingredients on cigarette smoke composition and biological activity: a literature overview. Beitr Tabakforsch Int 2002;20:107–247.
2. Rodgman A. Some studies of the effects of additives on cigarette mainstream smoke properties. I. Flavorants. Beitr Tabakforsch Int 2002;20:83–103.
3. Baker RR, Massey ED, Smith G. An overview of the effects of tobacco ingredients on smoke chemistry and toxicity. Food Chem Toxicol 2004;42 Suppl:S53–83.
4. Werley MS, Coggins CR, Lee PN. Possible effects on smokers of cigarette mentholation: a review of the evidence relating to key research questions. Regul Toxicol Pharmacol 2007;47:189–203.
5. Garten S, Falkner RV. Role of mentholated cigarettes in increased nicotine dependence and greater risk of tobacco-attributable disease. Prev Med 2004;38:793–8.
6. Hebert JR, Kabat GC. Menthol cigarette smoking and oesophageal cancer. Int J Epidemiol 1989;18:37–44.
7. Kabat GC, Hebert JR. Use of mentholated cigarettes and lung cancer risk. Cancer Res 1991;51:6510–3.
8. Sydney S, Tekawa IS, Friedman GD, Sadler MC, Tashkin DP. Mentholated cigarette use and lung cancer. Arch Intern Med 1995;55:727–32.
9. Carpenter CL, Jarvik ME, Morgenstern H, McCarthy WJ, London SJ. Mentholated cigarette smoking and lung-cancer risk. Ann Epidemiol 1999;9:114–20.
10. Brooks DR, Palmer JR, Strom BL, Rosenberg L. Menthol cigarettes and risk of lung cancer. Am J Epidemiol 2003;158:609–16.
11. Stellman SD, Chen Y, Muscat JE, et al. Lung cancer risk in white and black Americans. Ann Epidemiol 2003;13:294–302.
12. Kreslake JM, Wayne GF, Alpert HR, Koh HK, Connolly GN. Tobacco industry control of menthol in cigarettes and targeting of adolescents and young adults. Am J Public Health 2008;98:1685–92.
13. Azzi C, Zhang J, Purdon CH et al. Permeation and reservoir formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P) across porcine esophageal tissue in the presence of ethanol and menthol. Carcinogenesis 2006;27:137–45.
14. Benowitz NL, Herrera B, Jacob P 3rd. Mentholated cigarette smoking inhibits nicotine metabolism. J Pharmacol Exp Ther 2004;310:1208–15.

15. Henningfield JE, Benowitz NL, Ahijevych K, Garrett BE, Connolly GN, Wayne GF. Does menthol enhance the addictiveness of cigarettes? An agenda for research. Nicotine Tob Res 2003;5:9–11.
16. Hyland A, Garten S, Giovino GA, Cummings KM. Mentholated cigarettes and smoking cessation: findings from COMMIT. Community Intervention Trial for Smoking Cessation. Tob Control 2002;11:135–9.
17. Muscat JE, Richie JP, Jr., Stellman SD. Mentholated cigarettes and smoking habits in whites and blacks. Tob Control 2002;11:368–71.
18. Harris KJ, Okuyemi KS, Catley D, Mayo MS, Ge B, Ahluwalia JS. Predictors of smoking cessation among African-Americans enrolled in a randomized controlled trial of bupropion. Prev Med 2004;38:498–502.
19. Pletcher MJ, Hulley BJ, Houston T, Kiefe CI, Benowitz N, Sidney S. Menthol cigarettes, smoking cessation, atherosclerosis, and pulmonary function: the Coronary Artery Risk Development in Young Adults (CARDIA) Study. Arch Intern Med 2006;166:1915–22.
20. Okuyemi KS, Faseru B, Sanderson Cox L, Bronars CA, Ahluwalia JS. Relationship between menthol cigarettes and smoking cessation among African American light smokers. Addiction 2007;102:1979–86.
21. Fu SS, Okuyemi KS, Partin MR, et al. Menthol cigarettes and smoking cessation during an aided quit attempt. Nicotine Tob Res 2008;10:457–62.
22. Giovino GA, Sidney S, Gfroerer JC, et al. Epidemiology of menthol cigarette use. Nicotine Tob Res 2004;6 Suppl 1:S67–81.
23. Clark PI, Gardiner PS, Djordjevic MV, Leischow SJ, Robinson RG. Menthol cigarettes: setting the research agenda. Nicotine Tob Res 2004;6 Suppl 1:S5–9.
24. Richie JP, Jr., Carmella SG, Muscat JE, Scott DG, Akerkar SA, Hecht SS. Differences in the urinary metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in black and white smokers. Cancer Epidemiol Biomarkers Prev 1997;6:783–90.
25. Heatherton TF, Kozlowski LT, Frecker RC, Fagerstrom KO. The Fagerstrom Test for Nicotine Dependence: a revision of the Fagerstrom Tolerance Questionnaire. Br J Addict 1991;86:1119–27.
26. Wiener D, Fang JL, Dossett N, Lazarus P. Correlation between UDP-glucuronosyltransferase genotypes and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone glucuronidation phenotype in human liver microsomes. Cancer Res 2004;64:1190–6.
27. Coughtrie MW, Burchell B, Bend JR. A general assay for UDP glucuronosyltransferase activity using polar amino-cyano stationary phase HPLC and UDP[U-14C]glucuronic acid. Anal Biochem 1986;159:198–205.
28. Chen G, Dellinger RW, Sun D, Spratt TE, Lazarus P. Glucuronidation of tobacco-specific nitrosamines by UGT2B10. Drug Metab Dispos 2008;36:824–30.
29. Muscat JE, Djordjevic MV, Colosimo S, Stellman SD, Richie JP, Jr. Racial differences in exposure and glucuronidation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Cancer 2005;103:1420–6.
30. Friedman GD, Sadler M, Tekawa IS, Sidney S. Mentholated cigarettes and non-lung smoking related cancers in California, USA. J Epidemiol Community Health 1998;52:202.
31. Murray RP, Connett JE, Skeans MA, Tashkin DP. Menthol cigarettes and health risks in Lung Health Study data. Nicotine Tob Res 2007;9:101–7.
32. Stoner GD, Shimkin MB, Kniazeff AJ, Weisburger JH, Weisburger EK, Gori GB. Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice. Cancer Res 1973;33:3069–85.
33. Ahijevych K, Garrett BE. Menthol pharmacology and its potential impact on cigarette smoking behavior. Nicotine Tob Res 2004;6 Suppl 1:S17–28.
34. Clark PI, Gautam S, Gerson LW. Effect of menthol cigarettes on biochemical markers of smoke exposure among black and white smokers. Chest 1996;110:1194–8.
35. Ahijevych K, Gillespie J. Nicotine dependence and smoking topography among black and white women. Res Nurs Health 1997;20:505–14.
36. Jarvik ME, Tashkin DP, Caskey NH, McCarthy WJ, Rosenblatt MR. Mentholated cigarettes decrease puff volume of smoke and increase carbon monoxide absorption. Physiol Behav 1994;56:563–70.
37. Caraballo RS, Giovino GA, Pechacek TF, et al. Racial and ethnic differences in serum cotinine levels of cigarette smokers: Third National Health and Nutrition Examination Survey, 1988-1991. JAMA 1998;280:135–9.
38. Collins CC, Moolchan ET. Shorter time to first cigarette of the day in menthol adolescent cigarette smokers. Addict Behav 2006;31:1460–4.
39. Heatherton TF, Kozlowski LT, Frecker RC, Rickert W, Robinson J. Measuring the heaviness of smoking: using self-reported time to the first cigarette of the day and number of cigarettes smoked per day. Br J Addict 1989;84:791–9.
40. Ren Q, Murphy SE, Zheng Z, Lazarus P. *O*-Glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by human UDP-glucuronosyltransferases 2B7 and 1A9. Drug Metab Dispos 2000;28:1352–60.
41. Lazarus P, Zheng Y, Aaron Runkle E, Muscat JE, Wiener D. Genotype-phenotype correlation between the polymorphic UGT2B17 gene deletion and NNAL glucuronidation activities in human liver microsomes. Pharmacogenet Genomics 2005;15:769–78.
42. Chen G, Blevins-Primeau AS, Dellinger RW, Muscat JE, Lazarus P. Glucuronidation of nicotine and cotinine by UGT2B10: loss of function by the UGT2B10 Codon 67 (Asp>Tyr) polymorphism. Cancer Res 2007;67:9024–9.
43. Bhasker CR, McKinnon W, Stone A, et al. Genetic polymorphism of UDP-glucuronosyltransferase 2B7 (UGT2B7) at amino acid 268: ethnic diversity of alleles and potential clinical significance. Pharmacogenetics 2000;10:679–85.
44. Jin C-J, Miners JO, Lillywhite KJ, Mackenzie PI. Complementary deoxyribonucleic acid cloning and expression of a human liver uridine diphosphate-glucuronosyltransferase glucuronidating carboxylic acid-containing drugs. J Pharmacol Exp Ther 1993;264:475–9.
45. Ritter JK, Sheen YY, Owens IS. Cloning and expression of human liver UDP-glucuronosyltransferase in COS-1 cells. J Biol Chem 1989;265:7900–6.
46. Devlin RB, Frampton ML, Ghio AJ. *In vitro* studies: what is their role in toxicology? Exp Toxicol Pathol 2005;57:183–8.