

ARTICLE

Use of a modeling framework to evaluate the effect of a modifier gene (*MBL2*) on variation in cystic fibrosis

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Variants in mannose-binding lectin (*MBL2*; protein MBL) have shown association with different aspects (eg, lung function, infection, survival) of cystic fibrosis (CF) in some studies but not others. Inconsistent results may be due to confounding among disease variables that were not fully accounted for in each study. To account for these relationships, we derived a modeling framework incorporating *CFTR* genotype, age, *Pseudomonas aeruginosa* (*Pa*) infection, and lung function from 788 patients in the US CF Twin and Sibling Study. This framework was then used to identify confounding variables when testing the effect of *MBL2* variation on specific CF traits. *MBL2* genotypes corresponding to low levels of MBL associated with *Pa* infection 1.94 years earlier than did *MBL2* genotypes corresponding to high levels of MBL ($P=0.0034$). In addition, *Pa*-infected patients with *MBL2* genotypes corresponding to low levels of MBL underwent conversion to mucoid *Pa* 2.72 years earlier than did patients with genotypes corresponding to high levels of MBL ($P=0.0003$). *MBL2* was not associated with the time to transition from infection to conversion or with lung function. Thus, use of a modeling framework that identified confounding among disease variables revealed that variation in *MBL2* associates with age at infection with *Pa* and age at conversion to mucoid *Pa* in CF. *European Journal of Human Genetics* advance online publication, 13 January 2010; doi:10.1038/ejhg.2009.226

Keywords: infection; confounding; immunity

INTRODUCTION

Studies of twins and siblings affected with cystic fibrosis (CF) show that genetic modifiers have a major role in variation in CF pulmonary disease.¹ One of the first genes proposed to modify CF was mannose-binding lectin 2 (*MBL2*).² *MBL2* encodes mannose-binding lectin (MBL), a collectin with a central role in the innate immune response. MBL is responsible for pathogen recognition, opsonization, and activation of complement.³ Low MBL levels are associated with increased rates of infection, especially in susceptible populations.⁴ Infection with a variety of bacteria and fungi, in particular *Pseudomonas aeruginosa* (*Pa*), has an important role in the course of CF pulmonary disease.⁵ Thus, modulation of lung infection owing to variation in MBL levels provides an attractive mechanism for a genetic modifier of CF. Variation in the *MBL2* gene was initially associated with lung function measures (forced expiratory volume in 1 s; FEV₁) in *Pa*-infected CF patients and with survival of CF patients.² Later studies associated variation in the *MBL2* gene with infection with *Pa* and *Burkholderia cepacia*, as well as with age of *Pa* infection.^{6–14} However, numerous other studies failed to find an association with one or more of these attributes.^{2,6,7,9–15} Thus, the role of *MBL2* variation as a genetic modifier of CF has been of some debate.¹⁶

Lack of consistency among association studies can have many sources including population stratification, insufficient power, and variation in environmental factors.¹⁷ Another reason for inconsistency may be the presence of unaccounted confounding among different

clinical variables. If the patient groups being compared differ by a variable that affects the exposure and the outcome, that variable may be a confounder of the relationship between the exposure and the outcome.¹⁸ Therefore, in a disease state with many complex inter-related variables such as CF pulmonary disease, careful consideration of how these variables relate to each other is essential. Taking into account relationships between the exposures and outcomes through a modeling framework can facilitate recognition of and adjustment for confounding variables. We therefore developed modeling frameworks that incorporated variables commonly used in previous *MBL2* analyses and tested for association between each variable and variation in the *MBL2* gene.

PATIENTS AND METHODS

Subjects and clinical data

Pulmonary function, growth parameters, infection, and ethnicity/race data were derived from the CF Twin and Sibling Study¹⁹ supplemented using data from the Cystic Fibrosis Foundation Data Registry (Bethesda, MD, USA). Informed consent was received from all participants and/or guardians. All patients had DNA available for genotyping. Complete infection information (a recorded positive or negative culture and age of first positive culture, if applicable) for both *Pa* and mucoid *Pa* was available for all patients. In CF patients, *Pa* infection rates are relatively constant within the first 4 years of life, but rise quickly thereafter with increasing age.²⁰ Therefore, patients were excluded from the infection analysis if they were diagnosed ≥ 4 years of age

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Received 4 September 2009; revised 18 November 2009; accepted 19 November 2009

and had a positive *Pa* culture within 1 year of diagnosis. Respiratory cultures were obtained by throat swab, expectorated or induced sputum, or bronchoalveolar lavage. There was no difference in the number of clinic visits in the most recent year by MBL-sufficient/deficient status in uninfected patients. Over 30 000 cultures were available for the patients in this study (ranging from 2 to 112 per subject) and there was no difference in the mean number of cultures per subject by MBL-sufficient/deficient status. Age of patient at last pulmonary function test (PFT) was used as the age variable and only infection data collected before that age were used. Any data collected after lung transplant were dropped.

Pulmonary function was an average of the best PFT from each quarter of the most recent year of data as previously described.¹ No PFT data were used from patients who were less than 6 years of age or who had received a lung transplant. The average was available in two forms: FEV₁% predicted²¹ and a CF-specific percentile for FEV₁.²² As CF-specific percentiles account for age as well as sex and height, the longitudinal decline in lung function seen with traditional predictive equations is corrected for and patients of different ages can be easily compared.¹ In all analyses of lung function that required age correction to prevent potential confounding, the CF-specific percentile for FEV₁ was used.²² If age correction was not necessary, the FEV₁% predicted was used to enable comparison with previous studies. Lung function data were used for patients 6 years of age up to 40 years of age to account for the cohort used in deriving the CF-specific percentile.

Genotyping

Genetic variation in *MBL2*, including three amino-acid substitutions in exon 1 that prevent formation of the active MBL oligomer and promoter mutations that reduce transcription levels, tends to lower levels of active circulating MBL.^{23–26} *MBL2* alleles 'B' (rs1800450), 'C' (rs1800451), 'D' (rs5030737), and promoter variation 'X/Y' (rs7096206) were genotyped by hybridization of PCR-amplified DNA fragments encompassing each SNP to sequence-specific oligonucleotides in linear arrays as previously described.^{27,28} An allele that does not contain a B, C, or D coding variant was designated as 'A,' whereas the presence of any variant was classified collectively as allele 'O'. The range of MBL levels corresponding to *MBL2* diplotypes in CF patients has been well described.^{2,7,11,14} YO/YO and XA/YO individuals can be considered to have a 'deficiency' of MBL, whereas YA/YO, XA/XA, XA/YA, and YA/YA individuals have a high enough level of MBL to be considered 'sufficient'.^{2,7,14}

Multiplex PCR with biotinylated primers was performed as previously reported.^{27,28} Arrays were hybridized and washed using a BeeBlot machine (product number 1003; Bee Robotics Ltd, UK) with BeeBlot programmable software version 2.1b. Assay conditions were as follows: hybridization at 53°C for 10 min, wash at 25°C for 1 min, conjugation at 53°C for 10 min, wash at 25°C for 1 min, wash at 25°C for 10 min; citrate at 25°C for 5 min, substrate at 25°C for 10 min, deionized water at 25°C for 1 min (3×). Genotypes were determined using image analysis software (StripScan 5.8.0) provided by Roche Molecular Systems Inc (Pleasanton, CA, USA). Haplotype and diplotypes were constructed on the basis of previously reported haplotype data and by family data. *TGFB1* genotyping for codon 10 was performed as previously reported.²⁹ All SNPs meet the Hardy–Weinberg equilibrium requirements in parents. For *MBL2* allele frequencies (Table 1).

Statistical analysis

All analyses were performed in Intercooled Stata 8.2 and corrected for related individuals using the 'cluster' command (www.stata.com/). In this study, models of CF pulmonary disease were built using previously reported relationships to establish which variable was designated as 'exposure' and which variable was designated as 'outcome' for each pairwise relationship. Potential confounders were identified from pairwise statistical associations in the CF Twin and Sibling Study population in concert with background knowledge of causal networks that link exposure variables, outcome variables, and potential confounder variables.³⁰ To be considered a confounder in the exposure–outcome relationship, a variable had to be associated with exposure in our population, associated with outcome in our population, and not assumed to be in the causal pathway between exposure and outcome.³⁰ Associations between variables were evaluated by simple linear regression for two continuous

Table 1 *MBL2* genotype frequencies and breakdown of *MBL2* analysis variables in 788 Caucasian CF patients

Infection and pulmonary analyses	<i>MBL2</i> genotypes	Number of patients	Percent of patients
MBL sufficient	YA/YA	235	29.8
	YA/XA	183	23.2
	XA/XA	29	3.7
	YA/YB	121	15.4
	YA/YD	64	8.1
	YA/YC	25	3.2
MBL deficient	XA/YB	40	5.1
	XA/YD	30	3.8
	XA/YC	6	0.8
	YB/YB	19	2.0
	YB/YD	20	2.5
	YB/YC	4	0.5
	YD/YD	5	0.6
	YD/YC	6	0.8
	YC/YC	1	0.1

This chart contains individuals who are related to each other. For monozygous twins, one individual per set was used. Family relatedness was corrected for in all statistical analyses.

variables, ANOVA for dichotomous and continuous variables, and logistic regression for two dichotomous variables. Power was computed using UnifyPow.³¹

RESULTS

Modeling effects on lung disease in a CF population

Individuals in this study consist of 848 Caucasian siblings, dizygous twins, and monozygous twins enrolled in the US CF Twin and Sibling Study.^{1,32,33} For each of the 60 monozygous twin pairs, one twin was randomly removed from analysis. The remaining 788 individuals are 53.0% male, 50.8% $\Delta F508$ homozygotes, mean age 16.0 ± 7.8 with a median age of 14.4 years. In addition, 85.4% had a positive culture for *Pa* and 70.3% of the patients with a positive *Pa* culture had a culture positive for mucoid *Pa*. The prevalence of *Pa* is comparable to the rates seen in studies of unrelated CF patients.¹⁵

To construct a model to examine the genetic and nongenetic effects on CF pulmonary disease, we analyzed the pairwise relationships among four key variables, *CFTR* genotype, age, *Pa* infection and lung function, and displayed them in a graphical framework to guide our statistical modeling. However, as these variables are related, we first evaluated and adjusted for potential confounding effects in each pairwise relationship using a combination of *a priori* knowledge and statistical analysis.³⁰

As *CFTR* genotype was the only constant variable in these models, the first set of tests explored relationships between *CFTR* genotype (classified as $\Delta F508$ homozygote or non- $\Delta F508$ homozygote) and the three key outcome variables: age, *Pa* infection, and lung function. $\Delta F508$ homozygotes are 2.56 times more likely to be infected with *Pa* than non- $\Delta F508$ homozygotes (95% confidence interval 1.62, 4.03; $P=0.0001$), but *CFTR* genotype was not associated with age or lung function (Figure 1). Age was associated with *Pa* infection status as CF patients infected with *Pa* are on average 2.44 years older than uninfected CF patients ($P=0.0066$). Next, we considered the relationship between *Pa* infection status and lung function. As age was also associated with lung function, we adjusted for age using the CF-specific percentile for FEV₁.²² For every year increase in age, there is a decrease of 1.53 units of FEV₁% predicted ($P \leq 0.0001$). Finally, CF

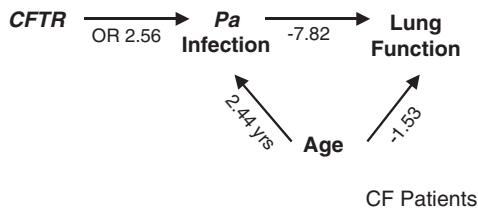


Figure 1 Model of the relationships between age, *CFTR*, *P. aeruginosa* (*Pa*) infection, and lung function in 788 cystic fibrosis (CF) patients. Significant associations between variables in a CF population are represented by arrows. The arrows indicate the assumed directionality of the effect, showing the 'exposure' variable having an effect on the 'outcome' variable. Values shown below each arrow quantify the effect and are explained fully in the text.

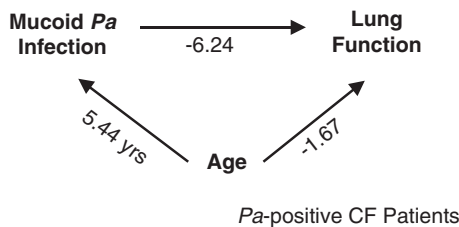


Figure 2 Model of cystic fibrosis (CF) pulmonary disease in 630 *P. aeruginosa* (*Pa*)-positive patients. Significant associations between variables in patients with at least one positive culture for *Pa* are represented by arrows. The arrows indicate the assumed directionality of the effect, showing the 'exposure' variable having an effect on the 'outcome' variable. Values shown below each arrow quantify the effect and are explained fully in the text.

patients infected with *Pa* have a decrease in lung function of 7.82 CF-specific percentile points on average when compared with uninfected patients once age has been corrected for ($P=0.0025$; Figure 1).

Modeling effects on lung disease in a *Pa*-positive CF population

The above model is relevant for *Pa* infection. However, many CF patients infected with *Pa* convert over time to a mucoid form. Conversion to mucoid *Pa* in patients infected with *Pa* is correlated with a significant decrease in lung function.⁵ To evaluate the role of *CFTR* and age at mucoid *Pa* conversion, we developed a second modeling framework specifically for CF patients infected with *Pa*. In CF patients infected with *Pa*, *CFTR* genotype did not associate with patient age, infection with mucoid *Pa*, or lung function. In pairwise analysis, CF patients infected with mucoid *Pa* are on average 5.44 years older than uninfected patients ($P \leq 0.0001$; Figure 2). Age was associated with lung function in patients infected with *Pa*. To address confounding attributed to patient age, we used the age-corrected FEV₁% predicted²¹ measurement when evaluating whether presence of mucoid *Pa* altered lung function. For every year increase in age, there is a decrease of 1.67 units of FEV₁% predicted ($P \leq 0.0001$). Further, conversion to mucoid *Pa* is associated with reduced lung function. CF patients infected with mucoid *Pa* have a decrease in lung function of 6.24 CF-specific percentile points on average when compared with uninfected patients once age has been corrected for ($P=0.0071$; Figure 2).

Incorporating MBL2 into models of CF pulmonary disease

Our primary objective was to establish the relationship between *MBL2* diplotype and CF disease in the context of the other variables evaluated so far. We used the models of CF pulmonary disease

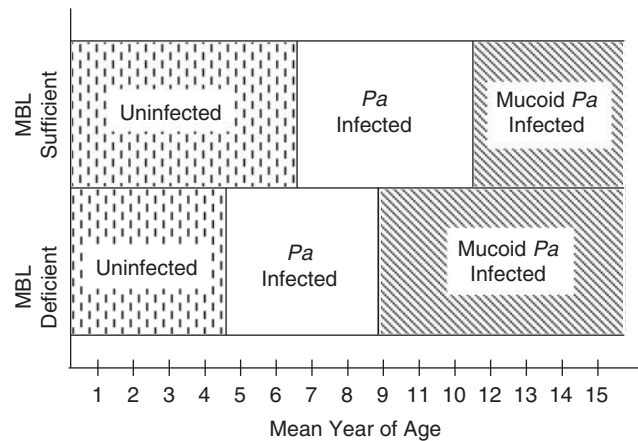


Figure 3 Effect of mannose-binding lectin 2 (*MBL2*) genotype groups on mean age of acquisition of *P. aeruginosa* (*Pa*) and conversion to mucoid *Pa* in 630 cystic fibrosis (CF) patients. MBL-deficient CF patients acquire *Pa* at a mean age of 4.67 ± 5.76 years of age compared with 6.61 ± 5.82 years of age for MBL-sufficient CF patients. MBL-deficient CF patients convert to mucoid *Pa* at a mean age of 8.90 ± 4.89 years of age compared with 11.62 ± 7.00 years of age for MBL-sufficient CF patients. The mean transition time between *Pa* and mucoid *Pa* is 4.58 ± 4.00 years for MBL-deficient CF patients and 4.98 ± 4.54 years for MBL-sufficient CF patients. The *P*-values are corrected for family structure but estimated means are not.

described above as a framework in which to analyze *MBL2* diplotypes for effect on survival, infection, and lung function. To identify potential confounders, we performed analyses in the same order as the CF pulmonary disease models.

Age

We first considered the relationship between *MBL2* genotype and age, given that earlier studies suggesting CF patients with *MBL2* genotypes corresponding to low levels of MBL have reduced survival.^{2,6} MBL-deficient CF patients in our study had a lower average age at their last PFT than MBL sufficient CF patients (2.25 years younger; $P=0.0014$).

Infection and age at infection

No association was found between *MBL2* genotype (sufficient/deficient) and *Pa* infection status. However, when we analyzed age at first positive *Pa* culture, MBL-deficient CF patients acquired *Pa* infection 1.94 years of age earlier than did MBL sufficient patients ($P=0.0034$). We then considered the association between *MBL2* genotype and mucoid *Pa* only in patients who were infected with *Pa*. *MBL2* was not associated with mucoid *Pa* status, but was associated with age at first positive mucoid *Pa* culture ($P=0.0003$). CF patients who are MBL deficient convert to mucoid *Pa* an average of 2.72 years of age earlier than MBL-sufficient patients. When analyzing the time between acquisition of *Pa* and conversion to mucoid *Pa*, there was no significant difference by *MBL2* genotype. We saw no difference in the relationship between age at infection and *MBL2* genotype when stratified by the *TGFB1* codon10 genotype. These results show that MBL-deficient patients both acquire *Pa* and convert to mucoid *Pa* approximately 2 years earlier than do MBL-sufficient patients (Figure 3).

Effect of shared environment

To determine whether cross-infection with a sibling infected with *Pa*^{34,35} confounded the effect of MBL deficiency, we analyzed only the first infected sibling from each family and evaluated for association with age at *Pa* infection, age at mucoid *Pa* infection, and the transition

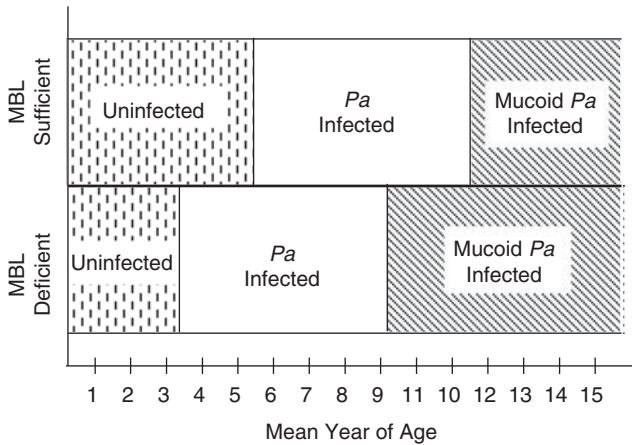


Figure 4 Effect of mannose-binding lectin 2 (*MBL2*) genotype groups on mean age of acquisition of *P. aeruginosa* (*Pa*) and conversion to mucoid *Pa* in 339 first infected cystic fibrosis (CF) siblings. MBL-deficient CF patients acquire *Pa* at a mean age of 3.43 ± 5.02 years of age compared with 5.52 ± 5.17 years of age for MBL-sufficient CF patients. MBL-deficient CF patients convert to mucoid *Pa* at a mean age of 9.27 ± 5.61 years of age compared with 11.25 ± 6.64 years of age for MBL-sufficient CF patients. The mean transition time between *Pa* and mucoid *Pa* is 5.83 ± 4.04 years for MBL-deficient CF patients and 5.64 ± 4.69 years for MBL-sufficient CF patients. None of the individuals in this analysis is related.

time between them. *MBL2* genotype was associated with age at *Pa* infection, with deficient individuals acquiring infection 2.08 years of age earlier than sufficient patients ($P=0.0067$; Figure 4). When analyzing *MBL2* genotypes and age at mucoid *Pa* infection, deficient patients showed a trend toward conversion to mucoid *Pa* earlier than did sufficient patients ($P=0.0982$). The transition time between *Pa* infection and conversion to mucoid *Pa* was not significantly different by *MBL2* genotype ($P=0.8214$). Thus, first infected siblings show the same association with age at *Pa* infection as do the entire cohort of CF siblings.

Pulmonary function measures

Our final step was to assess whether *MBL2* genotypes were associated with lung function in *Pa*-infected CF patients. Only infected patients were analyzed to avoid confounding by the relationship between *Pa* infection and lung function. We saw no association between *MBL2* and lung function (measured as FEV₁% predicted²¹ or as CF-specific percentile for FEV₁²²) in patients stratified by mucoid *Pa* status. Previous studies have suggested that the effect of *MBL2* on lung function occurs only in the adult and/or teenage years.^{2,7,11,12} To address this potential interaction with age, we stratified *Pa*-infected patients by mucoid *Pa* status if they were 15, 16, or 18 years or older. None of these separate age analyses showed association between *MBL2* genotype and lung function. Finally, we saw no difference in the relationship between lung function and *MBL2* genotype when stratified by *TGFB1* codon10 genotype.

DISCUSSION

To evaluate the role of *MBL2* in CF, we placed clinical variables underlying the progression of CF into modeling frameworks. The validity of the two models presented is supported by observations of similar relationships among the variables used here in other samples of the CF population. For example, it has been shown that increasing age correlates with decreasing lung function and increasing incidence of infection with *Pa*² as observed in our first modeling framework. In

addition, lung function has been shown to decrease at a faster rate after infection with *Pa*.^{36,37} A third example is the documented detrimental effect of mucoid conversion on lung function,^{5,38} which was replicated in our model of *Pa*-infected CF patients. This study of 630 *Pa*-infected patients had 86% power to detect association between *MBL2* genotypes and age at *Pa* infection.

Using the modeling frameworks, we observed a robust association between *MBL2* genotype and age at *Pa* infection. Correlation of low-producing *MBL2* genotypes with earlier age of infection is consistent with the findings of three earlier studies, including a recent study of 1019 patients reported by Dorfman *et al.*^{2,8,9} Although two other studies did not see a relationship between *MBL2* and age at *Pa* infection,^{11,13} it is possible that the conclusions drawn by the latter studies may have been due to low power to detect an association due to small sample size (162 and 116 *Pa*-infected patients^{11,13}). *MBL2* status did not modify *Pa* infection status or mucoid status in this study, consistent with the conclusions of earlier studies.^{2,9,11,12,14} This study is the first to show an association between *MBL2* variation and age of mucoid *Pa* conversion. We saw no effect of MBL status on the interval of time to transition between *Pa* infection and conversion to mucoid *Pa*. Furthermore, the rate of transition among patients was quite similar. Together, these findings suggest that MBL modifies age at *Pa* infection, but once infection has initiated, *Pa* advances to mucoid form at a rate relatively consistent among CF patients and independent of MBL status. Thus, the observed association between *MBL2* variants and age at conversion to mucoid *Pa* appears to be a consequence of the correlation between *MBL2* and initial *Pa* infection.

One important issue to address was the relationship between lung function measurements and *MBL2* genotype. Although association has been reported in seven studies,^{2,8-13} most observed association with lung function only after grouping the patients by infection status or age. For example, Garred *et al.*² and Trevisiol *et al.*⁹ saw a difference in lung function by *MBL2* genotype only in CF patients infected with *Pa* and Davies *et al.*¹¹ saw the association only in adults. Infection with *Pa* was associated with lower lung function in the first model (Figure 1). However, the lack of association between *MBL2* status and variation in lung function in this study is likely because of the identification and accounting of confounding factors (ie, infection status and age). Alternatively, a 2-year difference in age of acquisition of *Pa* infection may not result in statistically significant decreases in lung function for a number of years. Thus, our relatively young patient population (median age of our patients was 14.4 years compared with the 36.9 years median age of survival of the US CF population²⁰) may not have had sufficient time to manifest correlation between lung function and *MBL2* genotype. Indeed, in Davies *et al.*¹¹ the association of MBL with lung function was not seen in children with CF. A third possibility is that *MBL2* variation is not associated with variation in lung function, as shown in a well-powered study (Drumm *et al.*¹⁵ 808 patients) and two other studies.^{6,7}

In some studies, MBL deficiency has been associated with reduced survival.^{2,6} Although the design of our study did not allow assessment of the effect of *MBL2* on survival, *MBL2* genotypes corresponding to low levels of MBL were associated with a younger average age that would be consistent with a reduction in longevity. The work presented here, together with the bulk of the studies investigating MBL as a modifier of CF, strongly suggests that the primary effect of MBL deficiency is earlier *Pa* infection and earlier *Pa* conversion to mucoid status leading to reduced survival.

Although accounting for several important factors involved in CF infection and lung disease, our modeling approach is not exhaustive. Infections with other organisms, such as *B. cepacia*, and other

environmental modifiers, such as secondhand smoke or socio-economic status, have not been tested in this study. Extensive analysis by *CFTR* mutation variation has also not been performed. These variables will require a more complex version of the basic modeling framework shown in the paper and are worthy areas for future study. However, by establishing a basic modeling framework, we have created a foundation to test, quantify, and incorporate interactions among additional aspects of CF lung disease. Incorporating greater detail in the modeling frameworks should better reflect the interactions among these variables, thereby facilitating *a priori* hypotheses regarding modifier effects. Modeling frameworks such as those presented here may help reconcile inconsistent results generated by genetic association studies that evaluate different but correlated components of a complex phenotype.

CONFLICT OF INTEREST

GR Cutting is a consultant for Roche Molecular Systems, and received less than US\$1000 per year as consulting fees. M Grow and S Cheng are employed by Roche Molecular Systems.

ACKNOWLEDGEMENTS

We thank the North American Cystic Fibrosis Foundation for use of the Cystic Fibrosis Foundation Data Registry; Ase Sewall, Monica Brooks (both from Cystic Fibrosis Foundation), and the staff at the Data Registry; Nulang Wang for *CFTR* genotyping; Erica Senat for her assistance with *MBL2* genotyping; Michael Kulich (Charles University, Prague, Czech Republic), for providing conversion programs for cystic fibrosis-specific percentiles; Rita McWilliams, Julie Hoover-Fong, and Ada Hamosh, all from Johns Hopkins University, for designing questionnaires; and most importantly the patients with cystic fibrosis and their families, research coordinators, nurses, and physicians participating in the Cystic Fibrosis Twin and Sibling Study. This study was supported by the National Heart, Lung, and Blood Institute Grant HL68927. Genotyping reagents were provided by Roche Molecular Systems Inc (Pleasanton, CA, USA).

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