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ORIGINAL ARTICLE

Detection of Specific IgE against Molds Involved in Allergic Bronchopulmonary Mycoses in Patients with Cystic Fibrosis

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Abstract

Context Allergic bronchopulmonary mycoses (ABPM) can be due to molds other than *Aspergillus fumigatus* in patients with cystic fibrosis (pwCF). We aimed to develop immunoassays for the detection of specific IgE (sIgE) directed against five fungal species involved in ABPM: *Aspergillus terreus*, *Scedosporium apiospermum*, *Lomentospora prolificans*, *Rasamsonia argillacea*, and *Exophiala dermatitidis*.

Materials and Methods Serum samples ($n=356$) from 238 pwCF, collected in eight CF care centers in France, Germany, and Italy, were analyzed by dissociated enhanced lanthanide fluorescent immunoassay (DELFIA®) to assess levels of sIgE directed against antigenic extracts of each fungus. Clinical, biological, and radiological data were collected for each episode. One hundred serum samples from healthy blood donors were used as controls. Sera were classified into four groups depending on the level of sIgE according to the quartile repartition calculated for the pwCF population. A score of 4 for values above the 3rd quartile corresponds to an elevated level of sIgE.

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Results PwCF showed higher levels of sIgE than controls. Based on criteria from the ABPA-ISHAM working group, with an additional criterion of "a sIgE score of 4 for at least one non-*A. fumigatus* mold", we were able to diagnose six cases of ABPM.
Conclusions Using 417 IU/mL as the threshold for total IgE and the same additional criterion, we identified seven additional pwCF with "putative ABPM". Detection of sIgE by DELFIA® showed good analytical performance and supports the role played by non-*A. fumigatus* molds in ABPM. However, commercially available kits usable in routine practice are needed to improve the diagnosis of ABPM.

Keywords Specific IgE · DELFIA · Cystic fibrosis · ABPM · *Scedosporium* · *Exophiala* · *Rasamsonia*

Introduction

Allergic bronchopulmonary mycoses (ABPM), which mainly affect patients with cystic fibrosis (pwCF) or those with asthma, are characterized by hypersensitivity reactions due to allergens produced by fungi colonizing the airways of the patients. The symptoms are non-specific, such as cough, wheezing, or chest pain, and mimic clinical findings of pulmonary exacerbations in pwCF [1]. ABPM are principally due to *Aspergillus fumigatus* (called in this case, allergic bronchopulmonary aspergillosis; ABPA), of which the prevalence is estimated to be 10% among pwCF [2]. The prevalence of airway colonization by *A. fumigatus* in pwCF varies greatly between studies, from 3.2% to 56.7%, mainly due to differences in the population studied and the procedures used for mycological examination of respiratory secretions [3]. Aside from *A. fumigatus,* other molds, such as *Scedosporium* species and the closely related species *Lomentospora prolificans*, are frequently isolated from respiratory secretions of pwCF. In The Netherlands and Germany, *Scedosporium apiospermum* is most frequently identified in sputum sample cultures from pwCF [4, 5], whereas in France, *Scedosporium boydii* appears to be more common [5]. Other *Aspergillus* species, such as *Aspergillus terreus*, species of the *Rasamsonia argillacea* complex, and the black yeast *Exophiala dermatitidis* have also been reported to cause ABPM [2, 4, 6, 7]. Furthermore, several molds may be simultaneously involved [8]. Azole resistance is frequent among these fungi, and *Scedosporium/Lomento*spora species are considered to be intrinsically resistant to most currently available antifungal drugs [9, 10]. The diagnosis of ABPM is challenging because many clinical, radiological, and immunological characteristics are similar to CF symptoms. Identification of the etiological agents and early effective antifungal treatment according to the species involved are essential to avoid irreversible lung damage.

Several diagnostic criteria for ABPA have been proposed [1, 11]. In the most recent proposal from the ABPA-ISHAM working group [12], two criteria are mandatory: (*i*) an elevated serum total IgE (tIgE) level > 1000 IU/mL and (*ii*) positive skin tests to *Aspergillus* antigens or the presence of serum specific IgE (sIgE) against *A. fumigatus*. Two of the three following criteria are also required: (*i*) the presence of serum precipitins or IgG directed against *A. fumigatus*, (*ii*) a blood eosinophil count > 500 cells/µL in steroid-naive patients, and (*iii*) radiological pulmonary opacities consistent with ABPA [12].

A definition of ABPM for patients without CF was also recently proposed that included 10 criteria [13]: (*i*) current or previous history of asthma or asthmatic

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symptoms; (*ii*) peripheral blood eosinophilia (> 500 cells/µL), (*iii*) an elevated serum tIgE level (> 417 IU/mL), (*iv*) immediate cutaneous hypersensitivity or sIgE directed against filamentous fungi, (*v*) the presence of precipitins or specific IgG directed against filamentous fungi, (*vi*) the growth of filamentous fungi in cultures from sputum or bronchial lavage fluid, (*vii*) the presence of fungal hyphae in bronchial mucus plugs, (*viii*) central bronchiectasis by computed tomography (CT), (*ix*) the presence of mucus plugs in the central bronchi, based on CT or bronchoscopy, or the presence of mucus plus a history of expectoration, and (*x*) high attenuation mucus in the bronchi by CT. Patients who meet at least six of these criteria are considered to have ABPM. The filamentous fungi in criteria *iv* (sIgE) and *vi* (positive culture) should be identical.

These two definitions highlight positive skin tests or elevated serum sIgE levels as the main criteria for the diagnosis of ABPM. The detection of serum sIgE against *A. fumigatus* is widely performed using Phadia systems and ImmunoCAP™ tests (Thermofisher Scientific, Uppsala, Sweden). Although 25 mold allergens are available for the ImmunoCAP™ test, including a crude antigenic extract from *A. terreus* (ImmunoCAP *A. terreus* m36 allergen), only *A. fumigatus* whole allergens and recombinant antigens rAspf4 and rAspf6 are commonly used for the diagnosis of ABPM [14]. Until now, the presence of sIgE against other molds potentially involved in ABPM has not been assessed.

We have developed a dissociated enhanced lanthanide fluorescent immunoassay (DELFIA®) with high sensitivity to detect serum sIgE. With this tool, we were able to detect sIgE against purified and recombinant antigens of *A. fumigatus* for the diagnosis of ABPA in pwCF [15]. Titers of sIgE against *A. fumigatus* highly correlated with ImmunoCAP™ values [15]. In this first study, we demonstrated the performance and flexibility of the DELFIA® technique for the detection of sIgE against several types of *A. fumigatus* antigens [15]. This technique was then successfully used to detect sIgE against bacterial species not usually tested because of the lack of commercially available techniques, but possibly involved in allergic diseases [16, 17].

Several commercial kits are available for the detection and titration of *A. fumigatus* IgG antibodies against species involved in airway colonization of pwCF, mainly based on enzyme-linked immunosorbent assays (ELISAs). In addition, several in-house tests, such as ELISAs or dot immunoassays based on purified protein extracts or recombinant antigens, have been developed for the detection of IgG specific for *Scedosporium* spp. and *E. dermatitidis* [9, 18–20]. Here, we investigated the potential of DELFIA® to detect serum sIgE directed against uncommon molds that may be involved in ABPM in pwCF, *i.e.*, *S. apiospermum*, *L. prolificans*, *A. terreus*, *R. argillacea*, and *E. dermatitidis,* using whole protein extracts. In addition, we developed ELISA tests using the same protein extracts to detect specific IgG and assessed the utility of these new serological tools for the diagnosis of ABPM in a multicentric international cohort of pwCF.

Materials and Methods

Human Sera

Three hundred and fifty-six serum samples corresponding to 238 pwCF were collected retrospectively between 2016 and 2019 in eight hospitals in France (Angers, Besançon, Bordeaux, Brest, Rennes, Paris/Saint-Antoine), Italy (Children's Hospital and Research, Institute Banbino Gesù, Rome), and Germany (Centre Berlin-Charité, Berlin). Biological material was obtained only for standard diagnosis on the basis of the physicians' prescriptions. Clinical data were collected retrospectively from medical records and pseudonymized for the analysis. According to the article R1121-1 of the French Code of the Public Health (https://www.legifrance.gouv.fr/codes/article_lc/LEGIARTI000043723460), protocols of this type do not require approval from an ethics committee. This study respects the provisions of French law no. 78-17 dated 6 January 1978 relating to Information Technology, Files and Civil ("Commission Nationale de l'Informatique et des Libertés") and the General Data Protection Regulation (Regulation (EU) 2016/679).

Each date of serum sampling was considered to be a follow-up episode. Biological and clinical data were collected for the six months preceding or following each episode, including clinical signs (cough, fever, dyspnea, expectoration, or deterioration of the general condition), radiological images (tram track opacities,

nodules, finger-in-glove, mucoid impaction, fleeting opacities, bronchiectasis), and microbiological findings (sputum mycological cultures). Other criteria for ABPM were also recorded: eosinophil counts, tIgE levels, and the presence of *A. fumigatus* sIgE using the ImmunoCAP *A. fumigatus* m3 allergen, from Thermofisher Scientific, with a positive threshold of either 0.35 kIU/L according to the manufacturer or 1.91 kIU/L according to Agarwal et al. [21]. Any periods of antifungal treatment were also recorded. Data reporting precipitins or sIgG against *A. fumigatus* were not collected because of the diversity of in-house tests performed in the different participating centers.

One hundred sera from healthy blood donors, who volunteered to donate their blood specifically for research purposes, according to the procedures and ethical rules of the Bourgogne Franche-Comte Blood Transfusion Center (BTC), were used as controls. A control sample consisting of a pool of seven pwCF serum samples, chosen because of the quantity available, was initially used to determine the optimal conditions for DELFIA® and then for standardization of the ELISA test.

Antigen Production

Antigenic extracts were prepared from strains of *S. apiospermum*, *L. prolificans*, *E. dermatitidis*, *R. argillacea*, *A. terreus*, and *A. fumigatus* from the culture collection of the Mycology Department of the Besançon University Hospital. All strains were isolated from clinical samples and species identification was confirmed by ITS1, 18S, or beta-tubulin gene sequencing. They were grown for 7 to 10 days at 30 °C on Sabouraud dextrose agar plates. The obtained mycelia were gently scraped off with a scalpel and crushed with a mortar and pestle in liquid nitrogen. Crushed material (approximately 2 g) was then incubated in 0.1 M Tris–HCl buffer pH 7.5 with a mix of protease inhibitors (SIGMAFAST™ protease inhibitor cocktail; Sigma-Aldrich, Saint-Louis, MO, USA) for 1 h at room temperature with agitation. Cell debris was pelleted by centrifugation (10,000× *g* for 5 min at 4 °C) and the proteins precipitated from the supernatant by the addition of trichloroacetic acid and deoxycholate (7% and 0.1% final concentration, respectively). After centrifugation (10,000× *g* for 7 min), the protein pellet was washed with cold acetone and the proteins finally resuspended in PBS for assessment by SDS-PAGE (Supplemental data, Fig. 1).

Measurement of *specific IgE* by DELFIA®

DELFIA® was performed as previously described by Barrera et al. [15]. Various concentrations of the antigenic extracts and various dilutions of the control sera were tested to define the optimal conditions to give the lowest fluorescence background signal for each mold species. Finally, 96-well microtiter plates (OptiPlate-96 HB, Perkin-Elmer, Turku, Finland) were coated with the antigenic extracts at a final concentration of 0.125 µg/mL (except for the *A. terreus* antigenic extract, 1 µg/mL) and the serum samples were added in triplicate at a 1/10 dilution. Fluorescence was read after 5 min of incubation with enhancement solution with Synergy H1M (Biotek Instruments, Winooski, VT, USA) using a set of specific filters with an excitation/emission wavelength of 340/620 nm. The median of the three count values obtained was calculated. The results from the same plate, standardized according to the ratio obtained between the global background for all plates and the background of the analyzed plate, are reported as the count index (CI). All experiments were conducted over a short period by the same operator to limit inter-assay variability.

Measurement of Specific IgG by ELISA

Final concentrations of 2.5 µg/mL *A. fumigatus, S. apiospermum*, and *L. prolificans* antigenic extracts, 5 µg/mL *R. argillacea* and *A. terreus* antigenic extracts, and 1 µg/mL *E. dermatitidis* antigenic extract were used to coat the microtiter plates. The ELISA procedure was performed as described previously by Rouzet et al. [22], with revelation of the sIgG antibodies by horseradish peroxidase (HRP)-conjugated protein A. The control sample was included in each plate and used for standardization. Serum samples were deposited in three wells and the median of the three obtained values was calculated. The results are expressed as the optical density index (ODI), calculated as follows: (OD_{450nm} of sample—OD_{450nm} of blank)/(OD_{450nm} of control—OD_{450nm} of blank).

Statistical Analysis

For the pwCF population, the median and 1st and 3rd quartiles were determined from the CI (DELFIA®) and ODI (ELISA) values for each antigenic extract to score the sera as follows: 1 for values below the 1st quartile, 2 for values between the 1st quartile and the median, 3 for values between the median and the 3rd quartile, and 4 for values above the 3rd quartile.

For statistical analysis, three levels of serum tIgE were defined: low (< 417 IU/mL), medium (417–999 IU/mL), and high (≥ 1,000 IU/mL), according to published criteria for ABPM for patients with asthma [13] and pwCF [12], which define threshold values of 417 IU/mL and 1,000 IU/mL, respectively.

R version 4.0.2 software was used for statistical analysis. As the data were non-normally distributed, non-parametric tests were used for analysis. Statistical significance was analyzed using Kruskal–Wallis or Wilcoxon tests. For categorical variables (frequencies or percentages), statistical analysis was performed using the Chi-squared test, except for variables for which there were < 10 cases, which were analyzed using Fisher's exact test. Correlation between sIgG ODI or sIgE CI numerical variables was tested using Pearson's correlation test; a positive correlation was considered when the R coefficient was > 0.5. A p-value of < 0.05 was considered statistically significant.

Results

Demographic, Clinical and Biological Data for ABPM

The study population consisted of 238 pwCF, including 162 adults (median age of 28 years, [18–59 years]) and 76 pediatric patients (median age of 12 years, [0–17 years]), which corresponded to 123 male and 115 female patients. All patients with available data had radiological pulmonary opacities consistent with ABPM (Table 1). Cough and expectoration were the most frequent symptoms and bronchiectasis and mucoid impaction were the most frequently reported radiological findings. The eosinophil count was available for 212/356 episodes and was ≥ 500 cells/µL in 14% (29/212) of the episodes. The tIgE level was available for 310/356 episodes and was intermediate (between 417 and 1000 IU/mL) in 9.6% of cases and high (≥ 1,000 IU/mL) in 7.4%. Results from the *A. fumigatus* ImmunoCAP™ sIgE test were available for 315/356 episodes. This test was positive for 41% of the episodes when using 0.35 kIU/L as the cut-off value according to the manufacturer's recommendations and for only 29% of the episodes when using

Table 1 Clinical data and radiological and biological findings in the studied CF population

	Number of episodes (total number)	%
Clinical symptoms		
Cough	194/319	61
Expectoration	190/319	59
Deterioration of general condition	130/319	41
Dyspnea	59/319	18
Radiological findings		
Bronchiectasis	74/146	51
Mucoid impaction	42/146	29
Nodules	18/146	12
Tram track opacities	18/146	12
Fleeting opacities	8/146	5
Finger-in-glove	4/146	3
Microbiological findings		
Positive mycological culture	231/356	65
Aspergillus fumigatus	136/231	59
Aspergillus terreus	31/231	13
Scedosporium spp.	65/231	28
Lomentospora prolificans	9/231	4
Exophiala dermatitidis	37/231	14
Rasamsonia argillacea complex	20/231	9
Mixed (≥ 2 species)	64/231	28
Other biological data		
Total serum IgE		
Low level ≤ 417 IU/mL	257/356	83
Medium level [417–1,000] IU/mL	30/356	9.6
High level ≥ 1,000 IU/mL	23/356	7.4
A. fumigatus sIgE > 0.35 kIU/L*	128/315	41
A. fumigatus sIgE > 1.91 kIU/L**	91/315	29
Eosinophil counts > 500 cells/µL	29/212	14

Data are presented as the number of episodes and frequencies (n/N, %)

*ImmunoCAP *A. fumigatus* allergen m3 threshold according to the manufacturer

**ImmunoCAP *A. fumigatus* allergen m3 threshold according to Agarwal study (2014)

a cut-off value of 1.91 kIU/L according to Agarwal et al. [21].

A positive fungal culture from the sputum was reported for 231/356 (65%) episodes (positive culture for *A. fumigatus*, *Scedosporium/Lomentospora* species, *E. dermatitidis*, *A. terreus*, and the *R. argillacea* species complex for 136, 74, 37, 31, and 20 of the episodes, respectively) (Table 1). *Lomentospora prolificans* was identified in nine episodes of four different pwCF, one of whom was also co-colonized by a *Scedosporium* species. *Scedosporium* species were recovered for 65 episodes, but precise species identification was performed for less than half of the isolates (*S. apiospermum* for 17 pwCF and *S. aurantiacum* for one case). For *Rasamsonia* species (recovered for 20 episodes from 10 pwCF), only genus identification was carried out for eight pwCF and for the remaining two, the species *R. aegroticola* was identified.

For 64/231 (28%) episodes, two or more species were grown from sputum samples in the six months preceding or following the blood sampling (Table 2). *Scedosporium/Lomentospora* species were frequently associated with *Aspergillus* species (29/64, 45%) (Table 2).

DELFIA® specific IgE Against In-House Fungal Antigens

Detection of sIgE against the six antigenic extracts was only performed for 139 samples from pwCF (chosen according to the available quantity of serum, pertinence of the sampling date, and available data associated with the episode) and 75 of the samples from the blood donors (25 from the 100 samples with unvalidated data were retired to the analysis). Levels of sIgE against each of the six antigens, expressed as CI using the DELFIA® technique, were significantly higher in sera from pwCF than in sera from healthy blood donors (Fig. 1—Wilcoxon test, $p < 0.05$ for each antigenic extract). Levels of sIgE against *A. fumigatus* measured by ImmunoCAP™ and DELFIA were positively correlated ($p < 0.001$) for episodes associated with a level > 1.91 kIU/L.

We observed a score of 4 for at least one fungal species for 13% of the samples from the blood donors and 69% from those of the pwCF (Fig. 2A). None of the samples from the blood donors showed a score of 4 for more than two species, whereas 22% from those of the pwCF did (Fig. 2A). A score of 4 for only one species was observed for 30% of the samples from the pwCF (*A. fumigatus* for half of them) and for 12% of those from the blood donors (Fig. 2A).

Globally, the presence of high sIgE levels for *A. fumigatus*, *R. argillacea,* and *E. dermatitidis* was associated with a high tIgE level (> 1,000 IU/mL). Indeed, we observed a high tIgE level for 86% of the pwCF who obtained a sIgE score of 3 or 4 (36% and 50%, respectively) for *A. fumigatus.* Furthermore, 74% of pwCF who obtained a sIgE score of 3 or 4 for *R. argillacea* showed a high tIgE level (corresponding

Table 2 Details of fungal combinations detected by culture

Fungal combinations	Number of episodes (%)
A. fumigatus associated with other fungi ($N = 52$)	
Scedosporium/Lomentospora species	24 (46)
E. dermatitidis	19 (37)
R. argillacea complex	5 (10)
A. terreus	3 (6)
A. terreus + *Scedosporium/Lomentospora* species	1 (2)
Other combinations ($N = 12$)	
A. terreus + *Scedosporium/Lomentospora* species	2
A. terreus + *E. dermatitidis*	1
A. terreus + *Scedosporium/Lomentospora* species + *E. dermatitidis*	2
A. terreus + *R. argillacea* complex	4
E. dermatitidis + *Scedosporium/Lomentospora* species	1
Scedosporium/Lomentospora species + *R. argillacea* complex	2

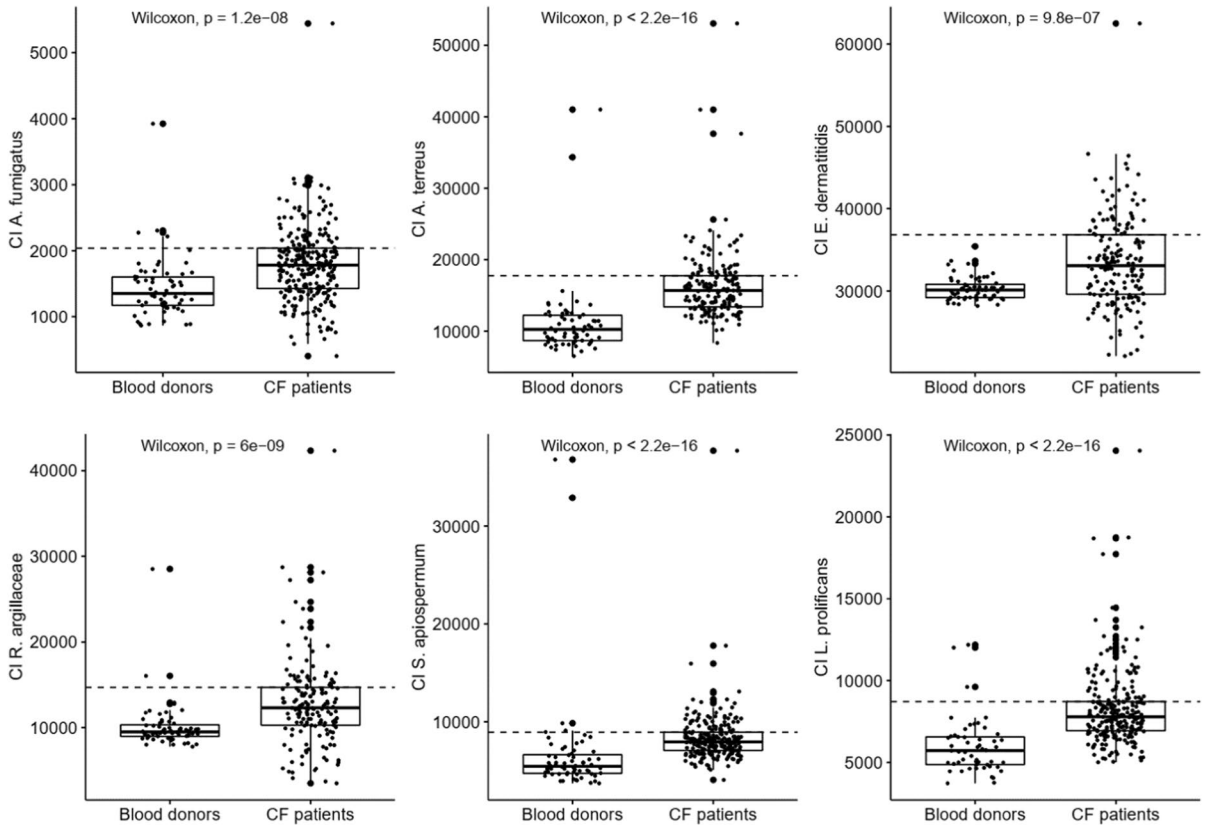

Fig. 1 Specific IgE levels expressed as the count index (CI) for blood donors and CF patients. DELFIA was used to analyze 75 serum samples from blood donors and 273, 190, 177, 162, 248, and 248 sera from CF patients using *A. fumigatus*, *A. terreus*, *E. dermatitidis*, *R. argillacea*, *S. apiospermum*, and *L. prolificans* antigenic extracts, respectively. The dashed line indicates the cut-off CI that defines the 3rd quartile in the CF population

to 63% and 11%, respectively) and 63% for *E. dermatitidis* (corresponding to 26 and 37%, respectively) (Table 3).

There was no positive association between a positive mycological culture from sputum samples and sIgE levels of the corresponding species (Supplementary Table I).

We also investigated correlations between the sIgE score and the age of the patients at the time of sampling. A lower median age was significantly associated with a higher score for *A. terreus* and *L. prolificans* but, inversely, with a lower score for *E. dermatitidis* (Supplementary Table II).

ELISA specific IgG Against In-House Fungal Antigens

We measured the sIgG against the six mold species for all serum samples (Fig. 3). There was a significant difference in ODI levels between the blood donors and pwCF for *A. fumigatus*, *E. dermatitidis*, *R. argillacea*, and *L. prolificans* (Wilcoxon test, $p < 0.05$). The percentage of samples that showed a score of 4 against one or two species was equivalent in the two populations: 33% for the blood donors and 34% for the pwCF, with low involvement of *A. fumigatus* (Fig. 2B). We observed a score of 4 for at least three species for 24% of the pwCF versus 4% of the blood donors (Fig. 2B).

A score of 3 or 4 for sIgG against *A. fumigatus*, *A. terreus*, and *R. argillacea* was obtained for 91%,

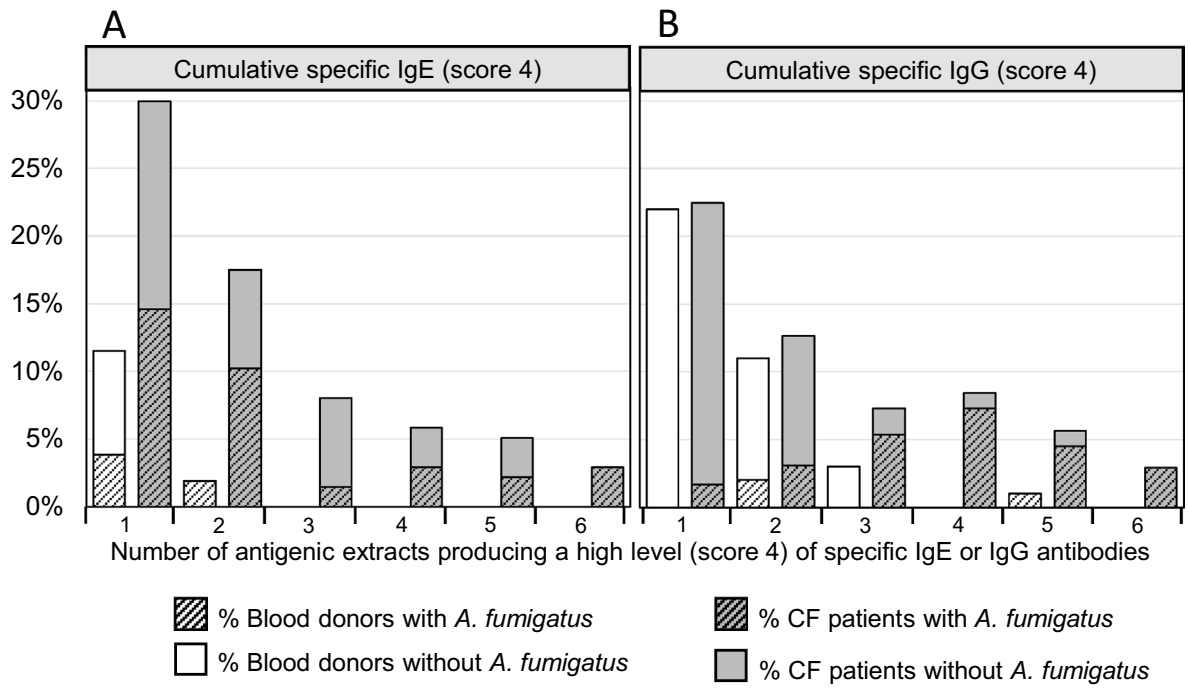

Fig. 2 **A** Percentage of serum samples exhibiting a high sIgE level against one, two, or more antigenic extracts. **B** Percentage of serum samples exhibiting a high sIgG level against one, two, or more antigenic extracts

Table 3 Comparison of the repartition of scores obtained for each antigenic extract according to total serum IgE levels

	Antigenic extracts tested	Total IgE levels			Khi2 *p*-value
		Low <417 IU/mL	Medium [417–1000 IU/mL]	Positive > 1000 IU/mL	
% of episodes with score 1/2/3/4 for sIgE	*A. fumigatus*	32/27/22/19	0/20/20/60	5/9/36//50	**<.001**
	A. terreus	30/26/21/23	17//35/22/26	11/21//42/26	0.308
	S. apiospermum	25/22/27/27	32/32/12/24	18/45/27/9	0.113
	L. prolificans	27/23/25/25	24/28/28/20	25/25/26/24	0.793
	E. dermatitidis	31/23/26/20	4/48/35/13	21/16/26/37	**0.025**
	R. argillacea	33/23/15/29	15/27/38/19	0/26/63/11	**<0.001**
% of episodes with score 1/2/3/4 for sIgG	*A. fumigatus*	25/27/25/23	13/23/37/27	4/4/30/61	**0.001**
	A. terreus	26/25/30/20	23/30/13/33	0/0/17/83	**<0.001**
	S. apiospermum	24/24/26/25	23/30/23/23	30/26/30/13	0.888
	L. prolificans	22/28/26/23	20/10/20/50	13/30/30/26	0.057
	E. dermatitidis	25/26/26/23	27/23/20/30	4/30/30/35	0.391
	R. argillacea	23/26/28/22	13/37/20/30	0/4/22/74	**<0.001**

sIgE serum-specific IgE, *sIgG* serum-specific IgG

p-value < 0.05 in bold

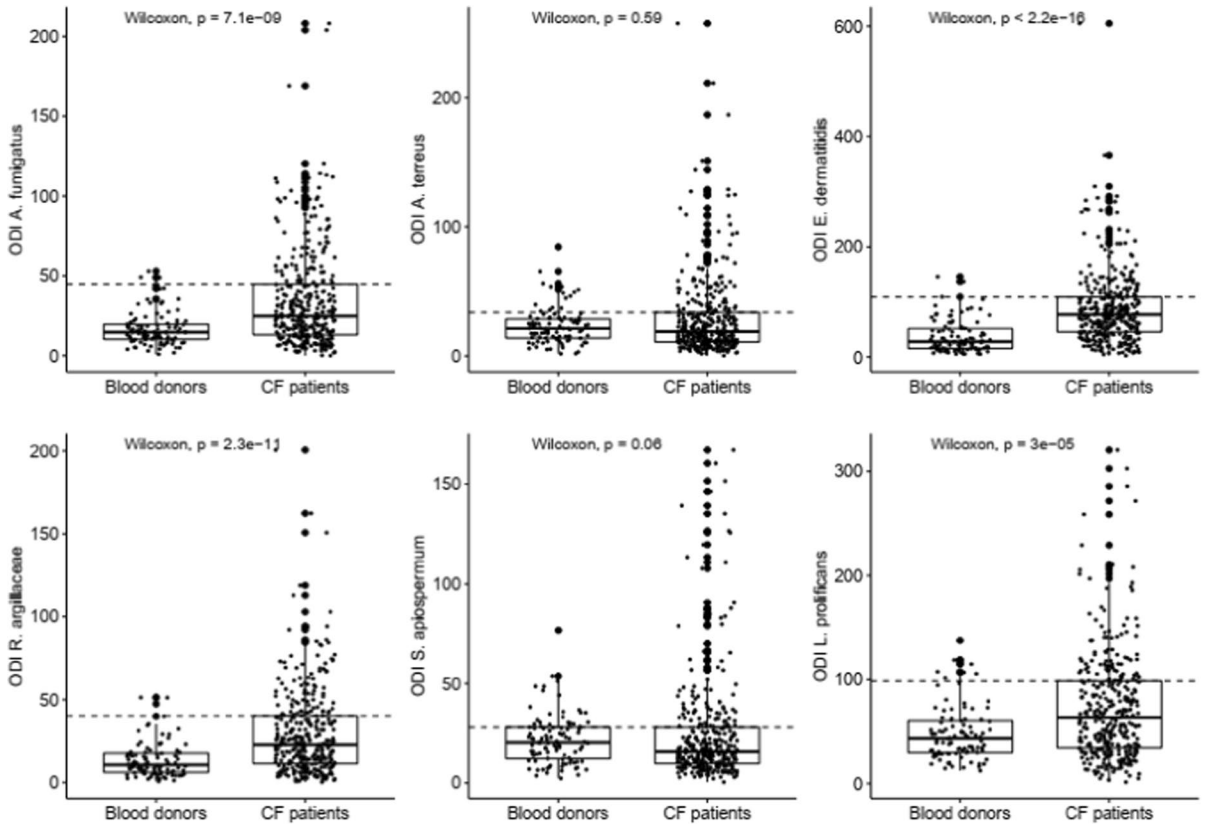

Fig. 3 Specific IgG levels expressed as the optical density index (ODI) for blood donors and CF patients. ELISA was used to analyze 100 serum samples from blood donors and 356 from CF patients using *A. fumigatus*, *A. terreus*, *E. dermatitidis*, *R. argillacea*, *S. apiospermum*, and *L. prolificans* antigenic extracts. The dashed line indicates the cut-off CI that defines the 3rd quartile in the CF population

100%, and 96% of pwCF with tIgE > 1000 IU/mL, respectively (Table 3).

There was a positive association between positive mycological culture in sputum samples and sIgG levels of the corresponding species for *Scedosporium/Lomentospora* ($p < 0.001$) (Supplementary Table I).

Low levels of sIgG (score of 1) against *S. apiospermum*, *L. prolificans*, *E. dermatitidis*, and *R. argillacea* in pwCF were associated with a lower median age, whereas scores of 2, 3, or 4 were observed for older patients (Supplementary Table II). No such association could be demonstrated for the two *Aspergillus* species.

Correlation between the Levels of Specific IgE and IgG Antibodies

There was a significant correlation between the levels of sIgE directed against *S. apiospermum* and *L. prolificans* ($R = 0.78$, $p < 0.05$), as well as those of the corresponding sIgG.

($R = 0.6$, $p < 0.05$) (Fig. 4B, 4D). Similarly, there was a significant correlation between the levels of sIgG against *A. fumigatus* and *A. terreus* ($R = 0.6$, $p < 0.05$) but not between those of sIgE directed against these two fungal species ($R = 0.31$) (Fig. 4A, 4C).

There was no correlation with the DELFIA® or ELISA results for any other combination of two antigenic extracts, demonstrating the absence of cross-reactions detectable by these techniques between the molds tested.

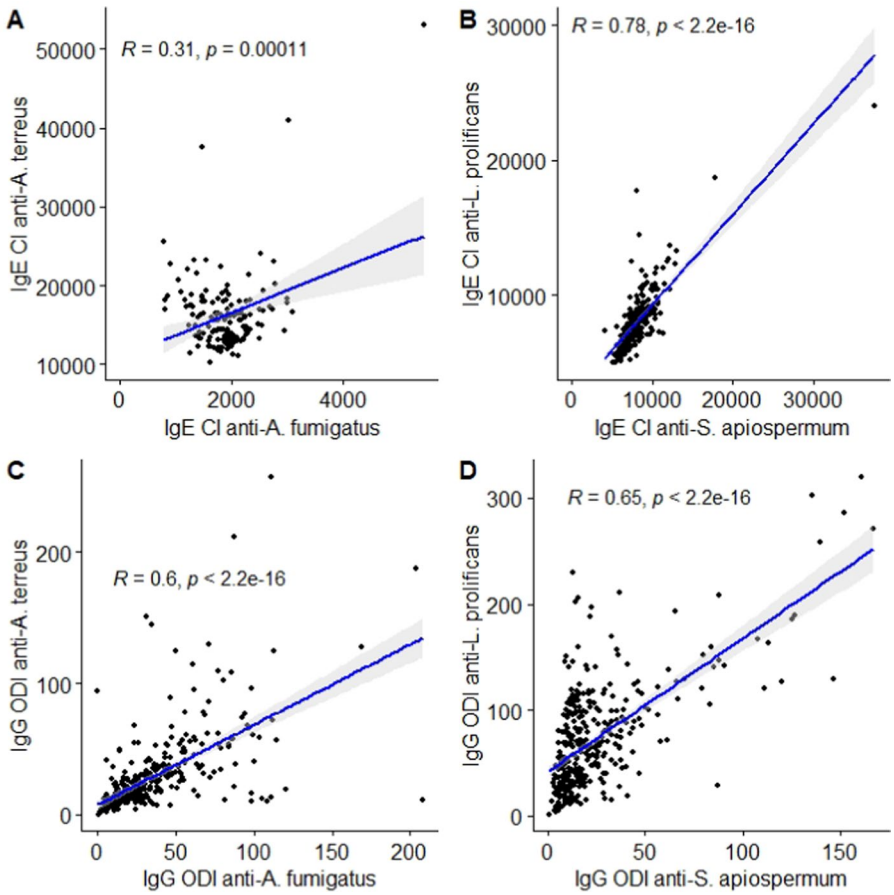

Fig. 4 Correlation between specific IgE and IgG levels directed against *Aspergillus fumigatus* and *Aspergillus terreus* (**A** for IgE, **C** for IgG) and against *Scedosporium apiospermum* and *Lomentospora prolificans* (**B** for IgE, **D** for IgG)

Specific Fungal IgE in CF Patients with a High Total Serum IgE Level

We performed a case by case analysis for patients who presented, at least for one episode, a tIgE level > 1000 IU/mL (17 patients), which is a mandatory criterion for ABPM diagnosis according to Agarwal et al. (2013), or a tIgE level between 417 IU/mL and 1000 IU/mL (19 patients among the 205 pwCF with available data, corresponding to 310 episodes), which is the cut-off value for ABPA for non-CF patients according to Asano et al. (2021).

The details for 17 pwCF presenting with a tIgE level > 1000 IU/mL are shown in Table 4. All patients with available data had a positive value for the *A. fumigatus* ImmunoCAP™ sIgE test (> 0.35 kIU/L according to the manufacturer), whereas only nine pwCF had a score of 4 for *A. fumigatus* sIgE using DELFIA. Three additional pwCF showed elevated sIgE towards other species. A decisional tree for the classification of the 17 pwCF using mandatory criteria for ABPM diagnosis, followed by secondary criteria, is shown in Fig. 5. Using this decisional tree, nine patients could be classified with ABPM, of whom two presented with anti-*A. fumigatus* sIgE exclusively (pwCF #2 and #17, therefore diagnosed with ABPA), and six with sIgE directed against *A. fumigatus* and another mold species (pwCF #4, #6, #7, and #13) or against two non-*A. fumigatus* mold species (pwCF #1 and #3), who were therefore classified as ABPM (Table 4).

Concerning the 19 pwCF with a tIgE level between 417 and 1,000 IU/mL (Table 5), 15 had a positive value for the *A. fumigatus* ImmunoCAP™ specific sIgE test (> 0.35 kIU/L). The test was negative for two pwCF and the results were not available for the two remaining patients. Fourteen presented with a score of 4 for sIgE against at least one species. Among them, five presented

Table 4 Data from the 17 pwCF (23 episodes) with a total serum IgE level > 1,000 IU/mL (only one episode is detailed in this table for each patient)

	Age (years)	tIgE (IU/mL)	ImmunoCAP *A. fumigatus* (kIU/L)	sIgE-DELFIA score						Sputum culture	Eosinophil count (cells/µL)[a]	Antifungal Treatment	Possible ABPM diagnosis
				A. fumigatus	*A. terreus*	*E. dermatitidis*	*R. argillacea*	*S. apiospermum*	*L. prolificans*				
#1	59	> 1000	na	3[b]	2[b]	4	3[b]	3[b]	**4[b]**	*Scedosporium* sp.	430	**PCZ**	ABPM
#2	33	2000	20	**4[b]**	2[b]	3	3[b]	2	3	*E. dermatitidis*	**630**	PCZ **ITZ**	ABPA
#3	26	1025	31	2[b]	**4[b]**	3	**4[b]**	4	4	–	**770**	**ITZ**	ABPM
#4	24	2140	48	4	3	4	3	2	2	*R. argillacea* complex	**600**	ITZ	ABPM
#5	19	1320	na	3[b]	2[b]	2[b]	3[b]	2[b]	3[b]	*S. apiospermum*	130	None	
#6	19	3496	68	4	**4[b]**	4	3	3	4	*E. dermatitidis*	150	None	ABPM
#7	18	3792	17	**4[b]**	3[b]	4	3[b]	2	2[b]	*L. prolificans*, *R. argillacea* complex	**840**	ITZ	ABPA
#8	19	3479	90	4	4	4	4	3	4	*A. fumigatus*	250	None	
#9	17	1068	20	4	3	3	3	2	1	–	210	None	
#10	17	3028	19	3[b]	**4[b]**	3[b]	3[b]	1	2	*A. fumigatus*	400	None	ABPM
#11	14	4397	28	4	3[b]	1	2[b]	2	3	*E. dermatitidis*, *A. fumigatus*	400	ITZ	
#12	12	2754	8	3[b]	1[b]	1[b]	3[b]	1	2	*A. fumigatus*	**1400**	None	
#13	13	1741	700	**4[b]**	**4[b]**	**4[b]**	3[b]	4	4	*A. fumigatus*	**560**	None	ABPM
#14	5	1010	10	3	3[b]	2	2	3	3	*A. fumigatus*	**1050**	None	
#15	27	2955	6	2[b]	nd[b]	nd[b]	nd[b]	2	2[b]	–	na	None	
#16	29	1936	41	1[b]	nd[b]	nd	nd[b]	1	1	–	na	None	
#17	12	2491	25	**4[b]**	nd[b]	nd[b]	nd[b]	1	1	–	na	Treated (no detailed information)	ABPA

IgE and IgG levels are expressed as a score from 1 to 4 according to the quartile as follows: 1 for values below the 1st quartile, 2 for values between the 1st quartile and the median, 3 for values between the median and the 3rd quartile, and 4 for values above the 3rd quartile

[a]Eosinophil counts higher than 500 cells/µL, thus compatible with the ABPM criteria, are indicated in bold font

[b]IgG score of 4 for the same mold species. "4[b]" results are highlighted in bold

Antifungal treatments ongoing at the date of sample collection are indicated in bold (as opposed to previously administered treatments)

ITZ itraconazole, *PCZ* posaconazole, *na* not available, *nd* not determined

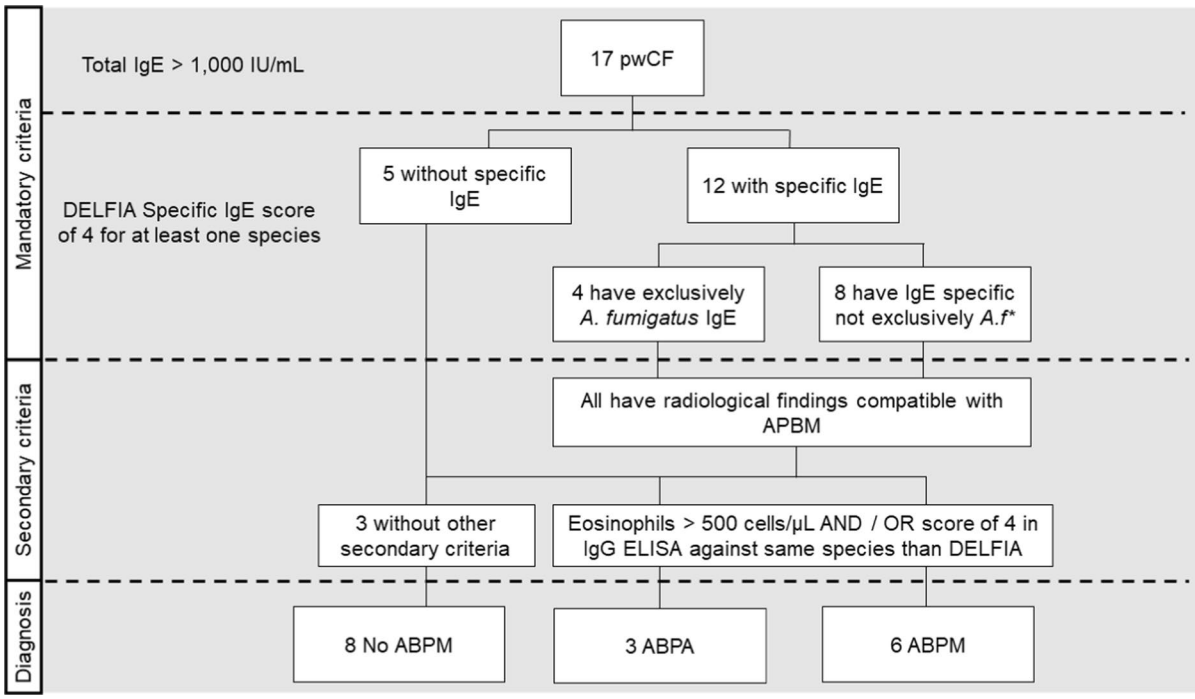

Fig. 5 Flowchart for the decisional diagnosis of ABPM according to DELFIA and ELISA results obtained in the present study and the criteria of ABPA for pwCF defined by the ABPA-ISHAM working-group. **A.f.: Aspergillus fumigatus*. The three cases of ABPA correspond to patients #7, #2, and #17 in Table 5 and the six cases of ABPM correspond to patients #4, #6, #13, #1, #3, #10 in the same table

with a score of 4 for *A. fumigatus* only, seven for *A. fumigatus* and at least one other species, and two for other species only. Finally, using a cut-off value of 417 IU/mL, three pwCF could be considered to have ABPA and seven putative ABPM as presented by the decisional tree in Fig. 6.

Among the 36 pwCF presented in Tables 4 and 5, 21 (58%) had a score of 4 for sIgE against *A. fumigatus*. A score of 4 for sIgE against other fungal species was found for 11 (30%), 10 (28%) 9 (25%), 8 (22%) and 6 (17%) pwCF for *A. terreus*, *L. prolificans, E. dermatitidis*, *S. apiospermum*, and the *R. argillacea* species complex, respectively. Concordance between presence of *A. fumigatus* in sputum and a high level of *A. fumigatus* ImmunoCAP™ sIgE was observed for only 13 of the 36 pwCF.

Discussion

This retrospective multicenter study gave us the opportunity to develop new fluorometric immunoassays to detect sIgE against *A. fumigatus* and five non-*A. fumigatus* molds considered to be potentially involved in ABPM in pwCF. We produced antigenic extracts from *A. fumigatus*, *A. terreus*, *S. apiospermum*, *L. prolificans, R. argillacea,* and *E. dermatitidis* and used them to detect sIgE in pwCF and control participants (blood donors). Our data suggest the potential involvement of some of these molds in ABPM. Thirteen percent of the serum samples from blood donors showed a high level of sIgE and up to one third sIgG against at least one of the molds tested, but we had no information on these donors and it cannot be ruled out that some of them had a fungal allergy.

Using the DELFIA® technique, we found that pwCF had frequently developed sIgE against several mold species simultaneously, and high levels of sIgE directed towards *A. fumigatus* only were infrequent. This result suggests that pwCF may have clinical symptoms linked to ABPA and ABPM simultaneously. We also found high sIgE and sIgG levels against *A. fumigatus, E. dermatitidis*, and the

Table 5 Data from the 19 pwCF (30 episodes) with a total serum IgE level ranging from 417 to 1,000 IU/mL (only one episode is detailed in this table for each patient)

	Age (years)	tIgE (lU/mL)	Immuno-CAP *A. fumigatus* (kIU/L)	sIgE-DELFIA score						Sputum culture	Eosinophil count (cells/µL)[a]	Antifungal Treatment	Putative ABPM diagnosis
				A. fumigatus	*A. terreus*	*E. dermatitidis*	*R. argillacea*	*S. apiospermum*	*L. prolificans*				
#1	60	446	16	3	2	2	3	1	1	*A. terreus, E. dermatitidis*	180	ITZ[c]	
#2	35	542	8	4	3	3[b]	3	3[b]	2[b]	*S. apiospermum*	70	none	
#3	34	806	10	4	**4**[b]	**4**[b]	**4**[b]	**4**[b]	**4**[b]	*A. fumigatus*	190	none	ABPM
#4	33	749	na	**4**[b]	2[b]	2	3[b]	2	3	*E. dermatitidis*	280	PCZ, ITZ[c]	ABPA
#5	25	717	24	**4**[b]	**4**[b]	3[b]	3[b]	4	4	*A. fumigatus, E. dermatitidis*	**910**	none	ABPM
#6	26	769	20	2	**4**[b]	3	3[b]	2	3	–	**500**	**ITZ**	ABPM
#7	25	770	0.18	3[b]	2	2[b]	2	2	1	*A. fumigatus*	**630**	none	
#8	20	424	na	4	3	4	4	**4**[b]	**4**[b]	*Scedosporium* sp.	**500**	**VCZ**	ABPM
#9	24	483	14	2[b]	1[b]	2[b]	2[b]	1[b]	2[b]	*S. apiospermum*	**550**	none	
#10	22	830	16	4	**4**[b]	4	4	4	4	*A. fumigatus, E. dermatitidis*	na	none	ABPM
#11	22	678	46	**4**[b]	2	2	2	2[b]	3[b]	*Scedosporium* sp.	**960**	none	ABPA
#12	22	706	15	4	4	3	3	4	4	*A. fumigatus*	630	none	ABPM
#13	20	904	4	**4**[b]	2[b]	2[b]	3[b]	**4**[b]	3[b]	*S. apiospermum*	na	**ITZ**	ABPM
#14	17	588	9	**4**[b]	2[b]	3	3[b]	2	2[b]	*L. prolificans*	**1090**	ITZ	ABPA
#15	11	706	24	4	3	3	4	3	3	*A. fumigatus*	na	na	

Table 5 (continued)

	Age (years)	tIgE (IU/mL)	Immuno-CAP *A. fumigatus* (kIU/L)	sIgE-DELFIA score						Sputum culture	Eosinophil count (cells/µL)[a]	Antifungal Treatment	Putative ABPM diagnosis
				A. fumigatus	*A. terreus*	*E. dermatitidis*	*R. argillacea*	*S. asperosperum*	*L. proliferans*				
#16	8	985	6	3	4	2	3	2	3	*A. fumigatus*, *E. dermatitidis*	na	none	
#17	8	964	1.3	3	2	2	2	1	2	–	90	none	
#18	19	701	0.1	4	nd	nd	nd	1	1	–	na	none	
#19	43	801	0.43	2	nd[b]	nd	nd	1	1	*A. fumigatus*	na	na	

IgE and IgG levels are expressed as a score from 1 to 4 according to the quartile as follows: 1 for values below the 1st quartile, 2 for values between the 1st quartile and the median, 3 for values between the median and the 3rd quartile, and 4 for values above the 3rd quartile

[a]Eosinophil counts higher than 500 cells/µL, thus compatible with the ABPM criteria, are indicated in bold font

[b]IgG score of 4 for the same mold species. "4[b]" results are highlighted in bold

Antifungal treatments ongoing at the date of sample collection are indicated in bold (as opposed to previously administered treatments)

ITZ itraconazole, *PCZ* posaconazole, *na* not available, *nd* not determined

R. argillacea species complex in pwCF with a high tIgE level (> 1000 IU/mL). Based on the "2013" criteria from the ABPA-ISHAM working group [12] (available at the time of the study design), with an additional criterion of "a sIgE score of 4 for at least one non-*A. fumigatus* mold", we were able to diagnose six cases of ABPM. It is generally accepted that the prevalence of ABPM is underestimated, so when using 417 IU/mL as the threshold for tIgE level (established for ABPA in asthmatic population) and the same additional criterion, seven other pwCF were identified with "putative ABPM".

Based on DELFIA®, 25% of the pwCF had score of 4 for *A. fumigatus* sIgE, whereas 41% of pwCF were positive by the ImmunoCAP™ test (> 0.35 kIU/L). Agarwal et al. [21] suggested increasing the threshold for ABPA diagnosis in pwCF population to 1.91 kIU/L. Application of this cut-off value decreased the frequency of pwCF with a positive sIgE level to 29%, which is more consistent with the 25% identified by DELFIA®.

We observed scores of 3 or 4 for both sIgE and sIgG against *R. argillacea* for patients with a tIgE level > 1000 IU/mL. However, sIgE and sIgG levels were not associated with a positive sputum culture for this species, which suggest an under-estimated role of *R. argillacae* in ABPM, possibly because of difficulties in its isolation from sputum in routine practice. Using an antigenic extract from the *R. argillacea* strain, we were able to detect sIgG and sIgE directed against *R. aegroticola,* which belongs to the same "complex", and is, indeed, more frequently isolated from pwCF samples than *R. argillacea* sensu stricto [10, 23]. Colonization of the airways by species of the *R. argillacea* complex can lead to the deterioration of lung function (10), but in most cases, the clinical significance of their isolation is still unclear due to their frequent association with other filamentous fungi. Therefore, the role of *Rasamsonia* species in ABPM requires further investigation.

Exophiala dermatitidis was more frequently isolated from sputum cultures of pwCF than the *Rasamsonia* species complex, but only one patient fulfilled the criteria of ABPM. Scores of 3 or 4 for *E. dermatitidis* sIgE were significantly more frequent when the tIgE was > 1000 IU/mL. There was no correlation between sIgE and sIgG levels and the recovery of *E. dermatitidis* from sputum in our study. On the contrary, while investigating the infectious role of *E.*

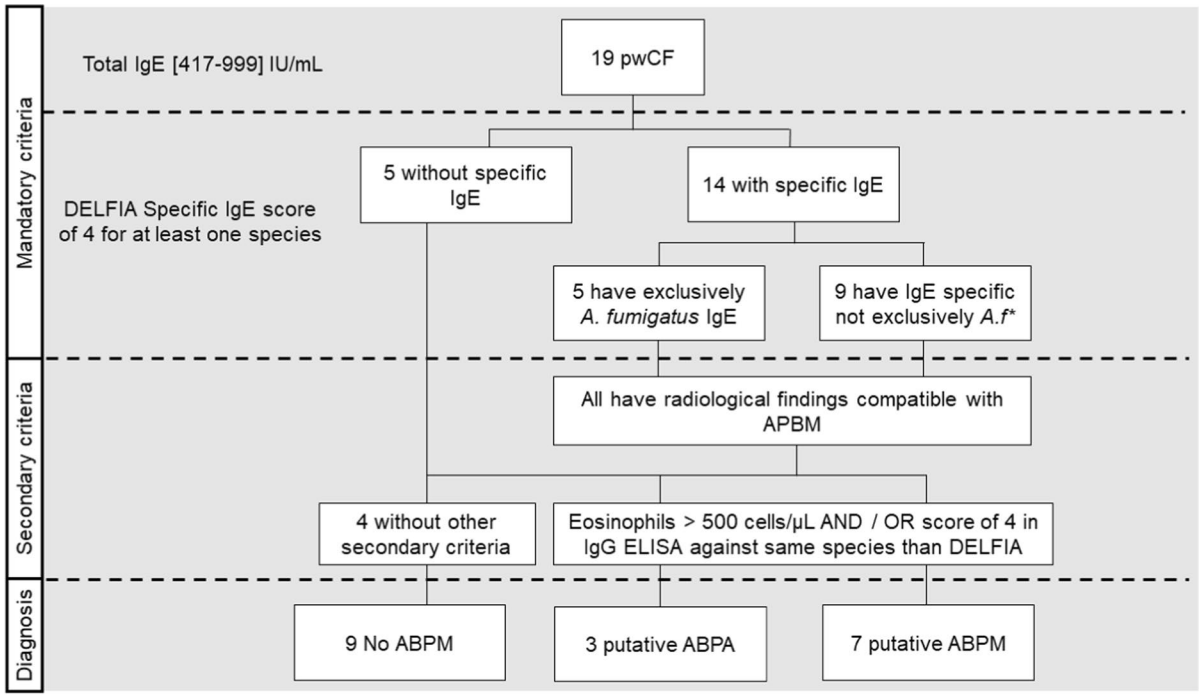

Fig. 6 Flowchart for the decisional diagnosis of ABPM according to DELFIA and ELISA results obtained in the present study and a cut-off value of 417 IU/mL for total, IgE as recommended for the diagnosis of ABPA in asthmatic patients. *A.f.: *Aspergillus fumigatus*. The three putative cases of ABPA correspond to patients #4, #11, and #14 in Table 5 and the seven putative cases of ABPM correspond to patients #13, #5, #12, #8, #3, #10, #6 in the same table

dermatitidis, Kondori et al. [24] showed a correlation between the recovery of this fungus from sputum cultures and positive results for *E. dermatitidis* sIgG by ELISA.

Aspergillus terreus was not commonly observed in the sputum cultures, but we found 12 pwCF who presented with scores of 4 for both sIgE and sIgG concomitantly. Among them, four showed all mandatory criteria of ABPM. Harada et al. [25] showed a link between the levels of *A. fumigatus* and *A. terreus* sIgG but did not investigate the sIgE responses. We found a high correlation between *A. fumigatus* and *A. terreus* sIgG levels, but not those of sIgE.

There was no association between the levels of sIgE and sIgG for *S. apiospermum* or *L. prolificans* and tIgE. To better assess the role of these species in ABPM, decreasing the threshold from 1000 to 417 IU/mL for the tIgE level as a diagnostic criterium for pwCF should probably be considered, as recently proposed for patients without CF [13]. Using this lower threshold, we were able to identify six cases of putative ABPM: six patients presented with a sIgE score of 4 and 3 with a concomitant score of 4 for sIgG directed against one *Scedosporium / Lomentospora* species or both a *Scedosporium* and *Lomentospora* species.

These species can cause severe infections in pwCF and three studies reported promising tests based on sIgG responses to identify infections with them [9, 18, 19]. Antigenic proteins revealed by 2D-western blotting using the sera of mice were shown to be different between animals that solely came into contact with *L. prolificans* and those that were infected (26). Mina et al. [19] developed an sIgG-ELISA using promising recombinant antigens from *Scedosporium* species that showed different antibody levels according to the infection or colonization status of the patients for these fungi. Further studies are needed to better understand the role of *Scedosporium / Lomentospora* in pwCF and detect ABPM and infections due to these species using serological tests.

The DELFIA® technique has the main advantage of being flexible, as it is adaptable to the detection of

sIgE directed against any mold species. The only limitation of this approach is the production of the antigenic extract. However, this method cannot be easily used in routine practice because it is relatively tricky to perform (difficulty in obtaining the lowest fluorescence background noise), costly (exclusive use of Perkin Elmer reagents to optimize the background) and only performed in series due to the use of 96-wells plate; this technique is only suitable for research purposes on series of samples.

Our study also highlights IgG cross-reactions between *Scedosporium* and *Lomentospora* species, which have been previously demonstrated [9]. By contrast, the use of a single antigenic extract to search for sIgE or sIgG from *Aspergillus* species does not appear to be possible. Detection of sIgE against *A. terreus* can be performed using the ImmunoCAP™ *A. terreus* m36 allergen. This technique developed by Thermofisher Scientific allows the switch between the detection of sIgG or sIgE according to the use of the appropriate reagents. This technology is already used for the detection of anti-*A. fumigatus* sIgE in routine practice and could be applied to the detection of anti-*A. terreus* sIgE.

The major limitation of our study was its retrospective design, with heterogeneous data and a large amount of missing information. However, its strength was the development of a new technique that allows the detection of sIgE against different molds involved in ABPM and the demonstration of high levels of sIgE directed against non-*A. fumigatus* molds in 35% of pwCF. In addition, our study suggests that a threshold < 1000 IU/mL should be used for the diagnosis of ABPA/ABPM. Recently, experts from the ABPA working group of the International Society for Human and Animal Mycology (ISHAM) re-evaluated the diagnostic criteria for ABPM and concluded that lowering the tIgE threshold from 1000 to 500 IU/mL was relevant for better monitoring of pwCF [26], which is consistent with our results. These new guidelines also define a probable diagnosis of ABPM when absence of sIgE against *A. fumigatus* and presence of other molds in sputum cultures (other clinical and biological criteria being met). In the absence of sIgE/sIgG commercially suitable tests, a positive culture was the only argument to support the diagnosis of ABPM. However, culture conditions are difficult to standardize, leading to a high variability from one laboratory to another [27]. In our study, we showed the few correlations between cultures and sIgE levels, but only a prospective study with a wide range of growth conditions could truly establish the relationship between culture and specific antibodies. In addition, of the 36 patients presented in Tables 4 and 5, only four presented tIgE > 500 IU/mL and *A. fumigatus* sIgE above 0.35 kIU/L or 1.91 kIU/L. So according to the new guidelines, only these four patients could be suspected of having ABPM, provided that other molds were found in the sputum. In the end, excluding the diagnosis of ABPM when a diagnosis of ABPA is established results in an underestimation of the number of ABPMs.

In conclusion, using the DELFIA® technique, we demonstrate, for the first time, high levels of sIgE directed against non-*A. fumigatus* molds in pwCF, a population at risk for APBM. Even if all ABPA criteria are met, a diagnosis of ABPA does not rule out a concomitant ABPM due to another mold. As a number of these molds are characterized by antifungal susceptibility that is different from that of *A. fumigatus,* serological surveillance using both sIgE and sIgG detection may contribute to alerting clinicians of their presence, even when they are not detected in sputum culture, and thus improve patient management. A prospective study based on the rigorous prospective collection of clinical and biological data may help in assessing the position of this new tool in the management of pwCF and in determining the frequency of serological surveillance to prevent ABPM. Finally, the development of commercial tests and antigens to detect both sIgE and sIgG against a panel of molds involved in ABPM is needed to make the surveillance strategy applicable in all CF care centers.

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Author's Contributions Bouchara JP, Millon L and Barrera C contributed to the study conception and design. Schwarz C, Delhaes L, Le Gal S, Ramel S, Gangneux JP, Guitard J, Hoffmann C provided serum samples and data collection. The experiments were conducted by Barrera C. Data analysis were performed by Barrera C, Millon L, Bouchara JP. The first draft of the manuscript was written by Barrera C, Millon

L, Bouchara JP and Bellanger AP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest All authors declared no conflicts of interest.

Ethical Approval This retrospective study does not require approval from an ethics committee according to the article R1121-1 of the French Code of the Public Health (https://www.legifrance.gouv.fr/codes/article_lc/LEGIARTI000043723460). This study respects the provisions of French law no. 78-17 dated 6 January 1978 relating to Information Technology, Files and Civil ("Commission Nationale de l'Informatique et des Libertés") and the General Data Protection Regulation (Regulation (EU) 2016/679).

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