

ORIGINAL ARTICLE

Asthma related to *Alternaria* sensitization: an analysis of skin-test and serum-specific IgE efficiency based on the bronchial provocation test

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Clinical & Experimental Allergy

Summary

Background It is difficult to find a causal relationship between exposure to *Alternaria* spores and the development of asthma symptoms in sensitized individuals due to the complexity of clinical situations in which positive diagnostic tests are often found.

Objective To analyse the diagnostic efficiency of skin testing (ST) and serum-specific IgE to *Alternaria*, based on the results of a bronchial specific challenge with *Alternaria* extracts.

Methods Seventy-four asthmatic patients sensitized to *Alternaria* underwent a specific bronchial challenge with this mould. Skin-testing weal sizes, serum-specific IgE values (CAP-system) and bronchial challenge results were analysed by receiver operating characteristics curves (ROC curves) and logistic regression. The sensitivity, specificity, positive and negative predictive values were calculated for different cut-off points.

Results Bronchial challenges to *Alternaria* elicited a positive result in 45 patients (61%). Skin prick testing almost perfectly predicted the outcome of bronchoprovocation tests (area under the ROC curve of 0.957), whereas intradermal skin testing had moderate efficacy. A negative result for skin prick test (SPT) showed a 4% probability of a positive bronchial challenge in the logistic regression analysis. However, weals around 5.5 mm in diameter had 90% probability of a positive challenge. Quantification of serum-specific IgE correctly classified 86% of the cases. In the logistic regression analysis, a CAP value ≥ 16 kU_A/L predicted a positive bronchial challenge result with 99% accuracy, whereas for a CAP value < 0.35 kU_A/L, this probability was 33%.

Conclusions and Clinical Relevance Most asthmatic patients with positive SPT results to *Alternaria* would have a positive bronchial challenge. As atmospheric mould levels may vary significantly with the weather conditions, sensitized patients should be instructed on the risk situations, environmental control measures and the importance of correct medication compliance. Immunotherapy with *Alternaria* could also be taken into account as a valid therapeutic option.

Keywords allergic asthma, alternaria serum-specific IgE, alternaria skin tests, logistic regression, ROC curve

Submitted 30 October 2009; revised 3 September 2010; accepted 27 September 2010.

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Introduction

A European multicentre study among patients with suspected respiratory allergy showed a global prevalence of allergic sensitization to *Alternaria* and *Cladosporium* of 9.4%, but ranging from 3% in Portugal to 20% in Spain [1]. The clinical relevance of this sensitivity is often uncertain. The specific diagnosis of *Alternaria* allergy can be made only by a combination of positive diagnostic tests and careful recording of the symptoms induced by *Alternaria* exposure. Positive skin tests (ST) and specific

IgE antibody assays have been shown to predict symptoms [2]. In fact, sensitization to *Alternaria* allergens appears to be a significant independent risk factor for asthma in children and adults [3, 4] and the frequency of sensitization to *Alternaria* increased significantly with increasing asthma severity [5–9]. Aerobiological studies show that the environmental levels of *Alternaria* spores depend on different geographical areas, weather and climate conditions, and therefore, they would vary from one place to another and from year to year in the same location [10]. Changes in the environmental levels can make it difficult

to establish a link between symptoms and exposure, which is further complicated by the fact that Alt a 1, *Alternaria* major allergen, is expressed in other members of the Pleosporaceae family as well. Moreover, high levels of this allergen can be detected in the air at times of the year when there are no *Alternaria* spores in the environment [11, 12]. Furthermore, patients with *Alternaria* sensitization are frequently sensitized to other inhaled allergens such as mould and/or pollen [1]. In the Madrid area, most patients also show grass sensitization. These pollens are present in the air together with *Alternaria* spores during May and June.

In some allergic processes, analysis of different cut-off point values for specific IgE and/or ST size can predict the actual clinical relevance of sensitization [13]. Specific serum IgE levels for mites can be a useful predictor of current allergen exposure and clinical sensitivity [4]; regarding cat allergy, the levels of serum-specific IgE to cat allergens are also useful [14].

In the case of asthma due to sensitization to airborne allergens, bronchoprovocation testing (BPT) yields useful information on the existence of specific and symptomatic bronchial reactivity, supplementing data from STs and specific serum IgE assessments [15]. When there are doubts on whether the allergen is really causing clinical allergy because of an inconclusive clinical history, we perform BPT as part of our standard diagnostic protocol. Based on the results obtained with BPT, we have analysed the diagnostic efficiency of STs and specific serum IgE (as determined by CAP) with *Alternaria* allergens in predicting the results of a bronchial-specific challenge with an *A. alternata* allergenic extract.

Material and methods

Type of study

A retrospective analysis was carried out of data from the clinical reports of asthmatic patients who underwent bronchoprovocation tests with *Alternaria*. This procedure is part of our current clinical protocol to establish an accurate diagnosis of specific bronchial hyperreactivity to *Alternaria*, when there is diagnostic uncertainty, especially in the case of multiple sensitizations and overlap in terms of seasonal exposure with other pollens/moulds. Before the bronchoprovocation, patients were informed about the procedure and signed an informed consent form approved by the Quality Committee of our hospital.

The patients' clinical data were used following the national law for this issue, and this was approved by the Ethics Committee of our hospital.

Subjects

Data from this study were obtained from the clinical reports of 74 asthmatic patients sensitized to *Alternaria*

allergens. As there was no clear-cut relationship between this sensitization and allergic symptoms, these patients underwent a BPT with *Alternaria*. Patients had been initially referred to our Allergy outpatient clinic by their general practitioner or other specialists. A thorough clinical history was obtained in order to confirm the diagnosis and severity of asthma, following the Global Initiative for Asthma Guidelines-2002 [16], and its possible relationship with allergy to inhaled allergens. In this regard, subjects were asked about worsening of their symptoms related to season, weather or climate changes. After the clinical history and physical examination were carried out, patients underwent ST with a battery of allergens (*vide infra*). Serum-specific IgE for *A. alternata* allergens was also assessed. These results were evaluated by an allergist, who further completed the study with a BPT in order to obtain a more precise diagnosis of the clinical relevance of *Alternaria* sensitization. Patients with severe asthma were excluded for BPT.

Skin prick test

Subjects underwent skin prick testing (SPT) with a battery of allergens including grass, tree and weed pollens, dust mites (*Dermatophagoides pteronyssinus* and *farinae*), dog and cat epithelium and moulds (*Aspergillus fumigatus*, *Alternaria alternata* and *Cladosporium herbarum*) (ALK-Abello, Madrid, Spain). The *Alternaria alternata* extracts contain 100 BU/mL and 25 mcg/mL of Alt a 1. The SPTs were performed on the forearm using an ALK-Lancet (ALK-Abello, Horsholm, Denmark).

Negative and positive control tests were performed with 50% glycerine-saline and histamine (10 mg/mL), respectively. After 15 min, the skin prick weal induced by allergens and the negative and positive controls were outlined and copied onto permanent records with adhesive cellotape; the orthogonal diameters were subsequently measured. A positive SPT result was defined as a weal at least 3 mm larger than the diameter of the negative control. All subjects avoided short-acting antihistamines for 72 h, long-acting antihistamines for 5 days and tricyclic antidepressants for 2 weeks before ST [17].

Intradermal skin test

In patients with negative SPTs, we also performed intradermal skin tests (IDST) with *Alternaria*, *Aspergillus* and *Cladosporium* extracts. In these cases, we injected 0.02 mL into the forearm with a 26-gauge hypodermic needle using the 1 BU/mL allergenic-extract concentrations. Control tests included a saline solution and histamine (0.1 mg/mL). A positive IDST result was defined as a weal at least 5 mm larger than the diameter of the negative control [18]. Also, weals were outlined and copied onto permanent records with adhesive cellotape.

Intradermal skin test titration

Immediately before BPT, all subjects underwent IDST titration with 10-fold dilutions of the 1 BU/mL extract (1, 0.1, 0.01 and 0.001 BU/mL).

Serum-specific immunoglobulin E

Specific serum IgE to *A. alternata* was assessed using the CAP system (CAP-FEIA, Phadia Diagnostics, Uppsala, Sweden). The positive cut-off value was ≥ 0.35 kU_A/L as provided by the manufacturer. The values for recombinant Alt a 1 were assessed in 21 cases, when this allergen was available.

Bronchial test

All patients underwent a BPT with *A. alternata* extracts using the hand-operated technique described by Yan et al. [19]. A DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, PA, USA) with a fitted output of 0.28 mL/min and intermittent aerosol generation was used [18]. The mouthpiece of the nebulizer was placed between the teeth of the subject, who exhaled to slightly below the functional residual capacity (FRC) and then inhaled slowly over 1–2 s towards total lung capacity, where the breath was held for 3 s. This manoeuvre was repeated five times, with each concentration of the allergenic extract being tested. The rest of the procedure was performed as described by Chai et al. [20]. The same extract as for ST, with concentrations ranging in tenfold increments from 0.001 to 10 BU/mL, was used. The dried allergenic extract (10 BU/mL: 2.5 mcg/mL Alt a 1) was reconstituted with a saline solution on the same day as the BPT. Flow-volume curves were carried out using a Jaeger Pneumotach (Viasys Healthcare GmbH, Hoechberg, Germany). Briefly, asymptomatic patients were studied when the baseline forced expiratory volume in 1 s (FEV₁) was 80% or greater with respect to the standard value. Pulmonary function studies were repeated 10 min after the inhalation of five breaths of saline solution. The specific bronchial test was carried out if a decline in the FEV₁ value < 10% was obtained. The challenge began with five breaths of the 0.001 BU/mL *A. alternata* extract. The next dilution was administered after 10 min if less than a 15% reduction from the control FEV₁ had taken place. If the reduction in FEV₁ was between 15% and 19%, an additional 10-min delay was implemented. Usually, the higher dose (10 BU/mL) was administered in a fractionated manner: two inhalations first, and if no FEV₁ changes were detected, 10 min later, the three remaining inhalations were administered. The test was completed, either when an FEV₁ decline $\geq 20\%$ was detected (positive result) or if no significant decline occurred after the inhalation of the most concentrated *A. alternata* extract (negative result). According to the nebu-

lizer calibration, every five extract inhalations are approximately equivalent to the administration of 100 µL. The cumulative nanogram dose of Alt a 1 was plotted on a semi logarithmic scale to assess the cumulative dose causing a 20% decline in FEV₁. Before the BPT procedure, all subjects avoided short-acting bronchodilators and inhaled steroids for 12 h, long-acting bronchodilators, theophylline and leukotriene antagonists for 24 h, short-acting antihistamines for 72 h, long-acting antihistamines for 5 days and tricyclic antidepressants for 2 weeks. In the case of a positive test, patients were given a dose of a short-acting bronchodilator and spirometry tests were repeated at 20-min intervals until normalization of pulmonary function. Patients remained in the hospital under observation for 6 h and spirometry was repeated hourly or sooner if the patient had any symptoms. In the case of a delayed reaction, oral corticosteroids (0.5 mg/kg) and inhaled Beta-2 agonists were given and patients were kept under observation until the symptoms reversed. A next-day evaluation was also performed. On a subsequent protocol modification, patients experiencing an acute reaction were also given oral corticosteroids, followed by home peak flow determinations and next-day hospital evaluation.

Statistical analysis

Quantitative variables were summarized by central tendency measures (mean or median), together with a dispersion measure such as standard deviation or range.

Qualitative variables were described by their distribution of frequencies and percentages, both globally and in special interest subgroups. A model of binary logistic regression was used to generate probability curves that depict the likelihood of patients with a particular size of ST and serum-specific IgE concentration to *Alternaria* allergens generating a positive outcome in BPT. The odds ratios are shown with Wald's 95% confidence intervals (CI). The receiver operating characteristic (ROC) analysis was used to quantify the accuracy of the diagnostic tests (STs and CAP) and their capacity to discriminate between a positive or a negative outcome in the BPT with *A. alternata* allergens. The area under the ROC curve is an overall index of diagnostic accuracy that does not depend on a decision threshold. An area of 0.5 indicates that the discriminatory ability of the test is not better than chance. An area of 1.0 indicates perfect discriminatory ability. For these analyses, an SAS 12.1 (SAS Institute Inc., Cary, NC, USA) statistical package was used. Assay performance was computed using the following definitions: where FN is a false-negative diagnostic test result, FP is a false-positive diagnostic test result, TP is a true-positive diagnostic test result and TN is a true-negative diagnostic test result. Sensitivity [TP/(TP+FN)100] is the percentage of positive tests in subjects with a positive BPT. Specificity [TN/(TN+FP)100] is the percentage of negative tests in

subjects with a negative BPT. The predictive value for a positive test $[TP/(TP+FP)100]$ describes the percentage of subjects with a positive test and a positive BPT. The predictive value for a negative test $[TN/(TN+FN)100]$ is the percentage of subjects with a negative test and a negative BPT. The association between qualitative variables has been analysed by means of the χ^2 test. The relationship between normally distributed continuous data and a dichotomic variable was assessed using Student's *t*-test, whereas the Mann-Whitney *U*-test was used for non-normally distributed continuous data. Comparisons were considered to be significant at a *P*-value of 0.05.

Results

Subject characteristics

The group included 43 females and 31 males, with ages ranging between 14 and 41 years (mean 22.6 years). Nine patients (12%) had intermittent asthma, 26 patients (35%) had mild persistent asthma and 39 (53%) had moderate persistent asthma. Sixty-four patients (86.5%) had other concomitant allergies: 57 (77%) patients had pollen allergy; 29 (39%) patients had positive STs for other moulds, mainly *Aspergillus*; 25 (34%) patients were ST-positive for dog and/or cat epithelium; and 14 (19%) patients were sensitized against dust mites. Table 1 shows how these data are distributed among patients with BPT-positive and -negative results. Fifty-seven patients reported worsening in spring that was mainly attributed to pollen allergy by the responsible physician. Other 28

patients associated their symptom worsening with humidity in general (mist, rainy days, humid environments), which could or could not be related to *Alternaria* allergy. In eight other patients, there were no suggestive symptoms of *Alternaria* allergy in their clinical records.

Skin-tests and serum-specific IgE

The results for SPTs with *A. alternata* were positive in 47 of the 74 patients (mean weal diameter 7.8 ± 2.9 mm, range: 3–14 mm). Serum-specific IgE results were positive in 55 of the 74 patients (mean 9.6 ± 17 kU_A/L range 0.35–100 kU_A/L). Twenty-six subjects with negative results for SPT with *A. alternata* showed a positive result for IDST (mean weal diameter 9.3 ± 3.2 , range 5–18.5 mm). The results are summarized in Table 1. Serum-specific IgE for rAlt a 1 ranged from <0.35 to 21.5 kU_A/L. One patient with positive results for CAP to *Alternaria* showed a negative result to Alt a 1 (Table 2).

Bronchial provocation test results

The BPT elicited a positive result in 45 patients (61%). The median FEV₁ decline was 28.8% (range between 20% and 57%) (Table 2). The majority of immediate reactions recovered with SABA administration in <30 min, including a female patient with an initial FEV₁ descent of 57%. Fifty percent of positive BPTs not treated with oral steroids presented with dual reactions. All but one recovered with SABA and oral corticosteroids also in <30 min. One patient had a prolonged reaction that required SABA and oral steroids' administration for 3 days. Once oral steroids were routinely given for immediate reactions dual responses were no longer observed. Three patients out of 47 with positive results for SPT showed a negative result for BPT. Twelve out of 55 patients with positive results for serum-specific IgE showed a negative BPT. In these 12 cases, the IgE values ranged from 17.8 kU_A/L (one patient) to 0.5 kU_A/L. Most IgE values in this group were under 2 kU_A/L. One out of 26 patients showing a negative SPT and positive IDST showed a positive result for BPT. One patient with only a positive result for serum-specific IgE (0.8 kU_A/L) showed a negative result for BPT.

Statistical analysis

Skin tests. The estimated area under the ROC curve for SPT-100 BU was 0.957 [95% CI 0.911–1.004] (Fig. 1). Predictive values for different cut-off points are shown in Table 3. In SPT logistic regression analysis (Fig. 2), a sigmoidal morphology curve was obtained. The probability of a BPT-positive result varied according to weal size: 4% when the SPT was negative and over 90% with weals around 5.5 mm in diameter. None of the curves obtained

Table 1. Clinical characteristics, ST sizes and CAP values in patients according to the *A. alternata* BPT outcome

Variables	Positive BPT patients <i>N</i> = 45 (61%)	Negative BPT patients <i>N</i> = 29 (39%)	Statistical significance (<i>P</i> < 0.05)
Age (years) (mean ± SD)	21.5 ± 6.7	24.4 ± 7.5	NS
Sex (female/male)	26/19	17/12	NS
Asthma severity			NS
Mild intermittent	4	5	
Mild persistent	14	12	
Moderate	27	12	
Other allergies			0.01
Yes	43	21	
No	2	8	
SPT-100 BU (mm) (mean ± SD)	7.7 ± 3.14	0.6 ± 1.9	0.000
IDST-1 UB/mL (mm) (mean ± SD)	11.4 ± 7.7	8 ± 4.4	0.01*
CAP (kU _A /L)	10.6 ± 18	1.4 ± 3.4	0.005

*Non-normally distributed data.

BPT, bronchoprovocation testing; SPT, skin prick test; ST, skin testing; IDST, intradermal skin testing.

Table 2. Characteristics of patients with positive *Alternaria* bronchoprovocation testing

	Age (year)	Sex	SPT (mm)	CAP <i>Alternaria</i> kU/L	CAP Alt a 1 kU/L	Total IgE kU/L	PD20-FEV ₁ Alt a 1 (ng)	Descent FEV ₁	Other allergies	Asthma severity
1	40	Male	10.5	28.6	ND	532	1	45	Yes	Moderate persistent
2	19	Male	4.5	5.8	ND	–	200	33	Yes	Moderate persistent
3	29	Female	12	1.6	0.93	13.6	17	24	Yes	Mild persistent
4	16	Male	3	0.8	ND	436	22	23	Yes	Moderate persistent
5	24	Female	5	<0.35	ND	–	–	20	Yes	Mild persistent
6	30	Male	12.5	5.1	2.7	125	15	22	Yes	Moderate persistent
7	30	Male	13.5	2.6	ND	37	14	20	No	Moderate persistent
8	23	Female	0	<0.35	ND	644	2	27	Yes	Moderate persistent
9	24	Female	9	2.9	ND	27	210	36	Yes	Mild persistent
10	14	Female	8	4.4	ND	–	45	30	Yes	Moderate persistent
11	16	Male	6	4.7	ND	149	11	37	Yes	Mild persistent
12	16	Male	3	2.5	ND	–	1	36	Yes	Mild persistent
13	32	Male	5	10.6	ND	613	–	36	Yes	Moderate persistent
14	24	Male	6.5	44.5	ND	653	11	26	Yes	Moderate persistent
15	16	Female	11	5.3	ND	70	2.2	20	Yes	Mild persistent
16	15	Male	11	11.6	ND	25.6	15	31	Yes	Moderate persistent
17	21	Female	11	4.8	ND	24.5	110	20	No	Mild persistent
18	17	Female	6.5	> 100	ND	1189	4.5	40	Yes	Moderate persistent
19	20	Male	6	4.9	ND	73	15	24	Yes	Moderate persistent
20	14	Male	7.5	1.2	ND	72	2	36	Yes	Moderate persistent
21	20	Male	4.4	1.1	ND	130	45	20	Yes	Moderate persistent
22	15	Female	3.5	61	ND	1107	10	39	Yes	Mild persistent
23	30	Female	10	7.1	6.24	166	11	20	Yes	Mild persistent
24	19	Female	11	7.7	2.03	230	0.8	32	Yes	Mild persistent
25	17	Female	5.4	11.2	<0.35	396	200	20	Yes	Mild persistent
26	14	Male	6	16.2	21.5	273	24	20	Yes	Moderate persistent
27	17	Female	9.5	2.7	1.58	164	20	21	Yes	Moderate persistent
28	25	Female	9	12.7	8.07	91.2	54	28	Yes	Moderate persistent
29	24	Female	8	3	1	63.8	55	33	Yes	Moderate persistent
30	31	Female	7	<0.35	<0.35	27	20	20	Yes	Mild persistent
31	34	Male	14	1.1	ND	6	12	29	Yes	Moderate persistent
32	17	Female	4	38.9	ND	–	20	30	Yes	Mild persistent
33	15	Male	9	8.4	7.89	140	24	20	Yes	Moderate persistent
34	29	Female	8	6	4	105	40.5	34	Yes	Mild persistent
35	22	Female	10	5	ND	131	81	23	Yes	Moderate persistent
36	17	Female	9	1.4	1.28	23.8	36	57	Yes	Moderate persistent
37	19	Female	7.5	6.1	2.61	104	112	20	Yes	Mild persistent
38	15	Female	7	7.4	3.28	244	112	20	Yes	Moderate persistent
39	19	Male	4	9.8	ND	–	70	21	Yes	Mild persistent
40	15	Male	12.5	3.7	ND	44.5	0.9	28	Yes	Mild persistent
41	22	Male	11.5	1	1.29	33.2	24.7	20	Yes	Mild persistent
42	15	Male	9	6	4.5	161	45	31	Yes	Moderate persistent
43	16	Female	6	10	4.6	103	11.2	33	Yes	Moderate persistent
44	43	Female	7.5	2.6	1.96	–	8.1	38	Yes	Moderate persistent
45	28	Female	6	2.2	ND	–	180	22	Yes	Moderate persistent

ND, not done; FEV₁, forced expiratory volume in 1 s; SPT, skin prick test.

with different extract concentrations used in IDSTs were better than the AUC value obtained with SPT (Fig. 1). These values were: IDST-1 BU/mL 0.763 [95% CI 0.606–0.866], IDST-0.1 BU/mL 0.777 [95% CI 0.663–0.891] and IDST-0.001 BU/mL 0.782 [95% CI 0.669–0.895].

Serum-specific IgE. The estimated area under the ROC curve for serum-specific IgE was 0.859 [95% CI

0.768–0.951] (Fig. 1). Analysis of different cut-off points is shown in Table 3. The optimal point for CAP is 1 kU_A/L, with 91% sensitivity and 72% specificity. In the logistic regression analysis, a hyperbolic morphology curve is obtained (Fig. 3). A low or a negative result for CAP is less useful than a higher result: when a CAP value is negative, the estimated probability of having a positive BPT is still 33%. On the other hand, an increasing CAP value

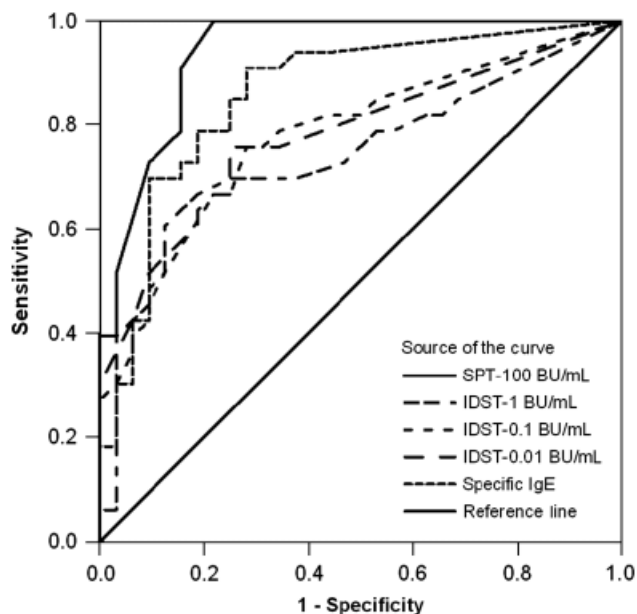


Fig. 1. Receiver operating characteristics (ROC) curves: skin prick test (SPT), intradermal skin test (IDSTs) and serum-specific IgE vs. bronchoprovocation results.

Table 3. Predictive values of several skin tests and CAP cut-off points

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SPT-100 BU				
≥ 3.0 mm	98	89.7	94	96
≥ 5.0 mm	82	93	95	77
≥ 8.0 mm	47	100	100	55
IDST-1BU				
≥ 5.0 mm	81	31	56.5	60.0
≥ 11 mm	56.0	93	90	66
IDST-0.1BU				
≥ 5.0 mm	80	55	68.3	69.6
≥ 12 mm	25.7	100	100	52.7
IDST-0.01BU				
≥ 5.0 mm	65.7	79	79	66
≥ 9.5 mm	31	100	100	54.7
CAP				
≥ 0.35 kU _A /L	93	58	78	85
≥ 1.00 kU _A /L	91	72	82	83
≥ 18.00 kU _A /L	11	100	100	42

IDST, intradermal skin tests; SPT, skin prick test.

corresponds to a higher probability of a positive BPT, being 90% for 8 kU_A/L and 99% for values of 16 kU_A/L.

Discussion

In the present study, SPTs have been proven to be very accurate to predict results for bronchial provocation tests with *Alternaria*. (AUC 0.957) (Fig. 1) In the case of a negative SPT, the probability of a negative BPT result is very high. In contrast, when SPT results are positive, it is almost certain to obtain a positive BPT result (Fig. 2). This

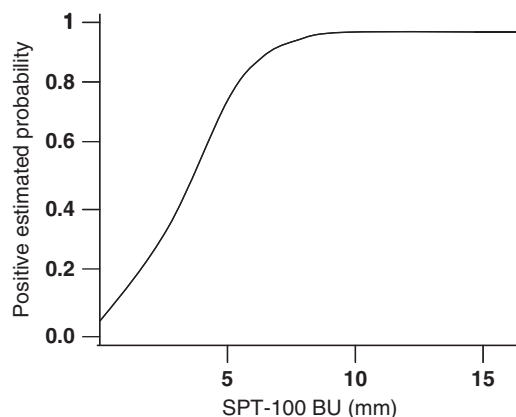


Fig. 2. Predicted relationship between skin prick testing and positive bronchoprovocation results with *Alternaria* allergens. The odds ratio estimate was 2.56 (95% Wald confident limits 1.69–3.80).

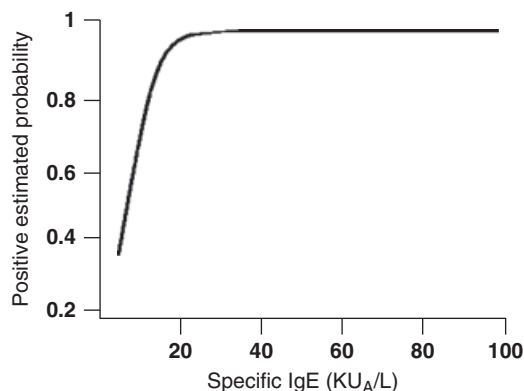


Fig. 3. Predicted relationship between CAP and positive bronchoprovocation results. The odds ratio estimate was 1.43 (95% Wald confident limits 1.15–1.79).

high diagnostic accuracy for SPT would explain why only one patient out of 26 SPT-negative and IDST-positive patients showed a positive BPT. In the patients included in this study, and with this particular extract, an additional IDST would mean a decrease in diagnostic accuracy, as SPT is an almost perfect diagnostic test. In comparison with SPT, specific serum IgE to *Alternaria* has less diagnostic accuracy, mainly because low CAP values have a low significance; however, as the anti-*Alternaria* IgE values increase progressively, the probability of a positive BPT increases as well: values over 8 kU_A/L predict a positive BPT result with 90% probability (Fig. 3). Different sources for SPT and BPT extract manufacture, on the one hand, and CAP, on the other, could account for the disparities between SPT and CAP in predicting BPT results. Recombinant Alt a 1 is currently available for detecting specific IgE; it is possible that better results could have been obtained for low serum CAP values with this allergen. However, in this study, we could only perform this test in 21 patients, which is an insufficient sample size to

perform a valid statistical analysis. In a previous work of our group, we studied patients with allergy to cat epithelium [14]. In that case, SPT had a lower capacity to predict the outcome of BPT than IDST and CAP. One of the possible explanations for this disparity may be the different standardization of each diagnostic extract. However, CAP analysis for cat epithelium showed data very similar to those obtained for *Alternaria*, both for ROC (AUC 0.850) and for logistic regression analysis. In the latter, a value of 17 kU_A/L predicted an over 90% probability for a positive BPT result [14]. *In vitro* studies of factors that may affect basophile degranulation have shown that increases of allergen-specific IgE values make patients more sensitive to allergens, thus increasing human basophile degranulation [21, 22]. Our *in vivo* results also show a good correlation between specific IgE levels and the outcomes of an allergen exposure test. Therefore, it is possible that the specific IgE serum concentration in asthma patients could be a useful predictor of the clinical relevance of a given sensitization, improving the diagnostic accuracy and treatment of these patients. More studies are clearly warranted in this regard.

In the case of airborne allergen-induced asthma, the bronchial provocation test can be used for research purposes, and also in the clinical settings when there are reasonable concerns about the clinical relevance of a particular sensitization [15]. Moreover, the bronchial provocation test provides the certainty of an allergen-induced bronchial hyperreactivity; in this case, immunotherapy could be a very specific therapeutic measure and a good adjuvant to anti-inflammatory therapy.

There is concern regarding the safety of BPTs with airborne allergens [17]. In our experience with this test being carried out for more than 20 years, it has proven to be a safe procedure, provided it is performed in an outpatient clinic hospital setting, with medical supervision, trained personnel in charge and checking at the beginning of the test that the patient's asthma is stable. In this group of patients, most of them with a confusing clinical history due to multiple sensitizations, we have obtained a high percentage of positive bronchoprovocations in positive *Alternaria*-SPT patients (93.6%). Some of these positive results might have been due to the presence of mycotoxins in the inhaled extract. These substances are biologically active, non-volatile, low-molecular-weight metabolites produced during fungal growth, some of which can be toxic [23]. However, BPT was more frequently positive in subjects with a positive SPT than those with a positive IDST, a fact that indicates specificity. Also in favour of a specific reaction is the observation that the probability of a positive BPT result increases with higher specific IgE levels or a larger ST size. Finally, bronchial reactions were typical immediate-response allergic reactions or classic delayed reactions that responded appropriately to specific treatments. Taken

together, all these data seem to discard a toxic or a non-specific reaction.

The elevated percentage of positive BPT in subjects sensitized to *Alternaria* may be in agreement with clinical data, showing that this type of sensitization is a risk factor for bronchial asthma [24]. The underlying mechanism could be related to the protease-activated pathways of inflammation [25]. It has been demonstrated that the total protease activity of *Alternaria alternata* extract is 2.18 U/mg. In studies carried out with A549 cells, human alveolar type II epithelium-like cell line cultures and primary human nasal epithelial cells, the *Alternaria alternata* extract was found to elicit both morphological and functional changes in cell cytokine production, all of which were abrogated by protease inhibitors [25]. The importance of proteases in the mechanism of allergic diseases and their relationship with IgE production are becoming increasingly apparent. A protease role in airway reactivity, inflammation and remodelling has been reported [26]. In this regard, *Alternaria* proteases have strong T helper 2-like adjuvant properties in the airways; consequently, inherently innocuous type 1 allergens acquire allergenic activity when applied to the bronchial system [27]. This immunogenic capacity could explain their strong association with asthma and allergic diseases in humans.

Since O'Hollaren et al. [5] reported in 1991 that *Alternaria* sensitization could be a risk factor for fatal or near-fatal asthma attacks, several clinical studies have confirmed the relationship between asthma (especially severe asthma) and sensitization against *Alternaria*. In many instances, studies were performed in ICU patients or patients with respiratory arrest [5, 8]. Our work is centred on a different group of patients without severe asthma and from the perspective of the results of a mould exposure test. Although this test is not similar to a natural exposure, it is very likely that the allergen dose given in BPT is much lower than that inhaled on a windy day or during a storm, when the atmospheric levels of *Alternaria* are very high. Under these circumstances, a sensitized patient can have a severe asthma attack [28, 29].

Clinical relevance

Given the clinical characteristics of *Alternaria*-induced asthma and the frequent difficulties in establishing a causal connection between sensitization and symptoms, our findings of a close relationship between SPT values and BPT outcomes could be very useful in evaluating these patients. In this regard, an important issue would be to advise sensitized patients about potential risk situations, strongly emphasizing the need to comply with the prescribed medication; patients should also be advised to always carry rescue medication. Immunotherapy with *Alternaria* could also be taken into account as a valid therapeutic option [30, 31].

Acknowledgements

To David Lora Pablo for statistical support.

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