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CYP2A6 metabolism in the development of smoking behaviors in young adults

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ABSTRACT

Cytochrome P450 2A6 (*CYP2A6*) encodes the enzyme responsible for the majority of nicotine metabolism. Previous studies support that slow metabolizers smoke fewer cigarettes once nicotine dependent but provide conflicting results on the role of *CYP2A6* in the development of dependence. By focusing on the critical period of young adulthood, this study examines the relationship of *CYP2A6* variation and smoking milestones. A total of 1209 European American young adults enrolled in the Collaborative Study on the Genetics of Alcoholism were genotyped for *CYP2A6* variants to calculate a previously well-validated metric that estimates nicotine metabolism. This metric was not associated with the transition from never smoking to smoking initiation nor with the transition from initiation to daily smoking ($P > 0.4$). But among young adults who had become daily smokers ($n = 506$), decreased metabolism was associated with increased risk of nicotine dependence ($P = 0.03$) (defined as Fagerström Test for Nicotine Dependence score ≥4). This finding was replicated in the Collaborative Genetic Study of Nicotine Dependence with 335 young adult daily smokers ($P = 0.02$). Secondary meta-analysis indicated that slow metabolizers had a 53 percent increased odds (OR = 1.53, 95 percent CI 1.11–2.11, $P = 0.009$) of developing nicotine dependence compared with normal metabolizers. Furthermore, secondary analyses examining four-level response of time to first cigarette after waking (>60, 31–60, 6–30, ≤5 minutes) demonstrated a robust effect of the metabolism metric in Collaborative Study on the Genetics of Alcoholism ($P = 0.03$) and Collaborative Genetic Study of Nicotine Dependence ($P = 0.004$), illustrating the important role of this measure of dependence. These findings highlight the complex role of *CYP2A6* variation across different developmental stages of smoking behaviors.

Keywords CYP2A6, genetics, nicotine dependence, smoking, young adults.

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INTRODUCTION

The development of nicotine dependence requires smoking initiation, conversion from experimental to daily use and finally the development of advanced smoking behaviors (Belsky *et al.*, 2013; Bierut, 2011). Although the majority of adult smokers initiate smoking during adolescence, rates of daily smoking substantially increase

during young adulthood (1 percent at ages 12–17, 12 percent at ages 18–25, 14 percent at ages 26 or more) (NSDUH, 2015). Furthermore, among those who report current daily smoking, the proportion of individuals who smoke a pack or more a day also dramatically increases with age (12 percent at ages 12–17, 23 percent at ages 18–25 and 33 percent at ages 26 or more) (NSDUH, 2015). Increasing our knowledge of what factors drive some young adults and not others to transition from initiation to daily smoking and then to advanced smoking behaviors is important for effectively preventing the progression toward nicotine dependence.

One genetic factor that plays an important role in the development of smoking behaviors is variation in the gene Cytochrome P450 2A6 (*CYP2A6*), which encodes the cytochrome P450 enzyme responsible for the majority of oxidation of nicotine to cotinine; this is the primary pathway of nicotine metabolism in humans (Hukkanen *et al.*, 2005). The *CYP2A6* locus is highly polymorphic, and alleles with reduced function have been associated with slower rates of nicotine metabolism. Common variants define multiple *CYP2A6* haplotypes in individuals of European ancestry (Haberl *et al.*, 2005), and the majority of inter-individual variation in the metabolism of nicotine to cotinine is explained by CYP2A6 genotypes in European Americans (Bloom *et al.*, 2011).

The region on chromosome 19 encompassing *CYP2A6* is genome-wide significantly associated with cigarettes per day in large meta-analyses of European ancestry adults (TAG, 2010; Thorgeirsson *et al.*, 2010). Among nicotine dependent adults, several studies demonstrate that slower metabolizers smoke fewer cigarettes per day (Benowitz, 2008; Malaiyandi *et al.*, 2005). This observation is thought to reflect that smokers naturally titrate cigarette consumption to maintain steady nicotine levels.

Studies in youth present conflicting results regarding the effect of nicotine metabolism on the development of nicotine dependence and other smoking behaviors (Audrain-McGovern *et al.*, 2007; Cannon *et al.*, 2016; Chenoweth *et al.*, 2016; Huang *et al.*, 2005; Moolchan *et al.*, 2009; O'Loughlin *et al.*, 2004; Rubinstein *et al.*, 2008; Rubinstein *et al.*, 2013). Some studies suggest that slow nicotine metabolism is associated with an increased risk of nicotine dependence (Chenoweth *et al.*, 2016; O'Loughlin *et al.*, 2004; Rubinstein *et al.*, 2013), possibly reflecting an increased sensitivity to initial nicotine exposure among youth who metabolize nicotine more slowly. In contrast, other studies suggest that slower metabolizers have a decreased risk for dependence and related symptoms (Audrain-McGovern *et al.*, 2007; Rubinstein *et al.*, 2008), paralleling findings in adults regarding reduced heaviness of smoking among slow metabolizers.

Our goal was to investigate the ways in which variation in *CYP2A6* relates to the development of smoking behaviors during the critical period of young adulthood in a sample of European Americans. A better understanding of how variation in nicotine metabolism contributes to the acquisition of smoking milestones will add to our fundamental knowledge of the developmental processes that lead to nicotine dependence and has the potential to identify individuals at increased susceptibility during this critical period.

MATERIALS AND METHODS

Primary sample description

The Collaborative Study on the Genetics of Alcoholism (COGA) is a United States multi-center, family study that aims to identify genes that contribute to alcohol use disorders and related phenotypes (Begleiter *et al.*, 1995). Since 2005, the adolescent and young adult study in COGA has used a longitudinal design to examine the development of substance use disorders in youth from high-risk (defined as recruited through alcohol dependent probands with multiple affected family members) and community comparison families. Members aged 12–22 years were recruited from six sites across the United States and interviewed approximately every 2 years with ongoing data collection. Detailed descriptions of the COGA prospective adolescent and young adult sample have been previously published (Chorlian *et al.*, 2015; Dick *et al.*, 2013).

Smoking behaviors in COGA

Assessments were performed using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA), which gathers detailed information on substance use with high reliability and validity (Bucholz *et al.*, 1994; Bucholz *et al.*, 1995; Hesselbrock *et al.*, 1999). Smoking initiation was evaluated with the question 'Have you ever smoked a full cigarette?' Daily smoking was defined as smoking at least 4 days per week for at least a month as performed in previous analyses (Kapoor *et al.*, 2012). This was assessed among individuals who had initiated smoking using the questions 'When you were smoking regularly, how many days per week did you usually smoke cigarettes?' and 'For how long did you smoke this many cigarettes at that rate?'

Among individuals who reported daily smoking ($n = 506$), several measures of more advanced smoking behaviors were examined in this analysis that focused on the period of heaviest smoking. Time to first cigarette after waking was derived from the question 'During this period when you were smoking the most, about how many minutes after you woke up did you smoke your first

cigarette?' and the four response options are the following: more than 1 hour, 31–60 minutes, 6–30 minutes and within 5 minutes. For the analyses, time to first cigarette was dichotomized into >5 minutes ($n = 338$, 67 percent) and ≤5 minutes after waking ($n = 168$, 33 percent). Cigarettes per day was evaluated with the question 'During the period of time when you were smoking the most, about how many cigarettes did you usually have per day?' and the four response options are as follows: 10 or fewer, 11–20, 21–30 and 31 or more cigarettes. Cigarettes per day was dichotomized into ≤20 ($n = 367$, 74 percent) and >20 cigarettes ($n = 131$, 26 percent) in the analyses as performed in previous studies (Belsky *et al.*, 2013; Broms *et al.*, 2006). A total Fagerström Test for Nicotine Dependence (FTND) score during the heaviest period of smoking was calculated at each interview using responses to these two questions as well as responses to questions assessing the four remaining criteria (Heatherton *et al.*, 1991). For the analyses, nicotine dependence was defined as a FTND score of four or more ($n = 306$, 61 percent), which is a sensitive and specific cut-off for smoking biomarkers (Huang *et al.*, 2008) and has been used in previous genetic studies (Bierut *et al.*, 2007; Saccone *et al.*, 2009).

Given the longitudinal design of this study, an endorsement of smoking initiation or daily smoking at any interview at age 30 years or younger was used to capture these behaviors during young adulthood. The highest FTND score across available interviews at age 30 years or younger was chosen to capture the lifetime maximum, and time to first cigarette as well as cigarettes per day were set at these interviews.

Genotyping

Bloom *et al.* (2011) developed a metric based on several genetic variants in *CYP2A6* to estimate nicotine metabolism. Cross-validation estimates that this metric predicts approximately 70 percent of the variance in metabolism of orally administered nicotine to cotinine in European Americans (Bloom *et al.*, 2012). Our goal was to use this *CYP2A6* metabolism metric to test whether *CYP2A6* variation predicts cigarette smoking behaviors in young adulthood.

Five *CYP2A6* single nucleotide polymorphisms (SNPs) (rs1801272, rs28399442, rs28399433, rs1137115 and rs28399435) were genotyped using the LGC (Teddington, Middlesex, UK) genomics competitive allele-specific polymerase chain reaction (KASP) (lgcgenomics.com). The *CYP2A6* copy number variant (CNV) was genotyped with TaqMan 5' Nuclease Assays (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The CNV assay was run in duplicate, and genotype calls were made using CopyCaller (Thermo Fisher Scientific, Waltham, MA, USA) software. The program PEDCHECK (O'Connell and Weeks, 1998) was used to examine Mendelian inheritance, and only individuals with no Mendelian inconsistencies were included in the analyzed sample. The metabolism metric was calculated based on the genotypes of the five *CYP2A6* SNPs and the CNV using an algorithm described in Table S1 (adapted from Bloom *et al.*, 2012).

A set of 64 ancestry informative markers was genotyped as part of a 96 SNP Biorepository Panel by the Rutgers University Cell and DNA Repository. These markers were used in SNPrelate, a function in R, to assign ancestry groups. HapMap populations were included as reference groups. There was high concordance (98 percent) between self-reported and genetically determined ancestry among European Americans. Only individuals with a genetic ancestry of European American were included in the analysis because the metric was optimized for this population (Bloom *et al.*, 2011).

Primary sample selection

The analysis was restricted to individuals who had reached young adulthood (19 years or older) because we were interested in transitions to daily smoking and advanced smoking behaviors, outcomes that often occur during this time. In the COGA adolescent and young adult study, 1209 European ancestry individuals with a last interview age of 19 years or older were genotyped for the *CYP2A6* variants, and participants for the analyses were drawn from this group (Fig. 1). The sample used to analyze daily versus non-daily smokers consisted of 776 individuals who had initiated smoking (64 percent of all subjects). For transitions to advanced smoking behaviors, we focused on the sample of 506 daily smokers (65 percent of initiators, 42 percent of all subjects, described in Table 1).

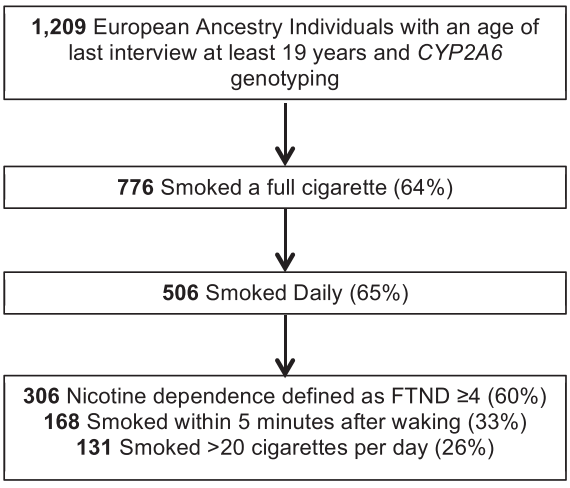

Figure 1 Primary Collaborative Study on the Genetics of Alcoholism (COGA) sample selection. *CYP2A6*, Cytochrome P450 2A6; FTND, Fagerström Test for Nicotine Dependence

Table 1 Characteristics of primary and replication samples of European American young adults.

Characteristic	*COGA young adult European American daily smokers (n = 506)*	*Replication: COGEND young adult European American daily smokers (n = 335)*
Sex, *n* (percent)	–	–
Males	288 (57 percent)	129 (39 percent)
Females	218 (43 percent)	206 (61 percent)
Age at last interview examined, years	–	–
Mean ± sd	24.4 ± 3.3	27.8 ± 1.7
Range	19–30	25–30
No. of interviews examined	–	–
Mean ± sd	4.0 ± 1.4	–
Range	1–6	1
Family status, *n* (percent)	–	–
From high-risk families	464 (92 percent)	–
From comparison families	42 (8 percent)	–
Lifetime DSM-IV alcohol dependence, *n* (percent)	185 (37 percent)	35 (11 percent)
No. of extended families	310	–
No. of nuclear families (full siblings)	431	–
FTND score	–	–
Mean ± sd	4.2 ± 2.6	3.0 ± 3.3
Range	0–10	0–10
Nicotine dependence (FTND ≥ 4), *n* (percent)	306 (60 percent)	166 (50 percent)
Time to first cigarette after waking	–	–
More than 1 hour	80 (16 percent)	168 (50 percent)
31–60 minutes	73 (14 percent)	19 (6 percent)
6–30 minutes	185 (37 percent)	67 (20 percent)
Within 5 minutes	168 (33 percent)	81 (24 percent)
Cigarettes per day	–	–
10 or fewer	182 (36 percent)	171 (51 percent)
11–20	185 (37 percent)	78 (23 percent)
21–30	81 (16 percent)	45 (13 percent)
31 or more	50 (10 percent)	41 (12 percent)
Metabolism metric*	–	–
Mean ± sd	0.86 ± 0.07	0.86 ± 0.07
Range	0.44–0.90	0.44–0.90
Metabolism status	–	–
Low (Metric ≤ .85)	134 (26 percent)	103 (31 percent)
Normal (Metric > .85)	372 (74 percent)	232 (69 percent)

*Distribution of metabolism metric in COGA and COGEND young adult daily smokers provided in Fig. S1. COGA, Collaborative Study on the Genetics of Alcoholism; COGEND, Collaborative Genetic Study of Nicotine Dependence; FTND, Fagerström Test for Nicotine Dependence.

Replication COGEND sample

The Collaborative Genetic Study of Nicotine Dependence (COGEND) is a multi-center case-control study designed to identify genes that contribute to nicotine dependence (Saccone *et al.*, 2007). Community based recruitment enrolled participants ages 25–45 years old. Cases were required to be current smokers and have an FTND score of four or more. Controls were required to have smoked at least 100 cigarettes and have a lifetime maximum FTND score of zero or one. For this analysis, only subjects who self-reported as being of European ancestry were examined (previous analyses using EIGENSTRAT have shown a high correspondence with genetic ancestry groups; Saccone *et al.*, 2009). Genotyping of variants to calculate the metabolism metric in COGEND has been previously described (Bloom *et al.*, 2012). We focused on the subsample of 377 COGEND young adults ages 25–30 that overlapped with the ages of the primary COGA sample. From this group, 335 (89 percent) reported smoking every day or nearly every day for at least 2 months and were considered daily smokers. Replication sample characteristics of these daily smokers are described in Table 1.

Primary data analysis

Data were analyzed using the Statistical Analysis System. Logistic regression was used to model dichotomous outcomes of smoking initiation, daily smoking, nicotine

dependence, time to first cigarette and cigarettes per day. In the primary analyses in COGA and COGEND, the continuous metabolism metric, sex, study site and last interview age were included as predictor variables. In COGA, family structure was accounted for using generalized estimating equations via PROC GENMOD. Results from the COGEND replication sample were meta-analyzed with the primary COGA results (Table 2) using a publically available Statistical Analysis System macro (>http://www.hsph.harvard.edu/donna-spiegelman/software/metaanal/). Meta-analyses results were based on fixed effect models to determine the evidence for association within the collected samples. In these analyses, we did not observe heterogeneity between the two studies based on the Q statistic ($P > 0.1$).

Secondary data analyses

Secondary analyses were performed to further explore our primary findings. First, individuals were divided into slow and normal metabolizers using a cut-off of ≤0.85 on the metabolism metric as previously described (Chen *et al.*, 2014). This cut-off captures the lowest quartile of metabolizers, and this dichotomous variable was used in logistic regression models of smoking behaviors. Second, because the majority of the COGA sample was recruited from families at high-risk for alcoholism, the primary analyses examining the continuous metabolism metric and smoking milestones were repeated with the covariate of lifetime DSM-IV alcoholism dependence. Third, after observing an association between the metabolism metric and the time to first cigarette dichotomous variable (>5 and ≤5 minutes), the four-level variable of time to first cigarette after waking (>60, 31–60, 6–30, ≤5 minutes) was also investigated in cumulative logistic regression models. These analyses were performed to assess whether the continuous metabolism metric predicted response across the four ordinal categories.

Ethics statement

Institutional review boards at all COGA and COGEND sites approved the study design and the studies were carried out in accordance with the Declaration of Helsinki. Written consent was received from all study participants.

RESULTS

Participant characteristics

Demographic, behavioral and metabolism metric characteristics of the COGA and COGEND samples are presented in Table 1. The primary COGA sample of young adult daily smokers consisted of 506 European American individuals from 431 nuclear families from 310 extended families. The mean age at last interview was 24 years, 43 percent were female, and 92 percent came from families at high-risk for alcoholism, with 37 percent meeting the criteria for lifetime DSM-IV alcohol dependence. Among these daily smokers, 61 percent were nicotine dependent, 33 percent smoked within 5 minutes after waking and 26 percent smoked greater than 20 cigarettes per day (Fig. 1 and Table 1). A total of 26 percent of the young adults were slow metabolizers, and the distribution of the metabolism metric (Fig. S1) was similar to that seen in other samples (Bloom *et al.*, 2012; Chen *et al.*, 2014).

The COGEND replication sample of young adult daily smokers consisted of 335 European Americans with an

Table 2 Logistic regression models examining the association of a continuous *CYP2A6* metabolism metric and smoking milestones in young adults.

	Metabolism Metric in COGA young adults			*Replication: metabolism metric in COGEND young adults*			*Meta-analysis of results*		
	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value
Among all young adults (COGA $n = 1209$)	–	–	–	–	–	–	–	–	–
Smoking initiation	0.46	0.97	0.63	–	–	–	–	–	–
Among young adult ever-smokers (COGA $n = 776$)	–	–	–	–	–	–	–	–	–
Daily smoking	−0.92	1.16	0.42	–	–	–	–	–	–
Among young adult daily smokers (COGA $n = 506$; COGEND $n = 335$)	–	–	–	–	–	–	–	–	–
Nicotine dependence	3.49	1.62	0.03	4.36	1.86	0.02	3.86	1.21	0.002
Smoked five or fewer minutes after waking	2.44	1.34	0.07	4.63	1.82	0.01	3.21	1.07	0.003
Smoked more than 20 cigarettes per day	−1.10	1.59	0.49	1.53	1.85	0.41	0.01	1.17	0.99

All models include sex, study site and age of last interview as covariates. Analyses with Collaborative Study on the Genetics of Alcoholism (COGA) were also adjusted for familial clustering.

average age at interview of 28 years and the majority were female (61 percent). Among COGEND young adult daily smokers, 50 percent were nicotine dependent, 24 percent smoked within 5 minutes after waking, 25 percent smoked greater than 20 cigarettes per day and 31 percent were slow metabolizers (distribution in Fig. S1).

CYP2A6 metabolism metric and early smoking behaviors

The continuous *CYP2A6* metabolism metric was not associated with smoking initiation ($P = 0.63$) nor with the development of daily smoking ($P = 0.42$) in the COGA young adults (Table 2). Of the 270 young adults who initiated smoking but did not transition to daily smoking, essentially all of them (98 percent) failed to develop any of the more advanced smoking behaviors, including nicotine dependence and smoking within 5 minutes after waking. This supports the notion that daily smoking is a prerequisite for the development of advanced smoking behaviors. Therefore, subsequent analyses of advanced smoking milestones focused on the 506 daily smokers.

CYP2A6 metabolism metric and advanced smoking behaviors in daily smokers

The *CYP2A6* haplotypes predictive of slower metabolism were associated with an increased risk of nicotine dependence in both the primary COGA and replication COGEND samples of young adult daily smokers (Table 2, Fig. 2). In multivariate models adjusting for age, sex and study site, the continuous *CYP2A6* metabolism metric had a significant effect in COGA ($P = 0.03$) and COGEND ($P = 0.02$), where a slow predicted metabolism was associated with an increased risk of nicotine dependence defined by an FTND score ≥ 4 (Table 2). Secondary analyses showed that slow metabolizers (defined by a metric of ≤ 0.85) had a 53 percent increased odds (OR = 1.53, 95 percent CI 1.11–2.11, $P = 0.009$) of developing nicotine dependence as compared with normal metabolizers (metric > 0.85) in meta-analyses of COGA and COGEND studies (Table S2). Figure 2 illustrates this association by showing that a larger proportion of slow metabolizers in both COGA and COGEND developed nicotine dependence as compared with normal metabolizers.

Consistent with the nicotine dependence results, a lower metabolism metric was associated with an increased risk of smoking within 5 minutes after waking (Table 2, Fig. 2). The continuous *CYP2A6* metabolism metric had a trending effect in COGA ($P = 0.07$) and a significant effect in COGEND ($P = 0.01$). In secondary meta-analysis, slow metabolizers had a 57 percent increased odds (OR = 1.57, 95 percent CI 1.13–2.18, $P = 0.007$) of smoking within 5 minutes after waking compared with normal metabolizers (Table S2). The *CYP2A6* metabolism metric was not associated with smoking more than 20 cigarettes per day in either sample or meta-analysis (Table 2, Fig. 2). Secondary analyses examining the effect of the metabolism metric on smoking behaviors after controlling for DSM-IV alcohol dependence illustrates similar results (Table S3), supporting that the associations are not dependent on alcoholism status.

Robustness of effect of *CYP2A6* metabolism metric on time to first cigarette after waking

Secondary analyses using all four responses of time to first cigarette after waking (>60, 31–60, 6–30, ≤5-minutes) demonstrated a more robust effect of the metabolism metric in both COGA ($P = 0.03$) and COGEND ($P = 0.004$) as compared with the dichotomous time to first cigarette (>5 and ≤5 minutes) used in our primary analysis (Table S4 and Table 2, respectively). Figure S2 illustrates that across the four categories, there was an increased proportion of slow metabolizers at shorter times to first cigarette after waking among COGA daily smokers. In COGEND daily smokers, we observed a similar trend, except in the category of 31–60 minutes that only had 19 individuals (6 percent of sample, Table 1).

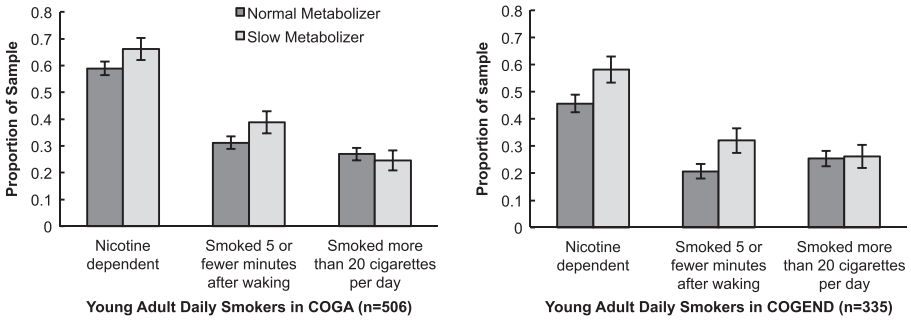

Figure 2 Association between predicted metabolism and smoking behaviors in two studies of European American young adult daily smokers. Error bars reflect standard errors adjusted for sample size. COGA, Collaborative Study on the Genetics of Alcoholism; COGEND, Collaborative Genetic Study of Nicotine Dependence

Taken together, these results support a possible 'dosage effect' where predicted slower metabolism was correlated with smoking sooner after waking.

DISCUSSION

Young adulthood is a critical developmental period for the progression from initiation to more advanced smoking milestones (NSDUH, 2015). This study links variation in a genome-wide significant gene, CYP2A6, with the development of smoking behaviors in two independent samples of European American young adults. Using specific *CYP2A6* polymorphisms, we calculated a nicotine metabolism metric, which has been previously shown to account for approximately 70 percent of the variance in metabolism of orally administered nicotine to cotinine in European Americans (Bloom *et al.*, 2012; Bloom *et al.*, 2011). Our primary finding is that slower nicotine metabolism is associated with an elevated risk of developing nicotine dependence among young adult daily smokers, adding important insight into the role of variation in *CYP2A6* across stages of smoking development, as illustrated in Fig. 3.

Despite having an important role in the development of nicotine dependence among daily smokers, variation in *CYP2A6* was not associated with smoking initiation nor the progression to daily smoking (step 1 in Fig. 3). Previous twin studies support that environmental influences primarily drive early adolescent nicotine use, and that the role of heritable factors on smoking behaviors increases throughout young adulthood (Kendler *et al.*, 2008; Koopmans *et al.*, 1999). Our results are consistent with this model by providing evidence of a specific gene that impacts the transition from daily smoking to nicotine dependence, without influencing initiation and daily smoking.

The observation that decreased predicted nicotine metabolism is associated with increased risk of nicotine dependence in young adult daily smokers also builds on previous studies conducted in adolescents (step 2 in Fig. 3). O'Loughlin *et al.* (2004) followed 228 non-dependent smokers in grade seven over approximately 30 months and found that individuals with less active genetic variants in *CYP2A6* were more likely to develop nicotine dependence but smoked fewer cigarettes per day once dependent. In a follow-up study examining 421 adolescents who had ever smoked a cigarette, Chenoweth *et al.* (2016) similarly found that slow metabolism conferred by *CYP2A6* variation was associated with increased risk of nicotine dependence in adolescence. Huang *et al.* (2005) examined variation in *CYP2A6* in 1518 adolescents enrolled in a longitudinal study in the United Kingdom and similarly found that individuals with variants associated with slower metabolism were more likely to be current versus former smokers at age 18 years compared with normal metabolizers. Rubinstein *et al.* (2013) assessed a biomarker of the rate of nicotine metabolism (the nicotine metabolite ratio) in 164 adolescent smokers and found that slower metabolizers showed greater symptoms of

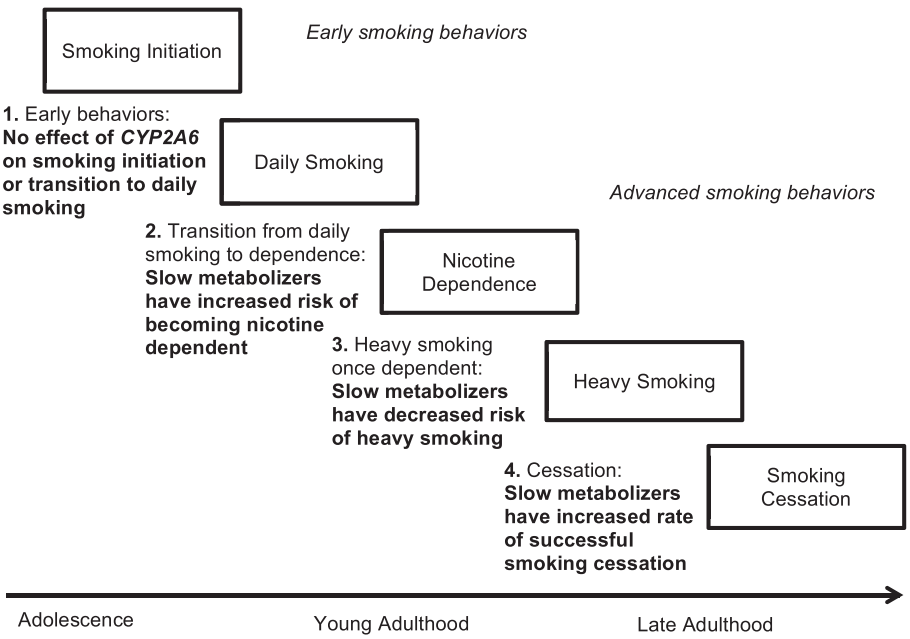

Figure 3 A theoretical framework of the development of smoking behaviors in relation to *CYP2A6*, Cytochrome P450 2A6 (CYP2A6) variation. Steps 1 and 2 are supported by this paper and previous studies. Steps 3 and 4 are supported by previous studies (reviewed in Benowitz, 2008; Malaiyandi *et al.*, 2005)

dependence. Our findings in two independent samples expand on these earlier results by demonstrating that during early young adulthood, when many advanced smoking behaviors develop, slow metabolizers who smoke daily continue to have a greater risk of lifetime dependence.

The increased susceptibility to developing nicotine dependence encountered by youth who are slow metabolizers compared with normal metabolizers has been hypothesized to reflect prolonged exposure to nicotine because of its longer half-life during initial smoking experiences (Chenoweth *et al.*, 2013; Malaiyandi *et al.*, 2005; Rubinstein *et al.*, 2013). Although accumulating evidence supports this role, it is important to note that a few studies show the opposite effect where slow metabolism is associated with decreased risk of smoking behaviors in youth (Audrain-McGovern *et al.*, 2007; Moolchan *et al.*, 2009; Rubinstein *et al.*, 2008). For example, Audrain-McGovern (2007) examined 222 adolescent ever-smokers of European ancestry and found that normal *CYP2A6* metabolizers developed symptoms of dependence at a faster rate than slower *CYP2A6* metabolizers. Other studies suggest that the increased risk of slower metabolizers for developing nicotine dependence in adolescence disappears by young adulthood (Chenoweth *et al.*, 2016). Cannon *et al.* (2016) followed 296 participants across ages 16–24 years and found that using a *CYP2A6* diplotype predictive metric, intermediate metabolism compared with slow and normal was a risk factor for smoking frequency and nicotine dependence. By the end of the study at age 24 years, however, the individuals with predicted normal metabolism were at greatest risk for these smoking behaviors. Many possible explanations exist for these discrepant results. One hypothesis is that the effect of slower nicotine metabolism transitions rapidly from increasing risk to being protective (Cannon *et al.*, 2016 O'Loughlin *et al.*, 2004), and previous studies may have observed different developmental periods in the fast-changing early course of smoking behaviors. The ascertainment of subjects and baseline smoking behaviors also varies across studies, which may influence the power to detect associations. Furthermore, previous studies use different measures of smoking behaviors and nicotine dependence, and it is possible that they capture different components of dependence that are differentially influenced by *CYP2A6* metabolism.

Our results suggest that time to first cigarette after waking is a critical contributor to the association between the *CYP2A6* metabolism metric and development of nicotine dependence assessed by the FTND criteria among daily smokers. Little consensus exists on the best measure of nicotine dependence, but research supports that two items from the FTND score, time to first cigarette after waking and cigarettes smoked per day, are strong, valid, reliable predictors of quitting behaviors, which are key indicators of dependence (Baker *et al.*, 2007; Borland *et al.*, 2010; Hyland *et al.*, 2006). Studies also suggest that these two measures are distinct predictors of addiction (Borland *et al.*, 2010; Lessov *et al.*, 2004), chronic obstructive pulmonary disease (Guertin *et al.*, 2015) and lung cancer (Gu *et al.*, 2014), suggesting the possibility that different genetic factors may contribute to urgency to smoke and levels of cigarette consumption. In a sample of over 1000 young adults, Haberstick *et al.* (2007) found that time to first cigarette was the most informative measure of heritable factors from the FTND score. Our results complement these findings by illustrating that necessity to smoke measured by time to first cigarette after waking at least partly drives the association of the *CYP2A6* metabolism metric and nicotine dependence in young adult daily smokers. Although the physiologic mechanism underlying this association remains unknown, slow metabolizer daily smokers likely have more consistent nicotine levels throughout the day compared with fast metabolizer daily smokers, which may contribute to a feeling of greater necessity to smoke in the morning when nicotine levels are low.

These findings in young adults should be considered in the context of the literature about adult smoking. Previous studies of adults demonstrate that once dependent, slower metabolizers smoke fewer cigarettes to reach target blood nicotine levels (Benowitz, 2008) (step 3 in Fig. 3). Although we did not observe an effect of slow metabolism on risk of smoking more than 20 cigarettes per day among daily smokers (Table 2, Fig. 2), only 26 percent of these young adults were heavy smokers, and heaviness of smoking continues to increase throughout adulthood (NSDUH, 2015). In the entire COGEND sample ages 25–45 years, a previous analysis demonstrated that among nicotine dependent smokers, slower metabolism is associated with decreased cigarette consumption (Bloom *et al.*, 2012). It is possible that slow metabolism is primarily protective at high levels of cigarette consumption, which is most evident in older populations of adults. Overall, these findings underscore that variation in *CYP2A6* has a variety of effects on smoking behaviors across stages of development: slow metabolism leads to increased risk for developing nicotine dependence in young adult daily smokers through time to first cigarette, but once dependent, slow metabolism is protective against heaviness of smoking.

Another important consideration is that the fraction of slow metabolizers in the population of smokers has been observed to decrease with age, suggesting that slow metabolizers are more likely to quit smoking (Benowitz, 2008) (step 4 in Fig. 3). In the COGEND dataset, among current nicotine dependent smokers ages 25–30 years,

we found that 36 percent (60/166) were slow metabolizers. However, among current nicotine dependent smokers over 30 years old, only 28 percent (250/883) were slow metabolizers (Chi-square, $P = 0.04$), supporting that proportionally more slow metabolizers have quit by this time. Furthermore, other studies directly support that slow nicotine metabolism, measured by *CYP2A6* genotypes or the nicotine metabolite ratio, is associated with increased cessation rates in both youth (Chenoweth *et al.*, 2013) and adults enrolled in clinical trials (Chen *et al.*, 2014; Ray *et al.*, 2009). Taken together, these findings suggest that across development, slow metabolizers may quit smoking more easily. Therefore, the observation that slow metabolism is associated with increased risk of nicotine dependence may be most pronounced in samples of youth when symptoms of dependence are first developing and before cessation attempts occur.

The findings reported here have limitations. First, this study focused on individuals of European Ancestry because the metabolism metric was optimized for this population (Bloom *et al.*, 2011). Second, the precise timing of length of transitions between smoking behaviors could not be examined in these analyses because the smoking questions did not assess age of onset. Third, the majority of the COGA participants were from families at high risk for alcoholism and rates of DSM-IV alcohol dependence are high in this sample, which may affect the generalizability of the findings. Secondary analyses that include DSM-IV alcohol dependence as a covariate and replication of the primary findings in a community-based recruitment sample (COGEND), however, support the conclusion that the findings are not specific to a high-risk population.

In summary, using a validated *CYP2A6* metabolism metric, this study demonstrates that slower nicotine metabolism is associated with an increased risk of nicotine dependence in two independent samples of young adult daily smokers. These findings add important knowledge about the complex role of *CYP2A6* variation across different developmental stages of smoking behaviors.

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Disclosure/Conflict of Interest

LJB, AG, and the spouse of NLS are listed as inventors on Issued U.S. Patent 8,080,371, 'Markers for Addiction' covering the use of certain SNPs in determining the diagnosis, prognosis, and treatment of addiction. JN is an investigator for Assurex and an investigator and consultant for Janssen. The other authors declare no conflict of interest.

Authors Contributions

LJB, AG, JPR, DMD, HJE, VMH. JRK, SK, JN, BP, MAS and JAT contributed to the conception and design of COGA. LJB, AG, JPR, EOJ, NLS, NB, DH and JS contributed to the conception and design of COGEND. AB and JAT managed the DNA biorepository. JPB performed the genotyping. SB cleaned the genetic data. EO performed the statistical analyses. All authors assisted with analysis design and interpretation of findings. EO and LJB drafted the manuscript. All authors critically reviewed the manuscript, provided important intellectual feedback and approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Predicted metabolism metric and *CYP2A6* diplotypes based on copy number and 5