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A singleplex IgE test to a mixture of molecules from multiple airborne allergen sources: Innovating in vitro screening of respiratory allergies

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Abstract

Background: In vitro immunoglobulin E (IgE) tests can be better standardized if based on molecules rather than extracts. However, singleplex screening tests for respiratory or food allergies are still based on extracts only.

Target: To validate a novel singleplex IgE screening test for respiratory allergies, based on a mix of major allergenic molecules Der p 1, Der p 2, Fel d 1, Can f 1, Can f 2, Can f 3, Can f 5, Bet v 1, Phl p 1, and Art v 1 (Molecular SX01, NOVEOS, HYCOR, USA), and requiring only four microliters (μl) of serum.

Methods: We examined six subsets of sera from participants of the German Multicenter Allergy Study (MAS) birth cohort enrolling 1314 newborns during 1990: (1) monosensitized ($n = 58$); (2) polysensitized ($n = 24$); (3) nonsensitized, with total IgE levels above ($n = 24$) or (4) below ($n = 24$) 300 kU/L; (5) sensitized to milk and/or egg but not to airborne allergens ($n = 24$); and (6) sera of children aged ≤5 years at their earliest IgE monosensitization to airborne allergens ($n = 41$). Sera were analyzed with the novel molecular SX01 test (NOVEOS) and with three categories of comparators: ImmunoCAP Phadiatop SX01, extracts, and molecules of *D. pteronyssinus*, cat, dog, grass, and birch. Sensitivity, specificity, positive and negative predictive values were calculated. Quantitative interrelationships were determined using Spearman's rank-order correlation coefficient and Bland–Altmann plots.

Results: The molecular SX01 test predicted the outcome of IgE tests based on molecules, extracts, or Phadiatop in 188 (96.4%), 171 (87.7%), and 171 (87.7%) of the 195 sera, respectively. Accordingly, sensitivity was 93.5%, 89.0%, and 82.4%, whereas specificity was 100%, 97.6%, and 96.1% when compared with molecular, extract, and Phadiatop tests, respectively. Inconsistent outcomes were largely confined to sera

Abbreviations: AR, Allergic rhinitis; ARIA, Allergic rhinitis and its Impact on asthma; CI, Confidence interval; CRD, component-resolved diagnosis; ESEP, Euroline Southern Europe Pollen; SAR, Seasonal allergic rhinitis; SD, Standard deviation; SPT, Skin prick test; tIgE, total IgE.

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with IgE-Ab levels around the cutoff value of 0.35 kU/L, except for 5/195 (2.5%) sera, containing high levels of IgE to Phl p 5 and/or Alt a 1 only. IgE levels measured by the molecular SX01 test and with IgE tests to molecules, extracts, and Phadiatop were highly correlated (rho 0.90; $p < .001$), (rho 0.87, $p < .001$), (rho 0.84, $p < .001$), respectively. The novel molecular SX01 test detected IgE-Ab in 27/28 (sensitivity 96.4%) of the sera of preschool children at their earliest IgE sensitization to the same molecules. **Discussion:** Our study validates the prototype of a novel category of IgE test, based on molecular mixes. The test's rather good precision and accuracy in early screening IgE sensitization to airborne allergens in German children may be further improved by adding a few other molecules, such as Phl p 5 and Alt a 1.

KEYWORDS
allergen-specific immunotherapy, component-resolved diagnostics, immunoglobulin E, pollen, precision medicine, seasonal allergic rhinitis

1 | INTRODUCTION

Allergic rhinitis and asthma are the most common chronic diseases in childhood in westernized countries and frequently start early in life.[1] In central and northern Europe, they are most often triggered by exposure to airborne allergens, including house dust mites, cat and dog dander, birch and grass pollen.[2] Early diagnosis is essential for early intervention (avoidance, pharmacotherapy, and immunotherapy) aimed not only at disease control,[3] but also at prevention of disease progression.[4,5]

IgE screening tests, based on mixes of most relevant allergen extracts,[6] have been used over the last few decades for early, cost-effective screening of respiratory allergies in children[7] and adults.[8] Qualitative[9] and quantitative[10] validation studies on these mixes were performed in the early 1990s. Threshold levels or decision (cut-off) points for positivity were also first thoroughly analyzed in the use of this test, leading to the introduction of the receiver operating characteristic (ROC) analysis in allergology,[10] a methodology extended thereafter also to the evaluation of patients with food allergies.[11]

Since the early 2000s, allergen-specific IgE tests have been made more precise and standardized using molecules, under the concept of component-resolved diagnostics (CRD).[12] Nevertheless, singleplex IgE screening tests are still based on mixes of extracts. Recently, the antinomial concept of "molecular extracts," that is, balanced mixes of allergenic molecules in substitution of allergen extracts, has been proposed.[13]

Over this same period, it became clear that IgE reactivity to airborne allergens begins with sensitization to one or few molecules and spreads progressively at later stages, following a process named "molecular spreading".[4,14] Studies in birth cohorts[14,15,16,17,18] have shown that a relatively small group of molecules, named allergy "initiator molecules"[19] are the first recognized by a specific IgE response in childhood. The list of known allergy initiator molecules includes Group 1 for grasses (e.g., Phl p 1 for Phleum pratense), PR.10 for many trees (e.g., Bet v 1 for birch), Fel d 1 for cat, and so on.[18]

Key Message

A qualitative and quantitative validation of a novel IgE screening test for respiratory allergies, based on the mix of allergy initiator molecules of the most frequent airborne allergens in Northern Europe. The new SX01 molecular test showed good precision and accuracy in IgE screening of airborne allergies in our cohort. It will allow diagnosis based on better standardized recombinant molecules, smaller volume of sera, and tailored composition according to specific needs.

A logical deduction of the above premises is that a singleplex IgE screening test might be based on a balanced mix of allergy initiator molecules, thus upgrading the detection properties of the mix of allergen extracts to the precision and standardization qualities of molecular IgE tests. To test this hypothesis, this study was aimed at the qualitative and quantitatively validation of an IgE screening test for respiratory allergies, based on the mix of allergy initiator molecules of the most frequent airborne allergens in Northern Europe.

2 | METHODS

2.1 | Study population

The Multicenter Allergy Study (MAS), a prospective birth cohort study, recruited a selection of 1314 of 7609 infants born in 1990 on six delivery wards in five German cities (Berlin, Dusseldorf, Mainz, Freiburg, and Munich).[20,21] The study was approved by local ethics committees. Each parent provided written informed consent at the time of enrollment. All children were asked to undergo a blood drawing during follow-up visits at the age 1, 2, 3, 5, 6, 7, 10, 13, and

20 years. Sera of consenting children were tested for total IgE and for specific IgE antibodies (ImmunoCAP, TFS, Uppsala, Sweden) to five airborne (mites, cat, dog, birch, and grass) and four food (milk, egg, soy, and wheat) allergen extracts. For the purposes of this study, we included six subsets of sera selected with the following criteria:

1. Monosensitized (58) or polysensitized (24): sera with IgE sensitization to one (monosensitized) or more (polysensitized) of the five airborne allergen extracts examined (mites, cat, dog, birch, and grass);
2. Nonatopic but high (24) or normal (24) IgE producers: sera with no IgE antibodies to any of the nine airborne or foodborne allergen extracts examined, but with total IgE levels above (high) or below (normal) 300 kU/L;
3. Animal-food sensitized (24): sera with no IgE to the five airborne allergen extracts, but with IgE sensitization to milk and/or egg (24);
4. At sensitization onset (41): sera of atopic children, aged 5 years or less, at their first detection of IgE sensitization to one of the five airborne allergen extracts.

2.2 | IgE chemiluminescent platform

The molecular SX01 test adopts a chemiluminescence detection system (NOVEOS, Hycor, USA) operating in a solid phase of fluorescently labeled and streptavidin-coated paramagnetic microparticles, already described elsewhere.[22] The microparticles are first incubated with a biotinylated allergen that binds the streptavidin molecules. After an extensive wash, the bound microparticles are then incubated with patient serum containing allergen-specific IgE, and the resulting bound complex is washed by aspirating unbound material from retained beads in the cuvette. They are subsequently incubated with an anti-IgE antibody conjugated to horseradish peroxidase and, after an incubation period, are washed to remove any unbound conjugate from bound material. The chemiluminescent signal is originated by adding a substrate solution. The concentration of allergen-specific IgE is directly proportional to the light intensity after correction (via fluorescence) for microparticle loss and is compared with an IgE reference curve traceable to World Health Organization (WHO) reference preparations (NIBSC 11/234). The sample volume used per test is 4 µl, and the time to first result is 104 min.[22]

2.3 | Molecular SX01 screening IgE test

This study examined the current configuration of SX01 on the NOVEOS system. The molecular SX01 IgE test utilizes a liquid phase test where biotinylated allergens are preincubated with a streptavidin solid phase before testing. It consists of a balanced mix of the following recombinant allergen molecules: Der p 1, Der p 2, Fel d 1, Can f 1, Can f 2, Can f 3, Can f 5, Bet v 1, Phl p 1, and Art v 1 (Table 1).

TABLE 1 Composition of the molecular SX01 IgE test

Allergen source	Molecules	Isoforms
D. pteronyssinus	nDer p 1	0111
	rDer p 2	0103
Cat	rFel d 1	0101
Dog	rCan f 1	0101
	rCan f 2	0101
	nCan f 3	0101
	rCan f 5	0101
Birch	rBet v 1	0101
Timothy grass	rPhl p 1	0101
Mugwort	rArt v 1	0101

The combination of molecules was verified for both concentration and potency using recombinant and native proteins available to the manufacturer. Based on the outcome of this study and clinical needs, it was advised to expand the mix to include Phl p 5 and Alt a 1 for Northern Europe and, in addition, Ole e 1, Cup a 1, and Par j 2, for Southern Europe. A novel method, the mix has its own internal threshold to determine the test positivity for each molecule.

2.4 | Other IgE tests

All the sera were tested for Phadiatop SX01 (*Dermatophagoides pteronyssinus*, cat, dog, Cladosporium herbarum, common rye, mugwort, timothy grass, and birch), total IgE, and for specific IgE with allergen extracts (mites, cat, dog, birch, grass, milk, egg, soy, and wheat) with ImmunoCAP-FEIA (Thermo Fisher Scientific [TFS]) and the results expressed in kU/L (cutoff for positivity 0.35 kU/L). Serum-specific IgE antibodies to individual allergen molecules (Der p 1, Der p 2, Fel d 1, Can f1, Can f 2, Can f 3, Can f 5, Bet v 1, Phl p 1, and Art v 1) were measured with the NOVEOS system[23] and, in some cases, also with the ImmunoCAP. When necessary, inconsistent results were examined for IgE to other molecules, such as Phl p 4, Phl p 5 and Alt a 1, or analyzed for IgE to Bromelain for cross-reactive carbohydrate determinant (CCD) reactivity.

2.5 | Statistics

Age was summarized as mean and standard deviation (SD). Categorical data were summarized as numbers (*n*) and frequencies (%). Sensitivity, specificity, positive and negative predictive values were calculated with their exact confidence interval at 95% (95% CI). Accuracy, positive and negative likelihood ratios were also calculated to evaluate the diagnostic performance of NOVEOS in detecting IgE sensitization, compared with the other IgE tests. Pearson's correlation was used to evaluate relationship between tests. Bland–Altman plots were used to investigate the agreement between quantitative values of IgE detected with the two different methodologies

(molecular SX01 vs. other IgE tests). They were applied using the log values of only positive samples (>0.1 kU/L). Mean difference (Bias), 95% CI, number of subjects under or over-limit of agreement (LOA), Lin's concordance index (Lin) and Spearman's correlation between the difference and average were reported. A *p*-value of <.05 was considered statistically significant. Statistical analyses were performed with Stata 16.1.

3 | RESULTS

3.1 | Study population

Overall, we examined 195 serum samples from 167 participants in the MAS cohort. Of these, 144, 19, 3, and 1 contributed with one, two, three, or four samples, respectively, for this study. Major sociodemographic (age and gender) characteristics of the participants for each of the population subsets are shown (Table 2).

3.2 | Sensitivity and specificity

The diagnostic performance in identifying IgE sensitization to airborne allergens through the SX01 method in comparison with allergen molecules (NOVEOS), extracts, and Phadiatop (ImmunoCAP) have been thoroughly analyzed in terms of sensitivity, specificity, accuracy, positive and negative predictive value in the whole set of 195 examined sera, or within each of the different sample subsets (Table 3). Overall, a concordant outcome between the molecular SX01 test and the singleplex tests has been observed in 188 (96.4%) (molecules), 171 (87.7%) (extracts), and 171 (87.7%) (Phadiatop) of the 195 serum samples examined. Sensitivity global values were 93.5% (101/108, molecules), 89.0% (97/109, extracts, NOVEOS), 81.3% (100/123, extracts, ImmunoCAP), and 82.4% (98/119, Phadiatop). Specificity global values were 100% (87/87, molecules), 97.6% (81/83, extracts, NOVEOS), 98.6% (71/72, extracts, ImmunoCAP), and 96.1% (73/76, Phadiatop). When the subsets for the individual allergen extracts were considered, the sensitivity of the molecular

TABLE 2 Characteristics of study population

Subset	*n*	Age (mean ± SD)	Males *n*	Males %
Monosensitized	58	20 ± 6	28	*48*
Polysensitized	24	20	16	*67*
Nonatopic but total IgE ≥ 300 kU/L	24	7 ± 5	15	*63*
Nonatopic but total IgE < 300 kU/L	24	20	8	*33*
Animal-food sensitized	24	6 ± 4	8	*33*
Sensitization onset[a]	41	5 ± 1	28	*68*

[a]Atopic children, ≤5 years, first detection of IgE monosensitization to one of the five airborne allergen extracts.

TABLE 3 Reciprocal comparison of molecular SX01 NOVEOS (cutoff ≥ 0.35 kU/L) versus molecular and extract-based IgE tests (cutoff ≥ 0.35 kU/L)

Allergens	*n*	X + N+	X + N-	X-N+	X-N-	Sensitivity %	Sensitivity (95% CI)[a]	Specificity %	Specificity (95% CI)[a]	PPV %	PPV (95% CI)[a]	NPV %	NPV (95% CI)[a]	Accuracy%	LR+	LR-	r_s
Phadiatop (ImmunoCAP)	195	98	21	3	73	82.4	74.3–88.7	96.1	88.9–99.2	97.0	91.6–99.4	77.7	67.9–85.6	87.7	20.863	0.18	0.84*
Molecules (Noveos)	195	101	7	0	87	93.5	87.1–97.4	100.0	95.8–100	100.0	96.4–100	92.6	85.3–97.0	96.4	-	0.065	0.92*
Extracts (Noveos)	192	97	12	2	81	89.0	81.6–94.2	97.6	91.6–99.7	98.0	92.9–99.8	87.1	78.5–93.2	92.7	-	0.113	0.90*
Extracts (ImmunoCAP)	195	100	23	1	71	81.3	73.3–87.8	98.6	92.5–100	99.0	94.6–100	75.5	65.6–83.8	87.7	-	0.19	87.4*

Note: X+, Methodology being compared in the column positive; X-, Methodology being compared in the column negative; N+, NOVEOS positive; N-, NOVEOS negative (positivity cutoff value ≥0.35 kU/L); (positivity cutoff value ≥0.35 kU/L).

Note: r represents the Spearman's rank correlation coefficient, significant differences were highlighted (*$p < .001$).

Abbreviations: PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio.

[a]Exact binomial confidence limits (95% CI).

IgE screening test was 100% for grass (Phl p 1), D.pt (Der p 1 and Der p 2), birch (Bet v 1) and cat (Fel d 1) initiator molecules, and 66.7% for dog molecules (Can f 1, Can f 2, Can f 3, Can f 5) (Table 4). Somewhat lower performances were obtained when the extract-based SX01 mix (Phadiatop) (Table 3) and the extract-based tests (Table 4) were used as a comparator. The overall specificity of the molecular SX01 reached 96% (23/24), 100% (24/24), and 100% (24/24) among the subsets of nonatopic patients with high or normal total IgE or in those with IgE to milk and/or egg only, respectively.

3.3 | Quantitative correlation between the molecular SX01 and the other tests

A good correlation was found between the IgE levels measured by the molecular SX01 test and the cumulative IgE levels measured by the IgE tests to individual molecules (rho 0.90; $p < .001$), extracts (rho 0.87, $p < .001$), Phadiatop (rho 0.84, $p < .001$), reflecting the good correlation observed in most cases when individual sera subsets were examined (Figure 1, **panels a–d**). The interpretation of these values took into account the specificity of the internal threshold in the mix, and the subsequent difference of unit scales between the comparisons. However, a few outliers were observed when the molecular SX01 was compared with the ImmunoCAP extract-based tests (see below for details). A total of 18 samples with inconsistent results were analyzed with ImmunoCAP for the individual molecules, and the obtained results matched the IgE sensitization profiles of the patients. In the population subset of sera with IgE monosensitization to dog extract, a weaker quantitative correlation was found, but the IgE-Ab levels of most these sera were weak (range 0.3–3 kU/L) (Figure 1). A Bland–Altmann correlation analysis showed that the correlation of the quantitative levels of the molecular SX01 mix was high across the whole range of detection, from 1 to 100 kU/L (Figure 2) and (Table 5).

3.4 | Detection of first IgE-positive outcome in the MAS cohort

An important target of the molecular SX01 IgE test is the early identification of IgE sensitization at preschool age. Hence, we selected from the MAS cohort biodata-bank the sera and data of 41 children at their earliest IgE monosensitization stage, already detected previously with ImmunoCAP extracts. The IgE-Ab content of these sera has been further examined with the molecular SX01, molecules and extracts of the NOVEOS system, and with the extract SX01 mix (ImmunoCAP Phadiatop) (Table 6). IgE to the individual molecules contained in the molecular SX01 tests were found in 28 of the 41 (68.3%) sera. Of these 28, 27 (96.4%) were also identified by the molecular SX01 test and 27 (96.4%) by the extract SX01 mix (Phadiatop).

TABLE 4 Sensitivity of molecular SX01 NOVEOS (cutoff ≥ 0.35 kU/L) versus molecular and extract-based IgE tests (cutoff ≥ 0.35 kU/L)

Allergens	*n*	*X* + *N*+	*X* + *N*-	%	Sensitivity (95% CI)[a]
Molecules (NOVEOS)					
Der p 1/Der p 2	13	13	0	100.0	75.3–100
Fel d 1	10	10	0	100.0	69.2–100
Can f 1, 2, 3, 5	9	6	3	66.7	29.9–92.5
Bet v 1	9	9	0	100.0	66.4–100
Phl p 1	11	11	0	100.0	71.5–100
Extracts (NOVEOS)					
Mites	14	13	1	92.9	66.1–99.8
Cat	10	10	0	100.0	69.2–100
Dog	10	6	4	60.0	26.2–87.8
Birch	9	9	0	100.0	66.4–100
Grass	11	10	1	90.9	58.7–99.8
Extracts (ImmunoCAP)					
Mites	15	13	2	86.7	59.5–98.3
Cat	11	10	1	90.9	58.7–99.8
Dog	10	6	4	60.0	26.2–87.8
Birch	9	9	0	100.0	66.4–100
Grass	13	11	2	84.6	54.6–98.1

Note: *X* = Methodology being compared; *N* = NOVEOS; *(cutoff value ≥ 0.35* kU/L*)*.

Note: *r* represents the Spearman's rank correlation coefficient, significant differences were highlighted (*$p < .001$).

[a]Exact binomial confidence limits (95% CI).

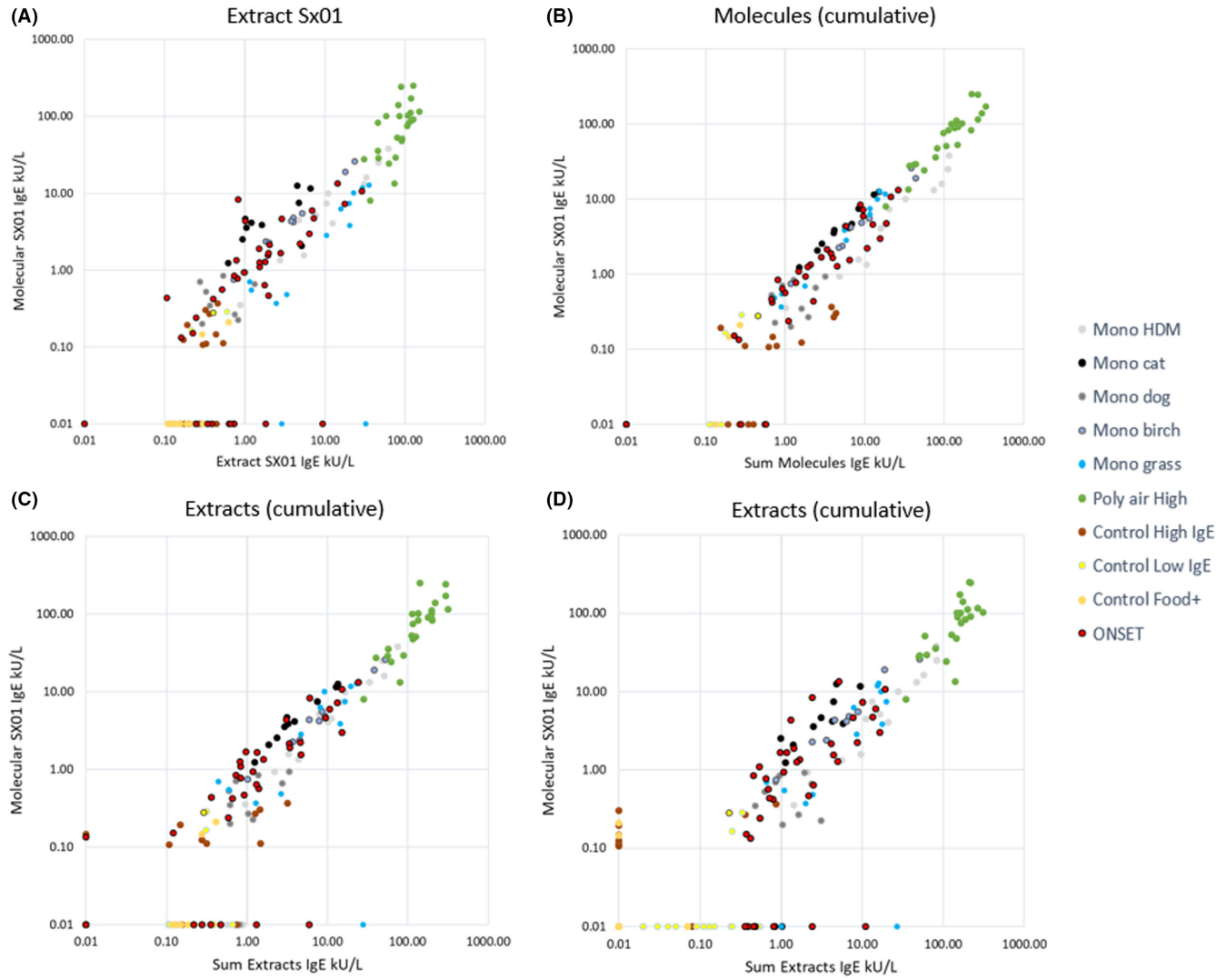

FIGURE 1 Correlation between molecular SX01 and other methodologies: Scatter diagrams showing the correlation between IgE levels detected with molecular SX01 mix (y axis), and IgE levels detected with: (A) extract SX01 mix; (B) molecules (cumulative); (C) extracts (NOVEOS); (D) extracts (ImmunoCAP).

Of the remaining 13 sera none (0%) were positive to the molecular SX01 mix, while seven (53.8%) were positive to the extract SX01 (Phadiatop). Of these seven, five were sensitized to grass pollen, one to mite and one to birch (Table 6).

3.5 | Inconsistent data and their putative explanation

We examined in more detail the 24 sera with inconsistent outcomes between the molecular SX01 and extract SX01. In 16 of the 21 sera with a positive outcome to extract SX01 and a negative outcome to molecular SX01, the IgE values measured by the extract SX01 were below 0.75 kU/L. Of the remaining five, three had moderate or high titers of IgE to Phl p 5 (but not to Phl p 1), one had high levels of IgE to Alt a 1, and the last had low levels of IgE to cross-reactive carbohydrate determinants (CCD). Among the three sera with a positive outcome to the molecular SX01 and a negative outcome to the extract SX01, two had IgE to dog extract, Can f 1, and Can f 2, while low levels of IgE to Phl p 1 and to Bet v 1 were found in the other.

4 | DISCUSSION

In this study, we present and investigate a novel molecular, in-vitro, singleplex test for the screening of IgE sensitization to respiratory allergies in childhood. To our knowledge, this is the first prototype of a new category of IgE assays, based on the mix of major allergen molecules from different and locally relevant airborne allergen sources, combined in a singleplex test. Our results show that this novel approach is reasonable; the new test is not only qualitatively sensitive and specific in early detection of IgE sensitization at preschool age, but also quantitatively accurate.

The overall sensitivity and specificity of SX01 in detecting sIgE was high, when compared with the outcome of a panel of single IgE tests to the same set of molecules presented in the SX01 mix,

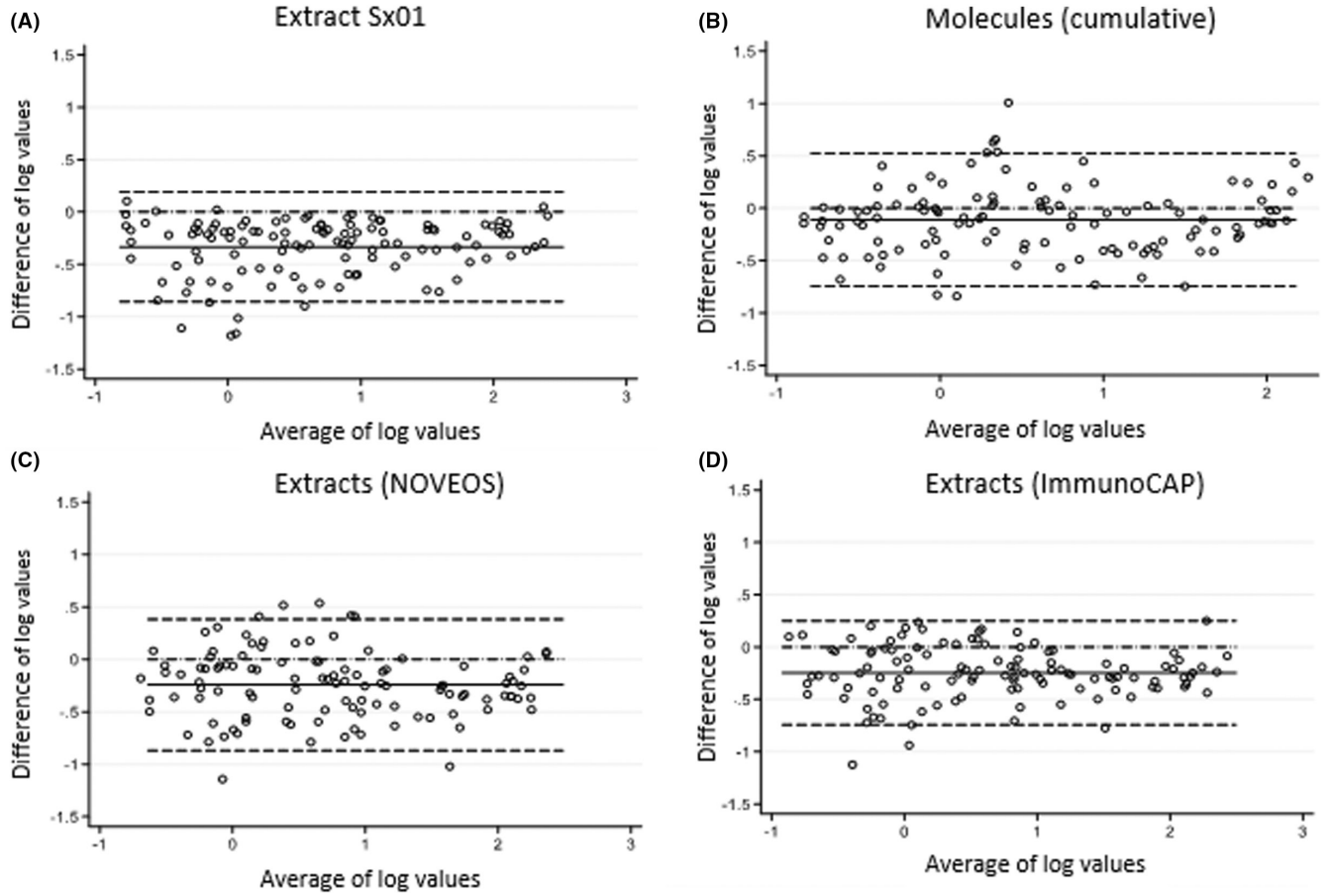

FIGURE 2 Bland–Altman plot: Bland–Altmann plots showing the correlation between IgE levels detected with molecular SX01 mix (y axis), and IgE levels detected with: (A) extract SX01 mix; (B) molecules (cumulative); (C) extracts (Noveos); (D) extracts (ImmunoCAP). In the Bland–Altman plot, the difference and the mean of the ImmunoCAP and the NOVEOS IgE log values (kU/L) are reported in the *y*- and *x*-axis, respectively. The continuous and the dashed lines parallel to the *X*-axis mark the population mean level and its bias, respectively, while the other two dash lines are the upper and lower limits of agreement, respectively.

TABLE 5 Correlation between molecular SX01 mix vs. comparators: Summary of quantitative data; Lin's CCC and Bland–Altman methods

	N	Bias[a]	#over limit[b]	#under limit[c]	SD	SE	CI (95%)	Lin[d]	Spr[e]
Phadiatop	117	−0.11	5	2	0.32	0.03	−0.17; −0.05	0.93	0.05
Noveos molecules	119	−0.33	0	6	0.27	0.02	−0.38; −0.28	0.89	0.08
Noveos extracts	115	−0.25	1	4	0.25	0.02	−0.29;-0.20	0.92	−0.05
ImmunoCAP extracts	111	−0.24	5	2	0.32	0.03	−0.30;-0.18	0.89	−0.11

[a]Bias, in Bland–Altman, calculated as the mean of the difference of values. Obtained with the two methods.
[b]Number of cases over the limit in Bland Altman.
[c]Number of cases under the limit in Bland Altman.
[d]Lin's concordance correlation coeficient.
[e]Spearman correlation between difference and average (*r*).

run in the same NOVEOS platform. The assay specificity was also high in sera with high total IgE levels or with IgE antibodies against foodborne, but not airborne allergens. This implies that mixing the individual molecules or testing them separately does not substantially modify their capacity to precisely bind serum IgE antibodies, even when they are at low concentrations. This evidence is not new, considering that molecular mixes of molecules belonging to *the same allergen source* (which could be named "homologous molecular mixes") are commercially available in the last 2 decades.[24] For example, ImmunoCAP IgE tests combining Phl p 1 with Phl p 5 or Phl p 7 with Phl p 12 are routinely used in many allergy laboratories and contributed to some scientific publications.[25] Interestingly, the results of the present study shows that this property is extended also to molecules coming from *different allergen sources* ("heterologous

TABLE 6 Outcome of molecular SX01 IgE test and of IgE tests to molecules, extracts, and extract-based SX01 in sera of children participating in the MAS cohort at their earliest sensitization to airborne allergens

Patients				Mol. Sx01		Extr. Sx01		tIgE	IgE to molecules (Noveos)						
Code[#]	Age	Sex	AR[a]	IgE	+/−	kU/L	+/−		Der p 1	Der p 2	Fel d 1	Can f 1	Can f 2	Can f 3	Can f 5
A1	3	m	no	13.31	+	14.32	+	128	0.19	0.03	0.03	0.02	0.02	0.05	0.03
A2	5	m	no	10.70	+	29.01	+	78	0.02	0.02	0.01	0.01	0.02	0.03	0.02
A3	3	m	no	8.35	+	0.82	+	26	0.05	0.04	**8.14**	0.04	0.15	0.04	0.04
A4	5	m	AR	7.24	+	17.60	+	279	**0.37**	0.04	0.07	0.03	0.05	0.04	0.06
A5	3	m	AR	5.98	+	6.98	+	53	0.00	0.02	0.01	0.01	0.02	0.01	0.02
A6	5	m	no	4.73	+	7.24	−	56	**18.24**	0.04	0.02	0.02	0.02	0.02	0.03
A7	5	m	no	4.62	+	2.88	+	54	0.20	**12.43**	0.02	0.02	0.01	0.01	0.02
A8	2	m	no	4.36	+	1.02	+	16	0.02	0.02	**5.58**	0.02	0.27	0.02	0.03
A9	3	m	no	2.99	+	6.36	+	154	0.05	**15.31**	0.02	0.43	0.02	0.02	0.02
A10	5	m	no	2.22	+	4.88	+	292	**10.81**	0.05	0.04	0.03	0.03	0.02	0.03
A11	5	m	no	2.15	+	2.03	+	157	0.04	0.03	0.03	0.03	0.03	0.03	0.04
A12	2	m	no	1.89	+	1.50	+	147	0.15	0.10	**1.47**	**1.59**	0.36	0.06	0.19
A13	3	m	no	1.67	+	2.79	+	10	0.00	0.02	0.02	0.02	0.02	0.02	0.02
A14	3	m	no	1.66	+	1.98	+	6	0.02	0.02	0.01	0.01	0.01	0.01	0.02
A15	3	f	no	1.55	+	1.96	+	95	0.03	**6.29**	0.01	0.13	0.02	0.01	0.03
A16	5	f	no	1.35	+	0.79	+	25	0.07	0.03	0.02	0.03	0.02	0.03	0.03
A17	3	m	AR	1.28	+	1.78	+	109	0.11	0.04	0.03	0.04	0.03	0.03	0.03
A18	3	m	AR	1.26	+	1.54	+	26	0.03	0.03	0.02	0.02	0.02	0.02	0.03
A19	3	m	no	1.09	+	1.53	+	28	0.17	0.04	0.02	0.02	0.02	0.02	0.03
A20	2	f	no	0.93	+	0.98	+	302	0.30	0.10	0.08	0.08	0.07	0.06	0.09
A21	5	m	no	0.84	+	0.74	+	77	0.02	0.03	0.01	0.01	0.02	0.01	0.02
A22	5	f	AR	0.78	+	0.82	+	27	0.02	0.01	0.01	0.02	0.02	0.01	0.01
A23	5	f	AR	0.64	+	1.77	+	32	0.02	0.02	0.02	0.01	0.01	0.01	0.02
A24	5	m	no	0.56	+	0.52	+	48	0.02	0.02	0.12	0.33	0.02	0.02	0.02
A25	5	f	no	0.47	+	1.98	+	149	0.04	0.04	0.03	0.04	0.05	0.03	0.04
A26	5	m	no	0.42	+	0.40	+	96	0.03	0.02	0.01	0.02	0.02	0.02	0.02
B1	5	m	no	0.24	−	0.25	−	203	0.16	**0.95**	0.06	0.06	0.07	0.05	0.05
B2	5	f	no	0.15	−	0.23	−	26	0.02	0.01	0.01	0.01	0.01	0.01	0.02
B3	2	m	no	0.13	−	0.16	−	248	0.03	0.02	0.02	0.02	0.02	0.02	0.03
B4	5	m	no	0.07	−	0.34	−	23	0.02	0.02	0.02	0.01	0.02	0.01	0.02
B5	5	m	no	0.03	−	0.05	−	4	0.02	0.04	0.02	0.02	0.01	0.01	0.02
B6	5	m	no	0.03	−	0.04	−	21	0.04	0.03	0.02	0.01	0.01	0.01	0.02
B7	5	f	no	0.02	−	0.25	−	85	0.06	0.04	0.01	0.02	0.02	0.01	0.02
C1	5	f	no	0.03	−	0.39[d]	+	99	0.06	0.02	0.02	0.02	0.01	0.01	0.02
C2	3	m	no	0.07	−	0.64	+	556	0.17	0.16	0.06	0.05	0.05	0.06	0.06
C3	1	f	no	0.01	−	0.66	+	4	0.02	0.01	0.01	0.01	0.01	0.01	0.01
C4	5	f	no	0.09	−	0.72[e]	+	51	0.03	0.28	0.02	0.02	0.03	0.01	0.02
C5	3	f	no	0.02	−	0.74	+	27	0.02	0.01	0.01	0.01	0.01	0.01	0.01
C6	5	m	AR	0.05	−	1.84[b]	+	107	0.17	0.06	0.04	0.14	0.25	0.08	0.06
C7	5	f	no	0.05	−	9.43[c]	+	48	0.02	0.07	0.02	0.02	0.02	0.02	0.02
D1	1	m	no	0.44	+	0.11	−	114	0.27	0.17	0.14	0.11	0.12	0.24	0.13

[a]AR = allergic rhinitis, according to the diagnostic criteria adopted in the MAS birth cohort.

[b]In this serum, IgE to Phl p 5 (3.6 kU/L) and to Alt a 1 (9.2 kU/L) were detected with immunoCAP.

[c]In this serum, IgE to Phl p 5 (25 kU/L) and to nPhl p 4 (7.58 kU/L) were detected with immunoCAP.

[e]In this serum, IgE to nPhl p 4 (2,2 kU/L) were detected.

[d]In this serum, IgE to nPhl p 4 (0.67 kU/L) were detected with immunoCAP.

[#]Sera are divided in four groups and ordered in declining extract SX01 mix IgE value: A, both molecular and extract SX01 mix positive (≥0.35 kU/L); B, both negative; C, extract SX01 positive and molecular SX01 negative; D, extract SX01 negative and molecular SX01 positive.

[f]n.d—not done, serum volume too low.

Positive IgE values to molecules and extracts were made bold.

				IgE to extracts (Noveos)						IgE to extracts (immunoCAP)					
Phl p 1	Bet v 1	Art v 1	+/−	Dpt	Cat	Dog	Birch	Grass	+/−	Dpt	Cat	Dog	Birch	Grass	+/−
0.57	**25.78**	0.05	+	0.03	0.03	0.03	**24.06**	0.25	+	<0,35	<0.35	<0.35	**5.15**	<0.35	+
21.38	0.02	0.09	+	0.05	0.02	0.02	0.04	**15.10**	+	<0.35	<0.35	<0.35	<0.35	**19.10**	+
0.24	0.04	0.26	+	0.04	**5.65**	0.25	0.05	0.11	+	<0.35	**2.43**	<0.35	<0.35	<0.35	+
9.01	0.23	0.05	+	0.07	0.04	0.09	0.16	**13.01**	+	<0.35	<0.35	<0.35	<0.35	**10.20**	+
0.12	**9.46**	0.06	+	0.02	0.01	0.02	**10.61**	0.07	+	<0.35	<0.35	<0.35	**14.70**	<0.35	+
0.46	0.02	0.01	+	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]		**13.60**	<0.35	<0.35	<0.35	<0.35	+
0.04	0.02	0.03	+	**9.56**	0.02	0.06	0.10	0.02	+	**7.81**	<0.35	<0.35	<0.35	<0.35	+
0.04	0.02	0.04	+	0.01	**2.93**	0.15	0.04	0.01	+	<0.35	**1.33**	<0.35	<0.35	<0.35	+
0.03	0.02	0.06	+	**15.15**	0.04	0.02	0.04	0.02	+	**16.40**	<0.35	<0.35	<0.35	<0.35	+
0.06	0.03	0.03	+	**4.64**	0.02	0.04	0.05	0.04	+	**8.76**	<0.35	<0.35	<0.35	<0.35	+
0.10	**3.38**	0.03	+	0.04	0.04	0.03	**3.34**	0.08	+	<0.35	<0.35	<0.35	**4.16**	<0.35	+
0.09	0.06	0.10	+	0.12	**0.98**	**2.03**	0.14	0.16	+	<0.35	**1.44**	nd[f]	<0.35	<0.35	+
2.86	0.05	0.02	+	0.05	0.01	0.02	0.04	**0.97**	+	<0.35	<0.35	<0.35	<0.35	**1.18**	+
3.92	0.06	0.01	+	0.09	0.06	0.06	0.16	**0.96**	+	<0.35	<0.35	<0.35	<0.35	**0.97**	+
0.04	0.02	0.04	+	**4.69**	0.02	0.03	0.04	0.02	+	**4.45**	<0.35	<0.35	<0.35	<0.35	+
0.07	**2.09**	0.03	+	0.02	0.02	0.02	**1.60**	0.04	+	<0.35	<0.35	<0.35	**1.70**	<0.35	+
0.08	**4.36**	0.04	+	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]	+	<0.35	<0.35	<0.35	**5.02**	<0.35	+
1.92	0.06	0.02	+	0.02	0.02	0.02	0.04	**0.82**	+	<0.35	<0.35	<0.35	<0.35	**1.55**	+
1.31	0.04	0.02	+	0.05	0.01	0.02	0.04	**0.84**	+	<0.35	<0.35	<0.35	<0.35	**0.54**	+
0.10	**1.49**	0.06	+	0.08	0.07	0.08	**1.18**	0.06	+	<0.35	<0.35	nd[f]	**1.08**	<0.35	+
0.40	**0.41**	0.02	+	0.02	0.02	0.02	**0.37**	**0.37**	+	<0.35	<0.35	<0.35	**0.46**	<0.35	+
0.98	**0.39**	0.02	+	0.02	0.02	0.02	**0.36**	**0.47**	+	<0.35	<0.35	<0.35	<0.35	**0.65**	+
0.92	0.01	0.01	+	0.02	0.02	0.01	0.04	**1.31**	+	<0.35	<0.35	<0.35	<0.35	**2.49**	+
0.04	**0.54**	0.02	+	0.03	0.19	**0.71**	**0.50**	0.03	+	<0.35	<0.35	<0.35	**0.69**	<0.35	+
0.67	0.04	0.04	+	0.14	0.05	0.03	0.07	**0.78**	+	<0.35	<0.35	<0.35	<0.35	**2.20**	+
0.04	**0.69**	0.02	+	0.02	0.02	0.03	**0.66**	0.02	+	<0.35	<0.35	<0.35	**0.79**	<0.35	+
0.06	0.05	0.06	+	**0.59**	0.03	0.04	0.08	0.05	+	**0.55**	<0.35	<0.35	<0.35	<0.35	+
0.23	0.01	0.02	−	0.05	0.01	0.02	0.04	0.12	−	<0.35	<0.35	<0.35	<0.35	**0.37**	+
0.26	0.02	0.02	−	0.03	0.02	0.02	0.05	0.08	−	<0.35	<0.35	<0.35	<0.35	**0.42**	+
0.10	0.02	0.01	−	0.02	0.01	0.02	0.04	0.28	−	<0.35	<0.35	<0.35	<0.35	**0.46**	+
0.04	0.01	0.02	−	0.05	0.02	0.02	0.04	0.02	−	<0.35	<0.35	<0.35	**0.47**	<0.35	+
0.07	0.01	0.01	−	0.01	0.01	0.01	0.04	0.03	−	<0.35	**0.38**	<0.35	<0.35	<0.35	+
0.03	0.02	0.02	−	0.02	0.01	0.02	0.05	0.02	−	<0.35	<0.35	<0.35	<0.35	**0.36**	+
0.05	0.05	0.02	−	0.01	0.01	0.02	0.07	0.22	−	<0.35	<0.35	<0.35	<0.35	**0.48**	+
0.24	0.05	0.04	−	0.10	0.04	0.05	0.08	0.08	−	**0.81**	<0.35	<0.35	<0.35	<0.35	+
0.03	0.01	0.01	−	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]		<0.35	<0.35	nd[f]	**0.36**	<0.35	+
0.04	0.02	0.01	−	0.20	0.01	0.02	0.04	0.27	−	<0.35	<0.35	<0.35	<0.35	**0.84**	+
0.05	0.01	0.01	−	0.01	0.01	0.02	0.03	**0.36**	+	<0.35	<0.35	<0.35	<0.35	**0.39**	+
0.07	0.04	0.04	−	0.09	0.03	0.03	0.07	**1.32**	+	<0.35	<0.35	<0.35	<0.35	**2.43**	+
0.04	0.02	0.02	−	0.07	0.04	0.07	0.04	**5.96**	+	<0.35	<0.35	<0.35	<0.35	**11.10**	+
0.30	**0.57**	0.22	+	0.08	0.12	0.13	0.11	0.09	−	<0.35	**0.72**	<0.35	<0.35	<0.35	+

molecular mixes"), which opens up new avenues for in vitro diagnostic tools.

Our results also show that even the quantitative measure of IgE levels to a single specific allergen molecule is not affected whether the molecule is offered alone or, within certain limit, in combination with other allergenic molecules. This implies that the overall quantitative result obtained in molecular mixes is a reliable proxy of the sum of the result obtained by testing the same molecules, one by one. The real limits of this property are unknown, but it can be predicted that it is a function of multiple factors, including the concentration of IgE antibodies to different components in the patient's serum, the binding capacity of the solid phase of the assay, the putative interference (steric, reciprocal binding, enzymatic, etc.) among the molecules. Of note, the specific addition ("spiking") of allergen molecules to an allergy extract has been proposed many years ago[24] and validated in the routine allergy practice.[25] Our approach is an important step forward in the same direction, with the important difference that only molecules are contained in the solid phase of the IgE test and not added to extracts.

On the contrary, it is also easy to predict that screening tests based on molecular mixes will increasingly show many advantages on extract mixes, such as the increased freedom of composition design, precision in titration, batch-to-batch standardization, lack of interference by nonallergenic molecules, comparability between products of different companies, adaptability to the molecular composition of allergen immunotherapy preparations, as previously discussed.[13] Therefore, the present study, by demonstrating the flexibility and precision offered by IgE tests on heterologous molecular mixes, adds a new asset to the in vitro diagnostics of IgE-mediated allergies.

The selection of the allergen components to be included in a molecular SX01 mix is crucial. In our opinion, a "perfect" composition does not exist. The decision must be driven by a profound knowledge of the purposes of the molecular mix and of the epidemiological scenario of the geographical area where the test will be used in the clinical practice or scientific studies.[10] The mix examined in our study is designed for Germany and neighboring countries but will not be useful for example in Southern Europe, where olive, pellitory, cypress, and other pollen is relevant.[26,27] Moreover, it might be debated whether the test should include major allergenic proteins of molds, such as Alt a 1[28] for Alternaria. Further studies will be necessary to answer these questions. Finally, our results also show that the mix does not identify the very small minority of grass pollen-allergic patients who become sensitized to Phl p 5 before they become sensitized to Phl p 1.[29] Although Phl p 5 is only very infrequently an initiator molecule, it is a very important major allergen molecule of grass pollen[30] so that its inclusion in the solid phase of the molecular SX01 may further increase the sensitivity of the assay. Similarly, the lack of Der p 23 in the molecular mix would prevent the identification of the small minority of mite allergic patients who develop their mite-specific IgE response starting with this molecule but not with the Der p 1 or Der p 2.[17,31] This also extends to patients sensitized to Can f 4, a major dog allergen and the most amply detected one. The addition of this molecule will improve the test's reliability, while also screening for monomolecularly sensitized patients.

A molecular SX01 IgE screening test can be very important in pediatrics as a tool for early detection of infants and children at risk of respiratory allergies, especially, if other risk factors such as atopic eczema are already present. Indeed, the need of only four μl of serum for the test is facilitating the test in early childhood.[22] Moreover, the capacity of identifying the participants of the MAS cohort as positive at their first onset of IgE sensitization to airborne allergens is of great clinical relevance. This implies that a molecular SX01 test can be efficiently used in a pediatric environment for early detection of respiratory allergies, at least in Germany and in areas with similar epidemiological and environmental characteristics. On the other side, our biobank included many sera with quite low levels of IgE to dog extract, and some of these sera were not detected as positive with the molecular SX01 mix, resulting in a rather low sensitivity for this allergen. Interestingly, we[32] and others[33] have previously shown that the appearance of very low levels (i.e., 0.35–1.0 kU/L) of IgE antibody to airborne allergens in early childhood (2–5 years) is very often a transient phenomenon undergoing remission and of no clinical relevance. On the contrary, IgE levels higher than 3.5 kU/L tend to persist at least up to adolescence and are more frequently associated with allergic symptoms.[32,33] In this respect, a slightly lower sensitivity of the molecular-based SX01 IgE test, when compared with the extract-based SX01 mix, may be useful to classify as negative sera with clinically irrelevant, very low level IgE sensitization to airborne allergens. This hypothesis deserves now to be tested in a real-life, routine pediatric clinical setting.

The new molecular test will allow for a diagnosis based on better standardized recombinant molecules and a smaller volume of serum. Its molecular composition may allow a better tailoring of the test to match the geographic and specific (age) needs, as well as diagnostic problems generated by cross-reactivity. Disadvantages of this approach may arise by testing patients with rare monomolecular sensitization, that is, patients with IgE only to molecules not included in the test and that may be instead present in the extract-based SX01. Whatever screening test is chosen, its outcome has always to be framed in the clinical history of the patient, and a further specific analysis of patient sensitization profile, in case of a positive outcome to the screening test, needs to be done before treatment.

In conclusion, this study showed that the accuracy of the SX01 molecular test in the screening of IgE to airborne allergies in German children is very promising and may be further improved by adding Phl p 5 and a few other molecules. This novel category of IgE screening test, based on a mix of major allergenic molecules from several allergen sources, may be particularly useful in the early screening of children with respiratory allergies.

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CONFLICT OF INTEREST

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