Title

Influence of *CYP2A6* genetic variation, nicotine dependence severity, and treatment on smoking cessation success

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Abstract (250/250 words)

Background: Genetic variation in CYP2A6, the major nicotine metabolizing enzyme, is associated with nicotine dependence and smoking cessation. Nicotine dependence severity also predicts smoking cessation. Our goals were to determine how *CYP2A6* variation and nicotine dependence alter smoking cessation, and whether dependence could refine *CYP2A6*-based treatment recommendations. **Methods:** Adult smokers treated for 12 weeks with placebo, nicotine patch, or varenicline (NCT01314001) were grouped as *CYP2A6* normal (n=567) or slow (n=432) nicotine metabolizers based on a *CYP2A6* weighted genetic risk score. FTND scores were measured at baseline and biochemically-verified smoking cessation was assessed at end-of-treatment. **Results:** Dependence neither mediated nor moderated an association between *CYP2A6* variation and smoking cessation overall, within any treatment arm, or after stratifying by ancestry (n=591 European, n=408 African ancestry) or sex (n=444 women, n=555 men). In within-treatment analyses, the mediation effect odds ratio (OR) ranged from 0.95-1.00 and the bias-corrected 95% confidence interval contained 1. Moderation (i.e., interaction) effect ORs ranged from 0.88-1.61 (P=0.397-0.828). For *CYP2A6* normal metabolizers, quit rates on varenicline were similar for those with high (41.1%) and low (43.4%) dependence, while quit rates were lower for those with high vs. low dependence on both patch (16.5 vs. 29.7%) and placebo (8.9 vs. 18.5%). *CYP2A6* slow metabolizers with high vs. low dependence had lower quit rates in all three treatment arms. **Conclusions:** While nicotine dependence severity neither mediated nor moderated *CYP2A6* associations with smoking cessation, incorporating information on dependence may optimize the choice of smoking cessation treatment aid in *CYP2A6* normal and slow metabolizers.

Implications: Variation in *CYP2A6* and nicotine dependence severity alter smoking cessation success. Our findings suggest that while nicotine dependence severity is unlikely to mediate or moderate *CYP2A6* associations with cessation, incorporating patient information on both *CYP2A6* and nicotine dependence severity may lead to improved smoking cessation strategies.

Introduction

Nicotine is the major psychoactive compound in cigarettes; it promotes dependence and chronic smoking (1). While ~70% of smokers express a desire to quit, many attempts are often required before successful smoking cessation is achieved (2). Twin studies show a 50-60% heritability estimate for smoking cessation (3). Genetic variation in *CYP2A6,* which encodes the major nicotine-inactivating enzyme, is associated with the rate of nicotine clearance and numerous smoking behaviors including smoking cessation outcomes (4). CYP2A6 inactivates nicotine to cotinine (5) and further metabolizes cotinine to 3'hydroxycotinine (6). The nicotine metabolite ratio (NMR; ratio of 3'hydroxycotinine to cotinine) is a biomarker of CYP2A6 activity, where higher NMR indicates faster CYP2A6 activity and nicotine clearance (7). Smokers with normal (vs. slow) CYP2A6 activity, measured by *CYP2A6* genetics or the NMR biomarker, smoke more cigarettes, inhale more deeply, experience greater nicotine reinforcement, and have a higher risk for lung cancer (4, 8-11). Variation in CYP2A6 activity is also associated with smoking cessation outcomes; for example, normal (vs. slow) metabolizers have lower quit rates on nicotine patch (12, 13), but show relatively higher quit success on varenicline (vs. nicotine patch) (14).

Nicotine dependence severity is also a robust predictor of smoking cessation outcomes; in general, greater nicotine dependence severity is associated with reduced cessation success (15). Moreover, variation in *CYP2A6* and the NMR is associated with nicotine dependence in some studies (8, 11, 16), and genetic variation may interact with nicotine dependence severity to alter smoking cessation outcomes (15). Smoking cessation outcomes also differ by ancestry and by sex/gender. For example, compared to European ancestry (EA) smokers, African ancestry (AA) smokers have lower smoking cessation success (17). In terms of sex/gender differences, compared to placebo, women have relatively lower quit success on bupropion and the nicotine patch vs. men, but equivalent quit success on varenicline (18).

We recently created ancestry-specific *CYP2A6* weighted genetic risk scores (wGRSs) in EA and in AA smokers by combining previously characterized and novel *CYP2A6* variants discovered in genome-

wide association studies (GWAS) of the NMR (>95% of hits mapped to the *CYP2A6* locus on chromosome 19) (19, 20). These wGRSs capture ~30-35% of the variability in the NMR and replicate NMR-based associations with smoking cessation treatment outcomes in both EA and AA smokers (19, 20).

While there are associations between *CYP2A6* variation and nicotine dependence (8, 11, 16), *CYP2A6* variation and smoking cessation (12-14, 21), and nicotine dependence and smoking cessation (15), the mechanism(s) by which *CYP2A6* variation and nicotine dependence alter smoking cessation is unclear; here we investigated mediation and moderation as two potential mechanisms in EA and AA smokers. To advance our goal of developing personalized smoking cessation approaches, we additionally examined whether information on nicotine dependence severity could be used to refine *CYP2A6*-based treatment recommendations.

Methods

Study Participants

This was a secondary analysis of data from the Pharmacogenetics of Nicotine Addiction Treatment (PNAT)-2 clinical trial (NCT01314001) in 1246 adult smokers (≥10 cigarettes/day) randomized 1:1:1 to placebo, nicotine patch, or varenicline based on their NMR, which was measured from blood samples collected at trial baseline (14). All participants received behavioral counseling. The current analyses were restricted to individuals of EA and AA ancestry, which was determined using multidimensional scaling in combination with HapMap 3 data and visualization; genetic ancestries were >95% concordant with self-reported ancestries (22, 23). Sex was determined using information from the X and Y chromosomes (22, 23). Participants who provided written informed consent for DNA collection and genotyping were analyzed, for a final analytic sample of n=999 participants. Procedures were approved by IRBs at all clinical sites and at the University of Toronto (14).

CYP2A6 wGRS Determination and Grouping Strategy

A *CYP2A6* wGRS comprising a combination of selected known functional *CYP2A6* alleles and variants tagging independent signals from a GWAS of the NMR was previously constructed separately for EA and AA participants (19, 20). An individual's overall *CYP2A6* wGRS was computed by summing the number of effect alleles weighted by their unstandardized effects on the NMR (19, 20). Participants were grouped as normal or slow CYP2A6 metabolizers based on a *CYP2A6* wGRS cut-point of 2.140 in EA (normal: wGRS ≥2.140; slow: wGRS <2.140) and 2.089 in AA (normal: wGRS ≥2.089; slow: wGRS <2.089) which optimally dichotomizes normal and slow CYP2A6 metabolizers; the wGRSs replicated NMR-based clinical outcomes alone or with both ancestries together (20).

Measurement of Nicotine Dependence and Smoking Cessation

Nicotine dependence was measured at baseline using the Fagerström Test for Nicotine Dependence (FTND; possible score range: 0-10), with higher scores indicating greater severity of dependence. Biochemically-verified (carbon monoxide (CO) ≤8 ppm=abstinent; CO>8 ppm=still smoking) 7-day point prevalence abstinence was assessed at week 12 (i.e., end-of-treatment) (14). Participants were analyzed as intention-to-treat; those with CO values >8 ppm and those lost to follow-up were considered to be smoking (14).

Mediation Path Analysis

We assessed an indirect effect of the *CYP2A6* wGRS (coded as 0=slow metabolism, 1=normal metabolism) on smoking cessation (coded as 1=quit, 0=still smoking) through raw FTND score. The indirect effect is the product of two unstandardized linear regression coefficients: the first is the effect of X (*CYP2A6* wGRS group) on the mediator (FTND score) (i.e., path a), and the second is the effect of the mediator on Y (smoking cessation) (i.e., path b) in a model controlling for X. The direct effect measured the effect of X on Y (i.e., path c') after controlling for the mediation effect (a*b) and covariates. We analyzed the three treatment arms together (controlling for ancestry, sex, age, and treatment arm) and separately (controlling for ancestry, sex, and age). We also conducted within-treatment analyses stratified by ancestry (controlling for sex and age) and by sex (controlling for ancestry and age). Model #4 in PROCESS (version 4.0; written by Dr. Andrew Hayes), a macro implementation of moderation and mediation analyses for SPSS (version 27; Armonk, NY: IBM Corp.), was used for analyses (24). A bias-corrected bootstrapping method was used to model the indirect effect and 10,000 replications were used to estimate the 95% confidence intervals. The total effect (path c) was calculated separately in SPSS using logistic regression models (controlling for covariates). Analysis code is available upon request.

Moderation (i.e., Interaction) Analysis

We also tested whether nicotine dependence severity interacts with the *CYP2A6* wGRS to influence smoking cessation (coded as 1=quit, 0=still smoking) using logistic regression. In the overall analysis, main effects of *CYP2A6* wGRS (coded as 0=slow metabolism, 1=normal metabolism), FTND (dichotomized as low and high dependence: ≤5 vs. >5, coded as 0 and 1, respectively), and treatment (coded as 0=placebo, 1=nicotine patch, and 2=varenicline), as well as all 2-way interaction terms and the 3-way interaction term, were included. Models controlled for ancestry, sex, and age. We also examined possible *CYP2A6* wGRS*FTND interactions in the three treatment arms separately. To identify potential sub-group differences, we performed ancestry- and sex-stratified analyses.

We also examined potential interactions between nicotine dependence severity and treatment on smoking cessation (coded as 1=quit, 0=still smoking) within *CYP2A6* normal and slow metabolizers using logistic regression. Models included main effects of FTND (dichotomized as low and high dependence: ≤5 vs. >5, coded as 0 and 1, respectively) and treatment (coded as 0=placebo, 1=nicotine patch, and 2=varenicline), and the treatment*FTND interaction term. Covariates included ancestry, sex, and age.

Mediation and Moderation Analyses Using the Heaviness of Smoking Index (HSI)

In smokers screened for participation in this trial, the NMR (CYP2A6 activity biomarker) was shown to be associated with another measure of nicotine dependence, the heaviness of smoking index (HSI) (25). Thus, we also tested whether the HSI mediates or moderates associations between *CYP2A6* variation and smoking cessation. The HSI (possible score range: 0-6) was calculated by summing two FTND items: time to first cigarette and cigarettes/day (25). HSI was used as the mediator (raw HSI score) in an analysis in the overall group (controlling for ancestry, sex, age, and treatment arm), and as the moderator (dichotomized as low and high dependence: ≤3 vs. >3, coded as 0 and 1, respectively) in analyses conducted in the three treatment arms separately (controlling for ancestry, sex, and age).

Results

Characteristics of the final analytic sample are shown in **Table S1**. We found no support for a mediating effect of FTND on an association between the *CYP2A6* wGRS and smoking cessation in the overall group, within any of the treatment arms, or when stratifying by ancestry or sex **(Figure 1, Table S2)**. Similarly, we found no evidence that FTND moderates an association between the *CYP2A6* wGRS and smoking cessation in the overall group, within any of the treatment arms, or when stratifying by ancestry or sex **(Figure S1, Table S3)**. Substituting HSI for FTND did not yield a significant mediating effect in the overall group (Indirect effect: Path a b: OR=0.96, 95% CI =0.90, 1.01). Similarly, no moderating effects of HSI were observed: the *CYP2A6* wGRS*HSI interaction ORR on cessation was 1.30 (95% CI=0.47-3.61), 1.05 (95% CI=0.34-3.23), and 0.72 (95% CI=0.22-2.34) in the varenicline, nicotine patch, and placebo arms, respectively.

Within *CYP2A6* normal metabolizers, there were significant main effects of treatment and FTND on cessation, as well as a trending treatment*FTND interaction effect **(Table S4, Figure 2)**. For *CYP2A6* normal metabolizers, the overall quit rates on varenicline, nicotine patch, and placebo were 42.3%, 23.8%, and 13.5%, respectively. Varenicline was similarly efficacious in those with high (41.1%) and low (43.4%) dependence, while quit rates were lower for those with high vs. low dependence on both patch (16.5 vs. 29.7%) and placebo (8.9 vs. 18.5%) **(Figure 2, Figure S1)**. Within *CYP2A6* slow metabolizers, there was a significant main effect of FTND on cessation, but no main effect of treatment or treatment*FTND interaction effect **(Table S4, Figure 2)**. Within *CYP2A6* slow metabolizers, the overall quit rates on varenicline, nicotine patch, and placebo were 30.1%, 26.9%, and 25.5%, respectively. Quit rates were lower for those with high vs. low dependence on varenicline (24.1 vs. 33.3%), nicotine patch (14.8 vs. 37.7%), and placebo (19.2 vs. 32.4%) **(Figure 2, Figure S1)**.

Discussion

In this secondary analysis of data from the PNAT2 clinical trial, we found no evidence that nicotine dependence severity mediates or moderates associations between *CYP2A6* and smoking cessation success overall, on varenicline, on nicotine patch, or on placebo. While we did not detect a significant association between the *CYP2A6* wGRS and nicotine dependence in this sample, the effect was in the anticipated direction (i.e., normal *CYP2A6* metabolizers had higher FTND (beta=0.147) and HSI (beta=0.125) scores). The inconsistent association between the NMR/CYP2A6 activity and dependence in clinical samples may be due to the selection of a more highly dependent sample with modest variation in nicotine dependence scores. Our study also tested whether information on baseline nicotine dependence severity could refine smoking cessation treatment recommendations for *CYP2A6* normal and slow metabolizers. In this trial, we previously showed that varenicline was more efficacious than nicotine patch for normal, but not slow, metabolizers (14). Our current findings support the use of varenicline as a frontline cessation therapy in *CYP2A6* normal metabolizers regardless of dependence level (**Figure 2**). Within *CYP2A6* slow metabolizers, quit rates on placebo (i.e., behavioral counseling), nicotine patch, and varenicline were similar, but consistently lower in those with high (vs. low) dependence. In general, *CYP2A6* slow metabolizers experience a more favorable side effect profile with nicotine patch therapy compared to varenicline (14). Together these findings support the use of behavioral counseling with or without nicotine patch therapy in *CYP2A6* slow metabolizers, however those with high dependence would likely benefit from additional cessation support.

Strengths of our study include the use of both mediation and moderation modelling, the use of two measures of nicotine dependence (FTND and HSI), sex- and ancestry-stratified analyses, and the evaluation of multiple cessation treatments. Our mediation models satisfied the temporality requirement, as *CYP2A6* genotype is determined before birth, nicotine dependence severity was measured at trial baseline, and abstinence was measured at 12 weeks. A limitation of our study is the relatively small sample

for sub-group analyses. Future studies could incorporate rare functional *CYP2A6* variants, which improves prediction of *CYP2A6* metabolizer status particularly among individuals of African ancestry (26).

In conclusion, we have provided preliminary evidence suggesting that nicotine dependence severity is unlikely to mediate or moderate associations between *CYP2A6* variation and smoking cessation success. Our findings also provide initial support for incorporating information on nicotine dependence severity to optimize the choice of smoking cessation treatment in *CYP2A6* normal and slow metabolizers. Optimized smoking cessation strategies in turn will reduce the enormous morbidity and mortality associated with cigarette smoking.

Data Availability Statement

Due to the type of informed consents given by student participants, individual level data cannot be made publicly available. Analysis code is available upon request.

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Declaration of Interests

R. F. Tyndale has consulted for Quinn Emanuel and Ethismos. The other authors declare no conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

MJC and RFT were responsible for the study concept and analysis plan. CL and RFT oversaw the design of the original study. MJC performed data analysis. MJC, CL, JK, and RFT interpreted the findings. MJC, CL, JK, and RFT obtained funding for the study. MJC and RFT drafted the manuscript and revised it based on co-author comments. All authors critically reviewed the manuscript and approved the final version for publication.

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Figure Legends

Figure 1. Nicotine dependence severity did not mediate an association between the *CYP2A6* wGRS and smoking cessation at end-of-treatment in the PNAT2 clinical trial. This model shows the relationship between the *CYP2A6* weighted genetic risk score and smoking cessation success at end-of-treatment via nicotine dependence severity measured at baseline using FTND. The (a) pathway shows the association between the *CYP2A6* wGRS and FTND score. The (b) pathways shows the association between FTND score and smoking cessation success. The mediating effect of FTND on smoking cessation success is shown in the (a b) pathway (i.e., indirect effect pathway) and is shaded in dark grey. The (c) pathway shows the direct effect of the *CYP2A6* wGRS on smoking cessation success adjusted for covariates, while the (c') pathway shows the direct effect of the *CYP2A6* wGRS on smoking cessation success adjusted for covariates and the indirect effect. Statistically significant effects (at $P<0.05$) are bolded. Ancestry, sex, and age were included as covariates in the treatment-stratified analyses (n=320 PLAC; n=332 PATCH; n=347 VAR). The overall analysis (n=999) additionally controlled for treatment; results are shaded in light grey. Abbreviations: wGRS = weighted genetic risk score, FTND = Fagerström Test for Nicotine Dependence, OR = Odds Ratio, CI = confidence interval, PLAC = Placebo, VAR = Varenicline.

Figure 2. There was a trending interaction effect between treatment and nicotine dependence severity on smoking cessation at end-of-treatment in *CYP2A6* normal metabolizers from the PNAT2 clinical trial. Full results from the moderation analysis are available in Table S4. Abbreviations: FTND = Fagerström Test for Nicotine Dependence; ORR = ratio of Odds Ratios.

Supplementary Information Titles

Table S1. Characteristics of the final analytic sample of participants from the PNAT2 clinical trial

Table S2. Mediation path analyses evaluating relationships between the *CYP2A6* wGRS, nicotine dependence severity, and smoking cessation at end-of-treatment (week 12) in the PNAT2 clinical trial: sub-group analyses

Table S3. Moderation analyses evaluating relationships between the *CYP2A6* wGRS, nicotine dependence severity, and smoking cessation at end-of-treatment (week 12) in the PNAT2 clinical trial

Table S4. Moderation analyses evaluating relationships between treatment, nicotine dependence severity, and smoking cessation at end-of-treatment (week 12) in the PNAT2 clinical trial

Figure S1. Nicotine dependence severity did not moderate an association between the *CYP2A6* wGRS and smoking cessation at end-of-treatment in the PNAT2 clinical trial. There were no significant interactions between the *CYP2A6* wGRS and baseline nicotine dependence severity (measured using FTND) on cessation in any of the treatment arms. Full results from the moderation analysis are available in Table S3. Abbreviations: wGRS = weighted Genetic Risk Score; FTND = Fagerström Test for Nicotine Dependence; ORR = ratio of Odds Ratios.