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ORIGINAL ARTICLE

Impact of *CYP2A6* genotype on pretreatment smoking behaviour and nicotine levels from and usage of nicotine replacement therapy

V Malaiyandi[1], C Lerman[2], NL Benowitz[3,4], C Jepson[2], F Patterson[2] and RF Tyndale[1]

[1]*Centre for Addiction and Mental Health and Department of Pharmacology, University of Toronto, Toronto, ON, Canada;* [2]*Department of Psychiatry and Abramson Cancer Center, Transdisciplinary Tobacco Use Research Center, University of Pennsylvania, Philadelphia, PA, USA and* [3]*Division of Clinical Pharmacology and Experimental Therapeutics, Medical Service, San Francisco General Hospital Medical Center, University of California, San Francisco, CA, USA and* [4]*Departments of Medicine, Psychiatry and Biopharmaceutical Sciences, University of California, San Francisco, CA, USA*

We investigated the effect of slow metabolism of nicotine, predicted by *CYP2A6* genotypes resulting in ⩽50% activity, on baseline smoking behaviours and treatment variables in an open-label nicotine replacement therapy (NRT) clinical trial. Caucasian smokers with CYP2A6 slow vs normal metabolism had lower metabolic activity, indicated by the 3-hydroxycotinine/cotinine ratio (0.23±0.17 vs 0.45±0.22, $P<0.01$, respectively). CYP2A6 slow metabolizers also smoked fewer cigarettes per day compared to normal metabolizers (20±7 vs 24±10, respectively, $P<0.04$). With nicotine patch use, slow metabolizers had higher nicotine plasma levels compared to normal metabolizers (22.8±4.6 vs 15.8±7.6 ng/ml, respectively, $P=0.02$) while using the same numbers of patches/week. With nicotine spray use, where like in smoking the nicotine intake can be easily adjusted to adapt to rates of metabolism, slow metabolizers achieved similar nicotine levels compared to normal metabolizers (5.8±4.1 vs 8.0±9.1 ng/ml, $P=0.82$), by using fewer doses of nicotine spray/day (4.8±3.6 vs 10.5±8.0, respectively, $P<0.02$). These findings indicate that *CYP2A6* genotype influences smoking behaviour in a Caucasian treatment-seeking population and that *CYP2A6* genotype affects plasma levels obtained from, and usage of, NRT.
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Introduction

Approximately 70–80% of nicotine consumed during smoking is metabolically inactivated to cotinine,[1] and roughly 90% of this conversion is mediated by CYP2A6.[2,3] Cotinine is subsequently oxidized, a step almost entirely catalyzed by CYP2A6 *in vivo* and *in vitro*, to form *trans*-3′-hydroxycotinine.[4,5] Presently, there are more than 20 known variants of the *CYP2A6* gene and these are listed on the *CYP2A6* Allele Nomenclature website, http://www.imm.ki.se/CYPalleles/cyp2a6.htm. Some of these have been characterized *in vitro* and/or *in vivo*, and result in absent, reduced, increased or normal enzyme activity. Data from our laboratories[6–8] indicate that the *CYP2A6* genotypes result in the expected increase and decrease in CYP2A6 enzymatic function. In addition, the 3-hydroxycotinine/cotinine (3HC/COT) ratio, derived from labelled nicotine and cotinine given orally ($n=62$), and in a small group of smokers ($n=14$) measured from natural nicotine, shows a good correlation with nicotine clearance, indicating its utility as an indicator of CYP2A6 activity.[4] One aim of the present study was to determine the impact of *CYP2A6* genotype on CYP2A6 activity measured by the 3HC/COT ratio in a larger number of smokers, using nicotine derived from pre-treatment baseline smoking rather than oral nicotine.

Factors potentially influencing nicotine plasma levels, such as high- vs low-yield cigarettes or different rate of removal from the body (e.g. acidification of the urine to increase nicotine clearance), have been shown to affect smoking behaviours.[9,10] Variability in the rate of nicotine metabolism owing to *CYP2A6* genetic polymorphisms may also influence nicotine plasma levels,[6,8,11] and a number of studies in adults have demonstrated that *CYP2A6* genetic variation, causing reduced or absent enzyme activity, is associated with a reduced risk of being a smoker, lower amount smoked, altered smoking intensity and

Correspondence: Dr RF Tyndale, Centre for Addiction and Mental Health and Department of Pharmacology, University of Toronto, Room 4326 Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8.
E-mail: r.tyndale@utoronto.ca
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increased quitting.[8,12–17] However, not all studies provide consistent results.[18–20]

Investigations of the impact of *CYP2A6* genotype on smoking behaviours usually recruit participants from the general smoking population or from cancer and health institutes.[12,14,15,17,21,22] Smokers who seek treatment, compared to the general smoking population, are more often heavier smokers, have higher Fagerstrom Test for Nicotine Dependence (FTND) scores, indicating greater nicotine dependence, and are more likely to be women, older in age and have higher levels of education.[23,24] In addition, smokers enrolled in cessation studies may differ from the general smoking population owing to the eligibility criteria (e.g. the amount smoked). The effect of *CYP2A6* genotype on smoking behaviours in Caucasian treatment seekers has not been investigated previously.

First-line drug treatments presently recommended for smoking cessation include nicotine replacement therapy (NRT) and bupropion.[25] The goal of NRT is to replace the nicotine normally acquired from smoking in a safe manner, without delivering toxins and carcinogens that are present in tobacco smoke, in order to alleviate withdrawal symptoms following smoking abstinence.[26,27] Limitations of NRT as a treatment for smoking cessation include the slower rate of nicotine delivery compared to smoking and lower levels of nicotine obtained from these delivery systems (i.e. under-dosing) compared to levels obtained with smoking.[28–30] Although several studies have shown that *CYP2A6* genetic variation alters the rate of nicotine metabolism,[6,8,11,31,32] the effect of these variants on plasma nicotine levels obtained with NRT in a clinical trial setting has not been determined. This is important to study as increasing the amount of nicotine replaced by NRT has been shown to improve abstinence rates and alleviate withdrawal symptoms, particularly on the nicotine patch.[33,34]

We investigated four *CYP2A6* genetic variants, *CYP2A6*2*, *CYP2A6*4*, *CYP2A6*9A* and *CYP2A6*12A*, which are common among Caucasians,[15] and have a known detrimental impact on enzyme function.[4,11,35–37] To assess whether *CYP2A6* genetic variants affect enzyme activity, smoking behaviours and plasma levels from treatments, *CYP2A6* activity groups were defined based on the impact of each *CYP2A6* allele.[8,11,35,37–39] Thus, our study aimed to assess the impact of *CYP2A6* genotypes on: (1) CYP2A6 activity using the 3HC/COT ratio derived from baseline smoking, (2) on pre-treatment baseline smoking behaviour in a treatment-seeking group and (3) on usage of nicotine patch and spray and resultant plasma nicotine and cotinine levels obtained.

Materials and methods

CYP2A6 genotyping

CYP2A6 primers were ordered from ACGT Corporation (Toronto, ON, Canada). Reagents used in the assays were purchased as a kit from Fermentas (Burlington, ON, Canada) with the *Taq* polymerase enzyme. The kit included *Taq* polymerase, 25 mM $MgCl_2$, 10 × *Taq* polymerase chain reaction (PCR) buffer containing 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl and 0.8% Nonidet P40. The dNTPs and 1-kb Gene Ruler® DNA ladder were also purchased from Fermentas (Burlington, ON, Canada). The dNTP set included 25 mM of each nucleotide. PCR amplifications were carried out on PTC-200 Peltier Thermal Cycler (BioRad, Toronto, ON, Canada). Agarose was purchased from ONBIO Inc. (Richmond Hill, ON, Canada). Two-step allele-specific PCR genotyping assays for *CYP2A6*2*, *CYP2A6*4*, *CYP2A6*9A* and *CYP2A6*12A* variants were conducted as described previously.[15,40]

Genotypes were grouped, as described previously,[15] based on the impact of the genetic variants into normal (100% activity), intermediate (75% activity) or slow (50% or less activity). Intermediate metabolizers included those having either one *CYP2A6*9A* or one *CYP2A6*12A* allele. Slow metabolizers included individuals having one or two copies of the inactive variants (*CYP2A6*2* and *CYP2A6*4*) or having two *CYP2A6*9A* and/or *CYP2A6*12A* alleles. Normal metabolizers included those individuals who did not have any of these four variants.

Participant recruitment and screening

Participants were recruited in Washington, DC and Philadelphia as described previously.[41,42] Participants completed an initial eligibility screening visit during which they received a brief medical history screening and physical exam by a registered nurse. Individuals meeting Diagnostic Statistical Manual 4th edition (DSM IV) criteria for psychiatric disorders or substance abuse other than smoking were not eligible to participate. Participants must have been at least 18 years of age and reported smoking at least 10 cigarettes per day for the past 12 months. Only those of Caucasian ethnicity (65% of those enrolled) were included in this analysis to reduce population stratification owing to interethnic variation in *CYP2A6* and other nicotine-metabolizing pathways such as those involved in glucuronidation.[15,43] A total of 394 Caucasian participants were included in the final analyses; of these, 193 received the nicotine patch and 201 received the nicotine nasal spray (Table 1).

Clinical trial design

This open-label clinical trial was conducted over a 12-month period including follow-up. Participants who met the eligibility criteria and provided consent were randomized to either the nicotine patch (Nicoderm® Glaxo Smith Kline, Research Triangle Park, NC, USA) or the nicotine nasal spray (Nicotrol® Pharmacia, Helsingborg, Sweden). NRT was initiated on the target quit date and delivered for 8 weeks, and all participants in both treatment arms received behavioural smoking cessation group counselling according to a standardized protocol.[41]

Table 1 Baseline participant characteristics for the nicotine patch, nicotine spray and the total group

Characteristic	*All*, n = *394*		*Nicotine patch*, n = *193*		*Nicotine spray*, n = *201*		P-*value*[a]
	Mean	*s.d.*	*Mean*	*s.d.*	*Mean*	*s.d.*	
Age (years)	46.7	11.3	46.8	11.8	46.6	10.8	0.87
Body mass index	27.5	5.4	27.3	5.4	27.7	5.5	0.56
FTND score	5.5	2.2	5.4	2.2	5.6	2.3	0.45
Cigarettes/day	24	9	23	9	24	9	0.34
Smoking duration (years)	30.3	11.5	30.3	11.9	30.3	11.2	0.98
Baseline nicotine (ng/ml)	17.2	9.6	17.3	9.2	17.1	10.0	0.82
Baseline cotinine (ng/ml)	252	121	247	111	256	130	0.60
Baseline 3-hydroxycotinine (ng/ml)	100	57	101	52	99	62	0.35
% Female	47		49		50		0.15
% College level education	52		50		54		0.37
% CYP2A6 NM	78		78		79		0.16
% CYP2A6 SM	6		4		8		
% CYP2A6 IM	16		18		14		

FTND, Fagerstrom Test for Nicotine Dependence; NM, normal metabolizer; SM, slow metabolizer; IM, intermediate metabolizer.

[a]Comparisons between the nicotine patch and nicotine spray treatment groups. The χ^2 test was used to compare categorical data between the two treatments. The Mann–Whitney test was used to compare group differences in baseline 3-hydroxycotinine plasma levels. Unpaired Student's *t*-tests were used to compare all other variables.

Plasma levels of nicotine, cotinine and 3-hydroxycotinine were measured by liquid chromatography–tandem mass spectrometry as described previously.[4] These levels were assessed at baseline to determine whether *CYP2A6* genotypes were related to CYP2A6 activity using the metabolite ratio (3HC/COT) described previously.[4] Plasma nicotine and cotinine levels were also measured 1 week following treatment initiation to determine the amount of nicotine and cotinine obtained from NRT. Participants provided a breath carbon monoxide (CO) sample at 1 week post-quit and those having breath CO values of $\leqslant$10 p.p.m. were considered as abstinent. Individuals having aberrant data values (>6 standard deviations (s.d.) from the genotype group mean) were excluded from CYP2A6 activity and post-treatment plasma analyses. These exceptionally high values may be a result of the presence of unknown inducers (e.g. oral contraceptives), unidentified *CYP2A6* gene variants[44,45] or a result of technical/methodological error. The exclusions included two individuals, one normal and one slow metabolizers, having 3HC/COT values of 16.1 (>10 s.d.) and 1.2 (>6 s.d.), respectively, who were removed from the CYP2A6 activity analysis. From the post-treatment plasma analyses, two individuals (both normal metabolizers for the gene variants tested), one in the nicotine patch and one in the spray group with plasma nicotine levels of 98ng/ml (>10 s.d.) and 118ng/ml (>10 s.d.), respectively, were also excluded. Exploratory analysis of side effect scores did not indicate toxicity. Such levels of nicotine, particularly on the nicotine patch, are likely owing to unidentified gene variants resulting in reduced enzyme activity. Individual with unidentified genetic variants would remain among the normal metabolizer genotype group.

Participants were asked to keep a daily log of their usage of NRT in a diary format and forms were collected at each weekly counselling session. For each week of treatment, nicotine patch usage was determined as the number of days the patch was worn in that week and nicotine spray usage was calculated as the average number of sprays doses (one dose = two sprays) used per day in that week. Analyses focused on usage during the first week on NRT, which corresponds to the time of blood sampling for post-treatment measures of plasma nicotine and cotinine.

Statistical analyses

To assess whether *CYP2A6* genotypes followed a Hardy–Weinberg distribution and whether allele frequencies in this study were similar to those reported in the literature, the χ^2 test was used. The Fisher's exact test was used when the criteria for the χ^2 test were not met. Categorical and continuous population characteristics were compared between the treatment groups (patch vs spray) using the χ^2 test or the Student's unpaired *t*-test, respectively. Covariates were compared across the three genotype groups by analysis of variance (ANOVA) and also between normal and slow groups alone by Student's unpaired *t*-test. CYP2A6 activity was compared across the three genotype groups by ANOVA followed by the Bonferroni multiple comparisons test. In all subsequent analyses (cigarettes per day, FTND components, post-treatment plasma measures and treatment usage),

only slow and normal metabolizers were compared. F-tests for the baseline plasma cotinine between group comparison (patch vs spray), for the cigarettes per day between genotype group comparison (slow vs normal) and for post-treatment nicotine spray plasma and usage between genotype group comparisons (slow vs normal) indicated that the variances were significantly different among groups. As such, these variables were log transformed to correct for the non-normality of the data. The Mann–Whitney test was used to compare baseline 3-hydroxycotinine between treatment groups as log transformation did not correct for unequal variances among groups. All statistical analyses were conducted using GraphPad Prism version 4.03 (San Diego, CA, USA).

Results

CYP2A6 *allele frequencies and baseline participant characteristics*

The allele frequencies for *CYP2A6*2*, *CYP2A6*4*, *CYP2A6*9* and *CYP2A6*12* alleles in this treatment-seeking population (Table 2) were similar to those reported in a large Caucasian study, except for *CYP2A6*4*.[15] The difference in *CYP2A6*4* allele frequencies is likely owing to the low frequency of this allele in Caucasians and the smaller size of the current study. *CYP2A6* genotypes followed a Hardy–Weinberg distribution.

There were no significant differences in the baseline participant characteristics between the nicotine patch and nicotine spray treatment groups, including the frequency of the genotype groups (Table 1). There were also no differences in age, baseline FTND score, body mass index, smoking duration, age of smoking initiation, baseline plasma nicotine and cotinine measures, or marital status by *CYP2A6* genotype group (Table 3). Compared to normal metabolizers, slow metabolizers were significantly more likely to be female and significantly less likely to have a college level education; they also had significantly lower baseline plasma 3-hydroxycotinine levels (Table 3).

CYP2A6 activity (3HC/COT)

The CYP2A6 activity (3HC/COT ratio) for participants in each *CYP2A6* genotype group is shown in Figure 1. The mean 3HC/COT ratio for normal metabolizers was significantly different from the slow metabolizer group ($P<0.01$); however, no differences were detected between the normal and intermediate or intermediate and slow genotype groups ($P>0.05$). As the intermediate genotype group was not significantly lower/higher in CYP2A6 activity, compared to normal/slow metabolizers, the focus of the remaining comparisons in this treatment-seeking group was between normal and slow metabolizers, paralleling our previous study of these genotype groups on smoking behaviours in a non-treatment-seeking population.[15]

Smoking behaviour

The number of cigarettes smoked per day at baseline was 24 ± 10 and 20 ± 7 for normal and slow metabolizers, respectively (Figure 2), indicating that slow metabolizers smoked fewer cigarettes per day than normal metabolizers ($P<0.04$). There were no differences in smoking duration or age of initiation between *CYP2A6* genotype groups (Table 3).

Differences between CYP2A6 slow and normal metabolizers were assessed for two items in the FTND: 'How soon after waking do you smoke the first cigarette?' and 'Which cigarette would you hate most to give up?' These two items were selected as they may be related to the rate of nicotine metabolism. To assess the effect of *CYP2A6* genotype group on the time to first cigarette, we examined two categories (the 0–5 and 6–30 min) within this item of the FTND as the majority ($\approx$80%) of normal and slow metabolizers in this study reported smoking within 30 min after waking. Slow metabolizers tended to be less likely to smoke within 0–5 min compared to normal metabolizers (OR = 0.48 (95% CI = 0.2–1.3) $P=0.07$), but this finding did not reach significance (Figure 3). Among slow metabolizers, only 6/19 (32%) reported smoking within the first 5 min of waking, whereas 13/19 (68%) smoked between 6 and 30 min after waking.

Table 2 *CYP2A6* allele frequencies observed in the present study compared to those reported previously

CYP2A6 *allele*	*Present study*		*Schoedel et al. (2004)*		P-*value*[b]
	Number of alleles[a]	*Observed frequency % (±95% CI)*	*Number of alleles*[a]	*Frequency % (±95% CI)*	
**2*[c]	788	2.0 (±0.9)	2300	2.2 (±0.3)	0.78
**4*	772	0.13 (±0.12)	2336	1.2 (±0.2)	0.01
**9A*	766	8.0 (±1.9)	1856	7.1 (±0.6)	0.45
**12A*	766	2.1 (±1.1)	1400	2.0 (±0.4)	0.72

[a]The number of individuals successfully genotyped for *CYP2A6*2*, *CYP2A6*4*, *CYP2A6*9A* and *CYP2A6*12A* alleles.
[b]χ^2 test or the Fisher's exact tests were used to compare the observed alleles to those reported by Schoedel *et al.* (2004).
[c]Only those having a *CYP2A6* genetic variant are included in the table. The remainder of the population did not have any of the genetic variants tested and were categorized as normal metabolizers.

Table 3 Baseline characteristics and covariates by *CYP2A6* genotype groups

Characteristic	*Normal*, n = *309* *Mean ± s.d.*	*Intermediate*, n = *63* *Mean ± s.d.*	*Slow*, n = *22* *Mean ± s.d.*	*ANOVA* P-*value*	*Unpaired* t-*test NM vs SM* P-*value*[a]
Age (years)	46.8 ± 11.2	44.8 ± 11.1	49.8 ± 12.1	0.18	0.28
BMI	27.5 ± 5.6	27.7 ± 5.0	26.7 ± 4.2	0.76	0.51
FTND score	5.6 ± 2.3	5.5 ± 2.1	5.4 ± 1.8	0.89	0.69
Smoking duration (years)	30.3 ± 11.3	28.9 ± 12.1	33.4 ± 12.6	0.29	0.11
Age of smoking initiation (years)	16.5 ± 3.3	15.9 ± 3.2	16.5 ± 3.3	0.47	0.97
Baseline nicotine (ng/ml)	16.6 ± 9.6	19.3 ± 9.6	18.8 ± 9.5	0.09	0.30
Baseline cotinine (ng/ml)	250 ± 123	271 ± 105	222 ± 130	0.22	0.30
Baseline 3-hydroxycotinine (ng/ml)	105 ± 58	89 ± 52	58 ± 44	< 0.01	< 0.01
				χ^2 *test*	
				P-*value*	P-*value*[a]
% Female	47	35	73	< 0.01	0.02
% College education	53	54	27	0.06	0.02
% Married	47	62	41	0.08	0.56

BMI, body mass index; FTND, Fagerstrom Test for Nicotine Dependence; NM, normal metabolizer; SM, slow metabolizer.
[a]Comparisons between slow and normal metabolizers alone.

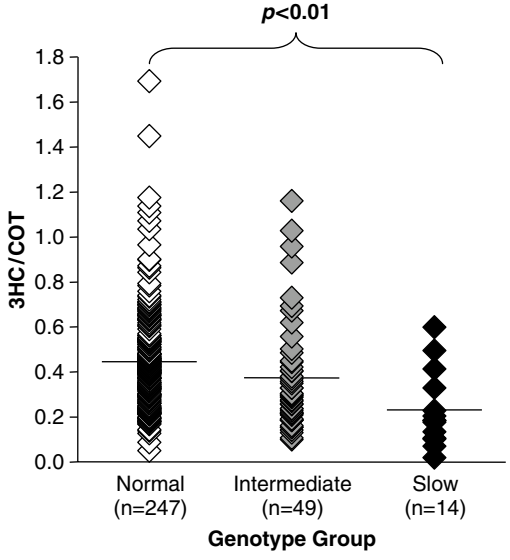

Figure 1 *CYP2A6* genotype groups alter CYP2A6 activity measured by the 3HC/COT ratio. Each point indicates the ratio for an individual. The horizontal bars indicate the mean for normal, intermediate and slow metabolizers (mean ± s.d. of 0.45 ± 0.22, 0.37 ± 0.25 and 0.23 ± 0.17, respectively). ANOVA followed by the Bonferroni multiple comparisons test was used to compare the three genotype groups.

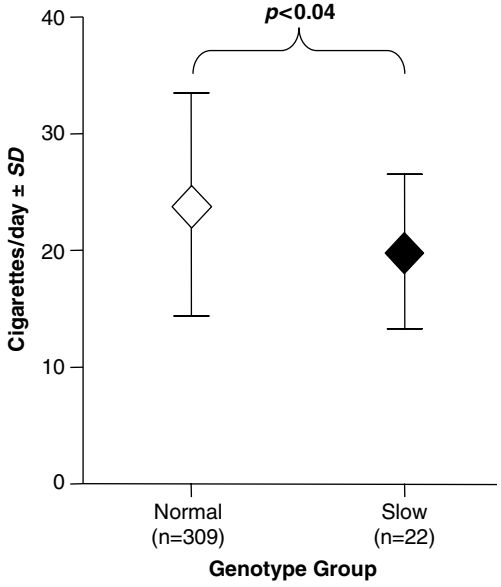

Figure 2 *CYP2A6* slow metabolizes smoked fewer cigarettes per day during pretreatment smoking. The self-reported mean numbers of cigarettes smoked per day ± s.d. at baseline is shown for normal and slow metabolizers. The data were log transformed for statistical analysis, following a failed F-test, and means were compared by the Student's unpaired *t*-test. Slow metabolizers smoked fewer cigarettes per day (20 ± 7) than normal metabolizers (24 ± 10) ($P < 0.04$). Untransformed means are shown and reported for clarity.

Among normal metabolizers, 118/241 (49%) smoked within 5 min and 123/241 (51%) within 6–30 min of waking. We found no difference in the proportions of slow and normal metabolizers willing to forgo the first cigarette of the day at whatever time this occurred (Figure 3).

Plasma levels obtained from NRT and treatment usage 1 week post-quit

Plasma nicotine and cotinine were measured 1-week post-quit (after 1 week of using NRT), a time when

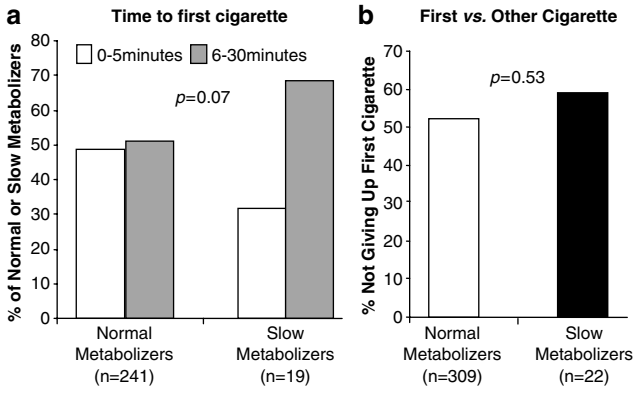

Figure 3 *CYP2A6* genotype tended to alter time to first cigarette, but not the willingness to give it up. (**a**) The percent of normal and slow metabolizers in each time to first cigarette category is shown. Slow metabolizers tended to be less likely to smoke within 5 vs 6–30 min of waking compared to normal metabolizers, OR = 0.48 (95% CI = 0.2–1.3, $P = 0.07$). χ^2 tests were used to compute the OR for this analysis. (**b**) The percent of slow and normal metabolizers who did not want to give up the first cigarette of the day based on the FTND question 'Which cigarette would you hate most to give up?' There were no differences ($\chi^2 = 0.40$, df = 1, $P = 0.53$) between the two groups.

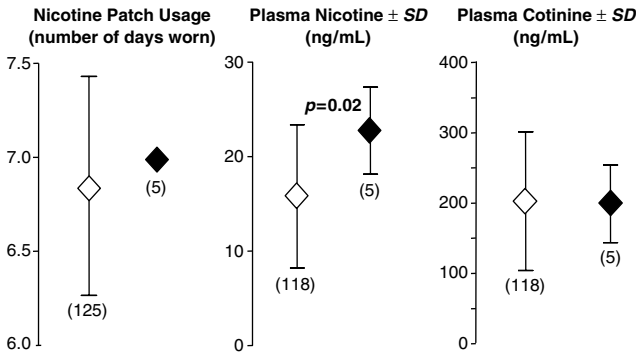

Figure 4 Nicotine patch: plasma nicotine, cotinine and usage by *CYP2A6* genotype groups. The mean values for nicotine patch usage, plasma nicotine and cotinine are indicated by open diamonds for the normal group and filled diamonds for the slow group. The bars represent ±s.d. of the mean, and the number of individuals in each group is indicated in parentheses. The number of days the nicotine patch was worn during the first week on treatment was the same ($P = 0.56$) for normal (6.8 ± 0.6) and slow (7.0 ± 0.0) metabolizers. The mean ± s.d. plasma nicotine levels differed for slow and normal metabolizers (22.8 ± 4.6 and 15.8 ± 7.6, respectively, $P = 0.02$). Plasma cotinine levels were not different between the two groups ($P = 0.92$) and were 207 ± 95 ng/ml in normal and 202 ± 53 ng/ml in slow metabolizers. Student's unpaired *t*-tests were used to compare means between slow and normal metabolizers.

steady-state nicotine and cotinine levels were expected (over five half-lives for both). Only participants who were confirmed as abstinent (breath CO ⩽10 p.p.m. to avoid cigarette smoke-derived nicotine and cotinine) and reported usage of NRT during the first week on treatments were included in the post-treatment plasma analyses. All participants who reported a value for nicotine patch or spray use 1 week post-quit (including those reporting zero) were included in the analysis of NRT use.

In the nicotine patch group, as illustrated in Figure 4, there was no difference ($P = 0.56$) in the reported usage of nicotine patch between normal (6.8 ± 0.6 days) and slow (7.0 ± 0.0 days) metabolizers. Plasma nicotine levels were significantly greater in slow metabolizers (22.8 ± 4.6 ng/ml) than in normal metabolizers (15.8 ± 7.6 ng/ml, $P = 0.02$). Plasma cotinine levels were not different ($P = 0.92$) in normal and slow metabolizers. Taken together, these findings indicate that the elevated levels of nicotine in the slow metabolizers was owing to reduced metabolism and not to lower nicotine intake.

For those in the nicotine spray group (Figure 5), slow metabolizers (4.8 ± 3.6) reported using significantly ($P < 0.02$) fewer doses/day than normal metabolizers (10.5 ± 8.0). This finding remained significant ($P < 0.02$) even after excluding those reporting zero usage (data not shown). Plasma nicotine levels were not significantly different ($P = 0.82$) between normal and slow metabolizers. Plasma cotinine levels tended ($P = 0.07$) to be lower among slow compared to normal metabolizers. Reduced nicotine spray use among slow compared to normal metabolizers is consistent with

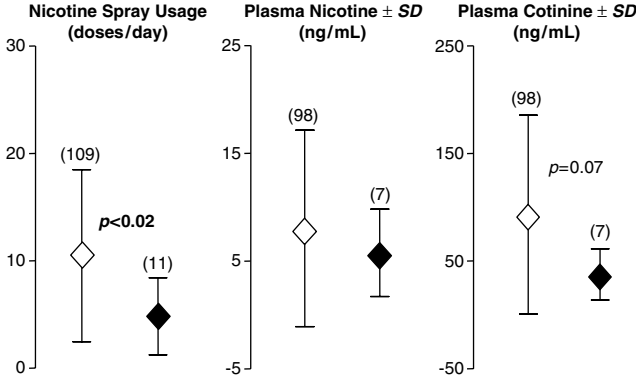

Figure 5 Nicotine spray: plasma nicotine, cotinine and usage by *CYP2A6* genotype groups. Usage and plasma data were log transformed to correct for unequal variance among groups indicated by F-tests. Student's unpaired *t*-tests were used to compare means between slow and normal metabolizers. The untransformed mean values, reported to facilitate interpretation, for nicotine spray usage, plasma nicotine and cotinine are indicated by open diamonds for the normal group and filled diamonds for the slow group. The bars indicate ± s.d. of the mean, and the number of participants in each group is shown in parentheses. Slow metabolizers (4.8 ± 3.6) used fewer doses/day than normal metabolizers (10.5 ± 8.0, $P < 0.02$). Plasma nicotine levels for normal (8.0 ± 9.1 ng/ml) and slow (5.8 ± 4.1 ng/ml) metabolizers were not different ($P = 0.82$), although cotinine plasma levels tended to be lower among slow compared to normal metabolizers (38 ± 24 vs 93 ± 92 ng/ml, respectively, $P = 0.07$).

the titration of the nicotine spray to maintain similar levels of plasma nicotine in the presence of slower metabolism (Figure 5).

Discussion

We have previously shown[15] a slow metabolizer frequency of 8–9% at smoking durations of 0–20 years, but a frequency of only 5–6% at 30 or more years of smoking. These findings are consistent with work by others suggesting that slow metabolizers are more successful at quitting[46] and may be under-represented among long duration smokers. This may explain the lower *CYP2A6*4* allele frequency reported here (Table 1) compared to a general smoking population.[15] The *CYP2A6*4* allele has been associated with lower risk for being a smoker, reduced cigarette consumption and reduced risk for lung disease,[12–14,17] and thus it may be under-represented among treatment-seeking smokers, as participants in this study were long duration heavier smokers with a mean smoking duration of 30 years. In addition, among slow metabolizers in this treatment-seeking population, there was a greater percentage of women and those with fewer years of education compared to normal metabolizers. Previous studies show that both gender and level of education influence quitting success,[47–51] and thus may influence the make-up of treatment-seeking smokers. This may suggest that slow metabolizers who remain in smoking populations for long durations, and eventually seek treatment for quitting, are more resistant to quitting compared to those who have quit on their own. It is possible that these individuals, in addition to having unique demographic characteristics, may also have additional genetic variants,[52–54] which may increase their risk of remaining as smokers. Owing to the low number of genetically identified slow metabolizers in this study, it was not possible to determine the impact of *CYP2A6* genotype on success of quitting on the nicotine patch and nicotine spray. However, when using the CYP2A6 phenotype (3HC/COT), the data indicate better quit rates for slow metabolizers relative to fast metabolizers on the nicotine patch (Lerman *et al.*, submitted), as would be expected given their higher levels of nicotine.[33,55]

The mean 3HC/COT ratio of the slow metabolizers was approximately 50% of that of the normal metabolizers (Figure 1). Those with a *CYP2A6* intermediate metabolizer genotype, predicting approximately 75% remaining activity, were indeed intermediate and were not significantly different from either the slow or normal metabolizers. These findings are consistent with our previous data,[6] and confirm that our genotype grouping methodology[15] is correct and consistent with the phenotype. This observation also indicates that genotypes predicting differences of only a 25% reduction in activity do not result in a significantly different metabolic activity from normal metabolizers; they are intermediate to the normal and slow metabolizers for both kinetic and smoking behaviours.[15,22] Owing to these observations, normal and intermediate metabolizers were not grouped as one; rather, all of the subsequent comparisons were conducted between normal and slow metabolizer groups where the difference in the impact on function would be expected to alter smoking behaviours, NRT use and plasma levels of the treatments.

CYP2A6 slow metabolizers smoked fewer cigarettes per day than normal metabolizers (Figure 2), consistent with several studies in adults[8,12,14,15,17] and one study in adolescents.[22] The effect of *CYP2A6* genotype on cigarette consumption was observed here among treatment-seeking smokers, whose characteristics and smoking behaviours tend to be different from those of the general smoking population.[23,24] In the current study, although the genotype association with smoking rate was significant, it was smaller (four vs seven cigarettes) compared to other studies in Caucasian adults.[8,15] It may be that slow metabolizers who smoke for long enough to be in treatment-seeking populations, and/or who have failed to quit on their own, are heavier or more dependent smokers than slow metabolizers in the general population. It is also possible that by excluding light smokers (for study inclusion smokers must smoke over 10 cigarettes per day), we excluded slow metabolizers with lower smoking, reducing the differences in levels of smoking between genotype groups. Also, longer term, heavier, treatment-seeking smokers may adjust consumption by altering the intensity of smoking,[56,57] again reducing the genotype difference in numbers of cigarettes smoked. A sub-study of this population indicated that CYP2A6 slow metabolizers take smaller puffs (puff volume) compared to normal metabolizers.[58] Together, these data suggest that, in addition to smoking fewer cigarettes per day, slow metabolizers may be adjusting their nicotine intake by altering smoking topography.

Slow metabolizers were half as likely, compared to normal metabolizers, to smoke within the first 5 min of waking compared to 6–30 min of waking (Figure 3). Smokers adjust their cigarette consumption to maintain steady levels of nicotine throughout the day; however, nicotine levels drop during the night, resulting in low levels in the morning.[59,60] Slower nicotine metabolism may result in somewhat higher morning nicotine levels and/or delayed receptor resensitization, thus postponing the time to the first cigarette among slow metabolizers. Consistent with other studies,[61,62] these findings suggest that rates of nicotine metabolism determine, at least in part, the amount smoked and timing of smoking. Smokers often report that the first cigarette of the day is the most satisfying;[63] this satisfaction with the first cigarette may be owing to the resensitization of nicotinic acetylcholine receptors in the morning following overnight abstinence.[59,64,65] There was no difference in the percent of slow and normal metabolizers reporting that they would not want to give up their first cigarette of the day. Taken together,

these findings suggest that although smoking of the first cigarette may be delayed in slow metabolizers, it is equally satisfying.

Considerable interindividual variation in plasma nicotine levels is observed when using NRT or when smoking. A large amount (75–99%) of this variation from the nicotine patch is accounted for by the systemic dose and the rate of metabolism of nicotine.[66] Likewise, when controlling for similar doses of nicotine delivered from smoking (16 cigarettes evenly spaced over 16 h), from the nicotine patch (15 mg/16 h) and from the nicotine spray (24×1 mg doses/day), clearance and dose accounted for the majority of this variability.[28] Specifically, individual nicotine clearance accounted for 45–57% of the variance and was the best predictor of plasma nicotine levels from cigarettes, nicotine patch or nicotine spray, whereas dose accounted for only 9–20% of the variance. In contrast, the majority of the variability in plasma cotinine from these delivery systems could be explained by the dose of nicotine (64–77% of variance), followed by cotinine clearance (15–30% of the variance).[28] In our study, among those subjects randomized to the nicotine patch, where usage, and likely the absorbed dose of nicotine, was similar between the two genotype groups, slow metabolizers had greater plasma nicotine concentrations consistent with reduced rates of nicotine clearance (Figure 4), but similar plasma cotinine concentrations. The fact that cotinine levels were similar in slow and normal metabolizers is most likely explained by balanced effects of a smaller percentage conversion of nicotine to cotinine and slower clearance of cotinine in slow metabolizers. In contrast to the nicotine patch, slow metabolizers using nicotine spray had similar plasma nicotine concentrations to the normal metabolizer group while using less of the spray. This is consistent with titration of nicotine spray usage to compensate for the slower rates of nicotine inactivation among slow metabolizers. Cotinine plasma concentrations tended to be lower among slow metabolizers compared to normal metabolizers consistent with a lower dosage of spray used among the slow compared to the normal group (Figure 5). Similar to other studies,[28] we also found a considerable amount of variation in plasma nicotine and cotinine levels in the nicotine spray. Although our findings are consistent with the concepts of titration and slow metabolism, these observations should be reproduced in a larger study.

Limitations of our study include the lack of detection of all *CYP2A6* genetic variants and the small sample size, particularly of slow metabolizers. There are likely many variants that have not been discovered, and several which have been identified but not yet characterized for their impact on metabolism.[44] The frequency of these alleles and their impacts on function are unknown, indicating that our *CYP2A6* genotype analysis is still incomplete; this is illustrated by the wide variation in the distribution of 3HC/COT ratios among the normal metabolizers (Figure 1). As novel variants are identified and characterized, it is likely that more of the individual variability in cigarette consumption, plasma levels obtained from the nicotine patch and amount of nicotine spray will be explained by genotype differences. With the present genotyping information, the frequency of known *CYP2A6* genetic variants among Caucasians is very low,[15,44] and thus is problematic for study design. This relatively low frequency of currently identified slow metabolizers prohibited analyses of the impact of genotype on cessation outcomes. One alternative is to use the 3HC/COT ratio, which is an indicator of the phenotype. Although the 3HC/COT ratio can be utilized as an indicator of CYP2A6 phenotype,[4] there are limitations to using this ratio as a measure of genetically determined CYP2A6 activity, as enzymatic activity is also influenced by diet, steroids, gender and environment. For instance, grapefruit juice can inhibit CYP2A6-mediated coumarin metabolism,[67] drugs such as phenobarbital and rifampin can induce CYP2A6,[68] and oral contraceptives can increase nicotine clearance.[45] Such dietary and drug histories were not available for the participants in this study. These environmental influences provide further impetus to determine the impact of novel *CYP2A6* genetic variants on CYP2A6 activity in order to distinguish genetic and environmental contributions to variation in activity. Having demonstrated an impact of *CYP2A6* genotype on smoking behaviours and NRT use, a subsequent study will be undertaken to confirm and extend these observations including assessment of variables that alter the CYP2A6 phenotype.

In conclusion, we have shown that *CYP2A6* genotype altered CYP2A6 activity measured by the 3HC/COT ratio derived from baseline smoking, and influenced pretreatment smoking behaviour. In addition, *CYP2A6* genotype altered plasma nicotine and cotinine levels and usage during NRT.

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