Cystic fibrosis (CF) is a common lethal autosomal recessive disease affecting the white population with an incidence of about 1 in 2500. Certain phenotypes of CF are clearly associated with different CF transmembrane conductance regulator (CFTR) genotypes such as pancreatic insufficiency, but pulmonary disease is strongly influenced by other factors. There are many genes that may affect the CF phenotype. These genes may affect secondary symptoms, such as genes encoding proteins involved in lung defence (immune system, inflammation processes, protection against oxidative stress) or genes encoding proteins which directly or indirectly interact with CFTR. Phenotypic differences among patients with the same CFTR genotype are most likely caused by differences in the function of such proteins and/or by environmental factors. Previous studies have shown an association between variant alleles and the severity of the CF phenotype—for example, it was found that polymorphisms in the mannose binding lectin (MBL) protein, which result in low or no functional MBL, contribute to more severe CF lung disease.

Tumour necrosis factor $\alpha$ (TNF$\alpha$), an endogenous factor of the lung involved in host defence, is therefore a possible modulator of CF pulmonary disease severity. TNF$\alpha$ is a proinflammatory cytokine largely produced by macrophages, whose gene is located on chromosome 6p21.3. It is prothrombotic, promotes leucocyte adhesion and migration, modulates haemato poiesis and lymphocyte development, induces other cytokines, and has an important role in macrophage activation and the immune response in tissues. Even though there are many polymorphisms in the promoter region of TNF$\alpha$, much attention has been given to the TNF$\alpha$ − 308g/a polymorphic locus. In a previous study of CF patients from a UK CF clinical centre, an association was found between the −308 allele and a more severe CF phenotype. A modulating role of a genetic factor with disease, based on association studies, should however only be treated as tentative until the finding of an association has been replicated in other studies. Moreover, we broadened the search with additional polymorphisms for an association between TNF$\alpha$ and severity of lung function in CF patients. We therefore investigated whether the TNF$\alpha$ − 308g/a polymorphic locus, together with the −851c/t, −238g/a promoter polymorphic loci and the polymorphic locus +691g ins/del in intron 1, are associated with pulmonary function, body mass index (BMI), and susceptibility to Pseudomonas aeruginosa infection in Belgian and Czech patients with CF.

**METHODS**

**Study subjects**

Only CF patients homozygous for the F508del CFTR mutation were included in the study in order to exclude variability in CF disease because of the CFTR genotype. 180 patients with CF were recruited to the study, 58 from the Belgium University Hospital Gasthuisberg and 122 from the Czech Republic CF Center Prague University Hospital Motol. The control population comprised 85 healthy adult blood donors.
from Belgium and 63 healthy adult blood donors and 55 newborn infants from the Czech Republic. Controls were ethnically matched to patients.

For Hardy-Weinberg equilibrium testing, all unrelated CF patients were included. For the actual association study of TNFα polymorphisms with the CF pulmonary phenotype, only 113 CF patients (41 Belgian and 72 Czech) between the ages of 12 and 15 years (mean age 13.4) were included; 49% were male. Indeed, a modulating gene may only become penetrant by age so it will only be detected in association studies using a well defined group of CF patients of a particular age or age range. All clinical data were retrieved from the patients’ case records. The BMI was measured and percentage predicted values of forced expiratory volume in 1 second (FEV1)2 were calculated according to Knudson et al.12

Statistical analysis of lung function was performed on the last FEV1 % predicted value between the ages of 12 and 15 years. The age of first infection with P aeruginosa was available from the case records of 119 of the total group of 180 CF patients. Patients were followed at the outpatient clinic at a minimum of 3 monthly intervals. At every visit, sputum samples (or throat swabs in patients who did not expectorate) were taken. The age at which P aeruginosa was isolated for the first time is referred to as first infection with P aeruginosa. The association studies were approved by the ethical committees of both universities.

PCR and single nucleotide extension assay

We developed multiplex single nucleotide primer extension assays in which the TNFα single nucleotide polymorphisms (SNPs) were included. This study is part of a much larger study involving semi-high throughput analysis of tens of SNPs, so gap filling experiments were needed for the samples in which a genotype could not be obtained for the first time. However, from a practical point of view, complete gap filling is not possible in such a large study because the DNA of some samples becomes exhausted. Thus, the number of genotypes/alleles obtained for each SNP may differ between different SNPs. For the single nucleotide primer extension assay, the SNP detection kit (SNaPshot; Applied Biosystems) was used. The primers used for amplification of the genomic regions covering the SNPs in TNFα and the single nucleotide extension oligonucleotides are shown in tables S1 and S2 respectively, available on the Thorax website at http://www.thoraxjnl.com/supplemental.

RESULTS

The TNFα promoter polymorphic loci −238g/a, −308g/a, −851c/t and the TNFα intron 1 polymorphic locus +691g ins/del were typed in 180 CF patients and 203 controls. We tested for the possibility of population stratification because of differences in ethnicity, age, and sex but found no significant differences. Furthermore, no differences were found between the Belgian and Czech cohorts with regard to the distribution of alleles/genotypes (see table S3 available on the Thorax website at http://www.thoraxjnl.com/supplemental).

FEV1 % predicted values of CF patients decline with age13 and females have a steeper decline in pulmonary function than males.14 FEV1 % predicted values calculated according to Knudson are corrected for sex and age.14 Nevertheless, we determined whether the FEV1 % predicted values were truly independent of sex and age in our age selected cohort of patients and found no influence of age (Pearson correlation coefficient r2 = 0.006) or sex (p = 0.21, mean difference 5.4%, 95% CI −13.7 to 3.0, two-sample t test) on FEV1 % predicted.

We then determined whether the values of FEV1 % predicted in CF patients were dependent on the distribution of TNFα genotypes. Given that a modifier effect may only become penetrant by age, as has been shown for MBL2,15 only CF patients aged 12–15 years were included in the actual association study. The mean FEV1 % of the age selected cohort of CF patients was approximately 70% predicted, so patients with an FEV1 less than 70% predicted were compared with those with an FEV1 of more than 70% predicted. The distribution of +691g ins/del was found to be significantly different between the two groups, with a higher proportion of those in whom the FEV1 was more than 70% predicted being heterozygous for the two alleles at the +691g ins/del locus (table 1). More importantly, the mean FEV1 % predicted values differed significantly between the two groups, patients with the +691g ins/+691g del genotype having a higher mean FEV1 % predicted value than those with the +691g ins/+691g ins genotype (table 2). Both these tests were significant even when a conservative Bonferroni correction (correction for multiple testing for four SNPs) was performed.

The distribution of −851c/t genotypes did not differ significantly between patients with FEV1 values above and below 70% predicted (table 1). However, in a dominant/recessive model, a significantly higher proportion of patients with an FEV1 lower than 70% predicted were homozygous for the −851c allele than in the other group of patients (table 1). The mean FEV1 % predicted values were not significantly different between the three genotypes of −851c/t separately or in a dominant/recessive model (table 2).

The −308g/a and −238g/a polymorphic loci were not significantly associated with FEV1 values above or below 70% predicted (table 1). Also, the mean FEV1 % predicted values were not significantly different between the three genotypes, separately or in a dominant/recessive model, for −308g/a or −238g/a (table 2).

Records of the age at first onset of P aeruginosa infection were available for 119 patients with CF. We examined whether the age of first infection with P aeruginosa was associated with SNP alleles or genotypes in TNFα and found that +691g ins/+691g ins homozygotes were colonised with P aeruginosa at a significantly earlier age than...
important to neutralise age variability because a modulating variability in treatment which might affect disease status and were included from only two centres in order to exclude variables that might affect the phenotype, the following precautions were taken. Only patients homozygous to exclude patients in the present cohort of age selected patients supports the credibility of the chosen age group. We also determined whether we could compare the disease severity between subjects without the influence of sex since female CF patients are known to have significantly lower FEV1 % predicted values than males.14 No significant difference in sex corrected FEV1 % values between male and female patients were seen in our cohort of age selected patients. The frequencies of the tested alleles/genotypes and FEV1 % predicted values were not significantly different between the two populations, allowing us to group the patient populations together. All genotype distributions were in Hardy-Weinberg equilibrium except for −238g/a in the total Czech patient subgroup. A technical artefact is unlikely, given the fact that this marker was in Hardy-Weinberg equilibrium for all control groups and the Belgian patients with CF. Since the Hardy-Weinberg disequilibrium is only observed in the Czech CF patients and not the Czech controls, and since it is caused by the presence of two homozygotes for the −238a allele instead of the expected 0 to 1 homozygote, it is possible genetic factor may only become penetrant by age,1 which can only be detected in studies in age selected groups of patients. Furthermore, FEV1 % predicted values of patients with CF decline more rapidly with age than in the general population,13 and therefore may interfere with results when testing pulmonary function as a dependent of genotype distribution. The fact that FEV1 % values did not differ with the age of the patients in the present cohort of age selected patients supports the credibility of the chosen age group. We also determined whether we could compare the disease severity between subjects without the influence of sex since female CF patients are known to have significantly lower FEV1 % predicted values than males.14 No significant difference in sex corrected FEV1 % values between male and female patients were seen in our cohort of age selected patients. The frequencies of the tested alleles/genotypes and FEV1 % predicted values were not significantly different between the two populations, allowing us to group the patient populations together. All genotype distributions were in Hardy-Weinberg equilibrium except for −238g/a in the total Czech patient subgroup. A technical artefact is unlikely, given the fact that this marker was in Hardy-Weinberg equilibrium for all control groups and the Belgian patients with CF. Since the Hardy-Weinberg disequilibrium is only observed in the Czech CF patients and not the Czech controls, and since it is caused by the presence of two homozygotes for the −238a allele instead of the expected 0 to 1 homozygote, it is possible for the different SNPs because gap filling of data could not always be done successfully.

### Table 1: Distribution of TNFα genotypes according to FEV1 % predicted

<table>
<thead>
<tr>
<th>TNFα SNP</th>
<th>Genotype*</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+691g ins/del</td>
<td>ins/ins (n = 100) ins/del (n = 13)</td>
<td>67.5 (23.0) 79.7 (12.8) 12.2 3.5 to 21.0 0.008</td>
</tr>
<tr>
<td>−851c/c</td>
<td>cc (n = 82) ct/c (n = 30)</td>
<td>66.9 (23.6) 72.6 (20.2) 5.7 −15.3 to 4.0 0.25</td>
</tr>
<tr>
<td>−308g/a</td>
<td>ga (n = 90) ga (n = 22)</td>
<td>66.9 (22.7) 74.8 (22.4) 7.9 −2.8 to 18.6 0.15</td>
</tr>
<tr>
<td>−238g/a</td>
<td>gg (n = 101) gg (n = 11)</td>
<td>68.2 (22.4) 70.4 (27.6) 2.2 −16.6 to 21.1 0.8</td>
</tr>
</tbody>
</table>

*For each genotype the number of individuals with an FEV1 higher or lower than 70% predicted is given with the percentage in parentheses. The analysis was performed by a likelihood ratio ² test, or by the Fisher exact probability test (F) when more than 20% of the cells had an expected count of < 5.

†Significant results are shown in bold and the odds ratio (OR) is given for significant values.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+691g ins/del</td>
<td>ins/ins (n = 59) ins/del (n = 3)</td>
<td>48 (81.4%) 11 (18.6%) 0.009 (OR = 6.0)</td>
</tr>
<tr>
<td>−851c/c</td>
<td>cc (n = 54) ct/c (n = 1)</td>
<td>52 (96.3%) 2 (3.7%)</td>
</tr>
<tr>
<td>−308g/a</td>
<td>ga (n = 57) ga (n = 1)</td>
<td>45 (81.8%) 10 (18.2%) 0 (0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+691g ins/del</td>
<td>ins/ins (n = 56) ins/del (n = 2)</td>
<td>37 (64.9%) 19 (33.3%) 1 (1.8%) 0.07 (F) 0.04 (OR = 2.4)</td>
</tr>
<tr>
<td>−851c/c</td>
<td>cc (n = 55) ct/c (n = 1)</td>
<td>47 (85.5%) 7 (12.7%) 1 (1.8%)</td>
</tr>
<tr>
<td>−308g/a</td>
<td>ga (n = 54) ga (n = 1)</td>
<td>51 (89.5%) 5 (8.7%) 1 (1.8%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TNFα SNP</th>
<th>Mean (SD) FEV1 (% predicted)</th>
<th>Mean difference 95% CI p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+691g ins/del</td>
<td>ins/ins (n = 100) ins/del (n = 13)</td>
<td>67.5 (23.0) 79.7 (12.8) 12.2 3.5 to 21.0 0.008</td>
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<td>68.2 (22.4) 70.4 (27.6) 2.2 −16.6 to 21.1 0.8</td>
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</tbody>
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*The analysis was performed by a two sample t test. There may be a difference in the number of genotypes/alleles typed for the different SNPs because gap filling of data could not always be done successfully.

†Significant results are shown in bold.

Table 1: Distribution of TNFα genotypes according to FEV1 % predicted

Table 2: Differences in mean FEV1 % predicted values according to TNFα genotypes

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that the Hardy-Weinberg disequilibrium was observed because of the small sample size. The mean FEV₁ of the age selected cohort of CF patients in the current study is approximately 70% predicted, so patients with an FEV₁ of less than 70% predicted were compared with those having an FEV₁ higher than 70% predicted. We found an altered distribution of two TNFα polymorphisms depending on pulmonary function. Specifically, the presence of the +691g del allele was more likely to be associated with better pulmonary function and −851c homozygotes were found to be associated with worse pulmonary function than −851c/−851t + −851t/−851t CF patients. In our cohort of CF patients the −308g/a and the −238a allele, which has been associated with increased levels of TNFα and the −238a allele which has been associated with lower levels of TNFα, although others have found that −308a and −238a do not affect the levels of TNFα.

Apart from the association of +691g del with better CF pulmonary function, this variant was also significantly associated with an older first recorded/observed age of infection with P. aeruginosa. The most common pulmonary infection in the lungs of patients with CF is P. aeruginosa, and progression of lung disease unequivocally accelerates after colonisation with this organism. Therefore, the younger the first age of infection with P. aeruginosa, the more rapid the decline in pulmonary function. Mucoid P. aeruginosa is a key factor in accelerating the decline in pulmonary function in patients with CF. TNFα plays an important role in the innate resistance to P. aeruginosa and its clearance from the respiratory tract. Polymorphisms in the TNFα gene may lead to changes in levels of TNFα which may, in turn, increase the ability of the lung to clear P. aeruginosa. On the other hand, increased concentrations of TNFα have been found in lung secretions of CF patients which may contribute directly to neutrophil influx and elastase activity that eventually destroy the CF lung. Furthermore, if a certain TNFα allele has a quantitative or qualitative effect on the TNFα protein, it would most likely have a knock on effect on the secretion and synthesis of other members of the cytokine system such as the TNFα gene. Interestingly, the variability in FEV₁ % predicted values in patients with the +691g del variant is small and in the mild range, but not the most mild (fig 1). This may be due to the balance between the beneficial and detrimental properties of the TNFα protein.

The −691g ins/del SNP was not associated with the BMI in the age selected cohort of patients; however, analogous to the results for FEV₁ % predicted and age of first infection with P. aeruginosa, patients with the +691g del allele tended to have a slightly better BMI than those homozygous for the +691g ins allele.

Both variants of the +691g ins/del and −851c/t polymorphic loci were found to be associated with the degree of severity of CF disease. Variants of the +691g ins/del locus were associated with the severity of CF lung disease in three separate tests. In contrast to +691g ins/del, −851c/t was associated with pulmonary function in only one test and was not associated with pulmonary defence. The association of +691g ins/del variants with the severity of CF pulmonary disease therefore seems to be more important than the −851c/t variants. Of course, it is possible that these observed associations with CF disease severity are caused by another mutation in linkage disequilibrium, either in this gene or in neighbouring genes. In this regard, TNFα alleles have been shown to be in linkage disequilibrium with HLA alleles—for example, +691g del is linked to DRB*11*13 and DQB*301. In a previous study by Hull and Thomson in which an association was found between the −308a allele and a more severe CF phenotype, no other TNFα polymorphisms were tested. There have also been a number of investigations into the possible association of the TNFα −308a/g polymorphic locus with susceptibility to chronic obstructive pulmonary disease, but their conclusions were conflicting. In the present study no significant association was found between

<table>
<thead>
<tr>
<th>TNFα SNP</th>
<th>Mean (SD) first age of infection with P. aeruginosa</th>
<th>Mean difference</th>
<th>95% CI</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+691g ins/del</td>
<td>11.4 (6.0)</td>
<td>3.1</td>
<td>0.5 to 5.6</td>
<td>0.018</td>
</tr>
<tr>
<td>−851c/t</td>
<td>9.2 (4.3)</td>
<td>0.5</td>
<td>−2.6 to 1.5</td>
<td>0.60</td>
</tr>
<tr>
<td>−308g/a</td>
<td>9.5 (5.2)</td>
<td>1.0</td>
<td>−1.1 to 3.0</td>
<td>0.37</td>
</tr>
<tr>
<td>−238g/a</td>
<td>7.9 (6.6)</td>
<td>0.9</td>
<td>−5.2 to 3.3</td>
<td>0.64</td>
</tr>
</tbody>
</table>

*The analysis was performed by a two sample t test. There may be a difference in the number of genotypes typed for the different SNPs because gap filling of data could not always be done successfully.

†Significant results are shown in bold.

Table 3: Distribution of mean first age of infection with P. aeruginosa according to TNFα genotypes

Figure 1: Box plot showing median FEV₁ percentage predicted values with 25–75% quartiles. The mean (SD) FEV₁ % predicted values of CF patients with the +691g ins/del TNFα genotype (79.7 (12.8)%, n = 13) and those with the +691g ins/ins TNFα genotype (67.5 (23.0)%, n = 100) were significantly different (p = 0.008, two-sample t test).
−308g/a polymorphic variants and severity of CF disease. Furthermore, we found that the −308g allele tended to be associated with more severe CF disease, although not significantly. Possible explanations for these dissimilar results might be explained by the fact that we selected only F508del homozygotes in order to exclude disease variability because of the CFTR genotype, while Hul and Thomson included patients who were either F508del homozygotes or heterozygotes. Also, we tested more than twice as many patients, and our patients were all aged between 12 and 15 years with a mean age of 13.4 years compared with the previous study where the patients were all aged 8 years.

The allele frequencies of the TNFα polymorphisms in this study were very similar to those found among French controls by Herrmann et al. In that study, six different haplotypes from these polymorphisms could be constructed. One haplotype contained the −691g/del allele, the −851c allele, and the −308a allele. The −691g/del allele is only observed on this haplotype while the −308a allele appears on this haplotype and an additional haplotype. This may explain the conflicting data of the association of TNFα −308a in CF lung disease in the study by Hul and Thomson, and possibly the association of TNFα −308a in COPD. Indeed, it is feasible that only one of the two haplotypes on which −308a resides is the causal one, while the other cannot be discriminated when typing the −308a SNP alone. This might also explain why several studies have found that the rare −308a allele is associated with increased levels of TNFα while others have found that −308a does not affect levels of TNFα.

Our findings might be of interest from a pharmacogenetic point of view. Our results show that particular polymorphic TNFα loci are associated with better lung function. Anti-inflammatory agents are currently being used for the treatment of CF—for example, ibuprofen was found to reduce the rate of decline in FEV1 values in CF patients under 13 years of age. Since ibuprofen seems to lessen the effects of TNFα, it may be important to determine the genotype of TNFα polymorphic variants of a patient before deciding on treatment with anti-inflammatory agents. Indeed, administration of such drugs to CF patients may be of therapeutic value in some patients but detrimental in others, based on the TNFα genotype. It should be noted that ibuprofen was not administered to any of the Belgian CF patients in this study but it was given to six Czech CF patients (+691g ins homozygotes) for a few weeks only.

In conclusion, we did not find any evidence that the −308a/g polymorphic locus is associated with CF lung disease in Belgian and Czech patients with CF. However, our results indicate an involvement of TNFα in the modulation of CF disease since the −691g/ins/del and −851c/t loci are associated with lung disease severity. Functional studies are needed to confirm this association and to unravel the mechanism of modulation.

Further data on the primers used for amplification of the genomic regions covering the SNPs in TNFα and the single nucleotide extension oligonucleotides (table S1 and S2 respectively), the distribution of alleles/genotypes in the Belgian and Czech cohorts (table S3), and the association of TNFα variants with BMI (table S4) are available on the Thorax website at http://www.thoraxjnl.com/supplemental.

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LUNG ALERT

Surfactant replacement does not reduce duration of ventilatory support in paediatric acute lung injury


The quality and composition of surfactant are abnormal in acute lung injury. In adult patients surfactant replacement has had little effect on outcomes, but preliminary studies in paediatric patients have supported further research. The importance of the constitution of the administered surfactant is increasingly being recognised. Calfactant is a modified bovine surfactant that closely resembles endogenous surfactant in composition and function.

This study is a randomised controlled trial in American paediatric intensive care units (ICU) with all patients receiving a protective ventilation strategy. The study was designed to recruit 300 patients in 2 years. The number of ventilator free days in the 28 days following study entry, a marker of the duration of respiratory failure, was the primary outcome. Because of difficulties recruiting adequate numbers of patients, the trial ended after 3 years with 152 patients randomised. Patients were randomised to two doses of intratracheal Calfactant or air placebo.

There was no difference in primary outcome between the groups (mean (SD) ventilator free days 13.2 (10) in the treatment group and 11.5 (10.5) in the placebo group; p = 0.21). However, while the study was not powered to detect a mortality difference, mortality was reduced in the Calfactant group (15/77 patients versus 27/75, OR 2.32, 95% CI 1.15 to 4.85) and oxygenation also improved after treatment. No patient was removed because of treatment complications.

This study failed to show a difference in duration of mechanical ventilation in paediatric ICU patients, but was underpowered and illustrates the difficulties in recruiting adequate patient numbers even to well designed trials in ICU.

A MacDuff

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