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Genetic determinants of C-reactive protein in COPD

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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). The present study sought to determine epidemiological 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 the surfactant protein B (*SFTPB*) gene were tested for association with CRP levels.

Increased age, female sex, higher body mass index, greater smoking pack-yrs and reduced forced expiratory volume in one second were all associated with increased CRP levels. There was a significant genetic influence on CRP (heritability=0.25). 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.

There is a genetic influence on C-reactive protein levels in chronic obstructive pulmonary disease patients. Preliminary evidence suggests an association of the surfactant protein B gene with systemic inflammation in chronic obstructive pulmonary disease.

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

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 *et al.* [2] found (geometric) mean CRP levels of 4.7 and 3.6 $mg \cdot L^{-1}$ in subjects with severe and moderate COPD, respectively, compared with 2.7 $mg \cdot L^{-1}$ in subjects without lung disease, among participants in the Third National Health and Nutrition Examination Survey (NHANES III). In a meta-analysis, GAN *et al.* [4] demonstrated a significant increase in CRP levels in COPD patients compared with controls (standardised 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, the present authors examined predictors of CRP levels in a unique population of extended families ascertained through a proband with severe chronic airflow obstruction at a young age. A family-based study design enabled the current authors to demonstrate a significant heritable component to CRP levels and to perform a genome-wide linkage analysis for CRP levels in COPD patients. The association between CRP levels and variants in a candidate gene, surfactant protein B (*SFTPB*), located near one of the regions identified as interesting in the linkage analysis was also investigated.

METHODS

Study subjects

Details of subject enrolment in the Boston Early-Onset COPD Study have been published previously [7]. Briefly, probands were aged ⩽52 yrs,

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with forced expiratory volume in one second (FEV_1) <40% predicted, and without severe α1-antitrypsin deficiency (*e.g.* proteinase inhibitor (PI) Z, PI null-null). Probands were recruited primarily from the lung transplant and lung volume reduction surgery programmes at Brigham and Women's Hospital and Massachusetts General Hospital (both in Boston, MA, USA), 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 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). Smoking pack-yrs were calculated as the product of smoking duration (in yrs) 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 (kg) by the square of the measured height (m). 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, Tokyo, 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, USA). For an STR near *SFTPB* (D2S388), fluorescently labelled PCR product sizes were determined by capillary electrophoresis on an ABI 3100 machine (Applied Biosystems, Foster City, CA, USA). Details of both assays are reported elsewhere [11].

Statistical analysis

The distribution of CRP levels had a rightward skew. Univariate and multivariate epidemiological predictors of natural log (ln) transformed CRP levels were analysed with multilevel models to account for familial clustering. Potential predictors included demographic measures, anti-inflammatory medications (oral and inhaled corticosteroids, theophylline), and other variables reported 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. Nonsignificant predictors were removed to achieve the most parsimonious model.

Narrow sense heritability was calculated using a variance component method [12]. Two-point and multipoint linkage analysis was performed using a variance component approach, 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 nonsmokers; a similar stratified analysis was performed in individuals with airflow obstruction (defined by FEV_1 <80% pred, with FEV_1/forced vital capacity (FVC) <90% pred).

Data for *SFTPB* were analysed with the extended pedigree family-based association test [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 BMI and FEV_1. The effect of airflow obstruction (FEV_1 <80% pred, with FEV_1/FVC <90% pred) on CRP levels across the relationship categories is demonstrated in fig. 1. Among siblings and older second degree relatives (aunts, uncles, grandparents), CRP levels were significantly higher in individuals with airflow obstruction compared with individuals without airflow obstruction. Smoking pack-yrs and history of ever-smoking were both significant predictors in univariate analyses, but current

TABLE 1 Characteristics of participants in the Boston Early-Onset Chronic Obstructive Pulmonary Disease Study

Characteristics	Probands	Family members
Subjects	72	516
Age yrs	47.7±5.3	46.5±18.5
Female sex	54 (75.0)	276 (53.5)
FEV_1 % pred	17.4±6.4	83.0±20.8¶
BMI kg·m^{-2}	24.4±5.9+	27.1±5.8¶
Smoking pack-yrs	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)¶
Current corticosteroid use		
Inhaled	61 (84.7)	28 (5.5)¶
Oral	26 (36.1)	6 (1.2)¶
Current theophylline use	38 (52.8)	11 (2.2)¶
CRP mg·L^{-1}#	2.87±3.29+	1.71±3.61¶

Data are presented as n, mean±SD or n (%). FEV_1: forced expiratory volume in one second; % pred: % predicted; BMI: body mass index; CRP: C-reactive protein. #: CRP level reported as geometric mean (±SD); ¶: n<516; +: n<72.

TABLE 2 Univariate predictors of C-reactive protein levels (log-transformed) in Boston Early-Onset Chronic Obstructive Pulmonary Disease Study participants

Predictor	β	p-value
Continuous		
Age yrs	0.026±0.0034	<0.0001
FEV1 % pred	-0.013±0.0019	<0.0001
BMI kg·m^{-2}	0.096±0.0080	<0.0001
Smoking pack-yrs	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
Current corticosteroid use		
Inhaled	0.48±0.12	<0.0001
Oral	0.56±0.21	0.009
Current theophylline use	0.71±0.16	<0.0001

Data are presented as mean±SE unless otherwise stated. FEV1: forced expiratory volume in one second; % pred: % predicted; BMI: body mass index.

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 to be explained by disease status (*i.e.* confounding by indication). In a model that controlled for FEV1 as a marker of disease severity, lnCRP levels were significantly lower in current users of inhaled steroids (β= -0.45±0.16, p=0.006). In similar models, CRP levels were not significantly lower in current users of theophylline and prednisone.

The multivariate model of CRP level is shown in table 3. Age, BMI, BMI^2 and FEV1 % pred remained strong predictors of CRP levels. Female sex continued to be associated with elevated CRP levels. The effect of smoking pack-yrs was attenuated, most likely because of the strong correlation with FEV1 (Pearson r=-0.48, p<0.0001), but remained significant. The effect of smoking pack-yrs was stronger in a model that excluded adjustment for FEV1 (β=0.0094±0.002, p<0.0001). In a model including FEV1, 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 FEV1 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 study families (h^2_N=0.25±0.07, p=0.00001). The covariates in the final model, age, sex, BMI, BMI^2 (pack-yrs; and FEV1 % pred) explained 36% of the trait variance.

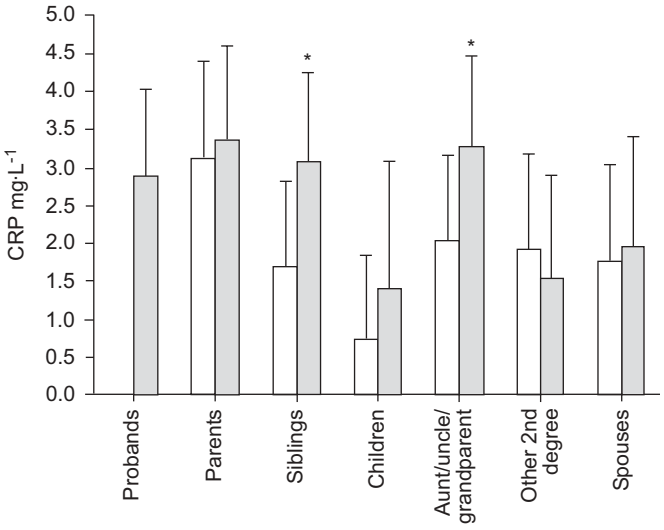

FIGURE 1. Effect of airflow obstruction on C-reactive protein (CRP) levels in participants in the Boston Early-Onset Chronic Obstructive Pulmonary Disease Study. Presence of airflow obstruction (■) is defined by forced expiratory volume in one second (FEV1) <80% predicted with FEV1/forced vital capacity <90% pred. Data are presented as geometric mean+SEM. *: p<0.05 compared with subjects without airflow obstruction (□).

In the genome-wide linkage analysis in all subjects, regions on chromosomes 2 and 7 had logarithm of the odds (LOD) scores >1 (table 4 and fig. 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 the present study. None of the other commonly

TABLE 3 Multivariate predictors of C-reactive protein levels (log-transformed) in Boston Early-Onset Chronic Obstructive Pulmonary Disease Study participants

Predictor	β	p-value
Age yrs	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
Smoking pack-yrs	0.0044±0.002	0.03
FEV1 % pred	-0.010±0.002	<0.0001

Data are presented as mean±SE unless otherwise stated. BMI: body mass index; FEV1: forced expiratory volume in one second; % pred: % predicted.

TABLE 4 Results of genome-wide linkage analysis for C-reactive protein levels (log-transformed) in the Boston Early-Onset Chronic Obstructive Pulmonary Disease Study families

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

Regions with LOD (logarithm of the odds) score >1 in either analysis are shown. Chromosomal locations are in Kosambi map units. #: airflow obstruction defined by forced expiratory volume in one second (FEV_1) <80% predicted, with FEV_1/forced vital capacity <90% pred.

studied COPD candidate genes was located near regions with higher LOD scores for CRP linkage [11]. The present authors have previously reported that a coding SNP in *SFTPB* (Thr131Ile) was associated with moderate-to-severe airflow obstruction (FEV_1 <60% pred with FEV_1/FVC < 90% pred) 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, BMI^2, smoking pack-yrs and FEV_1 % pred. None of the other alleles were significantly associated with CRP levels. The effect of the 263 bp allele appeared to be recessive (fig. 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 analysed in a dominant model.

Of note, the genotype completion rate for the *SFTPB* STR was 77%, which is lower than in the present authors' previous study [11]; however, there were only two 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, the present authors found that several predictors of CRP levels in the general population, including age and BMI, also affect CRP levels in COPD families. Furthermore, the association between reduced lung function and elevated CRP levels that has been demonstrated by several authors [2, 4] was confirmed. However, several findings in the present cohort differ with previous studies. Smoking pack-yrs 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. The present authors found that the variation in CRP levels has a significant familial component, which has been shown in families without COPD, but significant evidence for genetic linkage to any specific chromosomal regions was not found. However, a variant in a candidate gene, *SFTPB*, located near one of the regions of nominal linkage, was associated with CRP levels.

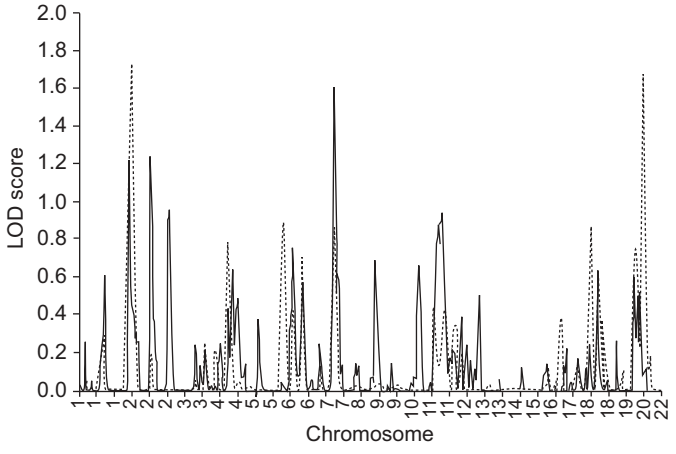

FIGURE 2. Genome-wide linkage analysis of C-reactive protein level (log-transformed) in the Boston Early-Onset Chronic Obstructive Pulmonary Disease Study, in all subjects (——) and in smokers only (·····). LOD: logarithm of the odds.

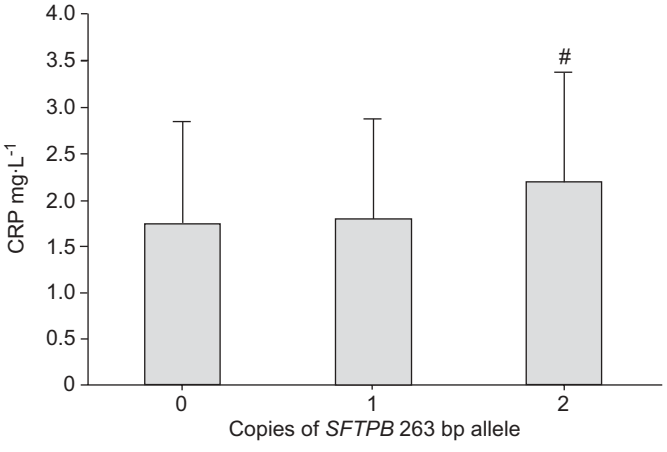

FIGURE 3. C-reactive protein (CRP) levels in the Boston Early-Onset Chronic Obstructive Pulmonary Disease Study subjects with 0, 1 or 2 copies of the 263 bp allele of the surfactant protein B short tandem repeat marker (D2S388). Data are expressed as geometric mean+SE. #: p=0.0004 for recessive model.

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 with never-smokers [5, 15]. However, current smoking status was not found to be a significant predictor of CRP levels in the present 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 also persists, which could explain the lack of effect of current smoking, in contrast to the effect of lifetime smoking (pack-yrs), on CRP levels in the present cohort.

The fact that CRP levels were higher in females than in males is an interesting finding in the current study. Several general population studies have failed to show a sex-related difference in CRP levels [10, 17], although a recent analysis of the NHANES data found higher levels in females [18]. Probands in the Boston Early-Onset COPD Study have been predominantly female, which suggests that females 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 females. The general population studies that did not find a sex-related effect on CRP levels [10, 17] have excluded post-menopausal females on hormone replacement therapy (HRT), a factor known to raise CRP levels. Information on menopausal status or HRT was not available for the current cohort, so the possibility that the sex-related increase in CRP could be due to these factors cannot be excluded.

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 ~40% in families from the general population participating in the Family Heart Study [6] and in families ascertained through a proband with hypertension [19]. A lower, but still significant, heritability was calculated in the present study, with 25% of the variability of CRP levels attributable to 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 significant evidence of linkage was not detected with the chromosomal regions that contain genes that have been associated with CRP levels, such as the *CRP* gene on chromosome 1 [22] and the interleukin (IL)-6 gene on chromosome 7 [19]. Significant association was found with *SFTPB*, 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, a coding variant in *SFTPB* was found to be associated with COPD and related spirometric phenotypes [11]. Other groups have also demonstrated association with *SFTPB* and COPD [23, 24]. Owing to these previous COPD associations, as well as the fact that *SFTPB* is located near one of the nominal linkage peaks, *SFTPB* variants were chosen to test 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-1β, 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-1α, IL-1β and the 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 *et al*. [28] have recently published a genome scan analysis of CRP and other vascular inflammatory markers in 1,054 individuals from 304 families participating in the Framingham Heart Study [28], which represents the first genome-wide linkage study of CRP. They estimated the heritability of CRP levels to be 28.2%, similar to the present 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 the current 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 the present authors' study of early-onset COPD, information on these comorbidities was not collected. Therefore, the possibility of residual confounding in the analysis cannot be excluded. A self-report of a doctor's diagnosis of ''heart trouble'' was not significant in the multivariate model, although 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]. Where possible, enrolment of COPD patients who were in the midst of or recovering from exacerbations was postponed as any residual confounding by recent infections may reduce the ability to detect genetic influences. The limited sample size of the present study may also reduce the power to find linkage for CRP levels, explaining the 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 subgroup, possibly enriched for genetic susceptibility for COPD. Caution is necessary when generalising 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) are applicable to other COPD patients.

In a family-based study, the significant heritability of CRP levels was demonstrated with 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 >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 chronic obstructive pulmonary disease 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 chronic obstructive pulmonary disease.

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