Associations of *IL6* polymorphisms with lung function decline and COPD

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ABSTRACT

Background: Interleukin-6 (IL6) is a pleiotropic pro-inflammatory and immunomodulatory cytokine which likely plays an important role in the pathogenesis of COPD. There is a functional single nucleotide polymorphism (SNP), -174G/C, in the promoter region of *IL6*. We hypothesized that *IL6* SNPs influence susceptibility for impaired lung function and COPD in smokers.

Methods: Seven and 5 SNPs in *IL6* were genotyped in two nested case-control samples derived from the Lung Health Study (LHS) based on phenotypes of rate of decline of forced expiratory volume in one second (FEV_1) over 5 years and baseline FEV_1 at the beginning of the LHS. Serum *IL6* concentrations were measured for all subjects. A partially overlapping panel of 9 *IL6* SNPs was genotyped in 389 COPD cases from the National Emphysema Treatment Trial (NETT) and 420 controls from the Normative Aging Study (NAS).

Results: In the LHS, three *IL6* SNPs were associated with FEV_1 decline ($0.023 \leq P \leq 0.041$ in additive models). Among them the *IL6*_-174C allele was associated with rapid decline of lung function. The association was more significant in a genotype-based analysis ($P = 0.006$). In the NETT-NAS study, *IL6*_-174G/C and four other *IL6* SNPs, all of which are in linkage disequilibrium with *IL6*_-174G/C, were associated with susceptibility to COPD ($0.01 \leq P \leq 0.04$ in additive genetic models).

Conclusion: Our results suggest that the *IL6*_-174G/C SNP is associated with rapid decline of FEV_1 and susceptibility to COPD in smokers.

INTRODUCTION

IL6 is a pleiotropic pro-inflammatory and immunomodulatory cytokine secreted by airway epithelial cells, alveolar macrophages, adipocytes and myocytes as well as other tissues and cells.[1,2] The potential importance of IL6 in the pathogenesis of COPD is suggested by studies showing that high levels of serum or sputum IL6 are associated with impaired lung function or a faster decline in lung function.[1,2] IL6 has been related to skeletal muscle weakness in COPD[3] as well as to exacerbations[4] and pulmonary infections[5] in COPD patients. In addition, IL6 overexpression in the murine lung resulted in airway inflammation and emphysema-like airspace enlargement.[6] Furthermore, IL6 is an important mediator of the acute phase response and can upregulate C-reactive protein (CRP) at the transcriptional level.[7] CRP has been associated with lung function levels in healthy individuals and/or lung function decline in smoking-induced COPD. [8,9] Taken together, these data support *IL6* as an appealing candidate gene for smoking-induced lung function impairment and COPD.

The *IL6* gene is located on chromosome 7p21. Previous studies have identified a functional SNP, -174G/C, in the promoter region of *IL6*.[10] Before initiation of the current study, a small study reported there was no association of an *IL6* SNP with COPD.[11] Recently, another group showed that the IL6_-572C allele was associated with COPD.[12] Large well-designed studies with carefully-defined COPD phenotypes are required to unravel the exact role of *IL6* genetic variants in the pathogenesis of COPD.

We investigated smokers with mild to moderate airflow obstruction who were participants in the Lung Health Study (LHS) cohort and hypothesized that there would be significant associations between SNPs and haplotypes in *IL6* with the rate of decline and/or the level of lung function and these association would be mediated through influencing IL6 serum concentrations. The LHS cohort provides an excellent opportunity to explore associations between gene polymorphisms and haplotypes with FEV_1% predicted[13,14] as well as the rate of decline in FEV_1.[15,16] To validate novel associations between *IL6* SNPs with lung function phenotypes, replication of results was sought in COPD cases from the National Emphysema Treatment Trial (NETT) with participants from the Normative Aging Study (NAS) serving as controls.[17,18]

METHODS
Study participants:
LHS participants: A total of 1488 subjects were selected from the ~ 4,800 LHS subjects for whom DNA and serum were available. The selection generated two nested case-control studies based on the extremes of rate of decline in lung function and baseline lung function. In the decline of lung function study, we selected the 266 and 293 non-Hispanic whites with the fastest and slowest rate of decline of lung function, respectively during the 5 year follow up (arbitrary cut-off points of $\geq$ 3.0% predicted decrease /year and $\geq$ 0.4% predicted increase /year in FEV_1 were used for rapid decliners and non-decliners, respectively). The rationale to select nested case-control studies with the indicated sample sizes is that 1) this approach has the advantage of reducing cost while keeping satisfactory statistical efficiency when compared with the full cohort approach;[19,20] 2) the Common Disease/Common Variants hypothesis (CD/CV) was suggested one decade ago which states that disease susceptibility alleles of common diseases will be present at high frequencies, [21] and 3) this sample size has relatively adequate power to detect common genetic risk variants as shown in our previous power analyses.[22] The baseline lung function study consisted of the 532 and 527 participants who had the highest and lowest baseline FEV_1% predicted, respectively (arbitrary cut-off points of FEV_1 % predicted $\geq$ 88.9% and $\leq$ 67.0% were used for the high and low baseline groups, respectively). There were 130 participants that overlapped between the two sets of nested cases and controls due to the fact that subjects in the rate of decline study group had baseline lung function within one of the categories for baseline lung function.

NETT-NAS participants: We selected 389 non-Hispanic white subjects who were enrolled in the NETT Genetics Ancillary Study. The control group was composed of 420 participants with normal spirometry from the NAS, a longitudinal study over the past four decades of healthy adult males that was initiated by the Boston Veterans Administration.

More information on the participants is included in the online supplement.

TagSNP selection and genotyping methods:
In the LHS, 5 tagSNPs were chosen from the SeattleSNPs database using the LDSelect program based on a relatively stringent LD threshold of $r^2 \geq 0.8$ and minor allele frequency cutoff of 10%. An additional 2 SNPs selected for the NETT-NAS study were subsequently chosen for genotyping in the decline of lung function study, in order to make the two studies more comparable. The nomenclature for the polymorphisms utilized in the study is summarized in Table E1 in the online supplement. SNP genotyping was performed using the TaqMan method (Applied Biosystems, Foster City, CA) for 5 tagSNPs and the Illumina Bead Array System for additional 2 SNPs. The positions of the selected and successfully genotyped 5 tagSNPs are shown in Figure 1.

In the NETT-NAS, the same criteria were used to select six LD-tagging *IL6* SNPs and three additional *IL6* SNPs were also selected for genotyping. The SNPs were genotyped on an Illumina BeadStation 500G System utilizing the GoldenGate assay technology (Illumina Golden Gate Assay, San Diego, CA).

SNP selection criteria are shown in more detail in the online supplement.

Measurements of serum IL6 concentration in the LHS participants:
After collection, the blood samples were separated into their various components and shipped to the LHS data co-coordinating center on dry ice and were kept in -70°C freezers until use. The serum samples were thawed once for IL6 measurements. The concentrations of IL6 were measured using a highly sensitive chemiluminescent multiplexed sandwich immunoassay (SearchLight Proteome Array System®, Rockford, IL).

Statistical Analysis:
In the LHS, Hardy-Weinberg equilibrium tests and linkage disequilibrium estimation were calculated using the genetics package for R (www.r-project.org). Multiple logistic regressions for rate of decline and baseline lung function were performed to test for the association with *IL6* SNPs and with IL6 serum levels. Confounding factors included body mass index (BMI), age, gender, pack years of smoking, and smoking status. Multiple linear regression was performed for the complete data set to test for association of *IL6* SNPs with log IL6 serum levels. Haplotype analysis was done using the R hapassoc package.

In the NETT-NAS, similar analyses were performed with SAS Genetics (Cary, NC).

Statistical Analysis is described in more detail in the online supplement.

RESULTS
Characteristics of the study participants:
In the total of 1488 participants from the LHS, genotyping success rates were 96.4% to 98.6% for the 5 studied *IL6* tagSNPs in all subjects and 97.9% for additional 2 SNPs in the rate of decline study. The demographic characteristics are shown in Table 1.

Table 1: The distribution of demographic characteristics for all subjects and those in the two nested case control study groups in the LHS.

	All participants (N = 1488)	Rate of decline study			Baseline lung function study		
		Fast Decliners (n = 266)	Non Decliners (n = 293)	p value	High Function (n = 532)	Low Function (n = 527)	p value
Men/Women	948/540	158/108	197/96	N/A	352/180	325/202	N/A
Age (years)	48.41 (0.177)	49.47 (0.397)	47.48 (0.399)	<0.001	46.21 (0.772)	50.76 (0.262)	<0.001
Smoking history (pack-yrs)*	40.41 (0.483)	43.23 (1.178)	38.35 (1.064)	0.002	35.33 (0.125)	45.24 (0.809)	<0.001
Smoking status during 5 years follow-up†							
Continuing smokers	979	266	293	N/A	264	286	N/A
Intermittent quitters	315	N/A	N/A	N/A	157	158	N/A
Sustained quitters	194	N/A	N/A	N/A	111	83	N/A
Body Mass Index (kg/m^2)	25.49 (0.099)	25.22 (0.248)	25.77 (0.210)	0.091	25.39 (0.150)	25.57 (0.183)	0.455
ΔFEV_1/yr (% predicted pre)‡	-0.98 (0.053)	-4.13 (0.066)	1.087 (0.042)	<0.001	-0.55 (0.067)	-1.22 (0.078)	<0.001
ΔFEV_1/yr (% predicted post)§	-0.85 (0.04)	-3.44 (0.078)	0.695 (0.054)	<0.001	-0.75 (0.064)	-0.74 (0.078)	0.949
Baseline FEV_1 (% predicted pre)‖	74.15 (0.302)	72.68 (0.542)	75.51 (0.472)	<0.001	86.49 (0.125)	61.07 (0.180)	<0.001
Baseline FEV_1 (% predicted post)**	77.57 (0.336)	75.00 (0.561)	79.80 (0.467)	<0.001	91.81 (0.099)	62.57 (0.144)	<0.001
IL6 concentration (pg/ml)	121 (99, 148)	125 (104, 152)	121 (97, 148)	0.1840	119 (97, 140)	121 (101, 152)	0.12

Values are means (SEM) for continuous data.

* Number of packs of cigarettes smoked per day × number of years smoking.

† Continuing smokers: participants who reported smoking at each annual visit. Sustained quitters: participants who were validated by salivary cotinine or exhaled CO as abstinent at every annual visit. Intermittent quitters: participants who were not sustained quitters or continuing smokers

‡ Change in lung function over a 5-year period per year as % predicted FEV_1 pre bronchodilator

§ Change in lung function over a 5-year period per year as % predicted FEV_1 post bronchodilator

‖ Lung function at the start of the Lung Health Study as measured by FEV_1(%) predicted pre bronchodilator

** Lung function at the start of the Lung Health Study as measured by FEV_1(%) predicted post bronchodilator.

There were significant differences in several potential confounding factors, such as age, gender, pack years of smoking, and smoking status between study groups. Therefore, multiple regressions were performed to adjust for relevant confounding factors.

In the total of 809 participants in the NETT-NAS, the genotype call rate for *IL6*_615A/G (rs2069832) was 85%; for all other SNPs the call rates were ≥ 97%. The demographic characteristics for the study groups are shown in Table 2.

Table 2: The distribution of demographic characteristics for NETT COPD cases and NAS controls.

Demographic Characteristics*	NETT (n = 389)	NAS (n = 420)	p-value
Age (years)	67 $\pm$ 6	68 $\pm$ 9	0.9
Pack-years	66 $\pm$ 30	39 $\pm$ 27	<0.001
FEV$_1$ (% predicted, post BD) †	28 $\pm$ 7	92 $\pm$ 11	
Modified BODE score (median $\pm$ IQR) ‡	5 $\pm$ 3	NA	
Gender (% male)	64%	100%	

*Values are $\pm$ standard deviation unless otherwise listed
†FEV$_1$ % predicted values for the NETT and NAS are based on the prediction equations of Crapo and Morris (47); however the NAS-1988 standards were used in the selection of the control group.
‡Modified BODE score incorporates the University of San Diego Shortness of Breath Questionnaire, IQR = interquartile range

The linkage disequilibrium pattern, Hardy-Weinberg disequilibrium and performance of tagSNPs:
The LD pattern of the five *IL6* tagSNPs in the full set of 1488 LHS study participants is shown in Figure 2A The r^2 values were from 0.04 to 0.89. It is worth noting that the r^2 values between *IL6*_-1479 (rs2069825) and *IL6*_-174 (rs1800795) as well as *IL6*_3331 (rs2069845) and *IL6*_-174 were greater than 0.86, which indicates that it is necessary to genotype only one of these 3 SNPs. The LD patterns of the low and high lung function subgroups were similar to that of all subjects (data not shown), as were those of the fast and non-decline subgroups, the LD pattern of all 7 SNPs genotyped in fast and non-decline subgroups are shown in Figure 2B. All the studied SNPs were in Hardy-Weinberg equilibrium.

More information on performance of tagSNPs is included in the online supplement.

Associations of SNPs and haplotypes in the *IL6* gene with rate of decline and baseline of FEV$_1$:
Three of 7 *IL6* SNPs were associated with FEV$_1$ decline ($0.023 \leq P \leq 0.041$ in additive genetic models) (Table 3). The well known functional SNP *IL6*_-174G/C (rs1800795) was among them. The frequency of the *IL6*_-174C allele was significantly higher in the group with rapid decline of FEV$_1$ than that in the non-decline group (45.2% versus 39.6%, OR 1.30, 95% CI = 1.01 – 1.66, P = 0.041). The association was more significant in the genotype-based analysis (P = 0.006) with 6 out of 7 SNPs reaching a significance level of P < 0.05 (Table 3).

Table 3: Associations of SNPs in *IL6* with rate of decline of FEV1 in the LHS and association with COPD in the NETT-NAS

Bin	SNP ID	SNP in gene	LD (r^2 with rs1800795)		Rate of FEV$_1$ decline study in the LHS				COPD case control study in the NETT-NAS			
			LHS	NETT-NAS	Fast decline MAF %	Non-decline MAF %	p-value‡ genotype based	p-value‡ additive	NETT MAF %	NAS MAF %	p-value§ genotype based	p-value§ additive
1	rs1800797†	-598G/A	NA	0.93					0.41	0.35	0.06	0.02
1	rs1800795*†	-174G/C	-	-	0.45	0.40	0.006	0.041	0.42	0.36	0.03	0.01
1	rs2069832†	615A/G	NA	0.98					0.42	0.37	0.1	0.09
1	rs1474348†	1090G/C	NA	0.97					0.41	0.36	0.1	0.04
1	rs1474347†	1306G/T	NA	0.98					0.42	0.36	0.06	0.03
2	rs1554606*†	1889G/T	0.84	0.87	0.47	0.43	0.011	0.103	0.44	0.38	0.02	0.01
2	rs2069845*	3331G/A	0.89	NA	0.47	0.42	0.007	0.078				
3	rs2069825*	-1479CT/-	0.90	NA	0.44	0.37	< 0.001	0.023				
4	rs2069840*†	1754C/G	0.36	0.33	0.32	0.37	0.005	0.064	0.35	0.36	0.3	0.7
5	rs1818879*	5909G/A	0.32	NA	0.29	0.34	0.012	0.035				
6	rs2069827*†	-1363G/T	0.12	0.14	0.08	0.08	0.786ll	0.669	0.09	0.09	0.9	0.4
other	rs2069849†	4338C/T	NA	0.02					0.03	0.02	0.2	0.2

* Genotyped in the LHS, † Genotyped in the NETT-NAS

‡ Adjustment for confounding factors such as age, gender, pack-years of smoking, and research center.

§ As the NAS controls were uniformly male smokers with normal lung function, the models were adjusted for age and pack-years.

ll The p values were from a dominant genetic model because the minor allele frequency of this SNP was very low.

It is worthwhile to note that the most significant association was found for the *IL6_*-1479CT in/del (rs2069825): the *IL6_*-1479CT deletion was associated with rapid decline of FEV$_1$ (p < 0.001) (Table 3). Three other *IL6 SNPs,* were not significantly associated in the additive model, but were significant in the genotype-based analysis (Table 3). The *IL6_*-1479 CT in/del and another 2 associated *IL6* SNPs were in high LD with *IL6_*-174G/C. Interestingly, the *IL6_*5909G/A and *IL6_*1754C/G, which were not in high LD with *IL6_*-174G/C and not in high LD each other ($r^2 = 0.52$), were also significantly associated with decline of lung function (Table 3).

No association was found for *IL6* haplotypes with rate of decline of FEV$_1$; *IL6* SNPs and haplotypes were not associated with the baseline level of FEV$_1$ (data not shown).

Associations of *IL6* SNPs and haplotypes with serum IL6 concentrations:
The associations of *IL6* SNPs with serum IL6 concentrations were analyzed in all LHS subjects for 5 tagSNPs and in rate of decline study subjects for 2 additional SNPs by linear regressions adjusted for BMI, age, gender, pack years of smoking, and smoking status (Table 4).

Table 4 Association of serum concentrations of IL6 and *IL6* genotypes (linear regression*)

SNP	Genotype	IL6 level [ln IL6 (pg/ml)]		
		N	Coefficient (SE)	p value
*IL6_*-1479 (rs2069825)	II	514		
	ID	681	-0.011 (0.046)	
	DD	229	-0.080 (0.063)	0.424†
*IL6_*-1363 (rs2069827)	GG	1185		
	GT+TT	234	0.000 (0.057)	0.997†
*IL6_*-174 (rs1800795)	GG	483		
	GC	691	0.013 (0.047)	
	CC	255	-0.056 (0.061)	0.486†
*IL6_*1754 (rs2069840)	CC	234		
	CG	234	0.000 (0.048)	
	GG	72	-0.030 (0.064)	0.820‡
*IL6_*1889 (rs1554606)	GG	169		
	GT	261	0.013 (0.044)	
	TT	110	-0.057 (0.054)	0.542‡
*IL6_*3331 (rs2069845)	AA	431		
	AG	689	0.015 (0.049)	
	GG	293	-0.020 (0.060)	0.819†
*IL6_*5909 (rs1818879)	AA	685		
	AG	593	0.012 (0.044)	
	GG	137	-0.025 (0.074)	0.877†

* P values were from genotype-based analysis (co-dominant genetic models) except for *IL6_*-1363 where p values were from a dominant genetic model because the minor allele frequency for this SNP was very low.

† Adjusted for BMI, age, gender, pack years of smoking, and smoking status;

‡ Adjusted for BMI, age, gender, pack years of smoking.

No significant association was found for *IL6* SNPs with IL6 concentrations. *IL6* haplotypes were also not associated with IL6 concentrations (data not shown).

Associations of serum IL6 concentrations with rate of decline and baseline FEV$_1$:
As shown in Table 1, there were no significant differences in IL6 concentrations between the rapid decline and non-decline groups or between high and low FEV$_1$ groups.

Replication of novel *IL6* associations in the NETT-NAS participants:
In the LHS, *IL6* SNPs were significantly associated with rate of decline of FEV$_1$ in mild COPD patients. Since rapid decline of lung function in smokers is the likely method of development of COPD we reasoned that the same SNPs would be associated with advanced COPD. To test this we used a case-control sample that has been very useful in revealing genes associated with COPD.[17,18] In the NETT-NAS study cases had advanced COPD requiring lung volume reduction surgery and controls were derived from a population of smokers who have not developed COPD. The *IL6_*-174G/C and another four *IL6* SNPs, which had high linkage disequilibrium with *IL6_*-174G/C, were associated with susceptibility to COPD ($0.01 \leq P \leq 0.04$ in additive genetic models). The *IL6_*-174C allele was associated with susceptibility to COPD (OR 1.3, 95% CI =

1.1 – 1.7, P = 0.01 in an additive genetic model). The frequency of the *IL6_*-174C allele was significantly higher in the NETT group than that in the NAS group (42.0% versus 36%). The association was also significant in genotype-based analysis (P = 0.03) (Table 3).

DISCUSSION

There are only three studies published on associations of *IL6* SNPs with COPD. Seifart et al. reported that there was no association of *IL6*_-174 with COPD.[11] Broekhuizen et al. did not find an association between *IL6*_−174 and a cachexia phenotype in COPD subjects.[23] Recently, Córdoba-Lanús et al. reported that *IL6*_−572 but not *IL6*_-174 was associated with COPD.[12] All three studies have relatively small sample sizes. The associations of *IL6* SNPs with FEV$_1$ decline in the current study are novel and are the most significant findings among all the studies we have published utilizing the LHS cohort.[13-16,24-27] To strengthen our initial finding in the LHS, we incorporated an association study of *IL6* SNPs with COPD in the NETT-NAS. All SNPs that were genotyped and in high LD with the *IL6*_-174G/C showed significant or borderline association with rapid decline of lung function in the LHS and with COPD in the NETT-NAS. We believe that the strength of the associations, the concordant results with several SNPs in high LD with the *IL6*_-174G/C SNP, the available previous functional data on *IL6*_-174G/C, the replication in a second population and the biologic plausibility for association provide strong evidence that this is a true association.

We examined the association of *IL6* SNPs with IL6 serum levels as well as relationships between IL6 serum levels and lung function decline. We did not find any associations. We also found that adjusting the associations between *IL6* SNPs and lung function for serum CRP levels in the LHS had no effect on the strength of the associations (data not shown). Therefore, we did not find evidence that the associations we report were mediated through an influence on production of IL6 or CRP.

Studies that have examined the effects of *IL6* SNPs on IL6 mRNA and protein expression have led to conflicting results. The first reporter gene study demonstrated that a construct containing the -174G allele had higher reporter gene expression in HeLa cells, both under basal conditions and after LPS or IL1 stimulation [10]. However, a second reporter gene study showed that a construct containing -174C had higher IL1-induced expression in HeLa cells than that of the -174G construct, although the difference did not reach statistical significance. [28] By comparison of the two different cell types, the authors concluded that there is a cell type-specific regulation of IL6 expression. [28] Nine of the most recently published studies of *IL6* SNPs with circulating IL6 concentrations are summarized in Table E2. A recent meta-analysis of 5659 subjects from seventeen studies concluded that the -174 IL6 SNP was not associated with circulating IL6 levels. [29] There are several explanations for the lack of consistent associations. First, the *IL6*_−174G/C polymorphism might not be a strong determinant of serum IL6 levels. Second, the serum half-life of IL6 is short. Serum IL6 levels show marked diurnal variability.[30] The blood samples for IL6 measurement in most studies, including our own, were not taken at a specific time of the day. A third explanation is that the SNPs studied may not be the actual functional SNPs. Recently, Samuel and colleagues have identified a novel *IL6* transcriptional regulatory region (-5307 to -5202) much farther from the transcription initiation site than *IL6*_-174 [31] This report coupled with more recent identification of a novel functional SNP, *IL6*_-6331T/C (rs10499563), with the T allele preferentially binding to Oct-1 transcription factor and producing higher reporter gene expression, provides evidence that additional functional SNPs do exist in *IL6*. [32] However, since IL6_-6331T/C is in low LD with IL6_-174, our finding is not likely to be explained by these new functional data.

If *IL6* SNPs are not related to IL6 levels then what is the basis for their association with FEV$_1$ decline and COPD? One possible explanation is that the association is truly driven via local

pulmonary IL6 expression or that it is driven by serum IL6 levels but that the variability and lability of serum IL6 levels obscures this relationship; FEV_1 may reflect the average IL6 levels and thus the degree of lung inflammation over the years of the study. In addition, the SNPs could influence IL6 levels and thus lung inflammation during exacerbations but not the constitutive levels during stable periods. IL6 is a pleiotropic cytokine which also modulates expression of many other genes.[33] It may be that it is the effect of the *IL6* variants on these genes that is the underlying mechanism for the associations we observed.

How can we explain the observation that *IL6* SNPs were not associated with baseline FEV_1 in the LHS but were associated with the presence of COPD in the NETT-NAS study? The mean age of the LHS participants was 48 years as opposed to a mean age of 68 years for the NETT-NAS participants. Baseline FEV_1 at age 48 is influenced both by maximal attained FEV_1 at ~ 25 years of age and by the rate of decline of lung function after age 25.[34] However, the relative contribution of rate of decline in lung function will be much greater by age 68 than at age 48. Thus, FEV_1 at age 68 in the NETT-NAS participants is likely to largely reflect the rate of decline of lung function during their long smoking history whereas there is likely a weaker relationship of FEV_1 decline and baseline lung function at age 48.

Compared with previous studies, strengths of this study include larger sample size and good power. This sample size has adequate power to detect common genetic risk variants as shown in our previous power analyses, for example, it has 80% power to detect a relative risk of 2.0 when the frequency of the risk factor is 10% or above.[22]

There are several potential limitations of this study. Firstly, population stratification could have led to false-positive results. However, it has been reported that significant false-positive associations are unlikely to arise from population stratification in the non-Hispanic white population, especially in well-designed, moderately-sized, case-control studies such as ours. [35] In addition, there was no significant evidence of population stratification in the NETT-NAS cases and controls.[17] Second, false positive results might have arisen from multiple comparisons. However the consistent results in the NETT-NAS replication study make false positive results unlikely. Third, we have not identified the causal SNP for the associations. The identification of a novel functional SNP *IL6*_-6331T/C (rs10499563), which has low LD with IL6_-174G/C (rs1800795) with r^2 of 0.169 in the CEU HapMap database, indicates that the control of IL6 transcription is likely to be complex.[32] We cannot exclude the possibility that SNPs other than the *IL6*_-174G/C are also causal SNPs. Finally, serum IL6 levels were measured at year 5 of the LHS, therefore it may not be appropriate to link IL6 levels at year 5 with the baseline FEV_1 as well as the rate of decline of FEV_1 during 5 year follow-up.

In summary, we report associations of *IL6* variants with rate of decline of lung function and with smoking-induced COPD.

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Figure legends

Figure 1: The *IL6* gene structure and position of single nucleotide polymorphisms genotyped in the Lung Health Study subjects. Numbered regions represent exons. A: adenine; C: cytokine; G: guanine; T: thymidine; UTR: untranslated region.

Figure 2: Linkage Disequilibrium of Single Nucleotide Polymorphisms (SNPs) of *IL6* in the Lung Health Study subjects using HAPLOVIEW. The linkage disequilibrium (r^2) between any two SNPs is listed in the cross cell. The darker the color indicates the higher the linkage disequilibrium between any two SNPs. Figure 2A: all subjects; Figure 2B: top: fast decline group; bottom: slow decline group.

REFERENCES

1. Donaldson GC, Seemungal TA, Patel IS*, et al*. Airway and systemic inflammation and decline in lung function in patients with COPD. *Chest* 2005; **128**(4)**:** 1995-2004.

2. Walston JD, Fallin MD, Cushman M*, et al*. IL-6 gene variation is associated with IL-6 and C-reactive protein levels but not cardiovascular outcomes in the Cardiovascular Health Study. *Hum Genet* 2007; **122**(5)**:** 485-94.

3. Yende S, Waterer GW, Tolley EA*, et al*. Inflammatory markers are associated with ventilatory limitation and muscle dysfunction in obstructive lung disease in well functioning elderly subjects. *Thorax* 2006; **61**(1)**:** 10-16.

4. Wedzicha JA, Seemungal TA, MacCallum PK*, et al*. Acute exacerbations of chronic obstructive pulmonary disease are accompanied by elevations of plasma fibrinogen and serum IL-6 levels. *Thromb Haemost* 2000; **84**(2)**:** 210-5.

5. Yende S, Tuomanen EI, Wunderink R*, et al*. Preinfection systemic inflammatory markers and risk of hospitalization due to pneumonia. *Am J Respir Crit Care Med* 2005; **172**(11)**:** 1440-6.

6. Kuhn C, III, Homer RJ, Zhu Z*, et al*. Airway Hyperresponsiveness and Airway Obstruction in Transgenic Mice . Morphologic Correlates in Mice Overexpressing Interleukin (IL)-11 and IL-6 in the Lung. *Am J Respir Cell Mol Biol* 2000; **22**(3)**:** 289-95.

7. Majello B, Arcone R, Toniatti C*, et al*. Constitutive and IL-6-induced nuclear factors that interact with the human C-reactive protein promoter. *Embo J* 1990; **9**(2)**:** 457-65.

8. Aronson D, Roterman I, Yigla M*, et al*. Inverse association between pulmonary function and C-reactive protein in apparently healthy subjects. *Am J Respir Crit Care Med* 2006; **174**(6)**:** 626-32.

9. Shaaban R, Kony S, Driss F*, et al*. Change in C-reactive protein levels and FEV(1) decline: A longitudinal population-based study. *Respir Med* 2006; **100**(12)**:** 2112-20.

10. Fishman D, Faulds G, Jeffery R*, et al*. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *J Clin Invest* 1998; **102**(7)**:** 1369-76.

11. Seifart C, Dempfle A, Plagens A*, et al*. TNF-alpha-, TNF-beta-, IL-6-, and IL-10-promoter polymorphisms in patients with chronic obstructive pulmonary disease. *Tissue Antigens* 2005; **65**(1)**:** 93-100.

12. Córdoba-Lanús E, de-Torres J-P, López-Aguilar C*, et al*. Association of IL-6 gene polymorphisms and COPD in a Spanish Population. *Resp Med* 2008; **102**(12.)**:** 1805-11.

13. He JQ, Burkett K, Connett JE*, et al*. Interferon gamma polymorphisms and their interaction with smoking are associated with lung function. *Hum Genet* 2006; **119**(4)**:** 365-75.

14.   He JQ, Connett JE, Anthonisen NR, *et al*. Glutathione S-transferase variants and their interaction with smoking on lung function. *Am J Respir Crit Care Med* 2004; **170**(4)**:** 388-94.

15.   He JQ, Connett JE, Anthonisen NR, *et al*. Polymorphisms in the IL13, IL13RA1, and IL4RA genes and rate of decline in lung function in smokers. *Am J Respir Cell Mol Biol* 2003; **28**(3)**:** 379-85.

16.   He JQ, Ruan J, Connett J, *et al*. Antioxidant gene polymorphisms and susceptibility to a rapid decline in lung function in smokers. *Am J Respir Crit Care Med* 2002; **166**(3)**:** 323-8.

17.   Celedon JC, Lange C, Raby BA, *et al*. The transforming growth factor-beta1 (TGFB1) gene is associated with chronic obstructive pulmonary disease (COPD). *Hum Mol Genet* 2004; **13**(15)**:** 1649-56.

18.   Hersh CP, Demeo DL, Lazarus R, *et al*. Genetic association analysis of functional impairment in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006; **173**(9)**:** 977-84.

19.   Allison DB, Heo M, Schork NJ, *et al*. Extreme selection strategies in gene mapping studies of oligogenic quantitative traits do not always increase power. *Human Heredity* 1998; **48**(2)**:** 97-107.

20.   Ernster VL. Nested case-control studies. *Prev Med* 1994; **23**(5)**:** 587-90.

21.   Lander ES. The new genomics: global views of biology. *Science* 1996; **274**(5287)**:** 536-9.

22.   Tanaka G, Sandford AJ, Burkett K, *et al*. Tumour necrosis factor and lymphotoxin A polymorphisms and lung function in smokers. *Eur Respir J* 2006; **29**(1)**:** 34-41.

23.   Broekhuizen R, Grimble RF, Howell WM, *et al*. Pulmonary cachexia, systemic inflammatory profile, and the interleukin 1beta -511 single nucleotide polymorphism. *Am J Clin Nutr* 2005; **82**(5)**:** 1059-64.

24.   He JQ, Shumansky K, Connett JE, *et al*. Association of genetic variations in the CSF2 and CSF3 genes with lung function in smoking-induced COPD. *Eur Respir J* 2008; **32**(1)**:** 25-34.

25.   He JQ, Shumansky K, Zhang X, *et al*. Polymorphisms of interleukin-10 and its receptor and lung function in COPD. *Eur Respir J* 2007; **29**(6)**:** 1120-6.

26.   Joos L, McIntyre L, Ruan J, *et al*. Association of IL-1beta and IL-1 receptor antagonist haplotypes with rate of decline in lung function in smokers. *Thorax* 2001; **56**(11)**:** 863-6.

27.   Sandford AJ, Chagani T, Weir TD, *et al*. Susceptibility genes for rapid decline of lung function in the Lung Health Study. *Am J Respir Crit Care Med* 2001; **163**(2)**:** 469-73.

28.   Terry CF, Loukaci V, Green FR. Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. *J Biol Chem* 2000; **275**(24)**:** 18138-44.

29.   Huth C, Illig T, Herder C*, et al.* Joint analysis of individual participants' data from 17 studies on the association of the IL6 variant -174G >C with circulating glucose levels, interleukin-6 levels, and body mass index. *Ann Med* 2008**:** 1-21.

30.   Sothern RB, Roitman-Johnson B, Kanabrocki EL*, et al.* Circadian characteristics of circulating interleukin-6 in men. *J Allergy Clin Immunol* 1995; **95**(5 Pt 1)**:** 1029-35.

31.   Samuel JM, Kelberman D, Smith AJ*, et al.* Identification of a novel regulatory region in the interleukin-6 gene promoter. *Cytokine* 2008; **42**(2)**:** 256-64.

32.   Smith AJ, D'Aiuto F, Palmen J*, et al.* Association of serum interleukin-6 concentration with a functional IL6 -6331T>C polymorphism. *Clin Chem* 2008; **54**(5)**:** 841-50.

33.   Wegrzyn P, Jura J, Kupiec T*, et al.* A search for genes modulated by interleukin-6 alone or with interleukin-1beta in HepG2 cells using differential display analysis. *Biochim Biophys Acta* 2006; **1762**(3)**:** 319-28.

34.   Tager IB, Segal MR, Speizer FE*, et al.* The natural history of forced expiratory volumes. Effect of cigarette smoking and respiratory symptoms. *Am Rev Respir Dis* 1988; **138**(4)**:** 837-49.

35.   Ardlie KG, Lunetta KL, Seielstad M. Testing for population subdivision and association in four case-control studies. *Am J Hum Genet* 2002; **71**(2)**:** 304-11.

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-1363G/T

-174G/C

3331G/A

5909G/A

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