Impact of Genetic Variants in the Nicotine Metabolism Pathway on Nicotine Metabolite Levels in Smokers

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ABSTRACT

Background: Nicotine metabolism is a major factor in nicotine dependence, with approximately 70% to 80% of nicotine metabolized to cotinine in Caucasians. Cotinine formation is catalyzed primarily by CYP2A6, which also converts cotinine to *trans*-3′-hydroxycotinine (3HC). The goal of the present study was to examine the effects of CYP2A6 deficiency on nicotine metabolism profiles *in vivo* and the importance of genetic variants in nicotine-metabolizing enzyme genes on urinary nicotine metabolites levels.

Methods: Urine samples from 722 smokers who participated in the Singapore Chinese Health Study were analyzed using UPLC-MS/MS to detect nicotine and eight of its urinary metabolites, and a total of 58 variants in 12 genes involved in nicotine metabolism were investigated in 475 of these subjects with informative genotyping data.

Results: Urine samples stratified by the ratio of 3HC/cotinine exhibited a 7-fold increase in nicotine-*N*'-oxide, a 6-fold increase in nicotine-Glucuronide (Gluc), and a 5-fold decrease in 3HC-Gluc when comparing the lower versus upper 3HC/cotinine ventiles. Significant ($P < 0.0001$) associations were observed between functional metabolizing enzyme genotypes and levels of various urinary nicotine metabolites, including CYP2A6 genotype and levels of nicotine, nicotine-Gluc, nicotine-*N*'-oxide and 3HC, UGT2B10 genotype and levels of cotinine, nicotine-Gluc and cotinine-Gluc, UGT2B17 genotype and levels of 3HC-Gluc, FMO3 genotype and levels of nicotine-*N*'-oxide, and CYP2B6 genotype and levels of nicotine-*N*'-oxide and 4-hydroxy-4-(3-pyridyl)-butanoic acid.

Conclusions: These data suggest that several pathways are important in nicotine metabolism.

Impact: Genotype differences in several nicotine-metabolizing enzyme pathways may potentially lead to differences in nicotine dependence and smoking behavior and cessation.

Introduction

Nicotine is the psychoactive alkaloid that stimulates the reward response in the brain upon the consumption of tobacco products. It is extensively metabolized *in vivo*, exhibiting a half-life of approximately 2 hours in humans. Nicotine addiction leads the user to self-titrate their nicotine plasma levels, with a steady usage of tobacco products throughout the day (1). Nicotine is mainly metabolized in the liver to cotinine and subsequently to *trans*-3′-hydroxycotinine (3HC), primarily by the cytochrome P450 (CYP) enzyme, CYP2A6 (see **Fig. 1**; ref. 2). Nicotine and cotinine are glucuronidated to nicotine-Glucuronide (Gluc) and cotinine-Gluc, respectively, primarily by the UDP-glucuronosyltransferase (UGT) 2B10 (3), whereas 3HC-Gluc is formed by several UGTs, including UGT1A9, UGT2B7, and UGT2B17 (4). Nicotine and cotinine are also *N*'-oxidized (5, 6), with nicotine-*N*'-oxide (NOX) formation catalyzed by flavin monooxygenase (FMO) 1, FMO2, and FMO3 (7–9), and cotinine-*N*'-oxide (COX) formation catalyzed primarily by CYPs 2C19, 2A6, and 2B6 (10). Another abundant metabolite of nicotine is 4-hydroxy-4-(3-pyridyl)-butanoic acid (HPBA) and it has been suggested that CYP2A6 may be involved in HPBA production (11).

Smoking behaviors vary widely among different ethnic populations and are heavily influenced by genetic factors (12). For example, smoking prevalence is highest among Native Americans and Alaskan Natives, with smoking rates up to 22%, whereas Asian and Hispanic Americans smokers exhibit smoking rates of between 7% and 15% (13, 14).

In smokers, the ratio of both plasma and urinary 3HC to cotinine, also known as the nicotine metabolic ratio (NMR), has been widely used as a biomarker of CYP2A6 activity (15–18). Recent studies have shown that the ratio of both urinary 3HC/cotinine and total urinary 3HC (3HC plus 3HC-Gluc)/cotinine are effective predictors of plasma NMR (19). It also has been shown that the NMR is highly variable among different demographic groups and that these differences could be affecting smoking cessation rates at the population level (20, 21). These differences have been associated with differences in nicotine metabolite profiles in the plasma and urine of smokers. For example, in populations with a higher frequency of active CYP2A6, 70% to 80% of nicotine is metabolized via CYP2A6 C-oxidation, whereas in

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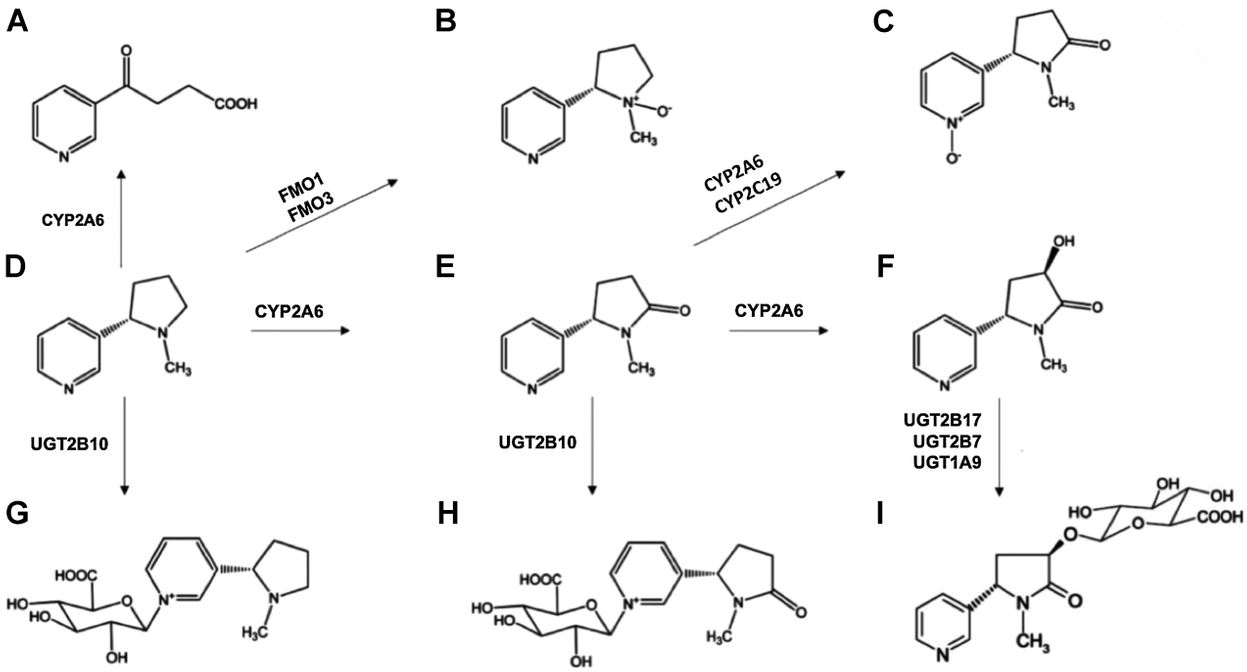

Figure 1.
Metabolic scheme for nicotine. **A,** 4-hydroxy-4-(3-pyridyl)-butanoic acid, (**B**) nicotine-*N'*-oxide, (**C**) cotinine-*N'*-oxide, (**D**) nicotine, **(E**) cotinine, (**F**) *trans-3'*-hydroxycotinine, (**G**) nicotine-Glucuronide, (**H**) cotinine-Glucuronide, and (**I**) 3HC-Glucuronide.

populations with defective CYP2A6 this percentage is decreased to approximately 50% (22). Studies examining the functional effects of genetic variation in nicotine-metabolizing enzyme genes on nicotine metabolite levels have been performed mainly in Caucasian and African American populations (22–24), leading to a lack of meaningful data in other populations.

The Chinese population has a relatively high frequency of CYP2A6-deficient alleles. The minor allele frequency (MAF) of the CYP2A6*4 (whole-gene deletion) allele is up to 25% in Chinese (25, 26) as compared with approximately 3% in Caucasians (27). CYP2A6*7 (rs5031016) has a missense SNP (I471T) that codes for a non-functional CYP2A6 allele with a MAF of up to 16% in Chinese populations but is almost absent in Caucasians (28). The intron variant (rs28399433) encoded by the CYP2A6*9 allele is associated with a 50% reduction in enzyme expression (29) and is prevalent in East Asian populations (MAF = 26%) with a much lower frequency among Caucasian populations (MAF = 7%). The CYP2A6*1A (rs1137115) has been associated with alternative splicing, lower mRNA expression, and slower nicotine metabolism in smokers (30) whereas CYP2A6*12 codes for a hybrid allele between the CYP2A7 and CYP2A6 intron 2 sequence and is associated with approximately 60% reduced activity *in vivo* (31); both of these alleles exhibit similar MAFs of 20% to 25% in the Caucasian and Chinese populations. As a result of the high prevalence of these defective CYP2A6 genetic variants, approximately 22% of Chinese smokers are considered poor metabolizers of nicotine and appear to exhibit different profiles as compared with other racial groups (26, 32).

Among smokers with CYP2A6-defective genotypes, nicotine-metabolizing enzymes other than CYP2A6 may play a more critical role in nicotine metabolism, but this has not been well-characterized. For example, it has been demonstrated that genetic variants in UGT2B10, the main enzyme involved in the formation of nicotine-Gluc and cotinine-Gluc, are associated with increased levels of free cotinine in African American smokers (33). Therefore, the presence of key genetic variants in nicotine-metabolizing enzymes other than CYP2A6 may play an important role in better informing smoking cessation therapy, leading to more tailored smoking cessation therapies particularly in CYP2A6-defective individuals. The goal of the present study was to analyze the topography of urinary nicotine metabolites in Chinese smokers with varying CYP2A6 metabolic capacity and to correlate the levels of urinary nicotine metabolites with genetic variants in major nicotine-metabolizing enzyme genes.

Materials and Methods

Chemicals and materials

LC/MS-grade ammonium acetate and acetonitrile were purchased from Thermo Fisher Scientific, whereas the ACQUITY UPLC BEH-HILIC (1.7 m 2.1 × 100 mm) column was purchased from Waters. Nicotine and eight of its major metabolites (cotinine, 3HC, NOX, COX, nicotine-Gluc, cotinine-Gluc, 3HC-Gluc, and HPBA) as well as their corresponding deuterium labeled (d3) internal standards were purchased from Toronto Research Chemicals.

Study population and collection of biological samples

Subjects were drawn from the Singapore Chinese Health Study (34); this study has been approved by the Institutional Review Boards of the National University of Singapore and the University of Pittsburgh. The present study was approved by the Institutional Review Board of the University of Pittsburgh and Washington State University, Spokane, Washington DC.

The Singapore Chinese Health Study enrolled 63,257 Chinese men and women in Singapore when they were 45 to 74 years old at the time of enrollment, from April 1993 to December 1998 (34). At baseline, each participant was interviewed in person by a trained interviewer using a structured questionnaire that asked for information on demographics, lifetime use of tobacco, and dietary habits during the prior 12 months using a validated semiquantitative food frequency questionnaire (35). Non-fasting blood (10 mL in a plain tube and 10 mL in a heparinized tube per subject) and single-void urine specimens were requested from a random 3% sample of cohort participants from April 1994 to December 1999. Immediately following blood collection, the vacutainer tubes were protected from light using aluminum foil and kept at 5°C–10°C in an ice box or refrigerator until further processing for separating serum or plasma from buffy coat and red blood cells within 4 hours. All blood components were aliquoted into 0.5 mL vials and stored at −80°C freezers. Urine was aliquoted into two urine bottles (20 mL, each containing 0.4 g ascorbic acid) and was stored at −80°C. Beginning in January 2000, requests for biospecimens were extended to all surviving cohort participants. Overall, biospecimens were obtained from 32,535 participants, representing approximately 60% of eligible subjects at the end of the biospecimen collection phase in April 2005. Half of all collected biospecimens were shipped on dry-ice to the University of Pittsburgh (Pittsburgh, PA) where they were continuously stored at −80°C until analysis. The present study included 722 urine samples from smokers that belonged to a case–control study of lung cancer within the Singapore Chinese Health Study.

Genomic DNA was extracted from individual buffy coats using QIAmp DNA mini kits (Qiagen Inc.). Quality and quantity of purified DNA were evaluated using a Nanodrop UV-spectrometer (Thermo Fisher Scientific Inc.). DNA samples were stored at −20°C until analysis immediately after extraction from buffy coats.

UPLC-MS/MS methods

In addition to urinary nicotine, eight major urinary nicotine metabolites (cotinine, 3HC, NOX, COX, nicotine-Gluc, cotinine-Gluc, 3HC-Gluc, and HPBA) were quantified using a previously described LC/MS method (36). Briefly, a UPLC-BEH-HILIC column (2.1 × 100 mm, 1.7 μmol/L) was used for separation of nicotine and its 8 metabolites using a mobile phase containing 5 mmol/L ammonium acetate (pH 5.7) in either 50% acetonitrile (buffer A) or 90% acetonitrile (buffer B), as follows: 20% buffer A for 1.5 minutes, a linear gradient to 100% buffer A from 1.5 to 2.5 minutes, maintenance at 100% buffer A for 3 minutes, and a re-equilibrium step to the initial 20% buffer A conditions from 5.5 to 7 minutes (flow rate 0.4 mL/min). The injection volume was 2 μL using a column temperature of 30°C. MS/MS detection was performed in an AB Sciex Triple-Quad 6500 instrument in MRM ESI^+ mode using a dwell time of 100 msec and a declustering, entrance, and collision cell exit potential of 30, 10, and 10 V, respectively. The mass transitions (Q1/Q3) and collision energies for each metabolite and their corresponding d3-labeled standard are listed in Supplementary Table S1. The nebulizing and drying gases were 50 psi and the optimal temperature of the ion source was 450°C. Observed metabolite retention times (see Supplementary Table S1) were compared with the retention times of corresponding d3 internal standards. Urinary creatinine was quantified as described previously (35) and used as a normalizer of renal function among individuals.

Standard curves were constructed by plotting the ratio of analyte peak area to peak area of the corresponding internal standard versus analyte concentration. For the nicotine and nicotine metabolites standards, a 10 ppm stock solution was made in water. The stock solution was serially diluted in water, and least 12 analyte concentrations were prepared and then mixed with an equal volume of urine from a nonsmoker. Standards at concentrations ranging from 0.005 to 10 ppm were used to establish standard curves. For creatinine, the stock solution (100,000 ppm) was serially diluted in water to concentrations ranging from 0.05 to 50 ppm and used to establish standard curves. Urinary analyte concentrations were determined by measuring the peak area ratios of analyte to internal standard and then calculating analyte concentration from the appropriate standard curve using AB Sciex's Muiltiquant software. The lower limit of quantification (LLOQ) was based on a signal/noise >10. The LLOQ observed were as follows: For nicotine, cotinine, cotinine-Gluc, and COX the LLOQ was 0.0001 ppm, for NOX and nicotine-Gluc it was 0.001 ppm, and for 3HC, 3HC-Gluc and HPBA it was 0.008 ppm. Approximately 9% of the samples were randomly selected for re-analysis for quality control (QC) purposes. A correlation value of $r^2 > 0.9$ was observed when comparing the levels of TNE in the original versus re-analyzed samples.

Candidate SNP genotyping

The methods for *CYP2A6* genotyping were described in detail in a previous report (26, 37). Briefly, there are two structural variant alleles for *CYP2A6*: **4* (gene deletion) and **12* (a hybrid allele derived from a crossover event that joins exons 1–2 of *CYP2A7* with exons 3–9 of *CYP2A6*). These structural variants were measured using quantitative PCR assays designed by ABI (Applied Biosystems). An initial assay using a probe specific to intron 1 of *CYP2A6* (hs0754274_cn) was used to determine the number of *CYP2A6* gene copies present relative to the housekeeping gene RNaseP, with all informative samples amplifying the RNaseP gene. Those samples with two copies of hs0754274_cn were designated (**1/*1*). Those samples with no amplification of hs0754274_cn would be designated as homozygous for the gene deletion (**4/*4*); this was not observed in the present study. Those samples with a single copy of hs0754274_cn (CYP2A6**1/x*) were carried forward to a second qPCR assay using ABI probe hs07545275_cn that is specific for intron 7 of the *CYP2A6* gene (i.e., does not cross-react with *CYP2A7*). Those samples with 1 copy of intron 7 in the second assay were designated (**1/*4*), and those with 2 copies of intron 7 were designated (**1/*12*). All qPCR assays were repeated 4 times.

Additional variants that do not alter the *CYP2A6* gene structure were then assessed. To maximize assay specificity and minimize DNA input, the **7* (rs5031016) and **9* (rs28399433) alleles were genotyped using nested PCR followed by pyrosequencing (using the Pyromark Q96MD Sequencer, Qiagen). The **1A* variant was captured using an allelic discrimination (TaqMan) assay for rs1137115 (Life Technologies).

The Sequenom MassARRAY iPlex platform was used to determine SNP genotypes for FMO3 (rs1736557, rs2266780, and rs226782) and UGT2B10 (rs294857, rs835316, rs844342, and rs909530). The UGT2B10 SNP rs11249509 was separately genotyped using a TaqMan assay (Life Technologies). The UGT2B17 deletion was assessed using qPCR as described by Gallagher and colleagues (38). For QC purposes, every 10th well of the genotyping plates contained a control DNA sample of Han Chinese ethnicity from the Coriell Institute (Coriell.org.).

Genome-wide SNPs

Genotypes of *CYP2B6, CYP2C19, and FMO1* were derived from genome-wide SNP analysis. The methods for the determination of genome-wide SNPs and imputation were described previously (39).

Briefly, DNA samples were genotyped using the Illumina Global Screening Array v1.0 or the Illumina Global Screening Array v2.0. Samples with a call-rate <95.0% and extremes in heterozygosity were excluded. We imputed for additional autosomal SNPs with IMPUTE v2 using the cosmopolitan 1000 Genomes haplotypes (Phase 3) as the reference panel. SNPs with an impute information score of <0.8, MAF <1.0%, Hardy–Weinberg Equilibrium P value of $<1 \times 10^{-6}$ as well as non-biallelic SNPs were excluded from subsequent analyses. Alleles for all SNPs were coded to the forward strand and mapped to Genome Reference Consortium Human Build 37 as previously described (39). In total, 6,407,959 genotyped and imputed SNPs were available for statistical analyses after stringent QC procedures were undertaken as previously described (39) in terms of genotyping completeness for genetic markers and individuals, and removal of samples showing evidence of duplication or contamination.

Metabolizing enzyme groups

For analysis, we examined SNPs with a MAF of >0.04 as described previously for Hans Chinese in the 1000 Genomes Database. The CYP2A6-metabolizing enzyme groups were classified according to a previously reported classification for the Chinese population (26). They included the *1A (40) and *9 (29) variant alleles, which are considered decreased activity alleles, the *4 deletion (26, 28, 37), and the *12 (31) and *7 (26, 28, 41) variants, which are considered null activity alleles; the *1 allele is considered to be wild type. CYP2A6-metabolizing enzyme groups were defined as follows: Extensive metabolizers EM_{2A6} (*1/*1; $n = 45$), intermediate metabolizers IM_{2A6} (*1/*1A, *1/*9,*1/*12; $n = 93$), slow metabolizers SM_{2A6} (*1A/*1A, *1A/*12, *1A/*9, *1A/*7,*1A/*4, *1/*4, *1/*7, *9/*9; $n = 171$), and poor metabolizers PM_{2A6} (*4/*7, *4/*9, *7/*7, *7/*9; $n = 49$). Other potential CYP2A6 genotype combinations were not observed for the alleles examined in the population studied.

The assessment of UGT2B10 genotypes was based on previously reported activities for the intronic variant rs835316 (42) and the splice variant rs2942857, which was in high linkage disequilibrium (LD $r^2 = 1.0$) with rs61750900 (43). UGT2B10 metabolizing enzyme groups were classified as follows: EM_{2B10} (*1/*1; $n = 101$), IM_{2B10} (*1/rs835316; $n = 188$), SM_{2B10} (rs835316/rs835316 and rs2942857/rs835316; $n = 58$), and PM_{2B10} (rs2942857/rs2942857; $n = 3$).

Single allele genotypes were analyzed for other metabolizing enzymes, including CYP2B6, CYP2C19, FMO1, and FMO3.

Statistical analysis

The levels of nicotine and its metabolites were analyzed in subjects that were statistically stratified by their urinary NMR (cotinine/3HC) into quartiles and ventiles (20 quantiles). The relative levels of individual nicotine metabolites, normalized by total nicotine equivalents (TNE, equivalent to the total level of urinary nicotine plus its eight measured metabolites), were compared within the different metabolizing enzyme groups. Because the distributions of the direct levels of the nine urinary biomarkers, or the biomarkers expressed as a ratio with TNE, were not normally distributed, statistical analyses for non-normally distributed samples was utilized. Spearman's (r) and Pearson's (r^2) correlation coefficients were calculated between the urinary NMR and the relative levels of individual nicotine metabolites. Z-scores were calculated to compare Spearman's correlation coefficients in men versus women, with a P value of 0.003 considered significant due to multiple comparisons of nine metabolites by gender (0.05/18). All analyses were two-sided and considered significant if $P < 0.05$ for all tests. Continuous outcome variables were analyzed for all genotype–phenotype associations using the Student t test (Mann–Whitney U test for non-parametric distributions), or the two-way Kruskal–Wallis test using a Dunn *post hoc* test for multiple corrections for all possible pairwise comparisons between metabolizing enzyme groups. All statistical tests were conducted using the statistics program GraphPad Prism version 6.01 (GraphPad Software).

Results

Nicotine urinary metabolites in smokers

Table 1 shows the levels of nicotine and its eight major metabolites in the urine of 722 Chinese smokers. Approximately 9% of the samples ($n = 64$) were randomly selected for re-analysis to assess metabolite level phenotyping error rates. A correlation of $r^2 = 0.91$ was observed when comparing the levels of TNE in the original versus re-analyzed samples. As described in previous studies (4, 44), 3HC (13.4 nmol/mg creatinine) was the major urinary metabolite observed in this population, followed by nicotine (6.3 nmol/mg creatinine) > cotinine (5.4 nmol/mg creatinine) ∼ 3HC-Gluc (5.2 nmol/mg creatinine) > cotinine-Gluc (4.4 nmol/mg creatinine) ∼ HPBA (4.4 nmol/mg creatinine) > NOX (3.2 nmol/mg creatinine) > nicotine-Gluc (2.5 nmol/mg creatinine) > COX (1.4 nmol/mg creatinine). No differences in this pattern or in the levels of these metabolites as a percentage of TNE were observed between patients with lung cancer and unaffected controls when analyzed independently (Supplementary Table S2).

To examine whether CYP2A6 activity affected the levels of urinary nicotine metabolites, subjects were stratified on the basis of their urinary NMR (cotinine/3HC). Because cotinine and 3HC comprise the two variables that are used to calculate the NMR, as expected, strong significant correlations were observed between the NMR and urinary cotinine ($r = -0.5802$, $P < 0.0001$) and 3HC ($r = 0.7755$, $P < 0.0001$) measured as a ratio with TNE (Supplementary Fig. S1A and S1B, respectively). Strong negative correlations were also observed between the NMR and urinary NOX/TNE ($r = -0.6158$, $P < 0.001$) and nicotine-Gluc/TNE ($r = -0.3080$, $P < 0.0001$; **Fig. 2A**). Significant negative correlations were also observed between the NMR and HPBA/TNE ($r = -0.2341$, $P < 0.0001$) and nicotine/TNE ($r = -0.2150$, $P < 0.0001$; Supplementary Fig. S1C and S1D, respectively) whereas a strong positive correlation was observed between the NMR and 3HC-Gluc/TNE ($r = 0.4968$, $P < 0.0001$; **Fig. 2A**). No significant correlations were observed between the NMR and either cotinine-Gluc/TNE ($r = 0.0677$) or COX/TNE ($r = 0.0148$; Supplementary Fig. S1E and S1F, respectively). Similar associations were observed when examining cases and controls independently (Supplementary Table S2) and when examining men versus women (Supplementary Table S3).

When stratifying subjects by NMR quartiles ($n = 180$–181/group), the mean NOX/TNE, nicotine-Gluc/TNE and 3HC-Gluc/TNE for each group (lowest to highest NMR) was 10.6%, 6.6%, 5.1%, and 3.0%; 12.0%, 7.3%, 7.1%, and 4.3%; and 8.7%, 14.3%, 16.3%, and 21.0%, respectively (**Fig. 2B**). These differences corresponded to significant 3.5- ($P < 0.0001$) and 2.8 ($P < 0.0001$)-fold decreases in urinary NOX/TNE and nicotine-Gluc/TNE, respectively, and a significant 2.4 ($P < 0.0001$)-fold increase in 3HC/TNE, when comparing the lowest versus highest NMR quartile groups. Urinary cotinine/TNE and 3HC/TNE exhibited expected decreases (19.4%, 16.2%, 12.5%, and 8.2%) and increases (11.6%, 21.0%, 27.1%, and 34.7%), respectively, in the lowest to highest NMR groups (Supplementary Fig. S2A and S2B). Furthermore, when subjects were stratified by urinary NMR ventiles ($n = 36$–37/group), significant differences were observed for the same five metabolites ($P < 0.0001$ for each), with 7.4- and

Table 1. Urinary nicotine metabolites in Chinese smokers (n = 722).

Metabolite	per Total-NIC-Eq[a] (%)		Creatinine-adjusted levels (nmol/mg creatinine)	
	Mean ± SD (95% CI)	Range	Mean ± SD (95% CI)	Range
Nicotine	12.7 ± 11.6 (11.8–13.6)	0–68.7	6.3 ± 9.6 (5.5–7.0)	0–112.3
Cotinine	9.1 ± 8.3 (8.7–9.6)	0–28.7	5.4 ± 8.0 (4.8–6.0)	0–69.3
3HC	29.3 ± 26.1 (27.3–31.4)	0–99.0	13.4 ± 18.3 (11.9–14.8)	0–115.9
Nicotine-Gluc	5.8 ± 5.4 (5.3–6.2)	0–40.6	2.5 ± 3.1 (2.2–2.7)	0–31.9
Cotinine-Gluc	10 ± 6.1 (9.5–10.5)	0.02–33.6	4.4 ± 4.7 (4.0–4.8)	0–69.3
3HC-Gluc	12.4 ± 11.0 (11.5–13.2)	0–58.8	5.2 ± 7.1 (4.7–5.8)	0–68.8
Cotinine-*N*-Oxide	3.2 ± 1.9 (3.1–3.4)	0–9.5	1.4 ± 1.3 (1.3–1.5)	0–18.3
Nicotine-*N*′-Oxide	7.6 ± 6.1 (7.1–8.0)	0.15–40.9	3.2 ± 3.8 (2.9–3.5)	0–43.7
HPBA	9.9 ± 7.6 (9.4–10.5)	0–85.7	4.4 ± 4.7 (4.0–4.7)	0–34.6

Abbreviations: CI, confidence interval; SD, standard deviation.
[a]Total-Nic-Eq, total nicotine equivalents, equal to the sum of all measured urinary nicotine and its eight major metabolites.

5.3-fold decreases observed for the mean NOX/TNE and mean nicotine-Gluc/TNE, respectively, and a 5.3-fold increase for the mean 3HC-Gluc/TNE, when comparing the lowest and highest NMR ventiles (Supplementary Fig. S3). Decreasing trends were also observed for both urinary nicotine and HPBA, with 1.3- and 1.6-fold decreases for nicotine/TNE and HPBA/TNE, respectively, when comparing the lowest with the highest NMR quartiles (Supplementary Fig. S2C and S2D). Similar results were observed when analyzing the case and control groups independently, with 4.5-, 5.7-, and 5.3-fold decreases observed for cotinine/TNE, NOX/TNE and nicotine-Gluc/TNE, respectively, and 7.2- and 6.9-fold increases for 3HC/TNE and 3HC-Gluc/TNE, respectively, observed when comparing the lowest with the highest NMR ventiles for cases, and 3.6-, 8.3- and 4.4-fold decreases observed for cotinine/TNE, NOX/TNE and nicotine-Gluc/TNE, respectively, and 6.6- and 5.5-fold increases for 3HC/TNE and 3HC-Gluc/TNE, respectively, when comparing the lowest with the highest NMR ventiles for controls. Cases and controls were thus combined for subsequent analysis.

Nicotine-metabolizing enzyme genotypes and urinary nicotine metabolite levels

A total of 58 genetic variants in 12 nicotine-metabolizing enzymes were analyzed in this study. This included five genetic variants in CYP2A6, three in CYP2B6, five in CYP2C19, 12 in FMO1, 11 in FMO3, five in UGT1A1, four in UGT1A4, one in UGT1A9, one in UGT1A10, three in UGT2B7, seven in UGT2B10, and one in UGT2B17. Significant (P < 0.05) associations for individual polymorphisms and the levels of urinary nicotine metabolites are shown for combined cases and controls in Supplementary Table S4. A total of 39 significant associations were identified between variants in CYP2A6, CYP2B6, CYP2C19, FMO1, FMO3, UGT2B10 and UGT2B17 and the

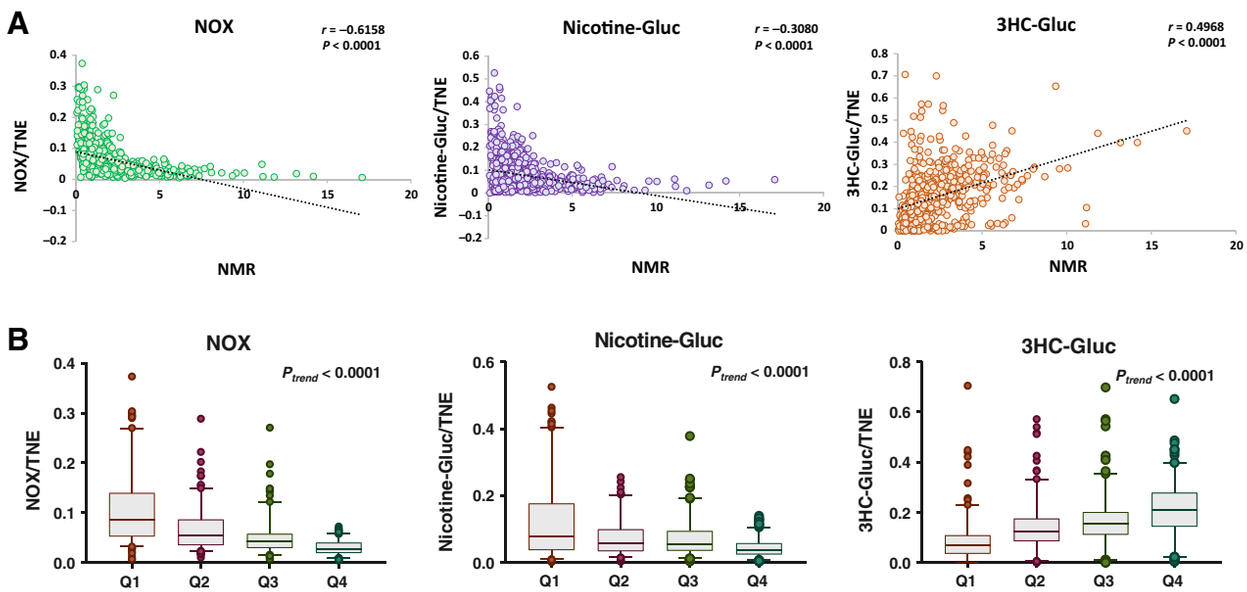

Figure 2.
Analysis of NOX, nicotine-Gluc, and 3HC-Gluc levels in Chinese smokers. **A,** Scatterplots of urinary NMR (3HC/cotinine) versus urinary NOX, nicotine-Gluc, and 3HC-Gluc. All nicotine metabolites were measured as a ratio of total nicotine equivalents (TNE). Black lines indicate the linear regression line of the data. The Spearman's correlation coefficient and P values are shown on the graphs. **B,** Box plots of urinary NOX, nicotine-Gluc, and 3HC-Gluc, measured as a ratio with TNE, are shown for samples stratified by quartiles of urinary NMR. The horizontal lines within each box plots are median values, whereas individual dots are considered outliers.

levels of different nicotine metabolites. No significant associations were observed for UGT1A1, UGT1A4, UGT1A9, UGT1A10 or UGT2B7.

CYP2A6 is involved in the formation of several nicotine metabolites, including cotinine, 3HC and HPBA (2, 11). The effects of CYP2A6 genotype on the levels of nicotine metabolites was examined using CYP2A6-metabolizing enzyme group stratifications classified according to CYP2A6 genotype as described in the Materials and Methods. Associations were observed between CYP2A6 genotype and the urinary levels of nicotine and 3HC (Supplementary Table S4), with significantly ($P < 0.0004$ for all comparisons) increased nicotine/TNE and decreased 3HC/TNE ratios observed in the PM_{2A6} ($n = 55$) group as compared with either the EM_{2A6} ($n = 65$), IM_{2A6} ($n = 114$) or SM_{2A6} ($n = 208$) groups (**Fig. 3A** and **C**, respectively). Similar to that observed for 3HC/TNE ratio, decreases in urinary NMR were also observed with CYP2A6 genotype (Supplementary Table S4), with significantly ($P < 0.0001$) decreased NMR observed in both the SM_{2A6} and PM_{2A6} groups as compared with either the EM_{2A6} or IM_{2A6} groups (**Fig. 3D**). Although significant associations were also observed for urinary cotinine/TNE ($P = 0.017$), nicotine-Gluc/TNE ($P < 0.0001$), cotinine-Gluc/TNE ($P = 0.0081$), 3HC-Gluc/TNE ($P < 0.0001$), and NOX/TNE ($P < 0.0001$; **Fig. 3B**, **E–H,** respectively), no significant associations were observed for urinary COX/TNE ($P = 0.34$) or HPBA/TNE ($P = 0.60$; **Fig. 3I** and **J**, respectively).

When combined groups were examined, the $EM_{2A6} + IM_{2A6}$ group ($n = 179$) exhibited significantly ($P < 0.01$ for all associations) decreased cotinine/TNE, nicotine/TNE, NOX/TNE, nicotine-Gluc/TNE, and cotinine-Gluc/TNE, and increased 3HC/TNE, 3HC-Gluc/TNE and NMR as compared with the $SM_{2A6} + PM_{2A6}$ group ($n = 263$); no significant associations were observed for urinary COX/TNE ($P = 0.13$) and HPBA/TNE ($P = 0.34$; Supplementary Fig. S4).

A similar analysis was performed for UGT2B10, which is the primary enzyme involved in the glucuronidation of both nicotine and cotinine (3, 45). Both urinary nicotine-Gluc and cotinine-Gluc levels were significantly ($P < 0.0001$) associated with UGT2B10 genotype in this study (Supplementary Table S4). Dramatic decreases in urinary nicotine-Gluc/TNE and cotinine-Gluc/TNE ratios were observed in PM_{2B10} ($n = 5$) subjects as compared with either EM_{2B10} ($n = 219$; $P = 0.0005$ and 0.0003, respectively) or IM_{2B10} ($n = 113$; $P = 0.0002$ and 0.0002, respectively) subjects (**Fig. 4E** and **F**). In contrast, UGT2B10 genotype was associated with significant increases in urinary cotinine/TNE ($P = 0.049$) and 3HC/TNE ($P = 0.0036$) ratios (**Fig. 4B** and **C**, respectively). No significant associations were observed for UGT2B10 genotype and levels of nicotine ($P = 0.41$), 3HC-Gluc/TNE ($P = 0.17$), NOX/TNE ($P = 0.72$), COX/TNE ($P = 0.23$), or HPBA/TNE ($P = 0.98$; **Fig. 4A**, **G–J**, respectively). The previously reported UGT2B10 D67Y SNP (rs61750900; ref. 3) exhibited a MAF = 0.002 in this study population, with only two heterozygous individuals present in the study population; both showed an approximately 50% decrease in the levels of both urinary nicotine-Gluc and cotinine-Gluc when compared with UGT2B10 (*1/*1) subjects.

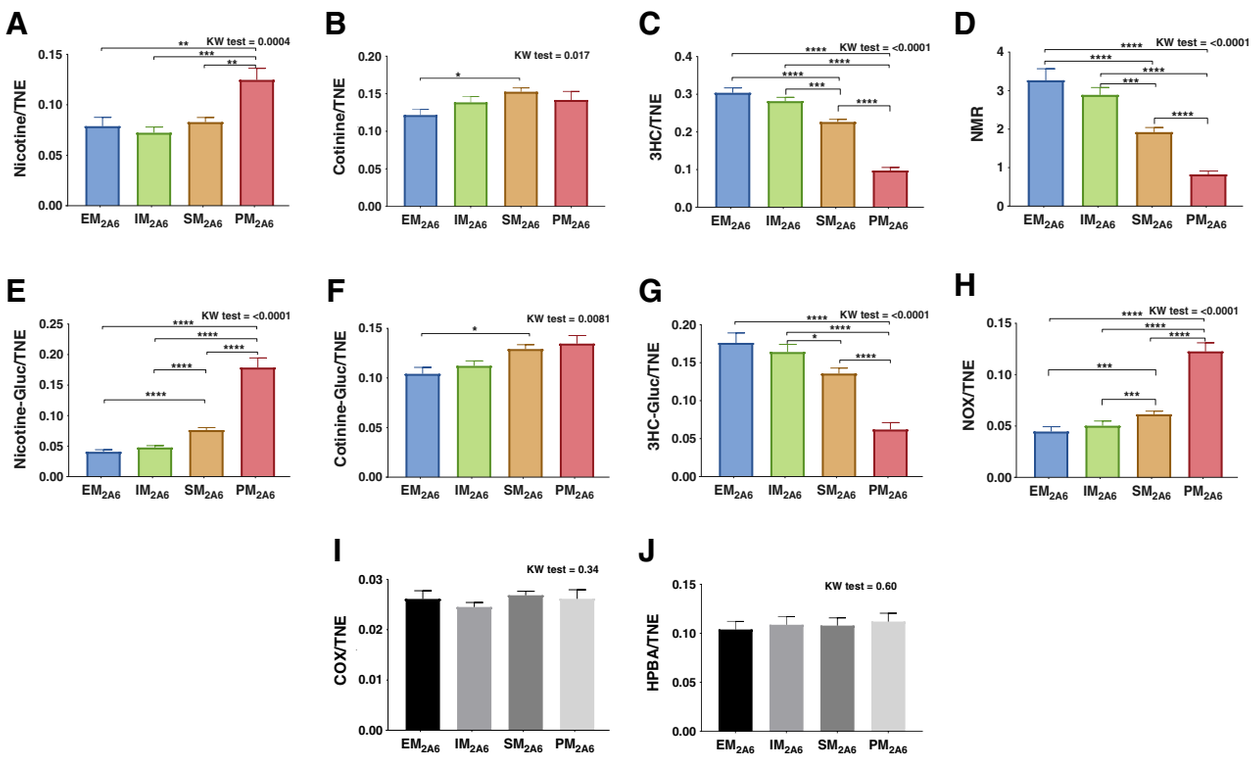

Figure 3.

Urinary nicotine metabolites in subjects stratified by CYP2A6-metabolizing enzyme groups. Urinary levels of **A,** Nicotine/TNE; **B,** Cotinine/TNE; **C,** 3HC/TNE; **D,** NMR; **E,** Nicotine-Gluc/TNE; **F,** Cotinine-Gluc/TNE; **G,** 3HC−Gluc/TNE; **H,** NOX/TNE; **I,** COX/TNE; and **J,** HPBA/TNE. Subjects were stratified into the following CYP2A6-metabolizing enzyme groups: Extensive metabolizers (EM_{2A6})–*1/*1; intermediate metabolizers (IM_{2A6})–*1/*1A, *1/*9, and *1/*12; slow metabolizers (SM_{2A6})–*1A/*1A, *1A/*4, *1A/*7, *1A/*9, *1A/*12,*1/*4, *1/*7, *9/*9, and *9/*12; and poor metabolizers (PM_{2A6})–*4/*7, *4/*9, *7/*7, and *7/*9. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Metabolites with significant differences are shown in color.

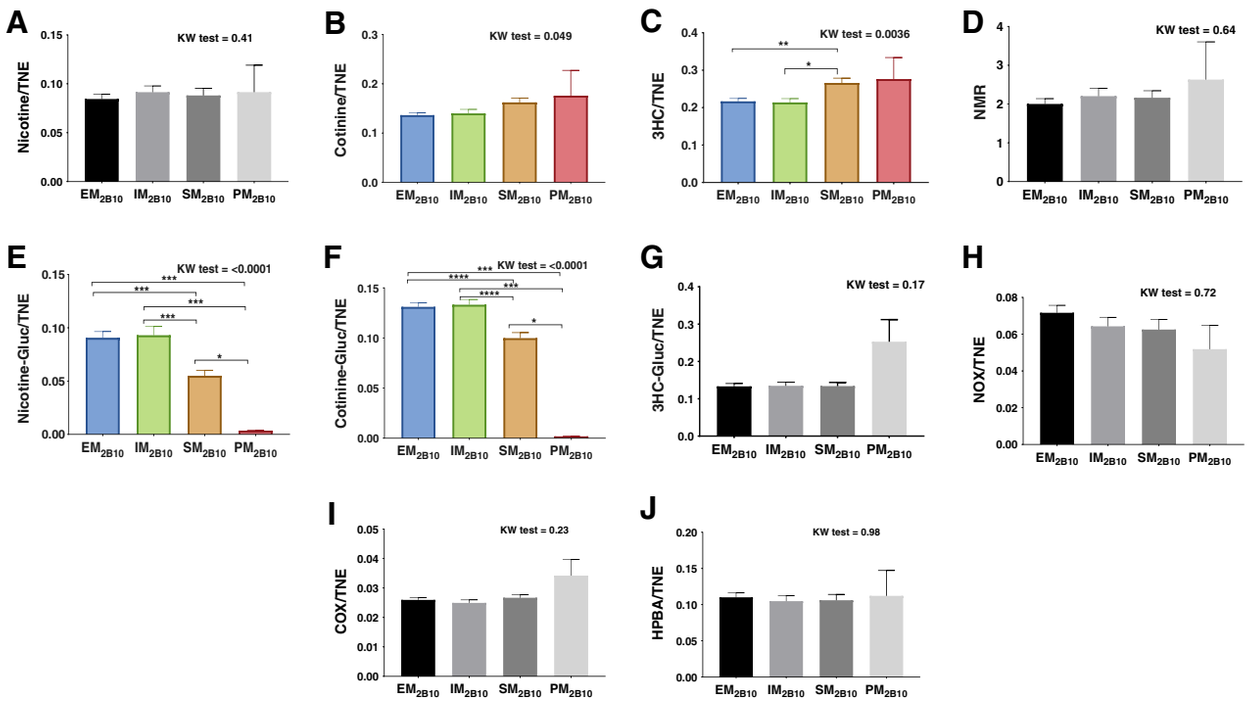

Figure 4.

Urinary nicotine metabolites in subjects stratified by UGT2B10-metabolizing enzyme groups. Urinary levels of **A,** nicotine/TNE; **B,** cotinine/TNE; **C,** 3HC/TNE; **D,** NMR; **E,** nicotine-Gluc/TNE; **F,** cotinine-Gluc/TNE; **G,** 3HC-Gluc/TNE; **H,** NOX/TNE; **I,** COX/TNE, and **J,** HPBA/TNE. Subjects were stratified into the following UGT2B10-metabolizing enzyme groups: Extensive metabolizers (EM_{2B10})–*1/*1; intermediate metabolizers (IM_{2B10})–*1/rs835316; slow metabolizers (SM_{2B10})–rs835316/rs835316 and rs2942857/rs835316; and poor metabolizers (PM_{2B10})–rs2942857/rs2942857. Allele designations have not been described for SNPs rs835316 and rs2942857 in the UGT2B10 gene, so the rs number was used as the allele designation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Metabolites with significant differences are shown in color.

UGT2B17 is one of three enzymes involved in the metabolism of 3HC to 3HC-Gluc (4, 6). Subjects homozygous for the UGT2B17 deletion allele ($n = 331$) exhibited significantly increased urinary 3HC-Gluc/TNE ($P = 0.0012$) ratios as compared with subjects with at least one wild-type UGT2B17 allele ($n = 142$; **Fig. 5A**). The small 1.1-fold increase in urinary 3HC/TNE observed in UGT2B17-deleted subjects versus subjects with at least one UGT2B17 allele was not significant ($P = 0.09$). No other nicotine metabolites exhibited significant associations with UGT2B17 genotype. The UGT isoforms 1A9 and 2B7 have also been reported to exhibit 3HC glucuronidation activity, but no significant associations were observed for rs28898622 for UGT1A9, or rs7439366, rs7434332, and rs10028494 for UGT2B7, and 3HC-Gluc/TNE ratio in this study.

The FMO3*2 allele, previously described as the *Hap allele (10, 46), includes the variants rs2266780 (31) and rs2266782 (9) that are in high LD ($r^2 = 0.837$) in East Asian populations (47). Although several enzymes are involved in NOX formation, only the FMO3*2 allele was associated with the levels of urinary NOX in this study. Subjects homozygous for the FMO3*2 allele ($n = 17$) exhibited a significant ($P < 0.027$) 1.6-fold decrease in urinary NOX/TNE ratio as compared with subjects with one or more wild-type FMO3*1 alleles ($n = 416$; **Fig. 5B**). Although no effect on NOX levels was observed for the FMO3 rs2075992 variant, this variant was significantly associated with increased TNE ($P < 0.014$) and decreased HPBA ($P = 0.035$; Supplementary Table S4).

For FMO1, the rs28384819 variant exhibited a significant ($P = 0.014$) increase in nicotine-Gluc formation, whereas the rs12044884 variant showed a significant ($P = 0.040$) increase in NOX/TNE and a decrease ($P = 0.018$) in 3HC-Gluc/TNE. The FMO1 variants rs7533889 and rs12044884 exhibited significant associations with TNE ($P = 0.026$) and NMR ($P = 0.019$), respectively, with the rs7533889 variant also exhibiting a significant ($P = 0.030$) association with decreased NMR (Supplementary Table S4).

CYP2B6 has been reported to play a role in the formation of cotinine, nornicotine, and COX (10, 48, 49). Subjects heterozygous for the CYP2B6 rs8192709 intron variant ($n = 49$) exhibited significant ($P = 0.04$) decreases in urinary COX/TNE (Supplementary Table S4); only one subject was homozygous for this variant and exhibited a 33.4% decrease in COX/TNE formation. Subjects homozygous for the CYP2B6 rs4803419 intron variant ($n = 75$) exhibited significantly decreased urinary NMR ($P = 0.015$) and HPBA/TNE ($P = 0.024$) ratios, and a near-significant ($P = 0.066$) increase in urinary nicotine-Gluc/TNE ratio, as compared with subjects with at least one copy of the wild-type CYP2B6*1 allele ($n = 378$; **Fig. 5C–E**, respectively). Although a role for CYP2C19 in COX formation was recently described (10), no significant associations were observed between CYP2C19 genotype and the levels of urinary COX in this study.

Discussion

In the present study, urinary nicotine metabolite profiles were examined in a large population of Chinese smokers to determine whether the levels of these metabolites are affected by deficiencies in CYP2A6 activity and genotypes in nicotine-metabolizing genes. This is

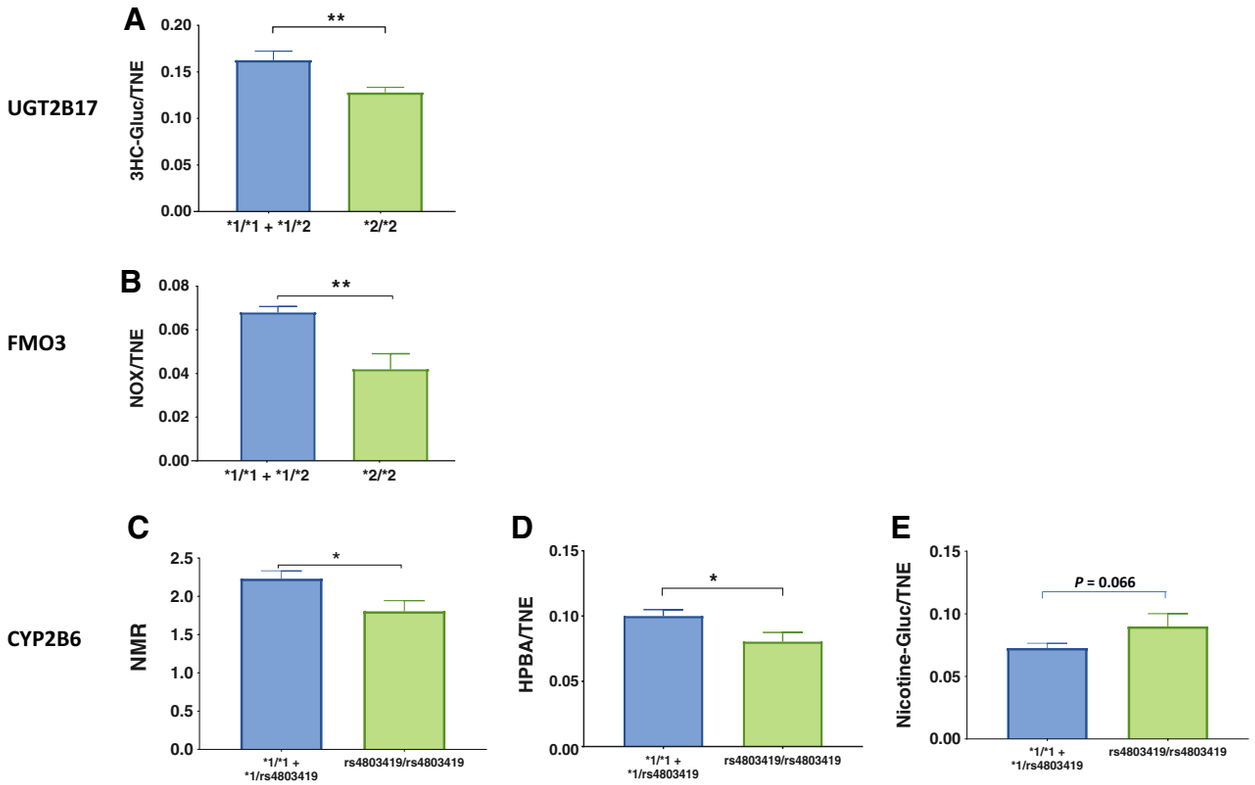

Figure 5.

Urinary nicotine metabolites in subjects stratified by UGT2B17, FMO3, and CYP2B6 genotypes. **A–E,** A dominant model was used for all comparisons, with urinary nicotine metabolites compared in subjects homozygous for the minor allele versus subjects with at least one copy of the major wild-type allele. Allele designations have not been described for SNP rs48034419 in the CYP2B6 gene, so the rs number was used as the allele designation. *, $P < 0.05$; **, $P < 0.01$.

the first study to undertake a comprehensive analysis of the effects of genetic variants in nicotine-metabolizing enzyme genes and nicotine metabolites in a Chinese population and, given the higher prevalence of CYP2A6 deficient alleles in this population, it allowed for a more thorough analysis of the importance of all pathways involved in nicotine metabolism. Overall, the levels of nicotine metabolites observed in this population were slightly different than that reported for Caucasian populations (36). A mean 58% higher level of urinary NOX and 35% higher level of nicotine-Gluc were observed in the Chinese smokers examined in this study than that reported previously for Caucasian populations (36). This corresponded with a mean 13% decrease in the percentage of urinary 3HC in Chinese smokers when compared with the levels reported for Caucasian smokers (36). This is likely due to the higher prevalence of functional variants in the CYP2A6 gene, including the *4, *7, and *9 alleles in the Chinese population as compared with Caucasian populations (31, 50, 51). In addition, the levels of cotinine-Gluc in the urine of the Chinese subjects examined in the present study were 37% higher than that observed in Caucasian and African American populations (36, 44). This corresponds with the lower prevalence of functional variants in UGT2B10 among the Chinese population as compared with either Caucasians or African Americans (23, 40, 43). The relative levels of free nicotine, free cotinine, COX, and HPBA observed in the present study were roughly similar to those observed previously in Caucasian populations (12.7% vs. 10.6%, 9.1% vs. 11%, 3.2% vs. 2.8%, and 9.9% vs. 8.9%, respectively) (36). The metabolite profile data obtained in this Chinese population were, however, similar to those reported for Japanese Americans, where the levels of NOX and nicotine-Gluc were both increased by approximately 10% as compared with Caucasians (23). Together, these data are consistent with a differential effect on nicotine metabolism by metabolizing enzyme allelic prevalence differences observed between Asians and other racial groups.

When stratifying subjects by CYP2A6 activity using the urinary NMR (cotinine/3HC) as a biomarker, a strong correlation was observed between urinary cotinine and 3HC with the urinary NMR, validating the use of the urinary NMR (the ratio of 3HC over cotinine) as a biomarker for CYP2A6 activity. Interestingly, strong negative correlations were also observed between the NMR and both urinary NOX and nicotine-Gluc, whereas a strong positive correlation was observed between the NMR and urinary 3HC-Gluc. When subjects were stratified on the basis of NMR quartiles or ventiles, large increases were observed in urinary NOX when comparing the highest versus lowest NMR activity quartiles (3.5-fold) or ventiles (7.4-fold). A similar pattern was observed for nicotine-Gluc, which exhibited 2.8- and 5.3-fold increases when comparing the highest versus lowest NMR quartiles or ventiles, respectively. In contrast, the levels of 3HC-Gluc were decreased by 2.4- and 5.3-fold when comparing the highest versus lowest NMR quartiles or ventiles, respectively. Together, these data suggest that when subjects exhibit low CYP2A6 activity, nicotine metabolism is rerouted to other nicotine metabolism pathways, including *N*-oxidation and glucuronidation, with the ultimate formation of 3HC-Gluc decreased due to lower levels of urinary 3HC in those

subjects. In addition, the fact that urinary nicotine was only moderately associated with urinary NMR in this population is consistent with nicotine metabolism being shunted to other pathways (e.g., by *N*-oxidation or glucuronidation) when CYP2A6 is deficient. These data are consistent with a small study of Chinese smokers ($n = 106$) where the levels of NOX were increased by 3.4-fold in the lowest versus highest NMR ventile groups (9) and are also consistent with a previous report indicating that NOX and nicotine-Gluc levels were as high as 31% and 46% of TNE, respectively, and 3HC was not detected, in two subjects deleted for CYP2A6 (52).

HPBA had previously been reported to be a direct metabolite of either nicotine or cotinine in a reaction mediated by CYP2A6 (11, 53, 54), and the present study is the first to examine HPBA levels in the urine of a panel of Chinese smokers. Interestingly, a significant negative correlation was observed between the NMR and HPBA levels in the present study, with a 54% decrease in HPBA observed in the lowest versus the highest NMR ventiles. Because the NMR is predictive of CYP2A6 activity, these data are consistent with previous studies, suggesting that CYP2A6 is likely to be an important enzyme involved in HPBA formation.

Ethnic and racial differences in the distribution of metabolizing enzyme genetic variants among different population groups, resulting in differences in overall enzymatic activities and ultimately in differences in nicotine metabolite profiles, have been well described previously (23, 33). Specifically, it has been reported that populations of Asian descent have an overall decreased amount of total nicotine metabolites excreted via the CYP2A6-mediated pathway (cotinine and 3HC) due to increased prevalence of CYP2A6-deficient alleles (22, 52). In the present study, a strong effect was observed for CYP2A6 genetic variation on nicotine metabolism profiles. For example, urinary NOX, nicotine-Gluc and cotinine-Gluc were 2.7-, 4.4-, and 1.3-fold higher, respectively, in the PM_{2A6} group as compared with the EM_{2A6} group, whereas urinary 3HC-Gluc was decreased by 65% in the PM_{2A6} group. A 1.6-fold increase was observed for nicotine and a decrease of 68% was observed for 3HC in the PM_{2A6} group versus the EM_{2A6} group. As discussed above, when CYP2A6 activity is impaired, nicotine (and cotinine) metabolism may be rerouted to non-CYP2A6 metabolism pathways with CYP2A6-catalyzed formation of metabolites like 3HC and its direct metabolite, 3HC-Gluc, decreased in the urine of smokers. Consistent with the important role of CYP2A6 in the metabolism of cotinine to 3HC, the NMR was decreased by 75% in the PM_{2A6} versus EM_{2A6} groups, a pattern consistent with previous reports within the Singapore Chinese Health study that demonstrated a decrease in NMR in "poor" versus "normal" CYP2A6-metabolizing enzyme groups (26).

When analyzed individually, the CYP2A6 (*4), (*7), and (*9) alleles showed significant associations with decreased levels of 3HC, increased levels of NOX, increased levels of nicotine-Gluc, decreased levels of 3HC−Gluc, and a significant effect on decreased urinary NMR when comparing subjects homozygous for any of these alleles with subjects containing no more than one of these alleles. This is in agreement with data previously reported for Japanese, Korean and Chinese populations (26, 28, 41, 55) and is also consistent with the loss-of-function associated with these alleles; the CYP2A6*4 variant codes for a whole deletion variant and is a gene knock-out (56, 57), the CYP2A6*9 variant is a TATA box polymorphism (-48 T>G) that is associated with reduced enzyme expression (29), and the CYP2A6*7 variant has an I471T amino acid substitution in the substrate recognition site in exon 9 that has been associated with a lack of CYP2A6 nicotine oxidation activity (58). In addition, the CYP2A6*1A allele showed a significant association with decreased levels of TNE in the present study, with subjects homozygous for this allele exhibiting a 28% reduction in TNE as compared with subjects who were homozygous wild-type or contained only one CYP2A6*1A allele. This is consistent with previous studies showing an association between this variant and reduced cigarette consumption (59) and late onset of smoking (60), and *in vitro* studies suggesting that this variant is associated with lower mRNA expression and slower nicotine metabolism (50, 61).

UGT2B10 has been previously shown to be the major enzyme involved in the glucuronidation of both nicotine and cotinine (3, 62), and UGT2B10 genotypes exhibited a significant impact on the levels of both urinary nicotine-Gluc and cotinine-Gluc in the present study. Greater than 25-fold decreases were observed for both urinary nicotine-Gluc and cotinine-Gluc in the PM_{2B10} versus EM_{2B10} groups and in subjects homozygous for the UGT2B10 splice variant (rs2942857 or rs116294140) versus subjects with at least one wild type UGT2B10 allele. These data further support UGT2B10 as the main enzyme catalyzing the formation of these metabolites (3, 36, 62), and are consistent with that observed previously for the UGT2B10 splice variant in African Americans (23) and that this variant codes for a non-functional UGT2B10 isoform (43). The data are also consistent with the pattern observed previously for the UGT2B10 D67Y variant, which is similarly associated with large decreases in nicotine-Gluc and cotinine-Gluc in Caucasians (33, 36, 40) and has been shown to be non-functional *in vitro* (63). Although the MAF of the D67Y variant is low in Chinese, two of the subjects examined were heterozygous for this SNP in the present study and exhibited approximately 50% decreases in levels of nicotine-Gluc and cotinine-Gluc as compared with subjects who were wild-type for UGT2B10.

Functionally deficient UGT2B10 genotypes also exhibited an important impact on cotinine levels. This is similar to that observed for African American and Caucasian smokers, where it was demonstrated that free plasma cotinine levels were up to 24% higher in carriers of defective UGT2B10 alleles as compared with subjects exhibiting the wild-type UGT2B10 genotype (64). In addition, significant increases in urinary 3HC were observed in the EM_{2B10} versus the combined $SM+PM_{2B10}$ groups in the present study, indicating some shuttling of cotinine metabolism to 3HC rather than cotinine-Gluc. UGT2B10-induced variability in cotinine and/or 3HC levels could potentially have important effects on the NMR because these are the two metabolites that are used in the calculation of the NMR. However, although we observed a 1.3-fold increase in the urinary NMR in the PM_{2B10} versus EM_{2B10} groups, this increase was not significant. The NMR has been used to assess nicotine dependence and smoking behavior (65) and to aid clinicians in prescribing the most efficacious pharmacotherapy for smoking cessation (66–68). The data from the present study suggest that although pathways other than CYP2A6 metabolism of cotinine to 3HC may affect the levels of these metabolites *in vivo*, thereby leading to potential variation in the NMR as a biomarker of CYP2A6 activity and potentially affecting treatment selection for smoking cessation therapy, variation in these pathways may result in minor overall NMR variability.

Marginal effects on the levels of 3HC and 3HC-Gluc and no overall effect on the urinary NMR were observed for the UGT2B17 deletion in this study. The lack of an effect on the urinary NMR is consistent with that observed in previous studies (24, 69) and is consistent with previous studies demonstrating that other UGTs, including UGTs 2B7 and 1A9, also play an important role in 3HC-Gluc formation (4, 6). However, recent studies suggest that urinary 3HC/cotinine as a measure of NMR may lead to some variability as a surrogate measure of CYP2A6 activity due to it not accounting for variability in 3HC-Gluc formation (19).

Similar to that observed in previous studies (9, 70), FMO3 genotype influenced the levels of NOX in the urine of smokers in the present study. Specifically, the FMO3*2 allele was associated with a 38% decrease in urinary NOX, a pattern consistent with the 36% to 44% decrease in urinary NOX observed previously in African Americans (23, 24) and Caucasians (32, 70). Together, these data are consistent with FMO3 playing an important role in nicotine metabolism.

FMO1 variants have been previously associated with nicotine dependence in both Caucasians and African Americans (8, 71). Although not linked to altered levels of specific nicotine metabolites including NOX in the present study, the FMO1 rs12044884 and rs7533889 variants were associated with marginally significant decreases in NMR, and the rs7533889 and rs28384819 variants were also associated with decreased TNE, a reflection of total nicotine exposure. Given the extra-hepatic expression of FMO1 in kidney and brain, the genotype-associated effects on urinary NMR are consistent with FMO1 playing a minor role in overall nicotine metabolism whereas an effect on TNE may be associated with a role for FMO1 metabolism of nicotine locally within the brain.

A role for CYP2B6 in nicotine metabolism has been reported previously, with it being suggested to play a more important role in individuals with impaired CYP2A6 activity (72). In addition, differences in CYP2B6 expression have been previously reported to have effects on nicotine metabolism (73, 74). An effect of CYP2B6 genotype on nicotine metabolite profiles was observed in the present study, with significant decreases in urinary NMR and HPBA observed in subjects homozygous for the rs4803419 SNP as compared with subjects with no more than one allele containing this CYP2B6 SNP. We observed that the rs4803419 variant was significantly associated with decreased urinary NMR (19.1%; $P = 0.015$) and HPBA/TNE (19.6%; $P = 0.024$ and near-significant increases in nicotine-Gluc (22.8%, $P = 0.066$). These data are also consistent with a previous report, suggesting that non-coding variation in CYP2B6 affects nicotine metabolism (73), and, similar to that observed for CYP2A6, is consistent with deleterious CYP2B6 genotypes resulting in a rerouting of nicotine metabolism to other pathways other than cotinine formation. In addition, the missense CYP2B6 rs8192709 variant was associated with a 33% decrease in the levels of urinary COX in the present study. This is consistent with *in vitro* results (10), suggesting that CYP2B6 is an important enzyme involved in COX formation.

Recent *in vitro* studies suggest that CYP2C19 is an important enzyme involved in the formation of COX, with functional CYP2C19 genotypes influencing COX formation in human liver microsomes (10). Although the known functional CYP2C19 *17 and *2 variants are infrequent in the Chinese population (MAF < 0.02) and were not analyzed in the current study, the three CYP2C19 variants that were examined (rs4304697, rs35390752, and rs10509677) were not associated with COX formation. No studies have as yet been performed examining the potential functionality of these variants *in vitro*.

In summary, the data presented in the present study demonstrate that when CYP2A6 activity is defective, nicotine metabolism is rerouted to alternate nicotine metabolism pathways, including the formation of NOX and nicotine-Gluc. It was also demonstrated that genetic variants in CYP2A6 and other nicotine-metabolizing enzymes play a significant role in the variation observed in individual nicotine metabolism profiles. Interestingly, neither UGT2B10 nor UG2B17 genotypes significantly affected the NMR in this study, suggesting that the urinary NMR (3HC/cotinine) is a relatively reliable biomarker for CYP2A6 activity *in vivo*. Future studies in a larger population should be performed to determine whether functional genotypes in non–CYP2A6-catalyzed nicotine metabolism pathways play a role in nicotine metabolite profiles in CYP2A6-deficient individuals.

Authors' Disclosures

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Authors' Contributions

Y.X. Perez-Paramo: Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **C.J.W. Watson:** Data curation, methodology, writing–original draft, writing–review and editing. **G. Chen:** Conceptualization, supervision, methodology, writing–review and editing. **C.E. Thomas:** Resources, data curation, writing–review and editing. **J. Adams-Haduch:** Resources, writing–review and editing. **R. Wang:** Data curation, formal analysis, validation, writing–review and editing. **C.C. Khor:** Resources, data curation, writing–original draft, writing–review and editing. **W.-P. Koh:** Resources, data curation, writing–original draft, writing–review and editing. **H.H. Nelson:** Resources, data curation, writing–original draft, writing–review and editing. **J.-M. Yuan:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing. **P. Lazarus:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, methodology, writing–original draft, project administration, writing–review and editing.

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References

1. Benowitz NL, Jacob P. Metabolism of nicotine to cotinine studied by a dual stable isotope method. Clin Pharmacol Ther 1994;56:483–93.
2. Nakajima M, Yamamoto T, Nunoya K, Yokoi T, Nagashima K, Inoue K, et al. Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. J Pharmacol Exp Ther 1996;277:1010–5.
3. Chen G, Blevins-Primeau AS, Dellinger RW, Muscat JE, Lazarus P. Glucuronidation of nicotine and cotinine by UGT2B10: loss of function by the UGT2B10 Codon 67 (Asp/Tyr) polymorphism. Cancer Res 2007;67:9024–9.
4. Chen G, Giambrone NE, Lazarus P. Glucuronidation of trans-3'-hydroxycotinine by UGT2B17 and UGT2B10. Pharmacogen Genomics 2012;22:183–90.

5. Park SB, Jacob P, Benowitz NL, Cashman JR. Stereoselective metabolism of (S)-(-)-nicotine in humans: formation of trans-(S)-(-)-nicotine N-1'-oxide. Chem Res Toxicol 1993;6:880–8.
6. Yamanaka H, Nakajima M, Katoh M, Kanoh A, Tamura O, Ishibashi H, et al. Trans-3'-hydroxycotinine O- and N-glucuronidations in human liver microsomes. Drug Metab Dispos 2005;33:23–30.
7. Cashman JR, Park SB, Yang ZC, Wrighton SA, Jacob P, Benowitz NL. Metabolism of nicotine by human liver microsomes: stereoselective formation of trans-nicotine N'-oxide. Chem Res Toxicol 1992;5:639–46.
8. Hinrichs AL, Murphy SE, Wang JC, Saccone S, Saccone N, Steinbach JH, et al. Common polymorphisms in FMO1 are associated with nicotine dependence. Pharmacogen Genomics 2011;21:397–402.
9. Perez-Paramo YX, Chen G, Ashmore JH, Watson CJW, Nasrin S, Adams-Haduch J, et al. Nicotine-N'-Oxidation by flavin monooxygenase enzymes. Cancer Epidemiol Biomarkers Prev 2019;28:311–20.
10. Perez-Paramo YX, Watson CJW, Chen G, Lazarus P. CYP2C19 plays a major role in the hepatic N-oxidation of cotinine. Drug Metabol Dispos 2022:DMD–AR–2021–000624.
11. Hecht SS, Hochalter JB, Villalta PW, Murphy SE. 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: formation of a lung carcinogen precursor. Proc Natl Acad Sci USA 2000;97:12493–7.
12. Perez-Stable EJ, Herrera B, Jacob P III, Benowitz NL. Nicotine metabolism and intake in black and white smokers. JAMA 1998;280:152–6.
13. Chae DH, Gavin AR, Takeuchi DT. Smoking prevalence among asian americans: findings from the National Latino and Asian American Study (NLAAS). Public Health Rep 2006;121:755–63.
14. American Lung Association. Tobacco use in racial and ethnic populations. 2020 [cited 2020 Jun 15]. Available from: https://www.lung.org/quit-smoking/smoking-facts/impact-of-tobacco-use/tobacco-use-racial-and-ethnic.
15. Ashare RL, Thompson M, Leone F, Metzger D, Gross R, Mounzer K, et al. Differences in the rate of nicotine metabolism among smokers with and without HIV. AIDS 2019;33:1083–8.
16. Benowitz NL, Pomerleau OF, Pomerleau CS, Jacob P III. Nicotine metabolite ratio as a predictor of cigarette consumption. Nicotine Tob Res 2003;5:621–4.
17. Dempsey D, Tutka P, Jacob P III, Allen F, Schoedel K, Tyndale RF, et al. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. Clin Pharmacol Ther 2004;76:64–72.
18. St Helen G, Novalen M, Heitjan DF, Dempsey D, Jacob P III, Aziziyeh A, et al. Reproducibility of the nicotine metabolite ratio in cigarette smokers. Cancer Epidemiol Biomarkers Prev 2012;21:1105–14.
19. Giratallah HK, Chenoweth MJ, Addo N, Ahluwalia JS, Cox LS, Lerman C, et al. Nicotine metabolite ratio: comparison of the three urinary versions to the plasma version and nicotine clearance in three clinical studies. Drug Alcohol Depend 2021;223:108708.
20. Baurley JW, Edlund CK, Pardamean CI, Conti DV, Krasnow R, Javitz HS, et al. Genome-wide association of the laboratory-based nicotine metabolite ratio in three ancestries. Nicotine Tob Res 2016;18:1837–44.
21. Fix BV, O'Connor RJ, Benowitz N, Heckman BW, Cummings KM, Fong GT, et al. Nicotine metabolite ratio (NMR) prospectively predicts smoking relapse: longitudinal findings from ITC surveys in five countries. Nicotine Tob Res 2017;19:1040–7.
22. Murphy SE. Nicotine metabolism and smoking: ethnic differences in the role of P450 2A6. Chem Res Toxicol 2017;30:410–9.
23. Murphy SE, Park SS, Thompson EF, Wilkens LR, Patel Y, Stram DO, et al. Nicotine N-glucuronidation relative to N-oxidation and C-oxidation and UGT2B10 genotype in five ethnic/racial groups. Carcinogenesis 2014;35:2526–33.
24. Taghavi T, St Helen G, Benowitz NL, Tyndale RF. Effect of UGT2B10, UGT2B17, FMO3, and OCT2 genetic variation on nicotine and cotinine pharmacokinetics and smoking in African Americans. Pharmacogen Genomics 2017;27:143–54.
25. Liu T, David SP, Tyndale RF, Wang H, Zhou Q, Ding P, et al. Associations of CYP2A6 genotype with smoking behaviors in southern China. Addiction 2011;106:985–94.
26. Yuan J-M, Nelson HH, Carmella SG, Wang R, Kuriger-Laber J, Jin A, et al. CYP2A6 genetic polymorphisms and biomarkers of tobacco smoke constituents in relation to risk of lung cancer in the Singapore Chinese health study. Carcinogenesis 2017;38:411–8.
27. López-Flores LA, Pérez-Rubio G, Ramírez-Venegas A, Ambrocio-Ortiz E, Sansores RH, Falfán-Valencia R. Data on polymorphisms in CYP2A6 associated to risk and predispose to smoking related variables. Data Brief 2017;15:86–91.
28. Minematsu N, Nakamura H, Furuuchi M, Nakajima T, Takahashi S, Tateno H, et al. Limitation of cigarette consumption by CYP2A6*4, *7, and *9 polymorphisms. Eur Respir J 2006;27:289–92.
29. Pitarque M, von Richter O, Oke B, Berkkan H, Oscarson M, Ingelman-Sundberg M. Identification of a single nucleotide polymorphism in the TATA box of the CYP2A6 gene: impairment of its promoter activity. Biochem Biophys Res Commun 2001;284:455–60.
30. Bloom J, Hinrichs AL, Wang JC, von Weymarn LB, Kharasch ED, Bierut LJ, et al. The contribution of common CYP2A6 alleles to variation in nicotine metabolism among European-Americans. Pharmacogen Genomics 2011;21:403–16.
31. Oscarson M, McLellan RA, Asp V, Ledesma M, Ruiz MLB, Sinues B, et al. Characterization of a novelCYP2A7/CYP2A6 hybrid allele (CYP2A6*12) that causes reduced CYP2A6 activity. Hum Mutat 2002;20:275–83.
32. Teitelbaum AM, Murphy SE, Akk G, Baker TB, Germann A, von Weymarn LB, et al. Nicotine dependence is associated with functional variation in FMO3, an enzyme that metabolizes nicotine in the brain. Pharmacogen J 2018;18:136–43.
33. Berg JZ, Mason J, Boettcher AJ, Hatsukami DK, Murphy SE. Nicotine metabolism in African Americans and European Americans: variation in glucuronidation by ethnicity and UGT2B10 haplotype. J Pharmacol Exp Ther 2010;332:202–9.
34. Yuan JM, Stram DO, Arakawa K, Lee HP, Yu MC. Dietary cryptoxanthin and reduced risk of lung cancer: the Singapore Chinese Health Study. Cancer Epidemiol Biomarkers Prev 2003;12:890–8.
35. Hankin JH, Stram DO, Arakawa K, Park S, Low S-H, Lee H-P, et al. Singapore Chinese Health Study: development, validation, and calibration of the quantitative food frequency questionnaire. Nutr Cancer 2001;39:187–95.
36. Chen G, Giambrone NE Jr, Dluzen DF, Muscat JE, Berg A, Gallagher CJ, et al. Glucuronidation genotypes and nicotine metabolic phenotypes: importance of functional UGT2B10 and UGT2B17 polymorphisms. Cancer Res 2010;70:7543–52.
37. Yuan JM, Nelson HH, Butler LM, Carmella SG, Wang R, Kuriger-Laber JK, et al. Genetic determinants of cytochrome P450 2A6 activity and biomarkers of tobacco smoke exposure in relation to risk of lung cancer development in the Shanghai cohort study. Int J Cancer 2016;138:2161–71.
38. Gallagher CJ, Muscat JE, Hicks AN, Zheng Y, Dyer AM, Chase GA, et al. The UDP-glucuronosyltransferase 2B17 gene deletion polymorphism: sex-specific association with urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronidation phenotype and risk for lung cancer. Cancer Epidemiol Biomarkers Prev 2007;16:823–8.
39. Dorajoo R, Chang X, Gurung RL, Li Z, Wang L, Wang R, et al. Loci for human leukocyte telomere length in the Singaporean Chinese population and trans-ethnic genetic studies. Nat Commun 2019;10:2491-.
40. Bloom AJ, von Weymarn LB, Martinez M, Bierut LJ, Goate A, Murphy SE. The contribution of common UGT2B10 and CYP2A6 alleles to variation in nicotine glucuronidation among European Americans. Pharmacogen Genomics 2013;23:706–16.
41. Xu C, Rao YS, Xu B, Hoffmann E, Jones J, Sellers EM, et al. An in vivo pilot study characterizing the new CYP2A6*7, *8, and *10 alleles. Biochem Biophys Res Commun 2002;290:318–24.
42. Ter-Minassian M, Asomaning K, Zhao Y, Chen F, Su L, Carmella SG, et al. Genetic variability in the metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Int J Cancer 2012;130:1338–46.
43. Fowler S, Kletzl H, Finel M, Manevski N, Schmid P, Tuerck D, et al. A UGT2B10 splicing polymorphism common in African populations may greatly increase drug exposure. J Pharmacol Exper Ther 2014;352:358–67.
44. Rangiah K, Hwang WT, Mesaros C, Vachani A, Blair IA. Nicotine exposure and metabolizer phenotypes from analysis of urinary nicotine and its 15 metabolites by LC-MS. Bioanalysis 2011;3:745–61.
45. Ghosheh O, Hawes EM. N-Glucuronidation of nicotine and cotinine in humans: formation of cotinine glucuronide in liver microsomes and lack of catalysis by 10 examined UDP-glucuronosyltransferases. Drug Metabol Dispos 2002;30:991–6.
46. Phillips I, Shephard E. Flavin-containing monooxygenases: mutations, disease, and drug response. Trends Pharmacol Sci 2008;29:294–301.
47. Xu M, Bhatt DK, Yeung CK, Claw KG, Chaudhry AS, Gaedigk A, et al. Genetic and nongenetic factors associated with protein abundance of flavin-containing monooxygenase 3 in human liver. J Pharmacol Exper Ther 2017;363:265–74.
48. Yamanaka H, Nakajima M, Fukami T, Sakai H, Nakamura A, Katoh M, et al. CYP2A6 AND CYP2B6 are involved in nornicotine formation from nicotine in humans: interindividual differences in these contributions. Drug Metab Dispos 2005;33:1811–8.

49. Yamazaki H, Inoue K, Hashimoto M, Shimada T. Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes. Arch Toxicol 1999;73:65–70.
50. Haberl M, Anwald B, Klein K, Weil R, Fu C, Gepdiremen A, et al. Three haplotypes associated with CYP2A6 phenotypes in Caucasians. Pharmacogen Genomics 2005;15:609–24.
51. Gu Y, Zhang S, Lai B, Zhan X, Zhang Y. Frequency of CYP2A6 gene deletion and its relation to risk of lung cancer. Zhongguo Fei Ai Za Zhi 2005;8:297–9.
52. Yamanaka H, Nakajima M, Nishimura K, Yoshida R, Fukami T, Katoh M, et al. Metabolic profile of nicotine in subjects whose CYP2A6 gene is deleted. Eur J Pharm Sci 2004;22:419–25.
53. Hecht SS, Hatsukami DK, Bonilla LE, Hochalter JB. Quantitation of 4-oxo-4-(3-pyridyl)butanoic acid and enantiomers of 4-hydroxy-4-(3-pyridyl)butanoic acid in human urine: a substantial pathway of nicotine metabolism. Chem Res Toxicol 1999;12:172–9.
54. Trushin N, Hecht SS. Stereoselective metabolism of nicotine and tobacco-specific N-nitrosamines to 4-hydroxy-4-(3-pyridyl)butanoic acid in rats. Chem Res Toxicol 1999;12:164–71.
55. Yoshida R, Nakajima M, Nishimura K, Tokudome S, Kwon JT, Yokoi T. Effects of polymorphism in promoter region of human CYP2A6 gene (CYP2A6*9) on expression level of messenger ribonucleic acid and enzymatic activity in vivo and in vitro. Clin Pharmacol Ther 2003;74:69–76.
56. N Ki, Yokoi T, Takahashi Y, Kimura R, Kinoshita M, Kamataki T. Homologous unequal cross-over within the human CYP2A gene cluster as a mechanism for the deletion of the entire CYP2A6 gene associated with the poor metabolizer phenotype. J Biochem 1999;126:402–7.
57. Oscarson M, McLellan RA, Gullstén H, Yue Q-Y, Lang MA, Luisa Bernal M, et al. Characterisation and PCR-based detection of a CYP2A6 gene deletion found at a high frequency in a Chinese population. FEBS Lett 1999;448:105–10.
58. Ariyoshi N, Sawamura Y-i Kamataki T. A novel single nucleotide polymorphism altering stability and activity of CYP2A6. Biochem Biophys Research Commun 2001;281:810–4.
59. Bergen AW, Michel M, Nishita D, Krasnow R, Javitz HS, Conneely KN, et al. Drug metabolizing enzyme and transporter gene variation, nicotine metabolism, prospective abstinence, and cigarette consumption. PLoS One 2015;10:e0126113.
60. Pérez-Rubio G, López-Flores LA, Ramírez-Venegas A, Noé-Díaz V, García-Gómez L, Ambrocio-Ortiz E, et al. Genetic polymorphisms in CYP2A6 are associated with a risk of cigarette smoking and predispose to smoking at younger ages. Gene 2017;628:205–10.
61. Pitarque M, von Richter O, Rodríguez-Antona C, Wang J, Oscarson M., Ingelman-Sundberg M. A nicotine C-oxidase gene (CYP2A6) polymorphism important for promoter activity. Hum Mutat 2004;23:258–66.
62. Kaivosaari S, Toivonen P, Hesse LM, Koskinen M, Court MH, Finel M. Nicotine glucuronidation and the human UDP-glucuronosyltransferase UGT2B10. Mol Pharmacol 2007;72:761–8.
63. Chen G, Dellinger RW, Gallagher CJ, Sun D, Lazarus P. Identification of a prevalent functional missense polymorphism in the UGT2B10 gene and its association with UGT2B10 inactivation against tobacco-specific nitrosamines. Pharmacogen Genomics 2008;18:181–91.
64. Murphy SE, Sipe CJ, Choi K, Raddatz LM, Koopmeiners JS, Donny EC, et al. Low cotinine glucuronidation results in higher serum and saliva cotinine in African American compared to white smokers. Cancer Epidemiol Biomarkers Prev 2017;26:1093–9.
65. Falcone M, Jepson C, Benowitz N, Bergen AW, Pinto A, Wileyto EP, et al. Association of the nicotine metabolite ratio and CHRNA5/CHRNA3 polymorphisms with smoking rate among treatment-seeking smokers. Nicotine Tob Res 2011;13:498–503.
66. Chen L-S, Horton A, Bierut L. Pathways to precision medicine in smoking cessation treatments. Neurosci Lett 2018;669:83–92.
67. Lerman C, Schnoll RA, Hawk LW Jr, Cinciripini P, George TP, Wileyto EP, et al. Use of the nicotine metabolite ratio as a genetically informed biomarker of response to nicotine patch or varenicline for smoking cessation: a randomised, double-blind placebo-controlled trial. Lancet Respir Med 2015;3:131–8.
68. Malaiyandi V, Lerman C, Benowitz NL, Jepson C, Patterson F, Tyndale RF. Impact of CYP2A6 genotype on pretreatment smoking behaviour and nicotine levels from and usage of nicotine replacement therapy. Mol Psychiatry 2006;11:400–9.
69. Zhu AZX, Zhou Q, Cox LS, Ahluwalia JS, Benowitz NL, Tyndale RF. Variation in trans-3'-hydroxycotinine glucuronidation does not alter the nicotine metabolite ratio or nicotine intake. PLoS One 2013;8:e70938–e.
70. Bloom AJ, Murphy SE, Martinez M, von Weymarn LB, Bierut LJ, Goate A. Effects upon in vivo nicotine metabolism reveal functional variation in FMO3 associated with cigarette consumption. Pharmacogen Genomics 2013;23:62–8.
71. Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PAF, et al. Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. Human Mol Gen 2007;16:36–49.
72. Lee AM, Jepson C, Shields PG, Benowitz N, Lerman C, Tyndale RF. CYP2B6 genotype does not alter nicotine metabolism, plasma levels, or abstinence with nicotine replacement therapy. Cancer Epidemiol Biomarkers Prev 2007;16:1312–4.
73. Bloom AJ, Martinez M, Chen LS, Bierut LJ, Murphy SE, Goate A. CYP2B6 non-coding variation associated with smoking cessation is also associated with differences in allelic expression, splicing, and nicotine metabolism independent of common amino-acid changes. PLoS One 2013;8:e79700–e.
74. Zanger UM, Klein K. Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): advances on polymorphisms, mechanisms, and clinical relevance. Frontiers Gen 2013;4:24.