

ORIGINAL ARTICLE

Epidemiology of Allergic Disease

β -1,3-glucanase rOle e 9 and MnSOD rAsp f 6 IgE reactivity are the signature of atopic dermatitis in the Mediterranean area

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Abstract

Background: Atopic dermatitis (AD) represents a chronic skin disorder seriously affecting patients' QoL and is often associated with immunological imbalance, disorders of the skin barrier function and environmental factors.

Objective: We extensively studied the proteomic IgE sensitization profile in a large AD Mediterranean cohort.

Methods: A total of 588 individuals with moderate-severe (70.6%) or mild and/or history of (29.4%) AD were evaluated in comparison to 1285 unselected atopic controls (AC) with a history of adverse reactions to foods, allergic rhinitis and/or bronchial asthma by means of ImmunoCAP ISAC₁₁₂[®] and Allergy Explorer-ALEX[®] microarray analysis.

Results: The olive tree pollen β -1,3-glucanase rOle e 9 and the manganese superoxide dismutase from *Aspergillus* rAsp f 6 were the molecules most significantly associated with AD occurrence and allowed to discriminate among the moderate and severe forms of disease. An IgE hyper-reactivity to cypress, grasses, olive tree, house dust mites (including rDer p 11), and to all cross-reactive components except profilin and polcalcin was observed. About 60% of adults with severe AD were sensitized to nsLTPs. Cross-reactive carbohydrate determinants (CCDs) IgE was found in about one-third of AD participants. Hen eggs nGal d 1 IgE sensitization was more prevalent in the paediatric population, whilst rAsp f 6 and rOle e 9 reactivity was found particularly in older patients. Despite the status of widespread IgE sensitization to both environmental and food allergens, a reduced frequency of patient-reported severe reactions to food or of asthma was observed in AD patients compared to AC, particularly in case of concomitant Ole e 9 reactivity.

Conclusion and Clinical Relevance: Testing IgE reactivity to a large panel of molecular components unveils important associations between IgE reactivity profiles and AD clinical presentation, highlights the allergens useful for a precise AD signature and allows the detection of interesting sensitisation patterns.

KEYWORDS

atopic dermatitis, CRD, IgE, MnSOD, Ole e 9

1 | INTRODUCTION

Atopic dermatitis (AD) is one of the most frequent causes of chronic inflammation of the skin; it is associated with intense itching, skin barrier deficiency and relapsing eczematous lesions.^{1,2} AD may be associated with a delayed worsening of skin condition after pollen, house dust mite,^{3,4} or food allergens exposure,^{5,6} but in some cases, no specific IgE sensitization can be detected, despite a clinical picture resembling typical AD.⁷ Chronic immune-mediated, or even autoimmune (sometimes IgE driven) reactivity,⁸⁻¹¹ genetic or environmental factors, including microbial agents,¹²⁻¹⁴ could play an important role in determining the onset of AD. Additionally, patients affected by severe AD may exhibit a significantly higher occurrence of depression.¹⁵

Bearing in mind the complexity of the pathogenesis, the diagnostic workup in AD assessment should take into consideration every possible variable, that is genetic, environmental exposure, or even the lifestyle, tailoring all those information to every single patient, with a customized approach known as "Precision Medicine."

Nowadays micro-arrayed allergen molecules systems, allowing IgE reactivity measurement to a large set of purified natural or recombinant components using a few microlitres of serum, are available.^{16,17} Such diagnostic approaches could be valuable in AD patients classified as "extrinsic,"¹⁸ showing specific IgE antibodies which possibly act as disease trigger factors, as shown by the worsening of symptoms after both environmental⁴ or food⁵ allergen exposure.

In this study, we sought to achieve a thorough assessment of IgE reactivity profiles in a large sample with present or with a history of AD, to collect evidence of relationship among specific IgE reactivity sensitization patterns and severity of AD, including the concomitant occurrence of food or respiratory symptoms.

2 | METHODS

2.1 | Recruitment and characterization of study subjects

The study was carried out on 1873 unselected participants born in Central or Southern Italy presenting with a history of adverse reactions to foods, allergic rhinitis, bronchial asthma and/or atopic dermatitis at the outpatient Allergy Unit of IDI-IRCCS in Rome, a National Reference Center for Dermatological diseases, between January 2018 and August 2019. Demographic details together with clinical data (food-related reactions, respiratory and dermatological symptoms) were recorded using the TD-Synergy[®] Laboratory Information System (Siemens Healthcare Diagnostics, Muenchen, Germany) and a customized electronic database.

Ethical Committee of IDI-IRCCS (492/1) approved the study and written informed consent was obtained before participation following the Declaration of Helsinki.

2.2 | Study design

Study patients were categorized based on clinical symptoms reported at the time of the first visit before IgE testing and were therefore blinded to microarray data. All patients with AD were classified according to the criteria of Hanifin and Rajka.¹⁹ Eczema severity was calculated using the objective SCORAD index.²⁰

Dermatological categorization was as follows: Group 1, absence of atopic dermatitis ("*allergic controls*"; AC); Group 2, patient with mild or reported AD in childhood ("*Remission/mild*";); Group 3, symptoms unequivocally suggestive of present moderate AD, with a objective SCORAD index between 15 and 40 ("*moderate*";); Group 4, severe AD with a SCORAD index above 41 ("*severe*").

For all patients, a further clinical categorization was as follows: [Food Allergy, FA] history of symptoms unequivocally suggestive of adverse reaction to a suspected plant food-derived trigger(s), milk and egg including urticaria and external angioedema, laryngeal angioedema, respiratory difficulty and/or pre-syncope/syncope in the last 6 months; [Respiratory Symptoms, RS] symptoms of rhino-conjunctivitis and/or bronchial asthma only.

Sera from all participants were collected and stored at the time of the first visit.

2.3 | IgE antibody measurements

Serum IgE reactivity was analysed using the ImmunoCAP ISAC₁₁₂ (Thermo Fisher Scientific) and Allergy Explorer-ALEX[®] (Macro-ArrayDX) platforms as per manufacturer's instructions.

The ISAC microarray in the current version is a fully commercial product.²¹ Allergens spotted on the chip are totally determined by the manufacturer, and currently mainly depending on molecule accessibility from several different providers being either research laboratories or companies.

In brief, ImmunoCAP ISAC₁₁₂ slides were washed, rinsed and dried at room temperature (RT). Undiluted serum (30 µL) from each patient was pipetted on to the slide and after 120 min incubation at RT in a humid chamber, slides were washed, rinsed and dried. IgE binding was detected by addition of an anti-human secondary antibody (Thermo Fisher Scientific). Slides were then washed, rinsed, dried and stored in the dark until scanning. Images were acquired by scanning allergen biochips with a CapitalBio LuxScan[™] 10K microarray scanner. IgE values were expressed as ISU arbitrary units (ISAC Standardized Units) corresponding to IgE antibody levels in the ng/ml range (detection limit: 0.3 ISU-E).^{16,22,23} Since, CCD moieties recognition may affect the IgE measurement of native glycosylated molecules (nCup a 1, nCry j 1, nPhl p 4, nCyn d 1, nJug r 2 and nPla a 2), all the values scoring below MUXF3 IgE levels for each patient were discarded and not considered.

In the Allergy Explorer-ALEX[®], different allergens and extracts are spotted onto a nitrocellulose membrane in a cartridge chip, which is then incubated with 0.5 mL of a 1:5 dilution of serum, containing a CCD inhibitor under agitation. After

incubation for two hours, the chips are washed three times and a pre-titred dilution of anti-human IgE labelled with alkaline phosphatase is added and incubated for 30 min. After another cycle of extensive washing, the enzyme-substrate is added, and after eight minutes, the reaction is stopped. The membranes are dried, and a charge-coupled device camera measures the intensity of the colour reaction for each allergen spot. The dedicated software digitalizes the images and prepares a report that lists the allergens and components and their score in kUA/mL. Finally, an arbitrary calibration curve is obtained by reacting four spots with decreasing concentrations of specific IgE corresponding to < 0.35, 0.35-1, 1-5, 5-15 kUA/L and > 15 kUA/L.^{24,25} We considered as positive a concentration ≥ 0.35 kUA/L.

2.4 | Statistics

All data were analysed using the SPSS/PC + statistical package for statistical evaluation (SPSS, version 15). The TD-Synergy Laboratory Information System was used to search and collect demographic (age and gender), clinical and laboratory data for Allergy Clinic patients who attended the outpatient Allergy clinic and underwent specific IgE testing.

The degree of relationship between the quantitative variables studied was analysed using Spearman's correlation (r) test, given the non-parametric distribution of the observed values.

The association between molecular reactivity to IgE and clinical symptoms recorded was studied in univariate and multivariate analyses. In univariate analysis, the Mann-Whitney U test (two groups) was first used to compare continuous IgE values in subjects with or without a given clinical involvement. Besides, each variable of interest was dichotomized (as negative or positive) to study the

proportion of subjects with organ involvement in the two groups thus obtained. Categorical variables were analysed using Pearson's χ^2 or Fisher's exact test when indicated. Finally, multiple logistic regression was performed for the clinical variables with dichotomous scores (present/absent) to investigate whether associations between clinical symptoms and IgE reactivity were present after simultaneously adjusting for other variables of interest. Separate modelling was performed for each condition including all molecules, in addition to sex and age. P values < .05 were considered significant.

Relatedness of IgE recognition profiles was examined with the Genesis 1.7.2 program by supervised Eisen hierarchical clustering²⁶ using the average linkage clustering as agglomeration rule. The supervised clustering involved sorting of both allergenic molecules and IgE recognition results reported in ISAC standardized units.²⁷ The IgE recognition tree was compiled based on a full data set, and the distance between samples was computed by using Pearson correlation as similarity measures. Molecular allergens with a similar IgE recognition were grouped as hierarchical clusters and presented as heat maps.

3 | RESULTS

3.1 | Study population

A total of 588 individuals (280 females; mean age 21 ± 15 years, range 1-80 years) with a present (70.6%) or patient-reported (29.4%) history of AD represented our study group. One thousand two hundred eighty participants with a history of adverse reactions to foods, allergic rhinitis and bronchial asthma acted as allergic controls (AC) (720 females, mean age 34 ± 18 , range 1-92).

TABLE 1 Demographic and clinical characterization of AD patients and controls

	n	F M	Age (years $\pm$ STD) Median (Range)	SCORAD Median (Range)	Rhino-conjunctivitis and/or Asthma n (%)	Food Allergy n (%)	Total IgE levels (kU/L) Geometric mean 95% CI
AD patients	588	280	21 ± 15	36.5	314	230	1096 ^a
		308	1-80	14.4-98.4	53.4% ^a	39.1% ^a	94-11,330
Remission/Mild	173	79	21 ± 15	-	109	74	505
		94	2-72		63% ^{a,b}	42.7% ^a	51-4,391
Moderate	257	125	19 ± 13	31.25	151	97	941 ^c
		132	1-78	14.4-40.9	58.7% ^a	37.7% ^a	97-8,720
Severe	158	76	24 ± 17	69.6	90	59	2175 ^a
		82	1-80	41-98.45	56.9% ^a	37.3% ^a	183-18,190
Allergic controls (AC)	1280	720	34 ± 18	-	976	814	265
		560	1-92		76.25%	63.5%	17-3,880

Abbreviations: AD, atopic dermatitis, F, Female, M, Male, n, number of positive individuals.

^a $P < .0001$ vs AC.

^b $P < .005$ vs severe AD.

^c $P < .003$ vs AC.

TABLE 2 (A) Linear-by-linear association among the most relevant species-specific molecules tested and AD subset in comparison with AC. The 10 top-ranked molecules are shown, data are sorted in descending order based on the chi-square values, and the ranking of the single-molecule in the respective subset is indicated. (n: native; r: recombinant). (B) Linear-by-linear association among homologous molecules tested and AD subset in comparison with AC. (n: native; r: recombinant)

AC	Remission/Mild		Moderate		Severe		χ^2 tests (linear-by-linear association)				
	Prevalence	Rank	Prevalence	Rank	Prevalence	Rank					
(A)											
rOle e 9	5.3%	54	11.0%	43	21.4%	30	36.7%	15	173.114	<i>p</i> : 1,546 ⁻³⁹	β -1,3-glucanase
rAsp f 6	2.8%	71	5.2%	68	15.6%	43	27.2%	32	151.090	<i>p</i> : 1,002 ⁻³⁴	Mn Superoxide Dismutase
nGal d 1	2.5%	77	6.4%	60	15.6%	45	22.2%	37	146.501	<i>p</i> : 1,009 ⁻³³	Hen eggs - Ovomuroid
nBos d 8	1.1%	103	3.5%	85	10.1%	58	17.1%	52	139.295	<i>p</i> : 3,796 ⁻³²	Cow Milk - Casein
nGal d 2	2.7%	73	11.6%	40	15.6%	46	21.5%	39	133.227	<i>p</i> : 8,064 ⁻³¹	Hen eggs - Ovalbumin
nAct d 1	6.7%	46	11.6%	37	22.6%	28	30.4%	27	122.913	<i>p</i> : 1,457 ⁻²⁸	Kiwi - Cysteine protease
rBlag 2	1.4%	99	4.6%	73	9.7%	60	15.8%	55	94.894	<i>p</i> : 2,008 ⁻²²	Cockroach - Aspartic protease
nDer f 1	28.5%	12	43.9%	8	51.0%	7	56.3%	8	87.721	<i>p</i> : 7,537 ⁻²¹	Mite - Cysteine protease
nDer p 1	28.0%	14	44.5%	7	49.0%	9	56.3%	9	85.948	<i>p</i> : 1,848 ⁻²⁰	Mite - Cysteine protease
nBos d 4	1.8%	90	4.0%	79	8.9%	66	12.7%	69	77.887	<i>p</i> : 1,091 ⁻¹⁸	Cow Milk - α -lactalbumin
(B)								χ^2 tests (linear-by-linear association)			
rAra h 9	18.9%	14.5%	24.1%	28.5%	9.593	<i>p</i> : 1,953 ⁻⁰³	nsLTP - 9k				
rCor a 8	15.5%	11.6%	19.8%	25.9%	10.045	<i>p</i> : 1,528 ⁻⁰³					
nOle e 7	11.0%	8.1%	14.4%	20.9%	12.891	<i>p</i> : 3,301 ⁻⁰⁴					
rPru p 3	25.2%	20.2%	33.5%	46.2%	29.413	<i>p</i> : 5,848 ⁻⁰⁸					
rCan f 1	8.8%	14.5%	27.6%	32.3%	109.374	<i>p</i> : 1,344 ⁻²⁵	Lipocalin				
rCan f 2	1.9%	2.3%	7.0%	13.3%	65.522	<i>p</i> : 5,747 ⁻¹⁶					
rEqu c 1	3.9%	9.2%	13.2%	19.6%	69.916	<i>p</i> : 6,189 ⁻¹⁷					
rFel d 4	4.8%	8.7%	14.8%	20.3%	64.006	<i>p</i> : 1,240 ⁻¹⁵					
nMus m 1	2.2%	6.4%	8.6%	15.8%	66.891	<i>p</i> : 2,869 ⁻¹⁶					
								(Continues)			

(Continues)

TABLE 2 (Continued)

	Allergic controls	Remission/Mild	Moderate	Severe	χ^2 tests (linear-by-linear association)	
rAct d 8	5.9%	5.8%	10.1%	16.5%	27.060	PR-10 protein
rAln g 1	12.9%	12.1%	23.0%	29.1%	38.870	
rApi g 1	4.5%	4.0%	9.7%	13.9%	32.139	
rAra h 8	8.4%	9.2%	18.3%	24.1%	46.843	
rBet v 1	16.6%	16.8%	27.6%	33.5%	36.297	
rCor a 1.01	16.1%	16.2%	27.6%	32.9%	38.164	
rCor a 1.04	13.5%	14.5%	24.1%	31.6%	44.702	
rGly m 4	6.6%	8.7%	17.9%	19.6%	53.439	
rMal d 1	14.5%	16.2%	25.3%	31.0%	38.709	
rPrp p 1	13.0%	12.7%	20.6%	31.6%	39.049	
nBos d 6	2.7%	4.6%	6.6%	10.1%	27.836	
nCan f 3	2.6%	3.5%	6.2%	9.5%	28.712	
nEqu c 3	1.8%	2.3%	4.3%	8.9%	27.629	
nFel d 2	4.8%	5.8%	7.4%	15.2%	25.626	
rAra h 2	0.9%	2.3%	3.9%	6.3%	31.680	
nAra h 6	1.5%	3.5%	4.3%	6.3%	21.852	
rBere 1	1.7%	2.3%	4.3%	7.6%	23.343	SSP, 2S albumin
nJug r 1	2.3%	8.7%	15.6%	12.7%	91.875	
rAna o 2	2.0%	3.5%	5.8%	10.8%	39.666	
rAra h 3	0.6%	0.3%	0.5%	0.5%	31.853	
nCor a 9	1.6%	4.6%	7.4%	12.7%	65.723	SSP, cupin, 11S globulin
nGly m 6	2.3%	5.2%	8.6%	20.9%	106.208	
nJug r 2	4.2%	8.1%	12.5%	19.0%	62.706	
rAra h 1	2.4%	4.0%	4.7%	5.7%	9.613	
nGly m 5	3.9%	5.8%	4.3%	13.3%	17.618	SSP, vicilin, 7S globulin
rAni s 3	4.1%	6.9%	7.0%	13.3%	20.830	
nBlag 7	3.8%	6.4%	8.9%	12.0%	25.327	
rDer p 10	6.6%	11.0%	15.6%	22.2%	52.471	
nPen m 1	5.1%	8.1%	9.3%	14.6%	21.617	Tropomyosin

Three hundred and fifty-four patients were < 14 years old (158 AC and 196 with AD). Demographic and clinical characteristics of study patients are summarized in Table 1. AD patients with moderate-to-severe skin involvement had significantly higher total IgE levels when compared to controls or patients with a history of AD in infancy. A lower occurrence of asthma, rhino-conjunctivitis and food allergies were recorded in AD participants in comparison to ACs (Table 1).

3.2 | Species-specific molecule recognition

A peculiar IgE sensitization profile was observed comparing AC to AD participants, even those with only a mild form or an history of atopic dermatitis in the first years of life. In fact, this subset showed a significantly higher prevalence of elevated IgE levels to: cockroach [rBla g 2 (χ^2 8.981; P : .003)], house dust mite molecules [nDer f 1 (χ^2 17.131; P < .001), rDer f 2 (χ^2 21.694; P < .001), nDer p 1 (χ^2 19.664; P < .001), rDer p 2 (χ^2 21.917; P < .001), and rBlo t 5 (χ^2 9.407; P : <.002)], lipocalins (rEqu c 1 [χ^2 10.031; P : .002] and nMus m 1 [χ^2 10.150; P : .001]), hen egg (nGal d 1 [χ^2 7.900; P : .005] and nGal d 2 [χ^2 32.600; P < .001]), seed storage proteins (SSP) (nJug r 1 [χ^2 20.320; P < .001] and nCor a 9 [χ^2 7.560; P : .006]) and the olive tree pollen allergen rOle e 9 (χ^2 8.704; P : .003).

Patients affected by moderate-to-severe AD had both increased prevalence and higher levels of IgE reactivity to the vast

majority (79/112) of tested components (Table 2A, B and Figure S1), when compared to AC. Interestingly, 59% of AD patients showed immunoreactivity to more than 10 allergenic sources, vs only 22% of AC.

The top-ranking frequencies were observed for molecules from cypress pollen, grass pollen, olive tree pollen and house dust mites (Figure 1). Table 2A documents the prevalence of the top 10 molecules most significantly associated with AD occurrence. More than 66% of the AD participants with moderate-to-severe skin involvement had IgE to the cypress pollen major allergen rCup a 1, thus representing the most common sensitization found. The major timothy grass pollen allergen, rPhl p 1, its homologous in Bermuda grass pollen, nCyn d 1, and group 2 mite allergens showed IgE levels above 60% in all AD affected subjects. A total of 55% of severe AD patients showed IgE specific for the major pellitory allergen rPar j 2, 52% for the cat major allergen rFel d 1, and 46% for the peach allergen rPru p 3.

IgE specific for the olive tree pollen protein, rOle e 9 and the manganese superoxide dismutase (MnSOD) from *Aspergillus* rAsp f 6 were found in 36.7% and 27% of severe AD patients respectively, a prevalence that was by far and significantly above the one observed in AC (5.3% and 2.8%, respectively). Accordingly, both molecules were the most significantly associated with AD occurrence, showing the highest Pearson χ^2 value (linear-by-linear association), followed by hen egg nGal d 1 and cow milk nBos d 8 casein (Table 2A). Also nGal d 3; rGad c

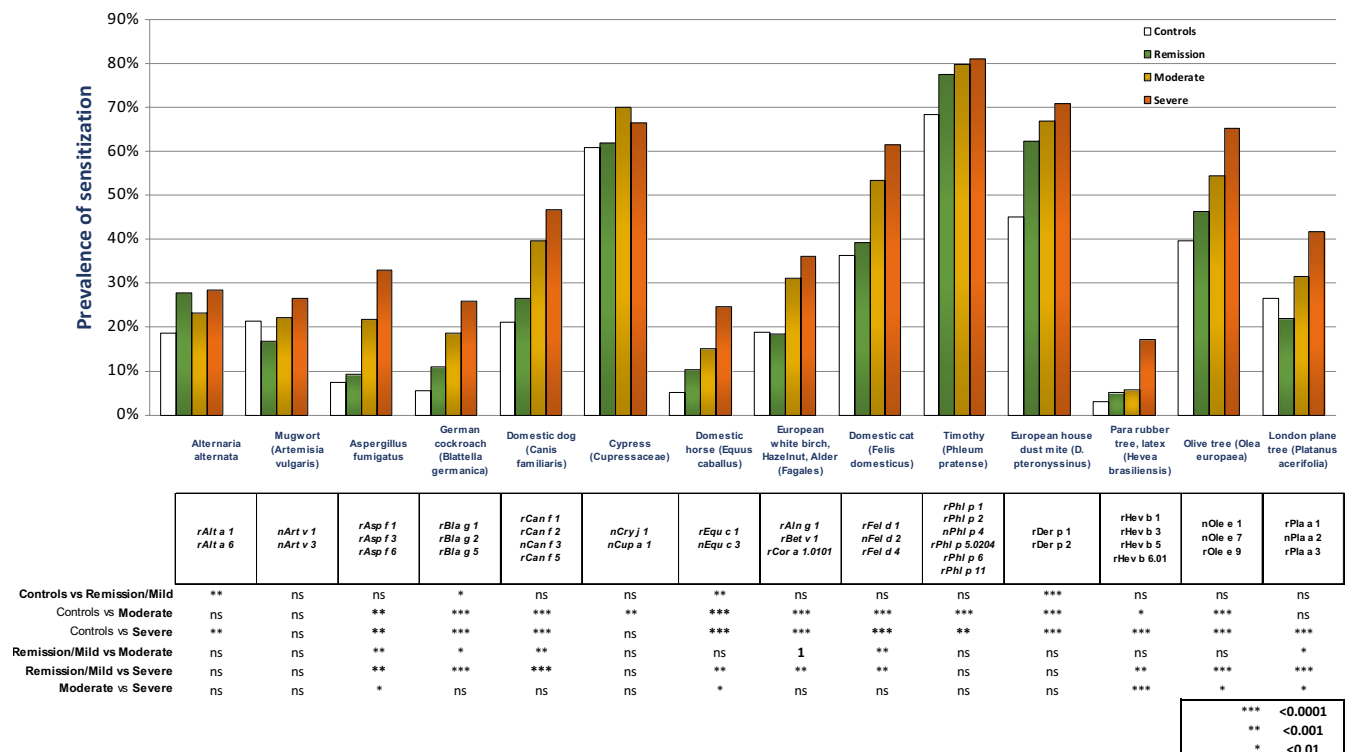


FIGURE 1 Environmental allergen IgE recognition profiles in AD patient and controls. Percentages of each biological source indicate the proportion of patients or controls reactive to that species within the distinct clinical category. IgE reactivity to at least one of the molecules belonging to the respective allergen source has been considered. The Pearson χ^2 -squared test was applied to determine the presence of a significant difference between the observed frequencies

1; rDer f 2; rBlo t 5; rDer p 2; nBos d 5; rLep d 2; nTri a 30; rAlt a 6; rHev b 6.01; nPla a 2; rChe a 1; rPar j 2; rFel d 1; nAct d 2; nPhl p 4; nPen m 2; nPen m 4; rBla g 1; rBla g 5; rCan f 5; nMUXF3; nCyn d 1; nOle e 1; rHev b 1; rHev b 5; nGal d 5; rHev b 3; rPhl p 11; nBos d Lactoferrin; nCry j 1 showed a significant linear-by-linear association with AD, with P values always $< .0001$ and Pearson χ^2 ranging from 70.362 to 5.96.

Comparing the IgE reactivity profile of patients with a moderate or with a severe form of AD, the molecules able to discriminate these two subset were the *Aspergillus* MnSOD rAsp f 6 (χ^2 8.301; P : .004), the soy bean cupins, nGly m 5 (χ^2 11.164; P : .001) and nGly m 6 (χ^2 12.930; P $< .001$), the *Lepidoglyphus destructor* group 2 molecule rLep d 2 (χ^2 12.225; P $< .001$), the olive tree rOle e 9 (χ^2 11.572; P : .001), the plane tree rPla a 1 (χ^2 8.933; P : .003) and the peach nsLTP rPrp p 3 (χ^2 6.719; P : .01).

We also evaluated additional molecules not present on the ImmunoCAP ISAC₁₁₂ test in a subset of 131 patients (65 AC and 66 with AD), by means of another micro-arrayed system (Allergy Explorer-ALEX®). The comparison between the 82 molecules shared by the two systems is shown in Table S1.

We observed an increased frequency of the *Malassezia sympodialis* MnSOD rMala s 11 (χ^2 16.567; P : .001), and rMala s 5 (χ^2 7.584;

P : .006) IgE sensitization in AD patients. rMala s 11 and rAsp f 6 sequences have a structure identity of 45.49% (identical position 111, similar position 59), and accordingly, a significant IgE cross-reactivity was found in AD patients (Pearson correlation 0.828 P : $< .001$).

House dust mite molecules rDer p 5 (χ^2 4.065; P : .044), rDer p 11 (χ^2 18.160; P $< .001$) and rDer p 23 (χ^2 4.902; P : .02) hypersensitivity was also more frequently observed in AD patients (Table S2).

Multiple logistic regression analysis, considering simultaneously all the molecules studied together with age and sex, showed a significant relationship between AD occurrence and rAsp f 6 (P $< .0001$; ORa = 3.581, 95% CI = 2.298-5.580), rOle e 9 (P $< .0001$; ORa = 2.248, 95% CI = 1.531-3.300), nJug r 1 (P $< .0005$; ORa = 2.508, 95% CI = 1.495-4.205), and nGal d 1 reactivity (P $< .01$; ORa = 2.306, 95% CI = 1.231-4.322).

3.3 | Panallergens

As shown in Figure 2, we detected an increased frequency of reactivity to all panallergens, apart from polcalcin and profilin homologous molecules, in both moderate and severe AD.

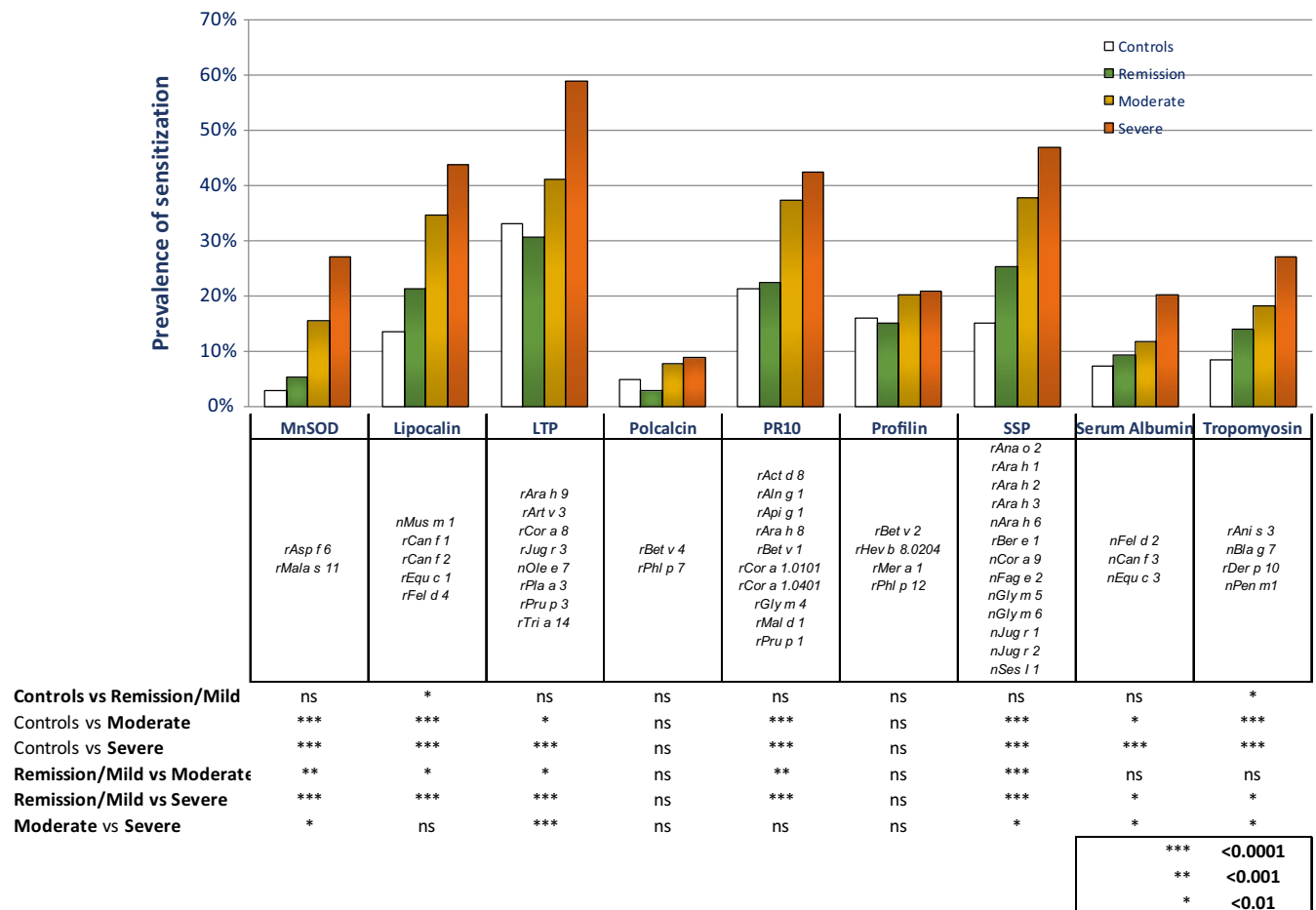


FIGURE 2 Cross-reactive allergen IgE recognition profiles in AD patient and controls. Percentages of each homologous subset indicate the proportion of patients or controls positive to that panallergen within the distinct clinical category. IgE reactivity to at least one of the molecules belonging to the respective panallergen superfamilies has been considered. The Pearson χ^2 -squared test was applied to determine the presence of a significant difference between the observed frequencies

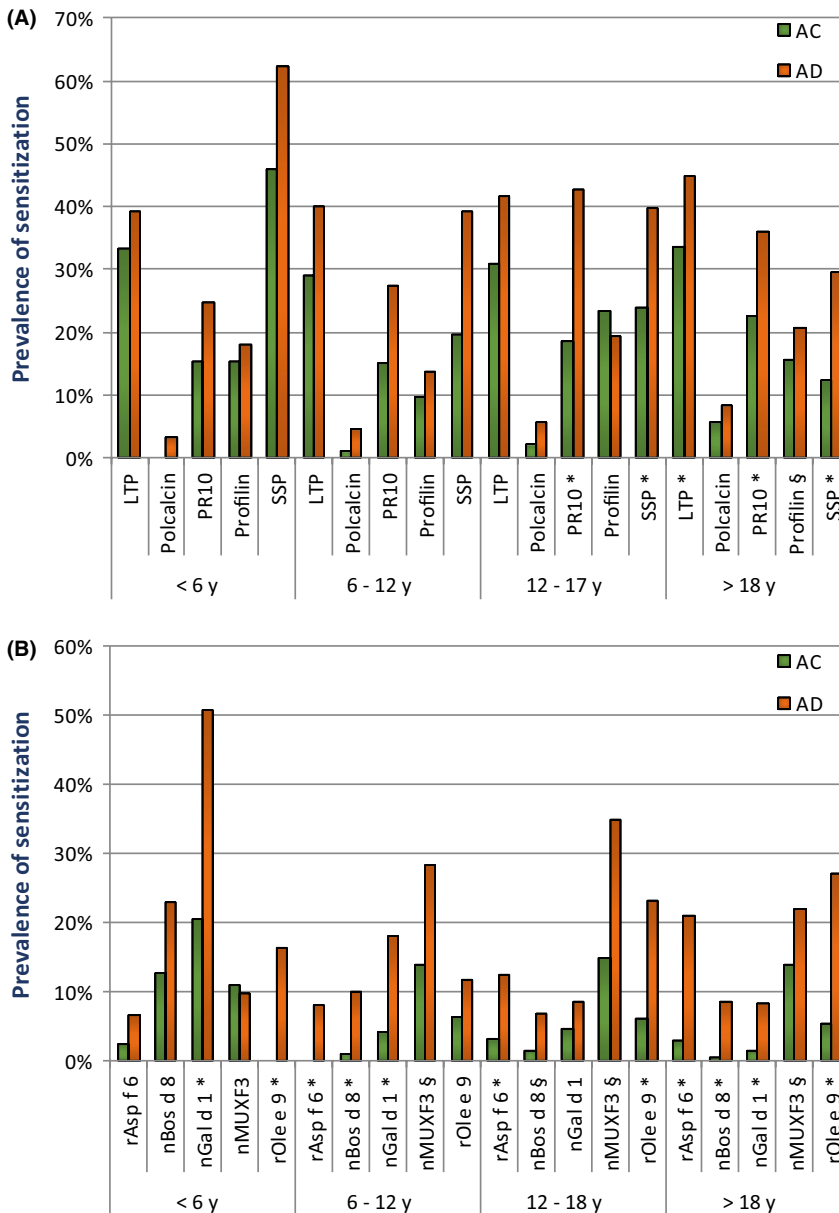
The increased prevalence of selective panallergen reactivity was even more marked in patients with severe AD, significantly higher than that observed in subjects with moderate AD. Accordingly, a significant linear-by-linear relationship was found with all the distinct component belonging to lipocalin, PR-10 protein, serum albumin, SSP and tropomyosin (2B).

No difference was observed comparing AC with participants who reported a history of AD, except for tropomyosin and lipocalin (Figure 2, Table 2B).

A significantly higher frequency of cross-reactive carbohydrate determinants (CCDs), as measured as MUXF3 reactivity, was observed in moderate and severe AD than in AC (24.1% and 28.5%, respectively, vs 15% in AC).

3.4 | Age distribution

Figure 3 summarizes the distribution of IgE reactivity to the most relevant homologous (A) or species-specific molecules (B) in AD, compared among different patients' age groups. Hen egg allergens (nGal d 1, nGal d 2 and nGal d 5) and SSP reactivity (11s globulins nGly m 6 and nCor a 9, and 2s Albumins nJug r 1 and rAra h 2) were more prevalent in the paediatric population, whilst in older patients, IgE recognition of *Aspergillus* Asp f 6 and pollen allergens (cypress, grass, olive tree rOle e 9 and pellitory) was predominant. Interestingly, the nsLTP from peach rPru p 3 reactivity was recorded in about 50% of adult with severe AD, and overall a higher frequency of PR10, LTP and SSP reactivity was observed in patients > 18 years. A significantly



* <0.01

§ <0.05

FIGURE 3 Distribution of IgE reactivity to the most relevant homologous (A) or species-specific molecules (B) in AD, compared among each age group. The p values for significance are shown

FIGURE 4 Respiratory symptoms (RS) and food allergy (FA) occurrence in AC vs AD, in total population and single age groups. In all cases, $P < .001$

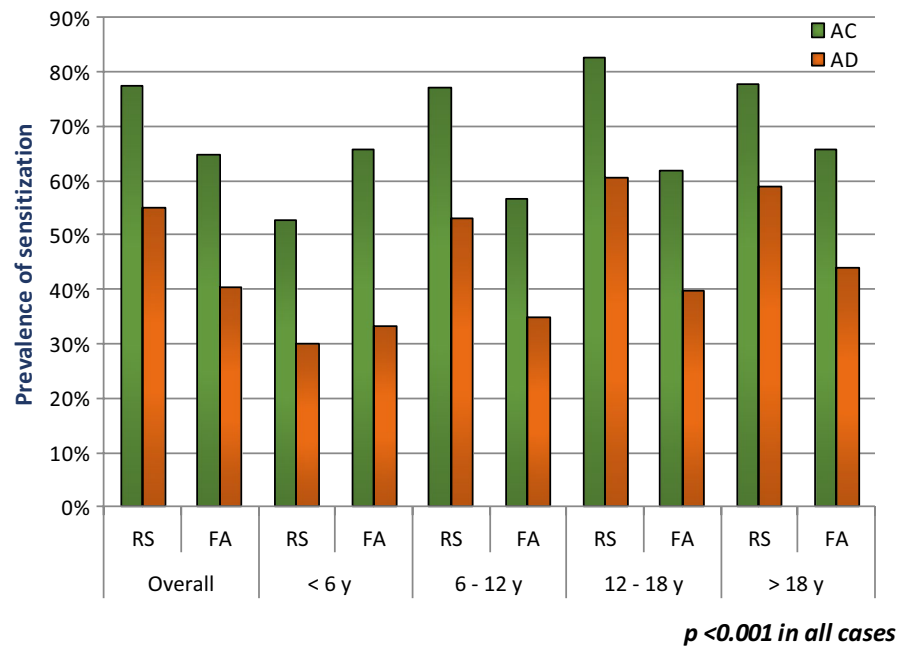
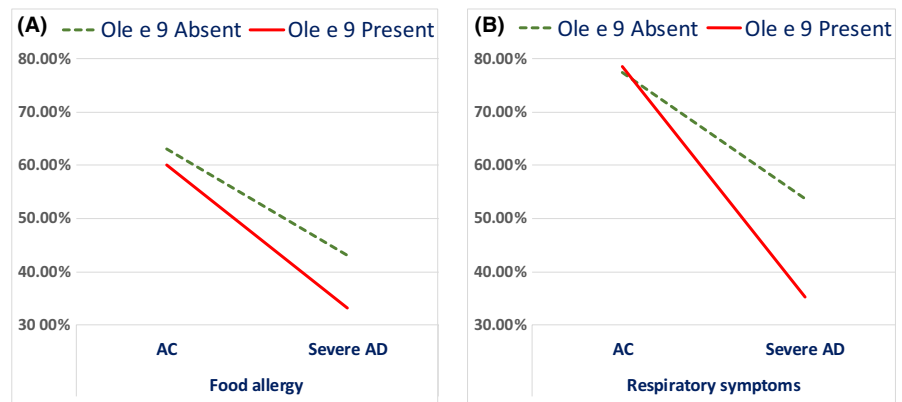


FIGURE 5 Food allergy (A) and bronchial asthma (B) occurrence in AC and severe AD participants with (solid red line) or without (dashed green line) rOle e 9 recognition



higher frequency of MUXF3 reactivity was also recorded in AD patients > 6 years (Figure 3).

3.5 | IgE reactivity and clinical symptoms

As shown in Table 1, from 59% to 63% of AD affected patients had also rhino-conjunctivitis and/or asthma, and around 40% of participants had a food allergy.

A history of rhino-conjunctivitis was mainly associated with pollen molecules (ie *Fagales*, grasses, cypress or plane tree) and mould (*Alternaria*) reactivity, whilst an inverse relationship was achieved by tropomyosin and the olive tree rOle e 9. Asthma was associated with polcalcin reactivity, group 1 mite allergens and cat rFel d 1. PR-10, profilin and nsLTP reactivity were directly associated with the occurrence of food allergy, whilst an inverse relationship was recorded for group 1 mite allergens and olive tree rOle e 9. AD participants showed a significantly reduced overall frequency of RS and FA when compared to AC (Table 1). Interestingly, Gal d and/or Bos d sensitized individuals with AD

showed significant lower prevalence of FA only in the paediatric population but not in adult, where the overall values were comparable (data not shown). The comparison across single age groups demonstrated that in all the age subsets considered a lower frequency of respiratory symptoms or adverse reaction to food was recorded in AD, from the childhood to the elderly (Figure 4). Interestingly, rOle e 9 reactivity was associated with a further significant reduction of either RS (35.2%) or FA (33.3%) occurrence in severe AD participants (Figure 5).

4 | DISCUSSION

In this study, we used two microarray-based detection systems, ImmunoCAP ISAC₁₁₂[®] ²⁸ and Allergy Explorer-ALEX[®] system ^{24,25} to identify and characterize specific IgE reactivity in a large cohort of unselected patients attending allergy clinics and correlated different reactivity profiles with the clinical expression of atopic dermatitis and with the concomitant occurrence of food allergy or respiratory symptoms. This investigation eventually produced several novel observations.

The *Aspergillus* MnSOD rAsp f 6, and the β -1,3-glucanase from the olive tree pollen, rOle e 9, represent the most reliable signatures of moderate-to-severe AD. When considered together, rAsp f 6 and rOle e 9 were found in 46% of severe AD (vs only 7.8% of controls) associated with a very large χ^2 value (245.856), confirming the significant relationship between such molecules and the occurrence of AD. Schmid-Grendelmeier et al²⁹ examined the reactivity to Asp f 6 and of the recombinant human MnSOD showing that only patients with AD or allergic bronchopulmonary aspergillosis had in vivo positive reactions. Taken together, rAsp f 6 recognition possibly represents the signature of an autoimmune process against human MnSOD, occurring in at least a subset of patients affected by AD.³⁰ The presence of autoantibodies of the IgE class and its association with disease severity has been suggested.³¹ In accordance with Mittermann et al,³² we also found a significantly higher frequency of IgE reactivity to *Malassezia sympodialis* rMala s 11 in severe AD, as a further confirmation of the presence of MnSOD reactivity in AD.

The olive tree molecule rOle e 9 has two domains immunologically and structurally independent: the N-terminal one displaying 1,3- β -endoglucanase activity and the C-terminal domain belonging to the pathogenesis-related protein family 2 (PR-2), implicated in latex-pollen-vegetable food allergy syndrome.³³ Such molecule has already been suggested to be linked with AD, evaluating a specific subset of olive tree allergic patients, particularly common in the Mediterranean area.³⁴ rOle e 9 reactivity could represent a distinctive signature of Mediterranean patients with AD. Interestingly, we found that AD patients with a severe form of skin involvement had a lower risk of severe reaction to food or bronchial asthma if co-sensitized to rOle e 9.

In keeping with some Authors,^{35,36} we observed a significant relationship among total IgE levels and AD severity, though such a relationship was not confirmed by others.^{37,38}

We found that, in the vast majority of cases, AD patients showed a widespread state of pan-reactivity to multiple biological sources and cross-reactive components, even in participants with mild AD or without currently active disease, and only with a history of AD. Accordingly, severe AD patients revealed a mean of 24 (range 1-74) distinct sensitizations/individual, and moderate AD participants had a mean of 18 (range 1-50), far above to the one found in ACs (12, range 1-54).

Patients affected by severe AD also differed from patients with moderate AD for several characteristics. In partial contrast with previous studies^{32,38} which were mainly performed on AD cohorts belonging to Northern Europe and therefore exposed to different environmental stimuli, we found a higher prevalence of IgE reactivity to several pollen sources (cypress, grasses, olive tree, pellitory) and to house dust mite, associated with a reduced overall prevalence of Fagales-related molecules. Mittermann et al,³² on the contrary, found that PR-10 molecules were associated with AD more commonly than grass pollen allergens rPhl p 1, rPhl p 5, rPhl p 2 and rPhl p 6. This observation may be the consequence of the different geographic location of the studied populations.

Nearly 70% of our AD participants had IgE against the major allergenic components of dust mites, nDer p 1, nDer f 1, rDer p 2 and rDer f 2. Among the mite molecules evaluated, rDer p 11 hypersensitivity was significantly associated with AD occurrence, as previously reported.³⁹

Interestingly, all homologous components, with the exclusion of profilin and polcalcin, were significantly associated with the occurrence of AD, in accordance with previous studies.^{32,38} We found, as already reported,⁴⁰ a very high frequency of hyper-reactivity to at least one nsLTPs in the allergic control population (33%). However, in the patients affected by severe AD, such frequency was almost double (ie 60%), thus representing the top-ranking panallergen reactivity observed, followed by SSP (46.8%), lipocalin (43.7%) and PR-10 (42.4%). Ott et al⁴¹ suggested that patients with severe AD had more frequently food adverse reaction than subjects with moderate AD whereas Röckmann et al⁴² did not find such association. Despite the presence of such a high level of reactivity to food-related cross-reactive components, we observed a reduced rate of patient-reported severe reactions to food compared to unselected ACs attending our allergy clinic. In this regard, it could be hypothesized that the presence of a massive cutaneous involvement of the T response could divert immunological resources from the adverse response to food, normally observed in subjects without atopic dermatitis.

A significantly higher prevalence of IgE to cross-reactive carbohydrate determinants (CCDs) was detected in moderate (24.1%) and severe AD (28.5%). Such observation bears practical relevance as the detection of IgE reactivity by the use of natural allergens may be influenced by the recognition of glycan moieties of glycoproteins⁴³ in about one-third of AD cases. Furthermore, it has been also demonstrated that false-positive results may occur even using recombinant molecules due to the presence of CCD epitopes of cellulose in the ImmunoCAP.⁴⁴ Thus, in patients with AD IgE reactivity should be preferentially detected using micro-arrayed systems, possibly after CCD removal.²⁵

In conclusion, dermatologists and allergologists should be aware that important associations between distinct clinical atopic dermatitis presentations and specific IgE sensitization profiles exist. Besides, some of these allergens may be potentially useful for AD staging and typing.

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CONFLICT OF INTEREST

Enrico SCALA has received consultant arrangements and speakers' bureau participation from Stallergenes and Thermo Fisher Scientific. The rest of the authors declare that they have no relevant conflicts of interest.

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REFERENCES

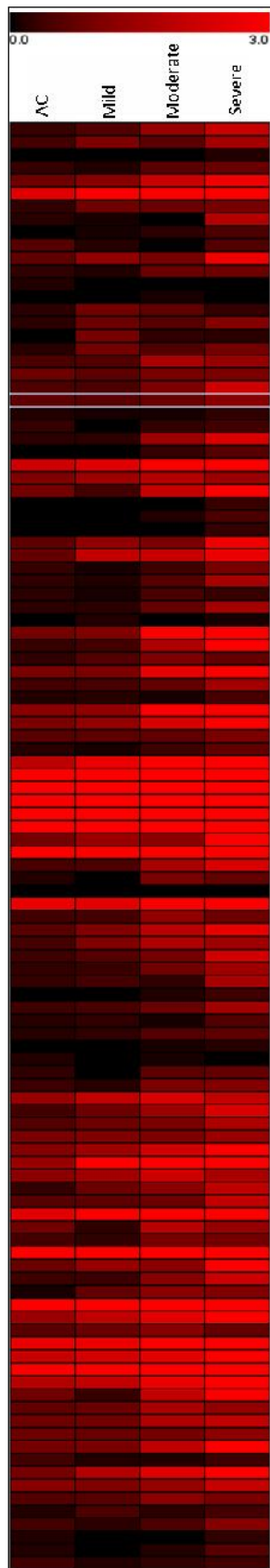
- Bieber T. Atopic dermatitis. *N Engl J Med*. 2008;358:1483-1494.
- Weidinger S, Novak N. Atopic dermatitis. *Lancet*. 2016;387:1109-1122.
- Tuft L. Importance of inhalant allergens in atopic dermatitis. *J Invest Dermatol*. 1949;12:211-219.
- Werfel T, Heratizadeh A, Niebuhr M, et al. Exacerbation of atopic dermatitis on grass pollen exposure in an environmental challenge chamber. *J Allergy Clin Immunol*. 2015;136:96-103.
- Reekers R, Busche M, Wittmann M, Kapp A, Werfel T. Birch pollen-related foods trigger atopic dermatitis in patients with specific cutaneous T-cell responses to birch pollen antigens. *J Allergy Clin Immunol*. 1999;104:466-472.
- Werfel T, Reekers R, Busche M, et al. Evidence for a birch pollen-specific cutaneous T-cell response in food-responsive atopic dermatitis. *Int Arch Allergy Immunol*. 1999;118:230-231.
- Flohr C, Johansson SG, Wahlgren CF, Williams H. How atopic is atopic dermatitis? *J Allergy Clin Immunol*. 2004;114:150-158.
- Gittler JK, Shemer A, Suarez-Farinas M, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J Allergy Clin Immunol*. 2012;130:1344-1354.
- Tang TS, Bieber T, Williams HC. Does "autoreactivity" play a role in atopic dermatitis? *J Allergy Clin Immunol*. 2012;129:1209-1215.
- Natter S, Seiberler S, Hufnagl P, et al. Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients. *FASEB J*. 1998;12:1559-1569.
- Valenta R, Mittermann I, Werfel T, Garn H, Renz H. Linking allergy to autoimmune disease. *Trends Immunol*. 2009;30:109-116.
- Caubet JC, Eigenmann PA. Allergic triggers in atopic dermatitis. *Immunol Allergy Clin North Am*. 2010;30:289-307.
- Schneider L, Tilles S, Lio P, et al. Atopic dermatitis: a practice parameter update 2012. *J Allergy Clin Immunol*. 2013;131:295-299.
- Løset M, Brown S, Saunes M, Hveem K. Genetics of atopic dermatitis: from DNA sequence to clinical relevance. *Dermatology*. 2019;235(5):355-364.
- Ahn HJ, Shin MK, Seo JK, et al. Cross-sectional study of psychiatric comorbidities in patients with atopic dermatitis and nonatopic eczema, urticaria, and psoriasis. *Neuropsychiatr Dis Treat*. 2019;15:1469-1478.
- Jahn-Schmid B, Harwanegg C, Hiller R, et al. Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. *Clin Exp Allergy*. 2003;33:1443-1449.
- Scala E, Alessandri C, Bernardi ML, et al. Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system. *Clin Exp Allergy*. 2010;40:911-921.
- Tokura Y. Extrinsic and intrinsic types of atopic dermatitis. *J Dermatol Sci*. 2010;58:1-7.
- Hanifin JM, Rajka G. Diagnostic features of atopic-dermatitis. *Acta Derm Venereol Suppl (Stockh)*. 1980;92:44-47.
- Kunz B, Oranje AP, Labreze L, Stalder JF, Ring J, Taieb A. Clinical validation and guidelines for the SCORAD index: consensus report of the European Task Force on Atopic Dermatitis. *Dermatology*. 1997;195:10-19.
- Phadia SAS. ImmunoCAP ISAC, an innovative tool in allergy diagnosis. *Eur Ann Allergy Clin Immunol*. 2008;40:106-107.
- Harwanegg C, Hiller R. Protein microarrays for the diagnosis of allergic diseases: state-of-the-art and future development. *Eur Ann Allergy Clin Immunol*. 2006;38:232-236.
- Harwanegg C, Laffer S, Hiller R, et al. Microarrayed recombinant allergens for diagnosis of allergy. *Clin Exp Allergy*. 2003;33:7-13.
- Heffler E, Puggioni F, Peveri S, Montagni M, Canonica GW, Melioli G. Extended IgE profile based on an allergen macroarray: a novel tool for precision medicine in allergy diagnosis. *World Allergy Organ J*. 2018;11:7.
- Buzzulini F, Da Re M, Scala E, et al. Evaluation of a new multiplex assay for allergy diagnosis. *Clin Chim Acta*. 2019;493:73-78.
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA*. 1998;95:14863-14868.
- Scala E, Villalta D, Uasuf CG, et al. An atlas of IgE sensitization patterns in different Italian areas. A multicenter, cross-sectional study. *Eur Ann Allergy Clin Immunol*. 2018;50:217-225.
- Hiller R, Laffer S, Harwanegg C, et al. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J*. 2002;16:414-416.
- Schmid-Grendelmeier P, Fluckiger S, Disch R, et al. IgE-mediated and T cell-mediated autoimmunity against manganese superoxide dismutase in atopic dermatitis. *J Allergy Clin Immunol*. 2005;115:1068-1075.
- Valenta R, Seiberler S, Natter S, et al. Autoallergy: a pathogenetic factor in atopic dermatitis? *J Allergy Clin Immunol*. 2000;105:432-437.
- Holmes J, Fairclough LC, Todd I. Atopic dermatitis and autoimmunity: the occurrence of autoantibodies and their association with disease severity. *Arch Dermatol Res*. 2019;311:141-162.
- Mittermann I, Wikberg G, Johansson C, et al. IgE sensitization profiles differ between adult patients with severe and moderate atopic dermatitis. *PLoS ONE*. 2016;11:e0156077.
- Huecas S, Villalba M, Rodriguez R. Ole e 9, a major olive pollen allergen is a β -1,3-glucanase. Isolation, characterization, amino acid sequence and tissue specificity. *J Biol Chem*. 2001;276:27959-27966.
- Scala E, Abeni D, Pomponi D, et al. Ole e 1, Ole e 7, and Ole e 9: Identifying distinct clinical subsets of olive tree-allergic patients. *J Allergy Clin Immunol*. 2015;137:629-631.
- Kiiski V, Karlsson O, Remitz A, Reitamo S. High serum total IgE predicts poor long-term outcome in atopic dermatitis. *Acta Derm Venereol*. 2015;95:943-947.
- Trzeciak M, Glen J, Bandurski T, Sokolowska-Wojdylo M, Wilkowska A, Roszkiewicz J. Relationship between serum levels of interleukin-18, IgE and disease severity in patients with atopic dermatitis. *Clin Exp Dermatol*. 2011;36:728-732.
- Kaminishi K, Soma Y, Kawa Y, Mizoguchi M. Flow cytometric analysis of IL-4, IL-13 and IFN-gamma expression in peripheral blood mononuclear cells and detection of circulating IL-13 in patients with atopic dermatitis provide evidence for the involvement of type 2 cytokines in the disease. *J Dermatol Sci*. 2002;29:19-25.
- Wojciechowska M, Zbikowska-Gotz M, Marek-Jozefowicz L, Przybyszewski M, Gocki J, Bartuzi Z. Allergic phenotypes in adult patients with atopic dermatitis, determined with the ISAC test (ImmunoCAP ISAC). *Postepy Dermatol Alergol*. 2018;35:351-359.
- Banerjee S, Resch Y, Chen K-W, et al. p 11 is a major allergen for house dust mite-allergic patients suffering from atopic dermatitis. *J Invest Dermatol*. 2015;135:102-109.
- Scala E, Till SJ, Asero R, et al. Lipid transfer protein sensitization: reactivity profiles and clinical risk assessment in an Italian cohort. *Allergy*. 2015;70:933-943.
- Ott H, Folster-Holst R, Merk HF, Baron JM. Allergen microarrays: a novel tool for high-resolution IgE profiling in adults with atopic dermatitis. *Eur J Dermatol*. 2010;20:54-61.
- Rockmann H, van Geel MJ, Knulst AC, Huiskes J, Bruijnzeel-Koomen CA, De Bruin-Weller MS. Food allergen sensitization pattern in adults in relation to severity of atopic dermatitis. *Clin Transl Allergy*. 2014;4:9.
- Aalberse RC, Koshte V, Clemens JG. Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and hymenoptera venom. *J Allergy Clin Immunol*. 1981;68:356-364.
- Hemmer W, Altmann F, Holzweber F, Gruber C, Wantke F, Wohrl S. ImmunoCAP cellulose displays cross-reactive carbohydrate

determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels. *J Allergy Clin Immunol*. 2018;141:372-381.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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	AC	R/M	M	S	Sig.
Act d 1	0.09	0.21	0.86	1.68	$p < 0.01$ [AC vs M and S - P vs S]
Act d 2	0.13	0.13	0.57	0.26	$p < 0.01$ [AC vs P and S]
Act d 5	0.01	0.00	0.00	0.03	n.s.
Act d 8	0.08	0.03	0.26	0.36	$p < 0.01$ [AC vs S]
Aln g 1	0.41	0.68	1.70	2.60	$p < 0.01$ [AC vs M and S]
Alt a 1	2.22	3.70	3.37	5.67	$p < 0.01$ [AC vs S]
Alt a 6	0.06	0.43	0.38	0.49	$p < 0.01$ [AC vs M and S]
Amh a 1	0.04	0.02	0.01	1.32	$p < 0.01$ [AC vs S]
Ana o 2	0.01	0.02	0.05	0.18	$p < 0.01$ [AC vs S]
Ani s 1	0.23	0.04	0.01	0.20	$p < 0.01$ [M vs S]
Ani s 3	0.30	0.81	0.49	2.63	$p < 0.01$ [AC vs S - M vs S]
Api g 1	0.06	0.03	0.37	0.42	$p < 0.01$ [AC vs M and S - P vs S]
Api m 1	0.03	0.00	0.01	0.01	n.s.
Api m 4	0.00	0.01	0.02	0.01	$p < 0.01$ [AC vs M]
Ara h 1	0.05	0.46	0.32	0.08	n.s.
Ara h 2	0.05	0.39	0.23	0.63	$p < 0.01$ [AC vs S]
Ara h 3	0.01	0.49	0.07	0.05	$p < 0.01$ [AC vs P and M]
Ara h 6	0.04	0.46	0.19	0.46	$p < 0.01$ [AC vs P and S]
Ara h 8	0.17	0.20	1.11	0.88	$p < 0.01$ [AC vs M and S - P vs S]
Ara h 9	0.41	0.29	0.50	0.53	n.s.
Art v 1	0.19	0.17	0.56	1.82	$p < 0.01$ [AC vs M and S]
Art v 3	0.29	0.26	0.36	0.69	$p < 0.01$ [AC vs S]
Asp f 1	0.03	0.02	0.02	0.07	$p < 0.01$ [AC vs S]
Asp f 3	0.08	0.01	0.07	0.12	n.s.
Asp f 6	0.05	0.05	0.97	2.10	$p < 0.01$ [AC vs M and S - P vs S]
Ber e 1	0.01	0.00	0.08	0.23	$p < 0.01$ [AC vs M and S]
Bet v 1	1.81	2.23	7.49	11.21	$p < 0.01$ [C vs M and S - P vs M and S]
Bet v 2	0.59	1.01	1.35	0.94	$p < 0.01$ [AC vs M]
Bet v 4	0.46	0.14	1.60	3.07	$p < 0.01$ [AC vs M and S]
Bla g 1	0.00	0.00	0.00	0.09	$p < 0.01$ [AC vs S]
Bla g 2	0.01	0.01	0.03	0.17	$p < 0.01$ [AC vs M and S]
Bla g 5	0.00	0.00	0.01	0.08	$p < 0.01$ [AC vs M and S]
Bla g 7	0.29	0.80	0.53	2.66	$p < 0.01$ [AC vs P and S - M vs S]
Blot 5	0.35	1.58	1.68	2.47	$p < 0.01$ [AC vs M and S]
Bos d 4	0.08	0.02	0.11	0.51	$p < 0.01$ [AC vs S]
Bos d 5 A	0.06	0.03	0.23	1.11	$p < 0.01$ [AC vs M and S]
Bos d 6	0.05	0.02	0.16	0.09	n.s.
Bos d 8	0.05	0.05	0.33	1.07	$p < 0.01$ [AC vs M and S]
Bos d Lat	0.01	0.03	0.01	0.02	$p < 0.01$ [AC vs P]
Can f 1	0.48	0.59	4.97	9.35	$p < 0.01$ [AC vs M and S - P vs M and S]
Can f 2	0.09	0.14	1.24	3.03	$p < 0.01$ [AC vs M and S - P vs S]
Can f 3	0.07	0.21	0.53	0.34	$p < 0.01$ [AC vs M and S]
Can f 5	0.53	0.54	2.44	3.65	$p < 0.01$ [AC vs M and S - P vs S]
Che a 1	0.14	0.16	0.32	1.05	$p < 0.01$ [AC vs S]
Cla h 3	0.03	0.04	0.02	0.07	$p < 0.01$ [AC vs S]
Cor a 1.0101	0.72	0.81	3.01	3.53	$p < 0.01$ [AC vs M and S - P vs S]
Cor a 1.0401	0.54	0.79	2.00	3.26	$p < 0.01$ [AC vs M and S - P vs S]
Cor a 8	0.25	0.28	0.30	0.54	$p < 0.01$ [AC vs S]
Cor a 9	0.04	0.02	0.11	0.31	$p < 0.01$ [AC vs S]
Cry j 1	1.47	2.45	3.57	4.41	$p < 0.01$ [AC vs M and S]
Cup a 1	8.10	11.63	16.34	20.85	$p < 0.01$ [AC vs M and S - P vs S]
Cyn d 1	4.86	6.28	9.19	13.02	$p < 0.01$ [AC vs M and S]
Der f 1	2.82	5.87	9.41	19.93	$p < 0.01$ [AC vs P, M and S - P vs S - M vs S]
Der f 2	6.40	12.48	18.43	32.98	$p < 0.01$ [AC vs P, M and S - P vs S - M vs S]
Der p 1	3.44	6.94	10.63	19.84	$p < 0.01$ [AC vs P, M and S - P vs S - M vs S]
Der p 10	0.47	0.91	0.71	3.84	$p < 0.01$ [AC vs S and S]
Der p 2	6.23	11.52	18.50	32.04	$p < 0.01$ [AC vs P, M and S - P vs S - M vs S]
Equ c 1	0.16	0.18	1.13	2.05	$p < 0.01$ [AC vs M and S - P vs S]
Equ c 3	0.03	0.01	0.52	0.32	$p < 0.01$ [AC vs S]
Fag e 2	0.01	0.00	0.00	0.00	n.s.
Fel d 1	2.47	2.32	8.01	13.43	$p < 0.01$ [AC vs M and S - P and S - M vs S]
Fel d 2	0.13	0.15	0.82	0.42	$p < 0.01$ [AC vs M]
Fel d 4	0.25	0.60	1.43	2.37	$p < 0.01$ [AC vs M and S]
Gad c 1	0.15	0.59	1.20	1.00	$p < 0.01$ [AC vs M and S]
Gad d 1	0.11	0.23	0.47	1.89	$p < 0.01$ [AC vs M and S - P vs S - M vs S]
Gad d 2	0.07	0.09	0.44	0.96	$p < 0.01$ [AC vs M and S]
Gad d 3	0.08	0.14	0.06	1.10	$p < 0.01$ [AC vs S]
Gad d 5	0.01	0.16	0.37	1.04	$p < 0.01$ [AC vs S]
Gly m 4	0.11	0.16	0.37	1.04	$p < 0.01$ [AC vs M and S - P vs S]
Gly m 5	0.04	0.06	0.02	0.22	$p < 0.01$ [M vs S]
Gly m 6	0.04	0.07	0.22	0.28	$p < 0.01$ [AC vs S]
Hev b 1	0.01	0.00	0.02	0.04	n.s.
Hev b 3	0.03	0.01	0.02	0.01	n.s.
Hev b 5	0.06	0.00	0.28	0.33	n.s.
Hev b 6.01	0.10	0.04	0.57	0.63	$p < 0.01$ [AC vs M and S]
Hev b 8.0204	0.88	1.53	2.11	1.56	$p < 0.01$ [AC vs M]
Jug r 1	0.13	0.39	0.92	2.11	$p < 0.01$ [AC vs M and S]
Jug r 2	0.22	0.41	0.52	1.23	$p < 0.01$ [AC vs M and S]
Jug r 3	0.52	0.59	0.58	0.90	n.s.
Lep d 2	0.65	1.01	1.88	6.85	$p < 0.01$ [AC vs M and S - P vs S - M vs S]
Mal d 1.0108	0.93	1.47	4.03	6.26	$p < 0.01$ [AC vs M and S - P vs S]
Mer a 1	0.74	0.99	1.80	1.14	$p < 0.01$ [AC vs M]
Mus m 1	0.08	0.38	0.69	1.48	$p < 0.01$ [AC vs M and S - P vs S]
MUXF3	0.25	0.29	0.41	1.61	$p < 0.01$ [AC vs S]
Ole e 1	2.31	4.26	6.21	10.65	$p < 0.01$ [AC vs M and S]
Ole e 7	0.45	0.06	1.39	0.86	n.s.
Ole e 9	0.14	0.06	0.48	0.45	$p < 0.01$ [P vs M and S]
Par j 2	4.70	5.08	8.39	13.11	$p < 0.01$ [AC vs M and S - P vs S]
Pen m 1	0.37	0.87	0.73	2.92	$p < 0.01$ [AC vs S]
Pen m 2	0.11	0.10	0.68	1.76	$p < 0.01$ [AC vs M and S]
Pen m 4	0.02	0.33	0.71	0.61	$p < 0.01$ [AC vs P, M and S]
Phl p 1	10.04	13.44	18.21	24.08	$p < 0.01$ [AC vs M and S - P vs S]
Phl p 11	0.92	1.56	2.25	4.27	$p < 0.01$ [AC vs M and S]
Phl p 12	0.27	0.38	0.69	0.35	$p < 0.01$ [AC vs M]
Phl p 2	2.45	2.55	6.06	5.70	$p < 0.01$ [AC vs M and S - P vs M and S]
Phl p 4	1.72	1.98	2.33	4.89	$p < 0.01$ [AC vs S - M vs S]
Phl p 5.0204	5.89	7.62	10.20	11.62	$p < 0.01$ [AC vs M and S]
Phl p 6	1.34	1.56	2.43	3.52	$p < 0.01$ [AC vs M and S]
Phl p 7	0.44	0.09	1.63	4.03	$p < 0.01$ [AC vs M and S - P vs S]
Pla a 1	0.31	0.37	1.12	0.81	n.s.
Pla a 2	0.42	0.44	1.23	1.56	$p < 0.01$ [AC vs M and S - P vs S]
Pla a 3	0.42	0.36	0.46	0.76	$p < 0.01$ [AC vs S]
Pla i 1	0.46	0.51	1.52	4.13	$p < 0.01$ [AC vs S]
Pol d 5	0.13	0.03	0.03	0.11	n.s.
Pru p 1	0.50	1.31	2.19	3.89	$p < 0.01$ [AC vs M and S]
Pru p 3	0.70	0.59	0.89	1.67	$p < 0.01$ [AC vs S]
Sal k 1	0.22	0.24	0.72	0.50	n.s.
Ses i 1	0.03	0.17	0.05	0.13	$p < 0.01$ [AC vs P]
Tri a 14	0.13	0.05	0.17	0.66	$p < 0.01$ [AC vs S]
Tri a 19.0101	0.03	0.00	0.01	0.06	n.s.
Tri a 30	0.03	0.01	0.03	0.24	$p < 0.01$ [AC vs S]
Ves v 5	0.12	0.03	0.07	0.12	n.s.

Figure. E1

	ALEX-ISAC+	ALEX-ISAC-	ALEX+ISAC+	ALEX-ISAC-	Pearson Chi-Square	Asymp. Sig. (2-sided)	Measure of Agreement [Kappa]	Cronbach's Alpha	Spearman's rho
Act d 1	24	1	10	96	26,363 ^a	0,000	0,364	0,517	,416 ^{**}
Act d 2	18	0	10	103	39,826 ^a	0,000	0,466	0,624	,355 ^{**}
Act d 5	1	0	1	129	64,996 ^a	0,000	0,316	0,758	,239 ^{**}
Aln g 1	10	3	32	86	77,280 ^a	0,000	0,762	0,440	,693 ^{**}
Alt a 1	5	0	25	101	104,017 ^a	0,000	0,885	0,704	,755 ^{**}
Amb a 1	4	4	2	121	11,895 ^a	0,001	0,301	0,473	,187 [*]
Ani s 1	6	0	1	124	17,851 ^a	0,000	0,240	0,552	0,141
Ani s 3	5	0	4	122	55,930 ^a	0,000	0,598	0,646	,226 ^{**}
Api g 1	4	2	8	117	65,291 ^a	0,000	0,702	0,708	,399 ^{**}
Api m 1	4	3	0	124	,097 ^a	0,756	-0,027	0,061	0,023
Ara h 1	4	2	5	120	48,172 ^a	0,000	0,601	0,782	,492 ^{**}
Ara h 2	1	1	3	126	72,148 ^a	0,000	0,742	0,582	,237 ^{**}
Ara h 3	2	3	4	122	46,750 ^a	0,000	0,595	0,790	,202 [*]
Ara h 6	2	0	3	126	77,372 ^a	0,000	0,743	0,725	,225 ^{**}
Ara h 8	17	1	10	103	36,268 ^a	0,000	0,462	0,603	,550 ^{**}
Ara h 9	8	2	30	91	86,187 ^a	0,000	0,806	0,890	,691 ^{**}
Art v 1	4	2	7	118	60,479 ^a	0,000	0,675	0,456	,387 ^{**}
Art v 3	24	0	9	98	28,699 ^a	0,000	0,359	0,321	,467 ^{**}
Asp f 3	5	0	2	124	35,978 ^a	0,000	0,431	0,462	,298 ^{**}
Asp f 6	9	1	9	112	53,121 ^a	0,000	0,604	0,342	,756 ^{**}
Ber e 1	No measures of association are computed								
Bet v 1	13	2	38	78	76,140 ^a	0,000	0,749	0,303	,769 ^{**}
Bet v 2	7	2	26	96	86,525 ^a	0,000	0,808	0,629	,733 ^{**}
Bla g 1	3	1	0	127	,024 ^a	0,878	-0,012	-0,011	-0,017
Bla g 2	14	0	3	114	20,589 ^a	5,7E-06	0,272	0,434	0,101
Bla g 5	No measures of association are computed								
Bos d 4	5	1	3	122	34,153 ^a	0,000	0,479	0,262	,291 ^{**}
Bos d 5A	3	2	7	119	67,436 ^a	0,000	0,716	0,479	,483 ^{**}
Bos d 6	7	0	3	121	37,151 ^a	0,000	0,442	0,833	,425 ^{**}
Bos d 8	5	0	5	121	62,901 ^a	0,000	0,649	0,559	,229 ^{**}
Can f 1	9	2	24	96	77,539 ^a	0,000	0,760	0,373	,739 ^{**}
Can f 2	2	0	10	119	107,362 ^a	0,000	0,901	0,536	,520 ^{**}
Can f 3	1	0	6	124	111,387 ^a	0,000	0,919	0,548	,370 ^{**}
Che a 1	15	3	3	110	6,975 ^a	0,008	0,195	0,382	0,066
Cla h 8	6	5	3	117	12,493 ^a	0,000	0,308	0,609	,214 [*]
Cor a 1.0101	21	1	30	79	57,150 ^a	0,000	0,620	0,409	,747 ^{**}
Cor a 1.0401	15	3	28	85	60,888 ^a	0,000	0,664	0,354	,658 ^{**}
Cor a 8	14	8	17	92	33,620 ^a	0,000	0,502	0,610	,493 ^{**}
Cor a 9	3	0	4	124	73,089 ^a	0,000	0,716	0,917	,246 ^{**}
Cupa 1	15	1	82	33	72,199 ^a	0,000	0,720	0,451	,913 ^{**}
Der f 1	11	1	53	66	89,338 ^a	0,000	0,816	0,374	,873 ^{**}

	ALEX-ISAC+	ALEX-ISAC-	ALEX+ISAC+	ALEX-ISAC-	Pearson Chi-Square	Asymp. Sig. (2-sided)	Measure of Agreement [Kappa]	Cronbach's Alpha	Spearman's rho
Der f 2	17	1	53	60	73,817 ^a	0,000	0,728	0,416	,882 ^{**}
Der p 1	8	2	58	63	94,870 ^a	0,000	0,847	0,526	,907 ^{**}
Der p 10	24	0	4	103	15,178 ^a	0,000	0,208	0,490	,441 ^{**}
Der p 2	12	1	59	59	86,868 ^a	0,000	0,803	0,449	,886 ^{**}
Equ c 1	7	1	11	112	67,681 ^a	0,000	0,700	0,325	,504 ^{**}
Fag e 2	No measures of association are computed								
Fel d 1	20	3	54	54	60,059 ^a	0,000	0,655	0,568	,876 ^{**}
Fel d 2	10	0	6	115	45,195 ^a	0,000	0,513	0,721	,216 [*]
Fel d 4	10	0	12	109	65,450 ^a	0,000	0,666	0,507	,580 ^{**}
Gad c 1	4	7	6	114	30,372 ^a	0,000	0,477	0,941	,318 ^{**}
Gal d 1	8	2	9	112	50,393 ^a	0,000	0,602	0,962	,383 ^{**}
Gal d 2	7	1	9	114	61,097 ^a	0,000	0,660	0,958	,590 ^{**}
Gal d 3	4	3	8	116	58,312 ^a	0,000	0,666	0,805	,468 ^{**}
Gal d 5	4	6	1	120	2,208 ^a	0,137	0,128	0,113	,245 ^{**}
Gly m 4	14	1	13	103	50,003 ^a	0,000	0,574	0,651	,625 ^{**}
Gly m 5	16	0	1	114	6,757 ^a	0,009	0,098	0,401	,271 ^{**}
Gly m 6	5	4	6	116	37,481 ^a	0,000	0,534	0,768	,249 ^{**}
Hev b 1	3	1	0	127	,024 ^a	0,878	-0,012	0,105	,172 [*]
Hev b 3	3	4	0	124	,097 ^a	0,756	-0,027	-0,031	0,011
Hev b 5	1	0	2	128	86,656 ^a	0,000	0,796	0,556	,211 [*]
Hev b 6.01	4	0	4	123	63,437 ^a	0,000	0,653	0,836	,267 ^{**}
Hev b 8.0204	10	0	26	95	85,601 ^a	0,000	0,790	0,633	,771 ^{**}
Jug r 1	3	2	9	117	76,184 ^a	0,000	0,762	0,483	,473 ^{**}
Jug r 2	9	14	6	102	8,010 ^a	0,005	0,244	0,483	,320 ^{**}
Mal d 1	19	0	24	88	60,133 ^a	0,000	0,629	0,353	0,679
Mus m 1	5	0	9	117	80,763 ^a	0,000	0,763	0,526	,454 ^{**}
Ana c 2.0101	No measures of association are computed								
Ole e 1	7	5	56	63	87,376 ^a	0,000	0,816	0,432	,928 ^{**}
Par j 2	13	4	44	70	71,475 ^a	0,000	0,731	0,343	,846 ^{**}
Pen m 1	9	2	3	117	16,147 ^a	0,000	0,316	0,596	0,141
Phl p 1	16	0	78	37	75,886 ^a	0,000	0,734	0,369	,938 ^{**}
Phl p 2	16	2	46	67	71,501 ^a	0,000	0,721	0,524	,817 ^{**}
Phl p 5.0204	6	0	60	65	109,027 ^a	0,000	0,908	0,591	,934 ^{**}
Ph p 6	14	1	45	71	79,806 ^a	0,000	0,764	0,724	,852 ^{**}
Phl p 7	3	1	7	120	77,080 ^a	0,000	0,762	0,422	,568 ^{**}
Pla a 1	5	0	6	120	68,596 ^a	0,000	0,687	0,610	,503 ^{**}
Pla l 1	6	1	11	113	72,432 ^a	0,000	0,730	0,789	,638 ^{**}
Pol d 5	14	0	2	115	14,598 ^a	0,000	0,201	0,018	,187 [*]
Prup 3	13	0	34	84	82,065 ^a	0,000	0,770	0,719	,738 ^{**}
Ses i 1	2	3	1	125	9,510 ^a	0,002	0,267	0,546	0,161
Ves v 5	17	1	3	110	11,380 ^a	0,001	0,210	0,572	0,130

Table E1

	Derp1	Derp2	Derp5	Derp7	Derp11	Derp23
Derp1	75,6%	60,0%	48,9%	33,3%	42,2%	51,1%
Derp2		75,6%	42,2%	31,1%	42,2%	46,7%
Derp5			48,9%	26,7%	33,3%	44,4%
Derp7				37,8%	17,8%	28,9%
Derp11					44,4%	33,3%
Derp23						57,8%

Table E2