

Hagen OTT¹
 Regina FÖLSTER-HOLST²
 Hans F. MERK¹
 Jens Malte BARON¹

¹ Department of Dermatology
 and Allergology, University Hospital
 Aachen, Pauwelsstrasse 30, D-52074
 Aachen, Germany

² Department of Dermatology, Venerology
 and Allergology, University Hospital
 Schleswig-Holstein, Campus Kiel, Germany

Reprints: H. Ott
 <hagen.ott@post.rwth-aachen.de>

Allergen microarrays: a novel tool for high-resolution IgE profiling in adults with atopic dermatitis

Microarray technology has recently been introduced into clinical allergology, but its applicability in adult patients with atopic dermatitis (AD) has not been investigated so far. We aimed to evaluate the utility of allergen microarrays in the diagnostic workup of adults with AD and to compare this new diagnostic tool with a conventional method of specific IgE (sIgE) measurement. In sera of 40 atopic adults, sIgE levels detected by microarray-analysis were correlated with the results of an established fluorescence enzyme immunoassay (FEIA). In a further 20 patients with AD, individual sIgE recognition patterns were established by microarray analysis and evaluated with regard to clinical features of AD. Allergen microarray and FEIA results were significantly correlated ($r = 0.72-0.99$), especially if recombinant allergens were employed. AD patients revealed a mean of 21 sensitizations per individual (range 1-46) and 65% displayed sIgE against cross-allergens, particularly pathogenesis related proteins such as the major allergen of birch pollen (Bet v 1), alder pollen (Aln g 1), apple (Mal d 1) or celery (Api g 1). The current study provides evidence that allergen microarrays represent a promising tool for component-resolved diagnosis in patients with AD. However, further large-scale studies in unselected patient populations are needed before the introduction of allergen microarrays into daily clinical practice.

Key words: microarray, atopic dermatitis, IgE, allergen component

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With a prevalence of up to 25% among children and 3% in adults, atopic dermatitis (AD) has become one of the most common dermatoses in industrialized countries [1]. Substantial scientific efforts have resulted in a better understanding of its complex pathogenesis, which involves genetically determined epidermal barrier dysfunction and characteristic immunological deviations. The latter include raised numbers of circulating regulatory T cells, eosinophilia, T helper type 2 (Th2)-skewed cytokine patterns and, in particular, elevated serum levels of total (tIgE) and allergen-specific IgE (sIgE) [2, 3]. Hence, a correct diagnosis of IgE-mediated sensitization is mandatory in patients affected by AD and if suspicion arises, several established *in vivo* and *in vitro* tools are readily available for sIgE determination [4, 5].

Furthermore, protein microarrays have recently been introduced into allergological research as promising tools

for the assessment of sIgE in patients with atopic diseases [6-9]. Moreover, we have previously demonstrated in a proof of principle study that microarray-based detection of sIgE against nutritive proteins is clinically useful in children with food allergy [10]. Still, the clinical implications of component-resolved diagnosis (CRD) including microarrayed inhalant and food allergens have hitherto not been investigated in adult patients suffering from AD. Thus, it was the principal objective of the present study to assess the clinical usefulness of an allergen microarray containing recombinant and highly purified natural allergen components for sIgE detection in adults with AD. Additionally, we aimed to correlate the assay performance of this allergen microarray to that of an established component-based fluorescence enzyme immunoassay (FEIA).

Materials and methods

Study design

The first part of this investigation was drafted as a retrospective proof-of-principle study performed in sera of adults with respiratory allergies. In order to assess the microarray's assay performance, we correlated sIgE titres against a panel of allergen components that were deter-

Abbreviations:

AD	atopic dermatitis
CRD	component-resolved diagnosis
FEIA	fluorescence enzyme immunoassay
SCORAD	severity scoring of atopic dermatitis index
sIgE	allergen-specific immunoglobulin E
tIgE	total serum immunoglobulin E

mined simultaneously by microarray and FEIA analysis. In the second part of this investigation, we prospectively evaluated the feasibility and clinical applicability of allergen microarrays in patients with AD.

Patients

The retrospective microarray performance analysis was performed in stored serum samples of 40 adult patients with previously diagnosed allergic airway disease who were known to exhibit at least one IgE-mediated sensitization against pollen or house dust mite allergens.

In the second part of this study, we prospectively recruited a total of 20 consecutive adults who had been referred to our centre of dermatology and allergology for the evaluation of suspected allergic skin disease. In all included patients, the diagnosis of AD was established by a dermatologist performing a thorough clinical examination and applying a structured questionnaire adhering to the Hanifin and Rajka diagnostic criteria for atopic dermatitis [11]. Grade of illness was classified according to the severity scoring of AD (SCORAD) index devised by the European Task Force on AD [12]. Data processing was performed in compliance with ethical standards on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the local ethical committee of the Medical Faculty of the RWTH Aachen.

Fluorescence enzyme immunoassays

Venous blood samples of all patients were collected during the first patient visit to our department and the serum probes obtained were aliquoted and stored at -80°C until further analysis.

Determinations of tIgE and sIgE levels were performed with a widely used, commercially available fluorescence enzyme immunoassay (FEIA) as proposed by the manufacturer (UniCAPTM, Phadia, Uppsala, Sweden). For comparison with microarray data, we determined sIgE titres against a panel of 6 timothy grass pollen (*Phleum pratense*, Phl p) and birch pollen (*Betula verrucosa*, Bet v) allergen components as well as a house dust mite (*Dermaphagoides farinae*, d. farinae) and a cat dander allergen extract purchased from the same supplier (manufacturer's code): Phl p 1 (g205), Phl p 5 (g215), Phl p 6 (g209), Phl p 7 (g210), Phl p 1 (g6), Bet v 1 (1215), d. farinae (d2), cat dander (e1). Total and specific IgE levels were quantified in protein units designated as kU/L with a lower detection limit of 0.35 kU/L.

Protein microarray test procedure

We employed a commercially available allergen microarray system (ImmunoCAP ISACTM, Phadia, Uppsala, Sweden), that has been shown to yield reliable analytical results when compared to fluorescence enzyme immunoassays in other clinical settings and with different sets of implemented inhalant or food allergen components [10, 13, 14]. This ambient analytical assay consists of a microscopy glass slide modified with a TeflonTM mask in order to create 4 individual reaction sites. These were coated with amine-reactive polymers allowing covalent immobilization of the 94 allergenic proteins investigated in the present study.

Microarray immunoassays were performed according to the manufacturer's recommendations as recently published by Deinhofer and co-workers [15]. Briefly, each microarray reaction site was incubated with 20 μL of undiluted patient serum for 180 minutes 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 five minutes, rinsed with de-ionised water and dried under nitrogen flow. Hereafter, microarray-bound IgE was marked with a secondary, fluorescence-tagged antihuman IgE antibody for 60 minutes 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). 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, Boston, MA) transforming the image information into numerical data, yielding four semi-quantitative classes of (negative, low, moderate, high) sIgE levels.

Statistics

The data obtained were expressed as mean $\pm$ standard deviation (SD) and range, unless otherwise indicated. The serum sIgE levels were used as response variables during linear regression analysis. Association of microarray and FEIA data was assessed by the Pearson correlation coefficient (r_p). Two-sided unpaired Students t-tests were performed for group comparisons and results were considered significant at $p < 0.05$, respectively. All statistical analyses were performed using the S PlusTM 6.1 statistical software package (Insightful Corp., Seattle, USA) and SigmaPlot 2004TM Version 9.01 (Systat Software, Erkrath, Germany) was used for graphing vertical bar charts as well as scatter plots.

Results

Correlation of conventional enzyme immunoassay and allergen microarray data

Sera of 40 atopic adult patients with extrinsic bronchial asthma and/or allergic rhinitis were investigated in the first part of our study. The mean age of individuals included (19 male, 21 female patients) was 38.4 ± 14.7 years (range 19-74 yrs). All patients showed increased sIgE levels against at least one inhalant allergen as detected by the UniCAPTM system, while tIgE levels ranged from 19.3 to 2000 kU/L (mean 704.1 kU/L, ± 759.1 kU/L). The complete panel of analyzed allergens and specific IgE reactivity patterns of this patient collective, assessed by FEIA testing, are displayed in table 1.

The diagnostic accuracy of the allergen microarray, as defined by correlation with the conventional FEIA system, was calculated by matching the respective recombinant allergen components in performance analysis, if possible. However, in the case of 2 allergens (*i.e.* d. farinae, cat dander) crude allergen extracts had to be used in the FEIA analysis because the corresponding recombinant

Table 1. Fluorescence enzyme immunoassay (FEIA) data obtained from venous serum samples of 40 atopic adults. Specific IgE (sIgE) serum levels are subdivided into 7 semiquantitative classes (CAP). Positive results are indicated for allergen components and FEIA classes respectively

CAP	sIgE (kU/L)	rBet v1	rPhl p1	rPhl p5	rPhl p6	rPhl p7	nDer f1	nFel d1	Total
0	< 0.35	4	11	15	19	31	8	19	107
1	0.35 ≤ 0.7	1	4	1	0	3	3	4	16
2	0.7 ≤ 3.5	6	2	2	8	3	7	4	32
3	3.5 ≤ 17.5	4	9	14	9	2	8	5	49
4	17.5 ≤ 50	15	6	2	2	0	5	6	36
5	50 ≤ 100	6	4	5	2	0	2	2	21
6	> 100	4	4	1	0	1	7	0	17
IgE pos ¹		36	29	25	21	9	32	21	173

¹ IgE pos: number of positive results for each allergen component.

Table 2. Correlation between fluorescence enzyme immunoassay (FEIA) and allergen microarray results in venous serum samples of 40 atopic adults. 5 recombinant (r) allergen components were matched in microarray and FEIA analysis, 2 natural (n) microarray components were correlated to their corresponding FEIA allergen extracts

Allergen source	Microarray allergen (component)	FEIA allergen (component)	FEIA allergen (extract)	r _p ¹
Birch pollen	rBet v 1	rBet v 1	-	0.81
Timothy grass pollen	rPhl p 1	rPhl p 1	-	0.92
	rPhl p 5	rPhl p 5	-	0.89
	rPhl p 6	rPhl p 6	-	0.89
	rPhl p 7	rPhl p 7	-	0.99
d. farinae	nDer f 1	-	d. farinae	0.72
cat dander	nFel d 1	-	cat dander	0.76

¹ r_p: Pearson correlation coefficient.

allergen components (Der f 1, Fel d 1) were not commercially available for UniCAP™ testing at the time of this investigation. Using this approach, the correlation coefficients obtained showed slight variations with regard to different allergen components, as shown in *table 2*. Nevertheless, a high degree of correlation ($r \geq 0.8$) could be demonstrated in the case of all recombinant allergen components, with a particularly high correlation coefficient in the case of timothy grass pollen components rPhl p 1 ($r = 0.93$) and rPhl p 7 ($r = 0.99$). Less correlation between the two methods was detected comparing the purified natural microarray allergens Der f 1 and Fel d 1 with their corresponding FEIA allergen extracts ($r = 0.72$ and $r = 0.76$ respectively).

Allergen-specific IgE profiling in adult patients with AD

20 adult patients (10 male, 10 female) with a mean age of 43 ± 18 years (range 19-73) entered this prospective study. All patients suffered from AD and 10 individuals (50%) displayed additional symptoms of allergic respiratory disease (bronchial asthma, allergic rhinitis) while contact allergy was observed in 5 patients (25%). Three individuals (15%) reported anaphylactic reactions after food ingestion: peanut (patient 1), fish (patient 8) and shrimp (patient 9). At a mean SCORAD score of 60 ± 23 (range 18-92), 6 patients (30%) displayed mild or moderate AD severity ($\text{SCORAD} \leq 50$), whereas a total of 14 patients (70%) suffered from severe AD ($\text{SCORAD} > 50$).

Microarray-based assessment of sIgE recognition patterns disclosed a mean of 21 ± 11 (range 1-46) sensitizations

per patient composed of 11 ± 7 (range 1-25) sIgE responses to plant allergens and 10 ± 6 (range 1-21) sensitizations to non-plant allergens. Positive sIgE results in more than 50% of the patients investigated were elicited by 9 plant proteins (nCyn d 1 [75%], rPhl p 1 [65%], rCor a 1 [55%], rPru p 1 [55%], rAln g 1 [55%], rBet v 1 [55%], Ara h 8 [50%]) and 8 non-plant proteins (rAlt a 1 [90%], rFel d 1 [65%], nDer p 2 [65%], rDer f 2 [65%], nDer f 1 [60%], nDer p 1 [60%], rCan f 1 [55%], rAsp f 6 [50%]). In contrast, sIgE could not be detected at all in the case of 6 microarrayed plant allergens (nAct d 1, nAct d 5, rBer e 1, rTri a 19, rHev b 3, rHev b 5) and 3 non-plant allergens (nBos d 5, nGal d 5, rBla g 5) (*figures 1A, B*). The total number of sensitizations was shown to positively correlate with SCORAD scores ($r_p = 0.46$) (*figures 2, 3*) and serum tIgE levels ($r_p = 0.45$), respectively.

We also analyzed sIgE repertoires against the following groups of plant and non-plant molecules that have previously been described as potentially cross-allergenic [16-18]: pathogenesis-related proteins (PR-10), profilins, lipid transfer proteins (LTP), parvalbumins, cysteine proteases, tropomyosins, as well as the house dust mite minor allergens nDer f 2 and nDer p 2, both belonging to the group of Niemann Pick type C2 (NPC2) proteins (*figures 1A, B*). As a result, 13 patients (65%) displayed sIgE cross-reactivity against PR-10 proteins. Simultaneously, cross-reactions were elicited by NPC2 proteins in 13 patients (65%), of whom 12 individuals (60%) also mounted sIgE against ≥ 2 cysteine proteases. Profilins, tropomyosins, LTP and parvalbumins elicited *in vitro* cross-reactivity in a minority of patients ($n \leq 3$), only.

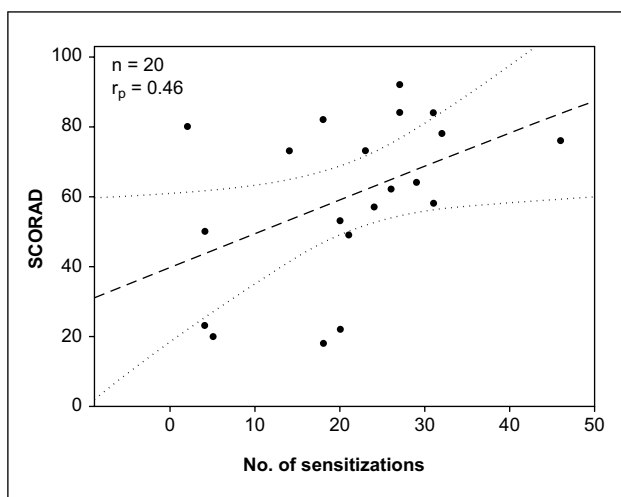


Figure 1. Correlation of SCORAD scores and the total number of sIgE-mediated sensitizations in 20 adult patients with AD. r_p = Pearson's correlation coefficient.

Group comparison of patients sensitized and non-sensitized to PR-10 proteins revealed a statistically significant difference with regard to disease severity, while this could not be observed for NPC2 (figure 4).

Discussion

Component-resolved diagnosis (CRD) has been reported to facilitate the determination of individual sIgE recognition patterns in patients with immediate-type allergic diseases [19-22] [3, 13-16]. In this context, proteomic microarrays have recently been proposed as diagnostic tools of high potential, suitable for large-scale CRD [13, 14]. However, studies directly comparing the test performance of microarrays and established *in vitro* sIgE detection methods are still scarce. Even more importantly, it has hitherto not been elucidated whether microarrays of recombinant and natural allergen components represent a viable mode of sIgE determination in adults suffering from AD.

Hence, in order to ensure feasibility and sufficient clinical performance of the allergen microarray employed, we compared the sIgE profiles obtained to those detected by an established FEIA. Viewing this first part of our investigation as a proof of principle study, we were able to further corroborate previous reports demonstrating high degrees of correlation between allergen microarray and FEIA data [19]. This was particularly evident in the case of sIgE antibodies against recombinant timothy grass pollen components (rPhl p 1, 5, 6, 7) and the recombinant birch pollen major allergen (rBet v 1). These findings are in accordance with a recent publication by Wöhrle and co-workers who also matched birch- and grass pollen-specific sIgE responses of both test systems and could not demonstrate any significant disparities [14]. These data also agree with another recent publication demonstrating equally high correlations of microarray, FEIA and ELISA results obtained with the same panel of recombinant pollen allergens [23]. Likewise, although not systematically evaluated in the current study, the inter-assay reproducibility of microarray testing has been

reported to be comparable to that of FEIA analysis with coefficients of variation ranging from 17% to 26% depending on the grass and tree pollen major allergen components (Phl p 1, Phl p 5, Bet v 1) implemented [23].

For the first time, the current study applied microarray technology for high-resolution sIgE detection in adult patients suffering from AD. We were able to simultaneously analyze *in vitro* reactivity to nearly 100 proteins of more than 40 allergen sources in minimal-volume serum samples. Consequently, pronounced inter-individual variations could be observed with regard to both the total number of specific sensitizations and the corresponding sIgE recognition patterns. Microarray-based CRD permitted the identification of a panel of 16 allergen components eliciting positive sIgE responses in the majority of patients. Of note, these proteins belonged to plant and non-plant allergens of well-known significance in AD pathogenesis. In particular, grass and tree pollen proteins as well as mould, animal dander and house dust mite components induced positive sIgE results. This is consistent with FEIA-based investigations describing similar sensitization rates, and it clearly implies that the allergenic proteins investigated in our study represent a comprehensive repertoire of clinically relevant allergen components [24-27].

In addition, the instated microarray indicated a possible clinical role of cross-allergens in the context of AD. While sensitization to some of these partially homologous proteins has been shown to be associated with a more severe course of food allergy, their impact on AD is still poorly understood [17, 28, 29]. In the current study, AD severity was elevated in patients who were sIgE-positive to PR-10 proteins, a family of highly cross-allergenic molecules contained in a wide range of flowering plants [18]. In like manner, the total number of sensitizations encountered in our patients was positively, albeit moderately associated with AD severity. Still, these data should be interpreted with caution, because pre-test probability could have been influenced by a selection bias in favor of individuals with serious AD who represent the majority of patients presenting to our tertiary care centre. Additionally, the facts that no control patients were analyzed and that there was a comparatively small number of patients included both hamper the transferability of the current results to other, non-selected patient populations in whom PR-10 sensitization or multiple sIgE responses might not be linked to the clinical grade of illness. Furthermore, the microarray investigated represents a diagnostic platform that can be loaded with virtually all allergen components of relevance in the context of AD. This would potentially empower future epidemiological investigations, especially cohort studies in search of marker allergens, other than hen's egg extract [30], for a longer disease duration or the later development of bronchial asthma, for example. Moreover, allergen microarrays represent a sensitive tool to differentiate the intrinsic variant of AD, characterised by a lack of IgE-mediated sensitization, from the extrinsic AD type, which is associated with the presence of allergen-specific IgE antibodies. A more reliable distinction of these two phenotypes would clearly enhance AD patient care, because both disease types are known to differ with regard to further diagnostic steps and effective allergological treatment [31, 32].

Additionally, if a strong suspicion of immediate-type food allergy arises which cannot be confirmed by extract-based

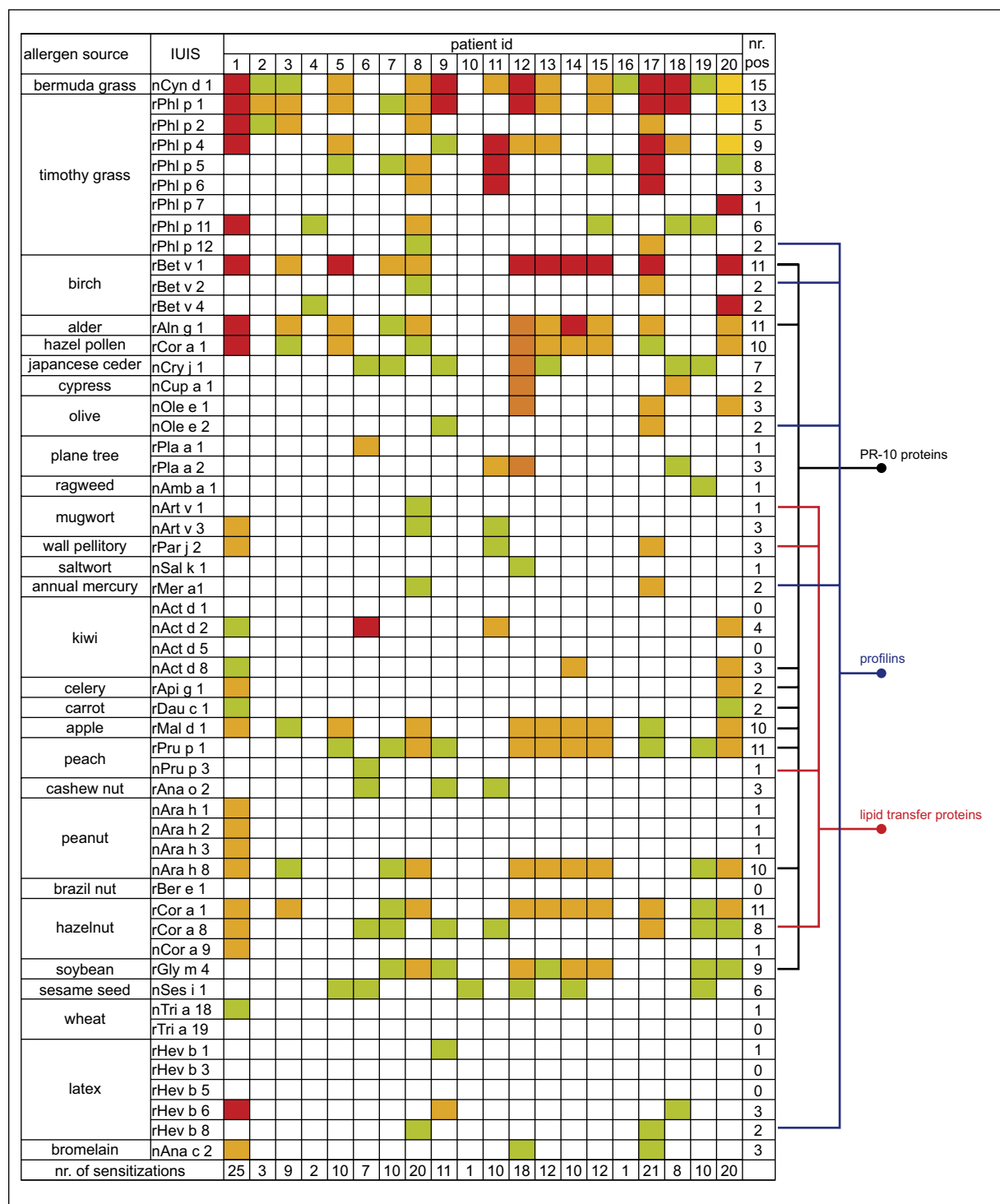


Figure 2. Microarray-based determination of sIgE recognition patterns in 20 adult patients with AD. Individual sIgE levels against *plant allergens* are represented semiquantitatively (red: high, yellow: medium, green: low, white: negative). Clusters of cross-allergenic proteins are displayed as non-hierarchical dendrograms. IUIS: designation recommended by the International Union of Immunological Societies.

skin prick testing or FEIA analysis, patient safety could be maximized by extensive scanning for markers of food-induced anaphylaxis, such as sIgE against wheat omega-5-gliadin (Tri a 19), peanut major allergens (Ara h 1, Ara h 2, Ara h 3) or LTP from different allergen sources

(nPru p 3, rCor a 8) [33–35]. This is of major importance, particularly in the context of AD where up to one third of affected children and up to 10% of adults additionally display IgE-mediated food hypersensitivity [36, 37]. Unfortunately, this could not be systematically explored

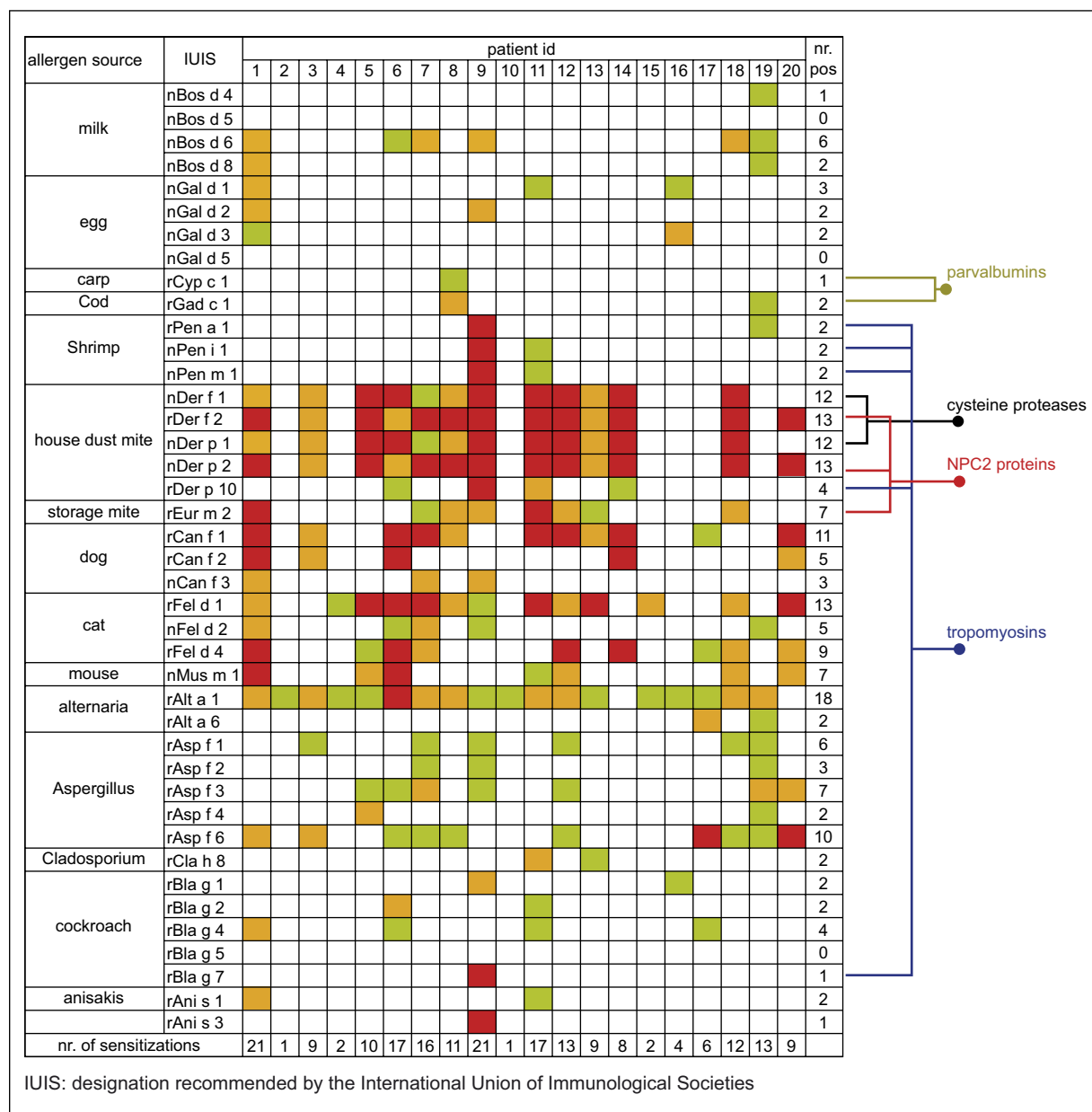


Figure 3. Microarray-based determination of sIgE recognition patterns in 20 adult patients with AD. Individual sIgE levels against *non-plant allergens* are represented semiquantitatively (red: high, yellow: medium, green: low, white: negative). Clusters of cross-allergenic proteins are displayed as non-hierarchical dendrograms. IUIS: designation recommended by the International Union of Immunological Societies.

in the present study due to the low prevalence of food hypersensitivity. Yet, out of 10 patients mounting sIgE against peanut proteins, microarray analysis identified sIgE to peanut major allergens (Ara h 1, Ara h 2) only in the patient with symptomatic peanut anaphylaxis, whereas a cross-allergenic PR-10 plant protein (Ara h 8) was found to be the culprit antigen in the remaining, asymptomatic individuals.

In conclusion, allergen microarrays provide a novel tool to diagnose IgE-mediated sensitization in adults suffering from AD. They show performance characteristics comparable to the current CRD-based sIgE detection method and

allow for the simultaneous detection of sIgE antibodies against multiple allergen components in minimal volumes of patient serum that can easily be obtained by capillary blood sampling. Thus, they might serve as sensitive and minimally-invasive sIgE screening tools improving patient care, especially in pediatric patients suffering from AD. However, further large-scale studies in unselected patient populations and in distinct high-risk groups such as AD patients with food-induced disease exacerbations are warranted before the allergen microarray can be introduced into daily clinical practice. ■

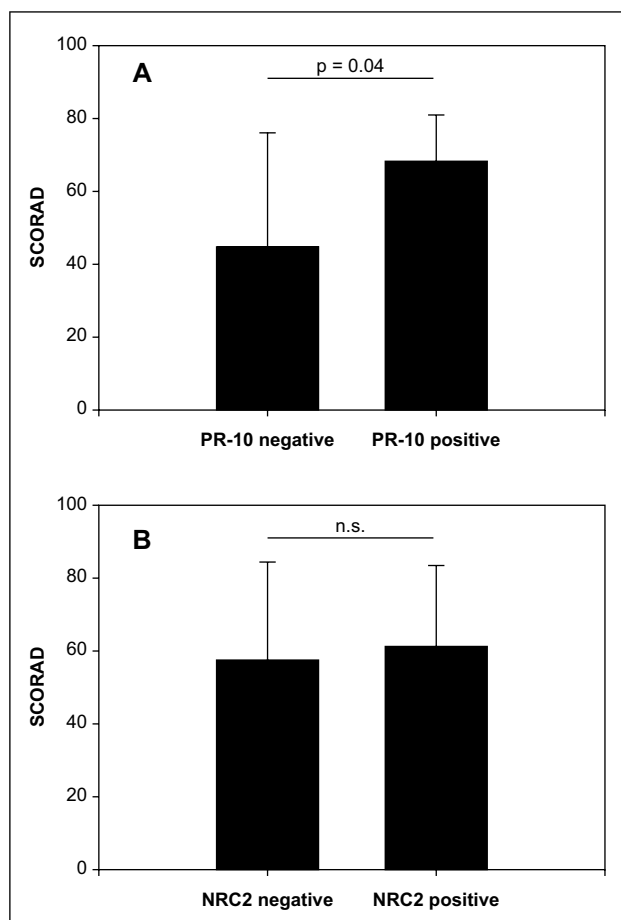


Figure 4. Group comparison of SCORAD scores in AD patients (n=20) with or without sIgE against cross-allergenic PR-10 proteins (**A**) and NPC2 (**B**).

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