CHRNA3/5, *IREB2*, and *ADCY2* Are Associated with Severe Chronic Obstructive Pulmonary Disease in Poland

Megan Hardin[1,2], Jan Zielinski[3], Emily S. Wan[1,2], Craig P. Hersh[1,2], Peter J. Castaldi[1,4], Eric Schwinder[1], Iwona Hawrylkiewicz[3], Pawel Sliwinski[3], Michael H. Cho[1,2], and Edwin K. Silverman[1,2]

[1]Channing Laboratory and [2]Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; [3]National Tuberculosis and Lung Disease Research Institute, Warsaw, Poland; and [4]Institute for Clinical Research and Health Policy Studies, Tufts Medical Center, Boston, Massachusetts

We examined the association between single-nucleotide polymorphisms (SNPs) previously associated with chronic obstructive pulmonary disease (COPD) and/or lung function with COPD and COPD-related phenotypes in a novel cohort of patients with severe to very severe COPD. We examined 315 cases of COPD and 330 Caucasian control smokers from Poland. We included three SNPs previously associated with COPD: rs7671167 (*FAM13A*), rs13180 (*IREB2*), and rs8034191 (*CHRNA 3/5*), and four SNPs associated with lung function in a genome-wide association study of general population samples: rs2070600 (*AGER*), rs11134242 (*ADCY2*), rs4316710 (*THSD4*), and rs17096090 (*INTS12*). We tested for associations with severe COPD and COPD-related phenotypes, including lung function, smoking behavior, and body mass index. Subjects with COPD were older (average age 62 versus 58 years, $P < 0.01$), with more pack-years of smoking (45 versus 33 pack-years, $P < 0.01$). *CHRNA3/5* (odds ratio [OR], 1.89; 95% confidence interval [CI], 1.5–2.4; $P = 7.4 \times 10^{-7}$), *IREB2* (OR, 0.69; 95% CI, 0.5–0.9; $P = 3.4 \times 10^{-3}$), and *ADCY2* (OR, 1.35; 95% CI, 1.1–1.7; $P = 0.01$) demonstrated significant associations with COPD. *FAM13A* (OR, 0.8; 95% CI, 0.7–1.0; $P = 0.11$) approached statistical significance. *FAM13A* and *ADCY2* also demonstrated a significant association with lung function. Thus, in severe to very severe COPD, we demonstrate a replication of association between two SNPs previously associated with COPD (*CHRNA3/5* and *IREB2*), as well as an association with COPD of one locus initially associated with lung function (*ADCY2*).

Keywords: chronic obstructive pulmonary disease; genetic association analysis; lung function; smoking; nicotine addiction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States, and its prevalence continues to grow (1). Although cigarette smoking is the most significant risk factor for COPD, cigarette smoking differentially affects lung function decline, and not all smokers will develop COPD (2). The individual response to cigarette smoke and other environmental factors is affected in part by genetic factors, and the development of COPD is the culmination of the environment acting in concert with a complex array of genetic traits (3).

Although the development of COPD is mediated by multiple genetic factors, to date few susceptibility genes other than α_1-antitrypsin (*SERPINA1*) have been convincingly identified. Early candidate gene studies yielded inconsistent results (4). These studies were limited by small sample sizes and a lack of uniformity in COPD phenotypes, including the degree of airflow obstruction and intensity of smoking exposure in subjects examined. However, several recent genome-wide association studies (GWAS) have identified genomic regions that demonstrate a highly significant and reproducible association with COPD in large studies including subjects with a range of impaired lung function and smoking history. These GWAS regions are located in *FAM13A* on chromosome 4q22 (5), on chromosome 4q31 near *HHIP* (6), and near several genes in the region of 15q25, including *IREB2* (7), *CHRNA3*, and *CHRNA5* (8). In addition to these studies examining COPD as an outcome, several recent large-scale GWAS meta-analysis identified genes associated with pulmonary function in general-population samples of subjects with a diverse range of lung function and smoking exposures (6, 9, 10). Because COPD is defined by a reduction in lung function, some of these genes seem likely to play a role in the pathogenesis of COPD. In fact, both *HHIP* and *FAM13A* were associated with lung-function levels in GWAS studies of general-population samples (6, 10). Using a candidate gene approach, several of these pulmonary-function GWAS regions recently demonstrated an association with COPD, including loci on 4q24 near *INTS12*, on 6p21 near *AGER*, on 5p15 near *ADCY2*, and on 15q23 near *THSD4* (11).

In a previous analysis, we demonstrated that *HHIP* single-nucleotide polymorphisms (SNPs) were also associated with COPD in a cohort of Polish subjects with severe COPD (12). Given this finding, we examined the relationship between previously reported COPD and lung function GWAS SNPs in this novel population comprised of cases with severe to very severe COPD. In addition, we examined the relationship between these SNPs and COPD-related phenotypes such as lung function, smoking behavior, and body mass index (BMI) in severe COPD. We hypothesized that a subset of SNPs previously associated with moderate to severe COPD and/or pulmonary-function levels would be associated with severe to very severe COPD. By investigating these associations in a homogeneous population with severe COPD, we believed we would be be better able to determine the genes responsible for COPD. In addition, by investigating COPD-related phenotypes such as lung function and smoking behavior, we hoped to differentiate more conclusively whether these genes were

CLINICAL RELEVANCE

These data demonstrate the association of several genes with chronic obstructive pulmonary disease (COPD) in a novel population with severe COPD. This work additionally replicates a novel finding of *ADCY2* with COPD. These data provide a platform for future investigations into the genetics of COPD.

(*Received in original form January 10, 2012 and in final form March 21, 2012*)

This work was supported by the National Institutes of Health grants T32 HL07427–29 (M.H.), R01HL084323 (E.K.S.), R01HL075478 (E.K.S.), P01 HL083069 (E.K.S.), P01 HL105339 (E.K.S.), and 5R01HL084323-04.

Correspondence and requests for reprints should be addressed to Megan Hardin, M.D., Channing Laboratory, Brigham and Women's Hospital, 181 Longwood Ave., Boston, MA 02115. E-mail: remeh@channing.harvard.edu

Am J Respir Cell Mol Biol Vol 47, Iss. 2, pp 203–208, Aug 2012

Originally Published in Press as DOI: 10.1165/rcmb.2012-0011OC on March 29, 2012
Internet address: www.atsjournals.org

directly acting upon COPD susceptibility, or were in fact mediated by COPD risk factors such as smoking behavior.

MATERIALS AND METHODS

Study Population

All subjects provided written, informed consent. Subjects participated at the Institute of Tuberculosis and Lung Disease (Warsaw, Poland), and genotyping and data analysis occurred at the Channing Laboratory of Brigham and Women's Hospital (Boston, MA). This study was approved by the institutional review boards at these institutions.

All subjects were white and from Poland, 40 to 80 years old, with at least 10 pack-years of smoking history. Cases manifested severe to very severe COPD (Guidelines for Obstructive Lung Disease Stage III or IV, post-bronchodilator $FEV_1 < 50\%$ predicted, and $FEV_1/FVC < 0.7$). Control subjects demonstrated normal lung function (post-bronchodilator FEV_1, $> 80\%$ predicted; FEV_1/FVC, > 0.7). Efforts were undertaken to frequency-match cases by age and sex. Subjects were excluded because of concomitant respiratory disorders, lung surgery, pregnancy, respiratory infection in the month before enrollment, or the presence of a first-degree or second-degree relative in the study.

Subjects performed standardized prebronchodilator and postbronchodilator spirometry, using the EasyOne Spirometer (NDD, Inc., Andover, MA), in accordance with American Thoacic Society/European Respiratory Society criteria (13). Postbronchodilator spirometry was performed approximately 20 minutes after administering 180 μg of albuterol via metered dose inhaler. In a few cases when subjects were unable to provide three acceptable maneuvers or slightly exceeded case spirometric criteria, those subjects' data were included at the investigators' discretion. FEV_1 percent predicted was calculated using Hankinson equations (14). Subjects completed a modified American Thoacic Society Respiratory Epidemiology questionnaire (15), and blood was collected for genetic analyses.

Genetic Analysis

We chose genomic regions based on a review of the literature, and used the most significant reported SNP for each gene. We examined three candidate genes associated with COPD in populations with moderate to severe COPD (*FAM13A*, *IREB2*, and *CHRNA3/5*) and SNPs in or near four candidate genes previously associated with lung function and COPD (*INTS12*, *ADCY2*, *THSD4*, and *AGER*). SNPs were genotyped using TaqMan assays (Applied Biosystems, Foster City, CA).

Statistical Analysis

We investigated the association between the chosen SNPs and COPD case/control status, average cigarettes smoked per day (CPD), pack-years, lung function, and BMI. All demographic data were analyzed using SAS version 9.2 (SAS, Inc., Cary, NC). All genetic analyses were performed using PLINK, version 1.07 (16). Logistic regression analysis was performed to test the association between each SNP with severe COPD case/control status, controlling for age, sex, and pack-years of smoking. Linear regression was performed to assess the relationship between SNPs and quantitative phenotypes. Analyses of CPD and pack-years were adjusted for age and sex, and analyses of FEV_1 percent predicted, FEV_1/FVC, and BMI were additionally adjusted for pack-years. We performed a parallel analysis among all study subjects, with the additional use of COPD case/control status as a covariate. A conditional analysis between SNPs from *CHRNA3/5* and *IREB2* with COPD was performed by including the SNP that was adjusted for as a covariate in the logistic regression analysis. To assess the relationship between smoking behavior, candidate genes, and COPD, subjects were divided into equal tertiles of pack-years. In each tertile, the relationship between the genetic variants (in *CHRNA3/5*, *IREB2*, and *ADCY2*) with COPD was examined by means of logistic regression, using all smoking control subjects as the reference group, and adjusting for age and sex.

RESULTS

Three hundred fifteen smoking patients with COPD and 330 smoking control subjects were included in the genetic association analysis (Table 1). More males than females participated in this study. However, sex was equally distributed between severe COPD cases and control subjects. Cases on average were older than control subjects (aged 62 years versus 58 years, $P < 0.0001$) and demonstrated a lower BMI (26.7 kg/m^2 versus 27.7 kg/m^2, respectively; $P < 0.05$). Cases had greater average smoking exposure, including pack-years (44.5 versus 33.5 pack-years, $P < 0.0001$) and average number of cigarettes per day (23.6 versus 20.8, respectively; $P < 0.0001$), and had started smoking at a younger age (age 18.7 years versus 19.5 years, respectively; $P < 0.0001$). Fewer cases than control subjects were active smokers at the time of study enrollment ($P < 0.0001$). By study design, cases had worse lung function than control subjects, including a lower FEV_1 percent predicted (30.36% versus 102.5%, respectively) and FEV_1/FVC (0.36 versus 0.77, respectively).

SNPs located in or near three genes previously associated with COPD (*CHRNA3/5* and *IREB2* on chromosome 15q25, and *FAM13A* on chromosome 4q22) and four genes previously associated with lung function (*INTS12* on chromosome 4q24, *ADCY2* on chromosome 5p15, *THSD4* on chromosome 15q23, and *AGER* on chromosome 6p21) were examined for associations with severe COPD case–control status (Table 2). Of these, the minor (C) allele of the *IREB2* SNP, rs13180 (odds raio [OR], 0.69; 95% confidence interval [CI], 0.53–0.88; $P = 0.003$) and the minor (C) allele from the *CHRNA3/5* SNP rs8034191 (OR, 1.89; 95% CI, 1.47–2.43; $P = 7.4 \times 10^{-7}$) were associated with COPD, in the same direction as previously reported. The minor allele of the *FAM13A* SNP, rs7671167, did not reach statistical significance when examining the association with COPD. However, the OR was in the same direction of effect as in previous studies (OR, O.82; 95% CI, 0.65–1.04; $P = 0.11$). Of the genes originally associated with lung-function levels, an SNP in *ADCY2*, rs11134242, was associated with an increased risk for COPD (OR, 1.35; 95% CI, 1.1–1.7; $P = 0.01$), which was in the same direction as previously reported in an analysis of COPD affection status (11).

The SNPs from *IREB2* (rs13180) and *CHRNA3/5* (rs8034191) were in weak linkage disequilibrium (LD) with each other ($R^2 = 0.21$), consistent with previous reports. To determine the independent effect of each SNP on COPD case status, we performed a conditional analysis examining the association of these SNPs with COPD. We examined the relationship between each SNP with COPD individually, while controlling for the presence of the other linked SNP (Table 3). When controlling for the presence of *IREB2*, *CHRNA3/5* remained significantly associated

TABLE 1. CHARACTERISTICS OF CASES AND CONTROL SUBJECTS

	Cases	Controls	*P* Value
n	315	330	
Age, yr	61.95 (7.33)	58.28 (7.26)	<0.0001
Male, %	220 (69.84)	222 (67.27)	0.50
Current smoker, *n* (%)	80 (25.40)	163 (49.39)	<0.0001
Pack-years	44.50 (22.33)	33.37 (14.94)	<0.0001
CPD	23.61 (9.3)	20.75 (8.13)	<0.0001
Age started smoking	18.68 (3.94)	19.49 (3.99)	0.01
FEV_1, L	0.89 (0.32)	3.13 (0.62)	NA
$FEV_{1\%}$ predicted, %	30.36 (9.60)	102.5 (12.11)	NA
FEV_1/FVC	0.36 (0.098)	0.77 (0.045)	NA
BMI, kg/m^2	26.68 (6.52)	27.72 (6.08)	0.037

Definition of abbreviations: BMI, body mass index; CPD, cigarettes per day; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; NA, not available.

Values are reported as means (standard deviations) or means (percentages). Sex and current smoking status were assessed according to the Fisher exact test. All continuous variables were compared using *t* tests.

TABLE 2. GENETIC ASSOCIATION: LOGISTIC REGRESSION BETWEEN SELECTED SNPs (WITH ADDITIVE CODING) AND COPD CASE–CONTROL STATUS

SNP	Chr	Gene	OR (95% CI)	*P* Value
rs7671167	4q22.1	*FAM13A*	0.82 (0.65–1.04)	0.107
rs13180	15q25	*IREB2*	0.69 (0.53–0.88)	$3.4 \times 10-3$
rs8034191	15q25	*CHRNA3/5*	1.89 (1.47–2.43)	$7.4 \times 10-7$
rs17036090	4q24	*INTS12*	0.84 (0.48–1.46)	0.54
rs11134242	5p15	*ADCY2*	1.35 (1.06– 1.7)	0.01
rs4316710	15q23	*THSD4*	1.16 (0.84–1.58)	0.37
rs2070600	6p21	*AGER*	1.41 (0.47–4.19)	0.54

Definition of abbreviations: Chr, chromosome; CI, confidence interval; COPD, chronic obstructive pulmonary disease; OR, odds ratio; SNP, single-nucleotide polymorphism.

All analyses were performed using logistic regression in PLINK, with age, sex, and pack-years as covariates. Each OR represents odds of COPD case status, with minor allele as the reference allele.

TABLE 3. CONDITIONAL ANALYSIS OF *CHRNA3/5* AND *IREB2*

Gene	SNP	Conditioned on	OR (95% CI)	*P* Value
IREB2	rs13180	rs8034191	0.89 (0.67–1.19)	0.45
CHRNA3/5	rs8034191	rs13180	1.75 (1.31–2.32)	1.4×10^{-4}

Definition of abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

Logistic regression was performed in PLINK, using conditional analysis with the conditioned SNP included as a covariate. Age, sex, and pack-years comprised additional covariates.

with COPD ($P = 0.0001$). However, when controlling for the presence of *CHRNA3/5*, the *IREB2* SNP was no longer significantly associated with COPD ($P = 0.45$).

We next examined the relationship between these SNPs and COPD-related phenotypes, including lung function (FEV_1 % predicted and FEV_1/FVC), smoking history (pack-years and average cigarettes per day), and BMI (Table 4). Regarding measures of lung function, the presence of the *FAM13A* minor allele was associated with a 1.39% increase in FEV_1/FVC ratio among all study subjects ($P = 8.0 \times 10^{-4}$), as well as a 1.8% increase in FEV_1/FVC among cases only ($P = 0.02$). Among all subjects examined together, the minor allele of the *ADCY2* SNP was associated with a decrease in FEV_1/FVC of 2.5% ($P = 0.03$). When examining control subjects only, no significant association was evident between these SNPs and any of the measures of lung function. No significant relationships were observed between any of these SNPs and measures of smoking behavior, FEV_1 percent predicted, or BMI (data not shown), both in cases only and in the entire cohort.

Because both *IREB2* and *CHRNA3/5* are located in a region that was also associated with nicotine addiction and smoking behavior (17–21), we investigated whether these SNPs demonstrated an independent effect on COPD case–control status, or if their effect was in fact the result of their impact on smoking behavior. When examining each SNP in relation to smoking behavior, neither *CHRNA3/5* nor *IREB2* was significantly associated with average cigarettes per day or pack-years, either in cases only or in all subjects.

We examined the relationship between each of the three SNPs significantly associated with severe COPD case–control status within tertiles of smoking intensity, based on pack-years of smoking, using control subjects as the reference group (Table 5). If the effect of the SNP is independent from smoking behavior, we might expect to see either a similar association within each tertile or potentially the strongest association in the lowest tertile of smoking behavior, in which COPD genetic susceptibility unrelated to nicotine addiction may be most important. The association between both *IREB2* and *CHRNA3/5* and COPD became more significant between tertiles 1 and tertiles 2–3 ($P = 0.18$, 0.004, and 0.008, respectively, for *IREB2*, and $P = 0.002$, 3.8×10^{-5}, and 7.4×10^{-5}, respectively, for *CHRNA3/5*). When the association between *IREB2* and COPD was conditioned on the presence of *CHRNA3/5*, the relationship was no longer significant ($P = 0.93$, 0.3, and 0.24), whereas the association between *CHRNA3/5* and COPD remained significant with increasing tertile ($P = 0.01$, 0.007, and 0.003008, respectively), despite conditioning for *IREB2*. We performed a similar analysis with *ADCY2* and smoking behavior, and found a significant association with COPD only in the lowest tertile of smoking behavior (OR, 1.49; 95% CI, 1.1–2.1; $P = 0.018$).

DISCUSSION

We have demonstrated that SNPs in *IREB2*, *CHRNA3/5*, and *ADCY2* are associated with severe COPD in a Polish case–control population. Although *FAM13A* was not significantly associated with COPD in this study, the effect measure was in the same direction as previously reported, and therefore the lack of significance may be related to our small sample size. This is the first demonstration, to the best of our knowledge, of an association between an SNP near *ADCY2* and severe COPD, and confirms a recent finding of an association between this SNP and COPD in a series that included a more heterogeneous population with COPD (11).

IREB2 and *CHRNA3/5* are located on a region of chromosome 15q25 that is particularly compelling for investigating the genetic components of COPD. This region contains a number of genes with biological plausibility for a role in the development of COPD. However, the significant linkage disequilibrium in this region complicates the ability to isolate causal genes and variants.

The gene cluster *CHRNA3*/*CHRNA5*/*CHRNB4* encodes nicotinic acetylcholine receptor subunits expressed in the central nervous system and bronchial epithelium, and is responsive to nicotine (22). These receptors have been implicated in nicotine addiction, and appear to be up-regulated with chronic tobacco use (23). *CHRNA3/5* has been associated with the risk for nicotine dependence through mRNA brain expression levels (24) and through candidate gene analyses (25), dense genotyping (19), and GWAS (17) (21, 26) in diverse populations, including smokers with normal lung function and lung disease as well as European and African-American populations. The *CHRNA3/5* SNP that we investigated, rs8034191, was found to be associated with heavier smoking behaviors (18) as well as increased susceptibility to lung cancer (27), COPD (8), and radiographically determined emphysema (28).

Although these studies suggest that *CHRNA3/5* acts on COPD case–control status by influencing smoking behavior, some evidence also exists for an independent effect of these genes on the development of COPD (8, 29). In the first GWAS of COPD, the *CHRNA3/5* SNP rs8034191 was associated with an increased risk for COPD (8). In that study, investigators did identify a gene-by-environment interaction between current smoking and this variant on COPD, indicating that some of the increased risk for COPD associated with this region could be mediated by smoking behavior. However, as in the present study, they did not identify an association between pack-years and this locus, and the association between *CHRNA3/5* and COPD remained robust even after adjusting for current smoking status and pack-years. Using mediation analysis, other investigators examined the relationship between rs1051730 and

TABLE 4. COPD-RELATED PHENOTYPES: ASSOCIATION BETWEEN SNPs SIGNIFICANTLY ASSOCIATED WITH COPD CASE–CONTROL STATUS AND ADDITIONAL SMOKING AND COPD-RELATED PHENOTYPES

SNP	CPD	Pack-Years	$FEV_{1\%}$ Predicted	FEV_1/FVC
Cases only				
FAM13A	0.06 (0.76), $P = 0.94$	0.85 (1.80), $P = 0.64$	0.19 (0.78), $P = 0.80$	1.79 (0.8), $P = 0.02$
IREB2	−0.55 (0.78), $P = 0.48$	−1.15 (1.83), $P = 0.53$	−0.45 (0.81), $P = 0.58$	−0.38 (0.8), $P = 0.65$
CHRNA3/5	0.37 (0.73), $P = 0.62$	0.70 (1.72), $P = 0.68$	−0.83 (0.75), $P = 0.27$	0.15 (0.8), $P = 0.85$
ADCY2	0.86 (0.73), $P = 0.24$	2.53 (1.69), $P = 0.14$	−1.10 (0.76), $P = 0.15$	−0.15 (0.78), $P = 0.85$
All subjects				
FAM13A	−0.53 (0.5), $P = 0.27$	0.29 (1.0), $P = 0.78$	−0.38 (0.61), $P = 0.54$	1.39 (0.41), $P = 0.0008$
IREB2	−0.62 (0.51), $P = 0.22$	−0.45 (1.1), $P = 0.67$	−0.82 (0.64), $P = 0.20$	−0.38 (0.45), $P = 0.40$
CHRNA3/5	0.16 (0.5), $P = 0.75$	−0.40 (1.1), $P = 0.71$	0.19 (0.63), $P = 0.77$	0.09 (0.4), $P = 0.83$
ADCY2	−0.33 (0.5), $P = 0.49$	−1.16 (1.0), $P = 0.25$	0.91 (0.61), $P = 0.13$	−2.5 (1.2), $P = 0.03$

Definition of abbreviations: COPD, chronic obstructive pulmonary disease; CPD, cigarettes per day; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; SNP, single-nucleotide polymorphism.

Data presented are β (standard error) with two-sided *P* values. Cigarettes per day and pack-years were adjusted for age and sex, FEV_1, FEV_1/FVC, and body mass indices were adjusted for age, sex, and pack-years. The analysis including all subjects was additionally adjusted for COPD case–control status.

COPD, which is in LD with the *CHRNA3/5* SNP we examined, and found that smoking may mediate the relationship between this SNP and COPD (30).

In our study, we demonstrated that the *CHRNA3/5* SNP is associated with an increased risk for COPD, even after adjusting for pack-years of smoking. In addition, we did not identify an association between the *CHRNA3/5* SNP and pack-years of smoking or cigarettes per day in cases, control subjects, or the entire group taken together. Although we are limited in our ability to assess the functional role that *CHRNA3/5* plays in increasing the risk for COPD, our findings suggest an effect that may be independent from smoking behavior. On the other hand, we note that the association between *CHRNA3/5* and COPD is strongest in the top tertile of smoking intensity, suggesting a gene-by-environment interaction. Further investigation, including the fine mapping of this region and improved smoking intensity phenotypes, may help tease apart these effects.

IREB2 is also located in the same genomic region as *CHRNA3/5*, and the SNP in our study was in moderate LD with rs8034191 ($r^2 = 0.21$). The protein product of this gene encodes the iron-response protein IRP2. This protein, together with IRP1, is involved in iron metabolism and the response to hypoxemia, and has been demonstrated to affect mitochondrial iron stores (31–33). *IREB2* protein concentrations are increased in emphysematous lungs when compared with control samples, as are *IREB2* mRNA concentrations, and *IREB2* was associated with FEV_1 level and COPD in candidate gene studies (7).

In this study, we demonstrated an association between an *IREB2* SNP and severe COPD case status. After adjustment for the presence of the *CHRNA3/5* SNP, this association is no longer significant, indicating that the association may be driven by linkage disequilibrium with *CHRNA3/5* SNPs. It is also possible that *IREB2* is not involved in severe COPD, at least in this Polish population. However, because the direction of effect remained the same despite a loss of significance, our study may more likely have been underpowered to demonstrate a persistent impact from *IREB2*.

Although we did not demonstrate a significant association between a *FAM13A* SNP and COPD, we did demonstrate that the minor allele of this SNP was associated with improved lung function, both in cases only and in the entire study population. These findings are consistent with those of a previous COPD association study that identified an association between *FAM13A* and lung function in two family-based studies of COPD, demonstrating a reduced odds of COPD associated with the minor allele of this SNP (5). This association was also evident in a recent population-based meta-analyses of lung function GWAS (10). However, other lung function GWAS have failed to identify this association (9).

When examining SNPs previously associated with lung function in general-population samples, we found that an *ADCY2* SNP was associated with COPD in our population. None of these previously reported lung function SNPs were associated with lung-function measures when examining cases only. However, when examining all subjects together, the *ADCY2* SNP demonstrated a significant association with spirometric measures of lung function.

ADCY2 encodes adenylyl cyclase–2, a member of the adenylyl cyclase family involved in G protein–coupled receptor signaling. The mRNA from this gene was found to be expressed in the human brain, smooth muscle, and testes, and mildly expressed in lung tissue (34). This gene was identified as part of a gene cluster associated with the ability to quit smoking in a previous

TABLE 5. COPD CASE–CONTROL LOGISTIC REGRESSION WITHIN EACH TERTILE OF SMOKING BEHAVIOR

Gene	Tertile 1	Tertile 2	Tertile 3
IREB2	0.79 (0.6–1.1), $P = 0.18$	0.59 (0.4–0.8), $P = 0.004$	0.6 (0.4–0.9), $P = 0.008$
*IREB2**	1.02 (0.68–1.53), $P = 0.93$	0.8 (0.52–1.22), $P = 0.3$	0.78 (0.51–1.19), $P = 0.24$
CHRNA3/5	1.7 (1.2–2.4), $P = 0.002$	2.1 (1.5–2.9), $P = 3.8 \times 10^{-5}$	2.1 (1.5–3.1), $P = 7.4 \times 10^{-5}$
*CHRNA3/5**	1.7 (1.1–2.5), $P = 0.01$	1.8 (1.2–2.6), $P = 0.007$	1.86 (1.2–2.8), $P = 0.003$
ADCY2	1.49 (1.1–2.1), $P = 0.018$	1.24 (0.89–1.7), $P = 0.2$	1.21 (0.9–1.7), $P = 0.26$

Definition of abbreviations: CI, confidence interval; COPD, chronic obstructive pulmonary disease; OR, odds ratio.

Data are presented as OR (95% CI), with *P* value (two-sided). All cases were divided into equal tertiles of smoking behavior (pack-years of smoking), and compared against all control subjects as the reference group for COPD case–control status.

* *IREB2* was additionally adjusted for the presence of the *CHRNA3/5* minor allele. *CHRNA3/5* was additionally adjusted for the presence of the *IREB2* minor allele.

genetic analysis (35). In their lung function meta-analysis, Hancock and colleagues (10) demonstrated that SNPs from this gene were associated with FEV_1 in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium, although their findings did not reach genome-wide significance. In a follow-up analysis, examining the *ADCY2* gene in greater depth, Castaldi and colleagues identified the minor allele of rs11134242 from *ADCY2* as significantly associated with COPD. Interestingly, this SNP is only in weak LD with the CHARGE SNPs (rs6555465 and rs7710510) near the same gene (11). To the best of our knowledge, the present study represents the first replication of the novel finding of association by Castaldi and colleagues (11).

We additionally examined the relationship of *IREB2*, *CHRNA3/5*, and *ADCY2* with COPD among tertiles of smoking behavior. Although we did not identify an association between these SNPS and smoking behavior when smoking intensity was measured as a quantitative variable (i.e., pack-years), we were also interested in determining if these associations were more significant in heavy versus light smokers. By dividing smokers into tertiles of smoking behavior, we attempted to distinguish whether the noted association between the SNPs was related to smoking behavior or COPD susceptibility. If the association were stronger in the lowest smoking tertile (light smokers), this would provide evidence of a greater relationship between the selected SNPs and COPD susceptibility. If the relationship became stronger with increased tertiles of smoking (heavy smokers), we felt this would indicate that smoking behavior was more likely to be driving the relationship. In our study, the association between *CHRNA3/5* and COPD was strongest among the upper two tertiles of smokers, which may indicate that this association is driven by smoking-related behavior. In contrast, the association between *ADCY2* and COPD was strongest among the lowest tertile of smokers. Taken in combination with our finding of an association between *ADCY2* and FEV_1/FVC in our entire study population, *ADCY2* appears to affect COPD case–control status through a nonsmoking-related effect on the development of airflow obstruction.

This study contains several limitations. As in any candidate gene analysis, we are limited by our *a priori* hypothesis in regard to which SNPs and genes to investigate, and which phenotypes to study. Using a Bonferroni correction for the multiple testing of seven individual SNPs and six separate outcomes, a P value of 6.25×10^{-3} would be required for statistical significance. With this more conservative approach, the associations between *IREB2* and *CHRNA3/5* and COPD remain significant, and the association between *CHRNA3/5* and COPD remained robust after adjusting for the *IREB2* association. The association between *FAM13A* and FEV_1/FVC also remained significant after adjusting for multiple comparisons. Although we were able to identify associations, we are not able to identify causal mechanisms. For example, although our data provide intriguing evidence for a role of *CHRNA3/5* and *IREB2* in the development of COPD, we were not able to demonstrate an independent effect of *IREB2* on COPD susceptibility. Although limited by our small sample size, we were still able to demonstrate several significant associations with COPD. A larger sample size may have allowed for greater evidence of the role of *FAM13A* in COPD. We also lacked an additional replication population of severe smokers. However, our study functions as a confirmation of prievous associations with COPD, and further strengthens the association of *CHRNA3/5*, *IREB2*, and *ADCY2* with COPD, especially given our more extreme case phenotype. Future studies should include investigations in additional populations of smokers with severe to very severe COPD.

We have attempted to distinguish distinct roles for *IREB2* and *CHRNA3/5* in the development of COPD that are independent from nicotine addiction. Here we demonstrate that *CHRNA3/5* is associated with COPD even while controlling for cigarette smoking exposure. However, our measures of nicotine addiction (i.e., cigarettes per day and pack-years) may not have been adequately replicated the toxic effects of tobacco smoke. Nevertheless, these same measures were used in many previous GWAS assessing smoking behavior, and we feel cigarettes per day to be a highly heritable, robust marker of nicotine addiction (26).

We examined the relationship between our SNPs and COPD-related phenotypes, including spirometry and smoking behavior, in our study population. We analyzed these traits in cases only as well as in our entire population using linear regression, including COPD case–control status as a covariate. Although this procedure is not a substitute for population-based sampling, it avoids potential biases in analyzing quantitative phenotypes in a case–control–based population (36, 37).

In conclusion, in a population with severe to very severe COPD, we confirm the previously identified association between *CHRNA3/5* and COPD, and this association remained robust after adjusting for smoking behavior. We also demonstrate that *ADCY2* is associated with severe COPD and with lung-function levels. Our results also provide some evidence that *IREB2* and *FAM13A* are associated with COPD. Further studies will be required to elucidate the functional variants that confer a risk for COPD in these associated genomic regions.

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Minino A, Xu J, Kochanek K. Deaths: preliminary data for 2008. *Natl Vital Stat Rep* 2010;59:1–52.
2. Wan ES, Silverman EK. Genetics of COPD and emphysema. *Chest* 2009; 136:859–866.
3. Silverman EK, Vestbo J, Agusti A, Anderson W, Bakke PS, Barnes KC, Barr RG, Bleecker ER, Boezen HM, Burkart KM, *et al*. Opportunities and challenges in the genetics of COPD 2010: an International COPD Genetics Conference report. *COPD* 2011;8:121–135.
4. Hersh CP, Demeo DL, Lange C, Litonjua AA, Reilly JJ, Kwiatkowski D, Laird N, Sylvia JS, Sparrow D, Speizer FE, *et al*. Attempted replication of reported chronic obstructive pulmonary disease candidate gene associations. *Am J Respir Cell Mol Biol* 2005;33:71–78.
5. Cho MH, Boutaoui N, Klanderman BJ, Sylvia JS, Ziniti JP, Hersh CP, DeMeo DL, Hunninghake GM, Litonjua AA, Sparrow D, *et al*. Variants in *FAM13A* are associated with chronic obstructive pulmonary disease. *Nat Genet* 2010;42:200–202.
6. Wilk JB, Chen TH, Gottlieb DJ, Walter RE, Nagle MW, Brandler BJ, Myers RH, Borecki IB, Silverman EK, Weiss ST, *et al*. A genome-wide association study of pulmonary function measures in the Framingham Heart Study. *PLoS Genet* 2009;5:e1000429.
7. DeMeo DL, Mariani T, Bhattacharya S, Srisuma S, Lange C, Litonjua A, Bueno R, Pillai SG, Lomas DA, Sparrow D, *et al*. Integration of genomic and genetic approaches implicates *IREB2* as a COPD susceptibility gene. *Am J Hum Genet* 2009;85:493–502.
8. Pillai SG, Ge D, Zhu G, Kong X, Shianna KV, Need AC, Feng S, Hersh CP, Bakke P, Gulsvik A, *et al*. A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci. *PLoS Genet* 2009;5:e1000421.
9. Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, Zhao JH, Ramasamy A, Zhai G, Vitart V, *et al*. Genome-wide association study identifies five loci associated with lung function. *Nat Genet* 2010; 42:36–44.
10. Hancock DB, Eijgelsheim M, Wilk JB, Gharib SA, Loehr LR, Marciante KD, Franceschini N, van Durme YM, Chen TH, Barr RG, *et al*. Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. *Nat Genet* 2010;42:45–52.

11. Castaldi PJ, Cho MH, Litonjua AA, Bakke P, Gulsvik A, Lomas DA, Anderson W, Beaty TH, Hokanson JE, Crapo JD, *et al*. The association of genome-wide significant spirometric loci with COPD susceptibility. *Am J Respir Cell Mol Biol* 2011;45:1147–1153.
12. Zhou X, Baron RM, Hardin M, Cho MH, Zielinski J, Hawrylkiewicz I, Sliwinski P, Hersh CP, Mancini JD, Lu K, *et al*. Identification of a chronic obstructive pulmonary disease genetic determinant that regulates HHIP. *Hum Mol Genet* 2012;21:1325–1335.
13. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, *et al*. Standardisation of spirometry. *Eur Respir J* 2005;26:319–338.
14. Hankinson JL, Odencrantz JR, Fedan KB. Spirometric reference values from a sample of the general U.S. population. *Am J Respir Crit Care Med* 1999;159:179–187.
15. Ferris BG. Epidemiology standardization project (American Thoracic Society). *Am Rev Respir Dis* 1978;118:1–120.
16. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559–575.
17. Thorgeirsson TE, Geller F, Sulem P, Rafnar T, Wiste A, Magnusson KP, Manolescu A, Thorleifsson G, Stefansson H, Ingason A, *et al*. A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature* 2008;452:638–642.
18. Stevens VL, Bierut LJ, Talbot JT, Wang JC, Sun J, Hinrichs AL, Thun MJ, Goate A, Calle EE. Nicotinic receptor gene variants influence susceptibility to heavy smoking. *Cancer Epidemiol Biomarkers Prev* 2008;17:3517–3525.
19. Saccone NL, Wang JC, Breslau N, Johnson EO, Hatsukami D, Saccone SF, Grucza RA, Sun L, Duan W, Budde J, *et al*. The *CHRNA5–CHRNA3–CHRNB4* nicotinic receptor subunit gene cluster affects risk for nicotine dependence in African-Americans and in European-Americans. *Cancer Res* 2009;69:6848–6856.
20. Saccone NL, Saccone SF, Hinrichs AL, Stitzel JA, Duan W, Pergadia ML, Agrawal A, Breslau N, Grucza RA, Hatsukami D, *et al*. Multiple distinct risk loci for nicotine dependence identified by dense coverage of the complete family of nicotinic receptor subunit (*CHRN*) genes. *Am J Med Genet B Neuropsychiatr Genet* 2009;150B:453–466.
21. Saccone NL, Culverhouse RC, Schwantes-An TH, Cannon DS, Chen X, Cichon S, Giegling I, Han S, Han Y, Keskitalo-Vuokko K, *et al*. Multiple independent loci at chromosome 15q25.1 affect smoking quantity: a meta-analysis and comparison with lung cancer and COPD. *PLoS Genet* 2010;6(8):e1001053.
22. Greenbaum L, Rigbi A, Teltsh O, Lerer B. Role of genetic variants in the *CHRNA5–CHRNA3–CHRNB4* cluster in nicotine dependence risk: importance of gene–environment interplay. *Mol Psychiatry* 2009; 14:828–830.
23. Dani JA. Overview of nicotinic receptors and their roles in the central nervous system. *Biol Psychiatry* 2001;49:166–174.
24. Wang JC, Cruchaga C, Saccone NL, Bertelsen S, Liu P, Budde JP, Duan W, Fox L, Grucza RA, Kern J, *et al*. Risk for nicotine dependence and lung cancer is conferred by mRNA expression levels and amino acid change in *CHRNA5*. *Hum Mol Genet* 2009;18:3125–3135.
25. Weiss RB, Baker TB, Cannon DS, von Niederhausern A, Dunn DM, Matsunami N, Singh NA, Baird L, Coon H, McMahon WM, *et al*. A candidate gene approach identifies the *CHRNA5–A3–B4* region as a risk factor for age-dependent nicotine addiction. *PLoS Genet* 2008; 4:e1000125.
26. Berrettini W, Yuan X, Tozzi F, Song K, Francks C, Chilcoat H, Waterworth D, Muglia P, Mooser V. Alpha-5/alpha-3 nicotinic receptor subunit alleles increase risk for heavy smoking. *Mol Psychiatry* 2008;13: 368–373.
27. Hung RJ, McKay JD, Gaborieau V, Boffetta P, Hashibe M, Zaridze D, Mukeria A, Szeszenia-Dabrowska N, Lissowska J, Rudnai P, *et al*. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature* 2008;452:633–637.
28. Pillai SG, Kong X, Edwards LD, Cho MH, Anderson WH, Coxson HO, Lomas DA, Silverman EK. Loci identified by genome-wide association studies influence different disease-related phenotypes in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;182: 1498–1505.
29. Schwartz AG, Cote ML, Wenzlaff AS, Land S, Amos CI. Racial differences in the association between SNPs on 15q25.1, smoking behavior, and risk of non–small cell lung cancer. *J Thorac Oncol* 2009;4: 1195–1201.
30. Wang J, Spitz MR, Amos CI, Wilkinson AV, Wu X, Shete S. Mediating effects of smoking and chronic obstructive pulmonary disease on the relation between the *CHRNA5–A3* genetic locus and lung cancer risk. *Cancer* 2010;116:3458–3462.
31. LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, Drake SK, Miller G, Abu-Asab M, Tsokos M, *et al*. Targeted deletion of the gene encoding iron regulatory protein–2 causes misregulation of iron metabolism and neurodegenerative disease in mice. *Nat Genet* 2001;27:209–214.
32. Leipuviene R, Theil EC. The family of iron responsive RNA structures regulated by changes in cellular iron and oxygen. *Cell Mol Life Sci* 2007;64:2945–2955.
33. Galy B, Ferring-Appel D, Sauer SW, Kaden S, Lyoumi S, Puy H, Kolker S, Grone HJ, Hentze MW. Iron regulatory proteins secure mitochondrial iron sufficiency and function. *Cell Metab* 2010;12:194–201.
34. Ludwig MG, Seuwen K. Characterization of the human adenylyl cyclase gene family: cDNA, gene structure, and tissue distribution of the nine isoforms. *J Recept Signal Transduct Res* 2002;22:79–110.
35. Uhl GR, Liu QR, Drgon T, Johnson C, Walther D, Rose JE, David SP, Niaura R, Lerman C. Molecular genetics of successful smoking cessation: convergent genome-wide association study results. *Arch Gen Psychiatry* 2008;65:683–693.
36. Monsees GM, Tamimi RM, Kraft P. Genome-wide association scans for secondary traits using case–control samples. *Genet Epidemiol* 2009;33: 717–728.
37. Lin DY, Zeng D. Proper analysis of secondary phenotype data in case–control association studies. *Genet Epidemiol* 2009;33:256–265.