

ORIGINAL ARTICLE

Clinical Allergy

Predicting reactivity threshold in children with anaphylaxis to peanut

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Summary

Background: Peanut allergy necessitates dietary restrictions, preferably individualized by determining reactivity threshold through an oral food challenge (OFC). However, risk of systemic reactions often precludes OFC in children with severe peanut allergy.

Objective: We aimed to determine whether clinical and/or immunological characteristics were associated with reactivity threshold in children with anaphylaxis to peanut and secondarily, to investigate whether these characteristics were associated with severity of the allergic reaction during OFC.

Methods: A double-blinded placebo-controlled food challenge (DBPCFC) with peanut was performed in 96 5- to 15-year-old children with a history of severe allergic reactions to peanut and/or sensitization to peanut (skin prick test [SPT] ≥ 3 mm or specific immunoglobulin E [s-IgE] ≥ 0.35 kUA/L). Investigations preceding the DBPCFC included a structured interview, SPT, lung function measurements, serological immunology assessment (IgE, IgG and IgG₄), basophil activation test (BAT) and conjunctival allergen provocation test (CAPT). International standards were used to define anaphylaxis and grade the allergic reaction during OFC.

Results: During DBPCFC, all 96 children (median age 9.3, range 5.1–15.2) reacted with anaphylaxis (moderate objective symptoms from at least two organ systems). Basophil activation (CD63⁺ basophils $\geq 15\%$), peanut SPT and the ratio of peanut s-IgE/total IgE were significantly associated with reactivity threshold and lowest observed adverse events level (LOAEL) (all $P < .04$). Basophil activation best predicted very low threshold level (< 3 mg of peanut protein), with an optimal cut-off of 75.8% giving a 93.5% negative predictive value. None of the characteristics were significantly associated with the severity of allergic reaction.

Conclusion and Clinical Relevance: In children with anaphylaxis to peanut, basophil activation, peanut SPT and the ratio of peanut s-IgE/total IgE were associated with reactivity threshold and LOAEL, but not with allergy reaction severity.

KEYWORDS

anaphylaxis, basophil, double-blinded placebo-controlled food challenge, food allergy, immunoglobulin E, immunologic tests, peanut allergy, threshold levels

1 | INTRODUCTION

Peanut allergy, the most common cause of severe and fatal allergic reactions in children in the western world,¹ has increased in prevalence over the last two decades^{2,3} currently affecting 1%-2% of the paediatric population.⁴ Once established, peanut allergy is likely to last a lifetime.^{5,6} The only established therapy is peanut avoidance and rescue medication, including intramuscular epinephrine auto injectors in case of severe systemic reactions. Nevertheless, accidental exposures frequently occur due to poorly labelled foods, manufacturing errors and peanut contamination.⁷ Consequently, quality of life is reduced in peanut-allergic children and their families.⁸⁻¹⁰

Diagnosing peanut allergy requires a convincing history of allergic reaction related to peanut exposure, supported by clinical and immunological investigations and/or an oral food challenge (OFC) to peanut. Clinical investigations include skin prick tests (SPT) and possibly the recently reported clinical conjunctival allergen provocation test (CAPT),¹¹ whereas immunological investigations include specific Immunoglobulin E (s-IgE) to peanut and its allergen components as well as the basophil activation test (BAT).¹²⁻¹⁴

Clinical reactions to peanut have been associated with weal size of SPT and s-IgE levels to peanut,^{15,16} while s-IgE to the peanut component allergen Ara h 2 has been shown to be the best predictor of allergic reactions¹⁷⁻¹⁹ with levels exceeding 1.00 kUA/L conferring a 97% probability of a systemic reaction.²⁰ Allergy reaction severity has been associated with basophil activation,²¹ and with peanut SPT and s-IgE in some^{13,22,23} but not all^{24,25} studies, whereas the ratio of s-IgG₄/s-IgE to peanut²⁶ and peanut s-IgE/total IgE²⁷ has been associated with diagnostic outcome of OFC. Finally, reactivity threshold has been associated with peanut SPT and s-IgE to peanut, Ara h 2 and basophil activation.^{23,24}

Reactivity threshold is necessary to personalize precautions related to peanut exposure, preferably determined by a double-blinded placebo-controlled food challenge (DBPCFC) and reported as the cumulated amount of peanut intake at the time of a positive OFC²⁸ or as the lowest amount of peanut protein that elicited mild, objective symptoms, that is the lowest observed adverse effect level (LOAEL).²⁸ However, children with the greatest likelihood of severe and life-threatening reactions are often excluded from DBPCFC and oral immunotherapy (OIT) due to the risk of systemic reactions.²⁹

Screening children with peanut allergy for enrolment in the open randomised controlled (RCT) peanut OIT trial; "Take-Away food allergy; inducing tolerance in children allergic to peanut" (the Take-Away trial), included clinical and immunological investigations as well as a DBPCFC to determine reactivity threshold and severity grade of allergic reaction.

The primary aim of this study was to determine whether clinical and/or immunological characteristics were associated with reactivity threshold in children with anaphylaxis to peanut, and secondarily to investigate whether these characteristics were associated with severity of the allergic reaction.

2 | METHODS

2.1 | Study design

This study setting was a 3-day investigation programme for children screened for eligibility for enrolment into the ongoing Take-Away trial, performed from February 2014 to June 2015 at the Department of Paediatric and Adolescent Medicine, Oslo University Hospital, Ullevål, Norway.

Children were recruited for screening investigations from the Oslo Peanut Allergy Study¹¹ as well as by referral from in-house and other paediatric allergy clinics in Oslo and the surrounding area.

Eligibility for enrolment in the Take-Away trial was determined by screening, consisting of a telephone interview to assess screening inclusion and exclusion criteria followed by a 3-day investigation programme. On day one, a structured interview, blood samples for cellular and immunology analyses, lung function measurements, SPT and titrated SPT (SPT_t), CAPT and BAT were performed, followed by a DBPCFC. Enrolment required a positive DBPCFC, whereas a positive food challenge at a level of ≤ 3 mg peanut protein precluded participation in the OIT.

Inclusion criteria for screening were age of 5-15 years, a history of systemic reactions to peanut or sensitization to peanut by a peanut SPT ≥ 3 mm or a s-IgE to peanut ≥ 0.35 kUA/L, living within acceptable distance from the Oslo University Hospital and willingness to participate in the peanut OIT study. Exclusion criteria were non-controlled asthma, allergy or intolerance to any other ingredients in the peanut DBPCFC vehicle (ginger bread), current or previous allergen-specific immunotherapy, cardiac disease, severe atopic skin disease, diabetes mellitus or other severe diseases that might interfere with adherence to the study protocol.

Written informed consent was obtained from both parents after detailed oral and written information. The Take-Away trial was approved by the Regional Committee for Medical and Health Research Ethics and monitored by a safety board with regular communications in case of severe or unexpected adverse events, and registered in the ClinicalTrials.gov (number NCT02457416).

2.2 | Study population

This study included 96 children with a positive DBPCFC. Of the 213 children referred for screening, 113 did not wish to enter the study, did not fulfil the inclusion and exclusion criteria or withdrew during screening and 4 had a negative DBPCFC (Figure 1).

2.3 | Methods

A structured parental interview including history of allergic reactions, allergic comorbidities and medical history of the child, use of medication and sociodemographic data was performed on day one.

Lung function was measured by maximal expiratory flow volume loops in accordance with international standards,³⁰ and reported by

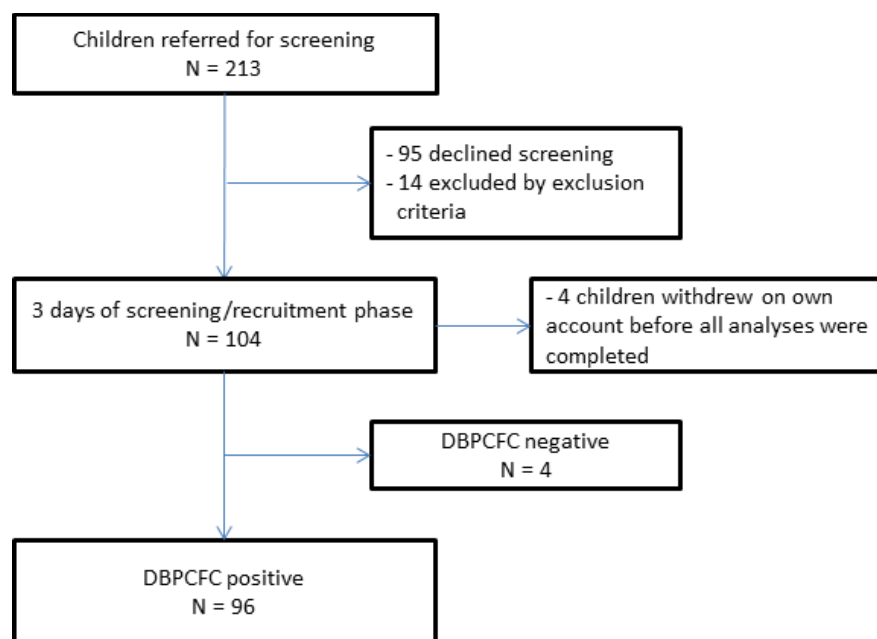


FIGURE 1 Flow chart of the recruitment phase in the Take-Away trial

percentage predicted forced expiratory volume in one-second (FEV₁%) using the reference values of Zapletal.³¹

Skin prick tests were performed using peanut and 11 other common food and inhalant allergens (see online supplement) according to international guidelines.³²

Titred SPT (SPT_t) to peanut was performed with dilutions of 1:20, 1:200, 1:2000 and 1:20 000 of the peanut allergen extract. Weal size was recorded after 15 minutes, and regarded positive if ≥ 3 mm greater than negative control.

Total IgE and s-IgE to peanut and peanut component allergens (Ara h 1, Ara h 2, Ara h 3, Ara h 8, Ara h 9) as well as other common allergens (see online supplement) were analysed in whole blood sampled in EDTA tubes and sent the same day to Furst Medical Laboratory using the Phadia CAP-System FEIA (ThermoFisher, Uppsala, Sweden). Specific IgE ≥ 0.35 kUA/L was considered positive.

Specific IgG (s-IgG) and IgG₄ (s-IgG₄) to peanut and to Ara h 2 were analysed in sampled frozen sera stored at -86°C using the Phadia CAP-System FEIA (ThermoFisher). According to the manufacturer's instructions, a positive test was defined as IgG >2.0 mg_A/L and IgG₄ of >0.07 mg_A/L.

Conjunctival allergen provocation test (CAPT) was performed double-blinded and placebo-controlled with commercially available extract of peanut (Greer laboratories, Lenoir, NE, USA) as described by Lindvik et al.¹¹ The NaCl 0.9% was used as placebo and as dilution fluid to make test solutions of 1:160, 1:80, 1:40, 1:20, 1:10 and 1:1¹¹ (further details in online supplement).

The BAT was performed in fresh EDTA blood stored at room temperature (usually 1–3 hours, maximum 24 hours). Whole blood aliquots were incubated with 20 μL staining solution as negative control, positive control, or with 2.5, 5 and 10 ng/mL peanut extract (further details in online supplement). For this study, basophil activation was only expressed as the % CD63-positive basophils (% CD63⁺) after stimulation of 5 ng/mL of the peanut extract, as all

children had a strong basophil activation already to the lowest allergen concentration of 2.5 ng/mL peanut extract, thus CD-sens (basophil allergen sensitivity—the allergen concentration eliciting half of the maximum basophil activation) could not be determined. Non-responders were defined as children responding with 0%–5% basophil activation.³³ In addition to analysing basophil activation as a continuous variable, the cut-off value for positive basophil activation in this study was set to $>15\%$ CD63⁺ basophils, whereas children with basophil activation of 5%–15% were classified as low responders, as suggested by Glaumann et al.³⁴ and the kit provider (Buhlmann Laboratories AG). In 10 children, BAT was not performed due to missed sampling ($n = 5$) or technical causes ($n = 5$) (technical failure of equipment [$n = 1$], incorrect handling of the blood sample [$n = 1$] or no available allergen extract [$n = 3$]).

The DBPCFC was performed according to international standardized procedure^{35,36} using peanut flour (Golden Peanut Company, Alpharetta, GA, USA) as active challenge ingredient in baked gingerbread according to a recipe by Vlieg-Boestra et al.³⁷ (further details in online supplement). The first challenge dose was chosen to 3 mg peanut protein, and the maximal cumulated dose was 6443 mg, distributed by 7 incremental steps (3, 10, 30, 100, 300, 1000, 5000 mg of peanut protein) of 30 minutes interval. In case of persistent subjective or mild objective symptoms, the interval was increased up to 60 minutes, in line with the modified Bock's criteria by Sampson et al.^{38,39} in which case the next step repeated the previous dose. The DBPCFC was regarded positive with the occurrence of moderate objective symptoms^{35,40} even if only part of the current dose had been eaten. Cumulated peanut protein (mg) intake at the time of positive DBPCFC was recorded. The LOAEL was recorded post hoc as the lowest cumulated amount of peanut protein that elicited the first mild, objective adverse reaction during food challenge was observed, according to the modified Bock's criteria.

Anaphylaxis was defined as moderate symptoms from at least two organ systems in line with European Academy of Allergy and Clinical Immunology (EAACI) task force position papers^{41,42} (Table S2), modified for children by Vetander et al.⁴³ Severity of anaphylaxis was also graded in line with the EAACI position papers^{41,42} scoring from 1 to 3 (mild-moderate-severe) in addition to the method of Sampson (Grading of Food-Induced Anaphylaxis According to Severity of Clinical Symptoms) ranging from one to five (extremely severe reaction).⁴⁴

2.4 | Outcomes and predictors

The primary outcome was reactivity threshold, defined as the cumulated peanut protein (mg) intake at positive DBPCFC. The LOAEL²⁸ was used as an additional threshold value and set to 3 mg for children with a positive DBPCFC at the first challenge dose.

Very low reactivity threshold was defined as a DBPCFC reactivity threshold of ≤ 3 mg of peanut protein.

The secondary outcome was severity of the allergic reaction during DBPCFC.

Potential predictors were baseline characteristics (age and gender, medical history including previous history of anaphylaxis, and parental atopic disease or food allergy) allergic comorbidities (current asthma, allergic rhinitis, previous or current atopic dermatitis and allergy to other foods or nuts), FEV₁%, clinical test results (SPT and SPT_t to peanut, peanut CAPT) and immunological test results (s-IgE, s-IgG and s-IgG₄ to peanut and Ara h 2), the ratios of peanut (s-IgE/total IgE and s-IgG₄/s-IgE to peanut), as well as basophil activation.

2.5 | Statistical analyses

Due to non-normal distribution, continuous baseline characteristics are presented by geometric mean (95% CI) and median (range), and categorical data are presented as number of cases (n) with percentage (%). Furthermore, bivariate unadjusted analyses were performed with Spearman correlation analyses (r_s = Spearman correlation coefficient). As the underlying assumptions for the standard mean regression analysis were not fulfilled, multivariate robust regression analyses with Huber's M-estimator were used to further assess associations between immunological parameters as the independent variables and both reactivity threshold and LOAEL as the dependent variables. Multivariate robust linear regression was used to assess the association between immunological parameters and severity of the allergic reaction.

A Hosmer step down multivariate analysis⁴⁵ included all variables significant at the 0.35 level in the bivariate analyses, and the final model was tested for confounding with all excluded variables.

Statistical analyses were performed using Statistical Analysis System (SAS, Version 9.3; SAS Institute Inc., Chapel Hill, NC, USA) and the IBM Statistical Package for Social Sciences (IBM SPSS Statistics, Version 21.0.1; IBM Corp., Armonk, NY, USA).

The level of significance was set to .05.

3 | RESULTS

All included 96 children (median [range] age of 9.3 [5.1, 15.2] years) were sensitized to peanut with s-IgE to Ara h 2 above 1.0 kUA/L in all but 4 patients (Table 1) who had levels of 0.96, 0.82 (two children) and 0.38 kUA/L, respectively. A prior history of anaphylaxis was reported by 83.0% with unknown time interval because the last anaphylactic event, whereas all children had anaphylaxis during the DBPCFC (Table 1). None of the children had an EAACI score of 3 or a Sampson score of 5 (Table 1).

The geometric mean (95% CI) for reactivity threshold and LOAEL was 33 (22.9, 48.4) and 13.8 (9.9, 19.5) mg of peanut protein, respectively (Table 1). Reactivity threshold level was <3 mg in five children, 3 mg in 14 children and >3 mg in 77 children. As anaphylaxis occurred at the first challenge dose in 19 children, their LOAEL was set equal to reactivity threshold. In 14 children with reactivity threshold >3 mg, the first objective symptom occurred at the first challenge dose, yet without a positive DBPCFC, resulting in a LOAEL of 3 mg. Consequently, five children were classified with a LOAEL <3 mg, 28 had the LOAEL set to 3 mg, and 64 had a LOAEL >3 mg.

In unadjusted bivariate analyses, basophil activation correlated significantly with reactivity threshold (correlation coefficient [r_s] = $-.30$, $P = .004$) and with LOAEL ($r_s = -.02$, $P = .032$). No significant correlations were found for reactivity threshold or LOAEL with peanut SPT or s-IgE to peanut, Ara h 2 or with the ratios of peanut s-IgE/total IgE and s-IgG₄/s-IgE to peanut (data not shown). Neither age, gender, allergic comorbidities nor FEV₁% were significantly associated with reactivity threshold, LOAEL or severity of allergic reaction in the bivariate regression analyses (data not shown).

In multiple robust regression analyses, reactivity threshold was significantly associated with basophil activation, peanut SPT, Ara h 2 and the ratios of peanut s-IgE/total IgE and s-IgG₄/s-IgE to peanut (Table 2). Neither peanut CAPT nor s-IgE or SPT_t to peanut was significantly associated with reactivity threshold. Repeating the analyses for LOAEL, significant association was found with peanut SPT, s-IgE to peanut, the ratio of peanut s-IgE/total IgE and basophil activation, but not with Ara h 2, the ratio s-IgG₄/s-IgE to peanut, peanut SPT_t and peanut CAPT (Table 2).

Subgroup analyses after excluding 23 children with a basophil activation $\leq 15\%$ CD63⁺ (non-responders [$n = 6$] and the low responders [$n = 17$]) from the model gave similar results for reactivity threshold and for LOAEL (data not shown).

Basophil activation was significantly associated with immunological and clinical test results with closest association with peanut CAPT ($\beta = -7.60$, $P = .02$) (Table 3) and with age among girls ($r_s = .31$, $P = .04$), but not among boys ($r_s = -.009$, $P = .95$). Additionally, basophil activation was the best predictor of very low reactivity threshold (<3 mg of peanut protein) (Figure 2), with an optimal cut-off of 75.8% giving a 93.5% negative predictive value (Table S1).

Neither peanut SPT or SPT_t nor CAPT to peanut nor any of the immunological parameters was significantly associated with severity of allergic reaction in the Hosmer step down multivariate robust regression analysis (data not shown).

TABLE 1 Baseline characteristics from screening in the Take-Away trial

	n (%)	Geometric mean (95% CI)	Median (min, max)
Age		9.3 (5.1, 15.2)	9.7 (5.6, 14.6)
Male sex	50 (52.1)		
Current asthma	52 (54.2)		
Allergic rhinitis	28 (29.2)		
Ever had eczema	74 (77.1)		
Allergy to other nuts than peanut	36 (37.5)		
Allergy to other food than nuts	49 (51.0)		
Parental atopic disease ^a	83 (86.5)		
Parental food allergy ^b	38 (39.6)		
History of anaphylaxis	80 (83.3)		
Mild	18 (18.8)		
Moderate	36 (37.5)		
Severe	26 (27.1)		
FEV1% predicted		97.1 (94.8, 102.1)	100 (42, 137)
Peanut SPT (mm)		9.8 (9.0, 10.7)	9.5 (4.0, 36.0)
S-IgE (kUA/L)			
Peanut		88.1 (62.4, 124.5)	108.5 (0.6, 2311.0)
Ara h2		44.6 (32.1, 61.9)	61.8 (0.38, 492.0)
Pos s-IgE (≥ 0.35 kUA/L)			
Other nuts ^c	87 (91.6)		
Other food ^d	92 (96.8)		
Ratio peanut s-IgE/total IgE (kUA/L)		4.0 (3.1, 5.3)	0.3 (0.0, 10.0)
Peanut s-IgG (mgA/L)		13.8 (11.5, 16.6)	13.9 (2.4, 80.9)
Peanut s-IgG ₄ (mgA/L)		2.1 (1.6, 2.6)	0.5 (0.1, 18.7)
Ara h 2 s-IgG (mgA/L)		6.3 (5.4, 7.3)	5.2 (0.0, 41.1)
Ara h 2 s-IgG ₄ (mgA/L)		8.9 (12.0, 6.6)	0.1 (0.0, 1.7)
Ratio s-IgG ₄ /s-IgE to peanut (ng/mL)		5.5 (3.9, 7.7)	5.0 (0.2, 564.6)
BA (%CD63 ⁺) pos ^e		69.5 (64.0, 75.4)	76.9 (16.1, 94.4)
BA (%CD63 ⁺) all children		44.9 (35.0, 57.5)	74.3 (0.5, 94.4)
CAPT positive (dilution)			
1/160	9 (9.4)		
1/80	24 (25)		
1/40	38 (39.6)		
1/20	20 (20.8)		
1/10	5 (5.2)		
Pre-OIT DBPCFC			
Use of adrenaline	42 (43.8)		
Anaphylaxis severity grade			
Modified EAACI ⁴³		1.7 (1.6, 1.8)	2 (1, 2)
Sampson ⁴⁴		2.6 (2.5, 2.8)	3 (2, 4)
LOAEL (mg)		13.8 (9.9, 19.5)	13.0 (0.8, 1610.0)
Reactivity threshold (DBPCFC positive) (mg)		32.4 (22.1, 47.5)	35.0 (1.0, 3943.0)

SPT, skin prick test; Ig, immunoglobulin; BAT, basophil activation test; CAPT, conjunctival allergen provocation test; OIT, oral immunotherapy; LOAEL, lowest observed adverse effect level; reactivity threshold—cumulated peanut protein eaten at positive DBPCFC.

^aAtopic disease includes asthma, allergic rhinitis, atopic dermatitis, allergic conjunctivitis.

^bFood allergy includes all food allergy including peanut and tree nut allergy.

^cIgE to other nuts ≥ 0.35 kUA/L includes hazelnut, almond, cashew nut, pistachio nut, walnut, pecan nut, Brazil nut and macadamia nut.

^dIgE to other food includes fenugreek, soya bean, pea, red kidney bean, lupin seed and wheat.

^eBAT results with basophil activation $\geq 15\%$ CD63⁺ basophils (positive).

TABLE 2 Multivariate robust regression analyses for significant associations between peanut reactivity threshold and the lowest observed adverse event level (LOAEL) in children anaphylactic to peanut

	Reactivity threshold		LOAEL	
	β -value (95% CI)	P-value	β -value (95% CI)	P-value
Peanut SPT (mm)	1.45 (0.08, 2.83)	.04	0.87 (0.43, 1.31)	.0001
Peanut s-IgE (kUA/L)	-		0.01 (0.002, 0.018)	.01
Ara h 2 s-IgE (kUA/L)	0.09 (0.02, 0.17)	.01	-	
Ratio peanut s-IgE/total IgE (kUA/L)	29.20 (22.57, 35.82)	<.0001	3.58 (1.44, 5.71)	.001
Ratio s-IgG ₄ /s-IgE to peanut (ng/mL)	1.69 (1.61, 1.78)	<.0001	-	
BAT all children (%) ^a	-0.45 (-0.73, -0.17)	.002	-0.09 (-0.17, -0.002)	.04

SPT, skin prick test; Ig, immunoglobulin; BAT, basophil activation given as percentage-activated CD63 cells test; -, no significant association.

Associations are given as the relative change (β) related to each mg increase in peanut threshold.

N = 86, BAT was not performed in 10 children due to missed sampling (n = 5) or technical causes (n = 5).

^aIncluded all children with a positive basophil activation (% CD63⁺ basophils).

4 | DISCUSSION

In this novel study including 96 children who all had primary sensitization to peanut and anaphylaxis during the DBPCFC, reactivity threshold as well as LOAEL was significantly associated with basophil activation, peanut SPT and the ratio of peanut s-IgE/total IgE. Reactivity threshold was further significantly associated with Ara h 2 and the ratio of s-IgG₄/peanut s-IgE to peanut, while LOAEL was significantly associated with s-IgE to peanut. Neither reactivity threshold nor LOAEL was significantly associated with age, gender, medical history, allergic comorbidities or lung function. None of the investigated clinical or immunological characteristics were significantly associated with severity of the allergic reaction during food challenge.

The significant association between reactivity threshold as well as LOAEL with basophil activation given by % CD63⁺ in our children with anaphylaxis to peanut is in line with previous reports^{21,24} in children

TABLE 3 Robust regression analyses for the associations between basophil activation and immunological and clinical parameters

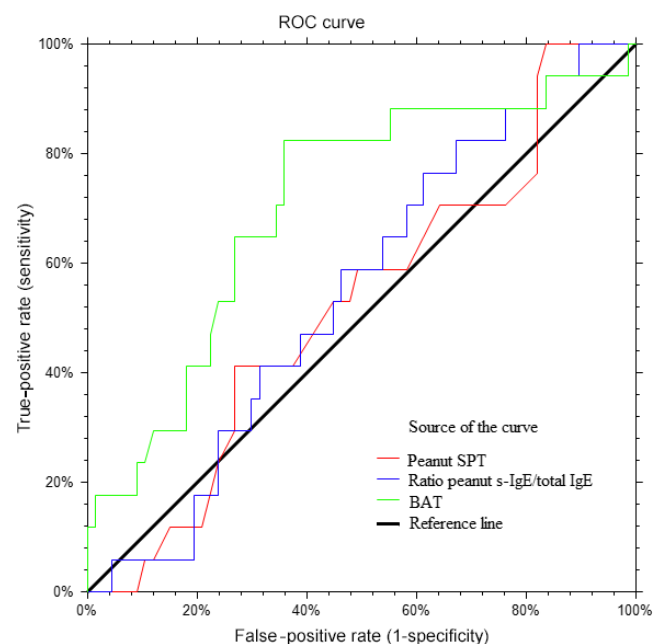
	Bivariate	
	Coeff (95% CI)	P-value
SPT to peanut (mm)	0.60 (-0.34, 1.54)	.21
Peanut s-IgE (kUA/L)	0.04 (0.02, 0.06)	<.01
S-IgE Ara h 2 (kUA/L)	0.08 (0.02, 0.13)	<.01
Ratio peanut s-IgE/total IgE (kUA/L)	58.2 (34.8, 81.7)	<.01
Ratio s-IgG ₄ /s-IgE to peanut (ng/mL)	-0.14 (-0.19, -0.09)	<.01
CAPT positivity level ^a	-7.60 (-14.06, -1.15)	.02

SPT, skin prick test; Ig, immunoglobulin; BAT, basophil activation test, CAPT, conjunctival allergen provocation test.

The coefficient represents the relative change related to percentage increase in activated basophils.

N = 80, BAT was not performed in 10 children due to missed sampling (n = 5) or technical causes (n = 5), non-responders are excluded (n = 6).

^aDilution steps at which the test was considered positive.

**FIGURE 2** Receiver operating characteristic curves predicting very low threshold (<3 mg of peanut protein). The basophil activation test used 5 ng/mL of allergen

with varying severity of peanut allergy. In 52 of 124 children with a median age of 5.4 years with a positive peanut challenge, Santos et al²¹ reported that basophil activation given by CD-sens as well as peanut SPT, s-IgE to peanut, Ara h 2 and the ratio of s-IgG₄/s-IgE to peanut was significantly associated with reactivity threshold, whereas basophil activation given by % CD63⁺ was the best predictor of allergy severity grade. In 63 peanut-allergic children with a median age of 6.5 years, Blumchen et al²⁴ found similar correlation between LOAEL and basophil activation for both % CD63⁺ ($r_s = -.32$) and CD-sens ($r_s = -.36$), which is also in line with other reports of correlation between CD-sens and % CD63⁺ ($r_s = .60$, $P = <.01$)²¹ and a 100% concordance has been shown between CD-sens and a positive DBPCFC.³⁴ In the present study, basophil activation expressed as % CD63⁺ best predicted very low threshold level (<3 mg of peanut

protein), whereas CD-sens could not be assessed due to strong basophil activation even at the lowest concentration of 2.5 ng/mL allergen extract, reflecting a study population with very low reactivity threshold.

Our finding of significant associations between reactivity threshold and peanut SPT and Ara h 2 is in line with previous reports in children^{21,24} and young adults.²³ In addition to correlation with basophil activation, Blumchen et al²⁴ found LOAEL also to correlate with peanut SPT, s-IgE to peanut and Ara h 2 ($r_s = -.20$ to $-.40$), in line with a Danish study²³ reporting correlation between reactivity threshold and Ara h 2 ($r_s = .30$) in 205 subjects 1-26 years of age, of whom 175 had a positive peanut challenge.

The associations between immunological and clinical test results and LOAEL were largely similar to those with reactivity threshold in the present study, with basophil activation, peanut SPT and peanut s-IgE/total IgE being significantly associated with both, although Ara h 2 was associated with reactivity threshold only, and peanut s-IgE with LOAEL only. The reasons for the different associations for reactivity threshold and LOAEL may be related to the observed larger variation in reactivity threshold compared to the LOAEL, or they may reflect different threshold entities with reactivity threshold requiring moderate objective symptoms while LOAEL appears more sensitive reflecting the lowest cumulated dose at which a mild objective symptom was noted. Additionally, the discrepancies may be due to the estimated rather than measured LOAEL in our study, with the first challenge dose of 3 mg. The estimated LOAEL may also explain why basophil activation was less strongly correlated to LOAEL than reactivity threshold and why the correlation is weak ($r_s = -.02$). On the other side, such a low correlation may also be irrelevant. It has been suggested that ideally, the highest dose that does not elicit objective symptoms (NOAEL = no observed adverse effect level) should be determined for defining the exact LOAEL,³⁵ but this was not possible in our study. The post hoc decision to also determine LOAEL was made to facilitate comparisons across studies with different stopping criteria of OFC.^{35,36,40} Some studies use only subjective symptoms that occur in consecutive doses as a sign of a positive OFC,^{17,46} although subjective symptoms may occur at doses 20-fold lower than the LOAEL⁴⁷ and some patients report no subjective symptoms prior to the objective symptoms.²⁴ Determining LOAEL might also be helpful in identifying the peanut exposure amount that elicited the first symptom or sign related to the importance of food labelling.²⁸

The severity of allergic reactions was not associated with any of the clinical or immunological characteristics in the present study, in line with the study of Blumchen et al,²⁴ but in contrast to the MIRA-BEL study,⁴⁸ in which baseline characteristics like age (teenagers and adults), asthma and not having atopic dermatitis were associated with more severe allergic reactions. Also, peanut SPT,¹³ s-IgE to peanut and Ara h 2,^{22,23} peanut SPT_t⁴⁹ and basophil activation (% CD63⁺)²¹ have been found to correlate to severity grades of allergic reactions. Song et al¹³ performed DBPCFC for different foods (42 of 44 positive with peanut DBPCFC) in 71 sensitized patients with a median age of 16, and found SPT ($r_s = .24$) and Ara h 2 ($r_s = .31$) to correlate with severity grade, in line with the correlation between Ara h 2 and allergy severity ($r_s = .60$) in 175 patients age of 1-

26 years with a clinical history of allergic reaction.²³ In 21 children (mean age of 60 months) with peanut allergy and 34 controls (28 tolerant and 6 non-anaphylactic reaction), Wainstein et al²² found both peanut SPT and s-IgE to peanut to predict anaphylaxis, whereas Tripodi et al⁴⁹ reported that the end-point titration in SPT_t differentiated between Sampson severity grades 3-5, grades 1-2 and negative food challenge in 20 of 47 children with OFC-proven hen's egg allergy. Methodological differences may explain some of the differences, including our reading of the SPT_t after 15 minutes vs 30 minutes in the Tripodi study. Finally, after multivariate analyses, Santos et al²¹ found that only basophil activation (% CD63⁺) and not SPT or other immunological test results was retained as the one marker closest associated with severity grade of allergic reaction. The lack of significant associations between biological markers and severity of allergy reaction in the present study is likely to reflect our relatively homogenous study population; all with primary peanut allergy and anaphylaxis^{42,44} during DBPCFC and 34.4% of the children with a LOAEL ≤ 3 mg of peanut protein, which exceeded the expected 10% with a very low reactivity threshold.⁵⁰ It also exceeded the 4.7% of the 63 peanut-allergic children with LOAEL at 3 mg of peanut protein reported by Blumchen et al,²⁴ although they suggested that their modified food challenge would result in a lower LOAEL.

Also, the DBPCFC was stopped when it was defined positive, which may have prohibited more severe reactions. Basophil activation on the other hand can be regarded as an "in vitro challenge"¹² that can proceed to higher doses. In the present study, it was associated with immunological and clinical test results shown to predict severity grade of allergic reaction,^{13,21-23} which may suggest a relationship between allergy severity and reactivity. This finding is in line with the previously reported association between severity of allergic reaction and reactivity threshold ($P = .027$).⁵¹ To our knowledge, the association between basophil activation in the present study with the peanut CAPT dilution at which subjects experienced allergy symptoms and signs¹¹ has not been shown previously, but is supported by the study of Varney et al⁵² where conjunctival provocation threshold increased significantly after specific immunotherapy to cat dander. Finally, we found basophil activation also to correlate with female gender, a finding we are not aware has been shown previously. The clinical relevance of the latter finding is not clear.

4.1 | Strengths and limitations

All 96 children had confirmed primary peanut allergy by DBPCFC, with moderate objective signs from at least two organ systems, excluding only skin symptoms and very mild symptoms as anaphylaxis^{41,42} in line with international standards, and the study included a novel CAPT to peanut to assess potential biological correlates. However, as all children had anaphylaxis this may limit the possibility to identify correlations across a wider variation of reactivity thresholds and severity reactions. Although we found immunological and clinical test results to correlate to reactivity thresholds and LOAEL within our study population, the generalizability of our study may be limited to children with similar peanut allergy severity. Further, to be

able to address biomarkers associated with very low reactivity threshold and LOAEL, the study would have benefited from setting a lower starting dose than was chosen for the study. Finally, a potential limitation to interpretation of the results may be that thresholds determined by OFC are not completely reproducible.^{53,54}

5 | CONCLUSION

In children with anaphylaxis to peanut, basophil activation, peanut SPT and the ratio of peanut s-IgE/total IgE were associated with reactivity threshold and LOAEL. None of the clinical or immunological markers appeared sufficient to substitute oral peanut challenge to determine reactivity threshold, and none were associated with severity of the allergic reactions.

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CONFLICT OF INTEREST

Magnus P. Borres is employed by Thermo Fisher Scientific, and the other authors declare no conflict of interest for the present study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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1 ONLINE SUPPLEMENTS

2 Methods

3 Skin prick test performed using 11 common food and inhalant allergens included hazelnut,
4 almond, soy, birch, grass (timothy), mugwort, cat, dog, mite and mold (cladosporium
5 herbarium) (ALK SQ extracts from ALK Abello (Hørsholm, Denmark)), pea and control
6 (Allergopharma (Reinbek, Germany)).

7 Specific IgE analysed to other common allergens included hazelnut and hazelnut allergen
8 components (Cor a 1, Cor a 8, Cor a 9, Cor a 14), almond, cashew nut, pistachio, walnut,
9 pecan nut, brazil nut, macadamian nut, fenugreek, soy bean, lupine seed, wheat, latex,
10 common silver birch, timothy and mugwort.

11 The CAPT was performed with incremental concentrations applied every 30 minutes with two
12 independent observers recording redness, itching, chemosis and/or lacrimation. The CAPT
13 was defined positive with the occurrence of ≥ 2 recorded symptoms (11). Photos were taken
14 before and after the test for documentation. Randomization was performed using Statistical
15 Analysis System (SAS, Version 9.3, SAS Institute Inc., Chapel Hill, NC, USA), and all tests
16 were un-blinded after the last child had completed investigations.

17 Basophil activation was determined in whole blood aliquots incubated for 15 minutes at 37
18 °C. Negative control was incubated with stimulation buffer (with IL-3, calcium and heparin)
19 and positive control with anti-Fc ϵ R1 or fMLP (n-Formyl-methionyl-leucyl-phenylalanine).

20 All reagents used including the peanut extracts were from the FlowCAST kit and CAST-
21 allergen peanut BAG-F13 (both from Buhlmann Laboratories AG, Schönenbuch,
22 Switzerland). The staining solution contained a mix of anti-CD63 FITC and anti-CCR3-PE
23 (Buhlmann Laboratories AG), in addition to 5 μ l anti-CD203c-APC monoclonal antibody
24 (BioLegend, San Diego, CA USA). After red blood cell lysis, centrifugation and resuspension
25 in wash buffer (Buhlmann Laboratories AG), the cells were analysed on a LSRII flow

cytometer with BD FACSDIVA software (all from BD Biosciences, New Jersey, USA).

Basophils were gated as side-scatter (SSC) low and CCR3⁺ cells.

The DBPCFC matrix was gingerbread, baked, allocated and blinded by a cook. The order of

active vs placebo days with randomization and block-size were unknown to all study

personnel present at the challenge, provided by the statistician using Statistical Analysis

System (SAS, Version 9.3, SAS Institute Inc., Chapel Hill, NC, USA), and un-blinded first at

the end of the recruitment phase.

To determine predictive performance of immunological parameters for very low threshold

levels, we used receiver-operating characteristic (ROC)-curve analyses, defining cut-off levels

for immunological parameters with significant association to threshold level.

Results

Basophil activation was found to be a better predictor of very low peanut threshold (≤ 3 mg)

with an (AUC, 95% C.I) of 0.71 (0.53, 0.82) compared to the corresponding results for

peanut s-IgE/total Ig-E (54.6, (0.39, 0.67)) and peanut SPT (52.5 (0.36, 0.66), respectively

(Supplementary Figure 1). However, the optimal basophil activation value (at 5 ng/mL

allergen) prediction cut-off level of 75.8 % gave an 82.4 % sensitivity and 64.2 % specificity

for predicting very low reactivity threshold, with a low positive and high negative predictive

value (Supplementary Table 1 and Figure 1).

Supplementary Table 1. Optimal cut-off values to predict very low peanut reactivity threshold (≤3 mg peanut protein)

Parameter	Cut-off value	Sensitivity	Specificity	PPV	NPV
Peanut SPT (mm)	10.0	57.9 (33.5, 79.8)	53.3 (41.5, 64.7)	23.4 (12.3, 38.0)	83.7 (70.3, 92.7)
Basophil activation (%CD63 pos cells)	75.8	82.4 (56.6, 96.2)	64.2 (51.5, 75.5)	36.8 (21.8, 54.0)	93.5 (82.1, 98.6)
Peanut s-IgE/total IgE (kUA/L)	0.36	58.8 (32.9, 81.6)	53.7 (41.1, 66.0)	24.4 (12.4, 40.3)	83.7 (69.3, 93.2)

Results are given as per cent with 95 % confidence interval

PPV: Positive predictive value

NPV: Positive predictive value

Supplementary Table 2. The clinical criteria for diagnosing anaphylaxis according to the EAACI position paper

1

Anaphylaxis is highly likely when any one of the following three criteria are fulfilled:

1. Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue or both (e.g. generalized hives, pruritus or flushing, swollen lips-tongue-uvula).

And at least one of the following:

a. Respiratory compromise (e.g. dyspnoea, bronchospasm, stridor, hypoxia).

b. Cardiovascular compromise (e.g. hypotension, collapse).

2. Two or more of the following that occur rapidly after exposure to a likely allergen for that patient (minutes to several hours):

a. Involvement of the skin or mucosal tissue (e.g. generalized hives, itch, flushing, swelling).

b. Respiratory compromise (e.g. dyspnoea, bronchospasm, stridor, hypoxia).

c. Cardiovascular compromise (e.g. hypotension, collapse).

d. Persistent gastrointestinal symptoms (e.g. crampy abdominal pain, vomiting).

3. Hypotension after exposure to known allergen for that patient (minutes to several hours)

2
3