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ARTICLE

Nicotine metabolism and its association with CYP2A6 genotype among Indigenous people in Alaska who smoke

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Abstract

Prevalence of smoking is higher in Alaska Native and American Indian (ANAI) populations living in Alaska than the general US population. Genetic factors contribute to smoking and cessation rates. The objective of this study was to compare *CYP2A6* genetic variation and CYP2A6 enzyme activity toward nicotine in an ANAI population. ANAI ($N = 151$) people trying to quit smoking were recruited. DNA samples were genotyped for *CYP2A6* variants **1X2A*, **1B*, **2*, **4*, **9*, **10*, **12*, and **35*. Multiple nicotine metabolites were measured in plasma and urine samples, including cotinine and 3′-hydroxycotinine used to determine CYP2A6 activity (e.g., nicotine metabolite ratio [NMR]). We calculated summary statistics for all of the genotypes and metabolites and assigned CYP2A6 activity scores based on known information. We studied the association of *CYP2A6* variants with the NMR and smoking histories. The overall frequency of the *CYP2A6*1B* gain of function allele was high in the ANAI versus non-ANAI populations in other studies. Both **4* null and **9* decrease of function alleles had frequencies similar to previous studies of ANAI populations. In a multivariate analysis, the genotype-inferred CYP2A6 activity score was associated with both plasma and urine NMR (p value = 8.56E-08 and 4.08E-13, respectively). Plasma NMR was also associated with duration of smoking (p value < 0.01) but not urinary total nicotine equivalents uncorrected for creatinine ($TNE9_{uc}$) or biological sex. Urine NMR was significantly associated (p value < 0.01) with $TNE9_{uc}$. Variation in NMR in this ANAI population is explained in part by *CYP2A6* genetic variation.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

CYP2A6 is a nicotine metabolizing enzyme that has been associated with smoking and cessation rates. Genetic variation in *CYP2A6* and CYP2A6 enzyme activity has not been thoroughly assessed in this Alaska Native and American Indian (ANAI) community.

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WHAT QUESTION DID THIS STUDY ADDRESS?
This study investigated whether inheritance of variants in *CYP2A6* affects the nicotine metabolite ratio (NMR) in plasma and urine in ANAI, which has subsequent implications on smoking cessation therapy for ANAI people.
WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
CYP2A6 genotype is associated with both plasma and urine NMR. Duration since participants started smoking is associated with plasma NMR. A gain of function allele (*CYP2A6*1B*) was at high frequency in this ANAI population and associated with higher NMR.
HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
Prospective pharmacogenetic screening for *CYP2A6* diplotypes or NMR testing could help guide smoking cessation treatment for ANAI populations.

INTRODUCTION

Smoking, a major contributor to morbidity and mortality worldwide, is high among the Indigenous people living in Alaska.[1] Estimates of smoking rates are 37% among Alaska Native or American Indian (ANAI) people living in Alaska, compared to 17% among non-native people living in Alaska[1,2] and 15% for the general adult US population.[3,4] Chronic smoking results from nicotine dependence,[5] which is influenced by an individual's metabolic capacity for eliminating nicotine from the body.[6] Nicotine metabolic clearance is largely determined by the function of a single enzyme, CYP2A6.[7] Inherited variation in the *CYP2A6* gene substantially contributes to interindividual differences in nicotine metabolic clearance and is commonly represented by the biomarker of nicotine metabolite ratio (NMR). The NMR is also associated with nicotine boost following smoking a cigarette and the frequency of cigarette smoking.[5]

The NMR has an impact on pharmacotherapy outcomes for smoking cessation, including in one NMR randomized trial.[8–13] Individuals with a low NMR (people with slow nicotine metabolism) randomized to receive treatment with a nicotine replacement patch were more likely to quit smoking than those with a high NMR (people with normal nicotine metabolism)[10]; this may be because systemic delivery of nicotine from the nicotine patch better mimics nicotine exposure following smoking in people with slow nicotine metabolism than in people with normal nicotine metabolism. Interestingly, individuals with slow nicotine metabolism receiving varenicline for smoking cessation treatment had lower quit rates and encountered adverse side effects more frequently than did those with normal nicotine metabolism.[13] Together, these results provide clinical rationale for NMR testing prior to the selection of pharmacotherapy, especially initial therapy, to improve long-term smoking quit rates.

The NMR to *CYP2A6* genotype relationship has been well-studied in the non-Hispanic White population of the United States,[14] but less so in racially and ethnically diverse populations, such as ANAI people.[15–18] Thus, we sought to characterize the relationship between NMR and *CYP2A6* variation in a diverse ANAI population of individuals who actively smoked. Claw et al. (2019) and Binnington et al. (2012) identified the commonly known *CYP2A6* allele frequencies in ANAI populations, with Tanner (2017) describing allele frequencies in Northern Plains and Southwest AI populations; each noted significant differences compared to non-native people.[15,17,18] In this study, we measured plasma and urinary concentrations of nicotine and its major metabolites, computed NMR values, and tested for associations with the major *CYP2A6* alleles and diplotypes, as well as with smoking history.

METHODS

The Alaska Area Institutional Review Board and Tribal research review boards approved the study. A certificate of reliance was obtained from the University of Washington, Institutional Review Board.

Study population

Southcentral Foundation (SCF) is a tribally owned and operated healthcare system that provides prepaid healthcare services to over 65,000 ANAI people in both urban and rural areas of Alaska.[19,20] Recruitment for this study

was from the SCF Quit Tobacco Program,[21] a comprehensive intervention program that provides tobacco cessation services to ANAI individuals.

Between May 2016 and October 2018, we recruited ANAI individuals, greater than or equal to 18 years of age, who smoked cigarettes at study enrollment and enrolled in the SCF Quit Tobacco Program. Exclusion criteria included simultaneous use of other tobacco products, including chew, iqmik (a form of smokeless tobacco used among AN people),[22] electronic cigarettes, pipes, and cigars. Additionally, potential participants were excluded if they were being treated for cancer, had hemophilia, or were pregnant according to self-report. Research staff obtained written informed consent from participants before data collection began.

Study design

The objective of this study was to characterize the relationship between NMR and *CYP2A6* in an ANAI population who actively smoke as part of an observational study examining factors associated with successful tobacco cessation. Participants completed a demographics and tobacco use history questionnaire and provided blood and spot urine samples at the end of the study visit. In previous studies, it has been reported that there is only minor variation in average daily NMR over a week-long period for daily smokers,[23] and NMR remains stable over a 44-week range.[24–26] Thus, a reliable estimate can be obtained from a single sample from a smoker. Plasma and the buffy coat were separated from blood cells by centrifugation and, along with an aliquot of urine, stored at −70°C or −80°C prior to analysis. Participants' electronic health records were queried for data elements, including age and biological sex.

DNA processing and allele-specific CYP2A6 genotyping

Genomic DNA was isolated from the buffy coat using QIAamp DNA blood kits (Qiagen). All samples (n = 151) were prepared for genotyping using nested real-time polymerase chain reaction (RT-PCR) and Taqman copy number assay methods. The following 10 high-frequency *CYP2A6* star alleles, both structural variants (SVs) and single nucleotide variants (SNVs), previously identified in this population,[18] were selected for genotyping analysis: **1X2A*, **1B*, **2*, **4*, **9*, **7*, **8*, **10* (composed of **7* and **8*), **12*, and **35*. The *CYP2A6* star alleles were identified in the 151 sample group by genotyping procedures using three methods: (1) a two-step, region-specific PCR reaction coupled with a variant-specific RT-PCR assay (*CYP2A6*1B*, **2*, **9*, and **35*), (2) Taqman copy number assays (*CYP2A6*1x2*, **4*, and **12* SVs), and (3) PCR and gel-based assays (*CYP2A6*7* and **8* to ascertain **10*).[27]

CYP2A6 genotypes were determined using manual calls of the assay output data. The SNV data were then phased into gene diplotypes using the PHASE2.1.1 software. Only the pairs of haplotypes for each individual with a probability greater than 0.75 were considered in further analysis. These phased haplotypes were combined with SV data to call a final genotype (referred to as the inferred genotype throughout).

Based on activity scores for other *CYP* genes (i.e., *CYP2D6*), we created activity scores for *CYP2A6* where, for each allele, none = 0, decreased = 0.5, normal = 1, and increased = 1.25 (i.e., *CYP2A6*1B*). The predicted activity score was calculated for a pair of alleles in a diplotype by adding the scores for each allele. An activity score of 1.25 distinguishes the activity of a homozygote *1B (activity score = 2.5) from someone with a single gene duplication (activity score = 3.0). To convert from predicted activity score to phenotype class, we used a method similar to that used for *CYP2D6*[28] and the following criteria to translate *CYP2A6* genotype into a qualitative measure of phenotype: poor (activity score = 0), slow (activity score >0 to <1), intermediate (activity score 1 to <2), normal (activity score 2 to <3), and fast (activity score ≥3).

Plasma and urine metabolite analysis

To quantify nicotine and its metabolites in plasma and urine, a modified bioanalytical procedure was used[29] and the full method that was developed is described in Supplementary File S1. Briefly, metabolites were purchased, and a single solution of internal standards contained 0.25 µg/ml of each analyte in methanol. For urine and plasma analysis, modified procedures[29] were used to prepare, quantify with liquid chromatography-mass spectrometry, and conduct quality control for all samples. The lower limit of quantification (LLOQ) values for plasma were: trans-3′-hydroxycotinine (3HC): 1.5 ng/ml, 3HC-GLUC: 1.2 ng/ml, COT-GLUC: 1.1 ng/ml, CNO: 1.4 ng/ml, COT: 1.4 ng/ml, NIC-GLUC: 1.2 ng/ml, NIC: 1.0 ng/ml, NNO: 1.2 ng/ml, and NNIC: 1.4 ng/ml. The limits of detection (LOD) in plasma were the same as the LLOQ. The LLOQ values for urine were: 3HC: 97 ng/ml, 3HC-GLUC: 14 ng/ml, COT-GLUC: 110 ng/ml, CNO: 8 ng/ml, COT: 11 ng/ml, NIC-GLUC: 58 ng/ml, NIC: 58 ng/ml, NNO: 40 ng/ml, and NNIC: 12 ng/ml. The LOD in urine were at most: 3HC: 20 ng/ml, 3HC-GLUC: 1.5 ng/ml, COT-GLUC: 23 ng/ml, CNO: 8 ng/ml, COT:

12 ng/ml, NIC-GLUC: 11 ng/ml, NIC: 12.5 ng/ml, NNO: 9 ng/ml, and NNIC: 4.5 ng/ml. For all compounds except NNIC, the concentration listed as the LOD was the lowest concentration checked. Five 3HC values in plasma and four 3HC and 108 3HC-GLUC values in urine below the LOD were replaced with the LOD divided by the square root of two before log transformation, as has been previously done.[30,31] COT, 3HC and concentrations, and NMR, were non-normally distributed and therefore the ratios were log-transformed for further analyses. In addition, logged NMR is more highly related to nicotine clearance rate than unlogged NMR.[32]

Plasma NMR was calculated by taking the ratio of free 3HC to COT concentrations. Urine NMR was calculated by taking the ratio of the sum of 3HC and 3HC-GLUC to the COT concentration.[15] The total nicotine equivalents uncorrected for creatinine ($TNE9_{uc}$), the molar sum of NIC, and eight major metabolites, was calculated and accounts for 85–90% of nicotine dose.[33] $TNE9_{uc}$ used throughout this paper refers to the absolute value, not creatinine corrected for variation in renal function and urine dilution. $TNE9_{uc}$, alongside cigarettes per day (CPD), was used as a measurement of nicotine intake and represents an alternative approach, especially in light or occasional smokers.

Statistical analysis

Descriptive statistics were calculated as percentages for categorical variables and mean with SD or median with interquartile range for continuous variables. We assessed *CYP2A6* variation through allele count and frequency. We calculated summary statistics for all measured metabolites and metabolic ratios reporting the mean, SD, and range. We compared the inferred *CYP2A6* diplotypes and predicted activity score assignments with NMR using various statistical tests to measure significance, the one-way analysis of variance (ANOVA) test and the Tukey Honest Significant Differences in R (version 3.3.1).[34] We also used the nonparametric Kruskal-Wallis rank sum test, which can be applied when ANOVA assumptions of normal distribution and variance are not met. We assessed the correlation between inferred diplotypes and NMR and further classified these into a phenotype class. We calculated an association between predicted activity scores and observed NMR using both of these tests. We used multivariate linear regression to assess the associations between both plasma and urine NMR and variables, such as predicted activity score, $TNE9_{uc}$, and years since regular smoking began (described here as smoking duration), age, and biological sex. We also tested whether NMR was associated with smoking history and correlations between CPD and $TNE9_{uc}$.

RESULTS

Study population

A total of 151 ANAI people who smoke was recruited into the study (Table 1). Of those participants, 58% ($n = 87$) were women and median age was 45 years old (range 18–80 years). All participants self-reported to smoke at the start of the study and had a mean cigarette intake of 12 CPD. Participants had smoked for a median duration of 26 years.

CYP2A6 allele frequencies

We were able to confidently call the 10 SNV genotypes for 139 of 151 (92%) participants (Table 2). Five participants had inconsistent genotype calls across three replicates, six had low confidence genotype calls across three replicates, and one did not provide DNA and thus was not genotyped. Most alleles in the 139 sample set were successfully genotyped, with the following exceptions; four *CYP2A6*2*, five participants that had failed or ambiguous calls, and in the phasing, we reported three participants with missing data points for the **2* allele. All of the remaining participants were genotyped for all variants. The phase of 139 participants was resolved with phased haplotype scores greater than 0.75. Overall, a total of 139 participants had final inferred genotypes that included SNV and SV data and were considered in further analyses.

TABLE 1 Demographics, tobacco use characteristics, and nicotine biomarkers of ANAI people trying to quit smoking in a tribal health setting

Characteristics or biomarkers	N	Mean ± SD
Age in years, median (IQR)	151	45 (34, 54)
Sex		
Female	87	58%
CPD	151	12 ± 10
Duration of smoking (years), median (IQR)	148	26 (18, 36)
Plasma biomarker NMR	143	0.50 ± 0.33
Urinary biomarker NMR	141	5.22 ± 4.69

Abbreviations: ANAI, Alaska Native and American Indian; CPD, cigarettes per day; IQR, interquartile range; NMR, nicotine metabolite ratio.

TABLE 2 Observed CYP2A6 alleles and frequencies among ANAI people trying to quit smoking in a tribal health setting ($N = 139$)

Variant	Allele count	Missing data	Total # chromosomes	Frequency (%)	Activity score
*1x2A	0	0	278	0.0	1.0
*1B	176	0	278	63.0	1.25
*2	5	10	268	2.0	0
*4	29	0	278	10.0	0
*7	0	0	278	0.0	0.5
*8	0	0	278	0.0	1.0
*9	57	0	278	21.0	0.5
*10	4	0	278	1.0	0.5
*12	2	0	278	1.0	0.5
*35	0	2	276	0.0	0.5

Abbreviation: ANAI, Alaska Native and American Indian.

TABLE 3 Plasma NMR stratified by inferred diplotypes ($N = 139$)

Phased diplotype	*N*	Frequency	Activity score	Average NMR[b]	Phenotype class[c]
*1B/*1B[a]	35	25.2	2.5	0.69[b]	Normal
*1B/*1A[a]	44	31.7	2.25	0.55[b]	Normal
*1A/*1A[a]	7	5.0	2	0.44	Normal
*1B/*1B+*9	1	0.7	2	0.90	Normal
*1B/*1B+*10	1	0.7	2	0.39	Normal
*1B/*1B+*35	1	0.7	2	0.41	Normal
*1B/*9[a]	11	7.9	1.75	0.44[b]	Intermediate
*1B/*12	1	0.7	1.75	0.91	Intermediate
*1A/*9[a]	4	2.9	1.5	0.27	Intermediate
*1A/*12	1	0.7	1.5	NA	Intermediate
*1B/*2[a]	2	1.4	1.25	0.88	Intermediate
*1B/*4[a]	18	12.9	1.25	0.31[b]	Intermediate
*1B/*9+*10	1	0.7	1.25	0.07	Intermediate
*1A/*2	1	0.7	1	0.41	Intermediate
*1A/*2+*9	1	0.7	1	1.58	Intermediate
*9/*9	1	0.7	1	0.15	Intermediate
*1B+*9/*4[a]	4	2.9	0.75	0.19	Slow
*1B+*10/*4[a]	2	1.4	0.75	0.18	Slow
*2/*4	1	0.7	0	0.00	Poor
*4/*4[a]	2	1.4	0	0.00	Poor

Abbreviations: NMR, nicotine metabolite ratio; NA, not available.

[a]The inferred diplotype is one of the 10 most common included in Figure 2.

[b]The average NMR excluded individuals with missing plasma NMR values.

[c]Poor = 0; 0 < Slow < 1; 1 ≤ Intermediate < 2; 2 ≤ Normal < 3.

Inferred diplotypes

The final inferred diplotypes of 139 participants, including both genotyped SVs and SNVs, and predicted activity scores and metabolism phenotype class are presented in Table 3. There were 20 unique diplotypes in the data set. The most frequent diplotype was **1B/*1A* at 32% in frequency, followed by **1B/*1B* at 25% frequency, **1B/*4* at 13% frequency, **1B/*4*9 at 8% frequency, and **1A/*1A* at 5%.

Based on the diplotype results, we were able to classify participants based on predicted activity scores and assign a predicted metabolism phenotype class (Table 3). A total of 4% and 2% were classified as people with slow nicotine metabolism and poor nicotine metabolizer phenotypes (with at least one null or decreased function genotype), respectively, 29% were classified as having an intermediate metabolizer phenotype (one or two decreased function genotypes), and 64% classified with a normal metabolizer phenotype. No one was classified with a fast metabolizer phenotype (activity score >3).

Plasma and urine metabolites

Plasma and urine samples from 149 participants and 145 participants (2 and 6 samples missing, respectively) were analyzed for nicotine and eight of its metabolites. Nicotine metabolites were not detected in five plasma and four urine samples, with two of those participants having low levels in both suggesting that these participants had not smoked recently at the time of sample collection. In total, 144 plasma and 141 urine samples had quantifiable analyte levels and are included in subsequent analysis. Summary statistics for metabolite concentrations are reported in Table 4. Total cotinine and 3-hydroxycotinine concentrations were low or undetectable in samples from the two participants with a *4/*4 genotype, as expected.[35,36]

Nicotine metabolite ratio distribution

A histogram of the plasma and urine NMR is presented in Figure 1. Using a previously determined NMR cutoff point of 0.31 for whole blood NMR to distinguish between people with slow and normal nicotine metabolism,[13] 30% of the ANAI study participants were classified with slow nicotine metabolism. A demarcation of urine NMR between people with normal and slow nicotine metabolism has not been reported, however, there was a positive and significant correlation ($r^2 = 0.32$, $p < 0.001$) between the logged urine and logged plasma NMR (Figure S1).

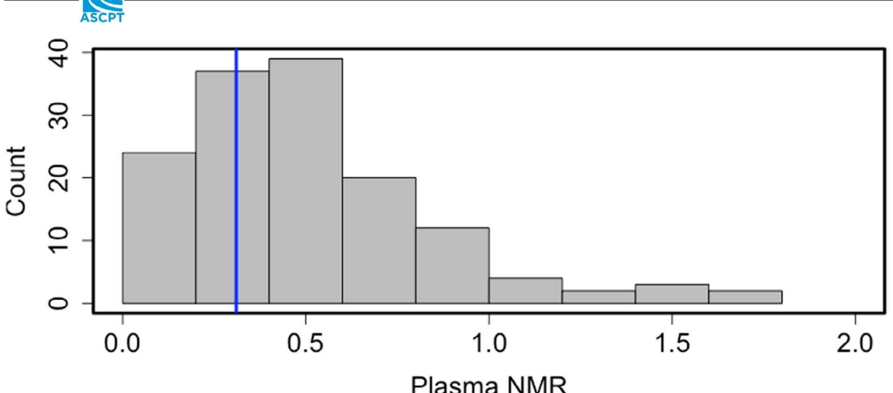
The proportion of nicotine metabolites, relative to $TNE9_{uc}$, in ANAI people who smoke is shown in Figure S2. NNO, NIC, and NIC-GLUC percentages (6, 15, and 5%, respectively) were similar to previously reported amounts in African American (AA), Native Hawaiian (NH), White (W), Latino (L), and Japanese American (JA) populations (5.4–10%, 10–20%, and 4.2–9.7%, respectively).[37] Total cotinine in ANAI (27%) was similar to JA population (31%) but lower than AA, NH, W, and L populations (40–53%). Total 3HC was higher in ANAI populations (43%) than AA, NH, W, L, and JA populations (24–30%). The number of CPD and $TNE9_{uc}$ are reported in Table S1. Although there was a positive trend for the relationship between CPD and $TNE9_{uc}$ values, this was not statistically significant.

TABLE 4 Profile concentration of nicotine metabolites in plasma and urine of ANAI who smoke

	Plasma (*N* = 144)		Urine (*N* = 141)	
List of metabolites	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
COT-GLUC	26.3 ± 20.7	0.3–91.6	3595.0 ± 3381.0	143.0–20,543.0
CNO	3.0 ± 1.6	1.2–10.8	365.2 ± 340.9	13.0–1691.0
COT	127.7 ± 98.1	0.7–519.8	989.5 ± 650.5	25.0–3260.0
NIC-GLUC	5.6 ± 5.2	0.3–27.3	978.3 ± 1088.0	62.0–7549.0
NIC	11.6 ± 8.0	1.4–46.3	1618.0 ± 3691.8	67.0–37,996.0
NNO	2.5 ± 1.4	0.6–7.3	640.0 ± 689.8	44.0–5385.0
NNIC	1.5 ± 0.4	1–2.5	91.0 ± 93.7	14.0–753.0
3HC	58.8 ± 55.5	0.6–253.8	4982.0 ± 4744.8	249.0–22,394.0
3HC-GLUC	—	—	69.2 ± 69.5	15.0–318.0
plasma NMR	0.50 ± 0.33	0.0–1.68	—	—
urine NMR	—	—	5.22 ± 4.69	0.03–26.6
$TNE9_{uc}$ (nmol/ml)	—	—	58.1 ± 54.9	2.8–427.0

All values reported in ng/ml unless otherwise specified.

Abbreviations: ANAI, Alaska Native and American Indian; NMR, nicotine metabolite ratio; $TNE9_{uc}$, total nicotine equivalents uncorrected for creatinine.



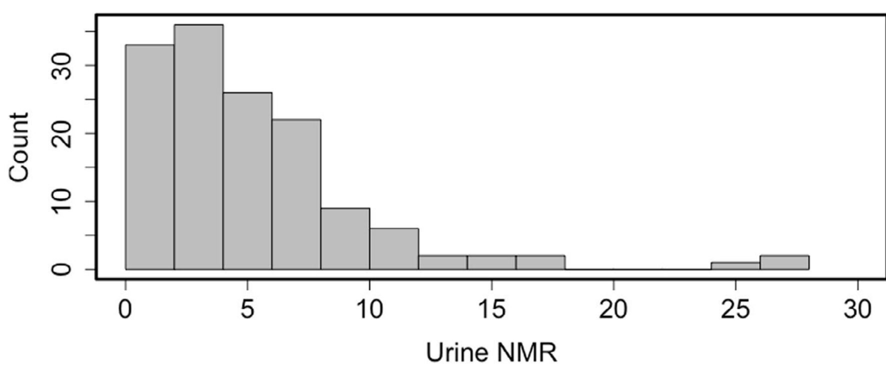


FIGURE 1 Histogram of plasma and urine NMR. Distribution of the plasma and urine NMR from 143 (plasma) and 141 (urine) Southcentral Foundation study participants. Blue line at 0.31 denotes the demarcation between people with slow versus normal nicotine metabolism. NMR, nicotine metabolite ratio

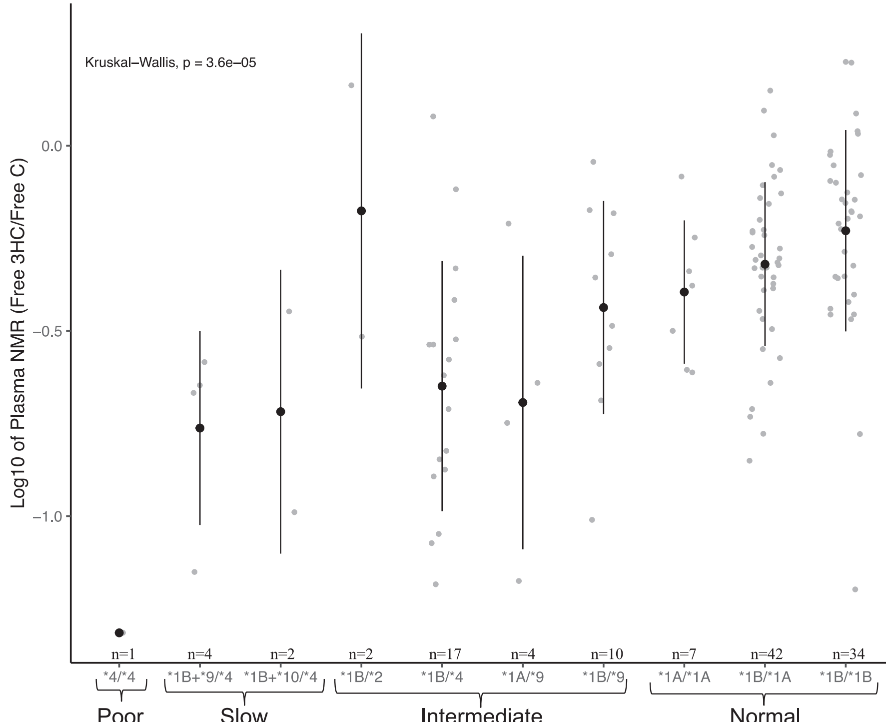


FIGURE 2 Plasma NMR segregated by individual *CYP2A6* diplotypes. NMR values for 123 study participants were stratified by inferred *CYP2A6* diplotype. NMR, nicotine metabolite ratio

NMR stratified by activity scores and diplotypes

Observed NMR values for study participants stratified by *CYP2A6* diplotype and inferred phenotype are also shown in Table 3. Considering only those diplotype subgroups with four or more counts, the average NMR values for different diplotypes among the inferred "normal" phenotype class were between 0.44 and 0.69, all greater than 0.31, a previously utilized cutoff point.[13] Those participants that

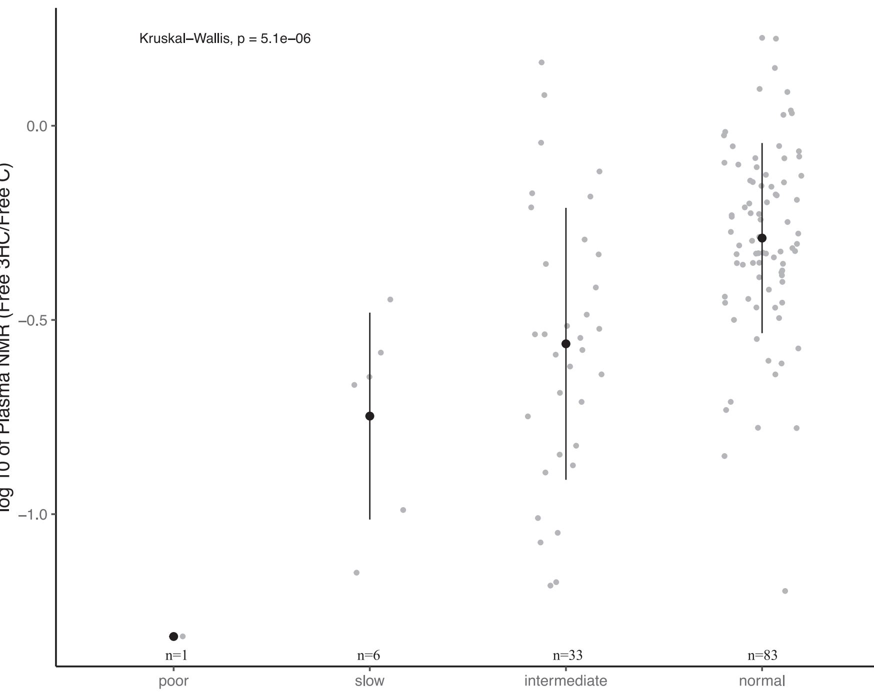


FIGURE 3 Plasma NMR segregated into poor, slow, intermediate and normal phenotypes. The activity phenotypes for 123 study participants are defined as follows: poor includes activity phenotypes equal to 0, normal includes activity phenotypes between 1 and 2, and enhanced includes activity phenotypes between 2 and 3. Group differences were evaluated by a comparison of means; Kruskal–Wallis *p* value = 5.1e-06. NMR, nicotine metabolite ratio

had diplotypes with an inferred "intermediate" phenotype showed average NMRs ranging from 0.27 to 0.44. The average NMR for those diplotypes within the "intermediate" phenotype class, including counts of one or two, were between 0.7 and 1.58, with the majority of observations falling between 0.7 and 0.44 (38 of 41 observations). There were three outliers within the intermediate phenotype, which included a **1/*2+*9* diplotype with an NMR of 1.58, a **1B/*12* diplotype with an NMR of 0.91, and a **1B/*2* diplotype with an NMR of 0.88. All "slow" and "poor" metabolizer phenotypes had low NMRs ranging from 0.00 to 0.19.

When comparing plasma NMR values for 10 common individual diplotypes (Figure 2), the average observed NMR was highest for the *CYP2A6*1B/*1B* group and lowest for the **4/*4* group, with a significant Kruskal-Wallis test between the mean of each diplotype (p = 3.6E-05). When we compared **1A/*1A* and **1B/*1B* diplotypes alone, the Wilcoxon rank sum test revealed a difference between the mean NMR of both groups (p = 0.053). The Wilcoxon rank sum test also revealed significant differences between the mean NMR for the **1B/*1B* group and that of diplotypes with **4* variants (**1B/*4* [p = 0.004] and **1B+*9/*4* diplotypes [p = 0.015]).

Given these results, we grouped all of the different diplotypes (including those with low frequency) into four metabolism phenotype classes: poor, slow, intermediate, and normal. There were significant differences between the means of observed NMRs for the different diplotype-inferred phenotype classes as well (Kruskal-Wallis p = 5.1E-06; Figure 3). When we performed a Wilcoxon rank sum test between the mean NMRs of the phenotype classes (Figure 3), each class was significantly different from the other, with the exception of the poor phenotype class, which had only one sample (p value ranged between 0.001 and 3.3e-5). The ANOVA test also showed significant differences between the means of observed NMRs for the phenotype classes (ANOVA p = 2.9E-08).

Multivariate regression

We performed multiple linear regressions to assess the association of plasma and urine NMR with diplotype predicted CYP2A6 activity score and other covariates (Table 5 and Supplementary File 2). Activity score had a strong association with both plasma and urine NMR. $TNE9_{uc}$ was also significantly correlated with plasma and urine NMR (p value = 0.04 and ≤0.001, respectively). Approximately 26% of variation in plasma NMR was explained by the model that included CYP2A6 activity score, age, sex, and smoking history ($TNE9_{uc}$ and smoking duration). Another model that excluded smoking history explained 23% of variation in plasma NMR. Conducting the same analysis with urine NMR and covariates, ~ 40% of the variation could be explained by a model that included activity score, age, sex, and smoking history. The model that excluded smoking history explained 30% of variation in urine NMR.

DISCUSSION

This study represents the first comprehensive investigation of associations between *CYP2A6* gene variation and

TABLE 5 Multivariate linear regression predicting NMR

Characteristics	*N*	Point estimate	*p* value	Variability explained, adjusted R^2
Plasma NMR (unconjugated 3HC/unconjugated COT)				
Univariate				
Predicted CYP2A6 Activity score	133	−0.93	≤0.001	0.224
Age	144	−0.62	0.030	0.026
Sex	144	−0.39	0.656	−0.006
Smoking history				
$TNE9_{uc}$	136	−0.48	0.043	0.023
Duration	141	−0.58	0.064	0.017
Multivariate				
Adjusted model including smoking history[a]	123	−1.36	≤0.001	0.264
Adjusted model excluding smoking history[b]	133	−1.09	≤0.001	0.231
Urine NMR (total 3HC+3HC-GLUC/ unconjugated COT)				
Univariate				
Predicted CYP2A6 activity score	129	−0.22	≤0.001	0.300
Age	141	0.38	0.180	0.006
Sex	141	0.62	0.019	0.032
Smoking history				
$TNE9_{uc}$	141	0.39	≤0.001	0.119
Duration	139	0.41	0.259	0.002
Multivariate				
Adjusted model including smoking history[a]	127	−0.54	≤0.001	0.398
Adjusted model excluding smoking history[b]	129	−0.28	≤0.001	0.301

Abbreviations: NMR, nicotine metabolite ratio; $TNE9_{uc}$, total nicotine equivalents uncorrected for creatinine.

[a]A fully adjusted model of NMR as the outcome variable and including all predictors in the table: predicted CYP2A6 activity score, smoking history, age, and biological sex.

[b]A fully adjusted model of NMR as the outcome variable and including all predictors in the table: predicted CYP2A6 activity score, age, and biological sex.

nicotine metabolism in a heterogenous group of ANAI people in Alaska who were attempting to quit smoking. A main finding was a strong association between *CYP2A6* diplotype and nicotine metabolism (i.e., plasma NMR). This was particularly evident when comparing the average observed NMR among slow, poor, intermediate, and normal phenotype classes, based on the observed diplotype (Figure 3). The association was primarily influenced by three common gene variations: the loss of function conferred by the **4* gene deletion, reduced function conferred by the **9* promoter variant and gain of function conferred by the **1B* variant in the 5′-flanking region of the gene. A similar but weaker association was observed between urine NMR and *CYP2A6* diplotype or predicted phenotype, consistent with the superiority of plasma NMR as a predictor of CYP2A6 activity.[15] Overall, 38% of the study group carried one or more reduced or nonfunctional alleles and we found that those alleles conferred a low NMR, suggesting a reduced capacity to metabolically eliminate nicotine from the body. The frequency of those individuals in the slow versus normal phenotype classes is similar to previously published data,[15,18] and the potential opportunity for clinical use of testing is high in this population. Because many people have normal nicotine metabolism, NMR testing would help guide tobacco cessation choice toward drugs with better efficacy. In this study, we included two types of NMR testing; NMR can be tested from saliva, blood, and urine. The collection of these samples varies in the degree of invasiveness for participants, and urine collection is not as invasive as blood collection and may be preferable for certain participants. In addition, with urine collection, specially trained phlebotomists would not be needed, and the sample would not need to be immediately spun with a centrifuge, as is the case for blood collection. This is especially important in the often rural communities where ANAI people reside.

The *CYP2A6* allele and diplotype frequencies observed in this sample of ANAI people who smoke was in agreement with frequencies reported in a previous study of the

same ANAI population but different sample set that included nonusers of tobacco products.[18] This included relatively high frequencies of the *CYP2A6*1B* and **9* alleles (63% and 21%, respectively), as well as the *CYP2A6*4* allele (10%) in the current study. These frequencies were also similar to those reported previously for a Yup'ik only AN population.[15] Importantly, individuals homozygous or heterozygous for the **1B* allele exhibited higher metabolic activity toward nicotine, on average, than those homozygous for the reference **1A* allele.[38] This noncoding change in the 5′-flanking region of *CYPA6*1B* could lead to increased mRNA stability and gene expression, and, as the 58 bp SV is part of a haplotype that includes a 3′-UTR variant, and the 3′-UTR variants have also been shown to stabilize *CYP2A6* mRNA, also increasing enzyme production.[39] A similar association between enhanced nicotine metabolism by *CYP2A6* and the *CYP2A6*1B* allele has been reported for European American and African American people who smoke,[38,40] but interestingly not Yup'ik people who smoke living in a southwestern Alaska community, although the trend for a higher plasma NMR in **1B* homozygotes in that study was the same as what we report.[15] In addition, individuals from both a Northern Plains and Southwestern AI tribal groups who were homozygous for the **1B* allele also exhibited a higher average plasma NMR than the corresponding subgroups homozygous for the **1A* reference allele.[17] As noted in the present study and by Tyndale et al.[15,17] and Claw et al.,[18] the **1B* allele frequency is appreciably higher in ANAI people from the Western United States and Alaska than is the frequency reported for other racial/ethnic groups of the world. There was also an association of plasma NMR with the duration of smoking, and this may result in increased difficulty quitting due to a mismatch between nicotine clearance (e.g., slow vs. fast) and initial treatment selection (i.e., patches).

Based on these findings among a subset of ANAI people receiving health care at SCF, many may have either the *CYP2A6*1B/*1B* or **1B/*1A* diplotype and are predicted to clear nicotine from the body more efficiently than those lacking the allele.[38] These frequencies approximate the frequencies reported in our previous study, with 31.6% for **1B/*1A*, 24.2% for **1B/*1B*, and 8.2% for **1A/*1A* for the SCF population.[18] Precision medicine NMR testing to identify people with slow versus normal nicotine metabolism could improve smoking cessation outcomes by reducing the use of an initial therapeutic treatment approach with a lower chance of success (nicotine replacement). A recent randomized clinical trial,[13] reported a higher smoking cessation success rate from varenicline therapy, compared to nicotine replacement therapy, among people with normal versus slow nicotine metabolism. In contrast, individuals who smoked that had slow nicotine metabolism did equally well on nicotine replacement therapy as varenicline and better tolerated nicotine replacement therapy compared to varenicline treatment. Whether these findings would extend to the noncontrolled setting of a tobacco cessation clinic in tribal health setting or elsewhere remains to be demonstrated through pragmatic research trials, but there is the possibility of significant benefit.

Although the association between *CYP2A6* diplotype and the metabolism of cotinine to 3-hydroxycotinine reported in this study and described by others is strong, most of the variance in CYP2A6 enzyme activity that we observed remains unexplained by the few SNVs tested here. Variation in other genes, such as the cytochrome P450 reductase (*POR*) that supports P450-mediated catalytic reactions and *AKR1D1*, which is a potential regulator of cytochrome P450 genes,[41] may contribute to the unexplained NMR variability. In addition, there are a number of NMR genomewide association study studies that have found genomewide significant signals in *CYP2A6* and in nearby genes, such as *CYP2B6*, *CYP2A7*, *EGLN2*, and *NUMBL*, among others.[42,43] Although much of the variability in nicotine metabolic clearance is heritable,[7,44] the environment is also expected to exert some effects.[7,45]

There are some limitations to the study design and results. We report that genetic and metabolism phenotype data was missing from some study participants. Some genotypic results were inconclusive. For the 11 individuals with inconsistent or low confidence calls, we repeatedly encountered assay difficulty across the genotyping panel for SNVs and copy number variants, perhaps because of unknown unique SNVs or structural variation, such as gene hybridization. We also were unable to quantify cotinine and 3-hydroxycotinine concentrations in some of the plasma and urine samples. The absence of these metabolites, in the presence of nicotine and other metabolic products, was assumed to be due to a complete loss of CYP2A6 activity conferred by loss of function alleles. However, there were also a few participants for whom we could not detect quantifiable levels of any nicotine metabolites. For these individuals, we assume that they had quit smoking cigarettes at least 1 day prior to sample collection. In addition, there were a few anomalous NMR-diplotype results likely due to random sampling and small sample numbers. These could also reflect uncontrolled nongenetic sources of variation in CYP2A6 metabolic activity. In addition, we did not test for rare *CYP2A6* variants or variation in other genes thought to contribute to differences in CYP2A6 activity. In addition, our urinary $TNE9_{uc}$ values are not creatinine corrected. Finally, our sampling was of ANAI people who smoke and were seeking cessation services and was not intended to represent all ANAI

who use tobacco products (e.g., chew or e-cigarettes). None of these limitations should have compromised our main study conclusions.

This study investigated whether variation in *CYP2A6* was associated with NMR in plasma and urine and has implications for smoking cessation therapy in an ANAI population. *CYP2A6* genotype was associated with both plasma and urine NMR among ANAI people who smoke. Moreover, a gain of function allele (*CYP2A6*1B*) was at high frequency and associated with a relatively higher NMR, compared to the reference (*CYP2A6*1A*) allele, in this ANAI population. Comprehensive care centers that provide a tobacco cessation program often have the support of a clinical laboratory service for measurement of cotinine in plasma and urine. The inclusion of 3HC as part of the analytical service can be readily adopted, as in the case of our institution, SCF. Results are typically reported within 24 h from sample collection and can be shorter if there is a compelling clinical need (e.g., cyclosporine or tacrolimus). In-house genotyping can be conducted on the same time-frame if the laboratory service is available. Unfortunately, such genetic testing is not routine outside of large academic medical centers. Prospective NMR testing, possibly with supplemental pharmacogenetic screening for *CYP2A6* diplotypes, could help guide smoking cessation treatment protocols, interventions, and programs in ANAI populations. We suggest these findings are extended to tribal health settings to demonstrate through pragmatic research trials the potential significant benefit and population impact of nicotine pharmacogenetics.

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CONFLICT OF INTEREST

R.F.T. has consulted for Quinn Emanuel and Ethismos Research, Inc. There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. All other authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

K.R.S., J.P.A., K.E.T., and K.G.C. wrote the manuscript. J.P.A., J.A.B., D.A.D., K.E.T., R.F.R., and K.G.C. designed the research. K.R.S., J.P.A., M.R.T., L.M.S., K.E.T., R.F.R., and K.G.C. performed the research. K.R.S., J.P.A., T.A.T., R.F.T., K.E.T., and K.G.C. analyzed the data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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