

Molecular-based allergy diagnosis of allergic bronchopulmonary aspergillosis in *Aspergillus fumigatus*-sensitized Japanese patients

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Summary

Background Distinguishing between patients with allergic bronchopulmonary aspergillosis (ABPA) and *Aspergillus fumigatus* (Af)-sensitized asthmatic patients without ABPA is sometimes difficult owing to the IgE-cross-reactivity between Af and other fungal allergens.

Objective To establish the usefulness of molecular-based allergy diagnostics using allergen components from Af in distinguishing ABPA from Af-sensitized asthma without ABPA.

Methods Sera from Japanese patients with ABPA ($n = 53$) and Af-sensitized asthma without ABPA ($n = 253$) were studied. The levels of IgE and IgG antibodies to allergen components from Af and IgE antibodies to different fungal allergen extracts were measured by ImmunoCAP. Comorbid atopic dermatitis (AD) was taken into consideration in the sensitization profile analysis.

Results Patients with ABPA possessed significantly higher levels of IgE antibodies to Asp f 1, and Asp f 2 than asthmatic patients without ABPA. The areas under the receiver operating characteristic curves for the levels of IgE to Asp f 1 and Asp f 2 as diagnostic markers of ABPA were 0.75 and 0.78, respectively. The presence of IgE positivity to Asp f 1 and/or Asp f 2 resulted in increased sensitivity while losing little specificity. Comorbid AD was associated with higher levels of IgE to Asp f 6 (manganese superoxide dismutase from Af, a ubiquitous pan-allergen in fungi) and low but positive levels of IgE to other Af-components, which hampered the serological discrimination of ABPA.

Conclusions and Clinical Relevance The levels of IgE to Asp f 1 and/or Asp f 2 can effectively differentiate ABPA from Af-sensitized asthma, suggesting that the amounts of IgE specific for these molecules are markers for genuine Af-sensitization in ABPA. However, comorbid AD must be taken into consideration in the interpretation of high IgE to Asp f 6. Establishing of IgE-sensitization profiles using panel of Af-allergen components provides valuable information for distinguishing genuine vs. cross-reactive sensitization in Af-sensitized patients.

Keywords allergen components, allergic bronchopulmonary aspergillosis, *Aspergillus fumigatus*, asthma, atopic dermatitis, component-resolved diagnostics, molecular-based allergy diagnostics, receiver operating characteristic analysis

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Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a pulmonary hypersensitivity disease characterized by uncontrolled asthma, recurrent transient radiographic infiltrate, peripheral and pulmonary eosinophilia and

bronchiectasis [1–4]. ABPA may lead to acute worsening of the respiratory condition and ongoing decline in lung function, ultimately progressing to a chronic state and lung fibrosis, if not treated adequately [5]. ABPA has to be diagnosed promptly, because early treatment is essential to prevent long-term damage. The diagnostic criteria

most widely accepted are those proposed by Rosenberg and Patterson [6], modified by Greenberger [4, 7]. Despite these criteria, it is sometimes difficult to differentiate patients with ABPA from Af-sensitized asthmatic patients without ABPA [2, 8].

As the most common fungal agent causing ABPA is *Aspergillus fumigatus* (Af), the diagnostic criteria of ABPA are based on the patient's immunological reactivity to crude extracts of Af. However, because of cross-reactivity between crude allergen extracts from different fungi [9], apparent sensitization to crude Af extract does not always indicate genuine Af-sensitization. Recently, allergen components purified from their native sources or produced as recombinant proteins have been introduced into the battery of tests available for the diagnosis of allergic diseases [10–13], generally known as component-resolved diagnostics (CRD) [14–16], more recently, molecular-based allergy (MA) diagnostics [17]. Such techniques and diagnostics may solve this problem; however, clinical studies in MA diagnostics for fungal allergy have thus far been limited [18]. Additionally, asthma is frequently complicated by atopic dermatitis (AD) [19]. AD is strongly associated with transdermal sensitization to *Malassezia* spp. [20], and studies have shown cross-reactivity between *Malassezia* spp. and Af [21, 22]. Therefore, comorbid AD should be taken into consideration in studies assessing MA diagnostics for fungal allergy.

In the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database [6], 23 allergenic proteins from Af have been registered. Among these proteins, it is possible to measure the levels of IgE and IgG antibodies (Abs) to recombinant Asp f 1, f 2, f 3, f 4 and f 6 using the commercially available ImmunoCAP system (ThermoFisher Scientific, Uppsala, Sweden). The aim of this study was to determine the utility of measuring the levels of IgE and IgG Abs to these Af-components to distinguish between ABPA and Af-sensitized asthma without ABPA. To determine the influence of AD-related fungal sensitization, Af-sensitized asthma patients were divided into 2 groups according to the presence or absence of comorbid AD, and sera from an additional control group of Af-sensitized AD patients without asthma were also included.

Materials and methods

Study design

We studied 53 adult asthma patients with ABPA who visited the Allergology Department of Sagami National Hospital, one of the largest tertiary hospitals for allergic diseases located in central Japan. The levels of IgE and IgG Abs to allergen components from Af in

patients with ABPA during exacerbations were compared with 3 control patient groups, namely 142 asthmatic patients without AD ('BA without AD', control group 1), 111 asthmatic patients with AD ('BA with AD', control group 2) and 34 Af-sensitized AD patients without asthma ('AD without BA', control group 3). The Ethics Committee of Sagami National Hospital approved the study protocol (No. 8 in 2010). Written informed consent was obtained from each patient.

Patients and diagnostic criteria for ABPA

Patients with ABPA and control asthmatic patients were recruited from the same cohort of consecutive outpatients at the Sagami National Hospital who tested positive for serum IgE to Af between 2002 and 2013. The hospital database with the results of intradermal testing and blood laboratory testing was used to identify those patients who were sensitized to Af. The medical records of patients positive for Af intradermal testing (Torii, Tokyo, Japan) between 2002 and 2008 or positive for serum IgE to Af (ImmunoCAP system, Thermo Fisher Scientific, Uppsala, Sweden) between 2009 and 2013 were reviewed in order to identify the candidates for this study. These patients were required to meet the following eligibility criteria: (1) to be patients with asthma diagnosed by pulmonologists and allergists according to American Thoracic Society criteria [23], (2) to possess serum IgE Abs reacting with Af (≥ 0.35 kU_A/L), (3) not to have any comorbid conditions other than asthma requiring systemic corticosteroid therapy (e.g. rheumatoid arthritis), (4) not to have undergone allergen-specific immunotherapy, (5) not to have any comorbid cardiopulmonary disease (non-tuberculous mycobacteria infection, malignancies, sarcoidosis, old tuberculosis, interstitial pneumonitis, idiopathic chronic eosinophilic pneumonia, eosinophilic granulomatosis with polyangiitis, or cardiac disease), (6) not to have a smoking history greater than 40 pack-years, (7) to have chest X-ray and peripheral eosinophil counts available and (8) not to be older than 79 years. According to these criteria, 361 patients with asthma were identified as candidates for this study.

By medical chart review, we additionally gathered clinical information important for the diagnosis of ABPA, namely serum total IgE (including historical data), precipitating antibodies (PA) to Af, chest X-rays with findings suggestive of ABPA (central bronchiectasis, infiltrates and mucus plugging), and total eosinophil count (including historical data). The most widely accepted diagnostic criteria suggested by Rosenberg-Patterson et al. [6, 24] include 8 criteria, namely (1) asthma, (2) immediate cutaneous reactivity to Af, (3) elevated total IgE > 417 kU/L, (4) elevated serum IgE to Af, (5) central bronchiectasis, (6) chest radiographic

infiltrate, (7) presence of PA to Af and (8) peripheral blood eosinophilia (> 1000 cells/ μL). Criteria (1) to (4) are also minimal essential criteria for seropositive ABPA proposed by Greenberger [4]. Because serum IgE Ab testing, not skin tests, is becoming routine for the screening of sensitization to respiratory allergens in patients with asthma, data from the latter were not available for some of the patients studied. Therefore, in this study, all patients testing positive for serum IgE to Af (≥ 0.35 kU_A/L) were regarded as meeting both criteria (2) and (4), also considering the low external validity of results from skin testing using fungal extracts, which is strongly influenced by variation in the concentration of allergenic proteins between test extracts [25]. We defined patients with ABPA as those who met all criteria (1) to (4) and more than 6 of the 8 ABPA criteria [2]. Patients with asthma who did not meet the ABPA criteria were taken as asthmatic controls. These patients with asthma were divided into two groups (group 1 and group 2) according to their current comorbid AD status.

Fifty-five patients were excluded from the study because their sera were unavailable. Two patients with ABPA were also excluded because their sera were not available during exacerbations. Finally, 53, 142 and 111 patients were included in the study as ABPA cases, BA without AD (control group 1) and BA with AD (control group 2), respectively. We also studied sera from 34 Af-sensitized (defined by positive Af-IgE Ab) AD patients without asthma as control group 4. Numbers of patients with ABPA who fulfilled each criterion are shown in Table S1 in Supporting Information.

Serological analysis

Serum samples from patients were stored at -20°C until assayed. If more than one serum sample taken from the same patient on more than one occasion was available, the sample with the highest levels of IgE Abs to Af was used for the analysis. Levels of serum IgE and IgG Abs specific for crude Af, rAsp f 1, f 2, f 3, f 4 and f 6 were determined using a commercially available ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden; allergen codes, m3, m218, m219, m220, m221 and m222). The levels of serum IgE Abs to *Alternaria alternata* (m6), *Candida albicans* (m5), *Cladosporium herbarum* (m2), *Penicillium chrysogenum* (m1), *Trichophyton rubrum* (m205) and *Malassezia* spp. (m227) were also measured. For patients with ABPA, IgE Abs to *A. flavus* (m228), *A. niger* (m207) and *A. terreus* (m36) were also measured. The sum of the levels of IgE Abs to Asp f 1 and Asp f 2 was also calculated and designated 'IgE to Asp f 1 + f 2'. The level of IgE Abs ≥ 0.35 kU_A/L was regarded as positive according to the manufacturer's recommendation.

Precipitation antibody testing

Precipitating antibodies (PA) to Af in patients' sera were evaluated by Ouchterlony double immunodiffusion testing using commercial crude extract of Af (HollisterStier, WA, USA).

Radiological findings and pulmonary function

A chest X-ray and high-resolution computerized tomography were performed, and central bronchiectasis (internal bronchial diameter greater than the diameter of the accompanying pulmonary artery), infiltrates and mucus plugging were evaluated. Diagnosis of these radiologic findings was based on the consensus of three independent blinded pulmonologists. Pulmonary function tests were performed using standard spirometry, and data acquired on the day nearest to the day when the serum sample was obtained (but not if this was during an exacerbation) were used in the analysis.

Statistical analyses

The collected data were analysed using SPSS version 21.0 (IBM, Tokyo, Japan). The levels of IgE and IgG Abs in sera from patients with ABPA were compared separately with those of control patients groups 1, 2 and 3. We also compared demographic variables, total IgE levels, pulmonary function, asthma duration, and asthma control levels and treatment steps on the basis of the GINA 2010 guidelines [26] between patient groups. The statistical significance of differences in the continuous variables between the groups was analysed by the Mann–Whitney *U*-test or Kruskal–Wallis test. The chi-square test was used to determine whether distributions of categorical variables significantly differed from each another. A *P* value of < 0.05 was considered statistically significant.

Receiver operating characteristic (ROC) curves as diagnostic markers for ABPA were plotted for the levels of IgE and IgG Abs to Asp f 1, f 2, f 3, f 4 and f 6 separately, and the area under each ROC curve (AUC) was calculated. To explore the diagnostic value of the levels of IgE Abs to Af-components at different cut-off values, sensitivity and specificity were calculated from two-by-two tables at the cut-offs of 0.35, 0.70 and 1.00 kU_A/L. The cut-off values 0.35 and 0.70 kU_A/L were determined in accordance with the classification recommended by the manufacturer, and the cut-off value of 1.00 kU_A/L was selected because it is frequently used in the literature [27, 28].

Multivariate logistic regression models were used to evaluate associations of the levels of IgE Abs with each criterion in the Rosenberg–Patterson diagnostic assessment of ABPA, the diagnosis of ABPA and severity of

asthma after adjusting for possible confounders including age, gender and duration of asthma. Associations between the possession of IgE Abs and lung function tests were evaluated using multivariate linear regression analysis after adjusting for age, duration of asthma, gender, severity of asthma and smoking pack-years.

Results

The clinical characteristics of the patients studied here are shown in Table 1. Of the 53 patients with ABPA, 12 (23%) also had comorbid AD. The levels and reactivities of IgE and IgG Abs to allergens are shown in Fig. 1 and Table 2. Frequencies of positivity for IgE Abs to Asp f 1, f 2, f 3, f 4 and f 6 in patients with ABPA were 60, 68, 57, 36 and 38%, respectively. As shown in Fig. 1a, patients with ABPA were characterized by high levels of IgE Abs to Asp f 1, f 2 and f 3, whereas most of the control 'BA without AD' patients had no IgE Abs to any of the Af-components, with the exception of high levels to Asp f 1, f 2 and/or f 3 in some patients. In particular, 21 (15%) of the 142 'BA without AD' patients had antibodies reacting with Asp f 3 but not other Af-components. Some of them also had higher levels of IgE specific for fungi other than Af (data not shown), implying that in these patients, the primary sensitizing agents were not Af. Sensitization profiles of 'BA with AD' patients were different from those of 'BA without AD', and resembled those of 'AD without BA'. Some 'BA with AD' patients showed high levels of IgE

to Asp f 6 and low levels to Asp f 1, f 2, f 3 and f 4, possibly reflecting comorbid AD. Additionally, the levels of IgE Abs to *Malassezia* spp. in 'BA with AD' patients were higher than those to crude Af, suggesting that *Malassezia* spp., not Af, were the primary sensitizer. High levels of IgE Abs to Asp f 6 were also observed in patients with ABPA, especially in those with comorbid AD (see Fig. 2). Although significantly higher levels of IgG Abs to Asp f 1, f 2, f 3 and f 4 in patients with ABPA than in 'BA without AD' patients were observed (Table 2), there was considerable overlap between the groups for this antibody isotype except for IgG to Asp f 1 (Fig. S1 in Supporting Information).

Receiver operating characteristic analysis was performed to evaluate the ability of IgE Abs specific for Af-components to distinguish between patients with ABPA and Af-sensitized patients without ABPA (control groups 1 and 2) (Fig. 3a; 53 ABPA compared with 253 patients with asthma). The AUCs for the levels of IgE Abs to Asp f 1, f 2, f 3, f 4 and f 6 were 0.75 [95% confidence interval (CI), 0.67–0.84], 0.78 (0.70–0.86), 0.71 (0.62–0.80), 0.64 (0.55–0.74) and 0.55 (0.46–0.64), respectively, with the AUC for 'IgE to Asp f 1 + f 2' higher [0.84 (0.77–0.92)]. ROC curves and AUCs remained essentially the same when the subjects were limited to patients without AD (Fig. 3b; 42 ABPA compared with 142 asthma), and patients with comorbid AD (Fig. 3c; 12 ABPA compared with 111 asthma). ROC analyses for IgG to Af-components were also performed (Fig. S2 in Supporting Information). It was found that

Table 1. Characteristics of studied patients

	ABPA (<i>n</i> = 53)	Controls			<i>P</i> value
		Group 1 BA without AD (<i>n</i> = 142)	Group 2 BA with AD (<i>n</i> = 111)	Group 3 AD without BA (<i>n</i> = 34)	
Age (years), mean ± SD	57 ± 12	53 ± 15	35 ± 12	29 ± 10	< 0.001
Gender (female), <i>n</i> (%)	22 (42)	80 (56)	64 (58)	18 (53)	0.235
BA, <i>n</i>	42	142	111	0	
AD, <i>n</i>	12	0	111	34	
Total IgE (IU/mL), median (IQR)*	1170 (810–3760)	643 (277–1555)	2115 (963–4200)	5015 (2350–9522)	< 0.001
Peripheral eosinophil counts (cells/μL), median (IQR)*	580 (210–980)	350 (185–655)	480 (313–730)	N.A.	< 0.001
Duration of asthma (years), median (IQR)	31 (17–40)	18 (5–36)	20 (4–32)	N.A.	0.002
GINA treatment step, <i>n</i> (1 : 2 : 3 : 4 : 5)	3 : 5 : 8 : 28 : 9	12 : 29 : 21 : 71 : 9	15 : 20 : 23 : 52 : 1	N.A.	0.005
Asthma control, <i>n</i> (controlled: partly controlled: uncontrolled)	17 : 25 : 11	91 : 27 : 24	80 : 21 : 10	N.A.	< 0.001
Pulmonary functions†					
FEV1% predicted, mean ± SD	82 ± 21	90 ± 25	94 ± 18	N.A.	0.001
FEV1/FVC, mean ± SD	69 ± 14	73 ± 17	78 ± 9	N.A.	< 0.001
FEF (25–75) % predicted, mean ± SD	40 ± 19	53 ± 27	62 ± 24	N.A.	< 0.001

ABPA, allergic bronchopulmonary aspergillosis; BA, bronchial asthma; AD, atopic dermatitis; IQR, inter quartile range; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; FEF (25–75), forced expiratory flow between 25% and 75%; N.A., not assessed.

*Data of the day when the serum samples for the study were collected.

†Sample size of available data of pulmonary function = 51 (ABAP cases), 129 (group 1) and 104 (group 2).

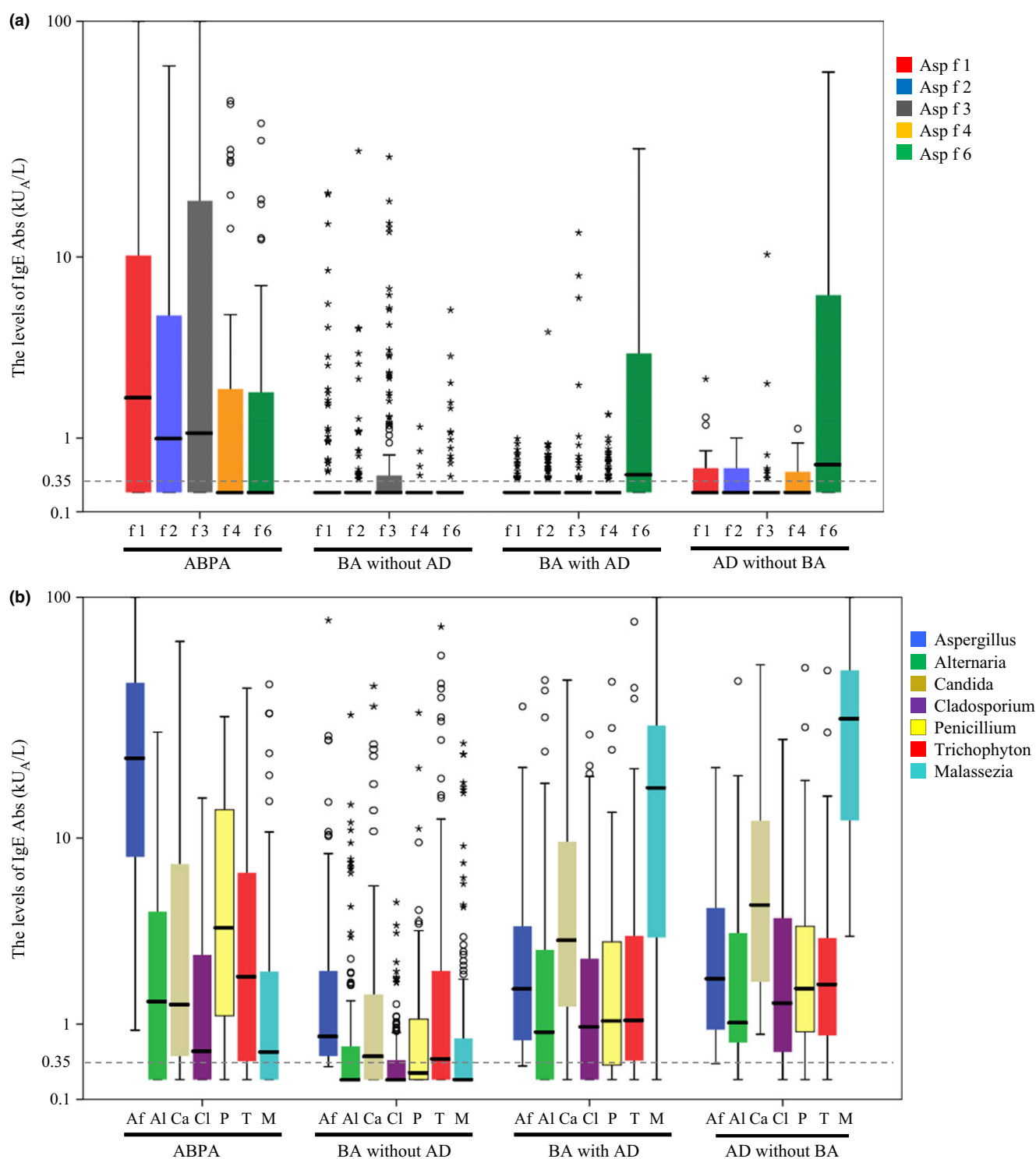


Fig. 1. Box plot of the levels of IgE Abs to Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 (a), and to crude fungal allergens (b) in sera from patients with ABPA and 3 control patient groups. The cut-off (0.35 kU_A/L) is indicated by the dotted line. ○ *, outlier.

although AUC for the levels of IgG to crude Af and Asp f 1 was relatively high, they were still lower than those of IgE to Af-components.

Table 3 shows the sensitivity and specificity of IgE Abs to Af-components at different cut-offs. IgE Abs to Asp

f 1 and f 2 performed well when using 0.7 kU_A/L as the cut-off value. IgE to Asp f 3 also showed good diagnostic performance, but its specificity was slightly lower than IgE to Asp f 1 and Asp f 2. When limiting the analysis to patients with comorbid AD, the specificity of Asp f 1, f 2,

Table 2. The levels and the frequencies of IgE and IgG Abs to crude Af, Af-allergen components and other fungi according to patient group

	ABPA (n = 53)	BA without AD (n = 142)	BA with AD (n = 111)	AD without BA (n = 34)	P value
Frequencies of IgE (≥ 0.35 kU _A /L) to crude Af and Af-components, n (%)					
Crude Af	53 (100)	142 (100)	111 (100)	34 (100)	N.A.
Asp f 1	32 (60)	25 (18) ^{††}	20 (18) ^{††}	14 (41) [†]	< 0.001
Asp f 2	36 (68)	23 (16) ^{††}	25 (23) ^{††}	14 (41) ^{††}	< 0.001
Asp f 3	30 (57)	38 (27) ^{††}	15 (14) ^{††}	8 (24)	< 0.001
Asp f 4	19 (36)	4 (3) ^{††}	22 (20) [†]	13 (38)	< 0.001
Asp f 6	20 (38)	13 (9) ^{††}	62 (56) [†]	21 (62) [†]	< 0.001
The levels of IgE Abs to crude Af and Af-components, median (IQR)					
Crude Af	21.97 (7.76–49.11)	0.79 (0.49–2.27) ^{**}	1.76 (0.72–3.98) ^{**}	2.04 (0.9–4.93) ^{**}	< 0.001
Asp f 1	1.93 (0.34–11.52)	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.53) ^{**}	< 0.001
Asp f 2	0.99 (0.34–5.49)	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.51) ^{**}	< 0.001
Asp f 3	1.10 (0.34–18.54)	0.34 (0.34–0.41) ^{**}	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.34) ^{**}	< 0.001
Asp f 4	0.34 (0.34–2.28)	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.34) ^{**}	0.34 (0.34–0.46)	< 0.001
Asp f 6	0.34 (0.34–2.22)	0.34 (0.34–0.34) ^{**}	0.42 (0.34–4.18) ^{**}	0.56 (0.34–7.35) [*]	< 0.001
The levels of IgG Abs to crude Af and Af-components, median (IQR)					
Crude Af	51.36 (33.01–86.55)	20.88 (11.12–37.49) ^{**}	25.26 (16.33–44.35) ^{**}	15.73 (9.55–24.51) ^{**}	< 0.001
Asp f 1	6.72 (3.96–11.77)	3.87 (3.12–5.36) ^{**}	4.26 (3.18–5.50) ^{**}	4.06 (3.06–6.07) ^{**}	< 0.001
Asp f 2	3.27 (2.49–4.08)	2.82 (2.14–3.33) [*]	2.89 (2.26–3.78)	3.34 (2.36–4.36)	0.023
Asp f 3	2.00 (2.00–3.31)	2.00 (2.00–2.00) ^{**}	2.00 (2.00–2.00) ^{**}	2.00 (2.00–2.68)	< 0.001
Asp f 4	2.46 (2.00–4.27)	2.04 (2.00–2.74) [*]	2.52 (2.00–3.20)	2.91 (2.24–4.28)	0.016
Asp f 6	2.89 (2.00–4.42)	2.71 (2.20–3.58)	3.23 (2.38–4.38)	5.01 (3.82–6.64) ^{**}	< 0.001
Frequencies of IgE (≥ 0.35 kU _A /L) to other fungi, n (%)					
<i>Alternaria alternata</i>	36 (68)	49 (35) ^{††}	79 (71)	28 (82)	< 0.001
<i>Candida albicans</i>	46 (87)	83 (59) ^{††}	104 (94)	34 (100) [†]	< 0.001
<i>Cladosporium herbaru</i>	33 (62)	43 (30) ^{††}	82 (74)	31 (91) ^{††}	< 0.001
<i>Penicillium chrysogenum</i>	52 (98)	71 (50) ^{††}	85 (77) ^{††}	31 (91)	< 0.001
<i>Trichophyton rubrum</i>	41 (77)	77 (54) ^{††}	90 (84)	32 (94)	< 0.001
<i>Malassezia</i> spp.	31 (59)	47 (33) ^{††}	104 (94) ^{††}	34 (100) ^{††}	< 0.001
The levels of IgE Abs to other fungi (kU _A /L), median (IQR)					
<i>Alternaria alternata</i>	1.46 (0.34–5.53)	0.34 (0.34–0.64) ^{**}	0.86 (0.34–2.99)	1.03 (0.61–3.65)	< 0.001
<i>Candida albicans</i>	1.39 (0.47–7.68)	0.49 (0.34–1.64) ^{**}	3.33 (1.32–9.78) [*]	4.94 (1.92–11.95) ^{**}	< 0.001
<i>Cladosporium herbarum</i>	0.56 (0.34–3.25)	0.34 (0.34–0.44) ^{**}	0.95 (0.34–2.66)	1.43 (0.55–4.36)	< 0.001
<i>Penicillium chrysogenum</i>	3.86 (1.05–13.63)	0.28 (0.34–1.10) ^{**}	1.06 (0.36–3.44) ^{**}	1.78 (0.85–4.1)	< 0.001
<i>Trichophyton rubrum</i>	2.10 (0.42–7.18)	0.45 (0.34–2.33) ^{**}	1.07 (0.43–3.58)	1.9 (0.79–4.05)	< 0.001
<i>Malassezia</i> spp.	0.55 (0.34–2.36)	0.34 (0.34–0.77) ^{**}	16.47 (3.37–30.47) ^{**}	31.98 (11.47–53.1) ^{**}	< 0.001

Af, *Aspergillus fumigatus*; ABPA, allergic bronchopulmonary aspergillosis; BA, bronchial asthma; AD, atopic dermatitis; IQR, inter quartile range; N.A., not assessed.

[†]P < 0.05, ^{††}P < 0.01; difference compared with values in ABPA group by chi-square test.

^{*}P < 0.05, ^{**}P < 0.01; difference compared with values in ABPA group by Mann–Whitney U-test.

f 3 and f 4 was greatly improved when the cut-off was set at ≥ 0.7 kU_A/L compared to when the cut-off was ≥ 0.35 kU_A/L, probably owing to non-specific low level IgE reactivity to Af-components in patients with AD complications. The combination of IgE to Asp f 1 and/or Asp f 2 contributed to increased sensitivity without losing much specificity, namely sensitivity and specificity of 77 and 85%, with the cut-off set to 0.7 kU_A/L.

In addition, associations of the levels of IgE Abs to Af-components with each diagnostic criterion and the diagnosis of ABPA were evaluated after adjusting for age, duration and severity of asthma (Table S2 in Supporting Information). The levels of IgE Abs to Asp f 1, Asp f 2 and Asp f 1 + f 2 were strongly associated with all the diagnostic criteria and the diagnosis of ABPA

even after adjustment. Weak but significant associations were also observed between the possession of IgE Abs to Asp f 3, f 1 + f 2 and poorer lung function (Table S3 in Supporting Information). In contrast, no associations between levels of IgE Abs to Af-components and asthma severity were observed or were marginal (Table S4 in Supporting Information).

We also evaluated the association between the sensitization profile and the presentation of the disease in patients with ABPA. Possession of IgE to Asp f 1 and/or f 2 was significantly associated with lower pulmonary function in patients with ABPA (Table S5 in Supporting Information). Possession of IgE to Asp f 1 and/or f 2 was associated with a larger number of Rosenberg–Paterson criteria being met, indicating that IgE reactive

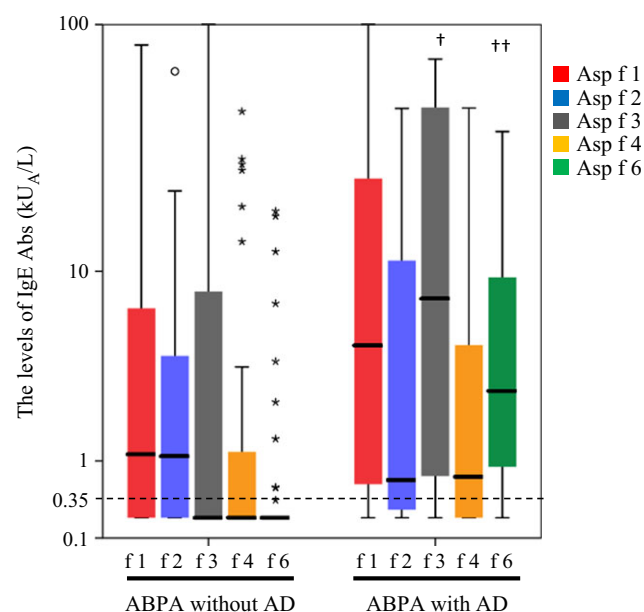


Fig. 2. Box plot of the levels of IgE Abs to Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 in sera from ABPA patients with or without AD. The cut-off (0.35 kU_A/L) is indicated by the dotted line. ○ *, outlier; †, $P < 0.05$, ††, $P < 0.01$, difference between the groups.

with these allergens was associated with more typical ABPA disease presentation (Fig. 4).

Of the 53 patients with ABPA, 42 (79.2%) possessed IgE to Asp f 1 and/or f 2, with 11 (20.1%) being negative for both. Detailed clinical information on these latter 11 patients is shown in Table S6 in Supporting Information. Two of them had higher levels of IgE Abs to *A. niger* than to Af and 5 had PA against *A. niger*, *A. flavus* or *Candida albicans* but not against Af. These findings may imply that fungi other than Af had contributed to the development of their disease.

Discussion

This study evaluated the utility of MA diagnostics using allergen components from Af to distinguish ABPA from Af-sensitized asthma without ABPA. Among the allergen targets studied, IgE to Asp f 1 and Asp f 2 showed good diagnostic performance, and combining them both even more so. The associations between the presence of IgE Abs to Asp f 1 and f 2 and the diagnosis of ABPA were significant even after adjustment for age, duration of asthma and severity of asthma. Comorbid AD in patients with asthma was associated with high levels of IgE to Asp f 6 and positive but low levels of IgE to other Af-components, which hampered serological discrimination of ABPA in Af-sensitized allergic patients. Comorbid AD must therefore be taken into consideration in MA diagnosis of ABPA.

One of the most important implications of MA diagnostics is the ability of this approach to distinguish genuine sensitization from sensitization due to cross-reactivity after evaluation of the sensitization profile to specific allergens and pan-allergens [17]. Asp f 1 has been identified as an 18-kD allergen, which is almost identical to restrictocin cloned from *A. restrictus*. In silico genomic analysis of fungal allergens reported by Bowyer et al. [29] showed that Asp f 1 has no homology with any known fungal genome (not including *A. restrictus*), indicating that Asp f 1 is a highly species-specific allergen for Af. Additionally, as it is not present in spores and is produced after their germination and growth of the fungi [30], sensitization to Asp f 1 can be considered as a specific marker for exposure to Af which has germinated in patients' respiratory tracts [31]. Asp f 2 [32] can also be regarded as a relatively specific allergen from Af and/or fungi other than Af in the genus *Aspergillus* [33, 34]. Together, both Asp f 1

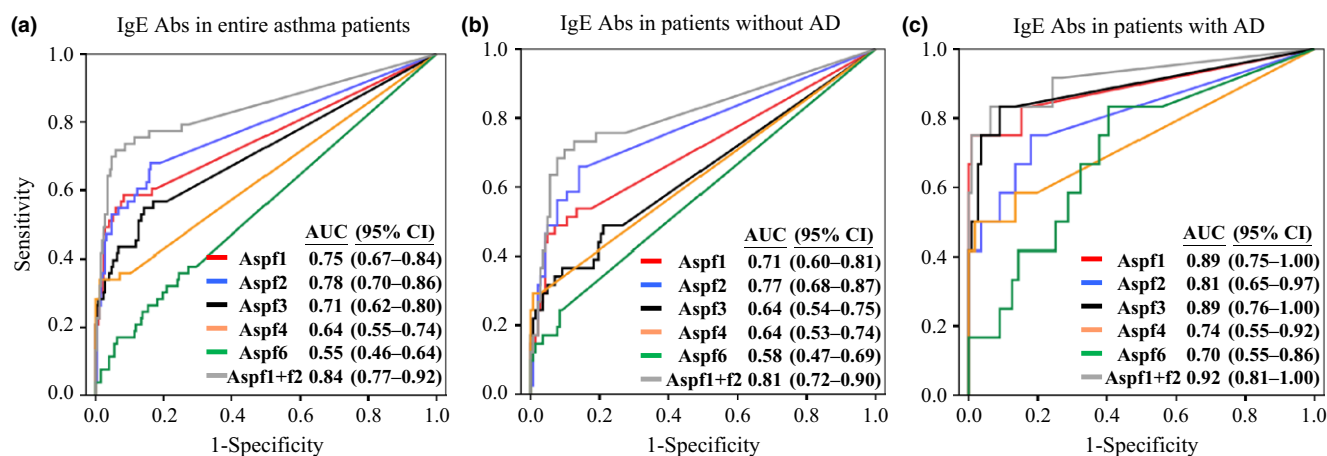


Fig. 3. Receiver operating characteristic curves for the levels of IgE Abs to Af-components in the diagnosis of ABPA. Analysis of all patients (a), in patients without AD (b), and in patients with AD (c). AUC, area under the curve; CI, confidence interval.

Table 3. Sensitivity and specificity of IgE Abs to Af-allergen components at different cut-off values for the diagnosis of ABPA

Allergen components from Af Cut-off values	All patients*		Subanalysis			
	Sensitivity (%)	Specificity (%)	Patients without AD [†]		Patients with AD [‡]	
			Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Asp f 1 (kU _A /L)						
0.35	60	82	54	82	83	82
0.70	59	91	54	87	75	96
1.00	55	94	51	89	67	100
Asp f 2 (kU _A /L)						
0.35	68	81	66	84	75	78
0.70	55	93	56	91	50	95
1.00	49	95	51	92	42	99
Asp f 3 (kU _A /L)						
0.35	57	79	49	73	83	87
0.70	55	85	49	78	75	95
1.00	51	87	41	80	75	96
Asp f 4 (kU _A /L)						
0.35	36	90	29	97	58	80
0.70	34	96	29	99	50	93
1.00	34	98	29	100	50	97
Asp f 6 (kU _A /L)						
0.35	38	70	24	91	83	44
0.70	32	78	17	94	83	57
1.00	28	81	17	95	67	62
Combination of the positivity						
Asp f 1 ≥ 0.35 and/or Asp f 2 ≥ 0.35	79	73	76	73	92	73
Asp f 1 ≥ 0.70 and/or Asp f 2 ≥ 0.70	77	85	76	81	83	91
Asp f 1 ≥ 1.00 and/or Asp f 2 ≥ 1.00	74	91	73	85	75	99

Af, *Aspergillus fumigatus*; AD, atopic dermatitis.

*53 ABPA compared with 253 asthma (control groups 1 and 2).

[†]42 ABPA compared with 142 asthma (control group 1).

[‡]12 ABPA compared with 111 asthma (control group 2).

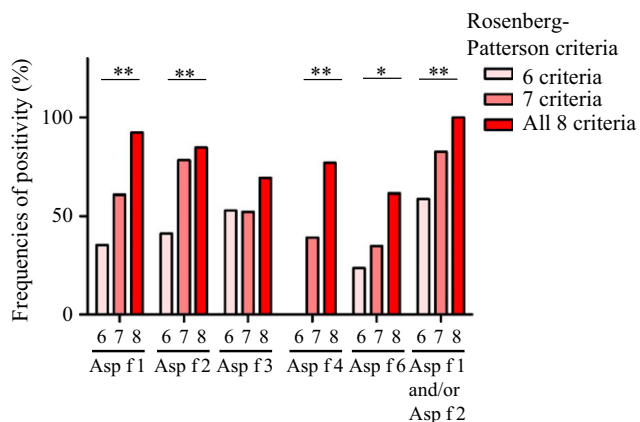


Fig. 4. Association between the frequencies of IgE to Af-components and the number of Rosenberg-Patterson criteria met. *, P -trend <0.05; **, P -trend < 0.01.

and f 2 can be regarded as specific allergens for genuine Af-sensitization. In contrast, Asp f 3 and f 6 can be regarded as pan-allergens in fungal allergy. Asp f 3 is peroxisomal membrane protein (PMP) and shows

high similarity to and identity with PMP in *Candida* spp. [35] and *Malassezia* spp. [22]. Asp f 6 is manganese superoxide dismutase (MnSOD), which is a ubiquitous allergenic protein in fungi [36]. In particular, Mala s 11, which is MnSOD from *Malassezia sympodialis* and a major allergen of *M. sympodialis* in AD, is 56% identical to Asp f 6 [21].

The frequencies of patients with IgE reacting with Asp f 1, f 2 and f 3 were 60, 68 and 57%, respectively, indicating that these three are the major allergens of Af for ABPA. This finding is in agreement with previous studies [37, 38]. ABPA was effectively identified by the presence of IgE to Asp f 1 and f 2, with AUCs of 0.75 and 0.78, respectively. This finding is compatible with the concept of MA diagnostics, in which both Asp f 1 and f 2 can be regarded as specific allergens for genuine Af-sensitization. The AUC of IgE to Asp f 3 was also as high as 0.71. However, we should be conservative and careful in the interpretation of this finding, because Asp f 3 is an even more cross-reactive allergen than most others. Additionally, we have to emphasize that the specificity of IgE to Asp f 3 was lower than to Asp f 1 and f 2, reflecting the

likelihood that some Af-sensitized asthmas were monosensitized to Asp f 3.

Eleven of 53 patients with ABPA were negative for IgE to both Asp f 1 and Asp f 2. Regarding these patients, the possibility remains that fungi other than Af may have contributed to the development of their disease. Therefore, it might be said that identification of the fungi colonizing the airways is useful, especially when patients' sensitization profiles are atypical for ABPA, namely negative for both Asp f 1 and f 2. Identification of colonizing fungi may be important information for the attending physicians especially when they plan ABPA patient treatment with antifungal drugs [39].

Studies have shown that IgE to Asp f 4 and f 6 is a highly specific marker for ABPA [37, 40, 41]. In our study, IgE to Asp f 4 was the most specific test for ABPA of all the Af-components. On the other hand, diagnostic specificity of IgE to Asp f 6 was not high, owing to the high frequency of sensitization to Asp f 6 in control asthma patients with AD. We consider that the major reason for the discrepancy between results of previous studies and our present study relates to the difference in the selection of control Af-sensitized patients. In our study, if the analysis was limited to patients without AD, the specificity of IgE to Asp f 6 was high (91%). Therefore, comorbid AD strongly influences the interpretation of the presence of IgE to Asp f 6. Indeed, the diagnostic specificity of IgE to Asp f 6 in some previous studies did not reach 100% [37, 40], although data regarding AD complications for each studied patient were not provided in those publications.

To the best of our knowledge, the present investigation is the first study also to evaluate the performance of IgG reactive to Af-components for the diagnosis of ABPA. IgG Abs to Asp f 1 also showed good diagnostic performance, but they were not superior to IgE Abs for this purpose.

The major limitation of this investigation relates to external validity of the study result in that all the subjects were Japanese. The sensitization profile of Af-sensitized asthma patients might be different in different populations, because of the difference in the predominant environmental fungi between communities [42]. This may affect the generality of our findings and applicability to other patient populations. Additionally, cystic fibrosis is extremely rare in Japan, which might influence the results. The possibility of misclassification of cases and controls cannot be completely excluded because CT scans and PA were not performed for every patient in the study. However, the effect of misclassification is assumed not to be very strong because this study was performed at a tertiary hospital specialized in allergic diseases, and because CT scans and PA are

routinely performed when attending physicians suspect the possibility of ABPA.

One other limitation may relate to the fact that we have not identified which fungi colonized the airways and did not have a way to determine the fungi genuinely sensitizing each patient. *A. flavus*, *A. niger* [43], *Candida albicans* [44] and *Schizophyllum commune* [45, 46] can also induce the same clinical condition of ABPA. However, identification of colonizing fungi by sputum culture may also have problems, because the culture positivity rate is not always high [47], and dichotomies between sensitizing and colonizing fungi have also been reported [39]. Additionally, transient findings in sputum culture may not reflect the long-term historical fungal colonization. In contrast, the production of IgE Abs usually results from long-term exposure to the fungal allergens, and there is a possibility that IgE Abs may be more likely to represent historical fungal colonization than transient sputum culture.

The reliability of the findings in this study was also confirmed using different diagnostic criteria for ABPA. The International Society for Human and Animal Mycology working group has recently proposed new diagnostic criteria for ABPA [2]. Even when we repeated the same analysis using these new criteria, instead of Rosenberg-Paterson's criteria, the results were almost identical (data not shown).

In conclusion, establishing of IgE-sensitization profiles using panel of Af-allergen components provides valuable information for identifying genuine vs. cross-reactive sensitization in Af-sensitized patients. ABPA can be effectively differentially diagnosed by measuring IgE Abs to Asp f 1 and f 2. Using a cut-off value of 0.7 kU_A/L, the combination of IgE positivity to Asp f 1 and/or f 2 showed good diagnostic performance, with a sensitivity and specificity of 77 and 85%, respectively. Comorbid AD should be taken into consideration in MA diagnosis of ABPA, because it hampers diagnostic performance of IgE to Af-components. More research in different populations is required to confirm the general validity of our findings.

Acknowledgements

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Conflict of interest

YF, HY, MT and KA have received fees for speaking and/or organizing education by Thermo Fisher Scientific.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Box plot of the levels of IgG Abs to crude Af, Asp f 1, Asp f 2, Asp f 3, Asp f 4, and Asp f 6 in sera from ABPA patients and 3 control patient groups.

Figure S2. Receiver operating characteristic curves for the levels of IgG Abs to crude Af and Af-components in the diagnosis of ABPA.

Table S1. Number of patients meeting each criterion in Rosenberg-Paterson diagnostic assessment of ABPA.

Table S2. Association between levels of IgE Abs to Af-components and Rosenberg-Patterson diagnostic criteria among patients with Af-sensitized asthma (ABPA cases, control group 1 and 2, $n = 306$).

Table S3. Crude and adjusted standardized coefficients (β) in a multiple regression model for the association between positivity for IgE Abs to Af-components and pulmonary functions in patients with Af-sensitized asthma (ABPA cases, group 1 and group 2, $n = 284$).

Table S4. Associations between levels of IgE Abs and severity of asthma among patients with Af-sensitized asthma (ABPA cases, group 1 and group 2, $n = 306$).

Table S5. Crude and adjusted standardized coefficients (β) in a multiple regression model for the association between positivity for IgE Abs to Af-components and pulmonary functions in patients with ABPA ($n = 50$).

Table S6. Sensitization profile and clinical characteristics of 11 ABPA patients who were negative for IgE to both Asp f 1 and Asp f 2.