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Alaska Native smokers and smokeless tobacco users with slower CYP2A6 activity have lower tobacco consumption, lower tobacco-specific nitrosamine exposure and lower tobacco-specific nitrosamine bioactivation

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Nicotine, the psychoactive ingredient in tobacco, is metabolically inactivated by CYP2A6 to cotinine. CYP2A6 also activates procarcinogenic tobacco-specific nitrosamines (TSNA). Genetic variation in *CYP2A6* is known to alter smoking quantity and lung cancer risk in heavy smokers. Our objective was to investigate how CYP2A6 activity influences tobacco consumption and procarcinogen levels in light smokers and smokeless tobacco users. Cigarette smokers (n = 141), commercial smokeless tobacco users (n = 73) and iqmik users (n = 20) were recruited in a cross-sectional study of Alaska Native people. The participants' CYP2A6 activity was measured by both endophenotype and genotype, and their tobacco and procarcinogen exposure biomarker levels were also measured. Smokers, smokeless tobacco users and iqmik users with lower CYP2A6 activity had lower urinary total nicotine equivalents (TNE) and (methylnitrosamino)-1-(3)pyridyl-1-butanol (NNAL) levels (a biomarker of TSNA exposure). Levels of N-nitrosonornicotine (NNN), a TSNA metabolically bioactivated by CYP2A6, were higher in smokers with lower CYP2A6 activities. Light smokers and smokeless tobacco users with lower CYP2A6 activity reduce their tobacco consumption in ways (e.g. inhaling less deeply) that are not reflected by self-report indicators. Tobacco users with lower CYP2A6 activity are exposed to lower procarcinogen levels (lower NNAL levels) and have lower procarcinogen bioactivation (as indicated by the higher urinary NNN levels suggesting reduced clearance), which is consistent with a lower risk of developing smoking-related cancers. This study demonstrates the importance of CYP2A6 in the regulation of tobacco consumption behaviors, procarcinogen exposure and metabolism in both light smokers and smokeless tobacco users.

Abbreviations: 3HC, trans-3′-hydroxycotinine; CI, confidence interval; COT, cotinine; CPD, cigarettes per day; NM, normal metabolizers; NMR, nicotine metabolite ratio; NNK, 4-(methylnitrosamino)-1-(3)pyridyl-1-butanone; NNN, *N*-nitrosonornicotine; NNAL, (methylnitrosamino)-1-(3)pyridyl-1-butanol; RM, reduced metabolizers; TNE, total nicotine equivalents; TSNA, tobacco-specific nitrosamines.

†The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the Centers for Disease Control and Prevention.

Introduction

Smoking is the largest preventable cause of lung cancer. American Indians and Alaska Native peoples have the highest prevalence of tobacco usage among all ethnic groups in the USA (1). On average, Alaska Native smokers consume fewer cigarettes per day (CPD) compared with Caucasians (2), yet the incidence of lung cancer among Alaska Native people is higher than the US national average (3,4). In addition to smoking, a significant proportion of Alaska Native people use commercial smokeless tobacco products and iqmik. The latter is a homemade smokeless tobacco product containing alkaline ash (5). We investigated whether genetic variation in *CYP2A6*, a nicotine- and tobacco-specific nitrosamine metabolizing enzyme, alters tobacco consumption and procarcinogen levels that could influence the risk of developing lung cancer in this population.

Nicotine is the main psychoactive ingredient in tobacco. Genetic variants influencing the pharmacokinetics and pharmacodynamics of nicotine are associated with altered tobacco consumption and lung cancer risk (6,7). In humans, the majority of nicotine is metabolized to cotinine (COT) by CYP2A6 (8). COT is then metabolized to *trans*-3′-hydroxycotinine (3HC) exclusively by CYP2A6 (9). The human *CYP2A6* gene is highly polymorphic: about 25% of Caucasians, 50% of African Americans and 60% of Asians have at least one copy of a reduced function *CYP2A6* allele (10–12). *CYP2A6* genotype significantly alters nicotine clearance, and has been associated with altered tobacco consumption and tobacco-related lung cancer risk (6,13). In addition to *CYP2A6* genotype, the ratio of 3HC to COT (also known as the nicotine metabolite ratio, NMR) is an *in vivo* endophenotype of CYP2A6 activity (14). The NMR is stable throughout the day and correlates highly with *in vivo* nicotine clearance (14,15). Caucasian smokers who have one or more reduced function *CYP2A6* allele(s) (i.e. *CYP2A6*-reduced metabolizers, RM), or have lower plasma NMR, generally smoke fewer CPD and are less nicotine dependent compared with genotypical normal metabolizers (NM) or smokers with higher plasma NMR (6,16). However, such differences in CPD and nicotine-dependence scores by *CYP2A6* genotype or NMR are not observed in some light-smoking populations, such as African Americans (17). This may be due to the limited sensitivity of tobacco consumption indicators like CPD and carbon monoxide in these light smokers (17,18). The influence of *CYP2A6* genetic variation on smokeless tobacco use has never been investigated.

CPD, carbon monoxide levels and plasma COT are widely used indicators of nicotine and tobacco consumption in smokers; however, they have significant limitations. For example, CPD is subject to reporting bias and does not account for the interindividual differences in the depth of smoke inhalation or other smoking topography measures. This is particularly relevant in light smokers as the level of nicotine intake per cigarette is inversely related to reported CPD (19). Carbon monoxide has a short half-life, which limits its utility in sporadic/light smokers (17), and it is not an indicator of smokeless tobacco use. Plasma COT is specific to nicotine exposure, has a relatively long half-life, and in heavy smokers it correlates with tobacco consumption (19). However, since COT is both formed and removed at different rates by CYP2A6, reduced function of *CYP2A6* variants may alter COT clearance more substantially than nicotine clearance, thus limiting the utility of COT as a biomarker of nicotine exposure. Recently, urinary TNE, which represents the summation of urinary nicotine and its metabolites (nicotine, nicotine glucuronide, COT, COT glucuronide, 3HC, 3HC glucuronide, nicotine-*N*-oxide, cotinine-*N*-oxide and nornicotine), has been used as an alternative biomarker of nicotine consumption (20,21). TNE accounts for about 90% of a transdermally administered nicotine dose (22), and creatinine-adjusted (e.g. per milligram Cre) spot urinary TNE correlates strongly with daily nicotine

consumption (23). The advantage of urinary TNE is that metabolites from non-CYP2A6 enzymatic pathways are also accounted for, such that TNE is not influenced by the rate of metabolism via CYP2A6. In this study, urinary TNE was used as the primary biomarker of nicotine consumption to evaluate the influence of CYP2A6 activity on nicotine consumption in smokers and smokeless tobacco users.

Tobacco use is associated with an elevated risk of developing cancer (24). Nicotine itself is not carcinogenic, but the structurally related tobacco-specific nitrosamines (TSNA) such as 4-(methylnitrosamino)-1-(3)pyridyl-1-butanone (NNK) and *N*-nitrosonornicotine (NNN) are carcinogenic (24). Once absorbed, these nitrosamines are bioactivated by cytochrome P450s to their α-hydroxyl metabolites, which are then spontaneously converted to diazonium ions and can result in DNA adducts. In the case of NNK, it is α-hydroxylated by CYP2B6 with an affinity roughly 10 times higher (i.e. a lower K_m) than that of CYP2A6 (25). In contrast, the α-hydroxylation of NNN is thought to be primarily mediated by CYP2A6 (26,27). Individuals vary extensively in their procarcinogen exposure and metabolism (24,28,29). Urinary biomarkers can provide information on the intake and metabolism of procarcinogens. In the case of NNK, it is rapidly metabolized in humans and has not been detected in urine (30). Hence total urinary (methylnitrosamino)-1-(3)pyridyl-1-butanol (NNAL), a reductive metabolite of NNK, is commonly used as a biomarker of NNK intake (24). NNAL has a long half-life, and urinary NNAL levels are stable and highly specific to tobacco exposure (24,31). The long half-life (7–10 days) is especially important in light smokers as it provides an estimate of procarcinogen exposure averaged over an extended period of time (32,33). Like other markers of tobacco smoke exposure, urinary NNAL levels in smokers are dose-dependently associated with the risk of tobacco-related cancers (34). In contrast to NNK, the procarcinogenic NNN can be directly measured in urine and its levels have been associated with the risk of developing esophageal cancer (35). Since CYP2A6 plays a larger role in the metabolic activation of NNN versus NNK, we used urinary NNAL levels as a biomarker of NNK intake and exposure, whereas we used urinary NNN levels as a biomarker of residual, unmetabolized NNN.

In this study, we used plasma NMR as the endophenotype of CYP2A6 activity. NMR accounts for both inherited and non-inherited variations in CYP2A6 activity (36), which makes it a more thorough representation of the current rate of nicotine clearance than *CYP2A6* genotype. As NMR can be influenced by exposure to inhibitors or inducers we also used *CYP2A6* genotype; this dual approach also confirms that genetic variation in *CYP2A6* is the major influence on the smoking behavior differences observed in individuals with different NMR endophenotypes. Using both the NMR endophenotype and *CYP2A6* genotype, we tested the hypothesis that light smokers and smokeless tobacco users with lower CYP2A6 activity (i.e. in the lower NMR stratum or *CYP2A6*-reduced metabolizers, RM) have lower tobacco consumption as measured by urinary total nicotine equivalents (TNE), a biomarker of daily nicotine consumption. We also tested the hypothesis that individuals with lower CYP2A6 activity will have lower urinary NNAL levels, reflecting lower tobacco consumption, and higher urinary NNN levels, reflecting reduced NNN metabolism.

Materials and methods

Study design

A detailed description of recruitment procedures, participant characteristics and tobacco-related behaviors has been reported previously (37). Briefly, 234 Yupik tobacco users were recruited in local villages near Bristol Bay, Alaska. Male and female (55%) current smokers (n = 141), commercial smokeless tobacco users (n = 73) and iqmik users (n = 20) aged between 18 and 65 were eligible for this study; these are the same subjects as described previously except that two smokers with a mixed Yupik ancestry were not included in the current genetic analysis (36). The research protocol was approved by the Alaska Area IRB, the Bristol Bay Area Health Corporation Board, the IRB at the University of California San Francisco, the IRB at the Centers for Disease Control and Prevention and the University of Toronto Ethics Review office.

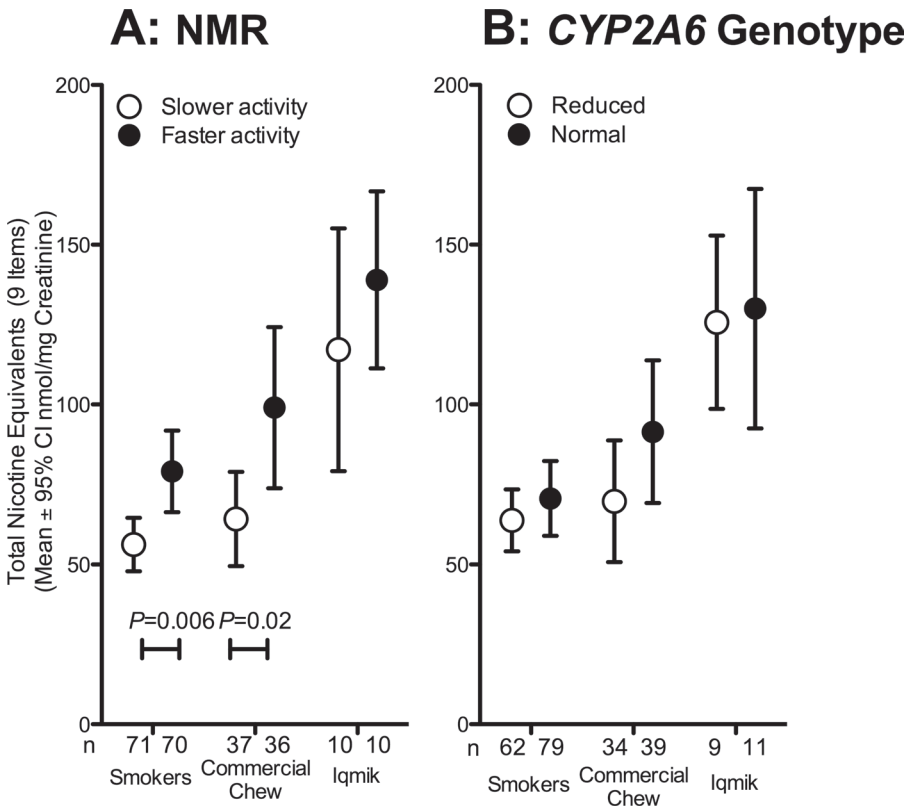

Fig. 1. Tobacco users with slower *CYP2A6* activity have lower nicotine intake: (**A**) plasma NMR strata and (**B**) *CYP2A6* genotypes. The statistical comparisons were made by Wilcoxon's test.

CYP2A6 genotyping

As described previously (36), DNA was extracted from blood (n = 234) using the Genelute purification kit according to the manufacturer's instructions (Sigma–Aldrich Ltd, Oakville, Ontario, Canada). Prevalent *CYP2A6* alleles with altered function (*CYP2A6: *2*, *4*, *7*, *9*, *10*, *12*, *17* and *35*) were genotyped by two-step-allele-specific PCR reactions according to previously reported methods (12,16,38). Those individuals with one or two copies of a reduced function allele (*2*, *4*, *7*, *9*, *10*, *12*, *17* and *35*) were classified as *CYP2A6* RM (12,39).

Nicotine metabolite and procarcinogen exposure measurements

Blood and urine samples were collected for the measurement of nicotine and its metabolites, as well as urinary total NNAL (i.e. NNAL and NNAL-glucuronide) and NNN (i.e. NNN and NNN-glucuronide) levels. Plasma COT and 3HC levels, and urinary nicotine, nicotine metabolites, NNAL and NNN levels were quantified by liquid chromatography-tandem mass spectrometry as described previously (14,40,41).

Statistical analyses

Statistical analyses were performed using Stata statistical package version 11 (StataCorp, College Station, TX). NMR stratification was performed by a median split of plasma NMR within each tobacco product group. Participants who were in the higher NMR stratum were considered the faster CYP2A6 activity group, whereas those in the lower NMR stratum were considered the lower CYP2A6 activity group. The biochemical measures (nicotine, nicotine metabolites, NNN and NNAL levels) were not normally distributed, thus pair-wise comparisons were performed by non-parametric Wilcoxon's tests. Also for this reason, regression analyses were conducted using log-transformed data.

Results

Alaska Native smokers with lower CYP2A6 activity exhibited lower tobacco consumption

Baseline demographics (e.g. age, gender and body mass index) did not differ between plasma NMR strata or *CYP2A6* genotype groups (Supplementary Table 1, available at *Carcinogenesis* Online). Lower tobacco consumption was observed in individuals with slower NMRs. Specifically, urinary TNE levels were significantly lower in smokers in the slower (n = 71) versus faster (n = 70) plasma NMR stratum (56.3 versus 79.2 nmol/mg Cre, respectively; P = 0.006; Figure 1 and Table I). Similar results by NMR strata were observed with a six-item TNE (i.e. nicotine, nicotine glucuronide, COT, COT glucuronide, 3HC and 3HC glucuronide), which is sometimes used as an alternative to the nine-item TNE (Table I) (19). The TNE levels did not significantly differ between *CYP2A6* genotypes (63.8 versus 70.7 nmol/mg Cre in *CYP2A6* RM and NM, respectively; Figure 1 and Table I).

Alaska Native commercial smokeless tobacco users with lower CYP2A6 activity exhibited lower tobacco consumption

Consistent with observations in smokers, commercial smokeless tobacco users in the slower (n = 37) versus faster (n = 36) plasma NMR stratum had 54% lower TNE (64.2 versus 99.0 nmol/mg Cre, respectively; P = 0.02; Figure 1 and Table II). Similar effects of NMR strata could be observed with the six-item TNE (Table II). The TNE levels did not significantly differ between *CYP2A6* genotypes (69.8 versus 91.5 nmol/mg Cre in *CYP2A6* RM and *CYP2A6* NM, respectively; P = 0.08; Figure 1 and Table II).

Alaska Native iqmik users with lower CYP2A6 activity had lower tobacco consumption

Among the small number of iqmik users, 19% lower TNE levels were observed in those in the slower (n = 10) compared with faster (n = 10) plasma NMR stratum (117.2 versus 139.0 nmol/mg Cre, respectively; P = 0.023; Figure 1 and Table II). However, no significant difference in TNE levels was observed by *CYP2A6* genotype group (Table II).

Table I. Urinary and plasma nicotine exposure biomarkers by plasma NMR stratum and *CYP2A6* genotype in smokers

Smokers (n = 141)	Plasma NMR strata						*CYP2A6* genotypes					
	Slower (n = 71)		Faster (n = 70)		$P_{absol.}$	$P_{\%}$	Reduced (n = 62)		Normal (n = 79)		$P_{absol.}$	$P_{\%}$
	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered			Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered		
Urinary (nmol/mg Cre)												
TNE (nine items)	**56.3 (35.3)**		**79.2 (53.6)**		**0.006**		63.8 (38.2)		70.7 (52.3)		0.681	
TNE (six items)[a]	**49.5 (31.7)**		**73.6 (50.1)**		**0.001**		56.3 (34.6)		65.5 (49.1)		0.416	
Total nicotine	12.0 (14.2)	**18.0 (14.6)**	7.3 (8.0)	**8.1 (6.3)**	0.070	**0.001**	12.5 (14.6)	**16.9 (15.2)**	7.5 (8.4)	**10.1 (8.2)**	0.082	**0.004**
Free	8.8 (12.3)	**12.5 (12.9)**	5.5 (7.1)	**5.9 (6.0)**	0.180	**0.001**	9.3 (12.7)	11.7 (12.9)	5.5 (7.4)	7.3 (7.8)	0.211	0.056
Glucuronide	**3.2 (3.4)**	**5.5 (4.2)**	**1.8 (1.5)**	**2.2 (1.4)**	**0.008**	**0.001**	**3.2 (3.5)**	**5.2 (4.5)**	**2.0 (1.7)**	**2.8 (2.0)**	**0.025**	**0.001**
Total COT	17.7 (13.0)	**31.4 (9.2)**	19.9 (14.8)	**24.3 (6.4)**	0.407	**0.001**	19.0 (13.4)	30.1 (9.7)	18.7 (14.4)	26.1 (7.5)	0.755	**0.01**
Free	8.9 (8.4)	**14.6 (6.7)**	9.4 (8.7)	**11.1 (4.5)**	0.473	**0.001**	9.5 (8.9)	13.9 (7.0)	8.9 (8.3)	12.1 (4.8)	0.813	0.205
Glucuronide	8.8 (5.6)	**16.8 (6.3)**	10.5 (7.0)	**13.1 (4.1)**	0.198	**0.001**	9.4 (5.6)	**16.1 (6.2)**	9.8 (7.0)	**14.0 (4.9)**	0.997	**0.034**
Total 3HC	**19.8 (13.3)**	**38.6 (15.4)**	**46.3 (33.2)**	**60.6 (11.8)**	**0.001**	**0.001**	**24.9 (17.8)**	**41.2 (17.6)**	**39.3 (33.3)**	**56.0 (14.6)**	**0.003**	**0.001**
Free	**17.2 (11.7)**	**33.8 (13.7)**	**39.4 (27.6)**	**51.5 (9.6)**	**0.001**	**0.001**	**21.7 (16.1)**	**35.8 (15.6)**	**33.4 (27.5)**	**47.9 (11.7)**	**0.004**	**0.001**
Glucuronide	**2.7 (2.7)**	**5.0 (4.6)**	**6.9 (6.6)**	**9.1 (4.9)**	**0.001**	**0.001**	**3.2 (3.1)**	**5.5 (4.8)**	**6.0 (6.5)**	**8.3 (5.1)**	**0.003**	**0.001**
Nornicotine	0.5 (0.4)	**0.8 (0.4)**	0.4 (0.3)	**0.5 (0.2)**	0.509	**0.001**	0.5 (0.4)	**0.8 (0.4)**	0.4 (0.3)	**0.6 (0.3)**	0.208	**0.001**
Nicotine-*N*-oxide	**4.5 (4.8)**	**7.7 (6.2)**	**2.8 (2.6)**	**3.4 (2.0)**	**0.025**	**0.001**	**5.0 (5.0)**	**7.6 (6.5)**	**2.6 (2.3)**	**4.0 (2.8)**	**0.001**	**0.001**
Cotinine-*N*-oxide	**1.8 (1.1)**	**3.5 (1.4)**	**2.4 (1.5)**	**3.1 (0.7)**	**0.019**	**0.044**	2.0 (1.2)	3.5 (1.4)	2.2 (1.5)	**3.2 (0.9)**	0.784	0.08
CPD	6.7 (4.4)		8.3 (5.9)		0.153		7.3 (4.9)		7.7 (5.5)		0.746	
Plasma												
COT (ng/ml)	171 (101)		170 (110)		0.795		177 (96)		165 (112)		0.26	
3HC (ng/ml)	**45.9 (33.6)**		**108.4 (77.2)**		**0.001**		**58.8 (46.3)**		**91.2 (76.8)**		**0.004**	
3HC/COT NMR	**0.263 (0.105)**		**0.685 (0.296)**		**0.001**		**0.316 (0.175)**		**0.595 (0.330)**		**0.001**	

$P_{\%}$, the P values from Wilcoxon's tests when the metabolites were normalized to percentage of urinary TNE. $P_{absol.}$ the P values from Wilcoxon's tests when the absolute urinary recovery values were compared. Bold values indicate statistically significant differences between the groups by Wilcoxon's test. Levels were compared between the normal metabolizers and the reduced metabolizers (individuals with at least one copy of reduced function *CYP2A6* alleles) or between the faster plasma NMR stratum and the slower plasma NMR stratum (median split).

[a]Compared with TNE (nine-items), TNE (six-items) did not included nornicotine, nicotine-*N*-oxide and cotinine-*N*-oxide.

Table II. Urinary and plasma nicotine exposure biomarkers by plasma NMR stratum and *CYP2A6* genotype in commercial smokeless tobacco users and imqik users

Commercial smokeless tobacco (n = 73)	Plasma NMR strata						*CYP2A6* genotypes					
	Slower (n = 37)		Faster (n = 36)		$P_{absol.}$	$P_{\%}$	Reduced (n = 34)		Normal (n = 39)		$P_{absol.}$	$P_{\%}$
	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered			Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered		
Urinary (nmol/mg Cre)												
TNE (nine items)	**64.2 (44.3)**		**99.0 (74.4)**		**0.022**		69.8 (54.6)		91.5 (68.7)		0.081	
TNE (six items)[a]	**57.2 (39.1)**		**91.7 (69.4)**		**0.012**		62.8 (49.4)		84.2 (64.1)		0.070	
Total nicotine	10.5 (11.1)	**15.0 (9.9)**	9.7 (11.7)	**8.5 (5.7)**	0.589	**0.001**	11.2 (11.8)	**15.2 (10.4)**	9.1 (11.0)	**8.9 (5.4)**	0.439	**0.002**
Free	7.5 (9.7)	9.9 (8.9)	7.1 (10.4)	6.0 (5.4)	0.757	0.051	8.0 (10.0)	**10.4 (9.3)**	6.7 (10.1)	**5.9 (5.0)**	0.493	**0.044**
Glucuronide	3.0 (2.1)	**5.1 (2.3)**	2.6 (2.6)	**2.6 (1.4)**	0.155	**0.001**	3.2 (2.8)	**4.8 (2.4)**	2.4 (1.7)	**3.0 (1.9)**	0.237	**0.001**
Total COT	22.1 (15.9)	**34.3 (8.0)**	24.4 (17.4)	**25.1 (6.8)**	0.699	**0.001**	23.1 (18.7)	**33.3 (8.5)**	23.4 (14.7)	**26.8 (7.7)**	0.658	**0.002**
Free	11.7 (10.3)	**17.7 (8.5)**	10.7 (7.9)	**10.9 (4.1)**	0.851	**0.001**	11.4 (10.3)	**16.8 (8.9)**	11.0 (8.2)	**12.2 (5.2)**	0.894	**0.026**
Glucuronide	10.3 (7.2)	**16.6 (6.0)**	13.8 (10.8)	**14.2 (5.3)**	0.208	**0.035**	11.7 (10.5)	16.5 (5.8)	12.3 (8.2)	14.5 (5.7)	0.439	0.091
Total 3HC	**24.6 (16.2)**	**39.8 (15.3)**	**57.6 (45.2)**	**59.2 (10.0)**	**0.001**	**0.001**	**28.4 (24.0)**	**41.2 (16.5)**	**51.7 (43.5)**	**56.5 (12.0)**	**0.002**	**0.01**
Free	**22.0 (14.9)**	**35.5 (14.4)**	**49.5 (37.9)**	**51.9 (11.4)**	**0.001**	**0.001**	**24.6 (20.6)**	**36 (15.4)**	**45 (36.4)**	**50.2 (12.1)**	**0.001**	**0.01**
Glucuronide	**2.7 (2.6)**	**4.5 (3.8)**	**8.2 (8.4)**	**7.4 (4.6)**	**0.002**	**0.005**	3.9 (4.7)	5.3 (4.4)	6.7 (7.9)	6.5 (4.4)	0.116	0.228
Nornicotine	0.6 (0.4)	**0.9 (0.4)**	0.6 (0.5)	**0.5 (0.2)**	0.604	**0.001**	0.6 (0.5)	**0.9 (0.5)**	0.6 (0.4)	**0.6 (0.3)**	0.666	**0.001**
Nicotine-*N*-Oxide	4.2 (4.0)	**6.1 (3.6)**	3.6 (3.1)	**3.5 (2.2)**	0.635	**0.001**	3.9 (3.7)	5.6 (3.6)	3.9 (3.4)	4.1 (2.8)	0.965	0.091
Cotinine-*N*-Oxide	2.3 (1.7)	**3.8 (1.3)**	3.1 (2.3)	**3.2 (0.6)**	0.103	**0.02**	2.5 (1.9)	**3.8 (1.3)**	2.8 (2.2)	**3.1 (0.6)**	0.514	**0.008**
Chew per day	6.3 (7.8)		5.2 (3.3)		0.911		6.6 (8.0)		4.9 (3.4)		0.588	
Can per week	1.7 (1.3)		1.6 (1.6)		0.307		1.8 (1.5)		1.5 (1.4)		0.387	
Plasma												
COT (ng/ml)	223 (116)		223 (148)		0.708		235 (129)		213 (135)		0.432	
3HC (ng/ml)	**58 (36)**		**137 (95)**		**0.001**		**71 (55)**		**119 (94)**		**0.009**	
3HC/COT NMR	**0.256 (0.092)**		**0.700 (0.275)**		**0.001**		**0.293 (0.135)**		**0.633 (0.319)**		**0.001**	
Iqmik users (n = 20)	Slower (n = 10		Faster (n = 10)		$P_{absol.}$	$P_{\%}$	Reduced (n = 9)		Normal (n = 11)		P_{absol}	$P_{\%.}$
	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered			Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered	Absolute values mean (SD)	Percentage of urinary nicotine equivalents recovered		
Urinary (nmol/mg Cre)												
TNE (nine items)	**117.2 (53.0)**		**139.0 (38.7)**		**0.023**		125.7 (35.3)		130.0 (55.7)		0.85	
TNE (six items)[a]	104.4 (47.9)		129.1 (37.3)		0.11		112.0 (30.9)		120.8 (53.1)		0.73	
Total nicotine	15.3 (8.1)	13.6 (6.1)	17.1 (10.2)	11.6 (5.7)	0.82	0.65	16.7 (3.2)	14.2 (4.8)	15.8 (12.1)	11.3 (6.5)	0.91	0.31
Free	10.3 (6.8)	8.5 (5.2)	13.4 (10.9)	8.8 (6.6)	0.50	1.00	10.7 (4.5)	9.1 (4.8)	12.8 (11.6)	8.3 (6.7)	0.91	0.73
Glucuronide	5.1 (3.7)	**5.1 (4.1)**	3.7 (3.1)	**2.8 (2.6)**	0.17	**0.04**	5.9 (4.3)	5.1 (4.4)	3.1 (1.7)	3.0 (2.6)	0.06	0.14
Total COT	34.3 (13.0)	32.0 (10.6)	31.2 (13.3)	23.0 (8.8)	0.60	0.07	38.1 (13.2)	30.3 (7.7)	28.4 (11.5)	25.2 (12.2)	0.06	0.10
Free	16.8 (9.3)	**14.5 (4.5)**	13.5 (8.3)	**9.5 (4.8)**	0.45	**0.02**	17.3 (9.9)	13.0 (5.0)	13.4 (7.8)	11.1 (5.3)	0.47	0.43
Glucuronide	17.5 (5.7)	17.5 (8.1)	17.7 (8.2)	13.5 (7.1)	0.76	0.17	20.9 (8.1)	17.2 (7.1)	14.9 (4.4)	14.1 (8.2)	0.10	0.16
Total 3HC	**54.8 (37.0)**	**43.6 (15.1)**	**80.8 (29.1)**	**58.2 (10.6)**	**0.04**	**0.02**	57.2 (23.0)	**44.7 (10.6)**	76.5 (41.5)	**56.0 (16.1)**	0.27	**0.02**
Free	**52.4 (38.6)**	40.9 (16.2)	**69.4 (26.2)**	50.1 (11.6)	**0.05**	0.20	52.4 (23.9)	41.0 (11.4)	67.9 (39.1)	49.3 (16.1)	0.38	0.12
Glucuronide	**2.4 (4.8)**	**2.6 (3.8)**	**11.4 (7.0)**	**8.1 (3.8)**	**0.01**	**0.01**	4.7 (8.0)	3.7 (5.3)	8.7 (6.9)	6.7 (3.7)	0.18	0.18
Nornicotine	1.0 (0.6)	**0.8 (0.2)**	0.8 (0.4)	**0.6 (0.2)**	0.71	**0.03**	1.0 (0.4)	0.8 (0.1)	0.8 (0.5)	0.6 (0.3)	0.43	0.31
Nicotine-*N*-Oxide	7.9 (4.3)	**6.8 (2.5)**	4.8 (2.7)	**3.5 (1.8)**	0.10	**0.01**	8.2 (4.0)	**6.5 (2.8)**	4.9 (3.1)	**4.0 (2.2)**	0.09	**0.04**
Cotinine-*N*-Oxide	3.8 (2.1)	3.3 (0.9)	4.2 (1.3)	3.1 (1.0)	0.33	0.71	4.6 (2.0)	**3.6 (0.8)**	3.5 (1.4)	**2.9 (1.0)**	0.27	**0.04**
Chew per day	4.1 (1.4)		4.6 (2.9)		0.97		4.2 (1.1)		4.5 (2.9)		0.67	
Can per week	1.3 (1.7)		1.0 (0.6)		0.75		0.8 (0.3)		1.5 (1.6)		0.39	
Plasma												
COT (ng/ml)	369 (198)		322 (201)		0.43		369 (244)		318 (140)		0.94	
3HC (ng/ml)	160 (108)		246 (230)		0.47		135 (126)		280 (214)		0.03	
3HC/COT NMR	**0.345 (0.144)**		**0.843 (0.367)**		**0.001**		0.459 (0.207)		0.704 (0.447)		0.24	

$P_{\%}$, the P values from Wilcoxon's tests when the metabolites were normalized to percentage of urinary TNE. $P_{absol.}$ the P values from Wilcoxon's tests when the absolute urinary recovery values were compared. Bold values indicate statistically significant differences between the groups by Wilcoxon's test. Levels were compared between the normal metabolizers and the reduced metabolizers (individuals with at least one copy of reduced function *CYP2A6* alleles) or between the faster plasma NMR stratum and the slower plasma NMR stratum (median split).

[a]Compared with TNE (nine items), TNE (six items) did not include nornicotine, nicotine-*N*-oxide and cotinine-*N*-oxide.

Urinary nicotine metabolite profiles differed between CYP2A6 activity groups in smokers

Figure 2A shows the total and proportional level of nicotine and each metabolite in smokers' urine (n = 141) by plasma NMR stratum, illustrating the dominant role of CYP2A6 in the metabolic removal of nicotine. Smokers in the slower plasma NMR stratum excreted 72% of their TNE via the CYP2A6-mediated COT pathways (i.e. COT and its metabolites, darker shades, Figure 2A), whereas >85% of TNE was excreted via the COT pathway among smokers in the faster plasma NMR stratum ($P < 0.0001$, Figure 2A). As a result, smokers in the slower plasma

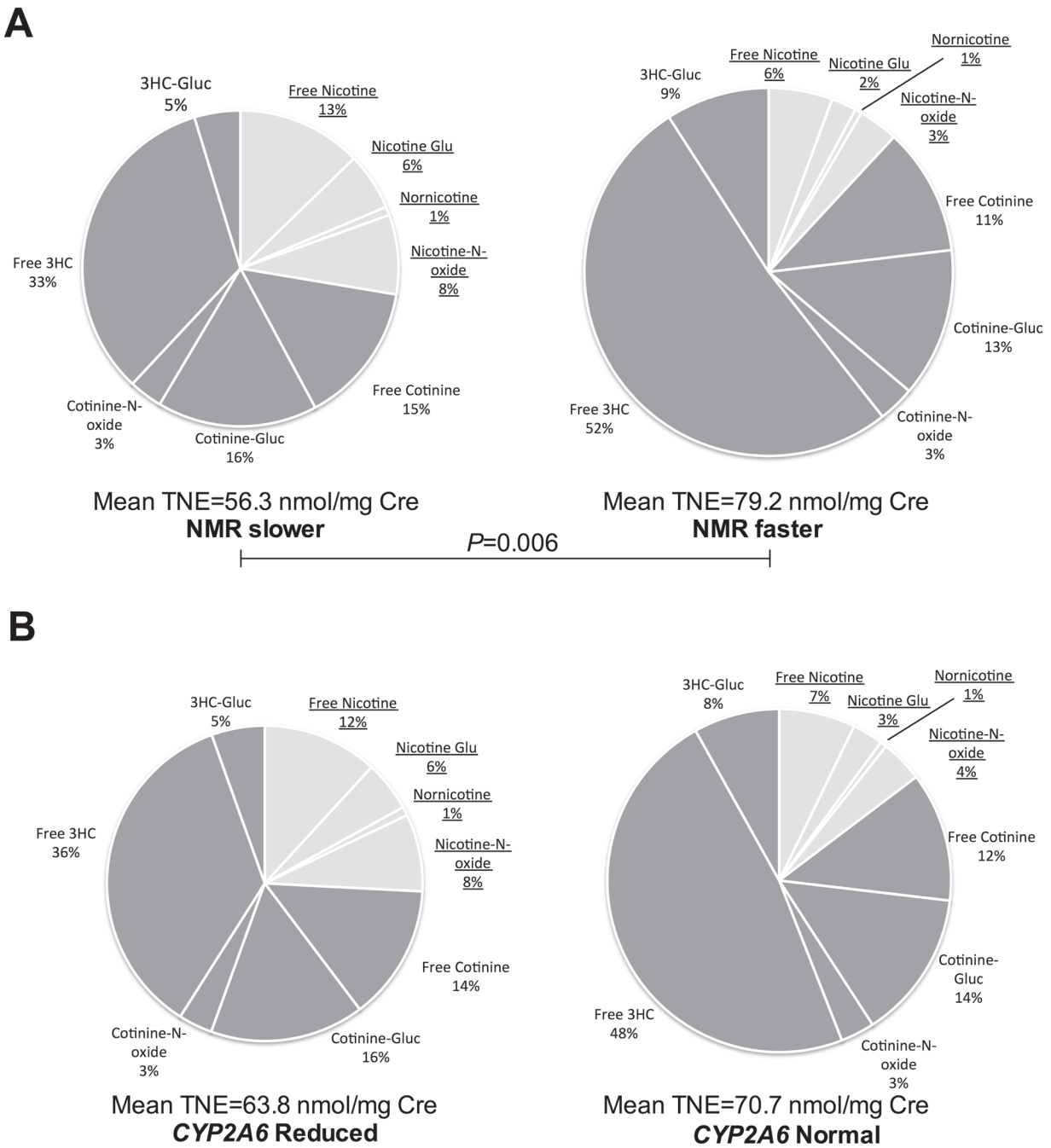

Fig. 2. Total tobacco consumption, and nicotine's metabolic profile, differed between (**A**) NMR strata and (**B**) *CYP2A6* genotypes in cigarette smokers ($n = 141$). The total size of pie charts represents the amount of urinary TNE. The percentage of TNE recovered as each nicotine metabolite is represented by the size of the section. Cotinine and its subsequent metabolites are represented by the darker shades, nicotine and the remaining metabolites are represented by the lighter shades.

NMR stratum excreted a significantly higher percentage of consumed nicotine (i.e. TNE) as free nicotine, nicotine glucuronide, nornicotine and nicotine-*N*-oxide ($P < 0.05$; Table I and Figure 2A) and a significantly lower percentage as 3HC and its glucuronide ($P < 0.001$; Table I and Figure 2A). Urinary nicotine metabolite profiles among smokers also differed between *CYP2A6* genotype groups (Figure 2B and Table I).

Urinary nicotine metabolite profiles differed between CYP2A6 activity groups in commercial smokeless tobacco and iqmik users

Similar to Alaska Native smokers, commercial smokeless tobacco users and iqmik users in the slower plasma NMR stratum excreted 78% of their TNE via the COT pathway, whereas >85% of TNE were excreted via the COT pathway in individuals in the faster plasma NMR stratum ($P < 0.01$; Table II). Similar to the results observed using plasma NMR stratum, urinary nicotine metabolite profiles also differed by *CYP2A6* genotype group in commercial smokeless tobacco users and iqmik users (Table II).

Neither plasma NMR strata nor CYP2A6 genotypes were associated with self-reported indicators of tobacco consumption or plasma COT levels

Despite the 41% difference in tobacco consumption as indicated by TNE, Alaska Native smokers in the slower versus faster plasma NMR stratum reported smoking similar to CPD (6.7 versus 8.3, respectively, non-significant) and had similar plasma COT levels (235 versus 213 ng/ml, respectively, non-significant; Table I). There were also no significant differences between plasma NMR strata in the number

of chews per day, the number of cans of smokeless tobacco used per week (standard Copenhagen-sized cans) or the levels of plasma COT in commercial smokeless tobacco users or iqmik users (Table II). Likewise, *CYP2A6* genotype was not associated with differences in self-reported indicators of tobacco consumption or plasma COT levels in smokers, commercial smokeless tobacco users or iqmik users (Tables I and II).

Urinary NNAL levels trended lower in individuals with lower CYP2A6 activity

There were 27 and 18% differences in urinary NNAL levels between NMR strata and *CYP2A6* genotype groups, respectively, but they were not significant (1.1 versus 1.4 pmol/mg Cre in the slower and faster plasma NMR stratum, respectively; 1.1 versus 1.3 pmol/mg in *CYP2A6* RM and NM, respectively; Table III). Among the commercial smokeless tobacco users, we observed 62% lower NNAL levels in the slower compared with the faster plasma NMR stratum (4.5 versus 7.3 pmol/mg Cre, respectively; $P = 0.01$; Table III). A similar difference in magnitude was also observed between *CYP2A6* genotypes (4.6 versus 7.1 pmol/mg Cre in *CYP2A6* RM and NM, respectively; $P = 0.006$; Table III). No difference in NNAL levels was observed between NMR strata or *CYP2A6* genotypes among the small iqmik group (Table III). As expected, the effects of NMR strata and *CYP2A6* genotypes on NNAL levels (a measure of exposure) were eliminated when nicotine dose (TNE) was controlled for (Supplementary Table 2A and B, available at *Carcinogenesis* Online).

Urinary NNN levels were higher in smokers with lower CYP2A6 activity

As total tobacco consumption (i.e. TNE) differed between NMR strata, we compared urinary NNN levels while controlling for TNE. NNN levels were higher in slower than in faster NMR strata [$B = 0.41$, $\beta = 0.17$, 95% confidence interval (CI) = 0.02–0.79, $P = 0.04$; (Table IVA). A 39% difference in NNN levels between NMR strata was observed when not controlling for tobacco dose, but this did not reach statistical significance (0.182 versus 0.110 pmol/mg Cre, respectively, non-significant; Table III). Significantly higher urinary NNN levels were observed in *CYP2A6* RM compared with *CYP2A6* NM (0.230 versus 0.083 pmol/mg Cre, respectively; $P = 0.05$; Table III), which was also greater when TNE was controlled for (by *CYP2A6* genotype: $B = 0.60$, $\beta = 0.24$, 95% CI = 0.21–0.98, $P = 0.003$; Table IVA). Among the commercial smokeless tobacco users, urinary NNN levels were moderately, but non-significantly, higher in those with slower compared with faster plasma NMR (0.126 versus 0.107 pmol/mg Cre, respectively; Table III), and the difference between the two groups increased when TNE was controlled for (by plasma NMR strata: $B = 0.41$, $\beta = 0.18$, 95% CI = –0.067 to 0.887, $P = 0.09$; Table IVB). No difference in NNN levels was observed between *CYP2A6* genotype groups among the commercial smokeless tobacco users (0.119 versus 0.113 pmol/mg Cre in *CYP2A6* RM and *CYP2A6* NM, respectively, $P = 0.67$; regression analysis by *CYP2A6* genotype: $B = –0.06$, $\beta = –0.03$, 95% CI = –0.544 to 0.418, $P = 0.793$, Tables III and IVB). No difference in urinary NNN levels was observed by NMR stratum or *CYP2A6* genotype group among iqmik users (Table III).

Discussion

This study revealed three novel findings, each supporting the key role of CYP2A6 in influencing tobacco consumption and the risk of developing tobacco-related disease. First, Alaska Native smokers, who smoked on average <10 CPD (i.e. light smokers), titrated their daily tobacco consumption (i.e. TNE) according to their CYP2A6 activities similarly to previous observations among Caucasian heavier smokers (6). Second, variation in CYP2A6 activity was associated with altered levels of tobacco use among commercial smokeless tobacco users and iqmik users. Third, Alaska Native smokers and smokeless tobacco users with lower CYP2A6 activities excreted lower levels of NNAL, reflecting lower NNK exposure as a result of their lower tobacco consumption and they may possess reduced NNN metabolic activation (inferred through higher residual urinary NNN levels). Together, lower tobacco and procarcinogen consumption and lower procarcinogen bioactivation would be expected to decrease the risk of tobacco-related cancers in individuals with lower CYP2A6 activities, consistent with a lower risk seen among heavy smoking Caucasians (6).

In this study, the levels of urinary TNE in Alaska Native smokers were comparable with those among Caucasian heavy smokers despite smoking <10 CPD (six-item TNE: 61.4 nmol/mg Cre in Alaska Native smokers versus 60–63 nmol/mg Cre in Caucasian smokers with ~20 CPD) (19,20). This suggests that Alaska Native smokers inhaled each cigarette more deeply and/or for a longer duration, resulting in similar amounts of nicotine (and presumably procarcinogens) exposure to that observed in Caucasian heavy smokers despite smoking fewer CPD (42). Several studies have observed that cigarette smokers with lower CYP2A6 activities smoke fewer CPD (6,16), but this difference in CPD has not been observed in light smoking populations such as African Americans (17), nor was it observed in this Alaska Native group. Smokers who consume comparatively fewer cigarettes typically inhale more intensely than smokers who consume more cigarettes, hence nicotine and procarcinogen intake per cigarette is inversely related to numbers of reported CPD (19). In this study, smokers with lower CYP2A6 activity (i.e. slower plasma NMR stratum or *CYP2A6* RM) had lower TNE levels while reporting similar daily cigarette use. This suggests that Alaska Native smokers with lower CYP2A6 activity reduced their nicotine consumption per cigarette to compensate for their slower nicotine inactivation, presumably by inhaling smaller and/or fewer puffs, a phenomenon observed previously in heavier smokers (43). The 41% reduction in TNE between NMR strata in Alaska Native smokers was larger than the 28% reduction in CPD observed in Caucasian heavy smokers between *CYP2A6* genotypes (6), suggesting consumption differences between CYP2A6 activity groups, when measured more accurately, could be larger than

Table III. Urinary procarcinogen biomarker levels in smokers, commercial smokeless tobacco users and iqmik users

	Plasma NMR strata			*CYP2A6* genotypes		
	Slower	Faster	*P*	Reduced	Normal	*P*
Smokers ($n = 141$)	$n = 71$	$n = 70$		$n = 62$	$n = 79$	
NNAL (pmol/mg Cre), mean (SD)	1.1 (0.8)	1.4 (1.2)	0.52	1.1 (0.8)	1.3 (1.2)	0.45
NNN (pmol/mg Cre), mean (SD)	0.182 (0.390)	0.110 (0.200)	0.57	**0.230 (0.450)**	**0.083 (0.093)**	**0.05**
Commercial smokeless tobacco users ($n = 73$)	$n = 37$	$n = 36$		$n = 34$	$n = 39$	
NNAL (pmol/mg Cre), mean (SD)	**4.5 (3.6)**	**7.3 (6.0)**	**0.01**	**4.6 (4.0)**	**7.1 (5.6)**	**0.006**
NNN (pmol/mg Cre), mean (SD)	0.126 (0.114)	0.107 (0.088)	0.68	0.119 (0.119)	0.113 (0.084)	0.67
Iqmik users ($n = 20$)	$n = 10$	$n = 10$		$n = 9$	$n = 11$	
NNAL (pmol/mg Cre), mean (SD)	0.8 (0.6)	0.7 (0.3)	0.65	0.9 (0.51)	0.7 (0.42)	0.34
NNN (pmol/mg Cre), mean (SD)	0.756 (1.95)	0.154 (0.195)	0.71	0.205 (0.211)	0.660 (1.87)	0.52

Bold values indicate statistically significant differences between the groups by Wilcoxon's test.

Table IV. NNN levels were significantly higher in smokers with slower *CYP2A6* activity when tobacco consumption was controlled for

A: Smokers	*B*	β	95% CI	*P*
Plasma NMR: $R^2 = 0.30$, $P < 0.001$				
TNE (increasing, per nmol)	**0.01**	**0.53**	**0.010,0.019**	**0.001**
NMR (slower strata)	**0.41**	**0.17**	**0.023,0.791**	**0.038**
Age (increasing)	0.01	0.08	–0.006,0.020	0.297
CYP2A6 genotype: $R^2 = 0.33$, $P < 0.001$				
TNE (increasing, per nmol)	**0.01**	**0.52**	**0.010,0.018**	**0.001**
CYP2A6 genotype (reduced)	**0.6**	**0.24**	**0.212,0.979**	**0.003**
Age (increasing)	0.00	0.03	–0.011,0.015	0.730
B: Commercial smokeless tobacco				
Plasma NMR: $R^2 = 0.36$, $P < 0.001$				
TNE (increasing, per nmol)	**0.01**	**0.63**	**0.007,0.015**	**0.001**
NMR (slower strata)	0.41	0.18	–0.067,0.887	0.091
Age (increasing)	0.00	–0.02	–0.021,0.017	0.825
CYP2A6 genotype: $R^2 = 0.33$, $P < 0.001$				
TNE (increasing, per nmol)	**0.01**	**0.58**	**0.007,0.014**	**0.001**
CYP2A6 genotype (reduced)	–0.06	–0.03	–0.544,0.418	0.793
Age (increasing)	–0.01	–0.05	–0.025,0.015	0.612

B is the unstandardized coefficient. β is the standardized coefficient (i.e. standardized to a variance of 1). The *B* and β provided refer to the variables listed in brackets beside each categorical predictor. The NNN levels were log-transformed to obtain a normal distribution.

those predicted by CPD (6). Substantial intersubject variation in TNE levels was observed between NMR strata and *CYP2A6* genotypes in the iqmik users. This is probably due to variation in the preparation procedures (e.g. content of the basic ash and premastication) and chewing behavior (chew duration and whether swallows the iqmik juice) (5). Due to these differences, biochemical markers such as TNE provide a better measure of nicotine exposure than the self-report iqmik consumption measures.

In this study, the effect of NMR strata on urinary TNE was stronger than the effect of *CYP2A6* genotype probably reflecting a closer relationship of NMR (versus *CYP2A6* genotype) to the phenotype of interest, CYP2A6 activity. This is most probably because of the influence of gender, body mass index and dietary inducers/inhibitors on CYP2A6 activity (12,44). Further, there may be novel *CYP2A6* genetic variants among the Alaska Native people, which were not investigated in our study (36).

To the best of our knowledge, this is the first demonstration of tobacco consumption titration by CYP2A6 activity among smokeless tobacco users. The consumption differences between plasma NMR strata among the commercial smokeless tobacco users were slightly larger than those observed in smokers (54 versus 41% lower TNE, respectively). This may be a reflection of slower nicotine absorption from smokeless tobacco versus cigarette smoking (45), which could allow for a longer period of time for metabolism to occur thereby increasing the effect of differences in the rates of nicotine metabolism.

Although urinary TNE levels differed by CYP2A6 activity group among Alaska Native smokers and commercial smokeless tobacco users, plasma COT levels did not. Plasma COT is often used as a biomarker of nicotine exposure, however, it is both metabolically formed and removed by CYP2A6. Lower CYP2A6 activity might differentially reduce the relative rates of COT formation versus COT removal resulting in the accumulation of COT among slower CYP2A6 metabolizers. Thus, plasma COT might overestimate nicotine dose in individuals with lower CYP2A6 activities.

In addition to influencing tobacco consumption, genetic variation in *CYP2A6* can also alter lung cancer risk (6). Some earlier studies in Caucasians did not find an association between *CYP2A6* and the risk for developing lung cancer (46). However, these studies did not genotype the majority of loss of function *CYP2A6* variants and suffered from reduced power (46). In recent studies, with more comprehensive genotyping, significant associations between *CYP2A6* and the risk for developing lung cancer have been found (6,47). This may be due to differences in both tobacco consumption and procarcinogen metabolic activation by *CYP2A6* genotype. Tobacco-specific nitrosamines such as NNK, NNN and NNAL are all potent procarcinogens (24). Commercial smokeless tobacco users with lower CYP2A6 activity had lower NNAL levels, which was eliminated when controlling for TNE levels, suggesting that the lower NNAL levels are a result of lower tobacco consumption rather than differences in NNK metabolism. In fact, we observed no evidence that variation in CYP2A6 activity had any effect on NNAL levels beyond the effect of CYP2A6 on tobacco consumption: the relative amount of NNK eliminated via the NNAL pathway did not differ between CYP2A6 activity groups in our study consistent with human genetic association studies (48).

Higher NNN levels were observed in the smokers with lower CYP2A6 activity, which was even greater after controlling for nicotine intake (i.e. TNE). We speculate that the lower CYP2A6 activity resulted in lower NNN clearance (i.e. lower bioactivation) resulting in higher levels of urinary NNN. CYP2A6 mediates the α-hydroxylation of NNN to genotoxic metabolites (26). If not detoxified, these genotoxic metabolites can covalently bind to DNA which can result in mutations. This is consistent with human transfection studies in which a higher degree of NNN-induced mutagenesis was observed in the CYP2A6-transfected cells than the control cells (27). However, more research is needed to understand the role of urinary NNN as a predictor of tobacco-related cancers cancer risk—to date, a dose-dependent relationship has only been seen for esophageal cancer (35).

There are limitations to our study. Our study only included Alaska Native individuals and the application of the findings to other race/ethnicities requires further investigation. Secondly, this study had a relatively small smokeless tobacco group, particularly for individuals who exclusively used iqmik.

Together our data indicate that Alaska Native light smokers and smokeless tobacco users titrate their tobacco consumption according to CYP2A6 activity. Previous failures to see evidence of titration by CYP2A6 activity among light smokers may be due to the poor sensitivity of the tobacco consumption indictors used (e.g. CPD). Furthermore, consistent with the lower tobacco consumption, individuals with lower CYP2A6 activities also had lower procarcinogen exposure (i.e. NNK and NNAL). In addition, lower CYP2A6 activity was associated with higher NNN levels suggesting reduced bioactivation. Together these findings support a reduced risk of tobacco-related cancers for slow versus fast, CYP2A6 metabolizers, in both smokers and smokeless tobacco users.

Supplementary material

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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