

The fraction of exhaled nitric oxide improves prediction of clinical allergic reaction to peanut challenge in children

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Summary

Background Retrospective studies of childhood peanut allergy demonstrate serum-specific IgE (IgE) levels against the peanut allergen Ara h2 may help predict a clinical reaction at food challenge. Fraction of exhaled nitric oxide (FeNO) is a non-invasive tool correlating to allergic airways inflammation and has been independently associated with increased food-specific IgE.

Objective To assess the validity of serum-specific Ara h2 IgE measured prospectively to diagnose peanut allergy and explore the utility of FeNO as a non-invasive screening tool for childhood food challenge.

Methods We recruited 53 participants from a cohort of consecutive children scheduled for an open-labelled peanut food challenge (OFC) by their paediatric allergist. Participants underwent skin prick test (SPT) measurement for sensitization to whole peanut extract, and serum was collected for Ara h2-specific IgE. FeNO was also measured in all cooperative children before the challenge. OFC and assessment of reaction were undertaken by clinicians blinded to test results.

Results Ara h2-specific IgE and FeNO each showed improved diagnostic accuracy when compared to SPT. Receiver operator characteristic curve analysis gave an area under the curve (AUC) for Ara h2 sIgE of 0.84 (95% CI, 0.72–0.96). The AUC for FeNO, 0.83 (95% CI, 0.71–0.95), was equivalent to that of Ara h2. Combined AUC for SPT, sIgE to Ara h2 and FeNO was 0.96 (95% CI 0.90–1.00). There was no correlation between FeNO and serum nitrite levels ($r_s = -0.13$, $P = 0.6$, $n = 18$).

Conclusion and clinical relevance Prospectively measured Ara h2-specific IgE improves diagnostic accuracy and reduces unsuccessful challenge to peanut. FeNO levels may provide improved diagnostic accuracy in a paediatric population undergoing OFC. The proposed FeNO-based diagnostic algorithm requires further validation studies.

Keywords Ara h2, diagnostic algorithm, FeNO, objective screening, peanut allergy diagnosis

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Introduction

Six to eight percent of children suffer from physician-diagnosed food allergy, and the prevalence is increasing worldwide [1–3]. Peanut allergy contributes 1–2% of childhood food allergies [4–6]. Perceived allergy is much greater with 20–30% of people altering diets on the basis of food allergy [2, 3]. While there are hypotheses, the true mechanisms that drive tolerance and

allergy remain elusive [7, 8]. As such, there is a growing need for improved diagnostics to clarify childhood food allergy and improve patient safety.

Allergic sensitization does not equal allergy. As many as one in ten 8-year-old children in the UK are sensitized to peanut, but only one in 50 has clinical manifestations of peanut allergy [9]. For many years, leading clinicians have suggested cut-off values of skin prick testing (SPT) and serum-specific IgE (sIgE) to diagnose

the likelihood of clinical reaction to peanut [10–12]. Those same authors recognize the difficulty with generalizing these values across different centres [3]. Interpretation of whole peanut SPT-positive and SPT-negative predictive values is also difficult as prevalence data within a certain population are required to accurately interpret the predictive values generated [13]. Further, there remains significant uncertainty with the large numbers of patients who fall between cut-off values requiring food challenge – the current gold standard for diagnosing clinical allergy. This can be resource intensive and sometimes a high-risk pursuit with substantial chance of anaphylaxis [14].

Children with food allergy are more likely to have related conditions such as asthma and atopic dermatitis [15]. Food allergy has been shown to be associated with future asthma development in children, with a fivefold increase reported [16]. Fraction of exhaled nitric oxide (FeNO) is increasingly being evaluated as a diagnostic tool in allergic airways disease [17] and atopy [18, 19] in children. Lower levels may suggest improved asthma control as measured by short-acting beta agonist use [17], and FeNO measurements have been used to titrate inhaled corticosteroid for patients with asthma [20]. Asthma is a known risk factor for severe reaction to food [21], and food allergy increases bronchial hyper-responsiveness independent of asthma diagnosis [22]. Population studies also suggest that a significant proportion of patients with current wheeze do not carry a physician diagnosis of asthma [23]. Recently, an independent association between raised FeNO levels and the presence of food-specific IgE was identified [24], suggesting perhaps a causal or consequential relationship between NO production and food protein sensitization. While the molecular and cellular mechanisms underpinning this association have yet to be identified, FeNO measurements may provide a non-invasive tool to predict outcome of patients undertaking food challenge.

Recently, diagnostic algorithms have been proposed to predict outcome at food challenge [25, 26]. Utilizing clinical patient data (symptoms at first exposure, age and sex) with available diagnostic tests (peanut SPT, total IgE and food-specific IgE), authors suggested accurate prediction of positive and negative outcome at food challenge [25]. Interpretation of allergic symptoms, unless witnessed by the clinician, is difficult and potentially inaccurate [27]. Klemens et al. [26] looked to validate the algorithm by retrospectively analysing hospital data, improving uniformity by removing reaction history. They also added sIgE to Ara h2 to the algorithm, an increasingly established peanut protein involved in clinical allergy [28–31]. Interestingly, sIgE to Ara h2 performed almost as well independently of the algorithm with a specificity of 98% and sensitivity

of 55% at a cut-off sIgE level of > 5 kU/L. Marginally better predictive values were reported by Dang et al. [31] with a specificity of 98% and sensitivity of 60% at a serum sIgE level of > 1.19 kU/L. For both studies [26, 31], serum measurements were taken retrospectively after food challenge and, while potentially accurate, there is the possibility that food challenge itself induces sIgE. Thus, it cannot be immediately extrapolated for use as a prospective diagnostic test. Use and cut-off values for sIgE has been recently reviewed [3, 32].

In this pilot study, we aimed to assess the value of FeNO in further improving prediction of a clinical allergic reaction in observed peanut food challenge. We also look to validate the use of serum sIgE to Ara h2 as a prospective diagnostic test. We propose a novel algorithm including FeNO measurements, which has the potential to improve diagnostic accuracy, potentially decreasing the need for food challenge testing in the paediatric peanut-allergic population.

Methods

Study population

Participants were recruited from sequential children offered open-labelled food challenge (OFC) at a tertiary referral paediatric allergy centre in Newcastle, Australia. The Hunter New England Health and University of Newcastle Human Research Ethics Committees approved all studies, and written informed consent was obtained from all parents before inclusion into the study. Food challenge was offered to (1) confirm a peanut allergy diagnosis, (2) to assess for the possibility of acquired tolerance, (3) to test for clinical reactivity in children who had not consumed peanut but were sensitized, or (4) had significant parental concern and anxiety. The cohort included 32 patients with a history of anaphylaxis as well as other episodes of IgE-mediated reactions to peanut, not within the last 12 months. Outpatient review and decision to challenge was within 4 months of OFC with patients excluded if SPT was ≥ 10 mm. Of 65 patients invited to participate, eight declined involvement and subsequently data were not obtained. One patient was unwell with asthma exacerbations on two separate occasions, resulting in OFC postponement. Three patients had equivocal challenge results and were excluded. All of these patients were sensitized to peanut without prior ingestion. Criteria for exclusion and equivocal result were unwillingness to co-operate with food ingestion or failure to complete entire challenge (2 patients) and immediate post-ingestion gag/vomit (1 patient). This is in keeping with previous publications [25, 31]. Data on the remaining 53 patients were analysed.

Pre-challenge assessment

Patients attended a pre-challenge clinic in the week prior to food challenge. Co-morbid atopic disease was assessed using modified versions of previously validated parental questionnaires [33, 34] and an interview data sheet. Allergic rhinitis was assessed based on published ARIA criteria recently validated in the paediatric population [35] where classification is based on symptom duration (intermittent vs. persistent) and severity (mild vs. moderate/severe). Diagnosis was then scored 1 – intermittent mild, 2 – intermittent moderate–severe, 3 – persistent mild or 4 – persistent moderate–severe. Eczema history was based on any previous medical diagnosis, and ‘active treatment’ included any current management aside from simple emollients. Visible eczema was scored using the well-established SCORAD system [36].

Subjects were clinically assessed and then underwent FeNO measurement, SPT, spirometry and blood collection. FeNO was measured in accordance with the American Thoracic Society and European Respiratory Society (ATS/ERS) guidelines [37]. Children were measured online by single-breath analysis (ECOMEDICS, Duernten, Switzerland). Expiratory flow rate of 50 mL/s was sustained for a minimum of 2 s during at least a 4-s expiration time. Nasal NO was prevented by a flow limiter maintaining constant minimum exhalation pressure of 5 cm H₂O. Repeated exhalations were performed until criteria were met (2 results within 5% or 3 within 10%) and mean recorded. SPT was conducted using standard whole peanut extract reagents, 1 : 10 w/v (Stallergènes®, Antony, France). A positive result was ≥ 3 mm greater than negative control, determined by averaging maximal perpendicular wheal diameters 15 min after applying the lancet. Positive control was with histamine base, 6 mg/mL (Stallergènes®, Antony, France) and had to be ≥ 3 mm [38]. Spirometry was conducted in accordance with current standardized procedures [39]. Finally, serum was collected and analysed with the ImmunoCAP™ 250 system (Phadia, AB, Uppsala, Sweden) for peanut-specific IgE and Ara h2-specific IgE.

Observed food challenge and outcome

Open-labelled food challenge to peanut was conducted in accordance with Australasian Society of Clinical Immunology and Allergy (ASCI) food challenge protocol [40] and was identical to that used in the HealthNuts study [41] (Data S1). Outcome of food challenge was designated as ‘clinical allergy, not anaphylaxis’ (CANA) if patients had a clinical reaction consistent with published pre-defined objective criteria [31, 42]. Anaphylaxis was determined as defined by the ASCIA guidelines [43]. One patient who reacted with generalized

urticaria approximately 6 hours after challenge completion was included as CANA in analysis. Medical supervision was present at all food challenges, and assessor was fully blinded to the results obtained during the pre-challenge clinic. Patients were declared tolerant if there was no reaction during the food challenge and over the following week with regular (daily) ingestion of peanut. For the purpose of this study, ‘clinical allergy’ includes all clinical reactions (CANA and anaphylaxis).

Quantification of serum nitrite

Blood was collected in a heparinized tube immediately prior to OFC. Serum was collected following centrifugation using a Ficoll gradient to separate cells. Nitrite levels were determined using modified Greiss reagent (Sigma-Aldrich, Castle Hill, New South Wales, Australia) with optical absorbance recorded at 540 nm using a Spectra-Max M5 (Molecular Devices, Sunnyvale, CA, USA) spectrophotometer. Values were quantified against a sodium nitrite (Sigma-Aldrich) standard curve as previously described [6].

Statistics

Patient characteristics are presented as means and standard deviations for continuous variables and frequency and percentages for categorical variables. Differences in patient characteristics between groups defined by the open food challenge were tested using analysis of variance (ANOVA) for continuous variables and Fisher’s exact test for categorical variables.

Receiver operator characteristic (ROC) curves were used to examine the sensitivity and specificity of each of the potential predictive measures in identifying clinical reaction to the peanut food challenge. The area under the ROC curve (AUC) was used to assess the accuracy of each measure in predicting the result of the peanut food challenge and to compare the predictive ability of different measures. Standard errors and hence confidence intervals for the AUC were estimated using the approach of DeLong et al. [44] as implemented in Stata. To estimate the predictive ability of a combination of measurements, a logistic regression model was fitted to the data with the result of the peanut food challenge as the outcome and the combination of measurements as the predictors. The probability of a positive result estimated from the logistic regression model was then used to estimate the AUC. Comparison of the predictive ability of each measurement was also performed using the approach of DeLong et al., but testing the added benefit of an additional measurement was performed using the likelihood ratio statistic.

Diagnostic performance of sIgE to whole peanut, sIgE to Ara h2, SPT to whole peanut and FeNO was analysed.

Results

Participant clinical features

Patients suffering anaphylaxis were older than those with peanut tolerance and CANA. Mean age in the group with clinical allergy was 11.3 years compared with 6.9 years in the tolerant group ($P = 0.03$, Table 1). There were statistically more boys in the group demonstrating tolerance ($P = 0.02$). All other clinical variables were similar across the three groups (anaphylaxis and CANA considered separately) and when tolerant participants were compared with those having clinical allergy. Importantly, history of past or current co-morbid allergic airways disease (allergic rhinitis and asthma) was comparable between groups.

Accuracy of SPT, sIgE and FeNO at predicting clinical outcome

In accordance with previous publications [30, 31], sensitivities and specificities to diagnose clinical allergy

are provided for each diagnostic test (Table 2). The AUC for whole peanut SPT was 0.73 (95% CI, 0.60–0.87). Using a cut-off value of > 8.75 mm on SPT gave a specificity of 100%, but a sensitivity of just 26.1% to predict allergy. If conventional SPT cut-off for food challenge was observed (≥ 8 mm), this would have classified four children (8%) as allergic when in fact they were tolerant to peanut. Ara h2-specific IgE, when prospectively acquired, showed a non-significant improved diagnostic accuracy when compared to SPT. The AUC for Ara h2 sIgE was 0.84 (95% CI, 0.72–0.96; Fig. 1). An Ara h2 cut-off of 0.67 kU/L gave 96.7% (95% CI, 82.8–99.9%) specificity with 73.9% (95% CI, .60–89.77%) sensitivity to predict clinical allergy. Specificity was improved to 100% (95% CI, 88.43–100.0%) if the cut-off was increased to 1.39 kU/L. Ara h2 sIgE levels of 0.03 kU/L were required to discriminate peanut-allergic patients with a sensitivity of $> 95\%$. We found no statistical difference between Ara h2 sIgE levels in those children who had anaphylaxis compared with those with any other clinical reaction ($P = 0.12$, data

Table 1. Patient characteristics grouped by the outcome of open food challenge

Characteristic		Outcome of peanut challenge			Clinical allergy		
		Tolerant ($n = 30$)	Clinical allergy ($n = 23$)	P -value	CANA ($n = 13$)	Anaphylaxis ($n = 10$)	P -value
Age (years)	Median (min, max)	6.9 (2.2, 15.2)	11.3 (2.1, 16.1)	0.0351	8.6 (2.1, 16.0)	11.7 (5.4, 16.1)	0.0858
Gender	Female	8 (27%)	14 (61%)	0.0234	9 (69%)	5 (50%)	0.4173
Previous Peanut ingestion (%)	No	13 (43%)	9 (39%)	0.7861	6 (46%)	3 (30%)	0.6693
Previous adrenaline required (%)	No	28 (93%)	20 (87%)	0.6421	12 (92%)	8 (80%)	0.5596
Other food allergy (%)	No	13 (43%)	12 (52%)	0.5862	4 (31%)	8 (80%)	0.0361
Parental smokers (%)	No	23 (77%)	21 (91%)	0.2699	11 (85%)	10 (100%)	0.4862
Allergic rhinitis (%)	No	13 (43%)	14 (61%)	0.2709	7 (54%)	7 (70%)	0.6693
Eczema ever (%)	No	4 (13%)	7 (30%)	0.1767	3 (23%)	4 (40%)	0.6500
Eczema Active treatment (%)	No	18 (60%)	15 (65%)	0.7794	7 (54%)	8 (80%)	0.3788
Asthma ever (%)	No	10 (33%)	7 (30%)	1.0000	5 (38%)	2 (20%)	0.4050
Current preventer (%)	No	15 (50%)	16 (70%)	0.1728	8 (62%)	8 (80%)	0.4050
Current reliever (%)	No	10 (33%)	9 (39%)	0.7750	5 (38%)	4 (40%)	1.0000
Percent predicted FEV1	Median (min, max)	98 (79, 153)	99 (80, 113)	0.4148	99 (90, 113)	96 (80, 111)	0.3555
Percent predicted FVC	Median (min, max)	102 (69, 143)	96 (76, 118)	0.3597	99 (89, 118)	92 (76, 115)	0.2271
Percent predicted FEV1/FVC	Median (min, max)	99 (84, 115)	99 (83, 111)	0.7676	99 (83, 110)	101 (84, 111)	0.7936
SCORAD for those with visible eczema	Median (min, max)	0.0 (0.0, 46.0)	0.0 (0.0, 37.0)	0.7963	0.0 (0.0, 37.0)	0.0 (0.0, 26.0)	0.4794
AR severity for those with AR	Median (min, max)	2.0 (1.0, 4.0)	2.0 (1.0, 4.0)	0.9113	2.5 (2.0, 4.0)	1.0 (1.0, 3.0)	0.2201
Whole peanut SPT (mm)	Median (min, max)	4.3 (0.0, 8.5)	7.0 (2.5, 11.0)	0.0056	6.0 (2.5, 11.0)	7.8 (4.0, 9.5)	0.3014
FeNO (p.p.b.)	Median (min, max)	21.0 (3.9, 66.3)	48.3 (9.1, 170.5)	0.0005	54.3 (9.1, 170.5)	39.3 (20.7, 97.7)	0.5774
Peanut sIgE (kU/L)	Median (min, max)	0.5 (0.0, 13.4)	5.0 (0.2, 100.0)	< 0.0001	1.6 (0.2, 29.4)	6.8 (0.8, 100.0)	0.1145
Ara h2 sIgE (kU/L)	Median (min, max)	0.2 (0.0, 0.9)	2.5 (0.0, 100.0)	0.0001	0.9 (0.0, 18.8)	6.0 (0.1, 100.0)	0.1961

For determination of rhinitis severity, see Methods.

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; AR, allergic rhinitis; SCORAD, SCORing Atopic Dermatitis; SPT, skin prick test; FeNO, fraction of exhaled nitric oxide; sIgE, serum-specific IgE.

Table 2. Cut-off values for Ara h2 sIgE, fraction of exhaled nitric oxide (FeNO) and peanut skin prick test (SPT) with associated sensitivity and specificity for clinical allergy

Cut-off	Sensitivity (%)	95% CI	Specificity (%)	95% CI	PLR
Peanut SPT					
> 1.5 mm	100	85–100	20	8–39	1.25
> 3.3 mm	96	78–100	30	15–49	1.37
> 7.8 mm	35	16–57	87	69–96	2.61
> 8.3 mm	30	13–53	97	83–100	9.13
> 8.8 mm	26	10–48	100	88–100	
FeNO					
> 7.7 p.p.b.	100	83–100	28	12–49	1.39
> 9.4 p.p.b.	95	75–100	28	12–49	1.32
> 20.3 p.p.b.	95	75–100	52	31–72	1.98
> 25.0 p.p.b.	90	68–99	64	43–82	2.50
> 40.7 p.p.b.	60	36–81	92	74–99	7.50
> 41.40 p.p.b.	60	36–81	96	80–100	15.00
> 44.30 p.p.b.	55	32–77	96	80–100	13.75
> 64.40 p.p.b.	20	6–44	96	80–100	5.00
Ara h2 sIgE					
> 0.02 kU/L	100.0	85–100	13	4–31	1.15
> 0.06 kU/L	96	78–100	20	8–39	1.20
> 0.09 kU/L	91	72–99	23	10–42	1.19
> 0.34 kU/L	74	52–90	63	44–80	2.02
> 0.57 kU/L	74	52–90	90	73–98	7.39
> 0.67 kU/L	74	52–90	97	83–100	22.17
> 0.93 kU/L	57	34–77	97	83–100	16.96
> 1.39 kU/L	57	34–77	100	88–100	
> 66.85 kU/L	20	6–44	100	86–100	

PLR, positive likely hood ratio; sIgE, serum-specific IgE.

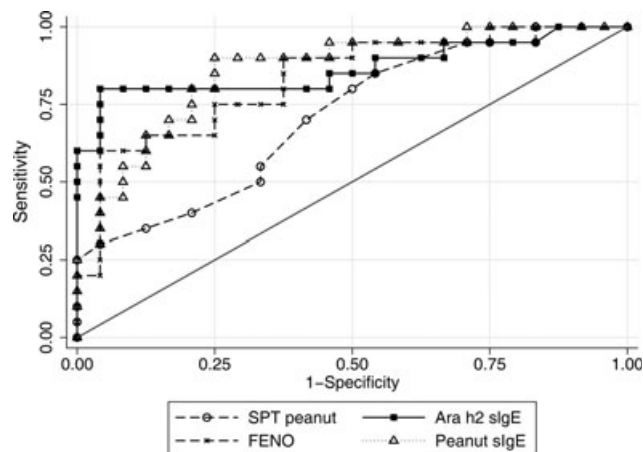


Fig. 1. Receiver operator characteristic (ROC) curves plotting sensitivity against false-positives (1-specificity) for four diagnostic modalities for peanut allergy. Outcome assessed was any clinical reaction to peanut (allergy) vs. no reaction (tolerant) in open food challenge. Each point represents one data point (patient result). Area under the curve for peanut skin prick test (SPT) Ara h2 serum-specific IgE (sIgE), fraction of exhaled nitric oxide (FeNO) and peanut sIgE are 0.74, 0.84, 0.84 and 0.85, respectively.

not shown). Whole peanut sIgE AUC was 0.85 (95% CI, 0.74–0.95), equivalent to Ara h2. Specificity was lost more rapidly as sensitivity increased requiring much higher cut-off levels to maintain low rates of false-positive classification (Fig. 1).

FeNO was measured on 44 patients able to meet ATS/ERS criteria representing patients generally over 4 years of age. Levels were significantly higher in the patients with clinical allergy to peanut than in those with peanut tolerance (median 48.3 p.p.b. vs. 21.0 p.p.b., $P = 0.0005$; Table 1). There was no significant difference in FeNO levels between patients with anaphylaxis vs. those with CANA (median 54.3 p.p.b. vs. 39.3 p.p.b., $P = 0.577$). The AUC for FeNO was 0.83 (95% CI, 0.71–0.95; Fig. 1). A FeNO of > 41.4 p.p.b. to predict allergy resulted in a specificity of 96% (95% CI 80–100%) and a sensitivity of 60% (95% CI, 36–81%).

The value of FeNO as an independent predictor for clinical allergy

Receiver operator characteristic curve analysis of the combined measures is presented in Fig. 2. Both Ara h2 sIgE and FeNO improve the diagnostic ability of SPT alone. When FeNO is added to the logistic regression model containing only SPT, the likelihood ratio statistic indicates an improvement in fit ($P < 0.001$) and the ROC curve analysis shows an improvement in the AUC ($P = 0.015$). Similarly, Ara h2 also improved the predictive power of SPT ($P < 0.001$ for likelihood ratio statistic and $P = 0.006$ for AUC). With all 3 measures in the predictive model, the AUC was 0.96 (95% CI 0.90–1.00; Fig. 2). The diagnostic tests each add to the predictive power when assessing children with peanut allergy.

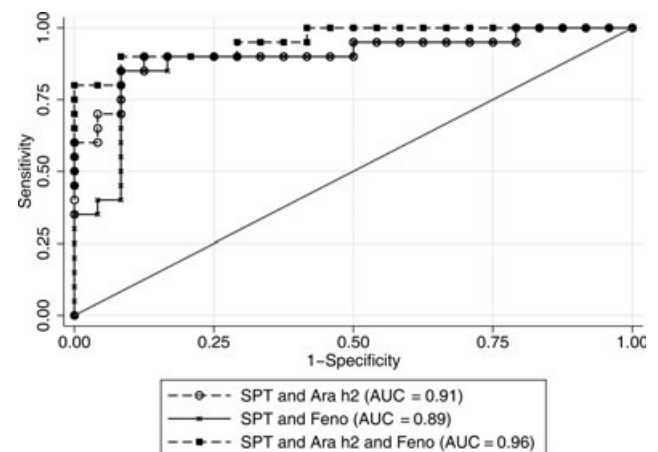


Fig. 2. Comparison of combined diagnostic methods by logistic regression analysis, then receiver operator characteristic (ROC) curve generation. Comparative area under the ROC curve (AUC) did not achieve statistical significance.

Prospective validation of a current clinical algorithm

Dang et al. [31] suggested a flow diagram to diagnose peanut allergy based on SPT result and retrospectively collected Ara h2 sIgE. Cut-off values proposed for SPT and Ara h2 sIgE are shown in Fig. 3. To assess cross-population validity, these cut-off values are applied to our cohort. Six patients are correctly identified as tolerant at Test 1, while four patients (8%) would be inappropriately labelled as peanut allergic with eight correctly identified and challenge avoided. Thirty-four (64%) would proceed to serological testing. Applying a cut-off of > 1.0 kU/L for Ara h2 sIgE would accurately identify seven patients as allergic with no false-positive results. The lower limit of < 0.1 kU/L identified six as tolerant with a 6% false-negative rate. The remaining 22 patients (41%) would proceed to food challenges. This is a reduction in OFC of 59%. The resulting challenge outcomes are presented in Fig. 3.

Modified Newcastle algorithm including FeNO

The pre-test probability for a reaction in our entire cohort was 0.43 (odds 0.75; 43% of all children; 23 of 53

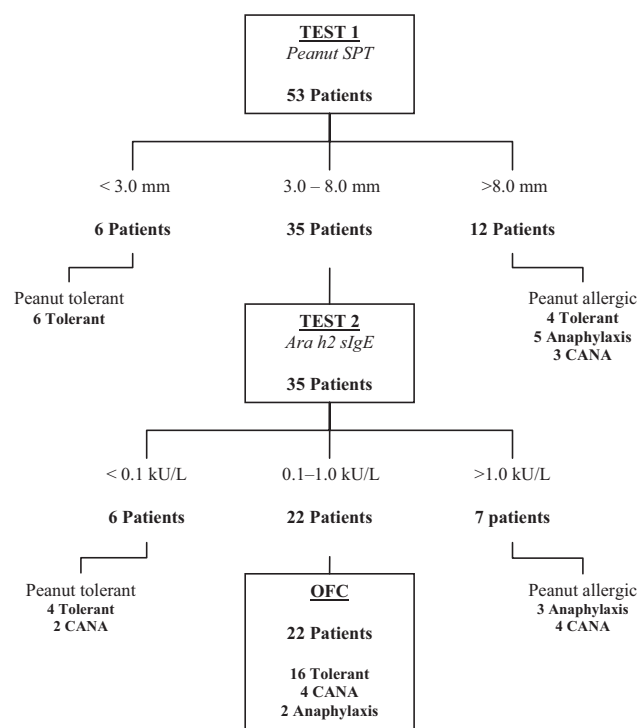


Fig. 3. Prospective validation of Ara h2 serum-specific IgE (sIgE) cut-off values. Skin prick test (SPT) and sIgE values are as published [31]. Presented in bold is patient allocation and the outcome at open-labelled peanut food challenge (OFC) for our cohort. CANA = reaction to ingested peanut that was indicative of clinical allergy *not* anaphylaxis.

subjects, Fig. 4). Improved specificity of peanut SPT in our population would be achieved by increasing a positive threshold of up to 9 mm (Fig. 4). The pre-test probability for a reaction in our cohort of children with a wheal size between 3 and 8.9 mm and available FeNO measurements was 0.49 (odds 0.96; 49%; 17 of 35 subjects, Fig. 4). In this cohort with a wheal size between 3 and 8.9 mm, we applied a cut-off value of > 45 p.p.b. and < 20 p.p.b. for FeNO. This resulted in a positive likelihood ratio (LR+) of 12.9 (sensitivity = 64%, specificity = 95%) for a clinical reaction if FeNO was > 45 p.p.b. and a negative likelihood ratio (LR–) of 0.14 (sensitivity = 93%, specificity = 50%) for no clinical reaction if FeNO was < 20 p.p.b.. Thus, the post-test odds of having a positive reaction was 12.4 if FeNO was > 45 p.p.b., and the post-test odds of having a positive reaction was 0.13 if FeNO was < 20 p.p.b. By introducing this step in screening, only 14 (40%) patients remain unclassified

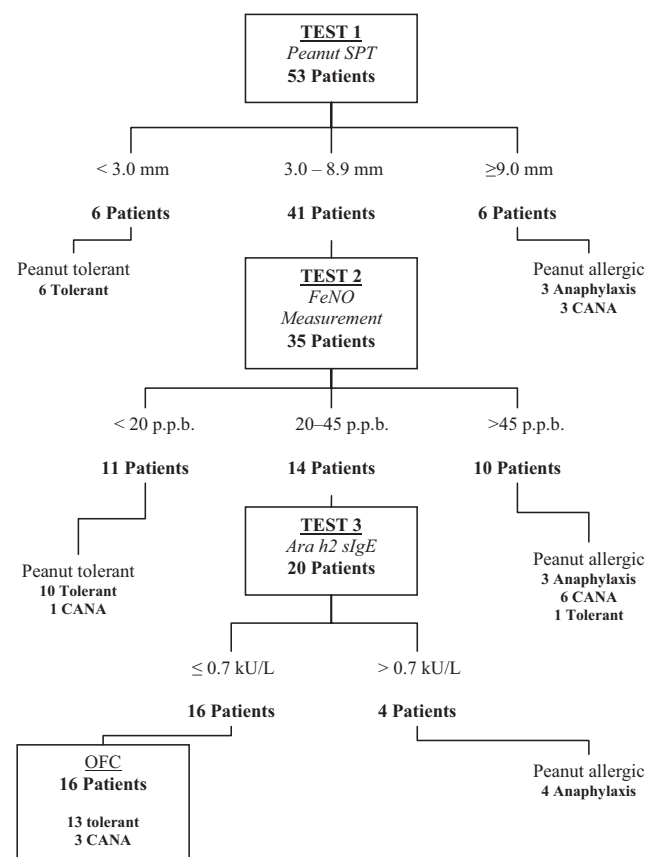


Fig. 4. Suggested three-tiered diagnostic Newcastle algorithm to evaluate children with peanut sensitisation. Non-invasive diagnostic modalities effectively screen 62% of patients into tolerant and allergic groups without requiring blood collection with $< 5\%$ falsely classified. In bold is patient allocation through the proposed algorithm and results at open-labelled peanut food challenge (OFC) in our cohort. Test 3 includes 6 patients unable to measure fraction of exhaled nitric oxide (FeNO) as per ATS/ERS guidelines. CANA = reaction to ingested peanut that was indicative of clinical allergy *not* anaphylaxis.

requiring serological testing. Six patients in our cohort were unable to satisfactorily complete FeNO measurement and would also require serological testing. With a focus on specific testing to identify allergy, an alteration in cut-off for Ara h2 sIgE level would also identify a greater proportion of children without altering the number required to challenge. By challenging only patients with a sIgE ≤ 0.7 kU/L, four patients avoid adverse reaction with no false-positive results.

With our three-tiered approach, 16 patients would remain unclassified requiring OFC. This represents 30% of the original population or a reduction in OFC requirement of 70%. Blood testing would be required in 20 patients rather than 34 following the approach suggested by Dang et al. [31]. Of the 16 challenges, 13 (81%) were tolerant, 3 had CANA and there were no episodes of anaphylaxis. This is a reduction in adverse events at food challenge of 87%. All of the 10 patients who suffered anaphylaxis were correctly identified as allergic.

FeNO levels are independent of serum nitrite levels

To determine whether FeNO measurements are influenced by systemic nitric oxide generation in this cohort (e.g. due to diet), we measured serum nitrite concentrations where available ($n = 18$ samples). There was no correlation between serum nitrite levels and FeNO (Fig. 5) nor total serum peanut IgE or Ara h2 IgE (data not shown).

Discussion

This study formally validates the use of Ara h2 sIgE as a prospective diagnostic test for clinical peanut allergy in a relevant target population. We also report the potential of FeNO as a non-invasive biomarker of clinical allergy

and tolerance in the paediatric population. The diagnostic approach proposed by Dang et al. [31] based on Ara h2 sIgE would decrease the requirement of oral food challenge in our population by 59%, close to the two-thirds reported. There is some weakness in the model with high false-positive rate (8%) at the initial stage of peanut SPT and false-negative rate (6%) using < 0.1 kU/L as a predictor of peanut tolerance. This is higher than commonly reported as an acceptable sensitivity and specificity for allergy diagnostic testing [11, 12]. There is also a requirement in this design to sample blood on all children that are to undergo food challenge.

The area under the ROC curve for Ara h2 sIgE was similar to that reported by Klemens et al. [26]. Interestingly, this group confirmed peanut allergy, based on double-blind placebo-controlled food challenge (DBPCFC) or open food challenge, in a cohort who had a mean peanut SPT of 14 mm with an interquartile range of 10–16 mm. As a consequence, in both the models presented in this study, children would have been excluded in the first step of diagnostic testing. It may represent a different group of (highly sensitized) peanut-allergic patients compared with the ones assessed at our centre. Food challenge in these children would be at much greater likelihood of clinical reaction/anaphylaxis [14, 45]. It may also explain why a much higher level of Ara h2 sIgE (> 5 kU/L) was required to reach adequate specificity. Conversely, a much lower cut-off of > 0.35 kU/L was reported to provide $> 95\%$ specificity in a population-based UK cohort [30]. The resulting ROC curve also had an improved AUC (0.99). Values in this cohort were seemingly extremely low with the 80 of 81 patients having measurable serum Ara h2 sIgE and 52 patients tolerant at challenge. Diagnostic accuracy of other Ara groups including Ara h8 and Ara h9 was poor [30], similar to results in our population (data not shown). A proposed feature of molecular allergology is that characteristics and component allergen relevance are unique to different populations [46]. The recent review by Sicherer et al. [3] presenting all currently published suggested cut-off values showed significant dichotomy. We attempt to improve reproducibility by presenting an algorithm (Fig. 4) with only an upper limit cut-off for Ara h2 sIgE. Lower limit of the test was difficult to define with two patients suffering generalized reaction with Ara h2 values < 0.1 , the level suggested by Dang et al. [31] as likely indicating peanut tolerance and low risk of reaction.

Southampton–Cork predictive models of peanut allergy diagnosis [25, 26] utilize clinical history of past reaction to peanut as a scored criteria for assessment. This is based primarily on parent or carer recollection and has the potential for subjective interpretation. One strength of our model is that history of

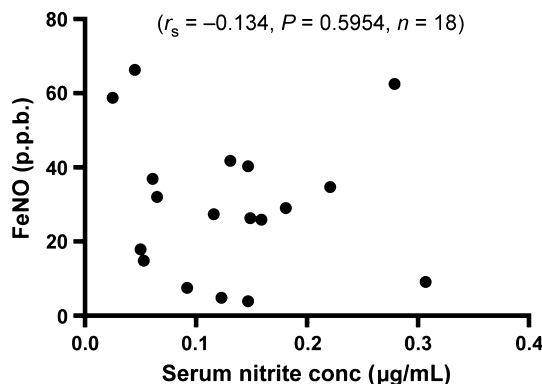


Fig. 5. Comparison of serum nitrite levels with exhaled nitric oxide demonstrating no correlation and ruling out systemic nitrite production or exogenous factors (e.g. diet) influencing fraction of exhaled nitric oxide (FeNO) variations.

reaction or atopic co-morbidities appear not to influence diagnostic accuracy. It uses validated and objective measurement modalities (Ara h2 sIgE and FeNO measurement) as the mainstays of diagnosis. This may allow a more efficient and accurate application to other populations.

FeNO is a quick and simple test to administer. Like spirometry, it also has a clearly established and validated guideline for test administration [37]. This makes it an attractive prospect for improving diagnostic accuracy and generalization in a field where user-dependent skin testing is the mainstay of objective diagnosis [1, 27]. Increased levels of FeNO have been seen in atopic asthmatic children compared with non-atopic asthmatic children, where atopy was determined by skin sensitization to a number of aeroallergens along with egg and peanut [18]. More recently, an independent association between increased FeNO levels and food sensitization, both plant and animal, has been shown [24]. In both studies, food sensitization (presence of food-specific IgE) rather than true food allergy was measured. We have demonstrated that FeNO has equivalent diagnostic accuracy as sIgE to Ara h2 in our population with comparable AUC on ROC analysis. FeNO is simple, immediate and non-invasive and therefore more attractive for the paediatric setting. As with Ara h2 sIgE, there was no difference between the two groups who showed clinical allergy. Furthermore, the diagnostic accuracy of each is improved if results are combined improving the AUC to 0.96. The positive and negative likelihood ratios in a cohort of children with a SPT wheal size between 3 mm and 8.9 mm to peanut allergen suggest that FeNO was an excellent biomarker for the prediction of a clinical reaction to peanut in this study.

Mechanisms underlying this finding remain undiscovered but are unlikely to be related to effects of diet on systemic nitric oxide generation. Nitric oxide plays a role in anaphylaxis, increasing guanylyl cyclase and causing smooth muscle relaxation [47]. Management of nitric oxide effects in refractory anaphylaxis with methylene blue has been proposed and demonstrated [48, 49]. Given the role of nitric oxide in anaphylaxis, one may have expected that the levels may have predicted anaphylaxis at challenge, but this was not the case. This suggests a role of nitric oxide in the allergic response perhaps beyond the role in anaphylaxis. It is unknown whether this method of assessment would be easily transferred to other types of food allergy (e.g. egg, milk and tree nut), and this is an area of ongoing investigation. While encouraging, the association of FeNO to challenge outcome was in a small study group and will need to be confirmed in a second cohort before any firm conclusion can be drawn on utility for clinical practice. Future work will also look to establish

trends over time perhaps predicting development of tolerance.

This is a proof of concept study. As such, a limitation is that measurements have not been taken for children that are not sensitized to peanut, that is, true controls in our population. Data on paediatric normal values for both FeNO [18, 50] and Ara h2 [29, 31] have been published elsewhere and fall well below proposed cut-off values for clinical allergy in our study. This study is also based in a tertiary referral allergy centre, and subsequently, the population will be skewed towards an allergic phenotype. The proposed diagnostic algorithm is for use in a tertiary centre due to the requirement of FeNO measurement, a relatively specialized investigative tool although handheld FeNO devices are available to be evaluated for the purpose of predicting allergic reaction to peanut in the future.

It is well documented that the demand on specialist services is increasing rapidly [51]. Improved diagnostic accuracy would decrease demand on the services required to house food challenges. Notionally, a selection bias is possible as decision to food challenge was at clinician discretion. The majority of patients challenged were on a wait list that pre-dated study commencement. Furthermore, the outcome of the challenges during the study time frame was in keeping with peanut OFCs conducted over the last 3 years at our centre, with approximately 50% having clinical reaction and half of whom had anaphylaxis. It follows that recruitment and offer of food challenge was representative of standard practice.

In summary, we show increasingly robust potential of Ara h2 as a prospective indicator of clinical peanut allergy in the paediatric population. Prediction of food challenge outcome could be substantially improved with regular use of Ara h2 sIgE and potentially FeNO in the process of planning OFC for peanut in children. While the overall number of food challenges is reduced, the performance is not perfect and OFC remains the gold standard of diagnosis. Further research is required to validate the clinical value of the proposed Newcastle algorithm in other allergy centres and to ensure cut-off values remain robust with greater numbers and across different populations. Evaluation of the underlying mechanisms may also uncover novel food allergy pathways and improve understanding in this constantly evolving field.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

K.Pr. and J.B. conducted measurements, recruited patients, and collected data. R.B. supervised oral food challenges. K.Pa. and A.C. provided laboratory support

and expertise. K.Pr., A.C., P.M. and J.M. analysed data. R.B., K.Pr., K.Pa. and J.M. conceived study design. K.Pr. and J.M. wrote manuscript, and all authors edited the final draft of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Food challenge protocols.

PEANUT CHALLENGE

CHALLENGE SUBSTANCE

Peanut Butter

Equivalency of Peanut butter to	(Oil) Roasted Peanuts
$\frac{1}{8}$ teaspoon peanut butter	0.625 grams
$\frac{1}{4}$ teaspoon peanut butter	1.25 grams
$\frac{1}{2}$ teaspoon peanut butter	2.5 grams
1 teaspoon peanut butter	5 grams

CHALLENGE PROTOCOL

Day 1

Time	Peanut butter
0	Smear inside lip (not to touch outside lip)
20 min	$\frac{1}{8}$ teaspoon
40 min	$\frac{1}{4}$ teaspoon
60 min	$\frac{1}{2}$ teaspoon
80 min	1 teaspoon (minimum last dose to call challenge negative)
100 min	2 teaspoon (if tolerated)
Daily total	3 $\frac{7}{8}$ teaspoons

OBSERVATION POST-CHALLENGE

Generally for 2 hours

HOME CONTINUATION

Days 2-7

2 teaspoons per day

After Day 7

Peanut products should be continued in diet at least weekly following a successful challenge.

NOTE: Reactions to smear inside the lip may be local (swelling at the site of application) or generalised (angioedema /urticaria at sites distant from application).

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COW'S MILK CHALLENGE

CHALLENGE SUBSTANCE

1. Less than 12 months old – cow's milk based infant formula
2. More than 12 months old - full cream cow's milk

CHALLENGE PROTOCOL

Day 1

TIME	ml milk
0	Drop inside lip (not to touch outside lip)
20 min	1 ml
40 min	5 ml
60 min	15 ml
80 min	40 ml
100 min	100 ml
Daily total	~160 ml

OBSERVATION POST-CHALLENGE

Generally for 2 hours

HOME CONTINUATION

Day 2

160 ml

Days 3-14

Increase amount as tolerated until all bottles in an infant (<12 months of age) are cow's milk based formula or daily amount is 200-300 ml (>12 months of age).

Note: Completely or partly hydrolysed (HA) formula should NOT be used for milk challenges.

SOY MILK CHALLENGE

CHALLENGE SUBSTANCE

1. Less than 12 months old – soy milk based infant formula
2. More than 12 months old - soy milk

CHALLENGE PROTOCOL

Day 1

TIME	ml milk
0	Drop inside lip (not to touch outside lip)
20 min	1 ml
40 min	5 ml
60 min	15 ml
80 min	40 ml
100 min	100 ml
Daily total	~160 ml

OBSERVATION POST-CHALLENGE

Generally for 2 hours

HOME CONTINUATION

Day 2

160 ml

Days 3-14

Increase amount as tolerated until all bottles in an infant (<12 months of age) are soy milk based formula or daily amount is 200-300 ml (>12 months of age).

WHEAT CHALLENGE

CHALLENGE SUBSTANCE

Plain wheat flour

Preparation instructions

1. Place 80 ml of milk or juice (which child is tolerant to) into a shaker.
2. Place 8 level teaspoons of wheat flour into the shaker. Note: 1 teaspoon (5mL) of wheat flour = 3 g of wheat.
3. Shake for 30 seconds.
4. Between each serve, shake the contents again for 30 seconds before administering.

CHALLENGE PROTOCOL

TIME	ml wheat in milk or juice	g of wheat
0	Touch lip	Touch lip
20 min	1 ml	0.3 g
40 min	2 ml	0.6 g
60 min	5 ml	1.5 g
80 min	10 ml	3.0 g
100 min	20 ml	6.0 g
120 min	42 ml	12.6 g
Total volume/g	80 ml	24 g

OBSERVATION POST-CHALLENGE

1-2 hours (2 hours observation is recommended in patients with a history of previous wheat reaction).

HOME CONTINUATION

1. No further wheat for 24 hours after challenge (in order to detect delayed reactions).
2. If no reaction 24 hours following a challenge, child should have wheat containing products on at least a weekly basis in their diet.

ALTERNATIVES IF CHILD REFUSES TO TAKE WHEAT FLOUR CHALLENGE

1. If using a milk or formula – try either
 - Adding 1-2 teaspoons of sugar into the mixture OR
 - Add the wheat flour into a fruit juice
2. Egg free pasta (note: 24 g of wheat flour = ~52 g of egg free pasta)
3. Semolina (note: 24 g of wheat flour = ~22 g semolina).

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COOKED EGG CHALLENGE

CHALLENGE SUBSTANCE

1 large (~50 g) egg (= 45 g of egg white and yolk)

Preparation instructions

- 1 Beat one large egg (yolk and white) with a fork for 1 minute
- 2 Place in microwave
- 3 Cook at 10 second intervals, mashing the egg mixture with a fork each time.
- 4 Cook egg until it is rendered semi-solid (approximately a total of 30 seconds of cooking is sufficient)

CHALLENGE PROTOCOL

TIME	Teaspoon amount	g of cooked egg
0	Touch lip	Touch lip
20 min	$\frac{1}{8}$	0.6
40 min	$\frac{1}{4}$	1.25
60 min	$\frac{1}{2}$	2.5
80 min	1	5.0
100 min	2	10.0
120 min	Rest of egg (~2 & 2/3 teaspoon)	13.1
Total volume/g	6 $\frac{1}{2}$	32 g of cooked egg*

* equivalent to 45 g of uncooked egg white and yolk

Note: cooking process reduces volume of egg white and yolk from 45 g to 32 g.

OBSERVATION POST-CHALLENGE

1-2 hours

HOME CONTINUATION

1. No further egg for 24 hours after challenge (to detect any delayed reactions)
2. If no reaction 24 hours following a challenge, cooked egg should be introduced into diet.

ALTERNATIVES IF CHILD REFUSES TO TAKE THE ABOVE CHALLENGE

Try mixing each subsequent dose of egg that the child has not yet taken with one of the following substances (which child is known to be tolerant to) such as:

1. Tomato sauce
2. Apple puree
3. Canadian maple syrup (contains water, sucrose, glucose, minute traces minerals, some B vitamins). Other maple syrups should not be used as they can potentially have other allergens, such as soy.

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BAKED EGG CHALLENGE

CHALLENGE SUBSTANCE

Muffin (parents required to make)

Muffin ingredients

1. 100 g of butter or Nuttalex margarine (or other milk free margarine if cow's milk allergic)
2. 160 g of sugar
3. 220 g of plain flour (if allergic to wheat, use appropriate flour – eg soy, rice)
4. 2 level teaspoons of baking powder
5. 125 ml of milk child is tolerant to (e.g. cow's, soy, rice, etc)
6. 2 large (50 g) sized eggs
7. Muffin tray (12 muffins) and muffin cups

Preparation instructions

1. Pre-heat conventional oven to 180°C
2. Mix baking powder and flour together in a bowl
3. Add butter and sugar and mix until a lumpy batter is formed
4. Add the two eggs and milk into the mix
5. Combine ingredients until a smooth batter is formed
6. Place muffin cups into the tray
7. Distribute batter evenly into each muffin cup
8. Bake for 20 minutes or until slightly golden brown and firm to touch

CHALLENGE PROTOCOL

Cut the muffin on the day of the challenge and administer as shown in the table. A maximum total of one muffin should be consumed.

Time	Amount of muffin	g of egg
0	$\frac{1}{16}$ of a muffin	0.47g
20 min	$\frac{1}{8}$ of a muffin	0.94 g
40 min	$\frac{1}{4}$ of a muffin	1.88 g
60 min	$\frac{1}{3}$ of a muffin	2.5 g
80 min	Rest of muffin	≈2.5g

OBSERVATION POST-CHALLENGE

1-2 hours

HOME CONTINUATION

1. No further muffin for 24 hours after challenge (to detect any delayed reactions).
2. If no reaction 24 hours after challenge, child can have foods containing baked egg (where a maximum 2 eggs has been used in a batch of 12 muffins or in a cake mixture).

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