ORIGINAL ARTICLE

Basophil Activation Test With Progressively Less Heated Forms of Egg Distinguishes Egg-Allergic From Egg-Tolerant Children

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Abstract

Background: Diagnosis of egg allergy based on basophil activation testing (BAT) has mainly been performed with an egg white extract or individual egg allergens rather than with clinically more representative whole-egg extracts. The impact of heating on the allergenicity of a whole-egg extract remains unassessed.
Objective: To validate BAT with gradually less heated whole-egg extracts in the diagnosis of egg allergy and as a marker of tolerance.
Methods: CD63-based BAT was performed with 5 progressively less heated extracts from cake, hard-boiled egg, omelet, soft-boiled egg, and raw egg in 10 egg-allergic (EA), 10 complete egg-tolerant (ET), and 12 non–egg-sensitized nonallergic (NEA) children. Cut-offs and diagnostic accuracy measures were established through receiver operating characteristic curve analysis. Changes in basophil response were assessed in 12 baked egg–tolerant children undergoing an 8-month gradual egg reintroduction protocol with BAT and oral food challenges prior to each reintroduction step.
Results: Basophil responses to all egg extracts were increased in EA children, but not in ET and NEA children. Responses decreased progressively with more heated egg extracts. Compared to ET children, EA children showed higher basophil sensitivity for all egg extracts. Negative BAT responses predicted clinical tolerance with 90%-100% sensitivity, 100% specificity, and a false positive rate of 2.78%. In comparison, the specificity of egg sIgE (<0.35 kU_A/L) was lower (50%-78%), with a false positive rate of 40%. Basophil reactivity and sensitivity tended to decrease in baked egg–tolerant children undergoing gradual egg reintroduction, concurrent with tolerance development.
Conclusion: BAT with progressively less heated egg preparations is a sensitive and highly specific tool to discriminate EA from ET children.

Key words: Basophil. Basophil activation test. Egg allergy. Baked egg tolerance. Heated egg. Pediatric. Egg sIgE.

Resumen

Antecedentes: El diagnóstico de la alergia al huevo mediante test de activación de basófilos (TAB) se ha realizado principalmente con un extracto de clara de huevo, o alérgenos de huevo individuales, en lugar de con extractos de huevo entero clínicamente más representativos. Aún no se ha evaluado el impacto del calentamiento en la alergenicidad del extracto de huevo entero.
Objetivo: Validar el TAB con extractos de huevo entero gradualmente menos calentados en el diagnóstico de la alergia al huevo y como marcador de tolerancia.
Métodos: Se realizó un TAB basado en la expresión de CD63 con cinco extractos de huevo progresivamente menos calentados (pastel, huevo duro, tortilla, huevo pasado por agua y huevo crudo) en 10 niños alérgicos al huevo (AH), 10 completamente tolerantes al huevo (TH) y 12 no alérgicos ni sensibilizados al huevo (NA). Se establecieron puntos de corte y medidas de precisión diagnóstica mediante análisis *Receiver Operating Characteristic* (ROC). Se evaluaron los cambios en el TAB en 12 niños que toleraban el huevo horneado sometidos a un protocolo de reintroducción gradual del huevo durante 8 meses con TAB y provocaciones orales previos a cada paso de la reintroducción.
Resultados: Las respuestas de basófilos a todos los extractos de huevo estaban aumentadas en los niños AH, pero no en los TH y NA. Las respuestas disminuyeron progresivamente con extractos de huevo más calentados. En comparación con los niños TH, los niños AH mostraron una mayor sensibilidad de los basófilos a todos los extractos de huevo. El TAB negativo predijo tolerancia clínica con una sensibilidad del 90-100%, una especificidad del 100% y una tasa de falsos positivos del 2,78%. En comparación, la IgE específica a huevo <0,35 kU_A/L tuvo una especificidad inferior del 50-78% con una tasa de falsos positivos del 40%. La reactividad y la sensibilidad de los basófilos tendieron a disminuir en los niños sometidos a la reintroducción gradual de huevo, en paralelo al desarrollo de tolerancia.
Conclusión: El TAB con preparados de huevo progresivamente menos calentados es una herramienta sensible y altamente específica para discriminar a los niños alérgicos a huevo de los tolerantes.

Palabras clave: Basófilo. Test de activación de basófilos. Alergia a huevo. Tolerancia a huevo horneado. Huevo calentado. Pediátrico. IgE específica a huevo.

Summary box

- **What do we know about this topic?**
  Diagnosis of egg allergy using basophil activation testing (BAT) has mainly been performed with an egg white extract or individual egg allergens rather than with clinically more representative whole-egg extracts. The impact of heating on the allergenicity of a whole-egg extract remains unassessed.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  BAT with gradually less heated whole-egg extracts discriminates egg-allergic from egg-tolerant children, with superior specificity compared to egg sIgE <0.35 kU_A/L. Progressive heating reduces the ability of whole-egg extracts to induce basophil activation in vitro.

Introduction

Diagnosis of childhood egg allergy is currently established based on the medical history and a first-line diagnostic test including measurement of serum specific IgE (sIgE) or skin prick testing (SPT) [1,2]. The specificity of these first-line tests can be suboptimal, as they often reflect irrelevant sensitization to hen's egg rather than clinical hen's egg allergy [1,2]. In a 2014 meta-analysis, egg sIgE levels ≥0.35 kU_A/L predicted egg reactivity with a mean specificity of 49% and sensitivity of 93% [3]. Increasing cut-off levels offered increased specificity at the cost of sensitivity [4]. Consequently, an in-hospital oral food challenge (OFC) is the gold standard for confirming the diagnosis of egg allergy or monitoring for resolution of allergy [5,6]. However, this procedure requires an experienced clinical team and well-equipped facilities, as life-threatening allergic reactions during OFCs have been described [5,6]. In this regard, basophil activation testing (BAT) has emerged as an alternative noninvasive ex vivo assay for IgE-mediated hypersensitivity that can be used to diagnose food allergy and monitor the development of natural or immunotherapy-induced tolerance [7-9].

The majority of studies that have assessed the performance of BAT to diagnose egg allergy or monitor for resolution used an egg white extract or the individual egg allergens, ovalbumin and ovomucoid, for basophil stimulation [10-12]. An ovalbumin-based BAT could diagnose egg allergy with 100% specificity and 77% sensitivity [10]. In another study, basophil reactivity following stimulation with egg white extract discriminated 2 clinical egg allergy phenotypes, in which baked egg reactive children had a higher percentage of CD63-positive (%$CD63^+$) basophils to egg white compared to baked egg–tolerant (BET) children [13]. Several studies evaluating BAT during egg oral immunotherapy also reported a decreased %$CD63^+$ basophils to pasteurized whole egg, egg white, ovalbumin, or ovomucoid at the end of the treatment [14-19]. However, few if any studies have examined BAT with clinically representative whole-egg extracts, and no studies have evaluated BAT as a noninvasive predictor of clinical tolerance during the gradual reintroduction of egg using an egg ladder [14,18].

In a recent study, we demonstrated the safe induction of tolerance to raw egg in a BET cohort through progressive introduction of less heated egg products over a 24-month period [20]. Having characterized extracts from these egg products (cake, hard-boiled egg, omelet, soft-boiled egg, and raw egg), we first aimed to investigate the diagnostic performance of BAT with these extracts in discriminating between true egg-allergic and egg-tolerant children with positive SPT or sIgE results for egg or egg components. Secondly, we assessed the evolution and predictive value of basophil responses in BET children undergoing a shortened 8-month gradual egg reintroduction protocol.

Methods

Basophil Activation Testing

BAT was performed on fresh heparinized whole blood samples (see Supplementary Material for full protocol). In brief, samples were stimulated for 20 minutes at 37°C with stimuli dissolved in an IL-3–containing buffer (final concentration, 9 ng/mL). Stimuli included 10-fold serial dilutions of 5 progressively less heated whole-egg extracts (0.1-100 µg/mL) including cake (35 minutes at 165°C), hard-boiled egg (10 minutes at 100°C), omelet (4 minutes at 120°C), soft-boiled egg (5 minutes at 100°C), and raw egg. Extracts were prepared as previously described and characterized in our previously published work (Supplementary Material) [20]. Mono- and polyclonal antihuman IgE (aIgE, 5 µg/mL), formyl-methionyl-leucyl-phenylalanine (fMLP, 2 µM) or buffer alone were used as positive and negative controls, respectively. Stimulation was halted on ice followed by staining with anti-CD123 PE, anti-HLA-DR AF647, and anti-CD63 FITC. After erythrocyte lysis, a minimum of 500 basophils (SSC^{low}/$CD123^+$/$HLA\text{-}DR^-$) was acquired on the LSR Fortessa flow cytometer running DIVA software and analyzed with FlowJo 10.8.1. Basophil activation was measured as %$CD63^+$ basophils, corrected for spontaneous CD63-expression by subtracting the %$CD63^+$ basophils in the unstimulated control condition. Children with <5% $CD63^+$ basophils to mono- and polyclonal aIgE were classified as nonreleasers, henceforth termed nonresponders [9].

Diagnostic Cohort

To explore the discriminative capacity of BAT between true egg allergy and tolerance, we recruited 10 egg-allergic (EA), 10 egg-sensitized but tolerant (ET), and 12 non–egg-sensitized nonallergic (NEA) children from the Pediatrics Department

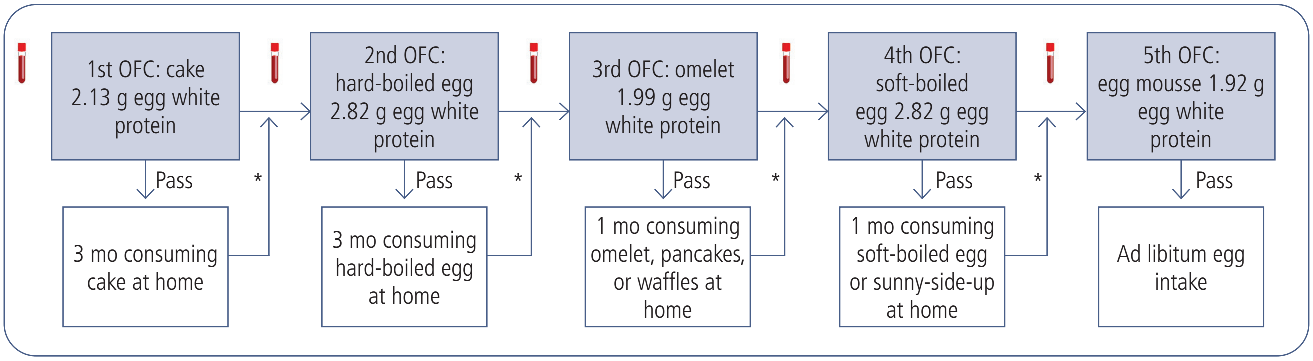

Figure 1. Eight-month gradual egg-introduction protocol. Blood samples were drawn prior to and 1 hour after each OFC to evaluate egg sIgE, tryptase level, and complement activation. During the home introduction, age-appropriate portions were incorporated into the child's diet 2-3 times per week. Parents monitored the frequency of consumption, allergic reactions, medication, and illness using a food diary. The child could proceed to the next OFC if no allergic reactions occurred during the introduction of the previous egg preparation at home (*). Adverse allergic events were categorized according to the CoFAR grading scale for allergic reactions (version 3.0) [32]. The amount of egg white protein indicated equals the cumulative dose administered during the OFC (Table S1). OFC indicates oral food challenge.

of UZ Leuven. Egg allergy was defined as a clinical type I hypersensitivity response to egg, along with a positive egg SPT (wheal >3 mm) or egg sIgE ≥ 0.35 kU_A/L (ImmunoCAP, lower limit of quantification of 0.10 kU_A/L). Children with a prior history of egg allergy who ingested foods containing raw egg without developing symptoms at the time of inclusion, independent of their egg sIgE levels, were considered complete egg-tolerant.

Gradual Egg Reintroduction Cohort (Pre-TETI-II Study)

Changes in BAT outcome were evaluated in an ongoing pilot study (pre-TETI-II) involving 12 BET children (L1-L12) who consecutively reintroduced cake, hard-boiled egg, omelet, soft-boiled egg, and raw egg over a period of 8 months [20] (Figure 1). This 8-month time period was supported by findings from our previous study, as several children progressed through the step-wise protocol at an accelerated pace (parental decision) and safely developed raw egg tolerance within 5-12 months [20]. Included children had proven egg allergy, along with an ovomucoid sIgE predicting at least a 75% chance of passing a baked egg OFC, but were still supposed to react to less heated egg products [21,22]. Prior to each reintroduction step, children underwent an in-hospital OFC with the corresponding egg preparation during which an additional blood sample was collected for BAT with all 5 egg extracts (Table S1). If OFCs were tolerated, cake and hard-boiled egg were further introduced for 3 months at home followed by a 1-month introduction of omelet and soft-boiled egg. Both studies were approved by the Ethics Committee Research UZ/KU Leuven, and written informed consent was obtained from parents with the accompanying assent of the child from the age of 6 years onwards.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.9.2.0 for Windows. Normality was determined using the D'Agostino and Pearson test. Continuous variables are reported as median (IQR) or mean (95%CI) and compared between groups using the *t* test or mixed-model analysis when appropriate. Cubic spline and nonlinear regression analyses were used to model the basophil CD63 dose-response for each extract. Receiver operator characteristic (ROC) curves were constructed to compare the area under the curve (AUC) for each extract concentration and to determine optimal cut-offs for BAT positivity based on optimal sensitivity and specificity values. Concentrations eliciting half-maximal basophil activation (EC_{50}) were derived from best-fit dose-response curves for each extract. Allergen threshold sensitivity (CD-sens) was calculated using the formula $1/EC_{50} \times 100$ [9]. Correlations between egg sIgE levels and BAT responses were evaluated using the Spearman or Pearson rank correlation test where appropriate. BAT nonresponders were excluded from the statistical analysis of all BAT data. A *P* value below .05 was considered statistically significant.

Results

Discriminative Capacity of the BAT Between Egg Allergy and Tolerance

Among the 10 EA, 10 ET, and 12 NEA children, 2 (6.3%) had nonresponder basophils to aIgE and were excluded from further analysis. The characteristics of ET and EA children are depicted in Table 1. Basophils of EA children showed high CD63-expression when stimulated with the 5 egg extracts, with the mean %$CD63^+$ basophils increasing in response to progressively less heated forms of egg (ie, increasingly allergenic) (Figure 2, Table S2). In contrast, the basophils of ET and NEA children showed low CD63 expression upon stimulation with these 5 egg extracts, with no significant difference between the groups.

Basophil dose-responses of EA children differed across the 5 extracts, with cake inducing a progressive increase in CD63 expression up to the maximum concentration of 100 µg/mL, whereas reactivity to hard-boiled egg reached a plateau at 0.10 µg/mL. Omelet, soft-boiled egg, and raw egg induced bell-shaped dose-responses, with decreasing CD63-expression from 1 µg/mL onwards (Figure 2). Compared to cake, the %$CD63^+$

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Table 1. Characteristics of the Egg-Allergic and Complete Egg-Tolerant Children at the Time of Performing the Basophil Activation Test.

	Age, y	Sex	Initial egg allergic reaction[a]	Total IgE, kU/L	IgE EW, kU_A/L	IgE EY, kU_A/L	IgE OVM, kU_A/L	IgE OVA, kU_A/L
EA 1	4	Female	Grade III	703	12.1	5.89	7.96	8.96
EA 2	2	Male	Grade I	864	3.02	0.90	2.04	1.00
EA 3	3	Male	Grade I	297	4.02	1.19	3.8	1.02
EA 4	4	Male	Grade I	939	14.9	17.2	4.44	5.31
EA 5+	<1	Female	Grade II	256	16.3	4.77	8.42	12.3
EA 6	2	Female	Grade I	1467	32	5.89	39.4	6.03
EA 7	2	Female	Grade II	187	13	1.7	20.8	2.31
EA 8	3	Male	Grade I	280	29	6.86	29.6	12.4
EA 9	1	Female	Grade I	19	1.15	0.32	1.08	0.62
EA 10	6	Male	Grade I	2018	5.43	2.16	4.46	3.31
Median (IQR)	2.5 (1.75-4)			500 (238.8-1071)	12.55 (3.77-19.48)	4.77 (1.7-5.89)	6.21 (3.36-23)	5.31 (1.67-10.63)
ET 1	8	Male	Grade I	278	0.82	0.1	0.64	0.1
ET 2	2	Male	Grade I	23	0.77	0.24	0.1	1
ET 3[b]	5	Female	Grade I	105	0.23	0.1	0.33	0.1
ET 4	2	Male	Grade I	8	0.1	0.1	0.1	0.1
ET 5	10	Male	Grade I	2814	1.2	0.91	0.49	1.36
ET 6	8	Male	Grade I	2133	0.16	0.12	0.16	0.13
ET 7	8	Male	Grade I	1232	0.4	0.13	0.52	0.15
ET 8	16	Female	Grade II	120	0.11	-	0.1	0.12
ET 9	4	Female	Grade I	51	0.32	0.1	0.25	-
ET 10	5	Male	Grade I	58	6.13	1.66	0.68	3.62
Median (IQR)	6.5 (3.5-8.5)			112.5 (44-1457)	0.36 (0.15-0.92)	0.10 (0.1-0.91)	0.25 (0.10-0.51)	0.11 (0.10-1.66)

Abbreviations: EA, egg-allergic; ET, completely egg-tolerant; EW, egg white; EY, egg yolk; OVM, ovomucoid; OVA, ovalbumin.
[a]Based on medical record, according to the CoFAR grading scale for allergic reactions (version 3.0) [32].
[b]Nonresponder to anti-IgE.

basophils was significantly higher in EA children when stimulated with 0.1 µg/mL of hard-boiled egg, omelet, and soft-boiled egg and 1 µg/mL of omelet and raw egg (Figure S1). CD63 expression differed significantly between EA and ET children across all concentrations for all tested extracts with the exception of 0.1 µg/mL of cake, which could not distinguish EA from ET children (Figure S2). The AUC of the BAT with each egg extract also differed significantly between EA and ET children (Figure S3). Accordingly, EA children showed higher basophil sensitivity for all egg extracts, as expressed by a lower EC_{50} compared to ET children (Figure 2).

Optimal %$CD63^+$ cut-off values for discrimination between EA and ET children were calculated for all concentrations tested (0.1-100 µg/mL) and all 5 egg extracts using ROC analysis (Table 2). The area under the ROC curve (AUROC) ranged between 0.98 and 1, indicating excellent discriminative capacity between both groups. Using these cut-offs, BAT sensitivity in discriminating EA from ET children ranged from 90% to 100%, with a specificity of 100% and false positive rate (FPR) of 2.78% (data not shown). Only the 0.1-µg/mL cake extract had a lower AUROC of 0.65, corresponding to 66.67% sensitivity and 55.56% specificity. When these findings were compared with the diagnostic accuracy of egg sIgE levels using the classic cut-off of 0.35 kU_A/L, the AUROC ranged from 0.89 to 1, with corresponding sensitivities between 90%-100% and specificities between 50%-78% (Table S3). Ovomucoid sIgE had the highest AUROC value (1.00), which could discriminate EA from ET children with 100% sensitivity and 60% specificity, resulting in an FPR of 40%. Alternatively, using the lower limit of detection of 0.10 kU/L for egg sIgE levels resulted in an even lower specificity between 20% and 44% (Table S3). Next, we sought to improve the diagnostic accuracy of egg sIgE levels by calculating optimal cut-offs for each sIgE based on the ROC analysis (Table S3). When these cut-offs were applied in our cohort, higher specificities, ranging from 80% to 100%, were achieved with minimal reduction in sensitivity. Additionally, egg white and ovalbumin sIgE levels correlated positively with the BAT dose-response AUC and

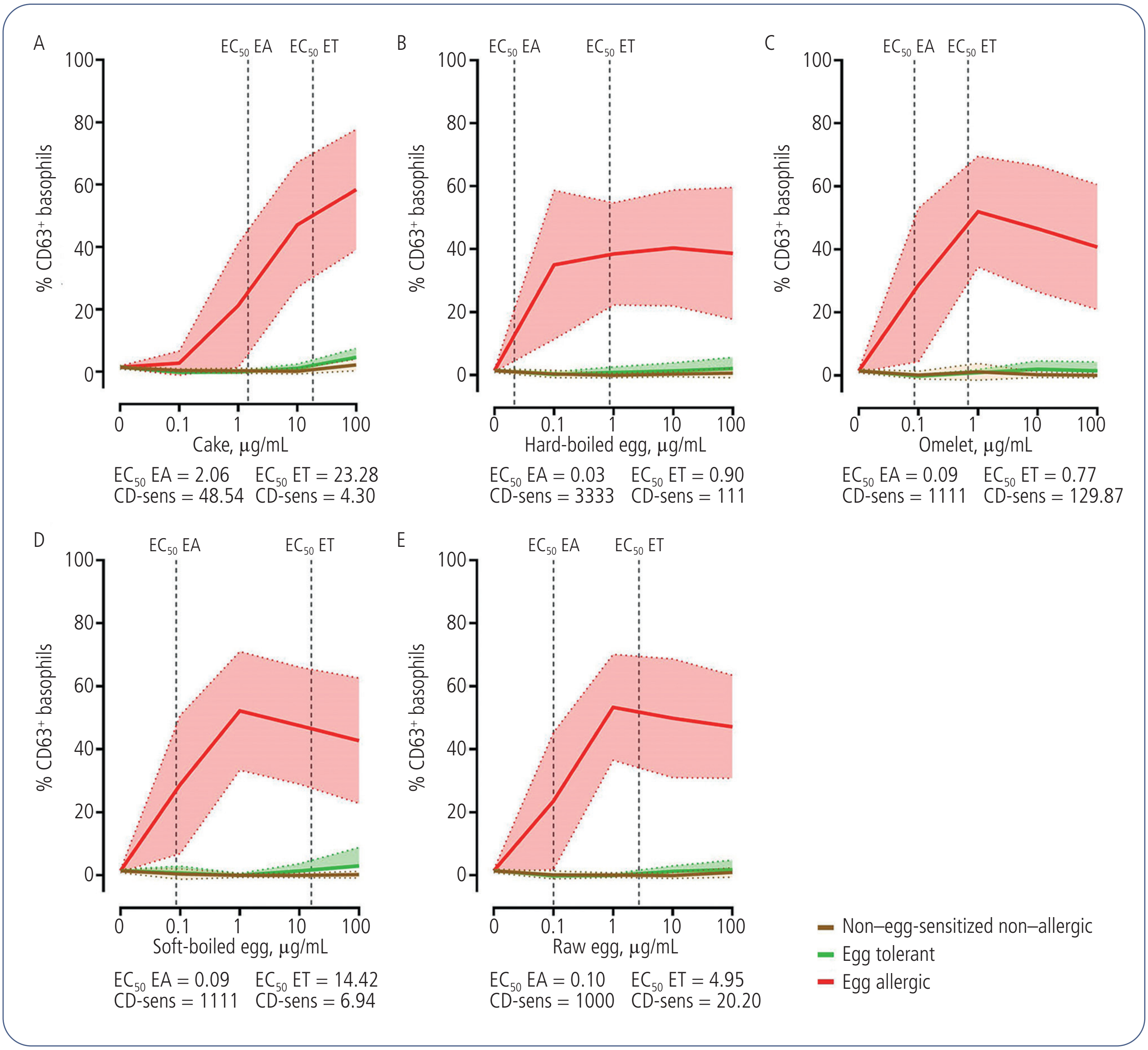

Figure 2. Cubic spline regression analysis of the basophil CD63 dose-response to increasing concentrations of cake (A), hard-boiled egg (B), omelet (C), soft-boiled egg (D), and raw egg (E) in egg-allergic (EA, red), complete egg-tolerant (ET, green), and non–egg-sensitized nonallergic (NEA, brown) children. The regression curves are shown in bold and represent the mean %CD63+ basophils for each group. The shaded area represents the 95% confidence interval. EC_{50} values were obtained from the nonlinear regression analysis for EA and ET children.

maximal %CD63+ basophils in response to cake, hard-boiled egg, and omelet (Table S4). Ovomucoid sIgE levels also correlated positively with the AUC of cake (r=0.51, *P*=.03).

Outcome of Gradual Egg Reintroduction and Comparison of OFC and BAT

Out of the 12 included BET children, 9 successfully passed all 5 OFCs and subsequent home-based introduction without major symptoms (Table S5). Two children stopped the study earlier owing to an allergic reaction during the hard-boiled egg OFC (L8: grade I) or the soft-boiled egg OFC (L2: grade II). No clinically significant changes in blood pressure, tryptase, or complement components were observed during the OFCs in any of the children (Table S6). Patients L9 and L11 had elevated tryptase levels before the OFCs, which was most likely attributable to hereditary α-tryptasemia given the absence of signs of primary mast cell disease. However, genetic testing was not available at that time. Based on current evidence, no interaction with food allergy or basophil responses was expected [24]. One child (L3) discontinued the study owing to an itchy tongue when eating runny egg yolk at home, despite passing the soft-boiled egg OFC. The clinical features of the study population are provided in Table 3.

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To evaluate whether BAT could predict OFC-associated symptoms, we applied the previously defined %CD63+ cut-off values at 10 µg/mL of each extract, which had the highest sensitivity and specificity, as well as the lowest workload (fewer dilutions), thus decreasing the risk of errors by manipulation (Table 2). Table 4 shows BAT responses to each extract with the outcome of the corresponding OFC. Three children (L8, 10, 11) were nonresponders at baseline, with 1 child (L11)

Table 2. Optimal Cut-off Values for %CD63+ Basophils to the 5 Egg extracts With the Largest Area Under the ROC Curve.

Extract	Concentration	AUROC	Cut-off, %CD63+ basophils	Sensitivity, % (95%CI)	Specificity, % (95%CI)
Cake	100 µg/mL	1 (1-1)	14.40	100 (84.54-100)	100 (70.09-100)
	10 µg/mL	1 (1-1)	12.14[a]	100 (84.54-100)	100 (70.09-100)
	1 µg/mL	1 (1-1)	2.14	100 (84.54-100)	100 (70.09-100)
	0.1 µg/mL	0.65 (0.41-0.89)	0.52	66.67 (45.37-82.81)	55.56 (26.67-81.12)
Hard-boiled egg	100 µg/mL	0.98 (0.96-1)	7.27	95.24 (77.33-99.76)	100 (70.09-100)
	10 µg/mL	1 (1-1)	10.75[a]	100 (84.54-100)	100 (70.09-100)
	1 µg/mL	1 (1-1)	9.59	100 (84.54-100)	100 (70.09-100)
	0.1 µg/mL	1 (1-1)	4.09	100 (84.54-100)	100 (70.09-100)
Omelet	100 µg/mL	1 (1-1)	13.94	100 (84.54-100)	100 (70.09-100)
	10 µg/mL	1 (1-1)	13.94[a]	100 (84.54-100)	100 (70.09-100)
	1 µg/mL	1 (1-1)	14.26	100 (84.54-100)	100 (70.09-100)
	0.1 µg/mL	1 (1-1)	3.27	100 (84.54-100)	100 (70.09-100)
Soft-boiled egg	100 µg/mL	0.99 (0.96-1)	10.21	95.24 (77.33-99.76)	100 (70.09-100)
	10 µg/mL	1 (1-1)	13.88[a]	100 (84.54-100)	100 (70.09-100)
	1 µg/mL	1 (1-1)	10.27	100 (84.54-100)	100 (70.09-100)
	0.1 µg/mL	0.98 (0.94-1)	2.30	90.48 (71.09-98.31)	100 (70.09-100)
Raw egg	100 µg/mL	1 (1-1)	16.44	100 (84.54-100)	100 (70.09-100)
	10 µg/mL	1 (1-1)	11.68[a]	100 (84.54-100)	100 (70.09-100)
	1 µg/mL	1 (1-1)	11.93	100 (84.54-100)	100 (70.09-100)
	0.1 µg/mL	0.99 (0.98-1)	1.74	95.24 (77.33-99.76)	100 (70.09-100)

Abbreviation: AUROC, area under the receiver operating characteristic curve.
[a]The concentration and cut-off were those applied in the pre-TETI-II study.

Table 3. Baseline Demographic and Clinical Characteristics of Patients Included in the Pre-TETI-II Study.

	Age, y	Sex	Atopy	Initial egg allergic reaction[a]	Total IgE, kU/L	IgE EW, kU_A/L	IgE EY, kU_A/L	IgE OVM, kU_A/L	IgE OVA, kU_A/L
L1	7	Male	AE, AA, AR	Grade I	487	0.48	0.12	0.52	0.12
L2	9	Male	AE, AA, AR	Grade III	2961	5.39	4.17	0.68	8.06
L3	4	Male	AE	Grade III	96	0.24	0.1	0.1	0.32
L4	2	Female	AE	Grade I	207	0.52	0.17	0.1	0.56
L5	2	Female	AE	Grade III	686	0.24	0.12	0.1	0.26
L6	2	Male	AE	Grade I	369	0.89	0.56	0.1	1.11
L7	2	Male	AE	Grade I	64	2	1.18	0.1	1.13
L8	10	Male	AE, AA	Grade III	576	4.01	1.54	0.8	3.77
L9	2	Female	AE	Grade III	929	0.24	0.2	0.1	0.22
L10	8	Female	AE, AA, AR	Grade III	509	1.25	0.53	1.15	0.64
L11	4	Male	N/A	Grade III	244	1.44	1.3	0.1	2.02
L12	4	Male	AE, AR	Grade I	1511	2.74	1.45	2.19	1.69
Median (IQR)	4 (2-7.75)				498 (216.3- 868.3)	1.070 (0.30-2.56)	0.55 (0.13-1.41)	0.10 (0.10-0.77)	0.88 (0.28-1.94)

Abbreviations: AA, allergic asthma; AE, atopic eczema; AR, allergic rhinitis; EW, egg white; EY, egg yolk; NA, not applicable; OVA, ovalbumin; OVM, ovomucoid.
[a]Based on medical records, according to the CoFAR grading scale for allergic reactions (version 3.0) [32].

Table 4. Basophil Response (%CD63+ basophils) to 10 µg/mL of the 5 Egg Extracts Compared With Their Corresponding Oral Food Challenge During the Pre-TETI-II Study.[a]

	Cake V1	Hard-boiled egg V2	Omelet V3	Soft-boiled egg V4	Raw egg V5
L1			6.21		3.75
L2		37.96			
L3		1.11		2.57	1.34
L4	0.11	5.64	6.26	3.76	0.09
L5		0	0	0	0.25
L6	7.14	1.15	2.9	6.16	0
L7	12.96	13.96	22.76	14.91	5.78
L8	9.49	0.61			
L9	0.09	0.24	0	0.51	0
L10	0	1.36	0.85	1.92	0
L11	1.33	0	2.45	9.94	5.29
L12		3.21		10.5	10.81
Cut-off	12.14	10.75	13.94	13.88	11.68

Abbreviation: V, visit.
[a]Red-bordered cells: children who experienced an allergic reaction during an oral food challenge. Orange cells: nonresponder basophils (L11 became a responder at V3). Green cells: concordant basophil activation test and oral food challenge results. Blue cells: %CD63+ basophils above previously defined optimal cut-off. White cells: the basophil activation test was not performed owing to practical difficulties, or results were not available owing to a technical error (L5 cake V1).

becoming a responder at visit 3. Overall, out of 45 study visits with available results for concurrent BAT and OFC, 9 (20%) were uninterpretable owing to nonresponding basophils. Of the remaining 36 informative BATs, 31 (86.1%) were concordant with the outcome of the OFC, whereas 5 (13.8%) were false positives.

Evolution of BAT Responses During Tolerance Development

No significant changes were noted in the %CD63+ basophils to aIgE or fMLP at the end of the study compared with baseline, including those with nonresponding basophils (Figure S4). Overall, we observed a decreasing trend in basophil responses to all 5 egg extracts between the first and last study visit, although these differences did not reach statistical significance (Figure 3, Figure S5). This decrease in the %CD63+ basophils was most pronounced in the BAT with omelet followed by soft-boiled egg, hard-boiled egg, raw egg, and cake, respectively (Figure 4). Additionally, basophil sensitivity decreased for all egg extracts over the course of the graduated protocol, as expressed by an increasing EC_{50} from the first to the last study visit (Figure 3). This increase in EC_{50} was most pronounced for raw egg, while only a small difference was found for cake. No significant changes were noted in the egg sIgE and total IgE levels from baseline to the end of the study (Figure S6). We found a positive correlation between egg white and ovalbumin sIgE levels and the dose-response AUC and maximal %CD63+ basophils to cake at the first study visit. Similarly, at the final study visit, the dose-response AUC of the BAT with soft-boiled egg correlated positively with the egg white sIgE levels (r=0.69, P=.04, Table S7).

Discussion

In this study, BAT was evaluated as a diagnostic tool for egg allergy using progressively less heated forms of egg. We demonstrated that our BAT protocol can discriminate clinically relevant from irrelevant IgE sensitization in egg-allergic versus complete egg-tolerant children, with a lower FPR and superior specificity to egg sIgE levels applying the classic 0.35 kU_A/L cut-off.

Several studies to date have evaluated the diagnostic accuracy of BAT to egg white or native egg proteins such as ovalbumin and ovomucoid [10-12]. Our study is the first to validate BAT with whole-egg extracts prepared under different heating conditions, which more closely approximate egg exposure in daily life. In our previous study, we characterized these extracts and demonstrated that heating led to the disappearance of ovalbumin due to formation of insoluble aggregates [20]. Recently, Claude et al [25] showed that heat-aggregated ovalbumin had a lower basophil degranulation ability than native ovalbumin. In our group of EA children, basophil reactivity was also higher to raw egg containing native ovalbumin and decreased in response to variously heated forms of egg containing heat-aggregated ovalbumin. Additionally, basophil responses to cake were significantly lower compared to several less heated egg extracts at 0.1 and 1 µg/mL. However, no significant differences were found between basophil responses to hard-boiled egg, omelet, soft-boiled egg, and raw egg, despite the difference in thermal processing and, consequently, allergenicity. This could indicate that these EA children were not close to acquiring tolerance to these less heated

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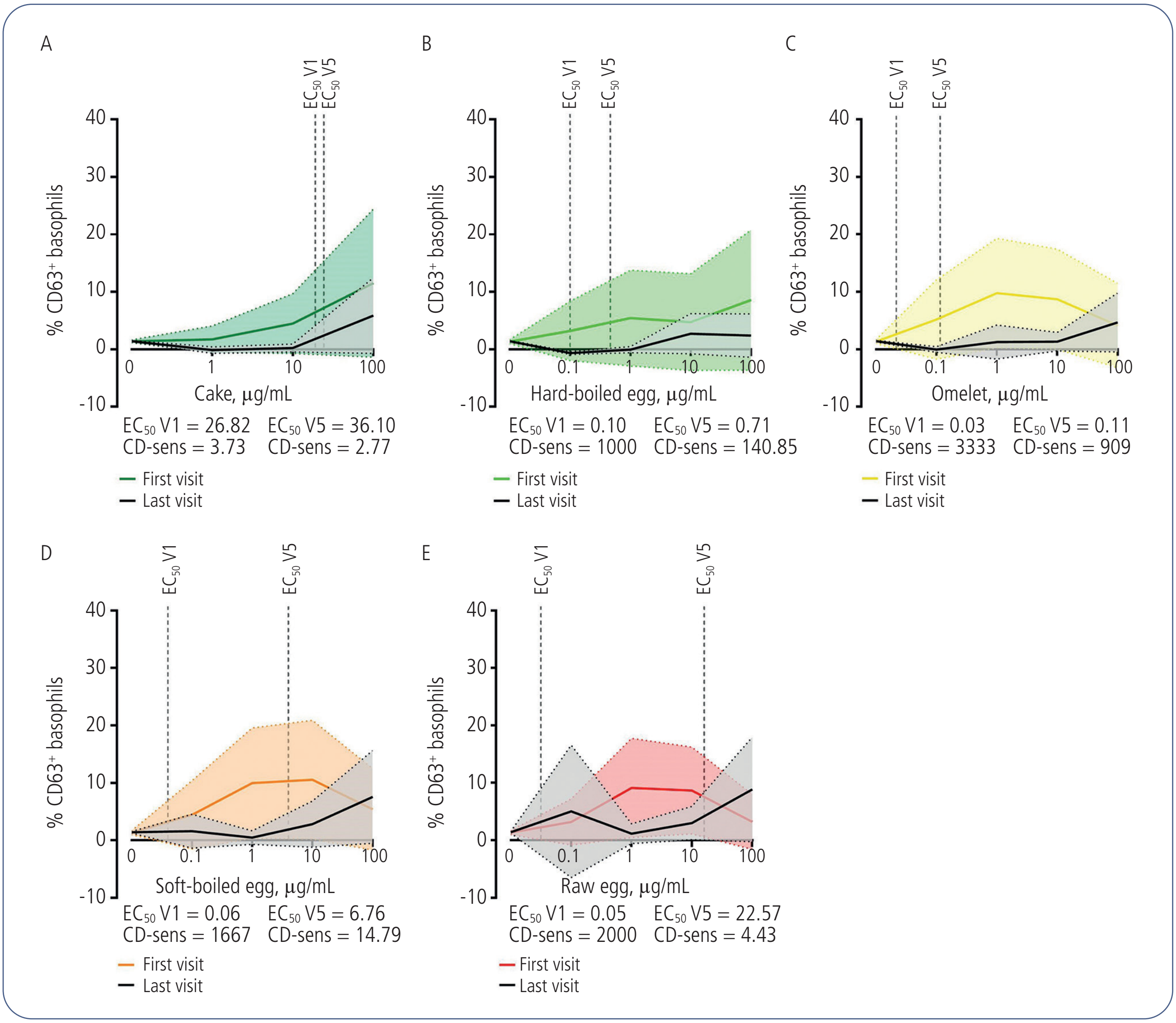

Figure 3. Cubic spline regression analysis of basophil CD63 dose-response to increasing concentrations of cake (A), hard-boiled egg (B), omelet (C), soft-boiled egg (D), and raw egg (E) during the first (V1) and last (V5) study visits of the pre-TETI-II study. Data from all participating children were included, excluding nonresponders (n=2). The regression curves are shown in bold and represent the mean %CD63+ basophils for each group. The shaded area represents the 95%CI. EC_{50} values were obtained from the nonlinear regression analysis for V1 and V5.

egg forms. Additionally, basophil reactivity at the maximal concentration of cake was higher than the degranulation observed at similar concentrations of more allergenic forms of egg. We hypothesize that at higher concentrations of cake, digestion becomes a more important factor in this extract-based set-up. Indeed, wheat further decreases the allergenicity of egg proteins by hampering their accessibility to digestion in vivo, which was not accounted for in our experimental set-up [26].

In comparison to the classic sIgE cut-off of 0.35 kU_A/L, BAT predicted egg reactivity with a superior specificity and lower FPR. Lowering the cut-off to 0.10 kU_A/L when analyzing the results offered an even lower specificity than 0.35 kU_A/L, although the clinical relevance of sIgE levels between 0.10 and 0.35 kU_A/L still remains a matter of debate for certain allergens, such as hen's egg [27]. However, when optimal cut-offs were selected from the ROC curve equivalent specificities to BAT were achieved with minimal loss in sensitivity. This shows that clinical decision points for the egg sIgE levels depend largely on the study population, with age, atopic comorbidities, and severity of the allergic reaction being influencing factors [28]. Ultimately, this demonstrates that a uniform cut-off of 0.35 kU_A/L has limited clinical relevance, although it remains widely applied in clinical practice. The optimal cut-offs for egg sIgE levels and BAT in this study should therefore also be validated in a larger cohort of egg-allergic and egg-tolerant children.

In the pre-TETI-II study, 9 out of 12 BET children developed complete raw egg tolerance within approximately 8 months, while 3 children experienced adverse events leading

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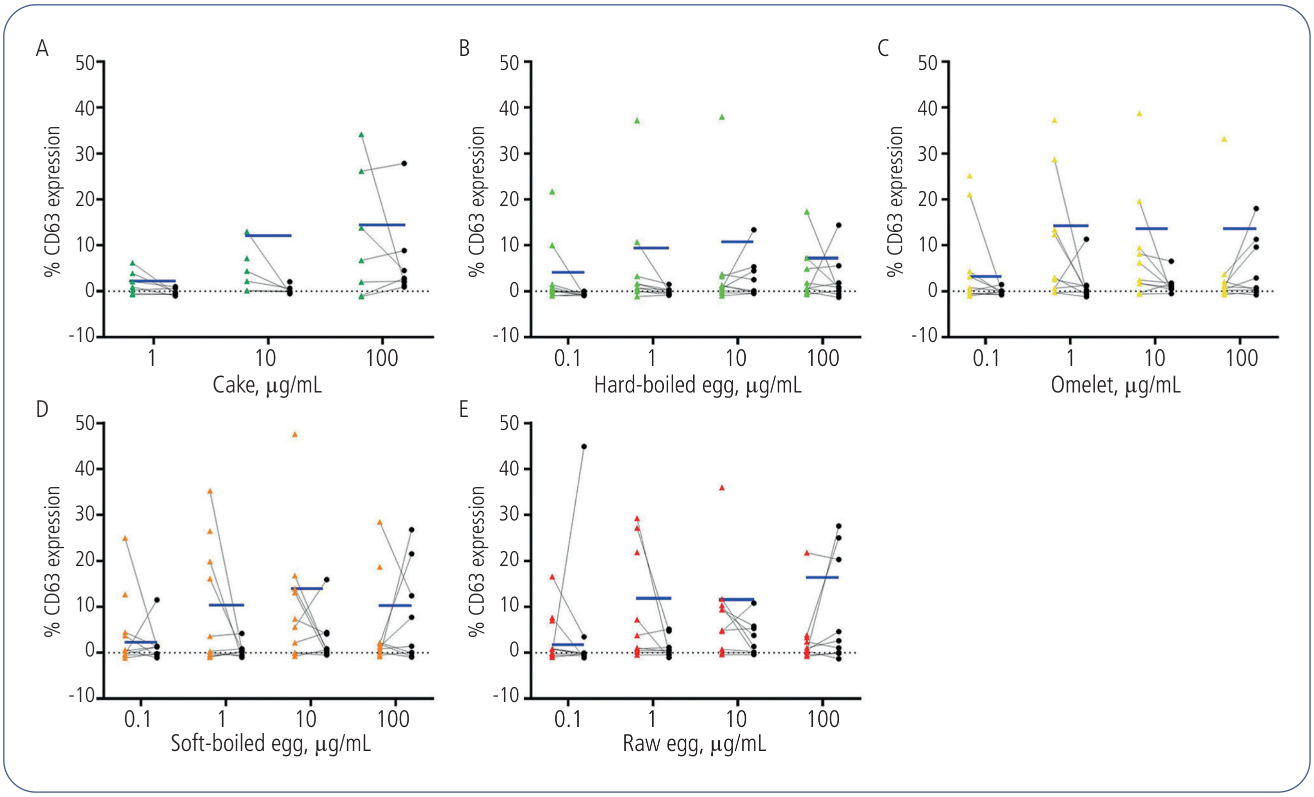

Figure 4. Basophil CD63 dose-response to cake (A), hard-boiled egg (B), omelet (C), soft-boiled egg (D), and raw egg (E) during the first and last study visits of the pre-TETI-II study (paired data). Data from all participating children were included, excluding nonresponders (n=2). The blue line represents the defined cut-off for each concentration (Table 2)..

to early withdrawal. Two allergic reactions took place during the hard-boiled and soft-boiled egg OFC, and 1 child reacted during home-based introduction of soft-boiled egg despite passing the corresponding OFC. Overall, BAT and OFC outcomes were concordant in over 86% of informative cases. Nevertheless, the small number of children experiencing allergic reactions during OFCs, and the absence of interpretable concurrent BAT results for the few in-hospital reactors preclude us from drawing a definitive conclusion on the ability of BAT to predict OFC-associated symptoms. It must be noted that 2 children passed OFCs, while BAT with the corresponding egg extract was positive. We cannot rule out that these children might have reacted during the OFC if a higher dose had been administered (eg, 4.4 g of egg white protein as per EAACI guidelines vs 2.82 g [Figure 1]) [29]. Indeed, in patient L2, the positive BAT at visit 2 might have been an early indication of decreased tolerance, which predisposed for the positive OFC at visit 4.

During the course of our gradual reintroduction protocol, basophil reactivity and sensitivity tended to decrease to all 5 egg preparations, although these differences did not reach statistical significance. Previous egg oral immunotherapy studies showed significant decreases in CD63-expression to egg (white), ovalbumin, or ovomucoid along with clinical evidence of tolerance development at the end of the treatment [14-19]. Possible explanations for our lack of significant difference in basophil reactivity include the small sample size, the relatively high percentage of nonresponders and the short duration of the gradual protocol. Indeed, studies have shown that basophil reactivity can be influenced by the duration of immunotherapy, as well as the dose of the food allergen [30]. The higher proportion of nonresponders in our egg reintroduction cohort (20%, 9/45 visits) and diagnostic cohort (6%, 2/32 children), compared to the 15% reported in literature, was likely due to coincidence given the limited sample size [9]. Additionally, we included BET children with a transient egg allergy phenotype who already had lower basophil reactivity to the 5 whole-egg extracts at baseline compared to EA children, despite only tolerating baked egg. However, these EA children were still far from acquiring baked egg tolerance. Similarly, Kim et al [17] found no significant decrease in %$CD63^+$ basophils to egg white after treatment with muffin over a 2-year period.

Clearly, validation of BAT as a marker of tolerance induction would require a larger cohort of BET children undergoing gradual reintroduction over a longer period. To this end, we are currently studying the evolution of the CD63-based BAT with gradually less heated egg preparations in a larger multicenter cohort of BET children undergoing a 12- or 20-month gradual egg-introducing protocol (TETI-II study, NCT04677790). A limitation of our study was reliance on a CD63/IL-3 based protocol with omission of CD203c as an additional activation marker, since IL-3 upregulates CD203c in an allergen-independent manner, limiting its interpretability [9,30,31]. Lastly, we did not evaluate the influence of natural egg-tolerance development on the BAT of EA children after 8 months, which could have influenced the BAT of BET children undergoing the 8-month protocol. Additional limitations to consider when implementing BAT in clinical practice are the prevalence of nonresponders, which inevitably results in uninterpretable BAT results, and the need for fresh blood, trained personnel, a flow cytometer, and standardization (protocol, extracts) [9,31].

Lastly, we found a significant correlation between egg white and ovalbumin sIgE levels and basophil responses to cake, hard-boiled egg, and omelet in EA and ET children. This is concurrent with earlier observations by Kim et al [12], who found that the %CD63$^+$ basophils to egg white positively correlated with the egg white sIgE levels of children with and without egg allergy. Additionally, the BAT dose-response AUC for cake correlated with ovomucoid sIgE levels, which is to be expected as low IgE levels to heat stable ovomucoid have been associated with a higher probability of tolerating baked egg [21,22]. Similarly, in the gradual reintroduction cohort, egg white sIgE and basophil response to either cake or soft-boiled egg correlated positively at the beginning and end of the treatment, respectively. This evolution could be seen as a reflection of development of clinical tolerance in EA children, which starts with tolerance to baked egg and ends with tolerance to lightly cooked and raw egg. In the future, it could be of interest to evaluate the integration of BAT results with egg sIgE levels in a larger cohort of children undergoing OFCs to construct a predictive model for tolerance development. From a diagnostic standpoint, similar to the approach proposed by Santos et al [23] in peanut allergy, use of BAT as a second-line diagnostic tool after sIgE measurement could be an accurate and cost-efficient diagnostic method in hen's egg allergy.

In conclusion, we demonstrate for the first time that BAT with progressively less heated egg extracts is a sensitive and highly specific tool to discriminate egg-allergic from egg-sensitized children who have completely outgrown their egg allergy. In the future, it would be interesting to compare these whole-egg extracts with classical egg white extracts and individual egg allergens to determine which strategy offers optimal discriminative capabilities. Measurement of egg sIgE remains a valuable first-line diagnostic tool. However, allergen- and patient-specific cut-offs are required to optimize diagnostic accuracy in distinguishing between sensitization and true allergy. As BET children evolved along the gradual process of tolerance development, basophil reactivity and sensitivity to progressively less heated forms of egg tended to decrease over time while tolerance was installed. Additional studies in larger cohorts of BET children undergoing gradual introduction over longer time periods are ongoing. These will help to determine the value of BAT with whole-egg extracts as a noninvasive tool for predicting clinical outcome and tolerance induction.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Conflicts of Interest

This work was presented at the EAACI Food Allergy School (2023, Taormina) and awarded a prize for best oral presentation.

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