

Distinct Loci in the *CHRNA5/CHRNA3/CHRNA4* Gene Cluster Are Associated With Onset of Regular Smoking

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ABSTRACT: Neuronal nicotinic acetylcholine receptor (nAChR) genes (*CHRNA5/CHRNA3/CHRN4*) have been reproducibly associated with nicotine dependence, smoking behaviors, and lung cancer risk. Of the few reports that have focused on early smoking behaviors, association results have been mixed. This meta-analysis examines early smoking phenotypes and SNPs in the gene cluster to determine: (1) whether the most robust association signal in this region (rs16969968) for other smoking behaviors is also associated with early behaviors, and/or (2) if additional statistically independent signals are important in early smoking. We focused on two phenotypes: age of tobacco initiation (AOI) and age of first regular tobacco use (AOS). This study included 56,034 subjects (41 groups) spanning nine countries and evaluated five SNPs including rs1948, rs16969968, rs578776, rs588765, and rs684513. Each dataset was analyzed using a centrally generated script. Meta-analyses were conducted from summary statistics. AOS yielded significant associations with SNPs rs578776 (beta = 0.02, $P = 0.004$), rs1948 (beta = 0.023, $P = 0.018$), and rs684513 (beta = 0.032, $P = 0.017$), indicating protective effects. There were no significant associations for the AOI phenotype. Importantly, rs16969968, the most replicated signal in this region for nicotine dependence, cigarettes per day, and cotinine levels, was not associated with AOI ($P = 0.59$) or AOS ($P = 0.92$). These results provide important insight into the complexity of smoking behavior phenotypes, and suggest that association signals in the *CHRNA5/A3/B4* gene cluster affecting early smoking behaviors may be different from those affecting the mature nicotine dependence phenotype.

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KEY WORDS: *CHRNA5*; *CHRNA3*; *CHRN4*; meta-analysis; nicotine; smoke

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Introduction

Tobacco use usually begins during adolescence; over 90% of those who smoke more than 100 cigarettes become regular smokers and one-third of those adolescents who experiment with tobacco go on to become nicotine dependent [Anthony et al., 1994; CDC, 2005; Mayhew et al., 2000]. Symptoms of dependence in these youths may appear within weeks of occasional tobacco use [DiFranza, 2008; DiFranza et al., 2000, 2002; Doubeni et al., 2010; Escobedo et al., 1993; O'Loughlin et al., 2002a, 2002b], particularly loss of control over their smoking [DiFranza et al., 2007; Scragg et al., 2008; Soteriades et al., 2011]. Early initiation of tobacco use is also associated with an increased number of daily cigarettes smoked in adulthood [Chen and Millar, 1998; D'Avanzo et al., 1994; Eisner et al., 2000; Everett et al., 1999; Fernandez et al., 1999] and a decreased rate of smoking cessation [Breslau and Peterson, 1996; Chen and Millar, 1998; Eisner et al., 2000; Lando et al., 1999]. Age of smoking onset in women is a significant predictor of cessation during pregnancy; a woman who initiates earlier is more likely to smoke while pregnant [Chen et al., 2006]. Individuals who initiate tobacco use early in life are also at greater risk for smoking-related lung cancer, in part because this tissue type continues to develop into early adulthood [Hirao et al., 2001; Wiencke and Kelsey, 2002; Wiencke et al., 1999]. In addition, smoking accelerates the rate of cer-

vical maturation and cell proliferation, therefore resulting in an increased risk of cervical cancer [Hwang et al., 2009; Ma et al., 2011]. Given the long-term health implications of early tobacco use, many studies have worked to identify and understand the underlying genetic and environmental risk factors for early smoking behaviors.

It is well established that genetic factors contribute to risk for tobacco initiation, quantity smoked, nicotine dependence, and smoking persistence [Li et al., 2003; Rhee et al., 2003; Rose et al., 2009; Schnoll et al., 2007]. Neuronal nicotinic acetylcholine receptor (nAChR) subunit genes are among the top candidates for several smoking-related phenotypes because a cluster of nicotinic receptor genes (*CHRNA5/CHRNA3/CHRNA4*) on chromosome 15q25 has been repeatedly associated with nicotine dependence, smoking behaviors, and lung cancer [Amos et al., 2008; Berrettini et al., 2008; Broms et al., 2012; Furberg et al., 2010; Greenbaum and Lerer, 2009; Hung et al., 2008; Liu et al., 2010; Rose, 2007; Saccone et al., 2010a, 2007; Thorgeirsson et al., 2008; 2010]. This region also contains the nonsynonymous risk variant rs16969968 G/A, that causes a functional amino acid change in the $\alpha 5$ subunit protein (Asp398Asn); the Asparagine (Asn) allele decreases response to a nicotine agonist [Bierut et al., 2008]. The high-risk $\alpha 5$ Asn 398 is associated with reduced Ca^{2+} permeability and desensitizes faster than the wild type Aspartic acid (Asp) at 398 ($\alpha 4\beta 2$) $_2 \alpha 5$ [Kuryatov et al., 2011]. In addition, Alpha5 knockout mice showed increased nicotine intake [Fowler et al., 2011]. This variant accounts for 4% of the variance in the serum levels of the long-term metabolite of nicotine, cotinine [Etter et al., 2009; Keskitalo et al., 2009]. In addition to this well-characterized functional variant, there have been other robust associations within the *CHRNA5/CHRNA3/CHRNA4* gene cluster to smoking quantity. These were reported in three genome-wide meta-analyses identifying SNPs in the cluster as top association signals with the outcome of cigarettes smoked per day (CPD) [Furberg et al., 2010; Liu et al., 2010; Thorgeirsson et al., 2010]. These reports support the prevailing view that smoking quantity/frequency as a proxy for nicotine dependence is most consistently associated with SNPs at the *CHRNA5/CHRNA3/CHRNA4* locus. A fourth meta-analysis [Saccone et al., 2010a] focused on four statistically distinct loci in the *CHRNA5/CHRNA3/CHRNA4* cluster tagged by SNPs rs16969968 (*CHRNA5*), rs578776 (*CHRNA3*), rs588765 (*CHRNA5*), and rs12914008 (*CHRNA4*) [Saccone et al., 2009a]. SNPs rs16969968 and rs588765 were each strongly associated with CPD in a joint model that adjusts for the effect of each, suggesting independent effects; rs578776 is also associated with CPD, but its effect size is smaller after adjustment for SNP rs16969968 in the association model [Saccone et al., 2010a].

Although the majority of the studies of *CHRNA5/CHRNA3/CHRNA4* variation and tobacco have targeted smoking behaviors in adulthood, a few reports specifically focused on early smoking behaviors. Weiss et al. found that a *CHRNA5/CHRNA3/CHRNA4* haplotype including SNPs rs16969968 and rs578776 was significantly associated

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with severity of nicotine dependence among individuals who began daily smoking at or before age 16, but not in those who began smoking after age 16 [Weiss et al., 2008]. Correlated SNPs rs1948 (*CHRNA4*) and rs8023462 (*CHRNA3/CHRNA4*) ($r^2 = 0.8$) were found to be associated with early age of tobacco initiation in two independent samples [Schlaepfer et al., 2008]. These two SNPs are not in high linkage disequilibrium with any of the four loci mentioned above (rs1696998 $r^2 = 0.32$, rs578776 $r^2 = 0.09$, rs588765 $r^2 = 0.56$, and rs12914008 $r^2 = 0.02$) (1000 Genomes Pilot) 1 CEU [Abecasis et al., 2012]. A second study targeting age of tobacco initiation in a Korean sample found that SNPs rs6495308, rs11072768, and rs951266 were significant for association [Li et al., 2010], but it is not clear whether in this sample these SNPs are in high linkage disequilibrium with the four previously described loci. Two genome-wide meta-analyses (with CPD as the primary phenotype) also included age of tobacco initiation as an outcome and did not find any association that exceeded genome-wide significance [Furberg et al., 2010;

Thorgeirsson et al., 2010]. In another large meta-analysis, age at onset of regular smoking modified the association between rs16969968 and CPD, with early onset of smoking being associated with a stronger gene-phenotype association. In that analysis, no association was seen between dichotomized age of onset (at age 16) and rs16969968 ($P = 0.77$) among 67,128 smokers [Hartz et al., 2012]. Thus, while some reports have found evidence for association in this region with age of tobacco initiation, others have not, and it has not been clear whether separate independent loci in these genes may be associated with different phenotypes related to early smoking behavior.

The goal of the current study was to perform a meta-analysis specifically targeting early smoking phenotypes and SNPs in the *CHRNA5/CHRNA3/CHRNA4* gene cluster. These analyses were undertaken to determine: (1) whether the most robust association signal in this region shown for other smoking behaviors (rs16969968) is also seen for age of smoking initiation and age of onset of regular smoking, and/or: (2)

Table 1. Summary of studies participating. Column 1 lists the acronym for each group, defined in the Supplementary Information (Dataset Descriptions). Column 2 indicates the number of subjects utilized for this meta-analysis. Columns 3 and 4 provide the mean age of initiation of tobacco (AOI) and onset of regular smoking (AOS) for groups that have those phenotypes. Columns 5–9 give minor allele frequencies at target SNPs for individual groups

Datasets	N	AOI	AOS	Recruitment location	rs				
					rs16969968 A	rs578776 T	rs588765 T	rs1948 A	rs684513 G
ACS-COPD	2,791		19.2	US	0.35	0.27	0.42	0.34	0.21
ACS-LCA	988		18	US	0.38	0.25	0.42	0.34	–
Add Health	690	12.8	15.5	US	0.31	0.26	0.41	0.34	0.22
ARIC	5,775		18.5	US	0.34	0.28	0.42	–	0.21
BOMA	828		18.1	Germany	0.36	0.27	0.41	0.34	–
CADD	397	14	15.4	US	0.32	0.24	0.43	0.35	0.23
COGA	1,704	14.3	21.9	US	0.34	0.29	0.41	–	–
COGEND	2,057	13.3	15.5	US	0.35	0.27	0.42	0.33	–
Dental Caries	459	13.8		US	0.36	0.27	0.42	0.33	–
EAGLE-PLCO	4,948		18	Italy	0.4	0.26	0.37	0.31	–
FINRISK	7,542		21.6	Finland	0.33	0.32	0.38	–	–
GEOS	475		16.9	US	0.37	0.3	0.39	0.31	–
KCI-WSU	945		16.4	US	0.38	–	–	–	–
LHS-Utah	1,943		17.4	US	0.39	0.24	–	–	–
LOLIPOP	643		17.6	UK	0.31	0.31	0.43	–	–
MDACC LCA	2,289		17.5	US	0.37	0.19	0.43	0.34	0.2
MDACC Melanoma	825		18.6	US	0.33	0.27	0.44	0.35	0.23
MUC12 cases	339		19.5	Germany	0.36	0.19	0.43	0.35	–
MUC12 controls	216		20.1	Germany	0.34	0.21	0.43	0.32	–
MUCMD cases	523		18.9	Germany	0.37	0.25	0.42	0.33	0.19
MUCMD controls	989		20.2	Germany	0.35	0.27	0.42	0.32	0.21
NAG OZALC	792		17	Australia	0.36	0.21	0.43	0.34	–
NAG FIN	714	15.4	18.1	Finland	0.37	0.3	0.36	0.31	–
NESDA	1,138		16.3	Netherlands	0.31	0.3	0.38	–	–
NFBC1966	2,243	16.1	18.3	Finland	0.32	0.27	0.37	0.34	–
NHS BRCA	1,198		19.5	US	0.35	0.27	0.42	0.34	–
NHS CHD	724		19.7	US	0.35	0.22	0.43	–	–
NHS T2D	1,590		19.6	US	0.34	0.24	0.4	–	–
NTR1	464	16.2	18.7	Netherlands	0.31	0.17	0.9	0.33	0.28
NTR2	593	15.9	18.1	Netherlands	0.31	0.3	0.46	0.36	–
NYS-FS	422	14.2	17	US	0.32	0.24	0.46	0.36	0.21
QIMR	258	16.4		Australia	–	0.29	–	0.34	–
SHIP	1,871		18.5	Germany	0.34	0.22	0.43	–	–
SMOFAM	398		17.8	US	0.32	0.28	0.46	0.38	0.18
STR	4,096		18	Sweden	0.34	0.28	–	0.34	–
UTAH	484	14.8	18	US	0.39	0.24	–	–	–
VA-Twin	1,960	14.4	16.5	US	0.33	0.28	–	–	–
WTCCC-CHD	1,219		17.7	UK	0.33	0.26	0.46	–	–
WTCCC-HT	766		17.3	UK	0.31	0.31	0.44	–	–
Yale	942	13.8	17.5	US	0.35	0.29	–	–	0.22
YFS	365	10.9	14.6	Finland	0.32	0.31	0.4	0.35	–

if additional statistically independent signals are important in adolescent initiation of tobacco use and progression to regular smoking.

Methods

Subjects

A total of 56,034 subjects from 41 datasets spanning nine countries were included in a meta-analysis. Sample sizes for each SNP and the two phenotypes differ (age of tobacco initiation $N \sim 12,662$, age of onset of regular smoking $N \sim 55,317$) depending on which of the studies, listed in Table 1, had contributed information. All but 11 of these datasets consisted of unrelated ever-smokers of European descent. Details regarding individual study characteristics are provided in supplementary Table S1. Eight (Dental Caries, VA-Twin, NAG-FIN, NAG-OZALC, NTR1, NTR2, QIMR, and STR) were subsets of unrelated subjects selected from family-based datasets. The Add Health, NYS-FS, and SMOFAM datasets included family members of European descent. Informed consent was obtained from all participants and was approved by the local institutional review boards. Inclusion into this study required ever smokers to be assessed for (1) Age of tobacco initiation (AOI); and/or (2) Age of onset of regular smoking (AOS). Mean AOI and AOS values are shown in Table 1. Phrasing of AOI and AOS questions is provided in supplementary Table S1.

SNPs

SNP rs1948 was chosen due to a previously established association with AOI [Schlaepfer et al., 2008]. Based on previous results [Saccone et al., 2010a; Saccone et al., 2009a], we also included three statistically distinct nicotine dependence loci in the *CHRNA5/A3/B4* gene cluster: rs16969968, rs578776, and rs588765. If a group did not have genotype data for SNPs rs16969968, rs578776, or rs588765 we chose a highly correlated ($r^2 > 0.8$) SNP proxy. Another option would have been to use imputed data. However, in that situation, it would have been important for all imputation to be performed consistently between groups which would have required some groups to reimpute specifically for this project. The advantage of using an imputed SNP instead of a high-LD proxy for this study was not compelling because the imputation quality of the SNP would need to be taken into account as well. Thus, imputed SNPs were unlikely to add a significant amount of information to this study and/or change the take home message. SNP proxies for applicable datasets are shown in Table 2. Information on r^2 was obtained using Haploview HapMap II+III CEU [Barrett et al., 2005] and the 1000 Genomes Pilot 1 CEU [Abecasis et al., 2012]. SNP rs684513 was included due to associations with nicotine dependence [Winterer et al., 2010] and lung cancer risk [Amos et al., 2010]; its r^2 is less than 0.8 with rs16969968, rs578776, and rs588765 in both Hapmap [Altshuler et al., 2010] and the 1000 genomes project (<http://www.1000genomes.org>) CEU

Table 2. Summary of SNP proxies for participating groups. Column 1 lists the acronym for each group, defined in the Supplementary Information (Dataset Descriptions). Columns 2–4 show the SNP proxies utilized by each group for rs16969968, rs578776, and rs588765 respectively. If a space is blank then the group genotyped the SNP of interest directly. A “–” indicates the group neither genotyped the SNP of interest directly, nor had an available proxy

Datasets	rs16969968 proxy	rs578776 proxy	rs588765 proxy
ACS-COPD			
ACS-LCA	rs8034191		rs6495306
Addhealth		rs11637630	rs680244
ARIC	rs17486278		rs6495306
BOMA	rs1051730		rs6495306
CADD		rs11637630	rs680244
COGA			rs6495306
COGEND			
Dental Caries	rs1051730		rs6495306
EAGLE	rs1051730		rs6495306
FINRISK			
GEOS			rs6495306
KCI-WSU		–	–
LHS-Utah			–
LOLIPOP			
MDACC LCA	rs1051730	rs6495309	rs6495306
MDACC Melanoma			rs6495306
MUC12 cases	rs1051730	rs6495309	rs6495306
MUC12 controls	rs1051730	rs6495309	rs6495306
MUCMD cases			rs680244
MUCMD controls			rs680244
NAG OZALC	rs1051730	rs6495309	rs680244
NAG FIN	rs1051730		rs6495306
NESDA			rs11637635
NFBC1966	rs1051730	rs6495309	rs6495306
NHS BRCA	rs1051730		rs6495306
NHS CHD	rs951266	rs6495306	rs938682
NHS T2D	rs951266	rs6495306	rs938682
NTR1			rs1163635
NTR2	rs1051730		rs680244
NYS-FS		rs11637630	rs680244
QIMR			
SHIP	rs951266	rs938682	rs6495306
SMOFAM	rs1317286		rs555018
STR			680244
UTAH	rs1051730		–
VA-Twin			–
WTCCC-CHD			
WTCCC-HT			
Yale			rs621849
YFS	rs1051730		rs6495306

Table 3. Linkage Disequilibrium values (as measured by r^2) utilized for the meta-analysis and taken from the 1000 genomes project Pilot 1 <http://www.1000genomes.org/>

SNP/Locus	rs16969968	rs578776	rs588765	rs1948
rs16969968				
rs578776	0.23			
rs588765	0.46	0.04		
rs1948	0.32	0.09	0.56	
rs684513	0.1	0.61	0.1	0.06

data [Abecasis et al., 2012]. Linkage disequilibrium (as measured by r^2) for all five loci is presented in Table 3. Allele frequencies for SNP proxies and target SNPs in each dataset are given in Table 1.

Quality Control

Quality control procedures were carried out to ensure proper coding of variables across studies. Specifically, this was carried out by examination of univariate and multivariate distributions for phenotypic variables within each study, allele frequencies for SNPs, and conformity with Hardy-Weinberg Equilibrium (HWE).

Statistical Analyses

Case/Control Data

To ensure consistent analyses across individual datasets, a unified script was created using SAS (SAS Institute, Cary, NC) and R (<http://www.r-project.org/>). These scripts were developed and distributed following protocols comparable to Saccone et al. [Saccone et al., 2010a]. Briefly, participating groups held several planning conference calls to decide on the analytical model and scripts were written through collaborative efforts between SHS at the University of Colorado and SMH at Washington University in St. Louis. Individual analyses were carried out by each participating group and summary results returned to the coordinating group.

AOS and AOI phenotypes were normalized within each dataset. Z-scores were calculated to correct for age and sex prior to analysis. Scripts were written to perform a linear regression using AOS or AOI as the dependent variable and each SNP as the independent variable. Each SNP was coded according to an additive model (0, 1, or 2 copies of the minor allele) and each SNP was analyzed separately.

Family Data

PLINK [Purcell et al., 2007] was used to analyze the three datasets drawn from families (Add Health, NYS-FS, and SMOFAM). The QFAM total association test (`-qfam-total`) was used to conduct a family-based analysis that partitions the between family variance and the within family variance [Fulker et al., 1999] and is similar to that incorporated in the QTDT package [Abecasis et al., 2000]. This linear regression approach was ideal for this study because beta values from each family study analysis could be included.

In order to maintain uniformity in individual analyses, SHS compiled detailed instructions and distributed these to the groups analyzing family data with information to Z-score AOS or AOI in SAS (SAS Institute, Cary, NC) and R (<http://www.r-project.org/>), to code additively with a consistent minor allele, and to perform the analyses in PLINK with the `-qfam-total` option. Results were returned to SHS.

Meta-Analyses

Meta-analysis was carried out using the meta-analysis procedure in PLINK [Purcell et al., 2007]. This procedure requires results files (for a quantitative trait) with beta estimates, standard errors, and an optional *P*-value. PLINK meta-analysis output includes a beta summary estimate, and

a summary *P*-value for both random and fixed effects models. The R package `rmeta` was used to create forest plots.

In addition, the PLINK meta-analysis output provides a measure of heterogeneity with a *P*-value for Cochran's *Q* statistic. This heterogeneity index in a meta-analysis refers to the variation in outcomes (betas) between studies. The *Q* statistic is calculated as the weighted sum of the squared differences between individual study effects (individual betas) and pooled effects (summary beta) across studies. *Q* has a chi-squared distribution with number of studies minus one degree of freedom.

A total of five SNPs and two phenotypes were examined, so the Bonferroni correction for multiple testing would be $0.05/10 = 0.005$. This threshold for significance is overly conservative because of a correlation that exists between certain of these SNPs (`rs684513/rs578776` $r^2 = 0.6$). However, the goal of this study was not to discover a novel association but to disentangle the well-established association between these genes and tobacco behaviors. Thus, the nominal *P*-values provided should be interpreted with this in mind.

Results

Descriptive

Allele frequencies were utilized to confirm correct coding of the minor allele for each locus and are presented in Table 1. Frequencies were similar among individual datasets and also to those of the target SNPs in the CEU population in HapMap. The mean AOI for the 15 contributing datasets was 14.4 years (range, 10.9–16.4). The mean AOS for the 39 contributing datasets was 18 years (range, 14.6–21.9).

Single SNP Analysis for AOI and AOS Phenotypes

Tables 4 and 5 show PLINK meta-analysis results for each single locus and the AOI and AOS quantitative phenotypes. Results were nearly identical for both the fixed effects and random effects models. Table 4 presents results of AOI analysis where column one shows the SNP/Locus and columns two and three are the number of contributing datasets and the number of subjects with genotypic and phenotypic information at that SNP/Locus. Columns four and five show the summary beta and *P*-value, respectively. Column six is the *P*-value for the Cochran's *Q* statistic. There were no significant associations for AOI at any of the five loci.

Table 5 shows the corresponding results of AOS meta-analyses. Locus `rs578776` was significantly associated with AOS (beta = 0.02, nominal *P* = 0.004, adjusted *P* = 0.04). Figure 1A shows the meta-analysis forest plot for the association between `rs578776` with a phenotype of AOI while Figure 1B shows these results for AOS. Although the `rs578776` locus was not significantly associated with AOI, both betas were 0.02 and suggest that for AOS, the minor allele confers a protective effect because it is associated with a later age of smoking onset. In addition, SNPs `rs1948` and `rs684513` were

Table 4. Results of single SNP analysis and the age of initiation of tobacco (AOI) outcome. Column 1 = SNP/Locus, column 2 = number of contributing datasets for each locus, column 3 = number of total subjects with genotypic and phenotypic information, column 4 = summary Beta, column 5 = summary P-value, column 6 = Cochran's Q statistic P-value. Random effects versus a fixed effects model produce nearly identical betas and P-values

SNP/Locus	Number of contributing datasets (AOI)	Number of Subjects	Summary beta (SE)	Summary P-value	Cochran's Q Statistic P-value
Locus 1: rs16969968	15	12,662	0.007 (0.013)	0.588	0.261
Locus 2: rs578776	15	12,662	0.02 (0.014)	0.233	0.723
Locus 3: rs588765	12	10,269	-0.019 (0.015)	0.198	0.084
rs1948	11	9,278	-0.027 (0.016)	0.362	0.138
rs684513	5	2,821	0.023 (0.034)	0.507	0.531

Table 5. Results of single SNP analysis and the age of onset of regular smoking (AOS) outcome. Column 1 = SNP/Locus, columns 2 = number of contributing datasets for each locus, column 3 = number of total subjects with genotypic and phenotypic information, column 4 = summary Beta, column 5 = summary P-value, column 6 = Cochran's Q statistic P-value. Significant summary P-values are indicated with bold. Random effects versus a fixed effects model produce nearly identical betas and P-values

SNP/Locus	Number of contributing datasets (AOS)	Number of Subjects	Summary Beta (SE)	Summary P-value	Cochran's Q Statistic P-value
Locus 1: rs16969968	39	55,317	0.0004 (0.004)	0.917	0.052
Locus 2: rs578776	38	54,796	0.02 (0.007)	0.004	0.601
Locus 3: rs588765	34	49,240	-0.009 (0.008)	0.297	0.01
rs1948	24	28,970	0.023 (0.009)	0.018	0.772
rs684513	12	15,950	0.032 (0.013)	0.017	0.676

associated with AOS (rs1948 beta = 0.023, $P = 0.018$; rs684513 beta = 0.032, $P = 0.017$). Figure 2A and B show meta-analysis forest plots for rs1948 with AOI and AOS, respectively. Figure 3A and B show forest plots for rs684513 AOI and AOS, respectively.

Cochran's Q statistic P-values (column six of Tables 4 and 5) indicate that there was one SNP/loci (rs588765 AOS only) showing modest heterogeneity.

Discussion

This meta-analysis was designed to examine the relationship of SNPs in the *CHRNA5/CHRNA3/CHRNA4* gene cluster with early smoking behaviors including AOI and AOS. Genetic variation in this genomic region shows robust association to nicotine dependence and tobacco use behaviors in adult samples [Furberg et al., 2010; Liu et al., 2010; Saccone et al., 2010a; Thorgeirsson et al., 2010]. Although previous studies examining association with tobacco initiation [Furberg et al., 2010; Thorgeirsson et al., 2010] and first regular use have been inconclusive, most have not found association with the locus including SNP rs16969968 [Furberg et al., 2010; Hartz et al., 2012; Thorgeirsson et al., 2010]. We hypothesized that a possible explanation for conflicting reports may be related to the multiple independent loci in this region [Saccone et al., 2009a; Saccone et al., 2010b], if different loci contribute to different smoking-related phenotypes. This analysis expands on previous work by examining two additional SNPs (rs1948 and rs684513) based on previous associations with smoking-related phenotypes [Amos et al., 2010; Schlaepfer et al., 2008; Winterer et al., 2010], in addition to three statistically independent nicotine dependence loci in the *CHRNA5/A3/B4* gene cluster: rs16969968, rs578776, and rs588765.

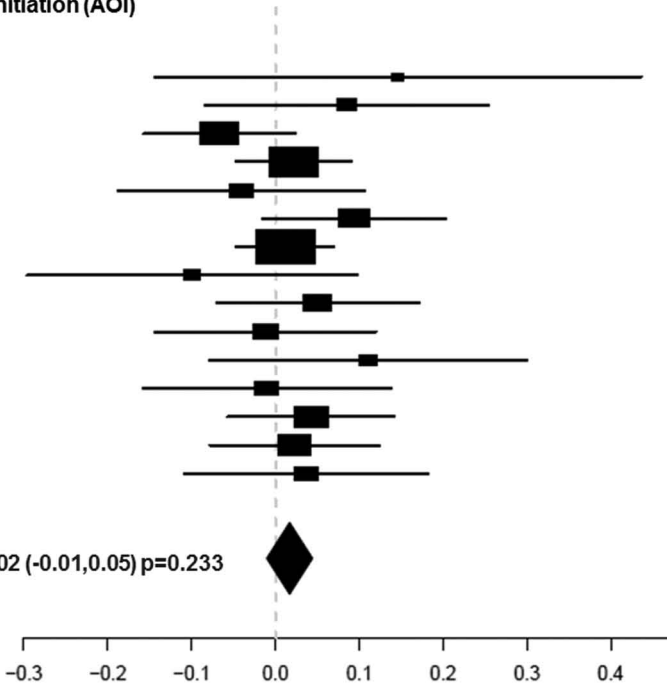
Results revealed evidence for association between AOS and three of the five target SNPs. Importantly, AOS shows no association with rs16969968, which is strongly and reproducibly associated with nicotine dependence, smoking quantity, and cotinine levels [Furberg et al., 2010; Greenbaum and Lerer 2009; Keskitalo et al., 2009; Saccone et al., 2010b; Saccone et al., 2007; Thorgeirsson et al., 2010]. This is consistent with previous reports [Broms et al., 2012; Ducci et al., 2011; Furberg et al., 2010; Thorgeirsson et al., 2010; Weiss et al., 2008]. One explanation put forward for the lack of findings with tobacco initiation and rs16969968 is a report by Hong et al where the "risk" allele (A) predicts a defect in a dorsal anterior cingulate-ventral striatum/extended amygdala circuit whose weakness predicts addiction severity [Hong et al., 2011]. One could also reason that rs16969968 is not associated with initiation and instead more strongly related to craving and ability to quit due to the action of *CHRNA5* to alter output of deep cortical layers [Picciotto and Kenny, 2012].

In the current report, the strongest evidence for association with AOS was detected with SNP rs578776 ($P = 0.004$). There was also evidence for association between SNPs rs1948 (beta = 0.023, $P = 0.018$) and rs684513 (beta = 0.032, $P = 0.017$) and AOS. Similar to rs578776, the betas for these SNPs were positive, signifying that having more copies of the minor alleles is associated with a later age of smoking onset. Results with rs578776 and rs684513 are in agreement with a previous study [Broms et al., 2012], where there was evidence for association of age of onset of smoking (AOS) and the Locus 2 signal (rs578776) and rs684513. In addition, significance with rs1948 in this report is supported by previous findings of significant association with "smoking at age 14" and suggestive evidence with age of onset of smoking [Broms et al., 2012].

A

rs578776 Age of Tobacco Initiation (AOI)

Add Health n=655 beta=0.15
CADD n=393 beta=0.085
COGA n=1058 beta=-0.067
COGEND n=2045 beta=0.022
Dental caries n=459 beta=-0.04
NAG-FIN n=714 beta=0.094
NFBC1966 n=2908 beta=0.012
NTR1 n=409 beta=-0.099
NTR2 n=593 beta=0.05
NYS-FS n=415 beta=-0.012
QIMR n=258 beta=0.11
UTAH n=484 beta=-0.01
VA-Twin n=974 beta=0.043
YALE n=932 beta=0.023
YFS n=365 beta=0.037

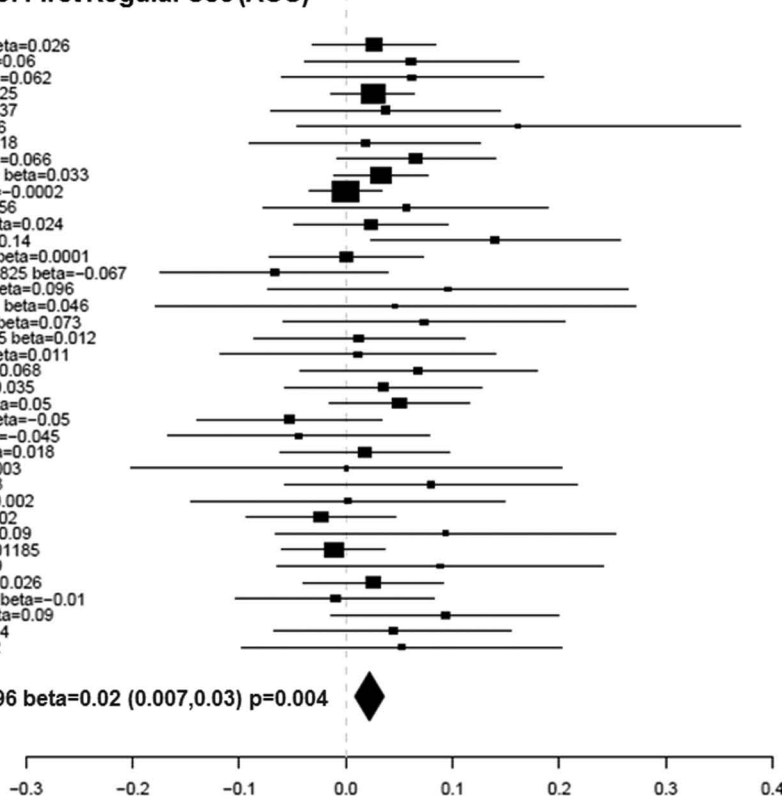


Summary n=12662 beta=0.02 (-0.01,0.05) p=0.233

B

rs578776 Age of First Regular Use (AOS)

ACS-COPD n=2787 beta=0.026
ACS-LCA n=987 beta=0.06
Add Health n=668 beta=0.062
ARIC n=5703 beta=0.025
BOMA n=828 beta=0.037
CADD n=251 beta=0.16
COGA n=743 beta=0.018
COGEND n=1764 beta=0.066
EAGLE-PLCO n=4949 beta=0.033
FINRISK n=6520 beta=-0.0002
GEOS n=475 beta=0.056
LHS-UTAH n=1943 beta=0.024
LOLIPOP n=643 beta=0.14
MDACC-LCA n=2288 beta=0.0001
MDACC-Melanoma n=825 beta=-0.067
MUC12 cases n=339 beta=0.096
MUC12 controls n=216 beta=0.046
MUCMD cases n=505 beta=0.073
MUCMD controls n=955 beta=0.012
NAG-OZALC n=731 beta=0.011
NAG-FIN n=706 beta=0.068
NESDA n=1097 beta=0.035
NFBC1966 n=2242 beta=0.05
NHS-BRCA n=1198 beta=-0.05
NHS-CHD n=724 beta=-0.045
NHS-T2D n=1590 beta=0.018
NTR1 n=390 beta=0.0003
NTR2 n=467 beta=0.08
NYS-FS n=415 beta=0.002
SHIP n=1868 beta=-0.02
SMOFAM n=397 beta=0.09
STR n=4096 beta=-0.01185
UTAH n=484 beta=0.09
VA-Twin n=1958 beta=0.026
WTCCC-CAD n=1218 beta=-0.01
WTCCC-HT n=766 beta=0.09
YALE n=731 beta=0.044
YFS n=329 beta=0.052



Summary n=54796 beta=0.02 (0.007,0.03) p=0.004

Figure 1. (A) Meta-analysis forest plot for rs578776 age of tobacco (AOI) initiation showing the individual betas (for each study), summary beta, individual number of subjects (*n*), summary *n*, and a summary *P*-value. Individual and summary betas are for the rs578776 T allele. (B) Meta-analysis forest plot for rs578776 age of first regular tobacco use (AOS) showing the individual betas (for each study), summary beta, individual number of subjects (*n*), summary *n*, and a summary *P*-value. Individual and summary betas are for the rs578776 T allele.

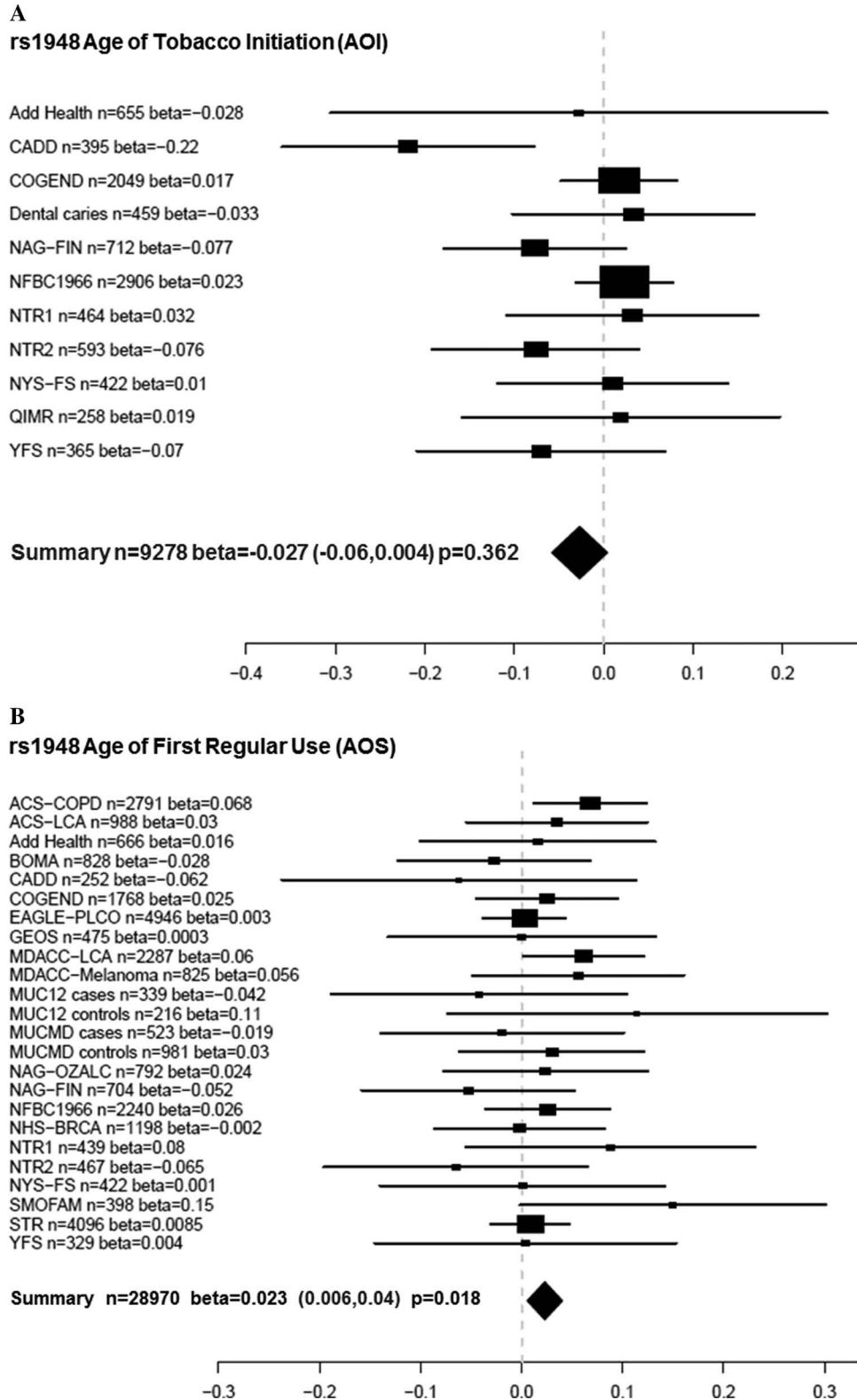


Figure 2. (A) Meta-analysis forest plot for rs1948 age of tobacco initiation (AOI) showing the individual betas (for each study), summary beta, individual number of subjects (*n*), summary *n*, and a summary *P*-value. Individual and summary betas are for the rs1948 A allele. (B) Meta-analysis forest plot for rs1948 age of first regular tobacco use (AOS) showing the individual betas (for each study), summary beta, individual number of subjects (*n*), summary *n*, and a summary *P*-value. Individual and summary betas are for the rs1948 A allele.

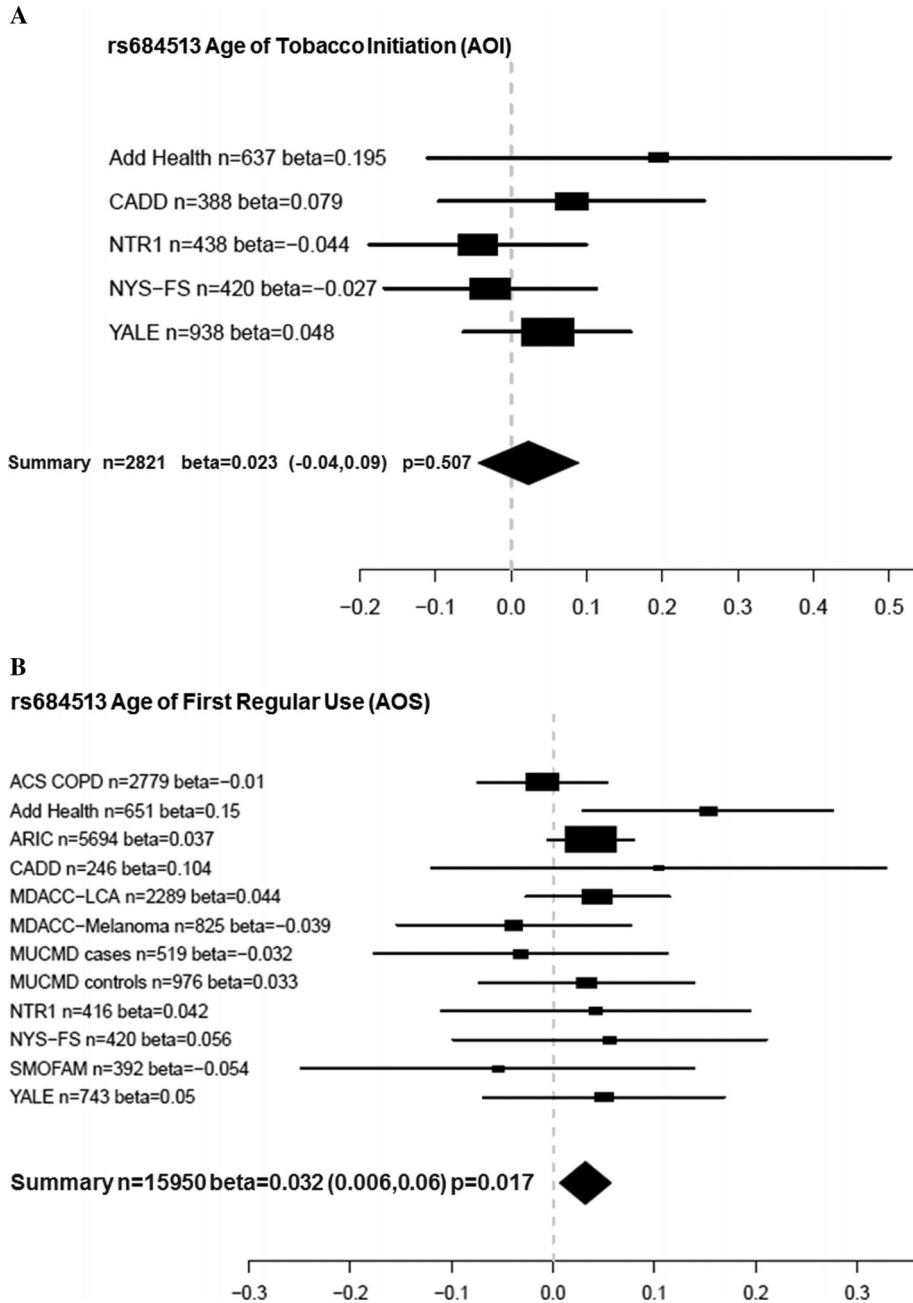


Figure 3. (A) Meta-analysis forest plot for rs684513 age of tobacco initiation (AOI) showing the individual betas (for each study), summary beta, individual number of subjects (n), summary n , and a summary P -value. Individual and summary betas are for the rs684513 G allele. (B) Meta-analysis forest plot for rs684513 age of first regular tobacco use (AOS) showing the individual betas (for each study), summary beta, individual number of subjects (n), summary n , and a summary P -value. Individual and summary betas are for the rs684513 G allele.

In contrast to the AOS findings, there was no evidence for association between AOI and any of the SNPs examined in the *CHRNA5/A3/B4* cluster. It might be intuitively expected that AOI and AOS would be highly correlated and that the associations with risk SNPs would not vary between them greatly even though there are differences in statistical power between AOI and AOS due to sample size. However, the correlations between AOI and AOS among the samples where

both measures are available are actually rather low (ranging from 0.20–0.65, data not shown). There is additional support for the hypothesis that a low correlation exists between these two phenotypes. Breslau et al. (1993) showed the lag time from first cigarette to daily smoking onset was significantly longer in those who initiated <14 years of age relative to those initiating ≥ 17 years of age. A social explanation was proposed for these findings in that early initiators were said to

face a nonpermissive environment (e.g. availability, community norms, adult supervision, etc.) [Breslau et al., 1993]. In addition, we performed an ad hoc exploratory analysis to determine whether there were any trends with the five loci and the delta between AOI and AOS. Utilizing a subset of samples with both phenotypes available (Add Health, CADD, COGA, COGEND, NYS-FS, YFS), we found no evidence for association between the AOI–AOS time differential and any of the loci used for this study (data not shown). The similarities and differences in results across SNPs for the two phenotypes are interesting also to consider in the context of work finding an association between externalizing behaviors and rs8040868, which is a locus not well-tagged by the SNPs investigated here [Stephens et al., 2012]. It is possible that some of the differences between AOI and AOS results are related to more general underlying behaviors, such as novelty-seeking and conduct disorder. Future studies aimed at disentangling possible unique genetic contributions to these phenotypes are merited. An alternative explanation for these findings could be biological. Age at initiation is related to behavioral traits such as Attention Deficit Hyperactivity Disorder (ADHD), Conduct Disorder (CD), Oppositional Deviant Disorder (ODD), delinquency, and criminal behavior [Armstrong and Costello, 2002; Flory et al., 2003; Han et al., 1999; Krueger et al., 2002; McGue and Iacono, 2008; McGue et al., 2001; Sihvola et al., 2011; Tarter et al., 2003; Young et al., 2000], while the time from initiation to regular smoking could be related to individual differences in neuroadaptation to nicotine and other psychoactive components of tobacco. Thus, it is reasonable to propose that nicotinic receptor properties are related to the neuroadaptation process and consequently to *CHRN* gene polymorphisms.

These findings corroborate and strengthen certain previous reports. Rs578776 (*CHRNA3*) has been previously associated with nicotine dependence in subjects of European ancestry [Saccone et al., 2009a, 2009b, 2010b; Weiss et al., 2008] and heavy smoking [Stevens et al., 2008] with the minor allele (T) acting in a protective manner against nicotine dependence. Our results also support the protective effect of the minor allele where a positive beta (0.02) indicated a later age of first regular smoking among individuals with 1 or 2 copies of the T allele. However, the SNP is not known to be functional, so the basis for the association needs to be investigated further. SNP rs1948 (*CHRNA4*) has previously been associated with tobacco and alcohol age of initiation in the CADD sample and NYS-FS samples [Schlaepfer et al., 2008], but few other studies have focused on this locus. SNP rs1948 was recently associated with the tolerance factor of the Nicotine Dependence Syndrome Scale (NDSS) ($P = 0.0071$) [Broms et al., 2012] and SNP rs684513 (major allele) has been previously associated with nicotine dependence [Winterer et al., 2010] and lung cancer risk [Amos et al., 2010]. Lori et al found an association with FTND of this *CHRNA5* SNP [Lori et al., 2011]. These meta-analysis results support previous findings showing a protective effect of the minor allele for AOS ($P = 0.015$, $\beta = 0.034$).

The PLINK meta-option reports the Cochran's Q statistic as a measure of heterogeneity for each SNP. One significant

heterogeneity P -value was obtained for SNP rs588765 ($P = 0.01$) in the AOS analysis only. It is unlikely this heterogeneity can be accounted for by differences in phenotype assessment between studies because this would be predicted to result in significant heterogeneity for all loci in the AOS analysis. Large differences in allele frequencies between groups could also produce heterogeneity. Examination of allele frequencies for all participating groups suggests one outlier (NTR1 T allele frequency is 0.9) within this SNP/locus. In order to determine whether this sample population was driving the significant Q statistic for rs588765 and AOS, the meta-analysis was performed excluding this sample. Results for AOS were similar and the Q statistic P -value was identical ($\beta = -0.004$, $P = 0.281$, Q P -value = 0.01) suggesting the NTR1 rs588765 T allele frequency is not the cause of observed heterogeneity at this locus. Furthermore, results from a random effects model (automatically generated by PLINK) were nearly identical for rs588765 (fixed effects $\beta = -0.009$, $P = 0.297$, random effects $\beta = -0.005$, $P = 0.402$). Thus, heterogeneity across samples is unlikely to be driving the association results for AOS with these two SNPs.

There are several limitations to this study. First, given the involvement of many groups across several countries and use of data collected for other purposes, methods for assessments were not consistent across all studies. The phrasing of questions differed across sites, and although an attempt was made to harmonize the phenotypes it would have been ideal to use the same tools and questions. Second, some groups did not have both AOI and AOS data, so direct comparison of results is incomplete because power to detect an association differs. Third, the exact same SNPs were not available for all groups, so we relied on the use of proxy SNPs based on the current HapMap data. It is possible that some of the SNPs used as proxies are not as well correlated with the target SNP signal as we believe, but given the large datasets now available, this is unlikely for most of the targets.

This study also features several strengths. First, this study was built on strong existing support for an important role of this gene cluster, enabling the efforts to be focused on characterizing the associations in the region. Second, this project began with multiple discussions by all participants about the goals for the study, after which a specific analytical plan was developed to test carefully defined hypotheses, thereby minimizing multiple testing. Third, the sample sizes are substantial and therefore have allowed us to separate the effects of unique loci in a manner not otherwise possible.

The most significant finding (rs578776) has been associated with nicotine dependence in multiple independent samples and the minor allele confers a protective effect. Results of this meta-analysis suggest the protective effect imparted by the minor allele may influence early smoking behaviors, resulting in a decreased risk of nicotine dependence later in life. Perhaps even more interesting is the fact that there was absolutely no evidence at the most highly replicated locus for smoking heaviness (rs16969968), which has been shown to confer a functional effect on receptor function [Bierut et al., 2008; Fowler et al., 2011; Kuryatov et al., 2011]. This is the third study to report nonsignificance for age of initiation or

age of onset of smoking at the rs16969968 [Furberg et al., 2010; Hartz et al., 2012; Thorgeirsson et al., 2010]. This work emphasizes the fact that early smoking behaviors are related to, but different from nicotine dependence. It also demonstrates the value in pursuing multiple SNPs in a region where one SNP has been definitively associated with behavior and has concomitant evidence for molecular function. It is likely that other genes found to be associated with complex behaviors will harbor multiple functional variants, which may be involved in different aspects in the development of the disorder. For example, the most significantly associated SNP in this study (rs578776) represents a possible target for early intervention, while SNP rs16969968 may be a more effective target for later treatment. Taken as a whole, these results underscore the complexity of smoking behavior phenotypes, and suggest different genetic signals in this region may contribute to different stages of tobacco use and dependence.

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