Relationships between the Nicotine Metabolite Ratio and a Panel of Exposure and Effect Biomarkers: Findings from Two Studies of U.S. Commercial Cigarette Smokers

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ABSTRACT

Background: We examined the nicotine metabolite ratio's (NMR) relationship with smoking intensity, nicotine dependence, and a broad array of biomarkers of exposure and biological effect in commercial cigarette smokers.

Methods: Secondary analysis was conducted on two cross-sectional samples of adult, daily smokers from Wave 1 (2013–2014) of the Population Assessment of Tobacco Use and Health (PATH) Study and baseline data from a 2014–2017 randomized clinical trial. Data were restricted to participants of non-Hispanic, white race. The lowest quartile of NMR (<0.26) in the nationally representative PATH Study was used to distinguish slow from normal/fast nicotine metabolizers. NMR was modeled continuously in secondary analysis.

Results: Compared with slow metabolizers, normal/fast metabolizers had greater cigarettes per day and higher levels of total nicotine equivalents, tobacco-specific nitrosamines, volatile organic componds, and polycyclic aromatic hydrocarbons. A novel finding was higher levels of inflammatory biomarkers among normal/fast metabolizers versus slow metabolizers. With NMR modeled as a continuous measure, the associations between NMR and biomarkers of inflammation were not significant.

Conclusions: The results are suggestive that normal/fast nicotine metabolizers may be at increased risk for tobacco-related disease due to being heavier smokers, having higher exposure to numerous toxicants and carcinogens, and having higher levels of inflammation when compared with slow metabolizers.

Impact: This is the first documentation that NMR is not only associated with smoking exposure but also biomarkers of biological effects that are integral in the development of tobacco-related disease. Results provide support for NMR as a biomarker for understanding a smoker's exposure and potential risk for tobacco-related disease.

Introduction

Nicotine, the main addictive substance in cigarettes (1), is metabolized primarily to cotinine (COT) by the cytochrome P450 2A6 (CYP2A6) enzyme (2, 3). CYP2A6 also metabolizes COT, the predominant metabolite of nicotine, to 3′-hydroxycotinine (3-HCOT; ref. 2). For this reason, the nicotine metabolite ratio (NMR) of 3-HCOT/COT is a phenotypic biomarker for CYP2A6 enzyme activity where lower ratios are reflective of low/null activity CYP2A6 variants and are indicative of a slower nicotine metabolism rate (4–6). Considering that an estimated 20% of the variability in NMR is due to environmental and hormonal factors, NMR provides a more complete picture of nicotine clearance than CYP2A6 genotype (2, 7).

Previous studies have shown that the NMR is associated with heaviness of smoking and exposure to some tobacco biomarkers (5, 8, 9). A systematic review found that normal/fast nicotine metabolizers smoke more cigarettes per day (CPD) than slow metabolizers (8). In line with these data are a topography study that observed fast metabolizers (quartile 4 of NMR) smoke each cigarette more intensely (i.e., larger puff volume) than slower metabolizers (quartile 1 of NMR; ref. 9). In a large study of smokers representing multiple races with varying levels of lung cancer (i.e., whites, African Americans, Japanese Americans), the association of NMR (modeled continuously) and CYP2A6 variants with heaviness of smoking, measured by the sum of urinary nicotine and its metabolites (i.e., total nicotine equivalents; TNE), was examined (5). The authors found a strong association between NMR and CYP2A6 variants with TNE (5). Their results provide evidence for decreased nicotine uptake and consequently decreased toxicant exposure from tobacco and tobacco smoke as the mechanism by which low or null activity CYP2A6 variants and a reduced NMR lead to the lower cancer risk in Japanese American smokers (5). Complementing these results are studies demonstrating metabolites of the lung carcinogen NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL), were lower among smokers with low/null activity CYP2A6 variants and considered slow metabolizers based on NMR (5, 9, 10). The evidence from these studies suggest that the higher levels of

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exposure to nicotine and the harmful constituents found in tobacco and tobacco smoke may result from normal/fast metabolizers smoking a greater number of CPD and/or smoking each cigarette more intensely than slow metabolizers.

Further study of the relationships between NMR and exposure levels to harmful consituents found in tobacco smoke, such as volatile organic componds (VOC) and polycyclic aromatic hydrocarbons (PAH), and biomarkers of biological effect such as inflammation and oxidative stress would provide a more comprehensive picture of a smoker's risk for tobacco-related disease based on NMR than have prior studies. The purpose of this study was to examine NMR's relation with nicotine dependence, smoking intensity, and an array of tobacco biomarkers of exposure and biological effect. The datasets analyzed, the U.S. Population Assessment of Tobacco Use and Health (PATH) study and baseline data from a randomized clinical trial (RCT) of daily cigarette smokers, were both selected for analysis because their combined use resulted in a greater array of biomarkers for study. The central hypothesis was that NMR would be associated with greater smoking intensity and nicotine dependence, and higher levels of biomarkers of tobacco-specific nitrosamines (TSNA), VOCs, PAHs, inflammation, and oxidative stress. If confirmed, findings will provide strong evidence for the utility of NMR in understanding exposure and potential health risks from smoking.

Methods

Data sources

The Population Assessment of Tobacco Use and Health (PATH) study is a nationally representative, longitudinal cohort study of adult and youth in the United States, aged 12 years and older (11). Wave 1 was conducted from September 2013 to December 2014 (11). Field interviewers visited the respondent's home to conduct the interview using audio computer-assisted self-interviews to collect self-report information such as tobacco use behavior (11). From adults who consented to provide them, urine and blood samples were collected. A urine sample was collected immediately following the completion of the interview, while blood was collected at a separate visit by a phlebotomist who visited the respondent's home after the interview at a time scheduled by the field interviewer (11). Address-based, area-probability sampling was used for recruitment (11). Data is weighted so that estimates are representative of the civilian, noninstitutionalized U.S. population. Additional information on the design and methods of the PATH study are available elsewhere (11).

For the current analysis, Wave 1 PATH adult data ($n = 32,320$) was restricted to daily established cigarette smokers ($n = 9,021$; i.e., defined as persons who have ever smoked a cigarette, have smoked more than 100 cigarettes in their lifetime, and currently smoke every day) who were included in the urine data collection ($n = 4,862$) and at the time of the urine sample reported having smoked sometime that day ($n = 4,575$). Data was further restricted to participants who were included in the blood data ($n = 3,012$). Next, data was restricted to those with a urinary COT level of >1,000 ng/mL to confirm regular smoking status ($n = 2,734$) and to facilitate comparison of the PATH study data to baseline data from the RCT (described below). Data were further restricted to participants reporting non-Hispanic white race/ethnicity ($n = 2,153$) to avoid racial differences in nicotine metabolism pathways that may affect the relationship between NMR and nicotine clearance (2, 12). Participants who were also established users of other tobacco (i.e., persons who self-reported using fairly regularly and

using every day or some day use) were excluded ($n = 1,436$) so that biomarker levels were not confounded. Experimental users of other tobacco products (i.e., persons who self-reported having never used fairly regularly) were included. Finally, participants considered outliers for cigarettes per day, reporting more than 80 CPD, or missing data on serum NMR were excluded. This resulted in a final unweighted analysis set of 1,422 participants.

Secondary data analysis was also conducted on baseline data from a RCT examining the effects of an immediate versus step down approach to reducing nicotine content levels in cigarettes. The RCT was conducted from 2014 to 2017 at 10 U.S. sites (clinicaltrials.gov Identifier: NCT02139930). To be eligible, individuals at the time of screening were of legal age to purchase tobacco; breath alcohol level <0.02%; smoked ≥5 cigarettes per day (CPD); had an expired carbon monoxide level of >8 ppm or if ≤8 ppm than a urinary COT level of >1,000 ng/mL. Exclusion criteria were breastfeeding, pregnant, or planning to become pregnant; intentions to quit smoking in the next 30 days; used roll-your own cigarettes exclusively or tobacco products other than cigarettes for >9 days of the past 30 days; had previously used reduced nicotine content study cigarettes; unstable or serious psychiatric or medical conditions; and positive urine toxicology for illicit drug use (excluding THC). Refer to Hatsukami and colleagues 2018 for additional details on the study design and participants (13). For this analysis, baseline data ($n = 1,250$) were restricted to smokers reporting non-Hispanic white race/ethnicity ($n = 758$) who provided a blood and urine sample at baseline ($n = 739$).

NMR

NMR was measured as the molar ratio of free (unconjugated) 3-hydroxycotinine to free cotinine (3HCOT/COT) in serum in both the PATH study and the RCT (13, 14).

Smoking intensity

CPD was assessed in the PATH and the RCT by the following question: On average, about how many cigarettes do you now smoke each day? In the RCT only, all participants provided a measurement of exhaled breath carbon monoxide (CO). In both the PATH study and the RCT, urinary total nicotine equivalents (TNE) overall and divided by CPD (TNE/CPD) were examined to estimate nicotine dose and nicotine dose per cigarette smoked, respectively. See below for further details on TNE.

Nicotine dependence

Heaviness of Smoking Index (HSI; ref. 15) and versions of the Brief Wisconsin Inventory of Smoking Dependence Motives (WISDM; ref. 16) were available in both the PATH and the RCT datasets and examined in this analysis. The HSI uses a 6-point scale calculated from the average number of CPD ($1–10, 11–20, 21–30, 30+$) and time to first cigarette upon waking (<5, 5–30, 31–60, >60 minutes; ref. 15). In PATH, 8 items of the Brief WISDM primary dependence motives subscale (PDM8), comprising items from each of the four domains encompassed by the PDM (i.e., automaticity, craving, loss of control, and tolerance) were available and were examined. Participants were asked to rate their level of agreement for each item using a Likert scale from 1 (not true of me at all) to 5 (extremely true of me; ref. 17). For each participant, PDM8 was calculated as the mean of the 8 items. In the RCT, all 16 items of the WISDM PDM (PDM16) were available and were examined. Participants were asked to rate their level of agreement for each item using a Likert scale from 1 (not true of me at all) to 7 (extremely true of me). For each participant, PDM16 was calculated as the mean of the 16 items.

Biomarkers of exposure and biological effect

Table 1 provides a summary of the biomarkers measured and their data source. In PATH, assays of TNE (molar sum of total nicotine, total COT, total 3-HCOT, total cotinine N-oxide, total nicotine N-oxide, total norcotinine, and total nornicotine; where "total" refers to the sum of unconjugated and glucuronide-conjugated compounds) and the following biomarkers of toxicant exposure have been previously described: (14) total NNAL, total NNN, 3-HPMA, 2-HPMA, SPMA, HMPMA, CEMA, AAMA, MHB3, PGA, HEMA, MADA, 1-HOP,2-FLU, and 1-NAP. In PATH, the following biomarkers of biological effect have also been described previously: (14) 8-iso-PGF$_{2\alpha}$, ICAM-1, IL6, and hsCRP. Assays of urinary biomarkers of exposure in the RCT were carried out in the Analytical Biochemistry Shared Resource of the Masonic Cancer Center, University of Minnesota as previously described for TNE (molar sum of total nicotine, total COT, and total 3-HCOT; ref. 18), total NNAL (19), 3-HPMA (20), 2-HPMA (21), SPMA (22), HMPMA (20), CEMA (23), and PheT (19). The Masonic Cancer Center also carried out assays of the following biomarkers of biological effect in the RCT: 8-iso-PGF$_{2\alpha}$ (24) and PGEM (25). Routine assays of the blood biomarkers of biological effect, hsCRP and WBC, in the RCT were conducted by the Advanced Research and Diagnostic Laboratory at the University of Minnesota and Health East Medical Laboratories.

Statistical analysis

Prior studies have observed the distribution of NMR to be nonlinear (26–31). Furthermore, these studies have observed NMR's validity for predicting nicotine exposure and smoking cessation to be greatest when the lowest quartile of NMR is compared with the highest three quartiles (26–31). On the basis of these studies, the lowest quartile of NMR in the U.S. representative PATH study was used to distinguish slow from normal/fast nicotine metabolizers in this study. The same cutoff was applied to baseline data from the RCT to distinguish slow from normal/fast metabolizers. In a secondary analysis, NMR (log-transformed) was modeled as a continuous variable in both datasets.

Self-report measures including demographics, nicotine dependence, and smoking history were summarized across NMR group (slow vs. normal/fast metabolizers) by means or proportions and 95% confidence intervals (CI). Urinary biomarker values were normalized for creatinine (biomarker/creatinine) to account for differences in urinary output across participants. All biomarkers had skewed distributions and were transformed using the natural logarithm to approximate normality and summarized using geometric means (GM) and 95% CI.

Linear regression modeling was used to assess statistically significant differences, at $P < 0.05$, between slow versus normal/fast metabolizers in terms of smoking intensity, nicotine dependence, and biomarkers of exposure and biological effect. Models were run with

Table 1. Biomarkers of tobacco exposure and biological effect.

Constituent	Biomarkers of exposure	Sample	Data source(s)
Nicotine	Total nicotine equivalents (TNE)[a]	Urine	PATH and RCT
Tobacco specific nitrosamines			
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone[b]	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL)	Urine	PATH and RCT
N'-Nitrosonornicotine[b]	N'-nitrosonornicotine and its glucuronides (total NNN)	Urine	PATH
Volatile organic compounds			
Acrolein[b]	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA)	Urine	PATH and RCT
Propylene oxide[b]	N-Acetyl-S-(2-hydroxypropyl)-L-cysteine (2-HPMA)	Urine	PATH and RCT
Benzene[b]	N-Acetyl-S-(phenyl)-L-cysteine (SPMA)	Urine	PATH and RCT
Crotonaldehyde[b]	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HMPMA)	Urine	PATH and RCT
Acrylonitrile[b]	N-Acetyl-S-(2-cyanoethyl)-L-cysteine (CEMA)	Urine	PATH and RCT
Acrylamide[b]	N-Acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA)	Urine	PATH
1,3-Butadiene[b]	N-Acetyl-S-(4-hydroxy-2-butenyl)-L-cysteine (MHB3)	Urine	PATH
Ethylbenzene[b]/Styrene[b]	Phenylglyoxylic acid (PGA)	Urine	PATH
Acrylonitrile[b]; Vinyl chloride[b]; Ethylene oxide[b]	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA)	Urine	PATH
Styrene[b]	Mandelic acid (MADA)	Urine	PATH
Polycyclic aromatic hydrocarbons			
Pyrene	1-Hydroxypyrene (1-HOP)	Urine	PATH
Fluorene	2-Hydroxyfluorene (2-FLU)	Urine	PATH
Naphthalene	1-Hydroxynaphthalene (1-NAP)	Urine	PATH
Phenanthrene	Phenanthrene tetraol (PheT)	Urine	RCT
Indication	**Biomarkers of biological effect**	**Sample**	**Data source(s)**
Oxidative stress	8-iso-prostaglandin F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$)	Urine	PATH and RCT
Inflammation	Prostaglandin E metabolite (PGEM)	Urine	RCT
Inflammation	Soluble Intercellular Adhesion Molecular (ICAM-1)	Serum	PATH
Inflammation	IL6	Serum	PATH
Inflammation	High-sensitivity C-reactive protein (hsCRP)	Serum	PATH and RCT
Inflammation	White blood cells (WBC)	Blood	RCT

[a]In the RCT, TNE is the molar sum of total nicotine, total cotinine, and total 3'-hydroxycotinine, where "total" refers to the unconjugated and glucuronide-conjugated forms. In PATH, TNE is the molar sum of total nicotine, total cotinine, total 3'-hydroxycotinine, total cotinine N-oxide, total nicotine N-oxide, total norcotinine, and total nornicotine.
[b]Included on the Food and Drug Administration list of harmful or potentially harmful constituents in tobacco or tobacco smoke (24).

NMR group as an independent variable and adjusted for the following covariates that have been shown to be associated with NMR and outcomes of interest: sex, age, duration of regular smoking, body mass index (BMI), and menthol status (2, 7, 32, 33). Analyses were repeated for NMR as a continuous variable. All analyses using PATH data were performed using survey procedures and weights that adjust for the complex sampling design. Blood biomarker weights were used in all analyses of the PATH data, because a smaller subset of respondents provided a blood versus a urine sample (14). PATH data variances were estimated by the balanced repeated replication method (34) with Fay adjustment set to 0.3 to increase estimate stability (35). All analyses were performed in SAS 9.4.

Results

PATH data presented in the tables and figures were weighted to reflect the U.S. adult population at the time of the Wave 1 (2013–2014) sample. In PATH, the first quartile, median, and third quartile values of NMR were 0.262 (95% CI: 0.246–0.277), 0.379 (95% CI: 0.361–0.396), and 0.506 (95% CI: 0.480–0.532), respectively. Using the first quartile value of NMR (0.262) as the cutoff, the unweighted sample sizes for slow and normal/fast nicotine metabolizers were $n = 343$ and $n = 1,078$, respectively. In the RCT, the first quartile, median, and third quartile values of NMR were 0.29, 0.40, and 0.55, respectively. Using the first quartile of NMR (0.262) in the PATH study as the cutoff, the sample sizes for slow and normal/fast nicotine metabolizers were $n = 148$ and $n = 591$, respectively, in the RCT.

Characteristics of the participants by NMR group are displayed in **Table 2**. A greater proportion of normal/fast nicotine metabolizers

versus slow nicotine metabolizers were female, which is consistent with prior studies that have demonstrated sex hormones influence nicotine metabolism (32). In the PATH study, normal/fast metabolizers had an older average age and a greater duration of regular smoking than slow metabolizers. In the RCT, normal/fast metabolizers had a lower average BMI than slow metabolizers.

The mean value of the HSI, after adjustment for age, gender, BMI, duration of smoking, and menthol use, was greater among normal/fast metabolizers than slow metabolizers (3.28 vs. 2.95; $P = 0.0178$) in the PATH study. Further examination of the two items comprising the HSI demonstrated that the significance observed in the PATH study was driven by number of CPD ($P = 0.0027$) and not time to first cigarette smoked upon waking ($P = 0.8985$). In the RCT, there was no difference in the HSI (3.35 vs. 3.34; $P = 0.9447$). The mean value of the WISDM PDM8 in the PATH study and the mean value of the PDM16 in the RCT did not differ by NMR.

Unadjusted results comparing frequency and intensity of smoking and biomarkers of exposure and effect across NMR group are displayed in Supplementary Tables S1 and S2. Results of the analyses examining smoking intensity measures by NMR group adjusted for covariates are displayed in **Fig. 1**. In the PATH Study, normal/fast nicotine metabolizers had greater CPD than slow metabolizers (18.07 vs. 15.69; $P = 0.0008$). In the RCT, normal/fast metabolizers and slow metabolizers did not differ in average CPD (19.34 vs. 18.42; $P = 0.2597$). In both datasets, normal/fast nicotine metabolizers versus slow metabolizers had higher average levels of TNE (PATH: 84.61 vs. 64.53 nmol/mg; $P < 0.0001$; RCT: 68.33 vs. 57.55 nmol/mg; $P = 0.0004$) and TNE per cigarette smoked (PATH: 5.33 vs. 4.67 nmol/mg; $P < 0.0001$; RCT: 3.92 vs. 3.48 nmol/mg; 0.0139).

Table 2. Means, geometric means (GM), or proportions (%) and 95% confidence intervals of covariates by NMR and data source.

	Slow metabolizers	Normal/fast metabolizers	P
PATH Wave 1			
	N = 4,642,172	N = 13,941,182	
Age (years), mean	40.23 (38.10–42.37)	45.47 (43.97–46.97)	**0.0002**
Female sex, %	40.79 (32.99–49.09)	59.51 (55.20–63.68)	**<0.0001**
Body mass index, mean	27.82 (26.94–28.70)	27.14 (26.54–27.75)	0.1864
Years smoking, mean	21.92 (19.51–24.34)	26.35 (25.16–27.55)	**0.0013**
Smokes menthol cigarettes, %	27.13 (21.99–32.97)	30.10 (24.54–36.32)	0.4344
Urinary creatinine, GM	1.11 (1.00–1.22)	0.88 (0.82–0.94)	**0.0004**
HSI, mean	3.02 (2.80–3.23)	3.27 (3.11–3.44)	0.0510
HSI[a], mean	2.95 (2.73–3.17)	3.28 (3.07–3.48)	**0.0178**
WISDM PDM8, mean	3.30 (3.16–3.43)	3.44 (3.35–3.54)	0.0778
WISDM PDM8[a], mean	3.35 (3.22–3.48)	3.43 (3.33–3.53)	0.2884
Baseline data from RCT			
	N = 148	N = 591	
Age (years), mean	43.41 (41.27–45.54)	45.36 (44.17–46.54)	0.1392
Female sex, %	33.78 (26.15–41.42)	47.88 (43.85–51.92)	**0.0020**
Body mass index, mean	29.71 (28.69–30.72)	28.50 (27.97–29.01)	**0.0376**
Years smoking, mean	26.20 (24.06–28.34)	28.23 (27.04–29.41)	0.1245
Smokes menthol cigarettes, %	30.41 (22.98–37.84)	23.35 (19.93–26.77)	0.0754
Urinary creatinine, GM	1.00 (0.90–1.12)	0.95 (0.90–1.01)	0.4085
Expired CO, mean	20.08 (18.64–21.51)	20.93 (20.12–21.73)	0.3431
HSI, mean	3.42 (3.19–3.65)	3.41 (3.29–3.52)	0.9315
HSI[a], mean	3.34 (3.10–3.57)	3.35 (3.21–3.48)	0.9447
WISDM PDM16, mean	4.26 (4.04–4.48)	4.23 (4.12–4.35)	0.8415
WISDM PDM16[a], mean	4.27 (4.04–4.50)	4.22 (4.08–4.35)	0.6739

Note: PATH data are weighted; parentheses include 95% confidence intervals; bold values indicate statistical significance at $P < 0.05$.
Abbreviation: GM, geometric mean.
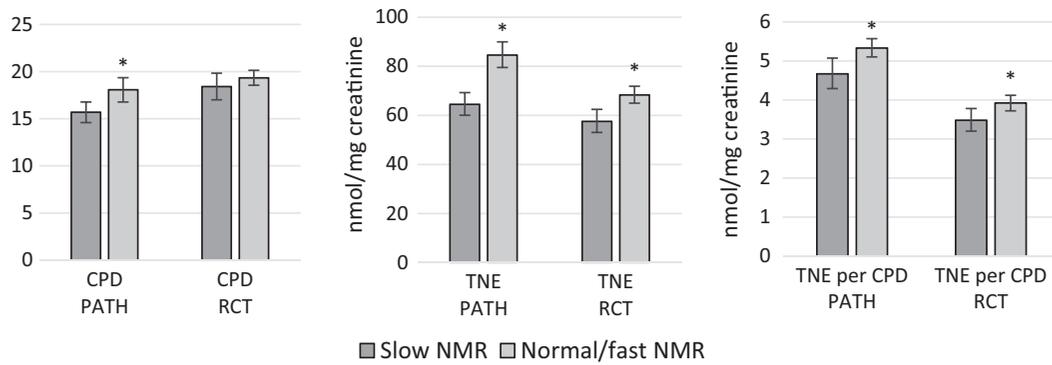[a]Estimates adjusted for age, sex, body mass index, duration regular smoking, and menthol use.

Figure 1.
Geometric means and 95% confidence intervals of heaviness of smoking measures by NMR strata. All estimates are adjusted for age, sex, body mass index, duration regular smoking, and menthol use. * Indicates a statistically significant difference ($P < 0.05$) in normal/fast nicotine metabolizers when compared with slow metabolizers.

Results of the adjusted analyses examining biomarkers of exposure across slow and normal/fast nicotine metabolizers are displayed in **Fig. 2**. In the PATH Study, normal/fast nicotine metabolizers versus slow nicotine metabolizers had higher average values of total NNAL (1.85 vs. 1.35 pmol/mg; $P < 0.0001$), 3-HPMA (7.64 vs. 6.14 nmol/mg; $P < 0.0001$), 2-HPMA (0.40 vs. 0.34 nmol/mg; $P = 0.0049$), HMPMA (15.25 vs. 12.06 nmol/mg; $P < 0.0001$), CEMA (0.99 vs. 0.73 nmol/mg; $P < 0.0001$), AAMA
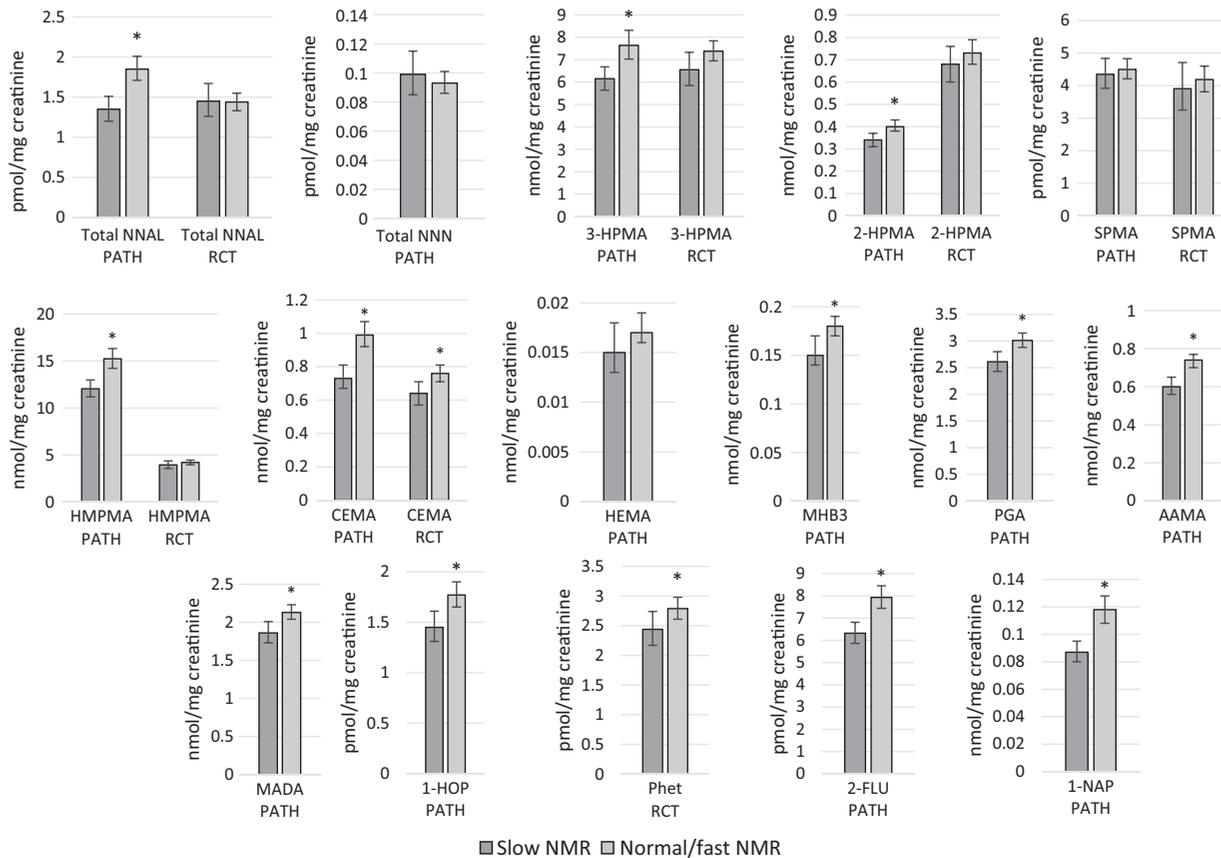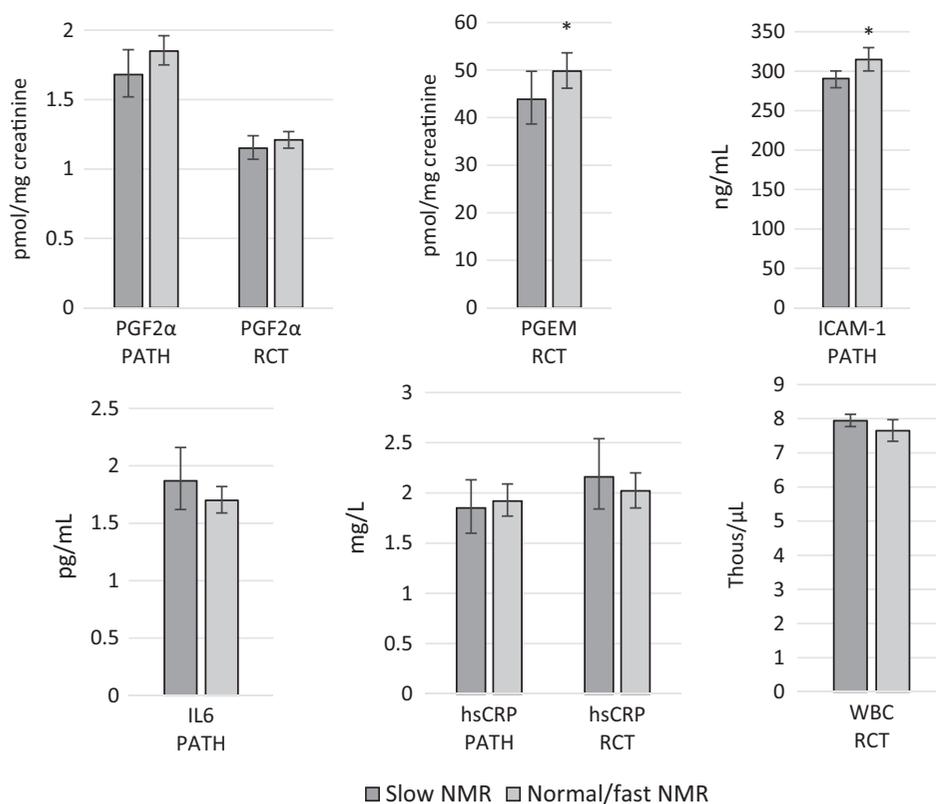

Figure 2.
Geometric means and 95% confidence intervals of biomarkers of tobacco-specific nitrosamines, volatile organic compounds, and polycyclic aromatic hydrocarbons by NMR strata. All estimates are adjusted for age, sex, body mass index, duration regular smoking, and menthol use. * Indicates a statistically significant difference ($P < 0.05$) in normal/fast nicotine metabolizers when compared with slow metabolizers.

Figure 3.
Geometric means and 95% confidence intervals of biomarkers of oxidative stress and inflammation by NMR strata. All estimates are adjusted for age, sex, body mass index, duration regular smoking, and menthol use. * Indicates a statistically significant difference ($P <$ 0.05) in normal/fast nicotine metabolizers when compared with slow metabolizers.

(0.74 vs. 0.60 nmol/mg; $P < 0.0001$), MHB3 (0.18 vs. 0.15 nmol/mg; $P = 0.00013$), PGA (3.01 vs. 2.61 nmol/mg; $P = 0.0010$), MADA (2.13 vs. 1.86 nmol/mg; $P = 0.0020$), 1-HOP (1.77 vs. 1.45 pmol/mg; $P = 0.0018$), 2-FLU (7.93 vs. 6.32; $P < 0.0001$), and 1-NAP (0.118 vs. 0.087; $P < 0.0001$). In the RCT, normal/fast nicotine metabolizers versus slow nicotine metabolizers had higher values of CEMA (0.76 vs. 0.64 nmol/mg; $P = 0.0074$) and PheT (2.79 vs. 2.44; $P = 0.0379$).

Results of the adjusted analyses examining biomarkers of effect by NMR group are displayed in **Fig. 3**. The inflammatory biomarker ICAM-1 measured in PATH was higher in normal/fast than slow nicotine metabolizers (314.76 vs. 290.73 ng/mL; $P = 0.0015$). The inflammatory biomarker PGEM measured in the RCT was higher in normal/fast than slow nicotine metabolizers (49.03 vs. 42.39 pmol/mg; $P = 0.0459$). The inflammatory biomarkers, IL6, hsCRP, and WBC, did not differ across NMR group. The oxidative stress biomarker PGF2α did not differ across NMR group (PATH: 1.85 vs. 1.68 pmol/mg; $P = 0.1075$; RCT: 1.21 vs. 1.15 pmol/mg; 0.2683).

Results of modeling NMR (log-transformed) as a continuous variable are displayed in Supplementary Tables S3 and S4. In adjusted analyses with the PATH Study, NMR was associated with increased levels of TNE ($P \leq 0.0001$), total NNAL ($P = 0.0017$), HMPMA ($P = 0.0079$), CEMA ($P = 0.0205$), PGA ($P = 0.0004$), MADA ($P = 0.0002$), 2-FLU ($P = 0.0041$), and 1-NAP ($P < 0.0001$). In the PATH Study, NMR was associated with decreased levels of total NNN ($P = 0.0028$). In the RCT, increased levels of NMR was associated with increased levels of 3-HPMA ($P = 0.0392$), CEMA ($P = 0.0001$), and PheT ($P = 0.0248$). In both datasets, continuous NMR was not associated with any of the biomarkers of effect.

Discussion

This study examined the association between NMR and intensity of smoking, nicotine dependence, biomarkers of TSNAs, VOCs, and PAHs, as well as the association between NMR and biomarkers of biological effect in two cross-sectional samples of non-Hispanic, white smokers. The results support the hypothesis that normal/fast nicotine metabolizers are heavier smokers, evidenced by an increase in levels of TNE by approximately 25 percent, and have higher levels of exposure to numerous carcinogens and toxicants found in tobacco and tobacco smoke when compared with slow metabolizers. A novel finding of the study was the observation that normal/fast nicotine metabolizers had higher levels of inflammation, measured by ICAM-1 and PGEM, than slow metabolizers. This study provides the first documentation that NMR is not only associated with smoking exposure but also biomarkers of biological effects that are integral in the development of tobacco-related disease.

Biomarkers of inflammation and oxidative stress have been observed to be affected by smoking (36, 37). ICAM-1 and PGEM were higher among normal/fast nicotine metabolizers. ICAM-1 has signaling properties in numerous cell types and is expressed in inflammatory conditions and chronic diseases including cancer and cardiovascular disease (38, 39). PGEM is a metabolite of the inflammatory mediator Prostaglandin E2 (PGE2; refs. 40, 41), which has been shown to promote tumor formation, growth, and metastasis and is the most abundant prostaglandin found in various types of malignancies including cancers of the lung, head, and neck (40). Higher levels of these inflammatory biomarkers among normal/fast metabolizers suggest greater disease risk and progression than slow metabolizers. Why ICAM-1 and PGEM were the only biomarkers of effect found to be

significantly elevated among normal/fast metabolizers may be due to their sensitivity to changes in smoking behavior. In a prior trial, levels of PGEM were found to decrease by 44% after smokers stopped smoking for 12 weeks, whereas levels of 8-iso-PGF$_{2\alpha}$ decreased by only 27% (42). The higher levels of inflammation in normal/fast metabolizers is consistent with prior studies that have shown levels of these biomarkers increase with pack-years and are correlated with cotinine levels (43–45). In a prior analysis of a Chinese cohort, NMR was significantly associated with lung cancer risk but not after adjustment for TNE (46). Indeed after adjustment for TNE, there was no difference between ICAM-1 and PGEM across NMR group in this study, suggesting that these relationships are driven by smoking intensity.

Unlike total NNAL and several other exposure biomarkers, total NNN was inversely correlated with NMR. A prior study among Alaska Natives also observed higher levels of total NNN in slow versus normal/fast nicotine metabolizers (47). The authors speculated that CYP2A6 may influence NNN metabolism and higher levels of NNN in slow metabolizers reflect lower bioactivation and therefore greater detoxification of NNN (47). However, another explanation is that this finding may reflect an artefact in the data as NNN measurements are highly prone to artefact formation during analysis (48, 49). Furthermore, only approximately 1% of the NNN dose is excreted in the urine and thus 99% of the NNN dose is not captured by the biomarker (48). This is in contrast to total NNAL, which is estimated to capture approximately 50% of the NNK dose (50).

Greater nicotine dependence has been examined as a potential explanation for why normal/fast nicotine metabolizers smoke more heavily than slow metabolizers (9, 27, 51–53). NMR was significantly associated with the HSI, but the relationship was primarily driven by CPD (and thus smoking intensity). There was no evidence for an association between NMR and the WISDM primary dependence motives subscale. Thus, the results of this study are consistent with the majority of prior studies that observed no association between NMR and various dependence scales (9, 27, 51–53).

While NMR modeled as the lowest quartile (slow metabolizers) versus the top three quartiles (normal/fast metabolizers) was associated with biomarkers of inflammation, NMR modeled as a continuous measure was not. In line with our results are prior studies that observed a threshold effect whereby mean plasma nicotine levels and quit rates were highest in the lowest quartile of NMR and then flattened out over the second, third, and fourth quartiles of NMR (27); thus the relationships were not linear. A limitation of prior studies is that the distribution of NMR was defined within each sample and this has resulted in varying NMR cutoff points to distinguish slow versus normal/fast nicotine metabolizers (26–31). A strength of this study was the use of a U.S. representative study of smokers to determine the NMR cutoff point (0.26 based on the molar ratio of free 3-hydroxycotinine to free cotinine in serum). Given the significant relationships observed in this study, the NMR cutoff point of 0.26 could serve as a measure for identifying non-Hispanic, white smokers who are potentially at the greatest risk for tobacco-related disease.

Another strength of the study was the use of data from two separate studies that allowed for a comprehensive array of biomarkers for study. The PATH Study and the RCT varied in inclusion/exclusion criteria and methods of recruitment, which likely resulted in the observed differences in the distribution of NMR across the two studies, as well as differences in participant characteristics. In addition, biomarkers were analyzed by different labs with different analytic approaches. For example, in the PATH study TNE was measured as a composite of seven metabolites (TNE7) while in the RCT TNE was a composite of three metabolites (TNE3). The ratio of TNE7 to TNE3 averages 1.18 (54). Thus, these two measures for TNE are correlated but have different means (as shown in the data with PATH participants' having a greater mean value for TNE). Despite these differences, levels of biomarkers and their patterns across NMR strata were similar across the two studies and demonstrate consistency of the results.

There are limitations to the current study. Variation in nicotine metabolism pathways, other than those catalyzed by CYP2A6 such as UGT2B10, can affect the accuracy of the NMR as measured in this study (free 3HCOT/free COT) as a measure of CYP2A6 activity (12, 55). Compared to 1% of whites, approximately 15% of African American smokers do not glucuronidate cotinine due to the presence of the UGT2B10 splice variant (12, 55). As a result, some African Americans could be misclassified as having a lower NMR when UGT2B10 is not also taken into account (12, 55). UGT2B10 genotype or its phenotype, ratio of total COT to free COT, was not available in the PATH data. Thus, this study was restricted to participants of non-Hispanic white race/ethnicity and findings may not be generalizable to other racial/ethnic groups. Another limitation is that the results are not generalizable to non-daily or light smokers, where NMR is less predictive of smoking behavior (56, 57), and the use of a urinary COT level of >1,000 ng/mL for inclusion may have hindered the generalizability of the results to some moderate smokers. In the clinical trial sample, several biomarkers of exposure were higher among normal/fast versus slow metabolizers, but the differences were not statistically significant. Given that several of these biomarkers reached significance in the PATH study, the lack of significance in the clinical trial sample could be due to the fewer number of slow metabolizers ($n = 148$ vs. $n = 343$). Finally, indicators of inflammation and oxidative stress and the metabolites studied, with the exception of total NNAL and NNN, are not specific to tobacco use and therefore may reflect environmental and/or endogenous exposures (23, 58).

In conclusion, the results are suggestive that normal/fast nicotine metabolizers of non-Hispanic white race may be at increased risk for tobacco-related disease due to greater smoking intensity, higher exposure to numerous toxicants and carcinogens, and higher levels of inflammation when compared with slow nicotine metabolizers of non-Hispanic white race. Results provide support for the NMR as a biomarker for understanding a smoker's exposure and potential risk for tobacco-related disease.

Disclosure of Potential Conflicts of Interest

N.L. Benowitz is a consultant for Pfizer and Achieve Life Sciences and has provided expert testimony against tobacco companies. M. Kotlyar reports receiving a Global Research Award for Nicotine Dependence (GRAND) program funded by Pfizer. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The contents and views in this manuscript are those of the authors and should not be construed to represent the views of the NIH or any of the sponsoring organizations and agencies of the U.S. government.

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