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IL1B Polymorphisms Modulate Cystic Fibrosis Lung Disease

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Summary

Rationale: Variability in pulmonary disease severity is found in patients with cystic fibrosis (CF) who have identical mutations in the CF transmembrane conductance regulator (*CFTR*) gene. We hypothesized that one factor accounting for heterogeneity in pulmonary disease severity is variation in the family of genes affecting the biology of interleukin-1 (*IL-1*), which impacts acquisition and maintenance of *Pseudomonas aeruginosa* infection in animal models of chronic infection. **Methods:** We genotyped 58 single nucleotide polymorphisms (SNPs) in the *IL-1* gene cluster in 808 CF subjects from the University of North Carolina and Case Western Reserve University (UNC/CWRU) joint cohort. All were homozygous for $\Delta F508$, and categories of “severe” (cases) or “mild” (control subjects) lung disease were defined by the lowest or highest quartile of forced expired volume (FEV₁) for age in the CF population. After adjustment for age and gender, genotypic data were tested for association with lung disease severity. Odds ratios (ORs) comparing severe versus mild CF were also calculated for each genotype (with the homozygote major allele as the reference group) for all 58 SNPs. From these analyses, nine SNPs with a moderate effect size, OR = 0.5 or > 1.5, were selected for further testing. To replicate the case-control study results, we genotyped the same nine SNPs in a second population of CF parent-offspring trios (recruited from Children's Hospital Boston), in which the offspring had similar pulmonary phenotypes. For the trio analysis, both family-based and population-based associations were performed. **Results:** SNPs rs1143634 and rs1143639 in the *IL1B* gene demonstrated a consistent association with lung disease severity categories ($P < 0.10$) and longitudinal analysis of

lung disease severity ($P < 0.10$) in CF in both the case-control and family-based studies. In females, there was a consistent association (false discovery rate adjusted joint P -value < 0.06 for both SNPs) in both the analysis of lung disease severity in the UNC/CWRU cohort and the family-based analysis of affection status. Conclusion: Our findings suggest that *IL1 β* is a clinically relevant modulator of CF lung disease.

Keywords

gene modifiers; cystic fibrosis; CFTR; IL-1 gene family

INTRODUCTION

Cystic fibrosis (CF) results from mutations in both alleles of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. However, none of the 1,521 mutations in *CFTR* identified thus far produces a predictable respiratory disease phenotype in CF patients, although homozygosity for certain alleles usually gives rise to more severe pulmonary disease. We hypothesized that one factor accounting for heterogeneity in pulmonary disease severity is variation in the family of genes related to the function of interleukin-1 (*IL-1*), which in an animal model of chronic lung infection¹ was shown to affect acquisition and maintenance of *Pseudomonas aeruginosa* infection, the major cause of morbidity and mortality in CF. Lack of functional *CFTR* has been shown to affect a number of responses within the lung whereby an ineffective innate immune response allows organisms, particularly *P. aeruginosa*, to establish a chronic infection. Chronic *P. aeruginosa* within the airway in CF patients is a well-known determinant of prognosis,² with several studies demonstrating associations between *P. aeruginosa* colonization and lung function decline.^{3,4}

Five components of the *IL-1* R type I pathway may have implications for resistance to *P. aeruginosa* lung infection and might also be involved in modulating airway inflammation and subsequent lung function decline: the receptor itself, the proinflammatory cytokines IL-1 α (gene *IL1A*) and IL-1 β (gene *IL1B*), the anti-inflammatory cytokine IL-1-RA (gene *IL1RN*), and the IL-1 type II receptor IL-1R1 (gene *IL1RI*).⁵⁻⁹ Certain genetic variations within the *IL-1* gene family can affect the expression and function of its gene products.¹⁰⁻¹⁴ Recent studies have shown the *IL-1* gene family to be an important participant in inflammatory signaling within the airway; both cultured human lung epithelial cells and the lung epithelium of mice homozygous for mutant or missing *CFTR* alleles fail to rapidly translocate nuclear factor kappaB (*NF-kB*) to the nucleus within 15 minutes of acute *P. aeruginosa* infection, a process that is critical for effective immunity to *P. aeruginosa* and which normally occurs in the presence of WT-*CFTR* alleles.¹⁵ *IL-1R* knockout mice developed chronic lung infection with *P. aeruginosa* following infection via drinking water, which also occurs with transgenic CF mice.¹⁶ Furthermore, cultured human airway epithelial cells from a $\Delta F508$ *CFTR* homozygous patient released considerably less IL-1 β in response to *P. aeruginosa* compared to the same cells transfected with a functional copy of WT-*CFTR*.¹ These studies suggest a relationship between *IL-1* responses and *CFTR*-dependent innate immune resistance to *P. aeruginosa* infection that could affect lung disease severity in CF. Therefore, we focused our analysis on the *IL-1* gene family because these studies have shown it to be an important participant in the innate immune response in the presence of wild-type (WT)-*CFTR* via activating signaling through the interleukin-1 receptor (*IL-1R*), which modulates the pulmonary immune response to *P. aeruginosa* infection.¹

In view of the important role of the *IL-1* family gene cluster in the early response to *P. aeruginosa* (which is also affected by *CFTR* genotype) and the potential role of the *IL-1* family in lung pathology, we sought to establish whether an association exists between variations in specific genes within the *IL-1* cluster of genes and lung disease severity in CF patients. We found that polymorphisms in the *IL-1* gene cluster in CF patients were associated with the severity of lung disease, implicating a contribution of genetic variation in these genes in the pathogenesis of lung function decline in CF.

METHODS

Study Populations

University of North Carolina and Case Western Reserve (UNC/CWRU) Cohort

—For this case-control study, we used the DNA and serum samples acquired by Michael Knowles, MD (UNC/CWRU) and Mitch Drumm, PhD, from 840 patients with CF, enrolled from 44 centers, who were initially determined to be homozygous for the $\Delta F508$ genotype.¹⁷ The diagnosis of CF was documented in the medical record by the pilocarpine iontophoresis sweat test (sweat chloride > 60 mmol/L). The 840 patients initially enrolled were chosen because their forced expiratory volume in 1 sec (FEV₁) measurements were in the lowest quartile or highest quartile for age among $\Delta F508$ homozygotes; of these, the lung function of 275 patients was classified as severely impaired (lowest quartile) and that of 565 patients as mildly impaired (highest quartile). Pulmonary function classifications are previously described by Schluchter et al.¹⁸ who define the mild CF patients as those who survived until 15 years of age and had an FEV₁ categorization consistent with mild CF lung disease. Severe patients are defined only as young as 8 years of age because of the need for at least 3 years of pulmonary function testing. Quartiles were defined by the Cystic Fibrosis Foundation registry data classification of pulmonary function testing for the UNC center when compared to US CF centers^{19,20} allowing for CF-specific lung function classification. Because FEV₁ is most predictive of survival in CF, it was used as the outcome for phenotype-genotype analysis.²¹ Furthermore, FEV₁% predicted was the outcome measurement for phenotype-genotype analysis in both the UNC/CWRU screening population and in the validation cohort, Children's Hospital Boston (CHB) (as below), for consistency among CF genetic modifier studies.¹⁷ The FEV₁ measurement was defined as percent predicted obtained from multiple FEV₁ measurements acquired during the previous 5 years (prior to enrollment) using a mixed model analysis as defined by Schluchter and colleagues.²² The FEV₁ measurement was obtained when the patient was clinically stable and not during a bronchitic exacerbation, defined as an FEV₁ change of greater than 15% predicted. A total of 32 patients were excluded because they had inadequate spirometers (2 patients), were found not to be homozygous for the $\Delta F508$ genotype on a subsequent evaluation (8 patients), or did not achieve more than 90% probability of being congruent with others in the severe or mild category. There were 808 patients in the final data set. No patient was excluded because of race or ethnic background; 96.7% of the patients were identified as Caucasian. Further description of this cohort can be found in the publication by Drumm and colleagues.¹⁷

Children's Hospital Boston (CHB) Cohort—To attempt to replicate the results of the case-control study using a different method of analysis and measurement of effect, we genotyped the single nucleotide polymorphisms (SNPs) in a second population of CF patients from CHB using a family-based design. All CF probands were registered in a clinical and laboratory database and followed at CHB from 1993 to 2005. The diagnosis of CF was documented in the medical record by the pilocarpine iontophoresis sweat test (sweat chloride > 60 mmol/L) and/or the presence of two *CFTR* mutations. Over 90% of the CF patients were evaluated in the CF clinic at least once per year. The yearly visits were

prospectively scheduled annual visits where clinical evaluation was performed and laboratory data obtained to include pulmonary function testing, sputum culture, and serum laboratory measures.

We evaluated 126 trios (ascertained by the proband) with a pulmonary phenotype similar to that used in the UNC/CWRU cohort, as described by Drumm and colleagues.¹⁷ We identified eligible CF patients and collected laboratory data from the clinical laboratory database at CHB. Specifically, for pulmonary function testing, microbiologic and genotype data were extracted and downloaded into an ORACLE database. A structured query language reporting tool was run to join the hospital-wide laboratory values requested to the CF patient population followed at CHB. In the CHB cohort, we had multiple laboratory and pulmonary function measurements spanning 13 years and were able to analyze the patients' PFT measurements in both categorical and longitudinal analyses. For the categorical lung function analysis, each patient's FEV₁ value was assigned a disease severity group based on FEV₁ values using the ESCF classification for patients in four age groups: 6–12 years (severe, FEV₁ 88.7% predicted; moderate, > 88.7–94.5%; mild, > 94.5–99.0%; very mild/normal, > 99.0%); 13–17 years (severe, FEV₁ 76.5% predicted; moderate, > 76.5–81.1%; mild, > 81.1–87.7%; very mild/normal, > 87.7%); 18–29 years (severe, FEV₁ 58.1% predicted; moderate, > 58.1–63.9%; mild, > 63.9–70.7%; very mild/normal, > 70.7%); and > 30 years (severe, FEV₁ 45.5% predicted; moderate, > 45.5–50.9%; mild, > 50.9–59.8%; very mild/normal, > 59.8%).^{19,20} For the analysis, a child aged 12.9 would have an age of 12, because we used a “floor” statistical function that rounds the age down to the integer. CF patients in the very mild/normal and the mild severity group were pooled for analysis and compared to moderate and severe disease groups, which were also combined. Methods for ascertaining sputum culture in the CHB cohort included both deep throat and sputum cultures. Colonization was defined as one positive microbiologic growth on culture. Given the method for extracting data from various electronic sources and merging them, it was not possible to obtain symptom history or medication information. However, as UNC/CWRU and CHB are accredited CF Care Centers, patients received standard CF care as outlined by the CF Consortium guidelines, and detection and eradication regimens for *P. aeruginosa* infection were similar. Most patients did not receive anti-staphylococcal prophylaxis. Anti-*Pseudomonas* antibiotics were routinely given to patients with two positive CF cultures (either documented by deep throat cultures or sputum culture) in an attempt to eradicate *P. aeruginosa*. Approval by the Institutional Review Board was obtained and we received informed consent from all subjects in the cohorts.

CFTR Genotyping

Genomic DNA isolated from each subject was evaluated for the presence of any of 1,500 *CFTR* gene mutations (Genzyme, Cambridge, MA or Ambry Genetics, Aliso Viejo, CA) as part of clinical evaluation.

SNP Genotyping

We investigated the IL1-gene cluster (gene *IL1A*, gene *IL1B*, gene *IL1RN*, and gene *IL1RI*) on chromosome 2, based on the National Center for Biotechnology Information (NCBI) dbSNP build 127 (refer to Supplementary Appendix Table for information regarding rs ID, alleles, minor allele frequency, HWE, and SNP function). We genotyped 58 SNPs in the *IL-1* gene cluster in 808 CF subjects from UNC/CWRU cohort. SNPs in candidate genes were selected for genotyping on the basis of one of three criteria: r^2 value of at least 0.7 (for linkage disequilibrium [LD]-tagging SNPs), allele frequency of at least 10% in European-American populations, and/or causing a non-synonymous alteration in the amino acid sequence of the coded protein. All SNPs were verified by review of documentation in several databases—dbSNP, the Innate Immunity (<http://innate.immunity.-net/SNPper>)

(<http://snpper.chip.org/>), and Seattle SNPs (<http://pga.mbt.washington.edu>) program for genomic applications (PGA) web sites. SNP genotyping was performed using the standard protocol for the iPLEX assay on a Sequenom MassARRAY MALDI-TOF mass spectrometer²³ (Sequenom, San Diego, CA) or TaqMan assays²⁴ (Applied Biosystems, Foster City, CA).

Association Analysis

Single SNP association analyses were conducted using logistic regression for dichotomous outcomes and linear regression for continuous phenotypes. All population-based statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC), where all family-based association testing was performed using FBAT²⁵ and PBAT.²⁶ In the two cohorts, we evaluated a potential association between IL1 polymorphisms and lung function in CF in the following way. In the screening step, we tested for association between the selected SNPs and lung disease severity using extremes of pulmonary function measurements, as defined by Drumm and colleagues, in a case-control analysis in the UNC/CWRU CF subjects.¹⁷ In the replication cohort (CHB), we tested for association with affection status (an allele transmission distortion) as defined by the ascertainment of the proband in a family-based analysis using the FBAT approach. As a secondary analysis, we also used the qualitative lung phenotype, with severity defined using extremes of pulmonary function measurement determined by ESCF classification in a family-based analysis.²⁷ Additionally, since quantitative lung function data were available in the CHB cohort, we evaluated the association between the selected SNPs and lung function in a longitudinal analysis, incorporating quantitative pulmonary function measurements over the first five study visits. Only the first five annual study visits were included to minimize the number of missing subjects (data completeness > 90% over the first five study visits) and minimize any potential cohort effect. The longitudinal analysis was conducted in SAS (version 9.1) using a mixed model with fixed effects for the SNP, subject age at baseline, and time under study. Random intercepts and slopes were modeled for each subject. The UNC/CWRU cohort had greater power to detect an association due to sample size and was used to screen SNPs while the smaller cohort (CHB) was used to test for replication. This process is the validated standard for analysis in genetic studies. We used the odds ratio (OR) and estimation of effect to screen 58 SNPs for those 9 SNPs we would evaluate in the smaller cohort. Nine SNPs were tested in the second cohort based on the OR because testing of all 58 SNPs would have diluted the power to detect any signal due to the multiple comparisons problem. In summary, we evaluated two CF populations (UNC/CWRU and CHB) and three phenotypes—analysis of extremes of lung function using dichotomized lung function severity (in UNC/CWRU and CHB cohorts), affection status/transmission distortion (CHB cohort), and longitudinal lung function measures (CHB cohort) to assess whether the *IL-1* family gene cluster has an effect on CF lung disease.

University of North Carolina (UNC/CWRU) Cohort Analysis

Analysis of Extremes of Lung Function in UNC/CWRU Cohort—SNP

characteristics (allele frequency and Hardy-Weinberg equilibrium) for each SNP were assessed. Selected SNPs in candidate genes were genotyped in 808 Δ F508 homozygotes with “severe” or “mild” lung disease, as defined by the lowest or highest quartile of FEV₁ for age. We tested for association between the selected SNPs and lung disease severity in subjects with “mild” (n = 545) and “severe” (n = 263) illness by both Fisher’s exact and Armitage trend testing. Multivariable associations between individual SNP and FEV₁ percent predicted in the presence of potential confounders (age and gender) were tested using a general linear model. Genotypes were coded assuming an additive genetic model. For complex trait statistical models in which two or more loci may be involved in disease susceptibility, additive models, in which the allele-specific risks of disease are associated

with the multilocus genotypes across different loci, can be modeled as sums of factors for each genotype at each locus. ORs comparing severe versus mild CF were also calculated for each genotype (with the homozygote WT as the reference group) for all 58 SNPs, to determine whether significant Fisher's exact and Armitage tests corresponded to a clinically meaningful change in the odds of severe CF. OR testing was conducted to assess the magnitude of effect and examine the effect of each genotyped on risk, without imposing any assumptions about the genetic model. Armitage tests with $P < 0.05$ have a 95% confidence intervals that do not include 1. A false discovery rate (FDR) correction was applied to the UNC cohort accounting for the 58 SNPs originally tested.²⁸ From these analyses, nine SNPs with OR = 0.5 or > 1.5 showing evidence of association and moderate effect sizes were selected for further testing in subjects recruited from CHB.

Children's Hospital Boston (CHB) Cohort Analysis

Affection Status Analysis (Transmission Distortion)—For the familial data (CHB), nine SNPs were tested using an additive model for evidence of association with the diagnosis of CF in the proband. The purpose was to determine whether any of the SNP alleles was significantly over- or under-transmitted to the proband.

Analysis of Extremes of Lung Function in CHB Cohort—We tested for association between the selected SNPs and lung disease severity in subjects with “mild” or “severe” illness as defined by an assigned disease severity group, based on FEV₁ values and using the ESCF patient classification for patients (described in Methods). Due to the small sample size in the CHB cohort, we were unable to analyze the subjects in any of the four “extreme” categories of normal/mild or moderate/severe lung impairment. Thus, the ESCF classification was collapsed into two categories: normal/mild and moderate/severe. In the CHB family analysis, P -values were obtained from an FBAT statistic comparing observed to expected allele transmission from parents to CF probands with normal/mild or moderate/severe lung function impairment at the first study visit.

Longitudinal Analysis of FEV₁% Predicted in the CHB Cohort—Longitudinally measured lung function phenotypes were available in this cohort. As some of the associations observed in the UNC/CWRU cohort displayed differential effects by gender, both overall and gender-stratified analyses were conducted.

To maximize the power to detect an association, we analyzed percent-predicted FEV₁ with a multivariate population-based analysis (the offspring genotype, rather than parent-child allele transmissions, was the predictor of interest). A mixed model (SAS) was fit, including a random effect for each subject and for time since recruitment. Each SNP was tested separately, assuming either an additive, dominant, or recessive genetic model as a fixed effect, along with age at recruitment, gender, and time since recruitment. As the $\Delta F508$ mutation in the *CFTR* gene is the most common cause of CF in the Caucasian population, the analysis was repeated with adjustment for the presence or absence of two copies of $\Delta F508$ alleles using a recessive genetic model.

Finally, we examined the relationship between the two SNPs of interest, rs1143634 and rs1143639, and the presence of non-mucoid or mucoid *P. aeruginosa* in the CHB cohort. Methods for ascertaining culture included both deep throat and sputum cultures. Colonization was defined as one positive microbiologic growth on culture. Two sets of analyses were conducted. Initially, logistic regression models were fit to determine whether rs1143634 or rs1143639 genotypes predicted the presence or absence of non-mucoid or mucoid *P. aeruginosa*, after adjusting for age at first study visit. Second, survival analyses (also adjusting for age at first study visit) were performed to test whether specific rs1143634

or rs1143639 genotypes predicted time of onset of non-mucoid or mucoid *P. aeruginosa*. The analysis was conducted using a Cox-proportional hazards model fit (using proc phreg in SAS) to test this hypothesis in both the overall group and gender-stratified groups.

RESULTS

Population Baseline Characteristics

A total of 808 UNC/CWRU subjects were analyzed (Table 1). The mean age for 263 patients classified with severe lung disease was 16.2 ± 4.1 years, and the mean age for 545 patients classified with mild lung disease was 28.6 ± 9.7 years (Table 1). Males made up 49.4% of the severe disease group and 55.6% of the mild disease group. All of the analyzed patients were $\Delta F508$ homozygous and therefore pancreatic-insufficient. Over 80% of the cohort had positive cultures for *P. aeruginosa*. The mean FEV₁% predicted was 46.6 ± 16.1 in the severe group and 72.4 ± 28.1 in the mild group. The reported rate of FEV₁ decline (percent/year) in the severe group was 3.65 ± 2.20 and 1.35 ± 1.51 in the mild group.¹⁷

In the replication cohort from CHB, a total of 126 trios were analyzed, with a mean age of 10 ± 6.46 years for the CF patients when the first clinical values were analyzed (Table 2). This cohort was 53% male and 47% female. Forty-one percent of the patients were $\Delta F508$ homozygous and 40% were heterozygotes. The other 12% of the *CFTR* mutations included G551D, G542X, G85E, W1282X, and N1303K. Ninety-five percent of the cohort were pancreatic-insufficient, and 92% had positive cultures for *P. aeruginosa*, of which 70% were positive for mucoid *P. aeruginosa*. The mean predicted rate of decline in FEV₁ was -2.29 ± 3.76 (percent/year), consistent with an average decline in FEV₁ percent predicted of 2.5–2.6 (percent/year) for CF patients.¹⁸ Table 3A illustrates the pulmonary function data available for the 126 CF subjects obtained stratified by age. Table 3B illustrates the pulmonary function data for the 126 CF subjects obtained at the first five clinical visits and stratified by age. In Table 3B, there is a value for each subject per year. For example, if a subject had data each year from the age of 11–14, the subject was represented four times in the table. They were represented twice in the 6–12 age category and twice in the 13–17 age category.

We confined our analyses to self-described Caucasians to minimize the chance of identifying false associations due to population stratification in the case-control analysis. In addition, power to detect associations in other racial groups is low due to small sample sizes, particularly in CF, where the affected cohort is overwhelmingly Caucasian.²⁹

Genotyping Quality Control

A total of 58 SNPs in the *IL-1* gene cluster were initially investigated in the UNC/CWRU cohort; 9 SNPs were then investigated in the CHB cohort. Across both cohorts, none of the selected SNPs showed significant overall departure from Hardy-Weinberg equilibrium. Genotyping quality for both Sequenom and TaqMan assays was high, with an average completion rate of 98%, no discordances on repeat genotyping of a random 10% of the cohort, and a low rate of Mendelian inconsistencies.

Associations Between *IL-1* SNPs and Lung Function

Results From the University of North Carolina (UNC/CWRU) Cohort

Analysis of extremes of lung function: We tested for an association between the selected SNPs and lung disease severity in subjects with “mild” ($n = 545$) or “severe” ($n = 263$) illness by Fisher’s exact and Armitage trend testing. As shown in Table 4, three SNPs in the gene for IL1- β (*IL1B*), rs1143633, rs1143639, and rs3917356, as well as a SNP in IL1-RN (the gene for the receptor antagonist for *IL-1*), rs4252019, were suggestive of an association

with lung disease severity in CF ($P < 0.10$). While not significant in the non-stratified analysis, when stratified by gender, other SNPs were associated with lung disease severity: another SNP, rs17561, in the gene for *IL-1 α* (*IL1A*) ($P < 0.05$ in the males), one SNP in the *IL1B* gene rs1143634 ($P < 0.07$ in the females), and one SNP rs2228139 in the *IL1-R1* gene ($P < 0.05$ in the females). To further determine which SNPs to genotype in a family-based analysis, we fit a logistic regression model for each SNP, calculating the odds of severe versus mild CF for each genotype in all 58 SNPs initially screened. The analysis with the logistic regression models confirmed the Armitage testing. Eight SNPs showed an OR > 1.5 (rs 17561, 3917356, 1143633, 1143634, 1143639, 3917368, 2228139, and 4252019), indicating greater odds of having severe versus mild CF lung disease when comparing genotyping categories (data not shown). For an OR > 1 , the genetic variant is associated with disease and in this case worse pulmonary function. One SNP, rs2071374 in the gene for *IL-1 α* (*IL1A*), had an OR < 0.5 indicating greater odds of having mild CF lung disease when comparing genotyping categories. With these promising results, we proceeded to evaluate nine SNPs using a family-based analysis in a second population.

Results From the CHB Cohort

Affection status analysis (transmission distortion): We next evaluated 126 trios in a second population of CF patients from CHB using a family-based design. For the familial data (CHB), nine SNPs were tested using an additive model for evidence of association with the diagnosis of CF in the proband (Table 5). Due to the gender-specific differences observed for SNPs selected from the UNC/CWRU sample for replication, the association analysis of affection status in the CHB cohort was conducted in both the overall and gender-stratified cohort. Under an additive model, two of the SNPs associated with the *IL1B* gene, rs1143634 and rs1143639, were suggestive of an association in the gender-stratified analysis ($P > 0.05$). SNPs 1143634 and rs1143639 are in strong LD between the two markers ($D = -0.171$, $D' = 1.0$, and $R^2 = 0.98$). None of the other seven SNPs was nominally significant (all $P > 0.05$) in either the overall or stratified cohort.

As shown in Table 5, in the subgroup analysis, the P -values for the females are significant ($P < 0.05$), demonstrating a strong effect size, given the small number of informative trios in the CHB cohort (the number of informative trios for females was 29 for both SNPs). The direction of the association is consistent across the UNC/CWRU and CHB cohorts; the frequency of the minor allele is overrepresented in the UNC/CWRU severe CF cases (Table 4) and over-transmitted to female probands in the CHB cohort (Table 5). Therefore, the association is suggestive, given the consistency across studies and the relatively small number of female subjects ($n = 366$ in UNC/CWRU and informative trios = 29 in CHB), and P -values ($P < 0.10$).

Given the consistent association in females (we have demonstrated a consistent effect across cohorts), the P -values for rs1143634 and rs1143639 from Fisher's test of association in the UNC/CWRU cohort and P -values from the family-based association test in the CHB cohort were combined using Fisher's combined probability test. The joint P -values for SNPs rs1143634 and rs1143639 from the overall test of association for the two studies, as well as the analysis in males, were not significant. For females, the unadjusted joint P -values were 0.0086 and 0.0052 for rs1143634 and rs1143639 SNPs, respectively. After a FDR correction was applied to the UNC/CWRU cohort, accounting for the 58 SNPs initially tested,²⁸ the adjusted P -values were 0.059 (rs1143634) and 0.052 (rs1143639). While this does not meet significance at the strict $\alpha = 0.05$ level, these are suggestive given the small sample size in the female cohort. We report the P -value > 0.05 as evidence of a consistent association with lung disease severity because the analysis is exploratory and is nonetheless worthwhile to

report. There is replication in a second cohort, even with smaller power, and it has the same effect direction.

Analysis of extremes of lung function in the CHB cohort: We also evaluated a dichotomous phenotype in the CHB cohort, based on FEV₁ ESCF categories, similar to the categorization of the UNC/CWRU cohort. To maximize information from an increased sample size that has fewer quantitative measures, the UNC/CWRU analysis compared extremes of lung function, that is, severely impaired (lowest quartile) with mildly impaired (highest quartile). For the CHB family data, which are from a smaller sample than the UNC/CWRU cohort but include additional longitudinal quantitative FEV₁ measurements, *P*-values were obtained from an FBAT statistic comparing observed to expected allele transmission from parents to CF probands with collapsed ESCF categories of normal/mild or moderate/severe lung function impairment. There was no association in either the overall or gender-stratified analysis in the CHB cohort (data not shown). However, this may be due to the small sample size, particularly in the gender-stratified analysis. For the females, 37 children were classified as normal/mildly impaired, 14 were classified as moderately/severely impaired, and 8 were missing an FEV₁ measurement. For the males, these numbers were 35, 14, and 18, respectively (summing to 126 children/trios). The number of informative families in each analysis ranged from 55 (in the overall analysis) to 23 (in the gender-stratified analysis for females).

Longitudinal analysis of FEV₁% predicted in the CHB cohort: To extract the most information from our cohort, we also conducted a population-based longitudinal analysis. Due to the unavailability of comparable longitudinal lung function data in the UNC/CWRU cohort, additional analyses of lung function could be conducted only in the CHB cohort. The two SNPs of interest in the *IL1B* gene from the affection status analysis, rs1143634 and rs1143639, were tested for association using the FEV₁% predicted phenotype, measured at the first five study visits.

Figures 1a,b (confidence intervals not shown) and 2a,b (confidence intervals drawn) present the age- and gender-adjusted means for FEV₁% predicted for each genotype for *IL1B* SNPs rs1143634 and rs1143639. The average length of time from the first study visit to the second study visit was 1.4 years, and the mean length of time between subsequent visits was 1 year. The mean length of time over all five study visits was 4.5 years. The calculated means are limited to subjects with complete data for the first five study visits, to limit any potential cohort effects. Displayed above each study visit (on the *x*-axis) are the corresponding univariate effect size estimates and *P*-values for each genotype group. The beta estimates represent the mean change in FEV₁% predicted at each time point, for the AG/AA [CT/TT] genotype in comparison to the GG [CC] genotype, after adjustment for age and gender. The *P*-values are obtained from a Wald test, which tests the hypothesis that beta is equal to 0 at each of the time points separately. When evaluating all five time points in a multivariate analysis (Table 6), there is a marginally significant statistical difference in the FEV₁% predicted in subjects with one or two copies of the minor A or T allele for rs1143634 or rs1143639, respectively. In the overall group, for rs1143634, the mean difference in FEV₁ between the GG and AG/AA genotype groups across the five study visits was 5.6% after adjusting for age and gender (*P* = 0.06). For rs1143639, the mean difference in FEV₁ between the CC and CT/TT genotype groups across the five study visits was 6.7% after adjusting for age and gender (*P* = 0.05). We would expect similar results for these two SNPs because of the strong LD between the two markers (*D* = -0.171, *D'* = 1.0, and *R*² = 0.98). The longitudinal model was adjusted for age at the first visit as well as time in follow-up to reflect the variable ages and to account for the effect of age on FEV₁. Age was not significant across the different genotype groups. We also analyzed the means by genotype (homozygote major allele vs. heterozygote and homozygote minor combined) and *t*-tests of

the difference in ages (at each study visit) across groups. None of the differences were significant (data not shown).

Table 6 presents the multivariate results for the dominant model, adjusting for age and gender in the overall group, and for age in the gender-stratified analysis. There is a trend toward decreasing FEV₁% predicted for the heterozygote or homozygote minor allele. The overall *P*-value (a test of whether the mean FEV₁% predicted differs across the major allele homozygotes vs. the heterozygotes and minor allele homozygotes combined) was 0.06 for SNP rs1143634 and 0.05 for SNP rs1143639. Therefore, in the longitudinal analyses, having one or two copies versus none of the minor allele shows evidence of association (*P* < 0.10) for both of the SNPs within the *IL1B* gene and lung function decline. The analysis of the CHB cohort was repeated with adjustment for ΔF508 alleles, assuming a recessive genetic model. The presence of ΔF508 mutations was not associated with FEV₁% predicted and did not affect the relationship between SNPs rs1143634 or rs1143639 and FEV₁% predicted. The magnitude of the effect size estimates and *P*-values for SNPs rs1143634 and rs1143639 did not substantially change after inclusion of ΔF508 in the model (data not shown).

Finally, in an exploratory analysis, we examined the relationship between the two *IL1B*-associated SNPs of interest, rs1143634 and rs1143639, and the presence of non-mucoid or mucoid *P. aeruginosa*. Neither the logistic regression analyses nor the Cox model showed a relationship between rs1143634 or rs1143639 genotypes and the presence or onset of mucoid *Pseudomonas* (data not shown). There was no significant association between the SNPs and presence of non-mucoid *P. aeruginosa*. However, in the overall (non-stratified) analysis, for both rs1143634 and rs1143639, the presence of one or two copies of the minor allele was associated with a later onset of non-mucoid *P. aeruginosa* with hazard ratios of 0.624 and 0.661 and *P*-values of 0.039 and 0.068, respectively.

DISCUSSION

This study reports an association between genetic variants of the *IL1B* gene and lung disease severity in CF patients. We utilized the cohort from the UNC/CWRU-based, multicenter study involving 808 ΔF508 homozygote CF patients¹⁷ to screen for an association between lung disease severity and polymorphisms in the *IL-1* gene cluster. We initially found an association in the *IL1B* gene and lung disease severity. We then sought to replicate this finding in a different single center using a family-based association analysis: in a subgroup analysis, our findings are significant in females and suggestive in males based on population analysis. Collection of study subjects from a single center affords some control over variation in environmental factors, which are difficult to control in multicenter studies. Despite the fact that we used a relatively small family-based cohort, we were able to substantiate the results of the larger case-control study, probably because a single-center study can have higher power to detect genetic contributions to outcomes that have a substantial non-genetic component by reducing environmental heterogeneity.³¹

Replication of associations in multiple independent patient populations using different study designs is critical in confirming a potential pathologic role for a genetic variant in a disease process. To our knowledge, two family association (FBAT) studies pertaining to a CF cohort have been published; neither study explored an association among *IL-1* gene polymorphisms and lung function decline or *Pseudomonas* acquisition.^{32,33} We confirmed the association we found in the case-control analysis, using 126 trios and FBAT, which avoids spurious associations related to population stratification. In addition to testing an additional cohort to validate the observed associations in the primary cohort, we undertook the following measures for validation: population stratification was tested for in the primary cohort and none was found; only Caucasian subjects were analyzed, so that similarities in the ethnicity

of the subject and allele frequencies observed in the cohorts suggest adequate genetic comparability; and adjustments for identical covariates in the analyses ensured comparability between the cohorts.

Furthermore, our findings are consistent among several phenotypes. We found that *IL1B* SNPs rs1143634 and rs1143639 were overrepresented among the CF severity groups in both the UNC/CWRU and CHB cohorts at a statistically significant level. Specifically in the analysis of longitudinal changes in lung function, there was a negative relationship between the number of copies of the minor allele of both SNPs and FEV₁% predicted, indicating that the minor allele is associated with a reduction in this measure of lung function. While dichotomous pulmonary function severity analysis did not show a consistent association between the two cohorts, nor were the genetic models consistent, this may be due to the small sample sizes in the CHB-based gender-stratified analysis. Although we avoid population stratification and admixture by using the FBAT approach, we lose power because of homozygous parents who are uninformative. Limiting the study to only $\Delta F508$ homozygotes, as had been done by Drumm and colleagues in the UNC/CWRU cohort,¹⁷ likely minimized variability at the *CFTR* locus and might have contributed to phenotypic differences. Differing ascertainment schemes of a single-center study versus multicenter study and diagnostic criteria for defining the dichotomization of pulmonary impairment categories may also help explain the discrepancy. Yet, despite these limitations, in addition to showing that variation in the *IL1B* gene is associated with diminished lung function, we also found an association between these variants and CF affection status (transmission distortion). Our hypothesis, supported by the trio data, is that these two SNPs—rs1143634 and rs1143639—are more strongly associated with disease status in the CF cases (the proband) compared with the controls (their unaffected parents).

The results of the transmission distortion testing (TDT) in the FBAT analysis for affection status, corrected for multiple comparisons, suggest that these two SNPs are associated with CF independent of *CFTR* genotype or could be a surrogate marker of disease severity. A subgroup analysis suggested that the effect was present only in females, in whom there was over-transmission of these alleles. In the family-based analysis, the association in females was observed despite the small number of informative families. In the population-based analysis, the strength of the association is stronger among females as well (FDR adjusted $P < 0.06$ for females for both SNPs). This could be of importance if survival bias allowed for a protective *IL1B* allele in males but a non-protective one in females. Earlier acquisition of *P. aeruginosa* in CF females has been postulated to contribute to diminished survival.^{34,35} It is conceivable that *IL-1 β* release following infectious and other environmental exposures (influenced by hormones) may occur in a time-dependent manner to affect bacterial clearance and ultimately disease expression differentially in males and females. The timing and duration of the infectious and environmental stimuli remain unknown and will be the basis of future studies.

The *IL1B* genotype association is consistent with findings related to a role for rapid *IL-1 β* release in activating innate immunity following binding of *P. aeruginosa* to WT-*CFTR* and gender findings showing a poorer outcome in CF females.^{34–36} However, the precise relationship remains unclear. Although the *IL1B* locus harbors variants that contribute to CF diagnosis (based on transmission distortion results) as well as lung function decline (based on longitudinal lung function analysis), the relationship and mechanism are complex. SNP analyses detected associations between SNPs within exon 5 (rs1143634) and intron 6 (rs1143639) and lung disease severity. While the changes to the nucleotides within the *IL1B* gene are synonymous, it is likely that the associations observed are due to strong LD between associated SNPs and the true phenotype causal variant(s) of lung disease severity in CF. We speculate that the causative variants may influence lung disease severity by altering

susceptibility to *P. aeruginosa* infection. For both rs1143634 and rs1143639, the presence of one or two copies of the minor allele was associated with later onset of non-mucoid *P. aeruginosa*, which is in turn associated with better lung function over the lifetime of CF patients.³⁵ Thus, early acquisition of *P. aeruginosa* may be affected, suggested by the parallel but offset lung function curves depicted in Figures 1 and 2. For patients in our study with one or two copies of the minor allele and lower FEV₁, the *IL1B* gene polymorphisms may represent a hyperimmune response that is effective against infection but possibly destructive to the airway. When evaluating the two SNPs we found to be significant in the *IL1B* gene, we detected no relationship between them and acquisition of mucoid *P. aeruginosa*. Again, the small number of subjects may explain this. However, these two SNPs may be influencing lung function in CF subjects by a different mechanism.

Several molecular properties associated with *IL-1* release and responses to *P. aeruginosa* have been reported. In gene expression microarrays modeling *P. aeruginosa* infection, *IL1B* was increased, suggesting a role for *IL-1B* in *P. aeruginosa* acquisition and lung function.³⁷ *IL-1β* release, binding to the *IL-1R*, and signaling involving the myd88 adaptor protein are all needed for rapid NF-κB nuclear translocation following *P. aeruginosa* infection in the presence of WT-*CFTR*.^{38,39} This process is deficient in cells and mice lacking WT-*CFTR*.¹⁵ Furthermore, myd88 knockout mice have increased susceptibility to *P. aeruginosa* infection.^{5,40,41} Thus, cellular and animal data suggest a role for *CFTR*-dependent *IL-1* release and signaling through the *IL-1R* in modulating resistance to *P. aeruginosa*. Another component of CF lung disease that could be affected by *IL-1* is the chronic inflammation that results from *P. aeruginosa* infection and clearly contributes to disease pathogenesis. A rapid and early inflammatory response would likely be effective in clearing *P. aeruginosa*, whereas a delay, as could occur in CF, would lead to dysregulated production of *IL-1* and pathology from inflammation. In support of this view, neutralization of *IL1B* well into the course of an acute lung infection in mice ameliorates pathology and promotes bacterial clearance. Thus, *IL-1*-mediated responses to *P. aeruginosa* could influence CF lung disease by modulating the early and/or chronic response to infection. The hypothesis that the innate immune response contributes independently to progression of CF lung disease, possibly via intrinsic airway inflammation sustained by *IL-1β*, is also supported by the recent finding that the antibiotic moxifloxacin, used to treat *P. aeruginosa* infection in CF patients, selectively inhibits NF-κB as well as the ERK signaling pathway, which can also be modulated by *IL-1*.⁴²

Functional analysis is ongoing to determine whether the significant SNPs we identified affect *IL-1β* levels, protein folding, or effectiveness in promoting *P. aeruginosa* colonization. Preliminary results suggest that some inflammatory cytokines are produced and/or released in a *CFTR*-dependent manner in airway epithelial cells, and proper production of these proteins is associated with increased resistance to *P. aeruginosa* infection.¹ Rapid *IL-1* release is muted in cells with only ΔF508 *CFTR* alleles compared with the same cells with WT-*CFTR*, but the endogenous levels of *IL-1* are comparable in these cells. However, *IL-1* is released from CF airway cells after about 60 minutes of exposure to *P. aeruginosa* suggesting that both the timing and amount of *IL1β* released in response to *Pseudomonas* in the lung are critical determinants of rapid bacteria elimination and resolution of inflammation.¹ Furthermore, natural limitations in diffusion of inflammatory cytokines resulting from proper closing of gap-junctional signaling following release of cytokines in airway epithelial cells expressing WT-*CFTR* contribute to the regulated inflammatory response resulting in elimination of this pathogen in normal lungs.⁴³ Thus, an *IL-1* allele in a CF patient resulting in higher endogenous levels of this cytokine in airway epithelial cells could lead to sufficient, *CFTR*-independent *IL-1* release following *P. aeruginosa* to mediate clearance of the microbe in a manner comparable to individuals with WT-*CFTR*. Alternatively, an *IL-1R* allele more responsive to lower levels of *IL-1* could also

induce a more WTinnate immune response in CF patients with such a (hypothetical) allele. In contrast, a similar situation could also be pathologic, resulting in an increased inflammatory response if the *IL-1R* interactions occur later, and other regulatory factors contributing to CFTR-dependent resolution of inflammation are lacking. Overall, preliminary results from cell culture, animal, and human genetic studies can be viewed in the context of an emerging clinical phenotype and its impact on CF lung pathophysiology, and represent a logical starting point for focused longitudinal genetic studies and functional analysis. Further studies may yield new insights into the pathogenesis of inflammatory airway disorders such as CF and reveal new links among the production of *IL-1 β* , the innate immune response, and *P. aeruginosa* acquisition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Age and gender-adjusted means at each visit representing each of 99 patients once. Plots and longitudinal analyses were limited to subjects with all five measurements, to limit any potential cohort effect. a: Fifty-six patients are homozygous major for the GG genotype, and 43 are heterozygous/homozygous minor with either an AG or AA phenotype (rs1143634 without confidence interval shown). b: Fifty-seven patients are homozygous for the major allele, CC, and 42 patients are heterozygous/homozygous minor for the CT or TT genotype (rs1143639 without confidence interval shown).

Fig. 2.

a: Fifty-six patients are homozygous major for the GG genotype, and 43 are heterozygous/homozygous minor with either an AG or AA phenotype (rs1143634 with confidence interval shown). b: Fifty-seven patients are homozygous for the major allele, CC, and 42 patients are heterozygous/homozygous minor for the CT or TT genotype (rs1143639 with confidence interval shown).

TABLE 1

Characteristics of 808 Patients With Cystic Fibrosis Homozygous for the DF508 Mutation, According to the Phenotype for Severe or Mild Impairment of Lung Function and Age (Initial Study)

Variable	Degree of impairment		P-value
	Severe, N = 263	Mild, N = 545	
Age (years)			
Range	8–25	15–55	
Mean	16.2 ± 4.1	28.6 ± 9.7	< 0.001
Sex (% male)	49.4	55.6	0.10
FEV1 (% of predicted value)	46.6 ± 16.1	72.4 ± 28.1	< 0.001
FEV1 decline (%/year)	3.65 ± 2.20	1.35 ± 1.51	< 0.001
Median predicted survival (age in year)	31.4	56.6	< 0.001
Body mass index (percentile)	19.6 ± 21.7	44.0 ± 26.1	< 0.001
Positive test for <i>P. aeruginosa</i> (%)	89.0	86.1	0.25
Diabetes mellitus (%)	15.6	24.0	0.006
Asthma (%)	19.4	22.0	0.39

Adapted from *N Engl J Med*.¹⁷

Pulmonary function classifications are described by Schluchter et al.¹⁸

TABLE 2

Demographics of Children’s Hospital Boston, CF Population

	N	%	Mean	Min	Max
Trios	126				
Female	59	47			
Age					
At consent	126		18.5 ± 8.7	8	45
On first clinical values	126		10.0 ± 6.5	1	36
1–12	95	75			
13–17	17	13			
18–29	11	8			
30+	3	2			
Genotype					
ΔF508 homozygous	52	41			
ΔF508 heterozygous	51	40			
Other	15	12			
Unknown	8	6			
Pancreatic insufficient	120	95			
Sweat chloride	119	94	104.3 ± 16.8	40	143
FEV ₁ (% predicted)	559		92.9 ± 19.6	24	162
Percent change FEV ₁ /year	559		−2.3 ± 3.6	−14.3	0

FEV₁, forced expiratory volume in 1 sec.

TABLE 3A

Pulmonary Function Severity Data for the 126 CF Subjects

Disease severity ¹	Age and number of patients (%) ²				
	6-12	13-17	18-29	> 30 years	Row totals
Normal/very mild	41 (59%)	10 (63%)	10 (91%)	2 (67%)	63 (63%)
Mild	6 (9%)	2 (013%)	1 (9%)	0 (0)	9 (9%)
Moderate	7 (10%)	0 (0)	0 (0)	1 (33%)	8 (8%)
Severe	16 (23%)	4 (25%)	0 (0)	0 (0)	20 (2%)
Totals	70	16	11	3	100

¹ Each patient was assigned a disease severity group based on FEV₁ values using the Epidemiological Study of Cystic Fibrosis (ESCF) classification for patients in four age groups: 6-12 years (severe, FEV₁ 88.7% predicted; moderate, > 88.7-94.5%; mild, > 94.5-99.0%; very mild/normal, > 99.0%); 13-17 years (severe, FEV₁ 76.5% predicted; moderate, > 76.5-81.1%; mild, > 81.1-87.7%; very mild/normal, > 87.7%); 18-29 years (severe, FEV₁ 58.1% predicted; moderate, > 58.1-63.9%; mild, > 63.9-70.7%; very mild/normal, > 70.7%), and > 30 years (severe, FEV₁ 45.5% predicted; moderate, > 45.5-50.9%; mild, > 50.9-59.8%; very mild/normal, > 59.8%).

² Excludes 26 visits for subjects when FEV₁ % predicted was missing.

TABLE 3B
Pulmonary Function Severity Data for the 126 CF Subjects Obtained at the First Five Clinical Visits

Disease severity ¹	Age and number of patients visits (%) ²				Row totals
	6-12	13-17	18-29	> 30 years	
Normal/very mild	192 (34%)	53 (9%)	66 (12%)	17 (3%)	328 (59%)
Mild	48 (9%)	11 (2%)	6 (1%)	2 (0%)	67 (12%)
Moderate	38 (7%)	8 (1%)	1 (0%)	2 (0%)	49 (9%)
Severe	96 (17%)	14 (3%)	4 (1%)	1 (0%)	115 (21%)
Totals	374 (67%)	86 (15%)	77 (14%)	22 (4%)	559 (100%)

¹ Each patient was assigned a disease severity group based on FEV₁ values using the Epidemiological Study of Cystic Fibrosis (ESCF) classification for patients in four age groups: 6-12 years (severe, FEV₁ 88.7% predicted; moderate, > 88.7-94.5%; mild, > 94.5-99.0%; very mild/normal, > 99.0%); 13-17 years (severe, FEV₁ 76.5% predicted; moderate, > 76.5-81.1%; mild, > 81.1-87.7%; very mild/normal, > 87.7%); 18-29 years (severe, FEV₁ 58.1% predicted; moderate, > 58.1-63.9%; mild, > 63.9-70.7%; very mild/normal, > 70.7%), and > 30 years (severe, FEV₁ 45.5% predicted; moderate, > 45.5-50.9%; mild, > 50.9-59.8%; very mild/normal, > 59.8%).

² Excludes 26 visits for subjects when FEV₁ % predicted was missing.

Significant Associations of IL1 Genotypes and Mild (Highest Quartile) Versus Severe (Lowest Quartile) Pulmonary Function Impairment in the UNC/CWRU Cohort

TABLE 4

Gene	SNP location	SNP rs#	MA	MAF (European population)	MAF (CF population)	Fisher's P-value	Fisher's P-value, male	Fisher's P-value, female	Armitage P-value
<i>IL1α</i>	Intron 4	2071374	G	0.30	0.30	0.102	0.497	0.163	0.091
<i>IL1α</i>	Exon 5	17561	A	0.33	0.29	0.415	0.008	0.015	0.248
<i>IL1β</i>	Intron 3	3917356	C	0.43	0.47	0.093	0.290	0.306	0.035
<i>IL1β</i>	Intron 4	1143633	T	0.45	0.38	0.067	0.329	0.157	0.073
<i>IL1β</i>	Exon 5	1143634	A	0.25	0.23	0.120	0.350	0.069	0.064
<i>IL1β</i>	Intron 6	1143639	T	0.28	0.23	0.057	0.289	0.039	0.038
<i>IL1β</i>	3'UTR	3917368	T	0.41	0.38	0.109	0.523	0.125	0.105
<i>IL1R1</i>	Exon 4	2228139	G	0.07	0.07	0.186	1.0	0.035	0.057
<i>IL1RN</i>	Intron 4	4252019	T	0.09	0.14	0.048	0.572	0.005	0.015

SNP rs#, refSNP number; MA, minor allele; MAF, minor allele frequency in the European and CF populations; P, P-value obtained from linear regression under an additive model using Fisher's and Armitage testing.

¹ Quartiles are based on the Cystic Fibrosis Foundation Registry Classification of pulmonary function testing for the UNC/CWRU center when compared to US CF centers.¹⁹ For the UNC/CWRU case-control data, P-values were obtained from a Fisher's exact test and Armitage trend comparing genotype counts in cases (severe CF) to controls (mild CF). There is an over transmission of the minor allele in the severe CF cases for these SNPs. Information for the minor allele frequency in the general population comes from several sources: dbSNP (www.ncbi.nlm.nih.gov/projects/SNP), SNPper (<http://snpper.chip.org/>), and Seattle SNPs (pga.mbt.washington.edu).

TABLE 5

Analysis of Affection Status in Children's Boston Cohort

SNP	Minor allele	Minor allele frequency	Females		Males		Overall	
			No. of info. families	Nominal two-sided <i>P</i> -value	No. of info. families	Nominal two-sided <i>P</i> -value	No. of info. families	Nominal two-sided <i>P</i> -value
RS1143634	A	0.249	29	0.016	43	0.285	66	0.463
RS1143639	T	0.246	29	0.016	42	0.225	65	0.527

P-values were obtained from an FBAT statistic comparing observed versus expected (assuming Mendelian) transmission from parents to affected offspring. All probands in this analysis have CF. In females, the minor allele for both SNPs is over transmitted ($P = 0.016$). Info. means informative and the number of informative families is determined by the allele status of the parents and therefore may vary.

TABLE 6Population-Based Analysis for a Mean Decrease in FEV₁% Predicted

Time point	RS1143634 (AG/AA vs. GG)		RS1143639 (CT/TT vs. CC)	
	Beta	P-value	Beta	P-value
All five visits				
Female	-7.21	0.06	-7.05	0.06
Male	-3.29	0.32	-4.10	0.29
Overall	-5.17	0.06	-5.27	0.05

All analyses are adjusted for age. Overall analysis is also adjusted for gender.

The overall *P*-values test whether mean FEV₁% predicted differs across the major allele homozygotes versus the heterozygotes and minor allele homozygotes combined.