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ORIGINAL ARTICLE

Cross-sectional survey on immunoglobulin E reactivity in 23 077 subjects using an allergenic molecule-based microarray detection system

E. Scala, C. Alessandri, M. L. Bernardi, R. Ferrara, P. Palazzo, D. Pomponi, D. Quaratino, C. Rasi, A. Zaffiro, D. Zennaro and A. Mari
Centre for Clinical and Experimental Allergology, IDI-IRCCS, Rome, Italy

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Summary

Background The availability of allergenic molecules and high-throughput microtechnologies allow the collection of a large number of IgE results at the same time in a single test. This can be carried out applying the test in the routine diagnostic work-up.

Objective The aim of this study was to make a cross-sectional evaluation of the raw prevalence of IgE reactivity to allergenic molecules in serum samples from a cohort of Italian patients using an innovative tool.

Methods The ISAC, a microarray system, has been used for specific IgE detection using 75 different allergenic molecules. Sera were collected from 23 077 unselected consecutive individuals complaining about any allergic disease.

Results Sixteen thousand four hundred and eight of 23 077 patients had IgE to at least one of 75 allergenic molecules. The top-ranked molecules in this cohort were Cup a 1 (42.7%), Der f 2 (38.7%), and Phl p 1 (37.9%), whereas all the other allergens tested scored in a range between 36.8% and 0.04%, including the first food allergen, Pru p 3, ranked 15th (9.79%). Prevalence varied quite markedly depending on the age range considered, and showing a different behaviour in the lifetime sensitization process. Unsupervised two-way hierarchical clustering analysis generated distinctive patterns of reactivity as the result of IgE recognition of either homologous allergens belonging to different biological sources or non-homologous belonging to the same biological source.

Conclusions Allergen-based microarray is a tool for the detection of IgE-related sensitization to panels of allergens and gives a more precise and comprehensive evaluation for an IgE-based epidemiology. This insight brings data for better understanding of the sensitization process.

Correspondence:
Adriano Mari, Centre for Clinical and Experimental Allergology, IDI-IRCCS, Via dei Monti di Creta 104, Rome, Italy.
E-mail: a.mari@panservice.it

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Introduction

The increasing prevalence of allergic disease in the past decades has generated the need for more accurate and reliable allergy testing, hopefully addressing at the same time both diagnosis and epidemiology of the IgE-mediated diseases [1]. Furthermore, a worldwide assessment of the prevalence of atopic disorders revealed a wide variability in the global epidemiology of IgE-mediated diseases [2–4]. There is a great need to establish shared screening procedures to monitor allergic sensitization and let the collected data to be comparable. Heinzerling et al. [5] showed how routine skin test performed in 29 European centres were not homogeneously applied. The availability of a unique, reliable, and comprehensive diagnostic approach might be critical for that purpose.

Current tools for the detection of IgE-mediated sensitization are based on allergenic extracts, and used both for *in vivo* skin testing or *in vitro* IgE singleplex testing [6]. Several problems still affect the use of allergenic extracts in clinical practice. They derive from raw (or partially purified) biological sources or their tissues, thus containing an unknown concentration of a mixture of allergenic and non-allergenic molecules [7]. The detection of IgE reactivity to an allergenic extract gives no information about the nature of the molecules involved in the immunological recognition.

Over the past 20 years, a growing number of allergenic molecules from allergenic sources have been identified, characterized, and produced as recombinant forms [8, 9]. Taking advantage from the application of micro-technology, minute amounts of natural or recombinant allergens

can be spotted on activated biochip surfaces and negligible serum amount can be used for IgE detection for a huge number of allergen specificities in a single step [10, 11]. A commercial protein microarray bearing natural and recombinant allergenic molecules (ISAC) has been used for IgE detection in our centre since 2006. As stated in the reviewed edition of approved guidelines on 'Analytical Performance Characteristics and Clinical Utility of Immunological Assays for Human Immunoglobulin E (IgE) Antibodies and Defined Allergen Specificities', this testing system is a multiplex assay, defined as 'a test delivering more than one result based on a single addition of a test specimen' [12].

The aim of this paper was to analytically report the first extensive cross-sectional survey of the IgE reactivity detected by a microarray system and 75 allergenic molecules in more than 23 000 Italian subjects complaining about allergic symptoms.

Methods

Patients

Sera from a total of 23 077 (61.2% female) unselected consecutive subjects referred to us at the Centre for Clinical and Experimental Allergology (IDI-IRCCS, Rome, Italy) for a history of food allergy, rhinitis, asthma, atopic dermatitis, urticaria, or anaphylaxis from March 2006 to April 2008 were collected. Demographical and clinical data were recorded for all patients by using a customized allergy *e*-record (InterAll version 1.0, Allergy Data Laboratories s.c., Latina, Italy) [13]. Oral informed consent for blood sampling was obtained from patients or caregivers during the allergy consult as a routine procedure during the diagnostic work-up.

The study received the approval of the institutional ethical committee (35/IDI-CE/2006).

Purified recombinant and natural allergens

Table 1 reports the list of the allergenic molecules spotted on the microarray used in this study. They include some of the most common allergens from sources causing IgE sensitization in western countries. Allergens have been included in the panel used in the present study on the basis of their availability by several different commercial and research lab providers, and anyhow decided by the manufacturer ISAC (VBC-Genomics, Vienna, Austria). For unequivocal identification of the allergenic molecules, codes are given as in the Allergome web site [9, 13], where molecule characteristics and full referencing are available. The latter provides detailed information about characterization level of each allergen spotted on the ISAC microarray.

Allergen microarray for immunoglobulin E testing

Immunoglobulin E microarray assay. The ISAC microarray system, including all allergens and reagents, the spotted microarray slides, and the software, has been purchased from VBC-Genomics. The testing procedures have been carried out following the manufacturer's instructions [14]. IgE values were expressed semi-quantitatively as kU_A/L by interpolating the mean fluorescence value with a previously established reference curve [14]. At the end of each run, IgE data were exported to the InterAll software (Allergy Data Laboratories s.c.) by a real-time connectivity. Several ISAC biochips with different number of spotted allergens have been used during the study timeframe (ISAC76, ISAC79, ISAC85, and ISAC89). We analysed results from the 75 molecules present on all of them.

Statistical evaluation and clustering analysis

Data generated during the IgE survey and saved in the InterAll database were entered into the SPSS/PC+ statistical package for evaluation (SPSS, version 15, Chicago, IL, USA). Differences between prevalence were evaluated using the non-parametric Mann–Whitney *U*-test and the chi-square test. The degree of relationship between the quantitative variables studied was analysed using Pearson's correlation test. Statistical significance cut-off level has been set for $P < 0.01$.

For the management and evaluation of the huge amount of data generated, we applied the same approach used for genomics data analysis. Relatedness of IgE recognition profiles was tested by applying unsupervised Eisen Kendall's hierarchical clustering analysis methods to the data set [15], encompassing allergenic molecules across all samples and using as agglomeration rule the average linkage clustering as implemented in the Genesis software 1.7.2 [16]. Unsupervised clustering involves the sorting of both allergenic molecules and IgE recognition values (tested patients). The IgE recognition tree was computed on the basis of the full data set and the distances between samples were computed by using the Pearson correlation as for similarity measures. As a result, allergenic molecules with a similar pattern of IgE recognition were grouped as hierarchical clusters and presented as heat-maps. Each square in the heat-map represents the presence (red) or absence (black) of IgE recognition for any tested allergens. The red colour intensity of every square in the heat-map is directly proportional to the amount of measured IgE. Interpretation of the heat-map generated by the software can be done either visually, where clustering molecules tends to give more homogeneous red areas, or by taking into consideration the higher or lower dendrogram levels on allergen side of the graph (*y*-axis).

Results

Study group description

A total of 16 408 subjects, 71.1% of 23 077 tested with ISAC, having IgE reactivity to at least one allergenic

Table 1. Prevalence of IgE sensitization to allergenic molecules tested on ISAC microarray

Rank	Allergen	Biological function	Allergome code	No.	%	Rank	Allergen	Biological function	Allergome code	No.	%	Rank	Allergen	Biological function	Allergome code	No.	%
1	nCup a 1		256	7010	42.72	26	rPho d 2	Profilin	571	876	5.34	51	nBos d 8	Casein	167	223	1.36
2	nDer f 2		302	6355	38.73	27	rCyn d 12	Profilin	279	857	5.22	52	rHev b 6	Hevein-like protein	390	213	1.3
3	rPhl p 1	Expansin homolog	549	6226	37.94	28	rAln g 1	PR-10	7	741	4.52	53	nBos d	Lactoferrin	1065	206	1.26
													Lactoferrin				
4	nLol p 1	Expansin homolog	450	6048	36.86	29	nCan f 1	Lipocalins	174	704	4.29	54	nTri a 18	Agglutinin	650	195	1.19
5	nDer p 2		316	5420	33.03	30	rMal d 1	PR-10	464	696	4.24	55	nBos d 5.0101	β-Lactoglobulin A	2738	190	1.16
6	rPar j 2	Lipid transfer protein	507	4218	25.71	31	rPar j 3	Profilin	510	688	4.19	56	nBos d 8αS1	α-Casein	2734	189	1.15
7	nDer p 1	Cysteine proteases	310	4042	24.63	32	nArt v 1		53	671	4.09	57	nBos d 5.0102	β-Lactoglobulin B	2739	169	1.03
8	nDer f 1	Cysteine proteases	295	3928	23.94	33	nGal d 4	Lysozyme	362	604	3.68	58	rApi g 1	PR-10	40	163	0.99
9	rPar j 1	Lipid transfer protein	503	3638	22.17	34	nBos d 6	Bovine serum albumin	165	591	3.6	59	nBos d 8 β	β-Casein	2736	117	0.71
10	rPhl p 5	Ribonuclease	558	3606	21.98	35	rPhl p 12	Profilin	553	551	3.36	60	rHev b 5		389	113	0.69
11	rPhl p 2	Unknown	555	3400	20.72	36	rDer p 10	Tropomyosin	311	430	2.62	61	nBos d 8 κ	κ-Casein	2737	108	0.66
12	nFel d 1	Uteroglobins	345	3292	20.06	37	rPhl p 7	Calcium-binding protein	670	406	2.47	62	rHev b 11	Class I chitinase	384	77	0.47
13	nOle e 1	Trypsin inhibitors	482	3208	19.55	38	nAna c 2	CCD marker	694	402	2.45	63	nGal d 3	Conalbumin	361	76	0.46
14	nBos d 7	Bovine IgG	166	3005	18.31	39	nGal d 2	Ovalbumin	360	371	2.26	64	rAsp f 1	Ribotoxin	62	70	0.43
15	rPhl p 6	P-particle associated	569	2224	13.55	40	nBos d 4	α-Lactalbumin	163	327	1.99	65	rBla g 2		141	66	0.4
16	nPru p 3	Lipid transfer protein	503	1607	9.79	41	rPen i 1	Tropomyosin	527	326	1.99	66	rHev b 10	MnSO dismutase	380	64	0.39
17	rAlt a 1		722	1569	9.56	42	rAni s 3	Tropomyosin	37	313	1.91	67	rDau c 1	PR-10	287	59	0.36
18	rBet v 1	PR-10	89	1314	8.01	43	rPen m 1	Tropomyosin	872	313	1.91	68	Hev b 1	Elongation factor	379	56	0.34
19	nPla a 2		573	1204	7.34	44	rHel as 1	Tropomyosin	378	309	1.88	69	rTri a 19.0101	ω5-Gliadin	651	45	0.27
20	rMer a 1	Profilin	476	1181	7.2	45	nCan f 3	Dog serum albumin	176	308	1.88	70	rHev b 9	Enolase	404	28	0.17
21	rHev b 8	Profilin	397	1019	6.21	46	rPla a 1		572	291	1.77	71	rHev b 7	Patatin homolog	394	26	0.16
22	rBet v 2	Profilin	127	986	6.01	47	rPer a 7	Tropomyosin	542	272	1.66	72	nGal d 5	α-Livetin	363	21	0.14
23	rOle e 2	Profilin	490	977	5.95	48	nHor v 17	β-Amylases	429	242	1.47	73	rBla g 5		144	15	0.09
24	rCor a 1	PR-10	232	960	5.85	49	nGal d 1	Ovomucoid	359	239	1.46	74	nHor v 16	α-Amylases	419	13	0.08
25	rHel a 2	Profilin	377	889	5.42	50	rAlt a 6	Enolase	14	225	1.37	75	rHev b 3		387	6	0.04

Prevalence has been calculated on the total number of tested subjects (n = 16 408), being positive to at least one of the 75 allergens spotted on the ISAC microarray.
Allergen names appear as they are reported in the Allergome web site (http://www.allergome.org). The allergen name suffixes stay for n, natural; r, recombinant.

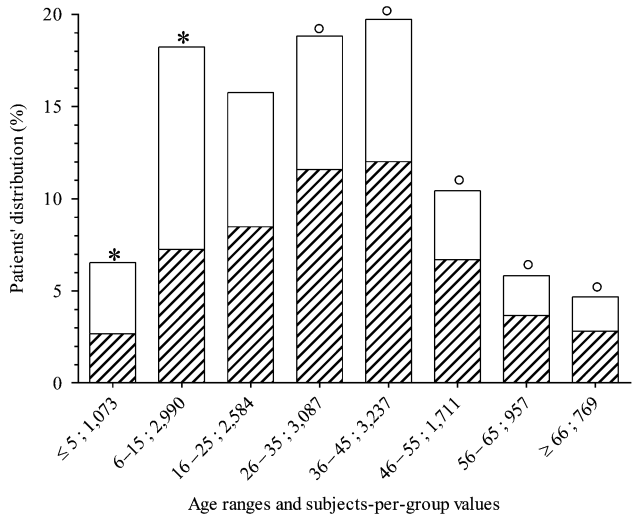

Fig. 1. Age group and gender distribution of the ISAC IgE-reactive population ($n = 16\,408$). Female: hatched bars; male: white bars. Statistically significant differences are marked as * for male vs. female and ° for female vs. male, all for $P < 0.0001$ excepting the over 60 age group ($P = 0.003$).

molecule represented our study group. Mean age and standard deviation was 30.8±18.3 years, ranging between 2 and 98 years old. The total IgE-positive cohort has been divided in eight consecutive age groups (Fig. 1). Male subjects, representing 44.78% of the study group, were significantly younger when compared with the female counterpart (27.66±18.56 vs. 33.37±17.72; Mann–Whitney rank-sum test $P < 0.0001$). As shown in Fig. 1, younger patients were prevalently male, whereas after 26 years old, the gender distribution was the opposite.

Prevalence of allergen immunoglobulin E recognition

Table 1 comprehensively reports prevalence for each of the 75 tested allergens. More than 42% of the studied Italian allergic population had IgE to cypress pollen allergen Cup a 1, thus representing the most common sensitization in the study area, followed by the mite allergen Der f 2 (38.73%), the timothy grass pollen allergen Phl p 1 (37.94%), and its highly homologous allergen in rye grass pollen Lol p 1 (36.86%). Der p 2, the homologous of Der f 2, was positive in 33.03% of the patients, whereas Der p 1 and Der f 1 followed with 24.63% and 23.94% of the patients, respectively. 25.71% of the patients had specific IgE to the parietaria allergen Par j 2, followed by the homologous Par j 1 with a 22.17% value. Cat major allergen Fel d 1 and the olive tree pollen Ole e 1 were found to be IgE reactive in 20.06% and 19.55% of the tested patients, respectively.

Among the first 20 positive allergens, IgE were detected for three more grass allergens (Phl p 2, Phl p 5, and Phl p 6), the birch allergen Bet v 1, plane tree pollen allergen Pla a 1, the Alternaria allergen Alt a 1, some representatives from the profilin allergen family (Mer a 1 and Hev b 8), and the lipid transfer protein (LTP) from peach, Pru p 3. The latter has been recorded positive in 9.79% of cases, thus representing the food allergen most frequently recognized in our cohort among all allergens, followed by the Bet v 1-like allergen from apple, Mal d 1 (4.24%), Gal d 4 from hen's egg (3.68%), and Pen i 1, the tropomyosin from a shrimp species (1.99%). Seven out of nine latex molecules spotted on the array (Hev b 1, Hev b 3, Hev b 5, Hev b 7, Hev b 9, Hev b 10, and Hev b 11) were recognized by < 1% of the total allergic population, whereas an IgE reactivity to the latex profilin Hev b 8 and the hevein Hev b 6 was recorded in 6.2% and 1.3% of patients, respectively.

Allergenic molecule mono-sensitization

Two thousand five hundred and sixty-five (15.63% of 16 408 allergic patients) patients recognized only one molecule out of the 75 spotted on the microarray and were considered as 'molecule mono-sensitized' (Table 2). The female subset was more represented among the mono-sensitized subjects (62.6% female) when compared with the multiple allergenic molecule-sensitized individuals (55.2% female, $P < 0.0001$, χ^2 49.53). About one-third of molecule mono-sensitized subjects ($n = 842$) had IgE to cypress pollen allergen Cup a 1, thus ranking cypress group 1 allergen first also among this subset as well. IgE reactivity to the peach LTP, Pru p 3, was recorded in 7.1% of the mono-sensitized cohort, thus being ranked third in this subset. Interestingly, 70.9% of the Pru p 3^+ patients were female. Mite, Alternaria, olive tree pollen, cat, parietaria, grasses, and mugwort have their group 1 allergen among the first 10 allergens, prevalence ranking between 9.5% and 1.32%. Other 48 IgE-positive allergens had prevalence below the latter value, most of them having just few subjects, whereas 15 allergens were never found positive without other allergen positivities (Table 2).

Gender distribution of allergen immunoglobulin E recognition

Table 3 reports IgE reactivity to molecules showing a statistically significant difference in gender distribution. Male individuals showed a significant higher prevalence of IgE reactivity to grass (Lol p 1, Phl p 1, Phl p 2, Phl p 5, and Phl p 6), mite (Der f 1, Der f 2, Der p 1, and Der p 2), olive tree (Ole e 1), plane tree (Pla a 2), and Alternaria (Alt a 1) allergens though the male gender was less represented in the studied population. Only IgE reactivity to Hev b 5 and Hev b 6 from latex was recorded higher in the female subset of patients. All other allergens had no or negligible differences when comparing male with female prevalence.

Cluster analysis

Unsupervised two-way hierarchical clustering analysis yielded several patterns of IgE reactivity in our population

Table 2. Prevalence of IgE mono-sensitization to allergenic molecules tested on ISAC microarray

Rank	Allergen	Allergome code	No.	%*	%†	Rank	Allergen	Allergome code	No.	%*	%†	Rank	Allergen	Allergome Code	No.	%*	%†
1	Cup a 1	256	842	0.0513	32.82	26	Bet v 1	89	8	0.0005	0.31	51	Dau c 1	287	1	0.0001	0.03
2	Der f 2	302	245	0.0149	9.55	27	Gal d 2	360	8	0.0005	0.31	52	Gal d 3	361	1	0.0001	0.03
3	Pru p 3	603	182	0.0111	7.09	28	Asp f 1	62	7	0.0004	0.27	53	Hev b 9	404	1	0.0001	0.03
4	Alt a 1	722	142	0.0087	5.53	29	Pla a 1	572	7	0.0004	0.27	54	Hor v 17	420	1	0.0001	0.03
5	Ole e 1	482	105	0.0064	4.09	30	Hev b 5	389	6	0.0004	0.23	55	Mal d 1	464	1	0.0001	0.03
6	Fel d 1	345	95	0.0058	3.7	31	Ani s 3	37	5	0.0003	0.19	56	Mer a 1	476	1	0.0001	0.03
7	Par j 2	507	83	0.0051	3.23	32	Phl p 2	555	5	0.0003	0.19	57	Ole e 2	490	1	0.0001	0.03
8	Phl p 1	549	40	0.0024	1.55	33	Bla g 2	141	4	0.0002	0.15	58	Phl p 12	553	1	0.0001	0.03
9	Art v 1	753	34	0.0021	1.32	34	Gal d 5	363	4	0.0002	0.15	59	Pho d 2	571	1	0.0001	0.03
10	Par j 1	503	34	0.0021	1.32	35	Hev b 1	379	4	0.0002	0.15	60	Api g 1	40	0		
11	Phl p 5	558	30	0.0018	1.16	36	Bet v 2	127	3	0.0002	0.11	61	Bla g 5	144	0		
12	Lol p 1	450	29	0.0018	1.13	37	Pen i 1	527	3	0.0002	0.11	62	Bos d 7	166	0		
13	Can f 3	176	27	0.0016	1.05	38	Phl p 6	569	3	0.0002	0.11	63	Bos d 8	167	0		
14	Bos d 6	165	26	0.0016	1.01	39	Phl p 7	570	3	0.0002	0.11	64	Bos d 8 κ	2737	0		
15	Der f 1	295	23	0.0014	0.89	40	Bos d 5.0102	2738	2	0.0001	0.07	65	Cor a 1	232	0		
16	Ana c 2	694	22	0.0013	0.85	41	Bos d Lactoferrin	1065	2	0.0001	0.07	66	Gal d 4	362	0		
17	Der p 1	310	20	0.0012	0.77	42	Cyn d 12	279	2	0.0001	0.07	67	Hel a 2	377	0		
18	Hev b 6	390	17	0.001	0.66	43	Hev b 7	394	2	0.0001	0.07	68	Hel as 1	378	0		
19	Bos d 4	163	13	0.0008	0.5	44	Pen m 1	972	2	0.0001	0.07	69	Hev b 10	380	0		
20	Can f 1	174	13	0.0008	0.5	45	Tri a 18	650	2	0.0001	0.07	70	Hev b 11	384	0		
21	Alt a 6	14	11	0.0007	0.42	46	Tri a Gliadin	651	2	0.0001	0.07	71	Hev b 3	387	0		
22	Der p 2	316	11	0.0007	0.42	47	Aln g 1	7	1	0.0001	0.03	72	Hev b 8	397	0		
23	Gal d 1	359	10	0.0006	0.38	48	Bos d 5.0101	2739	1	0.0001	0.03	73	Hor v 16	419	0		
24	Der p 10	311	9	0.0005	0.35	49	Bos d 8 αS1	2734	1	0.0001	0.03	74	Par j 3	510	0		
25	Pla a 2	573	9	0.0005	0.35	50	Bos d 8 β	2736	1	0.0001	0.03	75	Per a 7	542	0		

Allergens are ranked by prevalence. Prevalence has been calculated on either the total tested subjects (*n = 16 804) or the total mono-sensitized subjects (†n = 2565).

as a consequence of IgE recognition of either homologous structures belonging to different biological sources or non-homologous molecules belonging to the same biological source (Figs 2 and 3).

The simultaneous recognition of structures having most of the times also different functional activity, but belonging to the same biological source and defined as 'genuine' allergens, generated distinct cluster of IgE reactivity, as in the case of timothy grass allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6), parietaria allergens (Par j 1 and Par j 2), or mite allergens (Der p 1, Der p 2, Der f 1, and Der f 2) (Figs 2 and 3a). Pan allergens belonging to these biological sources (i.e. the polcalcin Phl p 7, the tropomyosin Der p 10 or the profilins Phl p 12 or Cyn d 12 from grasses, Par j 3 from parietaria, and Ole e 2 from olive tree) generated a distinct cluster of reactivity with other representative pan allergens from the group they belong to, instead of clustering with allergens belonging to their own biological source (Figs 2 and 3b). Pan allergen IgE recognition is therefore independent from 'genuine' markers of biological source sensitization. Unless this also applies to Bet v 2 vs. Bet v 1, a different clustering has been recorded for Bet v 1-like molecules belonging to distant taxonomical sources. Pollen Bet v 1-like (i.e. Bet v 1, Aln g 1, and Cor a 1) form the first two clusters, whereas Mal d 1 forms a third cluster, and Dau c 1 from carrot and Api g 1 from celery form a fourth and quite a rare cluster (Figs 2 and 3b).

Looking at the food allergen IgE reactivity, it was noticed that cow's milk-related allergens generate two distinct reactivity clusters: the first including Bos d 4, Bos d 5.0201, and Bos d 5.0101, and the Bos d 8 casein fraction and its isolated proteins, the second including Bos d 7, Bos d 6, and Bos d lactoferrin (Fig. 2). Hen's egg allergens Gal d 1, Gal d 2, and Gal d 3 showed a similar pattern of IgE recognition, thus resulting in a common cluster of reactivity (Fig. 2).

Age distribution profiles of different allergen sensitizations

Patients' age distribution profiles have been evaluated, taking advantage from the distinct IgE reactivity patterns identified by the hierarchical clustering analysis.

Cow's milk and hen's egg allergens. Analysing the age distribution profiles of IgE recognition to cow's milk and hen's egg allergens, we recorded the highest prevalence in patients below 5 years of age, as showed in Figs 4a and b. Patients having IgE to at least one cow's milk allergen were 975 (50.1% female) and to at least one hen's egg

Table 3. Gender distribution of allergen IgE recognition

Rank	Allergen	Female		Male		Chi-square test†
		No.	%*	No.	%*	
1	Phl p 1	3152	34.79	3074	41.83	85.5
2	Lol p 1	3062	33.8	2986	40.64	81.56
3	Pla a 2	519	5.73	685	9.32	77.06
4	Alt a 1	709	7.83	860	11.7	70.56
5	Phl p 2	1665	18.38	1735	23.61	67.67
6	Phl p 5	1776	19.6	1830	24.9	66.51
7	Phl p 6	1054	11.63	1170	15.92	63.7
8	Der p 1	2022	22.32	2020	27.49	58.47
9	Ole e 2	430	4.75	547	7.44	52.74
10	Bet v 2	444	4.9	542	7.38	44.02
11	Der f 1	1989	21.95	1939	26.39	43.82
12	Der p 2	2799	30.89	2621	35.67	41.83
13	Hev b 8	470	5.19	549	7.47	36.33
14	Cyn d 12	388	4.28	469	6.38	36.17
15	Mer a 1	555	6.13	626	8.52	34.79
16	Der f 2	3331	36.77	3024	41.15	32.92
17	Pho d 2	404	4.46	472	6.42	30.97
18	Par j 3	310	3.42	378	5.14	29.97
19	Ole e 1	1635	18.05	1573	21.41	29.13
20	Per a 7	108	1.19	164	2.23	26.91
21	Ani s 3	128	1.41	185	2.52	26.47
22	Phl p 12	246	2.72	305	4.15	25.78
23	Pen i 1	137	1.51	189	2.57	23.42
24	Ana c 2	176	1.94	226	3.08	21.8
25	Gal d 1	97	1.07	142	1.93	20.99
26	Hel a 2	426	4.7	463	6.3	20.24
27	Hel as 1	133	1.47	176	2.4	18.87
28	Pen m 1	135	1.49	178	2.42	18.85
29	Gal d 2	165	1.82	206	2.8	17.71
30	Der p 10	195	2.15	235	3.2	17.38
31	Hev b 6	146	1.61	67	0.91	15.5
32	Hor v 17	104	1.15	138	1.88	14.88
33	Bos d 7	1568	17.31	1437	19.56	13.72
34	Hev b 5	81	0.89	32	0.44	12.47

*Prevalence has been calculated on the female and male subset for each tested allergen.

†Results are based and ranked on the Chi-square two-sided tests, with significance level of $P<0.001$. Grey background cells indicate a greater female vs. male prevalence.

allergen 935 (50.5% female). The mean ages were 19.1±19.38 and 23.2±21.4 years, respectively. They were significantly lower when compared with the previously reported groups of patients sensitized to inhalant allergens ($P<0.001$). For comparative purposes, we choose IgE recognition of Pru p 3 that showed a completely different age distribution profile. The mean age of this group of patients (1607 subjects, 52.8% female) was higher than milk- and egg-sensitized subjects (27.02±14.4 years, $P<0.0001$), and the highest IgE recognition prevalence is reached in 15–25 years patients' group. Anyhow, in the group of patients aged 6–15 years, we recorded a prevalence of IgE-sensitized subjects to Pru p 3, close to those of cow's milk- and hen's egg-derived allergens.

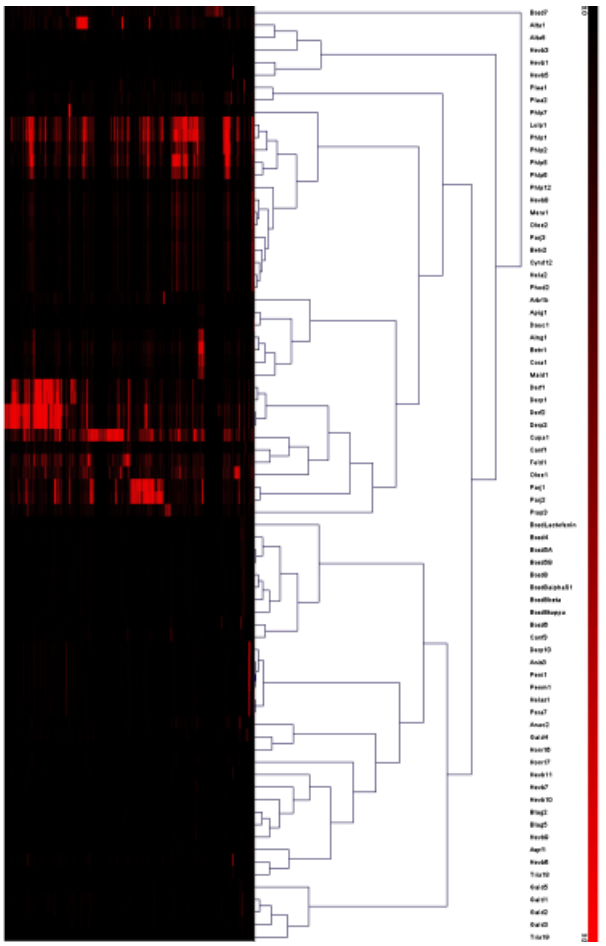

Fig. 2. Unsupervised two-way Eisen Kendall's hierarchical clustering analysis of IgE values for 75 allergenic molecules (*y*-axis) tested on a total of 16 408 ISAC IgE-reactive individuals (*x*-axis). Further explanations are in 'Methods'.

Mite allergens. The cluster including the largest group of patients was the one generated by IgE recognition of mite allergens. Seven thousand two hundred and seventy-two individuals (44.32% of the total cohort; 52.2% female) reacted to at least one of the allergens released by mites, Der f 2, representing the molecule most frequently and almost always recognized, as reported above. The mean age in this group was 27.9±16.9 years. 38.73% of patients between 6 and 35 years of age reacted against Der f 2 molecules (Fig. 4c). The prevalence of IgE recognition of Der f 2 significantly decreased in patients older than 45 years ($P<0.001$). A similar trend, even though with a lower prevalence of IgE recognition, was observed for all the other mite genuine allergens. As a result, all the molecules belonging to a given biological source had a common behaviour of IgE recognition throughout the age groups. By contrast, the tropomyosin Der p 10 showed a distinct pattern of IgE recognition throughout the age groups examined.

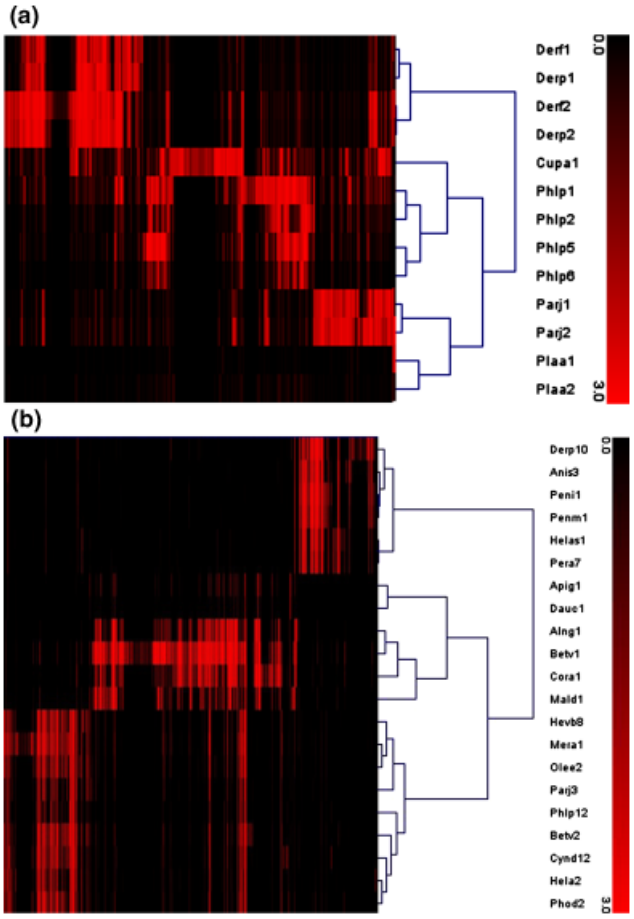

Fig. 3. Supervised hierarchical clustering analysis applied to genuine non-homologous molecules belonging to the same biological source (a), or to homologous allergens belonging to distinct biological sources (b). IgE-positive subjects to at least one of the allergens under evaluation were analysed for clustering. Allergens are reported on the *y*-axis, patients on the *x*-axis. Further explanations are in 'Methods'.

Grass pollen allergens. Seven thousand two hundred and forty-six patients (44.16% of the total allergic cohort; 51% female) were IgE reactive on ISAC to at least one of the timothy grass pollen allergens as representative markers of other homologous grass allergens [17]. Phl p 1 was the allergen most frequently recognized (Fig. 4d). The mean age was 30.1±16.5 years, significantly higher if compared with mite allergen IgE-reactive subjects ($P<0.001$), with the highest value of IgE recognition reached between 15 and 25 years of age (>50% of subjects within this group were reactive to Phl p 1). Phl p 2, 5, and 6 showed a similar behaviour of IgE recognition, whereas the pan allergens Phl p 7 (polcalcin) and Phl p 12 (profilin) showed a different pattern of IgE recognition, as in the case of Der p 10 for mites (Fig. 4c).

Parietaria allergens. Four thousand eight hundred and thirty-two subjects (29.45% of the total allergic cohort; 54.4% female) have specific IgE to at least one allergen released by parietaria pollen. Interestingly, these patients were significantly older, when compared with the other groups described above (mean age 33.7±16.6 years, $P<0.001$). Par j 1 and Par j 2 ranked the top level of IgE recognition in patients between 36 and 65 years of age. Also in this case, the profilin Par j 3 showed a distinct age distribution profile when compared with the other parietaria-tested molecules (Fig. 4e).

Figure 4f shows age distribution profiles of the allergen groups as a whole, summarizing the IgE recognition trend in a single line. A different pattern of IgE reactivity for every single age group was thus recorded.

Discussion

This is the very first paper reporting extensive and comprehensive cross-sectional analysis of IgE reactivity to a broad panel of single allergenic molecules evaluated using the ISAC system, a microarray-, allergenic molecule-based *in vitro* assay for specific IgE [10].

We first report about the use of the ISAC microarray approach to have an IgE-negative diagnosis. We found 28.9% of the observed population to be IgE negative to all the 75 allergenic molecules spotted on the chip. These data, and all the followings, need to be carefully evaluated in the light of the limitation of the number and type of allergens available for testing at the time of the study. It is worth to say that some relevant allergens were missing on the ISAC microarray, i.e. peanut, fish, and soy molecules. To be underlined that this was a transient limitation as the current version of the ISAC microarray bears 103 allergens.

We describe the IgE reactivity pattern of allergen recognition in a large Italian allergic cohort. This approach allowed the evaluation of all the available molecules at the same time in all the subjects, and the study of IgE recognition to distinct groups of allergens, either structurally related or not, but belonging to the same biological source.

The evaluation of the allergen ranking in order to define the effective impact of a given allergen (or group of allergens) in a population was therefore achievable. Several meaningful examples of ranking sensitizations by using molecules have been recorded. Unless it was recorded in the lowest range of prevalence, sensitization to latex allergens do represent a good example of how our view might change by the use of a panel of latex allergens in diagnosis and epidemiology. We previously reported latex sensitization, detected using a latex extract, affecting 1.2% of a general allergic population [18]. Similar prevalence has been reported by other authors in comparable cohorts [19, 20]. These figures are very close to what we obtained in the present study with the best-ranked latex genuine allergen Hev b 6 (1.3%), whereas the pan allergen Hev b 8, a profilin, would lead such prevalence up to 6.2%. By chance such higher prevalence of latex sensitization has not been recorded before

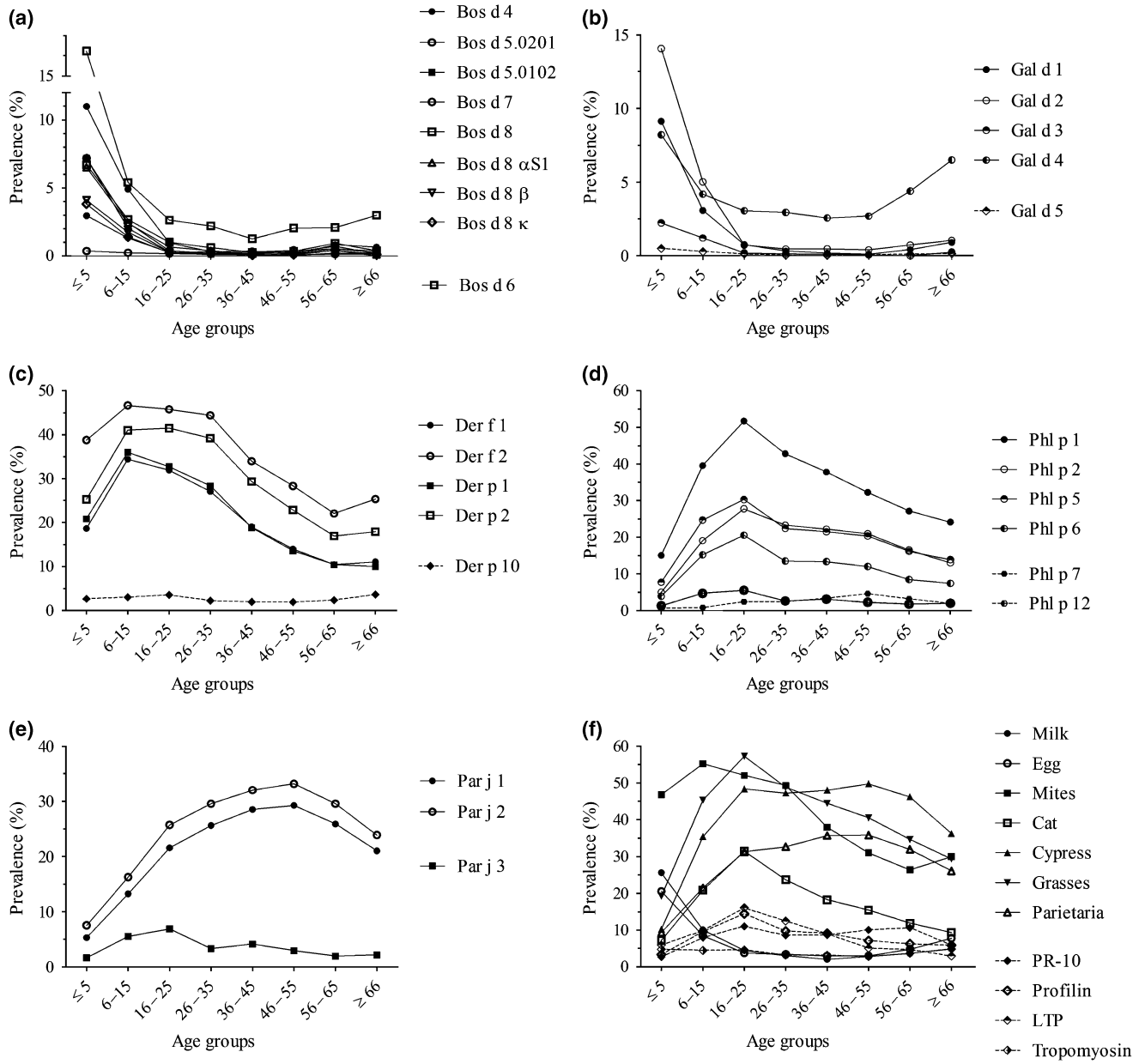

Fig. 4. Age distribution profiles of the allergen groups as a whole, summarizing the IgE recognition trend in a single line. A different pattern of IgE reactivity for every single age group was thus recorded. (a) Milk allergens; (b) hen's egg allergen; (c) mite allergens; (d) grass pollen allergens; (e) parietaria pollen allergen; (f) all major allergenic sources and homologous protein groups. PR-10, pathogenesis-related proteins, group 10; LTP, lipid transfer protein.

because latex extract preparations can lack or contain very small amount of Hev b 8 [21, 22], whereas the Hev b 6 content of the same diagnostic preparations granted for a good correlation between latex allergen sensitization detected by means of the extract and the clinical reactivity [22, 23]. A second example is cypress/cedar/juniper pollen allergy that has been described as an increasing and often underestimated winter pollinosis worldwide [24]. In our study, the genuine cypress pollen allergen Cup a 1 was recorded as the most frequently recognized molecule. Homologous group 1 cypress allergens like Cry j 1 and Jun a 1 [25] could act as sensitization markers in area where other *Cupressaceae* species are present [26]. Their availability on the microarray would increase the value of our findings obtained in our area, establishing the clinical and immunological relevance of geographically distant allergenic sources for patients sensitized to closely related allergens.

A further example is group 1 mite allergens. Der p 1 has been the first mite allergen described ever [27] and is considered to be the most important allergen among the 18 described so far [9]. It has been reported to have a high

intrinsic sensitization risk because of its proteolytic activity [28]. Our study ranks prevalence of Der p 1 and Der f 1 after the group 2 mite allergens, being the latter frequently recorded as mono-sensitizers. Recently, Der p 2 has been described to be a target by the adaptive immune responses because of its auto-adjuvant properties [29].

From all the reported data, we might also argue about allergen exposure and, for the most frequent inhalant sensitizations, the risk of getting symptoms when in a certain geographical area. An evidence of this phenomenon comes with the already reported finding of Bet v 1 IgE-positive patients in an area where almost no birch pollen is present [30]. Sensitization caused by exposure to pollen homologous Bet v 1-like molecules tested positive in this study (i.e. Aln g 1 and Cor a 1) confirm the previous findings, whereas the associated clustering sensitization with plant-derived food allergens of this group (i.e. Mal d 1, Dau c 1, and Api g 1) has been recorded by us in two subsets of patients.

We correctly established and confirmed the prevalence of grass, olive, birch, parietaria, cypress, and mite allergen sensitization, by getting rid of the pan allergen interference in estimating prevalence. For allergenic sources where several allergens were available (i.e. grass, mite, and latex), we ranked source-related allergens when an allergen multi-sensitization exists within single subjects.

The divergent gender sensitization to defined allergens is a further point to focus on. Mono-sensitized subjects were more prevalent in the female gender, whereas several groups of molecules were most frequently recognized by the male counterpart (i.e. grasses, mite, olive tree, cypress, and *Alternaria*) (Table 3). No substantial differences could be observed for other sensitizer such as parietaria or cat allergens. Furthermore, we observed that younger allergic patients in our cohort were prevalently male, whereas the gender distribution was the opposite after 26 years of age [31] (Fig. 1). As a result a female prevalence was observed, considered the whole population studied. A higher prevalence of mite sensitization within the male subset was already observed by other authors using allergenic extracts [32], but our results support for the first time the previous findings because the use of genuine markers of sensitization. Contrasting data or no sex differences have been reported for the prevalence of specific IgE to other common aero-allergens [33]. It is most likely that these previous studies evaluated the IgE sensitization by means of allergenic extracts, containing both genuine markers and pan allergens or even contaminants.

We also studied the trend of IgE recognition of all the different allergens, taking advantage from computational tools, up to now used for evaluating genomics microarray data [16]. Hierarchical clustering analysis, one of the most widely used techniques for the analysis of gene-expression data, has been applied to obtain a visualization of the huge amount of data we produced [34]. Two major branches of the hierarchical tree closely associated with the effective prevalence of IgE recognition of the molecules spotted on the array were observed (Fig. 3). The first branch includes the molecules most frequently recognized, whereas the allergens whose IgE reactivity was most rarely observed are clustered in the second branch. Among the sub-clusters observed in the first branch, those generated by mite allergens and timothy grass molecules represented the most expressed ones. That is mainly due to the IgE recognition of structurally unrelated components belonging to the same biological source (Fig. 3a). Interestingly, the pan allergens, generally tested using the mite and grass extracts (Der p 10, Phl p 12, and Phl p 7) did not generate any cluster with the molecules belonging to their parental biological source. Pan allergens showed clustering trends with their homologous molecules thus suggesting different pathways of immunological primary sensitization (Fig. 3b). As a result, three sub-clusters of homologous allergens belonging to taxonomically distant biological sources, namely tropomyosins, profilins, and Bet v 1-like molecules, have been observed. Detailed data will be reported elsewhere (Scala et al., manuscript in preparation).

From the analysis of the age distribution IgE recognition profile, every single group of genuine molecules belonging to the same biological source (i.e. mite or grasses) showed the same behaviour throughout different groups of age. As reported in Fig. 4, mite-related molecules reached the maximum level of IgE recognition between 6 and 25 years of age (Fig. 4c), whereas timothy grass pollen and cypress pollen Cup a 1 did the same between 16 and 25 years of age (Fig. 4f). Parietaria molecules showed a different profile of IgE recognition: these molecules, in fact, ranked the top level of recognition in patients older than 35 years, maintaining a steady level of IgE recognition up to 65 years of age (Fig. 4e). On the other hand, cow's milk and hen's egg allergens were mainly recognized by paediatric patients, whereas after 6 years of age, most of those allergens were recognized by $<5\%$ of the total allergic population (Figs 4a and b), unless Bos d 6 (serum albumin) and Gal d 4 (lysozyme) tend to be recognized by a small but higher proportion of our patients, possibly because of incurring different exposures [35, 36]. Pru p 3 IgE recognition has been recorded with a higher prevalence in the mono-reactive-sensitized group (Table 2). We also observed a different age distribution profile when compared with milk- and egg-related molecules, with the highest IgE recognition level recorded in patients between 16 and 25 years of age. In this age group, $>17\%$ of allergic patients were reactive to this potentially harmful molecule, often causing severe allergic reactions [37] (Mari et al., manuscript in preparation). As summarized in Fig. 3f, each age group shows its own characteristic profile of IgE molecule recognition.

Assuming that all the allergens are present in the environment, several factors must play in the induction of IgE sensitization leading to the recorded different profiles. Intriguing implication on the immune system function should be hypothesized after the observation of an age-related distinct IgE reactivity to molecules present at the same time, in the same environment. These findings are of course challenging for current hypothesis explaining the increase of allergic disease prevalence occurred in the last decades [38, 39].

Overall reported data should be further evaluated for the possible interference of clinically irrelevant IgE results induced by cross-reactive carbohydrate determinants (CCD). As reported in literature [40, 41], IgE reactivity to CCD can strongly influence the results of *in vitro* IgE detection. This phenomenon has been carefully evaluated in the present study, unless the low prevalence of IgE to Ana c 2 (bromelain) as CCD marker, the presence of just three known glycosylated allergens on the microarray (i.e. nCup a 1, nLol p 1, and nOle e 1) whose prevalence would change little even considering the possible CCD–IgE reactivity, and the higher prevalence value for the non-glycosylated rPhl p 1, the second group 1 grass pollen genuine marker with nLol p 1, lead us to rule out a strong impact of CCD–IgE reactivity on the overall IgE survey reported in the present study.

In conclusion, allergenic molecule-based IgE diagnostic systems based on a multiplex technology, like the ISAC microarray, open unpredictable diagnostic and epidemiological perspectives in allergology, allowing the analysis of a large number of allergens at the same time in a single test with minimal amount of serum and reagents. In this study, we investigated the pattern of IgE reactivity in a large Italian cohort using a panel of natural and recombinant proteins, describing their behaviour using descriptive statistics and new clustering analysis. IgE reactivity profiles according to patients' age, gender, and the type of allergen tested have been reported for the first time and suggest different sensitization pathways for each allergen and groups. This approach could be extremely useful in the study of IgE sensitization and cross-reactivity patterns of larger and representative samples of the population worldwide. The clinical relevance of single molecule IgE reactivity and clustering behaviours requires additional studies.

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