

ORIGINAL ARTICLE

Clinical Allergy

A new molecular multiplex IgE assay for the diagnosis of pollen allergy in Mediterranean countries: A validation study

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Summary

Background: The identification of the primary sensitizing pollen is difficult in Southern European patients with Seasonal Allergic Rhinitis (SAR) if sensitized to various pollen sources with overlapping seasonality. A more precise diagnosis is obtained by IgE assays to allergen molecules, currently available as singleplex or microarrays.

Objectives: To test the analytical performance of a multi-parameter immunoblot molecular "Pollen Test" specifically designed to test IgE antibodies to pollen extracts and molecules clinically relevant in Southern Europe.

Methods: Sera were obtained from 101 children and 98 adults with SAR and tested with a customized multiplex immunoblot assay (EUROLINE Southern European Pollen Profile [ESEP]; EUROIMMUN AG, Luebeck, Germany) containing a comprehensive panel of allergen extracts and molecules. ESEP's outcomes were then compared in selected sera (ESEP positive to negative = 2:1) with those of singleplex IgE assays (ImmunoCAP; ThermoFisher Scientific, Uppsala, Sweden). For each of the examined reagents, qualitative (sensitivity, specificity, accuracy), semi-quantitative (classes) and quantitative (Spearman's rank correlation, Bland-Altman plots) comparisons were performed.

Results: Compared to ImmunoCAP, cumulative ESEP's sensitivity and specificity were 87% (95% CI 84%-90%) and 88% (83%-93%) for extracts and 99% (98%-100%) and 87% (83%-91%) for molecules. Cohen's kappa coefficients (κ_c) ranged for extracts from 0.18 (Pellitory) to 0.50 (Cypress) and for molecules from 0.21 (Ole e 1) to 0.68 (Phl p 7). The quantitative outcomes of the two diagnostic tests were highly correlated, with Spearman's rank correlation coefficients always exceeding 0.80. Bland-Altman plots showed a tendency of ESEP to overestimate serum specific IgE levels, when compared to ImmunoCAP.

Conclusions and clinical relevance: Sensitivity and specificity of ESEP in testing serum IgE antibodies against pollen allergen extracts and molecules, in Italian patients with SAR, both exceeded 85%. The advantages and limitations of a multiplex customized immunoblot assay, in the routine clinical use of molecular diagnostics in Southern European pollen allergic patients, deserve to be tested.

KEYWORDS

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

1 | INTRODUCTION

Allergic rhino-conjunctivitis due to pollen allergy reduces the quality of life for millions of people around the globe,¹ especially children.² Until now, allergen-specific immunotherapy (AIT) is the only disease-modifying treatment and requires precise matching of the therapeutic preparation against the pollen allergens inducing or eliciting the symptoms. Especially in the Mediterranean area, it is often difficult to identify the primary sensitizing allergen, as a variety of plants pollinate at the same time of the year, and many patients with pollen allergy are nowadays sensitized simultaneously to various pollen sources with overlapping seasonality.³ Another challenge for an accurate diagnosis is the frequent sensitization to cross-reactive molecules shared by apparently unrelated plants and their extracts.⁴ Thus, an extract-based identification of the eliciting source, for example, via skin prick testing (SPT) or IgE antibody determination is often difficult.⁵

This diagnostic imprecision can be bypassed by using recombinant or highly purified allergenic molecules in a single- or multiplex assay format.⁶ While the latter allows a determination of IgE antibodies against a broad (>100) set of allergens in a single test run with small sample volumes, it may also provide unwanted information not directly focused on pollen allergy and is cost intensive. On the other hand, singleplex testing may also become cost intensive if the correct molecules to be tested cannot easily be identified and bigger serum volumes are required, both factors decreasing the usability as a screening tool, especially in paediatrics.⁷ These limitations would be overcome by a customized multiplex test precisely tailored to investigate IgE responses to molecules relevant for pollen-related allergic rhinitis (AR), rather than to a broadest array of molecules (including those from insect venom, drugs, milk, egg,) relevant in other IgE-mediated allergies.

A new component-resolved, multi-parameter immunoblot test system (EUROLINE Southern European Pollen Profile [ESEP], EURO-IMMUN AG, Luebeck, Germany) contains major allergens from different pollen sources and the corresponding extracts on one test strip. The design of this customized test combines therefore some of the advantages of singleplex (tailored to the patient's needs) and multiplex (comprehensive information) assays. This approach is based on previous experiences in diagnostics for autoimmune diseases^{8,9} and may provide an ideal solution for an affordable, yet accurate allergy-screening tool on a customized base. Yet, a comprehensive analytical validation comparing this assay with the long since established ImmunoCAP (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) standard has still not been performed. Therefore, we tested the sera of 199 allergic children and adults from two Italian allergy

centres with both analytical systems in order to assess their inter-methods comparability.

2 | METHODS

2.1 | Study population

Patients were consecutively recruited between November 2016 and February 2017 in the outpatient clinic of the Department of Pediatrics, "Sandro Pertini" Hospital, in Rome and of the Department of Allergy, "S.Maria degli Angeli" Hospital, in Pordenone (North-East Italy). Both centers use immunoCAP (ThermoFisher) to test IgE in their routine practice. The inclusion criteria were (a) age 10-18 years (Rome), 18-60 years (Pordenone); (b) a history of Seasonal Allergic Rhinitis (SAR) with/without asthma in one of the two last pollen seasons; (c) Italian-speaker; (d) smartphone use. The exclusion criteria were (a) previous pollen-allergen immunotherapy (AIT); (b) any other severe chronic disease (eg, immune-mediated disorders, immune deficiencies.); (c) living >20 km from/outside the climatic area served by the local aerobiological centre. Patients (or their parents, when children aged <14 were examined) were interviewed and underwent SPT and a blood drawing. All participants and/or their parents or tutors gave their informed written consent. The study design and procedures have been approved by the local Ethical committees. The present paper focuses only on the serological aspects of the study, while the results concerning the *in vivo* tests and the clinical aspects are reported elsewhere.

2.2 | Questionnaires and diagnostic criteria

Selected questions obtained from the following internationally validated questionnaires were administered to all participants: International Study of Allergy and Asthma in Childhood (ISAAC).^{2,10} Allergic Rhinitis and its Impact on Asthma (ARIA)¹¹ and Global Initiative for Asthma (GINA).¹² A "traditional" diagnosis of pollen-induced AR was made as previously described¹³ in the presence of (a) nasal and/or eye symptoms (apart from cold) ² for at least 3 weeks during one of the two last pollen seasons and (b) positive SPT (wheal reaction ≥ 3 mm) in accordance with clinical history and local pollination period. Pollen-induced AR was classified as mild or moderate/severe as well as intermittent or persistent according to the ARIA questions and classification.¹¹

2.3 | Skin prick tests

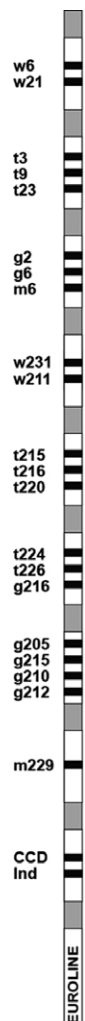
Skin prick testings were performed with a panel of commercial extracts (ALK-Abello, Lainate, Italy) of airborne allergens such as

timothy grass, bermuda grass, olive tree, cypress, mugwort, pellitory, birch, ragweed, plane tree, Russian thistle. Histamine 0.1 mg/mL and glycerol solution were the positive and negative controls, respectively. Morrow Brown needles were used to prick the skin. Readings were taken at 15 minutes, and a wheal ≥ 3 mm, after subtraction of the negative control, was regarded as positive.

2.4 | IgE assays

Serum total IgE and specific IgE have been tested with ImmunoCAP-FEIA (Thermo Fisher Scientific) and the results expressed in kU/L. Serum-specific IgE antibodies have been measured with ESEP (EUROLINE Southern European Pollen Profile). ESEP is a multiplex immunoblot assay (EUROIMMUN AG) specifically designed for the in vitro identification of specific IgE to pollen extracts in the Mediterranean area and their allergenic molecules and of *Alternaria alternata* and Alt a 1. The test strip (Figure 1) consisted of nine membrane chips with different allergen extracts or components immobilized in parallel lines which are mounted on a carrier foil. The ESEP included the following allergen extracts and native (n) and recombinant (r) molecules from pollen: birch (*Betula verrucosa*), rBet

v 1, rBet v 2, rBet v 4, olive tree (*Olea europaea*), rOle e 1, cypressus (*Cupressus arizonica*), nCup a 1, bermuda grass (*Cynodon dactylon*), rCyn d 1, timothy grass (*Phleum pratense*), rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12, mugwort (*Artemisia vulgaris*), nArt v 1, pellitory (*Parietaria judaica*), rPar j 2, alternaria (*A. alternata*), rAlt a 1 and a cross-reactive carbohydrate determinant (CCD) marker. The ESEP strips were incubated according to the manufacturer's instructions. In brief, 100 μ L of patients' sera diluted 1:11 were incubated at room temperature on a rocking shaker. After first three washing steps, the strips were incubated with alkaline phosphatase-conjugated anti-human and after second three washing steps with chromogen/substrate solution. The reaction was stopped by washing with distilled water. The strips were placed on the evaluation protocol, dried and evaluated with the "EUROLineScan" programme. The EUROLINE technique is a semi-quantitative method and therefore able to connect response magnitude (measured as band intensity) to an antibody concentration range (as exemplified by the EAST-class system). The range of intensity values corresponding to a certain EAST class is predefined in the EUROlineScan software. Basis for this definition is the use of pre-characterized (with regard to sIgE measured by ImmunoCAP) samples, which were used to correlate the band



Position	Allergen code	Allergen name
1	w6	Artemisia vulgaris
2	w21	Parietaria judaica
3	t3	Betula verrucosa
4	t9	Olea europaea
5	t23	Cupressus arizonica
6	g2	Cynodon dactylon
7	g6	Phleum pratense
8	m6	Alternaria alternata
9	w231	nArt v 1
10	w211	rPar j 2
11	t215	rBet v 1
12	t216	rBet v 2
13	t220	rBet v 4
14	t224	rOle e 1
15	t226	nCup a 1
16	g216	rCyn d 1
17	g205	rPhl p 1
18	g215	rPhl p 5
19	g210	rPhl p 7
20	g212	rPhl p 12
21	m229	rAlt a 1
22	CCD	CCD marker
a	Ind	Indicator band

FIGURE 1 An example of the multi-parameter immunoblot test system (EUROIMMUN AG). The list of allergen extracts and allergen molecules included in the ESEP and whose results are described in the present study is shown in the figure. ESEP, EUROLINE Southern European Pollen Profile

intensity to the respective EAST-class result, covering the whole measurement range from class 1 to 6 (EUROIMMUN). The quality of the test has been also tested by the manufacturer, and some data on this analytical validation are summarized in the electronic repository. The intensity of the bands was measured and converted into EAST classes of 0-6 which will be divided into the following concentrations (all expressed in kU/L): class 0 < 0.35; $0.35 \leq \text{class } 1 < 0.7$; $0.7 \leq \text{class } 2 < 3.5$; $3.5 \leq \text{class } 3 < 17.5$; $17.5 \leq \text{class } 4 < 50.0$; $50.0 \leq \text{class } 5 < 100.0$; class 6 ≥ 100.0 . These EAST classes 0-6 are therefore identical to the CAP classes (Thermo Fisher Scientific, Phadia). Class 1 or superior was the positivity criterion. All the sera were first tested with the ESEP. Then the sera were selected according to a 2:1 (pos:neg) randomization procedure for each allergen extract or molecule, in order to select the sera, whose ESEP outcome had to be compared with that of the CAP. The sets of sera used for different allergens were therefore different. Fifty ESEP-positive sera and 25 negative sera were randomly selected. If the maximum number of positive sera were <50, the proportion of negative sera were selected leaving constant the pos:neg ratio (2:1).

2.5 | Statistical analysis

Data were summarized as numbers (n) and frequencies (%) if they were categorical and as mean/median and standard deviation (SD)/interquartile range (IQR) if quantitative. Sensitivity, specificity, positive and negative predictive values, accuracy and positive and negative likelihood ratios were calculated to compare the diagnostic performance in detecting IgE sensitization of ESEP test vs ImmunoCAP test. Bland-Altman plots were used to investigate the agreement between quantitative values of IgE detected with the two different methodologies (ESEP vs ImmunoCAP). In this analysis, ESEP IgE value was subtracted from the CAP IgE value. To quantify the degree to which two tests were related, Spearman's rank correlation was calculated for each allergen extract and molecule considered. In order to measure the inter-rater agreement for semi-quantitative outcomes (classes) of ESEP vs ImmunoCAP in measuring serum IgE antibodies to allergen molecules, Cohen's kappa coefficient (κ) was computed. A *P*-value <0.05 was considered statistically significant. Statistical analyses were performed with R Core Team (2014), version 3.2.3.

2.6 | Ethical approval

The study was approved by the ethic committees "Comitato Etico Indipendente Lazio 2" (Study 10-16, Protocol number 9871—01/02/2016) and by "Comitato Etico Regionale Unico (Friuli Venezia Giulia)" (Protocol number 22/2016/Os—08/03/2016).

3 | RESULTS

3.1 | Study population and study design

Overall, 101 children and 98 adults with an average age of 13.5 (95% CI: 12.9-4.1) and 33.6 (95% CI: 30.8-36.4) met the inclusion

criteria for Rome and Pordenone samples, respectively. Male gender was more frequent for both populations: 62.4% for Rome and 55.6% for Pordenone. Roman children were characterized by a variable severity of AR, 49.5% had mild symptoms (18.8 intermittently vs 30.7 persistently) while 50.5% had moderate-severe symptoms (10.9% intermittently vs 39.6 persistently). Ninety-four Pordenone's adults presented a moderate-severe allergic rhinitis with a most frequent persistent status (78.6%). Prevalence of allergic asthma was similar in both groups (around 28% for Rome and Pordenone, respectively), while the paediatric population in Rome was more frequently affected by oral allergic syndrome, urticaria, atopic dermatitis and anaphylaxis episodes (Table 1).

3.2 | Inter-assay comparison: extracts

The comparison of the outcomes of IgE to allergen extracts was performed at qualitative (positive and negative; Table 2), semi-quantitative (classes; Figure 2A) or quantitative (units/volume; Figure S1A) level.

Compared to ImmunoCAP, cumulative ESEP's sensitivity and specificity for IgE to allergen extract were 87% (95% CI 84%-90%) and 88% (83%-93%; Table 2). The proportion of correctly classified subjects exceeded the 80% for each considered allergen extract. The highest and lowest levels of sensitivity were 98% for alternaria

TABLE 1 Characteristics of study population

	Rome ^a (n = 101)		Pordenone ^a (n = 98)	
	n	%	n	%
Male	63	62.4	54	55.6
Age (y) (mean, SD)	13.5	3.1	33.6	14.3
Allergic rhinitis				
Age at onset (y) (median, IQR)	6	(4-8)	15	(8-22)
Mild intermittent ^b	19	18.8	2	2.0
Mild persistent ^b	31	30.7	2	2.0
Mod./Severe intermittent ^b	11	10.9	17	17.3
Mod./Severe persistent ^b	40	39.6	77	78.6
Allergic asthma	28	27.8	26	28.0
Age at onset (median, IQR)	6.5	(3-10)	14	(7-29)
Other allergic comorbidities				
Oral allergic syndrome	32	32.3	26	26.5
Urticaria/Angioedema	19	19.2	8	8.2
Atopic dermatitis	28	28.3	13	13.3
Gastrointestinal disorders	4	4.0	2	2.0
Anaphylaxis episode	10	10.1	1	1.0
Other	5	5.1	2	2.0

IQR, interquartile range.

^aSporadic missing values for a few variables examined.

^bAccording to ARIA classification.

Data were summarized as numbers (n) and frequencies (%) if they were categorical and as mean/median and SD/IQR if quantitative.

TABLE 2 Diagnostic performance results in identifying IgE sensitization to allergen extracts through EUROLIN Southern European Pollen Profile (ESEP) method in comparison with ImmunoCAP method (gold standard)

Extract	N	C+E+	C+E-	C-E+	C-E-	Sensitivity		Specificity		PPV		NPV		Accuracy %	LR+	LR-	r_s
						%	95% CI ^a	%	95% CI ^a	%	95% CI ^a	%	95% CI ^a				
Alternaria	75	46	1	4	24	98	89-100	86	67-96	92	81-98	96	80-100	93	6.9	0.0	0.92*
Bermuda grass	75	45	7	5	18	87	74-94	78	56-93	90	78-97	72	51-88	84	4.0	0.2	0.93*
Birch	75	49	5	1	20	91	80-97	95	76-100	98	89-100	80	59-93	92	19.1	0.1	0.92*
Cypress	75	47	6	3	19	89	77-96	86	65-97	94	83-99	76	55-91	88	6.5	0.1	0.81*
Mugwort	74	47	8	2	17	85	73-94	89	67-99	96	86-100	68	46-85	86	8.1	0.2	0.80*
Olive tree	75	50	11	0	14	82	70-91	100	68-100	100	90-100	56	35-76	85	∞	0.2	0.84*
Timothy grass	75	47	5	3	20	90	79-97	87	66-97	94	83-99	80	59-93	89	6.9	0.1	0.95*
Wall pellitory	75	49	12	1	13	80	68-89	93	66-100	98	89-100	52	31-72	83	11.2	0.2	0.83*
All extracts ^b	599	380	55	19	145	87	84-90	88	83-93	95	93-97	73	66-79	88	7.5	0.1	0.86*

C+, ImmunoCAP positive; C-, ImmunoCAP negative; E+, ESEP positive; E-, ESEP negative; (positivity cut-off value ≥ 0.35 kU/L); LR+, positive likelihood ratio; LR-, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

^aExact binomial confidence limits (95% CI).

^bIncluding outcomes of all allergen extracts considered.

r_s represents the Spearman's rank correlation coefficient, significant differences were highlighted (* $P < 0.001$).

allergen extract and 80% for wall pellitory. Furthermore, the highest and lowest levels of specificity were 100% for olive tree extract and 78% for bermuda grass. Except for alternaria extract, negative predictive values were lower than positive predictive values for each allergen extract. The two diagnostic tests measurements were highly correlated in a positive way (Table 2).

The inter-rater agreement of the semi-quantitative outcomes (classes) of ESEP vs ImmunoCAP in measuring serum IgE antibodies to allergen extract was statistically significant different from zero for each extract. Specifically, among allergen extracts, Cohen's kappa coefficients (κ_c) varied from a minimum value of 0.18 for pellitory to a maximum value of 0.50 for cypress showing an inter-rater agreement from fair to moderate (Figure 2A).

Bland-Altman plots showed (a) a good agreement between the two methods for alternaria, cypress, mugwort and olive tree; (b) a linear trend of error across average of measurement (with no error for low value) for bermuda grass; (c) an overestimation at the central values (10-50 kU/L), with a trend to zero difference at lower or higher values, for timothy grass and pellitory; (d) errors around zero at lower value, overestimation in the central values and underestimation at the higher values for birch (Figure S1A).

3.3 | Inter-assay comparison: molecules

The comparison of the outcomes of IgE to allergen molecules was performed at qualitative (positive and negative; Table 3), semi-quantitative (classes; Figure 2B) or quantitative (units/volume; Figure S1B) level.

Compared to ImmunoCAP, cumulative ESEP's sensitivity and specificity for IgE to allergen molecules were 99% (95% CI: 98%-

100%) and 87% (83%-91%; Table 3). Overall, sensitivity values were higher than 89%. Moreover, 10 of the 13 examined molecules reached 100% of sensitivity values (rAlt a 1, nArt v 1, rBet v 2, nCup a 1, rOle e 1, rPar j 2, rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12). The highest and lowest levels of specificity were 100% (rBet v 1, nCup a 1 and rPhl p 5) and 56% (rBet v 4). The proportion of correctly classified subjects was above 72% for each considered allergen molecule, particularly, for nCup a 1 and rPhl p 5, no subject was incorrectly classified. Negative and positive predictive values reached an average value of 97% and 89%, respectively (Table 3).

The degree of concordance between the semi-quantitative results (classes) of ESEP vs ImmunoCAP in measuring serum rIgE antibodies was significantly different from zero for each molecule. Cohen's Kappa coefficient varied from a minimum value of 0.21 for Ole e 1 to a maximum value of 0.68 for Phl p 7 showing a modest to substantial concordance (modest for rAlt a 1, rPhl p 1, nArt v 1, nCup a 1, rOle e 1, rBet v 1, rPar j 2, rPhl p 12, rCyn d 1, moderate for rBet v 4, rPhl p 5, rBet v 1, substantial for rPhl p 7; Figure 2B).

Spearman's rank correlation coefficients showed a strong and positive relationship between quantitative values of the two diagnostic tests examined for each allergenic molecule (Table 3). Accordingly, Bland-Altman plots showed (a) a good agreement between the two methods for rBet v 4, rPhl p 7 and rPhl p 12; (b) a linear trend of error across average of measurement (with no error for low value) for nArt v 1, rBet v 2 and rOle e 1; (c) an overestimation at the central values (10-50 kU/L), with a trend to zero difference at lower or higher values, for rAlt a 1, rCyn d 1, rPar j 2 and rPhl p 1; (d) errors around zero at lower value, overestimation in the central values and underestimation at the higher values for rBet v 1, rPhl p 5 and nCup a 1 (Figure S1B).

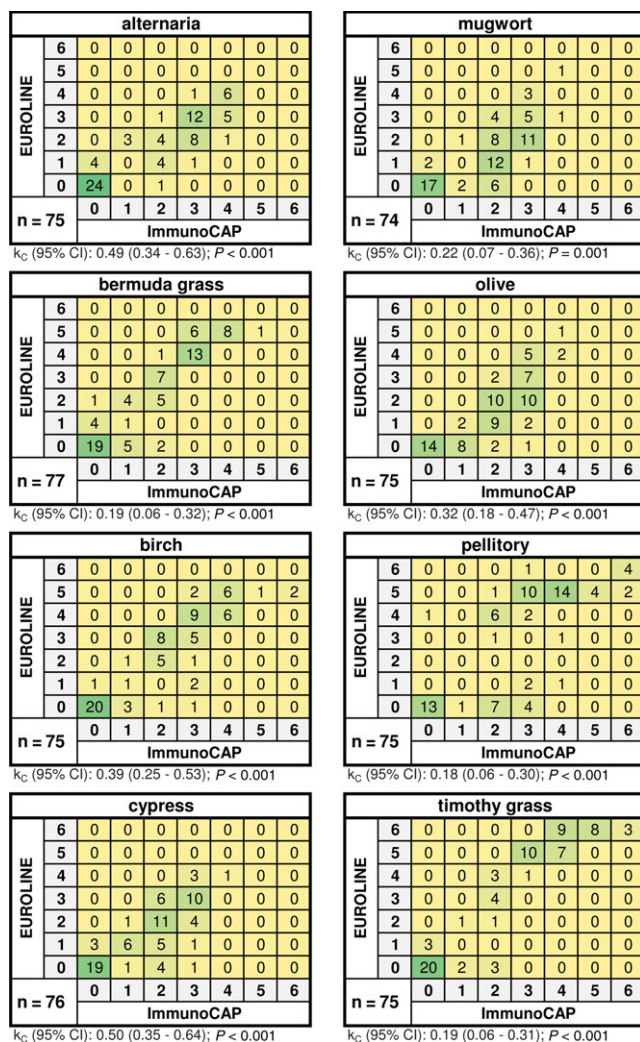


FIGURE 2 Comparison of the semi-quantitative outcomes (classes) of ESEP vs ImmunoCAP in measuring serum IgE antibodies to allergen (A) extracts and (B) molecules. κ_C (95% CI) represents Cohen's kappa statistics for agreement and its confidence intervals followed by testing "null hypothesis" that the extent of agreement is same as random, kappa statistic equals zero. ESEP, EUROLINE Southern European Pollen Profile

3.4 | CCD determinants and total IgE

IgE to CCD were found in 28.9% of the 199 patients, with declining prevalence from 10% with EAST class 1 to 2.5% with EAST class 6. The rate of outcomes negative to CAP but positive to ESEP ("false positivity") was not affected by the level of total IgE in the serum (Table S2).

4 | DISCUSSION

Our study extensively investigated the performance of a new multi-parameter immunoblot test (ESEP) aimed at detecting IgE to pollen allergen molecules and extracts. In a large series of sera from Italian children and adults with pollen allergy, the analytic qualitative and

quantitative performance of ESEP, when compared to ImmunoCAP, was valuable. To our knowledge, this is the first study investigating the analytical value of a multiplex pollen test specifically designed to detect IgE sensitization to pollen allergenic molecules in a specific world area.

4.1 | IgE to pollen molecules

The sensitivity of ESEP, when compared to ImmunoCAP, reached 100% in 10 of the 13 examined molecules, while its specificity exceeded 80% in 9/13 molecules tested. The overall accuracy of the test, examined on 763 comparisons with ImmunoCAP at molecular level, was 94%. The use of recombinant molecules in the in vitro IgE diagnosis started many years ago and has expanded and improved over years.¹⁴ Microarrays, which provide only low antigen-binding capacity, have already proven to be efficient enough in detecting even relatively low concentrations of IgE antibodies,¹⁵ at least in the absence of high concentrations of interfering IgG antibodies. The antigen-binding capacity of ESEP is much higher than that of a single spot of a microarray, thus justifying the sensitivity shown in our study for most of the allergen molecules examined.

4.2 | IgE to pollen extracts

The differences observed between the two tests can be due to (a) the different reagents used in the solid phase and (b) the intrinsic differences in the test method. It is difficult to discriminate which of the two elements in the assay is more important in a comparison between CAP and ESEP. However, it should be highlighted that ESEP showed a slightly lower sensitivity in detecting IgE to extracts, rather than major allergen molecules contained in those extracts. This result is not surprising, if we consider that the antigen-binding capacity of the ESEP is much smaller than that of the ImmunoCAP. Therefore, a lower antigen-binding capacity prevents the detection of IgE with lower avidity (eg, typical of cross-reacting antibodies) and concentration in the serum. The ImmunoCAP test has therefore a clearly higher sensitivity that allows detection of low levels of IgE antibodies with low affinity.

4.3 | Semi-quantitative and quantitative association

The results of our qualitative analysis, based on a positive-negative outcome, were confirmed by the semi-quantitative and quantitative analyses. However, the classes and concentrations of IgE antibodies measured with the ESEP tended to be higher than those measured with the ImmunoCAP. This happens especially in the ImmunoCAP range between 0.35 and 35 kU/L (classes 1-3). High total IgE levels did not interfere with the ESEP outcome; therefore, the observed tendency may be due to differences between the two tests in the methodologies and/or in the calibration systems. The qualitative (positive vs negative) interpretation of the ESEP test was, however, not affected by this semi-quantitative discrepancy.

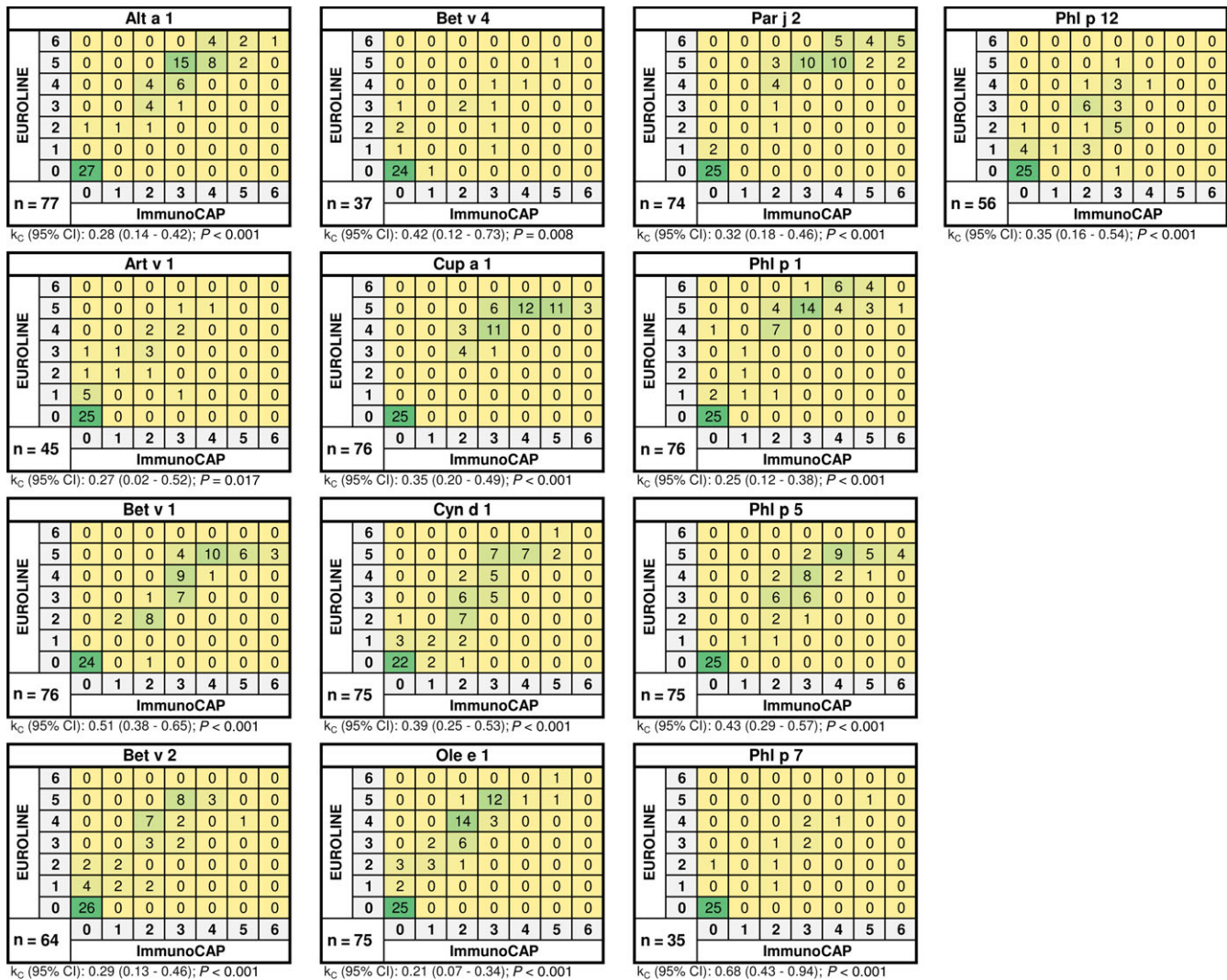


FIGURE 2 Continued

4.4 | Clinical implications

Our results prompt us to consider the use of customized multiparameter assays as an interesting alternative to the use of panels of singleplex tests and of large microarrays: (a) theoretically (ie, without considering administrative constraints), the ESEP can be easily prescribed as a “pollen test” not only by allergy specialists, also by doctors with a reduced expertise in allergy; (b) the need of only 100 µL of serum to provide results for over 30 reagents makes this test useful especially in paediatrics; (c) tests like ESEP, by offering all and only the necessary information on the patient's IgE sensitization profile, may help identifying primary sensitizers in Southern European patients who are very frequently polysensitized to multiple pollens with overlapping seasons.^{13,16,17} Overall, we may speculate that the use of ESEP or similar assays may facilitate the implementation of a more precise, molecular diagnosis in the daily practice. This progress may also facilitate a more precise prescription of allergen immunotherapy. The relative complexity of the molecular results provided by the ESEP, however, suggests that expert systems or

clinical decision support systems (CDSS) may be necessary to make user-friendly the assay in settings not specialized in molecular allergology.

4.5 | Study limitations

We acknowledge a few limitations of our study. First, the population samples of children and adults investigated in two Italian hospitals and cities are not representative of the patient population of Southern Europe. However, further studies are already in progress to investigate the diagnostic performance of ESEP in different Mediterranean countries. Second, as shown by our Table S1, the way the sera are selected has invariably an impact on the results; this means that the generalizability of our results is limited, as always in this type of studies. However, by testing a fixed proportion of ESEP positive and negative samples (2:1), we have chosen standard conditions for all the examined allergen extracts or molecules. This choice makes our study reproducible so that other laboratories can use the same study design in other settings and geographic areas,

TABLE 3 Diagnostic performance results in identifying IgE sensitization to allergen molecules through EUROLINE Southern European Pollen Profile (ESEP) method in comparison with ImmunoCAP method (gold standard)

Molecule	N	C+E+	C+E−	C−E+	C−E−	Sensitivity		Specificity		PPV		NPV		Accuracy %	LR+	LR−	r_s
						%	95% CI ^a	%	95% CI ^a	%	95% CI ^a	%	95% CI ^a				
rAlt a 1	75	49	0	1	25	100	89-100	96	80-100	98	89-100	100	80-100	99	26.00	0.00	0.97*
nArt v 1	29	13	0	6	10	100	66-100	63	35-85	68	43-87	100	59-100	79	2.67	0.00	0.82*
rBet v 1	75	50	1	0	24	98	90-100	100	80-100	100	90-100	96	80-100	99	∞	0.02	0.97*
rBet v 2	57	32	0	6	19	100	84-100	76	55-91	84	69-94	100	75-100	89	4.17	0.00	0.93*
rBet v 4	18	8	1	4	5	89	52-100	56	21-86	67	35-90	83	36-100	72	2.00	0.20	0.66*
nCup a 1	75	50	0	0	25	100	90-100	100	80-100	100	90-100	100	80-100	100	∞	0.00	0.97*
Cyn d 1 ^b	75	46	3	4	22	94	83-99	85	65-96	92	81-98	88	69-97	91	6.10	0.07	0.95*
rOle e 1	75	45	0	5	25	100	88-100	83	65-94	90	78-97	100	80-100	93	6.00	0.00	0.97*
rPar j 2	74	47	0	2	25	100	89-100	93	76-99	96	86-100	100	80-100	97	13.50	0.00	0.95*
rPhl p 1	75	47	0	3	25	100	89-100	89	72-98	94	83-99	100	80-100	96	9.33	0.00	0.96*
rPhl p 5	75	50	0	0	25	100	90-100	100	80-100	100	90-100	100	80-100	100	∞	0.00	0.97*
rPhl p 7	15	9	0	1	5	100	55-100	83	36-100	90	55-100	100	36-100	93	6.00	0.00	0.95*
rPhl p 12	45	25	0	5	15	100	80-100	75	51-91	83	65-94	100	70-100	89	4.00	0.00	0.87*
All molecules ^c	763	471	5	37	250	99	98-100	87	83-91	93	90-95	98	95-99	94	7.1	0.01	0.94*

C+, ImmunoCAP positive; C−, ImmunoCAP negative; E+, ESEP positive; E−, ESEP negative; (positivity cut-off value ≥ 0.35 kU/L); LR+, positive likelihood ratio; LR−, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value.

^aExact binomial confidence limits (95% CI).

^br (recombinant) for ESEP and n (native) for ImmunoCAP.

^cIncluding outcomes of all allergen molecules considered.

r_s represents the Spearman's rank correlation coefficient, significant differences were highlighted (* $P < 0.001$).

independently from the prevalence and profile of IgE sensitization in their study populations. Third, the test is not including all the molecules that play a clinically relevant role in pollen allergy in Southern Europe. However, most of the major allergenic molecules, whose investigation is essential for a precise prescription of allergen immunotherapy in Southern Europe, are included in the ESEP panel. Patients with “rare” or “very infrequent” pollen allergies, whose IgE sensitization profile goes beyond the diagnostic possibilities of the current ESEP, require a broader panel of extracts and molecules, which can easily be adapted in a similar assay format. On the other hand, a 2nd step complementation with clinically targeted singleplex assays can also be a solution to this problem.

5 | CONCLUSIONS

In conclusion, the analytic qualitative and quantitative performance of ESEP in testing serum IgE antibodies against pollen allergen molecules is valuable. The hypothesis, that a multiplex, customized immunoblot assay overcomes some limitations of singleplex assays or microarrays in the routine use of molecular diagnostics in Southern European pollen allergic patients, deserves to be tested in further studies. The use of disease-oriented multiplex tests, focusing on a few crucial molecules essential for the diagnosis of one allergic disease only (eg, pollen allergy), may be an alternative solution to the excess of information provided by the allergen microarrays.

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CONFLICT OF INTEREST

Dr. Matricardi reports grants and personal fees from EUROIMMUN AG, during the conduct of the study; grants and personal fees from Thermo Fisher Scientific, grants and personal fees from Hycor Biomedical Inc, outside the submitted work. All other authors declared no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure e1
a)

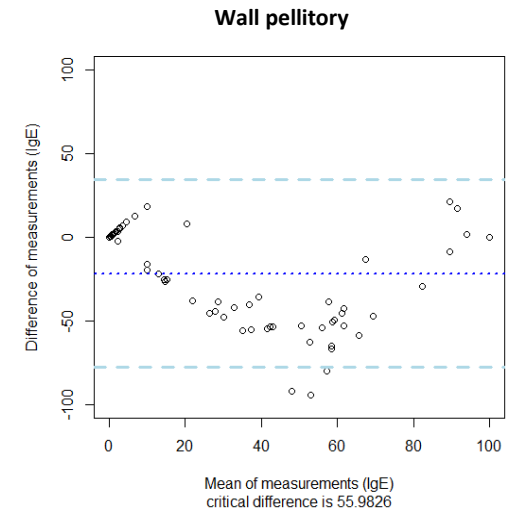
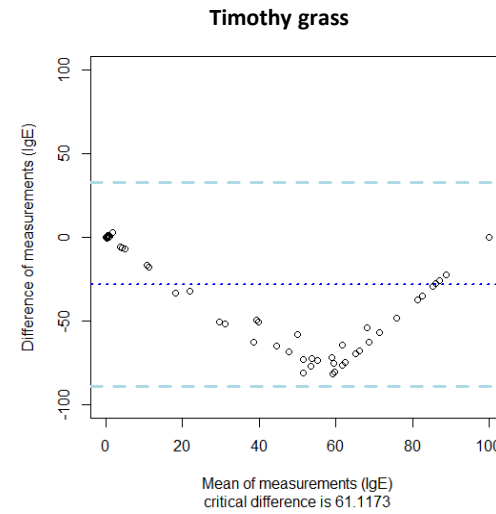
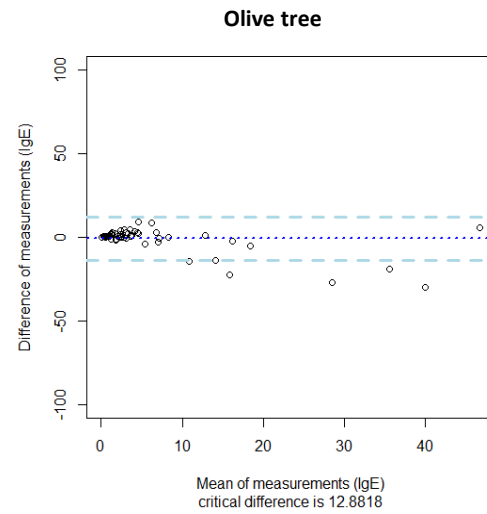
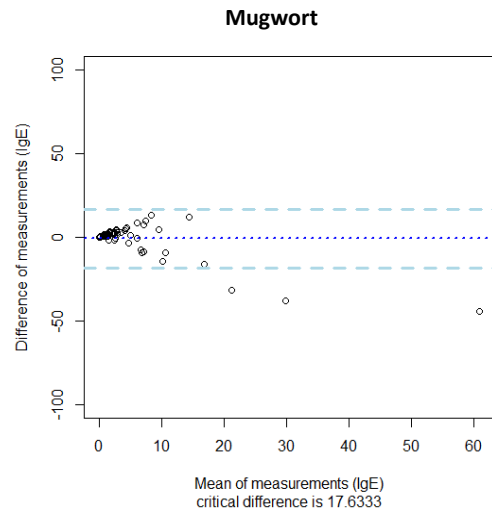
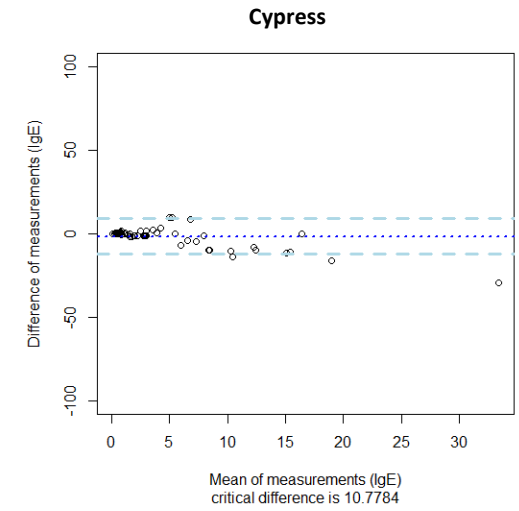
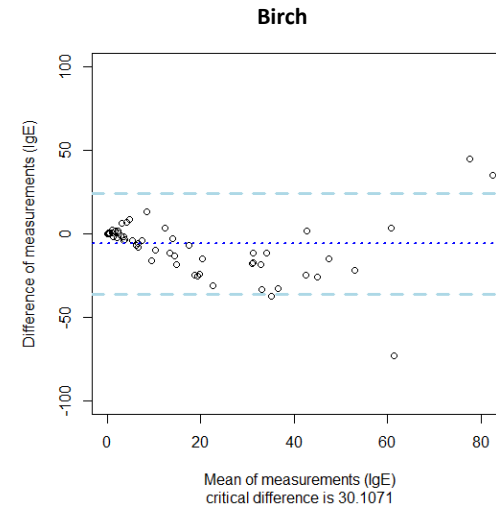
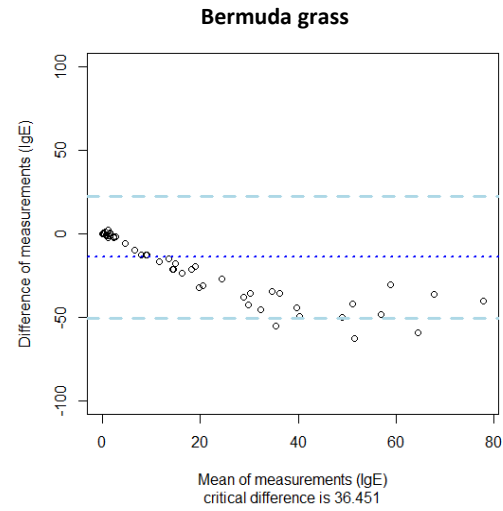
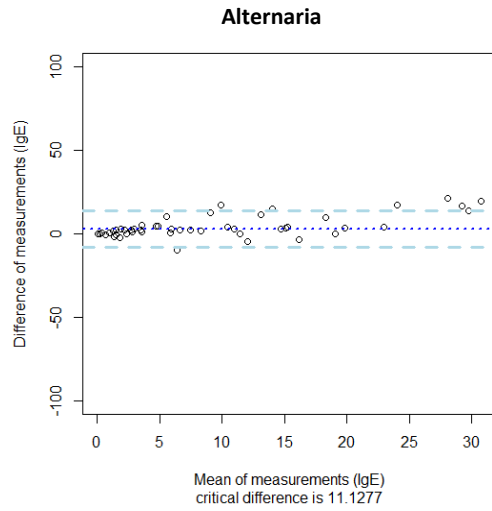


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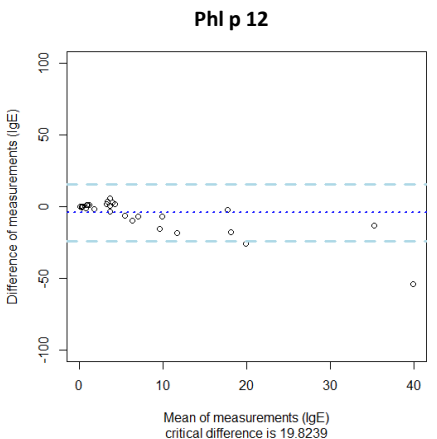
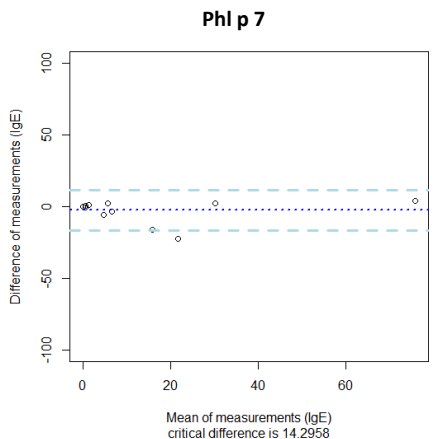
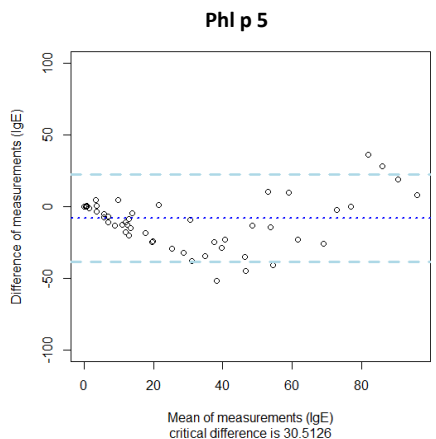
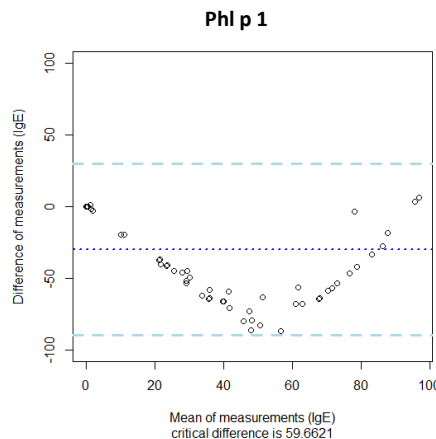
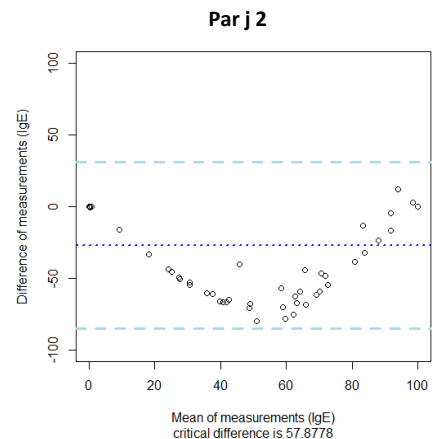
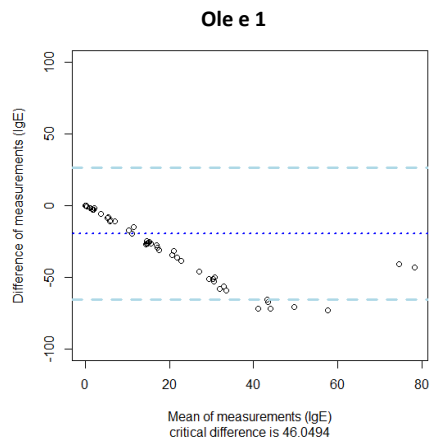
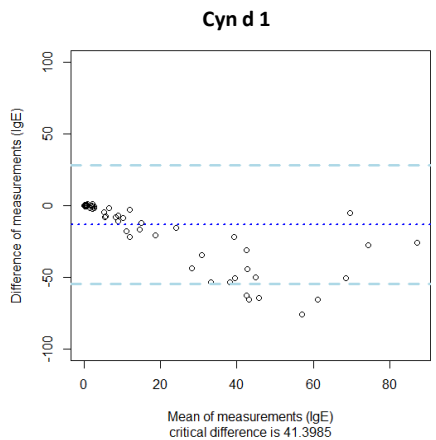
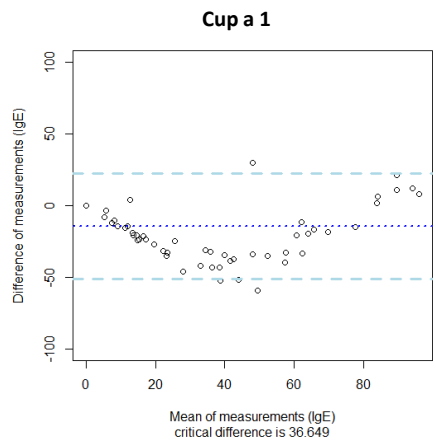
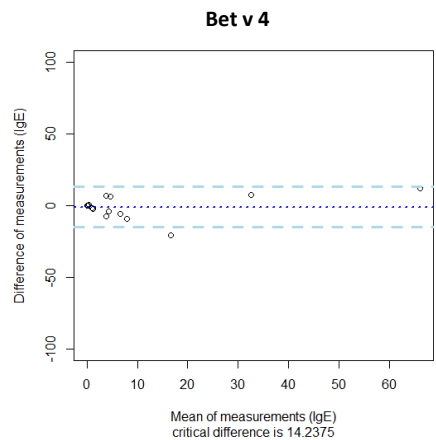
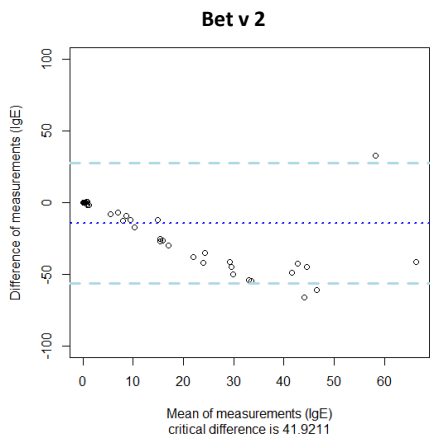
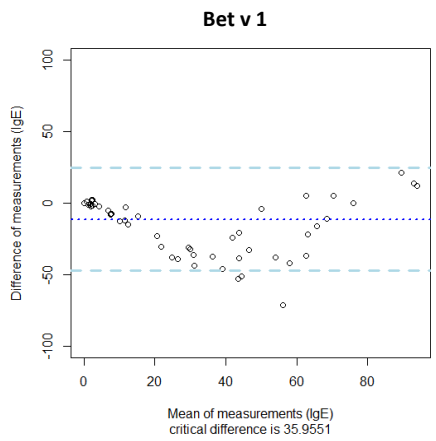
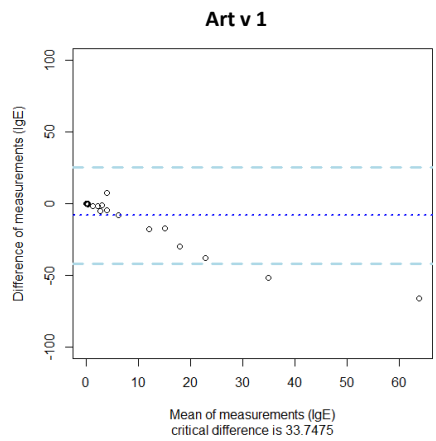
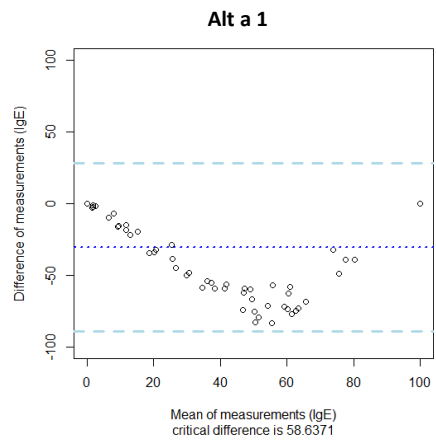
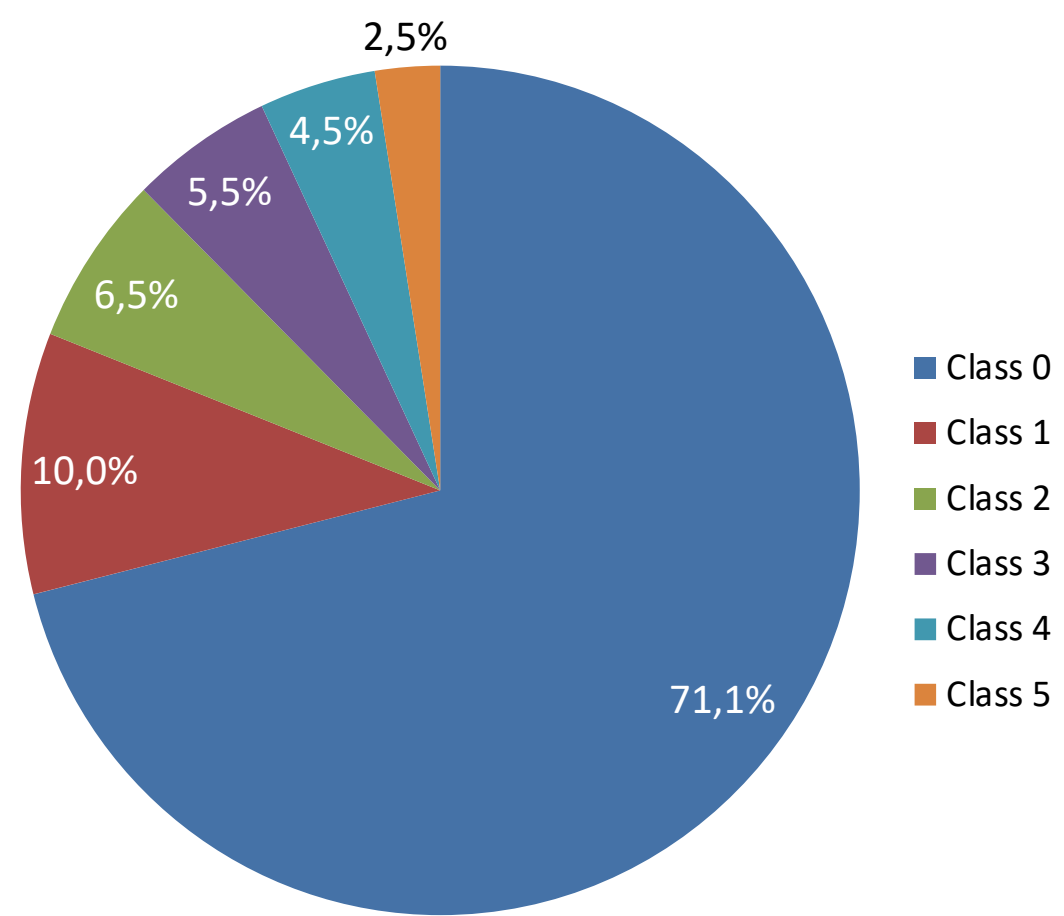


Figure e2



ELECTRONIC REPOSITORY

Title: A new molecular multiplex IgE assay for the diagnosis of pollen allergy in Mediterranean countries: a validation study

Running title: Multitest for pollen allergy

Tables: 2

Figures: 2

The work was carried out in: Charité Medical University, Dept. of Paediatric Pneumology and Immunology, Augustenburgerplatz 1, 13353, Berlin, Germany

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METHODS

Analytical validation of ESEP

Data on assay interference showed no interference by high concentrations of hemoglobin, triglycerides and bilirubin on the analytical results of the test. Use of human serum or EDTA, heparin or citrate plasma showed no differences between the sample matrices. Anti-human IgE-alkaline phosphatase conjugate was tested for cross-reactivity against human IgG, IgA and IgM and showed no unspecific binding at physiological concentrations of these human Immunoglobulins. Reproducibility was tested with 6 pos. samples over 2 days in 14 determinations. Intra-assay CV was <10% for all allergens, while Inter-assay CV was <15%. EUROLinScan, the intensity values for the class borders have been established through comparison studies with the ImmunoCAP specific IgE system using samples with known IgE antibody levels.

FIGURE LEGENDS

Figure E1 – Bland-Altman plot showing the differences between the ImmunoCAP and ESEP values (y-axis) against the mean of the two measurement (x-axis) for each allergen **(a)** extract or **(b)** molecule. The dotted blue line represents the average difference. The ESEP-kU/L-values were subtracted from the CAP-values. The critical difference equals half the difference of lower limit and upper limit. Sample size for each extract or molecule is shown in Table 2 and 3, respectively.

Figure E2 – Distribution of IgE to CCD determinants in ESEP.

Table e1. Diagnostic performance results in identifying IgE sensitization to allergen molecules through ESEP method in comparison to ImmunoCAP method (gold standard) - For each molecules the results are shown for the randomly selected sera or the whole population sample (see methods for details).

Molecule	N	C+nE+	C+nE-	C-nE+	C-nE-	Sensitivity		Specificity		PPV		NPV		Accuracy	LR+	LR-	r_s
						%	(95% CI)†	%	(95% CI)†	%	(95% CI)†	%	(95% CI)†	%			
rOle e 1	75	45	0	5	25	100	(88-100)	83	(65-94)	90	(78-97)	100	(80-100)	93	6.0	0.0	0.97 *
rOle e 1	199	100	0	9	90	100	(95-100)	91	(83-96)	92	(85-96)	100	(94-100)	95	11.0	0.0	0.95 *
rPhl p 1	75	47	0	3	25	100	(89-100)	89	(72-98)	94	(83-99)	100	(80-100)	96	9.3	0.0	0.96 *
rPhl p 1	199	160	0	12	27	100	(97-100)	69	(52-83)	93	(88-96)	100	(82-100)	94	3.3	0.0	0.94 *
rPhl p 12	56	25	1	5	25	96	(80-100)	83	(65-94)	83	(65-94)	96	(80-100)	89	5.8	0.0	0.83 *
rPhl p 12	199	25	6	5	163	81	(63-93)	97	(65-94)	83	(65-94)	96	(92-99)	94	27.0	0.0	0.61 *

C+, ImmunoCAP positive; C-, ImmunoCAP negative; E+, ESEP positive; E-, ESEP negative; (positivity cut-off value ≥ 0.35 kU/l).

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

‡ r (recombinant) for ESEP and n (native) for ImmunoCAP.

† Exact binomial confidence limits (95 % CI).

r_s represents the Spearman's rank correlation coefficient, significant differences were highlighted (* $p < 0.001$).

• Including outcomes of all allergen molecules considered.

Table e2. Number of false positive ESEP test results vs ImmunoCap test by quartile of tIgE distribution

Molecule	C-∩E+	First n=50	Second n=49	Third n=50	Fourth n=50	p*
rOle e 1	12	4	3	5	0	0
rPhl p 1	5	1	1	3	0	1
rPhl p 12	56	25	1	5	25	0

Total IgE quartiles: first <103,7 kU/l; second ≥ 103,7 kU/l & <253,6 kU/l; third ≥ 253,6 kU/l & <508,2kU/l; fourth ≥ 508,2 kU/l.

C-∩E+ : number of false positive; - : ImmunoCap negative; E+ : ESEP positive (positivity cut-off value ≥ 0.35 kU/l).

* : Chi-squared Test for Trend in Proportions.