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Research Article

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UGT2B10 Genotype Influences Nicotine Glucuronidation, Oxidation, and Consumption

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Abstract

Background: Tobacco exposure is routinely assessed by quantifying nicotine metabolites in plasma or urine. On average, 80% of nicotine undergoes C-oxidation to cotinine. However, interindividual variation in nicotine glucuronidation is substantial, and glucuronidation accounts for from 0% to 40% of total nicotine metabolism. We report here the effect of a polymorphism in a UDP-glucuronsyltransferase, UGT2B10, on nicotine metabolism and consumption.

Methods: Nicotine, cotinine, their *N*-glucuronide conjugates, and total *trans*-3′-hydroxycotinine were quantified in the urine (n = 327) and plasma (n = 115) of smokers. Urinary nicotine *N*-oxide was quantified in 105 smokers. Nicotine equivalents, the sum of nicotine and all major metabolites, were calculated for each smoker. The relationship of the UGT2B10 Asp67Tyr allele to nicotine equivalents, *N*-glucuronidation, and C-oxidation was determined.

Results: Individuals heterozygous for the Asp67Tyr allele excreted less nicotine or cotinine as their glucuronide conjugates than did wild-type, resulting in a 60% lower ratio of cotinine glucuronide to cotinine, a 50% lower ratio of nicotine glucuronide to nicotine, and increased cotinine and *trans*-3′-hydroxycotinine. Nicotine equivalents, a robust biomarker of nicotine intake, were lower among Asp67Tyr heterozygotes compared with individuals without this allele: 58.2 (95% confidence interval, 48.9-68.2) versus 69.2 nmol/mL (95% confidence interval, 64.3-74.5).

Conclusions: Individuals heterozygous for UGT2B10 Asp67Tyr consume less nicotine than do wild-type smokers. This striking observation suggests that variations in nicotine *N*-glucuronidation, as reported for nicotine C-oxidation, may influence smoking behavior.

Impact: UGT2B10 genotype influences nicotine metabolism and should be taken into account when characterizing the role of nicotine metabolism on smoking. *Cancer Epidemiol Biomarkers Prev; 19(6); 1423–31.* ©2010 AACR.

Introduction

Tobacco use is associated with cancers of the lung, larynx, nasal cavity, oral cavity, esophagus, liver, pancreas, bladder, and cervix, and leukemia (1). Therefore, cancer etiology and epidemiology studies frequently require assessment of tobacco exposure (2-5). Error in estimating tobacco exposure can obscure important observations, whereas accuracy in assessment is useful to identify tobacco exposure as either a causal or confounding factor. Many approaches have been used to evaluate tobacco exposure broadly, including: self-report of smoking history (cigarettes per day, pack-years of smoking, time spent around smoke, etc.), collection of used products (cigarette butts, discarded chew), topography measures (puff volume, frequency), and biomarkers (exhaled carbon monoxide, nicotine metabolites, tobacco-specific nitrosamines; refs. 6-11). Selecting a measure(s) to assess exposure is not a trivial choice and depends on the goals of a particular study.

Quantifying one or more nicotine metabolites is an objective measure of current exposure from smoking, smokeless tobacco use, or environmental/secondhand smoke. Plasma or urinary cotinine are the most frequently utilized biomarkers of tobacco exposure, other than exhaled carbon monoxide (12, 13). The only significant source of cotinine is metabolism from nicotine, and it is highly correlated with nicotine intake (14, 15). However, interindividual differences in nicotine metabolism also influence cotinine levels (15, 16). The role of ethnicity in interindividual variation is addressed in a recent publication that recommends the use of racial/ethnic-specific plasma cotinine levels to distinguish smokers from

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nonsmokers (13). A potentially more robust biomarker of nicotine exposure is nicotine equivalents, defined as the sum of nicotine and its major metabolites in urine (8). Total nicotine (nicotine + nicotine glucuronide), total cotinine (cotinine + cotinine glucuronide), and total *trans*-3′-hydroxycotinine (*trans*-3′-hydroxycotinine + its glucuronides) can be assessed with minimal sample preparation (e.g., a single solid phase extraction) by liquid chromatography/mass spectrometry with high throughput capacity (17). Therefore, assessing nicotine equivalents is feasible in the time typically used to quantify a single nicotine metabolite. Nicotine equivalents directly account for >80% of nicotine intake, and because it is a sum of metabolites, its value will not change if metabolism shifts from one pathway to another (14, 17, 18). Nicotine equivalents correlate with cigarettes per day, and have been used to better characterize the link between polymorphisms in nicotinic acetylcholine receptor A subunits, smoking, and lung cancer (8, 19).

In smokers, nicotine is metabolized by three pathways (Fig. 1). The major pathway is CYP2A6-catalyzed 5′-oxidation, which after a second oxidation results in the formation of cotinine. Nicotine may also be metabolized by *N*-glucuronidation and *N*-oxidation. On average, these two pathways each account for <10% of the nicotine dose (15). However, urinary nicotine *N*-glucuronide concentrations vary significantly among smokers and can account for as much as 40% of the total nicotine metabolites excreted (6).

Polymorphisms are common in nicotine metabolism genes and their regulatory regions, although only a minority of the known variants have been fully characterized (20). Enzyme amount and relative activity affect the metabolic fate of nicotine. Several polymorphisms in CYP2A6, the hepatic enzyme responsible for oxidation of nicotine to cotinine and a major catalyst of cotinine oxidation to *trans*-3′-hydroxycotinine, are associated with lower intensity smoking (21-23). For example, CYP2A6 polymorphisms that influence smoking behavior include CYP2A6*4, a deletion allele, CYP2A6*2, an inactivating point mutation, and CYP2A6*9, which alters the TATA box promoter region (21, 24, 25). Furthermore, low nicotine oxidation, low concentrations of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, and low nicotine equivalents have been observed among Japanese Americans, a population with a high prevalence of CYP2A6 deletion alleles, compared with European Americans (26). UGT2B10 and UGT1A4 catalyze the *N*-glucuronidation pathways of nicotine metabolism *in vitro*, and the genes encoding these enzymes are also polymorphic (27-30). UGT2B10 is a more efficient catalyst of nicotine *N*-glucuronidation than is UGT1A4, and it seems to be the key catalyst *in vivo* (28, 31). Recently, we reported that the UGT2B10 polymorphism Asp67Tyr was associated with a 20% decrease in the excretion of cotinine and nicotine as their glucuronide conjugates in the urine of 84 smokers (31). This UGT2B10 variant was characterized *in vitro* by Chen et al. as

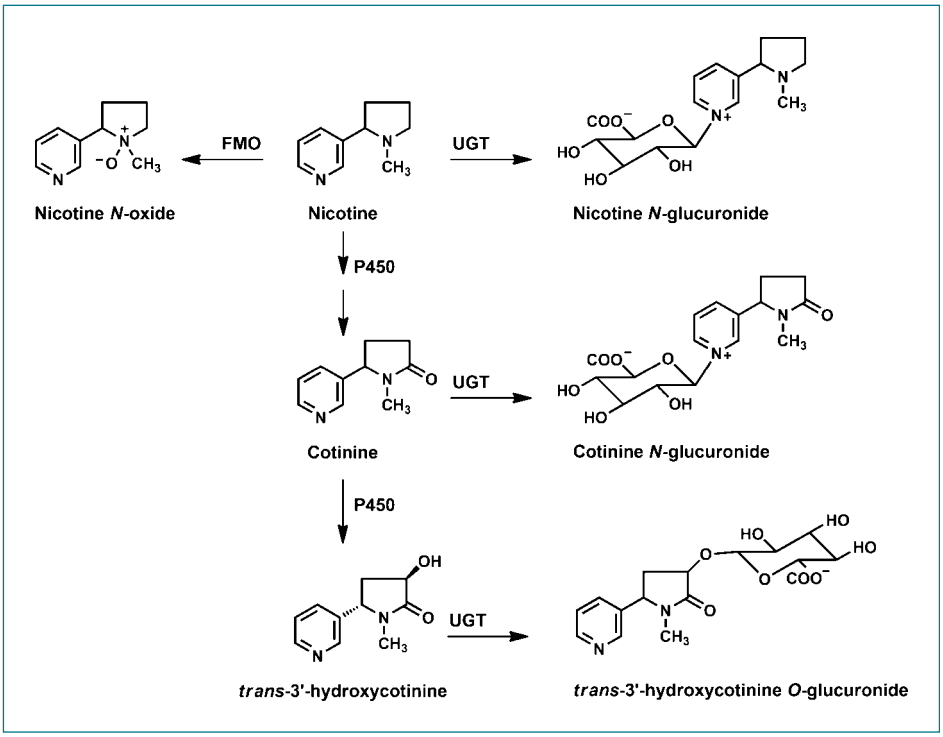

Figure 1. Nicotine metabolism pathways.

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having decreased nicotine and cotinine glucuronidation activity (27).

The main goals of the current study were to confirm the relationship between Asp67Tyr and lower nicotine glucuronidation, and to assess if glucuronidation was associated with a shift in nicotine metabolism through other pathways, including nicotine *N*-oxidation. In addition, the effect of the Asp67Tyr polymorphism on nicotine equivalents as a pathway-independent measure of tobacco exposure was determined. The measure of nicotine equivalents does not routinely include nicotine *N*-oxide. However, in the present study liquid chromatography/tandem mass spectrometry analysis was used to quantify nicotine *N*-oxide in a subset of subjects.

Materials and Methods

Study populations

This research was approved by the University of Minnesota Institutional Review Board. A total of 327 adult smokers who were recruited from the Twin Cities metropolitan area and who participated in one of three tobacco research studies conducted at the University of Minnesota were included in this genotype-phenotype investigation. Participants were recruited through advertisements on campus and local media, were screened by telephone, and were willing to participate in a smoking reduction or cessation study. Participants were in good physical and psychiatric health, had no contraindications to nicotine replacement therapy, were not pregnant or nursing, and were not using barbituates or anticonvulsants. For each study, participants were asked to provide a first void morning urine sample while they were smoking as usual, and this sample was collected at least 2 weeks before any study intervention (and before randomization to an intervention group). A blood sample was also collected from participants. Study 1 included 122 participants ages 18 to 65 years who smoked >10 cigarettes per day and who subsequently participated in a cessation trial of traditional nicotine replacement versus smokeless tobacco. Study 2 included 145 participants ages 18 to 70 years who were smokers of 10 to 40 "light" cigarettes per day (0.7-1.0 mg nicotine per cigarette), and who then participated in a study of low and no nicotine cigarettes (19). Study 3 included 84 smokers ages 18 to 70 years who smoked 15 to 45 cigarettes per day and subsequently participated in a smoking reduction trial (6, 31). Initial genotype-phenotype analysis of UGT2B10 Asp67Tyr genotype, and the percent of nicotine and cotinine excreted as glucuronide conjugates in urine was conducted on study 3 samples and was recently reported (31).

Nicotine and metabolite analysis

Urine was aliquoted and stored at −20°C until the time of analysis. Urinary nicotine and nicotine metabolite concentrations were analyzed by gas chromatography-mass spectrometry as previously described (6). Free nicotine, total nicotine (free nicotine + *N*-glucuronide), free cotinine, total cotinine (free cotinine + *N*-glucuronide), and total *trans*-3′-hydroxycotinine (*trans*-3′-hydroxycotinine + its glucuronides) were quantified. Analyses were done in duplicate for each urine sample and repeated if values differed by >10%, and the average duplicate error was 3%. Repeat analysis was required on <10% of the samples; no samples were excluded due to measurement error. Nicotine *N*-oxide was analyzed in the urine of study 1 samples by liquid chromatography/mass spectrometry. In brief, urine (20 μL) with added (d_3–methyl)-nicotine *N*-oxide (Toronto Research Chemicals) as an internal standard was processed by solid phase extraction with an Oasis MCX column eluted with 400 μL 2% ammonium hydroxide in methanol. Liquid chromatography/tandem mass spectrometry was done with the use of a Finnigan Discovery triple quadrupole mass spectrometer (Thermo Electron) with an electrospray source in positive ion mode. Selective reaction monitoring of the mass transitions for d0-nicotine *N*-oxide (*m/z* 179→130 and *m/z* 179→117) was done.

Plasma was separated from blood and stored at −20°C for study 1 samples. Plasma free cotinine, total cotinine (free + *N*-glucuronide), and total *trans*-3′-hydroxycotinine were quantified by gas chromatography-mass spectrometry as done for urinary metabolites, except for the addition of an initial solid phase extraction with the use of an Oasis MCX column (Waters Corporation; ref. 32). Analyses were done in duplicate and repeated if values differed by >10%.

UGT2B10 genotyping

DNA was isolated from blood with the use of the DNeasy kit (Qiagen). PCR-RFLP analysis was done targeting UGT2B10 at the codon 67 position with Hinf*I* digestion, as previously described (Chen et al., 2008). A second RFLP for the single nucleotide polymorphism rs7657958, which is linked with UGT2B10 Asp67Tyr in Caucasians (haplotype C), was done in a subset of samples (N = 107; 32%; ref. 29). Genotyping results were concordant between the two assays, as well as with the DNA sequence analysis of 10 samples.

Statistical analysis

Statistical analyses were done with the use of SAS (SAS Institute) and Excel (Microsoft). Wilcoxon two-sided *t* approximation statistics were calculated to compare the means of continuous variables that did not have a normal distribution, and a *P*-value of <0.05 was considered significant. Nonparametric Spearman correlation was used to assess univariate correlations. A general linear model was used to compare phenotypes by the UGT2B10 genotype and to adjust for nicotine equivalents. Individuals with the highest and lowest 1% of urinary nicotine equivalents, <9 and >236 nmol/mL, respectively, were excluded from analyses. Nicotine and cotinine *N*-glucuronidation were assessed as the percent

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of the analyte that was present as its *N*-glucuronide and as the square root–transformed ratio of glucuronide conjugate to free analyte. The square root–transformed ratio of total *trans*-3′-hydroxycotinine to free cotinine in plasma or in urine was used as an estimate of C-oxidation.

Results

A total of 327 participants were included in the genotype-phenotype analyses, which included samples collected at baseline while participants were smoking as usual from three studies. The study participants consisted of 87% European Americans and 8% African Americans; the remainder were of Asian, American Indian, or mixed race. The mean ages ± SD by study were 1) 43 ± 11.6 years, 2) 41 ± 14 years, and 3) 46 ± 10.6 years. The mean ± SD diary-recorded cigarettes per day at baseline were: 1) 21 ± 6.5, 2) 21 ± 8.8, and 3) 26 ± 6.9. No effect of UGT2B10 Asp67Tyr genotype on cigarettes per day was observed. The mean ± SD cigarettes per day (wild-type, Asp67Tyr heterozygotes) by study were (*a*) 20.2 ± 6.1, 22.8 ± 7.7; (*b*) 20.7 ± 8.7, 22.3 ± 9.1; and (*c*) 26.2 ± 7.1, 25.6 ± 7.0. The total allele frequency of the UGT2B10 Asp67Tyr variant was 10.6%, and this was consistent with Hardy-Weinberg equilibrium. Correspondingly, individuals who were heterozygous for the Asp67Tyr allele represented 20% of the participants: study 1, 17.4%; study 2, 18.3%; and study 3, 22.2%. The percent of cotinine excreted as a glucuronide conjugate in first morning urine was significantly decreased among individuals who were heterozygous for the Asp67Tyr allele compared with individuals without this allele in each of the three studies. Subsequently, analyses were conducted on the combined data unless indicated.

The distribution of urinary nicotine and its metabolites by UGT2B10 Asp67Tyr genotype is presented in Table 1. Analytes are presented as molar percentages of the sum of nicotine and its metabolites in urine, and reflect the relative contribution of different pathways to the fate of nicotine. The molar percentages of nicotine and cotinine *N*-glucuronides in urine were 30% lower for Asp67Tyr heterozygous individuals than for wild-type, 4.2% versus 6.1% ($P = 0.003$) and 17.6% versus 24.2% ($P < 0.0001$), respectively. Similarly, the fractions of nicotine and cotinine excreted as their glucuronide conjugates (i.e., percent glucuronidation) were lower among Asp67Tyr heterozygotes than among wild-type, 29.5% versus 38.2% ($P = 0.007$) and 51.3% versus 62% ($P < 0.0001$), respectively. As might be expected, a decrease in the molar percent of cotinine glucuronide was accompanied by an increase in excretion of free cotinine, 16% versus 13.4% ($P = 0.003$) for Asp67Tyr heterozygotes compared with wild-type. Moreover, a significant increase was observed in the molar percent of total *trans*-3′-hydroxycotinine in urine of Asp67Tyr heterozygotes compared with wild-type, 51.1% versus 44.3% ($P = 0.001$). In summary, the Asp67Tyr allele was associated with relatively lower urinary abundance of nicotine and cotinine *N*-glucuronides, higher free cotinine, and higher total *trans*-3′-hydroxycotinine.

There were three individuals whose DNA yielded a restriction digest pattern that would be consistent with homozygosity for UGT2B10 Asp67Tyr. No urine samples were available for the first individual, the second individual excreted a low percentage of cotinine as its glucuronide conjugate (7.1%) and had undetectable nicotine glucuronidation; and the third individual had a normal-to-high glucuronidation phenotype (73% cotinine glucuronidation, 67% nicotine glucuronidation). DNA sequence analysis of UGT2B10 is planned for these samples, who were excluded from the analyses that were focused on characterizing heterozygous carriers of UGT2B10 Asp67Tyr.

Table 1. Distribution of urinary metabolites by genotype as a molar percentage of nicotine and its metabolites

Analyte	Wild-type mean percent (SD), *n* = 263	UGT2B10 Asp67Tyr* mean percent (SD), *n* = 62	*P*
Nicotine	11.8 (9.5)	11.8 (8.5)	0.75
Nicotine glucuronide	6.1 (5.1)	4.2 (3.3)	0.003
Cotinine	13.4 (6.8)	16.0 (7.4)	0.003
Cotinine glucuronide	24.2 (10.7)	17.6 (8.0)	<0.0001
Trans-3′-hydroxycotinine	44.3 (16.7)	51.1 (14.6)	0.001
Percent nicotine glucuronidation†	38.2 (23.6)	29.5 (18.8)	0.007
Percent cotinine glucuronidation†	62.8 (18.2)	51.3 (18.5)	<0.0001

NOTE: Molar percentage = (analyte/∑ nicotine + cotinine + *trans*-3′-hydroxycotinine + glucuronides) × 100.
*UGT2B10 Asp67Tyr heterozygous individuals.
†Percent of analyte excreted as its glucuronide conjugate.

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Table 2. Nicotine *N*-oxide as a molar percentage of nicotine and its metabolites in urine by C-oxidation and glucuronidation phenotype (study 1)

Phenotype*	Quartile ratio values	*N*	Molar % of nicotine *N*-oxide mean (95% CI)
Cotinine glucuronide to cotinine†			
Low	<0.89	27	6.8 (5.7-7.9)
Average	0.89-1.5	54	6.1 (5.4-6.9)
High	>1.5	27	5.5 (4.4-6.6)
Trans-3′-hydroxycotinine to cotinine‡			
Low	<0.45	25	8.7 (7.7-9.7)
Average	0.45-0.69	52	6.1 (5.4-6.8)
High	>0.69	25	4.3 (3.3-5.3)

*Phenotype was assessed by metabolite ratio: low, lowest quartile; average, interquartile range; high, highest quartile.
†Cotinine glucuronide to free cotinine ratio quantified in first morning urine. Nicotine *N*-oxide means did not differ by phenotype. χ^2 test for trend: $P = 0.055$.
‡Ratio of total *trans*-3′-hydroxycotinine to free cotinine quantified in plasma. Mean nicotine *N*-oxide values differed significantly within each phenotypic group with *P*-values < 0.005.

In addition to C-oxidation and *N*-glucuronidation, nicotine may be metabolized by *N*-oxidation. The product of *N*-oxidation, nicotine *N*-oxide, although typically a minor metabolite, may be increased in individuals with decreased metabolism through other pathways (33). Therefore, we evaluated the influence of UGT2B10 genotype, and *N*-glucuronidation and C-oxidation phenotype on nicotine *N*-oxide abundance in urine. Urinary nicotine *N*-oxide was quantified in study 1 participants ($N = 108$). The concentration of nicotine *N*-oxide excreted by smokers who were heterozygous or wild-type for the UGT2B10 Asp67Tyr allele was not significantly different, 4.1 versus 3.8 nmol/mL, respectively ($P = 0.34$). The molar percentages of nicotine *N*-oxide excreted by these two groups were also not different, 6.27 ± 2.83 (range, 1.11-12.7) and 5.53 ± 3.16 (range, 0.76-11.8), respectively. Likewise, the excretion of nicotine *N*-oxide did not differ significantly by *N*-glucuronidation phenotype, the urinary ratio of cotinine glucuronide to free cotinine (Table 2). The phenotype was categorized as low, high, or average based on the lowest and highest quartiles of the metabolite ratio distribution and the interquartile range. The mean nicotine *N*-oxide abundances were 5.5%, 6.1%, and 6.8% for high, average, and low *N*-glucuronidation phenotype, respectively; and the χ^2 test for trend was $P = 0.055$. In contrast, the molar percent of nicotine *N*-oxide excreted increased as the ratio of plasma *trans*-3′-hydroxycotinine to free cotinine decreased (Table 2); that is, nicotine *N*-oxide excretion was significantly related to C-oxidation phenotype. The abundance of nicotine *N*-oxide increased with successively lower *trans*-3′-hydroxycotinine to free cotinine ratios, with means of 4.3%, 6.1%, and 8.7% for high, average, and low C-oxidation phenotype groups, respectively ($P < 0.005$ between each group).

The effect of the UGT2B10 Asp67Tyr allele on plasma concentrations of nicotine metabolites was evaluated in study 1 participants (Table 3). Free plasma cotinine was higher among Asp67Tyr heterozygotes than among wild-type individuals, 1.31 (231 ng/mL) versus 1.09 nmol/mL (192 ng/mL; $P = 0.03$). No difference in plasma cotinine glucuronide concentration was detected, 0.13 versus

Table 3. Plasma nicotine metabolites by UGT2B10 genotype (study 1)

Analyte	Wild-type (*n* = 95) mean (SD)	Asp67Tyr* (*n* = 20) mean (SD)	Range
Free cotinine (nmol/mL)	1.09 (0.49)	1.31 (0.40)†	0.19-2.75
Cotinine glucuronide (nmol/mL)	0.15 (0.14)	0.13 (0.14)	0.00-0.54
Percent cotinine glucuronide	11.3 (9.7)	8.72 (9.0)	0.0-36.4
Total *trans*-3′-hydroxycotinine (nmol/mL)	0.39 (0.32)	0.46 (0.24)‡	0.04-1.92
Total *trans*-3′-hydroxycotinine to free cotinine	0.38 (0.28)	0.37 (0.18)	0.07-1.29

*UGT2B10 heterozygous individuals.
†$P = 0.03$.
‡$P = 0.04$.

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Table 4. Urinary nicotine equivalents and metabolite ratios by UGT2B10 genotype: geometric means and 95% CI

UGT2B10 Genotype	Cotinine glucuronidation ratio*	Nicotine glucuronidation ratio*	Total *trans*-3′-hydroxycotinine to free cotinine†	Nicotine equivalents‡
Wild-type (N = 264)	2.03 (1.86-2.20)	0.77 (0.64-0.89)	3.80 (3.96-4.15)	69.2 (64.3-74.5)
Asp67Tyr§ (N = 63)	1.26 (1.00-1.56)	0.41 (0.25-0.62)	3.96 (3.35-4.64)	58.2 (48.9-68.2)
	$P < 0.0001$‖	$P = 0.005$‖	$P = 0.715$‖	$P = 0.048$

*Glucuronide conjugate to unconjugated analyte ratio.
†Total *trans*-3′-hydroxycotinine to total cotinine ratio was higher among Asp67Tyr heterozygotes compared with wild-type: 1.73 (1.46-2.03) versus 1.29 (1.18-1.41), $P = 0.003$, adjusted for nicotine equivalents.
‡Sum of nicotine, cotinine, *trans*-3′-hydroxycotinine, and their respective glucuronides in nmol/mL, quantified in first morning urine. Urinary nicotine *N*-oxide was quantified in a subset of individuals (n = 108; study 1) and did not differ by genotype: mean, 4.03 ± 2.8 nmol/mL; range, 0.08-14.5 nmol/mL.
§UGT2B10 Asp67Tyr heterozygous individuals.
‖Adjusted for nicotine equivalents.

0.15 nmol/mL for Asp67Tyr heterozygotes and wild-type individuals, respectively. Because the percent of conjugated cotinine present in plasma is low, the overall mean was 10%; it was difficult to quantify relatively small differences in cotinine *N*-glucuronide in plasma. Total *trans*-3′-hydroxycotinine was higher in the plasma of Asp67Tyr heterozygotes compared with that in wild-type, 0.46 versus 0.39 nmol/mL (P =0.04). Therefore, the effect of the Asp67Tyr allele on free cotinine and total *trans*-3′-hydroxycotinine was consistent in urine and plasma; both were higher among heterozygotes than wild-type.

The relationship between Asp67Tyr genotype and phenotype assessed as metabolite ratios is presented in Table 4. UGT2B10 Asp67Tyr genotype and *N*-glucuronidation ratios, as biomarkers of phenotype, were strongly associated. The urinary ratio of cotinine glucuronide to cotinine was significantly lower among Asp67Tyr heterozygotes than among wild-type individuals, with a mean of 1.26 [95% confidence interval (95% CI), 1.00-1.56] versus 2.03 (95% CI, 1.86-2.20; $P < 0.0001$. The ratio of urinary nicotine glucuronide to nicotine was also significantly lower among Asp67Tyr heterozygotes than among wild-type individuals (P = 0.005). The metabolite ratios in Table 4 were adjusted for nicotine equivalents. However, the relationship between Asp67Tyr and low *N*-glucuronidation was evident whether or not the glucuronide ratio was adjusted for nicotine equivalents. No statistical difference was observed in the urinary ratio of total *trans*-3′-hydroxycotinine to free cotinine between Asp67Tyr variant and wild-type individuals. A higher ratio of total *trans*-3′-hydroxycotinine to total cotinine was observed for Asp67Tyr heterozygotes, with a mean of 1.73 (95% CI, 1.46-2.03) versus 1.29 (95% CI, 1.18-1.41) for wild-type (P = 0.048).

To investigate the stability of the urinary glucuronide ratio and the effect of nicotine dose, we evaluated the ratio among individuals who decreased their smoking. The ratio of cotinine glucuronide to cotinine was assessed in a group of individuals (n = 27) at baseline and after 12 weeks, at which point they had reduced their nicotine intake by 60%, as assessed by nicotine equivalents. The ratio of cotinine glucuronide to cotinine decreased by 40% as they decreased their nicotine intake, with a mean of 2.59 (95% CI, 2.05-3.24) at baseline versus 1.51 (95% CI, 1.04-2.10) at week 12 (P = 0.008). However, when the ratio was adjusted for nicotine equivalents, this difference disappeared, with means of 1.99 (95% CI, 1.49-2.52) and 2.07 (95% CI, 1.58-2.63) for baseline and week 12, respectively (P = 0.81). A decrease of similar magnitude was observed in the urinary ratio of total *trans*-3′-hydroxycotinine to free cotinine as individuals decreased their nicotine intake, and adjusting for nicotine equivalents eliminated this difference.

During the evaluation of urinary nicotine metabolites, it became clear that nicotine equivalents were lower among Asp67Tyr than among wild-type individuals (Table 4). Nicotine equivalents were decreased 15% for Asp67Tyr heterozygotes relative to wild-type, with means of 58.2 (95% CI, 48.9-68.2) versus 69.2 nmol/mL (95% CI, 64.3-74.5), respectively (P = 0.048). Nicotine equivalents were also lower among Asp67Tyr individuals compared with wild-type when a nested comparison was used to take into account the effect of the study group, and there was no difference in nicotine equivalents for Asp67Tyr individuals by study. Nicotine equivalents account for >80% of nicotine intake, and therefore this biomarker is useful to estimate nicotine intake during *ad libitum* smoking. The only significant pathway of nicotine metabolism not accounted for by nicotine equivalents is *N*-oxidation. However, as discussed above, the excretion of nicotine *N*-oxide was not influenced by *N*-glucuronidation phenotype (Table 2). Moreover, we showed in study 1 that there is no substantial shift in nicotine *N*-oxidation among Asp67Tyr heterozygotes.

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Discussion

Interindividual variation in glucuronidation of nicotine and cotinine is substantial, and the contribution of glucuronidation to total nicotine metabolism ranges from <1% to 40% based on nicotine and its metabolites recovered in smokers' urine (6). On average, an estimated 25% of nicotine is metabolized to *N*-glucuronide conjugates of nicotine or cotinine (15). Variation in *N*-glucuronidation influences the distribution of nicotine metabolites and therefore may affect nicotine exposure assessment with the use of nicotine metabolite biomarkers. We report here that smokers who are heterozygous for the Asp67Tyr allele compared with individuals without this allele have decreased nicotine glucuronidation, increased nicotine C-oxidation, and decreased nicotine consumption as measured by excreted nicotine equivalents, a robust biomarker of nicotine intake. The observed increase in nicotine glucuronidation is consistent with our previous report and predicted based on *in vitro* studies (27, 31). The parallel increase in nicotine C-oxidation as measured by significant increases in the levels of both free cotinine and total *trans*-3′-hydroxycotinine in plasma and urine suggests that nicotine glucuronidation may play a more important role in circulating nicotine and cotinine levels than was previously appreciated. The observed decrease in total nicotine consumption is consistent with an influence of decreased glucuronidation on circulating nicotine levels. The striking observation that smokers who are UGT2B10 Asp67Tyr heterozygotes consume less nicotine suggests that variations in nicotine *N*-glucuronidation, as with C-oxidation (21), may influence nicotine consumption by smokers.

UGT2B10 is the most efficient catalyst of nicotine and cotinine *N*-glucuronidation *in vitro*, followed by UGT1A4 (28, 34). Both UGT2B10 and UGT1A4 are polymorphic, and for UGT2B10 there are three missense single nucleotide polymorphisms and >100 synonymous single nucleotide polymorphisms reported in the National Center for Biotechnology Information single nucleotide polymorphism database. The UGT2B10 Asp67Tyr allele was identified by Chen et al. because it was linked to a haplotype that was associated with decreased *N*-glucuronidation of the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol by human liver microsomes (29). Subsequently, a 20% to 30% decrease in nicotine and cotinine glucuronidation was observed among human liver microsomes that were heterozygous for the Asp67Tyr allele compared with wild-type (27).

In this study, we corroborated our prior finding that UGT2B10 Asp67Tyr is associated with decreased nicotine and cotinine *N*-glucuronidation *in vivo* (31). Significantly less nicotine and cotinine were excreted as glucuronide conjugates by individuals who were heterozygous for the Asp67Tyr allele compared with wild-type; the mean values were 22% and 17% lower for the Asp67Tyr group, respectively. In addition, the UGT2B10 genotype clearly differentiated glucuronidation phenotypes, assessed as the ratio of urinary cotinine glucuronide to cotinine or nicotine glucuronide to nicotine. The glucuronide ratio was 63% lower for cotinine and 53% lower for nicotine among Asp67Tyr heterozygotes compared with wild-type (Table 4).

The distribution of urinary metabolites was distinct for individuals with the Asp67Tyr allele compared with wild-type, as these individuals had a lower fraction of nicotine and cotinine *N*-glucuronides, but higher free cotinine and *trans*-3′-hydroxycotinine. Hence, low glucuronidation phenotype is compensated by an increase in the clearance of cotinine through C-oxidation. However, there was no significant increase in nicotine *N*-oxidation, the third nicotine metabolism pathway (Fig. 1). Higher plasma concentrations of free cotinine and *trans*-3′-hydroxycotinine were also observed in Asp67Tyr heterozygotes. Overall, we observed that the Asp67Tyr allele affects the concentrations of both plasma and urinary nicotine metabolites, and that the effect is consistent with a decrease in *N*-glucuronidation.

The dynamic nature of metabolite pathways may influence both biomarkers of nicotine exposure and phenotypic measures of nicotine metabolism. Specifically, when consuming similar amounts of nicotine, smokers with decreased nicotine glucuronidation will typically have higher plasma cotinine concentrations relative to smokers with average levels of glucuronidation. Therefore, UGT activity is one of several factors contributing to plasma cotinine levels. However, because both total *trans*-3′-hydroxycotinine and free cotinine increased when *N*-glucuronidation decreased, the C-oxidation ratio (total to free) was not significantly affected by UGT activity, and variations in this ratio likely still reflect variations in P450 2A6 activity.

We report here that urinary ratios of nicotine metabolites, both the ratio of cotinine glucuronide to cotinine and of *trans*-3′-hydroxycotinine to cotinine (total to total or total to free), are influenced by nicotine dose. Among individuals who significantly reduced their nicotine intake in a 12-week smoking reduction study (study 3), urinary metabolite ratios were significantly lower when the individuals were smoking less. This difference disappeared after adjusting for nicotine equivalents. Therefore, nicotine equivalents used as a measure of nicotine dose allow one to compare nicotine metabolism phenotypes across different levels of tobacco consumption. In contrast, adjusting for nicotine equivalents had no effect or actually increased the strength of the reported associations between UGT2B10 genotype and nicotine metabolite ratios (Table 4).

The most significant finding of this study was that individuals who were heterozygous for the Asp67Tyr allele had lower nicotine equivalents. When stratified by UGT2B10 genotype, the nicotine equivalent was 58.2 nmol/mL among heterozygotes compared with 69.2 nmol/mL for individuals who did not have an

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Asp67Tyr allele ($P < 0.05$). The only pathway of nicotine metabolism not routinely included in the measure of nicotine equivalents is nicotine *N*-oxidation (8). We saw no difference in the level of nicotine *N*-oxide excreted by Asp67Tyr UGT2B10 heterozygotes compared with wild-type smokers. Because the study participants were smoking *ad libitum*, the observed difference in nicotine equivalents represents a moderate but significant decrease in nicotine consumption that is likely attributable to the Asp67Tyr polymorphism in UGT2B10. The nicotine equivalent was 84% of that among wild-type, a change that is similar in magnitude to what has been reported for the influence of the CYP2A6 genotype on smoking. In two recent relatively large studies of smoking and CYP2A6 genotype, the percent decreases in reported cigarettes per day was 24% (35) and 17% (36) in individuals defined as slow nicotine inactivators. This is the first report showing that a polymorphism in UGT2B10 may influence smoking behavior. In different populations, the frequency of polymorphisms in CYP2A6 and in UGT2B10 may influence the relative contribution of these alleles to variation in nicotine metabolism and potentially to tobacco consumption.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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