

ORIGINAL ARTICLE

Performance of a component-based allergen-microarray in the diagnosis of cow's milk and hen's egg allergy

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Summary

Background The food challenge test (FCT) is the gold standard for the diagnosis of food allergy. This procedure is time consuming, costly and can induce potentially severe symptoms. An ideal *in vitro* test should allow to avoid the FCT.

Objective To assess the clinical performance of microarray for specific IgE (sIgE) detection in children with challenge-proven/excluded cow's milk (CM) or hen's egg (HE) allergy.

Methods One-hundred and four children with suspected IgE-mediated hypersensitivity to CM or HE were studied. In all patients, skin prick test, ImmunoCAP, microarray and FCT were performed.

Results The microarray components Bos d 8 for CM (27/58 patients) and Gal d 1 (20/46 patients) and Gal d 2 (24/46) for HE were the most frequently recognized allergens. Using the FCT results as the reference parameter, sIgE to Bos d 8 and Gal d 1 had the highest area under the curves. These were not significantly different from those obtained using the ImmunoCAP. Use of 95% clinical decision points (CDP) for sIgE to Bos d 8 and Gal d 1 resulted in higher negative predictive values (78% and 79%, respectively) than those obtained with the ImmunoCAP (57% and 59%).

Conclusions Our results show that in children with suspected CM or HE allergy, the microarray has a good ability to predict the FCT results. In a clinical application perspective, the microarray could be used as a second-level assay, if the ImmunoCAP sIgE is <95% CDP. This approach would lead to a decrease in the number of the FCT to be performed, as well as of positive FCTs with a subsequent decrease in severe reaction risk.

Keywords cow's milk allergy, food challenge test, hen's egg allergy, microarray, specific IgE

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Introduction

Food allergy is a common disease and affects up to 6% of the children in the first few years of life [1–3]. Cow's milk (CM), hen's egg (HE) and peanut account for most of the documented allergic responses [1]. The diagnosis of food allergy is based on patient history [2], skin prick tests (SPTs) and/or measurement of allergen-specific IgE [4]. Presently available methods for measuring specific IgE (sIgE) to food extracts do not have clinically relevant predictive values [5–7]. Currently, the double-blind placebo-controlled food challenge test (FCT) is the gold stan-

dard for the diagnosis of food allergy. This procedure places the patient at risk for severe anaphylactic reactions, and moreover, is time consuming and can be difficult to perform in a busy office setting. An ideal *in vitro* diagnostic test should allow to avoid the FCT. Indeed, Sampson [8], and subsequently several other groups [9–16], defined 95% predictive clinical decision points (CDP) and suggested that an FCT may not need to be performed in patients with sIgE levels exceeding these values. However, the established levels vary widely in different studies, and there is a large grey zone between high levels with good positive predictive values (PPV) and undetectable levels that have good negative predictive values (NPV).

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Protein microarrays have recently become available for measuring sIgE. This technology has two main advantages: it assesses simultaneously sIgE to many recombinant or highly purified allergens and it requires small amounts of blood, which is relevant in children. Establishing the clinical performance of this technology is important in the perspective of a wide-spread use.

The aim of this study was to assess the clinical performance of a component-based allergen microarray with highly purified allergens [17–21] for sIgE detection in children with challenge-proven/excluded CM and/or HE allergy, in comparison with traditional sIgE assay [22–25].

Methods

Study population

We studied infants and children referred to our Allergy Unit for evaluation of suspected IgE-mediated food hypersensitivity. We included only patients with an history related to CM or HE consumption, of severe and/or immediate reactions. These included skin manifestations (lips/periorbital oedema, urticaria/angioedema; $n = 44$, 42%), respiratory symptoms (bronchial asthma, stridor, throat tightness, rhinitis; $n = 6$, 6%), gastrointestinal symptoms (vomiting, diarrhoea; $n = 17$, 16%), combination of skin, respiratory and gastrointestinal symptoms ($n = 33$, 32%) and anaphylactic shock ($n = 4$, 4%). Patients with atopic eczema (AE) as the only manifestation of suspected allergy were excluded. In all patients SPT, sIgE (ImmunoCAP and microarray) and FCT were performed. The study was approved by the Hospital Ethical Committee and informed consent was obtained before enrolment. Laboratory personnel were not informed about the results of the SPT and the FCT.

Skin prick test

SPTs were performed with natural food and with commercial natural extracts to milk, α -lactalbumin, β -lactoglobulin, casein, egg white, egg yolk and with sodium chloride (0.9%) and histamine hydrochloride (Lofarma, Milan, Italy). The response was read 15 min after puncture and results were expressed as the mean weal diameter (mm). A diameter > 3 mm with erythema, was defined as a positive reaction.

Fluorescence enzyme immunoassays

Routine determination of sIgE antibodies against milk, α -lactalbumin, β -lactoglobulin, casein, egg white and egg yolk was performed with a widely used fluorescence enzyme immunoassay according to the manufacturer instruction (ImmunoCAP SystemTM, Phadia Diagnostics, Uppsala, Sweden). sIgE titres were quantified

in protein units designated as kU/L, as proposed by the manufacturer.

Allergen microarray assay

Commercially available allergen chips were purchased from Phadia-VBC Genomics (Vienna, Austria) and the assay was performed according to the instruction provided by the manufacturer [17]. We used a customized version of the microarray (ISACTM version CRD89) containing the following purified natural CM and HE allergen components: ovomucoid (Gal d 1), ovalbumin (Gal d 2), conalbumin (Gal d 3), lysozyme (Gal d 4), serum albumin (Gal d 5), α -lactalbumin (Bos d 4), β -lactoglobulin A (Bos d 5.0102), β -lactoglobulin B (Bos d 5.0101), bovine serum albumin (Bos d 6), IgG bovine (Bos d 7), casein (Bos d 8), α -casein (Bos d 8 alpha S1), β -casein (Bos d 8 beta), κ -casein (Bos d 8 kappa) and lactoferrin (Bos d lactoferrin). Chips were washed for 1 h in the washing buffer provided by the manufacturer, rinsed with deionized water and dried. Twenty microlitre of undiluted serum was applied onto each reaction well. Chips were incubated for 2 h at room temperature in a humid chamber, rinsed and washed twice in washing buffer and once in deionized water. Chips were incubated for 1 h at room temperature with 20 μ L of an Alexa 546-labelled anti-human IgE antibody (Phadia-VBC Genomics, Vienna, Austria) provided by the manufacturer, washed as above dried and stored in the dark until scanning. We used a Scan Array Gx Scanner (Perkin-Helmer, Boston, MA, USA) with two laser power settings to achieve a maximum dynamic range across different levels of IgE reactivities. Images were analysed using the MIA software (version 3.1; Phadia-VBC Genomics). sIgE were quantified as ISAC standardized units (ISU) as proposed by the manufacturer.

Food challenge test

Oral food challenges with either CM and/or HE were performed in all patients. Antihistamine drug and leukotriene receptor antagonists were not allowed in the preceding 1 week and oral corticosteroids in the preceding 2 weeks. Children were admitted to the day clinic in the morning in a fasting state without allergic symptoms. FCT were performed in an open fashion [5]. The FCT material was pasteurized CM and cooked egg (boiled for 10 min) or raw egg in the case of negative FCT to cooked egg. When the patient tolerated the first dose, the subsequent doses were given every 15 min. The FCT was interrupted when objective symptoms developed or when the entire FCT dose was ingested (for HE, the total amount corresponding to the equivalent of one egg; for CM, the total amount corresponding to the dose adapted according to the age of the subject up to 250 mL). FCT was performed under the control of an allergist. FCT were scored as positive if

anaphylactic shock or two or more of the following objective clinical reactions were noted: bronchial asthma, lips/periorbital oedema, urticaria/angioedema, rhinitis, conjunctivitis, diarrhoea and repetitive vomiting [9].

Statistical analysis

Multiple logistic regression analysis was performed in order to assess the determinants of positive FCT among the performed diagnostic tests and demographic information. The accuracy of different tests (SPT, ImmunoCAP and microarray) was evaluated through the receiver operator characteristics (ROC) curves and the area under the curves (AUC). Predictive values (PPV and NPV) were also calculated. As further clinically relevant assay parameters for all test systems, we determined the 95% CDP for a positive FCT at a given IgE level using the logistic regression analysis. The CDP was determined through the formula $P = 1/e^{-\alpha - \beta x} + 1$ where x is the continuous diagnostic test parameter, P is the probability for a positive diagnosis given the value x and α and β are the model parameters of the corresponding logistic regression model. Statistic analysis was performed by using STATA 10.1 (StataCorp 1984–2009).

Results

We enrolled 104 patients (62 males and 42 females) with suspected IgE-mediated allergy to CM or HE with a median age of 4.9 years (range 0.7–15.1). Total serum IgE, CM or HE sIgE and the SPT results are shown in Table 1. FCT resulted positive in 32/58 (55%) patients with

suspected CM allergy and in 22/46 (48%) patients with suspected HE allergy. Symptoms elicited by the FCT were skin manifestations (lips/periorbital oedema, urticaria/angioedema: $n = 31$, 57%), respiratory symptoms (wheezing, stridor, rhinitis: $n = 45$, 83%), gastrointestinal symptoms (vomiting, diarrhoea; $n = 14$, 26%), combination of skin, respiratory and gastrointestinal symptoms ($n = 38$, 70%) and anaphylactic shock ($n = 2$, 4%).

sIgE levels for each CM or HE component of the microarray are shown in Fig. 1. IgE reactivities to Bos d 8 (27/58, 46.5%) and Bos d 4 (16/58, 27.6%) were more frequent, while few patients showed sIgE to Bos d 7 (6/58, 10.3%) or lactoferrin (6/58, 10.3%). IgE reactivities to Gal d 1 (20/46, 43.5%), Gal d 2 (24/46, 52.1%) and Gal d 4 (17/46, 36.9%) were more frequent, while fewer patients showed sIgE to Gal d 3 (6/46, 13.0%) or Gal d 5 (2/46, 4.3%).

Table 1. Specific IgE levels measured with the ImmunoCAP System in patients with suspected cow's milk or hen's egg allergy

Suspected cow's milk allergy	$n = 58$
Total serum IgE (kU/L)	470.3 (3.7–3586.3)
Specific IgE, cow's milk (kU/L)	1.90 (0.01–> 100)
Specific IgE, casein (kU/L)	2.32 (0.01–> 100)
Specific IgE, α -lactalbumin (kU/L)	0.23 (0.01–94.9)
Specific IgE, β -lactoglobulin (kU/L)	2.50 (0.01–70.3)
Suspected hen's egg allergy	$n = 46$
Total serum IgE (kU/L)	525.9 (4.5–> 5000)
Specific IgE, egg white (kU/L)	5.44 (0.01–> 100)
Specific IgE, egg yolk (kU/L)	1.46 (0.01–> 100)

The data are shown as median (interquartile range) or as number (%).

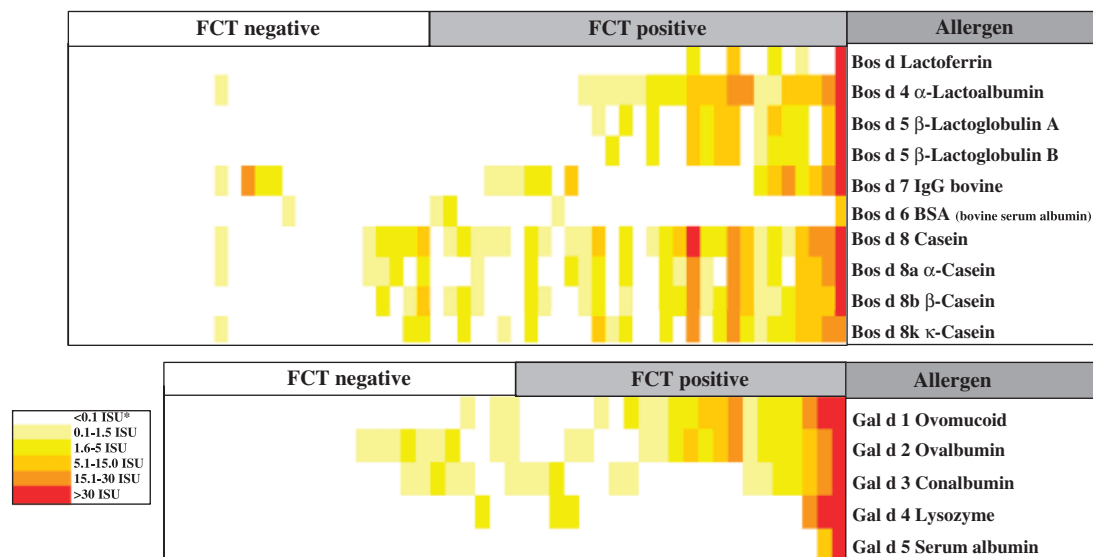


Fig. 1. Specific IgE to cow's milk or to hen's egg protein profiles measured with the allergen microarray. Specific IgE levels are categorized and indicated with different colours as shown in the lower panel. Each column represents the test results observed in a single patient. *ISU, ISAC standardized units; FCT, food challenge test.

Table 2. Summary of clinically relevant assay performance of allergen microarray and ImmunoCAP in suspected cow's milk allergy

	Microarray					
	AUC	95% CDP	PPV (%)	NPV (%)	LR ⁺ (%)	LR ⁻ (%)
Allergen						
Bos d lactoferrin	0.58 (0.51–0.64)	/	/	/	/	/
Bos d 4	0.70 (0.60–0.80)	2.27 (ISU)	100 (59–100)	51 (37–65)	100 (1–100)	0.1 (0.1–0.2)
Bos d 5.0102	0.66 (0.58–0.74)	/	/	/	/	/
Bos d 5.0101	0.64 (0.56–0.73)	/	/	/	/	/
Bos d 6	0.59 (0.48–0.70)	44.32 (ISU)	100 (2–100)	46 (32–59)	100 (1–100)	0.1 (0.1–0.2)
Bos d 7	0.51 (0.46–0.57)	/	/	/	/	/
Bos d 8	0.87 (0.79–0.95)	0.60 (ISU)	96 (80–99)	78 (60–91)	20 (3–140)	0.4 (0.2–0.8)
Bos d 8 α S1	0.81 (0.72–0.90)	0.64 (ISU)	95 (74–100)	64 (47–79)	15 (2–102)	0.2 (0.1–0.3)
Bos d 8 β	0.82 (0.74–0.91)	0.51 (ISU)	100 (82–100)	67 (50–81)	100 (1–100)	0.3 (0.2–0.4)
Bos d 8 κ	0.76 (0.67–0.86)	0.79 (ISU)	100 (78–100)	60 (44–75)	100 (1–100)	0.2 (0.1–0.3)
Sum ISAC	0.86 (0.77–0.96)	7.42 (ISU)	94 (71–100)	61 (45–76)	13 (2–92)	0.5 (0.4–0.7)
ImmunoCAP						
CM	0.92 (0.850.99)	16.6 (kU/L)	93 (66–100)	57 (41–72)	11 (1–76)	0.1 (0.1–0.2)
α -lactalbumin	0.77 (0.65–0.90)	34.27 (kU/L)	100 (40–100)	48 (34–62)	100 (1–100)	0.1 (0.1–0.2)
β -lactoglobulin	0.72 (0.58–0.86)	9.91 (kU/L)	100 (54–100)	50 (36–64)	100 (1–100)	0.1 (0.1–0.2)
Casein	0.93 (0.85–1.0)	0.78 (kU/L)	85 (68–95)	84 (64–96)	20 (3–140)	0.2 (0.1–0.4)

Data are shown with 95% CI in parentheses.

The units of the 95% CDP for microarray and for ImmunoCAP are shown between parenthesis. Sum ISAC shows the performance of the sum of IgE reactivities for each allergen components in the microarray for CM (Bos d 4, Bos d 5.0102, Bos d 5.0101, Bos d 6, Bos d 7, Bos d 8, Bos d 8 alpha S1, Bos d 8 beta, Bos d 8 kappa and Bos d lactoferrin).

AUC, area under the curve; CDP, clinical decision point; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio; ISU, ISAC standardized units; CM, cow's milk.

The outcome of the FCT was considered as the reference parameter. The performance of IgE reactivity to each component of the microarray and of the ImmunoCAP System was evaluated by ROC analysis. In children with suspected CM allergy, the highest AUC were observed for milk and casein in the ImmunoCAP System and for Bos d 8 in the microarray. The AUC for Bos d 8, Bos d 8 alpha S1 and Bos d 8 beta did not show significant differences when compared with the AUC for milk or casein in the ImmunoCAP System (Table 2 and Fig. 2). For HE, the AUC for Gal d 1 and Gal d 2 were not different from that for egg white in the ImmunoCAP System (Table 3 and Fig. 2).

Tables 2 and 3 show the 95% predicted probability (CDP) of a positive FCT for each test and each allergen for both CM and HE. It was not possible to calculate the 95% CDP for allergens to which sIgE were detected in a low percentage of patients. In the microarray, Bos d 8 for CM and Gal d 1 for HE yielded the highest PPV and NPV. Positive and negative likelihood ratios (LR) were calculated: sIgE to Bos d 8 and Gal d 1 were associated with an increase of 20- and 10-folds, respectively, of the pre-test probability (Tables 2 and 3). When we evaluated the sum of the IgE reactivities to the CM or HE allergens present in the chip, we did not observed advantages compared with the performance of sIgE to Bos d 8 and Gal d 1 (Tables 2

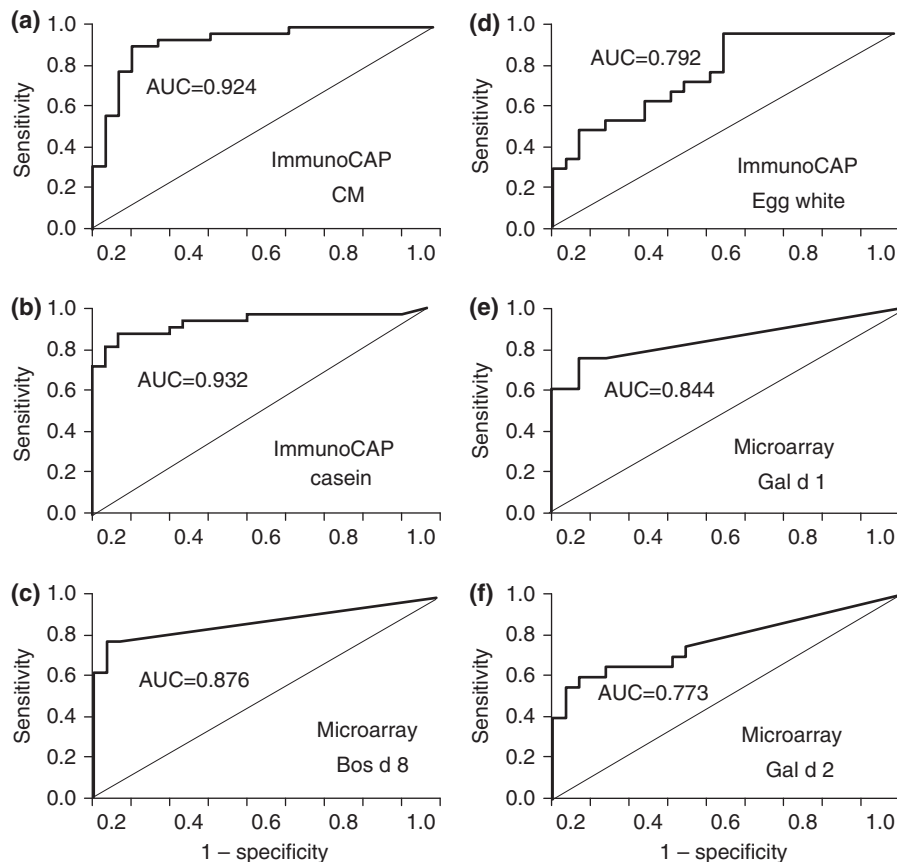


Fig. 2. The receiver operator characteristic curves for cow's milk (CM) ImmunoCAP extracts [(a) CM] [(b) casein] and for the allergen microarray component Bos d 8 (c) and for hen's egg ImmunoCAP extracts [(d) egg white] and for the allergen microarray components [(e) Gal d 1] [(f) Gal d 2]. AUC, area under the curves.

and 3); the AUC of the ROC curves were similar to those for Bos d 8 and for Gal d 1.

We next evaluated if the sequential use of the two tests would lead to a better prediction of the FCT result. The 95% CDP for sIgE to CM (≥ 16.6 kU/L) measured with ImmunoCAP resulted in the identification of 14 patients of whom 13 had a positive FCT (Fig. 3a), yielding a PPV of 93% and an NPV of 57% (positive LR = 11, negative LR = 0.1) (Table 4). The 95% CDP for sIgE to Bos d 8 (> 0.60 ISU) resulted in the identification of 26 patients of whom 25 had a positive FCT (Fig. 3b), yielding a PPV of 96% and an NPV of 78% (positive LR = 20, negative LR = 0.4) (Table 4). Sequential use of the two tests (Fig. 3c) led to a minor improvement in clinical performance (Table 4).

For suspected HE allergy, the 95% CDP for sIgE to egg white (≥ 25.3 kU/L), measured with ImmunoCAP, yielded a PPV of 86% and an NPV of 59% (positive LR = 4, negative LR = 0.5) (Fig. 4a and Table 4). The 95% CDP for sIgE to Gal d 1 yielded a PPV of 94% and an NPV of 79% (positive LR = 10, negative LR = 0.3) (Fig. 4b and Table 4). Sequential use of the two tests, as shown in Fig. 4c, did not lead to a further improvement in clinical performance (Table 4).

Discussion

In this study, we report that measurement of sIgE to CM or HE components by means of a microarray in children with clinically suspected CM and HE allergy has a good ability to predict the outcome of the FCT.

There are some methodological issues that should be considered. We investigated children with a clinical history of an immediate reaction (within 2 h) to CM or HE, therefore selecting children with most likely IgE-mediated events and excluding patients with delayed reactions possibly caused by mechanisms other than IgE. This represents the ideal setting to evaluate the clinical performance of tests based on sIgE measurement. We performed FCT in an open fashion. Although double-blind, placebo-controlled FCT represents the gold standard for diagnosis of food allergy, the EAACI 2004 position paper indicates a positive open FCT as sufficient when dealing with IgE-mediated acute reactions manifesting with objective signs [5]. In this study, we excluded patients with subjective signs following FCT.

Currently, no single test allows a definitive diagnosis of food allergy. Even if some studies showed that negative

Table 3. Summary of clinically relevant assay performance of allergen microarray and ImmunoCAP in suspected hen's egg allergy

	Microarray					
	AUC	95% CDP	PPV (%)	NPV (%)	LR ⁺ (%)	LR ⁻ (%)
Allergen						
Gal d 1	0.84 (0.73–0.96)	0.86 (ISU)	88 (62–98)	80 (61–92)	10 (3–38)	0.3 (0.1–0.6)
Gal d 2	0.77 (0.63–0.91)	2.22 (ISU)	92 (61–100)	73 (56–87)	8 (2–31)	0.4 (0.3–0.8)
Gal d 3	0.61 (0.5–0.72)	5.13 (ISU)	100 (29–100)	61 (44–75)	0 (0–0)	0.8 (0.6–0.9)
Gal d 4	0.63 (0.48–0.78)	7.23 (ISU)	100 (29–100)	60 (44–75)	2 (0.9–4)	0.7 (0.4–1.1)
Gal d 5	0.55 (0.48–0.62)	/				
Sum ISAC	0.88 (0.79–0.98)	2.36 (ISU)	79 (55–94)	74 (54–89)	4 (2–11)	0.4 (0.2–0.7)
ImmunoCAP						
Egg white	0.79 (0.66–0.92)	25.27 (kU/L)	86 (42–100)	64 (47–79)	4 (1–10)	0.5 (0.3–0.9)
Egg yolk	0.81 (0.69–0.94)	11.25 (kU/L)	86 (42–100)	64 (47–79)	5 (2–15)	0.5 (0.3–0.8)

Data are shown with 95% CI in parentheses.

The units of the 95% CDP for microarray and for ImmunoCAP are shown between parenthesis. Sum ISAC shows the performance of the sum of IgE reactivities for each allergen components in the microarray for HE (Gal d 1, Gal d 2, Gal d 3 and Gal d 4).

AUC, area under the curve; CDP, clinical decision point; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio.

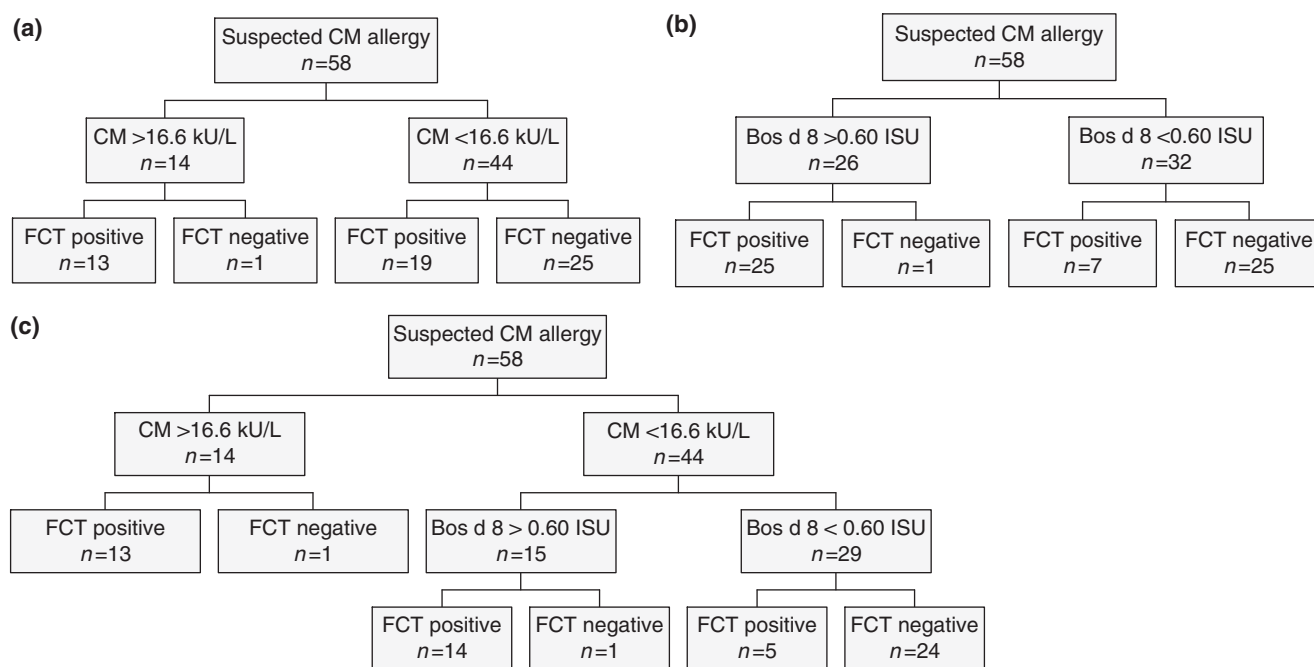


Fig. 3. Decision process regarding indication to perform the food challenge test (FCT) in patients with a suspected cow's milk (CM) allergy based on: (a) the 95% clinical decision points (CDP) of specific IgE (sIgE) to CM (ImmunoCAP) (b) on the 95% CDP of sIgE to Bos d 8 (microarray) (c) on the serial use of the 95% CDP of sIgE to CM (ImmunoCAP) and subsequently of the 95% CDP of sIgE to Bos d 8 (microarray). ISU, ISAC standardized units.

Table 4. Performance characteristics of the 95% clinical decision points (CDP) of specific IgE (sIgE) to cow's milk (ImmunoCAP), of the sIgE to Bos d 8 (microarray) and serial use of the two tests, and performance characteristics of the 95% CDP of sIgE to egg white (ImmunoCAP), of the sIgE to Gal d 1 (microarray) and the serial use of the two tests

	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)
Cow's milk (CM)				
sIgE to CM ≥ 16.6 kU/L (ImmunoCAP)	96 (80–99)	41 (24–60)	93 (66–99)	57 (41–72)
sIgE to Bos d 8 > 0.60 ISU (microarray)	96 (80–99)	78 (60–91)	96 (80–99)	78 (60–91)
Serial use of the two tests	92 (75–99)	84 (67–95)	93 (77–99)	83 (64–94)
Hen's egg				
sIgE to egg white > 25.3 kU/L (ImmunoCAP)	96 (79–99)	27 (11–50)	86 (42–99)	59 (42–74)
sIgE to Gal d 1 > 0.86 ISU (microarray)	96 (79–99)	73 (50–89)	94 (71–99)	79 (60–92)
Serial use of the two tests	92 (73–99)	73 (50–89)	89 (65–99)	79 (60–92)

PPV, positive predictive value; NPV, negative predictive value; ISU, ISAC standardized units.

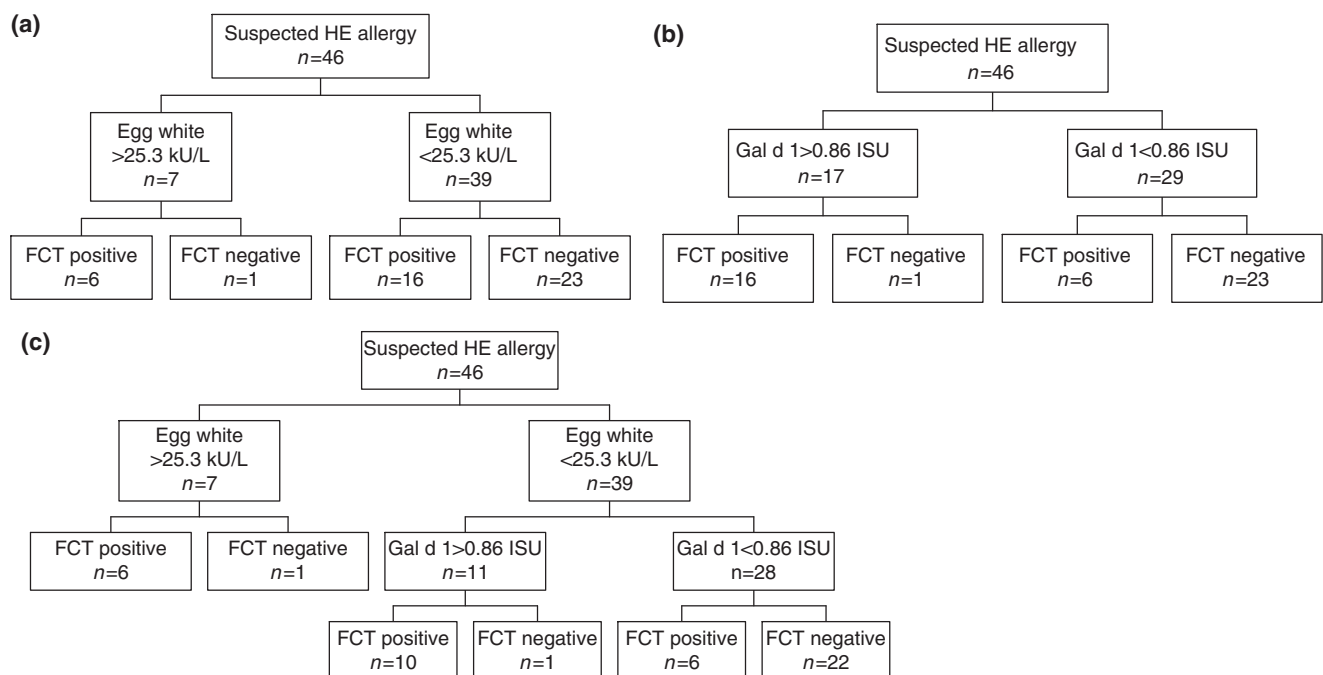


Fig. 4. Decision process regarding indication to perform the food challenge test (FCT) in patients with a suspected hen's egg (HE) allergy based on: (a) the 95% clinical decision points (CDP) of specific IgE (sIgE) to egg white (ImmunoCAP) (b) on the 95% CDP of sIgE to Gal d 1 (microarray) (c) on the serial use of the 95% CDP of sIgE to egg white (ImmunoCAP) and subsequently of the 95% CDP of sIgE to Gal d 1 (microarray). ISU, ISAC standardized units.

SPT responses have excellent NPVs for excluding the presence of IgE-mediated food allergy, SPTs are difficult to standardize because of variability in allergen preparations and modalities of administration [7, 26–28]. Moreover, although food-specific IgE levels, as measured by the most widely used test (ImmunoCAP) are predictive of clinical reactivity [8–16], their PPV and NPV are not sufficiently high.

In this study, the use of a component-based microarray showed that IgE to Bos d 8 and Gal d 1 were frequently detected in patients with positive FCT. It is known that sIgE to casein, measured with ImmunoCAP, are particularly frequent in patients with CM allergy [29–33]. Our results suggest that there is no significant advantage in

testing sIgE to casein fractions compared with total Bos d 8. The availability of recombinant caseins and the evaluation of reactivity to linear peptides [25] may help dissecting specificities and possibly provide better clinical performance. Regarding HE allergy, the observation that Gal d 1 is the most frequently recognized antigen was also expected. Ovomucoid (Gal d 1) comprises about 10% of the total egg white proteins and has been shown to be the dominant allergen [34–36]. Gal d 4 sIgE were also present in a remarkable percentage of patients, but were not associated with the outcome of FCT. Gal d 5 sIgE were present in only two patients. Gal d 5 is also present in chicken meat and indeed both patients showed FCT proven allergy to chicken meat.

In order to compare the performance of the various tests, we determined 95% CDPs. It should be pointed out that, using the ImmunoCAP System, 95% CDPs for CM and HE vary in different studies possibly due to different patients selection and different allergens used (i.e. CM vs. casein, HE vs. egg white). Nevertheless, for CM the 95% CDP was 16.6 kU/L, which is similar to that reported by Sampson (15.0 kU/L) [8]. On the contrary, the calculated 95% CDP for egg white (25.3 kU/L) was apparently higher than those reported by other studies [8–16]. It is well known that sIgE CDPs vary across age [8, 12]. The sample size does not allow an analysis in different age groups. However, after removing children with age < 2 years (11 with suspected CM allergy and six with suspected HE allergy), analysis in children with age > 2 years did not show significant differences in the performance of the microarray (data not shown).

Although the FCT still represents the diagnostic gold standard, it exposes patients to a significant risk for severe anaphylactic reactions, and moreover, has relevant costs and is time consuming. An ideal *in vitro* test should allow to avoid the FCT. In suspected CM allergy, our results suggest that the serial use of the two tests has a clinical performance very close to that of the FCT. Indeed, this two-step approach would have predicted a correct indication for a milk-free diet in 27 out of 29 children. Moreover, while the use of 95% CDP for CM sIgE, measured with ImmunoCAP, would have eliminated the need of the FCT in approximately 27% of the patients, the sequential use of the two tests (Fig. 3c) would have led to a 50% reduction in the number of FCT, and, even more notably, to a marked reduction in the number of positive FCT (5 vs. 17). In suspected HE allergy, the best performance was obtained using sIgE levels to Gal d 1, with the microarray. While use of 95% CDP for sIgE to egg white, measured with ImmunoCAP, would have eliminated the need of the FCT in approximately 15%, the use of 95% CDP for Gal d 1 sIgE (Fig. 4b) would have led to a 37% reduction, and to a reduction in positive FCT (6 vs. 16). To provide an estimate of the performance in other settings, we calculate LR, that are independent from prevalence: sIgE to Bos d 8 and Gal d 1 were associated with an increase of 20- and 10-folds, respectively, of the pre-test probability.

Our results differ from those obtained by Ott *et al.* [37], who reported the only available study on the clinical performance of a component-based microarray with respect to the outcome of the FCT in suspected CM or HE allergy. They found no advantage compared with the currently available test. The explanation of this apparent discrepancy appears to be provided by the different selection of the patients studied. The great majority (> 95%) of their patients had AE as the prevalent clinical manifestation of the suspected CM or HE allergy and exacerbation of AE was considered indicative of a positive FCT. AE results from complex mechanisms involving Th2-

dominated immune response with a Th17 component in acute skin lesions and a progressive conversion to a Th1-dominated response in chronic skin lesions [38]. Based on these considerations, we chose to include only patients in which clinical suspicion was based on a history, closely related to CM or HE consumption, of severe and/or immediate reactions and exacerbation of AE was not considered indicative of a positive FCT.

In order to provide a reasonable estimate of the pre-test probability, independently from the prevalence of allergy in our population, we calculated positive and negative LR for sIgE to Bos d 8 and Gal d 1. This resulted in an increase of 20- and 10-folds, respectively, of the pre-test probability. However, we believe that further studies on larger number of patients, in settings with different prevalence and severity of CM and HE allergy, are needed. Additional open issues to be addressed are: (a) the performance of the measurement of sIgE in the component-based microarray in children < 2 year of age; (b) predictive value of patterns of reactivity to specific CM and HE components; (c) identification of multi-step diagnostic procedures, which may include *in vitro* tests, as well as SPT; and (d) evaluation of the possible relationship between reactivity to specific CM or HE components with the dose eliciting a positive challenge.

In conclusion, in the setting of immediate CM or HE hypersensitivity, use of the 95% CDP for sIgE to Bos d 8 and Gal d 1, measured with a component-based microarray, may represent a relevant diagnostic improvement, compared with the ImmunoCAP system: it identifies a higher number of patients with true CM or HE hypersensitivity and yields a higher NPV. The possibility to identify patients with true CM or HE hypersensitivity with a low number of false positive suggests that the FCT could be eliminated from the diagnostic process of these patients. It should also be pointed out that the number of false negative remains relatively high (22% for CM and 21% for HE), suggesting that in patients with sIgE to Bos d 8 or Gal d 1 lower than the 95% CDP the FCT should still be performed. This approach would lead to a decrease in the number of the FCTs to be performed, as well as in the number of positive FCTs with a subsequent decrease in severe reaction risk. Furthermore, this would allow to significantly reduce costs related to the diagnostic process. The sequential use of the ImmunoCAP followed by the microarray does not significantly improve the PPV and NPV. However, the microarray-based technology is not yet widely available and still expensive. Therefore, in a clinical application perspective, serial use of the two tests could be envisaged based on the possibility to: (a) easily identify in first level centres patients with a very high probability of true allergy based on sIgE to CM or egg white greater than the 95% CDP with the widely available and low-cost ImmunoCAP assay, (b) screen with the microarray only the remaining patients

in second/third-level centres where the FCT is going to be performed.

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