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Evaluation of a new multiplex assay for allergy diagnosis

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ABSTRACT

Background: Molecular allergy has significantly improved the quality of allergy diagnosis; however, the positioning of singleplex and multiplex assays in the diagnostic algorithm is still a matter of debate.
Methods: Aim of the study was to test the analytical performance of the recently commercialized Allergy Explorer-ALEX® in a selected population (105 allergic patients and 15 negative controls), comparing it with the reference ImmunoCAP® method and with skin prick test (SPT).
Results: Inter-assay qualitative comparison showed a substantial agreement between ALEX® and SPT (k = 0.64). A substantial agreement between ALEX® and ImmunoCAP® was shown on the detection of IgE to extracts (k = 0.64 for inhalants and k = 0.51 for food allergens), whereas a higher agreement was shown on detection of molecular components (k = 0.92 for inhalants and k = 0.72 for food allergens). Quantitative comparison showed a poor correlation between ALEX® and ImmunoCAP®.
Conclusion: The simultaneous detection of both extracts and molecular components with ALEX® assay can potentially overcome some of the major limitations of the multiplex assay currently in use. However, before using ALEX® as routine method, the analytical performance (in particular for extracts) needs to be further investigated on a larger scale.

1. Introduction

Molecular allergy (MA) has significantly improved the quality of allergy diagnosis by giving the opportunity to distinguish genuine sensitizations from cross-reactions, with important cascade on the choice of the allergen specific immunotherapy and for the stratification of the risk in food allergy [1,2]. Nevertheless, to reach its full potential, it should be implemented on a clear diagnostic workup and based on a good level of knowledge of the diagnostic methods.

Currently MA testing can be conducted by singleplex or multiplex assays. The singleplex assays are, so far, the method of choice in a conventional "*top down*" approach; in this inductive strategy, the allergologist, moving from clinical history, skin prick test and/or IgE sensitization to extracts, chooses which molecules should be tested based on a precise clinical hypothesis. Singleplex assays have the value of being quantitative, highly automatized (with the possibility of dosing both extracts and molecules in the same test session) and they are cost effective if conducted on a solid diagnostic algorithm. On the other hand, some sensitization can be missed or underestimated. The multiplex assays, on their side, offer a broader vision of the sensitization profile of the patient. They are called for when there is a need to test > 12–13 molecules (carrying a clinical as well as an economic advantage) or in the pediatric setting because of the small quantity of serum needed. They are the first option in the case of a "*bottom up*" approach, for those clinicians that choose to move from broad laboratory testing to clinical, as well as in all those clinical conditions that require, after a first "*top down*" approach, to extend the panel of the molecules to be tested ("*U shaped approach*") [3]. However the available multiplex assays have the limit of being semi-quantitative and less sensitive than the singleplex approach. The first multiplex assay available on the market and currently in use is a microarray (ISAC®, Thermo Fisher, Sweden), that allows to test 112 different molecules, both recombinant and native, with a small amount of serum (30 µL) [4,5]. Recently, another macroarray (Allergy Explorer-ALEX®, Macro-ArrayDX Wien, Austria) has been commercialized. It differs from ISAC® because it contains both allergen extracts (157) and molecular components (125), it is commercialized as quantitative in nature, and the specificity of the array can be improved by the inhibition of Cross-reactive carbohydrate determinants (CCD) reactivity. From a technical perspective it performs one determination for each single allergen

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(ISAC performs a triple test on each molecular component) and it presents as a macroarray.

To our knowledge only one group has already published on this new array comparing ALEX® results with the on market microarray ISAC® [6]. In the present study we describe the performance of ALEX® and its potential contribution to a critical interpretation of the IgE sensitization profiles.

Aim of the present study was to test the analytical performance of ALEX® in a selected population comparing it with ImmunoCAP® (Thermo Fisher, Sweden). The two methods have been compared at qualitative level (inter-rater agreement), and quantitative level. A further analysis has compared each single method to skin prick tests (SPT) in terms of qualitative outcomes.

2. Material and methods

2.1. Population

Overall, 120 sera coming from two centers in Italy (Allergy and Immunology Unit, Santa Maria degli Angeli Hospital Pordenone, Italy and Laboratory Medicine Unity of Buccheri, la Ferla Hospital, Palermo, Italy) have been tested. Median age of the studied population was 29 years (range 3 years–69 years), with 58 males and 62 females. Of the reported population 56 were affected by respiratory allergy and 49 by food allergy. In the respiratory allergy subset forty-eight patients presented with rhinitis: 6 mild intermittent (12%), 2 mild persistent (4%), 5 moderate-severe intermittent (10%) and 35 moderate-severe persistent (70%). Fourteen patients were affected by allergic asthma. Of the food allergy population, 30 patients presented with lipid transfer protein (LTP) syndrome [5 patients (16.7%) with mild symptoms, 17 patients (56.6%) with moderate symptoms and 8 (26.7%) with severe symptoms] and 19 with allergy to walnut/hazelnut [5 patients with mild symptoms (26.3%), 12 patients with moderate symptoms (63.1%) and 2 patients (10.6%) with severe symptoms]. The clinical parts of the study, as well as specific IgE measurement, were part of the clinical routine of every participating centre. Patients gave informed consent to the use of their clinical data and serum in an anonymous form. The study was approved by the internal review board of the hospitals involved. In view of the essentially observational nature of the study, a formal approval by an external ethical committee was not required.

2.2. Methods

Sera were tested with ALEX® and the results were compared with data obtained from the singleplex IgE assay ImmunoCAP® and with SPT (ALK Abello for peach, and Stallergens for all other extracts) previously conducted on clinical indications following the conventional top down diagnostic approach. The decision to compare ALEX® to ImmunoCAP® arose from the need to look at ALEX® performance both in terms of qualitative and quantitative results and to evaluate both extracts and molecules.

In ALEX® different allergens and components 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 under agitation. Notably, serum diluent contains a CCD inhibitor; following this procedure without additional pre-incubation the manufacturer guarantees a CCD inhibition of 85%. After incubation for two hours, the chips are extensively washed, and a pretitered dilution of anti-human IgE labeled with alkaline phosphatase is added and incubated for 30 min. Following another cycle of extensive washing, the enzyme substrate is added, and after eight minutes, the reaction is complete. The membranes are dried, and the intensity of the color reaction for each allergen spot is measured by a couple-charged device camera.

The dedicated software digitalizes the images and prepares a report that lists the allergens and components and their score in kUA/mL. Total IgE is also measured. Finally, an arbitrary calibration curve is obtained by reacting four spots with decreasing concentrations of specific IgE corresponding to < 0.35 kUA/L, 0.35–1 kUA/L, 1–5 kUA/L, 5–15 kUA/L and > 15 kUA/L [6]. We considered as positive a concentration ≥0.35 kUA/L both for ImmunoCAP and ALEX®.

2.3. Statistical analysis

Inter-rater agreement between ALEX® and ImmunoCAP® was calculated for qualitative outcomes (positive-negative); Cohen's kappa coefficient (k), positive and negative agreement were assessed for each single extract and molecule considered. Additionally, each single method was evaluated in terms of qualitative agreement with skin prick test (SPT) results.

As conventionally assumed, kappa results have been interpreted as follows: k ≤0 no agreement, 0.01–0.20 none to slight, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial and 0.81–1.00 almost perfect agreement.

Bland Altman plots were used to investigate the correlation between quantitative values of IgE detected with the two different methods (ALEX® vs ImmunoCAP®).

All statistical analysis were performed using MedCal statistical software, version 10.4.5 (Mariakerke, Belgium).

3. Results

3.1. Inter-assay agreement for inhalants

ImmunoCAP® and ALEX® results comparison was first conducted at a qualitative level. The inhalant allergens (pollens + dust mites) showed an overall substantial inter rater agreement for extracts (k = 0.64). Among the inhalants, the lowest agreement was observed for mugwort (k = 0.26), ragweed (k = 0.16) and cypress (k = 0) (Table 1a). The inhalant molecular components showed overall an almost perfect agreement (k = 0.92), with the lowest performance observed for Amb a 1 (k = 0.35) according to the agreement profile observed on ragweed extracts. On the contrary, the agreement for Art v 1

Table 1a
Inter-assay agreement on inhalants: extracts.

	ICAP + AL+	ICAP + AL-	ICAP-AL+	ICAP-AL-	Pos agreem	95% CI	Neg agreem	95% CI	k
Grass pollen	22	4	0	7	0.92	0.83–0.99	0.78	0.56–0.99	0.7
Mugwort	2	8	0	23	0.33	0.0–0.67	0.85	0.75–0.95	0.26
Ragweed	2	12	0	19	0.25	0.0–0.5	0.76	0.62–0.89	0.16
Birch	20	3	0	10	0.93	0.85–1	0.87	0.72–1	0.8
Olive tree	15	2	0	16	0.93	0.85–1.02	0.94	0.85–1.02	0.88
Cypress	0	14	0	19	0	0–0	0.73	0.73	0
Pellitory grass	2	1	0	30	0.8	0.41–1.1	0.98	0.95–1	0.78
Alternaria	6	0	1	26	0.92	0.77–1	0.98	0.94–1	0.9
Dermatoph. Pt.	14	2	1	6	0.9	0.79–1	0.8	0.57–1	0.7
Dermatoph. Ph.	10	5	0	8	0.8	0.62–0.97	0.76	0.55–0.96	0.58
Overall	93	51	2	164	0.77	0.72–0.83	0.86	0.82–0.89	0.64

Table 1b
Inter-assay agreement on inhalants: molecular components.

	ICAP + AL +	ICAP + AL-	ICAP-AL +	ICAP-AL-	Pos agreem	95% CI	Neg agreem	95% CI	k
Phl p 1	26	0	1	6	0.9811	0.94–1	0.9231	0.77–1	0.9043
Phl p 5	17	0	0	16	1	1	1	1	1
Art v 1[a]	2	0	0	31	1	1–1	1	1–1	1
Amb a 1[a]	4	9	0	20	0.47	0.17–0.76	0.81	0.69–0.93	0.35
Bet v 1	18	0	1	14	0.97	0.92–1	0.96	0.89–1	0.93
Ole e 1[b]	12	0	0	21	1	1	1	1	1
Cup a 1[c]	19	1	1	12	0.95	0.882–1	0.92	0.81–1	0.87
Par j 2	3	0	0	30	1	1	1	1	1
Alt a 1	7	0	0	26	1	1	1	1	1
Der p 1	11	0	0	12	1	1	1	1	1
Der p 2	7	0	0	16	1	1	1	1	1
Der p 23	15	0	0	8	1	1	1	1	1
Overall	129	10	3	191	0.95	0.92–0.97	0.96	0.94–0.98	0.92

[a] Native in ImmunoCAP®.
[b] Native in ALEX®.
[c] Native in ImmunoCAP® and ALEX®.

and Cup a 1 respectively reached a k value of 1 and 0.87 (Table 1b).

3.2. Inter-assay agreement for food allergens

The overall agreement for food allergens resulted in a moderate agreement for extracts (k = 0.47) and substantial agreement for molecular components (k = 0.72).

In order to allow a critical interpretation of the data, the agreement for food allergens has been performed on two different population subsets.

The first is a population selected in Palermo and strictly characterized by lipid transfer protein (nsLTP) sensitization, noted as a typical Mediterranean fingerprint (30 samples) [7]. The second is a population selected for walnut/hazelnut sensitization (19 samples). In LTP sensitized population, peach extracts and Pru p 3 resulted in a perfect agreement; by contrast, both peanut extract (k = 0.25) and Ara h 9 (k = 0.63) showed lower agreement (Table 2a). In Walnut/Hazelnut sensitized patients the higher agreement was observed for Cor a 8 and the lowest for the 2S Albumin family with Cor a 14 (k = 0.21) (Table 2b). All results are detailed in Tables 2a and 2b but, notably, they are deeply influenced by the disequilibrium in term of positive/negative samples.

3.3. ImmunoCAP® and ALEX® vs SPT

In order to evaluate the methods in the context of the routine clinical practice we compared ALEX® and ImmunoCAP® results with SPT outcomes. The comparison between every single method vs SPT showed a substantial agreement for both ImmunoCAP® (k = 0.73, range 0.66–0.81) (Table 3a) and ALEX® (k = 0.62, range 0.53–0.70) (Table 3b).

The lowest agreement was observed for pellitory grass in the ImmunoCAP® vs SPT analysis and, as expected from the previous analysis, for mugwort ragweed and cypress in the ALEX® evaluation.

3.4. Quantitative correlation between extracts and molecules (ImmunoCAP vs ALEX)

The Bland-Atlman Plot showed an overestimation of ALEX® at low titres and of ImmunoCAP® at high titres, for both extracts and molecules (Fig. 1a and b).

3.5. Analysis of negative controls

An analysis was conducted on 15 negative controls accounting for 2335 tests on extracts and 1875 on molecular components. In all but one case ALEX® result was negative, giving a false positive rate of 0.00042%.

3.6. CCD analysis

Data for CCD analysis were available only for inhalant sensitized population (33 sera).

As expected, based on CCD inhibition on ALEX®, we found a low agreement on comparison of CCD qualitative results between ImmunoCAP® and ALEX® (k = 0.11, ICAP + ALEX + n = 1, ICAP + ALEX- n = 10, ICAP-ALEX + n = 0, ICAP-ALEX- n = 22).

11/33 sera (33.3%) resulted as CCD positive using ImmunoCAP® and a showed a complete CCD inhibition using ALEX® (Fig. 2).

4. Discussion

Our study investigated the performance of the new multiplex assay ALEX® in the detection of IgE antibodies to pollen, dust mites and food allergen extracts and molecules. A qualitative and quantitative analysis was performed comparing ALEX® to the singleplex test ImmunoCAP®.

4.1. Analysis of pollen extracts and molecules

The analysis of pollens extracts showed a substantial inter-assay

Table 2a
Inter-assay agreement on food allergens: LTP sensitized population.

	ICAP + AL +	ICAP + AL-	ICAP-AL +	ICAP-AL-	Pos agreem	95%CI	Neg agreem	95%CI	k
Peach extract	29	0	0	1	1	1–1	1	1–1	1
Peanuts extract	6	13	0	11	0.48	0.23–0.72	0.62	0.44–0.81	0.25
Overall	35	13	0	12	0.84	0.76–0.93	0.65	0.47–0.83	0.51
Pru p 3	29	0	0	1	1	1–1	1	1–1	1
Ara h 9	21	4	0	5	0.91	0.82–0.99	0.71	0.84–0.98	0.63
Overall	50	4	0	6	0.93	0.92–0.99	0.75	0.51–0.98	0.71

Table 2b
Inter-assay agreement on food allergens: Walnut/Hazelnut population.

	ICAP + AL+	ICAP + AL-	ICAP-AL+	ICAP-AL-	Pos agreem	95%CI	Neg agreem	95%CI	k
Walnut extract	18	1	0	0	0.97	0.92–1	0	0–0	0
Hazelnut extract	13	5	0	1	0.83	0.69–0.97	0.28	0.15–0.72	0.21
Overall	31	6	0	1	0.91	0.84–0.98	0.25	0.1–0.6	0.21
Jug r 1[a]	16	3	0	0	0.91	0.81–1	0	0.1–1	na
Cor a 8	4	0	0	15	1	1–1	1	1–1	1
Cor a 9[b]	7	1	0	11	0.93	0.8–1	0.95	0.87–1	0.89
Cor a 14[a]	6	9	0	4	0.57	0.31–0.82	0.47	0.17–0.76	0.21
Overall	33	13	0	30	0.86	0.77–0.94	0.85	0.75–0.93	0.70

[a] Native in ALEX®.
[b] Native in ImmunoCAP® and ALEX®.

agreement, with a k value > 0.81 in 3/10 extracts; the lowest agreement was observed for cypress (k = 0), mugwort (k = 0.26) and ragweed (k = 0.16). As reported in Table 1a, ImmunoCAP® is oftentimes positive whereas ALEX® remains negative. In order to explore the clinical relevance, if present, of such results, we analyzed the clinical data. In polysensitized patients the identification of the clinically relevant allergen is often challenging, however in the studied population, patients used the App *Allergy monitor* for reporting their symptoms, giving the possibility to match symptom scores with pollen counts. The analysis conducted on ragweed and cypress, the two allergens with major discrepancies, revealed the clinical relevance of ImmunoCAP results in 3/12 (25%) patients for ragweed and 3/14 (21.4%) for cypress. When comparing results obtained for IgE direct to molecular components, overall agreement was almost perfect (k = 0.92), with the lowest performance observed for Amb a 1 (ragweed major determinant). For all the other molecules, the results show a good consistency between the different molecules (*k* ranging from 0.87 to 1). Cypress pollen molecular component (Cup a 1) and mugwort molecular component (Art v 1) showed an almost perfect agreement, thus allowing the identification of cypress and mugwort sensitized patients missed at extract determination.

Resulting from the combined analysis on extracts and molecular components, we found some discrepancies. On comprehensive analysis, in three cases (2 for mugwort and 1 for olive extract) the presence of CCD could have generated false positive results in ImmunoCAP® extracts. In two other patients, the analysis showed disagreement between the two methods not only in terms of extracts but also in terms of molecular components, with ragweed and Amb a 1 positive in ImmunoCAP® and negative in ALEX®; it cannot be excluded that also in these cases CCD could have played a role, since Amb a 1 is *native* in ImmunoCAP® and recombinant in ALEX®. Thus far the low *k* observed for ragweed should carefully be interpreted in light of CCD interference.

Furthermore, as recently reported by Hemmer and co-workers, cellulose used as a solid-phase allergen carrier in ImmunoCAP can contain varying amounts of CCDs, sufficient to cause false positive test results with non-glycosylated recombinant allergens in patients with high levels of anti-CCD IgE antibodies [8].

In any case, for any of the reported considerations, it should be taken into account that the limitation in sample number, as reflected by the 95% CI, must be considered when looking at results.

4.2. Analysis of food allergens

In both populations, as expected, extracts showed a lower agreement compared to molecules.

In terms of molecular components the agreement was close to perfect for Pru p 3, and moderate for Ara h 9; however in the latter where ALEX® showed negative results, ImmunoCAP® was positive at low titers (< 1 kU/L).

The analysis conducted on the walnut/hazelnut sensitized population revealed an almost perfect agreement for the LTP family member (Cor a 8); among the storage protein family a good level of agreement was observed only for Cor a 9 (11S globulin) and not for Cor a 14 (2S albumin), with 6 patients resulting negative on ALEX® and positive at high titers on ImmunoCAP® (> 1 kU/l) for Cor a 14. It should be underlined that Cor a 14 is native in ALEX® and recombinant in ImmunoCAP ®. The higher sensitivity of ImmunoCAP® in the identification of nut allergy has been recently demonstrated also when compared to the multiplex method currently in use (ISAC®) [9].

4.3. SPT vs ImmunoCAP and ALEX®

The agreement between ImmunoCAP® and ALEX® vs SPT was reported as substantial for both methods. However, ALEX® showed a poor agreement on mugwort, ragweed and cypress, likely due to a low concentration of allergens in the extracts. It is worthy of note that both ImmunoCAP® and ALEX® present an agreement with SPT that is far from excellent. In the context of the debate between skin testing vs in vitro testing [10,11], this data raise up a concern on the sensitivity and the composition of SPT and, thus far, on their positioning as first step in the diagnostic algorithm. Literature reports for SPT a sensitivity of 70–95% and a specificity of 80–97% for inhalants with clinical history

Table 3a
Agreement between SPT and ImmunoCAP.

	SPT + CAP+	SPT + CAP-	SPT -CAP+	SPT-CAP-	Pos agreem	95% CI	Neg agreem	95% CI	k
Grass pollen	25	2	1	5	0.94	0.87–1	0.76	0.51–1	0.71
Mugwort	5	2	5	21	0.58	0.31–0.86	0.85	0.75–0.96	0.45
Ragweed	8	0	6	19	0.72	0.5–0.9	0.86	0.75–0397	0.6
Birch	18	1	5	9	0.85	0.74–0.97	0.75	0.55–0.94	0.61
Olive tree	12	1	5	15	0.8	0.64–0.95	0.83	0.7–0.96	0.63
Cypress	11	3	3	16	0.78	0.61–0.95	0.84	0.71–0.96	0.62
Pellitory grass	2	2	1	28	0.57	0.13–1.0	0.94	0.89–1	0.52
Alternaria	5	0	1	27	0.90	0.73–1	098	0.94–1	0.89
Dermatoph. Pt.	16	1	0	6	0.96	0.91–1	0.92	0.77–1.1	0.89
Dermatoph. Ph.	15	1	0	7	0.96	0.9–1	0.93	0.8–1	0.9
Overall	117	13	27	153	0.85	0.8–0.89	0.88	0.84–0.92	0.73

Table 3b
Agreement between SPT and ALEX.

	SPT+ AL+	SPT + AL -	SPT -AL +	SPT - AL -	pos agreem	95% CI	neg agreem	95% CI	k
Grass pollen	22	5	0	6	0.89	0.80–0.98	0.7	0.45–0.95	0.61
Mugwort	1	6	1	25	0.22	0.13–0.58	0.87	0.78–0.96	0.14
Ragweed	1	6	0	25	0.4	0–0.78	0.89	0.8–0.97	0.33
Birch	17	2	3	11	0.87	0.76–0.98	0.81	0.65–0.97	0.68
Olive tree	11	2	4	16	0.78	0.6–0.95	0.84	0.71–0.96	0.62
Cypress	0	14	0	19	0	0–0	0.73	0.59–0.86	0
Pellitory grass	2	2	0	29	0.66	0.23–1.1	0.96	0.92–1	0.63
Alternaria	5	0	2	26	0.83	0.6–1	0.96	0.91–1	0.79
Dermatoph. Pt.	15	2	0	6	0.93	0.85–1	0.85	0.66–1	0.79
Dermatoph. Ph.	10	6	0	7	0.76	0.58–0.94	0.70	0.47–0.92	0.50
Overall	85	45	10	170	0.77	0.69–0.81	0.86	0.82–0.89	0.62

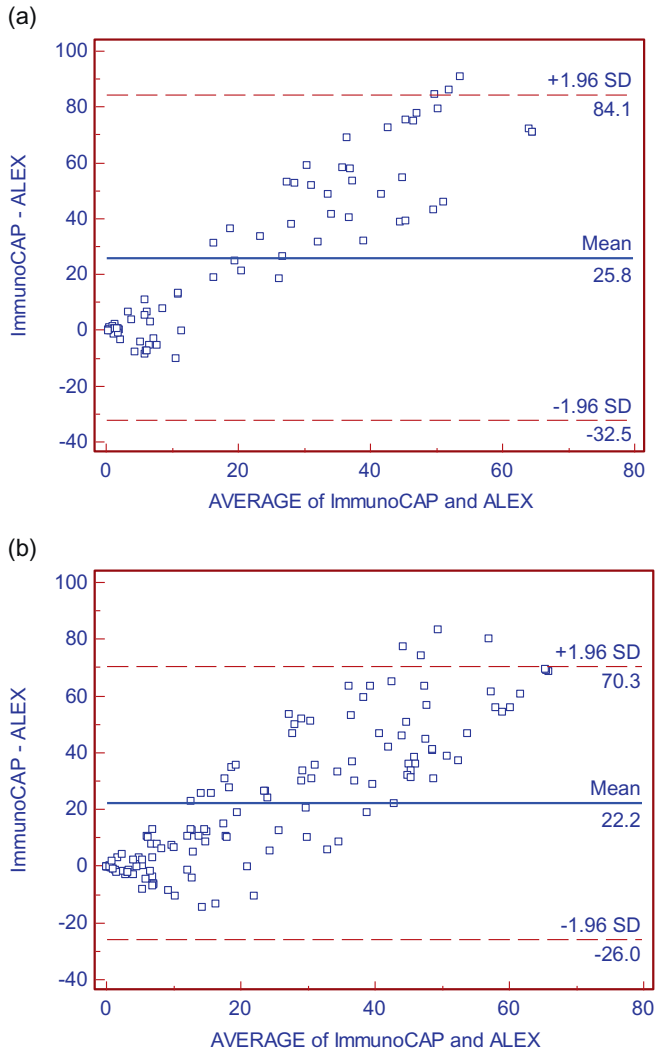

Fig.1. a. Altman Plot for extracts overall b. Altman Plot for molecular components overall.

that is warranty to increase SPT sensitivity up to 97–99. The performance on food allergens is lower with reported sensitivity and specificity of 30–90% and 20–60% respectively depending on the allergen and the type of approach (extract vs prick by prick test) [12,13].

However standardization of the major and minor allergenic determinants, batch to batch consistency and use of in-house reference material still represent formal bias for the interpretation of SPT results. [13–16]. In this scenario and on the basis of our data, the implementation of multiplex assay (containing both extracts and molecules) deserves at least to be critically investigated in their role as first line test, in particular in case of allergen of low abundance (in SPT extracts) or weak stability [11]. Nevertheless when interpreting data it is important to remember that, whatever the method, the demonstration of an IgE sensitization pattern remains only a proof of sensitization until clinical history rules out any correlate clinical symptoms.

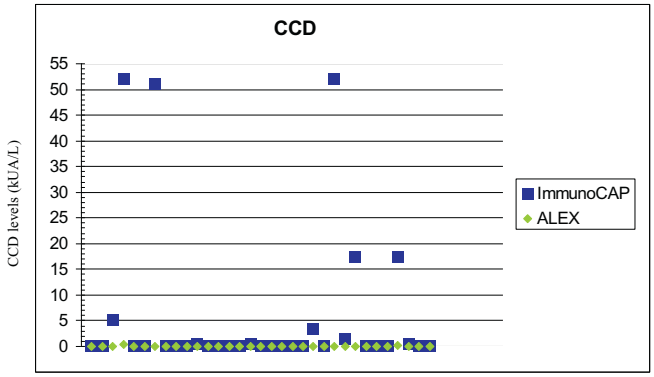

Fig. 2. CCD detection in pollen allergy with ImmunoCAP® and ALEX®.

4.4. Inter-assays quantitative comparison

Inter assay quantitative comparison showed a linear correlation but with diverse distribution in terms of titres, with ALEX underestimating higher levels and overestimating low titres. This is not surprising since discrepancies in terms of specific IgE values have already been demonstrated between consolidated assays even if calibration curves are interpolated with the same international standard [17,18].

5. Conclusion

Our study is, to our knowledge, the first reporting a direct comparison between the multiplex macroarray ALEX® and the singleplex test ImmunoCAP®.

Results showed a substantial agreement between the two methods, with slightly lower agreement on the detection of IgE to extract than to molecular components, both for inhalants and food allergens.

ALEX®, by performing a quantitative analysis, overcomes one of the major limits assigned to multiplex IgE antibody assays. The technical features, on their side, can potentially reduce the costs of instrumentation vs the first generation multiplex assays. In term of execution, ALEX® heightens all the advantages of multiplex assay technology. It presents as an easy to learn method, with a clear identification of the factors that can influence the final outcome. Compared to ISAC®, it requires a single determination on each allergen as opposed to a triple determination. However even if this characteristic was a pre-test concern in terms of error management and results

interpretation, in our setting the agreement with ImmunoCAP® results deposes for a substantial consistency of the data.

Nevertheless, the value of ALEX® in the routine clinical setting needs to be confirmed. In particular, further studies should focus on the optimization of some reagents, the analytical sensitivity (in particular for limit of detection-LoD), and the diagnostic sensitivity and specificity. As mentioned, the limit in sample size could have impacted the agreement rate, especially in the evaluation of the single extract/molecular component. In addition, it should be kept in mind that human immune-response mediated by IgE is polyclonal and subject to the effects of genetics. Allergen extracts may be heterogeneous and difficult to be standardized at fine molecular level and, as highlighted by Mueller [19], recombinant molecules may have an unpredictable behaviour. A comprehensive analysis conducted on the very large amount of data obtained by analyzing at least 300 different reagents, as well as evaluation for family-clustered components (e.g. LTPs, tropomyiosins) could help to reduce this bias.

The presence of both extracts and molecular components should play a role in facilitating and possibly accelerating the analytical performance, however before using ALEX® as the reference technique in a "bottom up" approach, data need to be confirmed on a large scale and interpreted in light of the clinical data.

In any case, the presence of a new multiplex test, when based on solid scientific data, gives a boost to research in the Molecular Allergy setting.

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Author's declaration

We confirm that the manuscript has been read and approved by all named authors and that the order of authors listed in the manuscript has been approved by all of us. All authors confirm that the present article is not under consideration for publication elsewhere. Any of the authors listed has reported his/her conflict of interest in the dedicated form.

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