

ORIGINAL ARTICLE

Diagnostic value of Alt a 1, fungal enolase and manganese-dependent superoxide dismutase in the component-resolved diagnosis of allergy to pleosporaceae

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Clinical & Experimental Allergy

Summary

Background Over the last decades, genomics and proteomics have contributed to the current knowledge of individualized allergenic components and their potential use in the diagnosis of IgE-mediated allergies. Recent investigations have demonstrated that Alt a 1 should be considered as a relevant allergen of the Pleosporaceae group and that enolase is the main allergen involved in the cross-reactivity to fungi. However, the real utility of these allergens as tools for the diagnosis of allergy to *Alternaria* is still unclear.

Objective To demonstrate the current value of the available fungal allergen panel and the need to build an accurate mould allergen array for the diagnosis of allergy to Pleosporaceae.

Methods Specific IgEs to individual mould allergens and allergenic mould extracts were evaluated using the ImmunoCAPTM system in 30 patients allergic to *Alternaria* and in 100 blood donors. Cross-reactivity studies were performed by Fluoro Enzyme ImmunoAssay (FEIA) and FEIA inhibition using individual allergens and allergenic extracts. Two-dimensional electrophoresis associated with a MALDI-TOF analysis was carried out to identify new allergen molecules.

Results All allergic patients had positive specific IgE responses to several moulds from different taxonomical families. Classic and molecular diagnosis demonstrated that 23% of patients had multi-sensitization. The current commercially available fungal allergen array was not sufficient to establish an accurate diagnosis. Unexpected correlations between *Alternaria* or Alt a 1 and *Curvularia* or *Cladosporium* stimulated the investigation of a more accurate allergen panel. A manganese-dependent superoxide dismutase (MnSOD) homologous to Asp f 6 was identified as a new IgE-binding molecule from *Alternaria alternata*.

Conclusions and Clinical Relevance Alt a 1 is the marker for allergy to Pleosporaceae, not including *Curvularia*. MnSOD can explain 6.6% of allergy to *Alternaria* without Alt a 1 sensitization and should be included together with Alt a 1 and fungal enolase in the molecular array for the diagnosis of allergy to Pleosporaceae.

Keywords allergy, Alt a 1, *Alternaria*, molecular diagnosis, Pleosporaceae

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Introduction

The application of recombinant allergens to *in vitro* tests for the diagnosis of allergies allows new individual reactivity profiles to be defined for each patient. This provides more accurate and reliable results for better management and more efficacious immunotherapy for allergic patients.

The component-resolved diagnostics (CRD) concept is an excellent tool that can be used to improve the current laboratory diagnoses of allergic diseases [1], including those that are caused by fungal species [2].

Recombinant Alt a 1 (rAlt a 1) was first described more than 10 years ago [3] and is well known as a main allergenic component of *Alternaria alternata*, sensitizing > 80% of allergic patients to *Alternaria* [4, 5]. Its capacity

as a tool for the diagnosis of allergies that are caused not only by *Alternaria*, but also by other Pleosporaceae, has been demonstrated previously [6, 7]. Thus, Alt a 1 is the main marker for evaluating sensitization to allergenic species belonging to the Pleosporaceae family [6, 7]. New *A. alternata* allergens were described previously [8–10]. Most of these allergens were intracellular housekeeping proteins. Nine of these allergens [e.g. heat shock protein 70, acid ribosomal protein P1 and P2, enolase, flavodoxin (YCP4 homolog), NADP-dependent mannitol dehydrogenase, aldehyde dehydrogenase, glutathione-S-transferase and nuclear transport factor 2] have not only been identified in *A. alternata* but have been identified in the closely related mould *Cladosporium herbarum*. Moreover, some *A. alternata* allergens showed orthologue proteins in non-fungal species (heat shock protein 70, enolase, aldehyde dehydrogenase and glutathione-S-transferase) [11]. All of these allergens will enhance the possibility of allergy diagnosis by components and will help to clarify important diagnostic questions regarding co-, poly-sensitization or cross-reactivity.

In addition to Alt a 1, the enolase Alt a 6 from the allergen array described for *Alternaria* also seems to have a high diagnostic value. Several authors have demonstrated that this allergen can play an important role in the cross-reactivity phenomenon among several fungi and among species that belong to different phyla [12, 13]. However, this protein can be considered to be a minor allergen because it is only able to sensitize approximately 15–22% of patients who are allergic to moulds [12, 13].

The incidence of mould allergies is high in the general population (6–24%), in the atopic population (44%) and in individuals with respiratory allergies (80%) [11]. Moreover, a high proportion of mould-allergic patients seem to be poly-sensitized to several mould species [11, 14].

The current availability of these allergens makes it possible to study the role of each molecule in the diagnosis and prognosis of mould allergy, thereby providing a solution for poly-sensitization or cross-reactivity.

The objective of this work was to analyse the importance of individualized mould allergens as markers for sensitization, cross-reactivity or clinical status in a general population and in a group of patients labelled as 'allergic to *Alternaria*'. The information obtained about the diagnostic margin of each allergen will enable the construction of an optimum fungal allergen panel. This panel should have the capacity to discriminate between different clinical pictures associated with mould allergy disease. Thus, the analysis of molecular sensitization profiles in mould-allergic patients should explain the differences between sensitization and a real allergy [15].

Methods

Sample

Two groups of sera were included in this study: Group 1: sera from 30 patients who were clinically defined to be allergic to *A. alternata* were used. These patients were selected from a larger group of patients having follow-up analysis of mould allergies at the University Hospital Miguel Hernández (Elche, Spain) from 1991 to 2006 ($N = 146$). The patients selected as subjects for the study had the same characteristics as the patients from the mould allergy follow-up programme.

Demographics, relevant clinical data and skin tests were recorded for each patient. Twenty-seven per cent of the patients were sensitized only to *Alternaria*, and the rest of the patients had a positive skin test to other allergens, as did the patients from the total sample in the follow-up programme (Table 1).

The subjects were not submitted to immunotherapy at this stage of the study.

Group 2: the sera from 100 blood donors were analysed. These sera were collected at the blood bank from the Hospital Miguel Hernández (Elche, Spain) following the rules for including criteria of blood donors.

Informed consent for participation in this study was obtained from the patients during the allergy follow-up study. The study was approved by the Ethical Committee of the Elche University Hospital.

Allergens

Six allergen extracts from moulds, including *A. alternata*, *Stemphylium botryosum*, *Ulocladium botrytis*, *Curvularia lunata*, *C. herbarum* and *Aspergillus fumigatus*, and nine individualized allergens, including rAlt a 1, rAlt a 6, rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, rAsp f 6, rHev b 9 and cross-reactive carbohydrate determinants (CCD), were used for the measurement of specific IgE with the ImmunoCAP™ System (Phadia AB, Uppsala, Sweden).

Labelling and binding of rAlt a 6 to Streptavidin ImmunoCAP

To activate rAlt a 6 with biotin, purified rAlt a 6 (Biomay AB, Vienna, Austria) was diluted in PBS at 1 mg protein/mL and biotinylated following the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Pre-washed solid-phase Streptavidin ImmunoCAP (Phadia AB, Uppsala, Sweden) was loaded with 50 µL of biotinylated rAlt a 6 and incubated for 30 min at 37 °C. The rAlt a 6 coupled to Streptavidin ImmunoCAP was used for the determination of specific IgE in the ImmunoCAP 100 System (Phadia AB) [16].

Table 1. Demographic, relevant clinical data and total and specific IgE values (kU/L) of sera from 30 allergic patients to *Alternaria*, tested with extracts from *Alternaria alternata* (A.a.), *S. botryosum* (S.b.), *U. botrytis* (U.b.), *C. lunata* (C.l.), *C. herbarum* (C.h.) and *A. fumigatus* (A.f.) and with individualized allergens (rAlt a 1, rAlt a 6, rAsp f 1, rAsp f 2, rAsp f 6 and CCD)

ID	Age	Sex	Clinical diagnosis	Relevant clinical allergens	Total IgE	Specific IgE (kU/L)											
						A.a.	S.b.	U.b.	C.b.	C.h.	A.f.	Alt a 1	Alt a 6	CCD	Asp f 1	Asp f 2	Asp f 6
24	22	F	Rhinitis and asthma	ALT	61.5	1.3	0.5	0.7	0	0	0.4	1.7	0	0	0	0	0
10	21	F	Rhinitis and asthma	ALT, POLLEN	117	1.3	2.2	4.7	0.8	1.2	0.4	1	0	0	0	0	0
20	19	M	Rhinitis and asthma	ALT, CAT, POLLEN	149	1.4	2	1.4	1.3	0.8	3.5	0.9	0	0	0	0	0
21	16	M	Rhinitis and asthma	HDM, ALT	248	1.6	0.4	1.6	0	0	0	1.1	0	0	0	0	0
23	28	F	Rhinitis	POLLEN, ALT	84.6	1.8	0.5	0.8	0.2	0	0.8	1.9	0	0	0	0	0
18	25	F	Rhinitis	POLLEN, ALT	220	2.2	1.2	1.8	0	0.2	0.1	1.3	0	0	0	0	0
28	21	F	Rhinitis and asthma	ALT, POLLEN	83.2	2.2	2.3	5	1.1	1.4	0.3	1.9	0	0	0	0	0
19	24	F	Rhinitis and asthma	ALT, POLLEN	86.8	2.3	1	0.6	0.5	0.4	1.8	5.1	0	0	0	0	0
11	28	F	Rhinitis and asthma	ALT, POLLEN	1464	3.1	11.8	0.2	21.7	10.9	4	0	0.2	0	0.2	1.1	13.2
14	27	F	Rhinitis and asthma	ALT, CAT, POLLEN	331	3.2	1.2	2.8	0.4	0.2	0.1	4.4	0	0	0	0	0
13	30	F	Rhinitis and asthma	ALT, CAT, POLLEN	222	3.4	4.2	3.5	0.9	0.3	0	3	0	0	0	0	0
17	23	M	Rhinitis and asthma	POLLEN, ALT	208	4.1	1	1.4	1.1	0.4	0.2	4.4	0	0	0	0	0
16	26	M	Rhinitis	ALT, POLLEN	254	5.5	3.4	3	1	0	0.1	6	0	0	0	0	0
7	18	M	Rhinitis	ALT, CAT, POLLEN	620	5.7	5.1	5.8	2.4	4.3	3.7	4.1	4.3	0	0.2	0	0
22	29	M	Rhinitis and asthma	ALT	177	5.8	3.2	1.5	3.6	2.9	1.9	2.8	3.5	0	0	0	0
12	26	F	Rhinitis and ABPA	ASP, ALT, POLLEN	1501	6.4	12.2	0.2	23.2	12.7	4.1	0	0.2	0	0.2	1.2	12.2
4	23	M	Rhinitis and asthma	ALT	372	7.1	4.1	3.8	6.7	9.9	12.2	1.8	6.7	0	0	0	0
15	20	F	Rhinitis and asthma	ALT, HDM	no data	8	3.9	5.2	1.2	1	0.9	9	0	0	0	0	0
2	23	M	Rhinitis and asthma	ALT, POLLEN	896	10.8	8.2	4	20.3	10.7	10.3	4.7	28	0	0	0	0
8	29	M	Rhinitis and asthma	ALT	1051	12.2	20.5	13.6	14.5	4.2	3.6	3.9	0	0	0	0	0
25	23	M	Rhinitis and asthma	ALT	1019	16.1	28.1	27.8	9.6	6.5	7.6	8.7	0	0	0	0	0
9	29	F	Rhinitis and asthma	ALT	666	18.4	16.4	8.6	14.4	10.2	5.1	10	29.5	0	0	0	0
3	20	M	Rhinitis and asthma	ALT, POLLEN	943	19.3	11.6	15.4	25.5	12.1	23.8	9.1	0.2	0.3	0.3	0	0
6	16	F	Rhinitis and asthma	ALT, HDM	980	19.8	22.7	32.4	24.2	11.3	18.5	7.2	0	0	0.1	0	0
26	23	M	Rhinitis and asthma	ALT, POLLEN	1736	23.6	26.4	34	29.4	15	21.9	7.6	0	0	0.2	0	0
30	32	M	Rhinitis and asthma	ALT, CAT, HDM	680	28.9	8.3	2.1	0.5	3.1	4	30	0	0	0	0	0
29	24	M	Rhinitis and asthma	ALT	1212	29.2	29.4	27.2	10.8	7.2	8.3	21.9	0	0	0.1	0	0
1	25	M	Rhinitis and ABPA	ASP, ALT, POLLEN	3705	38.7	13	32.1	4.8	8.5	61.9	19.4	5	0.9	0.1	38.6	0.2
5	22	F	Rhinitis	ALT	2087	65.2	37.6	46.5	31.7	28.5	11.6	25.9	17.6	0	0	0	0
27	20	M	Rhinitis and asthma	ALT, POLLEN	1696	75.1	49.6	58.9	36.4	35.9	13.9	29.3	11.5	0	0.3	0	0

ID, patient identification number; CCD, cross-reactive carbohydrate determinants; ABPA, allergic bronchopulmonary aspergillosis; ALT, *Alternaria* spp.; CAT, cat epithelium; HDM, house dust mites; POLLEN, pollens related to symptoms (grass, olive or Chenopodiaceae); F, female; M, male.

Specific immunoglobulin E measurements. The Fluoro Enzyme ImmunoAssay was carried out using ImmunoCAP 100 and ImmunoCAP reagents (Phadia AB) following the manufacturer's instructions.

Immunoassay inhibition. To determine the level of cross-reactivity among different mould extracts and/or individualized fungal allergens, immunoassay inhibitions were carried out according to Cardona et al. [17]. rAlt a 1, *A. alternata* and rAsp f 6 ImmunoCAP allergens were used as solid phases. Different extracts of *A. alternata*, *S. botryosum*, *U. botrytis*, *C. lunata* and *A. fumigatus* were used as inhibitors. A pool serum with positive IgE responses to *A. alternata* was generated to make inhibition assays with rAlt a 1 and *A. alternata* in solid phase. Sera from patients 11 and 12 giving strong specific IgE responses to Asp f 6 were used separately in inhibition immunoassays with rAsp f 6 in solid phase. A 1 : 5 dilution of these sera were pre-incubated overnight at 4 °C with different concentra-

tions of mould extracts (from 50 to 0.00005 mg/mL, 1/10 dilutions). After centrifugation, the specific IgE responses to *A. alternata*, rAlt a 1 and rAsp f 6 were measured using ImmunoCAP 100 (Phadia AB), and the percentage of inhibition was then calculated. The corresponding controls using positive serum to grass pollen were included.

Extracts

Culture filtrate extracts of each mould were obtained from 3-week fungal cultures in Czapek broth medium at 25 °C [18].

Two-dimensional electrophoresis and two-dimensional immunoblotting

The two-dimensional (2D) electrophoresis was performed following standard methods. Briefly, samples were loaded onto first-dimension IPG strips of pH 5–8 (BioRad,

Hercules, CA, USA) and were focused using the PROTEAN IF cell (BioRad), according to the manufacturer's instructions. The proteins were separated in the second dimension in 10% SDS polyacrylamide gels, and then they were either stained using Coomassie brilliant blue R-250 or transferred to a PVDF membrane [19].

The PVDF membrane was incubated overnight with the patient's sera (11 and 12) at 4 °C with agitation. After incubation for 1 hour with mouse anti-human IgE (Fc) horseradish peroxidase conjugate (SouthernBiotech, Birmingham, AL, USA), chemiluminescent reagents (ECL+, Western Blotting Detection Reagents, Amersham Biosciences, Bucks, UK) were used to detect the immune complex [20].

Mass spectrometry and database searching

The IgE-binding spots revealed by 2D immunoblotting were sent to the Proteomic Unit of the Foundation for the Cardiovascular Disease Research Centre Carlos III (Madrid, Spain) for identification by MALDI-TOF-MS and MALDI-TOF-MS/MS.

MALDI-MS and MS/MS data were combined through the BioTools programme (Bruker Daltonics, Billerica, MA, USA) to search a non-redundant protein database (NCBI nr; $\sim 4.8 \times 10^6$ entries; National Centre for Biotechnology Information, Bethesda, MD, USA; or SwissProt; $\sim 2.6 \times 10^5$ entries; Swiss Institute for Bioinformatics, Switzerland) using the Mascot software (Matrix Science, London, UK). MALDI-MS (MS) spectra and database search results were manually inspected in detail using the above programmes as well as homemade software and the Structural Database of Allergenic Proteins. The amino acid sequence was assessed by searching the NCBI nr database with fragment ion masses from the precursor ion at $m/z = 2710.286$. Protein scores greater than 81 were considered significant ($P < 0.5$).

According to the Structural Database of Allergenic Protein (SDAP), cross-reactivity between a query protein and a known allergen has to be considered when there is an identification of six contiguous amino acids between the query sequence and any allergen.

Statistical analysis

The statistical analysis was performed using GraphPad Prism[®] Version 4.03 software (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between the specific IgE values to *A. alternata* extracts or rAlt a 1 and the IgE values to the mould extracts or individualized allergens in the group of patients with an allergy to *Alternaria* were analysed by linear regression in order to predict one variable from another. To assess the strength of the association between variables, the non-parametric Spearman's correlation test was used (95% CI), and the r_s and P_s values were calculated.

Results

Table 1 shows the specific IgE responses to *A. alternata*, *S. botryosum*, *U. botrytis*, *C. lunata*, *C. herbarum* and *A. fumigatus* extracts together with the specific IgE responses to individualized allergens (rAlt a 1, rAlt a 6, rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, rAsp f 6 and CCD) of the 30 patients labelled as 'allergic to *Alternaria*'. The most relevant demographic data and the total IgE values of the patients are shown (Table 1). The distribution by sex was 46.7% women and 53.3% men. The average age of the patients was 23.7 years and ranged from 16 to 32 years.

The specific IgE response to *A. alternata* was 14 kU/L and ranged from 1.3 to 75.1 kU/L (Table 1). Of the 30 patients with a previous diagnosis of respiratory allergies caused by *Alternaria*, 28 showed positive IgE responses to rAlt a 1 (Table 1). Two of the patients (identification codes 11 and 12) with positive IgE responses to *A. alternata* (3.1 and 6.4 kU/L) and negative IgE responses to rAlt a 1 had very low IgE responses to rAlt a 6 (0.2 kU/L) and high IgE responses to *S. botryosum*, *C. lunata*, *C. herbarum* and rAsp f 6 (> 10 kU/L).

Of the 30 patients, 11 exhibited positive IgE responses to fungal enolase (Table 1). Of these, seven exhibited values equal to or higher than 3.5 kU/L. Four of the sera (identification codes 2, 7, 9 and 22) showed enolase values that were higher than or equal to those observed for fungal species not containing Alt a 1 (*C. lunata*, *A. fumigatus* and *C. herbarum*).

The results obtained with latex enolase (rHev b 9) were negative (< 0.1 kU/L) in all of the patients.

In Fig. 1, the distribution profiles of the specific IgE values and the frequency of positive results in the patient groups overlap perfectly. When 0.2 kU/L was considered as a cut-off point, the frequencies were 100% for *A. alternata* and *S. botryosum*, 93% for *U. botrytis* and rAlt a 1, 87% for *C. lunata*, 80% for *A. fumigatus* and *C. herbarum*, 27% for rAlt a 6, 10% for rAsp f 2, 7% for rAsp f 1, rAsp f 6 and CCD and 0% for rHev b 9, rAsp f 3 and rAsp f 4.

The prevalence of specific IgE antibodies to mould extracts and to individualized allergens in the blood donor group is shown in Fig. 2. The prevalence of individuals sensitized to recombinant Alt a 1 and rAlt a 6 was 6% and 1%, respectively. This result was similar to those obtained for *A. alternata* and other Pleosporaceae extracts. The specific IgE responses to *A. fumigatus* extracts in the blood donor group did not correlate with those obtained with individualized *Aspergillus* allergens. Only one individual was positive to CCDs. The detailed study of this subject revealed specific IgE to grasses (Phl p 1 and Phl p 5), *Salsola* (Sal k 1) and polcalcin allergens.

Cross-reactivity was measured by specific IgE-ImmunoCAP inhibition using rAlt a 1, *A. alternata* and rAsp f 6 in the solid phase. When *A. alternata*, *U. botrytis* and

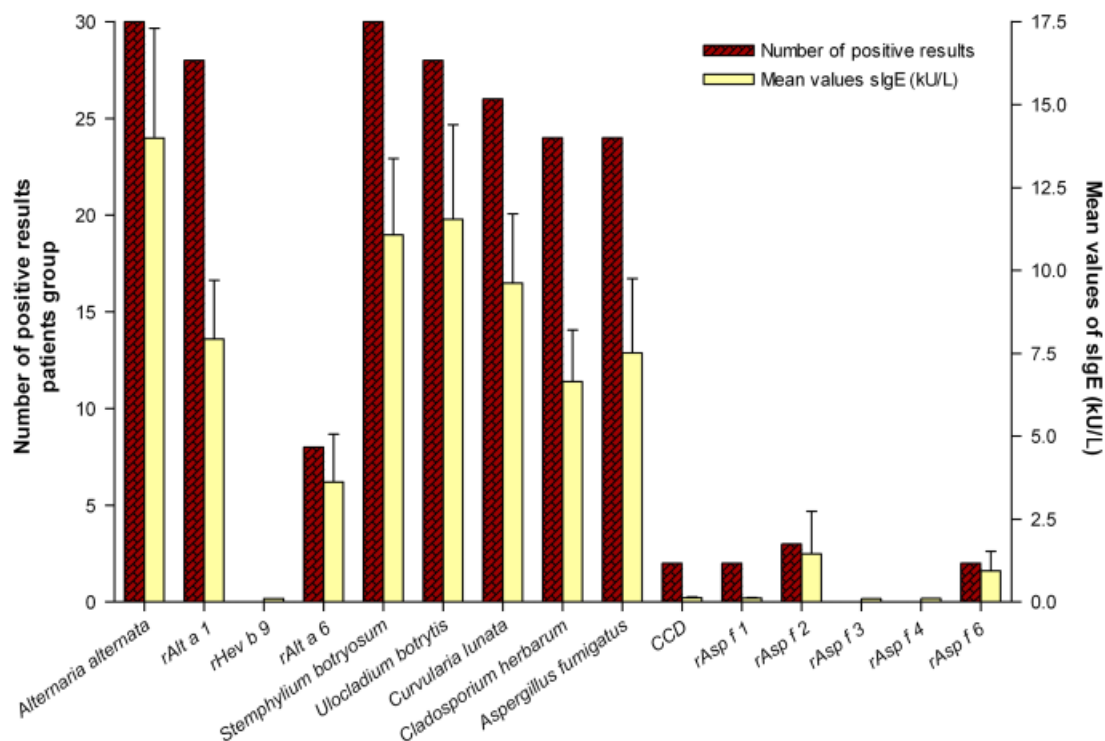


Fig. 1. Distribution profiles of the specific IgE values to allergens and frequency of positive results in the group of allergic patients to *Alternaria* ($n = 30$). Allergenic extracts: *Alternaria alternata*, *Stemphylium botryosum*, *Ulocladium botrytis*, *Curvularia lunata*, *Cladosporium herbarum* and *Aspergillus fumigatus*. Individualized allergens: rAlt a 1, rAlt a 6, rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, rAsp f 6, rHev b 9 and CCD. Filled bars indicate the number of positive results. Empty bars indicate the arithmetic mean of IgE values expressed in kU/L. CCD, cross-reactive carbohydrate determinants.

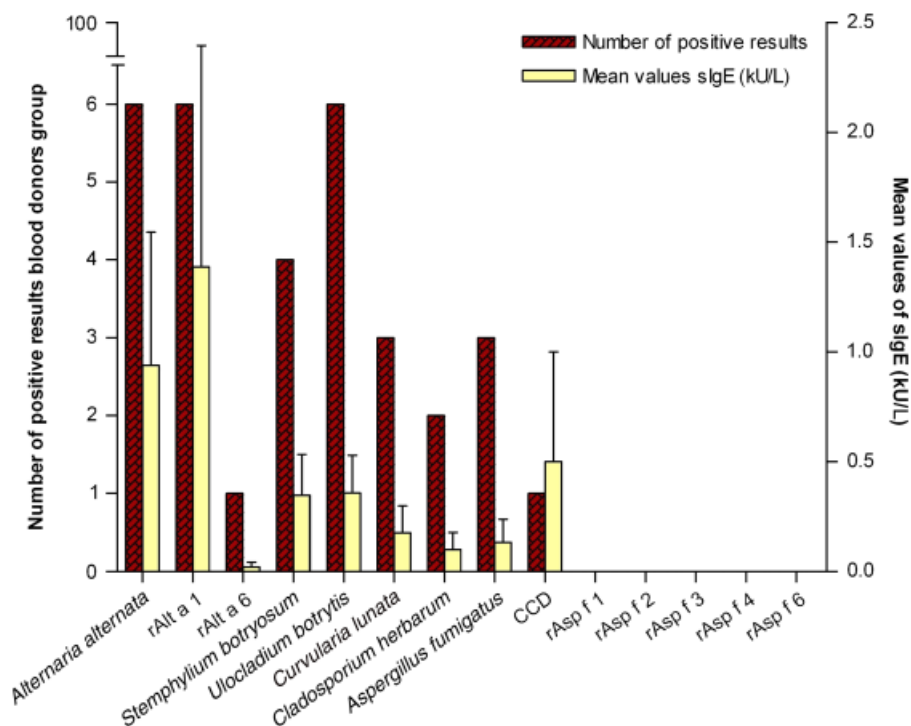


Fig. 2. Distribution profiles of the specific IgE values to allergens and prevalence of sensitization to mould allergens in the blood donors group ($n = 100$). Allergenic extracts: *Alternaria alternata*, *Stemphylium botryosum*, *Ulocladium botrytis*, *Curvularia lunata*, *Cladosporium herbarum* and *Aspergillus fumigatus*. Individualized allergens: rAlt a 1, rAlt a 6, rAsp f 1, rAsp f 2, rAsp f 3, rAsp f 4, rAsp f 6 and CCD. Filled bars indicate the number of positive results. Empty bars indicate the arithmetic mean of IgE values expressed in kU/L. CCD, cross-reactive carbohydrate determinants.

S. botryosum extracts were used as competitive inhibitors, the inhibition of solid-phase allergen-specific IgE reactions was equal to or greater than 90%. *C. lunata* was able to inhibit 4% of the rAlt a 1 solid-phase-specific IgE reaction and 65% of the *A. alternata* extract solid-phase-specific IgE reaction. *A. fumigatus* and *C. herbarum* did not show inhibition when rAlt a 1 was used in the solid phase, but showed 55% and 70% inhibition, respectively, when *A. alternata* extract was used in the solid phase.

The inhibition of solid-phase allergen-specific IgE reactions using rAsp f 6 in the solid phase and *A. alternata* extract as inhibitor was 80% in the case of patients 11 and 12. No inhibition was shown in the case of controls.

Table 2 shows the coefficients of the linear regression (r) and the Spearman's coefficients of correlation (r_s) between IgE to *A. alternata* extracts or rAlt a 1 and to the other mould extracts or individualized allergens in the group of patients with allergy to *Alternaria*. Significant correlations ($P_s < 0.05$) were demonstrated between the specific IgE values for *U. botrytis*, *S. botryosum*, *C. lunata*, *C. herbarum* or *A. fumigatus* and the specific IgE values for *A. alternata* extracts.

Similarly, significant correlations ($P_s < 0.05$) were demonstrated between the specific IgE values for *U. botrytis*, *S. botryosum* or *C. herbarum* and specific IgE values for rAlt a 1. No significant correlations ($P_s > 0.05$) were demonstrated between specific IgE values for *A. fumigatus* or *C. lunata* and specific IgE values for rAlt a 1.

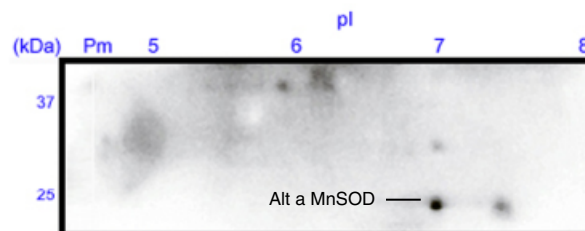
Also, significant correlations ($P_s < 0.05$) were demonstrated between specific IgE values for *A. alternata* extracts and specific IgE values for rAlt a 1 or rAlt a 1+rAlt a 6.

Figure 3 shows the 2D immunoblotting of the *A. alternata* extract using a 1:1 mix of sera from two

Table 2. Coefficient of linear regression (r) and Spearman's coefficient of correlation (r_s) between the IgE values to *Alternaria alternata* extracts or rAlt a 1, and the IgE values for other mould extracts or individualized allergens in the group of patients with allergy to *Alternaria*

Variables	r	r_s	P_s
<i>A. alternata</i> vs. <i>Ulocladium botrytis</i>	0.907	0.7465	<0.0001
<i>A. alternata</i> vs. <i>Stemphylium botryosum</i>	0.873	0.8701	<0.0001
<i>A. alternata</i> vs. <i>Curvularia lunata</i>	0.680	0.7345	<0.0001
<i>A. alternata</i> vs. <i>Cladosporium herbarum</i>	0.869	0.7498	<0.0001
<i>A. alternata</i> vs. <i>Aspergillus fumigatus</i>	0.509	0.7927	<0.0001
<i>A. alternata</i> vs. rAlt a 1	0.805	0.8324	<0.0001
<i>A. alternata</i> vs. rAlt a 6 (enolase)	0.397	0.4569	0.0111
<i>A. alternata</i> vs. rAlt a 1+rAlt a 6	0.790	0.8451	<0.0001
rAlt a 1 vs. <i>U. botrytis</i>	0.607	0.6971	<0.0001
rAlt a 1 vs. <i>S. botryosum</i>	0.610	0.5929	0.0006
rAlt a 1 vs. <i>C. lunata</i>	0.319	0.3821	0.0853
rAlt a 1 vs. <i>C. herbarum</i>	0.512	0.3899	0.0332
rAlt a 1 vs. <i>A. fumigatus</i>	0.358	0.5289	0.0518

r , coefficient of linear regression; r_s , Spearman's coefficient of correlation; P_s , Spearman's P -value.



Sample name: Alt a MnSOD
 Mw/pI experimental: 24.0 kDa/7.0
 Mw/pI theoretical: 21.4 kDa/7.1
 Protein description: manganese-dependent superoxide dismutase
 Sequence matched: K.FNAGGHINHSLFWQNLAPASSNEAK.S
 Sequence covered: 13%
 Mascot score: 150
 Mascot expect: 7.06E-09
 Mascot ion score: 131
 Registered in the databaseUniprotKB/SwissProt: P86254

Fig. 3. Two-dimensional immunoblotting of the *Alternaria alternata* extract revealed with a pool of patient's sera 11 and 12. Results of Mascot research. Mw, molecular weight (kDa); pI, isoelectric point.

patients (11 and 12). Mass spectrometry and database searching demonstrated that the sequence was matched with a high Mascot Score corresponding to a very low expect value ($7.6E-9$), indicating with a high probability that the IgE-binding protein detected in *A. alternata* belonged to the manganese-dependent superoxide dismutase (MnSOD) family (molecular mass = 24 kDa and pI = 7) with a very low probability of the sequence being a false positive. Other peptides were measured for this sample, but neither their masses nor their fragment ion masses matched any significant protein or peptide candidate by database searching. This IgE-binding protein of *A. alternata* was registered in the database UniprotKB/SwissProt with the accession number P86254.

SDAP searching results demonstrated that the sequence identified as MnSOD in *A. alternata* was the allergen Asp f 6, and according to the Allergenicity Rules based on Sequence Homology, more than six contiguous amino acids matched with the sequence of Asp f 6 [sequence query: GGHINHSLFW; PD Sequence similarity index: 0.00; z(PD, min): 7.5847; z(PD, all): 8.0129; start residue: 83; matching region: GGHINHSLFW; end residue: 92].

Discussion

Fungi are a common source of aeroallergens associated with asthma and allergic rhinitis. A specific diagnosis of a mould allergy can be made through a combination of aerobiological studies, a case history, positive diagnostic tests and a careful recording of symptoms elicited by causative allergens. However, the diagnosis is difficult and often inefficient, perhaps because of the wide variety of fungal species, the complexity of the complex allergenic extracts or the quality of the commercial fungal extracts [21].

The application of recombinant allergens to *in vitro* tests for the diagnosis of allergy allows new individual reactivity profiles to be defined for each patient and provides more accurate and reliable results for better management of allergic patients. In addition, the recombinant allergen-based concept of CRD is an excellent new tool for improving the current laboratory diagnosis of allergic diseases [1], including those caused by fungal species, and thus provides more specific measures of sensitization to fungi [22].

Several authors have demonstrated that *Alternaria* is the most important fungal genus causing respiratory allergies in Southern Europe, including Spain [21, 23, 24]. Several studies have also found a strong association between *Alternaria* sensitization and asthma severity [23, 25–27]. During allergy diagnosis, it is common to find sensitization to multiple fungi, making it difficult to define the role of each mould in primary sensitization or cross-reactivity. This makes the final diagnosis difficult; therefore, particularly in these cases, CRD is useful for reaching an accurate and reliable diagnosis.

As far as we know, only two clinical studies have been performed using *A. alternata* recombinant allergens [5, 28]. In both cases, Alt a 1 and Alt a 6 (enolase) were revealed to be promising candidates for a molecular-based approach to the diagnosis and therapy of *A. alternata*; however, no other recombinant allergens from other allergenic fungi were analysed, and no data about sensitization to individualized fungal molecules in the general population were recorded.

In this work, of the 30 patients with a previous diagnosis of a respiratory allergy caused by *Alternaria*, 28 showed positive IgE responses to rAlt a 1. The two patients with positive anti-*A. alternata* IgE values (3 and 6 kU/L) and negative anti-rAlt a 1, anti-rAlt a 6 (0.2 kU/L) or anti-CCD values showed high IgE responses to *S. botryosum*, *C. lunata*, *C. herbarum* and rAsp f 6 (> 10 kU/L). The high percentage of inhibition (80%) of IgE to Asp f 6 by the *A. alternata* extract using the serum from these two patients together with the results of the immunoblotting and subsequent MALDI-TOF analysis strongly suggested that the MnSOD of *A. alternata* is an allergen homologous to Asp f 6. Although a MnSOD has been described in *Curvularia lunata* (accession number AY291574), *Malassezia sympodialis* [29] and *Saccharomyces cerevisiae* [30], this has not yet been reported in *A. alternata*, and it could be a reason for the diagnostic errors initially made for allergies to *Alternaria*. The obvious involvement of Asp f 6 in the sensitization of these patients also suggested the possibility of allergic bronchopulmonary aspergillosis [31].

Based on our results, 17 of 30 patients could be considered to be allergic to *Alternaria* without the involvement of other significant allergenic sources. Cross-

reactions could be attributed to enolase in just four of the patients (subjects 2, 7, 9 and 22).

The results of the high specific IgE responses to the mould that did not contain Alt a 1 and the significant correlations between the IgE responses to *Alternaria* and *Cladosporium* or *Aspergillus* suggest that other allergenic molecules shared by several fungal species are commonly involved in the development of mould allergies. MnSOD and other allergen orthologous, such as proteases, mannitol dehydrogenases, acidic ribosomal proteins (P1/P2) or YCP4 proteins [32–34], are also present across the fungal kingdom and could explain the high specific IgE responses to *Curvularia* in patients showing significantly lower or negative IgE responses to Alt a 1 or Alt a 6. Similarly, this could explain the significant correlations found between *Alternaria* and *Curvularia* or *Cladosporium*, as well as the high inhibition percentages (> 50%) between *Alternaria* and *Curvularia*, *Aspergillus* or *Cladosporium*.

The frequencies and values of the most important sensitizing allergens of *Alternaria* and *Aspergillus* observed in the group of allergic patients indicate that Alt a 1 could be the most important primary sensitizer in mould allergy patients in our region. These data are additionally supported by the prevalence figures found for these allergens in the group of blood donors. In this group, the prevalence of individuals sensitized to rAlt a 1 was 6%, while the prevalence to *Aspergillus* allergens was insignificant.

The relevance of Alt a 1 and Alt a 6 to the study of the prevalence of subjects sensitized to reported allergens from *Alternaria* and other fungal species in the patient cohort (93% for Alt a 1, 27% for Alt a 6 and ≤ 10% for *Aspergillus* recombinant allergens) was comparable with results found by Asturias et al. [5], who reported prevalence data of 98% for Alt a 1 and 15% for Alt a 6; however, other individualized allergens from other fungal species were not evaluated.

Some authors suggest that several fungal species, such as *A. alternata*, *C. herbarum*, *Epicoccum nigrum*, *Fusarium roseum*, *Penicillium chrysogenum*, *A. fumigatus*, *Candida* spp. and *Malassezia* spp., should be considered as a minimal testing set when a fungal allergy is suspected [35]. According to the results obtained in this work, moulds such as *Curvularia*, and likely others, should also be included in such testing.

Crameri et al. [35] reported multiple sensitizations to several species in patients with a clinical history of an *A. fumigatus* allergy. A similar pattern was observed in this study in the group of patients labelled as allergic to *A. alternata*. Classical EIA inhibition studies or linear regression approaches can be used to define the cross-reactivity phenomenon, but, in many cases, the results are not convincing. Recombinant fungal allergens offer a new opportunity for a better understanding of sensitization,

pathogenesis, polysensitization or cross-reactivity. Therefore, through the results obtained in this work, we can establish the limits of cross-reactivity of enolase as a panallergen. The results suggest that 30% of the cross-reaction due to moulds can be attributed to fungal enolase. Similarly, it can be concluded that MnSOD plays a significant role in the cross-reactivity phenomenon. The negative IgE responses to CCD that were revealed in this study demonstrate that these allergens do not play a significant role in mould allergy.

Finally, from these results it is interesting to note the importance of Alt a 1, together with Alt a 6, as a tool for the diagnosis of fungal allergy in Spain. However, detailed analysis of allergic patients indicates that there is a significant lack of allergen molecules to define fungal allergic profiles for molecular diagnosis. In this sense, we

can conclude that MnSOD can explain 6.6% of allergy to *Alternaria* without Alt a 1 sensitization. So, this allergen should also be included together with Alt a 1 and fungal enolase in the molecular array for the diagnosis of allergy to Pleosporaceae.

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