

Glucuronidation Genotypes and Nicotine Metabolic Phenotypes: Importance of Functional UGT2B10 and UGT2B17 Polymorphisms

Gang Chen^{1,2}, Nino E. Giambone, Jr.^{1,3}, Douglas F. Dluzen^{1,3}, Joshua E. Muscat^{1,2}, Arthur Berg^{1,2}, Carla J. Gallagher^{1,2}, and Philip Lazarus^{1,2,3}

Abstract

Glucuronidation is an important pathway in the metabolism of nicotine, with previous studies suggesting that ~22% of urinary nicotine metabolites are in the form of glucuronidated compounds. Recent *in vitro* studies have suggested that the UDP-glucuronosyltransferases (UGT) 2B10 and 2B17 play major roles in nicotine glucuronidation with polymorphisms in both enzymes shown to significantly alter the levels of nicotine-glucuronide, cotinine-glucuronide, and *trans*-3'-hydroxycotinine (3HC)-glucuronide in human liver microsomes *in vitro*. In the present study, the relationship between the levels of urinary nicotine metabolites and functional polymorphisms in UGTs 2B10 and 2B17 was analyzed in urine specimens from 104 Caucasian smokers. Based on their percentage of total urinary nicotine metabolites, the levels of nicotine-glucuronide and cotinine-glucuronide were 42% ($P < 0.0005$) and 48% ($P < 0.0001$), respectively, lower in the urine from smokers exhibiting the *UGT2B10* (*1/*2) genotype and 95% ($P < 0.05$) and 98% ($P < 0.05$), respectively, lower in the urine from smokers with the *UGT2B10* (*2/*2) genotype compared with the urinary levels in smokers having the wild-type *UGT2B10* (*1/*1) genotype. The level of 3HC-glucuronide was 42% ($P < 0.001$) lower in the urine from smokers exhibiting the homozygous *UGT2B17* (*2/*2) deletion genotype compared with the levels in urine from wild-type *UGT2B17* subjects. These data suggest that UGTs 2B10 and 2B17 play important roles in the glucuronidation of nicotine, cotinine, and 3HC and suggest that the *UGT2B10* codon 67 SNP and the *UGT2B17* gene deletion significantly reduce overall glucuronidation rates of nicotine and its major metabolites in smokers. *Cancer Res*; 70(19): 7543–52. ©2010 AACR.

Introduction

Tobacco smoking causes 500,000 deaths annually in the United States, and nicotine is the pharmacologic agent responsible for tobacco addiction (1). The analysis of urinary nicotine metabolite profiles indicates that ~70% to 80% of nicotine is metabolized to cotinine and then cotinine is further metabolized to *trans*-3'-hydroxycotinine (3HC) and other compounds (ref. 2; see Fig. 1). Hepatic metabolism of nicotine to cotinine and then to 3HC is catalyzed primarily by CYP2A6 (3). Nicotine, cotinine, and 3HC undergo further phase II detoxification reactions by conjugation with glucuronic acid via catalysis by the UDP-glucuronosyltransferase (UGT) family of enzymes. Up to 31% of nicotine urinary metabolites are in the

form of phase II glucuronidated compounds, with nicotine-*N*-glucuronide (nicotine-Gluc), cotinine-*N*-glucuronide (cotinine-Gluc), and cotinine-3'-*O*-glucuronide (3HC-Gluc) comprising the majority of these conjugates (3). Both cotinine and nicotine are glucuronidated on the nitrogen of the pyridine ring, and *N*-glucuronidation of both compounds is observed in human liver microsomes (HLM) and in the urine of smokers (4–7). Approximately 90% of the systemic dose of nicotine is excreted through the urine, and although both *O*-glucuronidation and *N*-glucuronidation of 3HC were observed in HLM, only its *O*-glucuronide, 3HC-Gluc, was detected in the urine of smokers (8).

A high correlation exists between the *in vivo* urinary ratio of nicotine-Gluc/(nicotine + nicotine-Gluc) and the ratio of cotinine-Gluc/(cotinine + cotinine-Gluc) in smokers. By contrast, the *in vivo* urinary nicotine-Gluc/(nicotine + nicotine-Gluc) ratio is only moderately correlated with the ratio of 3HC-Gluc/(3HC + 3HC-Gluc; ref. 2). This suggests that the glucuronidation of nicotine/cotinine versus 3HC may be via different enzyme pathways. Previous studies have also shown a high correlation between the glucuronidation of nicotine and cotinine in HLM *in vitro* and that the hepatic UGT2B10 was the major enzyme responsible for their glucuronidation; no correlation exists between either nicotine-Gluc or cotinine-Gluc formation and 3HC-Gluc formation in HLM *in vitro*

Authors' Affiliations: ¹Molecular Epidemiology and Cancer Control Program, Penn State Cancer Institute and Departments of ²Public Health Sciences and ³Pharmacology, Penn State University College of Medicine, Hershey, Pennsylvania

Corresponding Author: Philip Lazarus, Department of Pharmacology, Division of Population Sciences and Cancer Prevention, Penn State Cancer Institute, Penn State University College of Medicine, 500 University Drive, Hershey, PA 17033. Phone: 717-531-5734; Fax: 717-531-0480; E-mail: plazarus@psu.edu.

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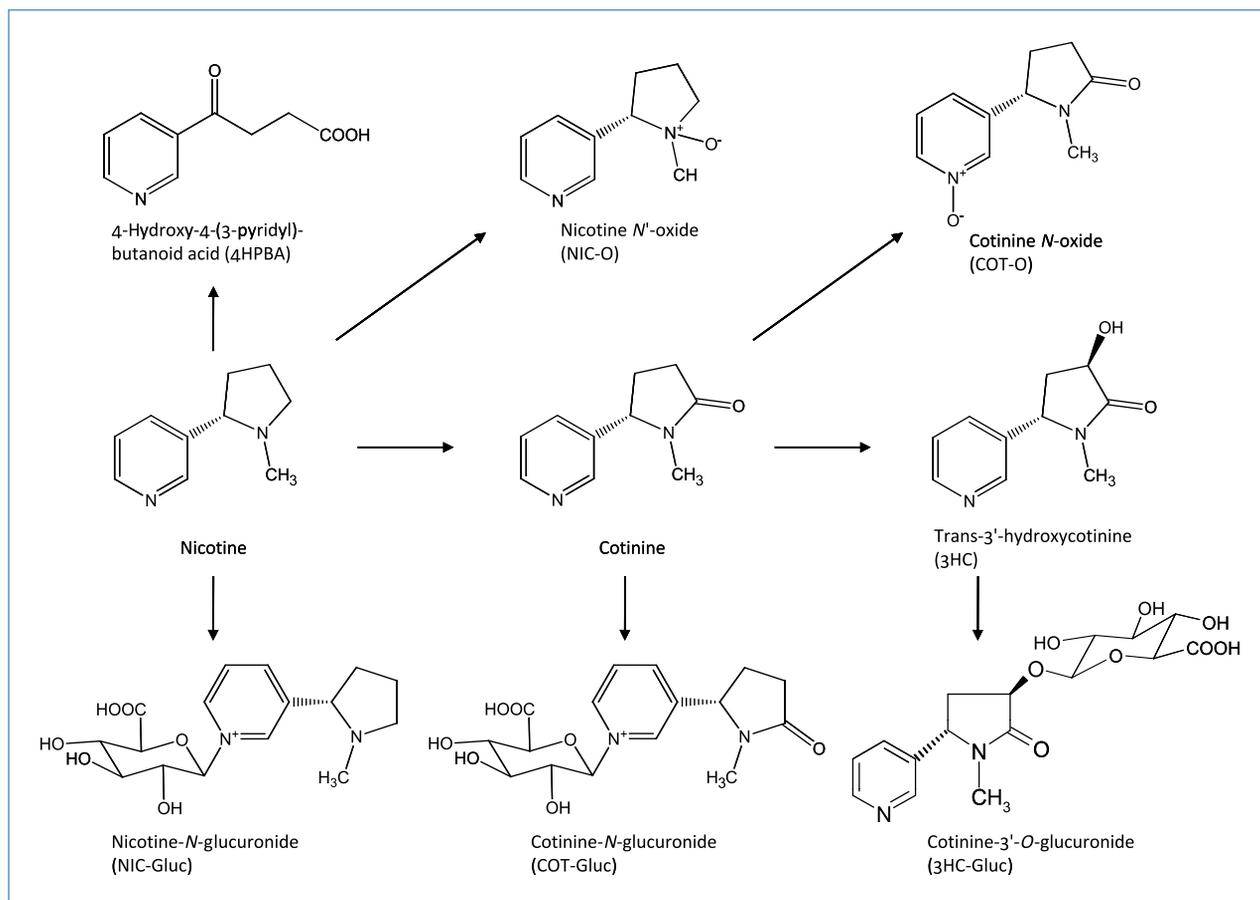


Figure 1. Major nicotine metabolites in the urine of smokers.

(9, 10). Whereas earlier studies identified UGTs 2B7 and 1A9 to be important in the glucuronidation of 3HC, more recent studies have shown that UGT2B17 exhibits the highest activity against 3HC *in vitro*.⁴

A functional polymorphism (rs61750900) at codon 67 (Asp > Tyr) of the *UGT2B10* gene with an allelic prevalence of ~10% in Caucasians has been reported (9). The *UGT2B10*^{Tyr} variant was shown to be associated with significantly reduced *N*-glucuronidation of nicotine and cotinine in HLM and *UGT2B10*-overexpressing cell lines *in vitro* (9, 11). The *UGT2B10*^{Tyr} variant was also shown to be associated with significantly reduced *N*-glucuronidation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the major metabolite of the nicotine-derived tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, in HLM and in *UGT2B10*-overexpressing cell lines *in vitro* (11). Similarly, a polymorphic whole-gene deletion of the *UGT2B17* gene, with an allelic prevalence of ~30% in Caucasians, has been shown to be associated with a significant decrease in the *O*-glucuronidation of 3HC in HLM *in vitro*.⁴ In similar studies, this polymorphism was also shown to be associated with a

significant decrease in the *O*-glucuronidation of NNAL (12). Together, these data suggest that both polymorphisms could significantly alter how nicotine is metabolized *in vivo*.

The goal of the present study was to assess the levels of nicotine and a panel of nicotine metabolites [cotinine, 3HC, nicotine-Gluc, cotinine-Gluc, 3HC-Gluc, nicotine *N'*-oxide, cotinine *N*-oxide, and 4-hydroxy-4-(3-pyridyl)-butanoic acid (4HPBA)] in the urine of smokers and to determine whether the relative levels of urinary nicotine-Gluc, cotinine-Gluc, and 3HC-Gluc are associated with the functional polymorphisms in the *UGT2B10* and *UGT2B17* genes *in vivo*.

Materials and Methods

Chemicals

Nicotine and creatinine were purchased from Sigma-Aldrich. Cotinine, 3HC, nicotine-Gluc, cotinine-Gluc, 3HC-Gluc, nicotine *N'*-oxide, cotinine *N*-oxide, and deuterium-labeled internal standards including nicotine-methyl-D₃, cotinine-methyl-D₃, 3HC-methyl-D₃, nicotine(methyl-D₃)-glucuronide, cotinine(methyl-D₃)-glucuronide, cotinine(methyl-D₃)-3'-*O*-glucuronide, nicotine *N'*-oxide-methyl-D₃, cotinine *N*-oxide-methyl-D₃, and creatinine-D₃ were purchased from Toronto

⁴ Chen and Lazarus, unpublished results.

Research Chemicals, Inc.. All other chemicals were purchased from Fisher Scientific.

Subjects and samples

Spot urine specimens and matching genomic DNA from buccal cells were from 107 subjects that were a subset of the group of healthy subjects that were recruited as controls as part of a case-control study conducted at the H. Lee Moffitt Cancer Center (Tampa, FL) from 2000 to 2003, as previously described (13). All of the 107 subjects in the present study were Caucasian and were self-reported current smokers. The age of the subjects ranged from 30 to 74 years with an average of 56.8 years, and 43% ($n = 46$) were female. All urine and DNA samples were stored at -80°C .

Sample preparation

A 10- μL aliquot of each urine sample was added to 5 μL of a mixture of deuterium-labeled internal standards, which included nicotine-methyl- D_3 , nicotine-oxide-methyl- D_3 , cotinine-methyl- D_3 , cotinine- N -oxide-methyl- D_3 , 3HC-methyl- D_3 , nicotine(methyl- D_3)-glucuronide, cotinine(methyl- D_3)-glucuronide, 3HC(methyl- D_3)-glucuronide, and creatinine- D_3 , each at a concentration of 10 ppm. Twenty-five microliters of 40 mmol/L NH_4AC (pH 6.7) and 160 μL of acetonitrile/methanol (75%:25%, v/v) were added to buffer the pH and to precipitate out proteins. After vortexing and subsequent centrifugation at $12,000\times g$ for 10 minutes at 4°C , 100 μL of supernatant were transferred to a sample vial for analysis by ultrahigh pressure liquid chromatography (UPLC)-mass spectrometry (MS) analysis.

UPLC-MS-MS conditions

Urine samples prepared as described above were analyzed using an Acquity LC-MS-MS system (Waters Corporation),

consisting of an Acquity UPLC pump, an autosampler, an ACQUITY UPLC BEH HILIC (2.1 mm \times 100 mm, 1.7 μm particle size; Waters Corporation) column at 30°C , and an Acquity TQ tandem mass spectrometer (Waters Corporation). UPLC was performed at a flow rate of 0.4 mL/min using the following conditions: 1.5 minutes in 20% solvent A, a linear gradient for 1 minute to 100% solvent A, and 3 minutes in 100% solvent A, where solvent A is 5 mmol/L NH_4AC (pH 6.7) and 50% acetonitrile (v/v) and solvent B is 5 mmol/L NH_4AC (pH 6.7) and 90% acetonitrile (v/v). The injection volume of each prepared urine sample was 5 μL , which represented 0.25 μL of urine collected from each subject. To verify the validity of values obtained from the analysis of one sample per subject, six sample preparations and analyses were carried out on urine specimens from five individual subjects. The interassay coefficient of variation was $<10\%$ for all five subjects.

The Waters Acquity TQ tandem mass spectrometer was equipped with an electrospray ionization probe operated in the positive ion mode, with capillary voltage at 0.64 kV. Nitrogen was used as both the cone and desolvation gases with flow rates maintained at 20 and 760 L/h, respectively. Ultra-pure argon was used as the collision gas with a flow rate of 0.1 L/h for collision-induced dissociation. The source and desolvation gas temperatures were 140°C and 450°C , respectively. For the assay of nicotine, nicotine N' -oxide, nicotine-Gluc, cotinine, cotinine-Gluc, cotinine N -oxide, 3HC, 3HC-Gluc, 4HPBA, and creatinine in urine samples, the mass spectrometer was operated in the multiple reaction monitoring mode (MRM), and the concentrations of all analytes were determined simultaneously. The dwell time for each ion was 100 ms with 5 ms of interscan delay. The ion-related parameters for the 19 transitions monitored are listed in Table 1.

Table 1. MRM transitions and ion optic parameters for the test compounds

Compound	ES+ MS transition (m/z)+	Cone voltage (V)	Collision energy (V)
Nicotine	163.1>106	35	15
Nicotine-methyl- D_3	166.1>106	35	15
Cotinine	177.1>98	40	20
Cotinine-methyl- D_3	180.1>101	40	20
3HC	193.1>80	40	20
3HC-methyl- D_3	196.1>80	40	20
Nicotine-Gluc	339.1>163.1	30	15
Nicotine-methyl- D_3 -Gluc	342.1>166.1	30	15
Cotinine-Gluc	353.1>177.1	30	15
Cotinine-methyl- D_3 -Gluc	356.1>180.1	30	15
3HC-Gluc	369.1>193.1	30	15
3HC-(methyl- D_3)-Gluc	372.1>196.1	30	15
Creatinine	114.1>44	30	15
D_3 -creatinine	117.1>47	30	15
Nicotine N' -oxide	179.1>117.1	35	30
Nicotine-(methyl- D_3)- N' -oxide	182>117.1	35	30
Cotinine N -oxide	193.1>96	40	25
Cotinine-(methyl- D_3)- N -oxide	196.1>96	40	25
4HPBA	182>109	35	25

Quantification

Standard curves were constructed by plotting the ratio of analyte peak area to peak area of the corresponding internal standard (described above) versus analyte concentration for

at least eight analyte concentrations. For the nicotine and nicotine metabolite standards, a 1,000-ppm stock solution was made in water. The stock solution was serially diluted in water and then mixed with an equal volume of urine from

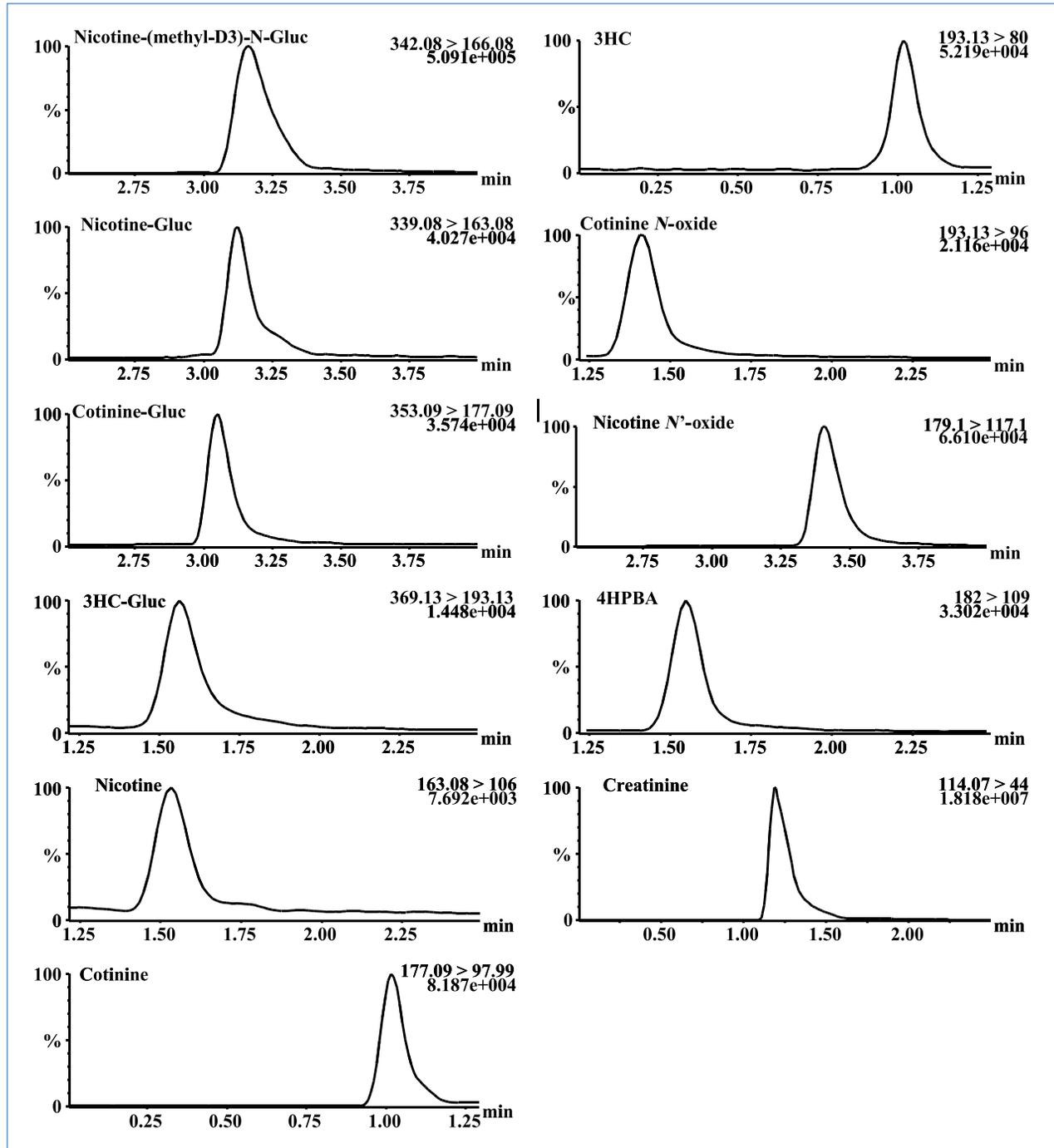


Figure 2. UPLC-MS-MS analysis of nicotine and its metabolites in the urine of smokers. Representative chromatograms for each analyte and a representative deuterated standard [nicotine (methyl-D₃)-Gluc] that was identified simultaneously from the MRM analysis of a single urine sample, as described in Materials and Methods. For each compound, the ratio of analyte peak area to that of the deuterated standard was used for quantification as described. The m/z transition monitored for each analyte is detailed on each graph.

Table 2. Urinary nicotine metabolite profile in 104 smokers

	% of Total-NIC-Eq*		Creatinine-adjusted levels (nmol/mg creatinine) [†]	
	Mean ± SD (95% CI) [‡]	Range	Mean ± SD (95% CI) [‡]	Range
3HC	33.6 ± 8.7 (32.0-35.4)	5.9-53.4	42.4 ± 25.2 (37.6-47.3)	0.78-145
3HC-Gluc	16.7 ± 7.8 (15.1-18.2)	3.3-43.6	21.8 ± 18.5 (18.2-25.3)	0.44-124
Cotinine	11.0 ± 6.6 (9.8-12.2)	2.9-32.0	14.2 ± 11.9 (11.9-16.5)	0.97-70
Nicotine	10.6 ± 7.3 (9.2-12.0)	0.58-31.7	12.9 ± 11.5 (11.0-15.2)	0.42-64
4HPBA	8.9 ± 3.7 (8.1-9.6)	3.1-32.3	10.6 ± 6.0 (9.4-11.8)	0.95-37
Cotinine-Gluc	7.3 ± 3.4 (6.6-8.0)	0-18.8	8.7 ± 5.3 (7.6-9.7)	0.00-26
Nicotine- <i>N'</i> -oxide	4.8 ± 2.5 (4.3-5.3)	1.4-13.4	5.6 ± 3.2 (4.9-6.2)	0.36-18
Nicotine-Gluc	4.3 ± 3.4 (3.6-4.9)	0-19.5	5.0 ± 4.6 (4.2-5.8)	0.00-31
Cotinine- <i>N'</i> -oxide	2.8 ± 0.77 (2.7-3.0)	1.2-5.0	3.4 ± 1.6 (2.7-4.9)	0.58-8.1

*Ratio of individual nicotine metabolite (μmol/L) measured as a percentage of total urinary nicotine metabolites [Total-NIC-Eq (μmol/L)].

[†]Ratio of individual nicotine metabolite (μmol/L) to urinary creatinine (mg/mL).

[‡]95% CI, 95% confidence interval.

a nonsmoker. Standards at concentrations ranging from 0.05 to 50 ppm were used to establish standard curves. For creatinine, the stock solution (100,000 ppm) was serially diluted in water to concentrations ranging from 10 to 10,000 ppm. 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 Waters' MassLynx software. Standard curves were included in each batch of analyses, as were aliquots of urine from both a smoker and a nonsmoker as quality controls. The quantification limits (signal/noise, >10) for each compound were ~0.01 ppm for cotinine-*N'*-oxide, nicotine-Gluc, and nicotine-*N'*-oxide; 0.05 ppm for cotinine and cotinine-Gluc; 0.1 ppm for 3HC and 3HC-Gluc; and 0.2 ppm for nicotine. The total nicotine equivalents (Total-NIC-Eq) present in each sample were determined as the sum of the micromolar concentrations of nicotine, nicotine-Gluc, nicotine *N'*-oxide, cotinine, cotinine-Gluc, cotinine *N'*-oxide, 3HC, 3HC-Gluc, and 4HPBA. The percentage of each analyte in the Total-NIC-Eq was calculated based on the equation: $C (\mu\text{mol/L}) / [\text{Total-NIC-Eq} (\mu\text{mol/L})] \times 100$, where *C* is the urinary metabolite concentration. Creatinine concentration in each sample was measured as a control for urine excretion and was also used for normalization of concentrations of nicotine metabolites from each sample.

Genotyping of UGTs 2B10 and 2B17

Genomic DNA was purified from oral buccal cell swabs collected from the same subjects who provided urine specimens as previously described (13). All subjects were recruited as healthy controls as part of a case-control study conducted at the H. Lee Moffitt Cancer Center from 2000 to 2003. Subjects were randomly selected from community residents attending the Lifetime Cancer Screening Facility of Moffitt Cancer Center, who underwent prostate-specific antigen testing, skin examinations, endoscopy, or mammography. Control

IDs were matched against the hospital patient database to identify subjects, if any, who might have developed cancer. All control subjects with a new cancer diagnosis were excluded from this study. Ninety-seven percent of subjects who were asked to participate signed a consent form approved by the institutional review board, and all were interviewed using a structured questionnaire.

The UGT2B10 codon 67 polymorphism was determined by real-time PCR using a custom designed TaqMan genotype assay (Applied Biosystems) on the ABI 7900 HT (Applied Biosystems). Real-time results were confirmed for 50% of the samples, including representative samples of each of the three UGT2B10 genotypes, by PCR-RFLP analysis using the *Hinf*I restriction enzyme, as described previously (9). Using the same two primers used for PCR amplification for RFLP, direct sequencing was performed on two subjects for each of the three potential *UGT2B10* genotypes [Asp/Asp (*1/*1), Asp/Tyr (*1/*2), Tyr/Tyr (*2/*2)], and genotypes were confirmed in all cases. The *UGT2B17* gene deletion polymorphism genotype was determined by real-time PCR using a previously described TaqMan assay (13).

Statistical analysis

All statistical analyses were performed using R (14). Linear regression analysis was used to measure the linear relationship between nicotine-Gluc, cotinine-Gluc, and/or 3HC-Gluc measured either as a percentage of Total-NIC-Eq or as creatinine-adjusted levels. The Student's *t* test and the regression trend test were used to compare levels of urinary 3HC-Gluc in smokers stratified by UGT2B17 deletion genotypes, whether measured as a percentage of Total-NIC-Eq, as creatinine-normalized levels, or as the ratio of 3HC-Gluc/(3HC + 3HC-Gluc) ratio. As the *UGT2B10* (*2/*2) genotype had limited sample size (*n* = 2), non-parametric rank-based statistical tests were used to reduce the assumptions made on the data. The Mann-Whitney *U* test and

the Jonckheere-Terpstra trend test (15) were used to compare the levels of urinary nicotine metabolites in smokers stratified by UGT2B10 codon 67 genotypes, whether measured as a percentage of Total-NIC-Eq or as creatinine-normalized levels.

Results

The levels of nicotine and its eight major metabolites (nicotine-Gluc, cotinine, cotinine-Gluc, nicotine-*N'*-oxide, cotinine-*N'*-oxide, 3HC, 3HC-Gluc, and 4HPBA) were determined simultaneously in the urine of 107 smokers. Following UPLC-MS-MS analysis, three subjects were excluded from further evaluation, because the Total-NIC-Eq value for each was $<5 \mu\text{mol/L}$ and individual nicotine metabolites from those samples could not be quantified reliably. Chromatograms obtained from a representative analysis of the urine of a single subject are shown in Fig. 2. The levels of each metabolite are shown in Table 2 and are expressed as both creatinine-adjusted concentrations as well as a percentage of Total-NIC-Eq. Similar to that observed in previous studies (16), 3HC was the major nicotine metabolite based on metabolite levels as a percentage of Total-NIC-Eq in this population. The ranking of the mean levels of nicotine metabolites as a percentage of Total-NIC-Eq was 3HC $>$ 3HC-Gluc $>$ cotinine $>$ nicotine $>$ 4HPBA $>$ cotinine-Gluc $>$ nicotine-*N'*-oxide $>$ nicotine-Gluc $>$ cotinine-*N'*-oxide. Identical results were obtained when comparing the levels of creatinine-adjusted metabolites (Table 2). Of the glucuronides, the mean Total-NIC-Eq-adjusted 3HC-Gluc levels were 2.3-fold and 3.9-fold higher than cotinine-Gluc and nicotine-Gluc, respectively, with nicotine-Gluc comprising an average 4.3% of Total-NIC-Eq. The levels of 3HC-Gluc and cotinine-Gluc comprised 16.7% and 7.3%, respectively, of the Total-NIC-Eq. Two subjects exhibited urinary nicotine-Gluc levels of $\geq 18\%$, and six exhibited urinary nicotine-Gluc levels of $\geq 10\%$ of Total-NIC-Eq (results not shown). Urinary nicotine-Gluc + cotinine-Gluc comprised $\geq 15\%$ of Total-NIC-Eq in 27 subjects, with two subjects exhibiting urinary nicotine-Gluc + cotinine-Gluc levels that were $>33\%$ of Total-NIC-Eq (results not shown). To determine whether any correlations existed between the amount of glucuronidated product formed for nicotine or its major metabolites, the levels of nicotine-Gluc measured as a percentage of Total-NIC-Eq were compared with the percentages of Total-NIC-Eq for cotinine-Gluc and 3HC-Gluc (Fig. 3). There was a significant ($P < 0.0001$, $r = 0.73$) correlation between the levels of urinary nicotine-Gluc and cotinine-Gluc in smokers (Fig. 3A). A weak negative association was observed between the percentages of Total-NIC-Eq for urinary nicotine-Gluc and 3HC-Gluc ($P < 0.05$, $r = -0.23$; Fig. 3B) or for urinary cotinine-Gluc and 3HC-Gluc ($P < 0.05$, $r = -0.23$; results not shown).

Informative genotyping data were obtained for all of the 104 subjects tested for the UGT2B10 codon 67 polymorphism. The UGT2B10 codon 67 (Asp $>$ Tyr) polymorphism genotype distribution was as follows: 80% ($n = 83$) exhibited the homozygous wild-type *UGT2B10* (*1/*1) genotype, 18% ($n = 19$) exhibited the heterozygous *UGT2B10* (*1/*2) genotype, and 2% ($n = 2$) exhibited the homozygous *UGT2B10* (*2/*2) genotype. This genotype distribution was consistent with Hardy-Weinberg equilibrium ($P = 0.5$), and the UGT2B10 allelic frequency

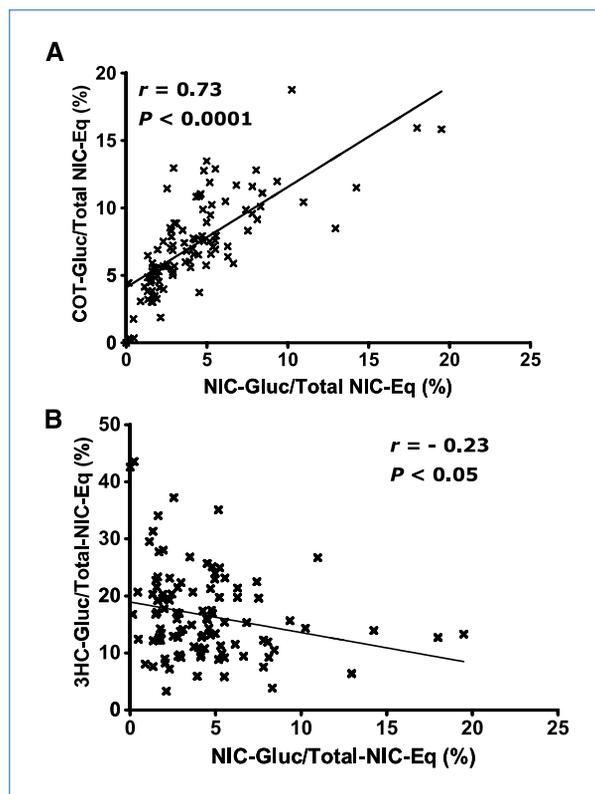


Figure 3. Correlation between percentages of Total-NIC-Eq for nicotine-glucuronide, cotinine-glucuronide, and 3HC-glucuronide in the urine of smokers. Nicotine-glucuronide, cotinine-glucuronide, and 3HC-glucuronide were determined for 104 smokers, as described in Materials and Methods. A, percentage of Total-NIC-Eq for nicotine-glucuronide and cotinine-glucuronide. B, percentage of Total-NIC-Eq for nicotine-glucuronide and 3HC-glucuronide.

(11%) was consistent with that observed in previous studies (9). The average percentage of Total-NIC-Eq determined for nicotine-Gluc in the urine from smokers with the wild-type *UGT2B10* (*1/*1) genotype was 4.7%, whereas that for smokers of each of the variant genotypes was significantly lower, at 2.8% for smokers with the *UGT2B10* (*1/*2) genotype ($P < 0.05$) and at 0.24% ($P < 0.001$) for smokers with the *UGT2B10* (*2/*2) genotype (Fig. 4A). A similar pattern was observed for the percentage of Total-NIC-Eq determined for cotinine-Gluc, with significant 1.9-fold ($P < 0.0001$) and 48-fold ($P < 0.0001$) lower levels in the urine from smokers with the *UGT2B10* (*1/*2; 4.2%) and *UGT2B10* (*2/*2; 0.17%) genotypes, respectively, compared with the level (8.2%; Fig. 4B) in the urine of smokers with the wild-type *UGT2B10* (*1/*1) genotype. There was a significant trend toward a decreased percentage of Total-NIC-Eq for nicotine-Gluc ($P < 0.001$) and cotinine-Gluc ($P < 0.0001$) levels in the urine of smokers with an increasing number of the variant *UGT2B10**2 allele. A similarly significant pattern was observed when examining the creatinine-adjusted levels of nicotine-Gluc or cotinine-Gluc (results not shown). No correlation was observed between the percentages of Total-NIC-Eq for 3HC-Gluc and UGT2B10 codon 67 genotype (Fig. 4C). Additionally,

no correlation was observed between UGT2B10 codon 67 genotypes and the percentage of Total-NIC-Eq for any of the other nicotine metabolites analyzed in this study (data not shown).

Of the 88 samples for whom informative UGT2B17 deletion genotype data were obtained, the UGT2B17 copy number distribution was as follows: 53% ($n = 47$) exhibited both copies of the wild-type *UGT2B17**1 gene, 39% ($n = 34$) exhibited one copy of the *UGT2B17**2 deletion allele, and 8% ($n = 7$) exhibited the *UGT2B17* (*2/*2) homozygous deletion genotype. This genotype distribution was consistent with Hardy-Weinberg equilibrium ($P = 0.5$), and the *UGT2B17**2 deletion allelic frequency (27%) was consistent with that observed in previous studies (12, 17, 18). The average percentage of Total-NIC-Eq for 3HC-Gluc in the urine of subjects exhibiting the wild-type *UGT2B17* (*1/*1) genotype was 19.2% but was significantly lower for subjects heterozygous (15.6%; $P < 0.05$) or homozygous (11.2%; $P < 0.001$) for the UGT2B17 deletion (Fig. 4D).

There was a significant trend toward a decreased percentage of Total-NIC-Eq for urinary 3HC-Gluc ($P < 0.05$) in subjects with increasing copies of the *UGT2B17**2 deletion allele. A similarly significant trend was observed when examining the levels of 3HC-Gluc as a ratio with (a) 3HC + 3HC-Gluc ($P < 0.001$) or (b) creatinine ($P < 0.05$) as the denominator (results not shown).

Discussion

The present study is the first to directly examine the role of functional variants in both UGTs 2B10 and 2B17 in nicotine metabolism in smokers. Previous studies have shown that, although UGTs 1A4 and 2B10 exhibit glucuronidating activity against both nicotine and cotinine *in vitro*, UGT2B10 exhibits a 3-fold to 37-fold lower K_m than UGT1A4 against these two compounds and a functional polymorphism at codon 67 of the *UGT2B10* gene results in a 5-fold and 16-fold

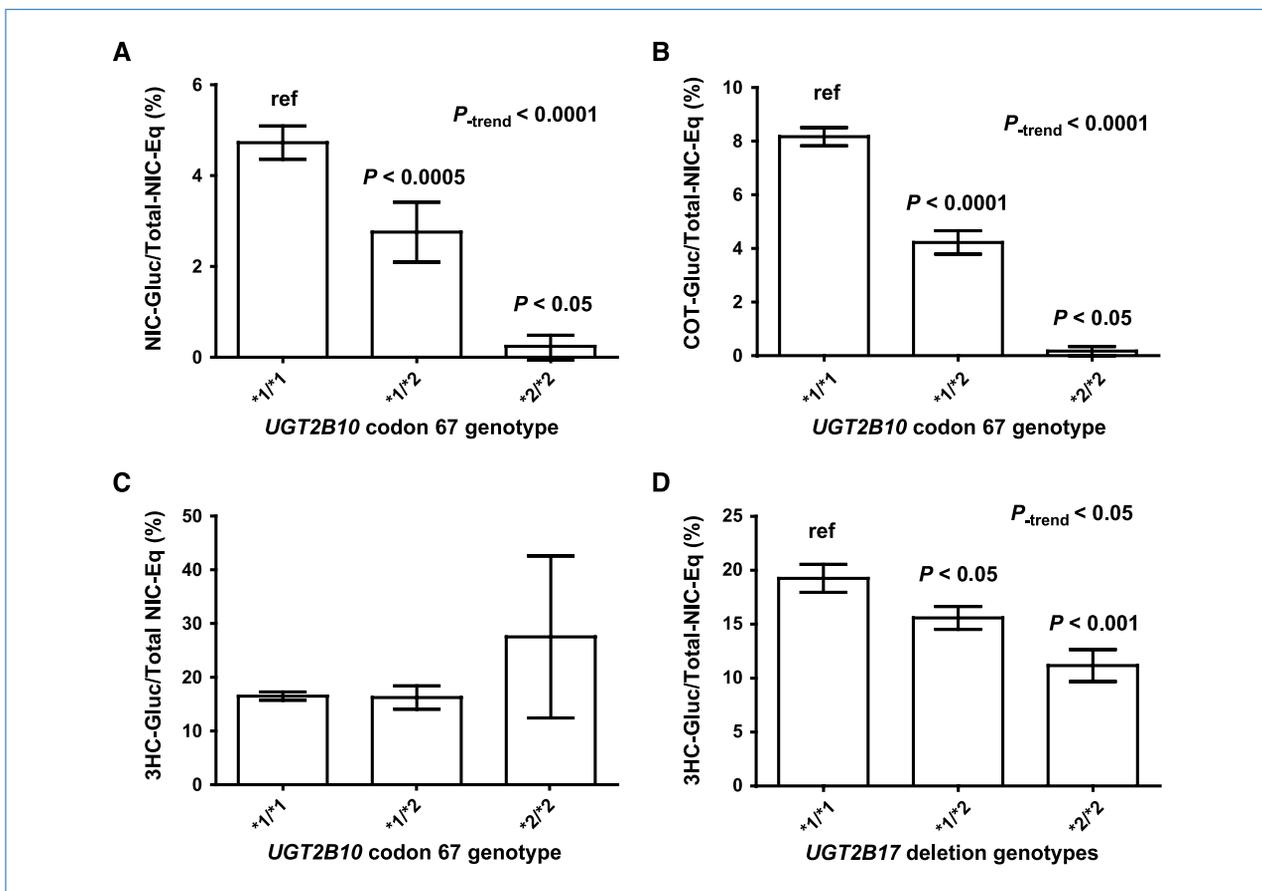


Figure 4. Effect of UGT2B10 and UGT2B17 polymorphisms on nicotine-Gluc, cotinine-Gluc, and 3HC-Gluc levels in urine samples from smokers. Percentage of Total-NIC-Eq for urinary nicotine and nicotine metabolite glucuronides for subjects stratified by UGT2B10 codon 67 or UGT2B17 deletion genotypes. A, UGT2B10 genotype versus urinary nicotine-Gluc. B, UGT2B10 genotype versus urinary cotinine-Gluc. C, UGT2B10 genotype versus urinary 3HC-Gluc. D, UGT2B17 genotype versus urinary 3HC-Gluc. Mann-Whitney U test was used to compare urine from subjects with the *UGT2B10* (*2/*2) or (*1/*2) genotypes with specimens from subjects with the wild-type *UGT2B10* (*1/*1) genotype, and the Jonckheere-Terpstra trend test was used to examine the overall effect of UGT genotypes on nicotine-glucuronide, cotinine-glucuronide, and 3HC-glucuronide levels determined as a percentage of Total-NIC-Eq. Student's t test was used to compare urine from subjects with the *UGT2B17* (*2/*2) or (*1/*2) genotypes with specimens from subjects with the wild-type *UGT2B17* (*1/*1) genotype, and the trend test was performed to examine the overall effect of UGT genotypes on nicotine-glucuronide, cotinine-glucuronide, and 3HC-glucuronide levels determined as a percentage of Total-NIC-Eq. Data are shown as the mean \pm SEM.

decrease in liver activity against nicotine and cotinine, respectively (9, 19, 20). In the present study, it was shown that the percentages of urinary nicotine-Gluc and cotinine-Gluc in smokers homozygous for the *UGT2B10**2 variant were decreased by 95% and 98%, respectively, compared with smokers exhibiting the wild-type *UGT2B10* (*1/*1) genotype. This *UGT2B10**2 allele-associated decrease in nicotine and cotinine glucuronidation was consistent with that observed in recent studies for urine specimens from subjects heterozygous for a tagSNP linked with the *UGT2B10* codon 67 SNP (21). This pattern is also consistent with previous *in vitro* studies in HLM and UGT-overexpressing cell lines, indicating that *UGT2B10* is the major enzyme active in the glucuronidation of these compounds (9). In addition, this confirms the functionality of the *UGT2B10*^{67Tyr} variant against nicotine and cotinine, an effect that is consistent with the drastic change in amino acid sequence from the acidic aspartic acid encoded by the *UGT2B10**1 allele to the bulky, phenolic tyrosine residue encoded by *UGT2B10**2.

Two previous studies (8, 22) have suggested that UGTs 1A9 and 2B7 are primarily responsible for 3HC-Gluc formation. For both of these studies, several UGTs, including *UGT2B17*, were not screened for activity against 3HC. Recent data indicate that *UGT2B17* exhibits a K_M that is lower than both UGTs 1A9 and 2B7 against 3HC and that the homozygous deletion of the *UGT2B17* gene results in a ~2-fold decrease in liver microsomal activity against 3HC *in vitro*.⁵ In the present study, it was shown that there was a 2.8-fold decrease in the levels of urinary 3HC-Gluc in smokers homozygous for the *UGT2B17* deletion variant compared with urine specimens from smokers with the wild-type *UGT2B17* (*1/*1) genotype. This is consistent with an important role for *UGT2B17* in 3HC glucuronidation. Based on the difference in urinary 3HC levels in *UGT2B17* (*1/*1) versus *UGT2B17* (*2/*2) smokers and the fact that the *UGT2B17* deletion is in effect a functional knockout of the *UGT2B17* gene, *UGT2B17* accounts for ~42% of all glucuronidated 3HC in humans, with UGTs 2B7 and 1A9 likely comprising the other UGTs active in this process (8, 22).

This is the first study to simultaneously and directly determine the levels of nicotine and eight of its major metabolites in the urine of smokers. Whereas the means of the percentage of Total-NIC-Eq for most of the nicotine metabolites examined in this study were similar to those described in previous reports, the relative levels of cotinine-Gluc and 3HC-Gluc were, on average, 1.7-fold lower and 2-fold higher, respectively, than the levels reported in other studies for these two metabolites in the urine of smokers (16, 23, 24). Whereas differences in the methodologies used for nicotine metabolite detection and quantification may contribute to the differences observed between studies, sample size variation could also play an important role. The current study included 104 subjects, whereas only up to 12 subjects were included in each of the two previous studies (16, 23). In addition, the method of urine sample collection was different

between studies. Spot urine samples were analyzed in the present study, whereas 24-hour urine specimens were used in previous studies. Due to the relatively short elimination half-life (~2 hours; ref. 25) of nicotine, the levels of nicotine metabolites in spot urine samples may vary in relation to when the last cigarette was smoked; 24-hour urine has the advantage of providing steady-state urinary nicotine excretion data. However, virtually identical trends were observed with respect to *UGT2B10* genotype and the formation of nicotine-glucuronide as well as cotinine-glucuronide, suggesting that the use of spot urine samples was not confounding the results observed in this study.

Interestingly, whereas the levels of glucuronide conjugates of nicotine and cotinine were low in most subjects analyzed in this study (mean percentage of Total-NIC-Eq of 4.3 and 7.3, respectively), higher relative levels of nicotine-Gluc and cotinine-Gluc were observed in some subjects, with the nicotine-Gluc reaching as high as 20% of total urinary nicotine metabolites and total nicotine-Gluc + cotinine-Gluc levels reaching as high as one third of total urinary nicotine metabolites. Therefore, the glucuronidation pathway may play an even more important role in the overall metabolism of nicotine and cotinine in a subset of the population than previously identified.

Alteration in the rate of clearance or any shift in the distribution of dose between nicotine and its metabolites and/or conjugates could have an effect on addiction and possibly carcinogen exposure resulting from altered smoking dose. The glucuronidation pathway may be particularly important in subjects for whom nicotine metabolism by hydroxylation/oxidation by CYP450 enzymes is low. CYP2A6 is the major hepatic enzyme involved in both the metabolism of nicotine to its nonaddictive metabolite cotinine and in the metabolism of cotinine to 3HC (3). It has been shown that ~20% of Caucasians and African-Americans as well as 70% of Asians exhibit a CYP2A6 enzyme phenotype that results in the reduction of CYP2A6 enzyme activity by at least 25% and thus results in the poor metabolism of nicotine and cotinine (26). In Asian smokers homozygous for the *CYP2A6**4 deletion allele and who, therefore, have no active CYP2A6 enzyme, the urinary levels of nicotine and nicotine-Gluc were shown to be as high as 45% and 55%, respectively, of total urinary nicotine metabolites (27). In subjects with a CYP2A6 enzyme phenotype that results in decreased metabolism of nicotine and cotinine, the percentage of the parent compounds would also likely be increased to even higher levels if that individual also had one or more *UGT2B10**2 variant alleles that resulted in less efficient glucuronidation of these compounds. Therefore, the *UGT2B10* codon 67 polymorphism could be a very important modifier of nicotine metabolism in subjects with CYP2A6 low-activity phenotypes and could therefore play an important role in inter-individual variations in nicotine addiction in this population.

The nicotine metabolite ratio of 3HC/cotinine has been used as an indicator of CYP2A6 activity (28, 29) and as a variable in clinical studies of smoking cessation (30–35). Because the *UGT2B17* deletion affects overall clearance of 3HC, this polymorphism could significantly affect the efficacy of this ratio as a determinant of CYP2A6 activity and variable in nicotine addiction. Further large-scale studies examining urinary

⁵ Chen and Lazarus, unpublished result.

nicotine metabolites in smokers of different combined CYP2A6 and UGT2B17 genotypes will be required to better assess these possibilities.

In conclusion, this study shows that UGT2B10 is the enzyme responsible for the majority of the *in vivo* glucuronidation of nicotine and cotinine, whereas UGT2B17 is an important enzyme responsible for the glucuronidation of 3HC *in vivo*. Additionally, this study has established that (a) the UGT2B10 codon 67 (Asp > Tyr) polymorphism is associated with large reductions in the levels of nicotine-Gluc and cotinine-Gluc and (b) the UGT2B17 gene deletion is associated with significant decreases in the levels of 3HC-Gluc in the urine of smokers. Population-based studies will be required to better assess their potential contribution in overall smoking behavior and nicotine dependency.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Gang Chen, Nino E. Giambone, Jr., Douglas F. Dluzen, et al.

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