

Original article

Clinical usefulness of microarray-based IgE detection in children with suspected food allergy

Background: Component-resolved diagnostics using microarray technology has recently been introduced into clinical allergology, but its applicability in children with food allergy has hardly been investigated so far. The aim of this study was to evaluate the utility of microarray-based IgE detection in the diagnostic workup of food allergy and to compare this new diagnostic tool with established methods of allergen-specific IgE detection.

Methods: We investigated 130 infants and children with suspected allergy to cow's milk (CM) or hen's egg (HE). Serum IgE measurements, skin prick tests, allergen microarray assays and controlled oral food challenges with HE and CM were performed.

Results: We analyzed 145 oral challenges that served as reference parameters for assay performance assessment. On this basis, the panel of microarrayed allergen components was shown to represent a comprehensive repertoire of clinically relevant CM and HE proteins. Additionally, the implemented CM and HE components respectively sufficed for equivalent test performance as compared to the corresponding fluorescence enzyme immunoassay extract and skin testing. However, component-resolved diagnostics for HE and CM allergy did not make oral food challenges superfluous. Clinical IgE decision points predicting positive oral food challenges could be calculated for both *in vitro* test methods.

Conclusions: Allergen microarrays provide a new tool to diagnose symptomatic CM and HE allergy. They show performance characteristics comparable to the current diagnostic tests and may be indicated in small children in whom only small blood volumes are obtainable. However, they are not capable of replacing double-blind, placebo-controlled food challenges in most cases.

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Key words: allergen components; allergen-microarray; children; food allergy; immunoglobulin E.

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Accepted for publication 13 March 2008

Food allergy is a common disease and affects up to 8% of children in western countries. Moreover, it accounts for the majority of anaphylactic reactions in this age group (1, 2). Infants and toddlers with atopic eczema (AE) are particularly susceptible revealing symptoms of IgE-mediated food allergy in as many as 35% of cases (3). Therefore, a correct diagnosis of food allergic disorders is crucial, especially in pediatric patients with AE.

If reasonable suspicion of food allergy arises, several established *in vivo*- and *in vitro*-diagnostic instruments are available. While double-blind, placebo-controlled food challenges (DBPCFC) remain the clinical and scientific gold standard *in vivo*, fluorescence enzyme immunoassays (FEIA) represent the *in vitro* reference method of food

allergen-specific IgE detection (4). However, protein microarrays have recently been introduced into allergological research as promising tools for the simultaneous assessment of specific IgE antibodies (IgE) against multiple recombinant or purified natural allergen components in low amounts of patient serum. The main advantage of this array technology is the low amount of serum required for analysis, which allows usage of capillary blood-derived samples for IgE detection (5–8). Still, only very few investigators have discussed clinical applications of component-resolved diagnostics including microarrayed food allergens and their use in pediatric allergy trials has hitherto been limited to the study of peanut and soybean hypersensitivity (9, 10).

Thus, the aims of the present study were to assess the clinical performance of an allergen microarray containing purified natural allergen components for IgE detection in children with challenge-proven food allergy and to compare clinically relevant assay performance parameters of this allergen microarray to those of an established extract-based FEIA and skin prick testing (SPT).

Abbreviations: AE, atopic eczema; AUC, area under the curve; CDP, clinical decision point; CM, cow's milk; DBPCFC, double-blind, placebo-controlled food challenge; FEIA, fluorescence enzyme immunoassay; HE, hen's egg; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating curve; SPT, skin prick test.

Methods

Study population

We retrospectively studied 130 infants and children referred to the Charité allergy center for evaluation of suspected IgE-mediated food hypersensitivity. Oral food challenges with either cow's milk and/or hen's egg (HE) were performed as previously described (11). The food challenges were scored as positive by a pediatric allergologist if one or more of the following objective clinical reactions were noted: urticaria, flushing, pruritus, angioedema, exacerbation of AE, vomiting, diarrhea, stridor or other respiratory symptoms.

The study was approved by the local ethical committees of the medical faculty of the RWTH Aachen and the University Children's Hospital of Charité – Universitätsmedizin Berlin.

Blood sampling

Venous blood samples of all patients were collected prior to oral provocation tests and obtained serum probes were aliquoted and stored at -20°C until further analysis.

Skin prick tests

Skin prick tests were performed in 39 out of 60 patients with suspected HE allergy (65%) and 58 out of 85 patients with suspected CM allergy (68%). One drop of either fresh cow's milk 3.5% fat or native HE (whisked white of egg and yolk) was applied to the patient's forearm and 1 mm single-peak lancets (ALK, Copenhagen, Denmark) were used for SPT. Saline solution and histamine hydrochloride 1% (ALK) served as negative and positive controls. All reactions eliciting a mean wheal diameter of 3 mm or greater than the negative control read 15 min after application were considered positive.

Fluorescence enzyme immunoassays

Routine determination of specific IgE antibodies against CM and HE allergen extract was performed with a widely-used fluorescence enzyme immunoassay (UniCAPTM, Phadia, Uppsala, Sweden) according to the product manual. Specific IgE titres were quantified in protein units designated as kU/l with a lower detection limit of 0.35 kU/l as proposed by the manufacturer.

Allergen microarray analysis

We employed a commercially available allergen microarray assay (ISACTM; VBC Genomics Bioscience Research, Vienna, Austria), that has been shown to yield reliable analytical results when compared to FEIA in other clinical settings and with different sets of implemented inhalant allergen components (5, 12, 13).

This ambient analyte assay consists of a microscopy glass slide modified with a TeflonTM mask in order to create four individual reaction sites. These were coated with amine-reactive polymers allowing covalent immobilization of nutritional allergenic molecules. In the present study, we focused on a customized version of the microarray (ISACTM version CRD 51) containing the following eight purified natural HE- and CM-allergen components that had been purchased from Sigma-Aldrich (Vienna, Austria) and spotted onto the microarray in vertical triplicates: ovomucoid (Gal d 1), ovalbumin (Gal d 2), lysozyme C (Gal d 4), α -lactalbumin (Bos d 4), β -lactoglobulin (Bos d 5) and the caseins (α -casein, β -casein,

κ -casein). As indicated by the manufacturer, the degree of purity of the respective allergenic proteins was as follows: 80% (Gal d 1), 85% (Bos d 4, α -casein), >90% (Bos d 5, β -casein, κ -casein) and 95% (Gal d 2, Gal d 4).

Microarray immunoassays were performed according to the manufacturer's recommendations as recently published by Deinhofer et al. (6) and Harwanegg and Hiller (14). Briefly, each microarray reaction site was incubated with 20 μl of undiluted patient serum for 180 min in order to capture allergen-specific serum IgE antibodies by their corresponding allergen molecules. In a second step, the microarray slides were washed with a conventional TBS-T (Tris buffered saline/Tween) buffer solution two times for 5 min, rinsed with deionised water and dried under nitrogen flow. Hereafter, microarray-bound IgE was marked with a secondary, fluorescence-tagged antihuman IgE antibody for 60 min at room temperature. After a second washing procedure with TBS-T, the corresponding fluorescence signals were scanned at a 10 μm resolution using a conventional biochip reader (Scan Array ExpressTM; Perkin Elmer Life Sciences, Boston, MA, USA). Based on calibration sera of known specific IgE content, raw data analysis of the corresponding digitized microarray images was performed with the QUANTARRAYTM 3.1 software (Perkin Elmer Life Sciences) transforming image information into numerical data quantified as dimensionless fluorescence intensity (FI) values.

Statistical analysis

Receiver operating characteristic (ROC) analysis was performed in order to study the effect of varying IgE thresholds on the numerical outcome of both the allergen microarray results and FEIA analysis. Corresponding ROC curves were obtained by plotting the true positive rate, i.e. the rate of correctly identified positive DBPCFC results, against the false positive rate for all possible IgE cutoff-points.

As a measure of clinical usefulness, i.e. the test's performance in separating patients with and without food allergy, the according AUC (area under the ROC curve) values were computed for each allergen microarray component and FEIA results respectively. Sensitivity and specificity as well as positive (PPV) and negative predictive values (NPV) of both test systems were calculated using IgE cutoff-points leading to the highest accuracy, i.e. the maximum value for the sum of sensitivity and specificity. Furthermore, corresponding 95% confidence intervals (CI) of these diagnostic measures were calculated. For comparison of the AUC obtained by microarray analysis and FEIA testing, nonparametric paired Wilcoxon tests were conducted for each component as proposed by Hanley and McNeil (15). The global significance level was chosen to $\alpha = 0.5$. As this was an explorative study, no α -adjustment for multiple testing was carried out. Thus, P -values < 0.05 could be interpreted as statistically significant test results with respect to the observed study group.

As further clinically relevant assay parameters for both test systems, we determined 90%, 95%, and 99% predictive decision points for a positive oral food challenge at a given IgE level using the logistic regression analysis method proposed by Sampson et al. (16) employing the formula $P = 1/e^{-\alpha - \beta x} + 1$ where x is the continuous diagnostic test parameter, P is the probability for a positive diagnosis given the value x and α and β are the model parameters of the corresponding logistic regression model.

All statistical analyses were performed using the s PLUSTM 6.1 statistical software package (Insightful Corp., Seattle, WA, USA) and MEDCALCTM Version 6.1.1 (MedCalc Software, Mariakerke, Belgium) was used for plotting of ROC curves.

Table 1. Patient characteristics and description of reaction types during oral food challenge

	Median (range)	n	%
Age at oral food challenge (months)	14 (5–150)		
Atopic eczema		124	95
Gender			
Male		70	54
Female		60	46
Open oral food challenges		42	29
DBPCFC		103	71
Suspected CM allergy		85	59
Total serum IgE (kU/l)	213.5 (3.5–12085)		
CM specific IgE (kU/l)	2.81 (0.36–872)		
Suspected HE allergy		60	41
Total serum IgE (kU/l)	159.0 (6.0–3920)		
HE specific IgE (kU/l)	4.08 (0.37–57.2)		
Symptoms elicited by provocation			
Urticaria		51	35
Flushing		47	32
Pruritus		30	21
Angioedema		2	1.4
Exacerbation of AE		12	8
Vomiting		23	16
Diarrhea		12	8
Stridor		2	1.4

Results

All 130 patients included in our study underwent either DBPCFC or, particularly in young infants, open oral food challenges (Table 1). During 145 oral provocations, CM was administered in 85 patients eliciting 42 positive reactions (49%) while oral application of HE induced clinical symptoms in 45 out of 60 individuals (75%). Interestingly, further data analysis revealed that the association of the number of recognized allergen components and the total extract-specific IgE was highly significant as determined by Kruskal–Wallis-testing (Fig. 1A,B).

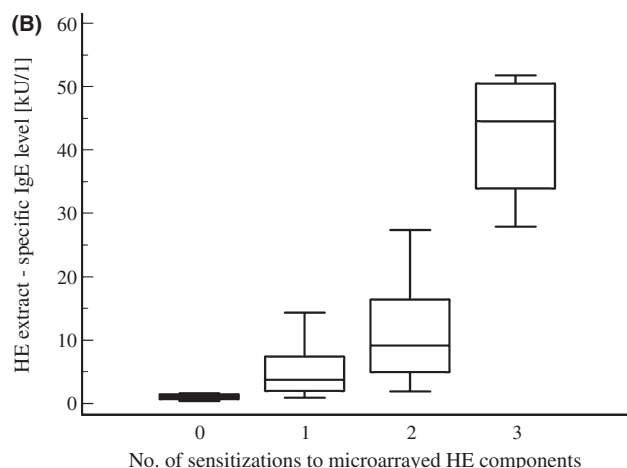
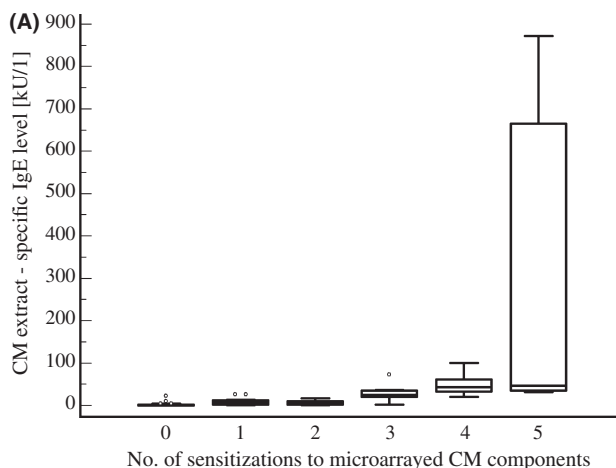


Figure 1. Box-and-whisker plots demonstrating the association of the number of recognized microarray allergen components and the total extract-specific IgE for CM (A) and HE (B). The mean ranks of extract-specific IgE levels are significantly different ($P < 0.05$) among all groups as determined by Kruskal–Wallis-testing.

The observed outcomes of oral food challenges were considered as reference parameters allowing performance evaluation by ROC analysis for both *in vitro* test systems and skin testing (Figs 2 and 3). Tables 2 and 3 display in detail all relevant assay performance parameters of FEIA analysis, microarray testing and SPT at the respective cut-off points related to optimal ROC accuracy values. The combination of all allergen microarray results generated the same or highly similar AUC values as compared to FEIA testing in both HE and CM allergy diagnosis. Comparison of FEIA results with each single microarray component by nonparametric Wilcoxon testing revealed no statistically significant differences between AUC values with the exception of microarray component Gal d 4 (HE lysozyme) yielding a manifestly smaller AUC ($P = 0.044$) than HE extract used for FEIA analysis. One single allergen component of each allergen source, i.e. Bos d 4 and Gal d 1, generated similar AUC results as the respective microarray component combination or FEIA testing while highest AUC values were obtained by SPT with native CM and HE preparations.

We additionally calculated clinical decision points (CDP) for a positive outcome of oral food challenges that have been considered valuable parameters in the diagnosis of pediatric food allergy (11, 16, 17). As a result, FEIA and combined HE and CM microarray data predictive for positive oral food challenges in 90%, 95% and 99% of patients in different age groups could be identified and are demonstrated in Table 4.

Discussion

Currently, no single *in vivo* or *in vitro* test system permits a clear-cut diagnosis of food allergy. Diagnostic decision points have been evaluated for few allergens; however,

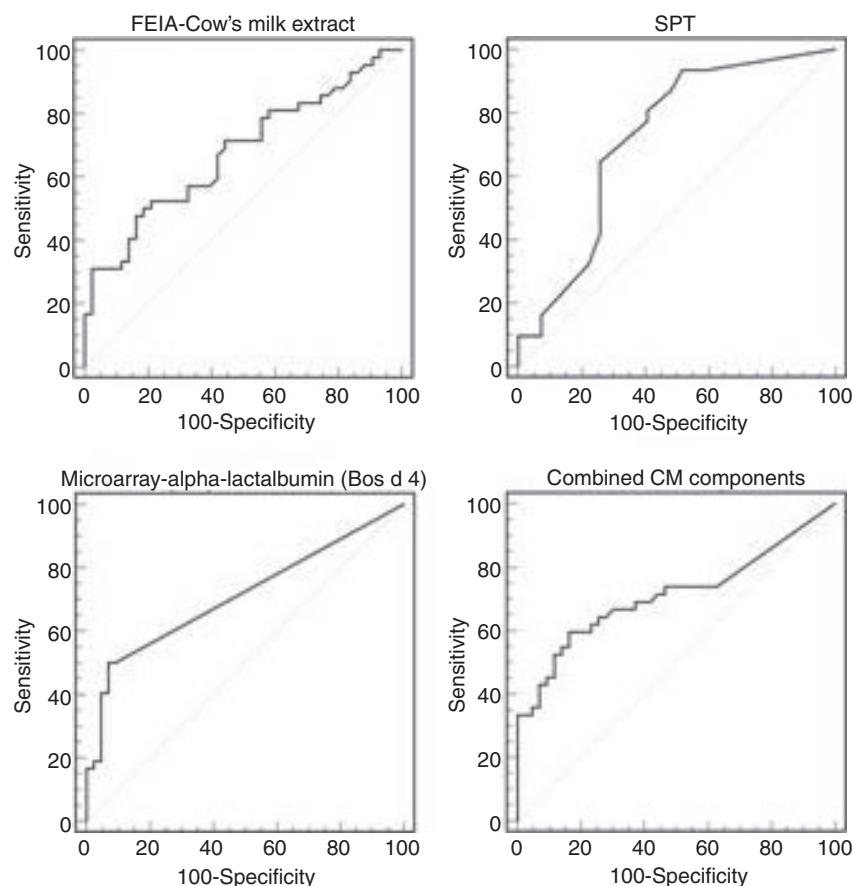


Figure 2. ROC curves for CM extract-based FEIA, allergen microarray component Bos d 4, combined CM microarray results and skin prick tests with native CM.

oral food challenge tests become superfluous only in a small number of individuals (18). In general, controlled oral provocation tests can be regarded as the most reliable indicator of food hypersensitivity (3). Therefore, the assay performance of a novel diagnostic *in vitro* tool is most accurately validated in patients whose allergy status has been established by this diagnostic gold standard (19).

Investigating 130 infants and children with challenge-proven/-excluded food allergy, we evaluated microarray-based IgE detection and directly compared this technique with conventional FEIA and SPT. Therefore we established individual IgE profiles demonstrating that microarrayed CM and HE proteins respectively sufficed for equivalent test performance as compared to the corresponding FEIA extract and skin testing. However, no single allergen component was found to be a superior discriminator between clinically irrelevant sensitization and genuine food allergy and there was no possibility to identify single marker allergens that significantly enhanced *in vitro* test performance. In contrast, HE lysozyme (Gal d 4) was identified as an insufficient indicator of clinical reactivity in our study which is in disagreement with Walsh and co-workers who described this HE component as an important allergen in a

subgroup of investigated patients. These conflicting results are most likely due to the fact that the aforementioned study only included patients with an unequivocal clinical evidence of egg hypersensitivity. Thus, a control group consisting of patients with negative food challenge outcomes was not included which might have caused an elevated rate of false positive results (20).

With one of the largest patient samples to date we assessed component-resolved diagnostics (CRD) in food allergy utilizing microarrayed HE and CM proteins. Interestingly, CRD-based investigations that have recently been undertaken for a broad range of nutritional allergens such as apple (21), cherry (22), hazelnut (23), carrot (24) and particularly wheat (25, 26) revealed that component-resolved IgE recognition patterns allowed to distinguish between patients with symptomatic food allergy and those who were sensitized but clinically tolerant. Unfortunately, data on CRD in CM and HE allergic patients are currently scarce. With regard to CM proteins, Garcia-Ara et al. identified CM-allergic patients developing tolerance by establishing their IgE antibody response to Bos d 4, Bos d 5 and Bos d 8 while Beyer and co-workers ascertained disease persistence by screening cow's milk allergic children with a panel of micro-

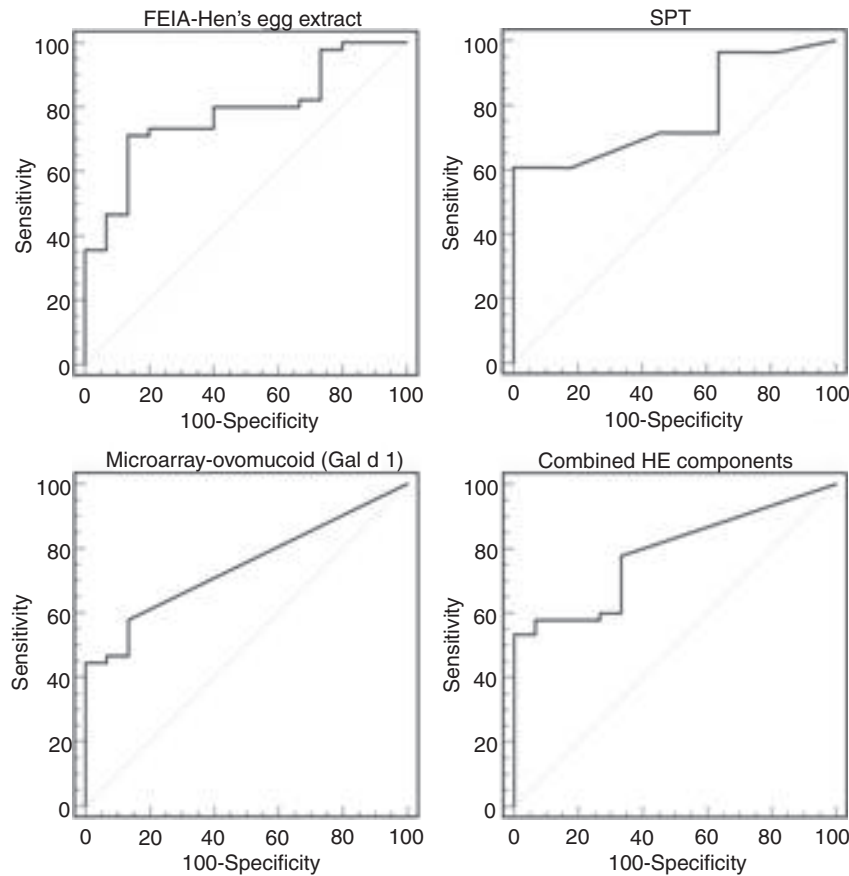


Figure 3. ROC curves for HE extract-based FEIA, allergen microarray component Gal d 1, combined HE microarray results and skin prick tests with native HE.

Table 2. Summary of clinically relevant assay performance parameters of allergen microarrays, fluorescence enzyme immunoassays and skin prick tests in the diagnosis of CM allergy

Allergen Microarray							FEIA	SPT
Allergen	α -casein	β -casein	κ -casein	Bos d 4	Bos d 5	Combination*	CM extract	Native CM
Cut-Off [†]	0.1	0.1	0.2	0.1	0.1	0.5	8.1	3.0
AUC	0.6 (0.5–0.7)	0.6 (0.5–0.7)	0.6 (0.5–0.7)	0.7 (0.6–0.8)	0.6 (0.6–0.8)	0.7 (0.6–0.8)	0.7 (0.6–0.8)	0.7 (0.6–0.8)
Sensitivity (%)	26.2 (13.9–42.0)	26.2 (13.9–42.0)	38.1 (23.6–54.4)	50.0 (34.2–65.8)	23.9 (12.1–39.5)	59.5 (43.3–74.4)	51.2 (35.5–66.7)	93.6 (78.5–99.0)
Specificity (%)	97.7 (87.7–99.6)	93.0 (89.9–98.5)	88.4 (74.9–96.1)	93.0 (80.9–98.5)	95.3 (84.2–99.3)	83.7 (69.3–93.2)	81.4 (66.6–91.6)	48.2 (28.7–68.0)
PPV (%)	91.7	78.6	76.2	87.5	83.3	78.1	73.3	67.4
NPV (%)	57.5	56.3	59.4	65.6	56.2	67.9	62.5	86.7
P-value [‡]	0.29	0.27	0.43	0.61	0.18	0.5	–	–

*Combination of all fluorescence intensity values of single CM allergen components.

[†]Allergen microarray (FI), FEIA (kU/l) and SPT (wheal diameter) results with 95 % confidence intervals corresponding to the highest accuracy value obtained by prior ROC analysis.

[‡]P-values of non-parametric paired Wilcoxon tests of FEIA and allergen microarray results with respect to AUC values.

arrayed casein peptides (17, 27). Additionally, Walsh and co-workers provided data on IgE sensitization against a comprehensive panel of egg white and egg yolk allergens in egg allergic children (20). Yet, these descriptive or prognostic studies did not address the association of IgE

recognition patterns with current clinical reactivity as defined by positive oral food challenges.

Although we could not show that testing with singular allergen components was superior to FEIA analysis, the respective combination of microarrayed CM and HE

Table 3. Summary of clinically relevant assay performance parameters of allergen microarrays, fluorescence enzyme immunoassays and skin prick tests in the diagnosis of HE allergy

Allergen Microarray					FEIA	SPT
Allergen	Gal d 1	Gal d 2	Gal d 4	Combination*	HE extract	Native HE
Cut Off	> 0	> 0	> 0	0.2	2.9	9.0
AUC [†]	0.8 (0.6–0.9)	0.77 (0.6–0.8)	0.6 (0.5–0.7)	0.8 (0.7–0.9)	0.8 (0.7–0.9)	0.8 (0.6–0.9)
Sensitivity (%)	57.8 (42.2–72.3)	57.8 (42.2–72.3)	17.8 (8.0–32.1)	53.3 (37.9–68.3)	71.1 (55.7–83.6)	60.7 (40.6–78.5)
Specificity (%)	86.7 (59.5–98.0)	80 (51.9–95.4)	100 (100)	100 (100)	86.7 (59.5–98.0)	100.0 (71.3–100.0)
PPV (%)	92.9	89.7	100	100	94	100.0
NPV (%)	40.6	38.7	28.8	41.7	50	50.0
P-value [‡]	0.61	0.2	0.044	0.98	–	–

*Combination of all fluorescence intensity values of single HE allergen components.

[†]Allergen microarray (FI), FEIA (kU/l) and SPT (wheal diameter) results with 95 % confidence intervals corresponding to the highest accuracy value obtained by prior ROC analysis.

[‡]P-values of non-parametric paired Wilcoxon tests FEIA and allergen microarray with respect to AUC values.

Table 4. Clinical decision points indicating a 90%, 95% and 99% probability of reacting to CM and HE on oral food challenge at a given allergen microarray or FEIA result

	CM (n = 85)				HE (n = 60)			
	n	90%	95%	99%	n	90%	95%	99%
FEIA (kU/l)								
All patients	85	52.66	66.90	98.39	60	7.72	10.18	15.62
≤ 24 months	62	46.67	61.66	94.78	41	6.12	8.55	13.90
>24 months	23	67.52	78.76	103.57	19	9.94	12.34	17.64
Microarray (FI)								
All patients	85	13.41	17.05	25.10	60	0.35	0.48	0.77
≤ 24 months	62	2.94	3.80	5.70	41	0.24	0.36	0.611
>24 months	23	35.54	41.45	54.51	19	0.63	0.80	1.18

proteins performed equally well as the corresponding extract. As illustrated by highly similar or even identical AUC values and the high degree of association between IgE reaction patterns obtained by the two *in vitro* test methods, the panel of allergen components investigated in our study can be regarded as a comprehensive repertoire of clinically relevant CM and HE proteins. This view is also strengthened by the fact that the number of sensitizations to single microarray components is closely correlated to the respective extract-specific serum IgE level as displayed in Fig. 1. Moreover, these findings are corroborated by other research groups who characterized the majority of allergenic structures implemented on the allergen microarray as potent antigens in prior allergenicity studies (20, 28).

Another crucial point of disease management in food allergic patients consists in the capacity to predict whether a potentially allergic patient will react after ingestion of the incriminated food allergen. In this context, positive predictive values (PPV), which could also be calculated for both IgE detection methods used in our study, are known to critically depend on the disease prevalence in the tested patient population. Hence, logistic regression

analysis was performed in order to define clinical decision points indicating the amount of allergen-specific IgE associated with a prechosen probability of positive oral food challenges in different age groups.

Interestingly, the obtained FEIA-based IgE level for a 95% probability of clinical reactivity to HE (10.18 kU/l) was similar to that identified previously by us and other study groups (7.0 and 12.6 kU/l) (11, 16), whereas 99% cut-off values for the same allergen were either not stated (16) or significantly higher (59.2 kU/l) in previous investigations (11). In contrast, clinical decision points predicting a 95% probability of allergy to CM were either markedly lower (15 kU/l) (17) as compared to our current data (66.9 kU/l) or could not be calculated at all (16). The main difference between our previously performed study and the current investigation is that in the previously performed study all children with suspected food allergy were included independent of the food-specific IgE level whereas in the current investigation all children had suspected IgE-mediated food allergy. Moreover, the variability of sIgE decision points is possibly due to an interaction of clinical factors influencing pretest probability. It can be hypothesized that study population

referral bias has led to an overrepresentation of children with moderate to severe atopic dermatitis, a group of patients known to suffer from food allergic reactions in up to one-third of cases (3). Moreover, in contrast to the majority of previous studies, the definite diagnosis of food allergy had been established by oral food challenges in all children included in our investigation. In addition, sIgE threshold levels have been found to be lower in younger patients indicating different degrees of pretest probability according to age (17). This is in agreement with our results obtained in children below and above two years of age (Table 4). As a consequence, these data clearly underline that clinical sIgE decision points still have to be interpreted with caution and that further large-scale studies are warranted before their routine application in clinical practice can be recommended.

The novel finding that IgE decision points can also be determined by using combinations of microarrayed HE and CM allergen components underlines the clinical potential of this new *in vitro* test method. In our study, microarray data qualitatively corresponded to FEIA results especially with regard to significantly higher cut-off points for CM as compared to HE proteins and similar intervals between the 90%, 95% and 99% probability thresholds. This clearly implies that microarray-based IgE quantification was accurate in predicting clinical reactivity to allergenic proteins in our study population. However, further investigations with a larger number of patients will have to evaluate the clinical implications of this finding especially with regard to the highly standardized and widely-used standard method of FEIA-based IgE detection.

These results are in accordance with data recently published by Wohrl et al. who assessed the clinical utility of a similar protein microarray containing inhalative allergen components (13). The authors performed ROC analyses matching combinations of microarrayed, recombinant birch pollen and grass pollen components with

corresponding FEIA allergen extracts. As in our investigation, the accordant AUC values did not differ clearly between the two diagnostic methods while the overall AUC levels of both test systems were distinctly higher reaching a maximum of 0.92 and 0.91, respectively. Besides the obviously distinct allergen sources, we believe that this disparity is best explained by different inclusion criteria, particularly because Wohrl et al. recruited patients on the basis of a suggestive history and positive SPTs alone. Additionally, unlike recombinant allergens, the natural HE and CM allergen components implemented on our microarrays were of limited purity which might have influenced test accuracy by contamination with other allergens or nonallergenic proteins.

Furthermore, our data demonstrate that the detection of specific IgE antibodies against multiple microarrayed allergen components can be performed with minimal volumes of patient serum that are easily obtained by capillary blood sampling (8). Thus, allergen microarrays might serve as low-invasive tools for allergen-specific IgE detection avoiding peripheral venipuncture which is known to cause distress especially in infants and children.

In conclusion, allergen microarrays provide a new tool to diagnose symptomatic CM and HE allergy. They show performance characteristics comparable to the current diagnostic tests and may be indicated in small children in whom only small blood volumes are obtainable. However, they are not capable of replacing double-blind, placebo-controlled food challenges in most cases.

Acknowledgments

This work was supported by the START program of the Medical Faculty of the Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen. Hagen Ott received a research grant from the Rotationsprogramm of the Medical Faculty of the RWTH Aachen.

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