CYSTIC FIBROSIS

Association of tumour necrosis factor alpha variants with the CF pulmonary phenotype

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Background: The pulmonary phenotype in patients with cystic fibrosis (CF), even in those with the same CF transmembrane conductance regulator (CFTR) genotype, is variable and must therefore be influenced by secondary genetic factors as well as environmental factors. Possible candidate genes that modulate the CF lung phenotype may include proinflammatory cytokines. One such protein is tumour necrosis factor α (TNFα), a member of the immune system.

Methods: Three polymorphic loci in the promoter (*−851c/t*, *−308g/a*, *−238g/a*) and one polymorphic locus in intron 1 (*+691g ins/del*) of the TNFα gene were typed by a single nucleotide primer extension assay in CF patients and healthy controls. Spirometric data and first age of infection with *Pseudomonas aeruginosa* were collected retrospectively from patients' medical records.

Results: An association was found between the TNFα *+691g ins/del* polymorphic locus and severity of CF lung disease. Patients heterozygous for *+691g ins* and *+691g del* were more likely to have better pulmonary function (mean (SD) forced expiratory volume in 1 second (FEV$_1$) 79.7 (12.8)% predicted) than patients homozygous for *+691g ins* (mean (SD) FEV$_1$ 67.5 (23.0)% predicted; p = 0.008, mean difference 12.2%, 95% CI 3.5 to 21.0). Also, patients heterozygous for *+691g ins* and *+691g del* were more likely to have an older first age of infection with *P aeruginosa* (mean (SD) 11.4 (6.0) years) than patients homozygous for *+691g ins* (mean (SD) 8.3 (4.6) years; p = 0.018, mean difference 3.1 years, 95% CI 0.5 to 5.6). An association was also found with the *−851c/t* polymorphic locus. In the group of patients with more severe FEV$_1$% predicted, a higher proportion of patients were homozygous for the *−851c* allele than in the other group of patients (p = 0.04, likelihood ratio χ^2, odds ratio = 2.4).

Conlusion: TNFα polymorphisms are associated with the severity of CF lung disease in Czech and Belgian patients with CF.

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Cystic fibrosis (CF) is a common lethal autosomal recessive disease affecting the white population with an incidence of about 1 in 2500.[1][2] 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.[3] 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.[4][5]

Tumour necrosis factor α (TNFα), an endogenous factor of the lung involved in host defence,[6] is therefore a possible modulator of CF pulmonary disease severity. TNFα is a proinflammatory cytokine largely produced by macrophages,[7] whose gene is located on chromosome 6p21.3.[8] It is prothrombotic, promotes leucocyte adhesion and migration, modulates haematopoiesis and lymphocyte development, induces other cytokines, and has an important role in macrophage activation and the immune response in tissues.[9] Even though there are many polymorphisms in the promoter

region of TNFα, much attention has been given to the TNFα *−308g/a* polymorphic locus. In a previous study of CF patients from a UK CF clinical centre, an association was found between the *−308a* allele and a more severe CF phenotype.[10] 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 TNFα and severity of lung function in CF patients. We therefore investigated whether the TNFα *−308g/a* polymorphic locus, together with the *−851c/t*, *−238g/a* promoter polymorphic loci and the polymorphic locus *+691g ins/del* in intron 1[11], 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

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FEV$_1$, forced expiratory volume in 1 second; MBL, mannose binding lectin; TNFα, tumour necrosis factor α

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 (FEV_1) were calculated according to Knudson et al.[12] Statistical analysis of lung function was performed on the last FEV_1 % 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.

Statistical analysis

Database management and statistical analysis were performed with the SAS statistical package release 8.01 (SAS Institute Inc, Cary, NC, USA) and the SYSTAT package release 7.0 (SPSS Inc, Chicago, IL, USA). Statistical tests were considered significant when their type I error was less than 0.05. Differences between the mean FEV_1 % predicted values of Czech and Belgian populations were assessed using a two-sample *t* test after checking whether the variances of the populations were equal or not. Comparison between one independent and one dependent qualitative variables were assessed using the χ^2 likelihood ratio test or by the Fisher exact probability test when more than 20% of the cells had an expected count of less than 5. When the independent variables were qualitative and the dependent one was quantitative, a parametric analysis of variance (ANOVA) was done. Pearson's correlation coefficient was calculated to test the goodness of fit of one independent quantitative value and one dependent quantitative value in a linear regression model.

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), mean FEV_1 (68.9% for Belgian patients and 68.4% for Czech patients, p = 0.91, mean difference 0.5%, 95% CI −9.3 to 8.3, two-sample *t* test) and mean FEV_1 % for each genotype ($-308g/a$: p = 0.71, $-851c/t$: 0.83, and $+691g$ ins/del: p = 0.65, ANOVA), so the data from the two populations were merged. In the combined groups there was no significant difference in allele and genotype frequencies between all CF patients compared with all controls (table S3).

FEV_1 % predicted values of CF patients decline with age[13] and females have a steeper decline in pulmonary function than males.[14] FEV_1 % predicted values calculated according to Knudson are corrected for sex and age.[12] Nevertheless, we determined whether the FEV_1 % 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 $r^2 = 0.006$) or sex (p = 0.21, mean difference 5.4%, 95% CI −13.7 to 3.0, two-sample *t* test) on FEV_1 % predicted.

We then determined whether the values of FEV_1 % 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,[5 15] only CF patients aged 12–15 years were included in the actual association study. The mean FEV_1 % of the age selected cohort of CF patients was approximately 70% predicted, so patients with an FEV_1 less than 70% predicted were compared with those with an FEV_1 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 FEV_1 was more than 70% predicted being heterozygous for the two alleles at the $+691g$ ins/del locus (table 1). More importantly, the mean FEV_1 % predicted values differed significantly between the two groups, patients with the $+691g$ ins/$+691g$ del genotype having a higher mean FEV_1 % 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 FEV_1 values above and below 70% predicted (table 1). However, in a dominant/ recessive model, a significantly higher proportion of patients with an FEV_1 lower than 70% predicted were homozygous for the $-851c$ allele than in the other group of patients (table 1). The mean FEV_1 % 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 FEV_1 values above or below 70% predicted (table 1). Also, the mean FEV_1 % 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

Table 1 Distribution of TNFα genotypes according to FEV$_1$ % predicted

TNFα SNP	Genotype*			p value†	
+691g ins/del	ins/ins	ins/del			ins/ins:ins/del
>70% FEV$_1$ predicted (n = 59)	48 (81.4%)	11 (18.6%)			**0.009** (OR = 6.0)
<70% FEV$_1$ predicted (n = 54)	52 (96.3%)	2 (3.7%)			
−851c/t	cc	ct	tt	cc:ct:tt	cc:ct+tt
>70% FEV$_1$ predicted (n = 56)	37 (64.9%)	19 (33.3%)	1 (1.8%)	0.07 (F)	**0.04** (OR = 2.4)
<70% FEV$_1$ predicted (n = 55)	45 (81.8%)	10 (18.2%)	0 (0%)		
− 308g/a	gg	ga	aa	gg:ga:aa	gg:ga+aa
>70% FEV$_1$ predicted (n = 57)	43 (75.4%)	14 (24.6%)	0 (0%)	0.15 (F)	0.18
<70% FEV$_1$ predicted (n = 54)	47 (85.5%)	7 (12.7%)	1 (1.8%)		
− 238g/a	gg	ga	aa	gg:ga:aa	gg:ga+aa
>70% FEV$_1$ predicted (n = 56)	51 (89.5%)	5 (8.7%)	1 (1.8%)	0.8 (F)	1.0
<70% FEV$_1$ predicted (n = 54)	50 (90.9%)	4 (7.3%)	1 (1.8%)		

*For each genotype the number of individuals with an FEV$_1$ higher or lower than 70% predicted is given with the percentage in parentheses. The analysis was performed by a likelihood ratio χ^2 test, or by the Fisher exact probability test (F) when more than 20% of the cells had an expected count of <5.
There may be a difference in 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 and the odds ratio (OR) is given for significant values.

+691g ins/+691g del heterozygotes (table 3). The mean first age of infection with *P aeruginosa* for the *+691g ins/del* genotypes did not differ between the Belgian and Czech CF cohorts (p = 0.21, ANOVA), indicating that there was no skewing to either of the two centres. No other variants were significantly associated with the first age of infection with *P aeruginosa* when the genotypes were tested separately or in a dominant/recessive model.

We also tested whether the BMI of the age selected cohort of patients was associated with SNP alleles or genotypes in TNFα. None of the TNFα variants was significantly associated with the BMI of these patients (see table S4 available on the *Thorax* website at http://www.thoraxjnl.com/supplemental).

DISCUSSION

The patients in this study were derived from two white European populations. We decided to group the patients in order to strengthen the statistical power of the study. In order to exclude variables that might affect the phenotype, the following precautions were taken. Only patients homozygous for the F508 deletion in the CFTR gene were included in order to exclude an effect of the CFTR genotype on disease. Patients were included from only two centres in order to exclude variability in treatment which might affect disease status and the measurement of clinical parameters of the patients. Only patients between 12 and 15 years of age were studied. It is important to neutralise age variability because a modulating genetic factor may only become penetrant by age,[5] which can only be detected in studies in age selected groups of patients. Furthermore, FEV$_1$ % 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 FEV$_1$ % 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 FEV$_1$ % predicted values than males.[14] No significant difference in sex corrected FEV$_1$ % values between male and female patients were seen in our cohort of age selected patients. The frequencies of the tested alleles/genotypes and FEV$_1$ % 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

Table 2 Differences in mean FEV$_1$ % predicted values according to TNFα genotypes

TNFα SNP	Mean (SD) FEV$_1$ (% predicted)*		Mean difference	95% CI	p value†
+691g ins/del	ins/ins (n = 100)	ins/del (n = 13)			
	67.5 (23.0)	79.7 (12.8)	12.2	3.5 to 21.0	**0.008**
−851c/t	cc (n = 82)	ct +tt (n = 30)			
	66.9 (23.6)	72.6 (20.2)	5.7	−15.3 to 4.0	0.25
−308g/a	gg (n = 90)	ga +aa (n = 22)			
	66.9 (22.7)	74.8 (22.4)	7.9	−2.8 to 18.6	0.15
−238g/a	gg (n = 101)	ga+aa (n = 11)			
	68.2 (22.4)	70.4 (27.6)	2.2	−16.6 to 21.1	0.8

*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 3 Distribution of mean first age of infection with *P aeruginosa* according to TNFα genotypes

TNFα SNP	Mean (SD) first age of infection with *P aeruginosa**		Mean difference	95% CI	p value†
+691g ins/del	ins/ins (n = 103) 8.3 (4.6)	ins/del (n = 16) 11.4 (6.0)	3.1	0.5 to 5.6	**0.018**
−851c/t	cc (n = 88) 8.6 (4.8)	ct+tt (n = 27) 9.2 (4.3)	0.5	−2.6 to 1.5	0.60
−308g/a	gg (n = 89) 8.5 (4.5)	ga+aa (n = 27) 9.5 (5.2)	1.0	−1.1 to 3.0	0.37
−238g/a	gg (n = 104) 8.8 (4.4)	ga+aa (n = 12) 7.9 (6.6)	0.9	−5.2 to 3.3	0.64

*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.

that the Hardy-Weinberg disequilibrium was observed because of the small sample size. The mean FEV_1 of the age selected cohort of CF patients in the current study is approximately 70% predicted, so patients with an FEV_1 of less than 70% predicted were compared with those having an FEV_1 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 *−238g/a* polymorphic loci were not associated with severity of pulmonary disease. It should be noted that the functional consequences of these polymorphisms on TNFα are not known; however conflicting results have been found for the *−308a* 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.[16–18]

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*,[19] and progression of lung disease unequivocally accelerates after colonisation with this organism.[20] 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.[21] TNFα plays an important role in the innate resistance to *P aeruginosa* and its clearance from the respiratory tract.[22 23] Polymorphisms in the TNFα gene may lead to changes in levels of TNFα[16] 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[7 24] which may contribute directly to neutrophil influx and elastase activity that eventually destroy the CF lung.[7] 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 cascade.[9] There is probably a balance between its activity in protecting the immune system against pathogens and its destructive side effects. It is feasible that this balance may be tipped in one or other direction when a patient has a certain polymorphic variant in an important mediator of the immune system such as the TNFα gene. Interestingly, the variability in FEV_1 % 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 FEV_1 % 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 DQB1*301.[25]

In a previous study by Hull and Thomson[10] 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 *−308g/a* polymorphic locus with susceptibility to chronic obstructive pulmonary disease, but their conclusions were conflicting.[26] In the present study no significant association was found between
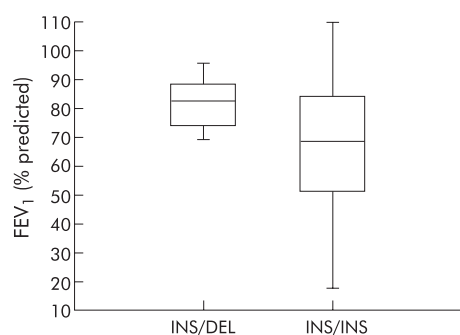

Figure 1 Box plot showing median FEV_1 percentage predicted values with 25–75% quartiles. The mean (SD) FEV_1 % predicted values of CF patients with the *+691g ins/del* TNFα genotype (79.7 (12.8)%, n = 13) and those with the *+691g ins/ins* TNFα genotype (67.5 (23.0)%, n = 100) were significantly different (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 Hull and Thomson[10] 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.[11] 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.[26] This may explain the conflicting data of the association of TNFα −308a in CF lung disease in the study by Hull and Thomson,[10] and possibly the association of TNFα −308a in COPD.[27–30] Indeed, it is feasible that only one of the two haplotypes on which −308a resides is the causal one, but which of them cannot be discriminated when typing the −308g/a 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.[16]

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[31]—for example, ibuprofen was found to reduce the rate of decline in FEV₁ values in CF patients under 13 years of age.[32] Since ibuprofen seems to lessen the effects of TNFα,[33] 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 −308g/a TNFα 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 (tables 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

▲ Willson DF, Thomas NJ, Markovitz BP, *et al.* Effect of exogenous surfactant (Calfactant) in pediatric acute lung injury: a randomized controlled trial. *JAMA* 2005;**293**:470–6

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.

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TABLE S1. PRIMERS FOR PCR AMPLIFICATION

SNP*	Primer†	Genbank	Nucleotide Sequence (5'→3')	Annealing Temperature	Length
	1840.5	Y14768	CTAAGTGTGTATGGAGTGAATG	57°C	296 bp
+691g ins/del	2135.3	Y14768	CCCTGAGGTGTCTGGTTTTC		
	196.5	Y14768	GAAGCAAAGGAGAAGCTGAGA	56°C	948 bp
-851c/t, -308g/a, and *-238g/a*	1143.3	Y14768	TCTGGAGGAAGCGGTAGTGG		

*SNP nomenclature according Herrmann et al. (1998) *Eur J Clin Invest* 28:59-66.

† The number preceding the decimal point refers to the nucleotide position of the Genbank DNA sequence of the specified accession number given in the neighboring column, which also corresponds to the 5' nucleotide of the oligonucleotide. The annotation '.5' refers to a forward primer and '.3' refers to a reverse primer. The annealing temperature for amplification and the length of the obtained PCR products are also given.

TABLE S2. SINGLE NUCLEOTIDE PRIMER EXTENSION OLIGONUCLEOTIDES

TNFα SNP*	Primer†	Genbank	Nucleotide Sequence (5'→3') ‡
+691g ins/del	1939.5	Y14768	AAAAGTGTGAGAAGAGAGATGGGG
-851c/t	394.3	Y14768	$(AAA)_{11}$ ACTCTACATGGCCCTGTCTTC ‡
-308g/a	943.5	Y14768	$(AAA)_9$ ACAATAGGTTTTGAGGGGCATG
-238g/a	1013.5	Y14768	$(AAA)_{13}$ AGAAGACCCCCCTCGGAATC

* SNP nomenclature according to Herrmann et al. (1998) *Eur J Clin Invest* 28:59-66.

† The number refers to the polymorphic nucleotide position of the Genbank DNA sequence of the specified accession number given in the neighbouring column. The annotation '.5' refers to a forward primer and '.3' refers to a reverse primer.

‡ The length of the oligonucleotide may be increased through addition of an A-tail; $(AAA)_n$ means 3 x n A nucleotides.

‡A reverse oligonucleotide is used.

TABLE S3. DISTRIBUTION OF ALLELES AND GENOTYPES OF SNPS IN TNFα IN CF PATIENTS AND CONTROLS

TNFα SNP	Genotype and Allele Frequency	Cystic Fibrosis Patients*			Control Subjects*		
		Czech	Belgian	Total	Czech	Belgian	Total
+691g ins/del	ins/ins n(%)	106(89.8)	48(85.7)	154(88.5)	96(94.1)	79(92.9)	175(93.6)
	ins/del n(%)	12(10.2)	8(14.3)	20((11.5)	6(5.9)	6(7.1)	12(6.4)
	del/del n(%)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	ins n(%)	224(94.9)	104(92.9)	328(94.3)	198(97.1)	164(96.5)	362(96.8)
	del n(%)	12(5.1)	8(7.1)	20(5.7)	6(2.9)	6(3.5)	12(3.2)
-851c/t	cc n(%)	87(75.7)	42(77.8)	129(76.3)	87(74.3)	64(78.1)	151(75.9)
	ct n(%)	27(23.5)	11(20.4)	38(22.5)	27(23.1)	17(20.7)	44(22.1)
	tt n(%)	1(0.9)	1(1.85)	2(1.2)	3(2.6)	1(1.2)	4(2.0)
	c n(%)	201(87.4)	95(88.0)	296(87.6)	201(85.9)	145(88.4)	346(86.9)
	t n(%)	29(12.6)	13(12.0)	42(12.4)	33(14.1)	19(11.6)	52(13.1)
-308g/a	gg n(%)	88(76.5)	42(76.4)	129(75.9)	92(78.0)	63(76.8)	155(77.5)
	ga n(%)	23(20.0)	13(23.6)	37(21.8)	23(19.5)	18(22.0)	41(20.5)
	aa n(%)	4(3.5)	0(0.0)	4(2.4)	3(2.5)	1(1.2)	4(2.0)
	g n(%)	199(86.5)	97(88.2)	295(86.8)	207(87.7)	144(87.8)	351(87.8)
	a n(%)	33(14.2)	13(11.8)	45(13.2)	29(12.3)	20(12.2)	49(12.2)
-238g/a†	gg n(%)	103(89.6)	47(85.5)	150(88.2)	106(90.6)	73(89.0)	179(90.0)
	ga n(%)	10(8.7)	8(14.6)	18(10.6)	11(9.4)	9(11.0)	20(10.0)
	aa n(%)	2(1.7)	0(0.0)	2(1.2)	0(0.0)	0(0.0)	0(0.0)
	g n(%)	216(93.9)	102(92.7)	318(93.5)	223(95.3)	155(94.5)	378(95.0)
	a n(%)	14(6.1)	8(7.3)	22(6.5)	11(4.7)	9(5.5)	20(5.0)

*For each genotype/allele, the number of individuals/alleles is given; the percentage is given between brackets. The distribution of alleles/genotypes in the Belgian controls, Czech controls, Belgian CF patients, Czech CF patients and total patient and control populations were not significantly different as tested by likelihood χ^2 ratio or by the Fisher exact probability test when more than 20% of the cells had an expected count of less than 5.

There may be a difference in number of genotypes/alleles typed for the different SNPs, since that gap-filling of data could not always be successfully completed.

† Czech patients were not in Hardy Weinberg equilibrium for this SNP, all other groups were in Hardy Weinberg equilibrium.

TABLE S4. DISTRIBUTION OF MEAN BMI ACCORDING TO TNFα GENOTYPES

TNFα SNP	Mean (SD) BMI [kg/m^2]*		Mean Difference	95%CI	p-value
+691g ins/del	ins/ins (n=100) 16.2 (2.1)	ins/del (n=13) 17.2 (1.8)	1.0	-0.2 to 2.1	0.11
-851c/t	cc (n=81) 16.3 (2.1)	ct+tt (n=30) 16.4 (1.9)	0.1	-1.0 to 0.8	0.85
-308g/a	gg (n=90) 16.3 (2.1)	ga+aa (n=22) 16.1(1.8)	0.2	-1.2 to 0.8	0.66
-238g/a	gg (n=101) 16.3 (2.1)	ga+aa (n=11) 16.4 (2.4)	0.1	-1.2 to 1.4	0.87

*The analysis was performed by a two-sample t-test. There may be a difference in number of genotypes/alleles typed for the different SNPs, since that gap-filling of data could not always be successfully completed.