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ORIGINAL ARTICLE

Food Allergy and Gastrointestinal Disease

Real-life evaluation of molecular multiplex IgE test methods in the diagnosis of pollen associated food allergy

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Abstract

Background: Diagnosis of food allergies is challenging, as combining information from specific IgE (sIgE)-sensitization pattern and skin prick tests (SPTs) with clinical history is necessary for a personalized management of allergic patients. The aim of this study was to compare two molecular tests, the ImmunoCAP ISAC (ISAC) and the Allergy Explorer, version 2 (ALEX^2) in the context of pollen food syndrome (PFS) diagnosis in a real-life scenario, to assess the benefit of multiplex testing in PFS patients.

Methods: Diagnosis of food allergy was performed in 53 patients. Allergen-sIgE concentrations were measured with ISAC and ALEX^2. Results for sIgE were statistically compared with each other, with SPT results and with clinical presentation of the patients.

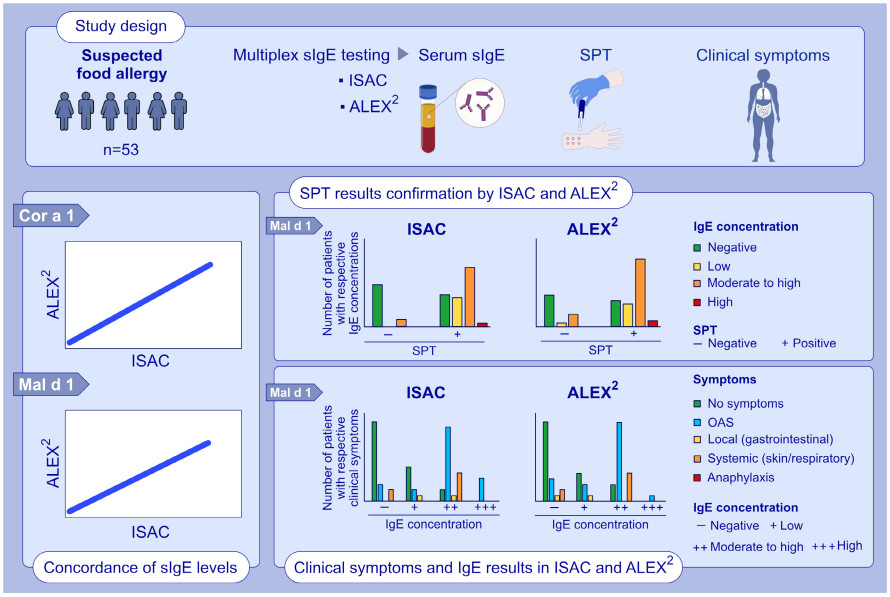
Results: Using ISAC as reference test for sIgE measurements, the average sensitivity of ALEX^2 for PR-10 allergens was 83.2% and the average specificity 88.0%. If only low sIgE concentrations were included, the sensitivity was 60.8% and the specificity

Abbreviations: ALEX^2, Allergy Explorer, version 2; Cor a 1, hazelnut allergen; ISAC, ImmunoCAP ISAC; Mal d 1, apple allergen; OAS, oral allergy syndrome; sIgE, specific IgE; SPT, skin prick test.

91.1%. Apple and hazelnut sensitizations were confirmed in most patients by concordance of sIgE and SPT results. Significant correlations were shown between clinical symptoms and Mal d 1- and Gly m 4-sIgE levels measured by both tests and for Cor a 1-sIgE levels measured by ALEX^2. In eight patients, profilin related symptoms were supported by Hev b 8-sensitization.

Conclusion: Multiplex testing is beneficial to understand patient-specific individual sensitization profiles and to providing personalized management recommendations. In the future, custom-designed test kits might enable reducing costs of multiplex testing for specific patient groups without compromising the diagnostic value.

KEYWORDS
food allergy, molecular diagnosis, multiplex testing, pollen food syndrome



GRAPHICAL ABSTRACT
This study compared two molecular tests, ISAC and ALEX^2, in the context of pollen food syndrome diagnosis in a real-life scenario and assessed the benefit of multiplex testing. When directly comparing sIgE levels determined by ISAC and ALEX^2, Spearman's correlation coefficient was significant for all six evaluated allergens. Mutiplex IgE testing supported diagnosis of pollen food syndrome patients and showed a good correlation with skin prick test results and clinical symptoms.
Abbreviations: ALEX2, Allergy Explorer, version 2; Cor a 1, hazelnut allergen; ISAC, ImmunoCAP ISAC; Mal d 1, apple allergen; OAS, oral allergy syndrome; sIgE, specific IgE; SPT, skin prick test

1 | INTRODUCTION

Molecular IgE test methods have substantially advanced allergy diagnostics.[1] Continuous evaluation of patients' sensitization profiles contribute to our mechanistic understanding of allergies and the characterization of major allergens.[2] Molecular IgE tests are an essential support for decision making of allergy immunotherapy (AIT) for IgE-mediated allergies and for choosing the right AIT preparation.[2,3] In food allergy, identification of specific IgE (sIgE)-sensitization pattern combined with the interpretation of the patients' history is essential for the management and guidance of patients. This information allows tailored recommendations regarding symptom severity based on the recognized allergen molecule.[1,4,5]

As commonly recommended, determination of sIgE is performed after detailed recording of clinical history and skin prick test (SPT) with culprit food.[4,6] If SPT is not possible, for example in patients with active atopic dermatitis, the "from molecules to clinic"-concept is preferable.[7] In multiplex testing, various allergens are tested simultaneously to provide clear sensitization profiles, which can predict potential reactions to foods, as well as possible cross-reactivity to homolog molecules. Food allergy is associated with a multitude of different symptoms, including anaphylaxis.[8] Especially patients with anaphylactic reactions

experience a substantial health burden, due to the severity of reactions. These patients might particularly benefit from early diagnosis and correct recommendations regarding restrictive diet.[9] Besides primary food allergy toward, for example, peanuts, tree nuts, and seafood, the pollen food syndrome (PFS) affects a large number of patients suffering from food adverse reactions due to cross-reactivity with airborne allergens. The estimated worldwide prevalence of PFS is 9.4%–35%.[10] The main clinical presentation is limited to local reactions in the oral cavity, such as itching and swelling of lips, oral mucosa, and tongue, in total known as the oral allergy syndrome (OAS), but reactions might also involve the gastrointestinal tract, and systemic symptoms may develop on skin, in the respiratory system, and in rare cases, the cardiovascular system, up to anaphylactic shock reactions.[10–13] Thus, a comprehensive diagnostic work-up of PFS patients is essential.[4] PFS-triggering allergens are mainly panallergens. These molecules are characterized by recognition of cross-reactive IgE due to amino-acid overlaps and similarities of the three-dimensional protein structure.[14] Among the allergens found in a large variety of different allergen sources, pathogenesis related (PR)-10 proteins and profilins found in most plants play a special role in PFS. Bet v 1 from birch is one of the best studied PR-10 proteins and homologous proteins, such as Mal d 1 from apple are termed Bet v 1-homologs.[14,15] Ten to twenty percent of patients with pollen allergy show reactivity to profilins.[16–18] The profilin in latex (Hev b 8) shows high homology to many other plant profilin proteins, such as Bet v 2 in birch and Cuc m 2 in melon.[4] Thus, testing of Hev b 8-sIgE can be used for diagnosis of profilin sensitization. Due to the high variability in the patients' sIgE repertoire, it is not possible to predict the cross-reactivity pattern with food allergens from pollen allergy diagnosis. Therefore, multiplex testing might be specifically of benefit to PFS patients. Moreover, multiplex testing might also implement precision medicine in AIT, as the AIT preparation can be specifically chosen based on the allergen patterns recognized by the patient as tested by multiplex molecular IgE tests, given that information regarding the specific AIT composition is available. ImmunoCAP ISAC (ISAC) and Allergy Explorer, version 2 (ALEX^2), are two molecular tests used for determining sIgE levels. ISAC has been licensed for medical diagnostics in 2011. Most of the allergens are produced recombinantly and 38% of the allergens are purified, natural allergens. For control purpose, cross-reactive carbohydrate determinants (CCDs)-containing proteins are spotted on the chip. The ALEX^2 multiplex test was licensed in 2018. ALEX^2 not only determines total IgE reactivity, but also sIgE reactivity to allergen components and to allergen extracts. In regards to the source of allergen molecules, 77% of the allergens are recombinantly produced and 23% are natural allergens. During the sample preparation, IgE antibodies specific for CCDs are removed from the serum.[4] The presence of whole food extracts and the high number of food proteins are an advantage of ALEX^2 when it comes to food allergy diagnosis.

Currently, only limited data are available on the usefulness of multiplex testing in PFS patients. Another hindering factor for the routine usage of the multiplex tests is that they are, at the time of writing this article, not reimbursed by the Austrian or any other national health insurance system and have to be paid privately by patients. When only testing few allergens, singleplex allergen tests are cheaper than the comprehensive multiplex tests. The aim of this study was to compare the two available molecular tests, ISAC and ALEX^2, in the context of PFS diagnosis in a real-life scenario. Patients seeking medical advice for food allergic reactions were included to evaluate the benefit of molecular multiplex testing in the diagnostic follow-up for suspected PFS.

2 | MATERIALS AND METHODS

2.1 | Patient characteristics

For this study, 53 consecutive patients seeking medical advice for suspected food allergic reactions were recruited. Patients were aged 18–63, with a male to female ratio of 22:31 and in general good health. At the time-point of inclusion into the study, none of the patients were undergoing immunotherapy and none were pregnant or lactating. Patients with alcohol or drug abuse and/or on medication impeding full understanding of the study protocol or interfering with skin prick test (SPT) results were excluded. Other exclusion criteria were active atopic dermatitis impeding SPTs, severe co-morbidities such as malignant tumors, immunological or endocrinological diseases, and chronic urticaria. Upon recruitment for this study, the study patients were not specifically screened for the presentation of PFS or IgE against specific allergens, but patients with suspected food allergy against different allergens with a wide variety of symptoms were included, to allow a better representation of the real-life, clinical situation. All patients gave written informed consent. The study was approved by the Ethics Committee of the Medical University of Vienna (EK No. 1207/2018) in accordance with the Declaration of Helsinki.

2.2 | Diagnostic procedure

The diagnosis of food allergy was performed based on international guidelines.[19] An elaborate patient history was recorded, addressing all aspects of food allergy, including symptoms when ingesting the food suspected to cause an allergic reaction and potential cofactors such as medication, timing, and form of consumption (e.g., raw, cooked, and pealed).

In patients with a clear medical history, SPTs with extracts of suspected causative foods were performed on the patients' forearm. If the patient tested positive in one or more SPTs with food allergens and consented in the participation in the study, a blood sample was taken.

2.3 | Multiplex IgE testing

Collected blood samples were centrifuged and sera were frozen according to good laboratory standards until further testing. As the last step of the diagnostic follow-up of the patients, sIgE concentrations were measured with ISAC and ALEX^2.

ImmunoCAP ISAC is an assay used for the semi-quantitative determination of allergen-sIgE in serum for extract components. On a chip, 112 allergen molecules are grouped in triplets, with 45 spots for food allergens. For testing, serum was applied to the chip and incubated for 120 min. Bound allergen-sIgE levels were measured via secondary fluorescent-labeled antibodies, which were applied in an extra step. The secondary antibodies were excited by laser light and the signals calculated into values. Results were expressed in ISU-E (standardized units for sIgE). Values under 0.3 ISU-E correspond to negative result, IgE levels between 0.3 and 0.9 ISU-E are considered as low concentrations. Values from 1 to 14.9 ISU-E indicate moderate to high concentrations and IgE titers over 15 ISU-E very high concentrations.[20]

The ALEX^2 assay simultaneously tests sIgE reactivity to 295 different allergens. The allergens were spotted on a nitrocellulose membrane on a chip as single spots. For sIgE testing, membranes were incubated with sera for 2 h, washed and thereafter incubated with alkaline phosphatase for 30 min. After repeated washing, the enzyme substrate was added, and the reaction was measured after several minutes. Based on reaction intensity, the results were translated into kUA/ml values. The same cut-off levels were used as with ISAC.[21]

2.4 | Statistical analysis

The data were evaluated using the SPSS Software (Version 27.0, IBM Deutschland GmbH). The data were analyzed in dichotomous (recognition of allergy) and metric (sIgE levels) form. For the calculation of sensitivity and specificity of the ALEX^2 test, the ISAC test was used as reference.

For further evaluation, six allergens relevant for PFS and present on both chips were chosen in accordance to their frequency in the study population. For these allergens, the Spearman's correlation coefficient was calculated to compare the measurements of ALEX^2 and ISAC, and data were depicted by scatterplots.

The measured sIgE values (ISU-E or kUA/ml, respectively), were divided into the categories negative (<0.3 ISU-E or kUA/ml), low concentration (0.3–0.9 ISU-E or kUA/ml), moderate to high (1–14.9 ISU-E or kUA/ml), and very high concentration (>15 ISU-E or kUA/ml). These ranges are predefined by the manufacturers of the multiplex tests (and see also[21]).

Results of SPTs were put into context with the multiplex test results. For easier interpretation, grouped bar graphs were created. To objectify the clinical presentation of the study population, clinical symptoms were divided into categories such as OAS, local gastrointestinal symptoms, systemic skin or respiratory symptoms, and anaphylaxis. To compare the sIgE results of ISAC and ALEX^2 with the SPT results and the clinical presentation of the patients, Spearman's correlation coefficient was calculated for each allergen component and SPT results or symptom category.

3 | RESULTS

3.1 | Patient demographics

In total, 53 patients were included in the study. Thirty-one female and 22 male patients had a mean age of 38.6 years (18–63 years). PFS was diagnosed in 41 of the included patients (77%) with apple as the most frequently recognized food allergy, followed by hazelnut and peanut (Table 1). The data of all 53 patients were included in the following statistical evaluations.

3.2 | Comparison of positive test results, sensitivity, and specificity of the two multiplex sIgE tests

To compare both molecular test methods, the frequencies of 40 common allergen components present on both tests were analyzed. By using the ISAC as reference, sensitivity and specificity of the ALEX^2 were calculated. Generally, the average sensitivity was 78.2% (range 33.3%–100%) and the average specificity 98.1% (range 60.9%–100%), when all 40 allergen components present on both chips were considered. Due to already reported differences in IgE detection between ISAC and ALEX specifically in the lower IgE range, we compared test sensitivity and specificity for the lower sIgE levels. If only data from patients with a sIgE concentration in the negative (<0.3 ISU-E or kUA/ml) or lowest range (between 0.3 and 0.9 ISU-E or kUA/ml, as suggested by the manufacturers of the multiplex tests) was used for the calculations, the average sensitivity dropped to 54.5% (range 0–100%) but the average specificity increased slightly to 98.7% (range 73.7%–100%).

To evaluate the sensitivity and specificity for PFS associated allergens specifically, we next included only the six most relevant allergens associated with PFS that were also observed in our study population (PR-10 proteins and the profilin Hev b 8). This resulted in an average sensitivity for ALEX^2 of 81.5% and a specificity of 90.1%.

If only all PR-10 proteins present on both chips were included in the calculation, the average sensitivity was 83.2% (range 75%–91.2%) and the average specificity 88.0% (range 60.9%–100%). When including only patients with negative (<0.3 ISU-E or kUA/ml) and low PR-10 sIgE concentrations (between 0.3 and 0.9 ISU-E or kUA/ml), the average sensitivity for ALEX^2 was 60.8% (range 25%–100%), and the average specificity was 91.1% (range 73.7%–100%).

For single allergens, the highest sensitivity in ALEX^2 was observed for Mal d 1 (91.2%). The lowest sensitivity in food allergens was observed for Ara h 8 (75%), and the sensitivity for Hev b 8 was generally the lowest (72.7%). Of interest, both Ara h 8 and Hev b 8 had the highest specificities with 100%. The lowest specificity was observed for Cor a 1 (Table 1).

TABLE 1 Number of positive sIgE reactivities and specificity and sensitivity of the molecular test methods and 95%-Clopper-Pearson Confidence Intervals (CI)

Allergen		No. of patients with IgE reactivity (>0)		No. of patients with IgE levels >0.3		Comparison of molecular test methods using ISAC as reference value			
								Only concentrations 0 to 0.9 ISU-E	
Allergen source	Allergen molecule (protein family)	ISAC	ALEX^2	ISAC	ALEX^2	Sensitivity in % (CI)	Specificity in % (CI)	Sensitivity in % (CI) No. of patients=*N*	Specificity in % (CI) No. of patients=*N*
Apple	Mal d 1 (PR-10)	37	37	34	32	91.2 (76.3–98.1)	94.7 (74.0–99.9)	62.5 (24.5–91.5) *N*=27	94.7 (74.0–99.9) *N*=27
Celery	Api g 1 (PR-10)	10	15	9	8	77.8 (40.0–97.2)	97.7 (88.0–99.9)	100 (15.8–100) *N*=46	97.7 (88.0–99.9) *N*=46
Hazelnut	Cor a 1.0401 (PR-10)	33	41	30	35	86.7 (69.3–96.2)	60.9 (38.5–80.3)	25 (0.6–80.6) *N*=23	73.7 (48.8–90.9) *N*=23
Peanut	Ara h 8 (PR-10)	26	21	20	15	75 (50.9–91.3)	100 (9.4–100)	50 (18.7–81.3) *N*=43	100 (89.4–100) *N*=43
Soy	Gly m 4 (PR-10)	17	19	14	17	85.7 (57.2)98.2	87.2 (72.6–95.7)	66.7 (9.4–99.2) *N*=41	89.5 (46.0–78.2) *N*=41
Latex	Hev b 8 (Profilin)	13	10	11	8	72.7 (39–94)	100 (91.6–100)	25 (0.6–80.6) *N*=46	100 (91.6–100) *N*=46

3.3 | Concordance of sIgE values measured in the molecular tests

The correspondence of the sIgE levels measured by both molecular tests was depicted by scatterplots (Figure 1). The Spearman's Correlation coefficient (r_s) was calculated. For all six included allergens, a significant correlation between the sIgE test results was detected (Figure 1). Within the six allergens relevant for PFS and present in both tests, the highest correlation of sIgE test results was shown for Mal d 1 and the lowest for the celery allergen Api g 1.

3.4 | Comparison of SPT results with allergen-sIgE levels evaluating food specific sensitization to PR-10 proteins

The next aim was to compare the SPT results to the measured allergen-sIgE values. For SPTs, a comprehensive panel of SPT substances were chosen based on the reported clinical symptoms of the patients. As no SPTs were performed with extracts containing profilin food allergens, we were only able to compare PR-10 IgE levels with skin reactivity. Generally, SPT with hazelnut revealed the highest number of positive test results with 38 out of 53 patients. The lowest number of positive test results in SPT were detected with soy extract (seven patients). SPT results were compared with the results from the molecular sIgE tests. While SPT extracts contain various allergen components, we compared these results with sIgE test results for single allergens in ISAC and ALEX^2. SPTs were performed for only five out of six evaluated PFS-relevant allergens (Mal d 1, Api g 1, Cor a 1, Ara h 8, Gly m 4). In Table 2, the number of positive sIgE test results in at least one of the multiplex tests are compared with positive SPT results.

When comparing the SPT results of apple extract with the test results for Mal d 1 in ISAC and ALEX^2, most positive SPT results were confirmed by either ISAC, ALEX^2, or both (82.6%). Several patients revealed low to moderate concentrations of Mal d 1-sIgE levels and remained negative in SPT with apple extract (Table 2, Figure 2A).

For celery sensitization, positive sIgE reactivity toward Api g 1 was confirmed only in a low number of patients (3/9 or 3/8, respectively; Table 2). Most celery SPTs remained negative in our patient cohort (Figure 2B).

The highest number of positive SPT results was recorded for hazelnut (Table 2). When comparing the positive SPT results to the sIgE levels measured for Cor a 1, ten SPT positive patients had a negative Cor a 1-sIgE test result with ISAC, and eight patients were negative with ALEX^2. Thus, different hazelnut allergens might be relevant for these patients. Most of the positive SPT results (98.5%) were confirmed by at least one molecular IgE test method. All patients with moderate to high or very high Cor a 1-sIgE concentrations had a positive SPT result (Figure 2C).

When evaluating the reactivity for peanut, a relatively low concordance of sIgE test and positive SPT results was observed (Table 2, Figure 2D). The number of patients with positive peanut

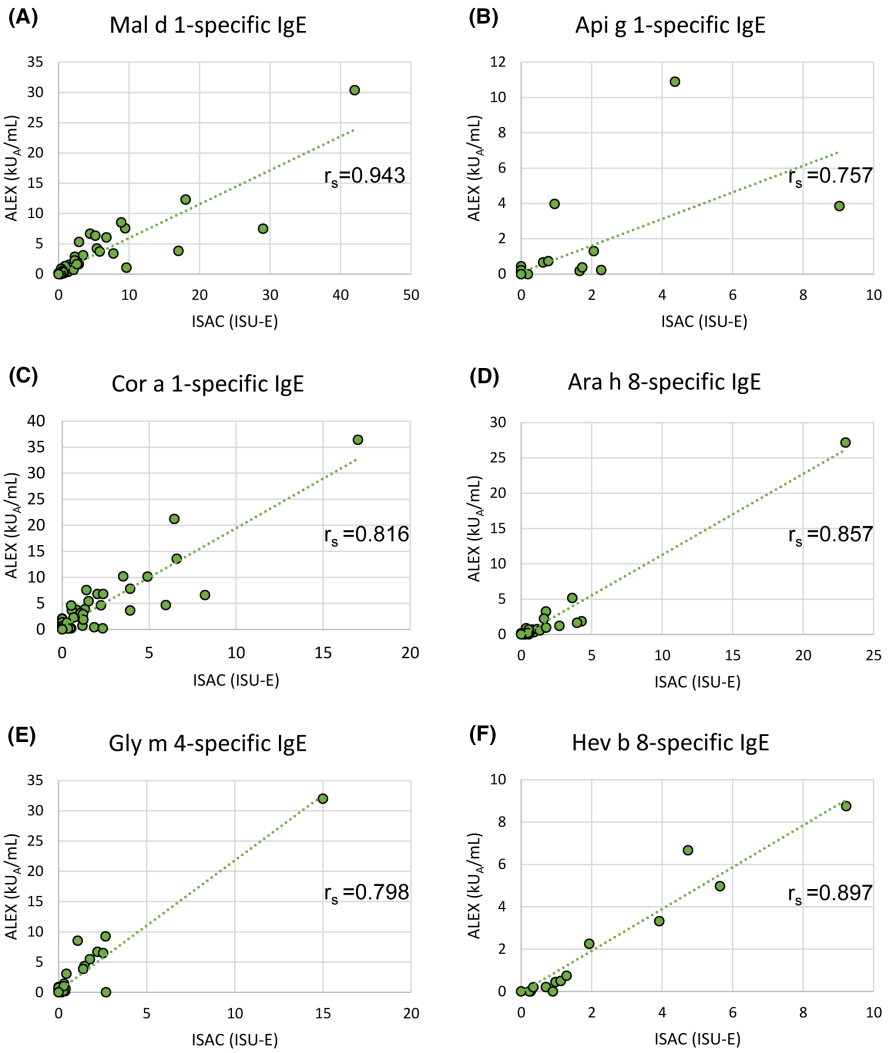


FIGURE 1 Comparison of measured allergen-sIgE concentrations by scatterplots and Spearman's correlation coefficient (r_s). *p*-values were <.0001 in all cases. (A) Mal d 1-sIgE, (B) Api g 1-sIgE, (C) Cor a 1-sIgE, (D) Ara h 8-sIgE, (E) Gly m 4-sIgE, (F) Hev b 8-sIgE. Values are indicated in ISU-E and kUA/ml

TABLE 2 Skin prick test results (SPT) to allergen extract compared with sIgE test results to major allergen compound in ISAC and/or ALEX^2

		No. of patients							
Allergen source	Allergen molecule	ISAC pos.	ALEX^2 pos.	SPT pos.	ISAC pos. & SPT pos.	ALEX^2 pos. & SPT pos.	SPT pos. & ISAC/ALEX^2 neg.	ISAC neg. & SPT pos.	ALEX^2 neg. & SPT pos.
Apple	Mal d 1	34	32	23	18	16	4	5	7
Celery	Api g 1	9	8	9	3	3	6	6	6
Hazelnut	Cor a 1	30	35	38	28	30	4	10	8
Peanut	Ara h 8	20	15	16	9	7	7	7	9
Soy	Gly m 4	14	17	7	2	2	5	5	5

SPTs without sIgE reactivity to the PR-10 protein Ara h 8 was high (35% Table 2) indicating the importance of other peanut allergens for these patients.

When soy reactivity in SPTs was compared with the results of the molecular tests for the allergen component Gly m 4, a similar number of patients with positive Gly m 4-sIgE results but negative SPTs was detected in both IgE tests. Moreover, out of seven patients with positive SPTs, only three patients had a positive result in at least one of the molecular tests for Gly m 4-sIgE, again indicating the relevance of other allergens in our patient cohort for soy allergens.

3.5 | Comparison of clinical reactions and sIgE test results for PR-10 allergens

To further evaluate the diagnostic value of the two molecular chip assays in PFS, the test results were compared with the symptoms

reported by the 53 patients. Most patients reported symptoms when consuming hazelnut or apple. In Table 3, the number of patients reporting food adverse symptoms are indicated in comparison with detected allergen-sIgE in molecular tests.

In a next step, the clinical presentation of the patients was divided into the following categories based on the predominant symptoms mentioned by the patients when ingesting different foods: OAS, local gastrointestinal symptoms, systemic symptoms involving the skin and/or the respiratory tract, and anaphylaxis. The number of patients with positive allergen-sIgE test results in comparison with clinical symptoms are indicated in Table 3.

Apple was the second most common food eliciting food-related allergic symptoms in our patient cohort. In 26 out of 31 patients, this was in accordance with the measurement of Mal d 1-sIgE antibodies. Only five patients reported symptoms but had a negative sIgE result in ISAC and ALEX^2. Most patients reported OAS and had moderate to high concentration of Mal d 1-sIgE (Figure 3A).

When comparing the Api g 1-sIgE results with the clinical presentation of the patients when ingesting celery, we observed limited overlap. None of the patients, who reported celery related symptoms, had a positive sIgE result in either ISAC or ALEX^2. Nine patients who tested positive for Api g 1-sIgE did not report allergic symptoms related to celery ingestion (Figure 3B).

In our study, most allergic symptoms were reported upon consumption of hazelnut, which correlates with the measured Cor a 1-sIgE concentrations in both or at least one of the molecular test methods. Six patients reported clinical symptoms but had a negative Cor a 1-sIgE test result in both ISAC and ALEX^2. One patient described an anaphylactic reaction after hazelnut ingestion and tested positive with moderate to high Cor a 1-sIgE levels in ALEX^2 test (Figure 3C).

When comparing the clinical symptoms upon peanut ingestion with Ara h 8-sIgE test results, the importance of non-PR-10 peanut proteins became apparent in our patient cohort. Patients reporting OAS upon peanut ingestion had low to moderately high concentrations of sIgE for Ara h 8 (Figure 3D).

Only one-third of the patients who had a positive Gly m 4-sIgE test result reported allergic symptoms when ingesting soy-containing food (Figure 3E). Most of these patients showed sensitization for Gly m 4, which is in line with the results for comparison with the SPT.

To correlate the metric sIgE levels with the five clinical symptom categories (no symptoms, OAS, local gastrointestinal symptoms, systemic skin and/or respiratory symptoms, and anaphylaxis), Spearman's correlation coefficient was calculated for the five allergens (Table 4).

A significant correlation was shown between clinical symptoms and sIgE levels measured with ISAC for the soy allergen Gly m 4 and the apple allergen Mal d 1. A significant correlation between clinical symptoms and Mal d 1-, Cor a 1-, and Gly m 4-sIgE was detected by ALEX^2. Thus, significant correlations between the clinical presentation and the measured sIgE levels could be confirmed for Gly m 4 and Mal d 1 for both molecular tests.

3.6 | Comparison of clinical reactions and sIgE test results for profilin allergens

The only profilin present on both molecular tests is the latex allergen Hev b 8. As PFS is related with profilin reactivity[16,18] and food profilins are present only on the ALEX^2 (Cuc m 2 from melon), Hev b 8 was used as a surrogate marker to compare the patients' profilin sensitization profiles (Table 5).

About 14 patients reported allergic symptoms to food, which might be related to profilin allergy, but did not have a positive Hev b 8 or Cuc m 2-sIgE result in ISAC or ALEX^2. Most of these patients described symptoms when ingesting tomatoes. Four patients had profilin sIgE results in at least one of the profilins tested but did not report any symptoms upon ingestion of profilin containing food. In eight patients, reported clinical symptoms were explained by the sensitization to profilins, in all cases, Hev b 8-sIgE was positive in both ISAC and ALEX^2 and in seven patients Cuc m 2-sIgE was additionally detected.

4 | DISCUSSION

Despite the low number of patients with severe, systemic reactions, PFS has a profound impact on the quality of life of affected patients. Affected patients seek medical advice for a diagnosis of food adverse reactions, which is unfortunately still not commonly accessible due to the necessary high medical expertize and the financial costs for allergy tests. As multiplex testing offers the opportunity to test various allergens simultaneously, we aimed to assess the diagnostic value of molecular test systems in patients with PFS. In our study, we observed 81.47% sensitivity and 90.02% specificity of PR-10 and profilin sIgE testing with ALEX^2 compared with ISAC. When directly comparing sIgE levels determined by ISAC and ALEX^2, Spearman's correlation coefficient was significant for all five evaluated allergens (Mal d 1, Api g 1, Cor a 1, Ara h 8, and Hev b 8).

For better evaluation of the diagnostic value of both multiplex tests in a real-life clinical setting, we not only aimed to compare sIgE reactivity patterns, but also correlated detected sIgE levels with SPT results and clinical symptoms. Positive SPT results were mostly confirmed by both molecular tests results for Ara h 8-, Cor a 1-, and Mal d 1-sIgE. The comparison with clinical symptoms revealed similar

FIGURE 2 Numbers of patients with positive or negative results in SPT in comparison to sIgE titers measured by ISAC (left panel) and ALEX^2 (right panel). (A) Mal d 1-sIgE and SPT with apple extract, (B) Api g 1-sIgE and SPT with celery extract, (C) Cor a 1-sIgE and SPT with hazelnut extract, (D) Ara h 8-sIgE and SPT with peanut extract, (E) Gly m 4-sIgE and SPT with soy extract. Color coding: green—negative; yellow—low IgE concentration; orange—moderate to high IgE concentration; red—very high IgE concentration

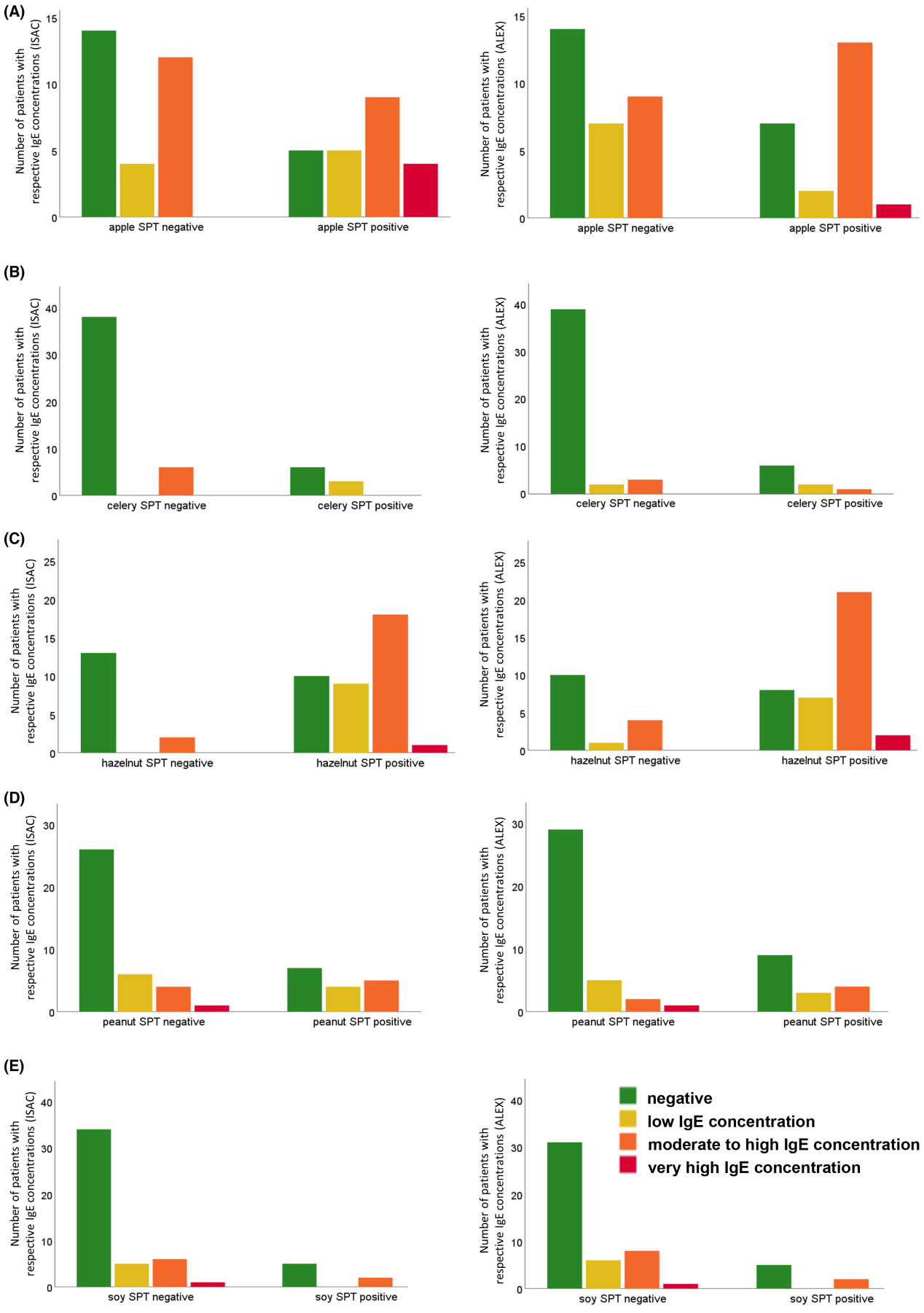

(A)
Number of patients with respective IgE concentrations (ISAC)
apple SPT negative
apple SPT positive
Number of patients with respective IgE concentrations (ALEX)
apple SPT negative
apple SPT positive
(B)
Number of patients with respective IgE concentrations (ISAC)
celery SPT negative
celery SPT positive
Number of patients with respective IgE concentrations (ALEX)
celery SPT negative
celery SPT positive
(C)
Number of patients with respective IgE concentrations (ISAC)
hazelnut SPT negative
hazelnut SPT positive
Number of patients with respective IgE concentrations (ALEX)
hazelnut SPT negative
hazelnut SPT positive
(D)
Number of patients with respective IgE concentrations (ISAC)
peanut SPT negative
peanut SPT positive
Number of patients with respective IgE concentrations (ALEX)
peanut SPT negative
peanut SPT positive
(E)
Number of patients with respective IgE concentrations (ISAC)
soy SPT negative
soy SPT positive
Number of patients with respective IgE concentrations (ALEX)
soy SPT negative
soy SPT positive
negative
low IgE concentration
moderate to high IgE concentration
very high IgE concentration

TABLE 3 Number of patients with clinical symptoms compared with IgE reactivity to major allergen compound in ISAC and/or ALEX^2

		No. of patients							
Food source	Allergen molecule	ISAC pos.	ALEX^2 pos.	Symptoms	ISAC pos. & symptoms	ALEX^2 pos. & symptoms	Symptoms & ISAC/ALEX^2 neg.	ISAC neg. & symptoms	ALEX^2 neg & symptoms
Apple	Mal d 1	34	32	31	26	24	5	5	7
Celery	Api g 1	9	8	3	0	0	3	3	3
Hazelnut	Cor a 1	30	35	32	22	24	6	10	8
Peanut	Ara h 8	20	15	14	6	4	8	8	10
Soy	Gly m 4	14	17	7	4	5	2	3	2

results. The correlation between the measured level of sIgE and the allergic reactions of the patients was significant for Gly m 4 and Mal d 1 in both tests. Moreover, a significant correlation of Cor a 1-sIgE levels detected by ALEX^2 in patients with clinical symptoms was observed.

In a recent study, three different multiplex testing platforms (ALEX, MeDALL-chip, and EUROLINE) were compared in relation to nut allergy, concluding that the test results were useable for pooling in meta-analyses. Their results, however, differed from our studies' findings regarding the comparison of SPT and sIgE.[22] Similar to Buzzulini et al,[23] who compared the two predecessors of ImmunoCAP and ALEX^2, in our study, the correlation of the two multiplex tests was high, but showed noticeable differences regarding the measured sIgE in the categories low and high to moderate concentration.

Multiplex testing of sIgE was criticized for over-estimating allergies, especially in tree nut allergic patients, due to high rates of negative oral food challenges despite sensitization as evidenced by SPT or presence of allergen-sIgE.[24] This highlights the importance of correlating test results with the clinical history.[25] Thus, we consider our results regarding significant correlation of sIgE levels and positive SPTs as well as symptoms to be of special importance. Comparison of the molecular test results with SPT results confirmed that for most PFS allergens a positive SPT correlates with sIgE detected in ISAC and/or ALEX^2, which was not the case in a previous study comparing SPT test results with sIgE levels detected by ISAC.[26]

Moreover, the sensitization pattern of the patients was determined not only by two molecular test assays, but the results were additionally correlated with the severity of clinical symptoms. A recently published study in adolescents in Japan compared PFS symptoms with sensitization status. In line with our study, the importance of PR-10 proteins and profilins was confirmed for PFS patients.[27] A Viennese study examining sensitization clusters highlighted a strong relationship between sensitizations to food and respiratory allergens.[28] Without any doubt, diagnosis of food allergy depends on clear clinical symptoms, while sIgE testing and positive SPTs only indicate sensitization. However, patients with food sensitization have a high risk of developing food allergy.[29] In doubt of clinical relevance of the detected sensitizations, the diagnosis of food allergy has to be validated through oral food challenge (OFC), in the best case by double-blind, placebo-controlled food challenge (DBPCFC).[30,31]

Generally, PFS is considered to induce anaphylaxis rarely. However, in accordance with other studies,[11] we observed severe symptoms in several PFS patients. There are multiple reasons for aggravation of allergic reactions such as age-related factors, concomitant diseases, medication, exercise, changes of the gastrointestinal physiology, or stress.[32–34] Risk factors for anaphylaxis in PFS were identified as the presence of atopic dermatitis, a high number of culprit foods and strong sensitization to specific pollens such as hazel, timothy, and ragweed.[11] In most cases, anaphylaxis in PFS occurs after ingestion of peanut or apple.[11]

Regarding profilin sIgE testing, our study underlines the importance of detecting sensitization by the high rate of patients with positive test results combined with clinical symptoms upon ingestion of profilin containing food. Our study suggests Hev b 8 to be a good marker for profilin sIgE reactivity in both molecular tests, with questionable clinical relevance for latex allergy. Previous studies suggested a risk for PFS patients sensitized to profilins for developing severe symptoms.[35] In another study, sensitization to tomato profilin tested through SPT revealed relevance in tomato allergic patients with a history of grass pollen allergy.[36] This was not confirmed in our study, as most of the patients reporting adverse reaction upon tomato ingestion did not have a positive result for Hev b 8.

We are fully aware of the limitations of our study. The pollen-associated food reactivities are only representative for a central European PFS patient cohort. Moreover, due to the sample size and the relatively low number of included patients, sensitivity and specificity calculations showed wide-ranging confidence intervals. Moreover, it is important to recognize that the performance (sensitivity and specificity) of the multiplex tests might vary within the

FIGURE 3 Clinical symptoms of patients and allergen-sIgE levels measured by ISAC (left panel) and ALEX^2 (right panel). (A) Mal d 1-sIgE in correlation with symptoms upon apple ingestion, (B) Api g 1-sIgE in correlation with symptoms upon celery ingestion, (C) Cor a 1-sIgE in correlation with symptoms upon hazelnut ingestion, (D) Ara h 8-sIgE in correlation with symptoms upon peanut ingestion, (E) Gly m 4-sIgE in correlation with symptoms upon soy ingestion. Color coding: blue—no symptoms; green—OAS; yellow—local (gastrointestinal symptoms); orange—systemic (urticaria/dyspnea); red—anaphylaxis

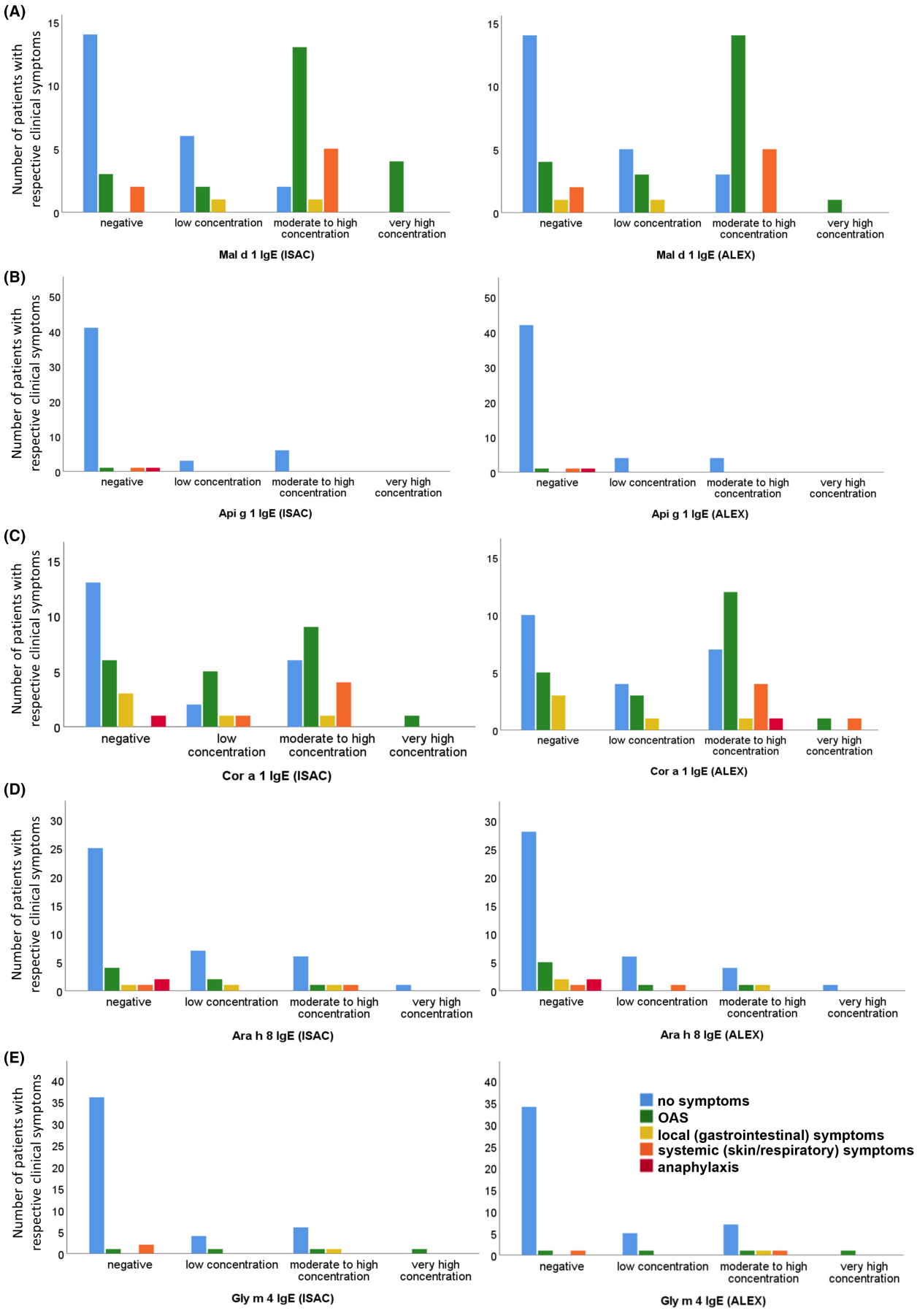

(A)
Number of patients with respective clinical symptoms
negative
low concentration
moderate to high concentration
very high concentration
Mal d 1 IgE (ISAC)
Mal d 1 IgE (ALEX)
(B)
Api g 1 IgE (ISAC)
Api g 1 IgE (ALEX)
(C)
Cor a 1 IgE (ISAC)
Cor a 1 IgE (ALEX)
(D)
Ara h 8 IgE (ISAC)
Ara h 8 IgE (ALEX)
(E)
Gly m 4 IgE (ISAC)
Gly m 4 IgE (ALEX)
no symptoms
OAS
local (gastrointestinal) symptoms
systemic (skin/respiratory) symptoms
anaphylaxis

TABLE 4 Spearman's correlation coefficient of ISAC and ALEX^2 correlated with the severity of symptoms

Food source	Allergen molecule	ISAC (ISU-E) vs. symptoms	ALEX^2 (kU$_A$/ml) vs. symptoms	ISAC (categories) vs. symptoms	ALEX^2 (categories) vs. symptoms
Apple	Mal d 1	0.521**	0.531**	0.522**	0.427**
Celery	Api g 1	−0.117	−0.151	−0.110	−0.103
Hazelnut	Cor a 1	0.195	0.297*	0.237	0.306*
Peanut	Ara h 8	−0.016	−0.083	0.049	−0.003
Soy	Gly m 4	0.346*	0.341*	0.279*	0.362**

Note: For calculations, metric data from ISAC and ALEX^2 as well as IgE-measurements in categories were compared with symptom categories (OAS, local (gastrointestinal) symptoms, systemic symptoms (urticaria/dyspnea), anaphylaxis).

*$p < .05$; **$p < .01$.

TABLE 5 Patients reporting symptoms after ingestion of profilin containing food in comparison to specific anti-Cuc m 2- and -Hev b 8-sIgE levels

Patient	Cuc m 2 (kUA/ml; ALEX^2)	Hev b 8 (ISU-E; ImmunoCAP ISAC)	Hev b 8 (kUA/ml; ALEX^2)	Clinical symptoms upon ingestion of profilin containing food (food source)
2	-	-	-	+ (melon)
9	-	-	-	+ (tomato)
11	4.78	4.73	6.66	+ (melon, citrus fruit)
12	-	-	-	+ (bell pepper)
16	-	-	-	+ (banana)
22	0.53	0.70	0.21	+ (melon)
25	0.40	0.97	0.44	+ (tomato, banana, melon, citrus fruit)
26	2.71	5.64	4.97	+ (tomato)
28	-	-	-	+ (tomato)
29	-	-	-	+ (pear)
30	-	-	-	+ (orange)
31	-	0.90	-	-
32	7.13	9.22	8.75	+ (banana, melon)
33	-	-	-	+ (banana)
36	-	-	-	+ (tomato)
37	-	-	-	+ (tomato)
38	-	-	-	+ (tomato)
39	-	-	-	+ (pear)
40	-	-	-	+ (tomato)
43	0.94	1.12	0.50	+ (melon)
45	-	0.34	0.20	-
49	-	3.92	3.32	+ (tomato)
50	-	-	-	+ (melon, banana, pear)
51	0.31	1.29	0.74	+ (tomato, melon)
52	1.33	-	-	-
53	-	1.93	2.25	-

population and might be overestimated in our current study. Our study patients were included based convincing clinical history indicative for food allergy. Thus, the results of our study were generated in a cohort with a high likelihood for the disease and might not reflect the situation in a random population. However, as the tests have to be paid privately, clinicians will only advice its use, if allergy is likely.

In conclusion, molecular tests such as the ISAC and ALEX^2 are supportive in the diagnosis of PFS. By usage of multiplex testing, clinicians can understand the patient's individual sensitization profile and can adapt and plan the tailored management including respiratory and future food immunotherapies and preventive measures. Based on the individual sIgE concentrations, the severity

of the symptoms and individual risk for anaphylaxis might be estimated. This information can be used for personalized management recommendations and limit the need for a broader food avoidance, which represent a major burden for patients. In the context of environmental changes, the impact of pollen related food as source of adverse food reactions is likely to increase in the future.[15] Adequate diagnosis and patient management will gain increasing importance. A study in the US showed that the rate of adult people believing to have food allergies is even higher (19%) than the previously mentioned approximate 10%, resulting in food restrictions and impaired quality of life, when insufficient diagnosis is provided.[37] In foresight of new therapeutic strategies with patient-centered immunological targets,[24] custom-designed test kits for specific patient groups such as with PFS, might enable reducing the costs of multiplex testing by reducing the costs of the kits themselves without compromising the highly informative value of multiplex tests, thus making them more accessible. As a next step, in-house point-of-care devices for multiplex testing should be a future goal.

AUTHOR CONTRIBUTION

UE involved in conceptualization, recruitment of patients, and project administration. LD, BN, RL, KL, and JJE involved in methodology. ZS and UE designed the study. LD, BN, and UE performed clinical investigation. LD, BN, RJ, and KL involved in read-out experiments. LD, BN, SZ, and UE performed validation. LD, SZ, and UE performed formal analysis and data curation. LD and UE performed writing original draft. BN, RJ, KL, AR, BK, DPA, FM, JG, TMJ, JJE, and SZ performed editing and reviewing of manuscript. LD and KL involved in visualization. AR, BK, DPA, FM, TMJ, and UE involved in supervision and funding acquisition. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

KB has received honoraria for talks and controlling webinar contents from ThermoFisher. The other authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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