**ABSTRACT**

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

**Methods:** Seven and five 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 1 s (FEV1) over 5 years and baseline FEV1 at the beginning of the LHS. Serum IL6 concentrations were measured for all subjects. A partially overlapping panel of nine IL6 SNPs was genotyped in 389 cases of COPD 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 decline in FEV1 (0.023 < \( p < 0.041 \) in additive models). Among them, the IL6 -174C allele was associated with a rapid decline in 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 < \( p < 0.04 \) in additive genetic models).

**Conclusion:** The results suggest that the IL6 -174G/C SNP is associated with a rapid decline in FEV1 and susceptibility to COPD in smokers.

Interleukin 6 (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. The potential importance of IL6 in the pathogenesis of chronic obstructive pulmonary disease (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. IL6 has been related to skeletal muscle weakness in COPD, as well as to exacerbations and pulmonary infections in patients with COPD. In addition, overexpression of IL6 in the murine lung resulted in airflow obstruction which is attributable to upregulation of C-reactive protein (CRP) at the transcriptional level. CRP has been associated with lung function levels in healthy individuals and/or lung function decline in smoking-induced COPD.

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 single nucleotide polymorphism (SNP), -174G/C, in the promoter region of IL6. Before initiation of the current study, a small study reported no association of an IL6 SNP with COPD. Recently, another group showed that the IL6 -572C allele was associated with COPD. 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 hypothesised 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 that these associations would be mediated by influencing IL6 serum concentrations. The LHS cohort provides an excellent opportunity to explore associations between gene polymorphisms and haplotypes with percentage predicted forced expiratory volume in 1 s (FEV1) as well as the rate of decline in FEV1. To validate novel associations between IL6 SNPs with lung function phenotypes, replication of results was sought in cases of COPD from the National Emphysema Treatment Trial (NETT) with participants from the Normative Aging Study (NAS) serving as controls.

**METHODS**

**Study participants**

LHS participants

A total of 1488 subjects were selected from approximately 4800 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 white subjects with the fastest and slowest rate of decline of lung function, respectively, during the 5-year follow-up period (arbitrary cut-off points of >3.0% predicted decrease/year and >0.4% predicted increase/year in FEV1 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...
Measurements of serum IL6 concentration in LHS participants

After collection the blood samples were separated into their various components and shipped to the LHS data coordinating centre on dry ice and kept at −70°C 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, Illinois, USA).

Statistical analysis

In the LHS, Hardy-Weinberg equilibrium tests and LD estimations were performed 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, 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, North Carolina, USA). The statistical analysis is described in more detail in the online supplement.

RESULTS

Characteristics of study participants

In the total of 1488 participants from the LHS, genotyping success rates were 96.4–98.6% for the five studied IL6 tagSNPs in all subjects and 97.9% for the additional two SNPs in the rate of decline study. The demographic characteristics are shown in table 1.

There were significant differences in several potential confounding factors such as age, gender, pack years of smoking and smoking status between study groups. Multiple regressions were therefore 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 (rs2069825) was 85%; for all other SNPs the call rates were ≥97%. The demographic characteristics for the study groups are shown in table 2.

Linkage disequilibrium (LD) 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 fig 2A. The r² values ranged from 0.04 to 0.89. It is worth noting that the r² values between IL6_-1479 (rs2069825) and IL6_-174, 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 three 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-declining and non-declining subgroups; the LD pattern of all seven SNPs genotyped in fast-declining and non-declining subgroups are shown in fig 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 FEV₁

Three of seven IL6 SNPs were associated with FEV₁ decline (0.023 ≤ p ≤ 0.041 in additive genetic models; table 3). The well-known functional SNP IL6_-174G/C (rs1800795) was among...

Fig. 1 The IL6 gene structure and position of single nucleotide polymorphisms genotyped in subjects in the Lung Health Study. Numbered regions represent exons. A, adenine; C, cytokine; G, guanine; T, thymidine; UTR, untranslated region.
The frequency of the IL6\_174C allele was significantly higher in the group with rapid decline of FEV1 than in the non-declining group (45.2% vs 39.6%, odds ratio (OR) 1.30, 95% CI 1.01 to 1.66, p = 0.041). The association was more significant in the declining group (45.2% vs 39.6%, odds ratio (OR) 1.30, 95% CI 1.01 to 1.66, p = 0.041). The association was more significant in the declining group (45.2% vs 39.6%, odds ratio (OR) 1.30, 95% CI 1.01 to 1.66, p = 0.041). It is worthwhile to note that the most significant association was found for the IL6\_174CT in/del (rs2069825): the IL6\_174CT deletion was associated with a rapid decline in FEV1 (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\_5909G/A and haplotypes were also not associated with the baseline level of FEV1 (data not shown).

Associations of IL6 SNPs and haplotypes with serum IL6 concentrations

The associations of IL6 SNPs with serum IL6 concentrations were analysed in all subjects in the LHS for five tagSNPs and in subjects in the rate of decline study for two additional SNPs by linear regressions adjusted for BMI, age, gender, pack years of smoking and smoking status (table 4). No significant association was found for IL6 haplotypes with rate of decline in FEV1; IL6 SNPs and haplotypes were not associated with the baseline level of FEV1 (data not shown).

Associations of serum IL6 concentrations with rate of decline and baseline FEV1

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 FEV1 groups.

Replication of novel IL6 associations in NETT-NAS participants

In the LHS, IL6 SNPs were significantly associated with rate of decline of FEV1 in patients with mild COPD. 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.\textsuperscript{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 IL\textsubscript{6} -174G/C and another four IL\textsubscript{6} SNPs, which had high LD with IL\textsubscript{6} -174G/C, were associated with susceptibility to COPD (0.01 ≤ p ≤ 0.04 in additive genetic models). The IL\textsubscript{6} -174 allele was associated with susceptibility to COPD (OR 1.3, 95% CI 1.1 to 1.7, p = 0.01 in an additive genetic model). The frequency of the IL\textsubscript{6} -174 allele was significantly higher in the NETT group than in the NAS group (42% vs 36%). The association was also significant in genotype-based analysis (p = 0.03, table 3).

DISCUSSION

Only three studies have been published on associations of IL\textsubscript{6} SNPs with COPD. Seifart \textit{et al} reported that there was no association of IL\textsubscript{6} -174 with COPD,\textsuperscript{11} Broekhuizen \textit{et al} did not find an association between IL\textsubscript{6} \textsubscript{-2174} and a cachexia phenotype in subjects with COPD\textsuperscript{23} and Córdoba-Lanús \textit{et al} recently reported that IL\textsubscript{6} -572 but not IL\textsubscript{6} -174 was associated with COPD.\textsuperscript{12} All three studies have relatively small sample sizes. The associations of IL\textsubscript{6} SNPs with decline in FEV\textsubscript{1} in the current study are novel and are the most significant findings among all the studies we have published using the LHS cohort.\textsuperscript{13–16 24–27} To strengthen our initial finding in the LHS, we incorporated an association study of IL\textsubscript{6} SNPs with COPD in the NETT-NAS. All SNPs that were genotyped and in high LD with the IL\textsubscript{6} -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 IL\textsubscript{6} -174G/C SNP, the available previous functional data on IL\textsubscript{6} -174G/C, the replication in a second population and the biological plausibility for association provide strong evidence that this is a true association.

We examined the association of IL\textsubscript{6} SNPs with IL\textsubscript{6} serum levels as well as relationships between IL\textsubscript{6} serum levels and lung function decline. We did not find any associations. We also found that adjusting the associations between IL\textsubscript{6} 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 IL\textsubscript{6} 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.\textsuperscript{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.\textsuperscript{28} By comparison of the two different cell types, the authors concluded that there is a cell type-specific regulation of IL6 expression.\textsuperscript{28} Nine of the most recently published studies of IL\textsubscript{6} SNPs with circulating IL6 concentrations are summarised in table E2 in the online supplement. A recent meta-analysis of 5659 subjects from 17 studies concluded that the -174G SNP was not associated with circulating IL6 levels.\textsuperscript{29} There are several explanations for the lack of consistent associations. First, the IL\textsubscript{6} -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.\textsuperscript{30} The blood samples for IL6 measurement in most studies, including our

Figure 2  Linkage disequilibrium (LD) of single nucleotide polymorphisms (SNPs) of IL6 in Lung Health Study subjects using HAPLOVIEW. The LD between any two SNPs is listed in the cross cell. The darker the colour, the higher the LD between any two SNPs. (A) All subjects. (B) Top: fast-declining group; bottom: slow-declining group.
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 (−25307 to −25202) much farther from the transcription initiation site than IL6−174. 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. 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 FEV1 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; FEV1 may reflect the average

### Table 4: Association of serum concentrations of interleukin 6 (IL6) and IL6 genotypes (linear regression*)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>IL6 level (ln IL6 (pg/ml))</th>
<th>N</th>
<th>Coefficient (SE)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6−1479</td>
<td>ID</td>
<td></td>
<td>514</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs2069825)</td>
<td>DD</td>
<td></td>
<td>681</td>
<td>−0.011 (0.046)</td>
<td>0.424†</td>
</tr>
<tr>
<td>IL6−1363</td>
<td>GG</td>
<td></td>
<td>1185</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs2069827)</td>
<td>GT+TT</td>
<td></td>
<td>234</td>
<td>0.000 (0.057)</td>
<td>0.997†</td>
</tr>
<tr>
<td>IL6−174</td>
<td>GC</td>
<td></td>
<td>483</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs1800795)</td>
<td>CC</td>
<td></td>
<td>691</td>
<td>0.013 (0.047)</td>
<td></td>
</tr>
<tr>
<td>IL6−1754</td>
<td>GG</td>
<td></td>
<td>234</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs2069840)</td>
<td>GG</td>
<td></td>
<td>255</td>
<td>−0.056 (0.061)</td>
<td>0.486†</td>
</tr>
<tr>
<td>IL6−1889</td>
<td>CC</td>
<td></td>
<td>169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs1554606)</td>
<td>GT</td>
<td></td>
<td>234</td>
<td>0.000 (0.048)</td>
<td></td>
</tr>
<tr>
<td>IL6−3331</td>
<td>AG</td>
<td></td>
<td>689</td>
<td>0.015 (0.049)</td>
<td></td>
</tr>
<tr>
<td>(rs2069845)</td>
<td>GG</td>
<td></td>
<td>293</td>
<td>−0.020 (0.060)</td>
<td>0.819†</td>
</tr>
<tr>
<td>IL6−5909</td>
<td>AG</td>
<td></td>
<td>685</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rs1818879)</td>
<td>GG</td>
<td></td>
<td>593</td>
<td>0.012 (0.044)</td>
<td></td>
</tr>
</tbody>
</table>
| SNP, single nucleotide polymorphism.  
* 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 body mass index, age, gender, pack-years of smoking and smoking status.  
‡ Adjusted for body mass index, age, gender and pack years of smoking.
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. 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 FEV1 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 compared with 68 years for the participants in the NETT-NAS study. Baseline FEV1 at age 48 is influenced both by maximal attained FEV1 at about 25 years of age and by the rate of decline in lung function after the age of 25. However, the relative contribution of rate of decline in lung function will be much greater by age 68 than at age 48. Thus, FEV1 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 to be a weaker relationship between FEV1 decline and baseline lung function at age 48.

Compared with previous studies, the 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. There are several potential limitations of this study. First, 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. In addition, there was no significant evidence of population stratification in the NETT-NAS cases and controls. 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_–63311T/C (rs10499563), which has low LD with IL6_–174G/C (rs1800795) with r2 of 0.169 in the CEU HapMap database, indicates that the control of IL6 transcription is likely to be complex. 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, so it may not be appropriate to link IL6 levels at year 5 with the baseline FEV1 as well as the rate of decline of FEV1 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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Competing interests: None.

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Vitamin D levels are inversely proportional to the “common cold”

The role of vitamin D in bone metabolism and associated pathology is well established. This paper is the first high-powered population study to demonstrate an inverse relationship between vitamin D levels and recent upper respiratory tract infection (URTI). Vitamin D levels were measured in 18,883 participants in the Third National Health and Nutrition Examination Survey in the USA and they were asked whether they had symptoms suggestive of an URTI in the preceding few days. The study was adjusted for diversity in age, sex, race, season, location, body mass index, smoking, asthma and chronic obstructive pulmonary disease (COPD); 24% of those with a vitamin D level <10 ng/ml had a recent URTI compared with 21% in those with levels of 10–30 ng/ml and 17% in those with vitamin D levels >30 ng/ml. Perhaps the most important finding was that patients with asthma had an odds ratio of 5.67 of recent URTI with vitamin D levels <10 ng/ml compared with those with vitamin D levels >30 ng/ml, and for COPD the odds ratio was 2.26.

The most important application of this research may be in those with asthma or COPD in whom an URTI may lead to lower respiratory tract infections or more life-threatening complications such as pneumonia. One of the limitations of this paper is that it is not clear whether vitamin D is a surrogate of poor nutrition in the context of chronic lung disease, and therefore susceptibility to URTI is due to that rather than pure vitamin D deficiency. More work is needed in the basic science of vitamin D and immunity which can then be translated to clinical trials.


W Thomas, R Quadery

Correspondence to: Dr W Thomas, F1 House Officer, Bedford Hospital, Bedfordshire, UK; william.thomas@bedfordhospital.nhs.uk