



# CTLA4 gene polymorphisms are associated with chronic bronchitis

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**ABSTRACT:** Chronic obstructive pulmonary disease (COPD) is characterised by chronic and progressive dyspnoea, cough and sputum production. T-lymphocytes may play a key role in the pathogenesis of COPD and chronic bronchitis. Cytotoxic T-lymphocyte antigen (CTLA) 4 is a potential candidate gene because it modulates T-cell activation.

Genetic association between nine *CTLA4* single nucleotide polymorphisms (SNPs) and chronic bronchitis was assessed in 606 pedigrees (1,896 individuals) from the International COPD Genetics Network (ICGN) population. We then replicated the associations in 342 COPD subjects with chronic bronchitis and 511 COPD subjects without chronic bronchitis from Bergen, Norway. Family-based association tests were used to analyse the ICGN cohort, and a logistic regression model was used for the Bergen cohort.

Six *CTLA4* SNPs were significantly associated with chronic bronchitis in the ICGN cohort ( $0.0079 \leq p \leq 0.0432$ ), with three being replicated with the same directionality of association in the Bergen cohort ( $0.0325 \leq p \leq 0.0408$ ). One of these replicated SNPs (rs231775) encodes the Thr to Ala substitution at amino acid position 17. Haplotype analyses supported the results of single SNP analyses.

Thus, *CTLA4* is likely to be a genetic determinant of chronic bronchitis among COPD cases.

**KEYWORDS:** Chronic bronchitis, chronic obstructive pulmonary disease, genetic association, replication, smoking, tobacco

Chronic obstructive pulmonary disease (COPD), one of the leading causes of death in developed countries, is characterised by progressive and poorly reversible airflow limitation [1]. Cigarette smoking is the main environmental risk factor for COPD, but only a minority of individuals who smoke cigarettes develop the disease [2, 3]. Twin studies and familial aggregation studies demonstrated that a genetic component is likely to be an important determinant of COPD susceptibility [4–8]. Genome-wide linkage analysis of the Boston Early-Onset COPD Study families showed a significant linkage peak for the airflow limitation on chromosome 2q [9–11]. Linkage to this same region of chromosome 2q to airflow limitation in general population pedigrees has also been demonstrated [12].

The chronic airflow limitation that is characteristic of COPD is caused by a mixture of small airway disease and emphysema and is often accompanied by chronic bronchitis [1]. The

activation and proliferation of T-lymphocytes is likely to be involved in the pathogenesis of COPD and chronic bronchitis, and the numbers of T-cells are correlated with the amount of alveolar destruction and the severity of airflow obstruction [13–16]. Activating naïve T-cells requires the presence of the antigen and co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells. The receptor on the T-cell for B7 is CD28. Activated T-cells express an additional receptor, the cytotoxic T-lymphocyte antigen (CTLA) 4, which binds to B7 with a much higher affinity than CD28 [17] and plays a potent role in downregulation of T-cell activation [18]. *CTLA4* is induced following T-cell receptor (TCR)/CD28 stimulation; it arrests activated T-cells, thereby keeping them in an anergic state. Arrested T-cells exit the lymph nodes and enter the circulation [19]. Blocking *CTLA4* can intensify T-cell responses and exacerbate autoimmune disease [20]; *CTLA4*-deficient mice die by 3–4 weeks of age due to multi-organ lymphocytic infiltration and tissue destruction [17]. *CTLA4*,

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which is located at chromosome 2q33, has been associated with several inflammatory diseases including asthma [21, 22]. Therefore, *CTLA4* is a candidate gene for COPD and/or chronic bronchitis because of its critical effect on inhibiting the activation of T-cells.

As *CTLA4* is located near the region on chromosome 2q that showed significant linkage with COPD-related phenotypes both in the Boston Early-Onset COPD Study [9–11], as well as in general population pedigrees [12], we hypothesised that *CTLA4* single nucleotide polymorphisms (SNPs) would be associated with COPD and COPD-related phenotypes, including the severity of airflow limitation and the presence of chronic bronchitis.

## METHODS

### Study design

The present study is a two-phase study. First, we explored whether *CTLA4* SNPs were associated with COPD and COPD-related phenotypes in a subset of the International COPD Genetics Network (ICGN) population, a large cohort of COPD probands ( $n=606$ ) and siblings ( $n=1,285$ ), whose methodological details have been described elsewhere [23–25]. Secondly, we attempted to replicate our findings in an independent population that included COPD cases ( $n=953$ ) and controls ( $n=956$ ) recruited in Bergen (Norway), whose characteristics have also been reported [23, 25].

### Populations and assessment

The clinical characteristics of the subjects in both cohorts included in the analysis of the chronic bronchitis phenotype are shown in table 1. Subject recruitment and clinical characteristics have been described previously [23–25]. Briefly, the first population represents the multicentre ICGN study; subjects with known COPD were recruited as probands, and siblings and available parents were ascertained through the probands. Inclusion criteria for probands were airflow limitation (post-bronchodilator forced expiratory volume in 1 s (FEV<sub>1</sub>) <60% predicted and FEV<sub>1</sub>/vital capacity (VC) <90% pred) at a relatively early age (45–65 yrs), a smoking history of  $\geq 5$  pack-yrs, and at least one eligible sibling (with a smoking

history of  $\geq 5$  pack-yrs). COPD in siblings was defined by a post-bronchodilator FEV<sub>1</sub> <80% pred and FEV<sub>1</sub>/VC <90% pred (generally consistent with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II or greater). 606 pedigrees with 1,896 Caucasian individuals were genotyped in this analysis. Chronic bronchitis was defined as chronic cough and sputum production for  $\geq 3$  months·yr<sup>-1</sup> for the past 2 yrs. Among the 1,193 COPD cases of the ICGN subjects analysed, 471 (39.5%) had chronic bronchitis.

The cohort recruited in Bergen included COPD cases and controls; all subjects were either current or ex-smokers with  $\geq 2.5$  pack-yrs of smoking history. COPD was diagnosed if post-bronchodilator FEV<sub>1</sub> was <80% pred with FEV<sub>1</sub>/forced vital capacity (FVC) <0.7. Controls were selected on the basis of FEV<sub>1</sub> >80% pred and FEV<sub>1</sub>/FVC >0.7. A total of 953 COPD cases and 956 controls were included. Out of the 953 cases with COPD, there were 342 (35.9%) with and 511 (53.6%) without chronic bronchitis.

### SNP selection

Linkage disequilibrium (LD) tagging of *CTLA4* SNPs was performed using an  $r^2$  threshold of 0.8 for the SNP selection with a pairwise method in the Haploview program [26]. The tagging SNP selection was based on HapMap data for European-Americans (US residents with northern and western European ancestry, i.e. CEPH (Centre d'Etude du Polymorphisme Humain) samples from Utah (CEU)) with a minor allele frequency >5% from the public database [27]. Accordingly, nine SNPs in *CTLA4* region were selected. These SNP locations and characteristics are summarised in table 2.

### Genotyping

Genotyping in the two cohorts was performed with the Illumina array-based custom SNP genotyping platform (Illumina Inc., San Diego, CA, USA). Deviation from Hardy–Weinberg equilibrium for each SNP was assessed in controls by using the goodness-of-fit test with SAS software version 8.2 (SAS Institute, Inc., Cary, NC, USA); Hardy–Weinberg equilibrium for each SNP was also tested in the family data using PBAT version 3.5 (Golden Helix Inc, Bozeman, MT, USA) [28].

**TABLE 1** Clinical characteristics of patients with and without chronic bronchitis (CB) in the chronic obstructive pulmonary disease (COPD) cases of the International COPD Genetics Network (ICGN) study and the Bergen cohort

	ICGN study			Bergen cohort		
	CB present	CB absent	p-value <sup>#</sup>	CB present	CB absent	p-value <sup>#</sup>
Subjects n	471	722		342	511	
Age yrs	58.33 ± 6.83	59.78 ± 7.83	0.0122	65.33 ± 9.97	65.73 ± 10.14	0.5435
Female	39.70	44.73	0.0743	34.79	41.86	0.0382
Post-FEV <sub>1</sub> L	1.37 ± 0.58	1.47 ± 0.67	0.1300	1.48 ± 0.66	1.65 ± 0.74	0.0007
Post-FEV <sub>1</sub> % pred	44.67 ± 16.61	48.96 ± 19.12	0.0816	46.41 ± 16.66	52.44 ± 17.49	<0.0001
Post-FEV <sub>1</sub> /FVC	0.42 ± 0.12	0.45 ± 0.14	0.0002	0.49 ± 0.13	0.53 ± 0.13	<0.0001
Smoking pack-yrs	53.27 ± 25.46	47.56 ± 25.51	0.0289	34.16 ± 21.12	30.62 ± 16.85	0.0102
Current smokers	59.23	33.98	<0.0001	55.43	41.76	<0.0001

Data are presented as mean ± SD or %, unless otherwise indicated. Post-FEV<sub>1</sub>: post-bronchodilator forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity. #: obtained from Chi-squared test or unpaired t-test, as appropriate.

**TABLE 2** Single nucleotide polymorphisms (SNPs) genotyped in the International Chronic obstructive pulmonary disease Genetics Network (ICGN) family data and Bergen chronic obstructive pulmonary disease (COPD) cases and controls in the *CTLA4* gene

SNP ID	SNP name	Region	Position bp	Alleles	MAF in COPD ICGN data	MAF in Bergen COPD cases	MAF in Bergen COPD controls
1	rs926169		204430997	G/T	0.385	0.500	0.496
2	rs733618	Promoter	204439189	A/G	0.099	0.088	0.100
3	rs11571316	Promoter	204439334	C/T	0.444	0.382	0.377
4	rs16840252	Promoter	204439764	C/T	0.183	0.166	0.156
5	rs231775	Thr/Ala	204440959	A/G	0.369	0.427	0.441
6	rs231777	Intron	204441833	C/T	0.163	0.145	0.144
7	rs231779	Intron	204442732	C/T	0.368	0.425	0.441
8	rs3087243	3' flank	204447164	A/G	0.469	0.404	0.401
9	rs231725	3' flank	204448920	A/G	0.313	0.370	0.369

MAF: minor allele frequency.

All *CTLA4* SNPs ( $p$ -values  $>0.05$ ) were in Hardy–Weinberg equilibrium in both the family data and the control data. Mendelian inheritance was assessed in the family data using the PedCheck program [29], and individuals with pedigree inconsistencies for a particular SNP were removed from analysis.

### Statistical analysis

In the ICGN study, we used Family-based Association Test (FBAT) version 1.7.3 [30] to analyse single-SNP associations with COPD and chronic bronchitis (qualitative phenotypes). The analyses of quantitative traits (FEV<sub>1</sub> and FEV<sub>1</sub>/VC) were performed with covariates including centre, age, sex, height and pack-yrs of cigarette smoking using PBAT version 3.5 (Golden Helix Inc, Bozeman, MT, USA) [28]. We performed analyses using an additive genetic model in order to reduce multiple statistical testing. The additive model is equivalent to the classic transmission disequilibrium test. The risk allele was determined from the FBAT Z statistic. Values of  $p < 0.05$  from the association analyses were considered nominally significant. We estimated the number of independent SNPs for nine SNPs using the SNP spectral decomposition (SNPSpD) approach [31], and five SNPs were found to be independent. Thus, the adjusted significant  $p$ -value is  $<0.01$  (*i.e.* 0.05/5) after correction for multiple testing. For the ICGN families, haplotypes were inferred and assessed using the HBAT command of the FBAT software with the use of Monte Carlo sampling [32]. A minimum of 10 informative families were analysed using both FBAT and HBAT. Using a SNP sliding window approach (adjacent sets of two and three SNPs), the results of global and haplotype-specific association statistics were determined. Values of  $p < 0.05$  from the analyses were considered the nominal significant levels. Considering that 15 sliding window haplotypes were evaluated in our study, the adjusted significant  $p$ -value is 0.0033 (*i.e.* 0.05/15) based on a global  $p$ -value after Bonferroni correction for multiple testing. In consideration of high correlation among COPD, FEV<sub>1</sub>, FEV<sub>1</sub>/(F)VC and chronic bronchitis phenotypes, we chose not to correct for multiple testing of the phenotypes in order to avoid being over-conservative. For chronic bronchitis data, we used PBAT version 3.5 [28] to estimate the power. At significance

level of 0.05, and penetrances of 0.7, 0.4 and 0.1 for three genotypes, our study had 97.32%, 75.20%, 97.51%, 92.43%, 97.23%, 90.52%, 97.23%, 97.81% and 96.67% power to detect association for SNP 1–9, respectively.

In the Bergen cohort, a logistic regression model was used to investigate relationships of *CTLA4* SNPs with COPD and chronic bronchitis. Analyses were performed using SAS software 8.2 with an additive genetic model. The odds ratios were estimated using the minor allele as the reference. Haplotype-based analyses were performed using the expectation-maximisation algorithm and score tests as implemented in Haplo.stats software [33]. As we used the Bergen case–control cohort for replication of the confirmed results from the ICGN family-based association analysis, we did not perform correction of multiple testing for the association analysis of both single SNPs and haplotypes. In the Bergen cohort, we estimated an inflation factor  $\lambda$  from the genomic control method [34] using a panel of 221 unlinked SNPs genotyped previously and did not detect significant evidence of population stratification; the mean inflation factor of the genomic control SNPs was 1.027 [23].

Visualisation of LD measures in the *CTLA4* region was performed with the Haploview program [26]. Haplotype blocks were defined with the confidence interval method described by GABRIEL *et al.* [35].

## RESULTS

### Individual SNP association analyses

We evaluated nine SNPs in the *CTLA4* region; the location and characteristics of those SNPs are summarised in table 2. We did not detect any significant genetic associations of *CTLA4* SNPs with the presence/absence of COPD in either the ICGN or the Bergen COPD cases and controls (table E1, online supplementary material). In addition, there were no significant associations between *CTLA4* SNPs and FEV<sub>1</sub> or FEV<sub>1</sub>/(F)VC in either the ICGN families or the Bergen COPD cases (data not shown).

In contrast, we found significant associations with the other COPD-related phenotype investigated, chronic bronchitis. In the ICGN families, six out of the nine *CTLA4* SNPs genotyped (SNPs

**TABLE 3** Association of individual *CTLA4* single nucleotide polymorphisms (SNPs) with chronic bronchitis phenotype in the International Chronic obstructive pulmonary disease Genetics Network (ICGN) family study and chronic obstructive pulmonary disease cases of the Bergen study

SNP ID	SNP name	ICGN family study			Bergen study		
		Risk allele	Informative families n	p-value	Risk allele	Effect estimates	p-value
1	rs926169	T	165	0.0432	T	0.956 (0.412–1.843)	0.9365
2	rs733618	A	60	0.6879	A	0.951 (0.634–1.442)	0.8143
3	rs11571316	C	176	0.0230	T	0.799 (0.649–0.983)	0.0359
4	rs16840252	T	100	0.4015	T	0.983 (0.762–1.269)	0.8669
5	rs231775	G	163	0.0081	G	1.247 (1.019–1.526)	0.0325
6	rs231777	T	91	0.7428	T	0.886 (0.674–1.166)	0.3791
7	rs231779	C	162	0.0112	C	1.221 (0.998–1.496)	0.0532
8	rs3087243	G	175	0.0079	G	0.805 (0.654–0.991)	0.0408
9	rs231725	G	152	0.0181	G	1.229 (1.002–1.508)	0.0401

Data are presented as OR (95% CI), unless otherwise indicated.

rs926169, rs11571316, rs231775, rs231779, rs3087243 and rs231725) were significantly associated with the chronic bronchitis phenotype (table 3;  $0.0079 \leq p \leq 0.0432$ ). Associations with SNPs rs231775 and rs3087243 ( $p=0.0081$  and  $0.0079$ , respectively) were significant even after a correction for multiple testing.

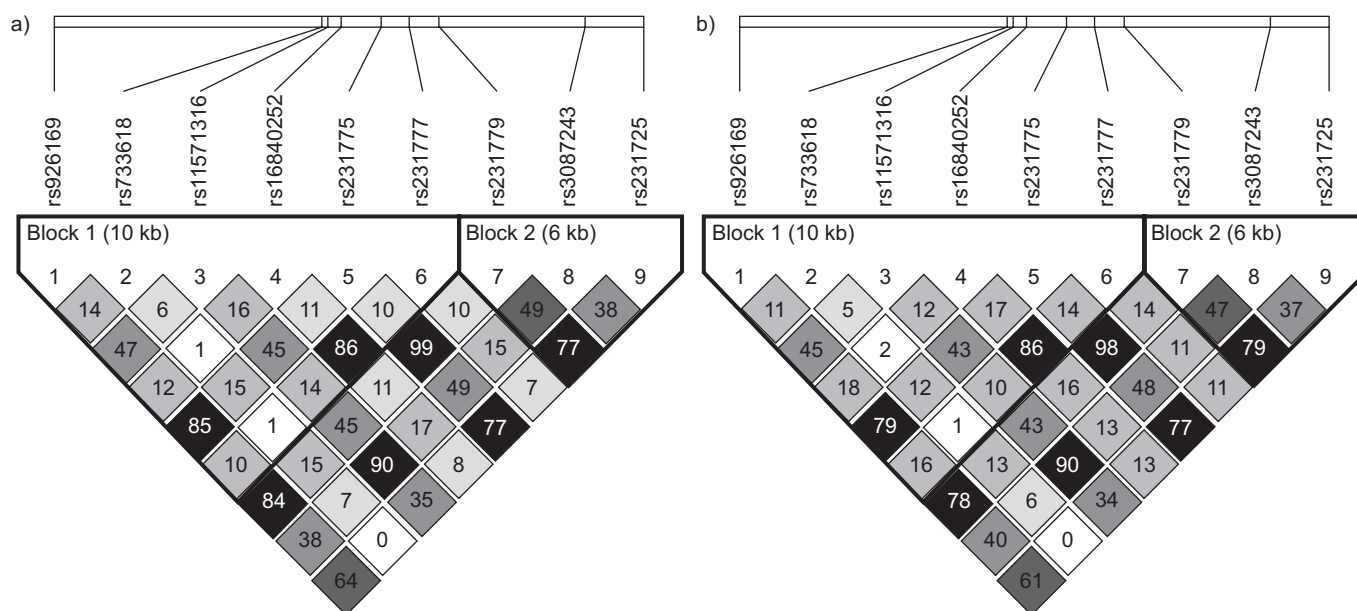
We replicated the significant association with the chronic bronchitic phenotype in the COPD cases of the Bergen cohort for four of the six *CTLA4* SNPs (rs11571316, rs231775, rs3087243 and rs231725;  $0.0325 \leq p \leq 0.0408$ ) identified in the ICGN study (table 3). After evaluating the risk allele (table 3), we found that three of the replicated significant SNPs

(rs231775, rs3087243 and rs231725) for chronic bronchitis have the same directionality of association in the two populations.

We did not detect any significant association with chronic bronchitis in control subjects without COPD in the Bergen cohort (data not shown), suggesting that *CTLA4* is associated with chronic bronchitis among COPD subjects.

#### LD block analysis

Figure 1 shows pairwise LD  $r^2$ -values for the nine SNPs in the *CTLA4* region. In the ICGN study, we identified two haplotype blocks. Several of the significantly associated SNPs (rs926169, rs11571316, rs231775 and rs231777) were located in block 1,



**FIGURE 1.** Linkage disequilibrium (LD) map across the cytotoxic T-lymphocyte antigen (*CTLA4*) region. Pairwise LD plots of nine single nucleotide polymorphisms (SNPs) within the *CTLA4* region in a) the International Chronic obstructive pulmonary disease Genetics Network family-based population and b) the Bergen case-control population. Values of  $r^2$  ( $\times 100$ ) are shown. ■:  $r^2=1$ ; □:  $r^2=0$ ; squares in shades of grey:  $0 < r^2 < 1$  (the intensity of the grey is proportional to  $r^2$ ). LD block structure was estimated using the Haploview program.

**TABLE 4** Significant results for chronic bronchitis from the haplotypes analyses in the International Chronic obstructive pulmonary disease Genetics Network (ICGN) cohort and chronic obstructive pulmonary disease cases of the Bergen cohort

Haplotype	ICGN cohort			Bergen cohort		
	Global p-value	Specific p-value	Haplotype frequency	Global p-value	Specific p-value	Haplotype frequency
2-3	0.0457	0.0286	0.477			
3-4				0.0402	0.0269	0.433
4-5	0.0033	0.0015	0.493	0.0481	0.0258	0.465
5-6	0.0072	0.0033	0.346	0.0304	0.0258	0.411
6-7	0.0309	0.0151	0.346			
7-8	0.0047	0.0032	0.346	0.0390	0.0385	0.368
8-9	0.0087	0.0065	0.287	0.0424	0.0382	0.369
3-4-5	0.0184	0.0125	0.352			
4-5-6	0.0029	0.0015	0.493	0.0302	0.0256	0.450
5-6-7	0.0199	0.0113	0.340	0.0291	0.0286	0.410
6-7-8	0.0260	0.0169	0.350	0.0282	0.0186	0.368
7-8-9	0.0108	0.0087	0.283	0.0472	0.0385	0.369

Haplotypes with a global p-value <0.05 are shown. The specific p-value represents the p-value of the most significant specific haplotype. Haplotype frequency represents the frequency of the most significant specific haplotype. The relative position of the single nucleotide polymorphisms (SNPs) in the haplotype combinations corresponds to the SNPs' identification numbers given in table 3.

while three other significantly associated SNPs (rs231779, rs3087243 and rs231725) were located in block 2. In block 1, there were strong pairwise correlations between SNPs rs926169 and rs231775 ( $r^2=0.85$ ), and between SNPs rs16840252 and rs231777 ( $r^2=0.86$ ). In the Bergen cohort, the same two haplotype blocks were identified, with SNPs significantly associated with chronic bronchitis in each block.

#### Haplotype association analyses

Finally, we conducted adjacent two and three SNP haplotype analyses for chronic bronchitis in both cohorts (table 4). In the ICGN data, 11 adjacent SNP combinations showed significant associations with chronic bronchitis ( $0.0029 \leq$  global p-value  $\leq 0.0457$  and  $0.0015 \leq$  specific p-value  $\leq 0.0286$ ). Haplotypes 4-5 (rs16840252-rs231775) and 4-5-6 (rs16840252-rs231775-rs231777) for chronic bronchitis remained significant after Bonferroni correction for multiple testing. Similar results were observed in the COPD cases of the Bergen cohort, where we found nine haplotypes showing significant association with chronic bronchitis ( $0.0282 \leq$  global p-value  $\leq 0.0481$  and  $0.0186 \leq$  specific p-value  $\leq 0.0385$ ).

#### DISCUSSION

The most salient finding of our study is the replicated association of several SNPs in the *CTLA4* gene with one of the most characteristic phenotypes of COPD, namely chronic bronchitis. These results suggest that *CTLA4* is a novel genetic determinant of chronic bronchitis among COPD cases.

#### Previous studies

Thus far, the most clearly documented genetic risk factor for COPD is severe  $\alpha_1$ -antitrypsin deficiency. Many previous studies have identified a variety of genetic variants in other genes that were associated with COPD, but these results have not often been replicated in other cohorts. To our knowledge, no previous study has investigated *CTLA4* in relation to

COPD-related phenotypes. Our results show that *CTLA4* is associated with chronic bronchitis among COPD cases. Interestingly, the *CTLA4* gene is located near the region of chromosome 2q with linkage to COPD-related phenotypes [9-12]. It is noteworthy that we did not find associations of *CTLA4* SNPs with the presence of COPD or the severity of airflow limitation assessed by FEV<sub>1</sub> and FEV<sub>1</sub>/FVC.

#### Potential mechanisms

T-lymphocytes play a key role in the pathogenesis of COPD and chronic bronchitis. *CTLA4* is a crucial gene in the control of T-cell activation [20]. We speculate that the *CTLA4* polymorphisms may compromise the regulatory function of *CTLA4*, and that a weakened inhibitory function of *CTLA4* could facilitate T-cell proliferation and differentiation, thus leading to airway inflammation and the symptoms of chronic cough and phlegm production that define chronic bronchitis [20]. This would be in keeping with the recently proposed hypothesis of an abnormal acquired immune response in the pathogenesis of COPD [36] and would be supported by the following documented findings. *CTLA4* controls a potentially damaging expansion of T-cells by arresting activated T-cells [20]. However, this *CTLA*-mediated anergic check-point can be released by simultaneous stimulation of TCR/CD28 and the polarising cytokines, interleukin (IL)-12 and IL-4, which lead to CD4+ T-cell differentiation towards T-helper cell (Th) type 1 and 2, respectively [20]. Th1 CD4+ cells and cytotoxic T-cell-1 CD8+ cells are increased in COPD and chronic bronchitis [13, 15, 16]. These cells secrete interferon (IFN)- $\gamma$  and tumour necrosis factor- $\alpha$ , which activate macrophages. Activated macrophages secrete IL-12, which in turn activates the transcription factor *STAT4* [37]. There is an increased number of activated *STAT4* and IFN- $\gamma$ <sup>+</sup> cells in bronchial biopsies and bronchoalveolar lavage fluid in patients with COPD, and their increasing expression correlates with decreasing lung function

[37]. If *STAT4* signals in the lung overcome the inhibitory role of *CTLA4* [38] or the latter is genetically compromised, tissue inflammation and damage may ensue and COPD including chronic bronchitis may develop. It is of interest that one of the SNPs associated with chronic bronchitis (rs231775) is a nonsynonymous coding polymorphism, which results in a change of the amino acid at position 17 from Thr to Ala. This polymorphism is in the signal peptide and is absent from the mature protein. It could affect the efficiency of post-translational modifications other than cleavage, potentially resulting in inefficient processing in the endoplasmic reticulum, leading to reduced surface expression [39]. There are several reports showing the functional effect of this polymorphism on T-cell function. MÄURER *et al.* [40] examined the T-cell response from healthy donors homozygous for either A or G at nucleotide position 49 (amino acid position 17). They found that the G allele affected the *CTLA4* downregulation of T-cell activation. In addition, blockade of *CTLA4* ligation using soluble anti-*CTLA4* monoclonal antibody did not augment proliferation as much in T-cells from G/G-expressing individuals as from A/A-expressing ones. Similar results have also been shown by SUN *et al.* [41]. All these studies show consistent results and it has to be noted that the association that we identified with chronic bronchitis is with the G allele. Thus, this polymorphism might result in reduced expression of *CTLA4*, which could lead to the development of chronic bronchitis. However, we note that this variant may also be a marker for an un-genotyped variant in tight LD with this variant in the *CTLA4* gene.

#### Strengths and limitations of our study

Our study has several strengths and limitations that deserve comment. Among the former is the fact that we can exclude population stratification because family-based association analyses, as we performed in the ICGN study, are robust against population stratification, and the genomic control methods used in our case-control cohort did not detect significant population stratification. A second strength of our experimental design is that the two study populations were phenotyped for chronic bronchitis using the same questionnaire, thus reducing possible phenotype heterogeneity. Thirdly, we replicated the genetic associations found in the ICGN cohort in an independent population, an approach that is considered the gold standard for demonstrating the relevance of a candidate gene for complex diseases [42, 43]. Finally, two SNPs (rs231775 and rs3087243) were still significantly associated with chronic bronchitis after conservative correction for multiple comparisons, thus reinforcing the validity of our observations.

Conversely, our study has a limitation that requires discussion. We did not find significant associations of *CTLA4* SNPs with the presence of COPD or the degree of airflow limitation assessed by FEV1 and FEV1/FVC. It is possible that the multiple mechanisms that could contribute to airflow obstruction in COPD impaired our ability to find significant associations with COPD; alternatively, it is possible that *CTLA4* is a genetic determinant of only chronic bronchitis, and not of airflow obstruction.

In conclusion, we performed a genetic association study and identified variants in the *CTLA4* gene that were associated with chronic bronchitis in COPD cases, although not with COPD

directly. Although the precise role of *CTLA4* in the pathogenesis of COPD including chronic bronchitis is still to be elucidated, our findings open a new avenue for COPD research.

#### STATEMENT OF INTEREST

Statements of interest for G. Zhu, P. Bakke, H. Coxson, E.K. Silverman and S.G. Pillai can be found at [www.erj.ersjournals.com/misc/statements.dtl](http://www.erj.ersjournals.com/misc/statements.dtl)

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