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A Prospective Validation of the NUT CRACKER Diagnostic Algorithm for Walnut and Pecan Allergy with Prediction of Severity

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Contributions of Authors:

Michael R Goldberg designed the study, prepared the SPT extract, interpreted the data, and drafted the manuscript. Michael Y Appel performed and analyzed the basophil activation tests and drafted the manuscript. Rachel Nega involved in analyzing and interpretation of data. Liat Nachshon, Michael B Levy, and Na'ama Epstein Rigbi involved in acquisition and interpretation of data and drafting of the manuscript. Arnon Elizur designed the study, analyzed and interpreted the data and drafted the manuscript.

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ABSTRACT

Background: We previously devised an algorithm utilizing skin prick tests (SPT), basophil activation tests (BAT) and co-allergy status to reduce the need for oral food challenges (OFC) in 63 patients with suspected walnut/pecan allergies.

Objective: To validate prospectively the NUT CRACKER diagnostic algorithm in a new cohort of patients (n=120) and to study the utility of SPT and BAT in predicting walnut-pecan allergy severity in both groups (n=183).

Methods: Patients (n=183) aged 8 (6-11) years, median, (IQR) with suspected tree nut allergy were studied. SPT utilized ground nut extract (10 mg/ml) and BAT assessed allergen-induced basophil CD63 expression. Allergy was determined based on a recent reaction or a positive oral food challenge (OFC) and tolerance was defined by regular consumption or a negative OFC.

Results: Walnut BAT was significantly higher in walnut/pecan dual compared to single walnut allergy ($p=0.003$) and predicted the need for epinephrine during positive walnut OFCs ($p=0.009$). Results of walnut and pecan-BAT correlated with the corresponding nut eliciting dose ($p=0.014$ and $p<0.001$, respectively). Receiver operating curves for walnut and pecan allergy, in the prospective cohort yielded area under the curves (AUC) ranging from 0.87-0.93 for SPT and BAT. Concordant with the original study, pecan allergic patients were all co-allergic for walnut. The algorithm decreased the need for OFCs by 78.8 % with 6/240 (2.5%) falsely positives and no false negatives.

Conclusion: The algorithm was validated prospectively and awaits broader testing across other populations. The use of BAT further associates with severity in walnut/pecan allergy.

Key Words: Diagnostic algorithm walnut/pecan allergy; Basophil activation test

51 **Abbreviations:**

52 *AUC - Area under the curves*

53 *BAT - Basophil activation tests*

54 *ED – Eliciting doses*

55 *OFC - Oral food challenges*

56 *ROC - Receiver operating characteristic*

57 *NUT CRACKER - NUT Co Reactivity – ACquiring Knowledge for Elimination Recommendations*

58 *NUT CRACKER-PS - NUT CRACKER-Prospective Study*

59 *sIgE - Specific IgE*

60 *SPT- Skin prick tests*

61 *QOL – Quality of life*

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64 The study was registered as a clinical trial (NCT02786914).

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Highlights

1. What is already known about this topic? We previously devised an algorithm utilizing skin prick tests, basophil activation tests and co-allergy status to reduce the need for oral food challenges (OFC) in patients with suspected walnut/pecan allergies
2. What does this article add to our knowledge? In a large scale prospective trial, this study validates a novel diagnostic algorithm for walnut and pecan allergy. It further demonstrates that basophil reactivity reflects the clinical manifestations of tree nut OFC.
3. How does this study impact current management guidelines? Given the high prevalence in tree nut allergic patients of cross-reactivities to several nuts, this algorithm will decrease the number of OFC needed to be performed, while maintaining accuracy of diagnosis.

Introduction

Tree nut allergy is estimated to be between 0.05-4.9% worldwide, with variations dependent on geographical location and mode of testing (1). Its increasing prevalence worldwide over the past decade and its risk of life-threatening anaphylactic reactions, gives importance for reliable testing modalities to detect affected individuals. Oral food challenges (OFCs) are the gold standard for diagnosis. However, OFCs are time-consuming and not without risk (2,3). The high rates of co-sensitization, which can range from 60.6-96.7% between pairs of nuts (4) together with the difficulty in performing numerous OFCs, lead clinicians to advise patients with confirmed allergy to one nut to avoid all tree nuts, which potentially can impact on the patients' quality of life.

An unmet goal to date in managing patients with suspected tree nut allergy is to minimize the need for OFCs and yet maintain accuracy to distinguish between sensitized individuals and those truly allergic. Laboratory assays such as specific IgE (sIgE), in terms of their sensitivity and negative predictive accuracy perform well, but their poor specificity and positive predictive accuracy limit their use for definitive diagnosis (2, 5-8). The use of component testing is an improvement in this regard, but is not yet comprehensively available (9,10).

In many European countries, Australia (11) and Israel, a skin prick test (SPT) is the first screening tool used for diagnosis of allergy. Ninety-five percent positive predictive value (PPV) measurements were demonstrated for milk, peanut, and egg utilizing commercial extracts, including in the infant population (12). For diagnosing allergy to tree nuts such as walnut, pecan and hazelnut, and to sesame, however, the sensitivity at the 95% PPV was unacceptably low for use in clinical practice (4,13). The use of high concentration extracts, which should mimic results obtained for prick to prick testing but allow for standardizing between lots has demonstrated diagnostic utility for walnut/pecan and sesame testing (4, 13). More recently, the Basophil

Activation Test (BAT), an ex vivo human blood assay that measures activation of basophils after cross-linking of allergen, has been examined (see 14 for review). The BAT assay was shown to be efficacious in diagnosing peanut (15,16), egg (17), milk (18), tree nut (4) and sesame (13) allergies and the combination of high-concentration SPT and BAT provided the highest accuracy in sesame (13) and peanut (15). The BAT was also shown to identify participants with severe allergic reactions during peanut OFC (16).

The NUT CRACKER study (NUT Co Reactivity – ACquiring Knowledge for Elimination Recommendations) evaluated and identified the cross-sensitizations and co-allergies (by OFC) between six nuts (walnut, pecan, hazelnut, cashew, pistachio and almond) in an Israeli cohort (4). It was noted that approximately 2/3 of patients allergic to walnut were allergic to pecan. Interestingly, all patients allergic to pecan were allergic to walnut (4) and *ipso facto* a patient tolerant to walnut was tolerant to pecan, as well. Utilizing this knowledge of co-allergenecity with a modified standardized SPT and BAT, the NUT CRACKER study devised a diagnostic algorithm that significantly reduced the need to perform OFC for diagnosis in 63 patients (4). The current NUT CRACKER-Prospective Study (NUT CRACKER-PS), prospectively evaluates the diagnostic algorithm in a new series of 120 patients. Furthermore, association of these diagnostic tests with reaction severity was evaluated in the entire patient population (n=183) from both studies.

Methods

Study Design

A cohort of 120 patients (NUT CRACKER-PS) with a suspected history of tree nut allergy was recruited prospectively from January 2017-August 2019 at The Institute of Allergy, Immunology and Pediatric Pulmonology at the Shamir Medical Center (formerly Assaf Harofeh). The study design is depicted in **Figure E1**. Patients were clinically evaluated and administered SPT and BAT. Validation of walnut allergic status was performed primarily through an OFC (n=97), except in cases of recent reactions (n=8) or if the evaluation uncovered recent walnut consumption (n=15) (**Table E1**). Patients were considered allergic if they had a positive OFC or a recent reaction (within 2 years) in the presence of IgE-reactivity. Patients were diagnosed as non-allergic if the OFC was negative or they were consuming the nut routinely (at least an amount equivalent to a whole nut in the 6 months preceding the study) without any adverse effects. For the first part of the analysis, this cohort was combined with the original NUT CRACKER cohort of 63 patients to evaluate the utility of SPT and BAT in predicting reaction severity of walnut and pecan allergy. For the second part of the study, the diagnostic algorithm for walnut/pecan allergy, proposed in the original NUT CRACKER study (4), was examined prospectively on the new NUT CRACKER-PS cohort.

The accuracy of the proposed algorithm (4) to diagnose walnut and pecan allergy using SPT and BAT was scored against the allergic status as defined above. A detailed description of the flow of the algorithm is presented in the online methods section. The study was approved by the institutional ethics committee and informed consent was signed by all study participants.

Diagnostic tests: Skin Prick Test and Basophil Activation Tests

The SPT and BAT have been previously described (4) and are detailed in the methods of the online repository. One non-responder in the BAT assay (not responding $\geq 5\%$ to control anti-IgE stimulation), was excluded at the outset of the study.

Oral food Challenges

OFC were performed as graded open challenges under an allergist's supervision, as has been previously described (4,19). Reaction criteria and treatment are detailed in the online methods. The OFC was evaluated according to the WAO allergy SAR grading system (20).

Statistics

Statistical analyses were performed using SPSS software (version 20; SPSS, Inc, Chicago, IL, USA). Shapiro-Wilk test was used to determine the normality of distribution of numerical variables. For categorical variables, the numbers are presented as n (%). Fisher's exact test was used to analyze differences in unpaired samples, and McNemar's test for paired samples. For non-normally distributed continuous variables the numbers are presented as median (IQR). The Mann-Whitney test was used to analyze differences in unpaired samples and the Wilcoxon test was utilized for paired samples. Rank correlations were evaluated by Spearman's rho test. All analyses were 2-tailed and a p value <0.05 was considered significant. Receiver operating characteristic (ROC) analysis using the EasyROC web interface (www.biosoft.hacettepe.edu.tr/easyROC/) evaluated for the ability of BAT and SPT to distinguish between allergic and non-allergic patient populations and to calculate area under the curve (AUC) values (21).

Results

Patient characteristics

The demographics and clinical characteristics of the entire study population (n=183, derived from 63 patients in the original NUT CRACKER cohort and 120 newly recruited patients in the NUT CRACKER-PS) as well as a comparison between the previous and current cohorts are presented in **Table 1**. The median age of subjects was 8 years (IQR, 6-11; range 3.6-27.3) and 57.9 % were male. In addition to tree nut allergies, patients (35%) reported other food allergies, the most prevalent being peanut (39.1% of those reporting, data not shown). Other allergic comorbidities included atopic dermatitis (48.9%) and asthma (35%). A previous reaction accounted for 55.4% of the cases for referral to the study (**Table 1**). The original NUT CRACKER cohort matched the NUT CRACKER-PS cohort in demographics and atopic co-morbidities (**Table 1**). However, patients in the NUT CRACKER-PS had a higher pecan SPT and induced %CD63 expression, reflective of the higher rate of co-allergy to pecan.

Distinguishing features between single walnut and dual walnut/pecan allergy

Following evaluation, 134/183 patients (47 from the NUT CRACKER and 87 from the NUT CRACKER-PS) were diagnosed with walnut allergy (120 based on a positive OFC and 14 based on a recent reaction) and 107 (79.9%) of them were also pecan allergic (99 based on a positive OFC and 8 based on a recent reaction). As in our previous NUT CRACKER cohort, all pecan allergic patients were also walnut allergic. Among all 134 walnut allergic patients, walnut BAT was significantly higher in those with dual allergy compared to single walnut allergy (median induced CD63% = 41.5% vs. 18.0%, respectively, $p=0.003$) (**Figure 1A**). This is consistent with the clinical finding that walnut ED in challenged patients with dual walnut/pecan allergy (n=95) was significantly lower compared to the walnut ED of single walnut allergic patients (n=25)

(median=60 mg vs. 900 mg, respectively, $p<0.001$). In contrast to the BAT, walnut SPT did not significantly differ between these patient groups (**Figure 1B**).

Distinguishing clinical reaction manifestations to walnut and pecan in dual allergic patients

The clinical characteristics of positive walnut and pecan challenges, in patients who had positive challenges for both ($n=87$), are presented in **Table 2**. While neither intramuscular administration of adrenalin nor grading severity differed between walnut and pecan challenges, there were several distinctive features between them. The median eliciting doses were significantly lower in walnut vs. pecan challenges (80 mg (40-210 mg) vs 180 mg (100-660 mg) median (IQR), respectively, ($p<0.0001$) (**Table 2**). Consistent with this, the median values for both SPT and CD63-BAT to walnut were significantly higher than their respectively paired values for pecan (SPT 9 mm vs. 7 mm, $p=0.005$; CD63-BAT 47.8% vs. 19.0%, $p<0.0001$) (data not shown). However, pecan OFC, compared to walnut, yielded a significantly greater number of patients with several clinical manifestations of reactions, such as systemic skin reactions, lower respiratory symptoms and treatment with bronchodilators (**Table 2**).

BAT and SPT as markers for severity

Given the utilization of BAT and SPT in the diagnostic algorithm, we next examined the association of these tests with particular walnut and pecan allergic manifestations in the context of OFC. Rank correlations between ED and both SPT and BAT, for all walnut ($n=120$) and pecan ($n=98$) positive OFCs, were performed. There was a mild inverse rank correlation between ED and SPT (Spearman's $\rho=-0.18$, $p=0.005$), and ED and BAT for walnut (Spearman's $\rho=-0.22$, $p=0.014$) (**Figure E2A-B**). In particular, walnut BAT was significantly higher in patients with severe allergy (walnut ED ≤ 100 mg protein) compared to those with milder allergy (ED >100 mg protein), ($p=0.018$) (**Table E2**). Furthermore, BAT was higher in patients with lower respiratory reactions and in those treated with bronchodilators ($p=0.035$ and $p=0.040$, respectively **Table E2**).

Furthering evidence that BAT relates to severity of reaction, patients who received EpiPen on OFC had a significantly higher BAT compared to those who did not ($p < 0.009$, **Table E2**). In agreement with this, only 13.3% of patients with CD63- BAT $< 25\%$ compared to 37.3% of those with CD63-BAT $> 25\%$ required epipen on OFC ($p = 0.006$) (**Figure 2**). No significant association was found between SPT with walnut ED, challenge reactions or treatment (data not shown). For pecan OFCs, stronger inverse ranked correlations compared to walnut, were found between both ED and SPT (Spearman's $\rho = -0.35$, $p < 0.001$) (**Figure E2C**) and ED and BAT (Spearman's $\rho = -0.42$, $p < 0.001$) (**Figure E2D**). Both BAT ($p = 0.002$) (**Table E3**) and SPT ($p = 0.004$, data not shown) were significantly higher in patients with severe ($ED \leq 100$ mg protein) compared to those with mild ($ED > 100$ mg protein) pecan allergy. BAT was also positively associated with vomiting during pecan OFC ($p = 0.046$) (**Table E3**). The association of BAT with severity as shown above provides a rationale for its integration into our diagnostic scheme.

Diagnostic accuracy of BAT and SPT in the NUT CRACKER-PS

The accuracy of the SPT and BAT were evaluated in the new NUT CRACKER-PS cohort. For both walnut and pecan, the allergic and non-allergic groups differed significantly in both SPT and BAT (**Figure 3A-D**). Receiver operating characteristic (ROC) analysis yielded area under the curve (AUC) values of 0.93 ± 0.03 and 0.92 ± 0.03 for walnut SPT and walnut-BAT respectively (**Figure 4A**). Receiver operating characteristic (ROC) analysis for pecan allergy yielded an AUC value of 0.87 ± 0.04 and 0.87 ± 0.03 for pecan SPT and pecan-BAT respectively (**Figure 4B**). The crossovers between the SPT and BAT curves in both walnut and pecan suggest use of both tests may complement one another to improve the diagnostic accuracy.

Diagnostic algorithm

Using the clinically-determined dispositions of patients in the NUT CRACKER-PS , we next sought to test the algorithm in this cohort. All 16 patients with an SPT less than 3 mm were validated to be non-allergic without any false negatives (**Figure 5**). Among the 56 patients with a walnut SPT ≥ 8 mm, 55 were true positives and one patient was diagnosed falsely positive. For those with a positive but intermediate SPT result ($3\text{mm} \leq \text{SPT} < 8 \text{ mm}$), a BAT was performed to clarify the diagnosis. The justification for this approach can be seen by ROC analysis, as the diagnostic utility of BAT was considerably stronger ($\text{AUC}=0.88\pm0.06$) compared to that of SPT ($\text{AUC}=0.73\pm0.08$) in diagnosing walnut allergy for patients with intermediate-positive SPT results (**Figure E3-A**), while the tests were similar at the two polar ends of testing ($<3 \text{ mm}$ or $\geq 8 \text{ mm}$) (**Figure E3-B**). A concordant positive BAT result ($\geq 5\%$) rendered the patient walnut-allergic and resulted in a true diagnosis in 25/27 patients with two false positives (**Figure 5**). A discordant negative BAT result yielded the need to utilize an OFC for diagnosis in 21 patients (**Figure 5**). Summarizing the disposition for the walnut arm of the diagnostic tree, 30 were non-allergic with no FN and 90 patients were considered walnut allergic including three false positives. All 87 walnut allergic patients (100% sensitivity) and 30 of the 33 walnut non-allergic patients (90.9% specificity) were identified ($\text{PPV}= 96.7\%$), with twenty-one of the 120 patients (17.5%) requiring a walnut OFC.

Regarding the pecan allergic state, the algorithm relies on the knowledge of co-allergenicity demonstrated previously (4). Similar to our original study all patients non-allergic to walnut ($n=30$) were validated to be pecan-tolerant. For those diagnosed as walnut allergic, a BAT was performed to evaluate pecan status. A positive pecan BAT ($\geq 5\%$) correctly classified 53/56 patients as having pecan allergy, along with three false positives. In order to be diagnosed pecan-tolerant, a negative BAT ($<5\%$) and a concordant negative SPT result ($<3\text{mm}$ wheal) were

required and correctly diagnosed four tolerant patients (**Figure 5**). Discordant SPT and BAT results were noted in 30 patients, requiring an OFC to determine the pecan status. Of these 30 patients, 24 were pecan allergic and 6 were pecan tolerant. Summarizing the disposition for the pecan arm of the diagnostic tree, 40 were non-allergic with no FN and 80 patients were considered pecan allergic including three false positives (**Figure 5**). All 77 pecan allergic patients (100% sensitivity) and 40 of the 43 pecan non-allergic patients (93% specificity) were identified (PPV=96.3%), with thirty of the 120 patients (25%) requiring a pecan OFC. Combining the walnut and pecan data, the diagnostic scheme decreased by 78.8% (189/240) the number of total OFC, yielding 2.5% (6/240) FP, and no FN. The categorization of allergic and tolerant patients as determined by each component (SPT, BAT and OFCs) of the algorithm for both walnut and pecan, is presented in **Figure 6**.

A post-hoc analysis was performed on the pecan arm of the algorithm with consideration that allergists often first perform a SPT due to its ease of application. The larger cohort of the current study demonstrated that a pecan SPT cutoff ≥ 8 mm provided a 95% PPV for diagnosis, which was not observed in the original NUT CRACKER study. In this scenario, utilizing SPT and BAT in the same manner as used in the walnut arm, the number of pecan challenges required would decrease to 15.8 % (19/120) (**Figure E4**). However, two additional patients would be categorized as FP. Combining this approach to the walnut data, the algorithm would decrease the number of required OFC by 83.3% (200/240) with 3.3 % (8/240) FP and no FN.

Discussion

One of the main challenges in diagnosing patients with tree nut allergy relates to the high degree of cross-sensitization between nuts which may not translate necessarily to co-allergy. Furthermore, there is a need to identify physiologic parameters to better predict potential reaction severity among patients. The NUT CRACKER study (4) proposed an algorithm to accurately distinguish between specific nuts to which a patient is allergic and minimize the need for OFC. The inclusion of basophil reactivity in the algorithm, facilitated its identification as a marker for allergic severity. In this prospective study utilizing an independent large set of patients, the algorithm significantly reduced the number of OFC required for diagnosis by approximately 80%, with a <3% false positive rate and no false negatives. The utility of the approach is based on several tenets. First, a standardized high concentration SPT formulation derived from ground nuts provides a useful first screening tool for diagnosis, since at the two polar extremes it allows to rule out or in walnut allergy, without the need for further testing. Second, this study confirms our previous findings (4) similarly observed by others (22), that all patients allergic to pecan were allergic to walnut, a finding consistent with them being in very closely related botanical groups. Conversely, therefore, patients ascertained to be non-allergic to walnut, were non-allergic to pecan, as well. Finally, the algorithm requires an OFC to be performed only in cases where SPT and BAT results were discordant, reducing significantly its need. Although an OFC is usually considered a safe test, it requires considerable expertise, and is not without risk. In addition, the algorithm minimizes false negatives that may subsequently be exposed inadvertently in an uncontrolled environment and false positives given the admittedly high cost to QOL and economic burden secondary to their misdiagnosis (for review see 23).

The BAT assay demonstrated its utility on two levels. BAT complemented SPT performance in cases where walnut-SPT results were in the mid-range. Both the reactivity of

different cells (mast cells versus basophils) and the differences in the protein make-up of the SPT (total protein) and BAT (soluble protein), potentially explain the complementary nature of SPT and BAT for diagnosis. The use of these two tests furthermore, safeguards against potential mishaps in technical application and/or reading of the SPT and performance of the BAT. In addition, the BAT also demonstrated its ability to identify walnut/pecan dual allergic patients and was associated with markers of reaction severity during the OFC, such as lower ED and adrenalin use. These data can assist clinicians to minimize OFCs, and be harnessed to identify individuals at risk for severe reactions in clinical setting.

The last decade has seen a substantial increase in the number of published reports concerning the clinical use of BAT, including for the diagnosis of food allergies (13,15,18,24). While standard practices for BAT have yet to be adopted, the identification of basophils by a CD123⁺ HLA-DR⁻ surface expression phenotype, with CD63 elevation as a marker for activation is a common strategy (14,25,26). The phenomenon of non-responding basophils has been reported to affect up to 17% of patients (27), but was a factor in only one patient excluded from our prospective study, perhaps due to addition of exogenous IL3 to the basophil stimulation mixture. The induction of CD63 on 5% of basophils as our diagnostic cutoff was in consonance with several previous studies (15,28,29). This value facilitates maximal identification of allergic patients, while remaining sufficiently above baseline to exclude most non-allergic subjects. While an appropriate diagnostic cutoff requires determination for each individual allergenic food, our findings support the adoption of BAT into a clinical allergy diagnostic framework, particularly in conjunction with other tests or with information on patient allergic background.

In comparison to the original data from which the algorithm was derived, a similar number of OFC were saved in this study, while the number of overall FP were more than halved from 5.6% to 2.5%. This may reflect the higher percentage of walnut-pecan dual allergic patients in the

current study, who are more sensitized than those with single walnut allergy (10). Additionally, in examining dual allergic patients, we noted that ED for walnut OFC were lower than for pecan, suggesting that walnut-pecan doubly allergic patients react to walnut from exposure to unique as well as allergens that are common to pecan. A further finding, while not completely understood, demonstrated stronger correlations between ED and BAT and SPT in pecan, compared to walnut. This may be reflective of the broader distribution of ED in pecan challenges.

There are several limitations to this study. First, availability of the high concentration SPT and resources to perform the BAT are not guaranteed. We would not expect, however, a difficulty to generate the SPT product which was derived from ground nuts and suspended at a fixed concentration for testing. This reduces the variability that may occur with prick to pick testing. Our SPT value for the walnut allergic cutoff ($\geq 8\text{mm}$) was $> 95\%$ PPV suggesting commercial preparations that achieve similar values, as demonstrated for walnut allergy (11), should perform equally well in the described algorithm. SPT of $< 3\text{ mm}$ was found to rule out allergy, in line with the original NUT CRACKER study (4). Given the high concentration of the SPT preparation, we expect this value to sufficiently protect against FN. However, given potential differences in the method of administration, in cases where the clinical history is highly suspect, it may be prudent to repeat the SPT to confirm results. The ease of preparation for the high concentration extract used in this study may additionally facilitate its use in other diagnostic schemes. Second, the BAT while available in hospital and research-based centers, in contrast, may not be readily available to the practicing physician. One possible scenario envisions that in the allergist's office the SPT can be performed and patients with mid-SPT values could subsequently be sent to a referral center for BAT. Furthermore, the use of blood collection tubes containing anticoagulants other than EDTA, allows BAT to be performed at least 24 hours after drawing of the blood (30). Third, while OFC or consumption in line with criteria in the EAT study (31) were used to define tolerance for an

overwhelming majority of walnut non-allergic patients, for four patients a minimal threshold of recent consumption of a single walnut was utilized. Given that only a minority of allergic patients reacted to an ED > 840 (the protein content of one walnut) there would be only a minimal effect on diagnosis. Third, the NUT CRACKER-PS cohort differed from the original NUT CRACKER cohort in the rate of walnut/pecan dual allergy. While this may have positively affected the pecan arm of the algorithm, this rate of co-allergy may reflect the actual rate of co-allergy between walnut and pecan, as reported by others (22).

In summary, using knowledge of co-allergy between walnut and pecan, in conjunction with modified SPT and BAT we were able to reduce the number of OFC required to diagnose walnut and pecan allergy by nearly 80% with <3% false positive rate and no false negatives. Basophil reactivity was also shown to reflect clinical manifestations of tree nut OFC. This algorithm may serve as a model for diagnosis of other closely related tree nuts (eg. cashew and pistachio). Future work on the algorithm should focus on testing its applicability to other populations.

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373 **Table 1. Demographics and clinical characteristics of original and current cohorts**

Parameter	All (n=183)	NUT CRACKER (n=63)	NUT CRACKER-PS (n=120)
Male	106 (57.9)	58.7%	57.5%
Age, years	8 (6 – 11)	8.7 (6.6 – 13.3)	7.8 (6 – 11)
With asthma	64 (35.0)	42.9%	30.8%
With atopic dermatitis	88 (48.9)	42.9%	50.8%
Other food allergies	64 (35%)	39.7%	32.5%
Walnut SPT, mm	7 (5 – 10)	7 (5 – 10)	7 (4.3 – 10)
Pecan SPT, mm	6 (4 – 8)	5 (2 – 6)	7 (5 – 10) [^]
Walnut %CD63	18 (1.4 – 54.7)	16.4 (1.3 – 49)	20.4 (1.4 – 58)
Pecan %CD63	2.7 (0.2 – 29.4)	1.4 (0 – 12)	3.9 (0.3 – 35.6) [^]
Reason for referral*:			
Hx of Reaction	98 (55.4%)	33 (57.9%)	65 (54.2%)
Positive SPT	38 (21.5%)	17 (29.8%)	21 (17.5%)
Other**	41 (23.1%)	7 (12.3%)	34 (28.3%)

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394 Categorical variables are presented as n (%).

395 Continuous variables are presented as medians and IQR

396 [^] Significant difference between NUT CRACKER and prospective study

397 * n=information available for 177 patients (120 NUT CRACKER-PS and 57 NUT CRACKER)

398 ** Referral due to reaction history to other foods, family history of allergy, or from fear of giving
399 allergens.

400 Hx, History

401

Table 2. Characterization of reactions and treatment in patients with both positive walnut and pecan OFCs

	Parameter	Walnut	Pecan	OR (95%CI)	P value
Reaction	Reaction dose, Median (IQR)	80 (40-210)	180 (100-660)	-	<0.0001
	Skin	49 (56.3%)	63 (72.4%)	2.1 (1.1-4.0)	0.038
	GI (abdominal pain/nausea)	37 (42.5%)	31 (35.6%)	0.7 (0.4-1.4)	NS
	GI (vomiting)	16 (18.4%)	12 (13.8%)	0.6 (0.2-1.7)	NS
	Respiratory (upper)	39 (44.8%)	30 (34.5%)	0.6 (0.3-1.2)	NS
	Respiratory (lower)	8 (9.2%)	19 (21.8%)	2.8 (1.1-7.2)	0.035
	Anaphylaxis	42 (48.3%)	48 (55.2%)	1.4 (0.7-2.6)	NS
Treatment	Antihistamine	72 (82.8%)	78 (89.7%)	2.0 (0.8-5.3)	NS
	Bronchodilator	11 (12.6%)	29 (33.3%)	4.0 (1.6-9.8)	0.001
	Adrenaline	25 (28.7%)	33 (37.9%)	1.6 (0.8-3.2)	NS
Grading	Grade I	25 (28.7%)	27 (31.0%)	1.1 (0.6-2.2)	NS
	Grade II	62 (71.3%)	58 (66.7%)	0.8 (0.4-1.5)	NS
	Grade III	0 (0.0%)	2 (2.3%)	-	-

n=87 patients with both positive walnut and pecan OFCs

OR (95%CI)=Pecan to Walnut McNemar's odds ratio (95%confidence interval)

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Figure Legends

Figure 1. Discrimination between walnut allergic patients without pecan allergy (n=27, WAL+/PEC-) and those with pecan allergy (n=107, WAL+/PEC+) for BAT (A) and SPT (B). Boxes show the IQR with the median value as the midline, with whiskers extending 1.5 times the IQR above and below. x= average values. Single dots represent outliers. p=0.003 for comparison of CD63 values between single vs. dual allergic patients.

Figure 2. The distribution of adrenalin treatments during walnut challenge across patients grouped by walnut-induced CD63 reactivity (p=0.006 by chi-square analysis).

Figure 3. Discrimination between walnut (A,B) and pecan (C, D) allergic and non-allergic patients by SPT (A,C) and BAT (B,D) in the NUT CRACKER-PS cohort. For walnut, n=87 allergic patients and n=33 non-allergic patients. For pecan, n=77 patients and n=42 non-allergic patients. Single dots represent outliers. P<0.0001 for comparisons between allergic and non-allergic for walnut and pecan SPT and BAT.

Figure 4. ROC curves for tree nut diagnostic tests. ROC curves plotting the proportion of the true positive patients (sensitivity) versus false positive patients for critical values of BAT, and SPT. The area under the curve (AUC) and the 95% confidence interval are presented. (A) Walnut allergy: n= 87 walnut allergic patients, n= 33 non-allergic patients. (B) Pecan allergy: n= 77 allergic patients, n= 42 non-allergic patients.

Figure 5. Algorithm depicting prospective evaluation of potential walnut-pecan-allergic patients. The algorithm utilizes the clinical findings of walnut pecan co-allergy, together with skin prick test (SPT) and basophil activation tests (BAT) for diagnosis. In cases where the SPT and BAT are discordant for diagnosis an OFC is required for diagnosis (shaded boxes). BAT +, %CD63 $\geq$ 5%; BAT -, %CD63 < 5%.

529 **Figure 6.** Categorization of allergic and tolerant patients as determined by SPT, BAT and OFCs
530 as directed by the diagnostic algorithms for walnut (A) and pecan (B).

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Figure 1.

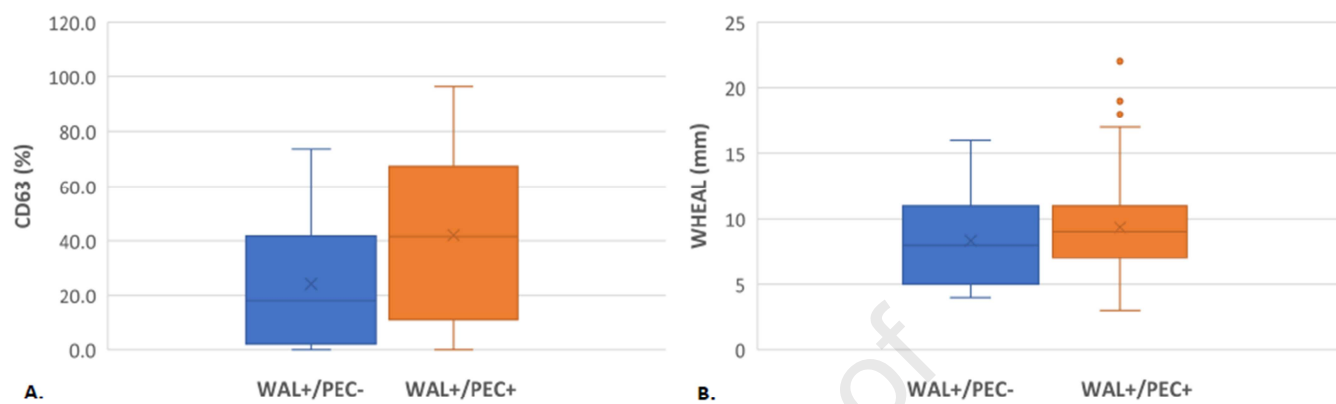


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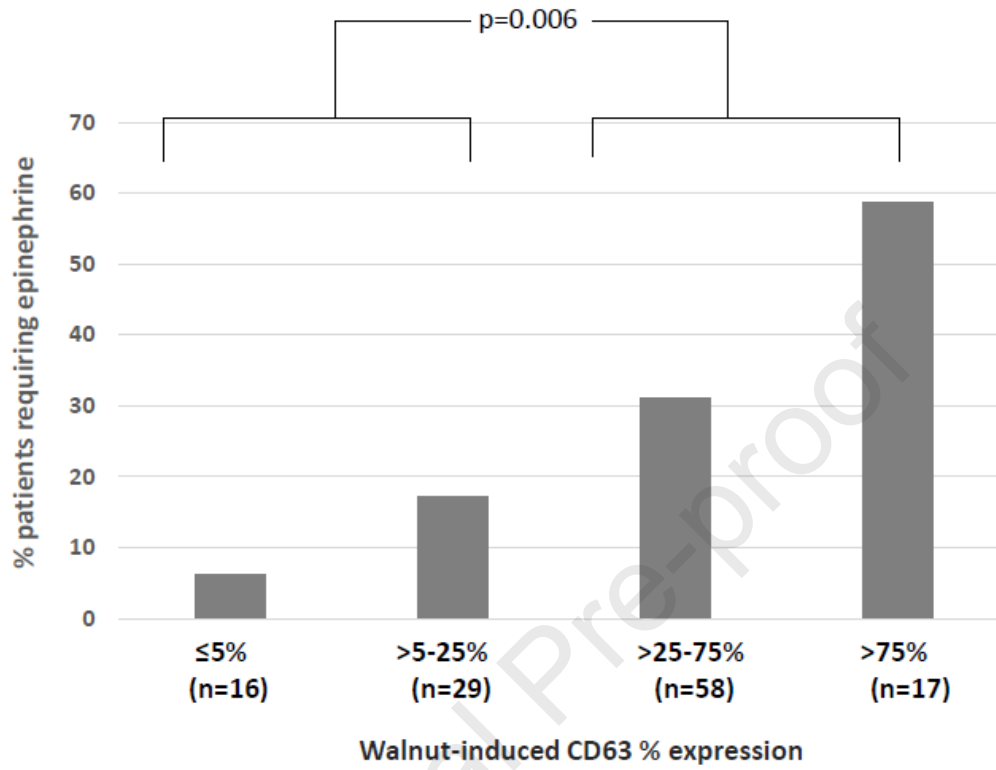


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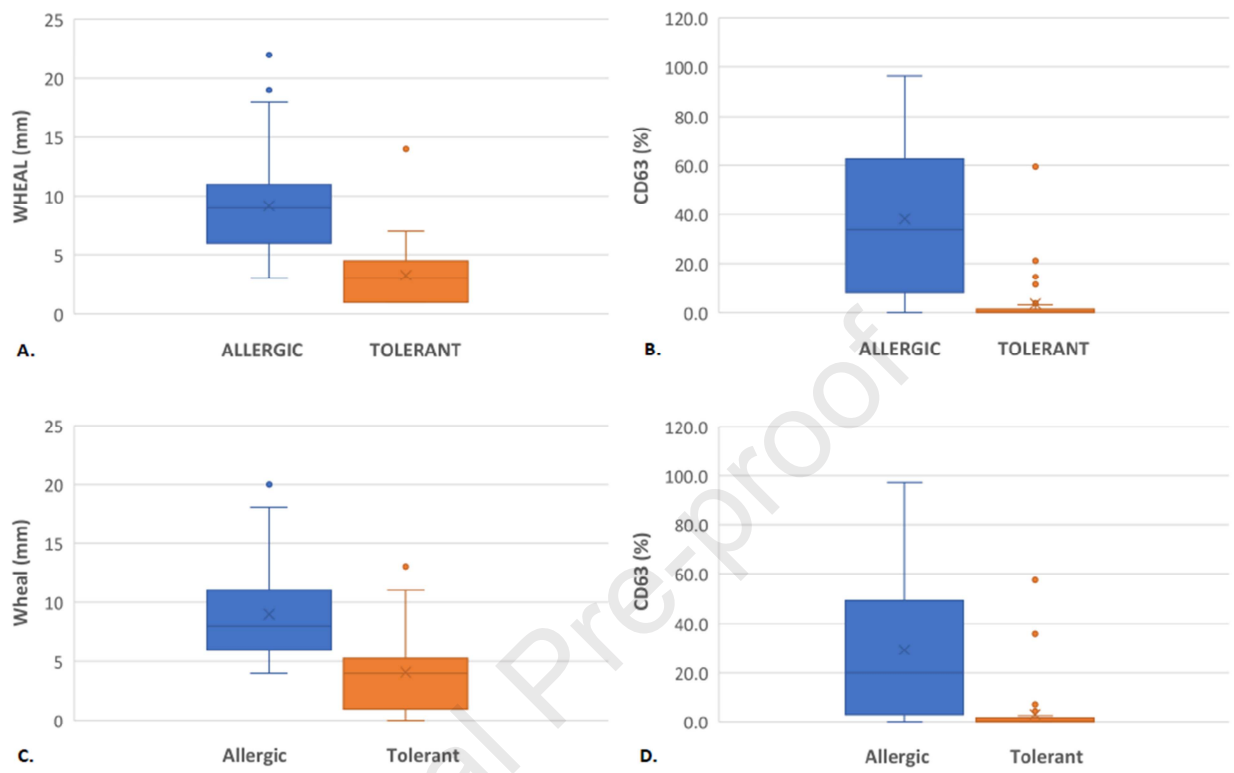


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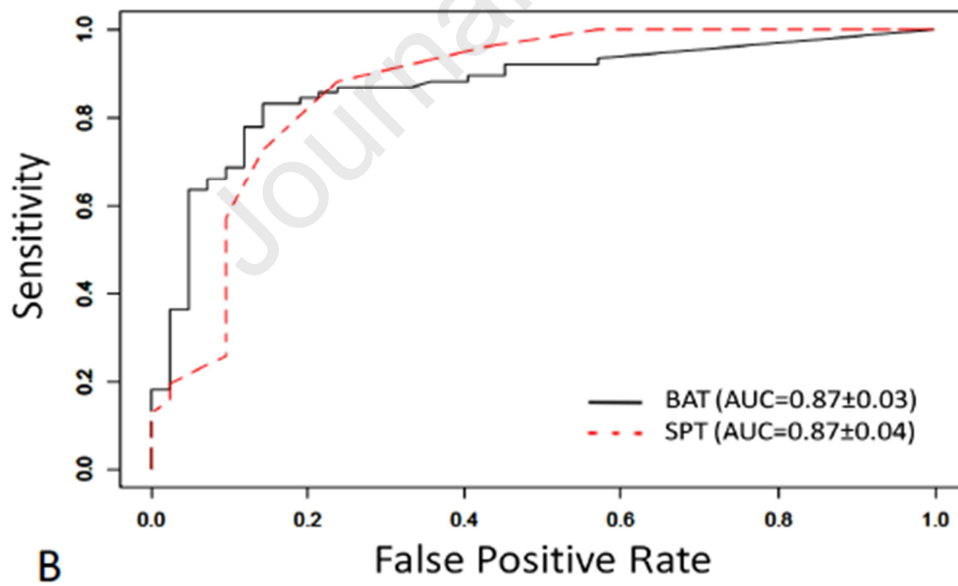
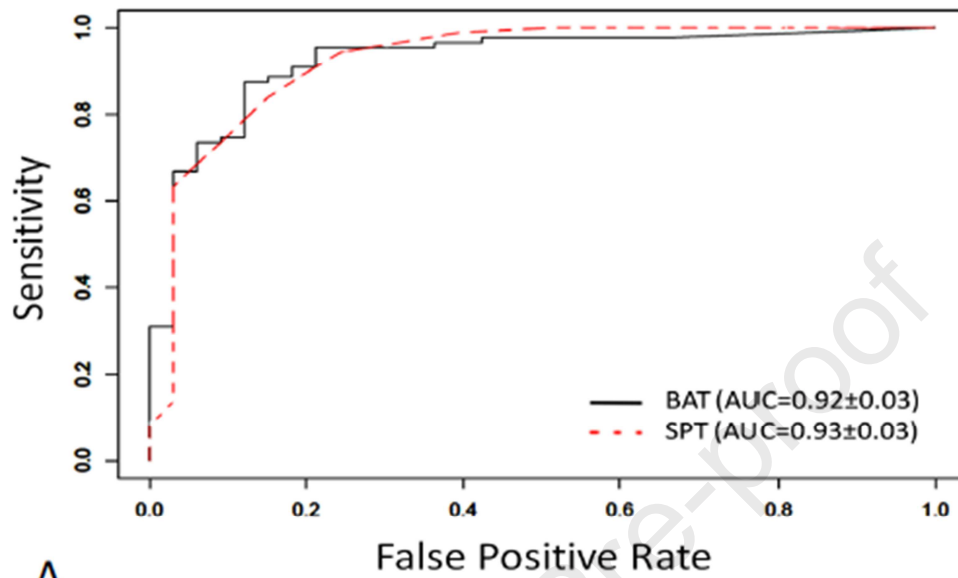


Figure 5

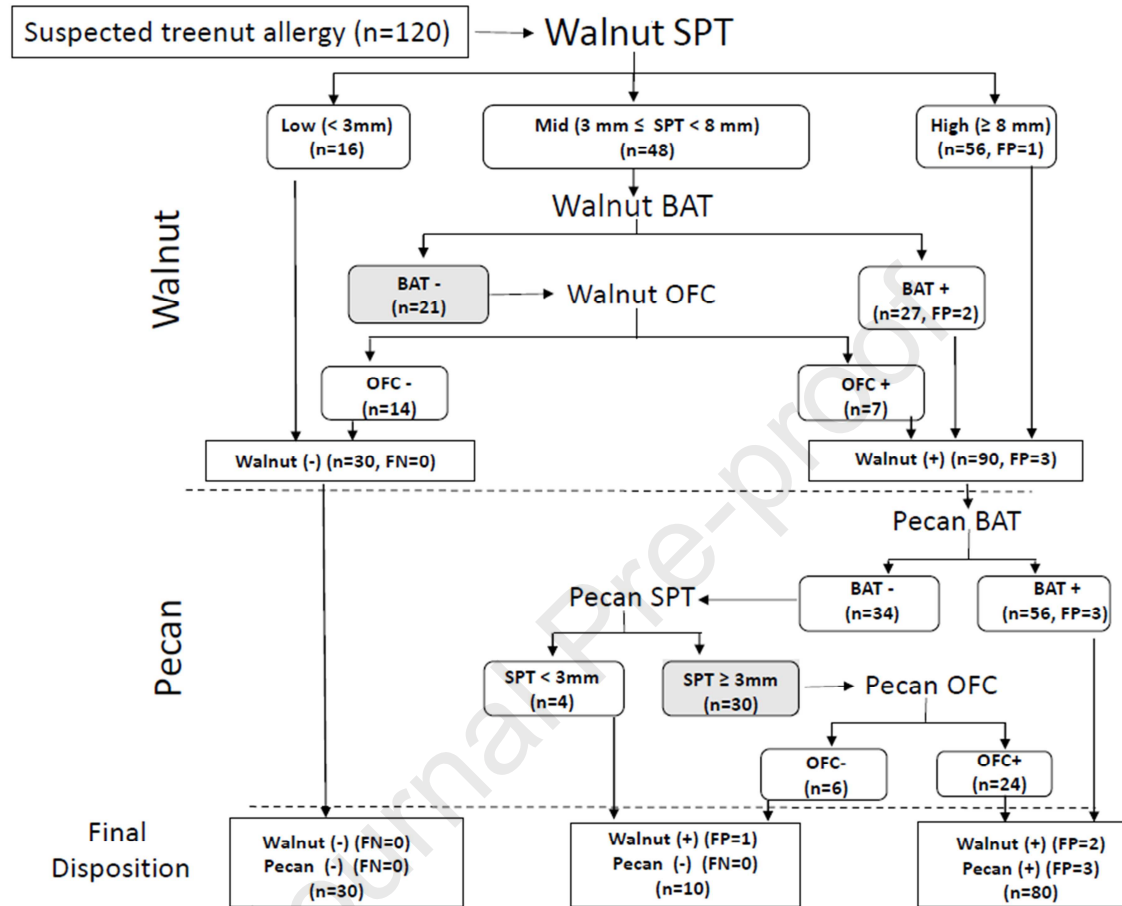


Figure 6

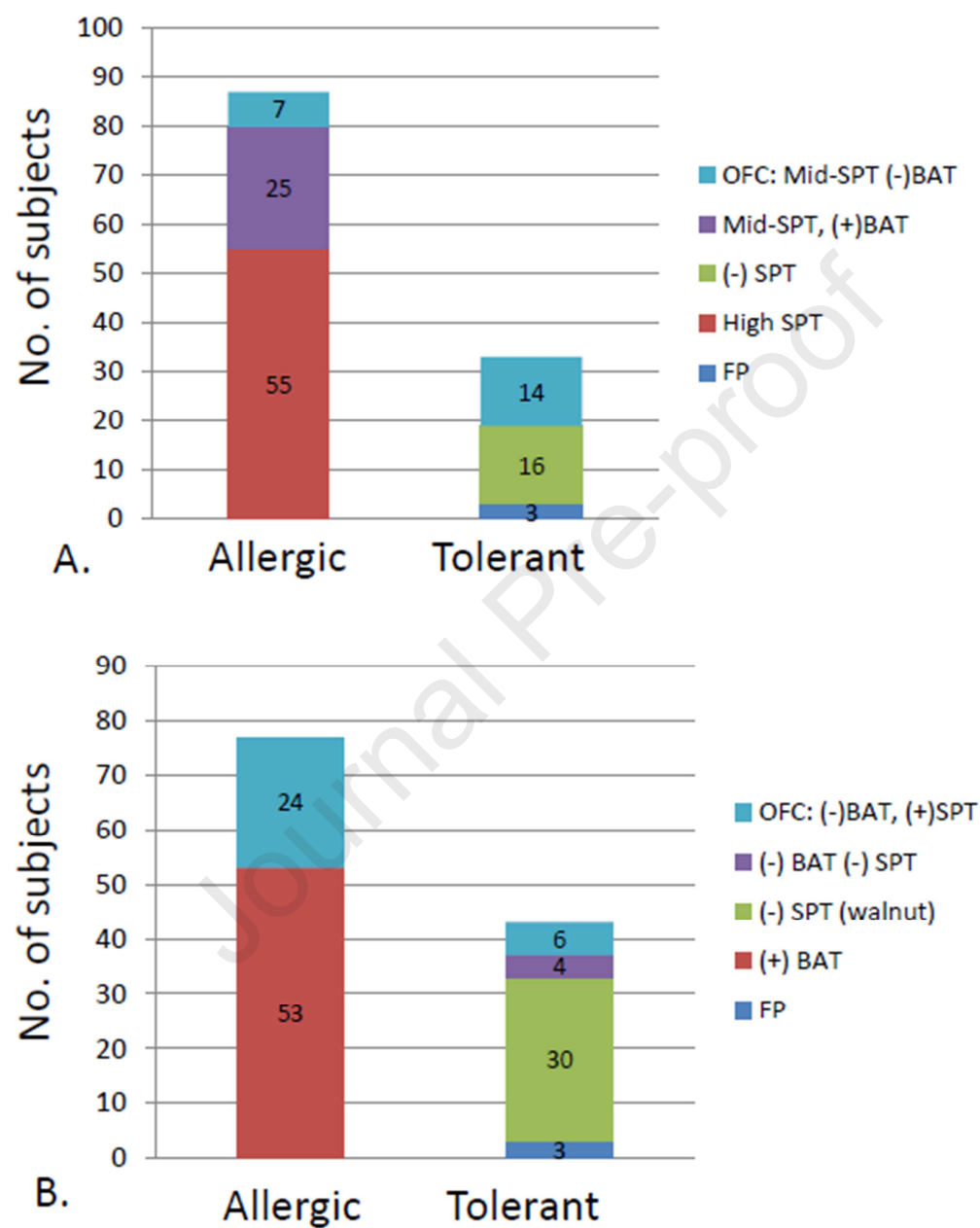


Figure E1.

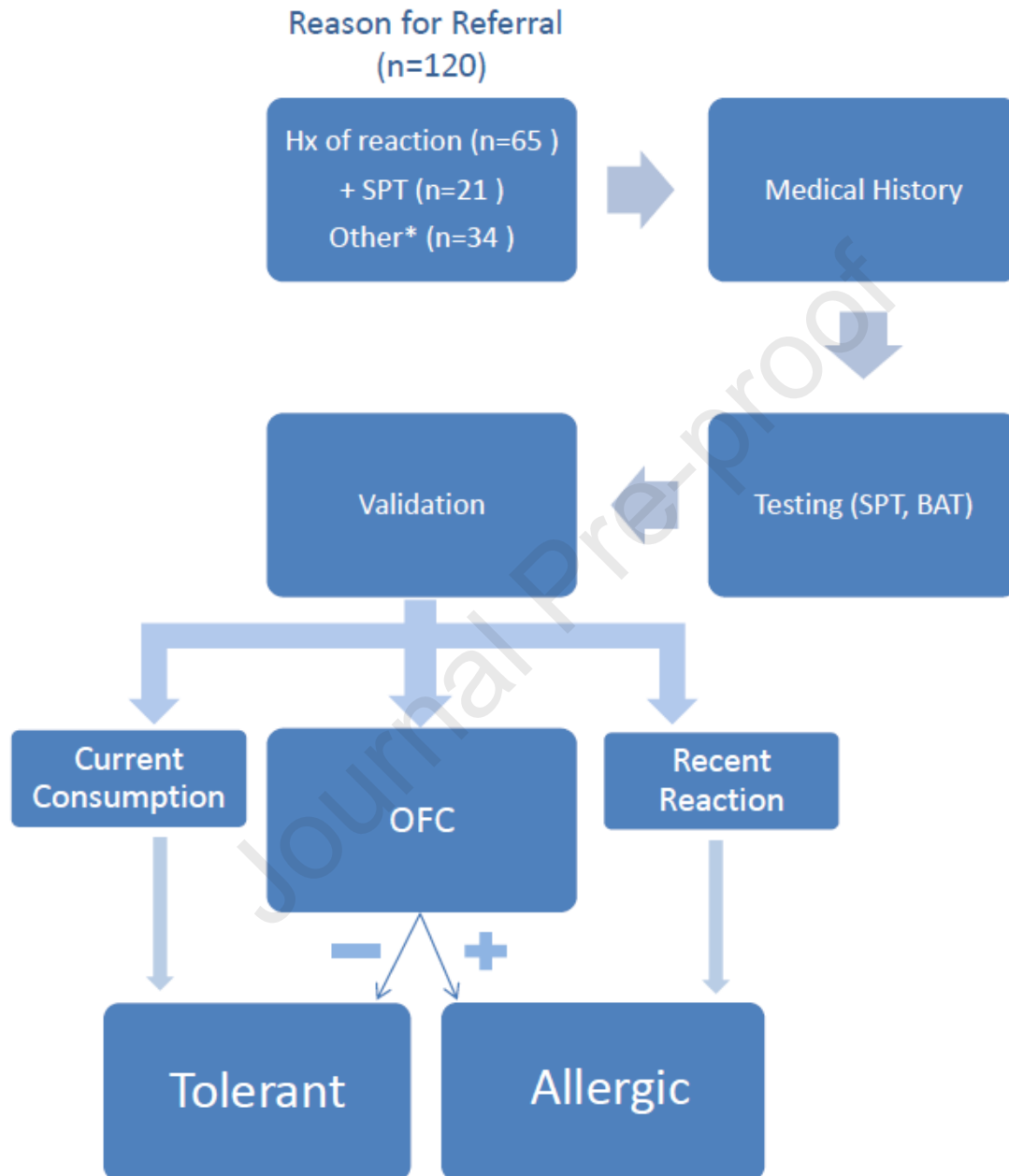


Figure E2.

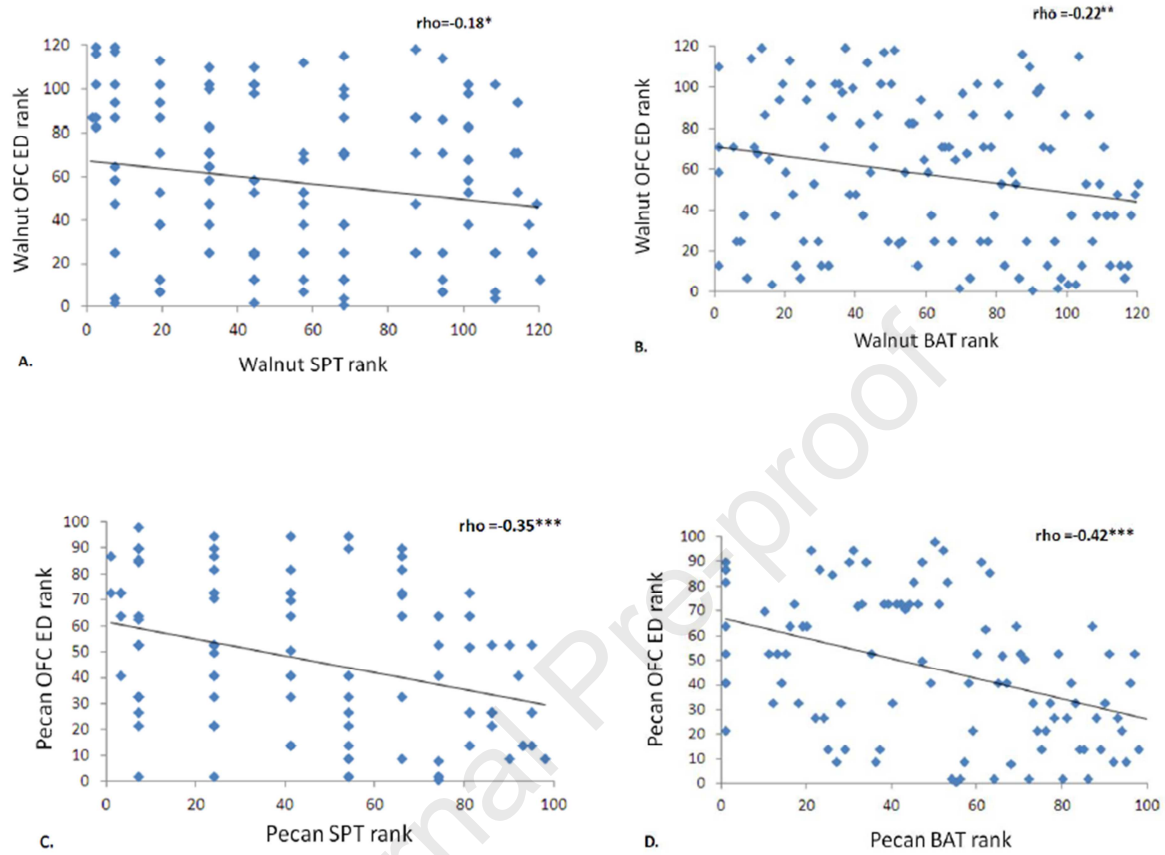
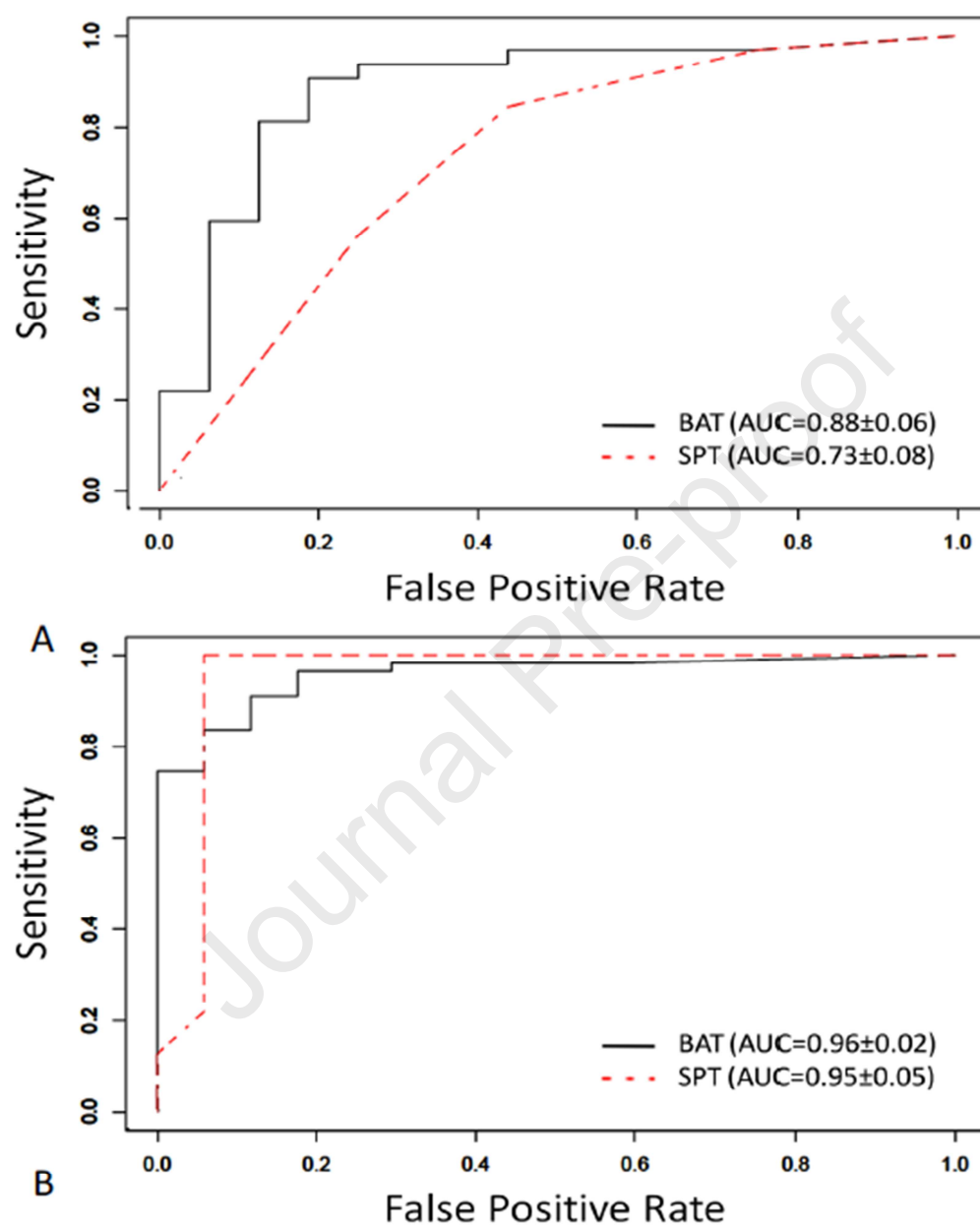


Figure E3.



1 Figure E4.

