

Research Article

The Ability of Plasma Cotinine to Predict Nicotine and Carcinogen Exposure is Altered by Differences in CYP2A6: the Influence of Genetics, Race, and Sex

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Abstract

Background: Cotinine, a nicotine metabolite, is a biomarker of tobacco, nicotine, and carcinogen exposure. However, a given cotinine level may not represent the same tobacco exposure; for example, African-Americans have higher cotinine levels than Caucasians after controlling for exposure.

Methods: Cotinine levels are determined by the amount of cotinine formation and the rate of cotinine removal, which are both mediated by the enzyme CYP2A6. Because CYP2A6 activity differs by sex (estrogen induces CYP2A6) and genotype, their effect on cotinine formation and removal was measured in nonsmoking Caucasians (Study 1, $n = 181$) infused with labeled nicotine and cotinine. The findings were then extended to *ad libitum* smokers (Study 2, $n = 163$).

Results: Study 1: Reduced CYP2A6 activity altered cotinine formation less than cotinine removal resulting in ratios of formation to removal of 1.31 and 1.12 in CYP2A6 reduced and normal metabolizers ($P = 0.01$), or 1.39 and 1.12 in males and females ($P = 0.001$), suggesting an overestimation of tobacco exposure in slower metabolizers. Study 2: Cotinine again overestimated tobacco and carcinogen exposure by 25% or more in CYP2A6 reduced metabolizers (≈ 2 -fold between some genotypes) and in males.

Conclusions: In people with slower relative to faster CYP2A6 activity, cotinine accumulates resulting in substantial differences in cotinine levels for a given tobacco exposure.

Impact: Cotinine levels may be misleading when comparing those with differing CYP2A6 genotypes within a race, between races with differing frequencies of CYP2A6 gene variants (i.e., African-Americans have higher frequencies of reduced function variants contributing to their higher cotinine levels), or between the sexes. *Cancer Epidemiol Biomarkers Prev*; 22(4); 708–18. ©2013 AACR.

Introduction

In humans, cotinine is metabolically formed from nicotine in a reaction catalyzed by CYP2A6 and is further metabolized to trans-3'-hydroxycotinine (3HC) by CYP2A6 (1, 2). Cotinine is routinely used as an objective index of tobacco and tobacco-derived carcinogen exposure. The use of cotinine is particularly useful because of

the wide individual variability in the relationship between self-reported cigarettes smoked per day and systemic exposure to tobacco and tobacco-derived carcinogen (3). Higher plasma cotinine levels have been associated with increased lung cancer risk (4), and variation in cotinine levels have recently been used as evidence that the mechanism mediating the association between genetic variation in *CHRNA5-A3-B4* and lung cancer risk is almost entirely through the modulation of tobacco consumption (5).

The systemic intake of nicotine is correlated with exposure to tobacco-derived carcinogen (3). When used as a biomarker of tobacco-derived carcinogen exposure, it is generally assumed that plasma cotinine levels reflect intake of nicotine, and that variability in the relationship between cotinine levels and nicotine intake is random among smokers. However, some research suggests that a particular level of cotinine does not predict the same level of tobacco exposure among different groups of smokers. For example, directional inconsistencies have been observed between plasma cotinine levels and other indicators of tobacco exposure in genetic association studies involving CYP2A6. Caucasian smokers with one or more reduced function CYP2A6 alleles (i.e., CYP2A6 reduced

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doi: 10.1158/1055-9965.EPI-12-1234-T

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metabolizers) smoke less cigarettes per day compared with the *CYP2A6* normal metabolizers, consistent with titration of smoke intake to obtain desired levels of nicotine in the body (6, 7). Yet despite smoking less cigarettes per day, smokers with reduced *CYP2A6* activity have similar plasma cotinine levels compared with the smokers with faster *CYP2A6* activity (8). Another example is found in studies of African-American light smokers, in which similar levels of cigarettes per day are reported between *CYP2A6* genotypes (i.e., *CYP2A6* reduced metabolizers have similar cigarettes per day compared with *CYP2A6* normal metabolizers), but the plasma cotinine levels are significantly higher in *CYP2A6* reduced metabolizers compared with *CYP2A6* normal metabolizers (9). Furthermore, gene variants at the *CYP2A6* loci (i.e., tag single-nucleotide polymorphisms; SNP) that are associated with decreased lung cancer risk are paradoxically associated with increased, rather than decreased, plasma cotinine levels (10). These observations suggest that *CYP2A6* reduced activity genotype may increase plasma cotinine levels in addition to its role in reducing nicotine inactivation, tobacco consumption, and nitrosamine metabolic activation (11).

Variation in the plasma cotinine levels is also observed between the sexes. The male participants of the National Health and Nutrition Examination Surveys have significantly higher plasma cotinine levels compared with the female participants even after adjusting for cigarettes per day, machine-determined nicotine delivery of cigarettes, race, age, body mass index, poverty status, and the use of either menthol or regular cigarettes (12). Because *CYP2A6* activity differs between the sexes, this systematic variation in cotinine levels between the sexes could be the result of the difference in *CYP2A6* activity (13).

Notable variation in cotinine levels is also observed between races. African-American smokers generally have higher plasma cotinine levels compared with Caucasian smokers after adjusting for the number of cigarettes smoked per day and the machine-determined nicotine delivery in cigarettes (14), which could be partially due to the slower cotinine clearance in African-Americans compared with Caucasians (15). Because the prevalence of *CYP2A6* reduced metabolizers is high in African-Americans compared with Caucasians, it is possible that the racial variation in cotinine levels originates from the difference in *CYP2A6* activities between racial groups.

Steady-state plasma cotinine levels are determined by 3 factors: the daily intake of nicotine, the fraction of nicotine converted into cotinine ($f_{\text{NIC} \rightarrow \text{COT}}$), and the systemic clearance of cotinine. The steady-state plasma cotinine level is described by the following equation:

$$[\text{COT}]_{\text{plasma}} = \text{Dose}_{\text{NIC}} \times \frac{f_{\text{NIC} \rightarrow \text{COT}}}{\text{Cl}_{\text{COT}}} \quad (1)$$

where $\text{COT}_{\text{plasma}}$ represents the steady-state plasma cotinine levels, dose_{nic} is the daily nicotine intake, $f_{\text{NIC} \rightarrow \text{COT}}$ is the fraction of nicotine converted into cotinine

(i.e., cotinine formation), and Cl_{COT} represents systemic plasma cotinine clearance (i.e., cotinine removal; ref. 16). Hence, the steady-state plasma cotinine levels are proportional to the ratio of $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ and the dose_{nic} . Cotinine is both formed and removed by *CYP2A6*. Thus, both $f_{\text{NIC} \rightarrow \text{COT}}$ and cotinine clearance are highly dependent on *CYP2A6* activity. We hypothesize that reduced *CYP2A6* activity will increase the ratio of $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ and alter the quantitative relationship between plasma cotinine and nicotine and tobacco-derived carcinogen exposure in cigarette smokers.

In this study, we use *CYP2A6* genotype as the primary indicator of *CYP2A6* activity. *CYP2A6* genetic variants are known to alter *in vivo* *CYP2A6* activity as well as nicotine and cotinine clearance (17–23). In addition to *CYP2A6* genotype, we also use the nicotine metabolite ratio (NMR) as a secondary indicator of *CYP2A6* activity (24). For the evaluation of nicotine intake in *ad libitum* smokers, we use urinary total nicotine equivalents (TNE; ref. 25). For the evaluation of tobacco-derived carcinogen in *ad libitum* smokers, we used total urinary 4-(methylnitrosamino)-1-(3) pyridyl-1-butanol (NNAL), 1-hydroxyfluorene, and 1-hydroxypyrene levels (26, 27).

Materials and Methods

Study design

Data for the present analysis were taken from 2 studies that have been previously published (21, 28).

Study 1. The pharmacokinetics of nicotine and cotinine were characterized in 181 nonsmoking Caucasian male and female sets of twins (both monozygotic and dizygotic). Demographic variable comparisons are shown in Supplementary Table S1. A comprehensive description of the study procedures has been published previously (29, 30). Briefly, participants received a simultaneous 30-minute infusion of deuterium-labeled nicotine and cotinine in the fasting condition. Blood samples were collected for the measurement of labeled nicotine and cotinine for determination of pharmacokinetic parameters, and DNA was extracted for *CYP2A6* genotyping. The cotinine and 3HC levels were measured in the 480-minute plasma sample for determination of the NMR (21). The pharmacokinetic calculations can be found in the Supplementary Materials and Methods.

Study 2. The relationship between plasma cotinine, nicotine intake, and carcinogen exposure was studied in daily *ad libitum* smokers (Supplementary Table S1). In a cross-sectional study of 163 Alaska Native smokers, the plasma cotinine levels, urinary TNE, total NNAL, and polycyclic aromatic hydrocarbon (PAH) metabolites were measured. A comprehensive description of the study procedures has been published elsewhere (28).

Tobacco and tobacco-derived carcinogen exposure biomarkers

To evaluate the quantitative relationship between plasma cotinine and tobacco exposure in cigarette smokers,

we used urinary TNE from Study 2 participants as the reference biomarker of nicotine and tobacco exposure as it is the summation of multiple nicotine metabolic pathways. TNE is the total urinary level of nicotine and 8 of its metabolites (i.e., nicotine, nicotine glucuronide, cotinine, cotinine glucuronide, 3HC, 3HC glucuronide, nicotine-*N*-oxide, cotinine-*N*-oxide, and nor nicotine). Together, the 9 analytes account for about 90% of nicotine (determined from a transdermal administered nicotine dose; ref. 31), and creatinine-adjusted spot urinary TNE correlates with daily tobacco consumption (32). TNE is not influenced by the different rates of nicotine and cotinine metabolism because it measures the nicotine metabolites generated via different metabolic pathways.

To investigate the relationship between plasma cotinine levels and tobacco-derived carcinogen exposure, we used total urinary NNAL levels as the biomarker for tobacco-specific nitrosamines exposure in Study 2 participants (33). NNAL is a reductive metabolite of the highly carcinogenic 4-(methylnitrosamino)-1-(3) pyridyl-1-butanone; prediagnostic levels are associated with subsequent lung cancer risk (34).

We also investigated the relationship between plasma cotinine levels and exposure to another class of tobacco-related carcinogens, which are not nicotine-derived, the PAHs (33). The urinary levels of 2 hydroxylated PAHs, 1-hydroxypyrene, and 1-hydroxyfluorene were used as biomarkers of PAHs exposure. 1-Hydroxypyrene levels have been directly associated with cancer risk (35), and recent evidence suggests that 1-hydroxyfluorene is more specific to tobacco exposure than 1-hydroxypyrene (27).

CYP2A6 Genotyping

Prevalent *CYP2A6* alleles with altered function were genotyped by 2 step allele-specific PCR reactions in the Pharmacogenetics laboratory at Centre for Addiction and Mental Health (CAMH; Ontario, Canada) and University of Toronto (Ontario, Canada) as previously reported (7, 20, 36, 37). Those individuals with 1 or 2 copies of reduced function allele (*2, *4, *7, *9, *10, *12, *17, and *35) were classified as *CYP2A6* reduced metabolizers (20, 36, 37).

The measurement of CYP2A6 activity

The *in vivo* *CYP2A6* activity was measured using NMR, which is the ratio between plasma 3HC and cotinine. NMR is widely used as an *in vivo* indicator of *CYP2A6* activity (24, 36, 38, 39). In the context of our data analysis, the use of NMR may be confounded with plasma cotinine because it uses plasma cotinine as the denominator. Thus, we use NMR to extend our findings with *CYP2A6* genotype as it is a continuous variable and for comparisons with the literature.

Tobacco and tobacco-derived carcinogen exposure measurements

Plasma nicotine, cotinine, and 3HC levels, as well as urinary nicotine and metabolites, total NNAL, and PAH

metabolites were quantified by gas/liquid chromatography-tandem mass spectrometry in the Clinical Pharmacology Laboratory at the University of California (San Francisco, CA) as previously described (24, 40–43). NMR stratification was done by a median split of plasma NMR within each study population; participants who were in the higher NMR stratum were considered the faster *CYP2A6* activity group, whereas those in the lower NMR stratum were considered the slower *CYP2A6* activity group.

Statistical analysis

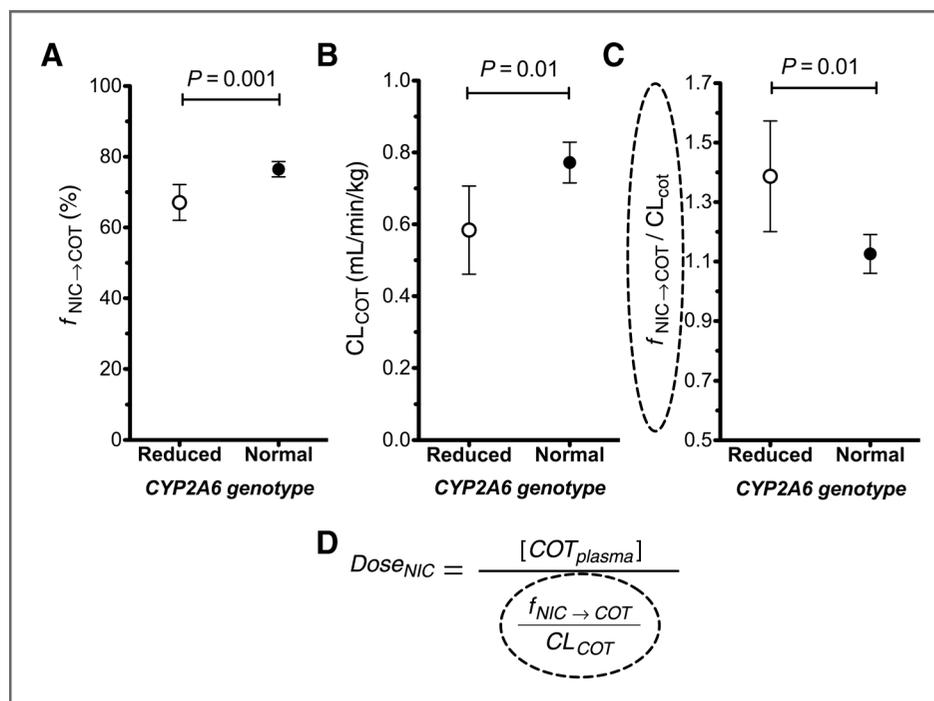
Statistical analyses were conducted using R statistical package (version 2.13, R foundation for statistical computing). In Study 1, statistical comparisons between *CYP2A6* genotypes and sex were conducted using mixed-effect linear regressions. All analyses controlled for nonindependence of data in twin pairs by modeling twinning as a random effect (21). Spearman's correlation was used to evaluate the relationship between (i) NMR and $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$, (ii) NMR and cotinine to TNE ratio, and (iii) plasma cotinine and urinary TNE, NNAL, 1-hydroxypyrene, and 1-hydroxyfluorene. Fisher *r* to *Z* transformation was used to compare the correlation coefficients. To minimize the influence of potential confounders such as race and smoking status on cotinine pharmacokinetics, the effects of *CYP2A6* genotype and sex were examined within nonsmoking Caucasians in Study 1.

Results

CYP2A6-Reduced function genetic variants decreased cotinine removal more than cotinine formation (Study 1)

Figure 1A compares cotinine formation ($f_{\text{NIC} \rightarrow \text{COT}}$) with *CYP2A6* genotypes in nonsmoking Caucasians. *CYP2A6* reduced metabolizers ($n = 33$) had significantly lower $f_{\text{NIC} \rightarrow \text{COT}}$ compared with *CYP2A6* normal metabolizers ($n = 148$). In *CYP2A6* reduced metabolizers, 67% of the nicotine dose was metabolized to cotinine compared with the 77% in *CYP2A6* normal metabolizers, a 15% relative reduction, $P = 0.001$ (Fig. 1A). *CYP2A6* reduced metabolizers also had lower cotinine clearance compared with the *CYP2A6* normal metabolizers (0.58 mL/min/kg in *CYP2A6* reduced metabolizers vs. 0.77 mL/min/kg in *CYP2A6* normal metabolizers; a 33% reduction; $P = 0.01$; Fig. 1B). The ratio of the fractional conversion of nicotine (reflecting cotinine formation) to cotinine clearance (reflecting cotinine removal), $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$, was significantly higher in *CYP2A6* reduced metabolizers compared with *CYP2A6* normal metabolizers (1.31 in *CYP2A6* reduced metabolizers vs. 1.12 in *CYP2A6* normal metabolizers; $P = 0.01$; Fig. 1C). Of note, the resulting $f_{\text{NIC} \rightarrow \text{COT}}/\text{Cl}_{\text{COT}}$ ratio is the conversion factor, or the average correction needed in plasma cotinine levels to accurately indicate the same nicotine dose (Fig. 1D). These genetic differences were

Figure 1. Reduced CYP2A6 activity had a greater impact on cotinine removal than cotinine formation (Study 1), which would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure. A, CYP2A6 reduced metabolizers ($n = 33$) metabolized a significantly lower amount of nicotine to cotinine compared with CYP2A6 normal metabolizers ($n = 148$). B, CYP2A6 reduced metabolizers ($n = 33$) had lower cotinine clearance (CL_{COT}) compared with CYP2A6 normal metabolizers ($n = 148$). C, the $f_{NIC \rightarrow COT}/CL_{COT}$ ratio was higher in CYP2A6 reduced metabolizers ($n = 33$) compared with CYP2A6 normal metabolizers ($n = 148$). Data presented as mean \pm 95% confidence interval. D, the $f_{NIC \rightarrow COT}/CL_{COT}$ ratio is the conversion factor between plasma cotinine levels and the actual nicotine dose.



consistent in both males and females (Supplementary Fig. S1A–S1F). In agreement with the findings between CYP2A6 genotypes, statistically significant differences in $f_{NIC \rightarrow COT}$, cotinine clearance, and $f_{NIC \rightarrow COT}/CL_{COT}$ were observed when comparing the faster with the slower NMR strata (Supplementary Fig. S1G and S1H). There was a significant inverse correlation between NMR (i.e., a continuous measure of CYP2A6 activity) and $f_{NIC \rightarrow COT}/CL_{COT}$, suggesting that the effect size is even larger when comparing the 2 extreme ends of CYP2A6 activity ($\rho = -0.57$; $P < 0.0001$).

Different relationships between plasma cotinine and tobacco exposure were observed between smokers with different CYP2A6 genotype groups (Study 2)

Next, the quantitative relationship between plasma cotinine and nicotine intake in smokers was investigated. Demographic variable comparisons are shown in Supplementary Table S1. Independent regression lines between urinary TNE and plasma cotinine were constructed in CYP2A6 reduced metabolizers and normal metabolizers ($n = 74$ and $n = 89$, respectively). As illustrated by Fig. 2, the slope of the regression line in CYP2A6 reduced metabolizers were significantly lower compared with the slope in CYP2A6 normal metabolizers (slope: 0.33 in CYP2A6 reduced metabolizers vs. 0.42 in CYP2A6 normal metabolizers; $P = 0.001$; Fig. 2). Statistical significance is shown by a significant interaction between CYP2A6 genotype and cotinine levels in the linear regression analysis presented in Supplementary Table S2A. A similar difference in regression line slopes was observed between the NMR strata (Supplementary Fig. S2). There

was a significant inverse correlation between NMR, a continuous measure of CYP2A6 activity, and the cotinine to TNE ratio ($\rho = -0.46$; $P < 0.0001$). These observations indicate that cotinine predicts nicotine intake, and therefore tobacco consumption, differently in smokers with reduced compared with normal CYP2A6 activity. As illustrated by Fig. 2, a 300 ng/mL (which is equivalent to 1,702 nmol/L) cotinine level was indicative of a 125 nmol nicotine equivalents exposure in CYP2A6 normal metabolizers, whereas the same cotinine level was indicative of roughly 100 nmol exposure in CYP2A6 reduced metabolizers. Alternatively, at 100 nmol tobacco exposure, CYP2A6 reduced metabolizers would have 300 ng/mL plasma cotinine levels, whereas the CYP2A6 normal metabolizers would have only 240 ng/mL. This is a 25% difference (60 ng/mL or 340 nmol/L) in cotinine levels between CYP2A6 genotype groups. Because reduced metabolizers include those with a range of decreased activity combined together, the 25% reflects an average difference (Fig. 3A). As illustrated by some specific genotypes or quartiles of NMR, these differences in cotinine estimates of dose can be approximately equal to 2-fold when comparing between the extremes of CYP2A6 activity or among different genotypes (Fig. 3B and C).

Different relationships between plasma cotinine and tobacco-specific nitrosamine exposures were observed in smokers with different CYP2A6 genotype groups (Study 2)

Next, we investigated the quantitative relationship between plasma cotinine and tobacco-specific

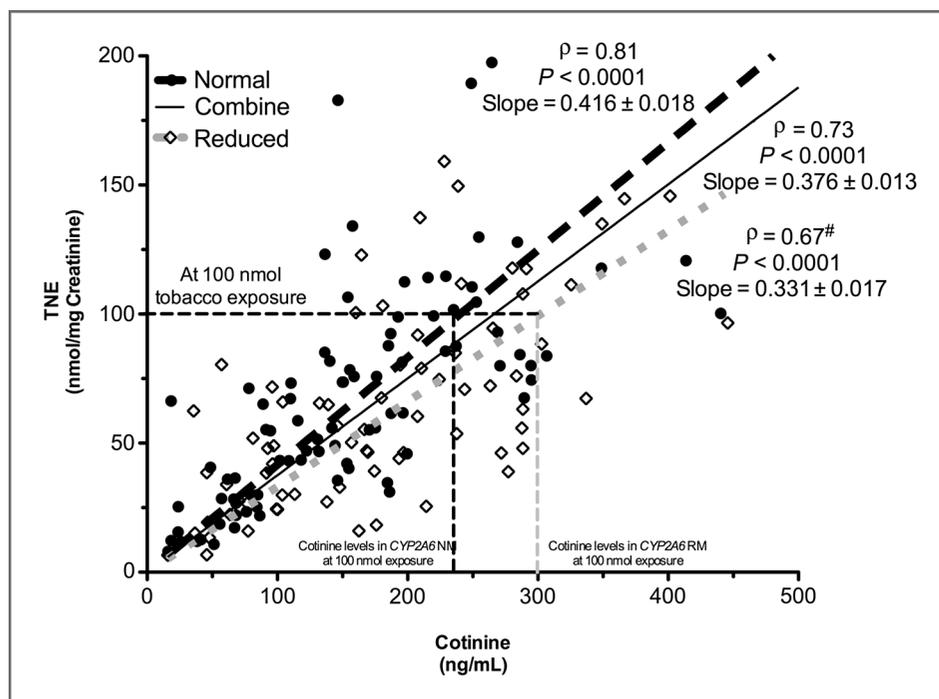


Figure 2. Cotinine's ability to predict tobacco exposure was different between *CYP2A6* genotypes (Study 2). The slope between urinary TNE and plasma cotinine was significantly lower in *CYP2A6* reduced metabolizers ($n = 74$) compared with that of *CYP2A6* normal metabolizers ($n = 89$; Supplementary Table S2A), suggesting that the quantitative relationship between cotinine and tobacco exposure (i.e., TNE) differed between *CYP2A6* genotypes. [#] indicates statistical significant difference in Spearman ρ compared with the *CYP2A6* normal metabolizers. The numbers after the slopes are SE. Of note, the strength of correlations between plasma cotinine and urinary TNE in the *CYP2A6* reduced metabolizers was significantly weaker than in *CYP2A6* normal metabolizers (Fisher r to Z transformation: $P < 0.01$).

nitrosamines exposure in smokers. As seen with TNE, the slope of the regression line between urinary NNAL and plasma cotinine in *CYP2A6* reduced metabolizers was significantly lower compared with the

slope in *CYP2A6* normal metabolizers (slope: 1.279 in *CYP2A6* reduced metabolizers vs. 1.687 in *CYP2A6* normal metabolizers; $P = 0.003$; Fig. 4. Statistical significance shown by a significant interaction term in the

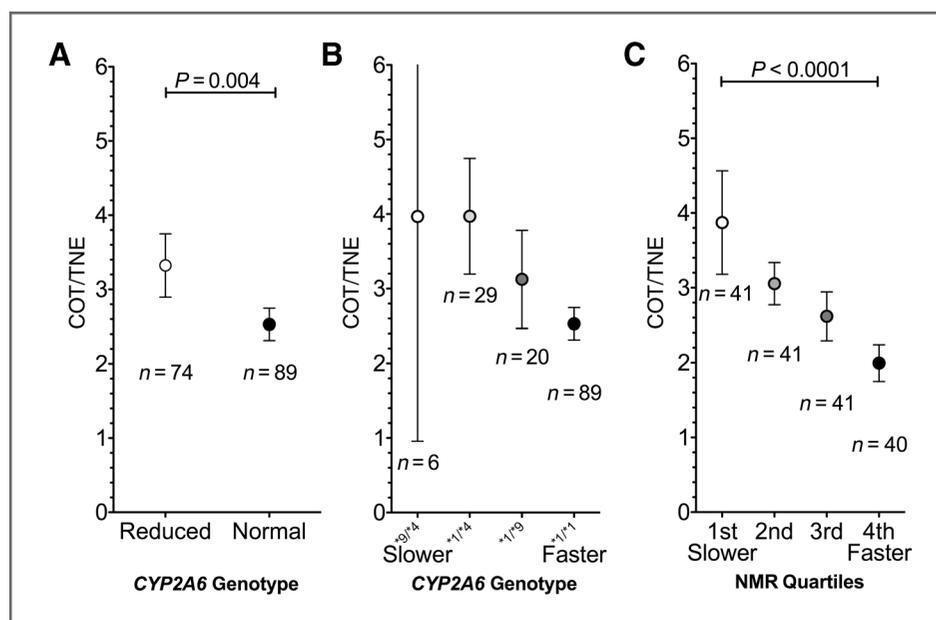
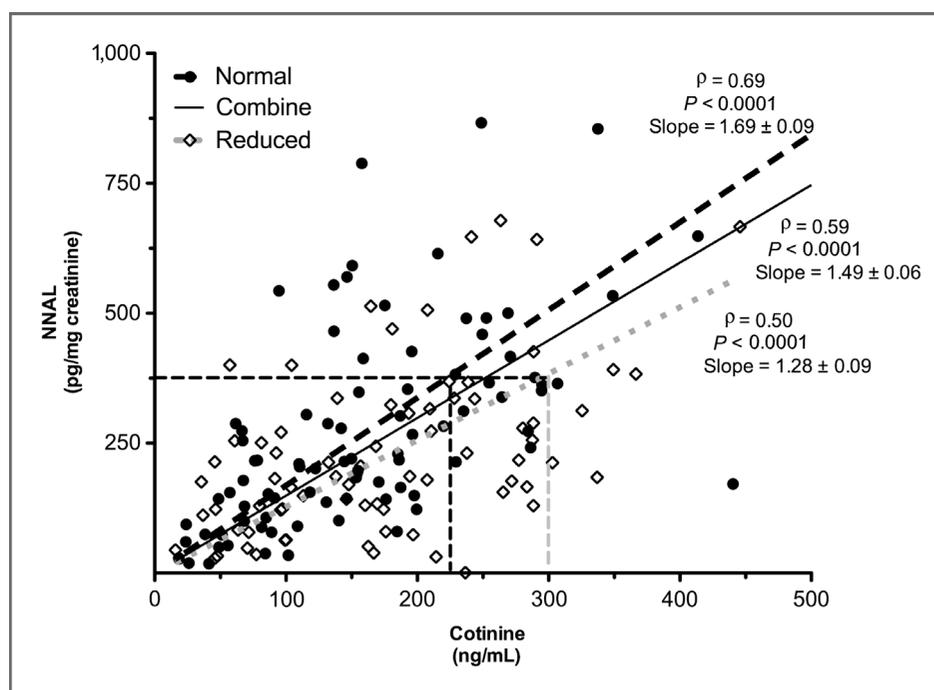


Figure 3. A, the cotinine to TNE ratio (i.e., cotinine levels per nicotine intake) was significantly lower in *CYP2A6* normal metabolizers compared with the *CYP2A6* reduced metabolizers (Study 2). B, the cotinine to TNE ratio decreased with *CYP2A6* genotypes with increasing activity (Study 2). As illustrated using some different *CYP2A6* genotypes, containing reduced function (*9) or loss of function (*4) allele compared with the wild-type individuals (*1/*1). Of note, individuals who are fully null for *CYP2A6* (i.e., these with 2 copies of gene deletions, *CYP2A6**4/*4) had unexpectedly lower COT to TNE ratio compared with the wild-type individuals (*1/*1, data not shown), suggesting the minor remaining cotinine formation pathway (likely *CYP2B6* or *CYP2A13*) is considerably slower than the low affinity cotinine removal pathway (likely *UGT2B10* glucuronidation or renal clearance). C, the cotinine to TNE ratio decreased by NMR quartiles (Study 2). Data presented as mean \pm 95% confident intervals. Statistical comparisons were conducted by Mann-Whitney or Kruskal-Wallis tests.

Figure 4. Cotinine's ability to predict tobacco-specific nitrosamines exposure (as indicated by NNAL levels) was different between *CYP2A6* genotypes (Study 2). The slope between urinary NNAL levels and plasma cotinine was significantly lower in *CYP2A6* reduced metabolizers ($n = 74$) compared with that of *CYP2A6* normal metabolizers ($n = 89$; Supplementary Table S2B). This suggested the quantitative relationship between cotinine and tobacco-specific nitrosamines exposure differed between *CYP2A6* genotypes. The numbers after the slopes are SE.



linear regression analysis is presented in Supplementary Table 2B. These results showed that cotinine levels predict NNAL levels, and therefore nitrosamine exposure, differently in smokers with reduced compared with normal *CYP2A6* activity. As illustrated by Fig. 4, a 300 ng/mL (which is equivalent to 1,702 nmol/L) cotinine level was indicative of a 510 pg/mg creatinine NNAL exposure in *CYP2A6* normal metabolizers, whereas the same cotinine level was indicative of roughly 375 pg/mg creatinine NNAL exposure in *CYP2A6* reduced metabolizers. Alternatively, at 375 pg/mg creatinine NNAL exposure, *CYP2A6* reduced metabolizers would have 300 ng/mL plasma cotinine levels, whereas the *CYP2A6* normal metabolizers would have only 225 ng/mL. The magnitude of the *CYP2A6* genotype effect (25%) on plasma cotinine levels was similar to that observed with TNE as the exposure marker; as with the TNE biomarker, this is an underestimation of effected size for some genotype or phenotype comparisons (see Fig. 3).

To determine whether the above observations were unique to plasma cotinine, independent regression lines between urinary NNAL and urinary TNE levels were constructed in *CYP2A6* reduced metabolizers and normal metabolizers. The slope of the regression lines did not differ between *CYP2A6* genotypes (Supplementary Fig. S3 and Supplementary Table S2C), indicating that as expected, TNE's relationship to tobacco-specific nitrosamines exposure in smokers was independent of the *CYP2A6* genotype. Together, this shows that the impact of *CYP2A6* genotype on plasma cotinine levels was specific to cotinine as an exposure biomarker.

Different relationships between plasma cotinine and polycyclic aromatic hydrocarbon exposure were observed in smokers with different *CYP2A6* genotype groups (Study 2)

Next, we investigated the quantitative relationship between plasma cotinine and PAH exposure in smokers. As illustrated by Supplementary Fig. S4 and S5, the slopes of the regression lines in *CYP2A6* reduced metabolizers were lower compared with the slopes in *CYP2A6* normal metabolizers (1-hydroxyfluorene slopes: 0.024 in *CYP2A6* reduced metabolizers vs. 0.027 in *CYP2A6* normal metabolizers; $P = 0.013$; Supplementary Fig. S4 and Supplementary Table S2D; 1-hydroxypyrene slopes: 0.010 in *CYP2A6* reduced metabolizers vs. 0.014 in *CYP2A6* normal metabolizers; $P = 0.079$; Fig. 5 and Supplementary Table S2E).

Males had a greater reduction in cotinine clearance than in cotinine formation (Study 1)

Because *CYP2A6* activity differs between the sexes, we investigated the impact of sex on cotinine formation and cotinine clearance. The $f_{\text{NIC} \rightarrow \text{COT}}$ did not differ between the sexes (74% in males compared with 75% in females, nonsignificant; Fig. 6A). However, males had a significantly lower cotinine clearance compared with females (0.58 vs. 0.81 mL/min/kg in males and females, respectively; $P < 0.001$; Fig. 6B). A significantly higher $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$ ratio, which is the conversion factor between plasma cotinine levels and the nicotine dose (Fig. 1D), was observed in males compared with females (1.39 vs. 1.12 in males and females, respectively; $P = 0.001$; Fig. 6C). As observed with the entire study population, similar impacts of sex on $f_{\text{NIC} \rightarrow \text{COT}}$, cotinine

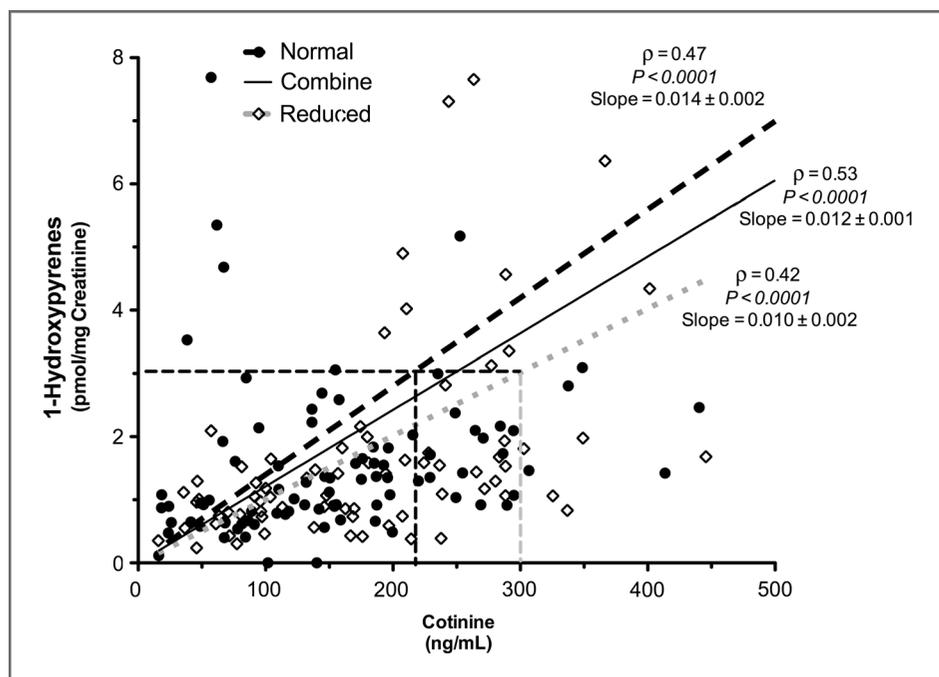


Figure 5. Cotinine's ability to predict PAH exposure (as indicated by 1-hydroxyppyrene) was different between *CYP2A6* genotypes (Study 2). The slope between 1-hydroxyppyrene levels and plasma cotinine was lower in *CYP2A6* reduced metabolizers ($n = 74$) compared with that of *CYP2A6* normal metabolizers ($n = 89$; Supplementary Table S2E). The numbers after the slopes are SE.

clearance, and $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ could be observed when examined only within the subgroup of *CYP2A6* normal metabolizers (i.e., excluding the *CYP2A6* reduced metabolizers; Supplementary Fig. S5).

Different relationships between plasma cotinine and tobacco exposure were observed in male smokers compared with female smokers (Study 2)

Independent regression lines between urinary TNE and plasma cotinine were constructed by sex. The slope of the regression line in males was significantly lower compared with the slope in females (0.34 in males vs. 0.42 in females; $P = 0.01$; Fig. 7 and Supplementary Table S2F). The magnitude of the sex effect on cotinine

levels was similar to the effect of *CYP2A6* genotype. At 100 nmol tobacco exposure, there is a 25% difference in plasma cotinine levels (60 ng/mL or 340 nmol/L) between the sexes. As observed with the entire study population, similar impacts of sex could be observed when examined within just the *CYP2A6* normal metabolizers (slopes: 0.36 ± 0.02 in males vs. 0.54 ± 0.03 in females; $P = 0.001$)

Discussion

Our analyses show that variation in *CYP2A6* metabolic activity, such as those that exist between *CYP2A6* genotypes or the sexes, may alter cotinine removal (i.e., cotinine clearance) to a different extent than cotinine

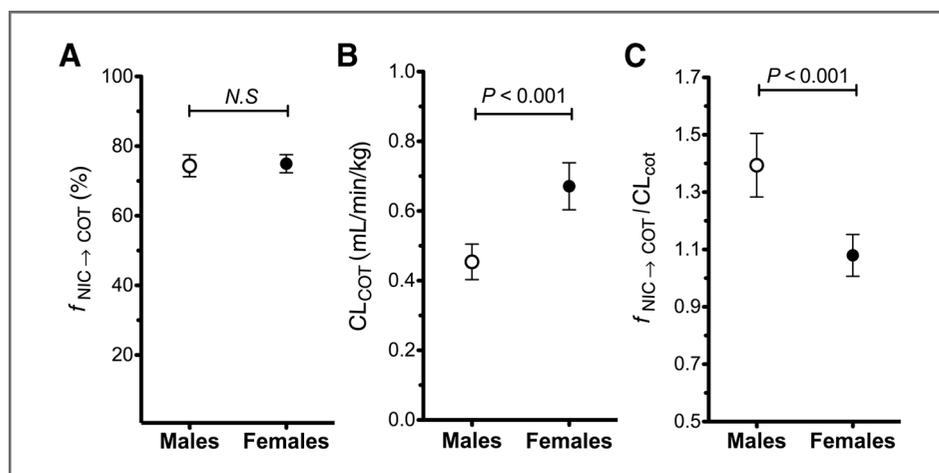
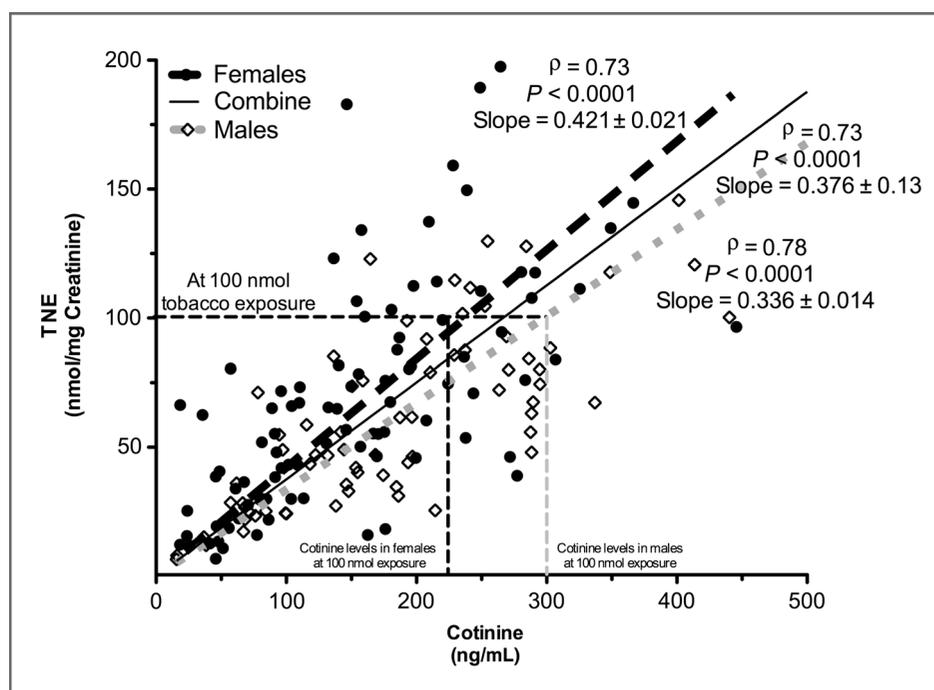


Figure 6. Males ($n = 54$), who have reduced *CYP2A6* activity compared with females ($n = 127$), had a greater ratio of cotinine formation over cotinine removal, which would result in the accumulation of cotinine and higher cotinine levels at a given tobacco exposure in male smokers (Study 1). A, the $f_{\text{NIC} \rightarrow \text{COT}}$ did not differ between the sexes. B, males had significantly lower CL_{COT} than the females. C, males had a higher $f_{\text{NIC} \rightarrow \text{COT}} / \text{CL}_{\text{COT}}$ compared with females indicating an overestimation of nicotine dose for their cotinine levels (see Fig. 1D). Data presented as mean \pm 95% confidence interval.

Figure 7. Cotinine's ability to predict tobacco exposure was different between the sexes. The slope between urinary TNE and plasma cotinine was significantly lower in males ($n = 71$) compared with that of females ($n = 92$; Supplementary Table S2F), suggesting cotinine overestimates tobacco exposure (i.e., TNE) in males compared with females (Study 2). The numbers after the slopes are SE.



formation (i.e., $f_{\text{NIC} \rightarrow \text{COT}}$). Individuals with lower CYP2A6 activities, such as CYP2A6 reduced metabolizers and males, have higher ratios of cotinine formation to cotinine removal (i.e., $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$) compared with individuals with faster CYP2A6 activity, such as CYP2A6 normal metabolizers and females. Thus, the quantitative relationship between plasma cotinine and tobacco and tobacco-derived carcinogen exposures in smokers varies between those with different CYP2A6 activity.

The pharmacokinetic mechanism

The greater effect of altered CYP2A6 activity on cotinine clearance compared with formation is likely related to differences in nicotine and cotinine's hepatic metabolism. Nicotine exhibits a higher hepatic extraction ratio compared with cotinine, which is largely due to differences in their intrinsic clearances. The intrinsic clearance (V_{max}/K_m) of nicotine is $1.69 \mu\text{L}/\text{min}/\text{mg}$ protein in human liver microsomes, which is 10 times faster than the $0.16 \mu\text{L}/\text{min}/\text{mg}$ protein of cotinine (1, 2, 44, 45). For drugs like nicotine, with high extraction ratios, clearance is determined in large part by liver blood flow and is less affected by changes in liver enzymatic activity. In contrast for low extraction drugs like cotinine, clearance is largely affected by changes in liver enzymatic activity. This differential impact of variation in CYP2A6 activity is consistent with previous observations on the effects of CYP2A6 induction. Estrogen induces CYP2A6 protein expression in humans (46). During pregnancy, when estrogen levels are elevated, the clearance of nicotine increases by 60%, whereas the clearance of cotinine increases by 140% (47). Thus, higher CYP2A6 activity during pregnancy

has a greater effect on cotinine clearance than nicotine clearance.

CYP2A6, Sex, and race

Consistent with a higher $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$ ratio predicting higher cotinine levels for the same tobacco and tobacco-derived carcinogen exposure (equation 1), we observed different quantitative relationships between cotinine and tobacco and tobacco-derived carcinogen exposure in smokers with reduced CYP2A6 activities (CYP2A6 reduced metabolizers and males). These observations provide a mechanistic explanation for the systematic variation in plasma cotinine levels previously observed between CYP2A6 genotypes, the sexes, and possibly races (9, 10, 12).

Our observations explain the directional inconsistencies between cotinine and other indicators of tobacco consumption in genetic association studies involving CYP2A6 (9, 10). Because of the higher $f_{\text{NIC} \rightarrow \text{COT}}/\text{CL}_{\text{COT}}$ ratio, CYP2A6 reduced metabolizers would have higher plasma cotinine levels upon the same tobacco exposure compared with the CYP2A6 normal metabolizers. This masks or reduces the true size of the effect of CYP2A6 genotype on tobacco consumption based on plasma cotinine (i.e., CYP2A6 reduced metabolizers have higher cotinine levels with lower tobacco consumption). These results could also explain why the same CYP2A6 genetic variants have been associated with lower lung cancer risk while having paradoxically higher plasma cotinine levels (10).

Similarly, during transdermal nicotine patch treatment (i.e., one week after starting treatment), steady-state plasma cotinine levels in biochemically confirmed

abstinent individuals (38) were inversely related to the pretreatment CYP2A6 activity (Supplementary Fig. S6). The 25% (50 ng/mL) average differences in cotinine levels between participants with faster and slower CYP2A6 activity (NMR) were in agreement with the effect size observed in smokers in Study 2. The difference double to 50% (100 ng/mL) when comparing those in the first with fourth quartile of CYP2A6 activity. Thus, for the same systemic nicotine exposure (delivered by 21 mg patch for all participants), the individuals with slower CYP2A6 activity had higher steady-state cotinine levels than the individuals with faster CYP2A6 activity (Supplementary Fig. S6).

Our results could explain the systematic variation in plasma cotinine levels between the sexes (48). Because of the lower average estrogen levels, males have lower CYP2A6 protein expression and activity compared with females (49). Thus, males have higher $f_{\text{NIC-COT}}/\text{CL}_{\text{COT}}$ ratios, which suggest that males would have higher plasma cotinine levels compared with females with similar tobacco exposure.

Finally, our observation could also explain the differences in plasma cotinine levels between Caucasians and African-Americans even when controlling for tobacco exposure. About 50% of African-Americans are CYP2A6 reduced metabolizers compared with the 20% in Caucasians (50). Therefore, African-Americans have, on an average, higher $f_{\text{NIC-COT}}/\text{CL}_{\text{COT}}$ ratios compared with Caucasians, resulting in higher plasma cotinine levels for similar tobacco exposure.

Implications for cancer research

Plasma cotinine is often used as an objective, non-self-report indicator of tobacco exposure in smoking-related cancer research. However, there could be a 25% or more variation in plasma cotinine levels following the same tobacco and tobacco-derived carcinogen exposure depending on CYP2A6 genotype or in the presence of other inducers/inhibitors of CYP2A6 activity. To put this difference in perspective, Munafò and colleagues reported that each risk allele in rs16969968, an SNP in the α -5 nicotinic acetylcholine receptor, is associated with a 138.4 nmol/L (or 24 ng/mL) increase in plasma cotinine levels, which in turn was predicted to increase lung cancer risk by 1.3 times (5). Our data suggest that the difference between CYP2A6 genotypes (or sex) would be approximately 340 nmol/L (or 60 ng/mL) at 100 nmol tobacco exposure (or roughly 375 pg/mg Cre NNAL). Hence, cotinine levels alone, without considering CYP2A6 activity, may not provide the most accurate information about tobacco and tobacco-derived carcinogen exposure in smokers, particularly when comparing groups with different frequencies of CYP2A6 genotypes (or race) or sex. Biomarkers of nicotine intake such as TNE that are less dependent on individual differences in nicotine metabolism or biomarkers of toxicants that are more directly

involved in the carcinogenic process, will enhance our understanding of the contribution of variable levels of tobacco consumption on lung cancer risk.

In conclusion, the quantitative relationship between plasma cotinine level and tobacco and tobacco-derived carcinogen exposure differs among groups with different levels of CYP2A6 activity. These comparison groups include the different CYP2A6 genotype groups within a race (e.g., CYP2A6 reduced metabolizers vs. CYP2A6 normal metabolizers among Caucasians) and different races with distinct prevalence of CYP2A6 reduced metabolizers (e.g., Caucasians with 20% CYP2A6 reduced metabolizers vs. African-Americans with 50% CYP2A6 reduced metabolizers). Furthermore, groups with different estrogen levels may also have variation in cotinine levels unrelated to differences in tobacco exposure. Those include different sexes, or those with varying pregnancy and menopause statuses. Therefore cotinine levels, as a quantitative marker of tobacco and tobacco-derived carcinogen exposure, are not directly comparable between CYP2A6 genotypes, sexes, and races.

Disclosure of Potential Conflicts of Interest

R.F. Tyndale has honoraria from Speaker's Bureau from multiple universities, is an associate editor for CPT, and is a consultant/advisory board member for Novartis and McNeil. D. Hatsukami has received research funding from Nabi Biopharmaceuticals to test a nicotine vaccine. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Clifford Watson and Connie Sosnoff for conducting the analytical chemistry.

Grant Support

This work was cosupported by the Indian Health Service, the National Institute for Drug Abuse at the NIH and the NCI at the NIH (DA02277, DA012353, DA020830, DA012353, DA11170, NARCH III U26IHS300012, HHSN261200700462P, UL1 RR024131, and CA114609); the Endowed Chair in Addiction for the Department of Psychiatry (to R.F. Tyndale); Canadian Institutes of Health Research (MOP86471 and TMH109787); Ontario Graduate Scholarship (to A.Z.X. Zhu); CAMH and the CAMH foundation; the Canada Foundation for Innovation (#20289 and #16014); and the Ontario Ministry of Research and Innovation.

Received November 5, 2012; revised January 9, 2013; accepted January 20, 2013; published OnlineFirst January 31, 2013.

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Cancer Epidemiol Biomarkers Prev 2013;22:708-718. Published OnlineFirst January 31, 2013.

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