

Original article

The performance of a component-based allergen-microarray in clinical practice

Background: Currently, the diagnosis of IgE-mediated allergy is based on allergen-specific history and diagnostic procedures using natural allergen extracts for *in vivo* and *in vitro* tests.

Objective: The aim of the study was to comparatively analyse a new component-based allergen-microarray and the 'quasi-standard' ImmunoCAP® for their clinical relevance in patients with allergic rhinoconjunctivitis to five aeroallergens [house dust mite (HDM), cat dander, birch, grass and mugwort pollen] in a prospective, double-centre study.

Methods: We enrolled 120 subjects at the two study centres. Allergic patients were defined as having an allergen-specific history plus a concomitant positive skin-prick test (SPT) to natural allergen extracts and specific serum IgE was measured by both methods. Each allergen was analysed separately.

Results: The microarray performed equally well in receiver-operating characteristic curve (ROC) analyses when compared with the CAP in cat (23 allergic vs 97 non-allergic, ROC area under the curve microarray 0.950 vs CAP 0.894, $P = 0.211$), birch (31/89, 0.908 vs 0.878, $P = 0.483$) and grass pollen (47/73, 0.923 vs 0.915, $P = 0.770$). It was slightly less sensitive in HDM-allergic subjects (26 allergic vs 94 non-allergic, ROC area microarray 0.808 vs CAP 0.911, $P = 0.053$) and displayed a reduced sensitivity in the mugwort pollen-allergic patients (17/103, 0.723 vs 0.879, $P = 0.032$).

Conclusions: Component-based testing and the whole-allergen CAP are equally relevant in the diagnosis of grass-, birch- and cat-allergic patients. Although slightly less sensitive, the microarray is sufficient for the diagnosis of HDM-allergic patients, but needs alternative and/or additional components for detecting mugwort allergy.

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The mainstay of diagnosing type-I allergy is obtaining a patient's detailed history which gives important information about the type of allergen eliciting the disease and the symptom severity (1). In routine settings, natural allergen extracts are used for further *in vivo* and *in vitro* diagnostic procedures. The results of *in vivo* tests (skin-prick and/or intradermal tests) are usually confirmed by *in vitro* measurement of allergen-specific IgE (sIgE) (2). The ImmunoCAP® assay (CAP; Pharmacia Diagnostics,

Uppsala, Sweden) is a well-established *in vitro* test for the detection of sIgE and the industrial 'quasi-standard' (3).

Naturally occurring allergens vary significantly in their composition (4, 5) and allergenicity (6). Most patients do not raise sIgE to all but only some allergenic components which is of special importance for the correct composition of allergen vaccines for specific immunotherapy (SIT). To cope with this, recombinant allergen components have been introduced in conventional *in vitro* testing such as CAP assays, a strategy termed component-resolved diagnosis (CRD) (7–11).

Based on the concept of DNA microarray technology in the early 1990s, protein microarrays have emerged as a promising approach towards the high-throughput large-scale profiling of protein interactions on a system-wide scale. Recently, the CRD concept has been combined

Abbreviations: CAP, ImmunoCAP®; CI, confidence interval; CRD, component-resolved diagnosis; EAACI, European Academy of Allergy and Clinical Immunology; HDM, house dust mite; rArt, recombinant mugwort pollen component; rBet, recombinant birch pollen component; rPhl, recombinant grass pollen component; ROC, receiver-operating characteristics; sIgE, allergen-specific serum IgE; SIT, specific immunotherapy; SPT, skin-prick test.

successfully with protein microarray technology to generate allergen-microarrays for the simultaneous monitoring of IgE antibodies towards a large number of allergy-eliciting molecules (12–14).

For the development of a novel diagnostic test, a comprehensive assessment of the application's analytical and diagnostic performance in comparison with established laboratory standards is mandatory. Therefore, allergen-microarrays were validated against the CAP/radioimmunoallergosorbent test (RAST) system with sera from sensitized patients in a routine diagnostic setting (13, 15). However, it is well known that not all patients with sIgE do suffer from clinically relevant allergies (16–19). Hence, only the combination of the patient's history with *in vivo* and *in vitro* tests allows the estimation of the clinical relevance of allergen sensitization.

To assess the clinical relevance of measuring sIgE with the new allergen-microarray, we matched results obtained with the microarray and the CAP with detailed patient histories and allergen-specific SPTs, respectively, to the five most common local aeroallergens in a comparative, prospective, double-centre study.

Patients and methods

Patients

A cohort of 120 adult subjects (70 F/50 M, mean age 35.9 ± 14.4 years) was enrolled from September to October 2004 after the end of the pollen season at the two study sites: 61 at the allergy outpatient clinic of the DIAID (MUW) and 59 at a private outpatient allergy clinic (FAZ). Clinical allergy was defined as the coexistence of a suggestive allergen-specific history and a concomitant positive SPT. Five locally most relevant aeroallergens (20) were chosen for this study, namely: HDM, cat dander (cat), birch pollen (birch), grass pollen mix (grass), mugwort pollen (mugwort). Each allergen was evaluated separately. Consequently, 76 of 120 individuals were classified as 'allergic' to at least one of the five study allergens including patients with mild, moderate and severe rhinoconjunctivitis and/or bronchial asthma as defined by the Allergic Rhinitis and its Impact on Asthma (ARIA) criteria (1) (Table 1). All subjects without allergen-specific history (atopics) and those

with additional negative SPTs (nonallergic) served as controls. Each allergen was analysed separately. Five additional patients had been excluded after detecting a total serum-IgE > 1000 kU/l in order to minimize nonspecific binding in the CAP system (21).

Allergen-specific history and SPTs with allergen extracts

Standardized histories were obtained in all subjects at both study sites by one of the authors (KV) using a questionnaire that gave special regard to the clinical relevance of the sensitization to each allergen (e.g. clinical relevance of the sensitization to birch pollen was affirmed by asking for an oral allergy syndrome to apple and other Rosaceae fruits). A copy of the questionnaire in German language is available on request from the corresponding author.

The distinct flowering seasons of the corresponding plants supported judging the clinical relevance of the sensitization to the different pollen allergens. SPTs were performed with commercial, natural extracts (MUW: HAL Allergie GmbH, Düsseldorf, Germany; FAZ: ALK, Horsholm, Denmark). Sodium chloride (0.9%) and histamine hydrochloride (ALK) served as negative and positive controls, respectively. SPTs were read after 20 min. Wheals and flares were pen-marked, transferred to a paper with transparent adhesive tape and analysed with an investigator-independent system calculating the wheal size in mm² (22). Wheals of ≤ 7 mm² (corresponding to wheals of 3 mm diameter) were considered positive as recommended by the EAACI (<http://www.eaaci.net>) guidelines (23). There was no centre bias as wheal sizes to SPTs of the study population did not differ between the study centres (ANOVA, data not shown).

Laboratory analysis

Sera of all patients and controls were analysed for the presence of sIgE to whole-allergen extracts by the CAP (ImmunoCAP®; Pharmacia Diagnostics) and to defined allergen components by the allergen-microarray (ISAC® version CRD-50, VBC-GENOMICS, Vienna, Austria) according to each manufacturer's recommendations. The allergen-microarray contained either highly purified natural allergen components (HDM, cat) or recombinant allergen components (birch, grass, mugwort) and the assays were performed as essentially described by Deinhofer et al. (24). Briefly, the microarray slides were washed in TBS-T buffer (150 mM sodium chloride, 10 mM Tris base, and 0.5% Tween 20, pH 8.0) for 120 min to remove noncovalently bound allergens from the microarray surface; 20 µl of undiluted patient serum was applied directly to the individual reaction sites of the microarray, generated

Table 1. Allergies and symptoms in the study population

	Allergic to at least one study allergen	Allergy to house dust mite	Allergy to cat	Allergy to birch pollen	Allergy to grass pollen	Allergy to mugwort pollen
Patients	76	26	23	31	47	17
Symptoms						
Conjunctivitis	64 (84)	23 (88)	21 (91)	30 (97)	40 (85)	16 (94)
Rhinitis	74 (97)	26 (100)	23 (100)	30 (97)	45 (96)	17 (100)
Bronchial asthma	27 (36)	4 (15)	11 (48)	14 (45)	6 (13)	8 (47)
Atopic dermatitis	7 (9)	3 (12)	2 (9)	3 (10)	4 (9)	1 (6)
Urticaria/angioedema	9 (12)	4 (15)	2 (9)	3 (10)	5 (11)	3 (18)
Pollen-associated food allergy	19 (25)	5 (19)	5 (22)	16 (52)	12 (26)	6 (35)
Atopic family history	40 (53)	11 (42)	13 (57)	19 (61)	24 (51)	8 (47)
Total IgE (kU/l; mean $\pm$ SD)	189.5 $\pm$ 187	182.3 $\pm$ 191.7	210.3 $\pm$ 148.6	182.1 $\pm$ 191.4	202.3 $\pm$ 186.2	245.7 $\pm$ 231.4

For each allergen, the patient sera were analysed separately. 'Allergic' patients had positive allergen-specific history and SPT. Values are given in *n* (%).

by a pre-affixed mask of Teflon®. Following incubation in a humid chamber for 180 min, excess serum was removed by a washing step in TBS-T buffer and 20 µl of fluorescence-labelled anti-human IgE detection antibody was applied. After a 60-min staining reaction and a second washing step to remove unbound detection antibody, the slides were scanned in an Affymetrix 428 microarray scanner (Affymetrix, Santa Clara, CA, USA) with two different laser power settings to achieve a maximum dynamic range across the IgE reactivities of the serum samples. Both images of each spot were analysed using the GenePix image analysis software (Version 3.0.6.89; Axon Instruments, Union City, CA, USA). The total serum IgE levels were determined in all individuals with the CAP system according to the manufacturer's recommendations (Pharmacia Diagnostics).

Statistical analysis

The receiver-operating characteristics (ROC) serves as a graphic tool which shows sensitivity over 1 – specificity for all possible cut-off points. ROC curves were plotted for each allergen separately and the area under the curve was computed. Furthermore, a nonparametric test (25) was conducted to compare the area under the curve between the CAP (in kU/l) and the microarray (in kUA/l) results for each allergen, assuming a null hypothesis without a difference between the two areas, both variables equally discriminating.

SAS 8.02 (SAS Institute Inc., Cary, NC, USA) was used for the ROC analysis. *P*-values < 0.05 were considered as significant. The ROC plots were produced with the MedCalc 8.0 for Windows software (MedCalc Software, Mariakerke, Belgium).

Ethical considerations

All patients gave informed written consent for participating in this study. The study was approved by the local ethics committee.

Results

Demographic and clinical characterization of the study population

Of the 120 individuals with a suspected allergic rhinoconjunctivitis, 76 had a clinically relevant allergy to at least one of the studied allergens and were defined 'allergic' (Table 1). Rhinitis (74%, 97%) and conjunctivitis (64%, 84%) were the dominating symptoms in the allergic patients. According to the ARIA criteria (1), the severity was judged as mild in 25 and moderate–severe in 51 of the subjects. Twenty-seven patients reported additional bronchial asthma. Only seven patients suffered from atopic dermatitis probably because we had excluded patients with total IgE > 1000 kU/l for the known CAP system's lack of specificity in high total IgE sera (26). As the majority of the 'allergic' patients (46/76; 60.5%) were sensitized to more than one study allergen, we had sera from 26 HDM-, 23 cat-, 31 birch-, 47 grass- and 17 mugwort pollen-allergic patients available for analysis (details in Table 1).

Comparative ROC analysis of the microarray vs the CAP

Our aim was to evaluate the performance of component-based allergen microarrays under routine diagnostic

conditions. Hence, patients were tested *in vivo* (SPT) as well as *in vitro* (CAP) with natural allergen extracts but not with recombinant allergens. On the contrary, the allergen-microarray measured sIgE either to highly purified natural allergen components (HDM, cat) or recombinant allergen components (birch, grass, mugwort pollen).

To assess diagnostic performance, we performed ROC analyses by comparing the CAP results (natural allergen extracts) with the sum of the individual allergen components (HDM: Der p1 & 2; birch pollen: recombinant Bet v1 & 2, grass pollen: recombinant Phl p1, 2, 5, 6 & 7).

Remarkably, allergen-microarray and CAP performed at the same excellent level in the *in vitro* confirmation of clinically relevant allergies to cat, birch and grass pollen. This finding was reflected by the similar shapes of the ROC curves for these particular allergens (Fig. 1B–D). Moreover, the area under the curve for the ROC plots did not differ in the case of the three allergens (Table 2). For the diagnosis of HDM allergy, the microarray ROC analysis resulted in a slightly reduced sensitivity when compared with the CAP (Fig. 1A), while this difference was not statistically significant (*P* = 0.053, Table 2). *In vitro* confirmation of mugwort allergy was less efficient when compared with the CAP (microarray vs CAP, *P* = 0.032), a problem originating from the low sensitivity of the microarray (< 50%, Table 2) in the detection of mugwort-specific IgE, while still retaining a high level of specificity.

CRD in allergic patients, nonallergic patients and in asymptotically sensitized patients

In case of the HDM, birch pollen and grass pollen allergens, the microarray contained more than one allergenic component (natural/recombinant protein) allowing CRD in the corresponding patients. In HDM-allergic patients, nine of 26 patients were sensitized to the main component Der p1 and five to Der p2 (Table 3). No sIgE to Der p1 and Der p2 was detectable in 12 HDM-allergic patients suggesting that the clinical symptoms in these patients were caused by other HDM-specific components.

rBet v1 (24/31; 77.4%) was by far the dominating component in the birch-allergic patients and rPhl p1 (36/47; 76.6%) was the most important component in patients with grass allergy (Table 3). rPhl p2, 5 and 6 were nearly equally important while rPhl p7 played only a minor role in the grass pollen-allergic patients studied (Table 3).

Interestingly, the component-specific sensitization patterns in the patients categorized as 'allergic' did not differ from those in asymptotically sensitized 'atopic' patients with a positive SPT and a negative allergen-specific history for all three allergens (chi-squared test; cf. Table 3).

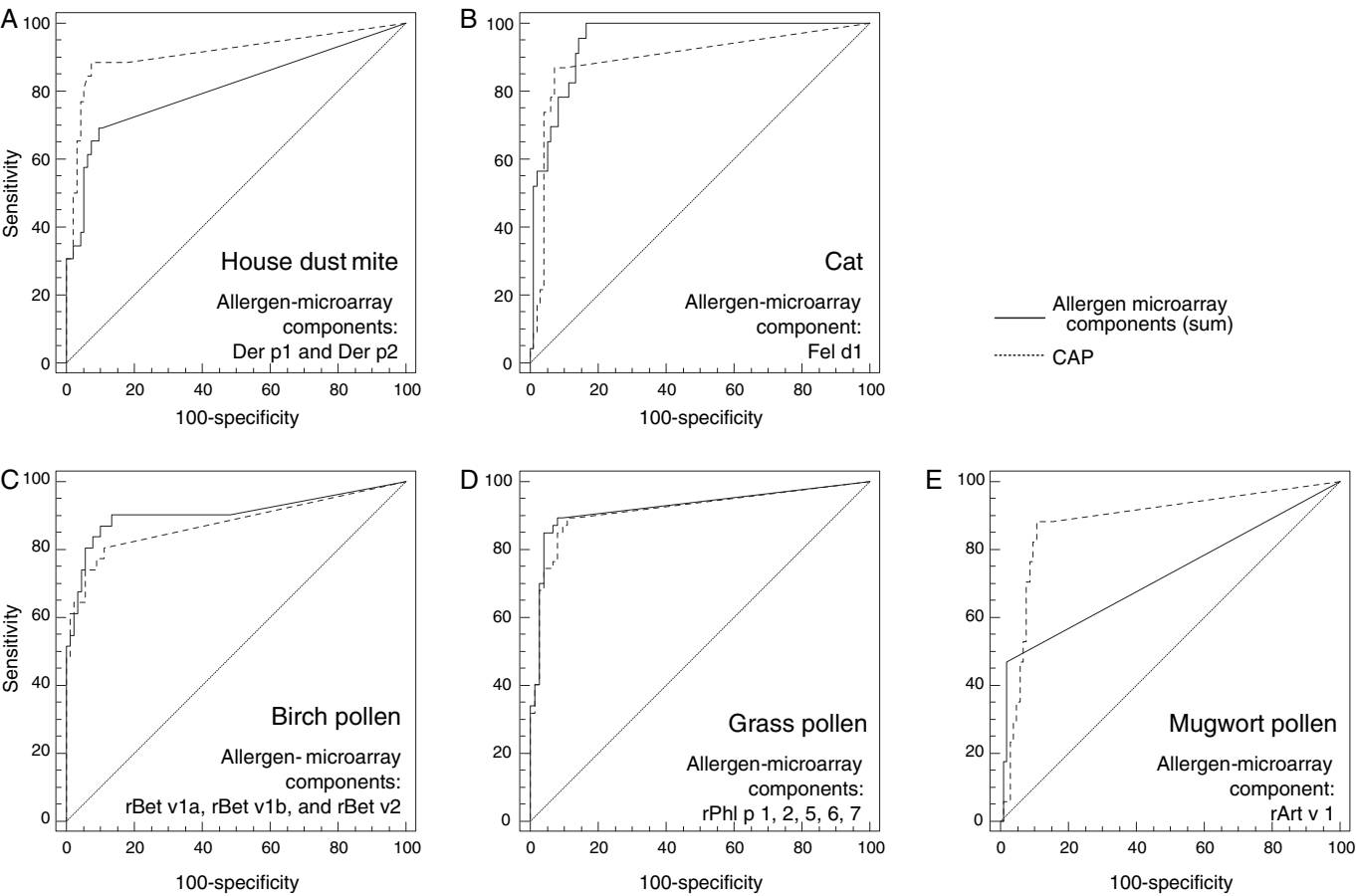


Figure 1. ROC curves for the five study allergens. The allergen-microarray and the CAP performed equally well in detecting clinical ‘allergy’ (according to Table 1) in cat-, grass pollen- and birch pollen-positive sera. The microarray was slightly less sensitive in house dust mite-allergic subjects and performed insufficiently in mugwort-allergic patients (details in Table 2).

Table 2. Comparative analysis of the performance of allergen-microarray vs CAP in allergic patients (plotted against ‘allergy’ defined according to the legend of Table 1)

Allergen	House dust mite	Cat	Birch pollen	Grass pollen	Mugwort pollen
Allergic/control (n)	26/94	23/97	31/89	47/73	17/103
ROC					
Microarray	0.808†	0.950	0.908†	0.923†	0.723
CAP	0.911	0.894	0.878	0.915	0.879
P-value	0.053	0.211	0.483	0.770	0.032
Microarray					
Sensitivity*	69.2 (48.2–85.6)†	78.3 (56.3–92.5)	87.1 (70.1–96.3)†	89.4 (76.9–96.4)†	47.1 (23.0–72.1)
Specificity*	90.4 (82.6–95.5)†	90.7 (83.1–95.7)	89.9 (81.7–95.3)†	90.4 (81.2–96.0)†	98.1 (93.1–99.7)
CAP					
Sensitivity*	88.5 (69.8–97.4)	87.0 (66.4–97.1)	77.4 (58.9–90.4)	87.2 (74.2–95.1)	88.2 (63.5–98.2)
Specificity*	90.4 (82.6–95.5)	90.7 (83.1–95.7)	88.8 (80.3–94.5)	90.4 (81.2–96.0)	89.3 (81.7–94.5)

*95% confidence interval.

†Sum of allergen components.

Upper part: The ROC curves were computed and a nonparametric test determined significant differences between the two methods (the ROC curves are displayed in Fig. 1). Lower part: The specificity was set as close as possible to 90% allowing to compare sensitivities between allergen-microarray and CAP.

Cut-off levels in the allergen-microarray system

For our study population, the standard cut-off value for the CAP system at 0.35 kU/l resulted in a sensitivity that

never exceeded 88.5% for any allergen when the specificity was set at 90% (Table 2). These findings suggest that the optimized cut-off level of the CAP system delivered false-negative results in about 10% of the patients for all

Table 3. Component resolved diagnosis in the three allergens (house dust mite, birch and grass pollen), where the allergen-microarray contained more than one component

Allergen component	House dust mite		Birch pollen		Grass pollen				
	Der p 1	Der p 2	Bet v 1	Bet v 2	Phl p 1	Phl p 2	Phl p 5	Phl p 6	Phl p 7
Allergic patients	26		31		47				
<i>n</i>	9	5	24	5	36	25	23	16	5
%	34.6	19.2	77.4	16.1	76.6	53.2	48.9	34.0	10.6
Atopic patients	11		6		6				
<i>n</i>	1	0	4	0	1	1	2	1	0
%	9.1	0.0	66.7	0.0	16.7	16.7	33.3	16.7	0.0
Controls	83		83		67				
<i>n</i>	1	1	1	2	1	1	1	1	0
%	1.2	1.2	1.2	2.4	1.5	1.5	1.5	1.5	0.0

Allergy defined by positive allergen-specific history plus positive SPT. *Atopic* denotes patients with negative history and an irrelevant positive SPT, whereas *Control* stands for negative history plus negative SPT.

Cut-off: 0.35 kUA/l.

Values are given in *n* (%).

five allergens when compared with the results obtained from positive history and SPT.

For the allergen-microarray, a CAP-derived arbitrary cut-off at 0.35 kUA/l was proposed by the manufacturer based on seroanalytical studies (27). Whether this cut-off value is ideal for the detection of clinically relevant allergies is unknown. To address this topic, we selectively analysed the single-component allergens – cat and mugwort pollen. According to our data – cat (Fel d 1): cut-off > 0.115 kUA/l, sensitivity 100.0 (95% CI: 85.0–100.0), specificity 83.5 (95% CI: 74.6–90.3); mugwort (Art v 1): cut-off > 0 kUA/l, sensitivity 47.1 (95% CI: 23.0–72.1), specificity 98.1 (95% CI: 93.1–99.7)] – lower cut-off levels appeared to increase the sensitivity without affecting the specificity.

Discussion

The component-based allergen-microarray was comparable with the ‘quasi standard’, the CAP system (3) in detecting clinically relevant allergies to the aeroallergens cat, birch and grass pollen. Although the sensitivity of the microarray was slightly lower in HDM allergy, when compared with the CAP, this difference did not reach statistical significance.

The study version’s layout of the allergen-microarray exhibited an insufficient sensitivity in detecting mugwort-allergic patients which in its current form cannot be recommended for routine outpatient settings. The low IgE reactivity observed for recombinant Art v 1 may result from the lack of crucial carbohydrate chains because of the expression of the protein in a bacterial host system. Recent data pointed out the importance of sugar side chains for IgE binding (28). On the other hand, the current microarray layout may require the addition of further allergens of mugwort pollen (e.g. Art v 2) to improve the sensitivity of the diagnosis (29).

In our investigation, the number of patients with clinically irrelevant positive sIgE was low (high specificity for all allergens without a loss in sensitivity; cf. Table 2). This may be explained by the highly preselected patient group and the exclusion of patients with high total serum IgE levels. The latter are known for having high levels of clinically irrelevant sIgE (21).

As our study aimed at comparing the overall sensitivity/specificity of the component-based microarray with CAP assays based on natural allergen, we made only limited use of the microarray’s immanent capacity of performing CRD. For three study allergens (HDM, birch, and grass pollen), the microarray contained more than one component and thus CRD was possible. In birch allergy, the predominance of rBet v1 over rBet v2 sensitization (Table 2) correlated well with previously published data from middle Europe (30). Notably, these two recombinant components sufficed for an equivalent sensitivity when compared with the whole-allergen CAP. The equal distribution among rPhl p1, 2, 5 and 6 in sera from grass-allergic patients was comparable with results from an Italian study by Mari (31). Remarkably, the lack of Phl p11, Phl p12 and Phl p4 did not reduce the sensitivity of the allergen-microarray in comparison with the extract-based CAP; 34.6% of the sera from HDM-allergic patients recognized highly purified Der p1 and 19.2% Der p2 (Table 3), which suggests that >40% of the patients were sensitized against many of the other known components (32). In a study on CRD in HDM-allergic subjects, Pittner et al. (33) showed that Der p4 constitutes another important component recognized by >70% of patient sera. Probably, the inclusion of Der p4 could further improve the microarray’s sensitivity in HDM-allergic subjects. The CRD in all three allergens did not differ between allergic and sensitized-only nonallergic patients (Fig. 2).

Overall, the allergen-microarray is a promising option not only for research but also for routine outpatient

settings. Twenty microlitres of serum is sufficient to determine a large number of sIgEs in a single measurement with high specificity. The allergen-microarray's immanent CRD may help to prepare customized formulations for SIT with allergen extracts and they will become advantageous as soon as recombinant allergens will become available for SIT.

Conclusion

Summing up the results of the single allergen components contained in an allergen-microarray test resulted in a sensitivity and specificity comparable with the well-validated whole-allergen CAP system in cat, birch and grass pollen allergy. The sensitivity of the allergen microarray was slightly reduced in HDM allergy whereas this was not statistically significant. The low sensitivity observed in mugwort-allergic sera showed that alternative (or, additional) mugwort allergen components have to be included in the test. Generally speaking, the clinical

relevance of each allergen needs to be validated separately prior to the implementation of multi-allergen panels into routine diagnostic settings.

Disclosure

This was an investigator-initiated study that was supported insofar that the determination of specific IgE with the ISAC[®] allergen-microarrays in all patients and with ImmunoCAP[®] in all patients from the FAZ was performed in the laboratories of VBC-GENOMICS, Vienna, Austria.

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