



The ImmunoCAP ISAC molecular allergology approach in adult multi-sensitized Italian patients with respiratory symptoms

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ABSTRACT

Objectives: To evaluate the performances of an allergen microarray in multi-sensitized allergic patients with respiratory symptoms.

Design and methods: 321 patients and 92 controls were included in this study. Specific serum IgE were assayed using ImmunoCAP ISAC, a microarray containing 103 components derived from 47 allergens and results were compared with extract-based ImmunoCAP Allergens slgE to 15 common airborne allergens.

Results: The reproducibility of ISAC was good. The Positive Percent Agreement (PPA) varied between 75% and 100% for slgE levels above 1 kUA/l. For samples with slgE levels below 0.1 kUA/l, the Negative Percent Agreement (NPA) ranged between 90% and 100%. Notably, 58% of respiratory allergy patients had IgE to food-specific proteins and 52% resulted sensitized to cross-reactive pan-allergens.

Conclusion: ImmunoCAP ISAC detects allergen sensitization at component level and adds important information by defining both cross and co-sensitization to a large variety of allergen molecules.

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Introduction

It is a common notion that in allergy diagnostic praxis only a limited number of allergens are routinely assayed using both Skin

Prick Test (SPT) [1] and specific IgE (slgE) detection [2]. This is in general related to the targeting of perceived relevant allergens on the basis of patient's history as well as to a suitable compromise between the numbers of tests. A large number of SPT may be disagreeable for the patient and a large number of slgE tests can be both expensive and blood sample volume demanding, the last being in particular problematic in small children. Another important factor is that many patients with allergic symptoms are multi-sensitized, and it is often difficult to assess whether this multi-sensitization is due to true species-specific co-sensitization or to cross-reactivity [3]. It is well known that many different allergens share common protein epitopes and the same slgE antibody can bind to proteins with similar structures present in different allergen sources, thus recognizing homologous allergens from different sources. This question cannot be examined and resolved with SPT or allergen-slge tests. The Molecular Allergology (MA) approach, also known as Component Resolved Diagnostic (CRD) [4], has been developed when highly purified or recombinant allergen molecules have become available. These molecules are the allergenic proteins toward which the specific and clinically relevant IgE immune-response is directed. This development has allowed identification of protein families and cross-reactivity patterns of importance in

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allergy [5]. The ability to differentiate between “less dangerous cross-reacting allergens” (Ca++ binding proteins, profilins) which seem to be less associated with severe clinical reactions and “more dangerous cross reacting allergens” (storage proteins; Ara h 2, Ber e1, lipid transfer proteins; Pru p 3, Cor a 8) which in many patients are associated with severe clinical allergic reactions has greatly improved the possibility for the clinician to give better advice to the patient. Some cross-reacting allergens (e.g. tropomyosins) are expressed both in aeroallergens, such as the Der p 10 of house dust mite, but are also expressed in certain foods, such as Pen a 1, Pen i 1 and Pen m 1 in shrimps and lobsters, thus explaining food allergies in some patients with a respiratory allergy. Thus, MA allows the detection of sIgE directed to “Primary/ species-specific allergens” (for example Phl p1, Der p1 and Der p 2), to “Cross reacting allergens” (such as Bet v 1, the prototype of the family of the homologous of PR-10 proteins, Phl p 12, a profilin or Ana c 2, a prototypic allergens belonging to the Cross-reactive Carbohydrate Determinant (CCD) family) and, finally, to “Minor allergens” that, although being species specific, are recognized only by a minority of allergic patients, such as Bla g 1 or Phl p 11. A new MA/CRD tool, ImmunoCAP ISAC, has recently become available [6–8] and basic characteristics of this technology have been already described [9–12]. The micro-array contains 103 different allergen components/molecules originating from 47 allergen sources. Some characteristics of this assay have been already published: indeed, the assay has been used to define the epidemiology of allergen sensitization [13] and, more recently, the comparison of results obtained using a routine specific IgE assay vs allergen microarray features has been reported on a small number of selected patients [14]. In order to evaluate the performance of this microarray, in particular the ability to detect pattern of true co-sensitization and cross-reactivity, a clinical multicenter observational trial was started and patients were included on the basis of presence of respiratory symptoms. The aim of the study was to evaluate if the addition of ImmunoCAP ISAC in the diagnostic program of allergic patients will offer important information not directly available using standard SPT and sIgE, and that this tool may have a role to improve the clinical management of polysensitized allergic patients. For this routine SPT, ImmunoCAP sIgE laboratory tests and ImmunoCAP ISAC for 103 allergen components were performed. In this report we describe the main characteristics of ImmunoCAP ISAC, its relationship with standard ImmunoCAP sIgE and its capacity of adding relevant information not accessible through traditional allergological tools.

Material and methods

Participants

A total of 321 patients (age 12 to 80 years, evenly distributed over both genders) with respiratory symptoms and multi-sensitized as assessed by Skin Prick Test (SPT) for 15 common airborne allergens, and 92 controls with no allergy-like symptoms, were recruited prospectively (in May 2009 to February 2010) in a cross-sectional observational Italian study. The study was performed at 5 sites distributed all over Italy: Ancona, Bari, Cuneo, Firenze and Genova. The geographic positions of the five centers assured a suitable representation of allergy in Italy. The protocol was approved by the Ethical Committees at each of the study sites. The patient information and informed consent form was signed by all subjects. Inclusion and exclusion criteria provided in the clinical protocol were met by all subjects. In particular, patients did not have infectious allergy-like respiratory symptoms, had not undergone immunotherapy within the last 3 years and were sensitized to at least 2 unrelated allergens. The controls were collected at the study sites and had no allergy-like respiratory symptoms. Both patients and controls were at least 12 years old and had no known HIV or Hepatitis B/C infection.

Serum samples, allergens tested and analysis of analytical performances

A blood sample was drawn from each subject at study inclusion visit. Serum samples were stored frozen until transported to a centralized trial laboratory in Genoa (Quality System certified according to the ISO 9000 rules since 2003, being accredited for excellence by the Joint Commission International in 2006). All sera were processed in this facility testing total IgE concentration, specific IgE (see below) and ImmunoCAP ISAC (Phadia, Milano, Italy), during a 9 month period. ImmunoCAP ISAC microarray contains 103 allergen components derived from 47 allergen sources. The assay had manual sample and reagent handling, followed by an automated fluorescence-detection scanner step. The testing procedure was carried out according to the manufacturer's instruction for use. Briefly, each microarray reaction site was incubated with 20 µL of undiluted patient serum for 120 min. After a washing step a fluorescence-tagged anti-human-IgE antibody was incubated for 60 min at room temperature and after subsequent washing the fluorescence signals were scanned using a conventional biochip reader. The final data processing was performed using the Phadia Microarray Image Analysis (MIA) Software. The IgE test results were reported in arbitrary semi-quantitative ISAC Standardized Units (ISU): 0.3 to <1 (low), 1 to <15 (medium) and 15 or above (high) ISU. Calibration procedures were performed according to the producer's recommendation. Briefly, a serum sample with a defined range of concentration of IgE to Bet v1, Can f1, Phl p1, Der p2, Phl p6, Art v1, Gad c1, Phl p12, Amb a1 and Gal d1 was used in every analytical session. If each result was within the expected range, the calibration was approved and the relevant results were accepted. If one or more results were out of the expected range, a calibration procedure performed via software on the Microarray reader was started to optimize the reading parameters, according to the producer's recommendation. Only in the presence of a suitable calibration curve, the clinical test results were accepted.

As stated before, sera collected during this study were also contemporarily analyzed for Total IgE and for sIgE using 15 complete extract-based ImmunoCAP Allergens (Table 1) in the Phadia250 system. Testing was performed according to manufacturer's instruction for use; quantitation range was from 0.1 to 100 kU_A/L for ImmunoCAP sIgE, and from 2 to 5000 kU/L for Total IgE.

Table 1

ImmunoCAP Allergens and ImmunoCAP ISAC components used in comparisons (when comparing ImmunoCAP ISAC with ImmunoCAP Allergens, both species specific and cross-reactive components were included).

ImmunoCAP allergens	ImmunoCAP ISAC species-specific components	ImmunoCAP ISAC allergen-related cross reacting components
<i>Alternaria alternate</i> (m6)	Alt a1, Alt a6	
<i>Ambrosia</i> (w1)	Amb a1	Mer a1, Phl p7, Ana c2
<i>Artemisia</i> (w6)	Art v1, Art v3	Mer a1, Phl p7, Ana c2
<i>Aspergillus fumigatus</i> (m3)	Asp f1, Asp f2, Asp f3, Asp f4, Asp f6	
<i>Betula alba</i> (t3)	Bet v1, Bet v2, Bet 4v	Ana c2
<i>Canis familiaris</i> (e5)	Can f1, Can f2, Can f3	
<i>Cynodon dactylon</i> (g2)	Cyn d1	Phl p12, Phl p7, Ana c2
<i>Cupressus arizonica</i> (t23)	Cup a1	Bet v2, Bet v4, Ana c2
<i>Dermatophagoides pteronyssinus</i> (d1)	Der p1, Der p2, Der p10	
<i>Dermatophagoides farinae</i> (d2)	Der f1, Der f2, Der p10	
<i>Felis domesticus</i> (e1)	Fel d1, Fel d4, Fel d2	
<i>Hevea brasiliensis</i> (k82)	Hev b1, Hev b3, Hev b5, Hev b6, Hev b8	Ana c2
<i>Olea europaea</i> (t9)	Ole e1, Ole e2	Phl p7, Ana c2
<i>Parietaria judaica</i> (w21)	Par j2	Mer a1, Phl p7, Ana c2
<i>Phleum pratense</i> (g6)	Phl p1, Phl p2, Phl p4, Phl p5, Phl p6, Phl p11, Phl p12, Phl p7	Ana c2

For ImmunoCAP ISAC, the within-assay and between-assay reproducibility were estimated by the Coefficient of Variation (CV). In detail, the within-assay reproducibility was evaluated in 5 repeated assays, for 4 different patients, in a single analytical session (corresponding to 2060 tests), and the between-assays reproducibility was evaluated over 3 different days using the sera of 4 different patients (corresponding to 1236 tests). A comparison between the present ISAC 103 component micro-array and previous ISAC 89 component micro-array was also performed, using 6 different sera originally assayed with the ISAC 89 micro-array. The interference of high anti-allergen IgG titers in the present ISAC IgE assay was evaluated by studying 5 samples with high IgG titers against components. Using Protein G (Sigma Aldrich, Milano, Italy), IgG were removed and the assay was repeated. Finally, for all the three assays (ISAC, total IgE and sIgE), in the absence of a formal External Evaluation of Quality managed by a third party, an internal quality control represented by a pool of human allergic sera fractionated in different tubes and stored frozen was used in every analytical session. According to the standard procedures of the centralized facility routine allergy laboratory, if an internal quality control is higher or lower than 15% of the average calculated result (based on previous analytical session), the test is repeated.

Comparison between ImmunoCAP ISAC and ImmunoCAP sIgE results

To evaluate the ability of the component based ImmunoCAP ISAC IgE to identify samples containing measurable levels of allergen sIgE as determined with the complete allergen extract based ImmunoCAP sIgE, the ISU values for corresponding components on ISAC were summed (Table 1). As shown the available species specific components were associated with the evaluated extract allergen. In addition for plants cross-reactive components present in the extract for a given allergen but absent on ISAC were substituted by highly homologue components available. Thus Ambrosia profilin was substituted by Mer a 1, the Calcium Binding Protein was substituted by Phl p 7 and the CCD was substituted by Ana c 2. A similar approach was used for Artemisia, Betula, Cynodon, Cupressus, Hevea, Olea, Parietaria and Phleum. Values below the detection limit for ImmunoCAP ISAC (<0.3 ISU) were set to zero. The ability of the microarray assay to detect a sensitized patient using a limited number of relevant components on ImmunoCAP ISAC (Table 1) was calculated for different cut off levels of the ImmunoCAP sIgE laboratory test: a) the Limit of Detection (LoD) $0.1 \text{ kU}_A/\text{l}$ and b) an arbitrary chosen higher cut off of $1 \text{ kU}_A/\text{l}$. For ImmunoCAP ISAC the (LoD), 0.3 ISU , was used in both cases and the percentage of subjects who were positive on ImmunoCAP ISAC out of all subjects who were positive on ImmunoCAP sIgE (Positive Percent Agreements (PPA)) was calculated. For evaluation of the specificity of the microarray the percentage of subjects who were negative on ImmunoCAP ISAC out of all subjects who were negative on ImmunoCAP sIgE (Negative Percent Agreement (NPA)) was calculated using the LoD (0.3 ISU) for ImmunoCAP ISAC and the LoD ($0.1 \text{ kU}_A/\text{l}$) for ImmunoCAP sIgE laboratory test. Confidence intervals of the agreements were estimated with the Clopper–Pearson method. The summed ISU values were also used to graphically display per allergen the quantitative correlation between the summed semi-quantitative ISU for ImmunoCAP ISAC and the quantitative kU_A/l for the complete extract based ImmunoCAP sIgE. In order to display zero values in the logarithmic scale graphs the sum was set to 0.15 ISU for ImmunoCAP ISAC if all components were below the detection limit and to $0.05 \text{ kU}_A/\text{l}$ for ImmunoCAP sIgE if values were below $0.1 \text{ kU}_A/\text{l}$. For comparison between sIgE measurements in ISAC with total IgE kU/l values, the ISU values for all ISAC components per patient were summed in a similar way. In order not to include the same cross-reactive IgE antibody several times, each protein family group was represented in the sum only by one or a few representative components. The following components were excluded: PR-10 s Act d 8,

Aln g 1, Ara h 8, Cor a 1.0101, Mal d1; LTP Cor a8; Profilin Hev b8, Mer a1, Ole e2, Phl p12; Tropomyosin Ani s3, Bla g7, Pen a1, Pen i1, Pen m1; Serum albumin Can f3, Equ c3; Parvalbumin Gad c1; Grass group 1 Cyn d1; Pectate lyase Cry j1; Polcalcin Phl p7; Cysteine protease Der f1; and NPC2 family Der f2, Eur m2. Relations between sIgE and total IgE were expressed both as a direct correlation between sum ISU sIgE and total IgE kU/l , and as the ratio of sum ISU sIgE/total IgE kU/l versus total IgE kU/l . Calculation of percent of patients having IgE antibodies to the different protein families and allergen groups were also made.

Results

During the period of the study, 15 calibrations were performed and only three of these required a further adjustment of the micro-array reader. After these adjustments, the assay results were all validated. For low ISU values (0.3 to 1) the within-assay CV, as expected, was very high ($>100\%$), for medium (1 to <15) and high (15 or higher) ISU values the CV was 17% and 8% respective. The corresponding between-assay CVs were $>100\%$, 33% and 13.2% respective. The internal quality control, performed using a pool of sIgE positive human sera, showed that the three different assays were under control during all the experimental period: indeed, the expected value for total IgE was $49 \text{ IU}/\text{mL}$ and the average result obtained during the 9 months of the study was $48 \text{ IU}/\text{mL}$ and the CV was 8% . Specific IgE were evaluated on ImmunoCAP sIgE reagents using a daily calibration curve and an external “home made” quality control. During the period of the study, the CV varied between 6.63% and 9.84% for all the allergens. Being all $<15\%$ and according to the abovementioned standard laboratory procedures, no analytical session was repeated and all results were validated. Quality controls for ISAC resulted in CV largely superimposable to those described for the intra and between assay CV, being high for low concentrations of specific IgE and always $<15\%$ for high ISAC values. Also in this case, the results obtained were accepted in all but one analytical session, when the tests were repeated. The updated ISAC micro-array showed a 2.7 (range 1.95 to 4.0) fold increase of the average signal compared to the older version. With the updated micro-array, 11 allergen components that were below limit of detection with the old version were now positive for the 6 samples. No effects of IgG masking of components could be detected.

The very large majority of the 92 control subjects with no allergy-like respiratory symptoms had no sIgE for ISAC components. The few positive of them expressed a substantially low ($<3 \text{ ISU}$) IgE concentration to ISAC molecules. The association of the two different platforms was highly linear on the log-scale (Fig. 1), for values above LoD (0.3 ISU for ImmunoCAP ISAC and $0.1 \text{ kU}_A/\text{l}$ for ImmunoCAP sIgE). The Positive Percent Agreement (PPA) of ImmunoCAP ISAC ranged from 13% to 87% , (Table 2). When the ImmunoCAP sIgE cutoff was elevated to $1 \text{ kU}_A/\text{l}$, the PPA of ImmunoCAP ISAC was substantially improved giving a range of 29% – 100% including all 15 evaluated allergens, and ranging between 75% and 100% for 12 of the allergens (Table 2). The specificity expressed as Negative Percent Agreement was 90% – 100% (Table 2). The association between the full ISU sum of sIgE antibodies to all ISAC components (calculated as described in Material and methods), and the ImmunoCAP Total IgE kU/l was also very linear on the log-scale, for full sum over 3 ISU (Fig. 2). The full ISAC sum was, in general, much lower than the Total IgE value. In addition to sensitization to the 15 common airborne allergens, co-sensitization with other important allergens (food, etc.) and sensitivity to various cross-reacting components were detected (Fig. 3). In particular, sensitization to cross-reactive pan-allergens was detected in 52% of the patients; 37% had IgE antibodies to the major birch component Bet v 1, and 93% of these were also having IgE antibodies to other PR-10 proteins, suggesting a potential food cross-reactivity. Further, 58% of the patients showed IgE antibodies to food-specific

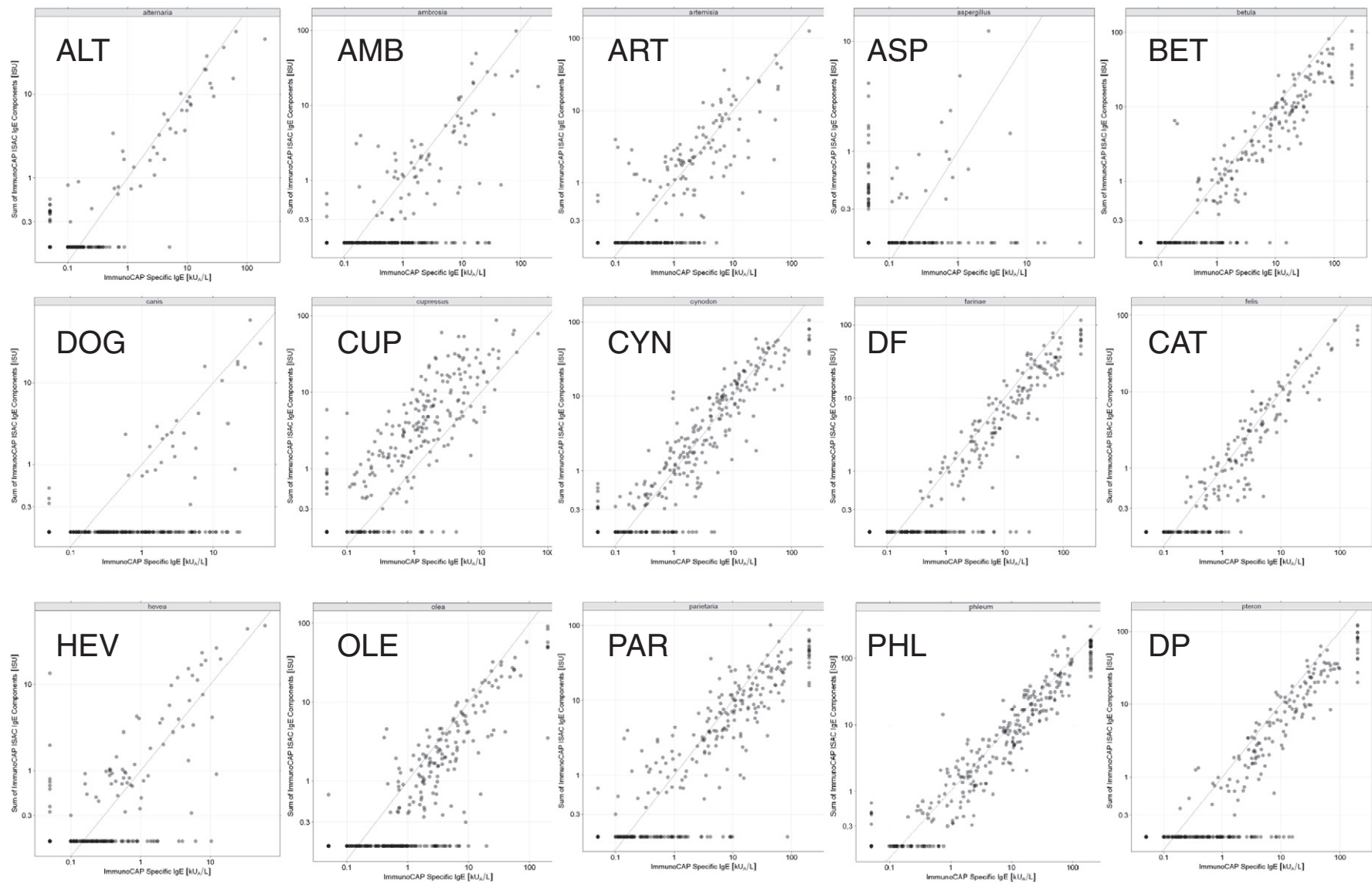


Fig. 1. The sum of the ImmunoCAP ISAC IgE components vs. the corresponding ImmunoCAP sIgE for different allergens evaluated in the 321 allergic patients. The dotted lines indicate where the two methods gave equal values. See Table 2 for number of results below detection limit per allergen.

Table 2

Negative Percent Agreement (NPA) and Positive Percent Agreement (PPA) of ImmunoCAP ISAC IgE compared to ImmunoCAP sIgE (with 95% confidence intervals), per allergen. Total number of evaluable subjects was 406.

	N	NPA ISAC 0.3 ISU sIgE 0.1 kUA/L	PPA ISAC 0.3 ISU sIgE 0.1 kUA/L	PPA ISAC 0.3 ISU sIgE 1 kUA/L
Phleum	406	96 (92–99)	87 (82–91)	100 (98–100)
Cupressus	406	93 (88–97)	80 (74–85)	95 (90–98)
Cynodon	406	94 (89–97)	76 (71–81)	95 (90–97)
Betula	406	100 (98–100)	68 (61–74)	90 (85–94)
Parietaria	406	99 (96–100)	68 (62–74)	89 (84–93)
Felis	406	100 (98–100)	67 (60–74)	97 (92–99)
<i>D. farinae</i>	406	100 (98–100)	60 (53–66)	88 (82–92)
Olea	406	99 (96–100)	55 (49–61)	81 (75–86)
<i>D. pteron</i>	406	100 (97–100)	55 (49–61)	83 (76–88)
Artemisia	406	99 (96–100)	47 (41–54)	83 (74–89)
Hevea	406	97 (93–99)	36 (29–44)	75 (60–86)
Ambrosia	406	98 (95–100)	30 (24–36)	57 (47–66)
Alternaria	406	95 (91–97)	25 (19–33)	97 (84–99)
Aspergillus	406	90 (86–93)	17 (10–25)	21 (8–43)
Canis	406	98 (96–100)	13 (9–19)	29 (21–39)

proteins; 18% to food LTPs (Pru p 3 and/or Cor a 8) and 8% to seed (storage proteins) whereof half (4%) to peanut storage proteins (Ara h 1, 2 and 3). sIgE levels to CCD (Ana c 2), Calcium-binding proteins and/or profilins were always accompanied with elevated IgE levels to grass or tree pollen components.

Discussion

A total number of 321 sera from multi-sensitized adult Italian patients with a well defined respiratory allergy and 92 control subjects with no history of allergy-like respiratory symptoms were studied using both conventional extract based allergen-sIgE and a Molecular Allergy diagnostic microarray tool in a prospective cross-sectional multicenter study. In the context of this clinical study our primary objectives were to evaluate the technical performance of the ISAC based CRD tool, the relation of ISAC results with extract based sIgE results and finally the ability to detect and resolve patterns of true co-sensitization and cross-reactivity. The first point to be discussed are the evident robustness of the assay (as supported by the

virtual absence of interference) with high concentrations of anti-component IgG, an immunoglobulin class that not only has no diagnostic significance [15] but can be easily observed in the large majority of patients with defects of the gastroenteric mucosa, such as celiac patients (unpublished observations). In addition, the clear increase of the sensitivity of the present 103 component assay, compared with that of the old 89 component, is extremely interesting. At user's level, no clear explanation of the method used by the producer to improve this feature is known. However, the clear advantage to be able to detect sensitization that, in the past, was negative, is evident. The results obtained clearly demonstrated that technical performances of ISAC and the correlation with "classic" sIgE assays were excellent. The within and between-assay precision of ImmunoCAP ISAC was very good for values above 1 ISU. As shown, the ISAC CV% were approximately 3 times higher compared to ImmunoCAP sIgE in the range 1 to <15 ISU, and similar to ImmunoCAP sIgE for ISU values above 15 [2]. In addition, the association of the two different platforms was highly linear on the log-scale, for values over detection limit, for most of the allergens. Indeed, using the LoD values of 0.3 ISU and 0.1 kU_A/L for the two assays respectively, the specificity of ImmunoCAP ISAC calculated as Negative Percent Agreement (NPA) was very high for all 15 allergens (90% to 100%), but Positive Percent Agreement (PPA) was above 75% for only 3 of the evaluated allergens; Phleum, Cupressus and Cynodon. When comparisons were made only for samples having above 1 kU_A/L as measured with ImmunoCAP sIgE, still using the 0.3 ISU LoD, the PPA for ImmunoCAP ISAC increased to 75% – 100% for all but 3 of the allergens (Ambrosia, Aspergillus and Dog (Canis)). These results are in good agreement with those observed by Gadisseur et al. [14], which demonstrated that 82 of the 384 results found to be positive with ImmunoCAP were negative with ISAC (concordance 78.65%), and of 171 negative results obtained with ImmunoCAP, 11 were positive with ISAC (concordance 93.57%). Our results also show that for some allergens such as Ambrosia, Aspergillus and Dog the extract-based ImmunoCAP Allergens contains important component(s) not contained in the present version of ImmunoCAP ISAC. For example, it is known that some allergens included in the complete extract used in the sIgE assays are not included in the current ISAC micro-array (e.g. Can f4, Can f5, Can f6, Amb a2, Amb a3, Amb a5, Amb a6, Amb a7, Amb a8, Amb a9, Amb a10 and many important IgE binding Aspergillus components). Also it has to be considered that the technology of the two platforms is widely different. The ImmunoCAP sIgE platform represents an established technology with a vast protein binding capacity, in the microgram range, being optimized for complete allergens and requiring 0.04 mL sample for each individual allergen test. The ImmunoCAP ISAC on the other hand is a new methodology still under development having a much lower allergen protein amount (even if highly homogeneous) on the solid phase, in the picogram range, which using only in total a minute volume (0.02 mL) provides discrete results for a vast number of allergen components (currently 103 components from 47 allergen sources). Of note, the allergen binding capacity of the solid phase is largely greater with the ImmunoCAP sponge technology compared to ImmunoCAP ISAC [12]. Nevertheless ImmunoCAP ISAC has the ability to measure IgE antibodies down to approximately 0.1 kU/L at least for some components and samples. When comparing the full sum of sIgE antibody values measured by the semi-quantitative ISU with total IgE values the achieved correlations both for a direct comparison and for the ratio sIgE/total IgE vs total IgE are comparable with published data [2] supporting the fact that ImmunoCAP ISAC is a useful tool for allergen sIgE measurements. Along this line, it is evident that in the presence of "discordant" results, in particular with low sIgE versus whole allergens and negative components, the question arises whether this is a real laboratory sign of "genuine" allergen sensitization. At present, the effect of cross-reacting components widely distributed in different allergen families cannot be ruled out: for this reason, excluding allergens poorly represented at component level

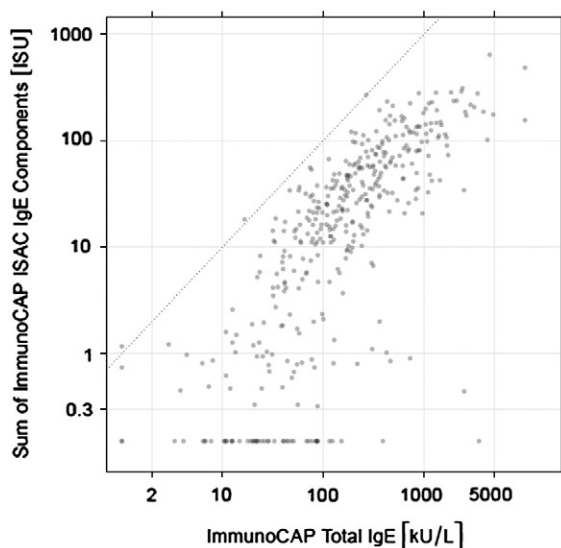


Fig. 2. The sum of ImmunoCAP ISAC IgE components vs. ImmunoCAP Total IgE. The dotted line indicates the identity line.

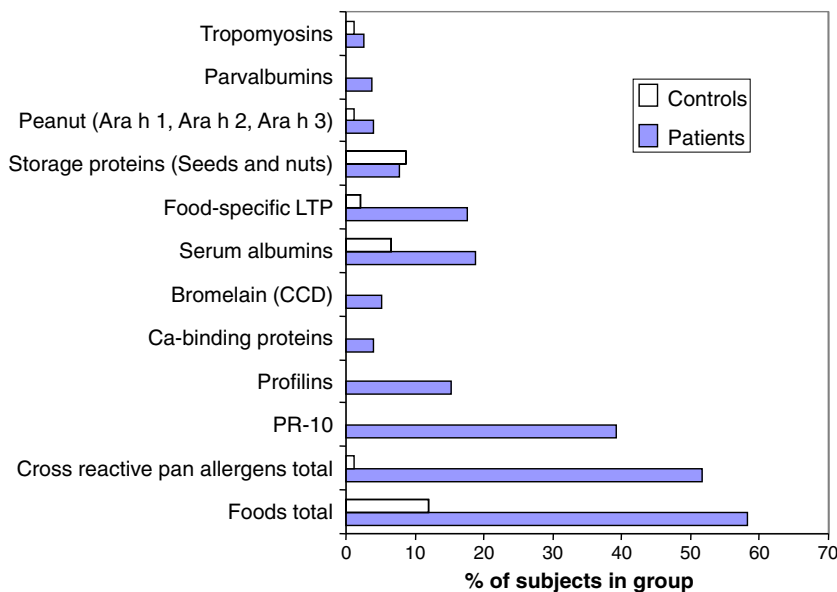


Fig. 3. Percent patients and controls with IgE antibodies to different protein families and important allergen-components, measured with ImmunoCAP ISAC.

(such as Dog, Ambrosia and Aspergillus, as stated before), a negative ImmunoCAP ISAC should be carefully considered as representative of the real IgE repertoire of the patient and only in the presence of clear clinical signs suggesting the sensitization, an ImmunoCAP ISAC false negative result should be suspected. However, the fact that ImmunoCAP ISAC, for certain allergens, does not cover a sufficient component repertoire, indicates that, nowadays, the possibility of its use as a first line allergy diagnostic tool is ruled out. Hopefully, new versions of the array, covering a wider spectrum of allergens, should overcome the present limitation.

Independently of the problem of “false negative results” (mainly in patients with very low concentrations of IgE antibodies), important information on co-sensitization to other allergens than the expected aeroallergens was obtained. In addition, sensitization to cross-reacting components explaining some of the perceived multi-sensitization was detected. Elevated IgE antibodies to Profilins, CCD (Ana c 2) and calcium-binding proteins were detected in 15%, 5% and 4%, of the patients respectively. IgE antibodies to these protein families were accompanied by, and probably caused by, sensitization to grasses and trees in this study. The possibility of identifying the presence of IgE antibodies to Profilins in patients with plant allergy is an interesting opportunity enabled by MA which may explain both numerous positive extract based tests, *in vivo* as well as *in vitro*, and Oral Allergy Symptoms to fruits and vegetables in the absence of IgE antibodies to PR10 proteins. Along this line, the possibility of excluding the presence of an allergy to profilins, in patients with plant allergy, is an interesting added value of this technique for the evident therapeutic outcomes that it implies. Finally, elevated IgE antibodies to Lipid Transfer Proteins were detected in 55% of the patients and Storage Proteins (seed) in 8%; these sensitizations imply an increased risk of severe systemic reactions, but this is currently not routinely searched for in respiratory patients.

It is evident that diagnostics using Molecular Allergy (CRD) offer possibilities not available with extract based standard techniques such as Skin Prick Test (SPT) and allergen sIgE tests. For example, Molecular Allergy effectively helps in distinguishing between specificity and cross-reactivity in patients with suspected multi-sensitization to various allergens. This may have a significant impact on the patient management in terms of risk assessment, advice

to avoid allergens, patient selection for immunotherapy, and immunotherapy regime. ImmunoCAP ISAC is also particularly powerful in poly-sensitized patients not only to detect the actual molecular component involved in the allergy, but also to rule out cross reacting allergens and other components (such as LTP) that can be responsible of adverse events.

ImmunoCAP ISAC is characterised by a diagnostic power clearly higher than that of sIgE performed using extract allergens. Due to the use of single recombinant components that unquestionably have a specific indication in selected cases, such as positivity to allergens characterised by the presence of pan allergens (e.g. Bet v1 for Betula v or Phl p12 for Phleum p.), ImmunoCAP ISAC can offer a wider representation of the IgE repertoire of the patients, with evident advantages in polysensitized patients with IgE specific for pan-allergens and/or cross-reacting allergens. However, the current version of ImmunoCAP ISAC cannot fully substitute the use of SPT or allergen sIgE tests, for its apparent inability to detect very low concentrations of IgE antibodies and for the absence of some important allergen components. Thus at present, a combined strategy of using SPT or allergen sIgE (complete allergens) together with Molecular Allergy tools (purified or recombinant allergen components) seems to offer the most complete approach.

In conclusion, ImmunoCAP ISAC is a very powerful tool, at least in polysensitized patients, for its capacity of detecting co-sensitization and cross-sensitization and, in the large majority of other cases, for its ability to contribute to an in-depth analysis of the results obtained with standard techniques such as SPT and sIgE.

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