
IMMUNOCAP ISAC MULTIPLEX MICROARRAY IGE TESTING – A TOOL FOR COMPONENT-RESOLVED DIAGNOSIS IN ALLERGY

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ABSTRACT

Introduction: Allergy has become a major health concern worldwide, affecting more than 20% of the population in the industrialized world. Allergy symptoms negatively impact quality of life of the sensitized individuals, increase in severity over time and with multiple allergen sensitizations. Component-resolved diagnosis can provide insights into patients' sensitization profiles, identifying primary sensitizers, cross-reactive components and risk to develop more severe symptoms in the future.

Aim: To identify IgE repertoires of polysensitized patients and to test whether multiplex ImmunoCAP ISAC assay can help to differentiate between genuine sensitization and cross-reactivity.

Methods: Three ragweed-allergic patients presenting sensitizations to at least two other allergen sources have been included in the study. Serum from these individuals was tested in ImmunoCAP ISAC containing 112 allergen components derived from 50 allergen sources according to manufacturer's instructions.

Results: All patients showed reactivity to the major allergen from ragweed Amb a 1 and had reactivity towards species-specific components from timothy grass. The main cross-reactive components towards which two patients showed low to moderate response were profilins, while one patient also had IgE towards tropomyosins. Highest IgE levels towards these cross-reactive components were for those contained in the same source as species-specific components.

Conclusions: ISAC multiplex testing helped to identify sensitization to an allergen source by reactivity towards the species-specific allergens, while also identifying reactivity towards cross-reactive allergens found in multiple sources. Additional allergens could improve the identification of primary sensitizers. For proper allergy diagnosis and treatment, patient history and exposure would also need to be evaluated by the allergist to determine the appropriate immunotherapy for the patient.

Keywords: allergy, IgE, polysensitized, cross-reactivity, ISAC multiplex

INTRODUCTION

Atopy is defined as the disposition to develop IgE antibodies against otherwise harmless external agents, which by cross-linking result in the release of inflammatory mediators like histamines or leukotrienes by the effector cells [1,2]. In the industrialized world, allergic disease occurs in around 20% of the population and appears to be increasing [3, 4]. The

most common symptoms of allergic diseases are rhinitis and conjunctivitis [5]. Rhino-conjunctivitis reduces the quality of life and implies increased health costs for sensitized individuals. Additionally, rhino-conjunctivitis can develop into more severe forms of allergic disease, like asthma [6], while rhinitis and asthma is more prevalent among patients sensitized to more than one allergen source [7]. Patients with multiple sensitizations and morbidities also have more

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persistent and more severe asthma symptoms [6]. Most frequent sensitizations are towards house dust mites (HDM) and pollen sources like timothy grass and short ragweed, while most severe allergic manifestation of allergy, anaphylaxis, may be caused by sensitization to allergens in food, insect venoms, and drugs [3], [8].

Allergen sources contain multiple proteins with sensitizing potential, some of which are considered species-specific major allergens and used as markers for sensitization while others are known panallergens (proteins with functional and structural homology belonging to the same protein family) (Fig.1). Proteins which are known as panallergens belong to the profilin, tropomyosin, polcalcine, nonspecific Lipid Transfer Protein (nsLTP) and Pathogenesis-Related protein (PR) families [9]. Allergy onset is triggered by sensitization towards the major and some minor allergens, while sensitization towards panallergens occurs after high exposure to allergen source and is sometimes an indicator of disease severity [9, 10]. When IgE antibodies are raised against a specific antigen, but are able to recognize sequences from other antigens based on their primary or tertiary structure, cross-reactivity occurs [11, 12]. Allergens with an amino acid identity above 70% are often considered to trigger reactions of cross-reactivity, while allergens with identities below 50% rarely cross-react [13]. Sequence homology is not always a prerequisite for cross-reactivity, since these reactions were also found between non-related proteins, i.e. soy Gly m 6 and cow milk Bos d 6 [12]. These reactions can be clinically relevant when both sources can trigger symptoms or they can be irrelevant to the patient [11]. Patients sensitized to certain pollens have often reported symptoms after ingestion of certain foods, known as pollen-food syndrome (PFS) e.g. birch pollen allergic patients, sensitized to the major allergen Bet v 1 report symptoms after ingesting apple, due to the homology between the two PR-10 proteins Bet v 1 and Mal d 1. Birch immunotherapy was also found to reduce the symptoms towards apple [14]. Similarly, patients with sensitization to plant food allergen Pru p 3, nsLTP from peach, also had rhinitis symptoms in response to mugwort nsLTP Art v 3 [15]. Another common PFS is ragweed-melon-banana which is probably induced by primary sensitization with reactivity towards nsLTP or profilin from ragweed [16]. Due to its predicted dispersal and increased allergenicity, ragweed has become a major health problem in Europe and worldwide [17]. Therefore it is important to investigate potential cross-reactive allergen sources among ragweed allergic patients to determine primary sensitization and select the appropriate immunotherapy [18].

Diagnosis of type I allergy requires the determination of allergen-specific IgE (sIgE) in the blood of patients or induction of immediate symptoms towards allergen in provocation test (nasal, skin, oral or bronchial provocation test) [19, 20]. Skin prick test (SPT) using allergen extracts were preferred by physicians and patients, since they were a fast, cost-efficient and easy way to diagnose allergy [21]. However, disadvantages include the lack of standardization of allergenic components when obtaining allergen extracts as well as the presence of cross-reactive carbohydrate determinants and panallergens in natural extract which could give false positive results [22]. To identify the specific sensitization profile of patients, production of recombinant allergens has proven to be an invaluable tool in the component resolved diagnosis of allergy and precision medicine [19, 23]. Two different techniques are most frequently employed in component-resolved diagnosis: singleplex ImmunoCAP and multiplex ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC). An important advantage of ImmunoCAP testing is obtaining quantified allergen-sIgE levels against an allergen coated on a cellulose matrix without interference from IgG. On the other hand, ISAC provides an overview of IgE sensitization patterns towards 112 allergens from 50 allergen sources coated on polymer-coated glass slide using only small quantities of serum. A drawback for ISAC being that it gives you only semiquantitative data at a relatively high cost per assay [24, 25]. In ImmunoCAP the bound IgE antibody is measured with an enzyme labelled anti-IgE antibody, where the fluorescence intensity is proportional to the amount of bound IgE. The principle behind the multiplex assay is similar, in ISAC the allergen-bound IgE is measured using fluorescently labelled anti-human IgE which is detected using a laser scanner. Both component-resolved assays can provide valuable information regarding genuine sensitization by the use of marker (species-specific) allergens or enabling to identify cross-reactivities by sensitization to panallergens. This assay provides also the opportunity to identify IgE reactivity profiles at risk of developing severe symptoms, e.g. anaphylaxis [26]. The ISAC assay has recently been evaluated by different operators in 23 sites worldwide, showing high repeatability across sites and variability estimated at 25.5% [27].

Aim of this study is to illustrate the effectiveness of component-resolved diagnosis in deciphering primary sensitization in ragweed allergic patients presenting multiple sensitizations in SPT. The patients chosen for this assay present multiple sensitizations towards at least three types of pollen. Therefore, the aim was to test whether ISAC multiplex assay can help

to differentiate between genuine sensitization and cross-reactivity in patients presenting multiple sensitizations.

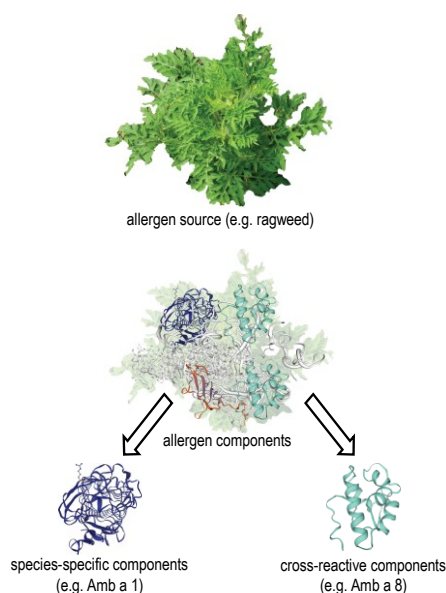


Fig. 1. Component-resolved diagnosis. Allergen sources contain multiple allergenic components, which can be either species-specific or cross-reactive.

MATERIALS AND METHODS

Patients sera

Three ragweed-allergic patients were selected for inclusion in this study after examination by an allergist at the Allergology Unit in Clinics, Timisoara, Romania. Inclusion criteria were reactivity towards ragweed pollen extract (SPT or blood IgE) as well as reactivity towards at least two other allergen sources (pollen from trees, grasses, other weeds, house dust mites, fungi, furry animals, latex or food) and symptoms of allergic rhinitis and conjunctivitis during allergy season. The participants in this study signed an informed consent to donate blood voluntarily. Sera samples were stored at -80°C until use. The study was approved by the local ethics committee of County Emergency Clinical Hospital „Pius Brînzeu” Timisoara (Ethical approval number 102, 10.01.2017).

Determination of IgE profiles using ISAC 112i

For this study four samples were measured: the calibrator sample (CTR03), provided by the manufacturer and three sera from polysensitized patients. IgE sensitisation to 112 allergen components was determined in triplicate by a semi-quantitative

allergen microarray assay using the ImmunoCAP ISAC 112i (ThermoFisher, Uppsala, Sweden) platform following the manufacturer's guidelines (Fig. 2). CTR03 is composed of known amounts of humanized chimeric monoclonal antibodies against 16 molecular components (Amb a 1, Art v 1, Art v 3, Bet v 1, Can f 1, Can f 2, Can f 5, Der f 1, Der p 1, Fel d 1, Gal d 1, Gal d 2, Mal d 1, Pla a 3, Phl p 5, Pru p 1, Pru p 3). The antibodies against these allergenic components are found in the calibrator in a range of 4 different concentrations expressed in ISAC Standardized Units (ISU-E) values for sIgE (1, 4, 15 and 50). The calibration curve is plotted from the 4 points of fluorescence intensity which correspond to the different ISU (Fig. 3 A, B).



Fig. 2. ImmunoCAP ISAC array layout. Layout shows positions and names of 112 allergen components (MIA software)

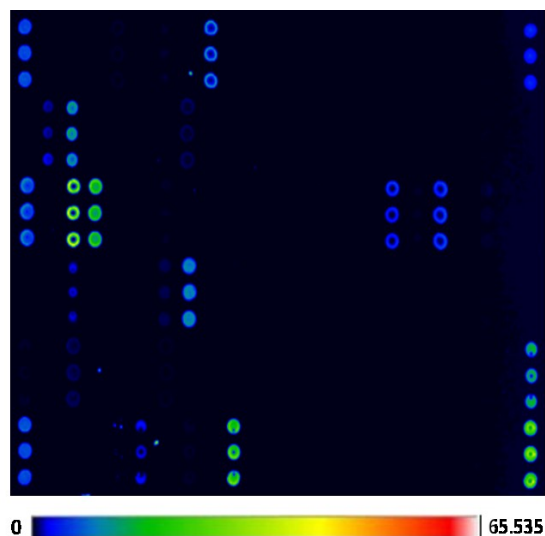


Fig. 3A. ImmunoCAP ISAC image scan of the calibrator sample (CTR03). Allergen components are arrayed in vertical triplicates. Image is shown in false colour display mode to better visualize the fluorescence output. Linear scale of false colour display is shown below the image.

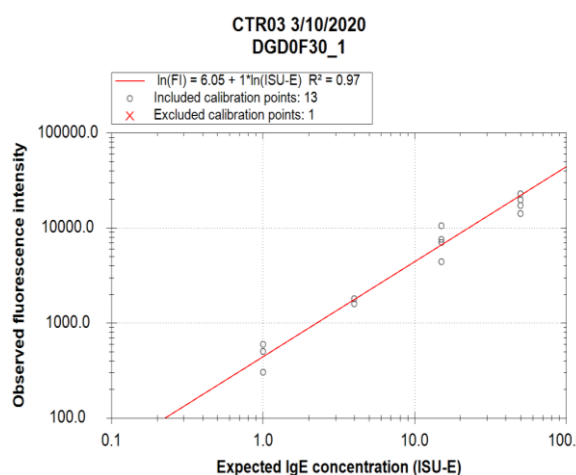


Fig. 3B. ISAC calibration curve used for this assay analysis (CTR03)

The microarray was incubated with 30 μ L of patient serum for 120 minutes at room temperature. Slides were then washed and 30 μ L fluorescence-labelled human IgE antibody was added on each microarray. After 30 min incubation in the dark humidity chamber, unbound antibodies were removed by washing and the fluorescence intensity was measured by microarray scanner (LuxScan 10K/A, CapitalBio, Beijing, China) with the following parameters: laser power (LP = 60) and photo-multiplier tube (PMT = 600). Results were interpreted using Phadia Microarray Image Analyzer software (MIA, ThermoFisher). The sIgE values expressed semiquantitatively as ISU-E units are assigned to one of the following four categories: Undetectable (<0.3 ISU-E), Low (0.3-0.9 ISU-E), Moderate/High (1-14.9 ISU-E), Very high ($\geq$ 15 ISU-E). Phadia Xplain add-on (ThermoFisher Uppsala, Sweden) was used for data interpretation.

RESULTS

All patients had a positive response in skin prick test/ blood IgE test towards weed pollen and grass pollen, while patients 2 and 3 were also reactive towards tree pollen (Table I.). Patient 1 also had positive results towards house dust mites (HDM), fungi, furry animals and food, while patient 2 was positive towards latex extract and HDM (Table I).

Table I. Patients characterization and sensitization to different allergen sources. Patients sensitization towards extracts as evaluated at the allergist clinic. (+ positive, - negative, NA not available)

P a t.	A g e	S e x	Allergen sources							
			T r e e	G r a s s	W e e d	Hous e Dust Mite	F u n gi	Furr y ani mal s	L at e x	F o o d
1	39	M	-	+	+	+	+	+	NA	+
2	63	M	+	+	+	+	-	-	+	-
3	16	F	+	+	+	NA	NA	NA	NA	NA

Component resolved analysis revealed that all three ragweed-allergic patients (patient 1, 2 and 3) were positive (ISU-E $\geq$ 0.3 ISU-E) for major ragweed component Amb a 1 with measured values between 8.8-20 ISU-E, corresponding to moderate – very high response (Fig. 4 A-F). Patient 2 reacted to major component from mugwort Art v 1 with a value of 3 ISU-E (Fig. 4 D). Patients 1 and 2 also reacted with profilin Mer a 1 from annual mercury with values of 0.5 and 8.7 ISU-E respectively (Fig. 4 B, D).

All three patients with allergy to grass pollen reacted to one or more species-specific components from timothy grass pollen as follows: patients 1 and 3 reacted to Phl p 1 with values of 2.3 and 71 ISU-E, to Phl p 2 with values of 0.9 and 17 ISU-E, patients 2 and 3 reacted to Phl p 4 with values of 1.6 and 2.3 ISU-E, patient 3 reacted to Phl p 5 and Phl p 6 with values of 25 and 5.2 ISU-E. Patient 2 also reacted to cross-reactive profilin Phl p 12 with a value of 1.7 ISU-E (Fig. 4 D). Cyn d 1 resulted in a moderate IgE response in patient 2 (2 ISU-E) and a very high response in patient 3 (36 ISU-E) (Fig. 4 C-F).

Among the patients sensitised to tree pollen, patient 3 showed reactivity to major allergen component from birch Bet v 1 with a value of 1.1 ISU-E, whereas patients 1 and 2 showed reactivity to profilin Bet v 2 with values of 0.4 and 7.6 ISU-E (Fig. 4 A-F). Patient 3 reacted slightly to major component from Japanese cedar Cry j 1 with value of 0.9 ISU-E.

Patient 2 showed a positive response to allergens from house dust mites (HDM) and mould (Fig. 4 D). Species-specific allergens Der f 2 and Der p 2 resulted in moderate response with values of 3.8 and 4.6 ISU-E, while the cross-reactive component tropomyosin, Der p 10, also had a moderate value of 1.7 ISU-E. Patient 2 also reacted to tropomyosins from three other animal sources: anisakis Ani s 3, cockroach Bla g 7 and shrimp Pen m 1 with low to moderate values of 0.8, 0.9 and 1.2, respectively (Fig. 4 D). Patient showed low reactivity to the enolase from mould Alt a 6 with a value of 0.6 ISU-E.

Other reactivities in ISAC were to profilin from latex Hev b 8 for patients 1 and 2 with values of 0.4 and 8.4 ISU-E and to food allergen from hazelnut Cor a 1.0401 for patient 3 with a value of 0.4 ISU-E. Also, patient 2 and 3 showed reactivity to CCD (cross-reactive carbohydrate determinant) MUXF3 with low levels of 0.7 and 0.9, respectively (Fig. 4 D, F).

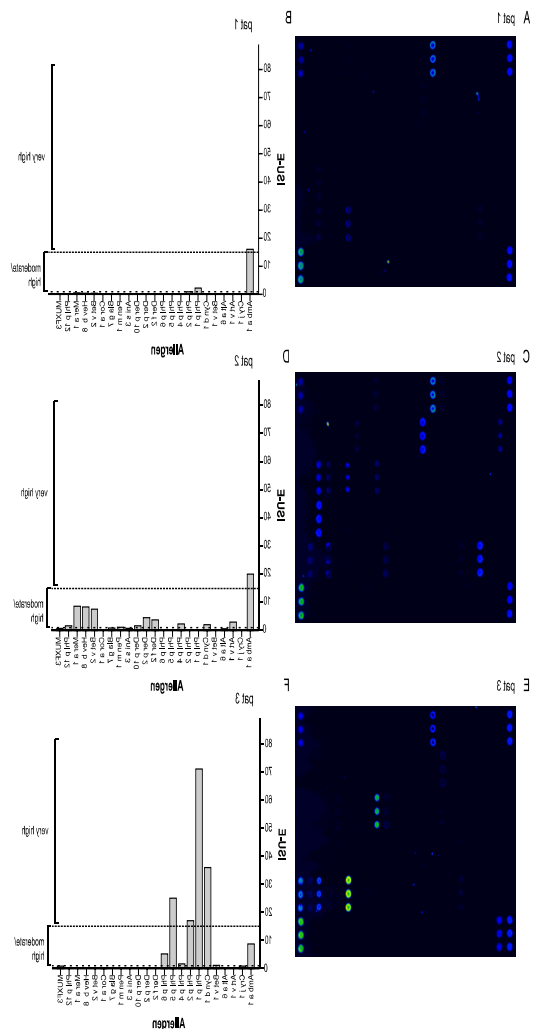


Fig. 4. ISAC IgE measurements for the three patients. ISAC readings and average ISAC Standardized Units (ISU-E) results for patient 1 (A, B), patient 2 (C, D) and patient 3 (E, F).

DISCUSSION

ImmunoCAP ISAC Molecular Allergy Test has proven to be a highly sensitive and specific method of diagnosis in allergy, showing a strong correlation with other methods as SPT and sIgE tests [27, 28]. Studies found that in Europe individuals are exposed to overlapping pollen seasons of a variety of allergenic plants [29]. Ragweed, mugwort, birch and timothy grass are only a few of the most common pollen sources which can be found in Western Romania [30, 31]. Previous studies conducted in Timisoara, Romania, showed that ragweed allergy has the

highest prevalence in both monosensitized and in polysensitized patients and ragweed pollen is considered to be the most significant outdoor allergen [32]. Therefore, three patients with ragweed allergy in addition to at least two other sensitizations were chosen to investigate allergen-specific IgE repertoire to 112 components from 50 allergen sources.

The sensitisation rate for the three patients included in our study was higher for pollen-derived components than for non-pollen derived components. All three ragweed-allergic patients showed reactivity to the species-specific component Amb a 1 from ragweed with high IgE levels, as expected, since older studies show that Amb a 1 is a highly allergenic molecule recognised by more than 90% of the ragweed-allergic patients and high IgE titres are found in patient sera [33]. Although other major and minor allergens are known from ragweed pollen to be clinically relevant contributing to the clinical picture of ragweed allergy, in this case, the patients are genuinely sensitised to ragweed pollen. In addition, all patients also reacted to at least one component from timothy grass.

Patient 1 showed specific IgE to major allergens Phl p 1 and Phl p 2 which indicate genuine sensitisation to timothy grass [34]. Low IgE reactivity to the profilins from annual mercury Mer a 1, latex Hev b 8 and birch Bet v 2, can indicate cross-reactivity due to the high sequence homology of these proteins. Studies found that Bet v 2 was an indicator of cross-reactivity with profilins from other pollen sources, plant-based foods and latex, similar to Mer a 1 which cross-reacted with other pollen profilins and Hev b 8 which was found to cross-react with other pollen profilins rather than to indicate genuine latex sensitization [34, 35]. All this data suggests that this patient may be sensitised to other profilins which are currently not spotted on the chip. The reactivity to the beforementioned profilins and not Phl p 12 (from timothy grass) could be attributed to sensitization towards profilin Amb a 8, a minor allergen from the ragweed pollen with an IgE frequency of 20-35 % in ragweed-allergic patients and a sequence identity of over 65% with other known allergenic profilins [36]. However, profilin sensitization was indicated to have little clinical relevance in allergic diseases [37].

Patient 2 appeared to be sensitised to Bermuda grass and timothy grass due to the positive response to natural components Cyn d 1 and Phl 4, respectively. This patient also had low IgE level to CCD. Proteins purified from extract can contain CCD components and IgE reactivity to Bermuda grass Cyn d 1, timothy grass Phl p 4, cypress Cup a 1, Japanese cedar Cry j 1 and plane Pla a 2 could be partially based on cross-reactivity to these CCDs [38, 39]. Although more than

20% of the allergic patients were found to have IgE that binds to CCDs, the clinical relevance of these molecules has yet to be proven [39]. Both Cyn d 1 and Phl p 4 are considered species-specific allergens and in this case, patient 2 is probably sensitised to both sources with only a small part of the IgE to Cyn d 1 and Phl p 4 being attributed to CCDs [34]. Art v 1 is the main specific mugwort component as at least 95% of the mugwort allergic patients react to this allergen and IgE to this marker shows sensitisation to mugwort pollen [34]. However, in ragweed pollen (towards which the patient is sensitized according to the high reactivity to Amb a 1) there is a minor allergen (defensin Amb a 4) 1 which is not included on the chip, showing 50% sequence identity with Art v 1 [36]. Pablos et al. found that between 52 and 74% of patients which reacted to Art v 1 (depending on region) also responded with a similar intensity towards the homologs in ragweed and *Parthenium* [40].

Patient 2 showed low reactivity to *Alternaria* enolase Alt a 6 minor allergen indicating probably weak sensitisation to mould [41]. Specific IgE towards marker allergens Der f 2 and Der p 2 indicated genuine sensitisation to house dust mites (HDM). Der p 2 and Der f 2 can cross-react. This patient also showed low-moderate reactivity to tropomyosins Ani s 3, Bla g 7 and Pen m 1, which could be due to cross-reactivity to Der p 10 from house dust mites towards which the patients is sensitized and which had slightly higher IgE level [34]. In order to determine the primary sensitizer in case of cross-reactivity, the highest IgE values for a protein from that family has been suggested as indicator of primary sensitization, while lower IgE values may be to cross-reactive proteins [38].

This patient also showed IgE reactivity to cross-reactive components from birch Bet v 2, latex Hev b 8, annual mercury Mer a 1 and timothy grass Phl p 12 indicating cross-reactivity between these profilins [34]. Based on the IgE levels towards these components, it is likely that patient 2 was primarily sensitized to annual mercury and this led to cross-reactivity with other profilins. On the other hand, given that patient 2 appeared to be sensitized to timothy grass, Phl p 12 could also be considered as primary sensitizer, although this could not explain why the IgE value to Phl p 12 was four or five times lower than to Bet v 2, Hev b 8 and Mer a 1. Another possibility could be that patient 2 was primarily sensitized to profilin from other allergen sources known to cross-react and towards which that patient showed high IgE reactivity, but which are unfortunately not available on the chip for testing (e.g. ragweed Amb a 8 or mugwort Art v 4). Patient 3 showed a high reactivity towards the species-specific components from grass pollen, five mainly species-specific components of timothy grass

(Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6) indicating genuine sensitization to timothy grass [42, 43]. High IgE values to Bermuda grass Cyn d 1 could also indicate a genuine sensitization to Bermuda grass, since Cyn d 1 is considered representative marker allergen for this species [34]. However, we also have to consider that this reactivity may be due to cross-reactivity to timothy grass Phl p 1. Previous studies have found a high degree of allergenic similarity or cross-reactivity between Bermuda grass pollen and timothy grass pollen and IgE to Cyn d 1 may cross-react to Phl p 1 [38, 44, 45].

Patient 3 showed a weak sensitization to tree pollen. IgE reactivity to Bet v 1 component indicates sensitization to birch [46]. IgE reactivity to hazelnut Cor a 1.0401 is associated with local allergic reactions in patients with pollen-food-syndrome caused by sensitization to birch (e.g. Bet v 1) or related trees [34]. In this case, sensitization to Cor a 1.0401 could be due to cross-reactivity to birch Bet v 1 belonging to the same protein family (PR-10 protein). Part of the IgE reactivity to components Cyn d 1 and Phl p 4 for this patient could be due to IgE against CCD, as mentioned before. Since the IgE values to Japanese cedar Cry j 1 and CCD are identical, it is possible that patient 3 was not primarily sensitized to Cry j 1 and the positive response could be entirely due to the CCD-specific IgE [34].

CONCLUSION

ISAC multiplex assay has proven to be an important tool to determine the IgE profiles of the polysensitized patients included in our study. After data interpretation, patients included in this study were sensitized to fewer allergen sources according to chip than was previously determined by extract testing. Therefore, ISAC measurements could help to explain certain reactivities obtained when testing the allergen extracts due to the cross-reactive allergen components or carbohydrate determinants within those allergen sources. Additional allergens on the chip could provide more information regarding primary sensitizer among cross-reactive allergens, especially when considering the exposure of the patients to the allergens. This information is very useful for precision medicine and personalized treatment in allergy. However, for the clinical diagnosis and selection of allergen immunotherapy, patient and symptom history as well as allergen exposure will have to be evaluated by the allergist to establish the appropriate treatment for each patient.

ACKNOWLEDGEMENTS: This research was financed by the INSPIRED (INovative Strategies for

Prevention, diagnosis and therapy of ragweed pollen Induced REspiratory Diseases) project, COP 2014-2020 92/09.09.2016, P_37_747.

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TESTAREA DE ANTICORPI IGE CU MULTIPLEX IMMUNOCAP ISAC MICROARRAY – O METODĂ DE DIAGNOSTIC MOLECULAR ÎN ALERGII

REZUMAT

Introducere: Alergiile au devenit o problemă majoră de sănătate în toată lumea, afectând mai mult de 20% din populația din lumea civilizată. Simptomele alergiei reduc calitatea vieții pacientului și manifestarea lor devine tot mai severă pe măsura creșterii ratei sensibilizării la alte alergene. Diagnosticul molecular oferă o înțelegere mai amplă a profilului de sensibilizare al pacientului, ajută la identificarea sursei sensibilizării primare, a componentelor alergenice care pot da reactivitate încrucișată și la evaluarea riscului de a dezvolta simptome mai severe pe viitor.

Scop: Studiul de față propune identificarea repertoriului de anticorpi IgE al pacienților polisensibilizați prin utilizarea tehnicii ImmunoCAP ISAC pentru a face diferența între sensibilizarea primară și reactivitatea încrucișată.

Metode: Trei pacienți alergici la polen de ambrozia care au prezentat și simptome la cel puțin alte două surse alergenice au fost incluși în acest studiu. Serul de la acești indivizi a fost testat în ImmunoCAP ISAC la 112 componente din 50 de surse alergenice conform instrucțiunilor producătorului.

Rezultate: Toți pacienții au prezentat reactivitate la alergenul major din ambrozia și la anumite alergene din timofică. Principalele alergene cu potențial de reactivitate încrucișată la care pacienții au prezentat un răspuns slab sau moderat au fost profilinele; un singur pacient a prezentat reactivitate la tropomiozine. În cazul pacienților care au răspuns la mai multe alergene omologe, răspunsul IgE cel mai crescut a fost pentru sursa la care pacientul s-a sensibilizat inițial la alergene majore.

Concluzii: Testarea cu ajutorul tehnologiei ISAC a ajutat la identificarea sursei de sensibilizare prin determinarea reactivității față de alergenele specifice unei surse, dar și la identificarea alergenelor care dau reactivitate încrucișată. Adăugarea suplimentară pe cip a altor alergene minore ar putea contribui la identificarea sursei alergenice primare în cazul reacțiilor de reactivitate încrucișată. În vederea stabilirii unui diagnostic corect al alergiei, istoricul pacientului și expunerea la diferite surse alergenice trebuie evaluată de medicul alergolog în cazul administrării imunoterapiei adecvate pentru fiecare pacient.

Cuvinte cheie: alergii, IgE, polisensibilizare, reactivitate încrucișată, ISAC multiplex