Association of the CFTR Gene With Asthma and Airway Mucus Hypersecretion

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Abstract

Introduction

Airway mucus hypersecretion is an undercharacterized phenotype of asthma.

Objectives

(1) To determine the presence of genetic variants of the *CFTR* gene in patients with asthma with and without airway mucus hypersecretion. (2) To identify the characteristics of the asthma phenotype with airway mucus hypersecretion.

Method

Comparative cross-sectional multicentre study including 39 hypersecretors and 61 non-hypersecretors asthma patients. Asthmatic hypersecretion was defined as the presence of cough productive of sputum on most days for at least three months in two successive years. Spirometry, fractional exhaled nitric oxide, induced sputum cell count, blood test and questionnaires were performed. Blood DNA samples were sequenced using a MiSeq sequencer and the Illumina platform was used for the *CFTR* gene analysis.

Results

Genetic differences were observed in the c.1680-870T>A genetic variant of the *CFTR* gene, significantly more evident in hypersecretors than in non-hypersecretors: 78.94% vs. 59.32% in the majority allele and 21.05% vs. 40.67% in the minority allele (p=0.036). Asthma hypersecretors were older (57.4 years vs. 49.4 years; p=0.004) and had greater asthma severity (58.9% vs. 23.7%; p=0.005), greater airway obstruction (FEV1/FVC% 64.3 vs. 69.5; p=0.041), poorer asthma control (60% vs. 29%; p=0.021), and lower IgE levels (126.4 IU/mL vs. 407.6 IU/mL; p=0.003).

Conclusion

Patients with asthma and with mucus hypersecretion may have a different disease mechanism produced by an intronic genetic variant in the *CFTR* gene (NM_000492.3:c.1680-870T>A). They present a more severe disease, poorer asthma control and a non-allergic inflammatory phenotype.

Introduction

In clinical practice, it is common to encounter patients with severe asthma who only partially respond to steroid treatment and who have marked airway mucus hypersecretion. The increase in mucus secretion may be mild or severe, marked only by chronic bronchitis or by recurring bronchial infections, respectively; in extreme cases, patients may develop infectious bronchiectasis and bronchiolitis. Probably because

"airway mucus secretion" is not usually included in cluster analyses of asthma, this condition is not recognized as a specific phenotype.

Wheaterall *et al* (1) evaluated patients with airflow obstruction and airway mucus hypersecretion, finding a poorer response to glucocorticoid treatment and more frequent hospital admissions due to exacerbations in patients with airway mucus hypersecretion secondary to chronic obstructive pulmonary disease (COPD) or asthma (bronchial hyperresponsiveness, elevated fractional exhaled nitric oxide (FeNO), rhinitis, dermatitis and blood eosinophilia). Mutations or polymorphisms in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene were detected in another study of four hypersecreting patients with asthma, with a neutrophilic inflammatory phenotype, bronchiectasis, pansinusitis and respiratory infections (2); this would suggest that a possible explanation for airway mucus hypersecretion may be that having asthma results in a combination of asthma and an attenuated form of cystic fibrosis (CF) in patients with a genetic alteration in the CF gene. By the 1990s, several studies had confirmed that carriers of a mutation for CF were at an increased risk of asthma and of greater lung function deterioration than patients with asthma without that mutation (3-4). However, several works published between 1998 and 2008 have been questioned since on the basis that they report contradictory results (5-6); furthermore, they had limitations that included incorrectly diagnosing asthma, using emergency registry databases, the detection of just a single *CFTR* gene mutation, and analyses of different populations with unequal comparisons in terms of sex and age (5-6). Furthermore, all types of asthma were included, i.e., no phenotype filtering was applied.

CF is the most common severe autosomal recessive hereditary disease reported for Caucasians; in Spain incidence is estimated as 1 in 5,352 full-term births (7) CF is caused by mutations in the *CFTR* gene residing on the long arm of human chromosome 7 and coding for the CFTR protein (8-9), which, in turn, acts as a chloride channel on the surface of epithelial cells in a wide variety of organs, thereby explaining the wide range of clinical manifestations of CF.

To date, of the some 1,900 genetic variants described for the *CFTR* gene, most frequent in the Caucasian population is the three-base-pair deletion NM_000492.3(CFTR):c.1521_1523delCTT (p.Phe508del) or F508del according to the classical nomenclature (10-11). Since the inheritance pattern for CF is autosomal recessive, both copies of the mutated *CFTR* gene must be present for the disease to develop, whereas only one copy is necessary to be an asymptomatic CF carrier. It is estimated that around 4%-5% of the general population are CF carriers (12).

The main line of work in our current project is the search for genetic variants (mutations or polymorphisms) in the *CFTR* gene using mass sequencing platforms and next-generation sequencing (NGS) and, more specifically, the investigation of airway mucus hypersecretors and non-hypersecretors with asthma and pre-defined clinical characteristics. The goal is, by considering the phenotype and genotype of the *CFTR* gene, to achieve better stratification in asthma classifications and so improve disease prognosis for hypersecreting patients and, ultimately, to enhance the efficiency of current treatments and develop targeted and personalized future treatments.

Materials And Methods

Comparative multicentre cross-sectional study designed to determine the presence of genetic variants (mutations or polymorphisms) of the *CFTR* gene in hypersecreting and non-hypersecreting patients with asthma and to identify the clinical and inflammatory characteristics of hypersecreting patients.

Included were 100 patients of both sexes, aged between 18 and 80 years, with and without airway mucus hypersecretion; complied with asthma diagnostic criteria according to the Global Initiative for Asthma (GINA) guidelines published in 2009 (13) and did not have a respiratory infection in the month before testing. Excluded were smokers and ex-smokers, patients with lung conditions (tuberculosis sequelae, bronchiectasis, CF, residual pleural diseases, interstitial diseases and severe associated comorbidities) and patients treated with oral corticosteroids or other immunomodulators for reasons other than asthma. Included patients were administered a questionnaire to evaluate symptoms and type of expectoration. Asthmatic hypersecretion was defined as the presence of cough productive of sputum on most days for at least three months in two successive years.

Ethical considerations

The study was conducted according to the principles of the Declaration of Helsinki (18th World Medical Assembly) and was approved by the Hospital de la Santa Creu i Sant Pau Clinical Research Ethics Committee (approval number COD: HBSP-CFT-2014-68; ClinicalTrials.gov Identifier: NCT02558127). All patients signed an informed consent prior to participating in the study and all patients were guaranteed the confidentiality of their data.

Method

Clinical and demographic data were collected for the 100 asthmatic patients included in the study. Tests were performed as follows: fractional exhaled nitric oxide (FeNO), spirometry, induced sputum cell count (conducted only at the Hospital de la Santa Creu i Sant Pau), total immunoglobulin E, peripheral blood eosinophil count, blood polymerase chain reaction (PCR), blood fibrinogen, blood albumin and skin prick test for common pneumoallergens. All patients completed the validated Spanish version of the Asthma Control Test (ACT) questionnaire (14) and the Mini Asthma Quality of Life (Mini-AQLQ) questionnaire (15).

FeNO was measured before spirometry using electrochemical equipment (NO Vario Analyzer, FILT Lungen and Thorax Diagnostic GmbH, Berlin, Germany) on the basis of expiration yielding a continuous flow of 50 mL/s of total lung capacity in accordance with American Thoracic Society (ATS)/European Respiratory Society (ERS) recommendations from 2005 (16). A high FeNO values was defined as ≥50 parts per billion (ppb) (17). Spirometry was performed with a Datospir-600 spirometer (Sibelmed SA, Barcelona, Spain) by an experienced pulmonary function technician who applied Spanish Society of Pulmonology and Thoracic Surgery (SEPAR) recommendations (18-19). Induced sputum samples were collected, according to the ERS consensus protocol (20), using an ultrasonic nebulizer (Omron NE U07,

HEALTHCARE Europe, Germany) – output 3 mL/s and mass mean aerodynamic diameter (MMAD) particle size 7 µm – to produce a hypertonic saline spray which the patient inhaled at increasing concentrations (3%, 4% and 5%). Sputum was processed within two hours of induction. Patients were classified as having neutrophilic asthma when neutrophil count was ≥61%, eosinophilic asthma when eosinophil count was ≥3%, paucigranulocytic asthma when neutrophil count was <61% and eosinophil count was <3%, and mixed asthma when neutrophil count was ≥61% and eosinophil count was ≥3% (21). Total serum IgE was measured by ImmunoCAP using the UniCAP 250 system (Phadia AB, Uppsala, Sweden), for a high value defined as >160 IU/mL. The prick test, performed as standard, was considered positive for wheal diameters >3 mm (22). Controlled asthma was defined as an ACT score ≥20. A high dose of inhaled glucocorticoids was defined as ≥1,000 mg/day of beclomethasone dipropionate or equivalent (23).

Study of genetic variants in the *CFTR* gene

A 10-mL peripheral venous blood sample was extracted from patients for analysis by the genetics service of the reference hospital. DNA extraction was by the saline method. DNA was analysed for genetic variants of the *CFTR* gene by means of NGS using the Illumina platform MiSeq sequencer. Used for this purpose were pre-designed Multiplicom *CFTR* gene panels (CFTR Master Dx kit) as diagnostic tests that are validated by a CE-IVD certificate and compatible with Illumina NGS sequencers. The NGS workflow consisted of five main steps: (1) library preparation using the CFTR Master Dx and MID Dx kits specific to the Illumina MiSeq for sample analyses; (2) cluster generation; (3) sequencing; (4) data analysis with the MiSeq Reporter software pre-installed in the MiSeq sequencer; and (5) data interpretation using computerized systems and databases incorporated in analytical software. Steps 2-4 were performed automatically by the MiSeq sequencer. The genetic variants identified using NGS were validated by Sanger sequencing.

Statistical analysis

Descriptive baseline values are reported as frequencies and percentages for qualitative data and means and standard deviation (SD) for quantitative data. The two asthma groups (hypersecretors and non-hypersecretors) were compared in terms of means and SD using the Student-t test for the main variable and the remaining quantitative variables. The categorical variables, described by means of contingency tables and tested for differences using the chi-square test, are reported as numbers of cases and percentages. Statistical significance was set to 5% (α=0.05) and statistical analyses were performed using SPSS (version 19.0) for Windows (SPSS, Inc., Chicago, Il, USA).

Results

Genetic results

Of the 100 DNA samples obtained from the peripheral blood of patients with asthma, 98 were analysed and 2 were excluded for being unsuitable for processing. A total of 50 genetic variants in the *CFTR* gene

were found, although significant differences between the two asthma groups were only observed in a single genetic variant, namely, NM_000492.3(CFTR):c.1680-870T>A, which was present in hypersecretors but not in non-hypersecretors: 78.94% vs. 59.32% in the majority allele (thymine nucleotide) and 21.05% vs. 40.67% in the minority allele (adenine nucleotide) (p=0.036) (see Table 1 of the supplementary material).

Of the 98 samples analysed, 4 showed pathological mutations (Table 1), already described in the literature and associated with CF. As all these mutations were detected in heterozygosis, patients who were carriers of the disease were informed. This finding confirms the internal validity of our results, since this percentage coincides with the 4%-5% risk of being a CF carrier described in the literature for the Caucasian population (12).

Table 1. Pathological mutations described in the analysed population with asthma.

Patient No.	Mutation	Exon
1	c.2047_2052delAAAAAAinsAAAAG [p.(Lys684Serfs*38)]	**E14**
2	c.3909C>G [p.(Asn1303Lys)]	**E24**
3	c.1521_1523delCTT [p.(Phe508del)]	**E11**
4	c.350G>A [p.(Arg117His)]; c.1521_1523delCTT [p.(Phe508del)]	**E4; E11**

The four patients with a pathological mutation in heterozygosis are considered asymptomatic FC carriers. Patient #4 presented with two heterozygous changes: pathological (c.1521_1523delCTT) and change c.350G>A, the latter classified as missense since an amino acid change is predicted, although it is described in the cystic fibrosis database as a genetic variant with variable clinical consequences.

As a secondary objective of the study, genetic variants were analysed in relation to asthma severity, with significant differences observed for two of them, namely, NM_000492.3 (CFTR):c.2506G>T (p.Asp836Tyr) and NM_000492.4(CFTR):c.3140-92T>C (p=0.024 and p=0.049, respectively), which were more frequently present in majority alleles in patients with severe persistent asthma and absent or present to a lesser extent in minority alleles (Table 2).

Table 2. Genetic variants in the *CFTR* gene that are significant according to asthma severity.

GENOTYPE	Intermittent asthma (N=25)	Persistent asthma		
		Mild (N=25)	Moderate (N=11)	Severe (N=37)
c.2506G>T [p.(Asp836Tyr)]*				
Homozygous majority allele (G/G)	23.70%	25.80%	11.80%	38.70%
Minority allele present (G/T & T/T)	75%	25%	0%	0%
c.3140-92T>C**				
Homozygous majority allele (T/T)	23.30%	25.60%	12.20%	38.90%
Minority allele present (T/C & C/C)	57.10%	28.60%	0%	14.30%

CFTR: cystic fibrosis transmembrane conductance regulator. * p=0.024; ** p=0.049.

Clinical hypersecretor and non-hypersecretor phenotypes

The clinical, inflammatory and functional characteristics of hypersecretor and non-hypersecretor patients with asthma are shown in Table 3; of the 100 patients analysed, 39 were hypersecretors and 61 were non-hypersecretors. To a significant degree ($p<0.05$), hypersecretors compared to non-hypersecretors were older, had severer asthma, experienced greater bronchial obstruction, had poorer asthma control (ACT <20), had received more short-term oral glucocorticoid treatments in the previous year, had lower peripheral blood albumin levels, induced sputum lymphocyte levels and IgE levels, and were less likely to have prick test-positive asthma.

Table 3. Demographic, clinical and functional characteristics of asthma with and without airway mucus hypersecretion.

Variables	Asthma with hypersecretion (N=39)	Asthma without hypersecretion (N=61)	p
Age (y)	57.43 (11.47)	49.44 (15.4)	**0.004**
Sex (% women)	61.5%	49.15%	0.159
Asthma diagnosis (% adults)	76.92%	71.18%	0.349
BMI (kg/m2)	27.41 (4.46)	27.24 (4.93)	0.864
Severe asthma (%)	58.97%	23.72%	**0.005**
FEV1/FVC (%)	64.39 (13.28)	69.55 (9.61)	**0.041**
FeNO (ppb)	32.45 (25.64)	39.81 (43.27)	0.291
Positive bronchodilator test (%)	23.68%	35.08%	0.483
Emergency visits, last 12 months	2.46 (3.08)	1.48 (2.24)	0.074
Oral glucocorticoid treatments, last 12 months	3.6 (3.7)	0.86 (1.3)	**0.002**
Medium-high oral glucocorticoid doses (%)	74.35%	64.4%	0.678
Rhinitis (%)	61.53%	64.44%	0.360
Polyposis (%)	20.55%	8.62%	0.172
High quality induced sputum (%)	61%	27.3%	0.100
Inflammatory phenotype in induced sputum (%)	Paucigranulocytic: 31.25% neutrophilic: 12.5% eosinophilic: 56.25% (N=16)	Paucigranulocytic: 26.32% neutrophilic: 26.32% eosinophilic: 47.36% (N=19)	0.596
Positive prick test (%)	46.15%	64.4%	0.216
Blood IgE (IU/mL)	126.4 (197)	407.59 (627.6)	**0.003**
Absolute eosinophils in peripheral blood (x10E9/L)	0.39 (0.32)	0.35 (0.27)	0.491
Blood polymerase chain reaction (mg/L)	4.26 (5.57)	4.20 (6.56)	0.969
Blood fibrinogen (g/L)	4.05 (1.02)	3.98 (0.93)	0.777
Blood albumin (g/L)	42.21 (3.06)	44.14 (3.12)	**0.008**

Lymphocytes in induced sputum (%)	0.71 % (0.52)	1.05% (0.38)	**0.023**
ACT <20 (%)	58.3%	29.09 %	**0.021**
AQLQ	3.55 (2.63)	2.6 (2.62)	0.113

Values are reported as means (standard deviation) or percentages, as indicated. **ACT**=Asthma Control Test; **AQLQ=**Asthma Quality of Life Questionnaire; **BMI=**Body mass index; **FeNO**=fractional exhaled nitric oxide; **FEV1**=forced expiratory volume in the first second; **FVC**=forced vital capacity; **IgE**=immunoglobulin E.

Discussion

Our study demonstrates that asthma associated with airway mucus hypersecretion is linked with an intronic genetic variant of the *CFTR* gene (NM_000492.3(CFTR):c.1680-870T>A). Patients with this genetic variant, who were older, had more severe asthma and showed poorer clinical control, represented a non-allergic predominantly eosinophilic inflammatory phenotype.

Airway mucus hypersecretion is a frequent symptom in patients with asthma, but unlike COPD, it is not usually taken into account in clinical practice or in clinical trials. A number of published works have demonstrated that airway mucus hypersecretion is associated with greater asthma severity, is a marker of reduced lung function in both smokers and non-smokers, is associated with an increased number of exacerbation episodes and is a predictor of a poorer response to anti-inflammatory treatment with glucocorticoids (24-26). Interestingly, in a 10-year follow-up study of 13,756 patients with obstructive lung conditions (chronic bronchitis, emphysema and asthma), Lange et al (27) observed that airway mucus hypersecretion increased the all-cause mortality risk and that hypersecretion combined with an altered lung function reflected a greater mortality risk for patients with asthma and COPD. Those results would point to the importance of airway mucus hypersecretion in influencing not only the natural course of an obstructive disease but also the associated mortality.

While airway hypersecretion may be an underappreciated condition, clinical practice guidelines for asthma (13) highlight mucus along with bronchoconstriction and inflammation as causes of airway obstruction and airflow limitation. Possible explanations are related to (1) the role played by mucins, (2) the function of toll-like receptors, and (3) the fact of being a carrier of a genetic variant in the *CFTR* gene, either a single-nucleotide polymorphism (SNP) or pathological mutations. Our findings point to clinical characteristics that differentiate patients with and without mucus hypersecretion, mainly that hypersecretors are older, have severer asthma, greater bronchial obstruction and poorer asthma control, have lower peripheral blood albumin and lower IgE levels, have lower induced sputum lymphocyte levels, are less likely to have prick test-positive asthma and, finally, needed more short-term oral glucocorticoid treatments in the previous year. Our results corroborate those of Martínez-Rivera et al (28), who reported that hypersecretion, common in patients with asthma, was associated with greater airway obstruction, poorer asthma control and more exacerbation episodes. Another European prospective respiratory health

study followed up over 9 years reported that one determinant of asthma severity was airway mucus hypersecretion (29). Furthermore, Wheaterall et al (1), who identified five phenotypes, observed that patients with phenotype 5 shared COPD and asthma characteristics (bronchial hyperresponsiveness, elevated FeNO, rhinitis, dermatitis and blood eosinophilia), airway mucus hypersecretion and a poorer response to glucocorticoid anti-inflammatory treatment (resulting in a greater need for treatment and more frequent hospital admissions due to exacerbation episodes).

Chronic airway mucus hypersecretion is classically associated with bronchiectasis and with smoking, although only a minority of smokers develop this symptomatology. A plausible explanation for a greater propensity to hypersecretion may be a genetic susceptibility. Dijkstra et al (30) conducted a genome-wide association study of habitual or former smokers (≥20 pack-years), with (n=2,704) and without (n=7,624) chronic mucus hypersecretion, reporting, for all cohorts, a strong association between chronic mucus hypersecretion and SNP rs6577641 located in intron 9 of the special AT-rich sequence-binding protein 1 locus (*SATB1*) gene on chromosome 3 ($p=4.25\times10^{-6}$; OR=1.17). Likewise reported was that the risk allele was associated with greater mRNA expression of *SATB1* in lung tissue (4.3×10^{-9}) and that airway mucus hypersecretion was associated with greater mRNA expression of SATB1 in bronchial biopsies from patients with COPD (30). Another genetic issue raised in the 1990s in relation to airway mucus hypersecretion and asthma was the correlation between being a CF mutation carrier, an increased asthma risk and a greater deterioration in lung function (3-4). Although the asthma-CF carrier relationship is well documented (3), studies conducted between 1998 and 2008 were widely debated, as some failed to find a higher incidence of asthma in CF carriers with the F508del mutation, although they did find a greater deterioration in lung function in patients with asthma who were CF carriers (5-6). It was also found that *CFTR* gene mutations alter RNA splicing and/or functional chloride conductance were likely to contribute to the susceptibility and pathogenesis of adult bronchiectasis and pulmonary non-tuberculous mycobacterial infection (31).

To date, of the some 1,900 genetic variants described for the *CFRT* gene, that most frequently encountered in the Caucasian population is p.Phe508del (or F508del following the classical nomenclature). Worldwide only 20 mutations occur with a frequency greater than 0.1% (9,10). Since the CF inheritance pattern is autosomal recessive, both copies of the mutated *CFTR* gene must be present for the disease to develop, whereas only one copy is necessary to be a CF carrier. It is estimated that around 4%-5% of the general population are CF carriers, and the fact that the NGS of 98 samples from patients with asthma resulted in 4% being CF carriers demonstrates the internal validity of our genetic study. One strength of this study, compared to studies that have typically sequenced just 39-50 *CFTR* gene mutations (3-6, 32-35), lies in the use of NGS technology, as it permitted sequencing of the entire coding region of the *CFTR* gene along with flanking introns and regions for which pathogenic mutations have been described.

Goodwin et al (2) reported, for four cases of asthma and airway mucus hypersecretion, a neutrophilic inflammatory phenotype accompanied by bronchiectasis, pansinusitis, respiratory infections and genetic variants in the *CFTR* gene (mutations and/or polymorphisms), posing the interesting possibility of an

association between mutations and a characteristic phenotype. The results of our NGS study would suggest that, in hypersecretors compared to non-hypersecretors with asthma, the NM_000492.3(CFTR):c.1680-870T>A polymorphism was present to a significantly greater degree: 78.94% vs. 59.32% in the majority allele (thymine nucleotide) and 21.05% vs. 40.67% in the minority allele (adenine nucleotide) (p=0.036). While this polymorphism is described as a SNP variant for CF (36), given that its role in patients with asthma is still unknown further studies based on larger samples and specific clinical variables are needed to confirm our findings.

The limitations of this study include the fact that the finding of a single polymorphism can potentially lead to spurious associations, among other reasons, because the genetic variant might be found in linkage disequilibrium with one or more other variants and so constitute a characteristic haplotype. Information about haplotypes is becoming more available online, so validating the prognostic or therapeutic utility of this polymorphism would be useful. Another limitation was the lack of a questionnaire that objectively evaluated airway mucus hypersecretion. Strengths include, as mentioned, DNA study using NGS, with the associated advantages. Furthermore, patients with a verified asthma diagnosis were included, i.e., not patients from a database – as in previous studies – for whom a confirmed objective diagnosis may not have been available. A final strength is that exhaustive phenotyping was possible because patients underwent extensive clinical, functional and inflammatory testing.

Conclusions

The findings of this study of patients with asthma and airway mucus hypersecretion demonstrate (1) that a significantly higher percentage expressed the genetic variant NM_000492.3(CFTR):c.1680-870T>A in the *CFTR* gene, and (2) that these patients were older, had greater asthma severity and poorer clinical control and had a non-allergic inflammatory phenotype. The results of this study point to a possible interesting explanation: the overlap of asthma and being a carrier of this genetic variant of the *CFTR* gene may cause a combination of asthma and an attenuated form of CF that would account for the component of bronchial mucosal hypersecretion. More studies are needed to validate the prognostic and therapeutic usefulness of these findings.

Declarations

Ethics approval and consent to participate. The study was conducted according to the principles of the Declaration of Helsinki (18th World Medical Assembly) and was approved by the Hospital de la Santa Creu i Sant Pau Clinical Research Ethics Committee (approval number COD: HBSP-CFT-2014-68; ClinicalTrials.gov Identifier: NCT02558127). All patients signed an informed consent prior to participating in the study and all patients were guaranteed the confidentiality of their data.

Consent for publication. All patients signed an informed consent prior to participating in the study including a consent for publication.

Availability of data and materials. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests.

AC has received fees in the last three years for talks at meetings sponsored by Chiesi, Esteve Laboratories, GlaxoSmithKline, Novartis, Ferrer, Zambón and Boehringer Ingelheim, has received travel and attendance expenses for conferences from Novartis, Bial, Teva and FAES Farma and has received funds/grants for research projects from several state agencies and non-profit foundations and from AstraZeneca.

EC reports non-financial support from Astrazeneca, personal fees from Boehringer-Ingleheim, personal fees and non-financial support from Chiesi, non-financial support from Novartis, non-financial support from Menarini, non-financial support from ALK, outside the submitted work.

SB, EdR, ER, **CM, NM, AP, SP, JG, AP, CC, JS** and **MB** have no conflicts of interest to declare.

VP has received fees in the last three years for talks at meetings sponsored by AstraZeneca, Boehringer-Ingelheim, MSD and Chiesi, has received travel and attendance expenses for conferences from AstraZeneca, Chiesi and Novartis, has acted as a consultant for ALK, AstraZeneca, Boehringer, MSD, MundiPharma and Sanofi, and has received funds/grants for research projects from several state agencies and non-profit foundations and from AstraZeneca, Chiesi and Menarini.

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Authors' contributions. All authors made substantial contributions to study conception, design, data acquisition, data analysis and results interpretation, and critically revised and approved the manuscript. More specifically, AC collected patient clinical data, built the database, performed statistical analyses and participated in data interpretation and manuscript writing; SB, EdR, ER and MB performed the genetic analysis of the samples and participated in data interpretation and manuscript writing; CM, NM, AbP, SP, JLC and AP enrolled patients; and JS, CC, EC and VP participated in data interpretation and manuscript writing.

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