Hum Genet (2001) 108 : 216–221
DOI 10.1007/s004390100467

ORIGINAL INVESTIGATION

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# *CFTR* gene mutations – including three novel nucleotide substitutions – and haplotype background in patients with asthma, disseminated bronchiectasis and chronic obstructive pulmonary disease

Received: 11 July 2000 / Accepted: 8 January 2001 / Published online: 28 February 2001


**Abstract** In order to investigate the incidence of cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations and unclassified variants in chronic pulmonary disease in children and adults, we studied 20 patients with asthma, 19 with disseminated bronchiectasis (DB) of unknown aetiology, and 12 patients with chronic obstructive pulmonary disease (COPD), and compared the results to 52 subjects from the general Greek population. Analysis of the whole coding region of the *CFTR* gene and its flanking intronic regions revealed that the proportion of *CFTR* mutations was 45% in asthma ($P<0.05$), 26.3% in DB ($P>0.05$), 16.7% in COPD ($P>0.05$), compared to 15.4% in the general population. Seventeen different molecular defects involved in disease predisposition were identified in 16 patients. Three potentially disease-causing mutations, T388 M, M1R and V11I, are novel, found so far only in three asthma patients. The hyperactive M470 allele was found more frequently in COPD patients (frequency 70.8%, $P<0.01$) than in the controls. The study of the TGmTnM470 V polyvariant *CFTR* allele revealed the presence of CFTR function-modulating haplotypes TG13/T5/M470, TG11/T5/M470, TG12/T5/V470 and TG12/T7, combined with M470 or V470, in six asthma patients, four DB patients ($P<0.01$), and two COPD patients ($P<0.05$). These results confirm the involvement of the *CFTR* gene in asthma, DB and possibly in COPD.

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## Introduction

Cystic fibrosis (CF) is a severe autosomal recessive genetic disease which affects various organ systems. It is the most common respiratory single gene disorder, and 4% of the Caucasoid population are asymptomatic carriers of mutations in the *CFTR* gene (Welsh et al. 1995). The phenotypic spectrum associated with mutations in the *CFTR* gene extends beyond the classically defined CF. Besides patients with atypical CF, there are large numbers of so-called monosymptomatic diseases associated with *CFTR* mutations, such as various forms of obstructive azoospermia, idiopathic pancreatitis and disseminated bronchiectasis. CFTR is expressed in several cell types of the airway, has multiple functions in epithelial cells and mutations in the *CFTR* gene have been found in several lung disorders.

Heterozygosity for the F508del mutation was identified in patients with disseminated bronchiectasis (DB) and bronchial hypersecretion (Dumur et al. 1990; Poller et al. 1991; Gervais et al. 1993). A more detailed screening of the *CFTR* gene revealed the presence of *CFTR* mutations or rare variants in 37.5% of patients with DB and in two of 12 patients with chronic obstructive pulmonary disease (COPD) (Pignatti et al.1995). Screening for the presence of the IVS8-5T allele showed it to be significantly increased in 31% of bronchiectasis patients, compared with normal control subjects (6%) (Pignatti et al. 1996). Another study of patients with DB found that 34% had one or two *CFTR* gene mutations. The IVS8-5T allele was not found in their group of patients (Girodon et al. 1997).

Studies to assess the association between asthma and the *CFTR* gene have led to conflicting results (Schroeder et al. 1995; Mennie et al. 1995; Dahl et al. 1998). A more complete study, characterizing the whole of the *CFTR* gene in adult patients with asthma, identified missense mutations in 15% and an additional 8%, without missense

mutations, which had the IVS8-5T allele. Based on the additional finding that almost all (90%) of the asthma patients with missense mutations had the hyperactive M470 allele, compared with 63% of the asthma patients without missense mutations, the authors suggested a putative role for a combination of *CFTR* mutations, including the M470 allele, in the genetic variability of asthma (Lazaro et al. 1999).

The allele present at the polymorphic locus M470 V affects the biogenesis of CFTR protein and the gating of the CFTR channel. The M470 CFTR proteins had a 1.7-fold increased intrinsic chloride activity compared with the V470 CFTR proteins. Also, the combination of the different alleles at the TGm and Tn loci of intron 8 splice branch/acceptor site modulated exon 9 skipping, thereby producing non- functional CFTR proteins, affecting the net chloride transport activity of CFTR expressing cells. These polyvariant alleles, in combination with mutations, can affect the final phenotypic expression of the mutation (Chu et al. 1993; Cuppens et al. 1998; Niksic et al. 1999).

We evaluated the frequency and nature of mutations, rare variants and polyvariant allele TGmTnM470 V in the *CFTR* gene in 51 patients with normal sweat chloride values, suffering from asthma, DB and COPD, compared to 52 subjects from the general Greek population.

## Materials and methods

### Sample composition

This study included unrelated Greek patients affected by asthma (20 patients, of whom 19 were children and 1 adult), disseminated bronchiectasis of unknown aetiology (19 patients, of whom 3 were children and 16 adults), and chronic obstructive pulmonary disease (12 patients, of whom 4 were children and 8 adults). The age range of the children was 9 months to 16 years and of the adults, 25–67 years. All 51 patients had normal sweat chloride levels (<60 mEq/l) and none had any of the classic symptoms of CF, including malabsorption. One asthma subject suffered from sinusitis, and two DB patients were positive by culture for *Pseudomonas* colonization. Familiar aggregation of respiratory symptoms was present in 4 out of 20 asthma patients.

The control group consisted of 52 subjects from the general Greek population, spouses of CF carriers and β-thalassaemia children, none of whom presented any signs of pulmonary disease.

### Mutation analysis

Genomic DNA was extracted from white blood cells as previously described (Miller et al. 1988). The presence of mutations in the 27 exons and neighbouring intronic regions of the *CFTR* gene was assessed by denaturing gradient gel electrophoresis (DGGE), following simple or multiplex PCR of patient DNA (Fanen et al. 1992; Costes et al. 1993; Tzetis et al. 1997). The cryptic splice mutation 3849+10KbC>T was analysed by restriction analysis (Highsmith et al. 1994).

All DNA samples showing a shift in DGGE mobility, and not presenting a pattern of a known mutation, were sequenced using an automatic DNA sequencer (Vistra, model 725-Molecular Dynamics) in order to identify the mutation (Kanavakis et al. 1998).

The intron 8 thymidine (IVS8-polyT) tract was analysed using an allele-specific PCR assay, which readily distinguished between the 5T, 7T and 9T alleles. The conditions for the allele-specific PCR were as described (Friedman et al. 1997), with the exception that AmpliTaq-Gold (Perkin Elmer–Roche) was used as a thermostable polymerase, and the internal control primer pair amplified a 323 bp fragment 3' to the $G_{\gamma}$-globin gene (Kanavakis et al. 1997).

The phase of the IVS8-(TG)mTn allele was determined (after the characterization of the Tn locus) by PCR amplification of the DNA samples with primers 9i5' (5'-TAATGGATCATGGGCCATGT-3') and E9R3 (5'-CTCAAATAATTCCCCAAATCCC-3'). Primer E9R3 was 5'-labelled with the fluorescent moiety Texas Red. Thirty PCR cycles (hot-start PCR, using AmpliTaq-Gold polymerase) were performed with annealing at 55°C. The size of the PCR products was determined after electrophoresis in a 6% denaturing acrylamide gel. The Macintosh computer software (Vistra 725 version 2.0 and Fragmentor analysis software) performed all the data collection and sizing (TG11/T7 allele: 148 bp).

### Statistical analysis

The frequency of mutations was determined by counts of patients. Differences between proportions were compared by the chi-square statistic or Fisher's exact test using the SPSS program. All *P* values were based on two-sided comparisons and values of less than 0.05 were considered to indicate statistical significance.

## Results

DGGE analysis of the whole coding and neighbouring intronic sequences of the *CFTR* gene was performed for the 51 patients with pulmonary disease (asthma, DB and COPD) and the 52 controls. A total of 17 different mutations deemed to be involved in disease predisposition, including three novel mutations (T388 M, M1R, V11I), were identified in 16 patients. More specifically, *CFTR* mutations were found in 9/20 patients with asthma ($P<0.05$), in 5/19 patients with DB ($P>0.05$) – one of whom was a compound heterozygote (F508del/S977F) – and in 2/12 patients with COPD ($P>0.05$). In the control group, CFTR mutations were detected in 8/52 subjects.

Of the 17 mutations in the patients, 9 (Y301C, I148T, R297Q, S1235R, T896I, S977F, L997F, F1052 V, A120T) have been listed by the Cystic Fibrosis Genetic Analysis Consortium (see website: http://www.cf.genet.sickkids.on.ca) as disease-causing mutations, while 3 (R668C, R75Q, I1027T) have been listed as sequence polymorphisms. R75Q and R668C have been reported with high frequencies in patients with DB, asthma and congenital bilateral absence of vas deferens (CBAVD) (Pignatti et al. 1995; Pignatti et al. 1996; Girodon et al. 1997; Lazaro et al. 1999; Kanavakis et al. 1998). Mutation D565G had previously been reported in a CBAVD patient (Kanavakis et al. 1998). Three novel mutations, T388 M, M1R and V11I, were first identified in this study, and were found only among asthma patients, not in the control group or in the other pulmonary disease patients.

The IVS8-5T allele was found in 2/20 asthma patients ($P<0.05$), in 1/19 DB patients ($P>0.05$), 1/12 COPD patients ($P<0.05$) and in 1/52 of the controls. Two of the 51 patients (1 asthma and 1 COPD) were homozygous for the 5T allele. The detailed distribution of all the *CFTR* gene mutations found in the subjects participating in the study are shown in Table 1 and Table 2.

**Table 1** *CFTR* genotypes of pulmonary disease patients and controls (*DB* disseminated bronchiectasis, *COPD* chronic obstructive pulmonary disease)

| Clinical status | Total tested | No. of cases | *CFTR* gene mutation[a] | IVS8-(T)n | IVS8-(TG)m | M470 V |
|---|---|---|---|---|---|---|
| Asthma | 20 | 1 | L997F, T338M[b] | 9/7 | 10/12 | M/V |
| | | 1 | Y301C | 7/7 | 11/11 | V/V |
| | | 1 | M1R[b], V11I[b] | 7/7 | 12/10 | M/M |
| | | 1 | I148T/– | 9/9 | 10/10 | M/V |
| | | 1 | L997F/– | 9/9 | 11/9 | M/V |
| | | 1 | R297Q/– | 5/5 | 13/11 | M/M |
| | | 1 | R297Q/– | 7/7 | 11/11 | V/V |
| | | 1 | R75Q/– | 7/7 | 11/11 | V/V |
| | | 1 | A120T/ | 5/7 | 11/11 | V/V |
| | | 1 | –/– | 7/7 | 11/12 | M/V |
| | | 1 | –/– | 7/9 | 11/11 | M/M |
| | | 2 | –/– | 7/7 | 12/10 | M/V |
| | | 7 | –/– | 7/7 | 11/11 | V/V |
| DB | 19 | 1 | F508del, I1027T | 9/9 | 10/10 | M/M |
| | | 1 | D565G, R668C | 7/7 | 11/11 | M/V |
| | | 1 | T896I/– | 7/7 | 11/10 | M/V |
| | | 1 | I148T/– | 7/9 | 11/10 | M/V |
| | | 1 | F508del/S977F | 5/9 | 12/10 | M/V |
| | | 1 | –/– | 7/9 | 12/10 | V/V |
| | | 1 | –/– | 7/9 | 10/10 | M/V |
| | | 1 | –/– | 7/7 | 11/12 | M/M |
| | | 2 | –/– | 7/7 | 11/10 | 1 M/V, 1 V/V |
| | | 2 | –/– | 7/7 | 12/10 | 1 V/V, 1 M/M |
| | | 3 | –/– | 7/9 | 11/10 | 1 M/M, 2 V/V |
| | | 4 | –/– | 7/7 | 11/11 | 1 V/V, 3 M/V |
| COPD | 12 | 1 | F1052 V/– | 7/7 | 11/10 | M/V |
| | | 1 | S1235R/– | 7/9 | 12/10 | M/M |
| | | 1 | –/– | 5/5 | 11/12 | M/V |
| | | 1 | –/– | 7/9 | 10/10 | M/M |
| | | 2 | –/– | 7/9 | 11/10 | 1 M/M,1 M/V |
| | | 3 | –/– | 7/7 | 11/10 | M/V |
| | | 3 | –/– | 7/7 | 11/11 | 1 M/V, 2 M/M |
| Controls | 52 | 1 | F508del/– | 7/9 | 10/10 | M/M |
| | | 1 | F1052 V/– | 5/7 | 10/11 | M/V |
| | | 1 | F1052 V/– | 7/7 | 11/11 | M/M |
| | | 1 | R668C, D565G/– | 7/7 | 11/11 | M/M |
| | | 1 | R688C, D565G/– | 7/7 | 11/10 | M/V |
| | | 1 | R75Q/– | 7/7 | 11/11 | V/V |
| | | 1 | R297Q/– | 7/7 | 11/10 | M/V |
| | | 1 | L997F/– | 7/9 | 10/10 | M/V |
| | | 1 | –/– | 7/7 | 10/10 | M/V |
| | | 1 | –/– | 7/9 | 10/10 | M/M |
| | | 1 | –/– | 7/9 | 12/10 | M/M |
| | | 4 | –/– | 7/9 | 11/10 | 1 M/M, 1 V/V, 2 M/V |
| | | 15 | –/– | 7/7 | 11/10 | 13 M/V, 2 V/V |
| | | 22 | –/– | 7/7 | 11/11 | 18 V/V, 3 M/V, 1 M/M |

[a]A minus sign (–) denotes absence of *CFTR* mutation after DGGE analysis of all 27 exons and adjoining intronic sequences

[b]Novel mutations, reported for the first time in this study

## Novel mutations

In this study three novel missense mutations in highly conserved residues (Tucker et al. 1992), T388 M, M1R and V11I, were found in the asthma patients.

The T388 M (C>T at nucleotide 1295 of exon 8) occurs in the vicinity of the sixth transmembrane segment, resulting in a nonpolar hydrophobic (methionine) substitution for a polar uncharged (threonine) amino acid. Moreover threonine at position 388 is conserved in human, bovine, *Xenopus* and fish but not in mouse (methionine at that position).

The M1R (T>G at nucleotide 134 of exon 1) missense mutation (Met nonpolar hydrophobic to Arg positively charged) occurs at the translation initiation codon, preventing translation initiation. Four other mutations have

**Table 2** Characteristics and allele frequencies of the 17 mutations found in the pulmonary disease patients and the control population (*n* represents the number of cases for each group). The proportion of CFTR alleles in each group is expressed as *c/d* (*e*), where *c* indicates the number of alleles with the genotype indicated at left, *d* indicates the number of total alleles examined in each group and *e* represents the percentage

| Mutation[a] | Control population (*n*=52) | Pulmonary disease patients | | | Greek CF patients (PS; PI) (*n*=426) |
|---|---|---|---|---|---|
| | | Asthma (*n*=20) | DB (*n*=19) | COPD (*n*=12) | |
| R75Q *(356 G/A, exon 3)* | 1 (0.96%) | 1 (2.5%) | – | – | 1 (0.1%) |
| R668C *(2134 C/T, exon 13)* | 2 (1.9%) | – | 1 (2.6%) | – | 1 (0.1%) |
| L997F *(3123 G>C, exon 17a)* | 1 (0.96%) | 2 (5%) | – | – | – |
| F508del | 1 (0.96%) | – | 2 (5.3%) | – | 465 (54.6%) |
| D565G *(A>G at 1825, exon 12)* | 2 (1.9%) | – | 1 (2.6%) | – | 1 (0.1%) |
| F1052 V *(T>G at 3286, exon 17b)* | 2 (1.9%) | – | – | 1 (4.2%) | 1 (0.1%) |
| R297Q *(G>A at 1022, exon 7)* | 1 (0.96%) | 2 (5%) | – | – | – |
| Y301C *(A>G at 1034, exon 7)* | – | 1 (2.5%) | – | – | – |
| I148T *(T>C at 575, exon 4)* | – | 2 (5%) | – | – | 1 (0.1%) |
| T388M[b] *(C>T at 1295, exon 8)* | – | 1 (2.5%) | – | – | – |
| M1R[b] *(T>G at 134, exon 1)* | – | 1 (2.5%) | – | – | – |
| V11I[b] *(G>A at 163, exon 1)* | – | 1 (2.5%) | – | – | – |
| I1027T *(3212 T/C, exon 17a)* | – | – | 1 (2.6%) | – | 1 (0.1%) |
| T896I *(C>T at 2819, exon 15)* | – | – | 1 (2.6%) | – | – |
| S977F *(C>T at 3062, exon 16)* | – | – | 1 (2.6%) | – | – |
| A120T *(G>A at 490, exon 4)* | – | 1 (2.5%) | – | – | – |
| S1235R *(T>G at 3837, exon 19)* | – | – | – | 1 (4.2%) | – |

[a]Mutation name according to the Cystic Fibrosis Genetic Analysis Consortium
[b]Novel mutations, reported for the first time in this study

**Table 3** Frequency of M470 and (TG)mTn alleles in pulmonary disease patients and controls *(DB* disseminated bronchiectasis, *COPD* chronic obstructive pulmonary disease, *n* number of cases, *ND* not detected)

| Clinical status | Allele | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M470 | TG11/T7 | TG10/T7 | TG12/T7 | TG10/T9 | TG11/T5 | TG12/T5 | TG13/T5 |
| Asthma[a] (*n*=20) | 13 (32.5%) | 23 (57.5%) | 3 (7.5%) | 5 (12.5%) | 3 (7.5%) | 2 (5%) | ND | 1 (2.5%) |
| DB (*n*=19) | 17 (44.7) | 18 (47.4%) | 6 (15.8%) | 4 (10.5%) | 9 (23.7%) | ND | 1 (2.6%) | ND |
| COPD (*n*=12) | 17 (70.8) | 12 (50%) | 5 (20.8%) | 1 (4.2%) | 4 (16.7%) | 1 (4.2%) | 1 (4.2%) | ND |
| Controls (*n*=52) | 37 (35.5%) | 71 (68.3%) | 23 (22.1%) | 1 (0.96%) | 6 (5.8%) | 1 (0.96%) | ND | ND |

[a]Alleles TG11/T9 (2) and TG9/T9 (1) also detected

been found that affect the same codon, of which M1 K affects the same nucleotide (T>A) (Cystic Fibrosis Genetic Analysis Consortium website).

The V11I (G to A at nucleotide 163 of exon 1) is a conservative amino acid substitution. Valine at that position is evolutionarily conserved only in human and bovine.

### Polymorphisms

The proportion of M470 (polymorphic variant 1540A/G, M470 V) was 32.5% (13/40 alleles) in the asthma patients ($P$>0.05), 44.7% (17/38 alleles) in the DB patients ($P$>0.05), 70.8% (17/24 alleles) in the COPD patients ($P$<0.01) and 35.5% (37/104 alleles) in the control population (Table 3).

For the polymorphic IVS8(TG)mTn, haplotype TG11/T7 was the most common in all groups studied. CFTR function-modulating haplotypes TG12/T7, TG11/T5, TG12/T5 and TG13/T5 showed statistically significantly higher frequencies in the pulmonary disease patients than in controls ($P$<0.05) (Table 3).

Eight silent mutations (no change in the coded amino acid) in the coding region of *CFTR* (492G/A, 1716G/A, 2694T/G, 3030G/A, 3417A/T, 4002 A/G, 4404C/T and 4521 G/A) and six nucleotide changes in the non-coding region (125G/C, 405+46G/T, 875+40A/G, 1001+11C/T, 3041-71 G/C and 4374+13 A/G) were detected in all the patient groups (asthma, DB and COPD) and the control population with similar frequencies, except for 2694T/G. Representation of G allele, was statistically significantly higher than expected in the patient group (controls T 73.1% vs G 26.9%, pulmonary disease patients T 58.3% vs G 41.7% [$P$<0.05]).

## Discussion

This study describes the characterization of *CFTR* mutations and unclassified variants in 20 asthma, 19 DB and 12 COPD patients. It confirms a statistically significant increase of *CFTR* gene mutations (45% heterozygotes, $P$<0.05) and of the IVS8-5T allele (10% carriers, $P$<0.05) in asthma. In all three pulmonary disease patient groups a statistically significant increase was found for the exon 9-modulating polyvariant alleles TG11/T5, TG12/T5 and TG13/T5 (Table 3). Additionally the IVS8-5T (8.3% carriers, $P$<0.05) and the M470 polymorphic variant (17/24

alleles, P<0.01) were both found more frequently in patients with COPD. Amongst the 52 controls, 15.4% were found to be carriers of *CFTR* mutations and unclassified variants, and 1.9%, carriers of the IVS8-5T allele (Table 1, Table 2 and Table 3).

### Asthma patients

Of the 20 asthma patients, 9 were carriers of *CFTR* gene missense mutations affecting evolutionary conserved residues, which are expected to determine changes in the amino acid sequence of the CFTR protein. Of them, one was homozygous for the IVS8-5T allele and also carried mutation R297Q, and another carrier of mutation A120T was heterozygous for the IVS8-5T allele. The phase of the IVS8-5T allele regarding the A120T mutation is not known, but the patient with the R297Q mutation could be considered a CF compound heterozygote.

Mutation L997F, previously reported with high frequency in asthma patients, was found twice in this study. It was first described in a patient with a borderline high sweat chloride value and features suggestive of CF (see Cystic Fibrosis Genetic Analysis Consortium website). It has also been associated with DB, CBAVD, sarcoidosis and recurrent idiopathic pancreatitis (Dork et al. 1997, Bombieri et al. 1998, Lazaro et al. 1999, Bombieri et al. 2000, Lira et al. 2000).

### *DB patients*

Four missense mutations and F508del were detected in five DB patients. Two patients had two mutations each (F508del and I1027T; D565G and R668C). Family members were not available in either of the above cases; therefore segregation analysis was not possible.

Mutation D565G is a novel Greek mutation previously reported by us in a CBAVD patient (Kanavakis et al. 1998). It is interesting that this CBAVD patient also carried R668C, had a negative sweat chloride test, but no pulmonary symptoms. Both the DB patient and the CBAVD subject were 7T homozygous. In this study, the 49-year-old male patient had pulmonary symptoms (FVC and FEV1 values are 66% and 37% of predicted, respectively) and frequent infections since the age of 3 years. He had a son aged 18 years, which indicated that he was fertile. The only genotypic difference between the CBAVD subject and the DB patient was the presence of variant 1716G/A (E528E), which has been reported to mildly affect normal splicing of exon 10 (Cuppens et al. 1998, Dork et al. 1997). This might explain the phenotypic difference between the CBAVD and DB patient.

Mutation T896I, previously reported in an asthma patient, was found in one DB patient. It involves an evolutionarily conserved residue and is a non-conservative amino acid substitution. It could therefore be considered a CF causative mutation (Lazaro et al. 1999). Mutation I148T is a CF-causing mutation, first reported in a pancreatic-insufficient CF patient (Cystic Fibrosis Genetic Analysis Consortium website).

One DB patient was a compound heterozygote, F508del/S977F, and also carried the IVS8-5T allele. This 53-year-old female patient had had bronchiectasis since childhood.

### COPD patients

Amongst the 12 COPD patients, two carried mutations F1052 V and S1235R. In addition, a single COPD patient was homozygous for the 5T allele but carried no other CF mutation. It is interesting that the hyper-functional M470 allele is over-represented in COPD patients (70.8% vs 35.5% in the control population, $P<0.01$). Similar results were found in a group of asthma patients (Lazaro et al. 1999).

### (TG)mTn allele

Five asthma, three DB and one COPD patient carried haplotype TG12/T7 combined with M470 or V470, from which 20–23% of *CFTR* transcripts produced lack exon 9 (Cuppens et al. 1998) (Table 3). This particular haplotype had a frequency of only 0.96% in the control population, associated with M470.

Two asthma patients (genotypes: R297Q/-, A120T/-) carried haplotypes TG13/T5/M470, TG11/T5/M470 and TG11/T5/V470, which have been found almost exclusively among CBAVD patients and not among those with normal *CFTR* genes (Costes et al. 1995). Haplotype TG11/T5 was found in the control population with a frequency of only 0.96%, while the other two were not. Exceptionally, haplotype TG13/T5/V470 has been reported in a PS-CF patient with only F508del in trans (Cuppens et al. 1998). In the case of the asthma patient, the milder mutation R297Q could, in association with the particular haplotype, produce the phenotype of asthma.

In conclusion, we have identified several *CFTR* mutations, unclassified variants and the presence of the $(TG)_{11–13}T_5$ exon 9-modulating allele in the pulmonary disease patients. These results therefore indicate that, at the molecular genetic level, there is a connection between CF and asthma, DB and possibly COPD. Due to the small sample size, these results are preliminary and need to be confirmed in a larger study, possibly requiring a multicentre collaboration. Patients found to be carrying CF mutations should receive genetic counselling and their partners' carrier status should be investigated, as their risk of having a child with CF is increased. Finally, for the specific respiratory diseases, environmental and/or other genetic factors could also play a definitive role in the onset and severity of the disease.

**Acknowledgements** The authors wish to thank K. Pouliou for technical assistance. S. Strofalis was supported by a Greek Ministry of Industry research programme (YPER) and Roche (Hellas) SA.

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