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Allergen extract- and component-based diagnostics in children of the ALLIANCE asthma cohort

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Abstract

Background: Current *in vitro* allergen-specific IgE (sIgE) detection assays measure IgE against allergen extracts or molecules in a single- or multiplex approach. Direct comparisons of the performance of such assays among young children with common presentations of allergic diseases regardless of sensitization status are largely missing.

Objectives: The aim of this study was a comparison of the analytical and diagnostic performance for common clinical questions of three commonly used technologies which rely upon different laboratory methodologies among children of the All Age Asthma (ALLIANCE) cohort (clinicaltrials.gov: NCT02496468).

Methods: Sera from 106 paediatric study participants (mean age 4 years) were assessed for the presence of sIgE by means of the ImmunoCAP™ sx1 and fx5 mixes, the ImmunoCAP ISAC™ 112 microarray and a Euroline™ panel.

Results: Total and negative concordance was high (>82%–>89%), while positive concordance varied considerably (0%–100%) but was also >50% for the most common sensitizations analysed (house dust mite and birch). All three test systems showed good sensitivity and specificity (AUC consistently > 0.7). However, no significant

Gesine Hansen and Anna-Maria Dittrich contributed equally to this study. †See appendix 1 ALLIANCE Study Group.

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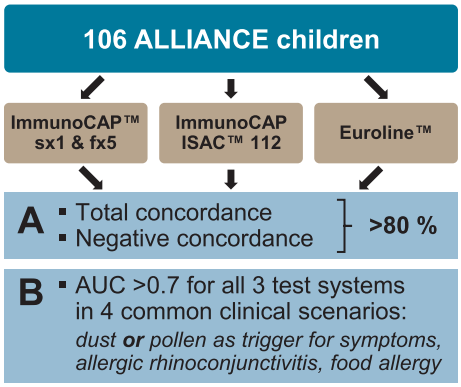
differences with regard to identifying sIgE sensitizations associated with symptoms in children with suspected pollen- or dust-triggered wheeze or presenting with symptoms of allergic rhinoconjunctivitis or food allergy were detected. Extending the number of allergens did not change the similar performance of the three assay systems.

Conclusion and Clinical Relevance: Among young children, the three sIgE assays showed good analytical and diagnostic concordance. Our results caution that the identification of larger numbers of sensitizations by more comprehensive multiplex approaches may not improve the clinical utility of sIgE testing in this age group.

KEYWORDS
allergen sIgE, analytical performance, diagnostic performance, *in vitro* allergy diagnosis, molecular allergology



GRAPHICAL ABSTRACT

Among 106 young children (mean age 4 years) of the All Age Asthma (ALLIANCE) cohort, ImmunoCAP™ sx1 and fx1 mixes, the ImmunoCAP ISAC™ 112 microarray, and a Euroline™ panel showed good analytical and diagnostic concordance in four common clinical scenarios. Our results caution that the identification of larger numbers of sensitizations by more comprehensive multiplex approaches may not improve clinical utility of sIgE testing in this age group.

1 | INTRODUCTION

In vitro test systems which detect allergen-specific IgE (sIgE) are a mainstay of diagnostic work-up in allergology, detecting sensitization and confirming clinically suspected allergies and/or cross-reactivities. They may guide treatment with regard to avoidance of allergens, application of seasonally directed anti-allergic therapy and allergen immunotherapy (AIT). Sensitization patterns have been shown to predict the development of allergic diseases, disease progression and severity.[1-8]

A multitude of laboratory methodologies to detect allergen sIgE are currently available, including multiplex approaches[9,10] which are particularly attractive in paediatric allergology since they require lower blood sample volumes for a comprehensive allergy work-up as compared to singleplex tests. Yet, the "by-product" is the detection of sensitizations against allergens whose clinical relevance remains unclear. Selection of a sandwich immunoassay versus a semiquantitative immunoblot- versus a chip (microarray)-based assay influences the number of allergens which can be assessed simultaneously but may also influence analytical and diagnostic performance. Testing for sIgE against allergen extracts is favoured in cases of mono- or oligo-sensitization, low clinical risk and highly abundant and/or stable allergens. Allergenic molecules (components) on the other hand are preferred among polysensitized patients who are suspected to be sensitized against low abundance or labile proteins in allergen sources/extracts or among oligosensitized patients with the aim to differentiate between genuine sensitizations and cross-reactivity.[11] Systematic comparisons of the analytical and clinical performance of

Key Messages

- Sera from children of the ALLIANCE cohort were assessed for the presence of allergen sIgE.
- ImmunoCAP™ mixes, ImmunoCAP ISAC™ 112 and a Euroline™ panel showed good analytical and diagnostic concordance.
- Identification of more allergen sensitizations may not improve the clinical utility of sIgE testing among children.

different multiplex test systems, particularly among young children, are scarce.

Allergen challenges, particularly food challenges have a high specificity to detect clinically relevant sensitizations. However, they are work-intensive and pose a non-negligible risk of anaphylaxis. Thus, pre-screening of possible triggering allergens by multiplex assays is typically performed to narrow the number of allergens consecutively employed in allergen challenges. Knowledge on the comparative performance of different multiplex systems is thus desirable to be able to gauge their interoperability.

We hypothesized that methodology and individual features of different allergen multiplex sIgE detection systems confer different utilities to their ability to detect sensitizations. We compared the performance of three different assay systems in a sub-group of children from the paediatric arm of the ALL Age Asthma (ALLIANCE) cohort with regard to their ability to detect sensitizations compatible with (i) trigger factors eliciting respiratory symptoms as reported by the parental questionnaire (pollen or dust respectively) or (ii) allergic diseases as per symptoms or doctors' diagnoses (allergic rhinoconjunctivitis or food allergy) typically triggered by allergen exposure (i.e. aeroallergens or food allergens). Sera were therefore tested for the presence of allergen sIgE by the ImmunoCAP™ (sx1 and fx5 allergen extract mixes), the Euroline™ (extract- and component-based immunoblot), as well as the ImmunoCAP ISAC™ 112 (ISAC, component-based microarray).

2 | METHODS

2.1 | Ethics approval and consent to participate

The study protocol for the ALLIANCE cohort was prepared according to the Declaration of Helsinki and CONSORT guidelines (http://www.consort-statement.org/) and was approved by the lead ethics committee (University of Lübeck, Ethics Committee, Ratzeburger Allee 160, 23538 Lübeck, Germany) and the local ethics committees. The paediatric arm of the ALLIANCE asthma cohort was registered at clinicaltrials.gov on July 14, 2015 (NCT02496468). Informed consent for the children was given by either parent or caretaker if aged <8 years, and additionally by the child if ≥8 years.

2.2 | Subjects

Serum samples from 82 children with asthma or wheeze (cases, 77.4%) and 24 children without recurrent asthma or wheeze (controls, 22.6%) were obtained from young participants of the ALLIANCE cohort, a prospective, multicentre-based cohort of children with wheeze and children, adolescents and adults with asthma as well as healthy controls.[12] Children are recruited into the ALLIANCE cohort if parents have reported ≥2 episodes of wheeze (children < age 6 years) or according to modified GINA (children > age 6 years). Acute illness and chronic lung disease other than asthma/wheeze are the most pertinent exclusion criteria. These apply similarly to controls which may exhibit atopic comorbidities, that is allergic rhinoconjunctivitis and food allergy in controls but no asthma or wheezy episodes.[12] For the analysis presented here, we included all children having received ImmunoCAP™ sx1/fx5 (mixes of aeroallergens and food allergens, respectively, see "Detection of allergen sIgE" and also Table S1 and S2 measurement at the doctor's discretion in addition to Euroline™ and ImmunoCAP ISAC™ 112 analyses which were performed for all participants.

2.3 | Clinical definitions

In addition to the clinical definitions of "recurrent wheeze" and "asthma" defined as above, we resorted to defined clinical definitions derived from questionnaires[12] as follows: trigger factors for "wheezing symptoms" were identified by parent-filled questionnaires on dust, pollen, animal dander, or food triggering wheezing "ever" or "in the past 12 months." *Food allergy* was defined as experiencing food as a trigger factor for wheeze "ever" or "in the past 12 months" or specific symptoms suggestive of an allergic reaction to food (21 specific questions referring to skin or gastrointestinal reactions, ocular or general (malaise, syncope, allergic shock) symptoms upon food ingestion) as per the questionnaire, or doctor's diagnosis of food allergy. *Allergic rhinoconjunctivitis* was defined as itchy eyes or nose "ever" or "in the past 12 months" as per the questionnaire or doctor's diagnosis of hay fever.

2.4 | Detection of allergen sIgE

For each patient, the presence of specific IgE antibodies to aeroallergens and food allergens was examined using three independent test platforms: ImmunoCAP™, ISAC (both by Thermo Fisher Scientific) and Euroline™ (Euroimmun), according to the manufacturers' instructions. ImmunoCAP™ is an extract- and single component-based assay system presenting results in kU/L (calibrated against the WHO Standard for IgE). We used two premixed assays with extracts from 14 allergen sources in total. The mix sx1 (not available in every country) contains eight aeroallergens (d1, e1, e5, g6, g12, m2, t3, w6), the mix fx5 comprises six food allergens (f1, f2, f3, f4, f13, f14). In case of a positive result, all included allergens were tested individually for this sample to confirm and define the specific sensitization. The ISAC is a multiplex microarray including in total 112 defined allergen components from 51 allergen sources, of which only food and aeroallergens (pollen, mite and other aeroallergens) were taken into account in this study (99 components from 45 sources). It is a manual, semiquantitative method with results given in ISU-E. The Euroline™ assay is a semiquantitative multiplex immunoblot, which, in our study, tested a total of 22 allergen extracts and 14 components. The results were calibrated by using the three indicator bands on each strip and are presented in kU/L.

For additional details regarding allergens and specific allergen sources included in a given test system, please consult Supplementary Information and Table S1 and S2.

2.5 | Data processing and statistical analyses

We used two thresholds for positivity for each test system: threshold 1 (analytical relevance) was defined as a sIgE ≥ 0.35 kU/L for ImmunoCAP™ sx1/fx5 and Euroline™ (Class 1) and ≥0.3 ISU/L for ISAC (lowest threshold for positivity). Threshold 2 (clinical relevance, used in the ALLIANCE cohort) was defined as ≥0.7 kU/L for ImmunoCAP™ sx1/fx5 and Euroline™ (Class 2), and ≥1.0 ISU/L for ISAC (moderate positivity). A test result was considered positive for a specific allergen when the concentration of any related extract or component exceeded the predefined threshold.

We calculated the overall concordance by dividing the number of samples with three equal test results by all 106 samples. Accordingly, positive and negative concordance represent the percentages of samples with three positive and negative test results among all samples tested positive and negative by at least one kit respectively. The comparisons of the test results were made using several measures, including the percentage of positive tests, percentage of negative tests, overall, positive, and negative concordance, sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and sensitization rate.

Two of the assay types (ISAC, Euroline™) cover more than one component for some allergen sources. We thus tested the following approaches: (i) either the sum of all allergen sIgE for each source or (ii) only the allergen with the highest response (maximum sIgE response) was considered. Our results showed no differences between the two approaches, so we opted to utilize the latter option within this publication (maximum sIgE response to any given allergen source).

For comparisons between the three analysis platforms with regard to defined clinical outcomes, we used the following approaches: first, we included only those allergens (only pollen allergens, only mite allergens, all aeroallergens (i.e. including pollen and mite allergens as well as assay-specific animal, mould and cockroach allergens, depending on the assay), or all food allergens) that are common to all three assay systems (Table 1). In a separate analysis, we included all allergens of a specific allergen group (as in the first approach) available in any of the test systems for comparisons (Table S1 and S2).

Diagnostic performance was illustrated by means of ROC curves by plotting the true-positive rate against the false-positive rate at different thresholds of specific allergen sIgE concentrations for the following determinants 1 = only pollen allergens, 2 = only mite allergens, 3 = all aeroallergens, 4 = all food allergens with the corresponding clinical outcomes: (i) the presence of wheezing symptoms upon trigger factor pollen defined per questionnaire, (ii) the presence of wheezing symptoms upon trigger factor dust defined per questionnaire, (iii) the presence of symptoms of or doctor's diagnosed allergic rhinoconjunctivitis, (iv) the presence of symptoms of or doctor's diagnosed food allergy. Comparison across systems was performed by assessing the area under the ROC curves (AUC). We ran a global ANOVA to detect differences in the diagnostic performance between all three test systems followed by bootstrapping with 10,000 bootstrap replications on the difference between each two AUC values. To assess the effect of one threshold over the other, or of considering individual allergens over all allergens, we either ran a McNemar's test (considering the paired nature of the data) or a binomial test between the two resulting 2x2 contingency tables which show the discrepancies between two given scenarios for each test. A p-value <.05 was considered as a statistically significant difference between both scenarios. We adjusted all p-values for age by stratification and for multiple testing according to the stepdown algorithm. Between the assays, any significance is related to the comparison of the ROC curves.

All computations were performed with the software R version 3.5.3 using the packages "openxlsx," "memisc," "surveydata," "survival," "ggplot2," "reshape2," "plyr," "dplyr," "doBy," "compareGroups," "Hmisc," "VennDiagram," "gridExtra," "plotROC," "pROC," "lsr," "caret," "xtable," "extrafont" and "boot."

3 | RESULTS

3.1 | Patient characteristics

Participants in this analysis had a mean age of 4 years (range 0–16 years), with the majority (69.8%) being <6 years of age. Almost two-thirds of all children were male (61.3%; Table 2). Cases and controls did not differ significantly in gender but cases were significantly younger (mean age 3 years) than controls (mean age 6 years, p = .001). Both groups did not differ significantly in doctor's diagnoses of atopic dermatitis or food allergy. Cases, however, had significantly higher rates of allergic rhinoconjunctivitis (19.5% vs. 0%, p = .02). They also had higher percentages of 1st degree relatives with asthma compared to controls (53.1%, 48/78 cases; 25.0%, 6/24 controls). 30.9% (25/81) of cases reported use of inhaled corticosteroids (ICS) use in the past four weeks. 41.2% (21/51) had ever received systemic steroids at least once and 23.0% (17/74) in the past 12 months. As per the use of salbutamol (a short-acting β_2 agonist), patients reported a fairly high number of exacerbations in the past 12 months (82.9%). 25.6% of cases had necessitated an emergency room visit and half of all patients had already been hospitalized for a wheezy episode (50.0%) yet most did not require oxygen supplementation (61.0%; Table 2). The most significant parental reported trigger factor for wheeze was pollen (20/82), followed by dust (14/82), animals (6/82) and food (5/82; Table 2). At threshold 2 but not at threshold 1 cases displayed significantly higher sensitization rates determined by Euroline™ than controls for comparable and all allergens (Table 3), differences being significant for threshold 2 also for the subgroup of pollen allergens, mite allergens, all aeroallergens and all food allergens (data not shown).

TABLE 1 Comparable aeroallergens and food allergens across all three *in vitro* diagnostic assays

ImmunoCAP™ sx1/fx5	Euroline™	ImmunoCAP ISAC™ 112	Allergen source
Aeroallergens			
d1	d1	Der.p.1	House dust mite
d1	d1	Der.p.2	House dust mite
d1	d1	Der.p.10	House dust mite
e1	e1	Fel.d.1	Cat dander
e1	e1	Fel.d.2	Cat dander
e1	e1	Fel.d.4	Cat dander
e5	e2	Can.f.1	Dog dander
e5	e2	Can.f.2	Dog dander
e5	e2	Can.f.3	Dog dander
e5	e2	Can.f.5	Dog dander
m2	m2	Cla.h.8	Cladosporium
t3	t3	Bet.v.1	Common silver birch
t3	t3	Bet.v.2	Common silver birch
t3	t3	Bet.v.4	Common silver birch
w6	w6	Art.v.1	Mugwort
w6	w6	Art.v.3	Mugwort
Food allergens			
f1	f1	Gal.d.1	Egg white
f1	f1	Gal.d.2	Egg white
f1	f1	Gal.d.3	Egg white
f2	f2	Bos.d.4	Milk
f2	f2	Bos.d.5	Milk
f2	f2	Bos.d.6	Milk
f2	f2	Bos.d.8	Milk
f2	f2	Bos.d. Lactoferrin	Milk
f3	f3	Gad.c.1	Fish (cod)
f4	f4	Tri.a.14	Wheat
f4	f4	Tri.a.19.0101	Wheat
f4	f4	Tri.a.aA.TI	Wheat
f13	f13	Ara.h.1	Peanut
f13	f13	Ara.h.2	Peanut
f13	f13	Ara.h.3	Peanut
f13	f13	Ara.h.6	Peanut
f13	f13	Ara.h.8	Peanut
f13	f13	Ara.h.9	Peanut
f14	f14	Gly.m.4	Soybean
f14	f14	Gly.m.5	Soybean
f14	f14	Gly.m.6	Soybean

3.2 | Qualitative concordance

Overall, the concordance of positive results of the comparable allergens between the three test systems depended on the threshold used to define positivity. When resorting to threshold 1 (see methods) 29/49 positive results were identified by all three test systems (Figure 1A). Euroline™ identified the highest number of positive samples (41.5% of all positive samples), while 31.1% were detected by ISAC and 34.0% by ImmunoCAP™ sx1/fx5 (Figure 1C).

TABLE 2 Population's characteristics

	All	Asthma+wheeze	Controls	
	n = 106	*n* = 82	*n* = 24	*p* overall
Age	4.39 ± 3.32	3.71 ± 2.92	6.71 ± 3.62	.001
Age group:				
0–6 years	74 (69.8%)	63 (76.8%)	11 (45.8%)	.008
>6 years	32 (30.2%)	19 (23.2%)	13 (54.2%)	
Gender				
Female	41 (38.7%)	31 (37.8%)	10 (41.7%)	.918
Male	65 (61.3%)	51 (62.2%)	14 (58.3%)	
Atopic dermatitis				
No	87 (82.1%)	64 (78.0%)	23 (95.8%)	.067
Yes	19 (17.9%)	18 (22.0%)	1 (4.2%)	
Food allergy				
No	96 (90.6%)	72 (87.8%)	24 (100%)	.112
Yes	10 (9.4%)	10 (12.2%)	0 (0.00%)	
Allergic rhinoconjunctivitis				
No	90 (84.9%)	66 (80.5%)	24 (100%)	.020
Yes	16 (15.1%)	16 (19.5%)	0 (0.00%)	
Trigger pollen				
No	86 (81.1%)	62 (75.6%)	24 (100%)	.006
Yes	20 (18.9%)	20 (24.4%)	0 (0.00%)	
Trigger dust				
No	92 (86.8%)	68 (82.9%)	24 (100%)	.036
Yes	14 (13.2%)	14 (17.1%)	0 (0.00%)	
Trigger animal				
No	100 (94.3%)	76 (92.7%)	24 (100%)	.333
Yes	6 (5.7%)	6 (7.3%)	0 (0.00%)	
Trigger food				
No	101 (95.3%)	77 (93.9%)	24 (100%)	.586
Yes	5 (4.7%)	5 (6.1%)	0 (0.00%)	
Asthma in 1st degree relative				
No	56 (53.3%)	38 (46.9%)	18 (75%)	.029
Yes	49 (46.7%)	43 (53.1%)	6 (25%)	
Allergic rhinoconjunctivitis in 1st degree relative				
No	37 (36.3%)	30 (38.5%)	7 (29.2%)	.558
Yes	65 (63,7%)	48 (61.5%)	17 (70.8%)	
Eczema in 1st degree relative				
No	69 (69.7%)	50 (65.8%)	19 (82.6%)	.201
Yes	30 (30.3%)	26 (34.2%)	4 (17.4%)	
ICS use in the past 4 weeks				
No	80 (76.2%)	56 (69.1%)	24 (100%)	.004
Yes	25 (23.8%)	25 (30.9%)	0 (0.00%)	
Systemic steroid use ever				
No	35 (50.0%)	30 (58.8%)	5 (26.3%)	.02
Once	8 (11.4%)	6 (11.8%)	2 (10.5%)	
More than once	27 (38.6%)	15 (29.4%)	12 (63.2%)	

(Continues)

TABLE 2 (Continued)

	All	Asthma+wheeze	Controls	
	n = 106	*n* = 82	*n* = 24	*p* overall
Systemic steroid use in the past 12 months				
No	84 (79.2%)	57 (77.0%)	27 (84.4%)	.551
Yes	22 (20.8%)	17 (23.0%)	5 (15.6%)	
SABA use in the past 12 months				
No	38 (35.8%)	14 (17.1%)	24 (100%)	<.001
Yes	68 (64.2%)	68 (82.9%)	0 (0.00%)	
Emergency room consultation for wheeze in the past 12 months				
No	85 (80.2%)	61 (74.4%)	24 (100%)	.003
Yes	21 (19.8%)	21 (25.6%)	0 (0.00%)	
Hospitalization for wheeze (ever)				
No	65 (61.3%)	41 (50.0%)	24 (100%)	<.001
Yes	41 (38.7%)	41 (50.0%)	0 (0.00%)	
Supplemental oxygen for wheeze (ever)				
No	74 (69.8%)	50 (61.0%)	24 (100%)	.001
Yes	32 (30.2%)	32 (39.0%)	0 (0.00%)	

Note: Basic demographic and clinical characteristics of the study cohort. Sera from 106 children from the ALLIANCE cohort were assessed for allergen sIgE by means of three commercially available test systems. Thresholds were ≥0.35 kU/L (threshold 1) and ≥0.7 kU/L (threshold 2) for ImmunoCAP™ and Euroline™ and ≥0.3 ISU/L (threshold 1) and ≥1.0 ISU/L (threshold 2) for ImmunoCAP ISAC™ 112.
Abbreviations: ICS, Inhaled corticosteroids; SABA, Short-acting β_2 agonists.

Results changed when defining a more stringent threshold 2 (see methods). The overall number of positive results was reduced to 39, of which 27 were identified by all three test systems (Figure 1B). This more stringent threshold led to ImmunoCAP™ sx1/fx5 identifying the highest percentage of positive samples (identifying 34.0% test samples as positive), followed by Euroline™ (28.3%) and ISAC (27.4%; Figure 1D).

Similarly, upon assessment of qualitative positive concordance for individual allergens, an increase in threshold for positivity led to important changes. Resorting to the lower threshold, ImmunoCAP™ sx1/fx5 showed the highest percentage of positive results for seven out of 12 comparable allergens. Euroline™ identified the highest percentage of positive samples for two out of 12 comparable allergens and ISAC showed the highest percentage of positive results for one out of 12 allergens (Figure 1E). Following application of the higher threshold, ImmunoCAP™ sx1/fx5 showed the highest percentage of positive results for nine allergens. ISAC remained the test system identifying the highest percentage of positive samples for peanut, and Euroline™ did not detect the highest percentage of positive samples for any comparable allergen (Figure 1F).

Overall, negative and positive concordance for all comparable allergens following application of the lower or higher positivity threshold is shown in Figure S1. Overall and negative concordances were uniformly high (at least 82%, regardless of threshold). Positive concordance was, however, more variable ranging between 0% (Cladosporium and wheat at both thresholds; dog dander and soybean at higher threshold) and 100%; the latter in the case of cod where one single positive sample was detected by all three test systems (Figure S1). Common silver birch and house dust mite, the allergens with the highest overall positive detection rates, showed comparatively high positive concordance (>50%) at both thresholds. All other allergens showed comparatively poor positive concordance (<50%) for both thresholds.

3.3 | Identifying pollen sensitization triggering wheezing symptoms

In order to compare the diagnostic performance of the three test systems, we examined four common scenarios according to the allergenic source suspected by their parents of triggering allergic symptoms, that is, young children who present with allergic symptoms of which the allergen triggers are at present unknown. We calculated sensitivity and specificity (plotted as ROC curves), as well as positive (PPV) and negative predictive values (NPV) for each of these scenarios.

As the first clinical scenario, we chose children whose parents reported wheezy symptoms triggered by pollen exposure (questionnaire-based item: trigger factor pollen "ever" or "within the last 12 months" for wheezing symptoms, positive for 20 of all 106 children). As determinants, we assessed sIgE values above the threshold for pollen allergens (defined in Tables 1, S1, Supplementary Information). Neither of the three assay systems displayed significant superiority for NPV, PPV, sensitivity or specificity for comparable or all pollen sensitizations, regardless of the thresholds used (Figure 2A-D).

Changing the threshold for positivity from threshold 1 to threshold 2 did not lead to significant changes of sensitivity, specificity,

TABLE 3 Overall sensitization rates

	Asthma+wheeze	Controls	*p* overall
ImmunoCAP™			
Threshold 1			
All allergens	33 (40.2%)	4 (16.7%)	<.001
Comparable allergens	33 (40.2%)	3 (12.5%)	<.001
Threshold 2			
All allergens	33 (40.2%)	3 (12.5%)	<.001
Comparable allergens	33 (40.2%)	3 (12.5%)	<.001
Euroline			
Threshold 1			
All allergens	42 (51.2%)	12 (50.0%)	.865
Comparable allergens	37 (45.1%)	7 (29.2%)	.209
Threshold 2			
All allergens	30 (36.6%)	3 (12.5%)	<.001
Comparable allergens	27 (32.9%)	3 (12.5%)	.004
ImmunoCAP ISAC™ 112			
Threshold 1			
All allergens	33 (40.2%)	4 (16.7%)	.011
Comparable allergens	31 (37.8%)	2 (8.3%)	<.001
Threshold 2			
All allergens	28 (34.1%)	3 (12.5%)	.002
Comparable allergens	27 (32.9%)	2 (8.3%)	<.001

Note: Thresholds for the determination of overall sensitization rates were ≥0.35 kU/L (threshold 1) and ≥0.7 kU/L (threshold 2) for ImmunoCAP™ and Euroline™ and ≥0.3 ISU/L (threshold 1) and ≥1.0 ISU/L (threshold 2) for ImmunoCAP ISAC™ 112.

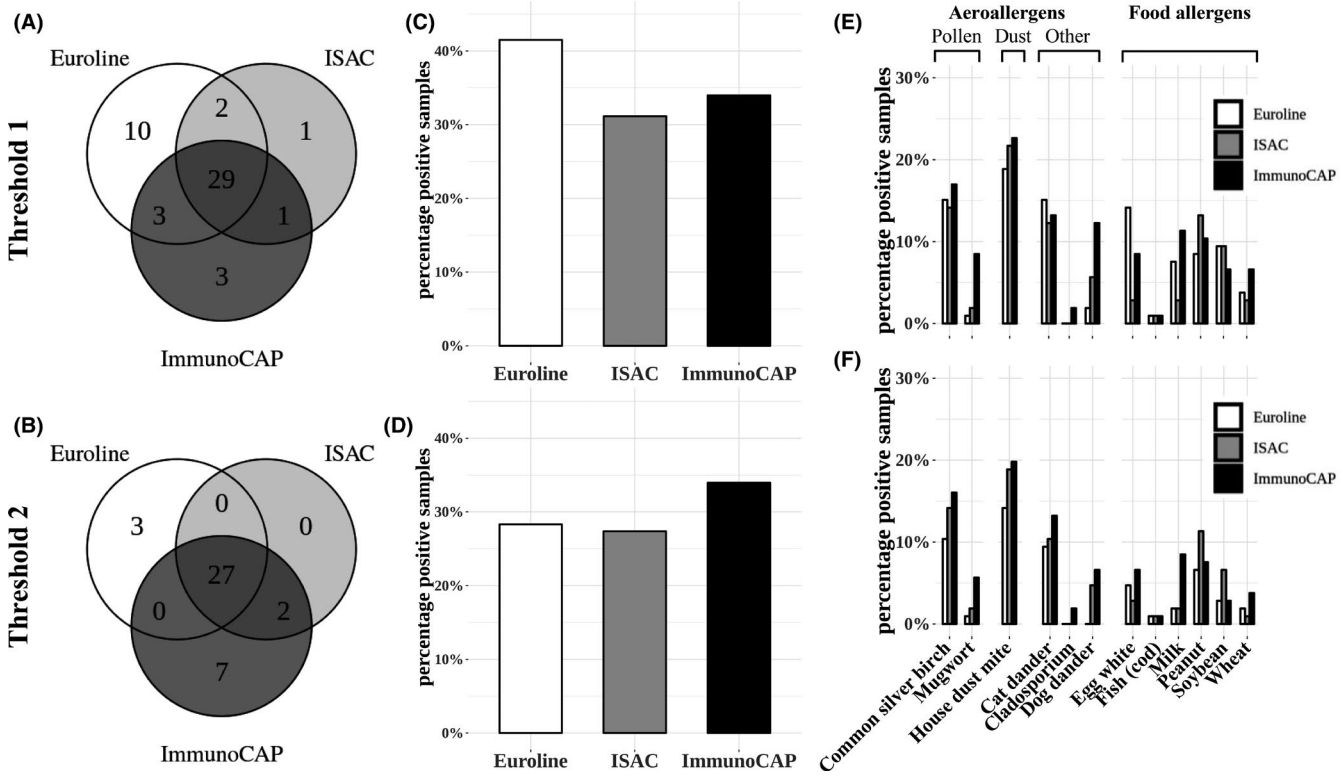


FIGURE 1 Analytical performance. Venn diagram and percentage of positive samples/assay for threshold 1 = ≥0.35 kU/L for ImmunoCAP™ and Euroline™ (Class 1), and ≥0.3 ISU/L for ImmunoCAP ISAC™ 112 (A, C) and for threshold 2 = ≥0.7 kU/L for ImmunoCAP™ and Euroline™ (Class 2) and ≥1,0 ISU/L for ImmunoCAP ISAC™ 112 (B, D); percentage of positive samples for comparable allergens only, for threshold 1 (E) and threshold 2 (F)

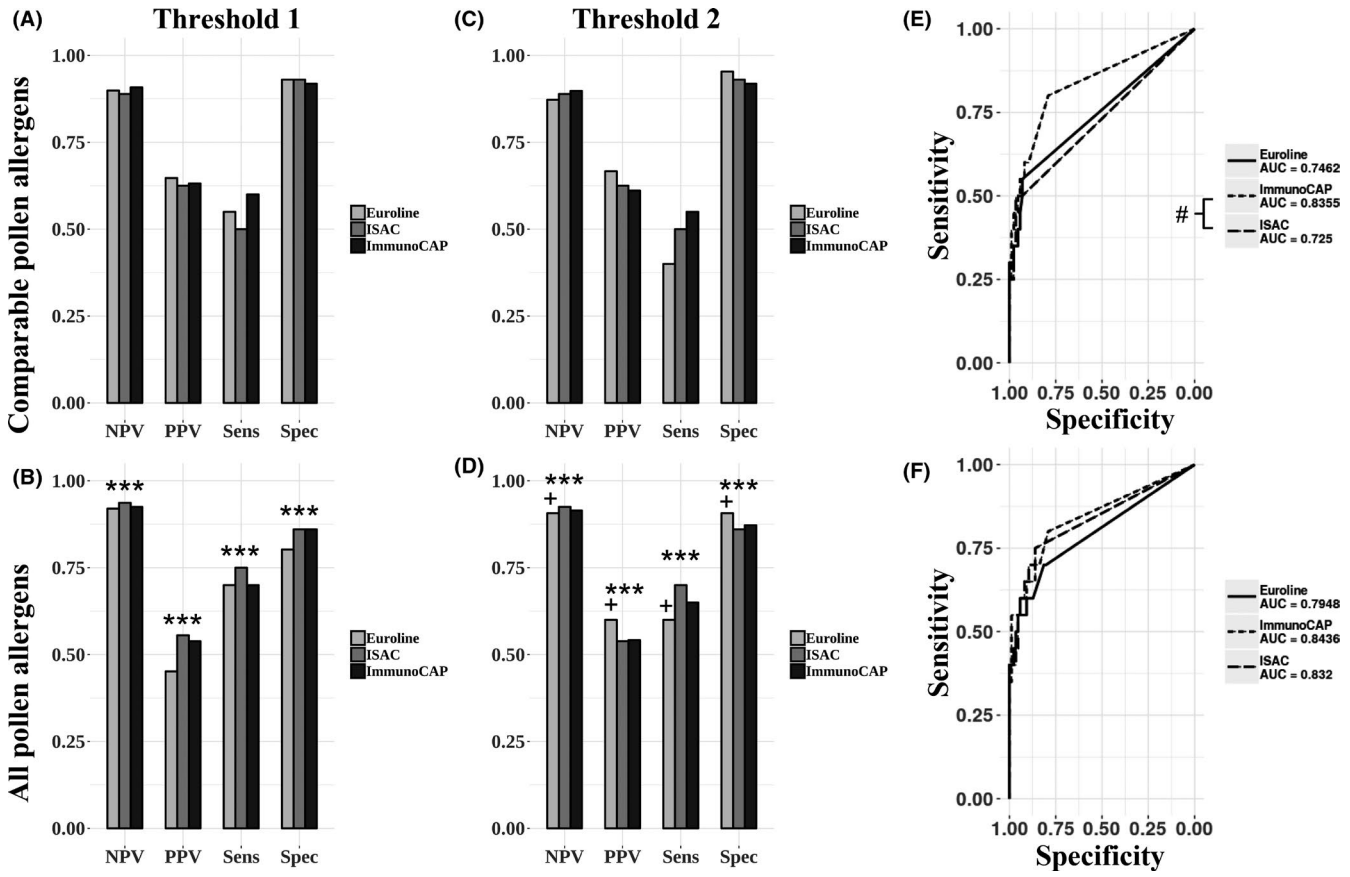


FIGURE 2 Negative (NPV) and positive predictive value (PPV), sensitivity and specificity for outcome respiratory symptoms, triggered by pollen. NPV, PPV, sensitivity and specificity for comparable pollen allergens and for all pollen allergens/assay for threshold 1 (A, B) and for threshold 2 (C, D). (E) ROC curves for outcomes "trigger pollen" for comparable and (F) all pollen allergens tested by the assays. For 20 of all 106 children, parents reported pollen as trigger for asthma/wheeze. $^{*}p < .05$ for all versus comparable allergens, $^{+}p < .05$ for threshold 1 versus threshold 2, $^{\#}p < .05$ between assays

NPV or PPV, except for the Euroline™ when assessing all pollen allergens included in the Euroline™ ($p = .001$, 2B vs. 2D).

As expected, enlarging the spectrum of allergens significantly increased sensitivity at the expense of specificity and NPV at the expense of PPV for all test systems ("comparable pollen allergens" vs. "all pollen allergens", Figure 2A vs. B, C vs. D; for details refer to Supplementary Information, Tables 1, S1). This was independent of the threshold used ($p < .001/p = .001/.016$ at threshold 1, $p = .008/.002/.031$ at threshold 2) for Euroline™ versus ISAC/ImmunoCAP™ sx1.

Considering only pollen allergens comparable across all three test systems, the ImmunoCAP™ sx1 test system showed significantly better performance for prediction of wheezing symptoms triggered by pollen exposure than ISAC but not compared to Euroline (Figure 2E, areas under the ROC curves (AUCs) for ImmunoCAP™, ISAC, Euroline™: 0.836, 0.725 and 0.746, respectively, $p = .041$ for ImmunoCAP™ sx1 vs. ISAC, $p = .080$ for ImmunoCAP™ sx1 vs. Euroline™). This difference was lost when including all pollen allergens contained in each test system into our analysis (Figure 2F, $p = .689$ for ImmunoCAP™ sx1 vs. ISAC, $p = .097$ for ImmunoCAP™ sx1 vs. Euroline™).

3.4 | Identifying mite allergens triggering wheezing symptoms

As the second scenario, we chose children whose parents reported that wheezing symptoms were triggered by dust exposure (questionnaire-based item: trigger factor dust "ever" or "within the last 12 months" for wheezing, positive for 14 of all 106 children), comparing the detected sensitizations against comparable versus all mite allergens as determinants (for details refer to Supplementary Information, Tables 1, S1).

Again, neither of the three assay systems displayed significant superiority for NPV, PPV, sensitivity or specificity for comparable or all mite allergens, regardless of the threshold used (Figure 3A-D).

There were mild increases in PPV and decreases in sensitivity (associated with mild increases in specificity) when comparing the lower to the higher threshold, however, these changes did not reach statistical significance (Figure 3A-D).

Unlike the trigger factor pollen, increasing the number of mite-specific allergens in a given test system did not induce consistent or significant changes in NPV, PPV, sensitivity or specificity (Figure 3A-D).

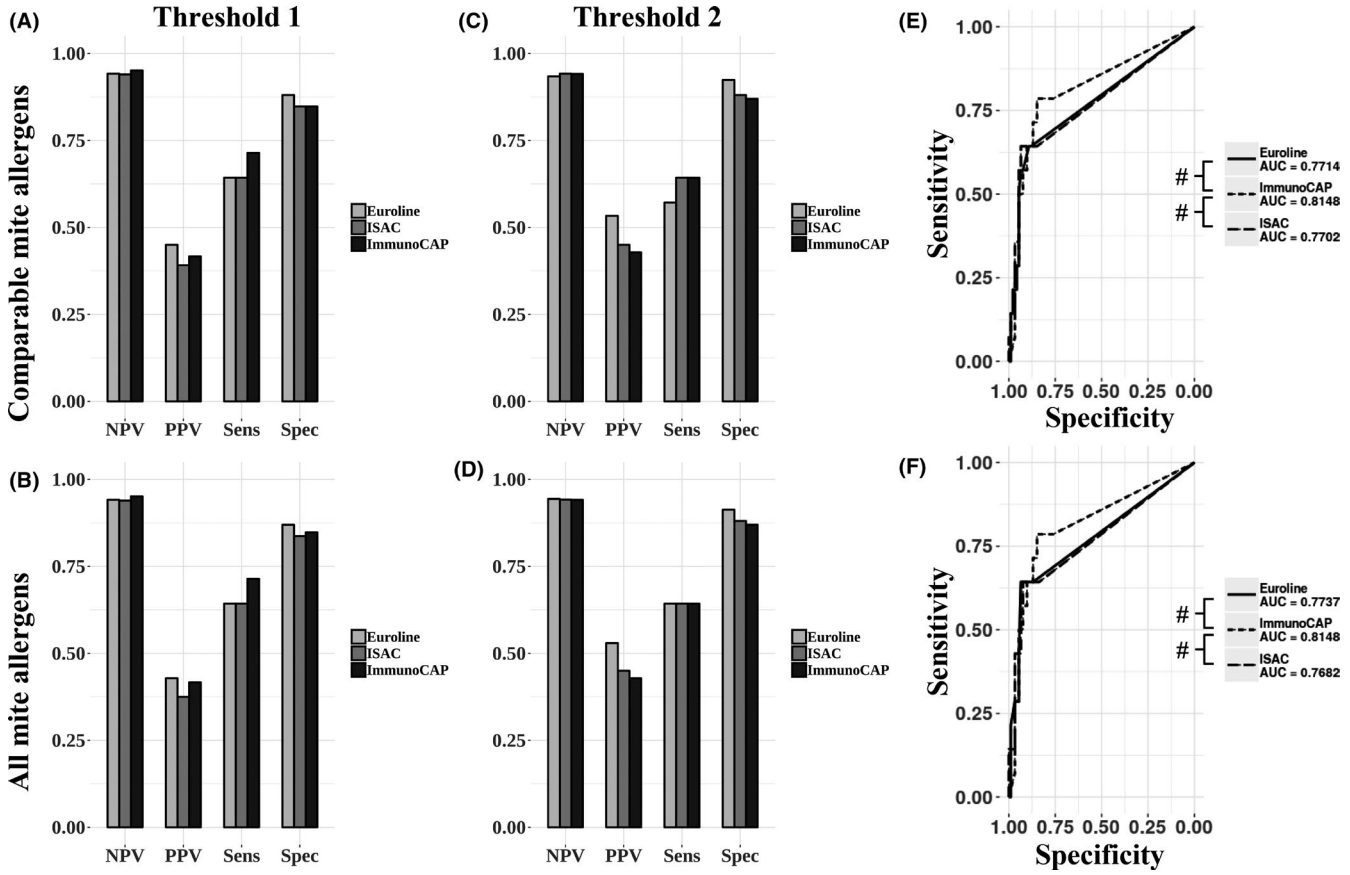


FIGURE 3 Negative (NPV) and positive predictive value (PPV), sensitivity and specificity for outcome respiratory symptoms, triggered by dust. NPV, PPV, sensitivity and specificity for comparable mite allergens and for all mite allergens/assay for threshold 1 (A, B) and for threshold 2 (C, D). (E) ROC curves for outcomes "trigger dust" for comparable and (F) all mite allergens tested by the assays. For 14 of all 106 children, parents reported dust as trigger factor for asthma/wheeze. $^{\#}p < .05$ between assays

AUCs of the ROC curves were the highest and significant for ImmunoCAP™ sx1 for both comparable and all house dust mite allergens included (0.815 for ImmunoCAP™ (both comparable and all mite allergens) versus 0.770 and 0.768 for ISAC (p = .029/.033) versus .771 and .774 for Euroline™ (p = .040/.040), Figure 3E-F).

3.5 | Identifying aeroallergen sensitization in children with allergic rhinoconjunctivitis

In the third scenario, we analysed children who presented with allergic rhinoconjunctivitis (questionnaire-based or doctor's-diagnosed, positive for 16 of all 106 children), comparing the detected sensitizations against all comparable versus all aeroallergens as determinants (for details refer to Supplementary Information, Tables 1, S1).

We did not observe any significant differences between the three assays' diagnostic performance in detecting clinically relevant aeroallergen sensitizations (NPV, PPV, sensitivity, specificity, Figure 4A-D).

Increasing stringency by moving from threshold 1 to threshold 2 consistently improved PPV and specificity for all three assays, yet these changes only attained statistical significance for the Euroline™ (Figure 4A vs. C and B vs. D, p = .031 and p = .002, for all comparable and all aeroallergens respectively).

Inclusion of all aeroallergens instead of only comparable allergens decreased PPV and specificity for all three assays for both thresholds but this change was only statistically significant for the Euroline™ at the lower threshold (Figure 4A vs. B, p = .031).

Furthermore, we could not detect any significant differences between the three assays in terms of predicting the outcome allergic rhinoconjunctivitis (questionnaire-based items: "itchy nose," "itchy eyes," or "itchy nose and eyes" "ever" or "within the last 12 months" or doctor's-diagnosed hay fever, determinants: all comparable or all aeroallergens in the three test systems) by ROC analyses of aeroallergens (Figure 4E-F).

3.6 | Identifying food allergen sensitization in children with food allergy

Finally, in the fourth scenario, we analysed the diagnostic performance in children with food allergy (questionnaire-based items: triggering food(s) for wheeze or specific symptoms suggestive of an allergic reaction to food, both "ever" or "within the past 12 months" or doctor's-diagnosed food allergy, positive for 5 of all 106 children),

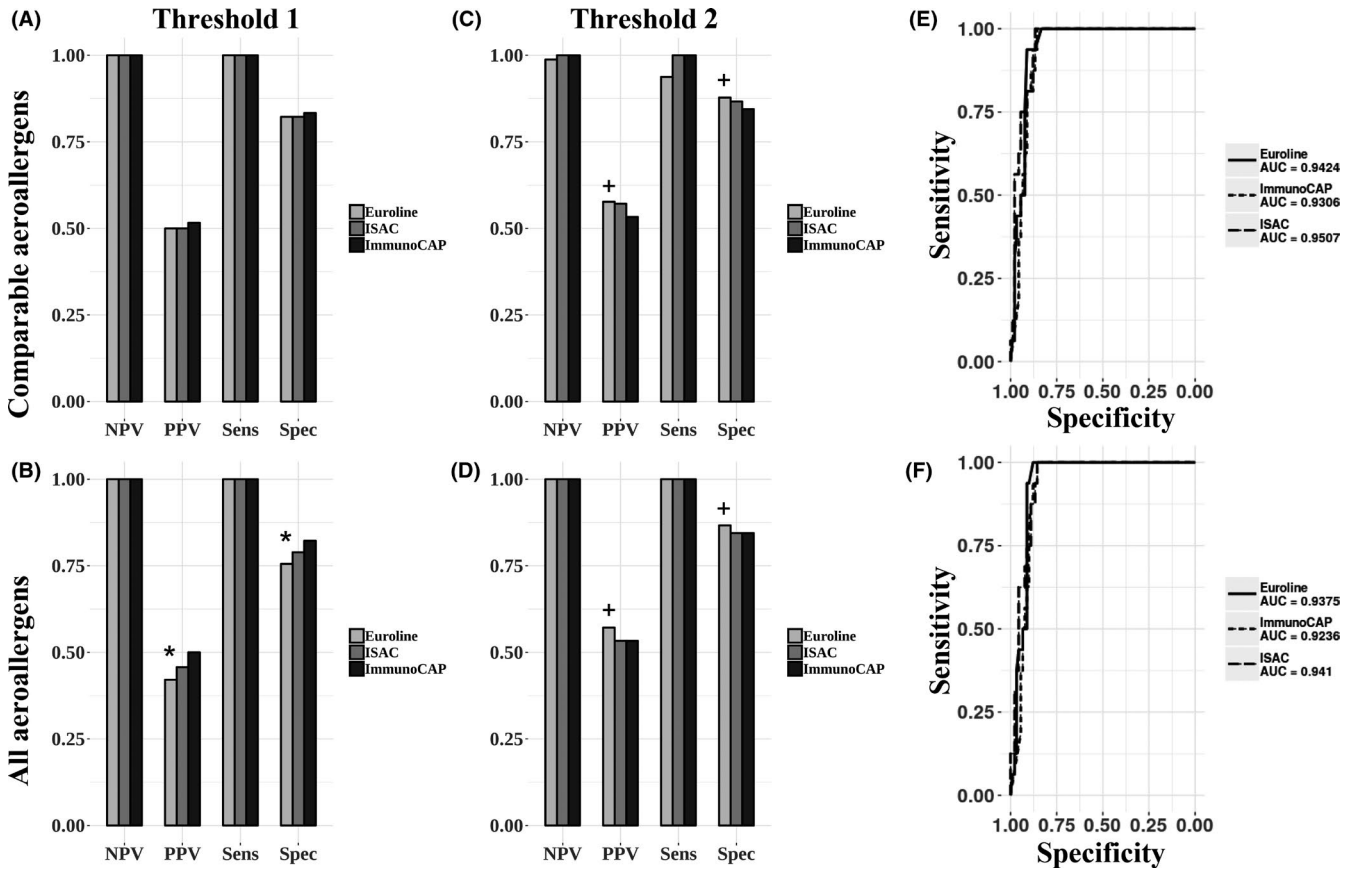


FIGURE 4 Negative (NPV) and positive predictive value (PPV), sensitivity and specificity for outcome allergic rhinoconjunctivitis. NPV, PPV, sensitivity and, specificity for comparable aeroallergens and for all aeroallergens/assay for threshold 1 (A, B) and for threshold 2 (C, D). (E) ROC curves for outcome "allergic rhinoconjunctivitis" for comparable and (F) all aeroallergens tested by the assays. Sixteen of all 106 children with asthma/wheeze had allergic rhinoconjunctivitis. $^{*}p < .05$ for all versus comparable allergens, $^{+}p < .05$ for threshold 1 versus threshold 2

assessing as determinants sIgE against comparable versus all food allergens (for details refer to Supplementary Information, Tables 1, S2). In these children, the overall diagnostic performance for determining sensitization against food allergens was similar with no consistent and significant superiority detected for any system (Figure 5A-D).

Increasing the threshold consistently increased PPV and specificity at the expense of sensitivity, however, these changes were statistically significant only for the Euroline™ for comparable and all food allergens (both $p < .001$, Figure 5A vs. C and B vs. D).

Increasing the number of allergens consistently resulted in decreased PPV and specificity, which was statistically significant for the Euroline™ and the ISAC at the lower threshold (Figure 5A vs. B, $p < .001$ and $p = .031$) and for the ISAC at the higher threshold also (Figure 5C vs. D, $p = .016$).

AUCs of ROC curves for sensitivity and specificity also showed no statistically significant differences in predicting food allergy (Figure 5E-F).

4 | DISCUSSION

In the analyses presented here, we compared the analytical and diagnostic performance of three test systems to detect sIgE in allergen mixes and in multiplex assays respectively. Although the three systems are based on different laboratory methodologies, our results show good and comparable overall and negative concordance and good positive concordance for the most common sensitizations found in our cohort (dust mite and birch). Similarly, the diagnostic performance of all tests was largely comparable regarding typical clinical scenarios such as the identification of clinically relevant food and aeroallergen sensitization. Regarding the outcomes “trigger factor pollen” and “trigger factor dust” where significant differences could be observed between the ImmunoCAP™ sx1/fx5 and the Euroline™ versus the ISAC (pollen) and the ImmunoCAP™ sx1/fx5 versus the Euroline™ and the ISAC (dust), these differences were small and most likely do not affect clinical utility. The comparatively larger allergen number included in the Euroline™ compared to the ISAC led to the detection of higher numbers of sensitization compared to the ImmunoCAP™ sx1/fx5. These differences, however, did not consistently identify more sensitizations associated with clinical symptoms, questioning the utility of larger allergen panels and/or molecular-based methodologies in this age group.

Allergen sIgE screening assays are a mainstay of paediatric allergology because trigger factors for young children's allergic symptoms are often more difficult to discern than in older children or adults

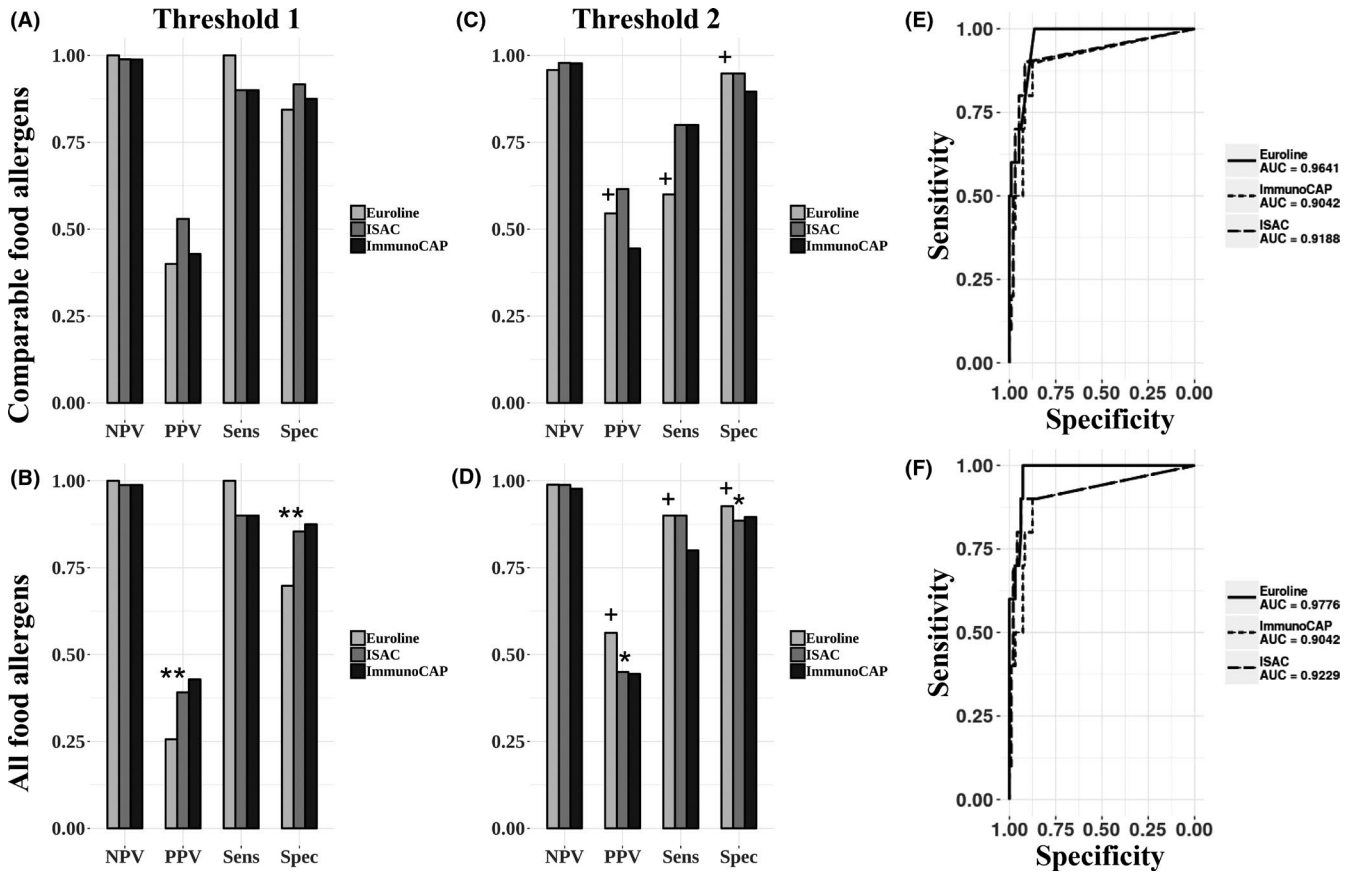


FIGURE 5 Negative (NPV) and positive predictive value (PPV), sensitivity and specificity for outcome food allergy. NPV, PPV, sensitivity and, specificity for comparable food allergens and for all food allergens/assay for threshold 1 (A, B) and for threshold 2 (C, D). (E) ROC curves for outcome "food allergy" for comparable and (F) all food allergens tested by the assays. For 5 of all 106 children, parents reported food as trigger factor for asthma/wheeze. $^{*}p < .05$ for all versus comparable allergens, $^{+}p < .05$ for threshold 1 versus threshold 2

and these assays require low blood volumes per number of detectable allergens. Whilst extended, molecular-based screening systems are clearly useful in an epidemiological context where patterns of sensitization have been shown to be associated with trajectories of disease,[1-6] their utility in clinical practice in young children remains unclear. Component-resolved diagnostics can provide information which cannot be obtained by extract-based assay systems.[13-15] However, depending on their composition, component-resolved assays may miss minor allergen specificities that are diagnostically relevant for individual patients compared to more comprehensive extract-based assays. Indeed, when we investigated which assay showed the most positive samples for any single allergen, the ImmunoCAP™ sx1/fx5 outperformed the Euroline™ and the ISAC, regardless of the threshold (Figure 1E,F). Published comparisons of sIgE assays often focus on analytical performance[13-17] which do not provide information on clinical utility. Available diagnostic performance studies typically include patients characterized by a specific sensitization and symptom profile.[18,19] In line with our findings, Wang et al. showed that the predictive values of allergen sIgE tests vary largely, depending on the allergen analysed.[20] Similarly, Griffiths et al. found that the ImmunoCAP™ performed better than the ISAC for single allergens but also on a diagnostic level analytically with regard to the identification of anaphylaxis. The ISAC outperformed the ImmunoCAP™ among patients with oral allergy syndrome.[19]

The uniqueness of our study lies in (i) the inclusion of children at a young age, (ii) the inclusion of children regardless of sensitization status and/or profile and (iii) the comparison of the analytical and diagnostic performance of three *in vitro* sIgE test systems commonly used in research or clinical settings. Indeed, to our knowledge, our report is the first including the Euroline™, the ImmunoCAP™ sx1/fx5 and the ISAC chip in a comprehensive comparative study.

Our analyses identify similar inter-assay agreement compared to studies by others,[20] including component-resolved panel systems.[13-17,21,22] We did find that, when considering overall positive samples, the Euroline™ suffered from a higher background signal as it detected the highest number of sensitizations at threshold 1, the less stringent threshold, but not at threshold 2, the higher and more stringent threshold. Along these lines, sensitivity, specificity, NPV and PPV of the three test systems we compared were similar to what others have previously reported,[19,20,23] reassuring the practicing allergologist and confirming an overall good concordance.

When increasing the number of considered allergens in our analyses from comparable allergens to all allergens within one assay, that is interrogating the value of extended (component resolved) allergen

panels, we detected up to 14 additional sensitizations with an average of four across all allergen groups and thresholds (Table S3). Yet, mostly, we identified non-significant decreases in PPV and specificity by these changes, regardless of the clinical scenario (Figures 2–5, A–D). Clinical prediction of specific allergens responsible for triggering clinical symptoms assessed by ROC curves did also not improve significantly by employing more comprehensive allergen panels (Figures 2–5, E–F). Rather contrary, for the association of the scenario of wheeze upon trigger dust with house dust mite sensitization, the extract-based ImmunoCAP™ sx1 mix outperformed both other more comprehensive assay systems (Figure 3F), suggesting that *Dermatophagoides pteronyssinus* (extract d1) constitutes the most important allergen source for clinically relevant sensitization to (dust) mite in our cohort. Thus, in the age group we analysed, where polysensitization is not common yet, the additionally identified sensitizations to d2 and d4 (Euroline™) and Der f1, Der f2 and Lep d2 (ISAC) appear to only inconsistently translate into symptoms and mostly seem to be "silent".

The only exception thereof was the analysis of pollen sensitization in the scenario of pollen-triggered wheeze where a more comprehensive allergen panel significantly improved PPV and specificity (Figure 2A vs. B, C vs. D). Grass sensitization could only be included in the analysis of all allergens (Figure 2B,D) but not in the analysis of comparable allergens (Figure 2A,C). The lack of comparable grass allergens across the three test systems (Table 1 vs. S1) thus hampered comparability. Another possible explanation could be that children with grass pollen sensitization were somewhat older than children with dust mite sensitization or food allergy. This supports the notion that a larger spectrum of allergens improves diagnostics for older individuals, who are more likely to be polysensitized or sensitized against multiple allergen components of a single source but not for young children. However, our p-values were age-adjusted, refuting age as a critical confounder in these analyses.

Particularly in food allergies, the eliciting trigger factors might not always be clinically discernable and oral challenges thus are the gold standard for diagnosis. In these situations, the detection of specific sensitizations via sIgE assays can guide the selection of allergens for food challenges. In that line, our results provide a firm basis, that neither of the three assay systems we analysed shows a distinct advantage in detecting sensitizations.

One important limitation of our study is that we resorted to parentally reported trigger factors and doctor's diagnosis of hay fever or food allergy, as indicative of clinically significant sensitizations. Allergen challenges similar to Käck et al.[24] certainly constitute the gold standard for assessing diagnostic performance. However, our approach was not restricted to a single allergen and would have therefore required several challenges, which were beyond our study group's capacity and would have subjected our young participants to substantial risks. Similarly, assessing other available test systems as well as consideration of the Clinical Laboratory Standards Institute (CLSI) I/LA-20 guideline was beyond the scope of this report.

The Euroline™ and the ISAC, which either test a mix of extracts and components (Euroline™) or include only components (ISAC), consistently detected more sensitizations than the ImmunoCAP™ sx1/fx5 when extending testing to all allergens from a given allergen group (Table S3). Others have stressed that component-resolved diagnostics may increase the number of identified allergen sensitizations[23,25-29] which we can corroborate by our findings (Table S3). However, the aforementioned studies did not address the clinical utility of their findings. Studies which have shown added clinical value for molecular allergen component-based microarrays have included older children and did not compare to extract-based systems making a comparison to our results difficult.[24]

Our study identified lower sensitization rates (depending on test system, threshold and allergens included: 27%–51% of all children and 33%–51% of children with wheeze/asthma identified as sensitized) (Table 3) compared to those found by Önell et al. (75% of all children and 100% of all asthmatic children identified as sensitized). This difference is most likely due to the fact that Önell et al.[23] included an older age range but we cannot exclude that the lower rates of sensitization may have affected our ability to find significant differences in analytical and/or diagnostic performance which could have only been addressed by including a larger group of children. It is of note, that upon the use of the lower threshold for overall sensitization rates Euroimmun™ does not discriminate between asthmatics/wheezers versus controls but only achieves this discrimination at the higher threshold while ImmunoCAP™ and the ISAC achieve such discrimination at both thresholds (Table 3). This further supports our choice within the ALLIANCE consortium on choosing the higher threshold for identifying children with atopy. Our results suggest, however, that among younger children where polysensitization across a multitude of allergen species is still rare, component-resolved sIgE test systems may not improve diagnostic performance in common clinical scenarios. This may be different in older, polysensitized patients.[27-29] In our study population, multiplex allergen sIgE testing increases the risk of revealing sensitizations of unknown clinical significance. Thus, careful history taking, physical examination, and—in the case of suspected food allergy—standardized food challenges remain indispensable for personalized treatment of different allergic patients.

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CONFLICT OF INTEREST

For CS: Consultancy and research funding, Hycor Biomedical and Thermo Fisher Scientific; Research Funding, Mead Johnson Nutrition (MJN); Consultancy, Bencard Allergie. For MVK: Speaker honorarium or consultant fees from Abbvie, ALK-Abello, Allergopharma, Chiesi, Infectopharm, Meda, Novartis Pharma, Sanofi-Aventis, Vertex GmbH. Funds for research from Allergopharma GmbH and

Vertex GmbH. For OF: Speaker honorarium or consultant fees from Vertex, aha! Allergiezentrum Schweiz, Menarini, Novartis, ALK-Abello, Vifor, Stallergenes Greer, Bencard Allergie. For BS: Speaker honorarium or consultancy for GlaxoSmithKline, Novartis, Sanofi. For CH: Grants from Novartis and PARI. For EvM: Personal fees from Pharmaventures, OM Pharma S. A., Springer-Verlag GmbH, Elsevier GmbH and Elsevier Ltd., Peptinnovate Ltd., Turun Yliopisto, Tampereen Yliopisto, Helsingin Yliopisto, European Respiratory Society, Deutsche Pharmazeutische Gesellschaft e. V., Massachusetts Medical Society, from Chinese University of Hongkong, European Commission, Böhringer Ingelheim International GmbH, Universiteit Utrecht, Faculteit Diergeneeskunde, Universität Salzburg, Georg Thieme Verlag and Japanese Society of Pediatric Allergy and Clinical Immunology (JSPACI), outside the submitted work. In addition, Dr. von Mutius has a patent LU101064 - Barn dust extract for the prevention and treatment of diseases pending, a patent EP2361632: Specific environmental bacteria for the protection from and/or the treatment of allergic, chronic inflammatory and/or autoimmune disorders with royalties paid to ProtectImmun GmbH, a patent EP 1411977: Composition containing bacterial antigens used for the prophylaxis and the treatment of allergic diseases licensed to ProtectImmun GmbH, a patent number EP1637147: Stable dust extract for allergy protection licensed to ProtectImmun GmbH, and a patent EP 1964570: Pharmaceutical compound to protect against allergies and inflammatory diseases licensed to ProtectImmun GmbH. All others declare no conflict of interest.

AUTHOR CONTRIBUTION

CS and AMD conceptualized and designed the work. NM, BS and IR provided samples for measurements. CS, AMD and KE collected the data. CS, AMD, PT, NT, KE and PPN analysed and interpreted the data. CS, AMD, PT and PPN drafted the manuscript, MW, CH, MVK, GH, EvM, HR, OF, BS, CS and AMD critically revised the article. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the ALLIANCE consortium (https://dzl.de/krankheitsbereiche/asthma-und-allergien/alliance/). Restrictions apply to the availability of these data, which were used under license for this study. Data are available with the permission of the ALLIANCE consortium.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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APPENDIX 1

ALLIANCE STUDY GROUP

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