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Basophil activation test shows high accuracy in the diagnosis of peanut and tree nut allergy: The Markers of Nut Allergy Study

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Statement of Contribution: LD collected and reviewed clinical data, contributed to data analysis, and wrote the manuscript. AC performed the statistical modelling and contributed to writing and critical review of the manuscript. JAH contributed to data analysis and wrote the manuscript. KS collected clinical data and generated BAT data, contributed to data entry, critically reviewed the manuscript. DS contributed to statistical modelling and performed statistical analysis and critically reviewed the manuscript. CMD, MP, JSL, LH, AB, SW, MAF, AM contributed to data collection, data entry and critically reviewed the manuscript. JEM, ARA, VHK, MA, DH contributed to data generation, clinical assessment and contributed to manuscript revision. HB and CP generated the allergen extracts and critically reviewed the manuscript. SRC, KM, MT, KN, and SJG contributed to data generation of the POISED and critically reviewed manuscript. AKR supervised and contributed to statistical modelling and critically reviewed the manuscript. ZS collected clinical data, acted a site PI Vienna, critically reviewed the manuscript. TE designed the study, contributed to generation of the manuscript, and supervised the project and collaboration. All authors reviewed the results and commented on the manuscript.

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Abstract

Background: Peanut and tree nut allergies are the most important causes of anaphylaxis. Co-reactivity to more than one nut is frequent, and co-sensitization in the absence of clinical data is often obtained. Confirmatory oral food challenges (OFCs) are inconsistently performed.

Objective: To investigate the utility of the basophil activation test (BAT) in diagnosing peanut and tree nut allergy.

Methods: The Markers Of Nut Allergy Study (MONAS) prospectively enrolled patients aged 0.5-17 years with confirmed peanut and/or tree nut (almond, cashew, hazelnut, pistachio, walnut) allergy or sensitization fromCanadian (n=150) and Austrian (n=50) tertiary pediatric centers. BAT using %CD63+ basophils (SSClow/CCR3pos) as outcome was performed with whole blood samples stimulated with allergen extracts of each nut (0.001-1000ng/mL protein). BAT results were assessed against confirmed allergic status in a blinded fashion to develop a generalizable statistical model for comparisonto extract and marker allergen-specificIgE.

Results: A mixed effect model integrating BAT results for 10 and 100 ng/mL of peanut and individual tree nut extracts was optimal. The area under the ROC curve (AUROC) was 0.98 for peanut, 0.97 for cashew, 0.92 for hazelnut, 0.95 for pistachio, and 0.97 for walnut. The BAT outperformed sIgE testing for peanut or hazelnut and was comparable for walnut (AUROC 0.95, 0.94, 0.92) ina sub-analysis in sensitized patients undergoing OFC.

Conclusions: BAT can predict allergic clinical status to peanut and tree nuts in multi-nut sensitized children and may reduce the need for high-risk OFCs in patients.

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Allergy diagnosis,Basophil, Challenge tests, Food allergy, Pediatrics, Molecular allergology, Multiple nut allergy

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Introduction

Peanut and tree nut allergy are among the most common food allergies in children across the globe.[1-4] The prevalence of peanut and tree nut allergy ranges between 1-5% and 0.05-4.9%, respectively, depending on the population, definition of allergy used, and type of study.[2,5,6]The rate of nut sensitization is also influenced by pollen sensitization, which has geographic variability. Peanut and tree nut allergies are not only common, but are the leading causes of life-threatening anaphylactic reactions.[7,8]These allergies also negatively impact the health-related quality of life (HRQL) of affected patients and their families.

The diagnosis of peanut and tree nut allergy is a result of the combination of the clinical history of an immediate allergic reaction, skin prick testing (SPT) and specific IgE (sIgE) testing. The diagnosis can become challenging when there is no clear history of peanut or tree nut consumption, or if the SPT and sIgE results are equivocal.[9]In addition, peanut allergic patients have increased rates of tree nut sensitization, and those presenting with a single tree nut sensitization are frequently sensitized to other tree nuts.[2,10]However, rates of tree nut sensitization are higher than the rates of true allergy.[2]

Oral food challenges (OFC) are the gold-standard procedure in the diagnosis of food allergy, but they can be associated with the risk of serious allergic reactions and familial anxiety.[11,12]OFCs also require medical resources that may not be readily available, especially in the context of multiple food allergies such as with individual tree nuts. OFCs are indicated when the clinical history, combined with results of SPT and sIgE testing, are equivocal, or in the absence of a clinical history. Although diagnostic decision values for SPT and sIgE have been reported in specific populations, there are patients who have results under these cut-offswho fail challenges, as well as those who can tolerate the foods despite having SPT or sIgE values above the cut-offs.[13-15] The growing need for OFCs identifies the requirement for more accurate diagnostic tests in food allergy.

Molecular allergy diagnosis (MolAD) has been investigated in food allergy with the intention to separate cross-reactivity with other allergen families and pollen allergens.[16]In particular, sensitization to the seed storage peanut protein Ara h 2 has been shown to be an important predictor of clinical reactivity.[17,18]Similar heat-stable proteins such Ana o 3(cashew and also pistachio), Cor a 9 and Cor a 14 (hazelnut), and Jug r 1(walnut) have been identified for tree nuts.[19-22]Studies investigating the relevance of these markers are encouraging but limited.

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The basophil activation test (BAT), an *in vitro* assay usingallergen stimulation in whole blood, has the potential to be used for diagnosing peanut and tree nut allergies.[23]Flowcytometry is used to evaluate degranulation and/or activation of basophils after stimulation with the respective food allergens.[24] It has been demonstrated to be useful due to its high specificity, and in particular when clinical history, SPT and sIgE results are equivocal.[23,25]

The purpose of The Markers of Nut Allergy Study(MONAS) was to determine,in a real-life setting in a large group of Canadian and Austrian children, the utility and accuracy of the BAT in the diagnosis of peanut and tree nut allergy.

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Methods

Patient Population

Patients from 6-months to 17-years with peanut and/or tree nut (almond, cashew, hazelnut, pistachio, walnut) allergy or sensitization were prospectively recruited from the Hospital for Sick Children, Toronto, Canada (n=150) and the Medical University of Vienna, Austria (n=50) between May, 2017 and November, 2019.

Clinical allergy was defined as positive OFC, or a pre-test probability of >90% of allergic reaction (SPT ≥8mm and/or sIgE≥15 kUA/L).[10,14,26] Almond allergy was defined solely based on positive OFCs or recurrent strong clinical reaction upon proven exposure. [23,27]Open OFCs, and not double-blind placebo-controlled food challenges(DBPCFCs), were performed as part of the clinical care provided at the allergy clinics at both sites. Non-allergic status was defined as tolerating an equivalent of a full serving of the food. Indeterminate allergic status was defined as sensitized individuals who were not known to tolerate the nut (no OFC, no history of tolerating nut).

Patients were eligible for enrollment to undergo further workup if they met the following inclusion criteria. 1) Peanut or tree nut allergy diagnosed based on an immediate allergic reaction to peanut or tree nut within the last 24 months, with corresponding positive sensitization, or a pre-test probability of >90% of allergic reaction (SPT≥8mm and/or sIgE≥15 kUA/L). 2) Sensitized to peanut or tree nut, where sensitization was defined by SPT>3mm and/or sIgE>0.35kUA/L,who were scheduled to undergo OFCs to at least one of the nuts. 3) Peanut and tree nut sensitized SPT>3 mm and/or sIgE>0.35 kUA/L, but who regularly consume nuts. Exclusion criteria were asfollows.1) Age <6 months. 2) Unclear history of peanut or tree nut allergy with no OFC in allergy clinic. 3) Sensitization and <90% pre-test probability of allergic reaction.

All patients were clinically assessedand clinical data was collected using a standardized data collection form.SPT was performed using peanut and individual tree nut extracts (ALK-Abello, Horsholm, Denmark) using lancets. sIgE level (peanut, individual tree nut, MOLAD) was determined using ImmunoCAP (Thermo-Fisher, Uppsala, Sweden).

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Oral food challenges

Open label OFCs were performed in accordance with internal standardized clinical protocols (see Online Repository).

Basophil activation tests

Heparinized whole blood was drawn and delivered to the research lab blinded. It was stored at room temperature and processed within <6 hours.Whole blood was stimulated with up to 7 serial 10-fold dilutions (0.001-1000ng/ml) with each ofpeanut extract (ALKAbelló), hazelnut, pistachio, cashew, walnut, and almond allergen extract. Tree nut extracts were made in house and characterized (seeOnline Repository).

The BAT was performed in accordance with the manufacturer's instructions (Bühlmann, Switzerland). Flow cytometric analysis was performed either with aCytoFLEXat the Canadian site (Beckman Coulter, United States) or a BD FACSCanto II (BD Bioscience, San Jose California) at the Austrian site. Basophils were gated as $SSC^{low}/CCR3^{+}$ cells. Basophil activation was determined as the percentage of activated basophils ($\%CD63^{+}$). Testing and analysis was performed in a blinded fashion.

In 9% of individuals who consented, eitherno blood was drawn(7.5%; n=15) or inclusion criteria were not met upon review (1.5%; n=3). 11.5% of BAT experiments were removed from analysis because of a baseline activation of >5% CD63 in the negative control(5%; n=10), because of a non-response to stimulation with either FMLP or FcƐRIcross-linking antibody(3.5%; n=7), or because of one with technical issues orfivehaving too low basophil counts (3%; n=6).

Assessment of allergen specific sensitization patterns

In addition to the clinically performed allergen testing,sIgE was measured with the Allergy Explorer (ALEX) array (Macro Array Diagnostics, Austria). Patient serum was diluted 1:5 and incubated on the ALEX chip containing 156 extracts and 126 molecular allergen markers as described recently.[28,29] The chips were imaged and quantified within the dynamic range of 0.3-40 kUA/L (Raptor, Macro Array Diagnostics).Positive sensitizations were defined as ≥0.30 kUA/L, as reported recently[28,30].

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Statistical analysis

Performance of BAT was examined against the allergic status to individual nuts using receiver-operating characteristic (ROC) curves via application of statistical models. All modeling and subsequent analyses were performed in R version 3.6.1 (R Core Team 2019). Figures were generated using the *ggplot2* package in R.[31]Bayesian logistic regression models were fit to predict the allergy status per nut per person using a variable slope, variable intercept mixed effect framework for each allergen concentration per allergen. Concentrations of 10 and 100 (ng/ml) were chosen forstatistical modelling as these models resulted with the greatest area under the ROC (AUROC) while maintaining minimal model complexity. Details of original model and model coefficients can be found in the Online Repository. The final equation used in modelling was:

Allergic=conc10+conc100+(1+conc10+conc100|allergen)

Where there is a variable slope for each concentration and variable intercept for each allergen.

An optimal threshold along the ROC curve was chosen using the Youden's Index[31], which corresponds to the maximum true positive rate (TPR) and minimum false positive rate (FPR) for each nut (*this is identical to maximizing both the sensitivity and specificity)*. The same methodology was applied to the performance of BAT against OFC status to individual nuts.

External validation cohort

To determine the applicability of the established statistical model in an assay using a similar outcome measurement and whole blood stimulation (after ~24 hours at 4^{o} C, with measurement of high CD63^{+} expression), with a different set of antibodies, and without IL-3 stimulation *in vitro*, external validation was performed in an independent cohort of peanut allergic patients with DBPCFC proven peanut allergy from the POISED trial (n=109 analyzed; n=9 non-responders; n=2 no data available).BAT results from the baseline visit were used. A detailed description of the patient population and methods was reported recently.[32,33]

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Ethics approval

Research ethics board (REB) approval was obtained at both Austrian (EK Nr.: 2302/2016) and Canadian (1000053791) study sites.

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Results

Study Population

Two-hundred patients were prospectively recruited for the study, 150 from Toronto and 50 from Vienna. Three were excluded because they had not undergone OFCs in the clinic by the end of the enrolment period (Figure 1). A total of 182 patients had blood samples available for MolAD, and 159 patients had BAT data for analysis.BAT analysis was conducted only with data that met the criteria defined above for clinical allergy or confirmed tolerance.The study population had a mean age of 8.5 years with male predominance. Fifty-two percent of patients had a diagnosis of asthma, 40% allergic rhino-conjunctivitis to environmental allergens, and 63% atopic dermatitis (Table 1).

Patients from the two sites were comparable with regards to sex, asthma, allergic rhino-conjunctivitis, concomitant egg allergy, andnumber of previous episodes of anaphylaxis (Table 1). Mean age of the children was higher in the Canadiangroup (9.4y vs. 5.9y) and Canadian individuals were more likely to have a history of atopic dermatitis (67% vs. 48%; p=0.01). Food allergies other than peanut, tree nut and sesame allergy,were more frequently diagnosed in the Canadian cohort (62% vs 38%, p=0.003 and 19% vs 2% p=0.004) respectively.

In total, 244 OFCs were performed in 107 patients. Results of 36 OFCs were positive. Mean SPT size and sIgE reported in Table E2 of the Online Repository. Individuals were most frequently sensitized to peanut (86%), hazelnut (76%) and pistachio (67%). No positive OFCs for pistachio or almond in either the Toronto or Vienna groups were reported. Astier scores based on challenge results indicated no difference regarding the severity of the clinical reaction between peanut, hazelnut, and walnut (2.6 ± 1.3; 2.0 ± 0.9 vs 2.4 ± 0.9; Table E2the Online Repository).

BAT discriminates between peanut and tree nut allergic and non-allergic patients

The main objective of this study was to determine whether the BAT could distinguish between allergic and non-allergic patients.The percentage of CD63 expressionwas used as the main outcome parameter. Activation via increasing concentrations of the individual nut extract (0.001-1000ng/mL) in whole blood from allergic and non-allergic children were compared. Patients who were peanut and tree nut allergic displayed a statistically significant higher %$CD63^+$ basophils (p<0.01)upon

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stimulation with 0.01-1000ng/mL (cashew, hazelnut, peanut, walnut) or 0.1ng-1000ng (pistachio; Figure 2). Sensitivity and specificity values for the BAT were good for peanut, hazelnut, walnut, cashew, and pistachio (Table E3 in the Online Repository). Results for peanut were most consistent with high sensitivity and specificity (95.3% vs. 93.2%), good positive predictive values (PPV), and negative predictive values (NPV; 96% vs. 91%). Although the sensitivity fortree nuts waslower (85.4-91.7%), specificity was 100% for pistachio and walnut.

Mixed effect model integrating 10 and 100ng/mL of peanut and individual tree nut stimulation is effective in diagnosing allergy

A mixed effect model integrating 10 and 100 ng/mL of peanut and individual tree nut extractswas selected to be optimalregarding specificity and sensitivity. A total of 429 observations over 147 participants were used in the model. Almond was excluded from statistical modeling because of the high rate of negative food challenges. The AUROC was 0.98 for peanut, 0.97 for cashew, 0.92 for hazelnut, 0.95 for pistachio, and 0.97 for walnut (Figure3A).Individual data of normalized %$CD63^+$ basophils of each nut for the concentrations 10 and 100 ng/mL are displayed in Figure S1 of the Online Repository.39/48 patients who underwent almond OFCs had BAT performed. The negative predictive value of BAT for almond reactivity was 100% (using positive BAT criteria of >10% $CD63^+$basophils at concentrations of 10 and 100 ng/mL).Age did not influence the performance of the BAT in our patient cohort.

BAT separates allergic from non-allergic status in patients who underwentclinically indicated OFCs

To determine if the BAT could distinguish between allergic and non-allergic patients in children with equivocal/absent history and specific IgE<15kUA/L and/or SPT<8mm,we investigated BAT results in patients who had positive OFCs versus those who had negative OFCs. For peanut, hazelnut, or walnut as a culprit allergen,those with positive OFCs had increased %$CD63^+$basophils at all nut concentrations compared to those who passed the OFC (Figure4). Cashew, pistachio, and almond were excluded from modelling because of paucity of positive OFCs. The AUROCwas 0.95

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for peanut, 0.94 for hazelnut, and 0.92 for walnut (Figure3B, Table E2the Online Repository). Sensitivity was 100% for peanut with a specificity of 92.3%. Hazelnut and walnut BAT displayed high specificity (95% vs 100%) with a slightly reduced sensitivity.

BAT outperforms extract-specificIgEtesting for peanut and hazelnut and is equally predictive for walnut allergy

While high sIgE levels (≥15kUA/L) or SPT ≥8mm to peanut and nut extracts are utilized in the diagnosis of food allergy, their high sensitivity combined with low specificity leads to overdiagnosis in case of equivocal history and lower sensitization values. In patients with clinical allergy, elevated levels of sIgE to extracts and molecular allergen markers were observed irrespective of the nature of the allergen (i.e. seed storage proteins, PR10, nsLTP; Tables E4-E9in the Online Repository).

In patients with positive OFCs and equivocal histories,increased sIgEwas demonstrated to the seed storage proteins Ara h 1, 2, 3, and 6 in peanut, Cor a 14 in hazelnut, and Jug r 1 and 2 in walnut, respectively (TablesE4b, E5b, E6b inthe Online Repository). In contrast, allergens from the PR-10 and nsLTP families in peanut and hazelnut did not differ significantly between groups with the exception of Cor a 8, which was only detected at low levels of unclear significance(Table E5b in the Online Repository).

In general, the BAT outperformed both extract and molecular allergen sIgE testing when assessingOFC. In patients requiring OFC confirmation for peanut allergy, Ara h 2 sIgE was the best performing molecular allergen marker test with an AUROC of 0.92 (Figure 5), which was most comparable to the performance of the peanut BAT with an AUROC of 0.95 (Figure 4). For hazelnut allergy, Cor a 14 sIgE was the best MolADmarker with an AUROC of 0.82 (Figure 5), albeit secondary to the performance of the hazelnut BAT(0.94, Figure 4B). For walnut allergy, Jug r 1 sIgE had a comparable AUROC of 0.93 (Figure 4B, Figure 5).

Validation of the mixed effect model in an external cohort of peanut allergic patients

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To determine the applicability of the established statistical model, external validation was performed in an independent cohort of peanut allergic patients with DBPCFC proven peanut allergyand BAT data from the POISED trial (n=109 analyzed; n=9 non-responders; n=2 no data available).[31]BAT values were compared with peanut allergic patients with positive OFC results from the MONAS cohort by utilizing the optimal cut-off determined in the MONAS cohort for the normalized fraction bound valuesof %$CD63^+$at 10 and 100 ng/mL of peanut extract. Utilizing this algorithm, the sensitivity in thePOISED cohortwas 0.88, as compared with sensitivity of 0.95 in the MONAS cohort (Figure S2 in the Online Repository). Specificity could not be calculated as data for negative peanut OFCs was not available from the POISED group.

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Discussion

Peanut and tree nut allergies are the most frequent causes of food-associated anaphylaxis.Patients with peanut allergy or a single tree nut allergy often have co-sensitization to multiple tree nuts. It is likely that the test related cross-reactivity is higher than clinical co-reactivity, resulting in unnecessary avoidance. The main purpose of the MONAS study was to prospectively investigate the utility of the BAT in discriminating between peanut and tree nut allergic and peanut/treenut sensitized individuals in a "real-life" setting in tertiary care allergy outpatient clinics. In addition, we compared BATand allergen sIgE testing.

In ourcohort of pediatric and adolescent patients from Canada and Austria, the BAT demonstrated theability to predictthe allergic clinical status in peanut and tree nut sensitized individuals.

Clinical allergy diagnosisis currently dominated by a combination of clinical history and extract-based SPT or sIgE testing.[34]While DBPCFCs are the gold standard if reaction thresholds, delayed reactions, or more subjective symptoms are evaluated, open OFCs represent the standard in clinical settings to assess immediate reactions using objective symptoms. Objective criteria were utilized in the open OFCs in this study.[15,35,36]The NUT CRACKER study,in line with our data,reported ahigher rate of co-sensitization thanco-allergy to tree nuts.With exception of highly cross-reactive tree nut pairs (pistachio/cashew and pecan/walnut), 30% of patients had a co-allergy.[37]The Pronuts study assessed open OFC proven rate,or a high pre-test probability,of coexistent peanut, tree nut, and sesame seed allergy,and compared it to conventional food allergy diagnostic tests.[38]We utilized a similar definition for the diagnosis of food allergy as the Pronuts study. They found that 60.7% of children had more than one nut or seed allergy. This is higher than previous studies which were mostly retrospective. This is comparable to our cohort with 52.6% of patients having an allergy to more than one nut. Using only BAT results,51.2% had a concurrent positive BAT to at least one more nut. Both the Pronuts and the MONAS cohorts, are derived from patients referred to tertiary centers and multi-sensitized complex patients are more likely to be seen in these settings.

Our study suggests that the BAT is more accurate than conventional allergy diagnostic methods, including SPT and sIgE testing, in the diagnosis of peanut and tree nut allergy. SPT is associated with false-positive results and sIgE can produce false-negative results.[39]For example, sIgE

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to peanut has a sensitivity of 75-100% and specificity of 17-63%.[17,18,26,40-44]The performance of BAT in the diagnosis of food allergy has been investigated in multiple foods, including peanut[25,44-47], hazelnut[48-50], cow's milk[51,52], egg[47,52], wheat[53,54], shellfish[55], sesame[56], and in pollen-food syndromes[57,58], with sensitivity and specificity ranging from 85-100%, and 75-100%, respectively.[23]The performance of the BAT in tree nut allergy is not well characterized. BAT use in food allergy diagnosis has been suggested to be more accurate than sIgE in several studies.[23,25,47,59,60] In one study of BAT use in peanut allergy, specificity and sensitivity was reported as between 94-96% and 95-100%, respectively.[25] BAT reduced OFCs by two-thirds, superior to Ara h 2-sIgE and SPT.[25]The BAT has also been studied to estimate the threshold of peanut allergic reactions.[61] In our study, BAT specificity and sensitivity for peanut were 93% and 95%, respectively, comparable to what has been reported in the literature.

Some utility of the BAT has been demonstrated for the diagnosis of hazelnut allergy.[48,49] In the recent NUT CRACKER study, for individual tree nuts (walnut, pecan, cashew, pistachio, hazelnut), BAT was superior to SPT with regards to specificity, but not sensitivity. However, comparability is limited, as only one concentration of allergen (1000ng/ml) was used. We developed a mixed-effects model integrating 10 and 100ng/mL of allergen. AUROC values were ≥0.95 for peanut and all tree nuts except hazelnut (0.92). In addition, the model was validated in an independent cohort of peanut allergic patients with positive OFCs. This is important for future approaches like meta-analysis of BAT data from clinical trials and comparable studies.

One of the strengths of the MONAS dataset is the direct comparison of conventional allergy testing (SPT, sIgE), MolAD, and BAT. MolAD data supporting the clinical value of peanut molecular allergen marker testing is strong.[17,18,62]Less data exists on the clinical value of Cor a 9 and Cor a 14 for hazelnut allergy, Jug r 1 for walnut allergy, and Ana o 3 for cashew allergy.[19-22,63]In our patients with equivocal history and sensitization, both Ara h 2 and peanut BAT were able to predict clinical reactivity (AUROC 0.92 vs 0.95). For the diagnosis of hazelnut allergy,the value of sIgE was limited(AUROC 0.65)whereas BAT was excellent (AUROC 0.94). Cor a 14 was the only relevant hazelnut allergen to predict allergy. BAT performed similarly to extract sIgE and Jug r 1 or Jug r 2 for walnut. DetailedMolADco-sensitization matrix in our patients is displayed in Figure S3in the Online

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Repository.Additional analyses combining MolAD with BAT data did not demonstrate significant superiority to the use of BAT alone (Table E10 in the Online Repository).

Our study differs from the Pronutsand the NUTCRACKER study as it was based on clinically relevant challenges. Challenges matched the clinical needs. The important message is that the majority of those patients who have an equivocal history or asymptomatic sensitization do not react during an OFC (14.8% MONAS, 26% Pronuts).[38]Given the limited availability of OFCs, this study highlights the importance of a novel tool to aid in clinical decision making about the appropriate use of these scarce resources. The BAT could be a very important additional tool to SPT, sIgE tests, and MolADtesting to avoid unnecessary OFCs.

Another approach involves pooled OFCs based on BAT results. Applying our model toa group of participants with indeterminate allergic status, 66% of walnut, pistachio, cashew and hazelnut and 93.3% of peanut sensitized individuals in this group had negative BATs (Figure S4 in the Online Repository). Using the BAT-based prediction for the complete cohort, there would be a justification to do an OFC to almost 80% of the nuts they are currently avoiding. The anticipated result would be a negative challenge.Prospective studies are needed to confirm this finding.

Our results demonstrate that the BAT has the potential to decrease the number of OFCsneeded, and allows for precision medicine in the diagnosis of peanut and tree nut allergy, in particular with multi-nut sensitizations. While there are technical limitations to performing the BAT in smaller clinical centres, patients who are being considered for an OFC could be referred for a BAT at specialized centres. Although indirect BAT tests have the potential to pool samples compared to direct BAT which uses fresh blood, they can require the same level of infrastructure (e.g. Need for cell cultures). In the case of a negative BAT,a low risk OFCcan be conducted at the smaller clinical centre, instead of in a more resource-intensive hospital setting.

Other strengths of the MONAS study include that it was a prospective study, and was conducted at tertiary pediatric centres on two different continents with comparable birch pollen exposure. The prospective nature is important as it reflects the value of the respective tests in a setting where testing and challenging is limited by timing and availability.

Based on recent retrospective[64] and prospective data[37,65]almond SPT/sIgE shows low specificity and no sIgE cut offs with >90% predictive value are available. No patients had a positive

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almond OFC in our patients despite sIgE values of up to 53.4 kUA/L and SPT results up to 8mm. Thus, in line with recent challenge proven data, routine testing to almond as part of panel testing should prompt OFCs even in the event of positive test results.

Disadvantages with the BAT include the need for rapid processing of the blood sample, although the POISED study used blood refrigerated for ~1 day before testing, and more resources. The non-responder rate was 4% in the MONAS cohort, comparable to what is reported in the literature.[23]While we do not suggest that BAT should become first-line in the routine diagnosis of food allergy, it does have the potential to transform food allergy diagnosis in clinical scenarioswith equivocal clinical history and multiple sensitizations. It can also be ofimportant value for safe food introduction in high-risk populations.

In conclusion, the MONAS study demonstrates that the BAT is effective in diagnosing peanut and tree nut allergy in a real-world setting, and may be useful in reducing the number of resource-intensive OFCs needed. Additional prospective studies to investigate the ability of the BAT to reduceOFCs are required.

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Tables

Table 1. Demographics of patient population.

Clinical characteristics	**All patients (n=197)**	**Toronto (n=149)**	**Vienna (n=48)**	***P* value**
Mean age (IQR)	8.53±4.70 (4.6-12.3)	9.37 ± 4.67 (5.8-17.9)	5.9 ± 3.77 (5.8-13.2)	**<0.001**
Male sex (%)	122 (62)	90 (60)	32 (67)	0.44
Asthma (%)	102 (52)	82 (55)	20 (42)	0.1
Allergic rhinoconjunctivitis (%)	80 (40)	64 (43)	16 (33)	0.22
Atopic Dermatitis (%)	123 (63)	100 (67)	23 (48)	**0.01**
Food allergy other than peanut/tree nut (%)	110 (56)	92 (62)	18 (38)	**0.003**
Previous anaphylaxis (%)	87 (44%)	61 (41)	26 (54)	0.13

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Figure Legends

Figure 1. Study design and analysis: Among the 200 participants who were recruited for the MONAS study (150 Toronto, 50 Vienna), 3 did not meet inclusion criteria and 15 did not have their blood drawn. Of the 182 available samples, BAT data could not be obtained from 23 participants due to several factors including technical issues (n=1), high background activity (n=10), non-responder basophils (n=7) and low cell count (n=5). BATs were performed on a total of 159 samples for the tested nuts: peanut (n=143), hazelnut (n=117), walnut (n=114), cashew (n=117), pistachio (n=110), and almond (n=121). BAT measurements from these 159 samples were used for subsequent data analysis.

Figure 2.BAT shows increased proportion of activated CD63+ basophils in peanut and tree nut allergic patients compared to non-allergic patients. Boxplots depicting the distribution of normalized %$CD63^+$ basophils for all samples of each nut and each concentration (0.001 to 1000 ng/mL). Allergic samples are depicted in red and non-allergic samples are depicted in blue(peanut allergic n=87, non-allergic n=46; hazelnut allergic n=48 non-allergic n=42, walnut allergic n=41, non-allergic n=35; cashew allergic n=50, non-allergic n=24; pistachio allergic n=45, non-allergic n=25). Dashed lines indicate the median values for each concentration. For each nut and concentration, the difference between allergic and non-allergic samples was statistically significant ($p<0.05$ Welch's t-test), except for pistachio at 0.01 ng/mL concentration.

Figure 3.3A: Mixed effect model is effective in distinguishing between peanut and tree nut allergic and non-allergic patients. Models are fit using data from 10 ng/mL and 100 ng/mL concentrations. Allergic samples are in red and non-allergic samples are in blue. ROC curves for each individual nut. All nuts show similar model performance (AUROC for each nut is depicted in the plot legend). **3B: Basophil activation test distinguishes between OFC-positive and OFC-negative patients in those who underwent OFCs.** The model was fitted using the same model equation using data from only OFC-confirmed samples at 10 ng/mL and 100 ng/mL concentrations. ROC curves for

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each individual nut with OFC-confirmed samples. All nuts show similar model performance (AUROC for each nut is depicted in the plot legend).

Figure 4. Basophil activation test distinguishes between OFC-positive and OFC-negative patients in those who underwent OFCs: Boxplots depicting the distribution of normalized %$CD63^+$ basophils for samples with OFC outcomes for peanut, hazelnut, or walnut at each concentration (0.001 to 1000 ng/mL). OFC positive samples are depicted in red and OFC negative samples are depicted in blue.

Figure 5. Allergen-specific IgE to Ara h 2, Cor a 14, and Jug r 1 are the best molecular allergen diagnostic markers to distinguish between OFC-positive and OFC-negative patients: Each line depicts a ROC curve for each allergen from peanut, walnut and hazelnut, obtained by thresholding the raw allergen-specific IgE concentrations (kU_A/L). The best performing sIgE markers for each nut were Ara h 2, Cor a 14, and Jug r 1 (AUROC for each allergen is depicted in the plot legend).

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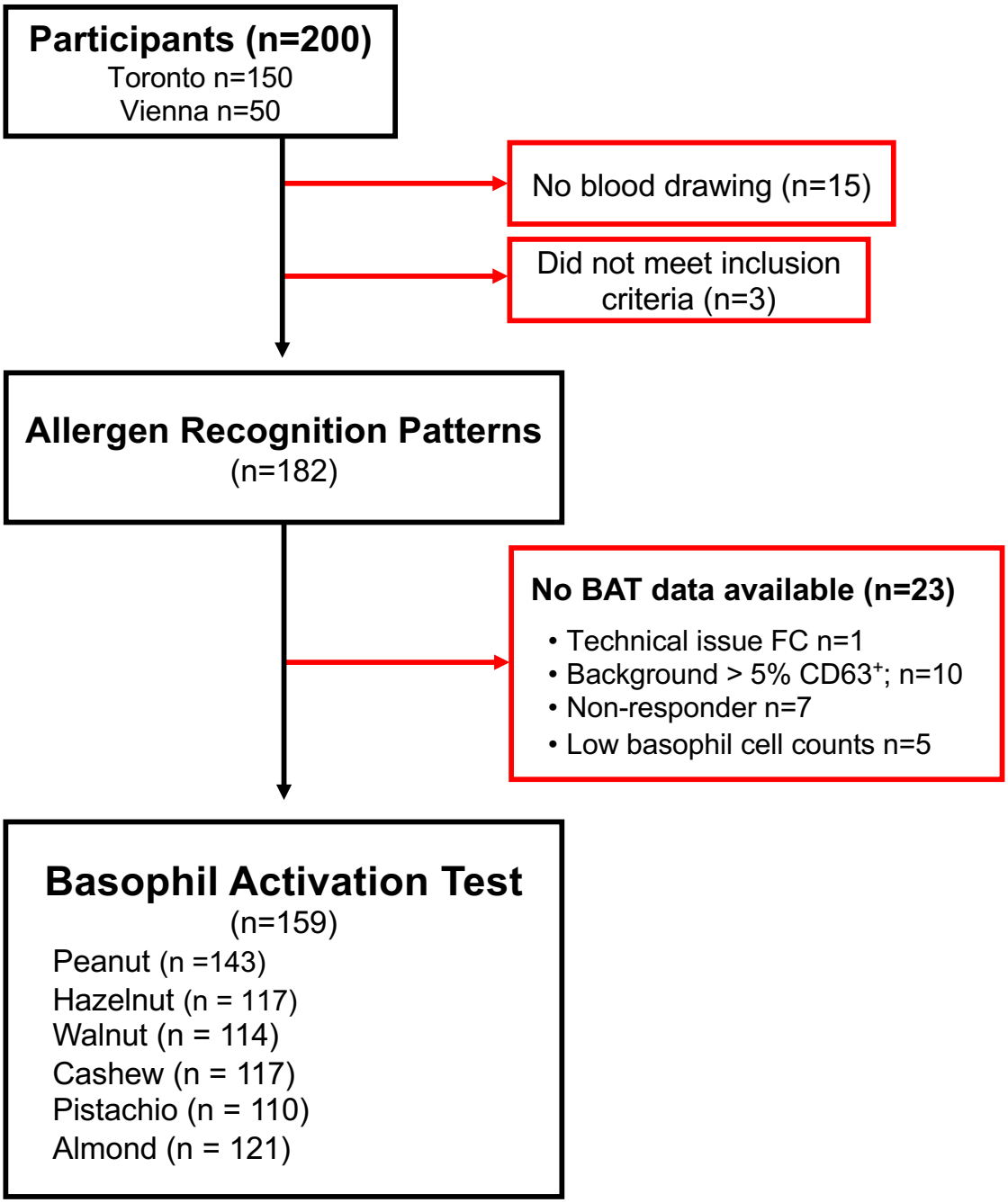


Figure 1

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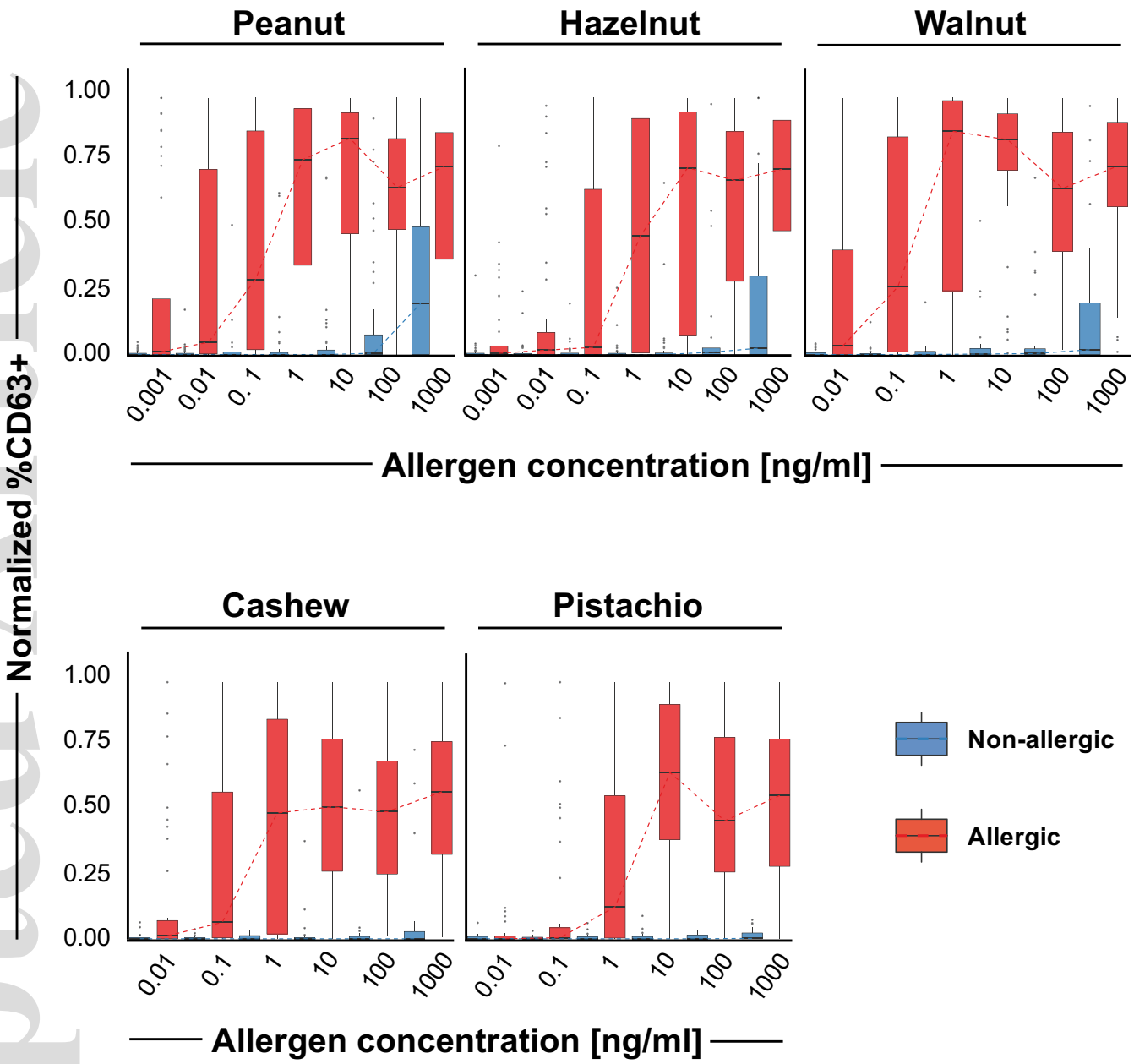


Figure 2

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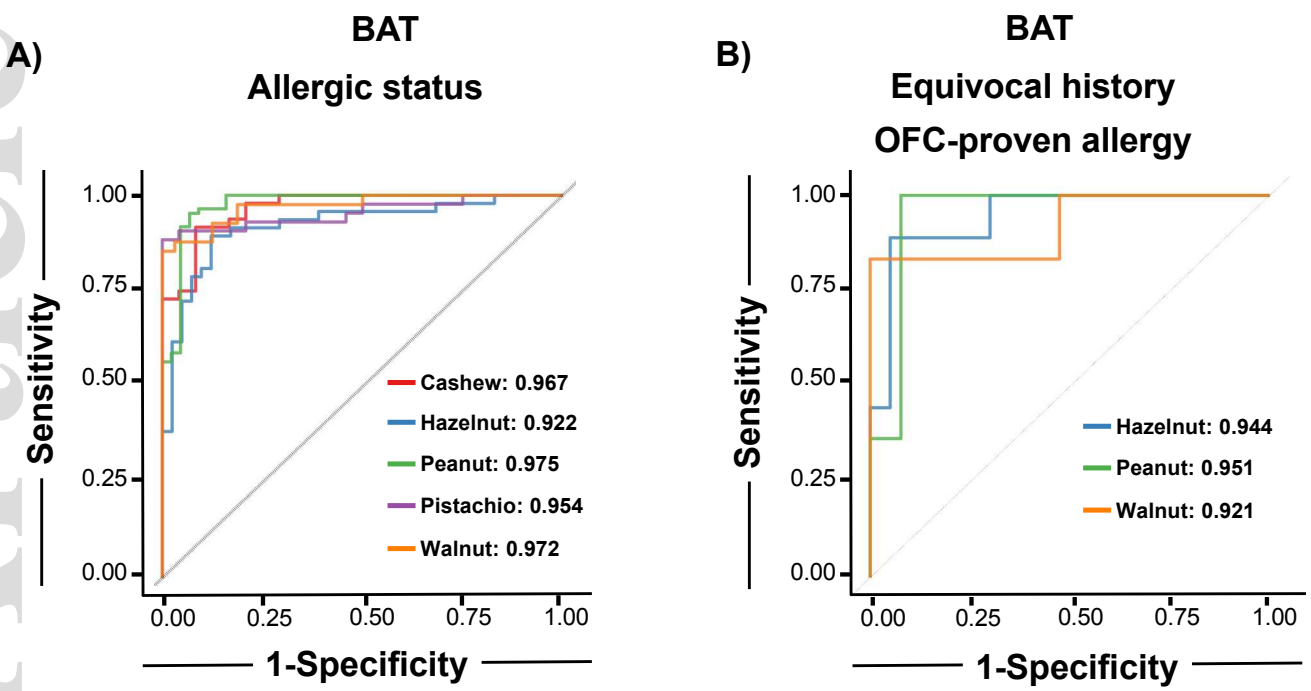


Figure 3

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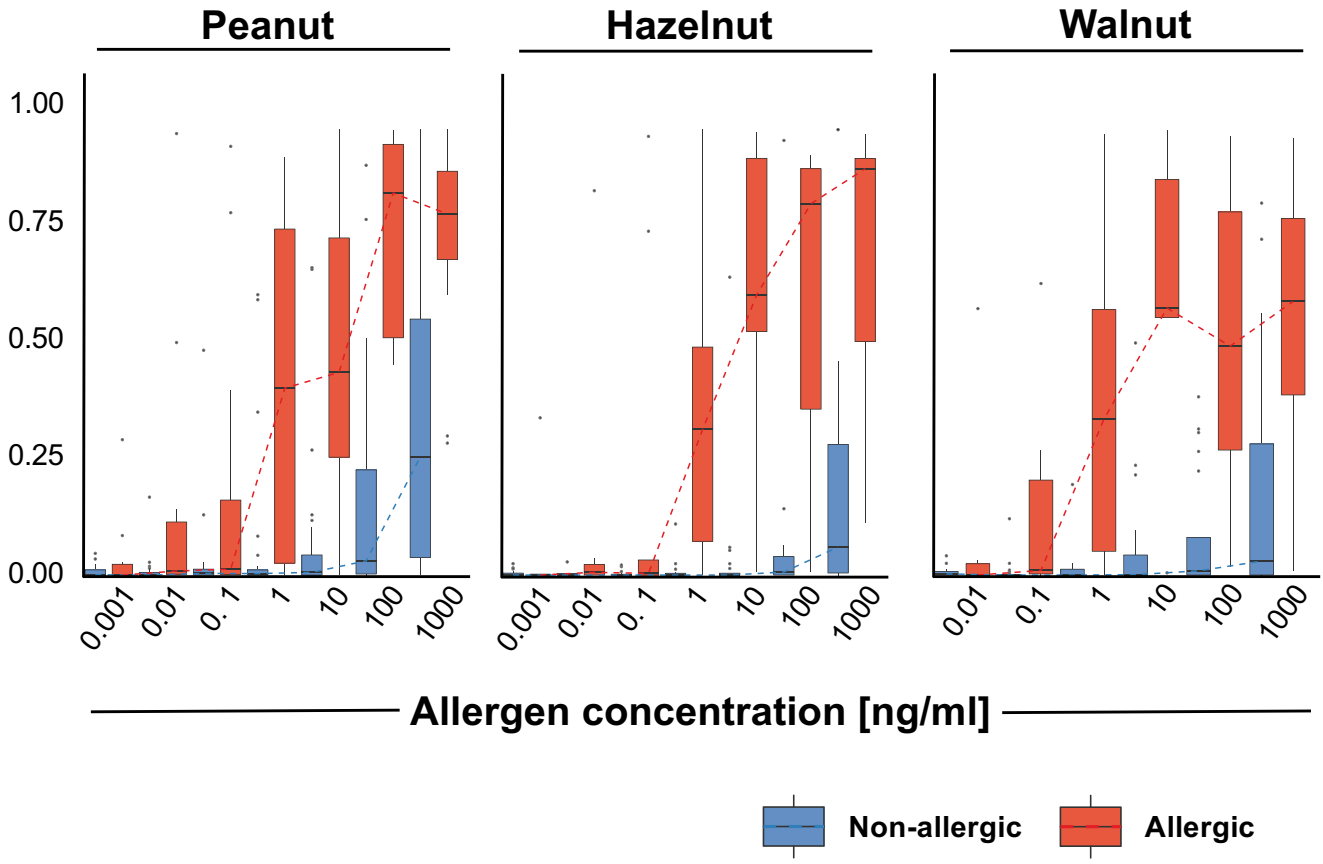


Figure 4

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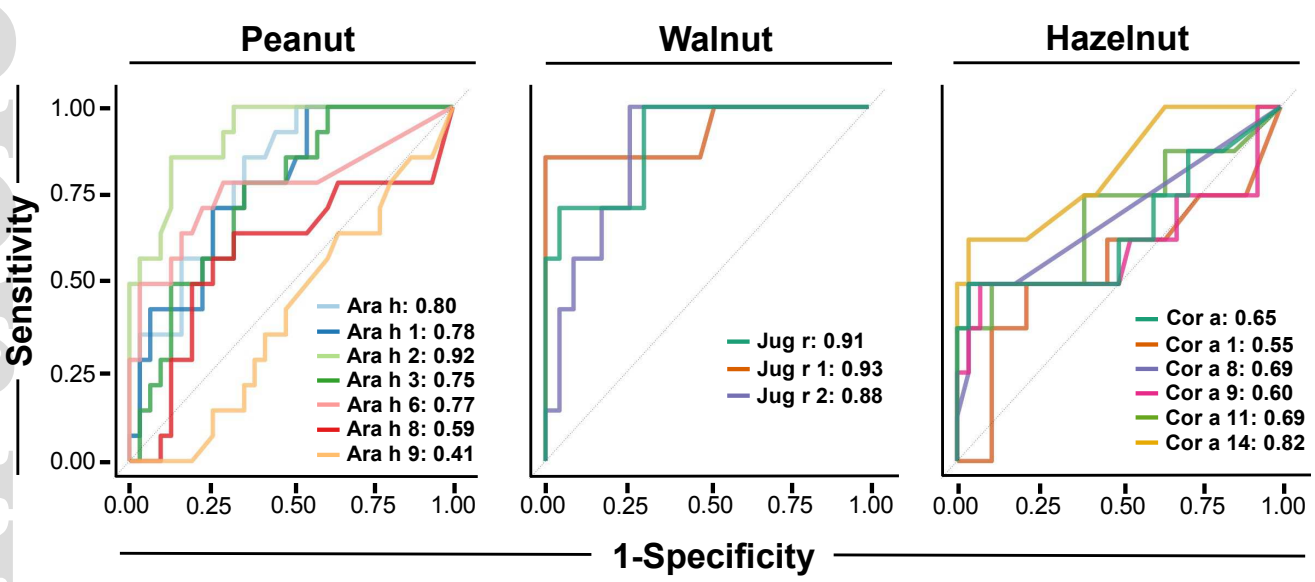
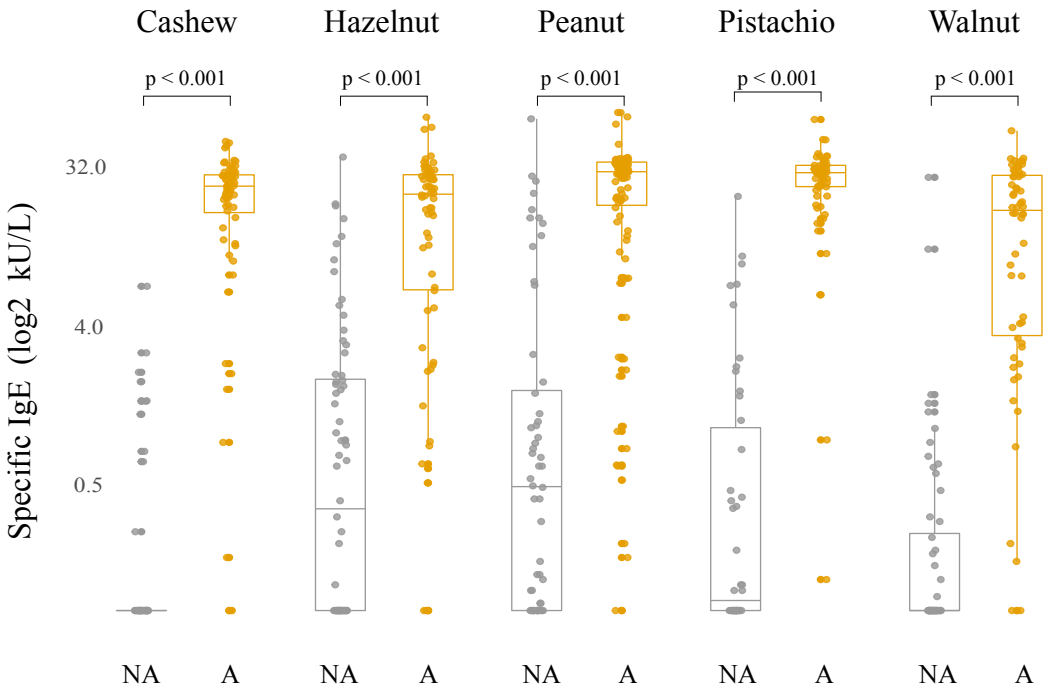
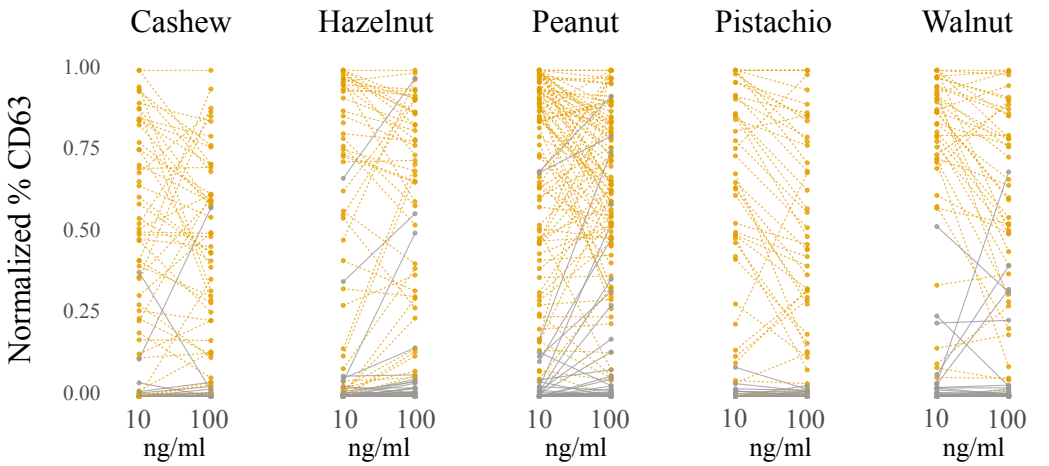


Figure 5

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Cashew
Hazelnut
Peanut
Pistachio
Walnut
p < 0.001
Specific IgE (log2 kU/L)
32.0
4.0
0.5
NA
A

A


Cashew
Hazelnut
Peanut
Pistachio
Walnut
Normalized % CD63
1.00
0.75
0.50
0.25
0.00
10
100
ng/ml

B

Figure S1

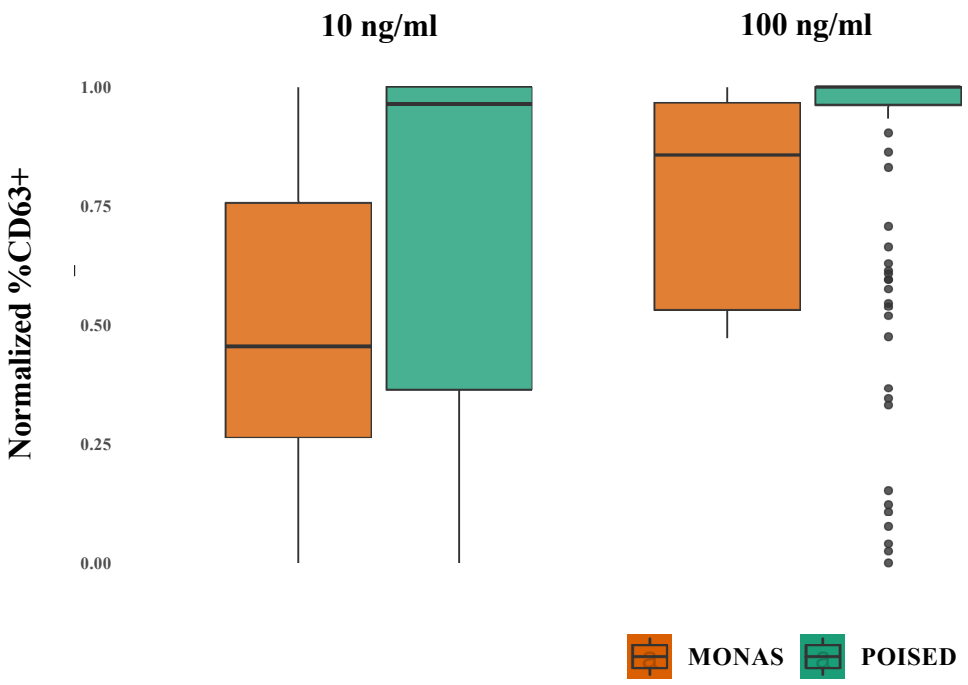

10 ng/ml
100 ng/ml
Normalized %CD63+
1.00
0.75
0.50
0.25
0.00
MONAS
POISED

Figure S2

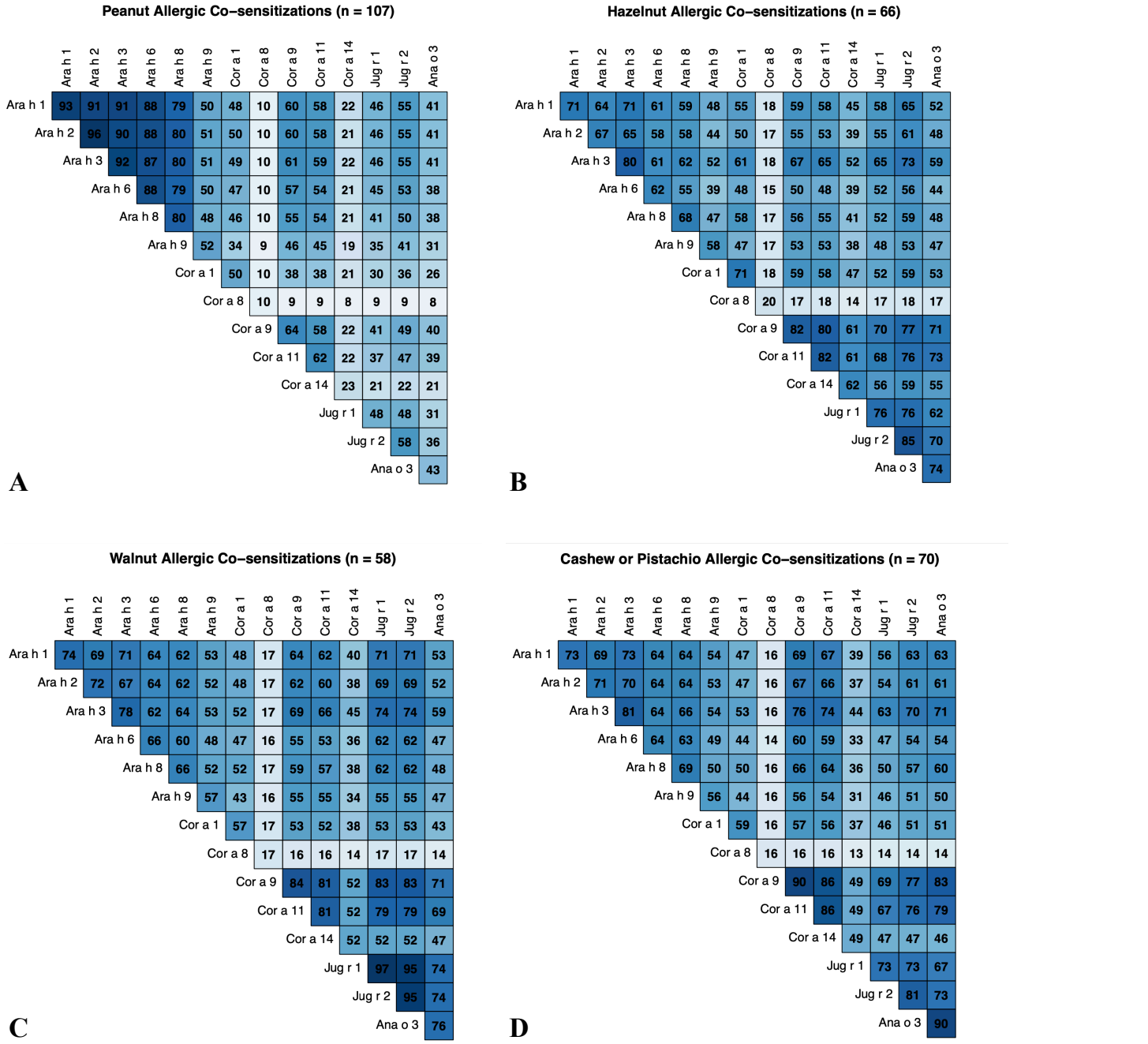
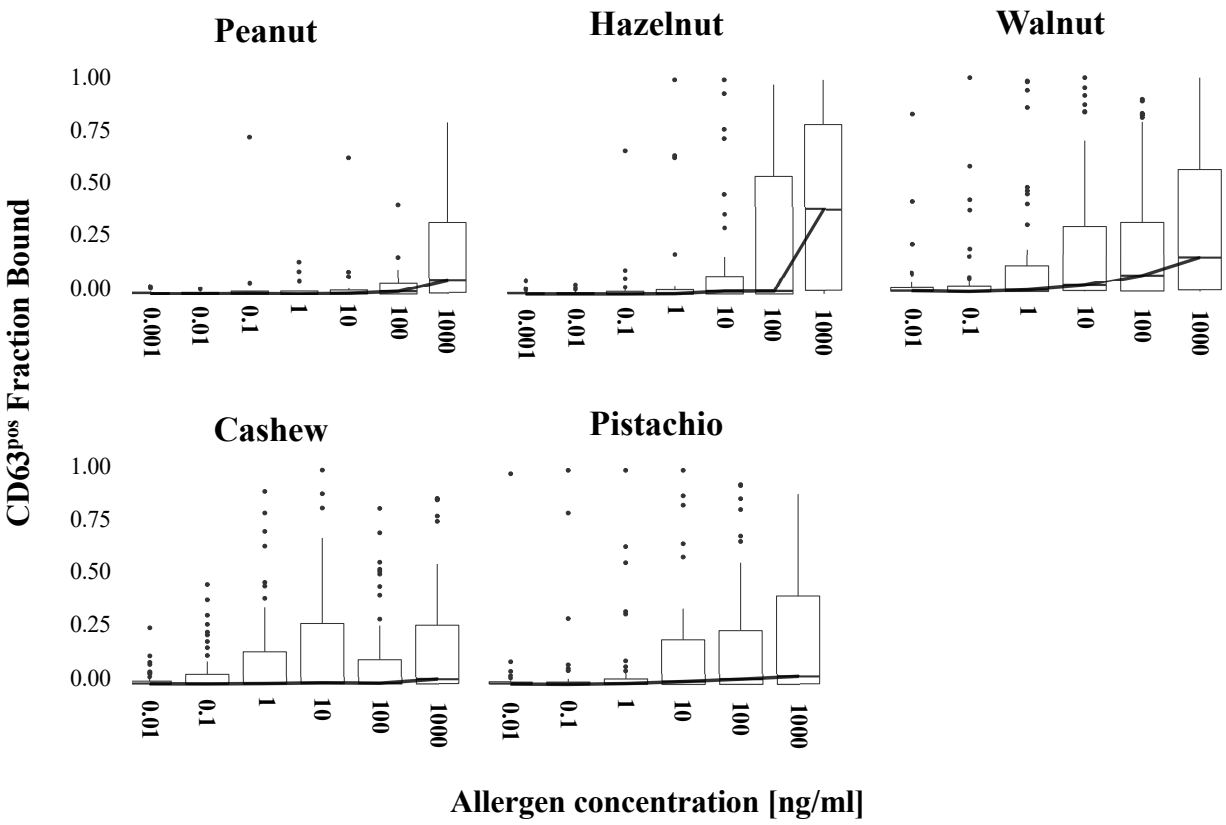
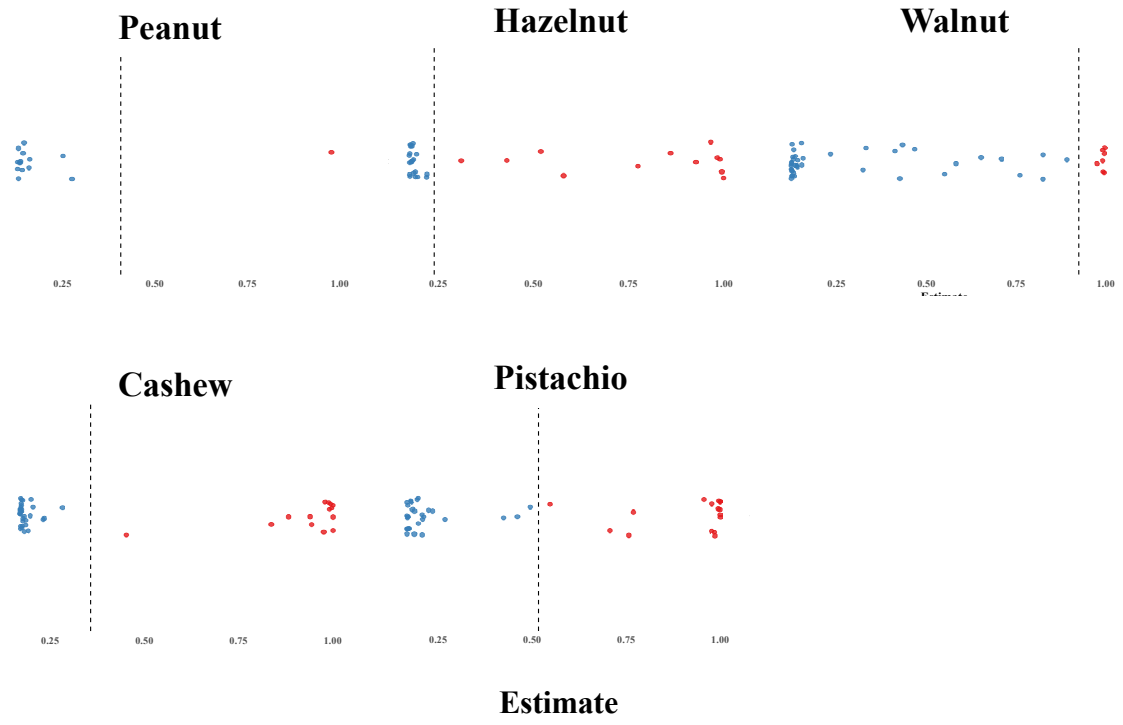

Peanut Allergic Co−sensitizations (n = 107)
Ara h 1 93 91 91 88 79 50 48 10 60 58 22 46 55 41
Ara h 2 96 90 88 80 51 50 10 60 58 21 46 55 41
Ara h 3 92 87 80 51 49 10 61 59 22 46 55 41
Ara h 6 88 79 50 47 10 57 54 21 45 53 38
Ara h 8 80 48 46 10 55 54 21 41 50 38
Ara h 9 52 34 9 46 45 19 35 41 31
Cor a 1 50 10 38 38 21 30 36 26
Cor a 8 10 9 9 8 9 9 8
Cor a 9 64 58 22 41 49 40
Cor a 11 62 22 37 47 39
Cor a 14 23 21 22 21
Jug r 1 48 48 31
Jug r 2 58 36
Ana o 3 43
A
Hazelnut Allergic Co−sensitizations (n = 66)
Ara h 1 71 64 71 61 59 48 55 18 59 58 45 58 65 52
Ara h 2 67 65 58 58 44 50 17 55 53 39 55 61 48
Ara h 3 80 61 62 52 61 18 67 65 52 65 73 59
Ara h 6 62 55 39 48 15 50 48 39 52 56 44
Ara h 8 68 47 58 17 56 55 41 52 59 48
Ara h 9 58 47 17 53 53 38 48 53 47
Cor a 1 71 18 59 58 47 52 59 53
Cor a 8 20 17 18 14 17 18 17
Cor a 9 82 80 61 70 77 71
Cor a 11 82 61 68 76 73
Cor a 14 62 56 59 55
Jug r 1 76 76 62
Jug r 2 85 70
Ana o 3 74
B
Walnut Allergic Co−sensitizations (n = 58)
Ara h 1 74 69 71 64 62 53 48 17 64 62 40 71 71 53
Ara h 2 72 67 64 62 52 48 17 62 60 38 69 69 52
Ara h 3 78 62 64 53 52 17 69 66 45 74 74 59
Ara h 6 66 60 48 47 16 55 53 36 62 62 47
Ara h 8 66 52 52 17 59 57 38 62 62 48
Ara h 9 57 43 16 55 55 34 55 55 47
Cor a 1 57 17 53 52 38 53 53 43
Cor a 8 17 16 16 14 17 17 14
Cor a 9 84 81 52 83 83 71
Cor a 11 81 52 79 79 69
Cor a 14 52 52 52 47
Jug r 1 97 95 74
Jug r 2 95 74
Ana o 3 76
C
Cashew or Pistachio Allergic Co−sensitizations (n = 70)
Ara h 1 73 69 73 64 64 54 47 16 69 67 39 56 63 63
Ara h 2 71 70 64 64 53 47 16 67 66 37 54 61 61
Ara h 3 81 64 66 54 53 16 76 74 44 63 70 71
Ara h 6 64 63 49 44 14 60 59 33 47 54 54
Ara h 8 69 50 50 16 66 64 36 50 57 60
Ara h 9 56 44 16 56 54 31 46 51 50
Cor a 1 59 16 57 56 37 46 51 51
Cor a 8 16 16 16 13 14 14 14
Cor a 9 90 86 49 69 77 83
Cor a 11 86 49 67 76 79
Cor a 14 49 47 47 46
Jug r 1 73 73 67
Jug r 2 81 73
Ana o 3 90
D

Figure S3


Peanut
Hazelnut
Walnut
Cashew
Pistachio
CD63pos Fraction Bound
1.00
0.75
0.50
0.25
0.00
0.001
0.01
0.1
1
10
100
1000
Allergen concentration [ng/ml]

A


Peanut
Hazelnut
Walnut
Cashew
Pistachio
0.25
0.50
0.75
1.00
Estimate

B

Figure S4

Online Repository

Supplemental Methods

Oral food challenges

Open label OFCs were performed in accordance with internal standardized clinical protocols. These protocols used dosing for children 6 months-17 years of age which were adapted and modified from PRACTALL guidelines.[1] The total protein dose of 3.90 g for 6-24 months was based on the AAAAI Work Group Report for Conducting an Oral Food Challenge to Peanut in an Infant.[2] Children 3-11 and 12-17 years of age were given a cumulative protein dose of 4.40 g and 7.30 g, respectively. A starting dose of 3 mg protein was used for all age groups. A total of five doses were provided, at 15-20 minutes intervals (Table E1 in the Online Repository). All children were required to stay for observations for a minimum of 1 hour following the fifth dose. Positive and negative challenges were categorized based on PRACTALL guidelines. Food materials used were either crushed whole shelled nuts or nut butters.

Basophil activation test:

Whole blood was collected in heparinized tubes and processed within 6 hours. Whole blood was stimulated with allergen or stimulation control at 37℃ in stimulation buffer containing calcium, heparin, and IL-3 and a staining reagent containing anti-CD63-FITC and anti-CCR3-PE (BÜHLMANN). Positive stimulation control was performed with anti-human FcεRI monoclonal antibody. Erythrocytes were then lysed with lysis buffer for 8 minutes as

room temperature, and cells were centrifuged at 500 G for 5 minutes then resuspended in wash buffer. Flow cytometry data was acquired with CytoFLEX (Beckman Coulter, United States) and analyzed with CytExpert software (version 2.3.0.84). Basophils were gated as $SSC^{low}/CCR3^{+}$ cells. Basophil activation was determined as the percentage of activated basophils ($\%CD63^{+}$ %).

Tree nut extracts were made in-house. In brief, shelled nuts were frozen at -20°C, ground in a blender, and defatted with 5 volumes (w/v) n-hexane at room temperature followed by drying overnight at 4 °C. Soluble proteins were extracted from the defatted nut flours by stirring with 7 volumes of PBS containing 3% polyvinyl polypyrrolydone and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany) for 4 h at 4 °C. Afterwards, the samples were centrifuged for 1 h at 14 000 rpm, the supernatants were cleared by centrifugation and filtration. The supernatants were freeze dried and stored at -80 °C. The total protein concentration of each nut extract was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA).

Statistical analysis

The chi-square and Fisher's exact tests were used to compare categorical variables. The Kruskall-Wallis and Mann-Whitney tests was used to compare age between more than two groups. Mean values, median values, and interquartile ranges (IQRs) were reported. A *p*-value of less than 0.05 was considered statistically significant. GraphPad Prism 8 was used for the aforementioned statistical analysis. Welch's *t*-test for unequal sample size and variances were used to compare normalized $\%CD63^{+}$ means between non-allergic and allergic groups.

Concentrations of 10 and 100 (ng/ml) were chosen for statistical modelling as these models resulted with the greatest area under the ROC (AUROC) while maintaining minimal model complexity. The original model consisted of concentrations 0.001-1000 ng/ml. 0.1 and 1

ng/ml were removed because not all samples had data points in these concentrations. 1000 ng/ml was removed because most samples showed a high degree of binding due to saturation. For the remaining 3 concentrations, combinations of 2 and 3 variable regression models were trialed before the final model was chosen. All final models were fit using the brms package with default priors.[3] Four chains and 20,000 iterations for each model were utilized, where 10,000 of them were discarded as warmup per chain. All models converged with either zero or negligible number of divergent iterations and showed good chain mixing upon visual inspection.

We employed a mixed-effects model to be able to account for different binding properties of different allergens while simultaneously utilizing information from variance and covariance structures between different concentrations and allergens. The default priors were chosen (student-t for intercept and standard deviation for allergen level intercept and Cholesky LKJ Correlation for group level random effects) because they are weakly informative priors and are easily overcome by data.

External validation cohort

One hundred nine individuals from the POISED study were selected out of 120 participants. One standard deviation above the baseline (unstimulated control) was considered a positive BAT result. To reflect the methodology in our study, we chose concentrations of 10 and 100 and normalized CD63% values using positive and negative controls. Welch's t-test for unequal sample sizes was used to compare the mean fraction bound (i.e., Normalized) CD63% values of the POISED cohort and the MONAs cohort. The mixed model fit on all patient data was used to obtain predicted probabilities for each individual and concentration in the POISED cohort. The threshold corresponding to Youden's J Index, maximizing TPR and FPR, was

chosen and applied to the predicted probabilities to obtain predictions of allergic and non-allergic status.

71 **Model Coefficients and Summary**

Model Coefficients and Summary

Model Summary

Population Level Summary

	Estimate	Est.Error	CI l-95%	CI u-95%	Rhat	Bulk_ESS	Tail_ESS
Intercept	-1.76	0.4	-2.59	-1.04	1	24044	24011
conc10	8.22	2.73	3.32	14.33	1	13927	11874
conc100	4.17	3.82	-2.29	13.34	1	8615	6779

Group level Summary

	Estimate	Est.Error	CI l-95%	CI u-95%	Rhat	Bulk_ESS	Tail_ESS
sd(Intercept)	0.54	0.56	0.02	1.95	1	13305	15209
sd(conc10)	3.67	3.26	0.13	12.28	1	12782	16680
sd(conc100)	6.11	4.74	0.39	18.18	1	8991	9047
cor(Intercept,conc10)	-0.09	0.5	-0.91	0.84	1	23411	22948
cor(Intercept,conc100)	0.12	0.48	-0.81	0.91	1	16201	19233
cor(conc10,conc100)	0.01	0.49	-0.86	0.87	1	17866	25866

Fixed effects

	Estimate	Est.Error	Q2.5	Q97.5
Intercept	-1.758025	0.4027687	-2.590986	-1.036006
conc10	8.224776	2.7255001	3.323435	14.330894
conc100	4.16659	3.816437	-2.285219	13.343462

Random Effects

allergen	Intercept			
	Estimate	Est.Error	Q2.5	Q97.5
cashew	0.146642	0.4464769	-0.627899	1.1797479
hazelnut	0.1914474	0.4386569	-0.51299	1.2168845
peanut	-0.176412	0.4439335	-1.201129	0.5709467
pistachio	0.1117816	0.4619499	-0.750062	1.1397062
walnut	-0.253165	0.4848968	-1.423997	0.4741769

allergen	conc10			
	Estimate	Est.Error	Q2.5	Q97.5
cashew	0.3363704	2.988441	-5.791454	7.017328
hazelnut	-2.236463	3.185032	-10.05611	2.302799
peanut	0.6298087	2.839898	-5.13838	6.844697
pistachio	1.8410354	4.373178	-4.575915	13.454095
walnut	-0.270442	2.861116	-6.674016	5.529833

allergen	conc100			
	Estimate	Est.Error	Q2.5	Q97.5
cashew	2.035326	4.160092	-6.037233	11.249231
hazelnut	-1.177243	3.920034	-10.45998	5.757788
peanut	-3.226155	3.940684	-12.67374	3.076117
pistachio	6.191176	7.185843	-1.994302	24.895639
walnut	-2.854157	4.107517	-12.68743	3.736786

Combined

Intercept				
	Estimate	Est.Error	Q2.5	Q97.5
cashew	-1.611383	0.3530397	-2.297289	-0.874891
hazelnut	-1.566577	0.3098167	-2.150714	-0.932954
peanut	-1.934436	0.3616066	-2.755263	-1.328815

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Model Coefficients and Summary

pistachio	-1.646243	0.3744461	-2.400603	-0.903009
walnut	-2.01119	0.4312128	-3.0393	-1.342364

conc10

	Estimate	Est.Error	Q2.5	Q97.5
cashew	8.561147	2.887956	3.4300496	15.21004
hazelnut	5.988313	2.442545	0.9968577	10.52575
peanut	8.854585	2.160682	5.2615488	13.75565
pistachio	10.065812	4.943447	3.178277	22.84823
walnut	7.954334	2.164862	4.1558434	12.71638

conc100

	Estimate	Est.Error	Q2.5	Q97.5
cashew	6.2019159	3.449164	0.8538304	14.045701
hazelnut	2.989347	1.863664	-0.363205	7.041394
peanut	0.9404349	1.403807	-1.908396	3.629052
pistachio	10.357766	7.936328	0.8890066	30.43102
walnut	1.3124327	2.124054	-3.097671	5.297074

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Supplemental Tables

Table E1. Amount of food in grams used for OFCs, by age group.

Ages 6-24 months						
Food	**Total Portion /protein**	**First dose (3 mg protein)**	**Second dose (100 mg protein)**	**Third dose (200 mg protein)**	**Fourth dose (1000 mg protein)**	**Fifth dose (2600 mg protein)**
Peanuts	16.65/3.90	0.01	0.42	0.84	4.21	10.93
Tree nuts	16.54[a]/3.90	0.01	0.42	0.84	4.21	10.93
Ages 3-11 years						
Food	**Total Portion /protein**	**First dose (3 mg protein)**	**Second dose (100 mg protein)**	**Third dose (300 mg protein)**	**Fourth dose (1000 mg protein)**	**Fifth dose (3000 mg protein)**
Peanuts	18.5/4.40	0.01	0.42	1.25	4.17	12.5
Tree nuts	18.5[a]/4.40	0.01	0.42	1.25	4.17	12.5
Ages 12-17 years						
Food	**Total Portion /protein**	**First dose (3 mg protein)**	**Second dose (300 mg protein)**	**Third dose (1000 mg protein)**	**Fourth dose (3000 mg protein)**	**Fifth dose (3000 mg protein)**
Peanuts (peanut butter)	30.43/7.30	0.01	0.42	4.17	12.5	12.5
Tree nuts	30.43[a]/7.30	0.01	0.42	4.17	12.5	12.5

[a] Volume was limited to 1 serving per nut versus total protein provided.

Table E2. OFC, SPT, sIgE, and Astier score results in patients who had OFC to peanut and individual tree nuts. OFCs shown as number of positive OFCs in total number of OFCs performed for individual nuts.

	Number of positive OFCs/OFCs performed (%)	**SPT size (Mean (min-max), in mm)**	**ImmunoCAP (Mean (min-max), in kUA/L)**	**Astier Score (Mean (min-max))**
Peanut	15/48 (31)	4.6 (0-15)	14.7 (0.1-100)	2.6 (1-5)
Hazelnut	9/38 (24)	2.7 (0-16)	11.39 (0.06-100)	2.0 (1-3)
Walnut	7/31 (23)	1.5 (0-5)	2.5 (0.1-15.7)	2.4 (1-3)
Cashew	1/23 (4)	1.6 (0-6)	1.1 (0.04-3.9)	3.0
Pistachio	0/18 (0)	0.8 (0-4)	2.1 (0.16-8.2)	0
Almond	0/48 (0)	2.5 (0-8)	5.0 (0.1-53.4)	0

Table E3. Diagnostic performance of BAT using optimal model threshold, determined per nut.

Population	Allergen	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[b] (%)	Threshold (probability)
Whole Population (n = 147)	Peanut (n = 129)	95.3	93.2	96.4	91.1	0.407
	Hazelnut (n = 88)	89.4	87.8	89.4	87.8	0.239
	Walnut (n = 73)	85.4	100.0	100.0	84.2	0.925
	Cashew (n = 72)	91.7	91.7	95.7	84.6	0.357
	Pistachio (n = 67)	88.4	100.0	100.0	82.8	0.516
OFC Confirmed (n = 70)	Peanut (n = 37)	100.0	92.3	84.6	100.0	0.356
	Hazelnut (n = 29)	88.9	95.0	88.9	95.0	0.348
	Walnut (n = 25)	83.3	100.0	100.0	95.0	0.641

[a] PPV=positive predictive value; [b] NPV=negative predictive value

Table E4. E4a: Peanut extract and peanut allergen-specific IgE in MONAS cohort with clinical allergy. E4b: Specific IgE to peanut allergen molecular allergen markers in patients with equivocal history who underwent OFC (n = 48).

Table E4a:

	Peanut Allergic (n=109)	Peanut Non-Allergic (n=59)	p-value
Ara h (Extract)			**<0.0001**
Mean (SD)	26.6 (14.3)	4.83 (10.7)	
Median [Min, Max]	30.9 [0.00, 67.1]	0.505 [0.00, 61.7]	
Ara h 1			**<0.0001**
Mean (SD)	15.3 (9.89)	2.15 (6.01)	
Median [Min, Max]	16.1 [0.00, 44.3]	0.0400 [0.00, 35.3]	
Ara h 2			**<0.0001**
Mean (SD)	27.6 (14.9)	1.92 (5.91)	
Median [Min, Max]	31.6 [0.0400, 72.2]	0.0850 [0.00, 30.2]	
Ara h 3			**<0.0001**

Mean (SD)	13.8 (11.1)	2.75 (8.76)	
Median [Min, Max]	11.9 [0.00, 43.3]	0.105 [0.00, 56.3]	
Ara h 6			**<0.0001**
Mean (SD)	18.2 (12.9)	0.955 (4.12)	
Median [Min, Max]	19.6 [0.00, 53.9]	0.00 [0.00, 27.4]	
Ara h 8			**<0.0001**
Mean (SD)	3.62 (5.73)	2.10 (5.40)	
Median [Min, Max]	2.11 [0.00, 32.2]	0.0750 [0.00, 29.2]	
Ara h 9			**0.0020**
Mean (SD)	1.39 (4.12)	2.30 (8.51)	
Median [Min, Max]	0.320 [0.00, 26.9]	0.180 [0.00, 51.2]	

Missing data: Allergic n = 2, Non-Allergic n = 3
P-values determined using 2-sided Mann-Whitney test.

Table E4b:

	OFC Negative (n=33)	**OFC Positive (n=15)**	**p-value**
Ara h (Extract)			**0.0008**
Mean (SD)	5.07 (11.9)	17.6 (21.6)	
Median [Min, Max]	0.740 [0.00, 61.7]	10.1 [0.670, 66.9]	
Ara h 1			**0.0026**
Mean (SD)	2.45 (6.60)	8.94 (13.1)	
Median [Min, Max]	0.170 [0.00, 35.3]	2.80 [0.0800, 44.3]	
Ara h 2			**<0.0001**
Mean (SD)	1.28 (3.51)	21.7 (22.9)	
Median [Min, Max]	0.100 [0.00, 18.3]	14.4 [0.240, 64.8]	
Ara h 3			**0.0071**
Mean (SD)	3.67 (10.7)	7.42 (10.6)	
Median [Min, Max]	0.250 [0.00, 56.3]	2.99 [0.140, 33.7]	
Ara h 6			**0.0028**
Mean (SD)	0.633 (2.55)	10.3 (16.4)	
Median [Min, Max]	0.0200 [0.00, 14.1]	1.86 [0.00, 51.8]	
Ara h 8			0.3520
Mean (SD)	1.95 (5.83)	1.18 (2.48)	
Median [Min, Max]	0.110 [0.00, 29.2]	0.330 [0.00, 9.42]	
Ara h 9			0.3716
Mean (SD)	1.24 (5.13)	0.210 (0.137)	
Median [Min, Max]	0.200 [0.00, 28.9]	0.190 [0.00, 0.480]	

Missing: OFC negative n = 2, OFC positive n = 1
P-values determined using 2-sided Mann-Whitney test.

Table E5. E5a: Hazelnut extract and hazelnut allergen-specific IgE in MONAS cohort with clinical allergy. E6b: Hazelnut extract and hazelnut allergen-specific IgE in patients with equivocal history who underwent OFC (n = 38).

Table E5a:

	Hazelnut Allergic (n=67)	**Hazelnut Non-Allergic (n=70)**	**p-value**
Cor a (Extract)			**<0.0001**
Mean (SD)	20.6 (14.4)	2.85 (6.22)	
Median [Min, Max]	23.1 [0.00, 63.0]	0.380 [0.00, 37.5]	
Cor a 1.0401			**<0.0001**
Mean (SD)	8.39 (12.3)	3.08 (9.71)	
Median [Min, Max]	1.07 [0.00, 52.1]	0.135 [0.00, 61.8]	
Cor a 8			**<0.0001**
Mean (SD)	1.57 (7.28)	0.217 (1.44)	
Median [Min, Max]	0.0200 [0.00, 56.4]	0.00 [0.00, 11.8]	
Cor a 9			**<0.0001**
Mean (SD)	8.97 (9.63)	1.19 (3.56)	
Median [Min, Max]	5.94 [0.00, 40.2]	0.195 [0.00, 26.6]	
Cor a 11			**<0.0001**
Mean (SD)	13.1 (12.9)	1.85 (4.33)	
Median [Min, Max]	9.66 [0.00, 49.0]	0.0700 [0.00, 29.0]	
Cor a 14			**<0.0001**
Mean (SD)	2.70 (4.11)	0.307 (2.05)	
Median [Min, Max]	0.915 [0.00, 17.2]	0.0300 [0.00, 16.9]	

Missing data: Allergic = 1, Non-Allergic = 2
P-values determined using 2-sided Mann-Whitney test.

Table E5b:

	OFC Negative (n=29)	**OFC Positive (n=9)**	**p-value**
Cor a (Extract)			0.1957
Mean (SD)	3.81 (5.50)	18.1 (21.0)	
Median [Min, Max]	1.37 [0.00, 20.3]	9.61 [0.00, 53.8]	
Cor a 1.0401			0.6737
Mean (SD)	4.06 (12.3)	2.98 (3.81)	
Median [Min, Max]	0.135 [0.00, 61.8]	0.740 [0.00, 9.25]	
Cor a 8			**0.0161**
Mean (SD)	0.02 (0.06)	0.15 (0.20)	
Median [Min, Max]	0.00 [0.00, 0.290]	0.05 [0.00, 0.540]	

Cor a 9			0.3835
Mean (SD)	1.18 (2.02)	7.90 (12.1)	
Median [Min, Max]	0.485 [0.00, 9.75]	2.27 [0.0100, 33.6]	
Cor a 11			0.1064
Mean (SD)	2.07 (3.17)	12.3 (17.4)	
Median [Min, Max]	0.485 [0.00, 11.0]	3.48 [0.00, 47.7]	
Cor a 14			**0.0037**
Mean (SD)	0.0739 (0.172)	3.79 (6.13)	
Median [Min, Max]	0.0400 [0.00, 0.920]	0.810 [0.0300, 17.2]	

Missing data: OFC negative n = 1, OFC positive n = 1

P-values determined using 2-sided Mann-Whitney test.

Table E6. E6a: Walnut extract and walnut allergen-specific IgE in MONAS cohort with clinical allergy. E6b: Specific IgE to walnut allergen extract and molecular allergen markers in patients with equivocal history who underwent OFC (n = 31).

Table E6a:

	Walnut Allergic (n=58)	**Walnut Non-Allergic (n=58)**	**p-value**
Jug r (Extract)			**<0.0001**
Mean (SD)	17.8 (13.5)	0.924 (4.08)	
Median [Min, Max]	18.7 [0.00, 52.8]	0.00 [0.00, 28.7]	
Jug 1			**<0.0001**
Mean (SD)	15.0 (12.6)	0.562 (3.58)	
Median [Min, Max]	14.1 [0.0200, 51.0]	0.0200 [0.00, 26.8]	
Jug 2			**<0.0001**
Mean (SD)	14.5 (10.9)	1.28 (4.11)	
Median [Min, Max]	13.4 [0.00, 43.3]	0.0550 [0.00, 26.1]	

Missing data: Non-Allergic n = 2
P-values determined using 2-sided Mann-Whitney test.

Table E6b:

	OFC Negative (n=24)	**OFC Positive (n=7)**	**p-value**
Jug r (Extract)			**0.0004**
Mean (SD)	0.289 (0.488)	2.17 (1.72)	
Median [Min, Max]	0.0400 [0.00, 1.68]	2.12 [0.190, 4.64]	
Jug 1			**0.0002**
Mean (SD)	0.0674 (0.137)	1.75 (2.16)	
Median [Min, Max]	0.0200 [0.00, 0.610]	0.840 [0.0200, 6.41]	
Jug 2			**0.0017**
Mean (SD)	0.696 (1.56)	3.67 (4.21)	
Median [Min, Max]	0.110 [0.00, 6.68]	2.01 [0.290, 12.3]	

Missing data: OFC negative n = 1
P-values determined using 2-sided Mann-Whitney test

Table E7. Cashew extract and cashew allergen-specific IgE in MONAS cohort with clinical allergy.

	Cashew Allergic (n=65)	**Cashew Non-Allergic (n=46)**	**p-value**
Ana o (Extract)			**<0.0001**
Mean (SD)	23.3 (11.4)	0.461 (1.22)	
Median [Min, Max]	25.6 [0.00, 46.0]	0.00 [0.00, 6.92]	
Ana o 3			**<0.0001**
Mean (SD)	21.3 (12.6)	0.133 (0.428)	
Median [Min, Max]	23.6 [0.00, 45.6]	0.00 [0.00, 2.10]	

Missing data: Allergic n = 1, Non-Allergic n = 2
P-values determined using 2-sided Mann-Whitney test.

Table E8. Pistachio extract and pistachio allergen-specific IgE in MONAS cohort with clinical allergy.

	Pistachio Allergic (n=59)	**Pistachio Non-Allergic (n=48)**	**p-value**
Pis v (Extract)			**<0.0001**
Mean (SD)	28.6 (10.2)	1.68 (4.00)	
Median [Min, Max]	30.5 [0.150, 61.2]	0.0950 [0.00, 22.4]	
Ana o 3			**<0.0001**
Mean (SD)	22.6 (11.1)	0.135 (0.438)	
Median [Min, Max]	25.1 [0.00, 43.3]	0.00 [0.00, 2.10]	

Missing Data: Non-Allergic n = 2
P-values determined using 2-sided Mann-Whitney test.

Table E9. Comparison of almond sIgE in MONAS cohort.

	Almond Allergic (n=3)	**Almond Non-Allergic (n=97)**	**Indeterminate (n=97)**	**p-value**
Pru du (Extract)				n/a*
Mean (SD)	9.69 (8.10)	1.01 (2.47)	2.89 (5.35)	
Median [Min, Max]	13.6 [0.370, 15.1]	0.00 [0.00, 18.1]	0.130 [0.00, 31.4]	
Missing	0 (0%)	3 (3.1%)	2 (2.1%)	

*n/a: p-value not calculated due to insufficient sample size (n<8).

Table E10. Comparison of BAT and BAT+MoldAD analysis.

	n	OFC+	Sensitivity	Specificity
Cor a 9	28	8	0.50	0.75
Cor a 14	28	8	0.63	0.95
BAT Hazelnut	**28**	**8**	**0.88**	**0.85**
Cor a 9 + BAT HN	28	8	0.50	0.95
Cor a 14 + BAT HN	28	8	0.63	0.95
Cor a 9 neg → BAT HN	28	8	0.88	0.65
Cor a 14 neg → BAT HN	28	8	0.88	0.85
Cor a 9 pos → BAT HN	28	8	0.50	0.95
Cor a 14 pos → BAT HN	28	8	0.63	0.95

Cor a 9 cut-off = 1.36 kUa/l Cor a 14 cut-off = 0.30 kUa/l

	n	OFC+	Sensitivity	Specificity
Ara h 2	37	12	0.83	0.92
BAT PN	**37**	**12**	**1.00**	**0.92**
Ara h 2 + BAT PN	37	12	0.83	0.96
Ara h 2 neg → BAT PN	37	12	0.92	0.88
Ara h 2 pos → BAT PN	37	12	0.71	0.96

Ara h 2 cut-off = 1.7kUa/l

	n	OFC+	Sensitivity	Specificity
Jug r 1	24	7	0.86	0.94
BAT WN	**24**	**7**	**0.71**	**1.00**
Jug r 1 + BAT WN	24	7	0.71	1.00
Jug r 1 neg → BAT WN	24	7	0.86	0.94
Jug r 1 pos → BAT WN	24	7	0.71	1.00

Jug r 1 cut-off = 0.30 kUa/l

Supplemental Figure Legends

Figure S1. Allergen specific IgE and normalized, individual BAT data for peanut and individual tree nuts:

S1A: Allergen extract specific sensitizations of allergic (A, yellow) and non-allergic (NA, grey) individuals to peanut and individual tree nuts were measured (peanut allergic n=87, non-allergic n=46; hazelnut allergic n=48 non-allergic n=42, walnut allergic n=41, non-allergic n=35; cashew allergic n=50, non-allergic n=24; pistachio allergic n=45, non-allergic n=25). S1B: Individual data of normalized %$CD63^+$ basophils of each nut for the optimal concentrations 10 and 100 ng/mL are displayed. Mann Wilcoxon *t*-tests were performed.

Figure S2. Mixed effects model validated in independent cohort of peanut allergic patients from the POISED study:

Boxplots depicting the distribution of normalized %$CD63^+$ basophils for peanut at 10 ng/mL and 100 ng/mL. MONAS peanut allergic samples are depicted in orange and POISED peanut allergic samples are depicted in green. Differences in mean normalized %$CD63^+$ basophils for each concentration between studies (MONAS vs POISED) were not significantly different at alpha = 0.05, p-values = 0.170 and 0.120, respectively.

Figure S3. Proportion of allergen component co-sensitizations in nut allergic patients:

Matrices depicting the allergen component sensitizations of individuals who are allergic to A) peanut (n=107), B) hazelnut (n=66), C) walnut (n=58), or D) cashew/pistachio (n=70). The

proportion (%) of allergic individuals with positive co-sensitizations are shown. Positive sensitization is defined as sIgE $\geq$ 0.3 kU_A/L.

Figure S4. Basophil activation dose-response curves and modelling for patients with indeterminate allergic status: Boxplots depicting the distribution of normalized %CD63+ basophils for samples with indeterminate allergic status for each nut at each concentration (0.001 to 1000 ng/µl).

Supplemental References

1. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, et al. Standardizing double-blind, placebo-controlled oral food challenges: American Academy of Allergy, Asthma & Immunology–European Academy of Allergy and Clinical Immunology PRACTALL consensus report. *Journal of Allergy and Clinical Immunology.* 2012;130(6):1260-1274.
2. Bird JA, Groetch M, Allen KJ, et al. Conducting an Oral Food Challenge to Peanut in an Infant. *The Journal of Allergy and Clinical Immunology: In Practice.* 2017;5(2):301-311.e301.
3. Bürkner B. Advanced Bayesian Multilevel Modeling with the R Package brms . *The R Journal.* 2018;10(1):395-411.