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NUT Co Reactivity - Acquiring Knowledge for Elimination Recommendations (NUT CRACKER) Study

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Short title – NUT Cracker study

ABSTRACT

Background. Ambiguities exist regarding the diagnosis of tree-nut allergy, necessitating either elimination or performance of oral food challenges (OFC).

Objective. To examine the co-incidences of allergies among tree-nuts and improve diagnostic testing to minimize the need for OFC.

Methods. Eighty three patients prospectively evaluated for walnut, pecan, cashew, pistachio, hazelnut and almond allergy. A history of previous reactions was obtained and standardized skin prick tests (SPT) using finely ground tree-nut solution and basophil activation tests (BAT) were performed. Patients underwent OFC for each tree-nut they eliminated and to which a reaction in the previous 2 years was not documented.

Results. While most patients were sensitized to 5-6 tree-nuts, over 50% were allergic to only 1-2 tree-nuts. The highest rate of allergy in sensitized patients was observed for walnut (74.6%) and cashew (65.6%). The rate of co-allergy for most tree-nuts was < 30%. Two thirds of walnut- and cashew-allergic patients were also allergic to pecan and pistachio, respectively, while all pecan- and pistachio-allergic patients were allergic to walnut and cashew, respectively. Receiver operating characteristic analysis for SPT and BAT was tree-nut dependent and yielded area under the curve (AUC) values ranging from 0.75-0.94. Knowledge of co-incident allergies in

these pairs along with the combination of SPT and BAT correctly distinguished allergic from tolerant patients for walnut (87%), pecan (66%), cashew (71%) and pistachio (79%).

Conclusion. The data presented here should assist in differentiating between allergic and tolerant patients, decrease the need for OFC and allow for appropriate elimination recommendations.

KEY WORDS

Allergy, Anaphylaxis, Basophil activation test, Eliciting dose, Skin prick test

INTRODUCTION

Tree nuts are among the most common food allergens, with an estimated prevalence ranging from 0.05% to 4.9%, depending on the geographical region tested and the diagnostic method used (1). Alarmingly, the prevalence of tree-nut allergy in children appears to be increasing (2). Tree-nuts are presumed to be a major cause of fatal and near fatal reactions, with walnut and cashew being most commonly associated with such reactions according to a US national self-reported registry (3, 4).

The prevalence of tree-nut sensitization ranges from 0.8% to 12.2% and is far more common than true allergy (1). In selected patient populations, however, such as those with peanut allergy (5,6), atopic dermatitis (7) and oral allergy syndrome (8), the prevalence of tree-nut sensitization and allergy is even higher. Cross-reactivity of tree-nut allergens with specific environmental allergens, such as the birch component (Bet v1) has been implicated in the oral allergy syndrome (9).

Patients with a single tree-nut allergy are frequently sensitized to other tree-nuts (2, 8). At least 19% of infants with a single tree nut allergy are already sensitized to additional tree-nuts

by age <2 years, with further tree-nut sensitizations developing over-time (10, 11). Certain tree-nuts (i.e. walnut / pecan and cashew / pistachio) share allergenic proteins that account in large part for the high rate of co-sensitization between them (12-14).

The main challenge in managing patients with suspected tree-nut allergy is to distinguish between sensitization and allergy. A review of the literature suggests that the accuracy of skin prick test (SPT) and ImmunoCAP are not sufficient to guide clinical decisions (15-18). Several studies reported SPT and sIgE cutoff points for diagnosing walnut, cashew and hazelnut allergy, but many patients had SPT/sIgE levels in “the gray zone” leading to uncertainty in the diagnosis (15, 16). Furthermore, the rate of false negatives and false positives were high and patients were not always challenged (17). Diagnostic cutoff points could not be determined for other tree-nuts, (15).

Component resolved diagnosis (CRD) and the basophil activation test (BAT) have been studied in order to increase the accuracy of *in vitro* allergy testing. For certain tree-nut allergies (cashew, pistachio and hazelnut), CRD has shown additional benefit over SPT and sIgE, but components for other tree-nuts have not been tested yet, and CRD for tree-nuts is not routinely available (13, 19). BAT has shown potential benefit in the diagnosis of peanut, milk, egg and wheat allergy, but data on tree-nuts is limited (20).

Oral food challenges (OFC) are still considered the gold standard for diagnosing food allergy. However, OFC are time- and personnel-consuming, especially in patients with multiple tree-nuts sensitizations, and are not without risk (21, 22). The high rate of co-sensitization between different tree-nuts and the difficulty in performing numerous OFC lead clinicians to often advise young children who have confirmed allergy to a single tree-nut to avoid all tree-nuts (23, 24). This could have significant impact on patients’ quality of life and potentially lead to the

development of new allergies (25, 26). The current study prospectively evaluated patients with one or more tree-nut sensitizations (SPT $\geq$ 3 mm) referred to the Institute of Allergy, Immunology and Pediatric Pulmonology at the Assaf Harofeh Medical Center, for suspected tree-nut allergy. The goal was to determine cross-sensitization and co-allergy between various tree-nuts and to examine whether SPT and BAT could reduce the need for OFC in these patients.

MATERIALS AND METHODS

Study Design

The study was a prospective single-center cohort study conducted at the Institute of Allergy, Immunology and Pediatric Pulmonology at the Assaf Harofeh Medical Center from April 2016-May 2017. Patients aged > 3 years old, referred for suspected IgE-mediated allergy to at least one of the following tree-nuts: walnut, pecan, cashew, pistachio, hazelnut or almond were included. Patients with chronic heart or lung disease other than asthma were excluded. A detailed medical history was obtained from each participant and patients' medical charts were reviewed. Information about tree-nuts patients were regularly consuming (tree-nuts which patients were told they were not allergic to and which they were consuming ad lib, and at least an amount equivalent to a whole nut in the 6 month preceding the study), reactions previously experienced, age at which a diagnosis of food allergy was made and the presence of other allergic comorbidities (other food allergies, atopic dermatitis, and asthma) was obtained. Each patients was skin-tested to all 6 tree-nuts and OFC were performed to each tree-nut which was not regularly consumed by the patient and to which no IgE-mediated reaction was documented within the last 2 years. Each patient was categorized for each tree-nut as being allergic (recent reaction or a positive OFC with evidence of sIgE reactivity based on SPT) or as tolerant (regular

consumption or a negative OFC). Blood was obtained from each patient for BAT. The study was approved by the institutional ethics committee and was registered as a clinical trial (NCT02786914). Informed consent was signed by all study participants.

Standardized skin prick tests

Tree-nut flour (walnut and cashew, Wellbees, Monsey, NY; hazelnut, Holmquist Hazelnuts, Lynden, Washington; and almond, Sukrin, Westwood Business Park Hassocks, BN6 8SG, United Kingdom) or tree-nuts (pecan and pistachio) were finely ground in a coffee grinder until a homogenous paste was generated. A different grinder was dedicated for the preparation of SPT for each tree-nut. This was then diluted to 10 mg/ml with mineral water pH 7.9 and frozen at -20 °C. Fresh aliquots were thawed weekly and stored at 4 °C for daily use.

SPTs were performed on the volar surface of the arm after application of the respective tree-nut solutions. A negative control, and histamine (1 mg/ml; ALK-Abelló, Port Washington, NY) as a positive control were used. The reaction was read after 20 minutes. A reaction of a 3-mm or larger wheal compared to the negative control was considered positive.

Oral food challenge

Open OFC were performed under an allergist's supervision according to international guidelines (27, 28). A standard protocol can be found in **Table S1** in the online repository. The challenge was terminated if objective cutaneous (urticarial rash), respiratory (rhinitis, cough or wheeze) or gastrointestinal (abdominal pain or vomiting) symptoms occurred within 2 hours of exposure. If no reaction occurred for at least 2 hours following the final dose, the OFC was deemed negative. The presence of itching or oral symptoms was not utilized to define a positive challenge.

Anaphylaxis was defined as involvement of 2 or more organ systems during an allergic reaction. Injectable epinephrine was used for severe reactions including diffuse flushing, stridor or

wheezing, severe abdominal pain, or reduced blood pressure and per the attending physician's discretion. Following a negative challenge, patients were advised to introduce the negatively challenged tree-nuts, and foods containing them, into the diet.

Basophil activation test

Whole blood was collected in EDTA-containing tubes and processed within four hours, as previously described (29). A detailed protocol can be found in Methods section in the online repository. Briefly, blood from each patient was divided into tubes containing RPMI-1640 +IL3 alone or with the addition of tree-nut allergens (1 μ g/mL), or polyclonal anti human IgE. A high dose of allergen (1 μ g/ml) was utilized since in preliminary dose response experiments for walnut and cashew, some patients had a maximum CD63 response at 1 μ g/ml, and others who reacted to lower doses still were positive at this dose (**Figure S1**). Patients (n=4) were excluded from the BAT analysis if the positive control (anti human IgE stimulation) yielded less than a 10% increase in CD63 on the cell compared to incubation with RPMI 1640+ IL3 alone.

Statistical analysis

Statistical analyses were performed using SPSS software (version 20; SPSS, Inc, Chicago, Ill). A student t-test was used to compare continuous variables. Fisher's exact test was used to analyze differences in categorical variables. All analysis with 2-tailed and a P-value of less than 0.05 were considered significant. Spearman coefficient was used to examine correlations. R software (version 3.3.3) was used with the pROC package (version 1.9.1) to generate AUC values and confidence intervals for the ROC curves. AUC values were assessed for statistical significance by utilizing the Winpepi (version 11.65) statistical package (30).

RESULTS

Co-sensitization rates and tree-nut elimination

A total of 83 patients were recruited. The median age was 8.7 years (range, 3 - 24) and 46 (55.4%) were male. Other allergic comorbidities included asthma (39.8%) and atopic dermatitis (37.3%). In this tree-nut allergic cohort, the most frequently other food allergies reported by the patients were peanut (24.1%), followed by sesame (7.2%), milk (6%), egg and fish (2.4% each). The rate of co-sensitization between the various tree-nuts was high, ranging from 60.6% to 96.7% (**Table S2**). Walnut, pecan and hazelnut comprised one group while cashew and pistachio comprised a second group, with a positive response among a member of each group being highly correlated both in SPT and BAT to the other members of their respective groups (**Table S3**).

Over-all, 232 OFC (113 positive and 119 negative) were performed. While sensitization rates (defined as wheal diameter of ≥ 3 mm on SPT) were high for all tree-nuts, ranging from 59% (49/83) for almond to 85.5% (71/83) for walnut, the rate of clinical allergy differed significantly between the various tree-nuts (**Table 1**). The highest rate of allergy among sensitized patients was observed for walnut and cashew (74.6% and 65.6% of sensitized patients, respectively). Only a single patient from the entire cohort was allergic to almond. Along with the high rates of sensitization, patients eliminated a mean of 4.2 nuts from the diet. While most patients eliminated 5-6 tree-nuts, and most were sensitized to 5-6 tree-nuts over 50% of the patients were allergic to only 1-2 tree-nuts (data not shown).

Characterization of tree-nut OFC

There were no significant differences in reaction type (lower respiratory tract reactions, anaphylactic reactions, or reaction requiring epinephrine) on OFC between the different tree-nuts

(Table S4). While cutaneous reactions were most frequently experienced during OFC to walnut, pecan and hazelnut, gastro-intestinal reactions were most frequently experienced during OFC to cashew and pistachio. Reactions, some requiring epinephrine, were documented following a very high dose (>2 grams of protein) for each of the nuts, as shown in Figure S2.

Co-allergies among tree-nuts

The extent of co-allergy between the various tree-nuts was much lower than the extent of co-sensitization (Table 2). Of note, some tree-nuts (walnut-pecan and cashew-pistachio) had high rates of co-allergy. While all pecan- and pistachio-allergic patients were allergic to walnut and cashew, respectively, two thirds of walnut- and cashew-allergic patients were also allergic to pecan and pistachio, respectively. Single walnut and dual walnut-pecan allergic patients had comparable walnut SPT wheal size. Patients with walnut-pecan dual allergy tended to have more GI reactions on walnut OFC (48.1% vs. 16.7%, $p=0.055$). Single cashew allergic patients had lower cashew SPT wheal size compared to those with cashew-pistachio dual allergy (8.5 ± 3.7 mm, vs. 12.2 ± 3.2 mm, respectively, $p=0.002$). Patients with cashew-pistachio dual allergy had more GI symptoms (72.7% vs. 28.6%, $p=0.016$) and less cutaneous (23.8% vs. 71.4%, $p=0.013$) and lower respiratory symptoms (13.6% vs. 50% $p=0.026$) on cashew OFC compared to those with single cashew allergy.

Distinguishing allergic versus tolerant patients using SPT and BAT

For all nuts, the mean SPT wheal size and the mean allergen-induced CD63 expression on basophils were significantly higher in allergic compared to tolerant patients (Figure 1). On Receiver-Operating Characteristic (ROC) curve analysis AUC values were not significantly different between SPT and BAT, except in the case of hazelnut ($p < 0.05$). AUC values for SPT

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ranged from 0.75 (hazelnut) – 0.94 (cashew), and for BAT the range was 0.78 (pecan)-0.94 (hazelnut) (**Figure 2**). In general, SPT outperformed BAT in terms of sensitivity, whereas BAT was superior to SPT with regard to specificity. SPT and BAT cutoffs providing 95% PPV were found only for walnut and cashew (**Table S5**)

Challenge determination utilizing SPT and BAT

We next examined how our clinical observations (walnut-pecan and cashew-pistachio co-allergy) integrated with our BAT and SPT results, distinguished allergy from tolerance. Patients who had available data for both tests (n=62), were utilized for analysis. All patients with walnut SPT wheal <3 mm (n=8) were non-allergic to walnut and all those with SPT wheal ≥ 8 (n=31) were walnut-allergic (**Figure 3**). Of patients with a walnut wheal between 3 and 8 mm and BAT value ≥ 5.0 % CD63 (n=13), 11 patients were walnut-allergic and 2 were not. Of these 52 cases, the combination of clinical data, SPT and BAT matched the clinical outcome in 50 (96.2%) with no false negatives and 2 false positives. In the remaining 10 patients (shaded boxes), the identification of allergy versus tolerance was low (3 patients were allergic and 7 were not). All 15 walnut tolerant patients were also tolerant to pecan (**Figure 3, bottom**). For pecan, BAT was the best initial criterion. Of patients with values $\geq 5.0\%$ CD63 (n=24), 19 were diagnosed as allergic while 5 were tolerant. Of the remaining patients, those with a pecan wheal < 3 mm (n=6) were non-allergic to pecan. Of these 45 cases, the algorithm matched in 40 (88.9%), had no false negatives and 5 false positives. In the remaining 17 cases (shaded boxes) matching was low (8 patients were allergic and 9 were not).

Similarly, cashew SPT was the best initial criterion for the cashew-pistachio testing. Patients with < 3mm wheal (n=15) were all non-allergic to cashew and patients with ≥ 12 mm wheal (n=12) were all cashew-allergic (**Figure 4**). In those with a cashew wheal between 3 and

12 mm and a BAT value of $\geq 5.0\%$ CD63 (n=15), 14 were cashew allergic whereas one patient was not. Of these 42 cases, the combination of clinical data, SPT and BAT matched the clinical outcome in 41 (97.6%) with no false negatives and 1 false positive. In the remaining 20 cases (shaded boxes) the matching was low, (5 patients were allergic and 15 were not). All 30 cashew-tolerant patients were also tolerant to pistachio. Of the 23 patients diagnosed as cashew-allergic who had pistachio SPT ≥ 8 mm, 19 were pistachio allergic and 4 were not (**Figure 4, bottom**). Of these 53 cases, the algorithm matched the clinical outcome in 49 (92.5%) with no false negatives and 4 false positives. In the remaining 9 patients the matching was low (6 were tolerant and 3 allergic). For hazelnut and almond, neither SPT nor BAT could provide a cutoff point yielding 95% PPV. However, BAT could predict tolerance and obviate the need for OFC in many sensitized patients (hazelnut; %CD63 $<3\%$, n=28/45 and almond; %CD63 $<6\%$, n=35/37, no false negatives, respectively).

DISCUSSION

The NUTCRACKER study was designed to examine the rate of co-sensitization and co-allergy between six different tree-nuts in the context of complete verification of patient allergic status for all the nuts tested. Cashew and walnut were most frequently found as allergenic in our population while almond allergy was rare. Differences between single walnut and single cashew allergic compared to walnut-pecan and cashew-pistachio dual allergic patients are described, including rate of co-allergies, and type of reactions. Optimal SPT and BAT cut-off points that correspond with clinical allergy for walnut and cashew are defined and, together with the knowledge of the co-incident allergies among the walnut-pecan and cashew-pistachio pairs,

might provide a framework, in the future, to accurately identify allergic patients with a minimum of OFC performed.

Previous studies, using a maximal dose of 875 mg or 1750 mg protein in OFC to a variety of allergens, have shown false negative rates of 5% and 12.7% to the two doses respectively (31, 32). The European guidelines recommend that the top dose administered during an OFC should be the normal daily intake in a serving of the food in question, adjusted for the age of the patient while the PRACTALL consensus report states that at least 2 grams of protein should be given (27, 28). In our study, 6.7% - 15.4% of patients with a positive OFC reacted after a dose >2 grams of protein. As the interval between the last 2 doses was 45 - 60 minutes, it is possible that patients reacted to a previous dose, which consisted of 1 – 2 grams of protein. Still, these doses are higher than a regular serving of tree-nuts.

Certain pairs of tree-nuts, i.e. walnut/pecan and cashew/pistachio, belong to the same family (Juglandaceae and Anacardiaceae, respectively) and their proteins can have a high percent of identity in their amino acid sequences (33, 34). High rates of co-sensitization between walnut and pecan (0.96) and between cashew and pistachio (0.98) have been described (6). However, OFC to the co-allergenic nuts are not typically performed (13). This study highlights several important features of the walnut/pecan and cashew/pistachio dual allergenic pairs that require confirmation in additional studies on other populations. We found in our population that while all pecan and pistachio allergic patients were allergic to walnut and cashew, respectively, only two thirds of patients who were allergic to walnut and cashew had allergy to pecan and pistachio. This allergic unidirectionality suggests that some components are shared by these pairs of tree-nuts while others are unique to cashew and walnut. Additionally, we found that patients allergic to these dual pairs experienced more GI reactions on OFC, perhaps related to the

2s albumins component (35), than patients singly allergic to walnut or cashew. It remains to be seen whether single walnut and cashew allergic patients develop dual allergy with time, although it is not supported by the fact that the single and dually allergic patients were comparable in age.

The diagnosis of tree-nut allergy is challenging. While SPT has high false positive rates, sIgE can often be negative even in allergic patients (7, 10, 16, 35). Commercial SPT extracts are highly variable in their composition of allergenic components (36). Clinicians often use prick to prick SPT to achieve better diagnostic accuracy, but they are not standardized. We report the use of a standardized SPT solution (10 mg/ml) made from tree-nut flour or ground tree-nuts. This SPT preparation provided reliable negative predictive value (NPV) for all tree-nuts and a good 95% positive predictive value (PPV) for walnut and cashew. BAT has emerged as another potentially promising tool for the diagnosis of peanut and egg allergy (37-39). Two previous studies have shown some utility for BAT in diagnosing hazelnut allergy, but those are limited by the small number of OFC performed and the low cumulative dose used (800 mg) (40, 41). In our study, SPT and BAT separately were limited in their ability to distinguish allergy from tolerance. Adding BAT to the clinical co-allergy knowledge and the standardized SPT, improved the ability to identify allergic and tolerant patients. This approach could potentially reduce the need for OFC in 97/124 (78.2%) walnut-pecan cases and 95/124 (76.6%) cashew-pistachio cases. These findings should be examined prospectively on a larger patient-population before more definitive conclusions can be drawn.

There are several caveats to this study. While the algorithms we provide for tree-nut allergy diagnosis would correctly identify a significant number of patients as allergic or tolerant, this scheme needs to be validated prospectively. Additionally, the varied prevalence of tree-nut allergy depending on the geographical region tested, could affect the allergy testing scheme.

While this is not an epidemiological study, our findings that allergy was most frequent for walnut, pecan and cashew, less frequent in the case of pistachio and hazelnut and rare for almond, matches findings from previous US-based studies (2, 42). This differs from European reports, however, and thus the algorithms may only apply to certain populations (1).

Regarding the performance of BAT, we chose to use the soluble protein component from our SPT preparation, but other extraction methods may provide for superior results. The accuracy of the BAT might have been limited because a dose response curves for each nut in every patient was not performed. However, our preliminary investigation did not demonstrate increased sensitivity at any higher or lower doses than what was utilized for this study. Another potential limitation of BAT is the requirement of a flow cytometer, which may not be routinely available in the clinic. Addressing this concern, newer protocols have been developed to allow for BAT to be performed greater than 24 hours after the blood was drawn (43). Finally, evidence of sIgE-sensitization was determined by SPT only, given previous descriptions that SPT is a better predictor for tree-nut allergy compared to sIgE (16, 18, 35). The use of CRD, on the other hand, might facilitate a better serum based IgE test, and it will be tested in the future.

In summary, the current study provides new insights into tree-nut allergy. The clinical information of rates of allergy for individual tree nuts, and co-allergenicity patterns provide important information for researchers and clinicians. SPT and BAT show promise in their ability to correctly distinguish allergic from tolerant patients, but prospective studies to confirm their use are required.

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CONFLICTS OF INTEREST

Arnon Elizur does not have any conflicts of interest to disclose.

Michael Y Appel does not have any conflicts of interest to disclose.

Liat Nachshon does not have any conflicts of interest to disclose.

Michael B Levy does not have any conflicts of interest to disclose.

Na'ama Epstein Rigbi does not have any conflicts of interest to disclose.

Keren Golobov does not have any conflicts of interest to disclose.

Michael R Goldberg does not have any conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Arnon Elizur contributed in conception and design of the study, acquisition of data and in analysis and interpretation of data and in drafting the manuscript.

Michael Y Appel contributes in performance and analysis of the basophil activation tests and in drafting the manuscript.

Liat Nachshon contributed in acquisition and interpretation of data and in drafting the manuscript.

Michael B Levy contributed in acquisition and interpretation of data and in drafting the manuscript.

Na'ama Epstein Rigbi contributed in acquisition and interpretation of data and in drafting the article

Keren Golobov contributed in acquisition of data.

Michael R Goldberg contributed in the design of the study, preparation of SPT extract, interpretation of data and in drafting the manuscript.

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Table 1: Rate of sensitization versus clinical allergy for each tree-nut

Tree-nuts (n=83)	Sensitization (SPT $\geq$ 3 mm)	Clinical allergy			Clinical tolerance		
		Total*	Reaction in last 2y**	OFC (+)**	Total*	Consumption [^]	OFC (-) [^]
Walnut	71	53 (74.6%)	22 (41.5%)	31 (58.5%)	18 (25.4%)	6 (33.3%)	12 (66.7%)
Pecan	57	34 (59.6%)	8 (23.5%)	26 (76.5%)	23 (40.4%)	2 (8.7%)	21 (91.3%)
Cashew	61	40 (65.6%)	13 (32.5%)	27 (67.5%)	21 (34.4%)	9 (42.9%)	12 (57.1%)
Pistachio	65	26 (40%)	8 (30.7%)	18 (69.3%)	39 (60%)	6 (18.5%)	33 (84.6%)
Hazelnut	61	14 (23%)	4 (28.6%)	10 (71.4%)	47 (77%)	34 (72.3%)	13 (27.7%)
Almond	49	1 (2%)	0	1 (100%)	48 (98%)	20 (41.7%)	28 (58.3%)

P value <0.0033 (after applying Bonferroni correction) is considered significant

* number (% of sensitized), significant differences for walnut vs. pistachio and hazelnut, pecan vs. hazelnut, cashew vs. hazelnut and for all nuts vs. almond.

** number (% of allergic)

[^] number (% of tolerant)

Table 2: Rate of co-allergy between tree-nuts

Tree-nut	Other tree-nuts allergies					
	Walnut	Pecan	Cashew	Pistachio	Hazelnut	Almond
Walnut (n=53)	-	34 (64.2%)	20 (37.7%)	11 (20.8%)	11 (20.8%)	0
Pecan (n=34)	34 (100%)	-	14 (41.2%)	6 (17.6%)	10 (29.4%)	0
Cashew (n=40)	20 (50%)	14 (35%)	-	26 (65%)	6 (15%)	0
Pistachio (n=26)	11 (42.3%)	6 (23.1%)	26 (100%)	-	3 (11.5%)	0
Hazelnut (n=14)	11 (78.6%)	11 (78.6%)	6 (42.9%)	3 (21.4%)	-	0
Almond (n=1)	0	0	0	0	0	-

FIGURE LEGENDS

Figure 1. SPT and BAT in tolerant versus allergic patients. SPT wheal size (mm) using 10 mg/ml tree-nut extract and induced CD63 expression by BAT in response to 1000 ng/ml tree-nut protein are shown for tolerant and allergic patients for each individual tree-nut. A t-test was used to analyze differences between tolerant and allergic patients.

Figure 2. ROC curves for SPT and BAT for individual tree-nuts. The area under the curve (AUC) and the 95% confidence interval are presented. Dashed line is generated curve for SPT and continuous line for BAT (induced CD63 expression).

Figure 3. Algorithm depicting evaluation of potential walnut-pecan allergic patients. A description of how the clinical finding of walnut-pecan co-allergy, together with SPT and BAT match OFC results in our cohort. Cases in which these parameters matched poorly with OFC results are presented in the shaded boxes.

Figure 4. Algorithm illustrating evaluation of potential cashew-pistachio allergic patient. A description of how the clinical finding of cashew-pistachio co-allergy, together with SPT and BAT match OFC results in our cohort. Cases in which these parameters matched poorly with OFC results are presented in the shaded boxes.

Figure Legend-Online Repository

Figure S1. Dose response curves of basophil activation in walnut and cashew allergic

patients. The increase in % CD63 expression over IL-3 alone after stimulation with walnut (A) or cashew (B) in the dose range from 4 ng/ml -10,000 ng/ml is presented.

Figure S2. Distribution of reaction doses for individual tree-nuts. The % of patients reacting following each dose (mg) during oral food challenges for each individual tree-nut. * refers to the last dose given prior to the reaction.

Methods-Online repository

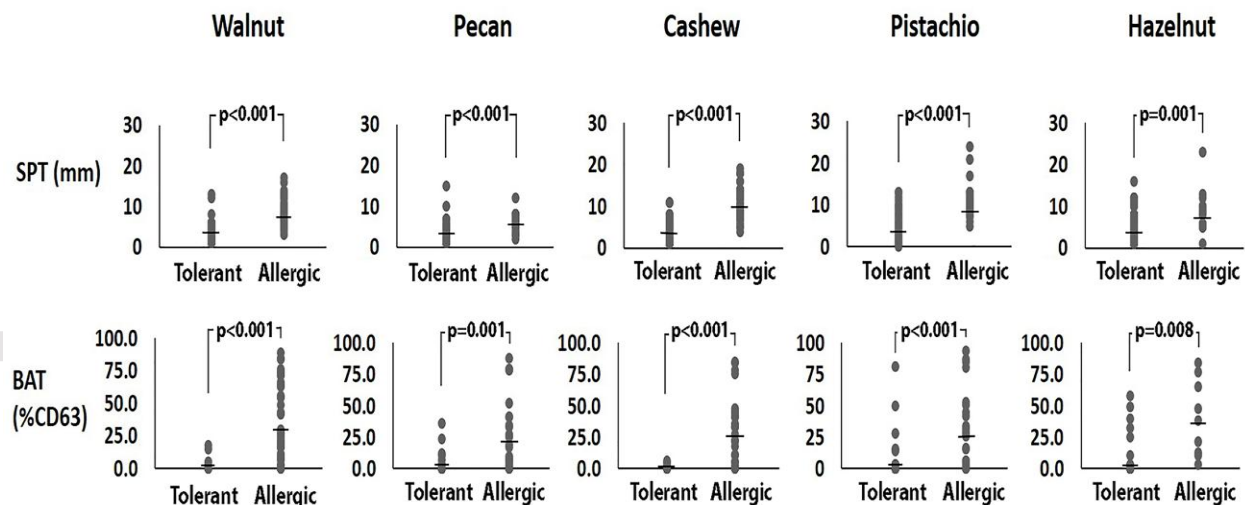
Oral Food Challenge

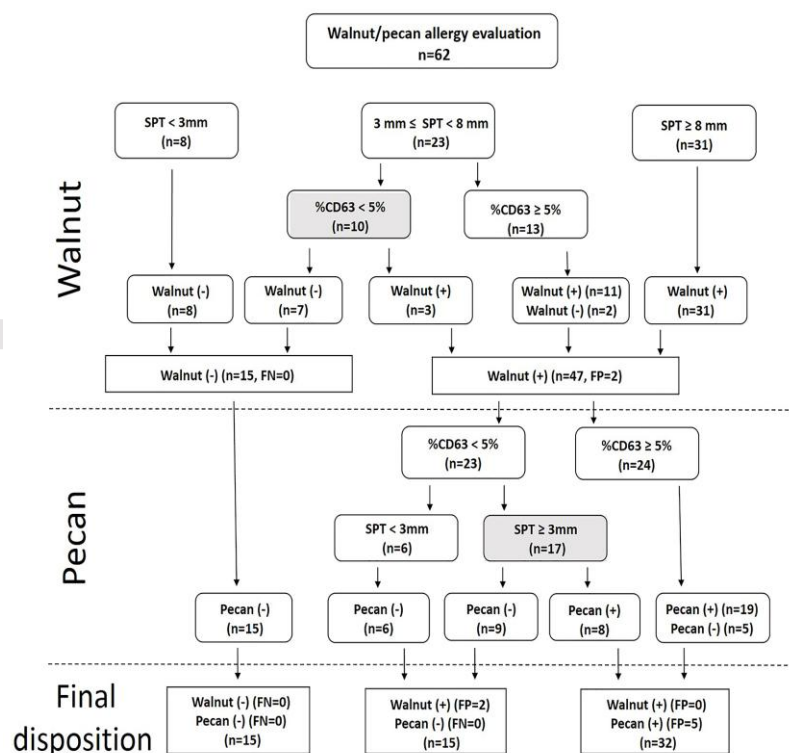
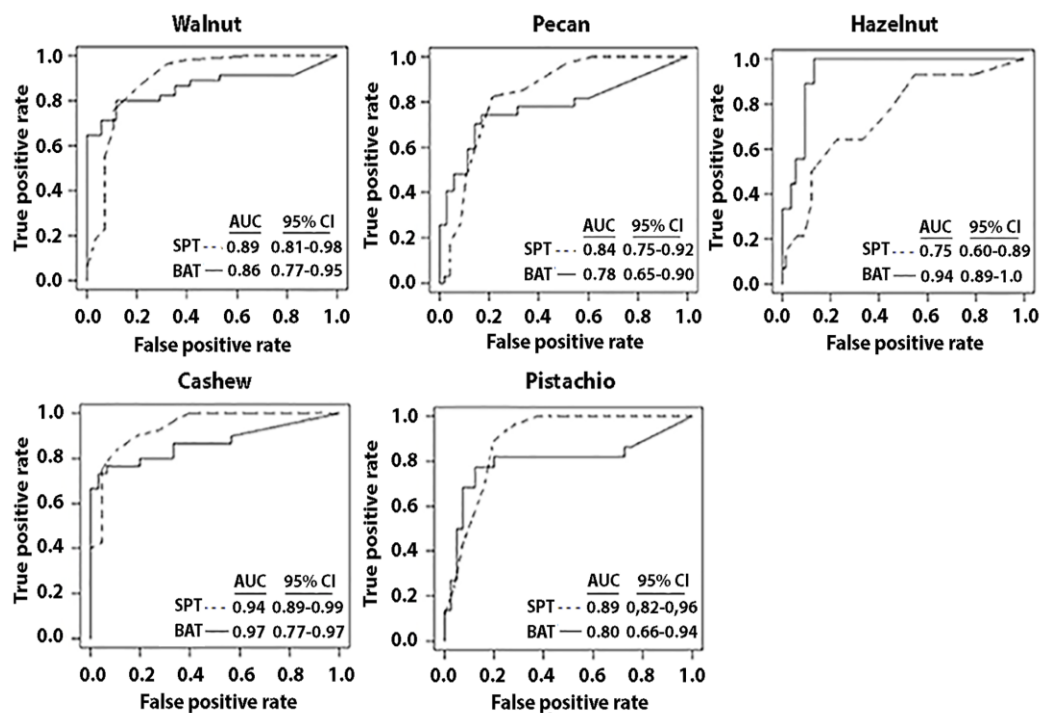
Each OFC started at a dose of 0.1 mg of tree-nut protein with escalation to 5 mg, 20 mg, 50 mg, and 100 mg, given as tree-nut flour reconstituted in water. Higher doses were given as tree-nuts up to a dose of 2.5 – 4 grams of tree-nut protein (see Table E1 in the online repository).

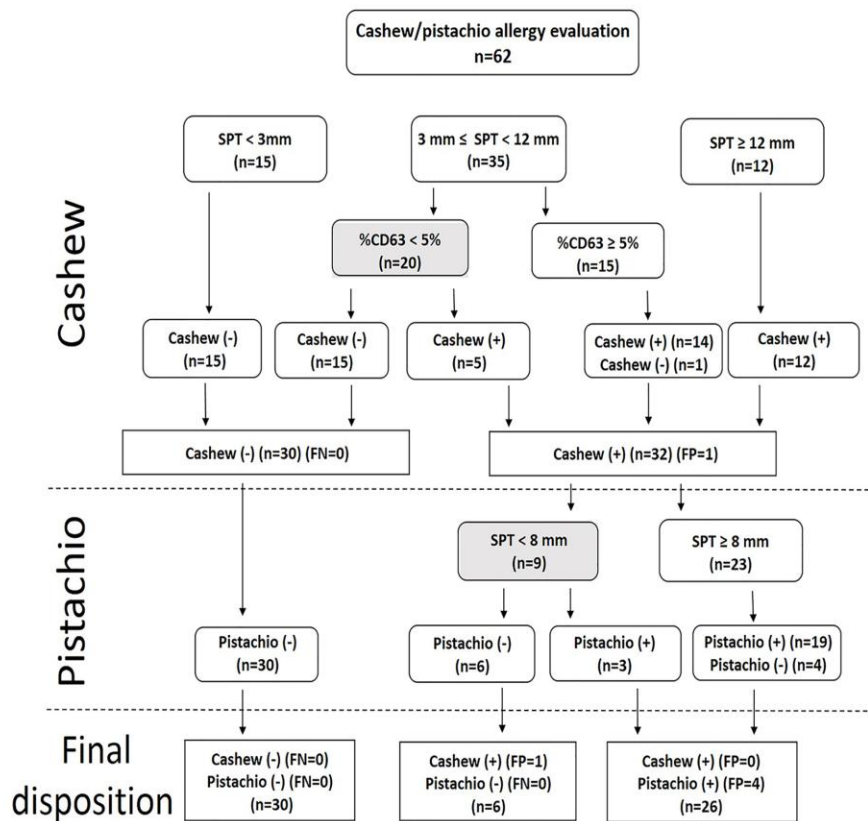
Basophil Activation Test

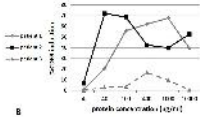
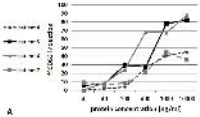
To generate solubilized extract for stimulation, treenut mixtures (from the same stock used for SPT) were centrifuged at 14,000 RCF for 15 minutes. The supernatants were collected, and quantified for protein concentration (DC Protein Assay, Bio-Rad, Berkely, CA). 1mg/ml stock tubes were prepared and stored at -20°C. Whole blood was collected in EDTA-containing tubes and processed within four hours. Whole blood was stimulated for 18 minutes at 37 °C with 1ng/ml recombinant human IL3 (R&D Systems, Minneapolis, MN) with or without each of the tree-nut proteins (1µg/ml) diluted in RPMI 1640 (Biological Industries, Beit HaEmek, Israel).

Stimulations with 1µg/ml polyclonal rabbit anti human IgE (Bethyl Laboratories, Montgomery, TX) were used as positive controls. Cells were incubated with fluorescent antibodies (Fluorescein isothiocyanate -anti human CD123 (clone 6H6), R-Phycoerythrin-Cy7 anti human HLA-DR (clone LN3), R-Phycoerythrin anti human CD203c (clone NP4D6) (All from eBioscience) and Allophycocyanin anti human CD63 (clone H5C6) (BioLegend, San Diego, CA)) during the stimulation, and on ice for an additional 10 minutes, and erythrocytes were lysed. Cells were centrifuged (500 G, 4 °C, 8 minutes), and were re-suspended in Phosphate Buffered Saline with 1% Bovine Serum Albumin and 2mM EDTA. Cells were acquired on a Beckman FC-500 or Navios flow cytometer. Basophils were identified as CD123+, HLA-DR- cells with approximately 500 basophils counted per sample. Analysis was performed with Flowing Software 2.5.1 (Turku Centre for Biotechnology, Finland).









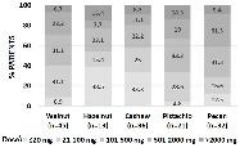


Table S1. Walnut oral food challenge protocol

* The first 5 doses were delivered as a ground nut solution in water. For the subsequent doses nuts were weighed and the total protein delivered based on the protein content for each respective nut. A representative example of a walnut protocol is presented.

Table S2. Rate of co-sensitization between tree-nuts

Sensitization (SPT $\geq$ 3 mm)*	Other tree-nuts sensitizations, n (%)					
	Walnut	Pecan	Cashew	Pistachio	Hazelnut	Almond
Walnut (n=71)	-	53 (74.6%)	51 (71.8%)	56 (78.9%)	59 (83.1%)	43 (60.6%)
Pecan (n=57)	53	-	42 (79.2%)	45 (84.9%)	43 (81.1%)	42 (79.2%)
Cashew (n=61)	51 (83.6%)	42 (68.9%)	-	59 (96.7%)	51 (83.6%)	39 (63.9%)
Pistachio (n=65)	56 (86.2%)	45 (69.2%)	59 (90.8%)	-	53 (81.5%)	42 (64.6%)
Hazelnut (n=61)	59 (96.7%)	43 (70.5%)	51 (83.6%)	52 (85.2%)	-	37 (60.7%)
Almond (n=49)	43 (87.8%)	42 (85.7%)	39 (79.6%)	42 (85.7%)	43 (87.8%)	-

* SPT performed with standardized ground nut solution (10 mg/ml).

Table S3. Correlation of test values (SPT and BAT) between tree-nut pairs

Tree-nut	Test	r-values					
		Walnut	Pecan	Cashew	Pistachio	Hazelnut	Almond
Walnut	SPT	-	0.42**	-0.03	0.04	0.47**	0.15
	BAT	-	0.75**	0.11	0.18	0.55**	0.34**
Pecan	SPT	0.42**	-	0.04	0.09	0.33**	0.45**
	BAT	0.75**	-	0.09	0.13	0.59**	0.46**
Cashew	SPT	-0.03	0.04	-	0.82**	0.25*	0.16
	BAT	0.11	0.09	-	0.76**	0.35**	0.17
Pistachio	SPT	0.04	0.09	0.82**	-	0.26*	0.28*
	BAT	0.18	0.13	0.76**	-	0.34**	0.18
Hazelnut	SPT	0.47**	0.33**	0.25*	0.26*	-	0.05
	BAT	0.55**	0.59**	0.35**	0.34**	-	0.48**
Almond	SPT	0.15	0.45**	0.16	0.28*	0.05	-
	BAT	0.34**	0.46**	0.17	0.18	0.48**	-

*Significant correlation with P value <0.05

**Significant correlation with P value <0.01

Table S4. Reactions and treatment in positive oral food challenges for each tree-nut

Tree-nuts	Positive oral food challenges results						
	Skin	Gastro-intestinal	Respiratory	Lower respiratory	Anaphylaxis*	Broncho dilator	Epinephrine
Walnut (n=45)	73.3%	35.6%	48.9%	11.1%	46.7%	15.6%	22.2%
Pecan (n=32)	71.9%	43.8%	56.2%	18.8%	59.4%	34.4%	25%
Cashew (n=36)	44.4%	55.6%	55.6%	22.2%	44.4%	27.8%	16.7%
Pistachio (n=21)	52.4%	66.7%	42.9%	19%	42.9%	19%	28.6%
Hazelnut (n=13)	61.5%	38.5%	61.5%	15.4%	69.2%	46.2%	23.1%

* Defined as a reaction involving 2 or more organ systems

Table S5. ROC analysis, sensitivity and specificity of SPT and BAT

Tree-nut	Test (OFC+/OFC-)	ROC AUC	Optimal Cutoff *	Sensitivity	Specificity	≥ 95% PPV
Walnut	BAT (45/17)	0.86	≥ 6.4% CD63	0.80	0.88	≥ 6.4% CD63
	SPT (53/28)	0.89	≥ 6mm	0.87	0.79	≥ 8 mm
Pecan	BAT (27/35)	0.78	≥ 2.7% CD63	0.74	0.83	--
	SPT (34/47)	0.84	≥ 5mm	0.83	0.79	--
Cashew	BAT (30/30)	0.87	≥ 2.0% CD63	0.77	0.93	≥ 5.5% CD63
	SPT (40/43)	0.94	≥ 8mm	0.83	0.91	≥ 12mm
Pistachio	BAT (22/40)	0.80	≥ 5.8%	0.77	0.88	--
	SPT (26/56)	0.89	≥ 7mm	0.92	0.77	--
Hazelnut	BAT (9/53)	0.94	≥ 3.4%	1	0.87	--
	SPT (14/66)	0.75	≥ 8mm	0.64	0.77	--

* Based on Youden's index

