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Research Article

Known and Novel Sources of Variability in the Nicotine Metabolite Ratio in a Large Sample of Treatment-Seeking Smokers

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Abstract

Background: The ratio of 3′hydroxycotinine to cotinine, or nicotine metabolite ratio (NMR), is strongly associated with *CYP2A6* genotype, CYP2A6-mediated nicotine and cotinine metabolism, and nicotine clearance. Higher NMR (faster nicotine clearance) is associated retrospectively with heavier smoking and lower cessation rates.

Methods: NMR as a predictive biomarker of cessation outcomes is being investigated (NCT01314001). In addition to strong *CYP2A6* genetic influences on NMR, demographic and hormonal factors alter NMR. Here, we analyzed, for the first time together, these sources of variation on NMR in smokers screened for this clinical trial ($N = 1{,}672$).

Results: Participants (mean age = 45.9) were 65.1% Caucasian, 34.9% African American, and 54.8% male. Mean NMR (SD) was higher in Caucasians versus African Americans [0.41 (0.20) vs. 0.33 (0.21); $P < 0.001$], and in females versus males [0.41 (0.22) vs. 0.37 (0.20); $P < 0.001$]. Among females, birth control pill use ($N = 17$) and hormone replacement therapy ($N = 14$) were associated with 19.5% ($P = 0.09$) and 29.3% ($P = 0.06$) higher mean NMR, respectively, albeit nonsignificantly. BMI was negatively associated with NMR (Rho = −0.14; $P < 0.001$), whereas alcohol use (Rho = 0.11; $P < 0.001$) and cigarette consumption (Rho = 0.12; $P < 0.001$) were positively associated with NMR. NMR was 16% lower in mentholated cigarette users ($P < 0.001$). When analyzed together in a linear regression model, these predictors (each ≤2%) accounted for <8% of total NMR variation.

Conclusions: Although these factors significantly affected NMR, they contributed little (together <8%; each ≤2%) to total NMR variation.

Impact: Thus, when using NMR, for example, to prospectively guide smoking cessation therapy, these sources of variation are unlikely to cause NMR misclassification. *Cancer Epidemiol Biomarkers Prev; 23(9); 1773–82. ©2014 AACR.*

Introduction

Tobacco use remains a leading cause of morbidity and mortality worldwide, and life expectancy is shortened by more than 10 years in smokers (1). If current trends in smoking prevalence continue, tobacco use is projected to kill one billion people worldwide during the 21st century (2), underscoring the need for improved smoking prevention and cessation strategies. One approach to improving smoking cessation rates and reducing the global burden of disease from tobacco may involve the personalization of smoking cessation pharmacotherapies using validated biomarkers that predict treatment success (3). A diagnostic and predictive biomarker of smoking cessation outcomes with potential clinical utility is the nicotine metabolite ratio (NMR; ref. 3).

Nicotine is the major psychoactive compound in cigarette smoke responsible for the reinforcing properties associated with cigarette smoking and the development

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of tobacco addiction (4). The majority (~80%) of nicotine is metabolically inactivated to cotinine, in a reaction predominantly catalyzed by CYP2A6 (5). Cotinine undergoes further metabolism to 3′hydroxycotinine, in a reaction exclusively mediated by CYP2A6 (6, 7). The ratio of 3′hydroxycotinine/cotinine, known as the NMR, is a biomarker of *CYP2A6* genotype activity, as well as nicotine metabolism rate, and it correlates strongly with total nicotine clearance (7, 8). The NMR has been shown retrospectively to be associated with smoking cessation success in multiple clinical trials involving heavy and light smokers, of Caucasian and African American descent, respectively (9–12). Individuals with lower NMR, indicative of lower CYP2A6 activity and slower nicotine clearance, displayed higher quit rates on transdermal nicotine (9, 10) and nicotine gum (12), relative to individuals with higher NMR. In contrast, there were no differences in quit rates on bupropion (a non-CYP2A6 substrate) between NMR groups; however, among those receiving counseling and placebo, those with lower NMR had higher quit rates (11).

In addition to cessation, NMR and *CYP2A6* genotype are associated with smoking acquisition, the level of cigarette consumption, as well as nicotine dependence (13–19). Those with slower nicotine metabolism rates, determined via NMR or *CYP2A6* genotype, display lower self-reported cigarettes smoked per day (15–17), lower total nicotine intake (20–23), lower nicotine dependence (18, 19), and lower total puff volumes resulting in lower carcinogen exposure (24). The relationship between lower NMR and reduced cigarette consumption/nicotine dependence scores may be more pronounced in men than in women (19) and in younger cohorts and smokers not seeking treatment (9, 15). *CYP2A6* genotype is also associated with lung cancer risk; those with reduced activity *CYP2A6* genotypes (i.e., slower metabolizers) have a lower risk of developing lung cancer (16, 25–27). The reduced lung cancer risk among slower metabolizers likely stems from both lower levels of smoking and lower metabolic activation of tobacco-specific nitrosamines (23).

One advantage to using NMR rather than *CYP2A6* genotype as a biomarker of nicotine metabolism rate is that it includes both genetic and environmental sources of variation in nicotine metabolism and clearance. Here, we investigate the influence of nongenetic sources (specifically non-*CYP2A6* genetic variation) of variation on NMR. If these sources of variation have a relatively small impact on NMR, and NMR is shown to prospectively predict cessation outcomes, this would further support the utility of NMR as a prospective biomarker to guide treatment assignment. In addition to *CYP2A6* genotype (28), a number of factors contribute to interindividual variability in NMR, including ethnicity (20, 29, 30), sex (31, 32), birth control pill use (31), body mass index (BMI; ref. 33), and potentially mentholated cigarette use (34, 35). NMR is higher among Caucasians relative to African Americans and Asians (8, 20, 29, 30), reflecting the lower frequency of slower-activity *CYP2A6* genetic variants in Caucasians relative to African and Asian populations (15, 36–38). NMR is also higher among premenopausal women relative to men, and even higher among women taking estrogen-containing birth control pills (31, 32). In contrast, there are no differences in NMR between men and menopausal or postmenopausal women (31).

Although several smaller studies have investigated individual influences on NMR, a comprehensive analysis to characterize these relationships simultaneously in one large population has not been performed. Moreover, to date the relationship between NMR and alcohol use has not been investigated, despite the common co-use of smoking and alcohol and the impact of alcohol on smoking cessation success (39, 40). In contrast with the well-characterized *CYP2A6* genotype contribution to variation in NMR, this article describes environmental influences that are less understood. We divided our analysis into three parts. We first examined previously known influences on NMR (i.e., ethnicity, gender, exogenous estrogen-based hormonal therapies, and BMI). We next characterized relationships between NMR, alcohol use, mentholated cigarette use, and the level of cigarette consumption. Our final objective was to quantify the overall influence of these predictors on NMR, to determine if they, alone or together, represent a substantial source of variation in this biomarker.

Materials and Methods

Study subjects and data collection

Treatment-seeking adults (ages 18–65) smoking ≥10 cigarettes per day for the past 6 months responded to advertisements for a smoking cessation clinical trial (NCT01314001). Exclusion criteria included the use of chewing tobacco, snuff or snus; recent treatment for substance abuse; current cocaine or opiate abuse; the consumption of >25 standard alcoholic drinks/week; current depression, mania, schizophrenia, or post-traumatic stress disorder; recent use of antipsychotics, antidepressants, prescription stimulants, metformin, cimetidine, cardiac medications, or other anticoagulants; and the daily use of prescription opiates/inhalers. Those interested in participating after meeting eligibility criteria provided a blood sample for NMR determination, collected when participants were smoking as usual. The detailed study protocol, including NMR determination, is described in a previous analysis of NMR and three self-report measures of nicotine dependence in a subset of the trial participants ($N = 833$ of 1,807 screened by NMR; ref. 19). Briefly, cotinine and 3′hydroxycotinine were assessed from whole blood by liquid chromatography–tandem mass spectrometry (LC/MS-MS) using a previously validated method (41, 42). NMR data were available on a total of 1,807 eligible participants screened at the four clinical sites: the University of Pennsylvania ($N = 487$), the Centre for Addiction and Mental Health (CAMH) at the University of Toronto ($N = 430$), the MD Anderson Cancer Center (Houston, TX; $N = 443$) and the University at Buffalo, SUNY (Buffalo, NY; $N = 447$). Survey data on demographic variables

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(including age, gender, and ethnicity) and smoking history were collected, as well as height and weight measurements to compute BMI. Data were also collected from female participants on the use of oral contraceptives and hormone replacement therapies. Data on mentholated cigarette use were collected from the subset of participants in the intent-to-treat (ITT) group ($N = 1{,}155$), assessed when they received their study medication and completed the first counseling session. Informed consent was obtained from each participant. The study was approved by Institutional Review Boards at each site.

Statistical analysis

All statistical analyses were completed using SPSS Version 22 (IBM Corporation). The Shapiro–Wilk test was used to determine whether continuous variables were normally distributed. Mann–Whitney *U* Tests (two-tailed) and χ^2 tests were used to compare continuous and categorical outcome measures, respectively, between two groups. The strength of correlation between continuous variables was assessed using Spearman rank correlation coefficient.

A univariate analysis of variance model was used to determine whether ethnicity and gender interact to influence NMR (2×2 factorial design). Hierarchical linear regression models were used to determine whether cigarette consumption confounds the association (i) between BMI and NMR, or (ii) between alcohol use and NMR. In these models, breath CO (carbon monoxide; a biomarker of cigarette consumption) was entered in block 1, and the predictor (BMI or alcohol use) was entered in block 2. Separate hierarchical linear regression models were also used to test whether BMI and alcohol use interact with ethnicity and/or gender to influence NMR. The single predictors (BMI and alcohol use) together with ethnicity or gender were entered in block 1, and the interaction term (e.g., BMI × gender) was entered in the second block. We used a univariate analysis of variance model to determine whether mentholated cigarette use and ethnicity interact to influence NMR (2×2 factorial design). A univariate analysis of variance model was also used to determine whether NMR stratum (faster vs. slower metabolism) interacts with either ethnicity and/or gender to influence cigarettes per day (CPD) ($2 \times 2 \times 2$ factorial design).

A linear regression model was also used to assess the variation in NMR accounted for by each of the predictors, and their combined overall contribution to NMR variability. The predictors [ethnicity, gender, birth control pill use, hormone replacement therapy (HRT) use, BMI, alcohol consumption (number of standard drinks/wk), and cigarettes/d) were entered simultaneously into the model. The overall contribution of the predictors to NMR variation was assessed by examining the model R^2 value. The unique and individual contribution of each predictor to overall NMR variation was assessed by squaring the part correlation coefficients and multiplying by 100%. A separate and similar model was also run in the ITT subgroup ($N = 1{,}155$), with and without the use of mentholated cigarettes as a predictor. A Pearson χ^2 test was used to determine whether the prevalence of mentholated cigarette use in African Americans and Caucasians was different.

Results

Participant demographics

Of the 1,807 eligible subjects with NMR, 55.7% were male with a mean age of 45.4 years and mean BMI of 29.4. The majority of the subjects were Caucasian (60.3%) and African American (32.3%), with a small number of subjects reporting Asian (3.4%), American Indian/Alaska native (0.3%), Hawaiian/Polynesian (0.1%), or "more than one" or "other" race (3.7%). We restricted all further analyses herein to Caucasians and African Americans with NMR ($N = 1{,}672$), as the small numbers of subjects from the four additional racial groupings ($N = 135$ total) precluded meaningful statistical analysis. Participant characteristics of the final analytic sample are shown in Table 1.

Factors (ethnicity, gender, exogenous estrogen, and BMI) known to influence NMR

Mean NMR was higher in Caucasians than in African Americans [0.41 (0.20) vs. 0.33 (0.21), $P < 0.001$] and in females compared with males [0.41 (0.22) vs. 0.37 (0.20), $P < 0.001$; Fig. 1A and B]. The interaction term (ethnicity ×

Table 1. Demographic characteristics of Caucasian and African American participants with NMR ($N = 1{,}672$)

Characteristic	Value
% Female, $N = 756$	45.2
Mean age (SD), $N = 1{,}672$	45.9 (11.0)
% Caucasian, $N = 1{,}089$	65.1
% African American, $N = 583$	34.9
Mean BMI (SD), $N = 1{,}672$	29.5 (6.5)
Mean number of drinks/wk (SD), $N = 1{,}672$	3.3 (5.2)
Mean cigarettes/d (SD), $N = 1{,}672$	18.7 (7.5)
Mean breath CO, ppm (SD), $N = 1{,}672$	23.3 (10.2)
NMR in total group, $N = 1{,}672$	
Mean (SD)	0.38 (0.21)
Median	0.35
Range	0.01–1.90
Skewness	1.51
Kurtosis	4.87
NMR in ITT group, $N = 1{,}155$[a]	
Mean (SD)	0.35 (0.20)
Median	0.30
Range	0.01–1.31
Skewness	1.39
Kurtosis	2.63

Abbreviation: ppm, parts per million.

[a]Participants with lower NMR were oversampled in the clinical trial, reflecting the lower NMR values observed in the ITT group versus in the total group.

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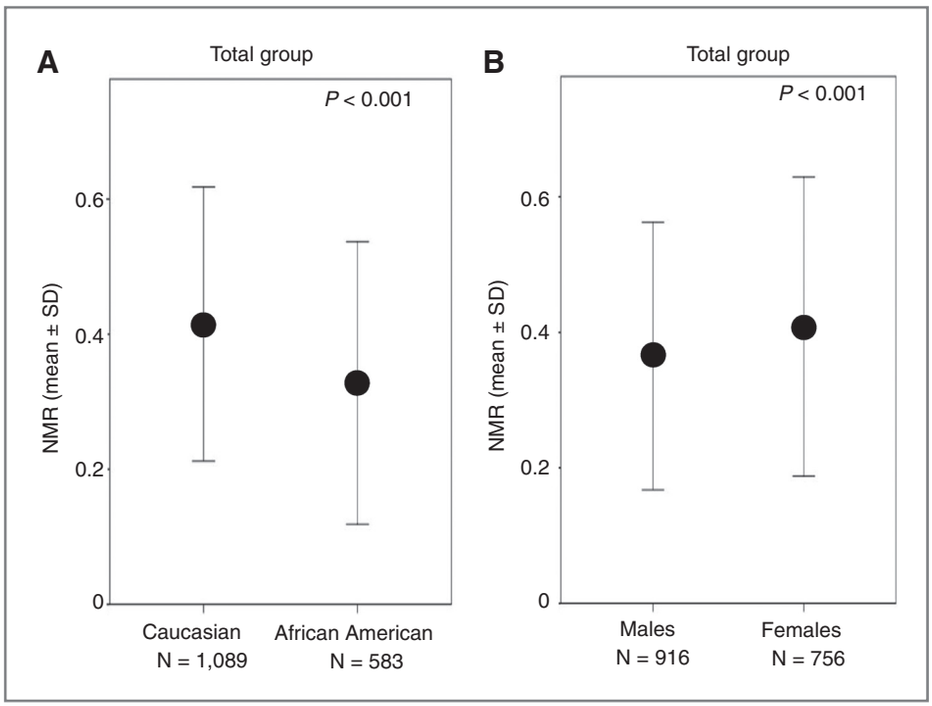

Figure 1. Variation in the NMR according to ethnicity and gender in Caucasian and African American adult smokers. NMR is shown as a function of ethnicity (A) and gender (B) in the total group (Mann–Whitney *U* tests).

gender) was not significant [$F(1, 1{,}668) = 1.06$, $P = 0.30$]. Next, we examined the influence of estrogen-containing birth control pill use and estrogen-containing HRT use on NMR in women. Relative to nonusers ($N = 739$), females that reported current birth control pill use ($N = 17$) had 19.5% higher mean (SD) NMR [0.49 (0.24) vs. 0.41 (0.22), respectively, $P = 0.09$; Fig. 2A]. Mean (SD) NMR was 29.3% higher among HRT users ($N = 14$) relative to nonusers [$N = 742$; 0.53 (0.29) vs. 0.41 (0.22), respectively, $P = 0.06$; Fig. 2B]. We noted similar relationships in Caucasian females (Fig. 2); in the African Americans, there were only 4 females in total using either birth control pills or HRT, precluding our ability to assess this impact.

In the total group, BMI was negatively correlated with NMR (Rho = −0.14, $P < 0.001$; Fig. 3A), which remained significant after controlling for breath CO, a biomarker of cigarette consumption (linear regression model R^2 change after controlling for CO = 0.018, $P < 0.001$). In a separate linear regression model, after controlling for main effects (BMI and ethnicity), the interaction term (BMI × ethnicity) was not significant ($P = 0.91$; model R^2 change = 0.0), suggesting that the relationship between BMI and NMR is similar in Caucasians and African Americans. Likewise, after controlling for main effects, BMI and gender did not interact to influence NMR ($P = 0.68$; model R^2 change = 0.0), suggesting a similar relationship between BMI and NMR in both males and females.

Associations of alcohol use, mentholated cigarette use, and cigarette consumption with NMR

Alcohol use (range = 0–25 standard drinks/wk) was positively associated with NMR in the total group (Rho = 0.11, $P < 0.001$; Fig. 3B), even after controlling for levels of smoking using breath CO (linear regression model R^2 change after controlling for CO = 0.008, $P < 0.001$).

In a linear regression model, after controlling for main effects (alcohol use and ethnicity), the interaction term (alcohol use × ethnicity) was not significant ($P = 0.73$; model R^2 change = 0.0), suggesting a similar relationship in Caucasians and African Americans. Similarly, after controlling for alcohol use and gender, the interaction term (alcohol use × gender) was not significant ($P = 0.65$; model R^2 change = 0.0), suggesting a similar relationship in males and females.

We next investigated the potential influence of mentholated cigarette use on NMR. Menthol inhibits CYP2A6 activity *in vitro* (34) and nicotine clearance *in vivo* (35), and, therefore, may result in lower NMR. In the ITT subgroup ($N = 1{,}155$), in which menthol use data were available, the prevalence of mentholated cigarette use was 22.7% and 85.6% among Caucasian and African American smokers, respectively ($P < 0.001$). Those smoking mentholated cigarettes ($N = 550$) displayed significantly lower mean (SD) NMR compared with those smoking nonmentholated cigarettes [($N = 601$; 0.32 (0.20) vs. 0.37 (0.20), respectively; $P < 0.001$; Fig. 3C]. We used a 2 × 2 factorial design to evaluate whether the association between mentholated cigarette use and NMR was similar across ethnicities. There was no significant main effect of mentholated cigarette use on NMR [$F(1, 1{,}147) = 1.62$, $P = 0.20$], whereas a significant effect of ethnicity [$F(1, 1{,}147) = 10.82$, $P = 0.001$] was observed. The interaction term (mentholated cigarette use × ethnicity) was not significant [$F(1, 1{,}147) = 3.51$, $P = 0.06$].

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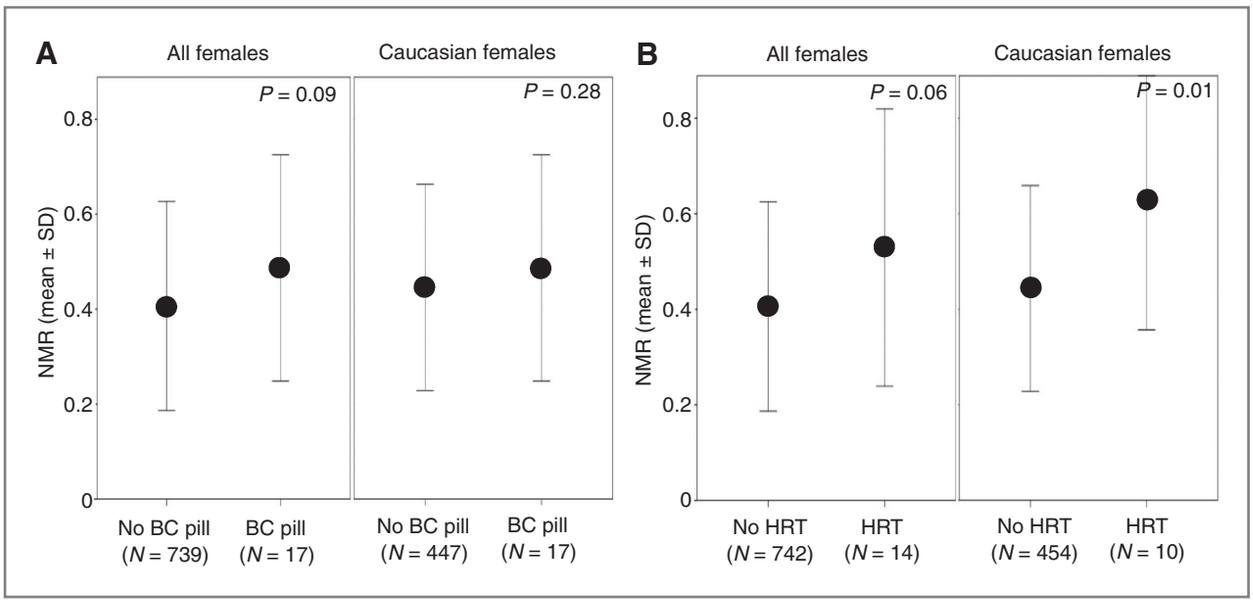

Figure 2. Association between exogenous estrogen-containing therapy and NMR among females. NMR is shown as a function of estrogen-containing birth control (BC) pill use in all females and Caucasian females (A) and as a function of estrogen-containing HRT use in all females and Caucasian females (B; Mann–Whitney *U* tests).

We next evaluated the relationship between NMR and CPD. In contrast with the influence of selected variables on NMR described above, variation in CYP2A6 activity, measured by *CYP2A6* genotype or NMR, has been previously shown to influence cigarette consumption (i.e., faster metabolizers smoke more heavily; refs. 13, 14, 16, 19). Thus, we were interested in investigating a potential effect of NMR (slow vs. fast nicotine metabolism) on CPD. We included NMR [median split; ≤0.350 ($N = 838$) vs. >0.350 ($N = 834$)] as the predictor variable and CPD (continuous measure) as the outcome variable. Mean (SD) CPD was 17.9 (6.8) and 19.5 (8.1) in those with lower versus higher NMR, respectively ($P < 0.001$; Fig. 3D). We ran a 2 × 2 × 2 factorial model to evaluate potential interactions between ethnicity, gender, and NMR on CPD. There were significant main effects of NMR [$F(1, 1{,}664) = 5.91$, $P = 0.02$], ethnicity [$F(1, 1{,}664) = 89.03$, $P < 0.001$], and gender [$F(1, 1{,}664) = 15.51$, $P < 0.001$] on CPD. The only significant interaction term in the model was ethnicity × gender [$F(1, 1{,}664) = 6.16$, $P = 0.01$]. The NMR cutoff point of 0.31 was used in the clinical trial (NCT01314001) to differentiate slower from faster metabolizers, to randomize participants to treatment based on NMR, and to compare treatment outcomes from this clinical trial. Thus, we also used an NMR cutoff point of 0.31 to stratify participants into slower (≤0.31; $N = 679$) and faster (>0.31; $N = 993$) NMR groups and to evaluate potential interactions between ethnicity, gender, and NMR (2 × 2 × 2 factorial model) on CPD. There were significant main effects of NMR [$F(1, 1{,}664) = 5.87$, $P = 0.02$], ethnicity [$F(1, 1{,}664) = 84.93$, $P < 0.001$], and gender [$F(1, 1{,}664) = 11.34$, $P = 0.001$] on CPD. The only significant interaction term in the model was ethnicity × gender [$F(1, 1{,}664) = 6.16$, $P = 0.01$].

To further illustrate these various relationships with NMR, we compared alcohol consumption, BMI, as well as CPD and CO across NMR groups using the cutoff point of 0.31. Consistent with the analyses above, slower metabolizers (lower NMR) displayed lower alcohol and cigarette consumption, but higher BMI, relative to faster metabolizers in the total group (Supplementary Table S1, also shown for the ITT subgroup).

Regression analysis identifying significant predictors of NMR

In the overall model, ethnicity, gender, HRT use, BMI, CPD, and number of alcohol drinks per week were significant predictors of NMR, whereas birth control pill use trended toward significance (Table 2, men were coded as "0" for birth control pill use and HRT). The overall model R^2 value was 0.076, indicating these variables accounted for 7.6% of the variation in NMR; each variable uniquely contributed ≤2% of the variation in NMR. We then ran the same model in females only (see footnote to Table 2). The overall R^2 value for the model, which included all predictors except gender, was 0.066. The impact of birth control pill use and HRT on NMR was of a similar magnitude in the female-only group, as for the total group.

We also ran a separate model examining the impact of these predictors on NMR among those in the ITT subgroup ($N = 1{,}155$; Table 3); a similar percentage of contribution (6.5%) to NMR variability was observed. Mentholated cigarette use (available in this subgroup) did not significantly contribute to NMR variation, and its inclusion in the model did not substantially alter the regression coefficients of the other variables (Table 3, footnote contains data on women only). Notably, HRT use was associated with a

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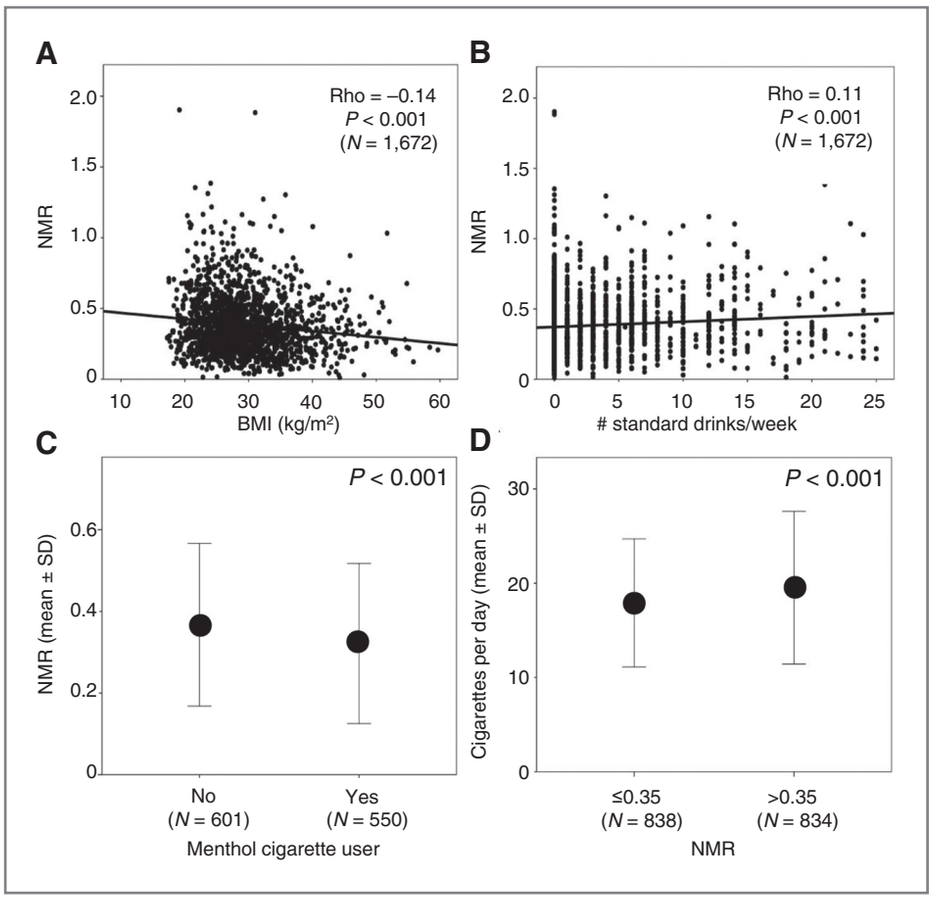

Figure 3. Associations for NMR with BMI, alcohol consumption, cigarette use, and menthol. Correlations between BMI and NMR (A) and alcohol use (# standard drinks/week) and NMR (B) are shown in the total group. The association between mentholated cigarette use and NMR in the ITT group (in which it was available) is shown in (C), whereas the association between NMR, as a median split, and CPD is depicted in the total group in (D; Mann–Whitney *U* tests).

relatively large unstandardized (B) coefficient in both the total (B = 0.11) and ITT (B = 0.16) groups, suggesting a potential impact on NMR in those receiving HRT.

Discussion

The NMR is currently being investigated prospectively as a predictive biomarker of response to smoking cessation treatments in an ongoing NMR-stratified clinical trial (NCT01314001). If NMR displays favorable efficacy and economic feasibility in predicting treatment response (3), the NMR could be used to tailor smoking cessation pharmacotherapy in treatment-seeking smokers. Although *CYP2A6* genotype, and potentially other genetic factors, cause substantial interindividual variability in NMR (28), within-person variability is relatively minor as the NMR is stable and reproducible over time in cigarette smokers (33, 42).

We first examined known influences (i.e., ethnicity, gender, exogenous estrogen-based hormonal therapies, and BMI) on NMR. The higher NMR observed among Caucasians relative to African Americans is likely largely due to the lower frequency of reduced *CYP2A6* activity variants among populations of Caucasian descent (36–38). Among individuals without *CYP2A6* variants (i.e., *CYP2A6*1/*1* wild-type individuals), there is no difference in NMR between Caucasians and African Americans (8), suggesting that the variability in the NMR observed between Caucasians and African Americans in this study is likely attributable to variation in *CYP2A6*. Interethnic variability in NMR may also arise when there are large differences between ethnicities in exposure to nongenetic factors that affect CYP2A6 expression and/or activity. For instance, the prevalence of mentholated cigarette use was much higher among African American relative to Caucasian smokers in our study, consistent with previous findings (43). Menthol has been shown to inhibit CYP2A6 activity *in vitro* (34) and nicotine clearance *in vivo* (35), and may be associated with lower average NMR in African Americans compared with populations with lower prevalence of menthol cigarette use. This effect on NMR, although not a significant predictor of variation in overall NMR (Table 3), may represent a source of variability in NMR under certain circumstances.

We observed higher NMR among women relative to men, and even higher NMR in women taking estrogen-containing birth control pills or HRT. The higher NMR is likely attributable to enhancement of *CYP2A6* transcriptional activity through estrogen binding of the estrogen response element located within the *CYP2A6* gene (44). We observed no interaction between gender and ethnicity on NMR, suggesting that the influence of estrogen on NMR is similar between ethnicities.

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Table 2. Linear regression analysis of the predictors of the NMR in the total sample ($N = 1,672$)

	NMR $R^2 = 0.076$[a]; $P < 0.001$				
Predictor	***B***	**Standard error**	β	***P***	**% of Variation[b]**
Ethnicity[c]	0.071	0.011	0.162	<0.001	2.3
Gender[d,e]	0.057	0.010	0.136	<0.001	1.7
Birth control pill use	0.036	0.050	0.017	0.47	0.03
HRT use	0.114	0.054	0.050	0.036	0.24
BMI	−0.003	0.001	−0.108	<0.001	1.1
Alcohol use (# drinks/wk)	0.003	0.001	0.070	0.004	0.46
Cigarettes/d	0.002	0.001	0.062	0.012	0.36

[a]Together the predictors account for 7.6% of the variation in NMR.
[b]Calculated by squaring the part correlation coefficient (not shown), and multiplying by 100.
[c]African Americans and Caucasians were coded as "0" and "1," respectively, in the model.
[d]Males and females were coded as "0" and "1," respectively, in the model.
[e]When we restricted the model to females only ($N = 756$), to further examine the effect of birth control pill and HRT use, the predictors (gender is excluded) together explained 6.6% of the variation in NMR. The standardized beta values for birth control pill and HRT use were 0.025 ($P = 0.48$) and 0.069 ($P = 0.052$), respectively, in the female-only group. They uniquely contributed 0.06% and 0.48% of the variation in NMR, respectively, in females.

BMI was negatively associated with NMR, consistent with previous reports (8, 12, 28, 33). Negative associations were also observed for plasma cotinine (Rho = −0.10; $P < 0.001$) and 3′hydroxycotinine (Rho = −0.18; $P < 0.001$) with BMI, in line with prior findings (45, 46). We postulate three potential explanations. First, when we controlled for the level of smoking (breath CO), as smoking is associated with lower body weight (47, 48), the negative association between BMI and NMR remained significant, suggesting that the lower BMI observed among those with higher NMR may not result from heavier smoking in those with faster nicotine metabolism. In African Americans (49), we observe no association between the *CYP2A6* genotype group and BMI, despite observing significant correlations

Table 3. Linear regression analysis of the predictors of the NMR in the ITT group ($N = 1,155$)

	NMR $R^2 = 0.065$[a]; $P < 0.001$				
Predictor	***B***	**Standard error**	β	***P***	**% of Variation[b]**
Ethnicity[c]	0.048 (0.053)	0.015 (0.012)	0.118 (0.130)	0.002 (<0.001)	0.81 (1.5)
Gender[d,e]	0.058 (0.058)	0.012 (0.012)	0.144 (0.144)	<0.001 (<0.001)	1.9 (1.9)
Birth control pill use	0.031 (0.030)	0.057 (0.057)	0.016 (0.015)	0.58 (0.60)	0.03 (0.02)
HRT use	0.158 (0.158)	0.062 (0.062)	0.074 (0.073)	0.010 (0.011)	0.53 (0.53)
BMI	−0.003 (−0.003)	0.001 (0.001)	−0.087 (−0.088)	0.004 (0.003)	0.69 (0.71)
Alcohol use (# drinks/wk)	0.002 (0.002)	0.001 (0.001)	0.056 (0.055)	0.062 (0.062)	0.29 (0.28)
Cigarettes	0.002 (0.002)	0.001 (0.001)	0.077 (0.077)	0.010 (0.011)	0.55 (0.53)
Menthol	0.007	0.015	0.018	0.627	0.02

NOTE: Numbers in parentheses indicate values when the mentholated cigarette use variable is removed from the model to facilitate comparison with the total group in Table 2 in which this variable is missing.
[a]Together the predictors account for 6.5% of the variation in NMR with and without mentholated cigarette use in the model.
[b]Calculated by squaring the part correlation coefficient (not shown), and multiplying by 100.
[c]African Americans and Caucasians were coded as "0" and "1," respectively, in the model.
[d]Males and females were coded as "0" and "1," respectively, in the model.
[e]When we restricted the model to females only ($N = 516$), to further examine the effect of birth control pill and HRT use, the predictors (gender and menthol are excluded) together explained 5.3% of the variation in NMR. The standardized beta values for birth control pill and HRT use were 0.021 ($P = 0.63$) and 0.10 ($P = 0.021$), respectively, in the female-only group. They uniquely contributed 0.04% and 1% of the variation in NMR, respectively, in females.

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between NMR and BMI in both the total population and in reduced *CYP2A6* metabolizers (A.Z.X. Zhu; unpublished data), further suggesting that this relationship is due to an effect of higher BMI (or obesity) on NMR rather than an effect of CYP2A6 activity or NMR on risk for obesity. Second, higher BMI may differentially affect the distribution pharmacokinetics of cotinine and 3′hydroxycotinine, resulting in an overall net reduction in NMR. However, it seems unlikely that this occurs through unique effects on the volume of distribution of cotinine versus 3′hydroxycotinine, as the pKa values of these compounds are similar (∼4.4 vs. ∼4.3, respectively; ref. 50). Likewise, it seems unlikely that higher BMI would differentially affect the urinary excretion of these compounds, because negative associations for BMI with urinary cotinine and 3′hydroxycotinine are similar to those for plasma cotinine and 3′hydroxycotinine (A.Z.X. Zhu; unpublished data from another study; ref. 23). Together these findings suggest that the relationship between higher BMI and lower NMR is not mediated by differential effects of obesity on the volume of distribution or excretion of cotinine and 3′hydroxycotinine. Third, higher BMI and adiposity may uniquely affect the enzymes involved in the metabolism of cotinine and 3′hydroxycotinine, for example, UDP-glucuronosyltransferases; however, this remains to be explicitly tested in a pharmacokinetic study.

NMR was positively associated with cigarette consumption. This is likely an effect of NMR on the level of smoking, in which faster metabolism is associated with heavier smoking and greater total nicotine intake (20, 23). Consistent with this, the inhibition of CYP2A6 activity using oral methoxsalen treatment lead to reductions in both smoking and CYP2A6-mediated metabolic activation of the procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (51). We also observed a positive relationship between alcohol consumption and NMR, which could suggest that CYP2A6 activity increases with alcohol use, or alternatively may represent an indirect effect of NMR on smoking, as nicotine is commonly used with alcohol (39, 52). When we controlled for smoking level, the association between higher alcohol use and higher NMR remained significant ($P < 0.001$), suggesting that the higher NMR in those consuming larger amounts of alcohol is not due exclusively to higher smoking among this group. In rodents, liver damage (53, 54) and 3-week ethanol treatment (55) induced CYP2A5, which is the murine ortholog of CYP2A6. In contrast, there was no impact on hepatic CYP2A6 levels or activity following 5 weeks of alcohol self-administration (∼24 mmol/L blood ethanol levels; equivalent to ∼4 standard drinks/d) in African green monkeys (56). The reason for the higher NMR in those with higher alcohol consumption remains to be determined. However, given that the number of drinks per week uniquely explained <1% of the total variation in NMR, typical variation in alcohol use is not likely to substantially alter the utility of NMR, in particular as a prospective biomarker to guide treatment.

In both Caucasians and African Americans, lower NMR is associated retrospectively with greater smoking cessation (9–12). This study used NMRs collected during participant screening for the first prospectively stratified study of NMR as a predictive biomarker of cessation. This trial is currently underway (NCT01314001). We studied multiple sources of variation in NMR, which together accounted for <8% of NMR variation; the greatest unique contribution made by any one factor to NMR variation was <2%, suggesting these factors contribute little to overall variation in NMR on a population level. When NMR is used prospectively to guide therapy, relatively permanent or long-term sources of variation (e.g., ethnicity, gender, and BMI) are unlikely to cause treatment misclassifications, whereas potentially more transient influences on NMR (e.g., HRT and birth control pill use) may need to be considered if they are likely to change during the course of treatment. Together, we extend our understanding of the type, and degree, of influence of demographic factors on NMR. Each factor examined, alone and together, contributed little variation to NMR, supporting the NMR as a stable, reliable, and independent biomarker with potential clinical utility to guide smoking cessation pharmacotherapy.

Disclosure of Potential Conflicts of Interest

Dr. George has consulted for Novartis. Dr. Tyndale has consulted for Apotex and McNeil. Dr. Schnoll has consulted for GlaxoSmithKline. Dr. Lerman has consulted for GlaxoSmithKline, Pfizer, AstraZeneca, and Gilead. Dr. Hawk has consulted on investigator-initiated smoking cessation studies funded by the state of Florida. Drs. Lerman and Schnoll have received medication and placebo from Pfizer. Drs. Lerman, George, and Cinciripini have received research funding from Pfizer. The remaining authors declare no conflicts of interest.

Authors' Contributions

Conception and design: T.P. George, P.M. Cinciripini, R.F. Tyndale
Development of methodology: L.W. Hawk Jr, R.F. Tyndale
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Novalen, L.W. Hawk Jr, R.A. Schnoll, T.P. George, P.M. Cinciripini, R.F. Tyndale
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.J. Chenoweth, L.W. Hawk Jr, T.P. George, P.M. Cinciripini, R.F. Tyndale
Writing, review, and/or revision of the manuscript: M.J. Chenoweth, M. Novalen, L.W. Hawk Jr, R.A. Schnoll, T.P. George, P.M. Cinciripini, C. Lerman, R.F. Tyndale
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Novalen
Study supervision: L.W. Hawk Jr, R.F. Tyndale
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