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Genetic determinants of C-reactive protein in chronic obstructive pulmonary disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is associated with a systemic inflammatory state, marked by elevations in serum inflammatory markers, including C-reactive protein (CRP). We sought to determine predictors of CRP levels, to estimate the genetic influence on CRP levels, and to identify genetic variants that affect CRP in a family-based study of COPD.

CRP was measured by a high-sensitivity assay in participants from the Boston Early-Onset COPD Study. Predictors of CRP level were determined using multilevel linear models. Variance component analysis was used to estimate heritability and to perform genome-wide linkage analysis for CRP levels. Two variants in surfactant protein B (*SFTPB*) were tested for association with CRP level.

Increased age, female sex, higher body mass index, greater pack-years of smoking and reduced forced expiratory volume in 1 second were all associated with increased CRP levels. There was a significant genetic influence on CRP (heritability = 0.25, p=0.00001). Genome-wide linkage analysis revealed several potentially interesting chromosomal regions, though no significant evidence for linkage was found. A short tandem repeat marker near *SFTPB* was significantly associated with CRP levels (p=0.007).

There is a genetic influence on CRP levels in COPD patients. Preliminary evidence suggests an association of *SFTPB* with systemic inflammation in COPD.

Keywords: C-reactive protein, emphysema, linkage analysis, smoking, surfactant proteins

Introduction

One of the hallmarks of chronic obstructive pulmonary disease (COPD) is the presence of inflammatory cells in the airways and in the lung parenchyma. Individuals with COPD also have evidence of a systemic inflammatory state. Several studies have demonstrated elevated levels of circulating inflammatory markers, including C-reactive protein (CRP) and fibrinogen, in patients with COPD.[1-3] For example, Mannino and colleagues found (geometric) mean CRP levels of 4.7 and 3.6 mg/L in subjects with severe and moderate COPD, respectively, compared to 2.7 mg/L in subjects without lung disease, among participants in the Third National Health and Nutrition Examination Survey (NHANES III).[2] In a meta-analysis, Gan and coworkers demonstrated a significant increase in CRP levels in COPD patients compared to controls (standardized mean difference = 0.53; 95% confidence interval: 0.34, 0.72).[4] Cigarette smoking by itself leads to systemic inflammation [5], but smoking and reduced lung function appeared to have independent effects on CRP levels in the NHANES III participants.[3]

Variation in CRP levels has been shown to have a significant genetic component in families from the general population [6], but the familial effect on CRP levels has not been investigated in COPD patients. In the Boston Early-Onset COPD Study, we examined predictors of CRP levels in a unique population of extended families ascertained through a proband with severe airflow obstruction at a young age. Using a family-based study design, we are able to demonstrate a significant heritable component to CRP levels and to perform a genome-wide linkage analysis for CRP levels in COPD patients. We also tested the association between CRP levels and a candidate gene,

surfactant protein B (*SFTPB*), located near one of the regions identified as interesting in the linkage analysis.

Methods

Study Subjects

Details of subject enrollment in the Boston Early-Onset COPD Study have been published.[7] Briefly, probands were aged 52 years or younger, with forced expiratory volume in 1 second (FEV_1) < 40% predicted, and without severe alpha 1-antitrypsin deficiency (e.g. PI Z, PI null-null). Probands were recruited primarily from the lung transplant and lung volume reduction surgery programs at Brigham and Women's Hospital and Massachusetts General Hospital (both in Boston), as well as from the pulmonary clinics at these hospitals and at the Brockton/West Roxbury Veterans Affairs Hospital. All available first-degree relatives, older second-degree relatives (aunts, uncles, grandparents), and other affected relatives were invited to participate. The present analysis included 585 subjects in 72 pedigrees as reported in the previous genome scan linkage analysis of COPD-related phenotypes [8,9]; three additional family members enrolled subsequently were also included in the epidemiological analysis. After providing written informed consent, subjects completed a study questionnaire, spirometry (pre- and post-bronchodilator), and provided blood samples in EDTA (for DNA extraction) and without anticoagulant (for serum). Pack-years of smoking was calculated as the product of smoking duration (in years) and average number of cigarettes per day, divided by 20 to convert to packs. Body mass index (BMI) was computed by dividing the self-reported weight (kilograms) by the square of the measured height (meters).

Smoking status, current medication usage, and doctor's diagnosis of "heart trouble" were determined by questionnaire. The study was approved by the Institutional Review Boards of Partners Healthcare.

Laboratory Methods

Serum CRP levels were measured using a high sensitivity assay (Denka Seiken, Japan).[10] As previously reported, genotyping of 377 autosomal short tandem repeat (STR) markers (average spacing 9.1 cM) was performed by the National Heart, Lung, and Blood Institute's Mammalian Genotyping Service, and pedigree and individual marker inconsistencies were resolved.[8,9] A single nucleotide polymorphism (SNP) in *SFTPB* (rs1130866, Thr131Ile) was genotyped with mini-sequencing reactions and mass spectrometry in Sequenom (San Diego, CA). For an STR near *SFTPB* (D2S388), fluorescent-labeled PCR product sizes were determined by capillary electrophoresis on an ABI 3100 machine (Applied Biosystems, Foster City, CA). Details of both assays are reported elsewhere.[11]

Statistical Analysis

The distribution of CRP levels had a rightward skew. Univariate and multivariate predictors of natural log (ln) transformed CRP levels were analyzed with multilevel models to account for familial clustering, using PROC MIXED in SAS (SAS Institute, Cary, NC). Potential predictors included demographic measures, anti-inflammatory medications (oral and inhaled corticosteroids, theophylline), and other variables known to affect CRP levels (BMI, smoking status). All univariate significant predictors ($p<0.05$)

were initially included in the multivariate models, including quadratic terms for continuous variables. Non-significant predictors were removed to achieve the most parsimonious model.

Narrow sense heritability was calculated using a variance component method, implemented in SOLAR, version 2.1.2.[12] Twopoint and multipoint linkage analysis was performed using the variance component approach in SOLAR, including an ascertainment correction for the single proband in each pedigree. Significant covariates from the multivariate model above were included as covariates in the linkage model. Stratified linkage analysis in smokers-only was performed by setting the CRP level to missing in lifelong non-smokers; a similar stratified analysis was performed in individuals with airflow obstruction (defined by FEV_1 <80% predicted, with FEV_1/FVC <90% predicted).

Data for *SFTPB* were analyzed with the extended pedigree family-based association test, using the software PBAT.[13] Markers were tested for association under the presumption of linkage, in models adjusted for relevant covariates.

Results

Predictors of CRP levels

Characteristics of included participants from the Boston Early-Onset COPD Study are listed in Table 1. Probands are predominantly female, as has been previously reported.[7] The majority of the cohort, including nearly all of the probands, reported a history of cigarette smoking.

In the univariate analyses, age was a significant predictor of CRP level (Table 2), as were body mass index and lung function, measured by the FEV_1. The effect of airflow obstruction (FEV_1 <80% predicted, with FEV_1/FVC <90% predicted) on CRP levels across the relationship categories is demonstrated in Figure 1. C-reactive protein levels were significantly higher in individuals with airflow obstruction among the siblings and among the older second degree relatives (aunts, uncles, grandparents), compared to individuals without airflow obstruction. Pack-years of cigarette smoking and history of ever-smoking were both significant predictors in univariate analyses, but current smoking status was not. Both female sex and questionnaire report of a doctor's diagnosis of "heart trouble" predicted a higher CRP level.

Current use of each of the three anti-inflammatory medications commonly employed in the treatment of COPD – inhaled corticosteroids, theophylline, and oral corticosteroids – was significantly associated with an increased CRP level. However, these paradoxical effects are likely explained by disease status (i.e. confounding by indication). In a model that controlled for FEV_1 as a marker of disease severity, lnCRP levels were significantly lower in current users of inhaled steroids ($\beta = -0.45 \pm 0.16$, $p=0.006$). In similar models, CRP levels were non-significantly lower in current users of theophylline and prednisone.

The multivariate model of CRP level is shown in Table 3. Age, body mass index (and BMI^2), and FEV_1 (% predicted) remained strong predictors of CRP level. Female sex continued to be associated with elevated CRP levels. The effect of pack-years of smoking was attenuated, likely because of the strong correlation with FEV_1 (Pearson $r = -0.48$, $p<0.0001$), but still remained significant. The effect of pack-years of smoking was

stronger in a model that excluded adjustment for FEV_1 (β=0.0094 ± 0.002, p<0.0001). In a model including FEV_1, in which smoking status was represented by ever-smoking status instead of pack-years, smoking was no longer significant. Ever-smoking status was significant when FEV_1 was excluded from the model (β=0.39 ± 0.11, p=0.0005).

Heritability and Linkage Analysis

When adjusted for the covariates in Table 3, estimated heritability of CRP levels was significant in the Boston Early-Onset COPD families (h^2_N = 0.25 ± 0.07, p=0.00001). The covariates in the final model – age, sex, BMI (and BMI^2), pack-years, and FEV_1 (% predicted) -- explained 36% of the trait variance.

In the genome-wide linkage analysis in all subjects, regions on chromosomes 2 and 7 had LOD (logarithm of the odds) scores greater than 1 (Table 4 and Figure 2). In no region did the LOD scores represent significant or even suggestive evidence of linkage.[14] In regions on chromosomes 2 and 21, the LOD scores increased in the linkage analysis of smokers-only, potentially implying the presence of a gene-by-environment interaction; in neither region was there significant or suggestive linkage evidence. The LOD score for chromosome 21q was also increased in an analysis limited to subjects with airflow obstruction, though not to the same degree as in the smokers-only analysis.

Association Analysis

The *SFTPB* gene is located on chromosome 2, near the linkage peak at 136 cM (Table 4), though this was not the highest linkage peak in our study. None of the other

commonly studied COPD candidate genes was located near regions with higher LOD scores for CRP linkage.[11] We had previously reported that a coding SNP in *SFTPB* (Thr131Ile) was associated with moderate-to-severe airflow obstruction (FEV_1 < 60% predicted, with FEV_1/FVC < 90% predicted) in the Boston Early-Onset COPD Study; an STR marker (D2S388) located near *SFTPB* (at 108 cM) was not associated with spirometric phenotypes.[11] The most common allele of this STR (263 bp, frequency = 0.39) was associated with lnCRP levels in the families (p=0.007), in an additive model that included adjustment for age, sex, BMI (and BMI^2), pack-years of smoking, and FEV_1 (% predicted). None of the other alleles were significantly associated with CRP level. The effect of the 263bp allele appeared to be recessive (Figure 3), and a recessive model showed stronger evidence for association (p=0.0004). The coding SNP in *SFTPB* (Thr131Ile) was not associated with CRP levels in the families under an additive model, but showed a trend for association (p=0.06) when analyzed in a dominant model.

Of note, the genotype completion rate for the *SFTPB* STR was 77%, lower than in our previous study [11]; however, there were only 2 pedigree inconsistencies among subjects in the current analysis. Completion rate for the *SFTPB* SNP was 96%. Both markers were in Hardy-Weinberg equilibrium in the founders.

Discussion

In a family-based study of COPD, we found that several predictors of C-reactive protein levels in the general population, including age and body mass index, also affect CRP levels in COPD families. We confirmed the association between reduced lung

function and elevated CRP levels that has been demonstrated by several authors.[2,4] However, several findings in our cohort are different than previous studies. Pack-years of cigarettes smoked were significant predictors of CRP level, but current smoking status was not. The effect of female sex on CRP level has not been consistently noted in other cohorts. We found that the variation in CRP levels has a significant familial component, which has been shown in families without COPD, but we did not find significant evidence for genetic linkage to any specific chromosomal regions. However, a variant in a candidate gene, surfactant protein B, located near one of the regions of nominal linkage, was associated with CRP levels.

Studies in the general population have demonstrated significant effects of cigarette smoking on CRP levels. In most studies, current smokers have the highest CRP levels, with former smokers also demonstrating elevations compared to never-smokers.[5,15] However, we did not find current smoking status to be a significant predictor of CRP levels in our cohort; ever-smoking status was significant in the univariate analysis, but not in the multivariate model. Studies have suggested that the inflammatory response in the airways in severe COPD patients may persist even after smoking cessation.[16] It is possible that the systemic inflammatory state persists as well, which could explain the lack of effect of current smoking – in contrast to the effect of lifetime smoking (pack-years) – on CRP levels in our cohort.

The fact that CRP levels were higher in women than in men is an interesting finding in our study. Several general population studies have failed to show a sex-related difference in CRP levels [10,17], though a recent analysis of the NHANES data found higher levels in women.[18] Probands in the Boston Early-Onset COPD Study have been

predominantly female, which suggests that women may have a greater risk of developing severe early-onset COPD, though the female predominance has not yet been fully explained.[7] A heightened inflammatory response to cigarette smoke, both in the airways and in the systemic circulation, may reflect the increased COPD risk in a subset of women. The general population studies that did not find a sex-related effect on CRP levels [10,17] have excluded post-menopausal women on hormone replacement therapy (HRT), a factor known to raise CRP levels. We did not have information on menopausal status or HRT in our cohort, so we cannot exclude the possibility that the sex-related increase in CRP could be due to these factors.

Twin studies and pedigree studies in families not ascertained due to COPD have demonstrated significant heritability of CRP levels.[6,19-21] Heritability has been estimated to be approximately 40% in families from the general population participating in the Family Heart Study [6] and in families ascertained through a proband with hypertension.[19] We calculated a lower, but still significant, heritability in our study; 25% of the variability of CRP levels could be explained by genetic factors in the Boston Early-Onset COPD Study families. Environmental factors may have a larger role in systemic inflammation in COPD than in systemic inflammation in the general population or in patients with cardiovascular disease, leading to the slightly lower heritability estimate in the Boston Early-Onset COPD Study.

Differences in study populations may also explain why we did not detect significant evidence of linkage with the chromosomal regions that contain genes that have been associated with CRP levels, such as the *CRP* gene on chromosome 1 [22] and Interleukin (IL)-6 on chromosome 7.[19] We found significant association with

surfactant protein B, a gene not previously associated with CRP levels. These findings will need to be replicated in other cohorts of COPD patients. In a previous analysis of the Boston Early-Onset COPD study and in a case-control study of COPD, we found a coding variant in *SFTPB* to be associated with COPD and related spirometric phenotypes.[11] Other groups have also demonstrated association with *SFTPB* and COPD.[23,24] Because of these previous COPD associations, as well as the fact that *SFTPB* is located near one of the nominal linkage peaks, we chose to test *SFTPB* variants for association with CRP levels. In an animal model, reduction in surfactant protein B levels in adult mice has been shown to cause increased concentrations of the inflammatory cytokines IL-6 and IL-1 beta, as well as increased numbers of inflammatory cells in the lung.[25] Variation in surfactant protein B levels, possibly due to genetic polymorphisms in the *SFTPB* gene, may modify local and systemic inflammation in COPD.

In the present study, only one of two *SFTPB* markers tested was significantly associated with CRP. This does not imply that the D2S388 STR is a functional variant. Linkage disequilibrium with variants in another gene (or genes) may explain the association results. The *IL-1* gene cluster (IL-1 alpha, IL-1 beta, IL-1 receptor antagonist) is located on chromosome 2, closer to the region of linkage than is *SFTPB*. Polymorphisms in genes in the *IL-1* cluster have also been reported to be associated with CRP levels.[26,27]

Dupuis and colleagues have recently published a genome scan analysis of CRP and other vascular inflammatory markers in 1054 individuals from 304 families participating in the Framingham Heart Study.[28] This is the first genome-wide linkage

study of CRP. They estimated the heritability of CRP levels to be 28.2%, similar to our results. And despite the larger sample size, they were not able to demonstrate significant or even suggestive evidence of linkage for CRP levels. The highest LOD score was 1.58, on chromosome 14q. Despite the confirmation of a genetic effect on CRP levels in the Framingham cohort and in our study, CRP levels may be influenced by many other factors. When adjustment for these covariates is not fully adequate, the ability to detect significant linkage will be reduced.

Other diseases, such as diabetes mellitus and cardiovascular disease, are known to be associated with higher CRP levels. In our study of early-onset COPD, we did not collect information on these comorbidities. Therefore, we cannot exclude the possibility of residual confounding in our analysis. A self-report of a doctor's diagnosis of "heart trouble" was not significant in the multivariate model, though this is an imprecise measure of cardiac disease. In the Boston Early-Onset COPD Study, body weight was self-reported. However, multiple studies have found self-reported body weight to be highly correlated with measured weight, and therefore suitable for epidemiology studies.[29]

High sensitivity CRP has been shown to be stable over serial measurements in healthy individuals.[30] Since CRP is an acute phase reactant, its levels may rise during COPD exacerbations.[31] We tried to postpone enrollment of COPD patients who were in the midst of or recovering from exacerbations. Any residual confounding by recent infections may reduce our ability to detect genetic influences. The limited sample size of our study may also reduce the power to find linkage for CRP levels, explaining our lack of significant or even suggestive linkage results. However, in previous linkage analyses

in the Boston Early-Onset COPD Study, LOD scores that represented genome-wide significance have been found for quantitative COPD-related traits.[9] Association testing is more powerful than linkage analysis, so the significant association result for *SFTPB* in the absence of significant linkage is not inconsistent. However, replication of the *SFTPB* association is required to confirm that this is not a false-positive finding.

Probands in the Boston Early-Onset COPD Study represent an extreme COPD phenotype, possibly enriched for genetic susceptibility for COPD. One must be cautious when generalizing findings from this study to patients with later-onset, less severe COPD. However, other studies in older COPD populations have found similar associations of CRP with clinical factors, such as age, sex, FEV_1, BMI, and smoking.[2,3,32] Although the probands in the Boston Early-Onset COPD Study were young, many of their affected relatives had COPD at later ages. In addition, genetic associations for COPD susceptibility found in the Boston Early-Onset COPD Study have been replicated in older patients with severe COPD from the National Emphysema Treatment Trial.[33,34] It is possible that other genetic associations (including associations with CRP levels) may be applicable to other COPD patients.

In a family-based study, we demonstrated significant heritability of CRP level and found important effects of female sex and lifetime smoking on this marker of systemic inflammation. Genome-wide linkage analysis did not reveal any chromosomal regions that were significantly linked to CRP levels, yet several regions had LOD scores greater than 1, including regions possibly influenced by gene-environment (smoking) interactions. A candidate gene, *SFTPB*, showed preliminary evidence for association. CRP levels in the general population and in COPD patients in particular are likely to be

influenced by multiple genetic and environmental factors. Candidate gene studies have found several positive associations with CRP levels in the general population; the only other reported genome-wide linkage analysis has also not been able to demonstrate significant evidence for linkage. Further candidate gene studies may help uncover determinants of systemic inflammation in COPD patients, but a more systematic approach, such as a genome-wide association study, may be required to better understand the multiple genetic effects on systemic inflammation in COPD.

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Figure Legends

Figure 1: Effect of airflow obstruction on C-reactive protein levels in participants in the Boston Early-Onset COPD Study. Presence of airflow obstruction is defined by FEV_1 <80% predicted with FEV_1/FVC <90% predicted. Geometric means (+ SEM) are shown. *p<0.05 compared to subjects without airflow obstruction.

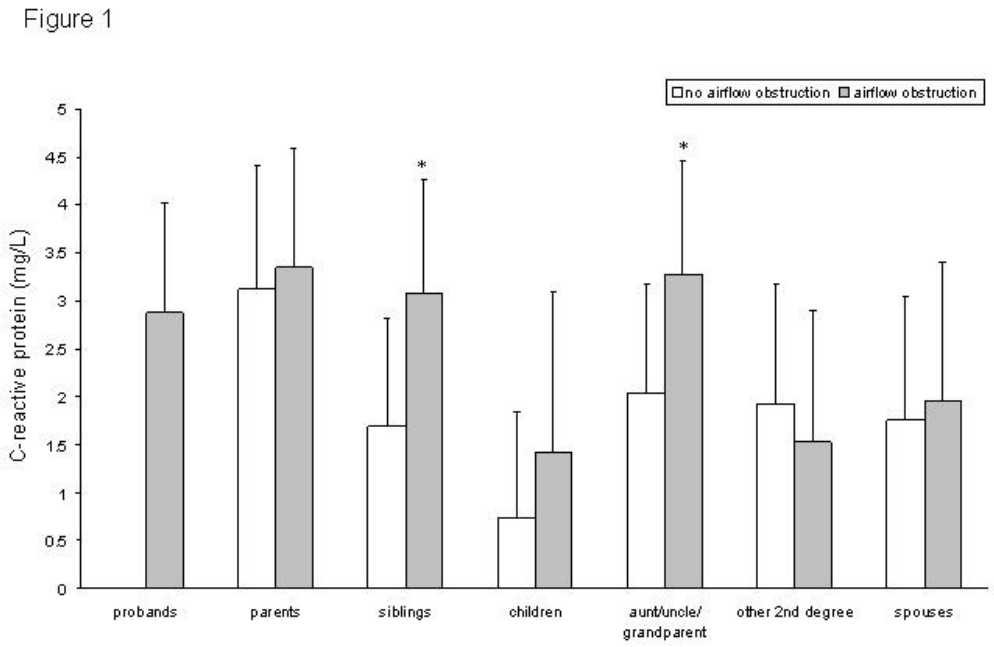

Figure 2: Genome-wide linkage analysis of C-reactive protein level (log-transformed) in the Boston Early-Onset COPD Study, in all subjects and in smokers only.

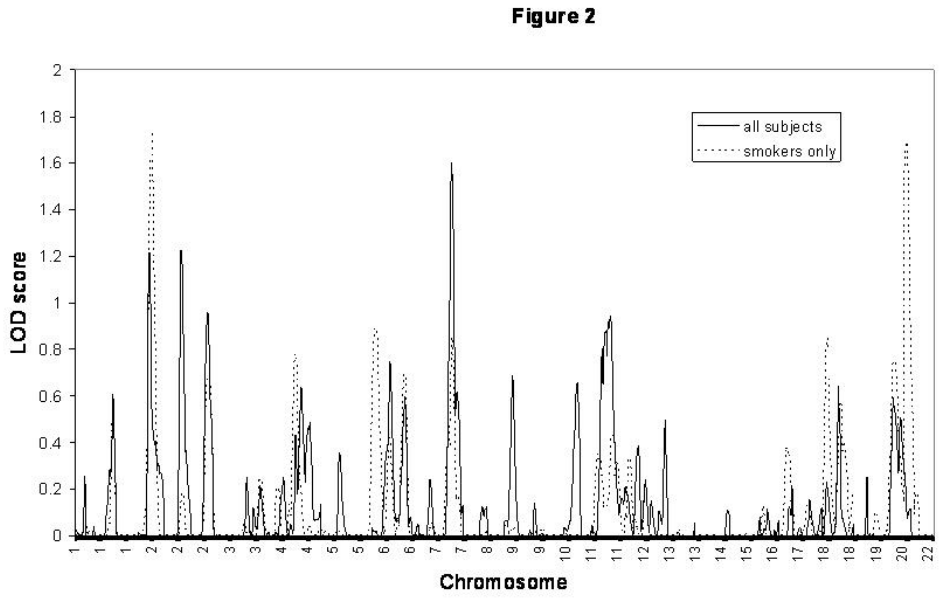

Figure 3: C-reactive protein levels in the Boston Early-Onset COPD Study subjects with 0, 1, or 2 copies of the 263 bp allele of the surfactant protein B short tandem repeat marker (D2S388). Geometric means (+ SEM) are shown. *p=0.0004 for recessive model.

Figure 3

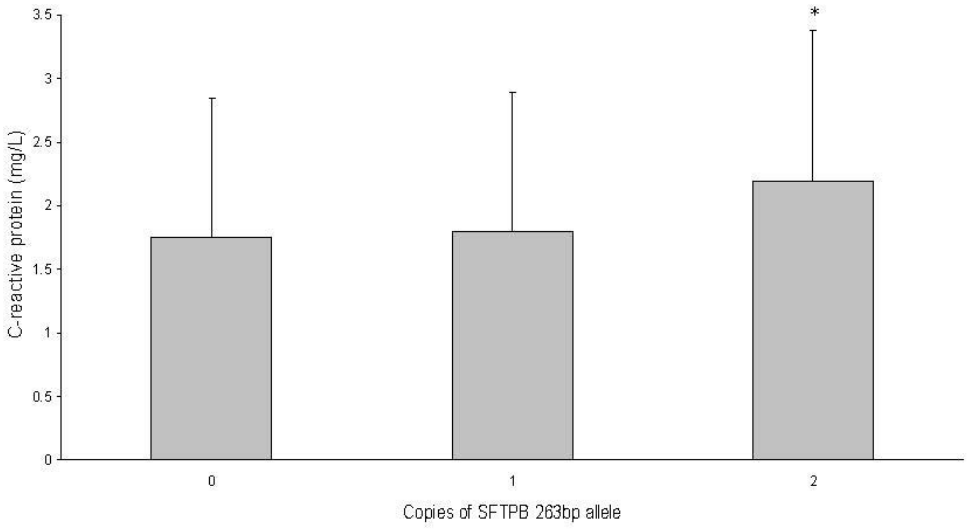
3.5
3
2.5
2
1.5
1
0.5
0
C-reactive protein (mg/L)
*
0
1
2
Copies of SFTPB 263bp allele

Table 1: Characteristics of participants in the Boston Early-Onset COPD Study.

Mean (± SD) or N (%). CRP level is reported as its geometric mean (± SD).

Characteristic	**Probands**	**Family members**
N	72	516
Age, years	47.7 (±5.3)	46.5 (±18.5)
Female sex	54 (75.0%)	276 (53.5%)
FEV_1, % predicted	17.4 (±6.4)	83.0 (±20.8)†
BMI, kg/m^2	24.4 (±5.9)*	27.1 (±5.8)†
Pack-years of cigarette smoking	38.9 (±21.6)	20.5 (±25.7)
Ever smoker	69 (95.8%)	342 (66.3%)
Current smoker	7 (9.7%)	168 (32.6%)
Doctor diagnosed "heart trouble"	10 (13.9%)	86 (16.7%)†
Inhaled corticosteroid use, current	61 (84.7%)	28 (5.5%)†
Oral corticosteroid use, current	26 (36.1%)	6 (1.2%)†
Theophylline use, current	38 (52.8%)	11 (2.2%)†
CRP, mg/L	2.87 (±3.29)*	1.71 (±3.61)†

*N<72 due to missing data.
†N<516 due to missing data.

Table 2: Univariate predictors of C-reactive protein levels (log-transformed) in Boston Early-Onset COPD Study participants

Predictor	**β (SE)**	**p-value**
Continuous		
Age, years	0.026 (0.0034)	<0.0001
FEV_1, % predicted	-0.013 (0.0019)	<0.0001
BMI, kg/m^2	0.096 (0.0080)	<0.0001
Pack-years of cigarette smoking	0.014 (0.0018)	<0.0001
Categorical		
Female sex	0.37 (0.095)	0.0001
Ever-smoker	0.51 (0.12)	<0.0001
Current-smoker	-0.044 (0.10)	0.67
Doctor diagnosed "heart trouble"	0.33 (0.13)	0.01
Inhaled corticosteroid use, current	0.48 (0.12)	<0.0001
Oral corticosteroid use, current	0.56 (0.21)	0.009
Theophylline use, current	0.71 (0.16)	<0.0001

Table 3: Multivariate predictors of C-reactive protein levels (log-transformed) in Boston Early-Onset COPD Study participants

Predictor	**β (SE)**	**p-value**
Age, years	0.011 (0.003)	0.001
Female sex	0.40 (0.08)	<0.0001
BMI, kg/m^2	0.11 (0.009)	<0.0001
$(BMI)^2$	-0.002 (0.0007)	0.007
Pack-years of smoking	0.0044 (0.002)	0.03
FEV_1, % predicted	-0.010 (0.002)	<0.0001

Table 4: Results of genome-wide linkage analysis for lnCRP levels in the Boston Early-Onset COPD Study families. Regions with LOD (logarithm of the odds) score >1 in either analysis are shown. Chromosomal locations are in Kosambi map units.

	All subjects		Smokers only		Subjects with airflow obstruction*	
Chromosome	**LOD score**	**Location**	**LOD score**	**Location**	**LOD score**	**Location**
2p	1.21	8 cM	1.74	16 cM	0.16	16 cM
2q	1.22	136 cM	0.19	143 cM	0.07	125 cM
7q	1.60	136 cM	0.85	137 cM	0.98	115 cM
21q	0.27	3 cM	1.69	8 cM	1.38	3 cM

*Airflow obstruction defined by FEV_1 <80% predicted, with FEV_1/FVC <90% predicted.