

UGT1A and UGT2B Genetic Variation Alters Nicotine and Nitrosamine Glucuronidation in European and African American Smokers

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Abstract

Background: Identifying sources of variation in the nicotine and nitrosamine metabolic inactivation pathways is important to understanding the relationship between smoking and cancer risk. Numerous UGT1A and UGT2B enzymes are implicated in nicotine and nitrosamine metabolism *in vitro*; however, little is known about their roles *in vivo*.

Methods: Within *UGT1A1*, *UGT1A4*, *UGT1A9*, *UGT2B7*, *UGT2B10*, and *UGT2B17*, 47 variants were genotyped, including *UGT2B10*2* and *UGT2B17*2*. The association between variation in these UGTs and glucuronidation activity within European and African American current smokers ($n = 128$), quantified as urinary ratios of the glucuronide over unconjugated compound for nicotine, cotinine, trans-3'-hydroxycotinine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), was investigated in regression models assuming a dominant effect of variant alleles.

Results: Correcting for multiple testing, three *UGT2B10* variants were associated with cotinine glucuronidation, rs2331559 and rs11726322 in European Americans and rs835309 in African

Americans ($P \leq 0.0002$). Additional variants predominantly in *UGT2B10* were nominally associated with nicotine ($P = 0.008-0.04$) and cotinine ($P = <0.001-0.02$) glucuronidation in both ethnicities in addition to *UGT2B10*2* in European Americans ($P = 0.01$, $P < 0.001$). *UGT2B17*2* ($P = 0.03$) in European Americans and *UGT2B7* variants ($P = 0.02-0.04$) in African Americans were nominally associated with 3HC glucuronidation. *UGT1A* ($P = 0.007-0.01$), *UGT2B10* ($P = 0.02$), and *UGT2B7* ($P = 0.02-0.03$) variants in African Americans were nominally associated with NNAL glucuronidation.

Conclusions: Findings from this initial *in vivo* study support a role for multiple UGTs in the glucuronidation of tobacco-related compounds *in vivo*, in particular *UGT2B10* and cotinine glucuronidation.

Impact: Findings also provide insight into ethnic differences in glucuronidation activity, which could be contributing to ethnic disparities in the risk for smoking-related cancers. *Cancer Epidemiol Biomarkers Prev*; 24(1); 94-104. ©2014 AACR.

Introduction

Cigarette smoking is the leading risk factor for lung cancer (1, 2), and is also associated with numerous other cancers includ-

ing those of the respiratory tract, digestive tract, bladder, pancreas, and kidney (3, 4). Genetic factors are associated with differences in the susceptibility to tobacco-related cancers including genes involved in the metabolism of nicotine and tobacco smoke carcinogens (5-7). For instance, *CYP2A6* gene variants are associated with reduced lung cancer risk within smokers of diverse ethnicities (8-13), as is a gene variant in *CYP2A13* (14), key enzymes in the nicotine inactivation pathway and in the activation pathway of tobacco-specific nitrosamines (TSNA) such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), respectively (15, 16).

UDP-glucuronosyltransferases (UGT) represent another group of enzymes with the potential to influence the relationship between smoking and cancer risk via their contribution to the metabolism of both nicotine and nitrosamines, including NNK (Fig. 1). In addition to the major metabolic pathway of nicotine to cotinine and further to trans-3'-hydroxycotinine (3HC) chiefly mediated by *CYP2A6*, nicotine, cotinine, and 3HC are also substrates of UGTs (17). Glucuronide conjugates account for 25% to 30% of recovered nicotine metabolites in urine (18-20). NNK is extensively metabolized by carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which, like NNK, is also carcinogenic (21, 22). NNAL is then metabolically detoxified by glucuronidation, and the noncarcinogenic glucuronide conjugates account for approximately 60% of NNAL detected in urine (23, 24).

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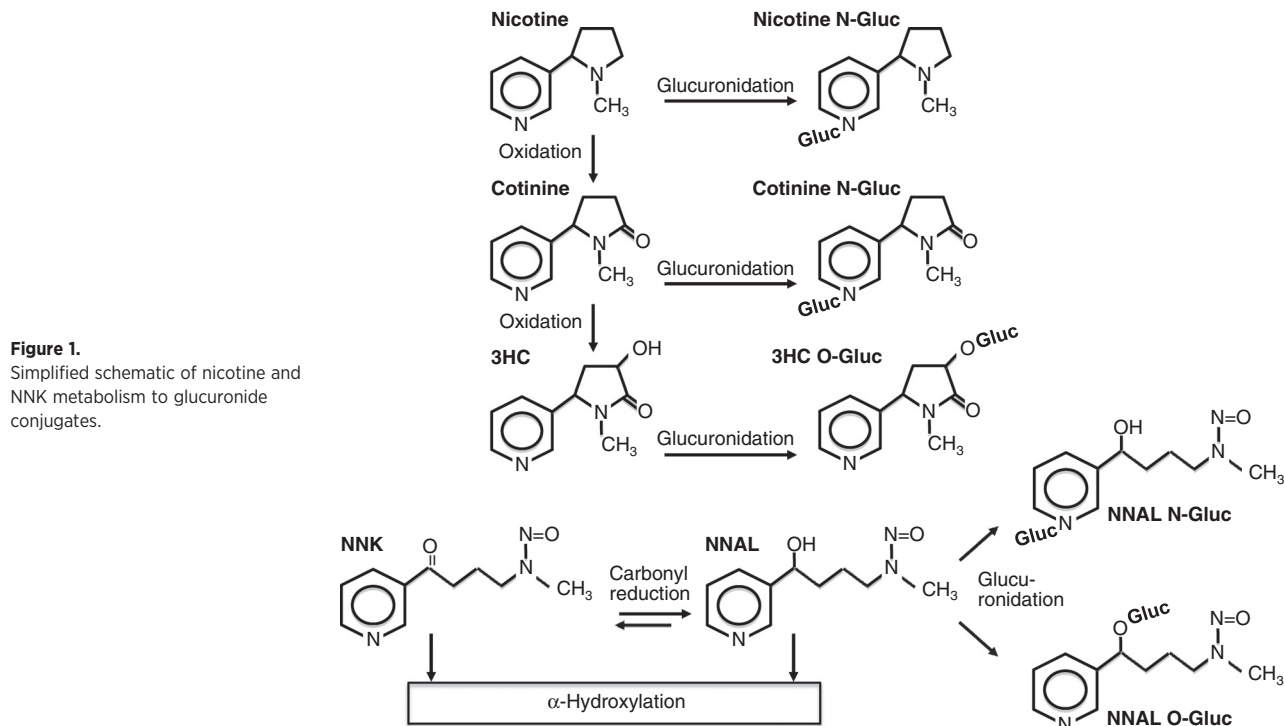


Figure 1. Simplified schematic of nicotine and NNK metabolism to glucuronide conjugates.

UGT gene variants may have utility in cancer risk prediction—deficient activity of some UGT enzymes enhances susceptibility to chemical carcinogenesis in animals (25, 26), and genetic variants in *UGT1A7*, *UGT1A10*, and *UGT2B17* are associated with the risk for tobacco-related cancers in humans (27–30). In addition, it is important to understand the impact of variable glucuronidation on biomarkers of nitrosamine exposure and on the ratio of NNAL-glucuronide to NNAL, a biomarker of nitrosamine detoxification (31) and a potential marker of cancer risk (32). Variable glucuronidation could also influence smoking through effects on nicotine clearance and could impact the interpretation of biomarkers of nicotine exposure and metabolism (33–35).

Many polymorphisms have been identified in the genes encoding the UGT1A and UGT2B enzymes (36); however, there are limited data on the impact of UGT variants on nicotine and nitrosamine metabolism *in vivo*, and in current cigarette smokers (37, 38). *In vivo* investigations have focused on two alleles, *UGT2B10*2* and *UGT2B17*2*. *UGT2B10*2* is associated with impaired nicotine and cotinine glucuronidation, whereas *UGT2B17*2* is associated with impaired 3HC and NNAL glucuronidation (20, 27, 34, 35, 39).

In vitro studies of human liver microsomes and expressed UGTs implicate additional UGT1A and UGT2B enzymes in the glucuronidation of nicotine, its metabolites, and NNAL. In human liver microsomes, *UGT2B10*2* is associated with reduced glucuronidation of nicotine and cotinine, but also 3HC and NNAL (33, 40, 41), *UGT2B17*2* is associated with reduced glucuronidation of 3HC and NNAL (33, 42), and *UGT1A4*2* and *UGT2B7*2* are associated with reduced glucuronidation of NNAL (42, 43). Inhibition studies in human liver microsomes implicate UGT1A1 in nicotine glucuronidation (44), UGT1A4 in nicotine, cotinine, and 3HC glucuronidation (44–46), and UGT1A9 in nicotine and cotinine glucuronidation (44, 46). Recombinant UGTs from

baculovirus-infected insect cells or overexpressed UGTs in human cell lines also implicate UGT1A4 in the glucuronidation of nicotine, cotinine, and NNAL (47, 48) and provide evidence for the involvement of UGT1A9 in the glucuronidation of 3HC and NNAL (45, 49) and for UGT2B7 in the glucuronidation of nicotine and 3HC (45, 47).

Given the data suggesting the involvement of multiple UGT1A and UGT2B enzymes *in vitro*, and the limited knowledge of the contribution of UGT gene variants to nicotine and nitrosamine glucuronidation *in vivo*, we investigated the association between variation in *UGT1A1*, *UGT1A4*, *UGT1A9*, *UGT2B7*, *UGT2B10*, and *UGT2B17*, and nicotine, cotinine, 3HC, and NNAL glucuronidation in European and African American current smokers. As few UGT gene variants have been characterized with respect to these substrates, and the functional impact of UGT gene variants can be substrate specific (50), we genotyped 43 tag SNPs within the candidate genes. We also genotyped two *UGT1A9* insertion/deletion variants and, importantly, *UGT2B10*2* and *UGT2B17*2*, the only alleles previously associated with impaired *in vivo* glucuronidation in smokers (20, 35, 39). To gauge the suitability of the dataset for genetic association analyses of urinary metabolite ratios, we genotyped participants for altered activity *CYP2A6* variants to confirm the well-established association between *CYP2A6* and the ratio of 3HC over cotinine, a biomarker for *CYP2A6* activity and nicotine clearance (Supplementary Results; ref. 51). In addition to identifying which UGT gene variants influence the glucuronidation of tobacco-related compounds among European Americans, a population in which sources of variation in glucuronidation are better characterized (*in vitro* studies almost exclusively utilize livers from individuals of European descent), the present study evaluated whether the same UGT variants were associated with glucuronidation among African Americans to provide pilot data focused on understanding the

higher risk of smoking-related lung cancer observed among African Americans compared with European Americans (52, 53).

Materials and Methods

Study description

Participant recruitment and characteristics are detailed elsewhere (54). Briefly, 128 current smokers required to be 18 to 65 years old, healthy, and to have smoked an average of 10 cigarettes per day or more for the past year or longer were recruited for a cross-sectional biomarker study. Subjects had to be self-identified non-Hispanic white (referred to as European American) or African American, with 4 grandparents of the same ethnicity. Smoking measures were collected and the time from smoking the last cigarette before urine sampling was recorded. One European American and one African American participant were excluded from the main genotype–phenotype analysis due to the absence of a urine sample and insufficient *UGT* genotyping results, respectively. Participant characteristics for the remaining 126 individuals are provided (Table 1). The study was approved by Institutional Review Boards at the University of California San Francisco (San Francisco, CA), the University of Chicago (Chicago, IL), and the University of Toronto (Ontario, Canada).

UGT Genotyping

Tag SNPs were selected within the candidate genes (± 10 kb) by determining the minimal set of common SNPs (minor allele frequency $\geq 5\%$) that captured the pairwise linkage disequilibrium ($r^2 > 0.8$) for all common SNPs within the HapMap CEU population. In HapMap2 release 24, the set of SNPs capture (at an $r^2 \geq 0.8$), on average, more than 80% of existing common SNPs within the CEU population, and in the YRI population they capture on average 37% of the existing common SNPs (Supplementary Table S1).

*UGT2B10**2 (rs61750900) was genotyped using a PCR restriction fragment length polymorphism in which the amplified product was subjected to digestion with *HinfI* (40). The *UGT2B17**2 deletion allele was assessed using the TaqMan gene expression assay, HS00854486_sH, with copy number reference assay TaqMan RNase P for the internal control, determined by TaqMan real-time quantitative PCR (55–57). Genotyping for 41 tag SNPs in *UGT1A4*, *UGT1A9*, *UGT2B7*, and *UGT2B10* was done

using the KASPar SNP genotyping system by LGC Genomics (formerly KBiosciences, LGC Limited). Genotyping for the *UGT1A9* indels, rs45625337 and rs10538910, was performed using the in-house GeneScan (PCR-Sizing) assays run on ABI 3700 as described previously (58). Genotyping for *UGT1A4*_135498 C>A (rs6755571) and *UGT1A4*_135876 T>C (rs12468274) was performed using a SNaPshot (Applied Biosystems, Life Technologies) single base extension 2-plex assay as described (59). The extension products were run on an ABI 3130xl (Applied Biosystems), and the data analyzed by the GeneMapper software (Applied Biosystems).

Analytical chemistry

Urine concentrations of nicotine and its metabolites cotinine, 3HC, and their respective glucuronide metabolites were measured by liquid chromatography/tandem mass spectrometry from spot urine with glucuronide conjugates calculated from the difference in total concentration before and after alkaline hydrolysis (nicotine, cotinine) or hydrolysis by β -glucuronidase (3HC, NNAL) as previously described (18, 51, 54, 60). Urine creatinine was measured in the San Francisco General Hospital clinical laboratory using a standard colorimetric assay.

Metabolite phenotypes

To investigate *UGT* genotype–phenotype relationships, the urinary ratios of nicotine-glucuronide over nicotine, cotinine-glucuronide over cotinine, 3HC-glucuronide over 3HC, and NNAL-glucuronide over NNAL were used as glucuronidation activity phenotypes (27, 35, 39). The urinary ratio of total 3HC (free and glucuronide conjugated) over free cotinine was used as a phenotype of CYP2A6 oxidative metabolism (51). Nicotine equivalents, a measure of the total intake of nicotine, were calculated as the molar sum of nicotine, cotinine, 3HC, and their glucuronide metabolites in urine corrected for creatinine concentration (61).

Statistical analyses

Comparison of demographic, smoking, and metabolite phenotypes in African versus European Americans was performed by Wilcoxon rank-sum test (continuous variables) or χ^2 (categorical variables). Correlations between metabolite phenotypes were performed on nontransformed values by Spearman. Geometric

Table 1. Characteristics of participants included in analyses, $n = 126^a$

Characteristic	European American, $n = 66$	African American, $n = 60$	P^b
Male, no. (%)	38 (58)	35 (58)	0.93
Age in years, median (IQR)	34 (25–45)	41 (36–49)	<0.01
Body mass index, median (IQR)	24 (22–28)	27 (24–33)	<0.01
Menthol cigarette use, no. (%)	18 (27)	41 (68)	<0.01
Cigarettes/day ^c , median (IQR)	20 (14–20)	15 (10–20)	0.04
Nicotine equiv. (nmol/mg-creat) ^d , median (IQR)	63 (54–75)	44 (37–53)	<0.01
Min from last cig, median (IQR)	80 (50–107)	90 (60–140)	0.08
Activity ratios (geometric mean, 95% CI)			
NIC-Gluc/free NIC	0.33 (0.25–0.44)	0.17 (0.13–0.23)	<0.01
COT-Gluc/free COT	1.03 (0.87–1.23)	0.40 (0.28–0.57)	<0.01
3HC-Gluc/free 3HC	0.19 (0.17–0.22)	0.26 (0.23–0.30)	<0.01
NNAL-Gluc/free NNAL	2.06 (1.87–2.27)	1.85 (1.61–2.12)	0.33

Abbreviation: IQR, interquartile range.

^a128 participants in the original study, 67 European American and 61 African American: One sample dropped due to absence of urinary biomarkers and another due to poor genotyping call rate.

^b P values: nonparametric Wilcoxon rank-sum test or χ^2 .

^cAverage of self-reported cigarettes/day in the three days before biomarker collection.

^dNicotine equivalents: urinary sum of nicotine, cotinine, trans-3-hydroxycotinine, and their glucuronide conjugates normalized to creatinine.

mean values are presented for non-normally distributed values unless indicated otherwise. Linkage disequilibrium between the *UGT1A* and *UGT2B* variants was assessed using Haploview (62), whereas other statistical analyses were performed using Stata13 (StataCorp). Hardy-Weinberg equilibrium tests were performed using the Stata program hwsnp (Mario A. Cleves, University of Arkansas for Medical Sciences, Little Rock, AR). Regression association analyses between *UGT* variants and glucuronidation phenotypes were performed using the Stata program qtlstnp assuming a dominant effect of the variant allele (Mario A. Cleves, University of Arkansas for Medical Sciences, Little Rock, AR). All regression models were performed separately within each ethnicity and were adjusted for age, gender, and menthol smoking. Because of missing genotypes and/or biomarker data of the individuals, the number of observations within each model ranged from 60 to 66 among European Americans and from 51 to 60 among African Americans. In total 36 *UGT* variants were tested against four phenotypes in European Americans and 43 *UGT* variants were tested against four phenotypes in African Americans, hence the significance thresholds were set at $P \leq 0.0003$ and 0.0002 , respectively, as determined by a Bonferroni correction for multiple testing. Nominally significant associations are also presented ($P \leq 0.05$).

Results

Glucuronidation phenotypes

The distribution of each of the four glucuronide phenotype ratios by ethnicity was assessed (Fig. 2A–D). African Americans had lower nicotine and cotinine glucuronide ratios compared with European Americans, as reported previously (39, 63). 3HC glucuronide ratios were higher among African Americans compared with European Americans, whereas no ethnic differences in NNAL glucuronide ratios were observed. Within-subject nicotine and cotinine glucuronide ratios were correlated in each ethnic group, but neither was correlated with the 3HC glucuronide ratio (Table 2), consistent with previous studies using 24-hour urine (18, 39). The NNAL glucuronide ratio was correlated with the cotinine glucuronide ratio and with the 3HC glucuronide ratio in each ethnicity (Table 2).

Demographic characteristics and urinary sampling parameters were evaluated as potential covariates of the glucuronide ratios. Demographic characteristics differed by ethnicity—African Americans were older ($P < 0.01$), had a higher BMI ($P < 0.01$), reported fewer cigarettes smoked per day ($P = 0.04$), and a greater prevalence of menthol cigarette use ($P < 0.01$; Table 1). Correlations between glucuronide ratios and time from last cigarette, creatinine

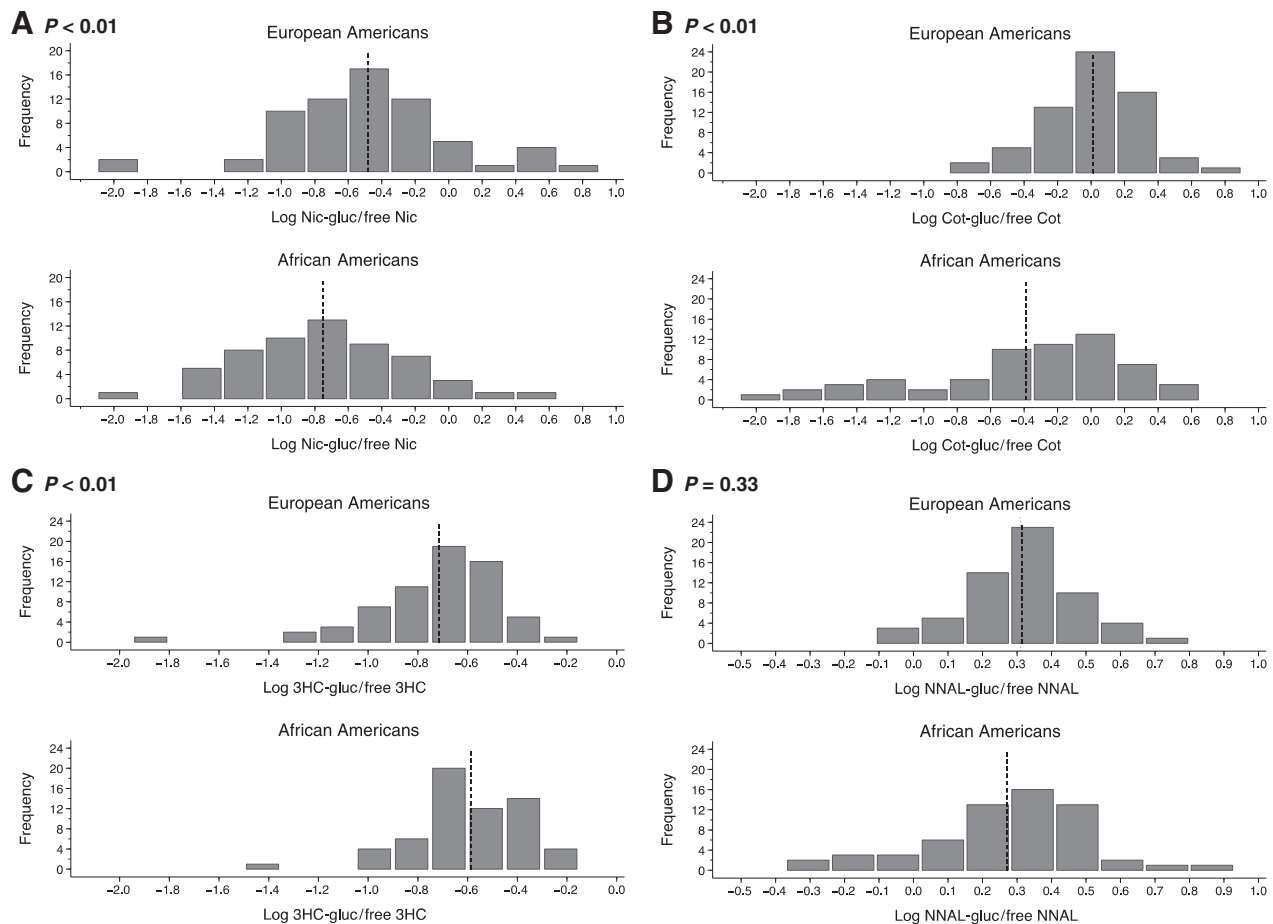


Figure 2. Glucuronidation phenotypes among European Americans and African Americans expressed as the logarithm transformed ratio of glucuronide conjugate over free nicotine in A, cotinine in B, trans-3-hydroxycotinine in C, and NNAL in D. Dotted lines indicate mean values of logarithm transformed phenotypes (refer to Table 1 for nontransformed means). *P* values, A–D, from nonparametric Wilcoxon rank-sum test comparing the phenotype distribution by ethnicity.

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Table 2. Spearman correlations between glucuronidation activity measures (nontransformed)

	ρ , <i>P</i> value	NIC-Gluc ratio	COT-Gluc ratio	3HC-Gluc ratio	NNAL-Gluc ratio	
EA	NIC-Gluc ratio	—	0.49, <0.001	-0.20, 0.13	0.19, 0.15	AA
	COT-Gluc ratio	0.34, 0.005	—	-0.05, 0.71	0.56, <0.001	
	3HC-Gluc ratio	0.10, 0.41	0.13, 0.31	—	0.32, 0.01	
	NNAL-Gluc ratio	0.04, 0.76	0.34, 0.007	0.38, 0.002	—	

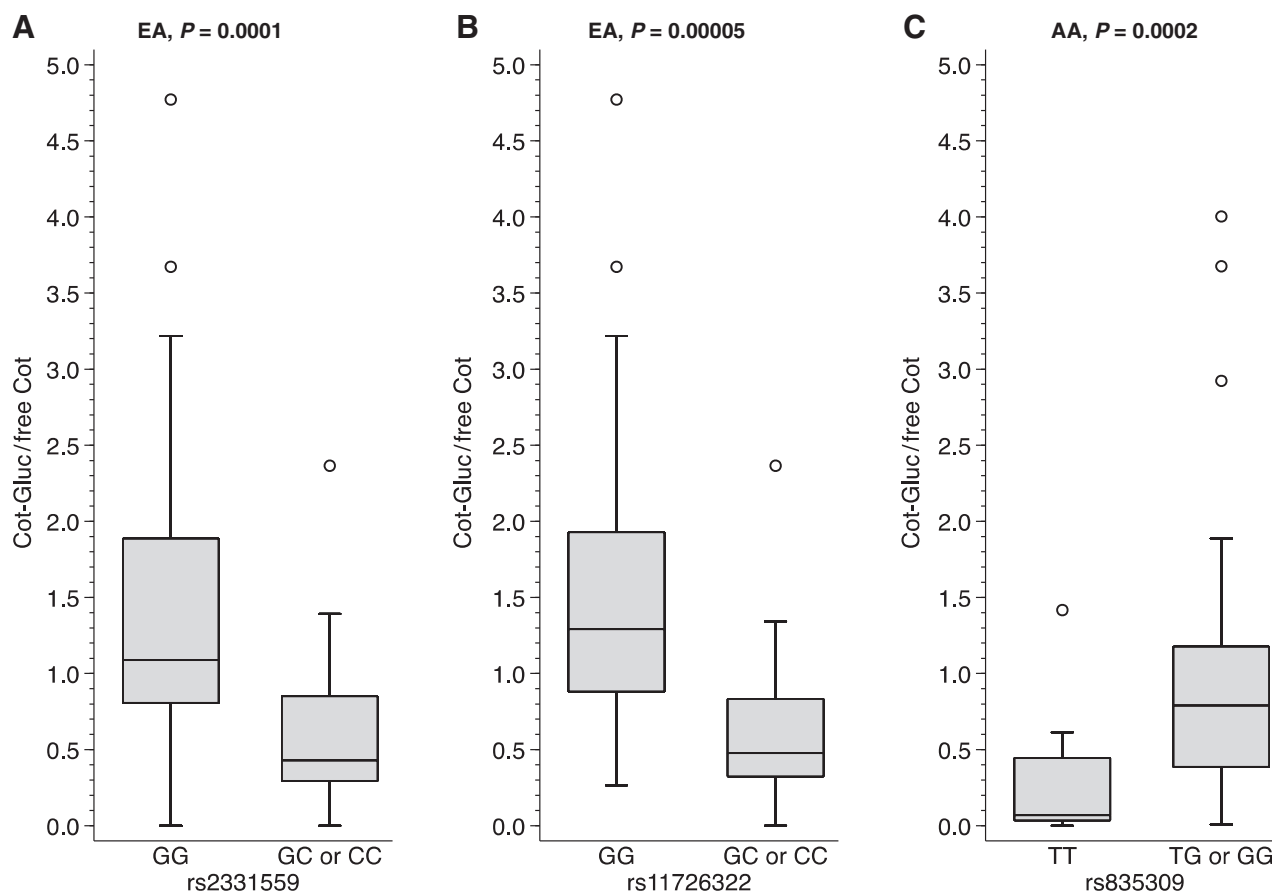
NOTE: Data for EA are in lower left side; AA are in upper right side (shaded). Abbreviations: EA, European American; AA, African American.

concentration, and nicotine equivalents were also assessed, as metabolites were quantified from spot urine. No consistent patterns emerged between glucuronide ratios and age, BMI, creatinine, nicotine equivalents, or time from last cigarette (Supplementary Table S2). No differences in glucuronide ratios by gender were noted among either ethnicity, but 3HC and NNAL glucuronide ratios were lower among African American menthol versus nonmenthol smokers (Supplementary Table S3). Menthol is glucuronidated by enzymes such as UGT2B7 and UGT2B17 (64, 65), which are also capable of glucuronidating 3HC and NNAL (33, 42); thus, menthol could act as a competitive inhibitor, and menthol was included as a covariate in genotype-phenotype analyses. Age and gender were also included as covariates due to

a priori evidence that these variables may influence the activity of specific UGTs (37, 66–68).

UGT genotyping results

The *UGT2B10**2 allele, rs61750900T, had a minor allele frequency of 9% and 4% among European and African Americans, respectively, and the *UGT2B17**2 allele had a minor allele frequency of 30% and 21% among European and African Americans, respectively, consistent with published frequencies (27, 35, 39). Among European Americans, rs3771342 and rs835310, and among African Americans, rs12468274 and rs12468543, were not consistent with Hardy-Weinberg Equilibrium and were excluded. Among European Americans, 3 variants in the *UGT1A*

**Figure 3.**

UGT2B10 variants significantly associated with cotinine glucuronidation following correction for multiple testing. Individuals heterozygous or homozygous for the minor alleles of rs2331559 in A and rs11726322 in B had lower cotinine glucuronide ratios, whereas individuals heterozygous or homozygous for the minor allele of rs835309 in C had higher cotinine glucuronide ratios. Genotype-phenotype relationships illustrated with box plots displaying the median value and interquartile range (25th to the 75th percentile) and whiskers displaying the upper and lower values within 1.5 times the interquartile range, and open circles displaying outlying individuals. *P* values from multivariate logistic regression modeling assuming a dominant effect of the minor allele. EA: European American; AA: African American.

locus and 6 variants in the *UGT2B* locus displayed high linkage disequilibrium ($R^2 > 0.9$; Supplementary Fig. S1A and S2A, respectively). Among African Americans, no variants in the *UGT1A* locus and only 2 variants in the *UGT2B* locus displayed high linkage disequilibrium (Supplementary Fig. S1B and S2B, respectively). Variants in high linkage disequilibrium were also excluded leaving 36 and 43 *UGT* variants in subsequent genotype-phenotype analyses in European and African Americans, respectively (Supplementary Table S4).

Glucuronidation associations

Three *UGT2B10* gene variants were statistically significantly associated with cotinine glucuronidation (Fig. 3) following correction for multiple testing (outlined in statistical methods). Among European Americans, individuals heterozygous or homozygous for the minor alleles of rs2331559 and rs11726322 had significantly lower cotinine glucuronide ratios compared with those homozygous for the major alleles (Fig. 3A and B). Among African Americans, individuals heterozygous or homozygous for the minor allele of rs835309 had significantly higher cotinine glucuronide ratios compared with those homozygous for the major allele (Fig. 3C). Nominally significant associations are also reported in the following sections, as this was the first investigation of multiple candidate genes chosen based on *in vitro* data with the goal of providing a relative sense of the potential importance of these candidate genes in the glucuronidation of nicotine and nitrosamines in smokers.

Nicotine glucuronidation

Two *UGT2B10* variants were nominally associated with impaired nicotine glucuronidation activity in European Americans, whereas in African Americans, two *UGT2B10* variants were nominally associated with enhanced and two with impaired activity (Table 3). A variant in the *UGT1A4* locus and two in the *UGT1A1* locus were also nominally associated with nicotine glucuronidation in African Americans.

Cotinine glucuronidation

In addition to the two *UGT2B10* variants significantly associated with impaired cotinine glucuronidation (Fig. 3A and B), *UGT2B10**2 was nominally associated with impaired cotinine

glucuronidation activity, whereas a single variant in *UGT2B7* was nominally associated with enhanced glucuronidation in European Americans (Table 4). Among African Americans, in addition to the *UGT2B10* variant significantly associated with enhanced cotinine glucuronidation (Fig. 3C), one other *UGT2B10* variant was nominally associated with enhanced and two with impaired activity (Table 4). Two variants in the *UGT1A4* locus and single variants in the common *UGT1A* exons and 3' flanking region of *UGT1A* were also associated with cotinine glucuronidation in African Americans (Table 4).

3HC glucuronidation

Among European Americans, the *UGT2B17* copy number variant was nominally associated with impaired 3HC glucuronidation activity (Table 5). Among African Americans, two variants in the *UGT2B7* locus were nominally associated with impaired 3HC glucuronidation activity (Table 5).

NNAL glucuronidation

No *UGT* variants reached nominal significance with NNAL glucuronidation activity among European Americans (Table 6). Among African Americans, two *UGT2B7* variants were nominally associated with NNAL glucuronidation, one with enhanced, and one with impaired activity (Table 6). Single variants in *UGT1A1*, the 3' flanking region of *UGT1A*, and *UGT2B10* were also associated with NNAL glucuronidation in African Americans (Table 6).

Discussion

This is the first study to investigate variation in multiple candidate *UGT1A* and *UGT2B* genes and glucuronidation activity within the nicotine and nitrosamine metabolic pathways among European and African American current smokers. Before this investigation, only *UGT2B10**2 and *UGT2B17**2 had been shown to influence the glucuronidation of tobacco-related compounds *in vivo* (20, 27, 34, 35, 39); whereas additional *UGT1A* and *UGT2B* enzymes were implicated in these pathways *in vitro* (33, 40–42, 44–49). As few *UGT* variants have been functionally characterized with respect to our substrates of interest, we focused this initial investigation on tag SNPs to provide a more comprehensive examination of the contribution of *UGT* genetic variation to

Table 3. *UGT* variants nominally associated with the nicotine glucuronide ratio

UGT variant	European American smokers				African American smokers			
	Alleles	MAF	P^{dom}	Coef (95% CI)	Alleles	MAF	P^{dom}	Coef (95% CI)
UGT1A4 locus								
rs13401281	T:G	0.409			T:G	0.475	0.012	0.35 (0.08–0.62)
UGT1A1 locus								
rs6742078	G:T	0.408			G:T	0.379	0.049	0.26 (0.001–0.52)
rs3771342 ^a	C:A	0.112			C:A	0.117	0.024	–0.38 (–0.72 to –0.05)
UGT2B10 locus								
rs61750900 (*2)	G:T	0.090	0.013	–0.42 (–0.76 to –0.09)	G:T	0.043		
rs2331559	G:C	0.119	0.015	0.38 (0.07–0.68)	C:G	0.362	0.008	0.34 (0.09–0.60)
rs835309 ^b	G:T	0.106			T:G	0.491	0.026	0.34 (0.04–0.64)
rs11726322	G:C	0.129			G:C	0.254	0.010	–0.34 (–0.60 to –0.09)
rs7673996	C:T	0.031			C:T	0.225	0.042	–0.27 (–0.53 to –0.01)

Abbreviation: MAF, minor allele frequency.

^aVariant excluded from analyses in European Americans.

^b P value not reported in European Americans as variant in high linkage disequilibrium with rs2331559. Alleles indicated as major:minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% confidence intervals from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < 0.05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*).

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Table 4. *UGT* variants nominally/significantly associated with the cotinine glucuronide ratio

UGT SNP/variant	Alleles	European American smokers			Alleles	African American smokers		
		MAF	P^{dom}	Coef (95% CI)		MAF	P^{dom}	Coef (95% CI)
<i>UGT1A4</i> locus								
rs3732220	C:T	0.092			C:T ↑	0.108	0.018	0.50 (0.09–0.91)
rs871514	G:A	0.478			G:A ↓	0.359	0.027	−0.36 (−0.68 to −0.04)
<i>UGT1A</i> exon 2–5								
rs1018124	T:C	0.056			T:C ↑	0.108	0.013 ^a	0.48 (0.10–0.86)
<i>UGT1A</i> 3' flanking								
rs10209214	T:C	0.313			T:C ↓	0.142	0.023 ^a	−0.39 (−0.72 to −0.05)
<i>UGT2B10</i> locus								
rs61750900 (*2)	G:T ↓	0.090	0.0004	−0.37 (−0.56 to −0.17)	G:T	0.043		
rs294765	G:A	0.015			G:A ↓	0.217	0.017	−0.39 (−0.71 to −0.07)
rs2331559	G:C ↓	0.119	0.0001	−0.36 (−0.53 to −0.19)	C:G ↑	0.362	0.006	0.43 (0.13–0.74)
rs835309 ^b	G:T	0.106			T:G ↑	0.491	0.0002	0.66 (0.33–0.99)
rs835310 ^c	C:G	0.023			C:G ↓	0.408	0.005	−0.46 (−0.78 to −0.14)
rs11726322	G:C ↓	0.129	0.00005	−0.35 (−0.51 to −0.19)	G:C	0.254		
<i>UGT2B7</i> locus								
rs4356975	C:T ↑	0.381	0.042	0.17 (0.01–0.33)	C:T	0.397		

Abbreviation: MAF, minor allele frequency.

^aAbsence of individuals homozygous for the minor allele.

^b P value not reported in European Americans as variant in high LD with rs2331559.

^cVariant excluded from analyses in European Americans. Alleles indicated as major: minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% CIs from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < 0.05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*).

variation in the glucuronidation of tobacco-related compounds in European Americans. We also examined whether these tag SNPs chosen in European Americans were associated with glucuronidation among African American smokers.

Our findings confirm an important contribution of genetic variation in *UGT2B10* to nicotine and cotinine glucuronidation with multiple *UGT2B10* variants nominally associated with nicotine and with cotinine glucuronidation in both ethnicities. Three variants in *UGT2B10* remained statistically significantly associated with cotinine glucuronidation following correction for multiple testing. Of note, the two variants significantly associated with cotinine glucuronidation in European Americans, rs2331559 and rs11726322, had similar effect sizes to *UGT2B10**2 (Table 4). *UGT2B10**2 likely did not reach the Bonferroni corrected threshold of $P \leq 0.0003$ due to the lower prevalence of the *2 allele (~9%) compared with rs2331559 (~12%) and rs11726322 (13%). The minor (less frequent) alleles of the *UGT2B10* variants surviving correction for multiple testing in European Americans were associated with impaired cotinine glucuronidation; whereas, among African Americans, the minor allele of the *UGT2B10* variant surviving correction for multiple testing was associated with enhanced activity. Hence, in contrast to European Americans, the major (more frequent) allele in African Americans would have reduced activity (versus enhanced activity) potentially con-

tributing to the lower levels of nicotine and cotinine glucuronide conjugates observed among African Americans (Fig. 2A and B; refs. 39, 63).

In addition to *UGT2B10*, we also observed nominal associations between variants in *UGT1A1*, *UGT1A4*, and *UGT2B7* and nicotine and/or cotinine glucuronidation. Consistent with inhibition studies of *UGT1A1* in human liver microsomes, which demonstrate an impact on nicotine but not cotinine glucuronidation (44), *UGT1A1* was only associated with the nicotine glucuronide ratio. *UGT1A4* is implicated in the glucuronidation of nicotine and cotinine *in vitro* and is considered to be the second most active UGT in the N-glucuronidation of these compounds after *UGT2B10* (44, 46, 47). We observed associations between *UGT1A4* variants and both the nicotine and cotinine glucuronide ratios potentially reflecting the minor contribution of this enzyme. *UGT2B7* is capable of nicotine glucuronidation *in vitro* (47); however, the nominal association that we report is between *UGT2B7* and cotinine glucuronidation and may represent a chance finding.

As the nicotine and cotinine glucuronide ratios were correlated, we anticipated that the same *UGT* variants might be associated with both glucuronidation phenotypes. While we observed overlap in variants associated with both pathways, particularly in *UGT2B10*, more *UGT* variants were associated with the cotinine

Table 5. *UGT* variants nominally associated with the 3HC glucuronide ratio

UGT SNP/variant	Alleles	European American smokers			Alleles	African American smokers		
		MAF	P^{dom}	Coef (95% CI)		MAF	P^{dom}	Coef (95% CI)
<i>UGT2B17</i> locus								
Copy number	1:0 ↓	0.303	0.028	−0.15 (−0.29 to −0.02)	1:0	0.215		
<i>UGT2B7</i> locus								
rs57216626	C:G	0.448			C:G ↓	0.250	0.016	−0.14 (−0.25 to −0.03)
rs4535394	C:T	0.440			C:T ↓	0.339	0.026	−0.13 (−0.25 to −0.02)

NOTE: *UGT2B17*0 allele: deletion allele. Alleles indicated as major:minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% CIs from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < 0.05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*).

Abbreviation: MAF, minor allele frequency.

Table 6. UGT variants nominally associated with the NNAL glucuronide ratio

UGT SNP/variant	European American smokers				African American smokers			
	Alleles	MAF	P^{dom}	Coef (95% CI)	Alleles	MAF	P^{dom}	Coef (95% CI)
UGT1A1 locus rs3771342 ^a	C:A	0.112			C:A ↑	0.117	0.012	-0.19 (0.04-0.34)
UGT1A 3' flanking rs10203853	A:T	0.431			A:T ↓	0.292	0.007	-0.16 (-0.38 to -0.05)
UGT2B10 locus rs835310 ^a	C:G	0.023			C:G ↓	0.408	0.019	-0.15 (-0.28 to -0.03)
UGT2B7 locus rs12506592	A:G	0.162			A:G ↓	0.275	0.030	-0.14 (-0.26 to -0.01)
rs4356975	C:T	0.381			C:T ↑	0.397	0.017	0.15 (0.03-0.28)

Abbreviation: MAF, minor allele frequency.

^aVariant excluded from analyses in European Americans. Alleles indicated as major:minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% CIs from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < 0.05$. Variants ordered by location along chromosome 2 (UGT1A) and chromosome 4 (UGT2B).

glucuronide ratio than with the nicotine glucuronide ratio and the only variants surviving correction for multiple testing were associated with cotinine glucuronidation. A greater genetic contribution to interindividual variation in cotinine versus nicotine glucuronidation has been proposed based on twin studies (69). Alternatively, this difference may be the result of a more stable glucuronidation phenotype for cotinine.

Comparatively few UGT variants were associated with the 3HC glucuronide ratio. We replicated the *in vivo* association of UGT2B17*2 with lower 3HC glucuronidation (20, 35) among European Americans. In line with the O-glucuronidation of 3HC by UGT2B7 observed *in vitro* (45), two UGT2B7 variants were associated with the 3HC glucuronide ratio among African Americans. We observed a high degree of linkage disequilibrium in the UGT2B7 locus among European Americans (Supplementary Fig. S2A); thus, the absence of association with UGT2B7 among European Americans could reflect a lower prevalence and/or diversity of altered activity UGT2B7 variants or inadequate tagging of functional variants.

Our findings do not support a dominant contribution of genetic variation in any single UGT to the overall level of NNAL glucuronidation, which may reflect the formation of both N- and O-glucuronide conjugates of NNAL *in vivo* (24, 43). Hence, multiple UGT enzymes, and their variants, may each make a relatively small contribution to the overall NNAL glucuronide ratio. Consistent with the correlations that we observed between the NNAL and cotinine glucuronide ratios, and the NNAL and 3HC glucuronide ratios (Table 2), the UGT2B10 rs835310 G allele showed impaired glucuronidation of both NNAL and cotinine and the UGT2B7 rs12506592 G allele showed impaired glucuronidation of both NNAL and 3HC (Tables 4 and 6). We did not replicate the *in vivo* or *in vitro* association of UGT2B17*2 with impaired glucuronidation of NNAL (27, 42). However, Gallagher and colleagues reported an association only among women (27), and our study size was insufficient to test for genotype-gender interactions, and Lazarus and colleagues measured O-glucuronide formation specifically (42), whereas our analytical method did not distinguish between N- and O-glucuronide conjugates. The N- and O-glucuronides are formed in near equal amounts *in vivo* (24), so a small genetic effect on either pathway may not be detectable in the current study. Consistent with a smaller effect of any one UGT on the overall level of NNAL glucuronidation, the effect sizes that we observed between UGT variants and NNAL glucuronidation (Table 6) were approximately half the magnitude of the observed associations with cotinine glucuronidation (Table 4). The overall low levels of NNAL

in the urine of smokers (free or glucuronidated) compared with cotinine may also have hindered genotype-phenotype associations.

The ratio of NNAL-glucuronide to NNAL is a biomarker of nitrosamine detoxification (31) and a potential marker of cancer risk (32). There is conflicting evidence regarding ethnic differences in this ratio, specifically whether it is lower among African Americans (70, 71). We did not observe a significant difference in NNAL glucuronide ratios by ethnicity, while replicating differences in other ratios (Fig. 2). However, 10% of African Americans had ratios below the lowest ratio observed among European Americans potentially putting these individuals at greater risk for cancer, as Chung and colleagues found that lower NNAL glucuronide ratios were associated with an increased risk of cancer (32). Variation in the UGT2B10 and UGT2B7 loci may be of particular interest in terms of cancer risk disparities, as these variants were only nominally associated with NNAL glucuronidation activity among African Americans.

In addition to altered carcinogen detoxification, variation in UGTs could influence lung cancer indirectly through altered smoking. Berg and colleagues reported significantly lower nicotine equivalents (a measure of nicotine intake) in smokers with the UGT2B10 *1*2 genotype in a study of European Americans (34) and in a mixed analysis of European and African American smokers (39), and speculated that slower nicotine glucuronidation may lead to reduced nicotine consumption. However, in a larger study of African American smokers, Zhu and colleagues did not find a significant association between UGT2B10*2 and lower nicotine equivalents (35). Consistent with glucuronidation as a minor pathway for nicotine inactivation (17), and with Zhu and colleagues, neither UGT2B10*2 genotype (data not shown), nor importantly the actual nicotine glucuronidation ratio (Supplementary Table S2), were associated with nicotine equivalents in either European or African Americans.

Many nominally significant associations between UGT variants and glucuronidation phenotypes were observed despite both the relatively small sample size for a genetic association study and the quantification of metabolites from spot urine with variable time from last cigarette. Biomarker assessment from spot urine is unlikely to have biased findings, since we observed similar correlations between glucuronide ratios as reported for 24-hour urine (18, 39). Furthermore, urinary cotinine, NNAL, and nicotine equivalents, which are all biomarkers of nicotine consumption, were correlated as expected (data not shown) and neither nicotine equivalents nor the glucuronide ratios were systemically associated with time from last cigarette (Supplementary Table S2). Only

one nominally significant variant, *UGT1A1* rs3771342, displayed an inconsistent direction of effect with the nicotine and NNAL glucuronidation phenotypes potentially reflecting an indirect genotype–phenotype association or simply a chance observation. The smaller sample size precluded interactions analyses. In particular, the interaction between menthol and *UGT2B7* and *UGT2B17* gene variants would be worthwhile exploring in a larger dataset given the conflicting evidence concerning menthol smoking and lung cancer risk (reviewed in ref. 72). More associations were observed among African Americans than among European Americans, as is seen in other genomic regions displaying lower linkage disequilibrium in African populations [e.g., chromosome 15q25 and lung cancer risk (73)]. Of note, the gene variants investigated were initially chosen to provide relatively good coverage of European smokers and provide relatively low coverage of common variation in Africans (Supplementary Table S1) suggesting that even more variation may be identified among this ethnic group. Alternatively, a single untested variant may underlie multiple associations observed in a gene region through linkage disequilibrium.

Overall, study findings confirmed a role for multiple UGTs in the glucuronidation of tobacco-related compounds *in vivo* and contributed to the understanding of sources of variation in the nicotine and nitrosamine metabolic inactivation pathways. Concurrently examining genetic sources of variation in European and African American smokers also provided insight into ethnic differences in glucuronidation activity, which could be contributing to ethnic disparities in the risk for smoking-related cancers.

Disclosure of Potential Conflicts of Interest

M.J. Ratain has ownership interest (including patents) in provisional patent application related to genomic prescribing and royalties related to *UGT1A1* genotyping. N.L. Benowitz is a consultant/advisory board member for Pfizer and GlaxoSmithKline, and has provided expert testimony for litigation against tobacco companies. R.F. Tyndale has received speakers' bureau honoraria from university talks, is a consultant/advisory board member for pharmaceutical

companies, and has provided expert testimony for *Clinical Pharmacology and Therapeutics* journal. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.A. Wassenaar, S. Das, P. Chen, N.L. Benowitz, R. F. Tyndale
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.L. Benowitz
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