Published in final edited form as:

Pharmacogenet Genomics. 2009 May ; 19(5): 388–398. doi:10.1097/FPC.0b013e32832a404f.

GENETIC AND ENVIRONMENTAL INFLUENCES ON THE RATIO OF 3’HYDROXYCOTININE TO COTININE IN PLASMA AND URINE

Gary E. Swan¹, Christina N. Lessov-Schlaggar², Andrew W. Bergen¹, Yungang He¹, Rachel F. Tyndale³, and Neal L. Benowitz⁴

¹Center for Health Sciences, SRI International

²Department of Psychiatry, Washington University School of Medicine

³Centre for Addiction and Mental Health, Department of Pharmacology, University of Toronto

⁴Division of Clinical Pharmacology, Departments of Medicine and Biopharmaceutical Sciences, University of California, San Francisco

Abstract

Objectives—The ratio of *trans*-3’hydroxycotinine/cotinine (3HC/COT) is a marker of CYP2A6 activity, an important determinant of nicotine metabolism. This analysis sought to conduct a combined genetic epidemiologic and pharmacogenetic investigation of the 3HC/COT ratio in plasma and urine.

Methods—One hundred thirty nine twin pairs (110 monozygotic [MZ] and 29 dizygotic [DZ]) underwent a 30-minute infusion of stable isotope-labeled nicotine and its major metabolite, cotinine, followed by an 8-hour in-hospital stay. Blood and urine samples were taken at regular intervals for analysis of nicotine, cotinine, and metabolites. DNA was genotyped to confirm zygosity and for variation in the gene for the primary nicotine metabolic enzyme, *CYP2A6* (variants genotyped: *1B, *1×2, *2, *4, *9, *12). Univariate biometric analyses quantified genetic and environmental influences on each measure in the presence and absence of covariates, including measured *CYP2A6* genotype.

Results—There was a substantial amount of variation in the free 3HC/COT ratio in plasma (6 hours post-infusion) attributable to additive genetic influences (67.4%, 95% CI = 55.9–76.2%). The heritability estimate was reduced to 61.0% and 49.4%, respectively, after taking into account the effect of covariates and *CYP2A6* genotype. In urine (collected over 8 hours), the estimated amount of variation in the 3HC/COT ratio attributable to additive genetic influences was smaller (47.2%, 95% CI = 0–67.2%) and decreased to 44.6% and 42.0% after accounting for covariates and genotype.

Conclusions—Additive genetic factors are prominent in determining variation in plasma 3HC/COT variation but less so in determining variation in urine 3HC/COT.

Keywords

pharmacogenetics; nicotine; cotinine; metabolism; *CYP2A6*; twins; genetics; heritability

INTRODUCTION

The liver enzyme cytochrome P450 (CYP) 2A6 is the major enzyme responsible for the metabolism of nicotine [1,2]. Nicotine is metabolized primarily by CYP2A6 via C-oxidation to cotinine (COT) which is, in turn, metabolized primarily or exclusively by CYP2A6 to *trans*-3'-hydroxycotinine (3HC). The 3HC/COT ratio is highly correlated with the oral clearance of nicotine [3] and smokers with CYP2A6 slow metabolism variants have a lower 3HC/COT ratio than normal metabolizers [4].

The 3HC/COT ratio can be measured in a spot sample of plasma, urine, or saliva in individuals exposed to tobacco smoke in the absence of an invasive nicotine infusion and complicated pharmacokinetic protocols, suggesting its utility for population-based studies [5–7]. The 3HC/COT ratio in urine has been studied in relation to measures of tobacco dependence in adult, mostly Caucasian [8] and younger, ethnically diverse smokers [9]. In the Benowitz et al. study, the 3HC/COT ratio was associated with cigarettes per day ($r = 0.33$, $P < 0.01$) but not with scores on the Fagerström Test for Nicotine Dependence (FTND; [10]). In the Kandel et al. study, the 3HC/COT ratio in urine was not associated with either the number of cigarettes smoked per day or nicotine dependence [9].

The 3HC/COT ratio in plasma has been examined in three different adult cohorts including smokers who participated in a clinical trial of two different forms of nicotine replacement therapy [11,12], smokers who had returned to smoking eight years after cessation with nicotine patch treatment [13], and *ad libitum* smokers who were not seeking treatment [4]. The 3HC/COT ratio in plasma has also been studied in one adolescent cohort [14]. Three of the studies found modest, but significant associations between the 3HC/COT ratio in plasma and number of cigarettes smoked per day [11–13] while the other two found no significant association with amount smoked [4,14]. No study has reported a significant association between 3HC/COT in plasma and formal measures of nicotine dependence.

The 3HC/COT ratio is viewed as a reliable indicator of CYP2A6 activity [4,15] and reflective of an individual's characteristic rate of nicotine metabolism [3]. Previously we have shown that the clearance of nicotine via the COT pathway (another measure of CYP2A6 metabolic activity) has substantial heritability even after adjusting for CYP2A6 genotype status (genotypes associated with a normal rate of metabolism as compared to variants associated with a reduced rate of metabolism), thereby suggesting that other genes are determinants of variation in this metabolic phenotype [16].

Given that the 3HC/COT ratio is gaining acceptance as a marker of CYP2A6 activity and may be predictive of cessation of tobacco use following treatment with nicotine replacement therapy for nicotine dependence [11], we decided to conduct heritability analyses of the 3HC/COT ratio as measured in two different body fluids, plasma and urine. The biometric analyses of the 3HC/COT ratio were conducted before and after adjustment for covariates and CYP2A6 genotype. Whereas the previous paper focused on the direct measurement of nicotine clearance in plasma only and included CYP2A6 genotypes known at that time [16], the current paper incorporates additional variants identified in the 3 years since. Another difference between the previous and current analysis is the focus on the 3HC/COT phenotype as measured in two different fluids, plasma and urine. The 3HC/COT phenotype is believed to reflect CYP2A6 activity, as it appears that this enzyme is the main, if not the exclusive, enzyme that metabolizes cotinine to 3HC. In contrast, nicotine clearance reflects not only CYP2A6 activity, but also the activity of other oxidative and conjugation enzymes that metabolize nicotine. While the ratios as estimated in the two fluids are substantially correlated, the relationship is not perfect, and the analyses in this paper compare and contrast inferences about the nature of the underlying genetic and environmental architecture for each. A final difference between the current and

the previous paper is its focus on the 3HC/COT ratio, a phenotypic marker of CYP2A6 activity that is widely available and used in multiple studies of adolescent and adult smoking behavior and in clinical trials of therapeutics for smoking cessation.

METHODS

Setting

The study involved two primary research centers: the Center for Health Sciences at SRI International (SRI; Menlo Park, CA) and the Division of Clinical Pharmacology and Experimental Therapeutics, University of California, San Francisco (UCSF), based at the San Francisco General Hospital Medical Center. SRI was responsible for the recruitment, screening, and scheduling of twin pairs drawn from the SRI Northern California Twin Registry (NCTR; described below) and was responsible for biometric analyses. UCSF was responsible for conducting the nicotine/cotinine infusion procedure in the General Clinical Research Center at San Francisco General Hospital (SFGH), for analytical chemistry procedures to determine nicotine, COT, and metabolite levels, and for estimation of pharmacokinetic parameters. Additional contributions were made by the University of Toronto, Department of Pharmacology (genotyping for *CYP2A6*), and by the Department of Neurology, UCSF (molecular determination of zygosity).

Participants

Twins were recruited from the NCTR and subjected to rigorous screening for conditions requiring exclusion from the study: *Inclusion Criteria*—To be eligible for inclusion in this study, volunteers needed to have a twin who was also willing to participate and be between the ages of 18 and 65 years. Both members of a twin pair needed to be in good general health. *Exclusion Criteria*—To minimize the effect of medical conditions and/or medication usage known to influence drug metabolism, individuals were excluded from participation if they met any of the following criteria: age of less than 18 years or older than 65 years; weight of more than 30% over ideal height-adjusted weight; pregnancy; the presence of any of the following conditions: use of drug metabolism-altering medications such as anticonvulsant drugs and barbiturates; uncontrolled hypertension or diabetes; a history of heart disease as indicated by self-report or history of bypass surgery, valve replacement, use of a pacemaker, or angioplasty procedures; Raynaud's disease; chronic diseases such as cancer, liver, and kidney diseases, or asthma, that were not stable or were not in remission for at least 1 year; migraine headaches, anemia, abnormal blood sugar levels that were not well-controlled by medication, substance abuse and/or dependence (other than tobacco), psychiatric disorders that could limit study compliance or require the use of metabolism-altering psychotropic medications, positive HIV status, hepatitis B or C, history of vasovagal reactions, discomfort with venipuncture procedures, or a self-reported history of "difficult veins." Because the study procedures could be seriously confounded or could lead to adverse events for the participants by the presence of the conditions described above, a three-tiered screening procedure (telephone, in-person, and in-hospital) was employed. All study procedures are described more completely elsewhere [17].

All methods for recruitment, informed consent, screening, data and DNA collection, and genetic analysis were reviewed and approved by the Institutional Review Boards of SRI International, UCSF, and the University of Toronto.

Questionnaire measures

Zygosity status was assessed by standard zygosity questionnaire items [18] and confirmed by DNA genotyping (see below). Zygosity questions included whether, as children, the twins were 'as alike as two peas in a pod', whether parents, siblings, or teachers had trouble telling them

apart, and the twins' own knowledge of their zygosity. A series of standard demographic questions were asked to determine date of birth, race/ethnicity, marital status, number of children, and educational attainment. In addition to information pertinent to the previously described exclusionary conditions, participants also provided a history of hospitalization for major medical illness or surgery. The timing of each female participant's menstrual cycle was determined following a previously published approach [19]. The infusion protocol was scheduled to occur in the mid- to late-follicular phase (operationally, between the end of the menses and day 11 of the cycle). A series of questions were also asked to ascertain smoking status (never, former, or current smoking). Current smoking status and the number of cigarettes smoked were important determinants of the dose of nicotine infused (see below).

In-hospital pharmacokinetic study procedures

The project physician prepared the nicotine/cotinine solution for each participant who arrived at SFGH for the infusion appointment. Two factors determined the nicotine dose level: body weight and screening plasma COT levels. Participants received 0.5 µg/kg/min if plasma COT levels were 50 ng/ml or lower (levels consistent with not smoking or smoking five or fewer cigarettes per day); 1.0 µg/kg/min if plasma COT levels were 50–150 ng/ml (levels consistent with smoking 5–15 cigarettes per day); and 2.0 µg/kg/min if plasma COT levels were >150 ng/ml (levels consistent with smoking 15 or more cigarettes per day). The dose was always based on the lower plasma COT level within a twin pair so that both twins of pairs discordant for smoking received the same dose. The dose of COT was the same as that for nicotine. In-dwelling catheters were placed in each arm. The catheter in the right arm was used to deliver the stable isotope-labeled nicotine/cotinine solution while the catheter in the left arm was used to obtain periodic blood samples.

Participants received a simultaneous intravenous infusion of deuterium-labeled nicotine (nicotine-d₂ = 3', 3'-dideuteronicotine) and COT (COT-d₄ = 2, 4, 5, 6-tetradeutercotinine). Labeled compounds are necessary for metabolic studies because individuals who use tobacco already have considerable levels of nicotine and COT in their bodies that would make measurement of clearance of unlabeled nicotine or COT impossible. The synthesis of these deuterium-labeled compounds and their preparation for infusion has been described previously [20]. During all infusions, participants underwent continuous cardiac monitoring and frequent BP measurements.

Prior to the start of the 30-minute nicotine infusion, baseline blood and urine samples were obtained from each participant. After the start of the infusion, a total of 10 blood samples (5 ml) were obtained at the following intervals: 10, 20, 30, 45, 60, 90, 120, 180 minutes, and at 4 and 6 hours. Blood samples were also collected on the morning of days 2, 3, 4, and 5 as described below. All urine was collected over the course of the 8-hour protocol. To make the lengthy 8-hour stay in the hospital as pleasant as possible, participants were provided with snacks, meals, and a TV/VCR with a large collection of popular movies on video.

Prior to discharge, post-hospitalization blood sample collection procedures and appointment times were reviewed with participants so that blood samples (5 ml) could be obtained on four subsequent consecutive mornings.

Analytical Chemistry and Pharmacokinetic measures—COT and 3HC

concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [21]. We computed the free 3HC/COT ratio in plasma (6 hours post-infusion) and in urine collected for 8 hours after the onset of the infusion by use of both the COT-d₂- and COT-d₄-derived compounds.

Genotyping for zygosity—Self-reported zygosity was confirmed by genotyping of highly polymorphic microsatellite markers on 11 chromosomes (D1S1612, D2S1788, D3S1764, D4S2368, D5S1501, D7S513, D8S1110, D13S1796, D14S608, D16S753 and D15S657) using the method described elsewhere [22]. Because microsatellites have a low, but detectable, somatic mutation rate, the zygosity of one twin pair could not be determined without additional marker testing. As a check on the accuracy of DNA genotyping for zygosity, a subset of DNA samples from 30 randomly selected pairs was sent to an independent laboratory (Rutgers University Cell and DNA Repository). All zygosity determinations from the first laboratory were confirmed by subsequent analysis from the independent laboratory.

Genotyping for CYP2A6—Genotyping of *CYP2A6*1B*, *CYP2A6*1*×2, *CYP2A6*2*, and *CYP2A6*4* was performed according to previously described protocols [2,23,24]. Methods to detect *CYP2A6*9* and *CYP2A6*12* alleles are described elsewhere [25]. Each allelic assay relies on a two-step PCR detection system, where the first amplification uses genomic DNA as the template with gene-specific primers and the second amplification uses DNA derived from the first PCR amplification with allele-specific primers. Taq polymerase was obtained from Gibco BRL (Life Technologies, Burlington, Canada) or MBI Fermentas (Burlington, Canada). For the genotyping procedures, each set of samples included three positive controls with the different genotyping combinations and a negative control lacking DNA. All PCR amplifications were performed using PTC-200 Peltier Thermal Cyclers, and the second amplification products were analyzed on 1.2% or 3% agarose gels (OnBio, Richmond Hill, Canada) containing ethidium bromide. A 1kb DNA ladder was used for each set of samples to confirm the appropriate amplicon sizes.

Statistical Analysis

Groupings were created on several covariates likely to influence the 3HC/COT ratio [1,26] including sex, age (≤ 37 years of age or > 37 years of age), ethnicity (Caucasian and/or Hispanic or Other), BMI (≤ 24.5 kg/m² or > 24.5 kg/m²), smoking status (current or not current), weekly alcohol consumption ($\leq$ three drinks per week or $>$ three drinks per week), oral contraceptive use (yes/no), and *CYP2A6* genotype status. Cutpoints for age, BMI, and weekly alcohol consumption were determined by median split. *CYP2A6* genotype was a 3-category variable defined based on the genotype association with rate of plasma nicotine clearance [15,27] and that captures normal metabolizers (**1A/*1A*, **1A/*1B*), variant or lower activity metabolizers (**2*, **4*, **9*, **12*), or increased activity metabolizers (**1B/*1B*, **1*×2). In covariate analysis, *CYP2A6* genotype was dummy coded (normal vs. all others and slow metabolizers vs. all others). Ten individuals were missing either plasma or urine pharmacokinetic measurements and were not included in this analysis.

Mean 3HC/COT in plasma and urine were compared for each of the covariate groups using the MIXED procedure in SAS [28]. Least square means and associated standard errors are reported in Table 2.

Twin analysis followed standard principles and methods [29]. Briefly, using data from twins reared together, total phenotypic variance was partitioned into: (1) the additive effects of genes (A), or heritable factors, which contribute to similarity between co-twins. Genetic effects are perfectly correlated in MZ co-twins who are 100% genetically related, and are correlated 0.5 in DZ co-twins who share on average, 50% of their genetic material; (2) shared environmental effects (C), such as family environment, peers, or school factors common to co-twins, which contribute to co-twin similarity. Shared environmental factors are assumed to influence MZ and DZ co-twins similarly and, are assumed to be perfectly correlated for both zygositys; and (3) non-shared environmental effects (E), which are individual specific and include measurement error, and contribute to co-twin dissimilarity.

The distribution of the 3HC/COT ratio in urine and in plasma was highly skewed (see Figure 1). Prior to statistical analysis, plasma 3HC/COT values were subjected to the square root transformation while those for 3HC/COT in urine were log transformed to minimize skewness and kurtosis for each measure. The values based on COT-d2- and COT-d4 were highly correlated, $r = 0.99$, $P < 0.0001$, as was the derived 3HC/COT ratio based on each, $r = 0.97$, $P < 0.0001$. Therefore, only COT-d2-derived values were used in the analysis.

As a first step in the biometric analyses, twin pair Pearson correlations patterns for the 3HC/COT ratio in both plasma and in urine were examined. MZ twin pair correlation that is twice that of DZ twin pairs suggests a contribution of genetic sources. An MZ twin pair correlation that is less than twice that of DZ twin pairs suggests the influence of both genetic and shared environmental effects. MZ and DZ twin pair correlations of similar magnitude reflect the influence of shared environmental effects.

Next, univariate models were fit to 2-group (MZ and DZ) twin pair covariance matrices using Mx software [30]. For covariate adjustment, models were fit to residualized covariance matrices. Model parsimony guided the choice of the best fitting model. The significance of the contribution of A or C was tested by equating each or both to zero and examining the fit of the “AE”, “CE”, or “E” reduced models against the fit of the full “ACE” model, using the likelihood-ratio chi-square difference test. A significant difference ($P < 0.05$) indicated significant deterioration of the reduced model fit relative to the full model and did not warrant the imposed constraints. To examine the genetic and environmental contribution to covariation of plasma and urine 3HC/COT measures, a correlated factor model was fit to 2-group cross-twin cross-phenotype covariance matrices. As in univariate analysis, bivariate models were fit to unadjusted and covariate-adjusted 2-group data.

RESULTS

Characterization of study participants has been reported elsewhere [17]. Generally, application of the exclusion criteria resulted in a younger, healthier study sample as compared to those who were deemed ineligible.

Demographic characteristics

Participants had a mean age of 37.7 years (Table 1). The majority were women (69.8%), MZ (79.1%), and Caucasian (76.3%). This was a well-educated sample with more than half (52.1%) having obtained at least a Bachelor's degree. About one-third of the twins (34.6%) were single, more than one-half were married or living with a partner (53.1%), with the remaining 12.4% being either divorced, widowed, or separated. Overall, 19.8% of the sample was smoking cigarettes at the time of the study and 24.1% were former smokers. Among women, 26.8% were using oral contraceptives. Table 1 also displays characteristics by zygosity. MZ and DZ twins did not differ significantly on any of the characteristics shown.

Characteristics of the 3HC/COT ratio in plasma and urine

The average value for the 3HC/COT ratio in the 6 hour plasma sample was 0.25 ± 0.13 (range = 0.04–0.79) while that for the 3HC/COT ratio in urine was 1.37 ± 0.78 (range = 0.20 to 6.16). The 3HC/COT ratio in plasma and in urine was significantly correlated, $r = 0.70$, $P < 0.01$.

CYP2A6 allele frequencies

Since MZ and DZ twins share 100% and 50% of their genome, respectively, inclusion of all twins in this portion of the analysis would lead to biased estimates of allele frequencies. The total number of pairs with complete genotypic and phenotypic data was 138. After one twin from each pair was randomly selected, the following allele frequencies were observed for the

sample of 138 unrelated individuals: *1A, $n = 148$, 53.6%; *1B, $n = 92$, 33.3%; *1 $\times$ 2, $n = 5$, 1.8%; *2, $n = 6$, 2.2%; *4, $n = 2$, 0.7%; *9, $n = 16$, 5.8%; *12, $n = 7$, 2.5%. These frequencies are similar to those reported previously for a sample of unrelated individuals of European descent [25] and are in Hardy-Weinberg equilibrium [27].

Comparison of 3HC/COT means in covariate subgroups

As shown in Table 2, males and females differed significantly from each other on 3HC/COT values in both plasma, $F = 15.68$, $P < 0.05$, and urine, $F = 10.67$, $P < 0.05$, with women having a higher 3HC/COT ratio than men. After adjustment for sex, individuals with a BMI of 24.5 kg/m² or less had a significantly higher 3HC/COT ratio than did those with a BMI greater than 24.5 kg/m² in plasma, $F = 14.58$, $P < 0.01$. Consistent with previous results, women who use oral contraceptives had significantly higher 3HC/COT ratios in plasma, $F = 14.14$, $P < 0.01$, and urine, $F = 8.93$, $P < 0.05$, than did those women who do not [31].

Comparison of 3HC/COT means in CYP2A6 genotype subgroups

As reported previously [15,27], the 3HC/COT ratio in plasma and urine was associated significantly with genotype status, $F = 15.1$, and, $F = 14.2$, respectively, both $P < 0.01$. The 3HC/COT ratio in both fluids was highest in participants who were either homozygous for *1B or had a *1 duplication (Table 2). Pairwise Scheffe comparisons adjusted for twinning indicate that mean transformed 3HC/COT in plasma for the group with “Decreased” metabolism is significantly lower than the mean ratio in the group with “Normal” and in the group with “Increased” metabolism, $P = 0.0009$ and 0.0011 , respectively. The “Normal” and “Increased” genotype groups did not differ significantly from each other, $P = 0.3689$. A similar pattern of pairwise metabolic group differences was seen for the transformed ratio in urine.

Twin pair correlations

For the 3HC/COT ratio in plasma, the greater MZ relative to DZ twin pair correlations suggested an important role for genetic, relative to shared environmental effects (Table 3). In comparison, the generally smaller difference between MZ and DZ correlations for the 3HC/COT ratio in urine suggested a larger role for shared environmental influences.

Univariate genetic models of the 3HC/COT ratio

Estimates of the relative contribution of genetic and environmental effects for the best fitting univariate genetic models are presented in Table 4. As suggested by the pattern of twin pair correlations and before adjustment for covariates, approximately 67% of the variability in the 3HC/COT ratio in plasma was attributable to additive genetic effects. The estimate of additive genetic variation in the 3HC/COT ratio in urine was more modest (47.2%). None of the models for the ratio measure in urine could distinguish the relative contribution of genetic and shared environmental effects, as either effect could be dropped from the models without significant deterioration of model fit. Dropping both effects together, however, resulted in model fit deterioration, suggesting a significant influence of the combined genetic and shared environmental (i.e., familial) influences. It is notable that the point estimates of the genetic parameter for the 3HC/COT ratio in urine were much higher than those of the shared environmental parameter in unadjusted and adjusted models.

Adjustment for covariates without considering *CYP2A6* genotype led to a modest reduction in the estimates of additive genetic variance for the 3HC/COT ratio in each fluid. In plasma, the estimate was reduced to 61.0% (a 9.5% reduction from 67.4%) while in urine it was reduced to 44.6% (a 5.5% reduction from 47.2%). Inclusion of *CYP2A6* genotype as a covariate led to further reduction in the estimates of additive genetic influence. In plasma, the estimate was reduced to 49.4% (a 19.0% reduction from the non-genotype, covariate-adjusted estimate of

61.0%) while that in urine was reduced to 42.0% (a 5.8% reduction from 44.6%). The lower reduction in the heritability estimate of genotype covariate-adjusted urine ratio could have been in part due to a relatively higher heritability point estimate because of lower estimated shared environmental effects (0%) compared to small shared environmental effects in the unadjusted (8.3%) and non-genotype covariate-adjusted models (7.3%). Still, these results suggest that, to the degree that variation in these proxy measures of nicotine metabolic activity is attributable to variation in *CYP2A6* allele status, the effect is relatively larger on the 3HC/COT ratio in plasma than on the 3HC/COT ratio in urine. In both fluids, however, a substantial amount of the heritable component of the 3HC/COT ratio is not accounted for by *CYP2A6* genotype status derived from the variants assessed.

Bivariate genetic analysis of 3HC/COT in urine and plasma

Bivariate genetic models helped explain some of the causal sources of the high phenotypic correlation between the 3HC/COT ratio in plasma and urine. As in univariate analysis, heritability for the 3HC/COT ratio in plasma was relatively higher than that for the 3HC/COT ratio in urine in unadjusted and adjusted models (Table 5). In all cases the genetic correlation could be equated to one suggesting that covariation in the 3HC/COT ratio in plasma and urine is entirely explained by common genetic factors. In all models, non-shared environmental correlations were significant (r_e range = 0.38–0.49) suggesting that some of the same individual-specific environmental factors affect the 3HC/COT measure in both fluids. The relative contribution of genetic and shared environmental effects for each ratio measure could not be distinguished in the model adjusted for covariates and *CYP2A6* genotype, however the point estimates suggested a relatively minor role of shared environmental effects. Accordingly, the shared environmental correlation could not be reliably estimated. A pattern similar to that seen in the univariate series of analyses of the plasma 3HC/COT ratio was observed for the relative reduction in the estimate of additive genetic variance in the presence of adjustment for covariates and genotype. In contrast, a more substantial impact of adjustment for genotype on estimated additive genetic variance was observed for the urine 3HC/COT ratio. Compared to the non-genotype adjusted estimate (48.3%), heritability was reduced to 32.8%. However, reduction in the heritability estimates was in part due to the presence of a small shared environmental effect (8.4%).

DISCUSSION

In our previous analysis we reported that the amount of additive genetic variation in the clearance of nicotine via the COT pathway ranged from 51.8 to 60.8% depending on the nature of the adjustment for covariates and genotype [16]. The univariate results reported here for the 3HC/COT ratio in plasma are consistent and ranged from 49.4 to 67.4%. In urine, the estimated heritability was generally lower and ranged from 42.0% to 47.2% with evidence for a small contribution from shared environmental factors (range 0–8.3%). The relative decrease in the estimated portion of additive genetic variance in the two phenotypes after adjustment for *CYP2A6* genotype was 19.0% for the 3HC/COT ratio in plasma (similar to the relative reduction in the estimated genetic portion of variance in measured clearance of nicotine via the COT pathway (15.9%; [16]) and 5.8% for the 3HC/COT ratio in urine. The relatively lower reduction of heritability after genotype adjustment for the 3HC/COT ratio in urine could be in part due to the small influence of shared environmental factors on the urine ratio. Heritability estimates are larger in the absence of evidence for significant contribution of shared environmental effects.

In the present analysis, the 3HC/COT ratio in plasma and in urine were highly correlated with one another ($r = 0.70$). The bivariate genetic analyses indicated that the same genetic factors that influence 3HC/COT variance in plasma also influence 3HC/COT variance in urine. In

other words, there was no evidence for genetic factors specific to the 3HC/COT ratio in urine. On the other hand, while there was overlap in the influence of individual-specific environmental factors, there was evidence for non-shared environmental effects specific to the ratio in urine. In addition to CYP2A6 activity, the ratio in urine is influenced by the relative renal clearance of COT and 3HC, which is in turn influenced by urine flow rate and urine pH. Urine flow rate and pH are affected by nonshared environmental factors, such as fluid intake and diet [1]. Our results indicate that the phenotypic correlation marks common genetic mechanisms but the size of the phenotypic correlation is lower than might be expected due to environmental factors influencing plasma and urine levels differently. Thus, while the 3HC/COT ratio in plasma and urine are both presumed to mark CYP2A6 enzyme activity, these measures are in part etiologically distinct. Univariate and bivariate analyses were consistent in showing a relatively larger heritability for the 3HC/COT ratio in plasma relative to the 3HC/COT ratio in urine, likely because of a relatively greater importance of shared and nonshared environmental effects on the 3HC/COT ratio in urine. We further note that total variation in the urine ratio (31-fold) is greater than in the plasma ratio (20-fold). Because the ratio in urine is more variable and subject to more influence from environmental factors, we conclude that the ratio in plasma is the more reliable of the two.

Consistent with a previous analysis of this data set as well as other published research [1,14, 15,26], women had a significantly higher 3HC/COT ratio in both plasma and urine (i.e., faster nicotine metabolism) than did men and, in women, those who used oral contraceptives had a higher mean level 3HC/COT ratio than non-users, indicating a higher level of CYP2A6 activity.

Age and alcohol consumption were not related to the 3HC/COT ratio in either fluid. The lack of association with age and alcohol consumption is consistent with findings from previous research [26] and is most likely to be due to the nature of the exclusions for participation in the study (e.g., very young and older individuals as well as heavy drinkers were excluded from participation). The observation that smoking status and race/ethnicity were not associated with 3HC/COT in this study differs from other studies that show both characteristics have an effect on CYP2A6 activity [26,32,33]. Because of the relatively small numbers of non-Caucasians and smokers in the present sample, the reader should view this observation with caution. That the 3HC/COT ratio in both plasma and urine appear to be influenced by environmental sources other than cigarette smoking might also provide an explanation of why most studies of the relationship between the 3HC/COT ratio in both fluids and number of cigarettes smoked report only very modest associations [8,9].

Even after adjustment for sex, higher levels of BMI were associated with a lower 3HC/COT ratio in plasma and in urine. While we are unaware of any previous published research on this relationship, a subsequent analysis of plasma data confirmed this association in another sample of 647 individuals. The association remained significant and negative in both men and women (unpublished data; Tyndale, personal communication, December 2007).

Evidence to date suggests that the metabolism of COT to 3HC is mediated entirely or nearly entirely by CYP2A6 [3]. Our observation that the amount of genetic variance in the plasma and urine 3HC/COT ratios remains significant and substantial after accounting for the effects of CYP2A6, leads us to the same conclusion as we did for clearance of nicotine via the COT pathway [16]. Either more variation in CYP2A6 genotype exists than what we have accounted for here or additional genes (so far unaccounted for in this analysis) are involved in regulation of the COT metabolic pathway. Likely candidates include CYP2B6 [34], CYP2E1 [35], several of the UDP glycosyltransferase (UGT) genes [33], nuclear receptors involved in the transcriptional regulation of CYP2A6 such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [36], cofactors that influence P450-catalyzed oxidation such as

cytochrome P450 oxidoreductase (*POR*) [37], and transporters involved in renal clearance such as organic cation transporter (*OCT2*) [38].

So far as we know, the amount of variance attributable to genetic sources in the 3HC/COT ratio as measured in saliva is unknown. Moreover, even though the amount of genetic variance in the clearance of nicotine via the COT pathway in plasma is known [16], current estimates of the 3HC/COT ratio were derived from a protocol designed to measure nicotine metabolism after infusion of known doses of nicotine and COT, with plasma and urine sampling at consistent points in time. In contrast, the amount of total genetic variance in spot measures of the 3HC/COT ratio (plasma, saliva, and urine), as would be collected in smokers in population studies, is unknown. Caution is recommended in assuming that spot measures of the 3HC/COT ratio are equally determined by the effects of genetically-mediated mechanisms.

Potential limitations of the study

Generalizability of the study sample—The characteristics of the NCTR and participant samples were similar with regard to sex and zygosity. However, most likely as a result of the method of recruitment for the NCTR, both samples had an over-representation of females and MZ twins. This situation is similar to other twin studies which have relied on recruitment through the media, and largely reflects a bias in the nature of volunteers for research [39].

The overall rate of nonwhite participation in this study is below the 2000 US Census estimate [40] for nonwhites residing in the San Francisco Bay Area (41.3%) and increasing participation among nonwhites in research studies, in general, remains a serious issue [41,42]. The rate of current smoking in study participants (19.8%) is lower than the national median prevalence (23.1%, [43]) and most likely reflects the effects of the California tobacco control campaign, one of the most aggressive smoking reduction programs in the US [44].

Limited number of DZ twin pairs—The number of DZ twin pairs recruited to this study was lower than anticipated. Nonetheless, given our sample size, and assuming no significant contribution from shared environmental sources, we had over 80% power to detect genetic effects of 30% or greater in univariate analysis of the 3HC/COT phenotype. The small number of DZ pairs may also have limited our ability to distinguish genetic from environmental influences in the models involving 3HC/COT in urine and the bivariate models involving both ratios.

Lack of racial/ethnic variation—The results from this investigation are generalizable to individuals of European descent. While there were too few non-Caucasian individuals to support meaningful analyses in the present study, there are data indicating that African Americans have a lower plasma 3HC/COT ratio than do Caucasians [26,32]. The extent to which these differences alter estimates of heritability is not known.

Limited number of CYP2A6 alleles—As of the date of this paper, as many as 31 variants of *CYP2A6* have been determined (www.cypalleles.ki.se). Many of these occur with very low frequency in the population and were not observed in the present, mostly Caucasian sample. Some are of unknown functional consequence. For this paper, we have assessed the main alleles found in Caucasians which are known to alter activity. Additional alleles will be found with resequencing and other techniques. As new alleles are identified, we will incorporate them into future analyses of this data. The approach to the present analysis was to categorize measured variants into three groups of increased, normal, or decreased activity. To the extent that additional alleles are discovered with functional consequences that are different from the functional categories to which they were assigned (i.e., misclassified) in the present analysis, the impact of the misclassification on heritability estimates after adjustment for genotype would

be underestimated. If there is no functional misclassification of new genotypes that become known, no net effect on heritability estimates would be expected. However, it is likely that a larger proportion of the genetic variance of the metabolite ratio would be explained as more functional *CYP2A6* genotype variants are discovered.

Acknowledgments

The authors wish to thank the following people for their work on this study (listed alphabetically): Faith Allen, MD (data manager), Dorit Carmelli, PhD (consultant), Delia Dempsey, MD (project physician), Brenda Herrera (clinical research associate), Ewa Hoffman, MSc (molecular biologist), Lori Karan, MD (project physician), Ruth E. Krasnow, MA (data analyst), Mary R. McElroy, MPH (project coordinator), Gunnard Modin (pharmacokinetic analysis), Sharyn E. Moore, MA, MS (recruiter, field assessment), Ovide Pomerleau, PhD (consultant), Jill Rubin (recruiter), Kerri Schoedel, Jill Mwenifumbo, and Bo Xu (molecular biologists), Michelle Wambach, MA (recruiter, field assessment), Kirk Wilhelmsen, MD, PhD, (genotyping for zygosity), (Sylvia Wu (chemist), and Lisa Yu (chemist).

We are deeply grateful to the twins for their participation, without whom this work would not have been possible.

Research supported by National Institute on Drug Abuse grants DA11170 (GES), DA02277 (NLB), DA12393 (NLB), and DA20830 (NLB, RFT, GES, AWB, and CNL). Carried out in part at the General Clinical Research Center at San Francisco General Hospital Medical Center with support of the NIH Division of Research Resources, grant RR00083, and at the Center for Addiction and Mental Health with support of grant CIHR MOP 86471 (RFT) and a CRC to RFT.

REFERENCES

1. Hukkanen J, Jacob P 3rd, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* 2005;57:79–115. [PubMed: 15734728]
2. Xu C, Goodz S, Sellers EM, Tyndale RF. CYP2A6 genetic variation and potential consequences. *Adv Drug Del Rev* 2002;54:1245–1256.
3. Dempsey D, Tutka P, Jacob P 3rd, Allen F, Schoedel K, Tyndale RF, Benowitz NL. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. *Clin Pharmacol Ther* 2004;76:64–72. [PubMed: 15229465]
4. Malaiyandi V, Goodz SD, Sellers EM, Tyndale RF. CYP2A6 genotype, phenotype, and the use of nicotine metabolites as biomarkers during ad libitum smoking. *Cancer Epidemiol Biomarkers Prev* 2006;15:1812–1819. [PubMed: 17035386]
5. Lea RA, Dickson S, Benowitz NL. Within-subject variation of the salivary 3HC/COT ratio in regular daily smokers: prospects for estimating CYP2A6 enzyme activity in large-scale surveys of nicotine metabolic rate. *J Anal Toxicol* 2006;30:386–389. [PubMed: 16872570]
6. Levi M, Dempsey DA, Benowitz NL, Sheiner LB. Prediction methods for nicotine clearance using cotinine and 3-hydroxy-cotinine spot saliva samples II. Model application. *J Pharmacokinet Pharmacodyn* 2007;34:23–34. [PubMed: 17206525]
7. Levi M, Dempsey DA, Benowitz NL, Sheiner LB. Population pharmacokinetics of nicotine and its metabolites I. Model development. *J Pharmacokinet Pharmacodyn* 2007;34:5–21. [PubMed: 17206524]
8. Benowitz NL, Pomerleau OF, Pomerleau CS, Jacob P 3rd. Nicotine metabolite ratio as a predictor of cigarette consumption. *Nicotine Tobacco Res* 2003;5:621–624.
9. Kandel DB, Hu MC, Schaffran C, Udry JR, Benowitz NL. Urine nicotine metabolites and smoking behavior in a multiracial/multiethnic national sample of young adults. *Am J Epidemiol* 2007;165:901–910. [PubMed: 17322544]
10. Heatherton TF, Kozlowski LT, Frecker RC, Fagerström KO. The Fagerström Test for Nicotine Dependence: a revision of the Fagerström Tolerance Questionnaire. *Br J Addict* 1991;86:1119–1127. [PubMed: 1932883]
11. Lerman C, Tyndale R, Patterson F, Wileyto EP, Shields PG, Pinto A, Benowitz N. Nicotine metabolite ratio predicts efficacy of transdermal nicotine for smoking cessation. *Clin Pharmacol Ther* 2006;79:600–608. [PubMed: 16765148]

12. Malaiyandi V, Lerman C, Benowitz NL, Jepsen 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;11:400–409. [PubMed: 16402128]
13. Johnstone E, Benowitz N, Cargill A, Jacob R, Hinks L, Day I, Murphy M, Walton R. Determinants of the rate of nicotine metabolism and effects on smoking behavior. *Clin Pharmacol Ther* 2006;80:319–330. [PubMed: 17015050]
14. Berlin I, Gasior MJ, Moolchan ET. Sex-based and hormonal contraception effects on the metabolism of nicotine among adolescent tobacco-dependent smokers. *Nicotine Tobacco Res* 2007;9:493–498.
15. Benowitz NL, Swan GE, Jacob P 3rd, Lessov-Schlaggar CN, Tyndale RF. CYP2A6 genotype and the metabolism and disposition kinetics of nicotine. *Clin Pharmacol Ther* 2006;80:457–467. [PubMed: 17112802]
16. Swan GE, Benowitz NL, Lessov CN, Jacob P 3rd, Tyndale RF, Wilhelmsen K. Nicotine metabolism: the impact of CYP2A6 on estimates of additive genetic influence. *Pharmacogenet Genomics* 2005;15:115–125. [PubMed: 15861035]
17. Swan GE, Benowitz NL, Jacob P 3rd, Lessov CN, Tyndale RF, Wilhelmsen K, Krasnow RE, McElroy MR, Moore SE, Wambach M. Pharmacogenetics of nicotine metabolism in twins: methods and procedures. *Twin Res* 2004;7:435–448. [PubMed: 15527659]
18. Cederlof R, Friberg L, Jonsson E, Kaij L. Studies on similarity diagnosis in twins with the aid of mailed questionnaires. *Acta Genet Stat Med* 1961;11:338–362. [PubMed: 13877601]
19. Pomerleau CS, Garcia AW, Pomerleau OF, Cameron OG. The effects of menstrual phase and nicotine abstinence on nicotine intake and on biochemical and subjective measures in women smokers: a preliminary report. *Psychoneuroendocrinology* 1992;17:627–638. [PubMed: 1287682]
20. Jacob P 3rd, Benowitz NL, Shulgin A. Synthesis of optically pure deuteriumlabeled nicotine, nornicotine and cotinine. *J Labelled Compd Radiopharmaceut* 1988;25:1117–1128.
21. Jacob P 3rd, Yu L, Benowitz NL. Determination of nicotine, its major metabolites, and deuterium-labeled isotopomers in human urine using LC-MS/MS. *Drug Metab Rev* 2002;34:160.
22. Weber JL, May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 1989;44:388–396. [PubMed: 2916582]
23. Goodz SD, Tyndale RF. Genotyping human CYP2A6 variants. *Methods Enzymol* 2002;357:59–69. [PubMed: 12424898]
24. Rao Y, Hoffmann E, Zia M, Bodin L, Zeman M, Sellers EM, Tyndale RF. Duplications and defects in the CYP2A6 gene: identification, genotyping, and in vivo effects on smoking. *Mol Pharmacol* 2000;58:747–755. [PubMed: 10999944]
25. Schoedel KA, Hoffmann EB, Rao Y, Sellers EM, Tyndale RF. Ethnic variation in CYP2A6 and association of genetically slow nicotine metabolism and smoking in adult Caucasians. *Pharmacogenetics* 2004;14:615–626. [PubMed: 15475735]
26. Mwenifumbo JC, Sellers EM, Tyndale RF. Nicotine metabolism and CYP2A6 activity in a population of black African descent: impact of gender and light smoking. *Drug Alcohol Depend* 2007;89:24–33. [PubMed: 17161559]
27. Mwenifumbo JC, Lessov-Schlaggar CN, Zhou Q, Krasnow RE, Swan GE, Benowitz NL, Tyndale RF. Identification of novel CYP2A6*1B variants: the CYP2A6*1B allele is associated with faster in vivo nicotine metabolism. *Clin Pharmacol Ther* 2008;83:115–121. [PubMed: 17522595]
28. SAS Institute. SAS for Windows, version 9.1. Cary, NC: SAS Institute, Inc; copyright 2002–2003
29. Neale, MC.; Cardon, L. Methodology for genetic studies of twins and families. Dordrecht/Boston/London: Kluwer Academic Publishers; 1992.
30. Neale, MC.; Boker, SM.; Xie, G.; Maes, HH. Mx: Statistical Modeling. 6th ed.. Richmond, VA: Department of Psychiatry, Virginia Commonwealth University; 2002.
31. Benowitz NL, Lessov-Schlaggar CN, Swan GE, Jacob P 3rd. Female sex and oral contraceptive use accelerate nicotine metabolism. *Clin Pharmacol Ther* 2006;79:480–488. [PubMed: 16678549]
32. Moolchan ET, Franken FH, Jaszyna-Gasior M. Adolescent nicotine metabolism: ethnoracial differences among dependent smokers. *Ethn Dis* 2006;16:239–243. [PubMed: 16599377]
33. Nakajima M, Yokoi T. Interindividual variability in nicotine metabolism: C-oxidation and glucuronidation. *Drug Metab Pharmacokinet* 2005;20:227–235. [PubMed: 16141602]

34. Yamanaka H, Nakajima M, Fukami T, Sakai H, Nakamura A, Katoh M, Takamiya M, Aoki Y, Yokoi T. CYP2A6 AND CYP2B6 are involved in normicotine formation from nicotine in humans: interindividual differences in these contributions. *Drug Metab Disposition* 2005;33:1811–1818.
35. Howard LA, Ahluwalia JS, Lin SK, Sellers EM, Tyndale RF. CYP2E1*1D regulatory polymorphism: association with alcohol and nicotine dependence. *Pharmacogenetics* 2003;13:321–328. [PubMed: 12777962]
36. Itoh M, Nakajima M, Higashi E, Yoshida R, Nagata K, Yamazoe Y, Yokoi T. Induction of human CYP2A6 is mediated by the pregnane X receptor with peroxisome proliferator-activated receptor-gamma coactivator 1alpha. *J Pharmacol Exp Ther* 2006;319:693–702. [PubMed: 16857725]
37. Hart SN, Wang S, Nakamoto K, Wesselman C, Li Y, Zhong XB. Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsomal P450-catalyzed drug metabolism. *Pharmacogenet Genomics* 2008;18:11–24. [PubMed: 18216718]
38. Zevin S, Schaner ME, Giacomini KM. Nicotine transport in a human choriocarcinoma cell line (JAR). *J Pharm Sci* 1998;87:702–706. [PubMed: 9607946]
39. Lykken DT, McGue M, Tellegen A. Recruitment bias in twin research: the rule of two-thirds reconsidered. *Behav Genet* 1987;17:343–362. [PubMed: 3675526]
40. 2000 Census Data. 2000 [cited 2004 March]. Available from: <http://factfinder.census.gov>
41. Britton A, McKee M, Black N, McPherson K, Sanderson C, Bain C. Threats to applicability of randomised trials: exclusions and selective participation. *J Health Serv Res Policy* 1999;4:112–121. [PubMed: 10387403]
42. Seto B. History of medical ethics and perspectives on disparities in minority recruitment and involvement in health research. *Am J Med Sci* 2001;322:248–252.
43. State-specific prevalence of current cigarette smoking among adults--United States, 2002. *MMWR Morb Mortal Wkly Rep* 2004;52:1277–1280. [PubMed: 14712175]
44. Pierce, JP.; Gilpin, EA.; Emery, SL.; Farkas, AJ.; Zhu, SH.; Choi, WS. Tobacco Control in California: Who's Winning the War? An Evaluation of the Tobacco Control Program 1989–1996. San Diego: University of California; 1999.

A. 3HC/COT in plasma. **B. 3HC/COT in urine.**

Figure 1. Frequency histogram of the 3HC/COT ratio in plasma (A) and urine (B) prior to transformation in 266 individuals.

Table 1

Demographic and smoking status characteristics overall and by zygosity.

Characteristic	ALL (n=276)	MZ (n=220)	DZ (n=56)
Age ($\pm$ SD) y	37.8 $\pm$ 11.9	37.8 $\pm$ 12.0	38.0 $\pm$ 11.8
BMI ($\pm$ SD) kg/m ²	25.0 $\pm$ 4.0	24.9 $\pm$ 4.0	25.4 $\pm$ 3.9
Women (%)	69.6	67.9	75.9
Zygosity (% MZ)	79.0		
Oral contraceptive use in women (%)	27.1	28.4	22.7
Race/Ethnicity (%)			
Caucasian	76.1	76.2	75.9
African American	3.6	3.7	3.5
Hispanic	12.3	11.9	13.8
Asian	5.1	6.4	0
Other	2.9	1.8	6.9
Education (%)			
Bachelor's degree or more	51.7	53.1	46.4
Marital status (%)			
Single/never married	34.1	33.8	35.1
Married/living together	53.5	53.2	54.4
Divorced/widowed/separated	12.4	13.0	10.5
Smoking status (%)			
Non-smoker	55.8	58.3	46.6
Former smoker	24.3	24.3	24.1
Current smoker	19.9	17.4	29.3
3HC/COT ($\pm$ SD)			
Plasma	0.25 $\pm$ 0.1	0.25 $\pm$ 0.1	0.27 $\pm$ 0.1
Urine (molar ratio)	1.38 $\pm$ 0.8	1.37 $\pm$ 0.7	1.41 $\pm$ 0.9

Table 2
Means and standard errors for 3HC/COT ratio in plasma and urine (untransformed) as a function of covariate status.

Covariate	3HC/COT (n=268)					
	Plasma			Urine		
	M	SE	P-value	M	SE	P-value
Sex						
Males	0.20	0.02		1.10	0.09	
Females	0.27	0.01	0.0286	1.46	0.06	0.0224
Age						
≤ 37 years of age	0.23	0.01		1.29	0.08	
> 37 years of age	0.26	0.01	0.1138	1.39	0.07	0.3617
Ethnicity						
Caucasian/Hispanic	0.25	0.01		1.35	0.06	
Others	0.20	0.03	0.0780	1.28	0.15	0.6656
BMI ^a						
≤ 24.5 kg/m ²	0.28	0.01		1.44	0.07	
> 24.5 kg/m ²	0.22	0.01	0.0009	1.26	0.07	0.0693
Smoking status						
Current	0.23	0.02		1.29	0.10	
Not current	0.25	0.01	0.1849	1.35	0.06	0.5848
Alcohol						
≤ 3 drinks/wk	0.25	0.01		1.35	0.07	
> 3 drinks/wk	0.25	0.01	0.9556	1.30	0.08	0.5914
Oral contraceptive use						
Yes	0.32	0.02		1.74	0.12	
No	0.25	0.01	0.0027	1.37	0.08	0.0111
CYP2A6 genotype						
Normal (*1A/*1A, *1A/*1B)	0.26	0.01		1.44	0.06	
Decreased (*2, *4, *9, *12)	0.16	0.02		0.81	0.11	

Covariate	3HC/COT (n=268)					
	Plasma			Urine		
	M	SE	P-value	M	SE	P-value
Increased (*IB/*IB, *I×2)	0.29	0.02	0.0013 ^b	1.56	0.11	0.0012 ^b

Means adjusted for effects of twinning. Statistical tests based on transformed values.

^a Adjusted for sex;

^b P value is presented for overall significance.

Table 3

Pearson correlations for the 3HC/COT ratio in plasma and urine in MZ and DZ twin pairs.

3HC/COT	Zygosity			
	MZ twins <i>n</i> _{pairs} = 97–104	<i>P</i> value	DZ twins <i>n</i> _{pairs} = 26–29	<i>P</i> value
Plasma				
Unadjusted	0.68	<.001	0.30	.143
Adjusted for covariates ¹	0.61	<.001	0.24	.240
Adjusted as above and for <i>CYP2A6</i> genotype ²	0.47	<.001	0.20	.327
Urine				
Unadjusted	0.59	<.001	0.36	.059
Adjusted for covariates ¹	0.52	<.001	0.36	.052
Adjusted as above and for <i>CYP2A6</i> genotype ²	0.36	<.001	0.27	.162

¹ Adjusted for age, sex, BMI, ethnicity (Caucasians and Hispanics vs. all others), current smoking status (current smoker vs. all others), and oral contraceptive use in women.

² *CYP2A6* genotype was dummy coded (normal vs. all others and slow metabolizers vs. all others).

Table 4

Best fitting univariate estimates for the effects of additive genetic (A), shared environmental (C), and non-shared environmental (E) influences on variability in the 3HC/COT ratio in plasma and urine before adjustment for covariates, after adjustment for covariates, and after adjustment for covariates and *CYP2A6* genotype.

3HC/COT	Source of variation		
	A (95% CI)	C (95% CI)	E (95% CI)
<i>Total sample (n = 260–262)</i>			
Plasma ¹	67.4 (55.9, 76.2)	ns	32.6 (23.8, 44.1)
Urine ^{1,*}	47.2 (0, 67.2)	8.3 (0, 56.3)	44.5 (32.8, 59.4)
<i>Adjusted for covariates (n = 260–262)</i>			
Plasma ²	61.0 (47.5, 71.4)	ns	39.0 (28.6, 52.5)
Urine ^{2,*}	44.6 (0, 64.6)	7.3 (0, 52.5)	48.2 (35.4, 64.7)
<i>Adjusted as above and for CYP2A6 genotype status (n = 260–262)</i>			
Plasma ³	49.4 (33.2, 63.4)	ns	50.1 (36.6, 66.8)
Urine ^{3,*}	42.0 (0, 57.9)	0 (0, 40.9)	58.0 (42.1, 78.8)

¹ Unadjusted for covariates.

² Adjusted for age, sex, BMI, ethnicity (Caucasians and Hispanics vs. all others), current smoking status (current smoker vs. all others), and oral contraceptive use in women.

³ *CYP2A6* genotype was dummy coded (normal vs. all others and slow metabolizers vs. all others).

* Either A or C but not both together could be equated to zero without significant deterioration of model fit.

Table 5

Best fitting bivariate estimates for the effects of additive genetic (A), shared environmental (C), and non-shared environmental (E) influences on variability in the 3HC/COT ratio in plasma and urine before adjustment for covariates, after adjustment for covariates, and after adjustment for covariates and *CYP2A6* genotype.

3HC/COT	Source of variation		
	A (95% CI)	C (95% CI)	E (95% CI)
<i>Total sample (n = 260–262)</i>			
Plasma ¹	66.9 (54.9, 76.0)	ns	33.1 (24.0, 45.1)
Urine ^{1,*}	50.8 (36.1, 63.4)	ns	49.2 (36.6, 63.9)
r_a		1.0	
r_c		0.38 (0.22, 0.52)	
<i>Adjusted for covariates (n = 260–262)</i>			
Plasma ²	60.8 (47.0, 71.5)	ns	39.2 (28.6, 53.0)
Urine ²	48.3 (32.4, 61.9)	ns	51.7 (38.7, 67.7)
r_a		1.0	
r_c		0.39 (0.24, 0.53)	
<i>Adjusted as above and for CYP2A6 genotype status (n = 260–262)</i>			
Plasma ^{3,*}	51.1 (11.3, 64.3)	0 (0, 0)	48.9 (35.7, 65.4)
Urine ^{3,*}	32.8 (2.2, 56.5)	8.4 (0, 35.9)	58.8 (43.3, 77.2)
r_a		1.0	
r_c^{**}		0 (0, 1.0)	
r_e		0.49 (0.33, 0.63)	

r_a : genetic correlation; r_c : shared environmental correlation; r_e : non-shared environmental correlation.

¹ Unadjusted for covariates.

² Adjusted for age, sex, BMI, ethnicity (Caucasians and Hispanics vs. all others), current smoking status (current smoker vs. all others), and oral contraceptive use in women.

³ *CYP2A6* genotype was dummy coded (normal vs. all others and slow metabolizers vs. all others).

* Either A or C but not both together could be equated to zero without significant deterioration of model fit.

** Correlation could be equated to either 0 or 1 without significant deterioration of model fit.