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UGT1A and *UGT2B* genetic variation alters nicotine and nitrosamine glucuronidation in European and African American smokers

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Abstract

Background—Identifying sources of variation in the nicotine and nitrosamine metabolic inactivation pathways is important to understanding the relationship between smoking and cancer

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risk. Numerous UGT1A and UGT2B enzymes are implicated in nicotine and nitrosamine metabolism *in vitro*; however, little is known about their roles *in vivo*.

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

Results—Correcting for multiple testing, three *UGT2B10* variants were associated with cotinine glucuronidation, rs2331559 and rs11726322 in European Americans and rs835309 in African Americans (P≤.0002). Additional variants predominantly in *UGT2B10* were nominally associated with nicotine (P=.008-.04) and cotinine (P=<.001-.02) glucuronidation in both ethnicities in addition to *UGT2B10*2* in European Americans (P=.01, P<.001). *UGT2B17*2* (P=.03) in European Americans and *UGT2B7* variants (P=.02-.04) in African Americans were nominally associated with 3HC glucuronidation. *UGT1A* (P=.007-.01), *UGT2B10* (P=.02) and *UGT2B7* (P=. 02-03) variants in African Americans were nominally associated with NNAL glucuronidation.

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

Impact—Findings also provide insight into ethnic differences in glucuronidation activity, which could be contributing to ethnic disparities in the risk for smoking-related cancers.

Keywords

UGT1A; UGT2B; nicotine; NNK; cigarette smoking

Introduction

Cigarette smoking is the leading risk factor for lung cancer (1, 2), and is also associated with numerous other cancers including those of the respiratory tract, digestive tract, bladder, pancreas and kidney (3, 4). Genetic factors are associated with differences in the susceptibility to tobacco-related cancers including genes involved in the metabolism of nicotine and tobacco smoke carcinogens (5-7). For instance, *CYP2A6* gene variants are associated with reduced lung cancer risk within smokers of diverse ethnicities (8-13), as is a gene variant in *CYP2A13* (14) – key enzymes in the nicotine inactivation pathway and in the activation pathway of tobacco specific nitrosamines (TSNAs) such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), respectively (15, 16).

UDP-glucuronosyltransferases (UGTs) represent another group of enzymes with the potential to influence the relationship between smoking and cancer risk via their contribution to the metabolism of both nicotine and nitrosamines, including NNK (Figure 1). In addition to the major metabolic pathway of nicotine to cotinine and further to trans-3'-hydroxycotinine (3HC) chiefly mediated by CYP2A6, nicotine, cotinine and 3HC are also substrates of UGTs (17). Glucuronide conjugates account for 25-30% of recovered nicotine

metabolites in urine (18-20). NNK is extensively metabolized by carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which, like NNK, is also carcinogenic (21, 22). NNAL is then metabolically detoxified by glucuronidation, and the non-carcinogenic glucuronide conjugates account for approximately 60% of NNAL detected in urine (23, 24).

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

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

In vitro studies of human liver microsomes and expressed UGTs implicate additional UGT1A and UGT2B enzymes in the glucuronidation of nicotine, its metabolites and NNAL. In human liver microsomes: *UGT2B10*2* is associated with reduced glucuronidation of nicotine and cotinine, but also 3HC and NNAL (33, 40, 41), *UGT2B17*2* is associated with reduced glucuronidation of 3HC and NNAL (33, 42), and *UGT1A4*2* and *UGT2B7*2* are associated with reduced glucuronidation of NNAL (42, 43). Inhibition studies in human liver microsomes implicate UGT1A1 in nicotine glucuronidation (44), UGT1A4 in nicotine, cotinine and 3HC glucuronidation (44-46), and UGT1A9 in nicotine and cotinine glucuronidation (44, 46). Recombinant UGTs from baculovirus-infected insect cells or over-expressed UGTs in human cell lines also implicate UGT1A4 in the glucuronidation of nicotine, cotinine and NNAL (47, 48) and provide evidence for the involvement of UGT1A9 in the glucuronidation of 3HC and NNAL (45, 49) and for UGT2B7 in the glucuronidation of nicotine and 3HC (45, 47).

Given the data suggesting the involvement of multiple UGT1A and UGT2B enzymes *in vitro*, and the limited knowledge of the contribution of *UGT* gene variants to nicotine and nitrosamine glucuronidation *in vivo*, we investigated the association between variation in *UGT1A1*, *UGT1A4*, *UGT1A9*, *UGT2B7*, *UGT2B10*, and *UGT2B17*, and nicotine, cotinine, 3HC and NNAL glucuronidation in European and African American current smokers. Since few *UGT* gene variants have been characterized with respect to these substrates, and the functional impact of *UGT* gene variants can be substrate specific (50), we genotyped 43 tag single nucleotide polymorphisms (SNPs) within the candidate genes. We also genotyped two *UGT1A9* insertion/deletion variants and, importantly, *UGT2B10*2* and *UGT2B17*2*,

the only alleles previously associated with impaired *in vivo* glucuronidation in smokers (20, 35, 39). To gauge the suitability of the data set for genetic association analyses of urinary metabolite ratios, we genotyped participants for altered activity *CYP2A6* variants to confirm the well-established association between *CYP2A6* and the ratio of 3HC over cotinine, a biomarker for CYP2A6 activity and nicotine clearance (Supplementary Results) (51). In addition to identifying which *UGT* gene variants influence the glucuronidation of tobacco-related compounds among European Americans, a population in which sources of variation in glucuronidation are better characterized (*in vitro* studies almost exclusively utilize livers from individuals of European descent), the present study evaluated whether the same *UGT* variants were associated with glucuronidation among African Americans to provide pilot data focused on understanding the higher risk of smoking-related lung cancer observed among African Americans compared to European Americans (52, 53).

Materials and Methods

Study description

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

UGT Genotyping

Tag SNPs were selected within the candidate genes (+/− 10 kb) by determining the minimal set of common SNPs (minor allele frequency ≥ 5%) that captured the pairwise linkage disequilibrium ($r^2 > 0.8$) for all common SNPs within the HapMap CEU population. In HapMap2 release 24, the set of SNPs capture (at an $r^2 \geq 0.8$) on average over 80% of existing common SNPs within the CEU population, and in the YRI population they capture on average 37% of the existing common SNPs (Supplementary Table 1).

*UGT2B10*2* (rs61750900) was genotyped using a PCR-restriction fragment length polymorphism in which the amplified product was subjected to digestion with HinfI (40). The *UGT2B17*2* deletion allele was assessed using the TaqMan gene expression assay, HS00854486_sH, with copy number reference assay TaqMan RNase P for the internal control, determined by TaqMan real-time quantitative PCR (55-57). Genotyping for 41 tag SNPs in *UGT1A4*, *UGT1A9*, *UGT2B7* and *UGT2B10* was done using the KASPar SNP genotyping system by LGC Genomics (formerly KBiosciences, LGC Limited, Middlesex, UK). Genotyping for the *UGT1A9* indels, rs45625337 and rs10538910, was performed using

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

Analytical Chemistry

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

Metabolite Phenotypes

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

Statistical Analyses

Comparison of demographic, smoking, and metabolite phenotypes in African versus European Americans was performed by Wilcoxon's rank-sum test (continuous variables) or χ^2 (categorical variables). Correlations between metabolite phenotypes were performed on non-transformed values by Spearman's. Geometric mean values are presented for non-normally distributed values unless indicated otherwise. Linkage disequilibrium between the *UGT1A* and *UGT2B* variants was assessed using Haploview (62), while other statistical analyses were performed using Stata13 (StataCorp, College Station, TX). Hardy-Weinberg equilibrium tests were performed using the Stata program hwsnp (Author: Mario A. Cleves, University of Arkansas for Medical Sciences). Regression association analyses between *UGT* variants and glucuronidation phenotypes were performed using the Stata program qtlsnp assuming a dominant effect of the variant allele (Author: Mario A. Cleves, University of Arkansas for Medical Sciences). All regression models were performed separately within each ethnicity and were adjusted for age, gender and menthol smoking. Due to individuals missing genotypes and/or biomarker data, the number of observations within each model ranged from N=60-66 among European Americans, and N=51-60 among African Americans. In total 36 *UGT* variants were tested against four phenotypes in European Americans and 43 *UGT* variants were tested against four phenotypes in African Americans, hence the significance thresholds were set at $P \leq .0003$ and .0002, respectively, as

determined by a Bonferroni correction for multiple testing. Nominally significant associations are also presented (P ≤.05).

Results

Glucuronidation phenotypes

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

Demographic characteristics and urinary sampling parameters were evaluated as potential covariates of the glucuronide ratios. Demographic characteristics differed by ethnicity – African Americans were older (P<.01), had a higher BMI (P<.01), reported fewer cigarettes smoked per day (P=.04) and a greater prevalence of menthol cigarette use (P<.01) (Table 1). Correlations between glucuronide ratios and time from last cigarette, creatinine concentration and nicotine equivalents were also assessed, since metabolites were quantified from spot urine. No consistent patterns emerged between glucuronide ratios and age, BMI, creatinine, nicotine equivalents or time from last cigarette (Supplementary Table 2). No differences in glucuronide ratios by gender were noted among either ethnicity, but 3HC and NNAL glucuronide ratios were lower among African American menthol versus non-menthol smokers (Supplementary Table 3). Menthol is glucuronidated by enzymes such as UGT2B7 and UGT2B17 (64, 65), which are also capable of glucuronidating 3HC and NNAL (33, 42); thus, menthol could act as a competitive inhibitor, and menthol was included as a covariate in genotype-phenotype analyses. Age and gender were also included as covariates due to *a priori* evidence that these variables may influence the activity of specific UGTs (37, 66-68).

UGT genotyping results

The *UGT2B10*2* allele, rs61750900T, had a minor allele frequency of 9% and 4% among European and African Americans, respectively, and the *UGT2B17*2* allele had a minor allele frequency of 30% and 21% among European and African Americans, respectively, consistent with published frequencies (27, 35, 39). Among European Americans, rs3771342 and rs835310, and among African Americans, rs12468274 and rs12468543, were not consistent with Hardy-Weinberg Equilibrium and were excluded. Among European Americans, 3 variants in the *UGT1A* locus and 6 variants in the *UGT2B* locus displayed high linkage disequilibrium ($R^2 > 0.9$) (Supplementary Figure 1A and 2A, respectively). Among African Americans, no variants in the *UGT1A* locus and only 2 variants in the *UGT2B* locus displayed high linkage disequilibrium (Supplementary Figure 1B and 2B, respectively). Variants in high linkage disequilibrium were also excluded leaving 36 and 43 *UGT* variants

in subsequent genotype-phenotype analyses in European and African Americans, respectively (Supplementary Table 4).

Glucuronidation associations

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

Nicotine glucuronidation

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

Cotinine glucuronidation

In addition to the two *UGT2B10* variants significantly associated with impaired cotinine glucuronidation (Figure 3A-B), *UGT2B10*2* was nominally associated with impaired cotinine glucuronidation activity, while a single variant in *UGT2B7* was nominally associated with enhanced glucuronidation in European Americans (Table 3B). Among African Americans, in addition to the *UGT2B10* variant significantly associated with enhanced cotinine glucuronidation (Figure 3C), one other *UGT2B10* variant was nominally associated with enhanced and two with impaired activity (Table 3B). Two variants in the *UGT1A4* locus and single variants in the common *UGT1A* exons and 3' flanking region of *UGT1A* were also associated with cotinine glucuronidation in African Americans (Table 3B).

3HC glucuronidation

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

NNAL glucuronidation

No *UGT* variants reached nominal significance with NNAL glucuronidation activity among European Americans (Table 3D). Among African Americans, two *UGT2B7* variants were

nominally associated with NNAL glucuronidation, one with enhanced and one with impaired activity (Table 3D). Single variants in *UGT1A1*, the 3' flanking region of *UGT1A*, and *UGT2B10* were also associated with NNAL glucuronidation in African Americans (Table 3D).

Discussion

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

Our findings confirm an important contribution of genetic variation in *UGT2B10* to nicotine and cotinine glucuronidation with multiple *UGT2B10* variants nominally associated with nicotine and with cotinine glucuronidation in both ethnicities. Three variants in *UBT2B10* remained statistically significantly associated with cotinine glucuronidation following correction for multiple testing. Of note, the two variants significantly associated with cotinine glucuronidation in European Americans, rs2331559 and rs11726322, had similar effect sizes to *UGT2B10*2* (Table 3B). *UGT2B10*2* likely did not reach the Bonferroni corrected threshold of P≤.0003 due to the lower prevalence of the **2* allele (~9%) compared to rs2331559 (~12%) and rs11726322 (13%). The minor (less frequent) alleles of the *UGT2B10* variants surviving correction for multiple testing in European Americans were associated with impaired cotinine glucuronidation; whereas, among African Americans the minor allele of the *UGT2B10* variant surviving correction for multiple testing was associated with enhanced activity. Hence, in contrast to European Americans, the major (more frequent) allele in African would have reduced activity (versus enhanced activity) potentially contributing to the lower levels of nicotine and cotinine glucuronide conjugates observed among African Americans (Figure 2A and 2B) (39, 63).

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

glucuronidation *in vitro* (47); however, the nominal association that we report is between *UGT2B7* and cotinine glucuronidation and may represent a chance finding.

As the nicotine and cotinine glucuronide ratios were correlated, we anticipated that the same *UGT* variants might be associated with both glucuronidation phenotypes. While we observed overlap in variants associated with both pathways, particularly in *UGT2B10*, more *UGT* variants were associated with the cotinine glucuronide ratio than with the nicotine glucuronide ratio and the only variants surviving correction for multiple testing were associated with cotinine glucuronidation. A greater genetic contribution to inter-individual variation in cotinine versus nicotine glucuronidation has been proposed based on twin studies (69). Alternatively, this difference may be the result of a more stable glucuronidation phenotype for cotinine.

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

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

The ratio of NNAL-glucuronide to NNAL is a biomarker of nitrosamine detoxification (31) and a potential marker of cancer risk (32). There is conflicting evidence regarding ethnic

differences in this ratio, specifically whether it is lower among African Americans (70, 71). We did not observe a significant difference in NNAL glucuronide ratios by ethnicity, while replicating differences in other ratios (Figure 2). However, 10% of African Americans had ratios below the lowest ratio observed among European Americans potentially putting these individuals at greater risk for cancer, as *Chung et al*. found that lower NNAL glucuronide ratios were associated with an increased risk of cancer (32). Variation in the *UGT2B10* and *UGT2B7* loci may be of particular interest in terms of cancer risk disparities, since these variants were only nominally associated with NNAL glucuronidation activity among African Americans.

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

Many nominally significant associations between *UGT* variants and glucuronidation phenotypes were observed despite both the relatively small sample size for a genetic association study and the quantification of metabolites from spot urine with variable time from last cigarette. Biomarker assessment from spot urine is unlikely to have biased findings, since we observed similar correlations between glucuronide ratios as reported for 24 hour urine (18, 39). Furthermore, urinary cotinine, NNAL and nicotine equivalents, which are all biomarkers of nicotine consumption, were correlated as expected (data not shown) and neither nicotine equivalents nor the glucuronide ratios were systemically associated with time from last cigarette (Supplementary Table 2). Only one nominally significant variant, *UGT1A1* rs3771342, displayed an inconsistent direction of effect with the nicotine and NNAL glucuronidation phenotypes potentially reflecting an indirect genotype-phenotype association or simply a chance observation. The smaller sample size precluded interactions analyses. In particular, the interaction between menthol and *UGT2B7* and *UGT2B17* gene variants would be worthwhile exploring in a larger dataset given the conflicting evidence concerning menthol smoking and lung cancer risk (reviewed in (72)). More associations were observed among African Americans than among European Americans, as is seen in other genomic regions displaying lower linkage disequilibrium in African populations (e.g. chromosome 15q25 and lung cancer risk (73)). Of note, the gene variants investigated were initially chosen to provide relatively good coverage of European smokers and provide relatively low coverage of common variation in Africans (Supplementary Table 1) suggesting that even more variation may be identified among this ethnic group. Alternatively, a single untested variant may underlie multiple associations observed in a gene region through linkage disequilibrium.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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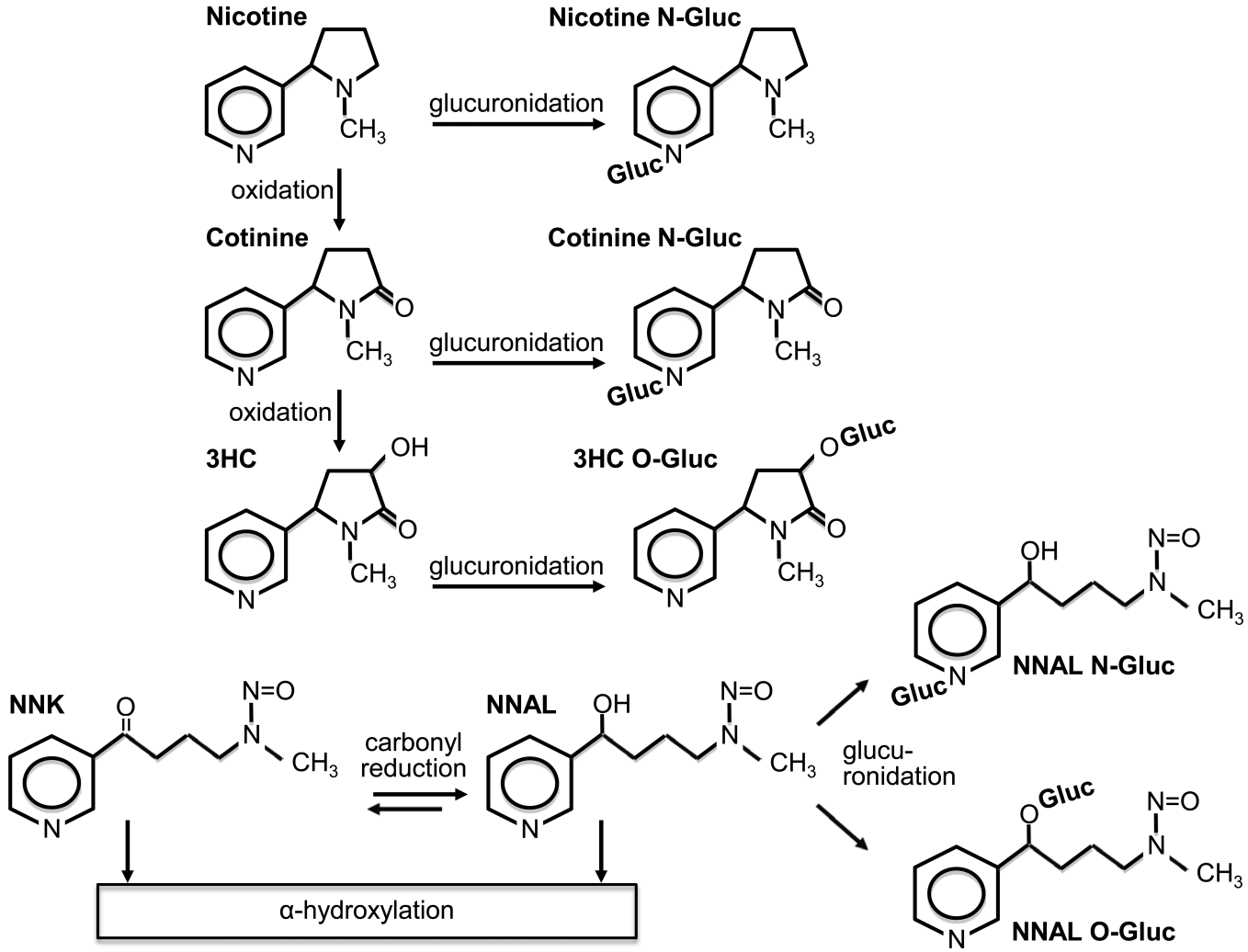

Figure 1.
Simplified schematic of nicotine and NNK metabolism to glucuronide conjugates.

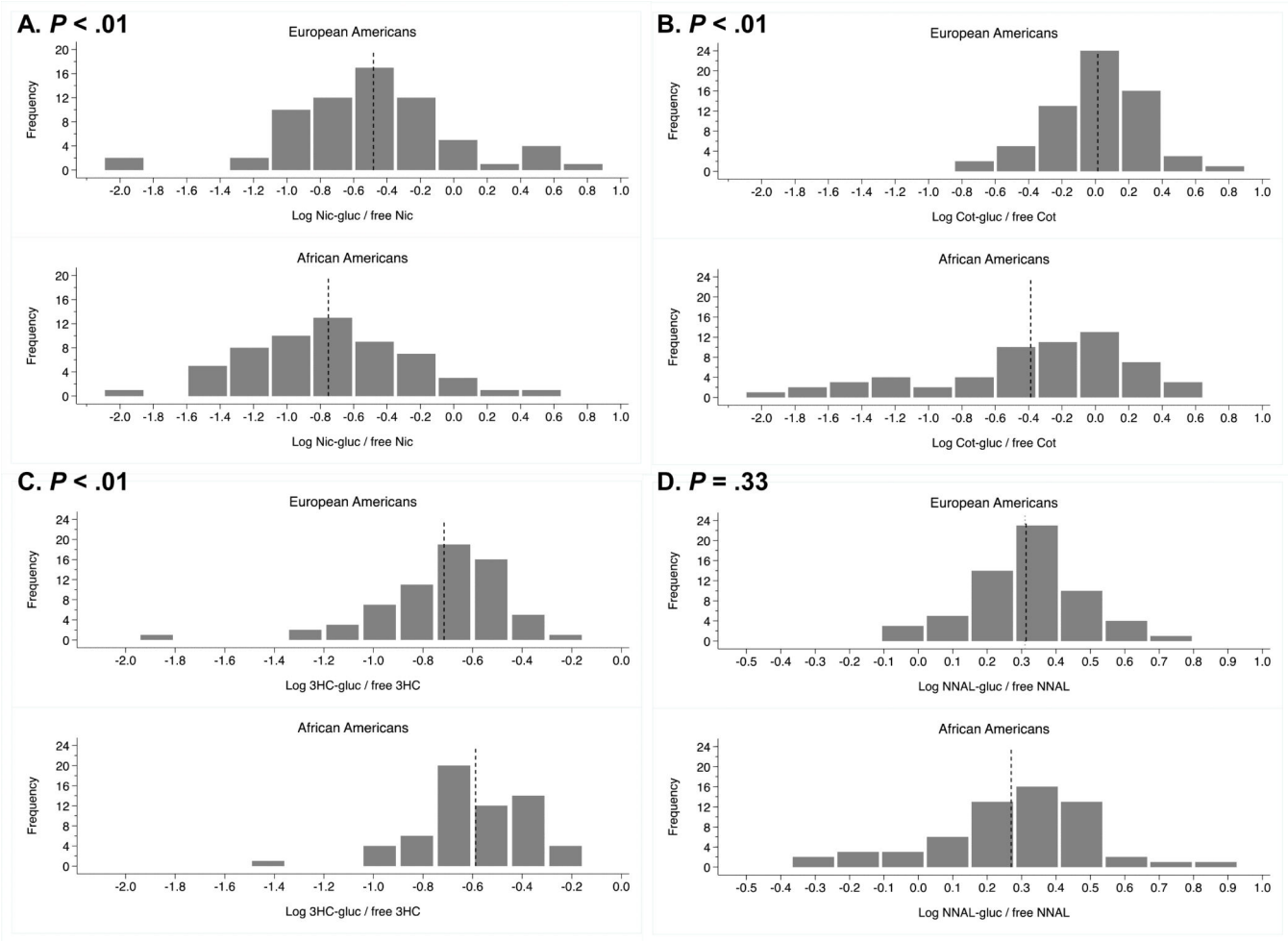

Figure 2.

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

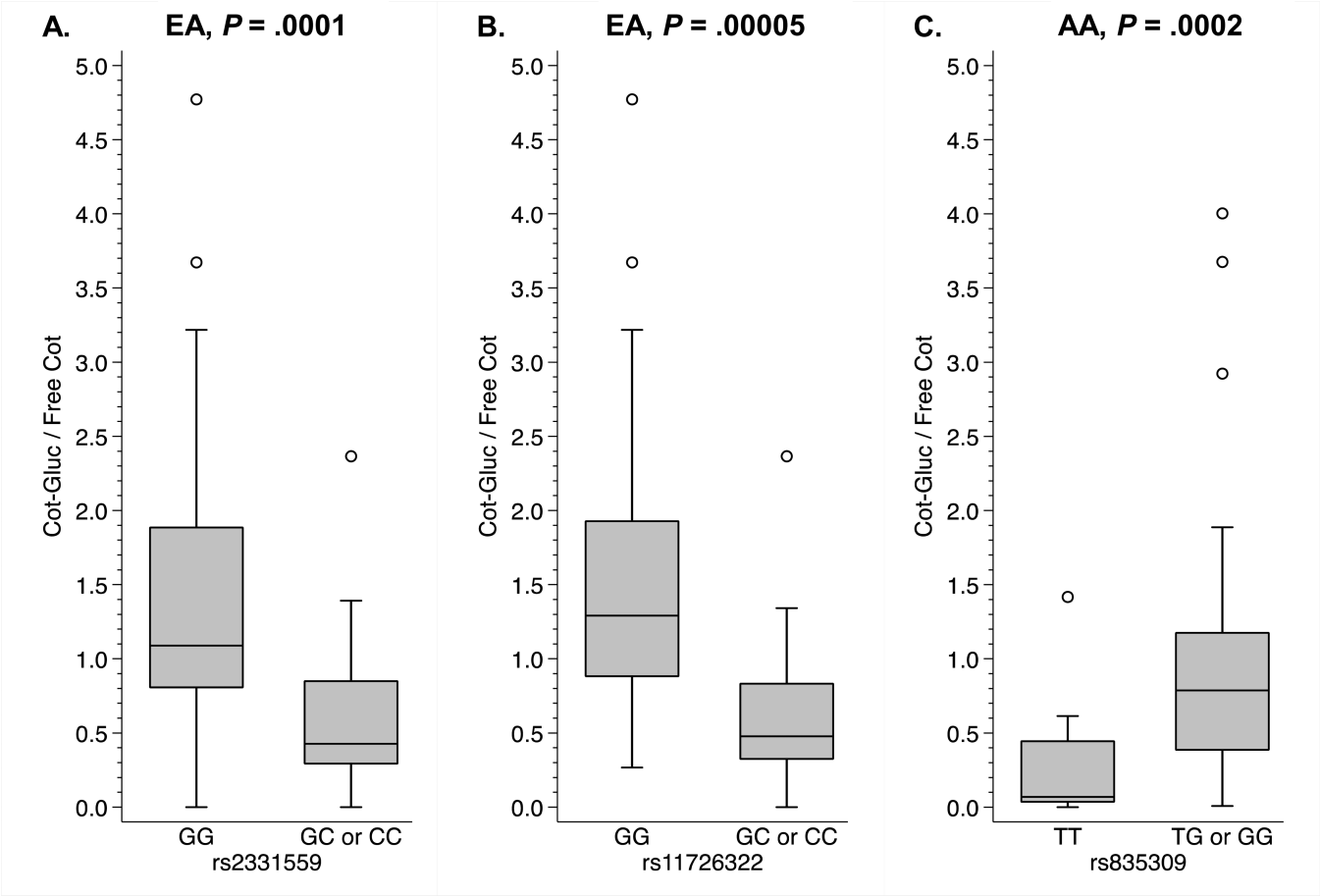

Figure 3.

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

Table 1

Characteristics of participants included in analyses, n = 126[a]

Characteristic	European American n = 66	African American n = 60	*P* value[b]
Male, no. (%)	38 (58)	35 (58)	.93
Age in years, median (IQR)	34 (25-45)	41 (36-49)	<.01
Body mass index, median (IQR)	24 (22-28)	27 (24-33)	<.01
Menthol cigarette use, no. (%)	18 (27)	41 (68)	<.01
Cigarettes/day[c], median (IQR)	20 (14-20)	15 (10-20)	.04
Nicotine equiv. (nmol/mg-creat)[d], median (IQR)	63 (54-75)	44 (37-53)	<.01
Min from last cig, median (IQR)	80 (50-107)	90 (60-140)	.08
Activity ratios (geometric mean, 95% CI)			
NIC-Gluc/ free NIC	0.33 (0.25-0.44)	0.17 (0.13-0.23)	<.01
COT-Gluc/ free COT	1.03 (0.87-1.23)	0.40 (0.28-0.57)	<.01
3HC-Gluc/ free 3HC	0.19 (0.17-0.22)	0.26 (0.23-0.30)	<.01
NNAL-Gluc/ free NNAL	2.06 (1.87-2.27)	1.85 (1.61-2.12)	.33

IQR: interquartile range

[a] 128 participants in the original study, 67 European American and 61 African American: One sample dropped due to absence of urinary biomarkers and another due to poor genotyping call rate

[b] *P* values: Non-parametric Wilcoxon ranksum test or chi square

[c] Average of self-reported cigarettes/day in the three days before biomarker collection

[d] Nicotine equivalents: Urinary sum of nicotine, cotinine, trans-3-hydroxycotinine and their glucuronide conjugates normalized to creatinine

Table 2

Spearman correlations between glucuronidation activity measures (non-transformed)

EA	rho, *P* value	NIC-Gluc ratio	COT-Gluc ratio	3HC-Gluc ratio	NNAL-Gluc ratio	AA
	NIC-Gluc ratio	--	0.49, <.001	−0.20, .13	0.19, .15	
	COT-Gluc ratio	0.34, .005	--	−0.05, .71	0.56, <.001	
	3HC-Gluc ratio	0.10, .41	0.13, .31	--	0.32, .01	
	NNAL-Gluc ratio	0.04, .76	0.34, .007	0.38, .002	--	

EA: European American; AA: African American

Table 3A

UGT variants nominally associated with the nicotine glucuronide ratio

UGT Variant	European American Smokers					African American Smokers				
	Alleles		MAF	P^{dom}	Coef (95% CI)	Alleles		MAF	P^{dom}	Coef (95% CI)
UGT1A4 locus										
rs13401281	T : G		.409			***T*** : G	↑	.475	.012	0.35 (0.08, 0.62)
UGT1A1 locus										
rs6742078	G : T		.408			***G*** : T	↑	.379	.049	0.26 (.001, 0.52)
rs3771342[a]	C : A		.112			C : ***A***	↓	.117	.024	−0.38 (−0.72, −0.05)
UGT2B10 locus										
rs61750900 (*2)	G : ***T***	↓	.090	.013	−0.42 (−0.76, −0.09)	G : T		.043		
rs2331559	G : C	↓	.119	.015	0.38 (0.07, 0.68)	***C*** : G	↑	.362	.008	0.34 (0.09, 0.60)
rs835309[b]	G : T		.106			***T*** : G	↑	.491	.026	0.34 (0.04, 0.64)
rs11726322	G : C		.129			G : ***C***	↓	.254	.010	−0.34 (−0.60, −0.09)
rs7673996	C : T		.031			C : ***T***	↓	.225	.042	−0.27 (−0.53, −0.01)

Alleles indicated as major : minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% confidence intervals from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < .05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*). MAF: Minor allele frequency

[a] Variant excluded from analyses in European Americans

[b] *P* value not reported in European Americans as variant in high linkage disequilibrium with rs2331559.

Table 3B

UGT variants nominally/significantly associated with the cotinine glucuronide ratio

UGT SNP/Variant	European American Smokers					African American Smokers				
	Alleles		MAF	P^{dom}	Coef (95% CI)	Alleles		MAF	P^{dom}	Coef (95% CI)
UGT1A4 locus										
rs3732220	C : T		.092			***C*** : T	↑	.108	.018	0.50 (0.09, 0.91)
rs871514	G : A		.478			G : ***A***	↓	.359	.027	−0.36 (−0.68, −0.04)
UGT1A exon 2-5										
rs1018124	T : C		.056			***T*** : C	↑	.108	.013[a]	0.48 (0.10, 0.86)
UGT1A 3' flanking										
rs10209214	T : C		.313			T : ***C***	↓	.142	.023[a]	−0.39 (−0.72, −0.05)
UGT2B10 locus										
rs61750900 (*2)	G : ***T***	↓	.090	.0004	−0.37 (−0.56, −0.17)	G : T		.043		
rs294765	G : A		.015			G : ***A***	↓	.217	.017	−0.39 (−0.71, −0.07)
rs2331559	G : ***C***	↓	.119	.0001	−0.36 (−0.53, −0.19)	***C*** : G	↑	.362	.006	0.43 (0.13, 0.74)
rs835309[b]	G : T		.106			***T*** : G	↑	.491	.0002	0.66 (0.33, 0.99)
rs835310[c]	C : G		.023			C : ***G***	↓	.408	.005	−0.46 (−0.78, −0.14)
rs11726322	G : ***C***	↓	.129	.00005	−0.35 (−0.51, −0.19)	G : C		.254		
UGT2B7 locus										
rs4356975	***C*** : T	↑	.381	.042	0.17 (0.01, 0.33)	C : T		.397		

Alleles indicated as major : minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% confidence intervals from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < .05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*). MAF: Minor allele frequency

[a] Absence of individuals homozygous for the minor allele

[b] *P* value not reported in European Americans as variant in high LD with rs2331559

[c] Variant excluded from analyses in European Americans.

Table 3C

UGT variants nominally associated with the 3HC glucuronide ratio

UGT SNP/Variant	European American Smokers					African American Smokers				
	Alleles		MAF	P^{dom}	Coef (95% CI)	Alleles		MAF	P^{dom}	Coef (95% CI)
UGT2B17 locus										
copy number	1 : ***0***	↓	.303	.028	−0.15 (−0.29, −0.02)	1 : 0		.215		
UGT2B7 locus										
rs57216626	C : G		.448			C : ***G***	↓	.250	.016	−0.14 (−0.25, −0.03)
rs4535394	C : T		.440			C : ***T***	↓	.339	.026	−0.13 (−0.25, −0.02)

UGT2B17 0 allele: deletion allele. Alleles indicated as major : minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% confidence intervals from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < .05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*). MAF: Minor allele frequency

Table 3D

UGT variants nominally associated with the NNAL glucuronide ratio

UGT SNP/Variant	European American Smokers				African American Smokers				
	Alleles	MAF	P^{dom}	Coef (95% CI)	Alleles		MAF	P^{dom}	Coef (95% CI)
UGT1A1 locus									
rs3771342[a]	C : A	.112			***C*** : A	↑	.117	.012	0.19 (0.04, 0.34)
UGT1A 3' flanking									
rs10203853	A : T	.431			A : ***T***	↓	.292	.007	−0.16 (−0.38, −0.05)
UGT2B10 locus									
rs835310[a]	C : G	.023			C : ***G***	↓	.408	.019	−0.15 (−0.28, −0.03)
UGT2B7 locus									
rs12506592	A : G	.162			A : ***G***	↓	.275	.030	−0.14 (−0.26, −0.01)
rs4356975	C : T	.381			***C*** : T	↑	.397	.017	0.15 (0.03, 0.28)

Alleles indicated as major : minor with impaired allele in bolded italics and an arrow indicating direction of effect for the minor allele. P^{dom} and coefficient with 95% confidence intervals from multivariate logistic regression modeling assuming a dominant effect of the minor allele and reported when $P < .05$. Variants ordered by location along chromosome 2 (*UGT1A*) and chromosome 4 (*UGT2B*). MAF: Minor allele frequency

[a]Variant excluded from analyses in European Americans.