

CYP2A6 Genotype, Phenotype, and the Use of Nicotine Metabolites as Biomarkers during *Ad libitum* Smoking

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Abstract

CYP2A6 inactivates nicotine to cotinine and cotinine to 3-hydroxycotinine. We investigated which of plasma nicotine and metabolites were most related to CYP2A6 genotype and smoking levels. We assessed demographic and smoking histories in 152 Caucasian *ad libitum* smokers, measured breath carbon monoxide (CO) levels, and determined plasma nicotine, cotinine, and 3-hydroxycotinine by high-performance liquid chromatography and CYP2A6 genotypes by PCR. Cigarettes per day was most closely related to CO ($r = 0.60$, $P < 0.001$) followed by plasma cotinine ($r = 0.53$, $P < 0.001$), whereas plasma cotinine was most strongly correlated with CO levels ($r = 0.74$, $P < 0.001$), confirming that cotinine is a good indicator of smoking levels; this was not limited by CYP2A6 variants. 3-Hydroxycotinine/cotinine is reported to be a good marker of CYP2A6 activity, and we found that the 3-hydroxycotinine/(cotinine + nicotine) ratio was most correlated with CYP2A6 genotype ($r = 0.38$,

$P < 0.001$). Inclusion of the CYP2A6*12A allele strengthened the correlation ($r = 0.46$, $P < 0.001$), suggesting that the identification of novel alleles will continue to improve this relationship. Nicotine metabolism is slower in smokers, and we have shown that CYP2A6 is reduced by nicotine treatment in monkeys. Here, we found that plasma nicotine levels were inversely correlated with CYP2A6 activity (3-hydroxycotinine/cotinine, $r = -0.41$, $P < 0.001$) among those without CYP2A6 variants, suggesting a reduction in metabolism with higher nicotine levels. Together, these findings (a) confirm the use of plasma cotinine and CO as indicators of Caucasians' smoking levels, and that this is not limited by CYP2A6 genetic variation; (b) indicate that 3-hydroxycotinine/cotinine and 3-hydroxycotinine/(cotinine + nicotine) are moderately good indicators of the CYP2A6 genotype; and (c) support that nicotine exposure may reduce its own metabolism. (Cancer Epidemiol Biomarkers Prev 2006;15(10):1812-9)

Introduction

In humans, the major nicotine-metabolizing (1) and cotinine-metabolizing enzyme is cytochrome P450 2A6 (CYP2A6; refs. 2-5). CYP2A6 exhibits a narrow substrate spectrum, having a major role only in the metabolism of coumarin, a major caffeine metabolite 1,7-dimethylxanthine, a few pharmaceutical compounds including nicotine, (+)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride (SM-12502), Tegafur, and tobacco-specific nitrosamines such as 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosornicotine (6, 7). In humans, ~70% to 80% of nicotine is metabolized to cotinine *in vivo* (5), and roughly 90% of this conversion is mediated by CYP2A6 (4, 8). Cotinine is subsequently oxidized, a step catalyzed virtually exclusively by CYP2A6 to form 3-hydroxycotinine (3, 9). Substantial variation in CYP2A6 activity and protein levels exist using coumarin (10, 11) and nicotine (5, 9) as substrates; this variation has been largely attributed to genetic polymorphisms in the CYP2A6 gene (12).

There are >20 established CYP2A6 alleles (<http://www.imm.ki.se/CYPalleles/cyp2a6.htm>); some have been characterized *in vitro* and/or *in vivo* and result in absent, reduced,

increased, or normal enzyme activity. Reduction in the rates of nicotine metabolism, due to CYP2A6 genetic polymorphisms (13, 14), has been shown to be associated with a reduced risk for being a current smoker, reduced amounts of daily smoking among smokers, reduced smoking intensity, and increased quitting (15-20). However, not all studies are in agreement with these findings (21, 22).

Carbon monoxide (CO) and cotinine are good indicators of smoking levels (23-25); however, CYP2A6 genetic variants alter nicotine and cotinine metabolism and smoking behaviors (12, 19). Our first aim was to evaluate measures of plasma nicotine, cotinine, 3-hydroxycotinine, and their ratios as biomarkers of smoking levels and to determine whether the CYP2A6 genetic variants limit the use of these as indicators in Caucasian *ad libitum* smokers.

A second objective was to determine whether plasma nicotine, its metabolites, or metabolite ratios, measured during *ad libitum* smoking, were related to CYP2A6 genotype and could serve as an indicator of phenotype. Typically, phenotyping involves the administration of a specific amount of a probe drug that is selectively metabolized by the enzyme of interest and is not present in the study participants before the administration. A plasma or urine sample is collected at a specific time; the parent drug and the metabolite levels are assessed; and the metabolite ratio calculated providing an index of their *in vivo* phenotype (26, 27). Coumarin 7-hydroxylation is mediated by CYP2A6; whereas coumarin could be used in smokers, it is a poor probe drug as it is highly extracted (28), limiting its use to distinguish intermediate, normal, and fast CYP2A6 activity. In addition, some CYP2A6 genetic variants alter coumarin metabolism differently than nicotine metabolism, and smokers have reduced rates of metabolism. The metabolic ratio of cotinine/nicotine is used as an indicator of CYP2A6 activity (5, 14); however, this ratio is not ideal for assessing CYP2A6 activity during *ad libitum* smoking as it is very sensitive to the timing of the last exposure

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to nicotine. This is due to the large difference in half-lives between nicotine and cotinine: 1 to 2 hours versus 13 to 19 hours, respectively (5). Recently, the 3-hydroxycotinine/cotinine ratio was found to be highly correlated with oral nicotine clearance ($r = 0.76-0.83$, $P < 0.01$; ref. 9); however, this was done in a controlled kinetic study using orally given labeled nicotine and cotinine, not during *ad libitum* smoking. Pilot data in 14 smokers in the same study suggested that the 3-hydroxycotinine/cotinine ratio derived from tobacco smoke nicotine was also correlated with oral nicotine clearance ($r = 0.70-0.95$, $P < 0.01$; ref. 9). Smokers have a difficult time abstaining for long periods of time (as cotinine is cleared), making controlled kinetic studies in abstainers difficult. In addition, the rates of nicotine metabolism are slower during smoking, suggesting that to understand nicotine metabolism in smokers, they have to be smoking. Therefore, our second aim was to assess the relationship between the *CYP2A6* genotype groups and measures of plasma nicotine, its metabolites (cotinine and 3-hydroxycotinine), and the metabolite ratios. We also assessed the effect of the *CYP2A6*12A* variant, which reduces coumarin metabolism (29), but its effect on nicotine metabolism has not yet been determined.

Lastly, nicotine metabolism is reduced during smoking, using deuterium-labeled (*S*)-nicotine (30, 31). CO (from tobacco smoke) and cotinine did not alter nicotine metabolism *in vivo* (31, 32); however, we have shown, in a monkey model, that chronic nicotine treatment alone results in decreased nicotine C-oxidation and *CYP2A6* protein levels (33). Therefore, a final objective of this study was to assess whether *CYP2A6* activity is inversely related to nicotine plasma levels during *ad libitum* smoking.

Materials and Methods

Study Population. Subjects were recruited at the Sunnybrook and Women's College Health Science Centre in Toronto, Canada by flyers and newspaper advertisements. Participants reporting having three or more grandparents of Caucasian ethnicity ($N = 152$) were included. Participants were 18 to 73 years of age and current smokers. Male subjects ($n = 82$) had an average age \pm SD of 38.3 ± 12.8 , and female subjects ($n = 69$) had an average age of 36.5 ± 12.0 . Gender data were missing for one subject. Participants reported smoking an average \pm SD of 19.5 ± 12.5 cigarettes per day (CPD). Participants signed a written consent form and answered a demographic questionnaire that also assessed their history and patterns of smoking as previously described (19). Participants provided blood samples between 4 and 8 PM for *CYP2A6* genotyping and for nicotine, cotinine, and 3-hydroxycotinine plasma measures and were assessed for breath CO using the Micro II Smokelyzer (Bedford Science Ltd., Upchurch, England). The study was approved by the Ethics Review Committee of the Sunnybrook and Women's College Health Science Centre.

Genotyping of Samples. Blood samples were stored at -80°C . Genomic DNA isolation was done using the Sigma kit for DNA extraction (GenElute Mammalian Genomic DNA kit, Sigma-Aldrich Co., Canada Ltd., Oakville, Ontario, Canada). *CYP2A6*2*, *CYP2A6*4*, *CYP2A6*5*, *CYP2A6*6*, *CYP2A6*7*, *CYP2A6*8*, *CYP2A6*9A*, *CYP2A6*10*, and *CYP2A6*12A* alleles were assessed by two-step allele-specific PCR assays as previously described (19, 34).

Measurement of Samples: Liquid-Liquid and Solid-Phase Extraction Procedures. Plasma samples were kept at -20°C and thawed at room temperature. Drug concentrations were assessed using high-performance liquid chromatography (HPLC) with UV detection (35-37). First, nicotine, cotinine, and 3-hydroxycotinine levels were measured with a solid-phase extraction assay modified from previously published

procedures (36, 37), and then, for a comparison of the results, the levels of nicotine and cotinine were also measured with a liquid-liquid extraction procedure, which has poor recoveries of 3-hydroxycotinine, described by Hariharan et al. (35). Plasma samples (0.5 mL) were incubated at 37°C for 24 hours with 0.25 mL of β -glucuronidase enzyme (type H-1, Helix Pomatia, Sigma, St. Louis, MO) diluted in acetate buffer (0.2 mol/L, pH 5; final concentration = 15 mg/mL) to allow for maximum deconjugation of the drugs. Briefly, for the solid-phase extraction assay, the samples received 50 μL of 5-methylcotinine (the internal standard, 1.2 $\mu\text{g}/\text{mL}$) and 0.6 mL of NaOH (0.2 mol/L) for a final sample volume of 1.45 mL. The plasma samples were then transferred to the VacMaster 20 sample processing station and allowed to absorb into the sorbent bed (modified form of diatomaceous earth) of the Isolute HM-N SPE (3.0 mL sample) column (Chromatographic Specialties, Inc., Brockville, Ontario, Canada) for 15 minutes. Methanolic hydrochloric acid (300 μL , 20 mmol/L) was added to the 12-mL conical polypropylene collection tubes, and the drugs were eluted under gravity with 13 mL of dichloromethane/2-propanol (24:1, v/v). Following the evaporation of the elutant under nitrogen at 40°C , the crystallized samples were reconstituted in 105 μL of 0.01 N HCl, and 90 μL were injected into a Supelco LC8DB steel column (150 \times 4.6 mm, 5- μm particle size) of the HPLC-UV system. The mobile phase was composed of a citric acid buffer/acetonitrile mixture (100:7, v/v). The citric acid buffer was prepared by combining 0.034 mol/L citric acid (BDH, Inc., Toronto, Ontario, Canada), 0.034 mol/L potassium phosphate monobasic (Mallinckrodt, St. Louis, MO), 671 mg/L of heptanesulfonic acid (Sigma), and 5 ml/L of triethylamine (Sigma) with water. KOH (5 N) was used to adjust the pH to 4.62. Acetonitrile (70 mg/L) was added to obtain the desired mobile phase solution. The flow rate was set at 1 mL/min, and UV detection was monitored at 260 nm. Plasma standard curves for nicotine, cotinine, and 3-hydroxycotinine were prepared using blank plasma samples (0.5 mL) added to 50 μL of drug (nicotine: 0-80 ng/mL, cotinine: 1-400 ng/mL, 3-hydroxycotinine: 0-160 ng/mL).

Statistical Analyses. The Kolmogorov-Smirnov test was conducted to determine the normality of the data. Measures that were not normally distributed were transformed into logarithmic form (all ratios), and those that were normally distributed were not transformed (plasma nicotine, cotinine, and 3-hydroxycotinine concentrations). Pearson's correlation analysis was done to assess whether plasma measures of nicotine, and log metabolite ratios were correlated with smoking level (assessed by level of CPD or CO). MedCalc for Windows, demo version 7.4.1.0 (MedCalc Software, Mariakerke, Belgium) statistical analysis software was used to determine whether differences between correlation coefficients reached significance. Unpaired Student's *t* tests were conducted to compare the mean log 3-hydroxycotinine/cotinine ratio between *CYP2A6*1*1* and *CYP2A6*1*12A* individuals. Student's *t* tests were preceded by F-tests to determine whether the variances were equal or unequal. ANOVA followed by Bonferroni's multiple comparison test was used to determine differences in 3-hydroxycotinine/(cotinine + nicotine) by *CYP2A6* genotype groups. Pearson's correlation analysis was also conducted to determine whether plasma measures of nicotine and metabolite ratios were related to *CYP2A6* genotype groups and whether a relationship existed between *CYP2A6* activity and nicotine levels. All statistical analyses were conducted using GraphPad Prism version 4.03 (San Diego, CA).

Results

Detection of Nicotine, Cotinine, and 3-Hydroxycotinine in the Plasma of Smokers. Sensitivity of the solid-phase

extraction HPLC-UV assay was found to be 5 ng/mL for nicotine, 12.5 ng/mL for cotinine, and 10 ng/mL for 3-hydroxycotinine. The between-day and within-day coefficients of variation were 9.4% and 15.0% at 5.0 ng/mL nicotine, 14.8% and 10.6% at 12.5 ng/mL cotinine, and 11.4% and 13.3% at 10 ng/mL 3-hydroxycotinine, respectively ($n = 5$). The assay remained linear for the following range of values: nicotine, 0 to 160 ng/mL ($y = 0.005x + 0.0132$, $R^2 = 0.999$); cotinine, 0 to 800 ng/mL ($y = 0.0065x + 0.0062$, $R^2 = 0.999$); and 3-hydroxycotinine, 0 to 640 ng/mL ($y = 0.006x - 0.0332$, $R^2 = 0.999$). The recoveries ranged from 80% to 90% for nicotine, 75% to 85% for cotinine, and 55% to 65% for 3-hydroxycotinine. The sensitivity of the liquid-liquid extraction HPLC-UV assay (35) was found to be 2.5 ng/mL for nicotine and 5 ng/mL for cotinine. The between-day coefficient of variation was 13.8% for nicotine at 2.5 ng/mL and 13% for cotinine at 5 ng/mL ($n = 5$), and the recoveries were 75% to 80% for nicotine and 70% to 76% for cotinine. It was found that the nicotine and cotinine levels measured by both assays were significantly correlated ($r = 0.85$ and $r = 0.92$, respectively; $P < 0.001$). Subsequent analyses shown in this article were based on the solid-phase extraction HPLC-UV assay.

CYP2A6 Genotype among Caucasian Smokers. The participants ($N = 152$) were genotyped for the CYP2A6*2, CYP2A6*4, CYP2A6*5, CYP2A6*6, CYP2A6*7, CYP2A6*8, CYP2A6*9A, CYP2A6*10, and CYP2A6*12A alleles. CYP2A6*5, CYP2A6*6, CYP2A6*8, and CYP2A6*10 alleles were not found. The allele frequencies (percent \pm 95% confidence interval) for CYP2A6*2, CYP2A6*4, CYP2A6*7, CYP2A6*9A, and CYP2A6*12A were 1.6 \pm 0.7%, 1.0 \pm 0.6%, 0.3 \pm 0.3%, 6.3 \pm 1.4%, and 2.6 \pm 0.9%, respectively. These data are consistent with previously characterized Caucasian smoker populations (19).

Indicators of Smoking Levels. Daily smoking for the entire group was average for Caucasians (mean = 19.4 CPD). Smoking level was defined as number of CPD or breath CO in parts per million. Measures tested as indices of CPD or CO levels included plasma nicotine, cotinine and 3-hydroxycotinine concentrations (ng/mL); cotinine/nicotine, 3-hydroxycotinine/cotinine, (3-hydroxycotinine + cotinine)/nicotine, and 3-hydroxycotinine/(cotinine + nicotine) ratios; and breath CO ppm (only for CPD). The relationships (r s and P s for correlations) between the indices and smoking levels indicated by CPD or breath CO levels (Table 1) are shown for those with only a CYP2A6*1/*1 genotype (excluding all subjects with CYP2A6 genetic variants) and for the total group (including those with variants). The inclusion of study participants carrying known CYP2A6 variants did not alter these results (Table 1).

CPD was most strongly correlated with breath CO levels ($r = 0.60$, $P < 0.001$) followed by plasma cotinine levels ($r = 0.53$, $P < 0.001$; Fig. 1A and B, respectively). Significant but weaker correlations were also found with plasma 3-hydroxycotinine, nicotine, cotinine/nicotine, and (3-hydroxycotinine + cotinine)/nicotine (Table 1). We found no relationship between the 3-hydroxycotinine/cotinine ratio and CPD level (Table 1). Breath CO levels showed the strongest correlation with plasma cotinine ($r = 0.74$, $P < 0.001$; Fig. 1C).

All indices that were significantly related to both CPD and CO levels showed a stronger correlation with the CO level than with reported CPD (Table 1). To investigate whether smokers reporting higher levels of smoking (20 to ≥ 25 CPD) were smoking with decreased intensity, providing one explanation for the plateau observed at the greater CPD levels, we assessed the ratio of CO to cigarette as an indicator of the intensity of inhalation (Fig. 1D). A negative correlation was found between the two measures in the total group ($r = -0.52$, $P < 0.001$); a similar relationship was observed excluding those with CYP2A6 variants ($r = -0.54$, $P < 0.001$).

Indicators of Genotype. The effect of the CYP2A6*12A variant on nicotine metabolism was assessed by the 3-hydroxycotinine/cotinine ratio, an indicator of CYP2A6 activity towards nicotine (9). Individuals with a CYP2A6*1/*12A genotype had lower log 3-hydroxycotinine/cotinine ratios (mean \pm SD) compared with those with a CYP2A6*1/*1 genotype (-0.69 ± 0.13 versus -0.31 ± 0.23 , respectively; $P < 0.001$; Fig. 2). Two subjects with a CYP2A6*12A allele also had additional decreased function alleles; their 3-hydroxycotinine/cotinine ratios were very low, indicating a gene-dose effect on this ratio (Fig. 2).

To investigate the association among CYP2A6 genotype, plasma measures, and metabolite ratios, participants were classified into genotype groups, based on the known *in vivo* and/or *in vitro* effect on enzyme function of each CYP2A6 allele (9, 38-40). Based on our present findings for nicotine and those reported previously for coumarin (29), participants who were heterozygous for the CYP2A6*12A variant were grouped among those expected to have 60% to 70% remaining activity. The four different genotype groups, the expected activity, and the genotypes included in each cluster are described in Table 2.

We found that the 3-hydroxycotinine/cotinine and 3-hydroxycotinine/(cotinine + nicotine) showed the strongest correlations with CYP2A6 genotype group ($r = 0.35$, $P < 0.001$ and $r = 0.38$, $P < 0.001$, respectively; Table 3). Figure 3 illustrates the relationship between CYP2A6 genotype groups and the 3-hydroxycotinine/(cotinine + nicotine) ratios after including the CYP2A6*12A genotyping. Correlations with CYP2A6 genotype group for both 3-hydroxycotinine/cotinine ($r = 0.43$, $P < 0.001$) and for 3-hydroxycotinine/(cotinine + nicotine; $r = 0.46$, $P < 0.001$) were improved, although the difference (with versus without CYP2A6*12A genotyping) in correlation coefficients did not reach significance (Table 3). There was a significant difference in the ANOVA among the four genotype groups ($P < 0.001$), with groups 1 ($P < 0.01$), 2 ($P < 0.001$), and 3 ($P < 0.001$) being significantly lower than group 4, the group without detected variants. The observed effect of each genotype group on activity [3-hydroxycotinine/(cotinine + nicotine); Fig. 3] is consistent with the predicted effects (Table 3) with groups 3, 2, and 1 having 63%, 48%, and 30%, respectively, remaining activity of group 4 (100% activity).

We have previously compared genetically slow metabolizers (those with $\leq 50\%$ activity; our Table 2, groups 1 and 2 combined) with normal metabolizers, those without detected variants (Table 2, group 4; refs. 19, 41). Using plasma ratios, we found that both the log 3-hydroxycotinine/cotinine ratio and the log 3-hydroxycotinine/cotinine + nicotine were significantly lower for slow metabolizers relative to normal metabolizers (-0.65 versus -0.31 , $P < 0.001$; -0.70 versus -0.35 , $P < 0.001$, respectively). The decrease in the ratio was $\sim 50\%$ as expected based on the activity of the slow metabolizers, indicating that this plasma ratio derived from *ad libitum* smoking distinguishes genetically slow and normal metabolic groups.

Effect of Levels of Smoking on CYP2A6 Activity. We tested whether the plasma nicotine levels were inversely related to CYP2A6 activity (indicated by the 3-hydroxycotinine/cotinine ratio) to determine whether *in vivo* CYP2A6 activity was inversely related to plasma nicotine among CYP2A6*1/*1 individuals (excluding all known CYP2A6 genetic variants causing reduced activity). A significant inverse relationship between plasma nicotine and the 3-hydroxycotinine/cotinine ratio was detected ($r = -0.41$, $P < 0.0001$), suggesting that as nicotine plasma levels increase, CYP2A6 activity decreases.

Discussion

The solid-phase extraction HPLC-UV assay for the simultaneous detection of nicotine, cotinine, and 3-hydroxycotinine

Table 1. Pearson's correlation analysis of indices of smoking level measured by levels of CPD and CO

| Index* | CYP2A6*1/*1 only (n = 120) | | Total group (n = 152) | | Between-group comparisons | |
|---|----------------------------|--------|-----------------------|--------|---------------------------|----------------|
| | r | P | r | P | P [†] | P [‡] |
| CPD level | | | | | | |
| CO (ppm) | 0.60 | <0.001 | 0.60 | <0.001 | 1.00 | |
| Nicotine (ng/mL) | 0.46 | <0.001 | 0.46 | <0.001 | 1.00 | |
| Cotinine (ng/mL) | 0.52 | <0.001 | 0.53 | <0.001 | 1.00 | |
| 3-Hydroxycotinine (ng/mL) | 0.47 | <0.001 | 0.47 | <0.001 | 1.00 | |
| Cotinine/nicotine | 0.32 | <0.001 | 0.30 | <0.001 | 0.85 | |
| (3-Hydroxycotinine + cotinine)/nicotine | 0.25 | <0.01 | 0.23 | <0.01 | 0.86 | |
| 3-Hydroxycotinine/cotinine | -0.19 | 0.05 | -0.15 | NS | 0.74 | |
| 3-Hydroxycotinine/(cotinine + nicotine) | -0.15 | NS | -0.11 | NS | 0.74 | |
| CO level | | | | | | |
| Nicotine (ng/mL) | 0.70 | <0.001 | 0.66 | <0.001 | 0.55 | 0.01 |
| Cotinine (ng/mL) | 0.73 | <0.001 | 0.74 | <0.001 | 0.86 | <0.01 |
| 3-Hydroxycotinine (ng/mL) | 0.58 | <0.001 | 0.55 | <0.001 | 0.72 | 0.35 |
| Cotinine/nicotine | 0.30 | <0.001 | 0.30 | <0.01 | 1.00 | 1.0 |
| (3-Hydroxycotinine + cotinine)/nicotine | 0.17 | 0.05 | 0.17 | NS | 1.00 | — |
| 3-Hydroxycotinine/cotinine | -0.34 | <0.001 | -0.32 | <0.001 | 0.86 | — |
| 3-Hydroxycotinine/(cotinine + nicotine) | -0.30 | <0.01 | -0.28 | <0.01 | 0.86 | — |

*All ratios were log transformed for these analyses.

[†]Statistical significance of the differences between correlation coefficients in the CYP2A6*1/*1 group and the total group.

[‡]Statistical significance of differences between correlation coefficients for CPD and CO level. Only indices that were significantly related to both CPD and CO levels were compared.

concentrations in the plasma of *ad libitum* Caucasian smokers was sensitive and reproducible; the between-day variations were within acceptable limits; and the assay remained linear in excess of the necessary ranges of detection. Nicotine and cotinine levels measured by this assay were highly correlated to the nicotine and cotinine levels measured by a traditional liquid-liquid extraction HPLCUV assay. Therefore, the solid-phase extraction HPLC-UV assay can be used to identify and quantify levels of nicotine, cotinine, as well as 3-hydroxycotinine in the plasma of smokers.

We aimed to identify biomarkers for the estimation of smoking levels in CYP2A6*1/*1 individuals and to determine whether the CYP2A6 variants limited the use of these indices. There are billions of smokers worldwide, and cigarette smoking was the cause of nearly five million premature deaths globally in the year 2000 (42). Although smoking has been clearly identified as a direct cause of multiple medical disorders, including cancers, cardiovascular, and respiratory diseases, people continue to smoke. To conduct studies of the pathologies associated with smoking, it is necessary to identify robust biomarkers of smoke exposure.

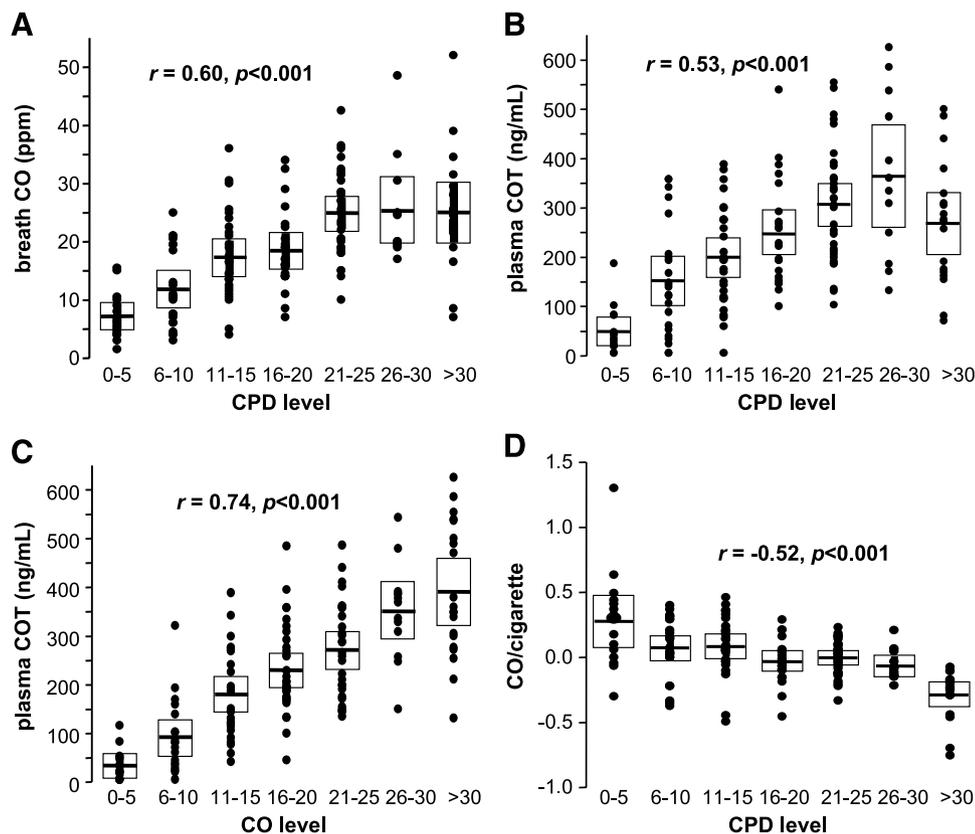


Figure 1. Indices of smoking levels and relationship between smoking intensity (CO per cigarette) and CPD levels ($N = 152$). The numbers on the x-axes (1-7) indicate levels of smoking measured by CPD (A, B, and D) or CO (C) in ppm. The upper and lower limits of the boxes indicate the 95% confidence interval of the mean. The horizontal bars in the boxes indicate the means for each smoking level. Pearson's correlation analysis was used to derive all r s and P s.

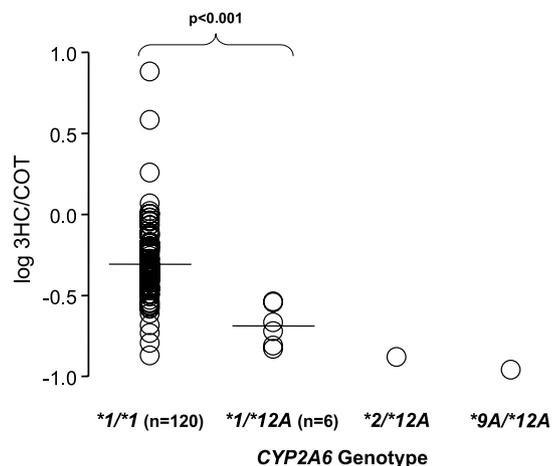


Figure 2. The *CYP2A6**12A variant has reduced enzymatic activity, measured as 3-hydroxycotinine/cotinine (3HC/COT) derived from *ad libitum* smoking. In this aligned scatter plot, each open circle represents the log 3-hydroxycotinine/cotinine ratio for an individual participant. The horizontal bars indicate the mean. Means were compared using an unpaired Student's *t* test. The *CYP2A6**1/*1 group had a greater mean \pm SD log 3-hydroxycotinine/cotinine ratio compared with the *CYP2A6**1/*12A group (-0.31 ± 0.23 versus -0.69 ± 0.13 , respectively; $P < 0.001$).

Multiple measures were tested as possible correlates of CPD in *CYP2A6**1/*1 Caucasian smokers. We found that breath CO and plasma cotinine were most strongly related to CPD level (Table 1; Fig. 1) as others have found (23-25). In addition, consistent with previous data (43, 44), we found that these relationships were not linear over the entire range of CPD but rather tended to plateau at greater levels of CPD (Fig. 1A and B), suggesting that variation in the intensity of inhalation may limit the use of breath CO and plasma cotinine to distinguish among those smoking >20 to 25 CPD. We found that CO per cigarette was inversely related to the level of CPD (Fig. 1D), and that this relationship seemed to be influenced mainly by the two extreme groups (0-5 and >30 CPD), suggesting that the heaviest smokers inhale each cigarette with less intensity compared with those smoking the least CPD. This has also been seen in the relationship between daily cigarette consumption and carboxyhemoglobin levels measured in plasma (45). Our results indicated that plasma cotinine showed the strongest correlation with CO levels. This relationship was greater than that observed with CPD level (Table 1) and remained linear throughout the range of CO levels that were examined (Fig. 1C). Thus, like other studies (23-25, 43, 46), we found that cotinine is a moderately good biomarker, which can be used to confirm self-reported CPD and a good indicator of smoke exposure (CO). In addition, when *CYP2A6* genetic variants were excluded, we found no differences in correlation coefficients between smoking levels (measured by CPD or CO

levels; Table 1). These findings suggest that among Caucasians, genetic variation in *CYP2A6* does not limit the use of plasma cotinine to indicate cigarette consumption or exposure to tobacco toxins. It should be noted that using the current genotyping the frequency of those having any known *CYP2A6* variant is low (~15%) among Caucasians. However, it is very likely that *CYP2A6* genetic variation will influence indicators of smoking level, such as cotinine, among ethnic groups where the frequencies of these variants are substantially higher, such as among Japanese and Chinese populations (19, 47).

The 3-hydroxycotinine/cotinine ratio, derived from urinary measures of cotinine and 3-hydroxycotinine, has been shown previously to correlate with the amount of cigarette consumption during *ad libitum* smoking (48), suggesting that rates of nicotine metabolism and clearance, at least in part, affect the amount smoked (19, 20, 49). In the present study, we found a relationship between 3-hydroxycotinine/cotinine and CPD level but only among the group without *CYP2A6* variants; the relationship was similar but not significant for the total group (Table 1). One notable difference between these two studies is the use of a urinary 3-hydroxycotinine/cotinine ratio in the former study (48) versus the plasma ratio used in the present study. It may be that the urinary ratio is a better measure of *CYP2A6* activity and therefore more closely related to CPD, as it controls for differences in rates of glucuronidation. Recently, we have found that genetically slow metabolizers have a reduced plasma 3-hydroxycotinine/cotinine ratio and lower levels of smoking compared with those without variants (41); however, a correlation across the entire group was not attempted in this analysis. In addition to the use of a plasma metabolic ratio, another reason for the apparent discordance between the 3-hydroxycotinine/cotinine ratio and CPD may be that individuals smoke with varying intensities (50). We have shown that *CYP2A6* genetically slow metabolizers reduce their smoking both through a reduction in CPD (18, 19) and also through decreased intensity, specifically by taking smaller mean puff volumes, resulting in a reduced overall puff volume (51). Thus, it is possible that differences in smoking topography may have masked the association between 3-hydroxycotinine/cotinine and CPD. This relationship would be poorer when slow metabolizers are included as they adjusted their smoking using intensity as well as CPD; this difference in intensity may account for the better relationship between the 3-hydroxycotinine/cotinine ratio and the *CYP2A6**1/*1 rather than the whole group (Table 1). This explanation is supported by the substantially better (2-fold) and significant relationships seen between the 3-hydroxycotinine/cotinine and 3-hydroxycotinine/(cotinine + nicotine) ratios and CO levels (Table 1), in both the *CYP2A6**1/*1 group and the total group, as this measure includes differing amounts and intensities of smoking.

Our second objective was to study the effect of the *CYP2A6**12A variant and investigate the relationships between *CYP2A6* genotype groups and nicotine, its metabolites, and metabolite ratios. We found that the *CYP2A6**12A variant resulted in reduced *CYP2A6* activity towards nicotine, measured

Table 2. Definition of *CYP2A6* groups, distribution of specific genotypes in each group, and predicted effect on enzyme function

| Groups | Definition | <i>CYP2A6</i> genotypes (n) | Predicted remaining <i>CYP2A6</i> activity*(%) |
|--------|---|---|--|
| 1 | Two inactive (*2 or *4) or one inactive and one reduced activity (*9A or *12A) variants | *2/*12 (1), *4/*7 (1) | <40 |
| 2 | One inactive (*2 or *4) or two reduced activity (*9 and/or *12) variants | *1/*2 (4), *1/*4 (2), *9A/*9A (1), *9A/*12A (1) | 40-50 |
| 3 | One reduced activity (*9 or *12) variant | *1/*9A (16), *1/*12A (2) | 60-70 |
| 4 | No variants | *1/*1 (120) | 100 |

*Remaining activity was predicted based on each *CYP2A6* genotype and the known *in vivo* and/or *in vitro* effect of each allele.

Table 3. Pearson's correlation analysis of CYP2A6 genotype groups with plasma drug and metabolite levels and ratios

| Index* | CYP2A6 genotype groups with no *12A | | CYP2A6 genotypes groups with *12A | | P [†] |
|---|-------------------------------------|--------|-----------------------------------|--------|----------------|
| | r | P | r | P | |
| Nicotine (ng/mL) | -0.14 | NS | -0.22 | 0.01 | 0.47 |
| Cotinine (ng/mL) | 0.01 | NS | -0.02 | NS | 0.80 |
| 3-Hydroxycotinine (ng/mL) | 0.18 | 0.02 | 0.21 | 0.01 | 0.79 |
| Cotinine/nicotine | 0.16 | 0.04 | 0.18 | 0.01 | 0.86 |
| (3-Hydroxycotinine + cotinine)/nicotine | 0.26 | <0.01 | 0.30 | <0.001 | 0.70 |
| 3-Hydroxycotinine/cotinine | 0.35 | <0.001 | 0.43 | <0.001 | 0.42 |
| 3-Hydroxycotinine/(cotinine + nicotine) | 0.38 | <0.001 | 0.46 | <0.001 | 0.40 |

*All ratios were log transformed for these analyses.

†Statistical significance of differences between correlation coefficients before and after inclusion of the CYP2A6*12A variant.

by the 3-hydroxycotinine/cotinine ratio, consistent with its effect on protein levels and coumarin 7-hydroxylase activity (29).

CYP2A6 genetic variants alter rates of nicotine and cotinine metabolism and resulting plasma levels of nicotine, cotinine, and 3-hydroxycotinine (9, 12, 13); genotyping has been used in a variety of epidemiologic association studies to investigate their role in smoking behaviours and nicotine dependence (18, 19, 52). However, the relationship between CYP2A6 genotype groups and plasma measures and metabolite ratios has not been assessed. Here, we found that during *ad libitum* smoking, in the absence of controlling the timing of the last cigarette, both plasma 3-hydroxycotinine/cotinine and 3-hydroxycotinine/(cotinine + nicotine) metabolite ratios were associated with the predicted activity of the CYP2A6 genotype groups. In addition, these relationships were improved after taking into consideration the CYP2A6*12A variant, although not statistically significantly. Using genotype grouping of slow versus normal metabolizer as previously described ($\leq 50\%$ activity versus 100% activity; refs. 18, 19, 51), we found that both log 3-hydroxycotinine/cotinine and 3-hydroxycotinine/cotinine + nicotine ratios distinguished these genotype groups. Taken together, these findings suggest that as novel CYP2A6

variants are identified and characterized, which has been occurring rapidly during the past few years, the group separation will improve. The 3-hydroxycotinine/(cotinine + nicotine) ratio showed a slightly better relationship with CYP2A6 genotype groups compared with the 3-hydroxycotinine/cotinine ratio. It might have been expected that the 3-hydroxycotinine/cotinine ratio would have shown the strongest relationship with CYP2A6 genotype as plasma nicotine levels are highly influenced by the time to last cigarette, and inclusion of nicotine might have weakened the relationship. However, the slight improvement observed with 3-hydroxycotinine/(cotinine + nicotine) compared with 3-hydroxycotinine/cotinine may be attributed to the role that CYP2A6 plays in the metabolism of nicotine to cotinine. Some genetic variants may differ slightly in their effect on the two pathways, therefore accounting for both may improve the relationship.

The 3-hydroxycotinine/(cotinine + nicotine) ratios among the four genotype groups suggested that the predicted effect on function was reasonable (Table 3) and supports the use of these groupings in genetic epidemiology studies (19, 52). In contrast, the cotinine/nicotine ratio was not related to CYP2A6 genotype group when measured during *ad libitum* smoking (Table 3), likely due to the sensitivity of this ratio to the last exposure of nicotine as a result of its short half-life. In addition, it is possible that enzymes such as CYP2B6 may contribute to the C-oxidation of nicotine (53), whereas the metabolism of cotinine to 3-hydroxycotinine seems to be almost essentially mediated by CYP2A6 (3, 9), making the ratio of the metabolites a more specific probe for CYP2A6 activity.

Although we found reasonable relationships with genotype for both the 3-hydroxycotinine/cotinine and 3-hydroxycotinine (cotinine + nicotine) ratios, there was a considerable amount of scatter within the genotype groups (Fig. 3), particularly among the group without known genetic variants (100% group). The very high values in this group suggest the possibility of increased function alleles, such as an alternative form of the gene duplication, or increased transcriptional variants not yet characterized. In addition to untested and unidentified genetic variants in CYP2A6 (<http://www.imm.ki.se/CYPalleles/cyp2a6.htm>), additional factors that may have limited the correlation include gender, CYP2A6 inducers (oral contraceptives), and inhibitors (grape fruit juice and menthol; ref. 6). However, given that the objective of this study was to observe these relationships during *ad libitum* smoking (relative to traditional phenotyping), we found fairly strong relationships between these metabolite ratios and CYP2A6 genotype group.

Lastly, we investigated the relationships between CYP2A6 activity (3-hydroxycotinine/(cotinine)) among the CYP2A6*1/*1 group without detected CYP2A6 genetic variants and nicotine plasma levels. Previous data suggest that smoking reduces nicotine clearance, and that this may be due to nicotine rather than CO or cotinine (31-33). CYP2A6 activity showed an inverse relationship with plasma nicotine, suggesting that

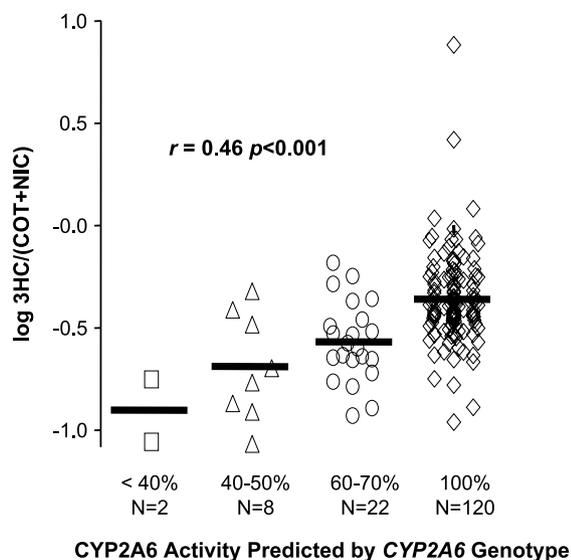


Figure 3. CYP2A6 genotype groups are related to the log 3-hydroxycotinine/(cotinine + nicotine) ratio derived from *ad libitum* smoking; the relationship improves following detection of the CYP2A6*12A allele. The horizontal bars indicate the mean log ratio for each genotype group, whereas N indicates the number of people per group. ANOVA ($P < 0.001$) followed by Bonferonni's multiple comparison test indicates that each group (1, 2, and 3) is significantly different from group 4.

increased levels of nicotine predicted reduced CYP2A6 activity. This inverse relationship tended to be stronger with nicotine than CO levels, suggesting that the reduction of CYP2A6 activity may be mainly due to levels of nicotine exposure itself rather than an alternative component of cigarette smoke. This is consistent with the observations in a primate model where we found that chronic nicotine treatment alone (no other smoke constituents) reduced CYP2A6 protein levels and activity (33).

Together, these findings confirm the use of plasma cotinine and CO as indicators of Caucasians' smoking levels, and that this is not limited by CYP2A6 genetic variation likely due to the low frequency of these variants in Caucasians and due to the early stage of gene variant identification and characterization at the current time. The data also indicate that 3-hydroxycotinine/cotinine and 3-hydroxycotinine/(cotinine + nicotine) are moderately good indicators of CYP2A6 genotype, which is likely to increase in strength as new variants are identified. Lastly, the data support the finding that smokers have slower nicotine metabolism, and that this may be related to nicotine exposure, resulting in a reduced rate of nicotine metabolism through either regulatory mechanisms or perhaps by acting as a suicide substrate.

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BLOOD CANCER DISCOVERY

CYP2A6 Genotype, Phenotype, and the Use of Nicotine Metabolites as Biomarkers during *Ad libitum* Smoking

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