



CFTR gene mutations and asthma in the Norwegian Environment and Childhood Asthma study[☆]

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Summary

Background: Several candidate genes have been implicated in the etiology of asthma, including the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR). Mutations in the CFTR gene result in derangements of mucociliary clearance. Homozygotes for CFTR mutations develop cystic fibrosis (CF), a disorder characterized mainly by lung and pancreas disease.

Objective: To investigate whether there was an increased frequency of CFTR mutations in asthma patients.

Methods: Seven hundred and three subjects aged 10–11 years from the environment and childhood asthma (ECA) study were included in the present study. Possible associations between asthma, reduced lung function, bronchial hyperresponsiveness (BHR), and increased or decreased nitrogen oxide (NO) levels (based on structural parental interview, spirometry, PD₂₀ methacholine challenge test and exhaled NO measurements), and the five most common CFTR mutations in Norway ($\Delta F508$, R117H, R117C, 4005+2T→C, 394delTT), the modulating polymorphisms IVS8(TG)_{mT_n} and the IVS8-5T were investigated.

Results: No association were found between asthma, reduced lung function, BHR or exhaled NO levels and CF heterozygosity. However, the IVS8(TG)_{11T₇} haplotype was associated with normal lung function.

Conclusions: Our results do not support the hypothesis that CFTR mutations or polymorphisms play a role in the pathogenesis of asthma in children. However, the

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distribution of Tn(TG)m haplotypes differed between individuals with reduced lung function and individuals with normal lung function.

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Introduction

Asthma is an inflammatory disease characterized by recurrent symptoms due to airway obstruction. Asthma prevalence has increased worldwide over the last decades, and there is increasing evidence of gene–environment interactions in disease development.¹ The Genetic Association Database (<http://geneticassociationdb.nih.gov>) alone has registered over 500 genetic association studies to asthma.² Among the candidate genes is the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7q32.

The CFTR gene codes for the cystic fibrosis (CF) transmembrane conductance regulator. Mutations in the CFTR alleles result in abnormal epithelial ion and water transport and subsequent disturbances in airway mucociliary clearance. More than 1400 CFTR mutations are described conferring different degrees of CFTR protein malfunctions (<http://www.genet.sickkids.on.ca/cftr/>). We have earlier determined CFTR mutations in 148 Norwegian patients with CF, and the most common mutations are presented in Table 1.³ Mutations on both copies of the CFTR results in classical CF, characterized by progressive lung disease with severe chronic infections, bronchiectasies, pancreatic exocrine insufficiency, infertility in males, and elevated concentrations of chloride in sweat.⁴ Other genetic variations such as the thymidine tract polymorphism (sometimes referred to as a mild mutation)⁵ and (TG)m repeats in intron 8 may also modify the disease, as the IVS8-5T causes inefficient splicing of intron 8 and thus reduces the amount of CFTR transcripts. CF heterozygote carriers (in Norway estimated to 3% of the population⁶) are generally disease free, although it is speculated whether or not heterozygosity predisposes for other inflammatory conditions such as disseminated bronchiectasies, bronchopulmonary aspergillosis, sinusitis and asthma.^{7–9}

Both CF and asthma are characterised by airway inflammation and smooth muscle contraction due to stimulation by host inflammatory mediators.¹⁰ Asthma-like symptoms have been reported in 18% and bronchial hyperresponsiveness (BHR) in 43% of CF patients.¹¹ However, exhaled nitrogen oxide (NO) levels are increased in asthmatic airway inflammation,^{12,13} but decreased among CF patients.¹⁴

Table 1 CFTR mutations in the Norwegian cystic fibrosis patients [3].

CFTR mutation	Alleles (%)
F508del*	184 (62.2)
R117C	12 (4.1)
R117H*	12
394delTT	11 (3.8)
4005+2T→C	11
G551D*	6 (2.0)
3659delC*	5 (1.7)
E60X	4 (1.4)
V232D	4
1525-2A-G	3 (1.0)
N1303K*	3
G542X*	2 (0.7)
E279X	2
R75X	2
S912X	2
E116X	1 (0.3)
L295Q	1
R347L	1
Q493X*	1
I506L	1
I507del*	1
R553X*	1
G576A	1
621-1G→T*	1
2183AA-G	1
S945L	1
R1162X*	1
I1234V	1
3849+10 kbC-T*	1
W1282X*	1
Unknown	18 (6.5)
Total alleles	296 (100%)

Mutations detected with OLA31 m kit—74%.

Bolded: five most frequent mutations—78%.

*Detected with OLA 31 m mutation kit.

Possible associations between CF genotypes and asthma are unclear. Reports range from CFTR heterozygosity conferring protection from asthma,¹⁵ showing no association,^{7,16,17} to predisposing for asthma.^{18–22} The variability in design, populations, statistical power, and clinical definitions of asthma, make comparisons and interpretation difficult. Thus, studies with sufficiently large sample sizes and power with detailed clinical phenotypes have been warranted.¹⁷

We aimed to assess whether primarily asthma, or variations in the asthma phenotype such as reduced lung function, BHR or exhaled NO levels, were associated with CFTR mutations and/or polymorphisms in a non CF population.

Subjects and methods

Study design

In the prospective birth cohort study "The Environment and Childhood Asthma study" in Oslo (ECA study) 3754 infants born during a period of 15 months from January 1, 1992 were recruited, as described elsewhere.²³ Briefly, inclusion criteria were: birth-weight >2000 g, absence of illness likely to impair respiration (severe respiratory, cardiovascular, neuromuscular or metabolic disease) and no requirement for assisted ventilation or oxygen therapy after 6 h of life. Exclusion criteria were plans to move out of Oslo within 6 months and insufficient language skills among the parents. Written informed consent was obtained from parents of all subjects, and the study was approved by the regional committee for medical ethics and the Norwegian Data Directorate.

In a subgroup of 803 children lung function was measured at birth.²⁴ During the first 2 years, a nested case-control study (ECA-1) was established to perform detailed examinations of children with recurrent (two or more) or persistent (>4 weeks) physician confirmed bronchial obstruction (rBO) ($n = 306$) and age-matched controls ($n = 306$) (the child born closest in time to the case without lower respiratory illness).²⁵ Two clinical investigations between 0 and 2 years were performed, with at least one investigation in 562 children, and in addition questionnaires including the child's health and diseases with specific and detailed questions related to all types of infectious diseases, family history of disease, socio-economic factors and environmental exposure from birth to their second birthday were completed by the parents every 6 months from birth through the child's second birthday.

A 10-year follow up, "The Environment and Childhood Asthma study part 2 (ECA-2)" was completed in 2004, including the children from the nested case control study at 2 years as well as all the children with lung function measurements at birth.²⁴ All traceable children were asked to attend an examination (two visits) including a detailed parental structured interview with core questions from International Study of Asthma and Allergies in

Children (ISAAC) related to airways symptoms of the child and detailed questions regarding environmental exposure, life-style and diseases. In addition, the examination performed at least 4 weeks after any suspicion of respiratory tract infection included blood tests (DNA), skin prick tests for allergic sensitization, spirometry, PD₂₀ methacholine challenge test, exhaled nitric oxide, urine sampling and clinical examinations on day one, and an exercise test by treadmill running within a week. The present study reports results from the structured interview, spirometry, PD₂₀ methacholine challenge test, and exhaled nitric oxide.

Subjects

The children included in the present study were the first 703 of the included 1019 children who attended the 10-year follow-up in ECA-2. This was not a random selection of children from the child birth cohort, since those with lung function measurements at birth and those included in the 2-year case-control follow up study were prioritized, and thus children with asthma were over-represented (see Table 2). Technical difficulties precluded DNA analysis in five subjects. Of the remaining 698 included subjects, one subject had insufficient information to establish an asthma diagnosis.²⁴ The related phenotypes were ascertained in the following number of subjects: FEV₁ measurements in 688 subjects, PD₂₀ methacholine challenge test for BHR in 682 subjects, and exhaled nitric oxide measurements in 431 subjects (Table 2).

Methods

Forced expiratory flow volume loops were measured according to European standard²⁶ (reference values of Zapf et al.²⁷) on a SensorMedics Vmax 20c (SensorMedics Diagnostics, Yorba Linda, CA, USA) on four occasions; prior to metacholine and exercise challenge tests and after salbutamol given at the end of challenge tests, respectively (on separate days), and at least 4 weeks after any suspected respiratory tract infection. The following medication was not taken for the given time prior to investigation; short acting bronchodilator for 12 h, long acting bronchodilator/leukotriene receptor antagonist for 72 h, long-acting antihistamine for 7 days, systemic steroids for a month. The reported values were the best baseline values obtained before either of the challenge tests.²⁴

BHR was assessed with a dosimetric methacholine challenge test according to international

Table 2 Clinical baseline characteristics of study population.

	No. of subjects analyzed	All children (n = 698)	Children with asthma (n = 236)	Children without asthma (n = 461)	P-value
Gender (male) n/%	698	377 (54%)	144 (61%)	233 (51%)	0.01
Mean lung function \pm SD (FEV ₁ (L))	688	2.10 \pm 0.31	2.03 \pm 0.29	2.13 \pm 0.31	<0.01
Reduced lung function n/% (FEV ₁ % pred <80%)	688	21 (3%)	9 (4%)	12 (3%)	0.37
BHR n/% (PD ₂₀ \leq 8 μ mol)	682	229 (34%)	106 (46%)	123 (27%)	<0.01
Mean FeNO level \pm SD (ppb)	431	8.52 \pm 7.32	10.66 \pm 8.87	7.40 \pm 6.09	<0.01
Increased FeNO levels n/% (FeNO > 15.6 ppb)	431	45 (11%)	31 (21%)	14 (5%)	<0.01
Low FeNO levels n/% (FeNO < 2.5 ppb)	431	15 (4%)	1 (1%)	14 (5%)	0.02
Parental asthma n/%	679	129 (19%)	68 (29%)	61 (13%)	<0.01
Allergy (SPT pos) # n/%	689	193 (28%)	85 (36%)	108 (24%)	<0.01

Baseline characteristics of the study population given as total numbers as well as among children with and without asthma.

The P-value refers to comparison of the groups of children with and without asthma. Information was lacking for asthma diagnosis in one child. BHR: refers to positive bronchial hyper responsiveness FeNO: exhaled nitric oxide.

Parental asthma includes at least one parent reporting asthma.

Allergy was defined as at least one positive skin prick test (SPT).

guidelines.²⁸ In short, methacholine was delivered in successive increasing doses ranging from 0.05 μ mol up to a cumulative dose of 22.4 μ mol. The dose (μ mol) at which a fall in FEV₁ of 20% (PD₂₀) was identified by linear interpolation on the dose response curve.

Exhaled NO levels were measured with Eco Medics CLD 88 analyzer (ECO MEDICS AG, Duernten, Switzerland) using single exhalation techniques with 50 ml/min flow in accordance to ERS/ATS guidelines.²⁹ The mean value of three expiratory NO concentrations was calculated from each subject.

Genotyping

DNA was isolated from peripheral blood by the use of a magnetic beads based method on the instrument MagnaPure LC (Roche). The CFTR mutations Δ F508, R117H, 4005+2T→C, 394delTT, IVS8 T_n(TG)_m were analyzed by different PCR-based methods, as described in details below.

PCR and fragment analysis for the 394delTT and Δ F508 were performed in a multiplex reaction with the following primers:

394F (forward) 5'-FAM-GCAGAGAATGGGATAGA-GAGC-, 394R (reverse) 5'-ATTCACCAGATTTTCGTAGTC- and F508F (forward) 5'-HEX-GCCTGGCACCATTAAAGAA—and F508R (reverse) 5'-AGTTGGCATGCTTTGATGAC-. Fragment representing a normal 394-allele was 199 bp while a normal F508-allele was 84 bp. The deletion specific alleles were 197 and

81 bp, respectively. Fragment sizes were detected on capillary electrophoresis on ABI310 (Applied Biosystems).

The variable number of T-bases and TG-repeats in intron 8 were analyzed by multiplex PCR and fragment analysis with a common forward primer 5'-TAATGG ATCATGGGCCATGT- and differently labelled reverse primers specific for the numbers of T's: 5TR 5'-FAM-CCCCAAATCCCTGTAAAAAC-, 7TR 5'-HEX-CCCCAA ATCCCTGTAAAAAA AC- and 9TR 5'-NED-CCCCAAATCCCTGT TAAAAAAA C—producing amplicons of approximately 136 bp, depending on the number of TG-repeats. The fragment lengths were determined by capillary electrophoresis on ABI310. In order to correlate the IVS8 T_n(TG)_m haplotype to the fragment lengths, a couple of samples were sequenced and used as standards.³⁰

R117C was analyzed by PCR with the forward primer 5'-M13-TTCACATATGGTATGACCCTC and reverse primer 5'- TTGTACCAGCTCACTACCTA followed by restriction digestion by BsmI and visualized on agarose gel. The fragment sizes from normal samples were 330 and 126 bp, while heterozygote samples got additional bands of 228 and 102 bp.³¹

R117H was analyzed in two separate PCR's with a common forward primer C: 5' TCACATATGGTATGACCCTC, and with normal specific arms reverse primer in one tube 5'-CTTATGCCTAGATAAATCGCGATAGAAC and mutated specific arms reverse primer 5'-CTTATGCCTAGATAAATCGCGATAGACT in the other tube. A R117H heterozygote sample would produce a 237 bp long fragment in both reactions.

4005+2T→C was analyzed by PCR with the forward primer 5'-GGTCAGGATTGAAAAGTGTGCA and the reverse primer 5'-CTATGAGAAAAGTCACTGGA, followed by restriction digestion by Hph I and visualized on agarose gel. The digested normal PCR product yielded fragments of 266, 137 and 70 bp, while the mutated PCR product with a loss of restriction site yielded fragments of 403 and 70 bp. The method is described earlier by Boman et al. CF consortium Newsletter #69.

Assay specific heterozygote control samples were included in every PCR run. Further details will be given upon request.

Outcomes

Asthma was defined by fulfilling at least two of the following three criteria:²⁴

1. Dyspnoea, chest tightness and/or wheezing ever.
2. A physician's diagnosis of asthma.
3. Use of asthma medication (β -2 agonist, sodium cromoglycate, corticosteroids, leukotriene antagonists and/or aminophylline).

The secondary outcomes were defined as:

Lung function: Reduced lung function was defined as FEV1% predicted below 80%.²⁷

BHR: Individuals with PD₂₀ value of ≤ 8.0 μ mol were defined as exhibiting BHR.

Increased or decreased exhaled NO levels: Healthy subjects in the present study (no asthma) were used to establish a normal reference interval (mean 7.34 ppb, standard deviation 6.12). Upper limit was set to the 95th percentile and lower limit to the 5th percentile, 15.6 and 2.5 ppb, respectively. Individuals with FeNO values above 15.6 ppb were defined as having increased FeNO, and those with FeNO values below 2.5 ppb as having low FeNO.

Genotypic outcomes were defined into following groups:

CF heterozygotes: those subjects that were heterozygote for one of the five CFTR mutations.
CF heterozygotes including the IVS8-5T polymorphism: those subjects that were heterozygote for either one of the five CFTR mutations or the IVS8-5T polymorphism, since this polymorphism, in some studies, is referred to as a mild mutation.

Tn(TG)m haplotypes: subjects were divided into haplotype groups depending on number of

TG repeats (10, 11 or 12) and T repeats (5T, 7T or 9T).

Statistical analysis

The study was designed to detect a difference of 6% or more in CFTR carrier status between asthmatic and non-asthmatic subjects, a difference based on frequency of CFTR carrier status in the general Norwegian population⁶ and CFTR carrier frequencies among asthmatics in Denmark.²⁰ Using Sample Power 2000 (<http://www.spss.com>) (comparison of proportions), proportions 0.03 (carrier status in general population) versus 0.09 (carrier status among asthmatics), and a desired 80% power with significance level P at 0.05, at least 200 asthmatics and 400 controls were required. P -values below 0.05 were considered statistically significant.

Possible associations between categorical variables were analyzed using Pearson's χ^2 analysis for dichotomous outcomes in Statistical Package for Social Sciences (SPSS) version 11.

Results

The mean age of the subjects included in the study was 10.4 years ($SD = 0.6$). Fifty-four percent were boys (Table 2). In the study group as a whole, 34% had asthma, 3% had FEV1% predicted below 80%, 34% exhibited BHR, 11% had increased FeNO levels and 4% had low FeNO levels. For detailed description of how these phenotypes varied between the asthma group and the non asthma group, see Table 2.

Five percent of the study population were CF heterozygotes and 3.7% carried the IVS8-5T polymorphism. There were no associations between asthma, reduced lung function, BHR, or FeNO levels and CF heterozygosity, both when including and excluding the IVS8-5T polymorphism (Fig. 1a and b). However, a significant association between normal lung function and the IVS8(TG)₁₁T₇ haplotype was found by haplotype analysis (Table 3).

Among children with asthma only, there were no associations between reduced lung function, BHR and FeNO levels and CFTR mutations or IVS8(TG)_mT_n haplotypes.

Discussion

The present study found no significant association between asthma, reduced lung function, BHR or

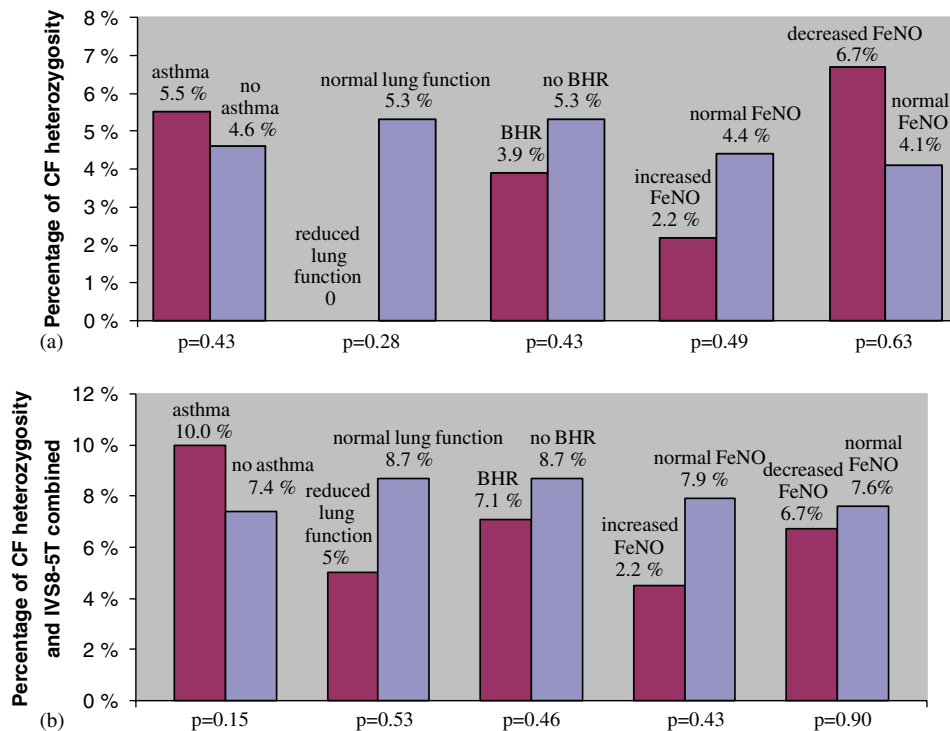


Figure 1 (a) Percentage of CF heterozygotes in asthma, reduced lung function, bronchial hyperresponsiveness (BHR) and exhaled nitric oxide (FeNO) levels. Bars represent percentage of CF heterozygotes in groups with or without asthma, reduced lung function, BHR, increased and decreased FeNO. The *P*-values for each group are indicated along the X-axis. (b) Percentage of CF heterozygotes and IVS8-5T polymorphism in asthma, reduced lung function, bronchial hyperresponsiveness (BHR) and exhaled nitric oxide (FeNO) levels. Bars represent percentage of combined CF heterozygotes and the IVS8-5T polymorphism in groups with or without asthma, reduced lung function, BHR, increased and decreased FeNO. The X-axis represents *P*-values for each group separately.

Table 3 Reduced lung function and the $T_n(TG)_m$ haplotypes.*

$T_n(TG)_m$	Reduced lung function no. of alleles (expected no.)	Normal lung function no. of alleles (expected no.)
$T_5(TG)_{10}$	0 (0)	1 (1)
$T_5(TG)_{11}$	0 (1)	22 (21)
$T_5(TG)_{12}$	1 (0)	2 (3)
$T_7(TG)_{10}$	10 (10)	309 (309)
$T_7(TG)_{11}$	17 (23)	733 (727)
$T_7(TG)_{12}$	7 (4)	105 (108)
$T_9(TG)_{10}$	6 (3)	101 (104)
$T_9(TG)_{11}$	1 (1)	45 (45)
Total # alleles	42	1318

Distribution of $T_n(TG)_m$ haplotypes in the subjects with reduced lung function ($FEV_1\%$ predicted below 80%) versus normal lung function. Significance of $P = 0.01$ for the χ^2 test as a whole, and the haplotype distribution that differs from the expected is bolded.

*Allele count.

FeNO levels and CF heterozygosity (both when including or excluding the IVS8-5T polymorphism) or the modulating polymorphism ($IVS8(TG)_mT_n$). However, the $IVS8(TG)_{11}T_7$ haplotype alone was associated with normal lung function.

The lack of significant association between CF heterozygosity and asthma found in the present study is supported by studies from the UK,¹⁶ France¹⁷ and Italy.³² Furthermore, a linkage study from Iceland failed to show evidence of linkage

between chromosome 7q32 and asthma.³³ This is in contrast to studies from Denmark,^{18–20} Greece²¹ and Spain²² that reported positive association between asthma and CF heterozygosity and one study from the US¹⁵ that concludes that CF heterozygosity confers protection against asthma.

To our knowledge, the relationship between IVS8(TG)_{mT_n} haplotypes and lung function has not previously been explored, although a recent study from Denmark found no association between the IVS8-5T polymorphism and annual decline in FEV₁ or asthma.³⁴ The IVS8(TG)_{11T₇} was the most common haplotype in our population, similar to findings in a Greek study by Tzetzis et al.²¹ The higher frequency of the IVS8(TG)_{11T₇} haplotype among our subjects with normal versus reduced lung function is in line with the findings in Tzetzis' study, where they detect a higher frequency of the IVS8(TG)_{11T₇} haplotype among healthy controls versus asthmatic patients.²¹ The IVS8(TG)_{12/13T₅} haplotype has been associated to congenital bilateral absence of vas deferens (CBAVD)³⁵ and IVS8(TG)_{11/12/13T₅} to pulmonary diseases.²¹ In the present study, only 21 subjects had reduced lung function (estimated by Zapletal's reference values), leaving insufficient power to determine whether this was associated with similar haplotypes. However, since the IVS8(TG)_{11T₇} haplotype possibly protected against reduced lung function, one might speculate whether these modulating polymorphisms exert an effect on lung physiology in general and not on lung function specifically in subjects with asthma.

The strength of the present study is the detailed and well described population that has been followed for 10 years with a design especially suited for investigating asthma and asthma phenotypes. Thus, the discrepancies concerning the asthma diagnosis are minimized. Furthermore, we anticipated that if CFTR mutations are clinically relevant for asthma, they should be found among the most common mutations in the population studied. Thus, we analyzed the five most common CFTR mutations, covering 78% of known CF mutations in our native population. Finally, the present study represents, to our knowledge, the largest case-control population with sufficient power especially designed to look at clinically ascertained asthma among the CFTR–asthma studies, and the results from the present study are supported by the comparable French EGEA study.¹⁷

In addition to asthma, reduced lung function, BHR and increased FeNO levels were analyzed separately. Although power estimates ascertained sufficient study power for asthma only, the lack of associations between CFTR mutations and lung function, BHR and increased FeNO levels support

the main conclusion of no association between asthma and CFTR genotypes. In fact, as can be seen in Fig. 1a and b, the percentage of CF heterozygosity (both including and not including the IVS8-5T polymorphism) was higher in subjects without reduced lung function, BHR or increased FeNO levels than in those with the clinical traits.

A limitation of the present study was that the entire CFTR gene was not screened to fully exclude association. Secondly, the subject population was recruited from a healthy birth cohort and included few subjects with low lung function (only 21 subjects with FEV₁% predicted below 80%), and the Bonferroni corrected *P*-value of significance for the association between IVS8(TG)_{11T₇} haplotype and reduced lung function is a borderline 0.04 (original *P*-value multiplied by the four phenotypes analyzed). Thus, the possible significance of IVS8(TG)_{mT_n} haplotypes for lung remains to be ascertained.

In conclusion, CFTR heterozygosity was not associated with asthma, reduced lung function, BHR or exhaled NO levels in children, whereas the IVS8(TG)_{11T₇} haplotype was associated with normal lung function. We suggest that future efforts to understand the role of CFTR genotypes should focus on possible associations with lung function rather than asthma.

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We declare no conflict of interest.

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