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Cell-based Functional IgE Assays are Superior to Conventional Allergy Tests for Shrimp Allergy Diagnosis

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Cell-based Functional IgE Assays are Superior to Conventional Allergy Tests for Shrimp Allergy Diagnosis

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ABSTRACT

Background: The diagnosis of shellfish allergy currently relies on patient history, skin prick test (SPT) and serum specific IgE (sIgE) quantification. These methods lack sufficient diagnostic accuracy, while the gold standard of oral food challenges are risky and burdensome. Markers of reactivity and severity of allergic reactions to shellfish will improve clinical care of these patients.

Objectives: This study compared the diagnostic performance of SPT, sIgE, basophil activation test (BAT) and IgE crosslinking-induced luciferase expression (EXiLE) test for shrimp allergy.

Methods: Thirty-five subjects with documented history of shrimp allergic reactions were recruited and grouped according to results of double-blind placebo-controlled food challenge (DBPCFC). In addition to routine diagnostics, BAT (Flow CAST) and EXiLE test with shrimp extract and tropomyosin were performed.

Results: 15/35 subjects were shrimp-allergic with pruritus, urticaria and itchy mouth on DBPCFC while 20/35 were tolerant to shrimp. Tropomyosin only accounted for 53.3% of sensitization among subjects with challenge-proven shrimp allergy. BAT using shrimp extract as stimulant showed the highest AUC value (0.88), Youden index (0.81), likelihood ratio (14.73), odds ratio (104) and variable importance (4.27) when compared with other assays and tropomyosin diagnosis. Results of BAT significantly correlated with those of EXiLE ($r=0.664$, $P<.0001$).

Conclusion: BAT is a more accurate diagnostic marker for shrimp allergy than SPT and shrimp sIgE, while EXiLE test based on IgE-crosslinking assay is a good alternative to BAT.

Tropomyosin may not be the most important shrimp allergen in Chinese, which warrants further investigation to search for other major allergens and diagnostic markers.

Highlight Box

1. What is already known about this topic?

Shrimp allergy is prevalent, but conventional diagnostic methods including SPT and sIgE measurements have low specificity. Testing for tropomyosin improves diagnostic accuracy for shrimp allergy in Caucasians, but this has not been replicated among Asians.

2. What does this article add to our knowledge?

BAT has superior diagnostic performance for shrimp allergy than SPT and sIgE measurement. Tropomyosin may not be the most appropriate diagnostic marker in the Chinese population. EXiLE test can be a good alternative to BAT.

3. How does this study impact current management guidelines?

A single step of BAT may replace SPT and sIgE in the diagnosis of patients with clinical history suggestive of shrimp allergy. EXiLE test can be a suitable alternative with respect to cost and sampling constraint.

Keywords:

Shrimp allergy; basophil activation test; IgE crosslinking-induced luciferase expression test; DBPCFC; tropomyosin; CD63; CCR3

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Abbreviations used:

AUC: area under curve

BAT: basophil activation test

%CD63⁺/anti-FcεRI: ratio of the percentage of CD63⁺ basophil stimulated by culprit allergen to that by anti-FcεRI antibody

DBPCFC: double-blind placebo-controlled food challenge

EXiLE: IgE crosslinking-induced luciferase expression

LH: likelihood ratio

NPV: negative predictive value

OR: odds ratio

PPV: positive predictive value

ROC: receiver operating characteristic

SCORAD: SCORing Atopic Dermatitis

sIgE: specific immunoglobulin E

SPT: skin prick test

INTRODUCTION

Shellfish is one of the most common food allergens, with shrimp being the most frequent offender causing anaphylaxis in both pediatric and adult populations¹. In the United States, 2.9% of the adult population and 1.3% of children suffered from shellfish allergy^{2, 3}. In Europe, shrimp was one of the three commonest food allergens among adults with IgE sensitization rate of 1.46%⁴. Shellfish was the dominant food allergen among Asian populations, with prevalence rates of shellfish allergy ranging from 0.9% to 6.9%⁵. In Hong Kong, Guangzhou and Shaoguan, the prevalence of probable shellfish allergy as defined by reported symptoms together with positive skin prick test or specific IgE (sIgE) was 1.05%, 0.18% and 0.65% respectively⁶. Shellfish allergy is a growing healthcare concern due to its increasing global prevalence and low resolution rate^{7, 8}.

Like other food allergies, double-blind placebo-controlled food challenge (DBPCFC) is the gold standard for shrimp allergy diagnosis. However, it is risky, labor-intensive and expensive. Although skin prick test (SPT) and sIgE can aid in diagnosis, concerns about their poor diagnostic accuracy and correlation with clinical allergic reactions limited their application in routine patient care⁹⁻¹¹. Therefore, there is an unmet need to identify objective biomarkers that can accurately diagnose shrimp allergy and reflect its severity.

Over the last decade, basophil activation test (BAT) has been advocated as “food challenge in test tube”¹². This functional assay quantifies fluorometrically the number of activated CD63-expressing basophils in response to IgE crosslinking with a given dose of the culprit allergen. BAT has been shown to closely reproduce patients’ clinical phenotype, and basophil response can be a reliable biomarker for the severity and threshold of allergic

reactions¹³⁻¹⁶. Incorporating BAT into the diagnostic workup was also shown to reduce the need for oral food challenges¹⁷. Nonetheless, the pitfalls of BAT include limited window of sample processing and presence of non-responder basophils¹². Mast cell activation test (MAT), histamine release assay (HR), passive HR^{18, 19} and a rat basophilic leukemia cell line-based approach called IgE crosslinking-induced luciferase expression (EXiLE) test were alternative methods adopting similar machinery²⁰. In the EXiLE test, a rat basophil leukemia cell line that overexpresses human FcεRI receptor and firefly luciferase reporter gene (RS-ATL8 cell line) is stimulated by the culprit allergen in human serum. The degree of allergen-IgE crosslinking was then quantified by the luciferase signals²¹. The area under curve (AUC) of EXiLE test correlated well with outcome of oral food challenges²², which was reported as a convenient and sensitive diagnostic biomarker for egg allergy.

To our knowledge, these cellular tests have not been evaluated for usefulness to diagnose shrimp allergy. This study aimed to evaluate and compare the diagnostic efficacy of SPT, sIgE measurement, BAT and EXiLE test on shrimp allergic and tolerant subjects as defined by DBPCFC.

METHODS

Subjects

Patients aged 5-50 years who exhibited documented history of immediate allergic reactions within two hours of shrimp consumption on at least two occasions over the past five years were recruited from our three regional hospitals (Prince of Wales Hospital [PWH], Princess Margaret Hospital and Yan Chai Hospital). All subjects underwent allergy evaluations and DBPCFCs according to the EuroPrevall protocol in PWH (Online Repository Method & Fig E1), who were classified into allergic and tolerant groups²³. The study protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (2018.484) and Kowloon West Research Ethics Committee (127.12).

Allergy tests

SPT was performed with commercial shrimp extract (ALK-Abelló) together with 10 mg/ml histamine (positive control) and normal saline (negative control). The concentrations of total IgE and sIgE to shrimp (f24) and rPen a 1 (f351) were measured by ImmunoCAP (ThermoFisher Scientific). *In vitro* BAT was performed on fresh venous blood using Flow CAST kit (BÜHLMANN Laboratories) and predetermined concentrations of *Penaeus monodon* protein extract and recombinant Pen m 1 (rPen m 1) (Fig E2). EXiLE test was performed by sensitizing the RS-ATL8 cells with 1:100 diluted serum, followed by

stimulation with a pre-determined concentration of *P. monodon* protein extract or rPen m 1²¹.

Please refer to Online Repository for details of these assays.

Statistical analysis

GraphPad Prism (version 8.0) and SPSS (version 26) were used for graphical presentation and statistical analyses. Differences in quantitative variables were analyzed by Mann-Whitney U test, and categorical data was compared by Fisher exact test. The diagnostic accuracy of allergy tests was evaluated by Area Under Curve (AUC) value on receiver-operating characteristic (ROC). Optimal cutoff values to identify shrimp allergy were determined. Sensitivity, specificity, odds ratio (OR), likelihood ratios (LH), and positive (PPV) and negative (NPV) predictive values were calculated using two-by-two contingency tables, while the Youden Index was calculated as (sensitivity + specificity -1). The random forest method implemented in the SPSS add-on package for R system version 3.5 was used to investigate the ability of each allergy test to correctly discriminate between shrimp allergic and intolerant subjects based on the calculated variable importance, in which the higher the variable importance, the more relevant will that variable be for disease discrimination. To obtain sufficiently stable variable importance estimates, each forest consisted of 10,000 trees and the unscaled variable importance was reported²³. The correlation between allergy tests was assessed by Pearson correlation. $P < .05$ was considered as statistical significance.

RESULTS

Subjects' allergy status

Table I summarized the demographics, SPT and sIgE results of 15 shrimp allergic and 20 shrimp tolerant subjects as defined by DBPCFC. The median age of first shrimp-allergic reaction was 5.5 (2-19) years and 7.0 (2-17) years for the allergic and tolerant groups respectively. These two groups were matched for age, gender, total IgE, other food allergies and allergic diseases. The scores of allergic reactions that shrimp-allergic subjects developed during DBPCFC ranged from 3 (mild reactions) to 7 (severe reactions) (Table E1). The majority of these subjects reported skin reactions such as pruritus (80.0%) and urticaria (73.3%) as well as oral symptoms such as itchy mouth (66.7%). On the other hand, all shrimp tolerant subjects did not report any objective or persistent and severe subjective reactions (Table E2).

SPT and sIgE results

The shrimp allergic and tolerant groups differed significantly with respect to SPT wheal ($P=.0082$; Fig 1A) but not the rate for positive SPT ($P=.196$, Fisher exact Test; Table I). Neither sIgE levels nor the sensitization rates to shrimp and rPen a 1 at 0.35 kUA/L cutoff differed significantly between allergic and tolerant groups (Table I and Fig 1B & C). The “optimal decision points” (i.e. highest Youden Index) for SPT and sIgE to shrimp and rPen a 1 were determined from ROC curves (Table II). At cutoff wheal size of 4.75 mm, 9/14

(64.3%) allergic subjects and only 1/18 tolerant subject were SPT positive ($P=.0006$, Fisher exact test). At a higher cut-off sIgE to shrimp of 0.89 kUA/L, 14/15 (93.3%) of allergic and 11/20 (55.0%) tolerant subjects were positive ($P=.022$, Fisher exact test). Similarly, a higher cut-off sIgE to rPen a 1 of 0.56 kUA/L differentiated shrimp allergic and tolerant subjects ($P=.027$, Fisher exact test) in which 8/15 (53.3%) of allergic subjects and only 3/20 (15.0%) of tolerant subjects were sIgE positive.

Basophil activation test

Our group optimized BAT to shrimp and recombinant shrimp tropomyosin rPen m 1 prior to subject testing according to methods described in the Online Repository and Figure E3.

Whole blood samples were collected from all shrimp allergic subjects and all except three tolerant subjects. Of the 32 analyzed samples, none of the patients were non-responders; all samples showed $>10\%$ CD63-expressing basophils upon anti-FC ϵ RI monoclonal antibodies stimulation. Among allergic subjects, the median (range) %CD63⁺basophil at 5,000 and 10,000 ng/mL of shrimp extract were 67.1 (0.7-96.8) and 72.9 (0.9-94.7) respectively (Fig 1D & E). In the tolerant group, the respective median (range) %CD63⁺basophil were 2.4 (0.2-80.3) and 4.4 (0.6-91.8). The inter-group differences in %CD63⁺basophil were significant at both tested concentrations ($P=.0001$ and $<.0001$, respectively). When stimulated with rPen m 1, the median (range) %CD63⁺basophil were 3.8 (0.2-57.8) and 12.7 (0.2-61.0) in the allergic group (Fig 1 F & G), and 0.8 (0-15.6) and 1.0 (0.2-38.7) among tolerant subjects at the two tested concentrations. Such differences in %CD63⁺basophil between the allergic and tolerant groups were also significant at both concentrations ($P=.0472$ and $.0495$, respectively).

Based on the Youden Index, the optimal cutoffs for %CD63⁺basophil were 22.1% and 38.8% at the two shrimp extract concentrations respectively (Table II). At these cutoffs, 13/15 (86.7%) shrimp allergic subjects were BAT-positive but only 1/17 tolerant subject showed positive results ($P<.0001$, Fisher exact test; Table II). Using 5,000 ng/mL and 10,000 ng/mL rPen m 1 as stimulants for BAT, the respective optimal cutoffs were 16.1% and 9.0% (Table II). BAT positivity was detected in 7/15 (46.7%) allergic subjects while negative BAT was found on all tolerant subjects at 5,000 ng/mL rPen m 1 ($P=.0019$, Fisher exact test; Table II).

EXiLE test

Serum samples from all shrimp allergic and tolerant subjects were analyzed under optimized condition described in methods of the Online Repository and Fig E3. At 100 ng/mL of shrimp extract, the median (range) fold changes of luciferase expression were 1.8 (1.2-2.6) and 1.0 (0.2-2.5) in the allergic and tolerant groups respectively ($P=.0002$; Fig 1H). However, the fold changes induced by the same concentration of rPen m 1 did not differ between the two groups ($P=.6848$; Fig 1I). BAT and EXiLE, both analyzed the degree of IgE cross-linking, showed significant correlation by Pearson test ($r=.664$, $P<.0001$). From ROC curve, the optimal cutoff for EXiLE with shrimp extract was a fold increase of 1.1 (Table II). All shrimp allergic and 6/20 (30.0%) of tolerant subjects were positive ($P<.0001$, Fisher exact test). On the other hand, at the optimal cutoff for rPen m 1 at fold increase of 1.2, 8/15 (53.3%) allergic subjects and 50% of tolerant subjects were positive ($P>.999$, Fisher exact test; Table II).

Diagnostic performance of different allergy tests

We only analyzed allergy tests that showed significant differences in positivity between allergic and tolerant groups. Accordingly, we excluded conventional tests of SPT at 3 mm cutoff, sIgE to shrimp and rPen a 1 at 0.35 kUA/L cutoff points, and rPen m 1-EXiLE (Fisher exact test $P > .05$; Table II).

Among remaining tests, shrimp-BAT had the highest Youden Index (0.81) with sensitivity and specificity of 0.87 and 0.94 respectively regardless of stimulant concentrations (Table II). This test also showed the highest LH ratio (14.73), high PPV (0.93) and NPV (0.89). Despite having the highest sensitivity (1.00), shrimp-EXiLE had lower Youden Index (0.7) and a much lower LH ratio (3.33). For SPT at 4.75 mm wheal cutoff, it had a low Youden Index (0.58) but comparable specificity (0.94), PPV (0.90) and LH ratio (11.57) to shrimp-BAT.

The ability of allergy tests to discriminate between allergic and tolerant subjects were then compared by AUC values, OR and variable importance from random forest method (Table II, Fig 2 & 3). Shrimp-BAT at both concentrations had the highest AUC values (0.87 and 0.88 respectively), OR (104) and variable importance (3.20 and 4.27 respectively). These results indicated that BAT, particularly with 10,000 ng/mL shrimp extract as stimulant, was the best method to diagnose shrimp allergy. Despite comparable AUC value (0.85), shrimp-EXiLE had much lower OR (69.2) and variable importance (2.59). SPT at 4.75 mm cutoff had weak discriminative power (AUC value 0.77, OR 30.6 and variable importance 1.82), and the discriminative power was even weaker for conventional sIgE measurements at 0.89 kUA/L (shrimp extract) and 0.56 kUA/L (rPen m 1) cutoffs (Table II, Fig 2 & 3).

Diagnostic algorithm for shrimp allergy

Given the good diagnostic accuracy of shrimp-BAT and comparable accuracy of shrimp-EXiLE, we analyzed if single step tests with either SPT (at 3 mm wheal cutoff),

shrimp sIgE (≥ 0.35 kUA/L), shrimp-BAT ($\geq 38.1\%$ CD63⁺ basophil response) and shrimp-EXiLE (fold change ≥ 1.1) would predict DBPCFC outcomes (Fig 4A). Only 56.6% of SPT-positive subjects and 61.9% of sIgE-positive subjects reacted during shrimp DBPCFC. Shrimp-EXiLE performed slightly better than 70% of shrimp-EXiLE-positive subjects reacted during shrimp DBPCFC. By contrast, 12/13 (92.3%) patients with positive BAT reacted in DBPCFC while 14/16 (87.5%) patients with negative BAT results passed DBPCFC. We also compared algorithms with sequential use of different allergy tests (Fig 4B). Because SPT was widely used in clinical practice, we chose this allergy test as the first step in both algorithms. For algorithm with SPT followed by shrimp-sIgE, 12/17 (70.6%) patients with positive tests reacted during DBPCFC whereas five (29.4%) subjects were tolerant. This workflow showed acceptable sensitivity but only 0.29 specificity. For algorithm with SPT followed by shrimp-EXiLE, 13/16 (81.3%) patients with positive SPT and shrimp-EXiLE reacted in DBPCFC. The algorithm showed higher sensitivity and specificity (0.40) than that of sequential SPT and sIgE. By contrast, 12/13 (92.3%) patients with positive SPT and shrimp-BAT reacted in DBPCFC. This stepwise algorithm with SPT followed by BAT had the best sensitivity (0.92) and specificity (0.83). However, this stepwise approach did not improve the overall diagnostic accuracy when compared with the single shrimp-BAT approach.

DISCUSSION

This study demonstrated that standard assessment methods for shrimp sensitization could not accurately identify shrimp allergy. Our data indicated that basophil parameters were possible biomarkers of the clinical outcome of patients with shrimp allergy.

While DBPCFC remains the gold standard diagnosing food allergy, the clinical utility is limited by the cost and safety considerations. Accurate alternative diagnostic methods are therefore important to circumvent the need for oral food challenges. SPT and measurement of sIgE levels to shrimp extract remain the standard clinical procedures for helping to diagnose shrimp allergy. From our data, SPT is a more accurate predictor of shrimp allergy compared with shrimp sIgE that is also similarly reported for sesame allergy²⁴. Our study also corroborated with published studies that SPT and sIgE levels had high sensitivity of >90% but low specificity of 28% (SPT) and 35% (shrimp sIgE) at the standard cutoff points, as well as low AUC values that indicate their low diagnostic power to shrimp allergy^{25, 26}.

IgE crosslinking assays were shown to closely resemble the clinical phenotype of food allergic patients and reflect the severity and threshold of allergic reactions^{15, 27, 28}, as these tests took into account the levels, specificity, diversity and affinity of allergen-specific IgE as well as possible interference by other allergen-specific antibodies such as IgG4. This type of assays involved liquid phase rather than immobilized allergens as in ImmunoCAP test, so the former would more closely reflect the physiological interaction between allergens and IgE²⁹. Implementation of BAT in cow's milk allergy diagnosis was reported to reduce 30-40% of oral food challenges¹⁷. However, BAT assay has intrinsic disadvantages of small window period (4-24 hours) for sample processing after collection, presence of non-releaser basophils, and high operational cost³⁰. With the possibility to overcome these obstacles, our data showed that EXiLE test significantly correlated with BAT results. EXiLE could also discriminate between shrimp allergic and tolerant subjects with comparable sensitivity and AUC value to BAT. A single step shrimp-EXiLE analysis could already improve diagnostic accuracy comparing to conventional allergy tests such as SPT and sIgE measurement. EXiLE test may thus be a suitable alternative to BAT in terms of providing a cheaper option and when fresh blood samples and flow cytometric facilities are not available. This test also allows longer time from blood sampling to testing because EXiLE is done on archived serum instead of viable cells as required by BAT. However, it is noteworthy that BAT remained superior as a diagnostic test with the highest Youden index (i.e. high sensitivity and specificity) and much higher LH ratio, OR and variable importance measure compared to EXiLE and other allergy tests. This discrepancy is attributed to the functional heterogeneity of basophils and existence of different allergic manifestations among different subjects that cannot be reflected from an engineered cell line used in EXiLE test.

We demonstrated herein, for the first time, that BAT to shrimp was the most accurate in diagnosing shrimp allergy in challenge-confirmed patients. Our data demonstrated that in

contrast to ImmunoCAP tests that only consider sIgE concentrations, BAT using shrimp extract as stimulant was able to discriminate true allergic patients from a pool of individuals positive for shrimp-sIgE. This reflects the functional nature of BAT that takes into account both the affinity of sIgE and presence and level of sIgE. The discriminative performance of BAT for shrimp allergy diagnosis was equivalent to those for sesame and peanut allergies, with AUC values being 0.88 in our analysis and 0.86 and 0.97 for the latter foods respectively^{13, 28}. Our study and other publications also similarly showed higher sensitivity and specificity of BAT over conventional allergy tests. On the other hand, our findings demonstrated that even a single diagnostic step involving shrimp-BAT could significantly improve the diagnostic accuracy for shrimp allergy. However, the diagnostic accuracy was comparable between a single diagnostic step with shrimp-BAT and when adding basophil response as a second diagnostic step to all patients with a positive SPT. Nevertheless, our algorithm clearly illustrated the incomparable discriminative ability of shrimp-BAT as both a rule-in and rule-out test. Our study thus sets the stage for more accurate diagnosis of shrimp allergy and reduction in use of oral food challenges.

Other studies suggested that measuring tropomyosin-sIgE level could better predict clinical reactivity to shrimp, with independent studies reporting >90% NPV with this parameter^{9, 10}. Although measuring rPen a 1-sIgE levels generates a specificity of 85% at 0.56 kUA/L decision point in this study, only 8/15 (53.3%) of our shrimp-allergic patients were tropomyosin sIgE-positive when compared with those by Pascal et al. (82.8%) and Yang et al. (71.4%)^{10, 25}. Although the utilization of shrimp extract-based BAT significantly improved the diagnostic accuracy comparing to ImmunoCAP test with shrimp extract, both allergy tests with shrimp tropomyosin only presented comparable performance (i.e. unremarkable differences in diagnostic properties including AUC values, sensitivity, specificity, LH ratio and variable importance). Consistently, only 7/15 shrimp-allergic subjects showed positive

basophil response to rPen m 1 at both tested concentrations. Taking together the relatively low IgE sensitization rate to shrimp tropomyosin similarly reported in Thai (34.2%) and Japanese (37%)^{26, 31}, it is possible that tropomyosin might not be the major allergen in Asians. This finding challenged the suitability of tropomyosin as a diagnostic biomarker in these populations.

We noted that both the concentrations of protein extract used and the optimal decision point detected in this study were higher than prior BAT studies in which up to 1,000 ng/mL allergen extract was often adopted with cutoff points of 4.78%-17%¹². However, these differences might be due to the fact that peanuts and tree nuts had higher basophil activation levels than other allergens³². However, we would like to emphasize that our BAT assay could accurately differentiate shrimp allergic from tolerant individuals with high sensitivity and specificity. Considering that our BAT was performed using commercial kit with shrimp extract and recombinant allergen being prepared by standardized methods, it would not be difficult for allergy groups in other populations to establish this diagnostic allergy test for shrimp allergy.

BAT results could indeed be interpreted by the absolute percentage of activated basophils, ratio of %CD63⁺ basophil stimulated by allergen to that by anti-IgE (%CD63⁺/anti-FcεRI), basophil threshold sensitivity (CD-sens) and effective dose at 50% of maximum dose response (EC50)¹⁴. Santos et al. reported that %CD63⁺/anti-FcεRI was the best basophil marker for predicting symptom severity in peanut allergy¹⁵. In contrary to their report, we did not detect an improved diagnostic performance when BAT results were interpreted as %CD63⁺/anti-FcεRI when compared to absolute percentage; with equal AUC values, Youden Indices, PPV, NPV and LH (data not shown). This might be attributed to similar basophil responses between shrimp allergic and tolerant subjects upon anti-IgE

stimulation, which was different from findings in prior reports³³. However, this study had not investigated the underlying mechanism behind this difference.

This study had several limitations. The EC50 values and CD-sens of BAT were not assessed due to the lack of CD63 dose-response curve for all tested samples. Only tropomyosin, being the major shrimp allergen in published studies, was analyzed in this study while a number of other clinically important allergenic components were identified³⁴. Our findings revealed that tropomyosin only contributed to ~50% of all shrimp sensitization among allergic subjects. Thus, further studies should investigate shrimp allergy diagnosis using other allergens, such as sarcoplasmic calcium-binding protein and myosin light chain that are associated with clinical reactivity to shrimp as well as arginine kinase and hemocyanin that denote cross-reactivity with tropomyosin²⁵. While BAT with shrimp extract may be a robust test, our present study only compared the magnitude of parameters such as AUC values and Youden indexes without making statistical comparison. Besides, our sample size calculation suggested that 30 participants would allow us to compare AUC values 0.7-0.9 for different diagnostic tests (see Online Repository Text). Nonetheless, this number might still be insufficient for other diagnostic parameters. Future studies need to recruit a larger sample size and test for more shrimp allergens in the diagnostic algorithms with conventional tests and cell-based functional IgE assays for shrimp allergy.

In conclusion, this is the first study to evaluate the diagnostic utility of BAT in shrimp allergic patients as defined by DBPCFC. Our results demonstrate the superior diagnostic accuracy of BAT over conventional allergy tests. Further studies are needed to test the combinatorial approach of BAT with other shrimp allergens in the component-resolved diagnosis for shrimp allergy as well as the optimal algorithm with different biomarkers that can minimize the need for oral food challenges.

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FIGURE LEGENDS

FIG 1. SPT, sIgE, BAT and EXiLE responses of shrimp allergic and tolerant subjects. Individual data points with median (line) are presented. Differences in the responses between two groups were assessed by Mann-Whitney U test. Bold face indicates statistically significant difference.

FIG 2. ROC curves plotting the proportion of the true allergic and tolerant subjects. The curve for each type of allergy test (SPT, BAT using 5,000 and 10,000 ng/mL of shrimp extract or 5,000 ng/mL of rPen m 1, as well as EXiLE test using shrimp extract) is depicted by a different color. AUC values (95% CI) are shown.

FIG 3. Variable importance plot. High positive values of mean decrease in accuracy mean a higher variable importance, whereas the small positive values indicate a variable that is less relevant for discrimination.

FIG 4. Stepwise diagnostic approach for shrimp allergy. (A) Schematic diagrams depicting single-step approach for the diagnosis of shrimp allergy. (B) Schematic diagrams depicting two-step approach with the complementary utility of SPT and sIgE level to shrimp, SPT and shrimp-EXiLE, as well as SPT and shrimp-BAT. Cut-off points for SPT, shrimp-sIgE, shrimp-EXiLE and shrimp-BAT are ≥ 3 mm wheal size, ≥ 0.35 kUA/L, ≥ 1.1 fold change and 38.8%, respectively.

Table I Characteristics of study participants

Demographic Features	Allergic Group	Tolerant Group	P-value
N	15	20	/
Gender: male, % (number)	66.7 (10)	45.0 (9)	0.306 [^]
Age (year): median [range]	25.0 [5-44]	30.5 [9-49]	0.443 [#]
Total IgE (kIU/L): median [range]	386 [25.9-2781]	464 [44.8-4081]	0.705 [#]
Other food allergies: % (number)	60.0 (9)	30.0 (6)	0.097 [^]
Eczema: % (number)	80.0 (12)	65.0 (13)	0.458 [^]
Asthma/allergic Rhinitis: % (number)	66.7 (10)	40.0 (8)	0.176 [^]
Age of shrimp allergy onset (year): median [range]	5.5 [2-19]	7.0 [2-17]	0.821 [#]
History of shrimp allergy	+	+	/
DBPCFC	+	-	/
SPT			
Wheal size (mm): median [range]	5.5 [0-12]	3.3 [0-5]	0.0082[#]
Sensitization rate: % (number)	92.8 (13/14)	72.2 (13/18)	0.196 [^]
sIgE to shrimp			
Level (kUA/L): median [range]	2.28 [0.24-33.2]	1.71 [0.01-11.1]	0.122 [#]
Sensitization rate: % (number)	93.3 (14)	65.0 (13)	0.101 [^]
sIgE to rPen a 1			
Level (kUA/L): median [range]	0.65 [0-24.5]	0.01 [0-7.04]	0.097 [#]
Sensitization rate: % (number)	53.3 (8)	20 (4)	0.071 [^]

Values are show as percentages (number) or median [range]. Statistical differences were assessed by Fisher exact test for categorial data ([^]) and Mann-Whitney U test for quantitative variables ([#]). Bold face indicates statistically significant difference. "+", positive; "-", negative; *DBPCFC*, double-blind placebo-controlled food challenge; *SPT*, skin prick test; *sIgE*, specific IgE.

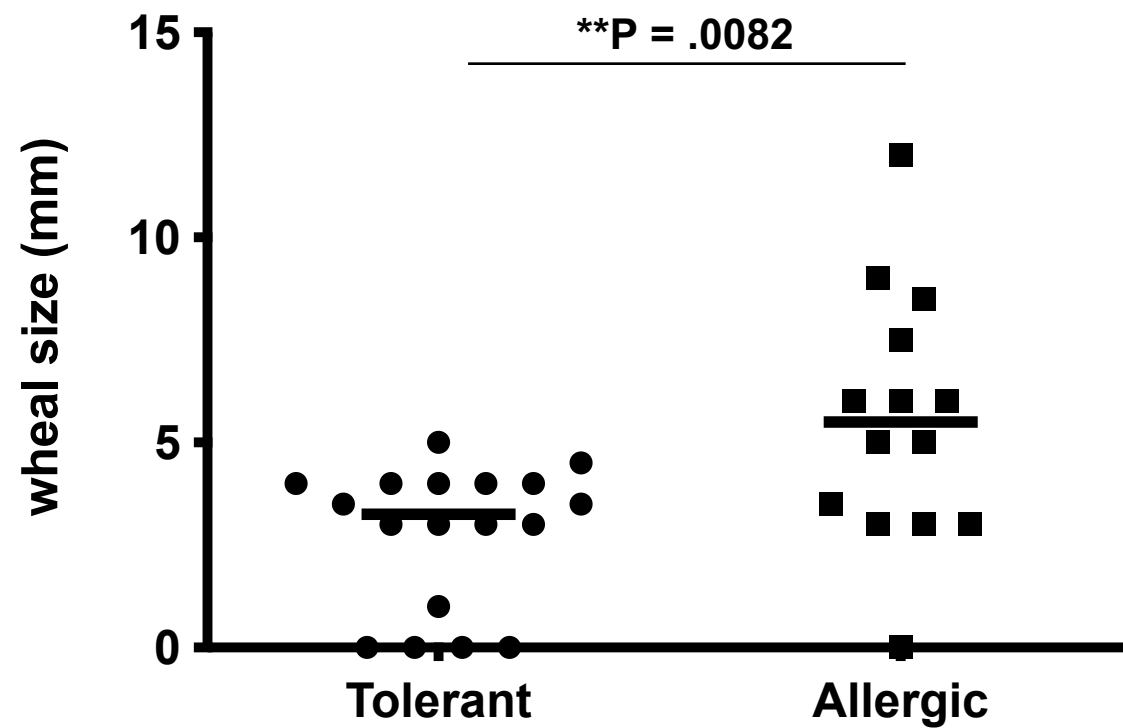
Table II Properties of SPT, ImmunoCAP, BAT and EXiLE test as diagnostic tests for challenge-confirmed shrimp allergy

Allergy Test	P-value by Fisher Exact Test	AUC	Odds Ratio	Cut-off	Sensitivity	Specificity	Youden's Index (J)	PPV	NPV	Likelihood ratio
SPT	0.196	0.77 [0.59-0.95]	5.00 [0.51-48.9]	3.0 mm	0.93 [0.66-1.00]	0.28 [0.10-0.53]	0.22	0.50 [0.30-0.70]	0.83 [0.36-1.00]	1.29
	0.001		30.6 [3.09-303.4]	4.75 mm*	0.64 [0.35-0.87]	0.94 [0.73-1.00]	0.58	0.90 [0.56-1.00]	0.77 [0.55-0.92]	11.57
slgE to shrimp	0.101	0.66 [0.47-0.84]	7.53 [0.81-69.9]	0.35 kUA/L	0.93 [0.68-1.00]	0.35 [0.15-0.59]	0.28	0.52 [0.32-0.71]	0.88 [0.47-1.00]	1.44
	0.022		11.5 [1.25-104.6]	0.89 kUA/L*	0.45 [0.23-0.68]		0.38	0.56 [0.35-0.75.6]	0.90 [0.56-1.00]	1.70
slgE to rPen a 1	0.071	0.67 [0.48-0.85]	4.57 [1.03-20.4]	0.35 kUA/L	0.53 [0.27-0.79]	0.80 [0.56-0.94]	0.33	0.67 [0.35-0.90]	0.70 [0.47-0.87]	2.67
	0.027		6.49 [1.39-26.2]	0.56 kUA/L*	0.53 [0.30-0.75]	0.85 [0.64-0.95]	0.38	0.73 [0.43-0.90]	0.71 [0.51-0.85]	3.56
BAT to shrimp (5,000 ng/ml)	<0.001	0.87 [0.74-1.00]	104 [8.46-1279]	22.1%*	0.87 [0.60-0.98]	0.94 [0.71-1.00]	0.81	0.93 [0.66-1.00]	0.89 [0.65-0.99]	14.73
BAT to shrimp (10,000 ng/ml)		0.88 [0.74-1.00]		38.8%*						
BAT to rPen m 1 (5,000 ng/ml)	0.002	0.71 [0.52-0.89]	30.9 [1.57-606.8]	16.1%*	0.47 [0.21-0.73]	1.00 [0.80-1.00]	0.47	1.00 [0.59-1.00]	0.68 [0.47-0.85]	NA [#]
BAT to rPen m 1 (10,000 ng/ml)	0.049	0.70 [0.52-0.89]	6.56 [1.10-39.3]	9.0%*		0.88 [0.64-0.99]	0.35	0.78 [0.40-0.97]	0.65 [0.43-0.84]	3.97
EXiLE to shrimp	<0.001	0.85 [0.72-0.98]	69.2 [3.57-1340]	1.1 FC*	1.00 [0.78-1.00]	0.70 [0.46-0.88]	0.70	0.71 [0.48-0.89]	1.00 [0.77-1.00]	3.33
EXiLE to rPen m 1	>0.999	0.54 [0.35-0.74]	1.14 [0.27-4.04]	1.2 FC*	0.53 [0.30-0.75]	0.50 [0.30-0.70]	0.03	0.44 [0.25-0.66]	0.59 [0.36-0.78]	1.07

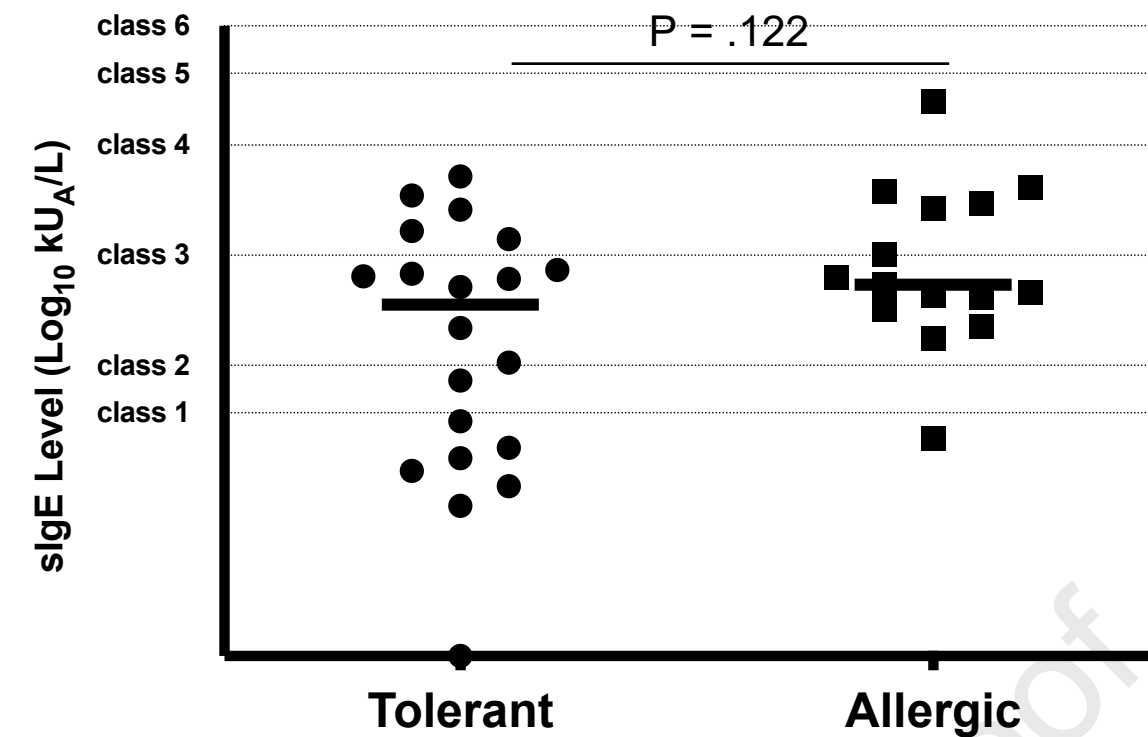
Values are shown 95% CI. Odds ratio, sensitivity, specificity, Youden Index, PPV, NPV and likelihood ratio were determined from the two-by-two contingency table. Boldface indicates statistically significant difference. *AUC*, area under curve; *PPV*, positive predictive value; *NPV*, negative predictive value; *SPT*, skin prick test; *BAT*, basophil activation test; *EXiLE*, IgE crosslinking-induced luciferase expression; *NA*, data not available. * Optimal cutoff point as determined by the ROC curve based on the highest Youden Index calculated as (sensitivity + specificity - 1). [#] *NA*: Likelihood ratio for BAT assay using rPen m 1 (5,000 ng/mL) could not be determined from two-by-two contingency table.

SPT

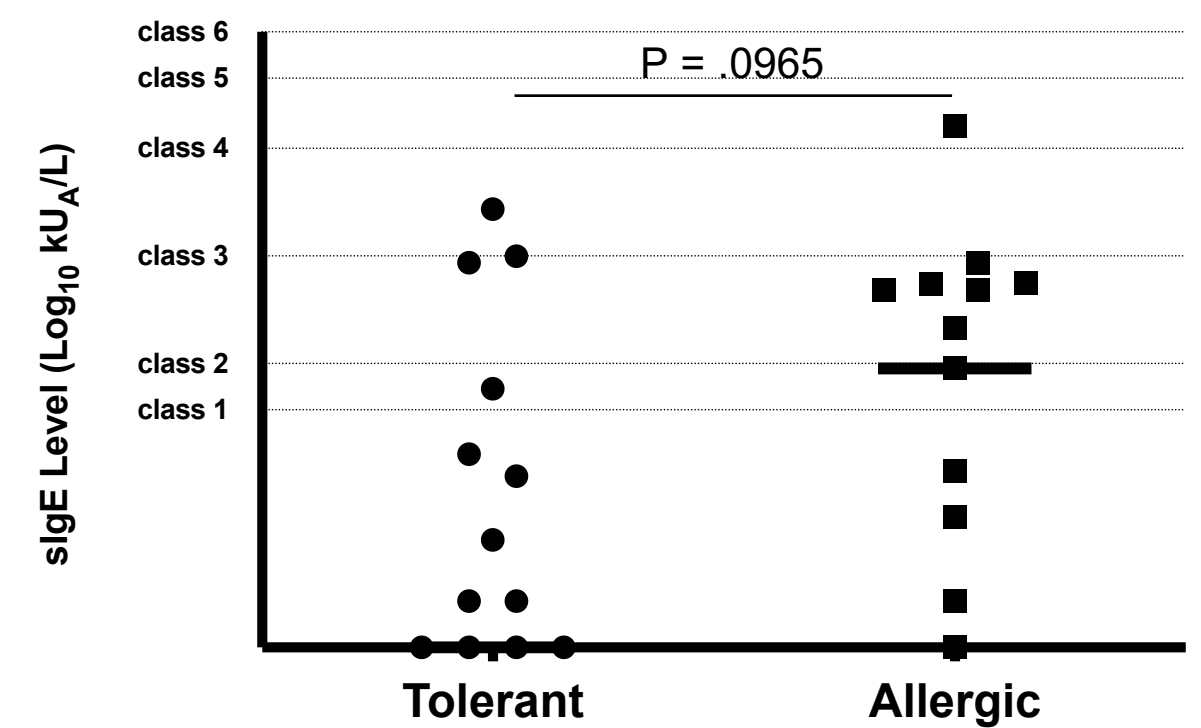
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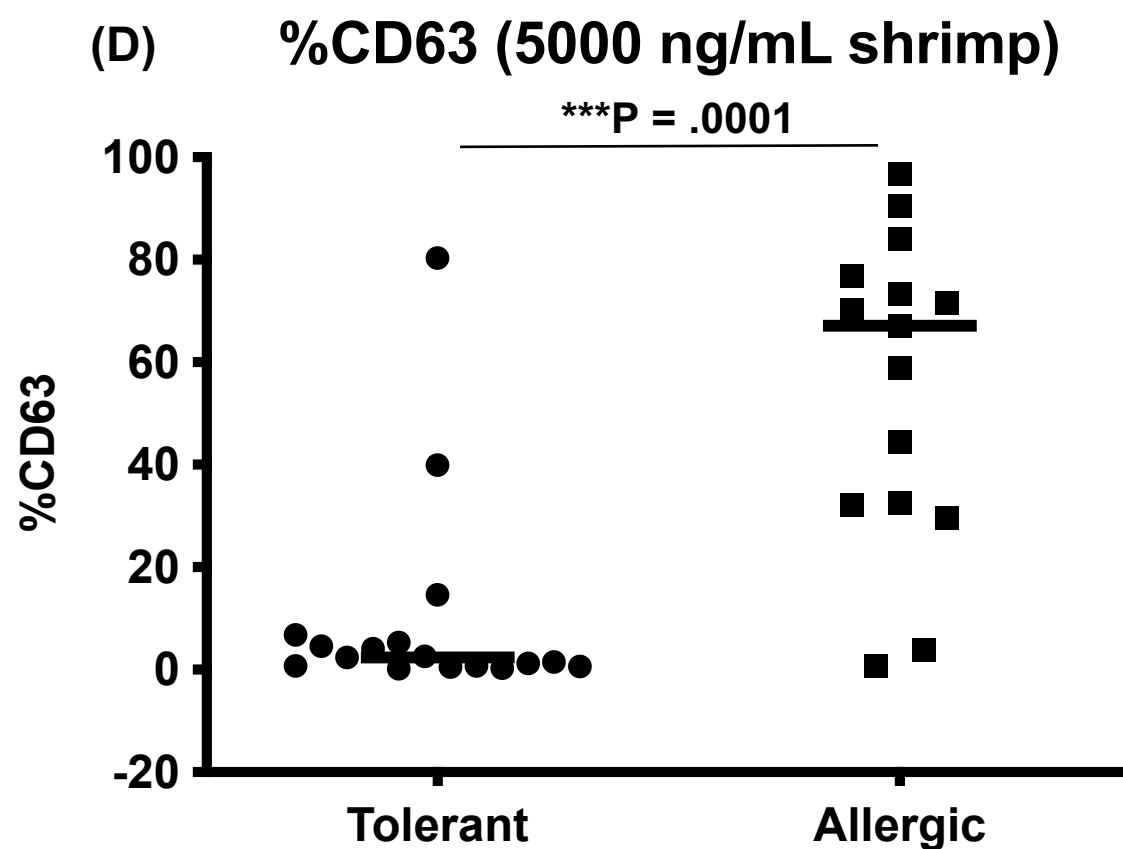
(B)



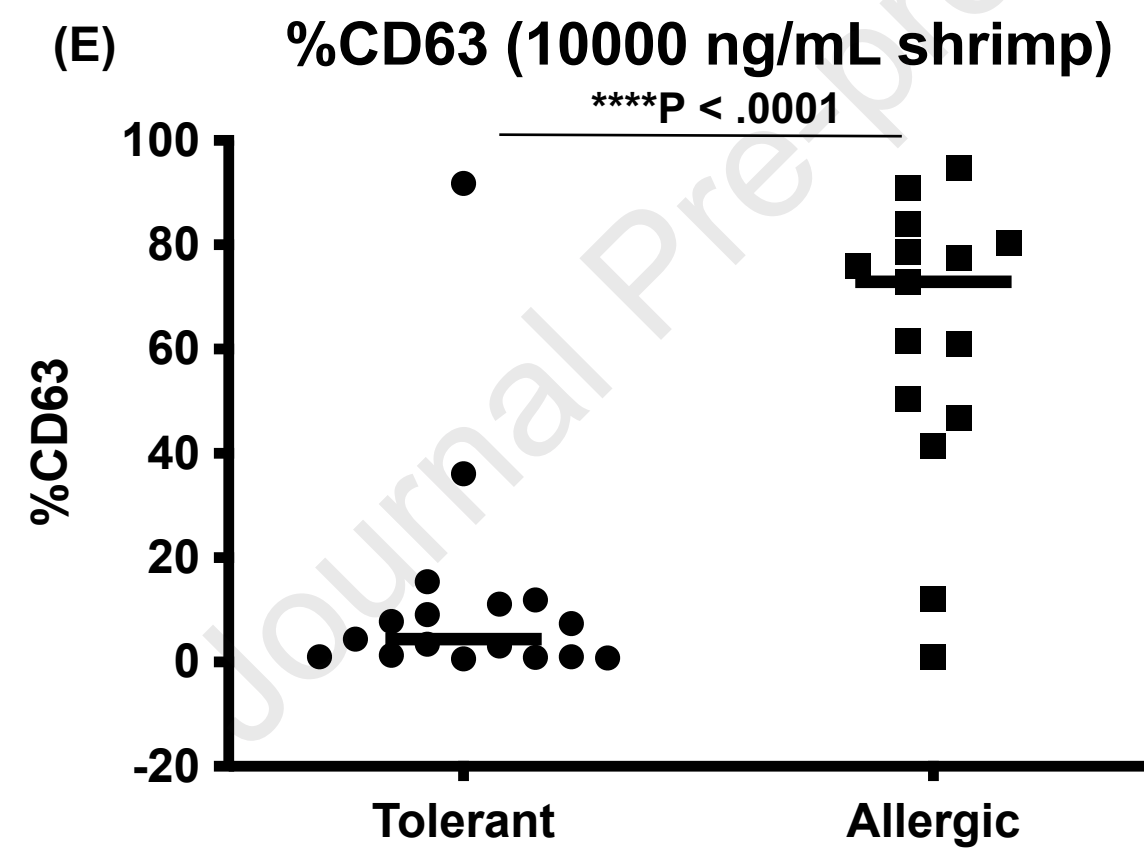
(C)



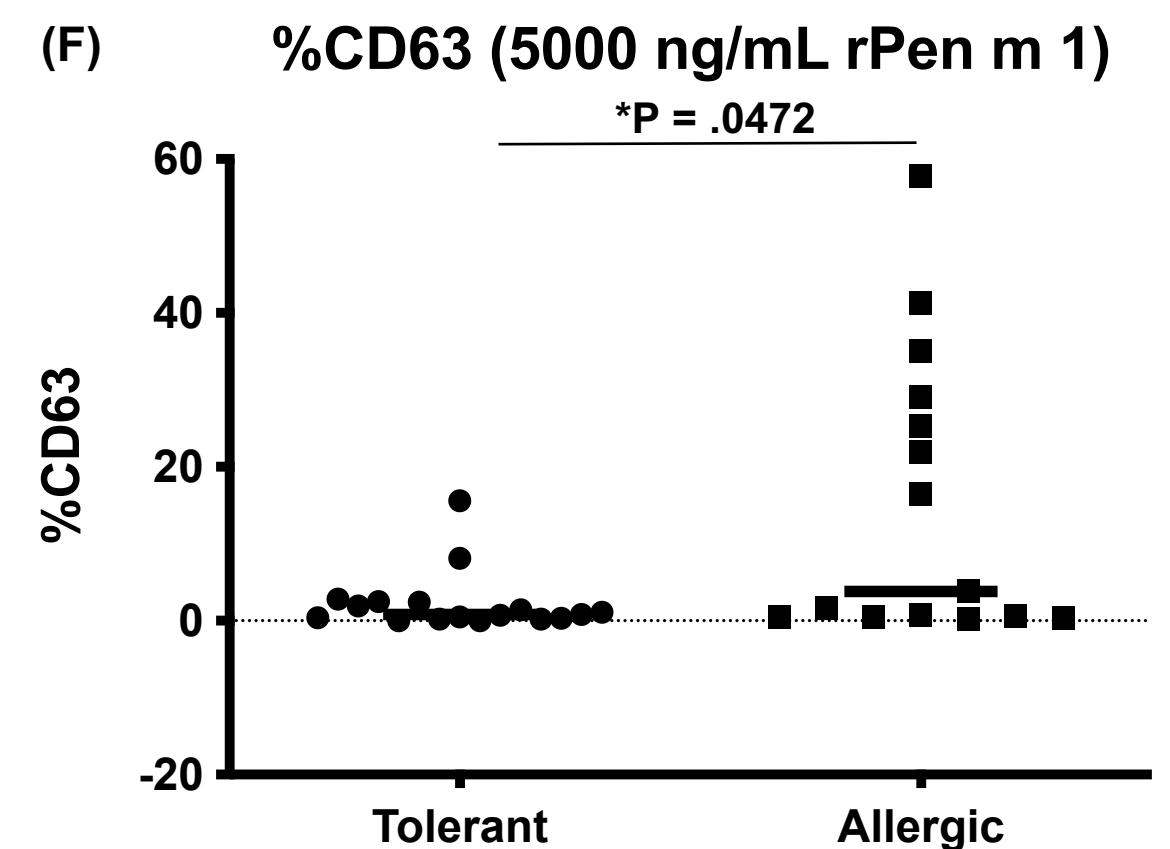
(D)



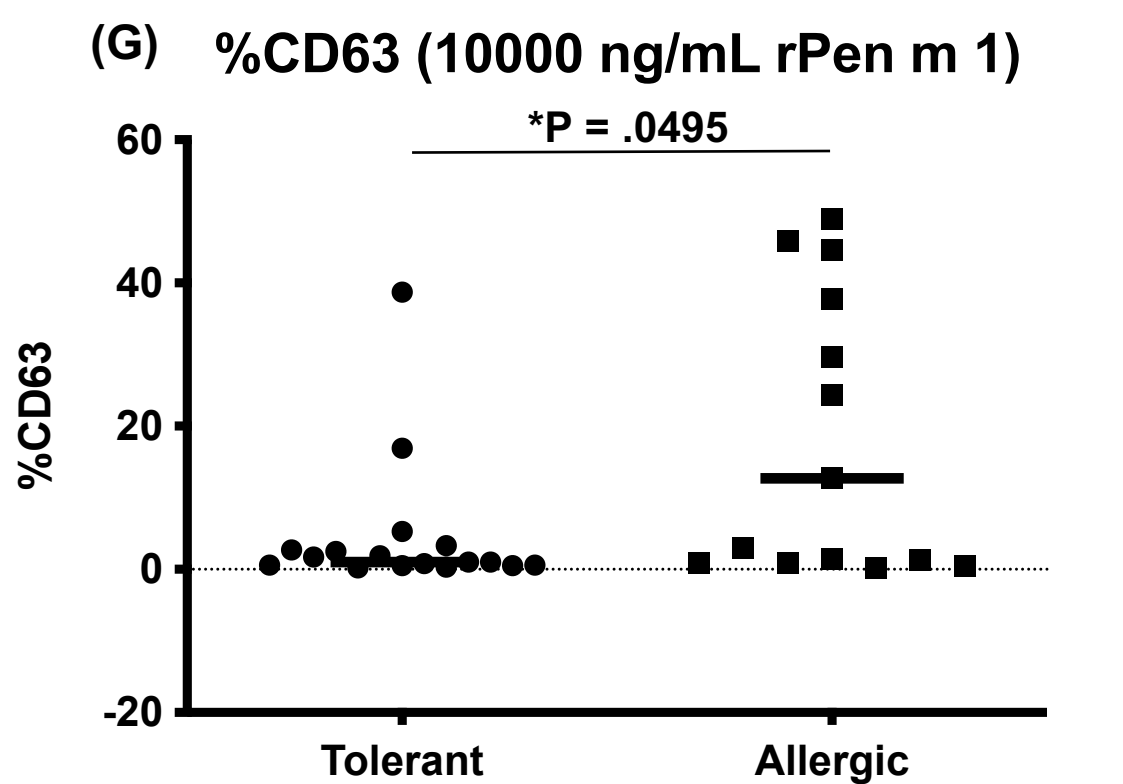
(E)



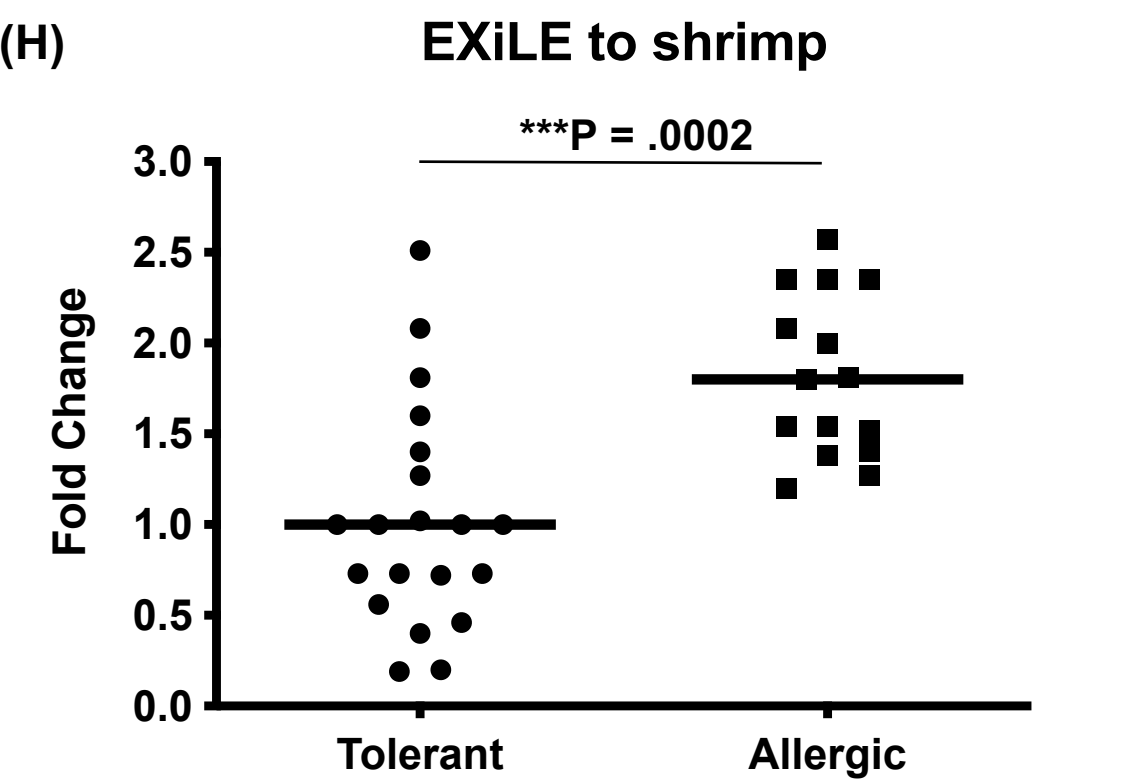
(F)



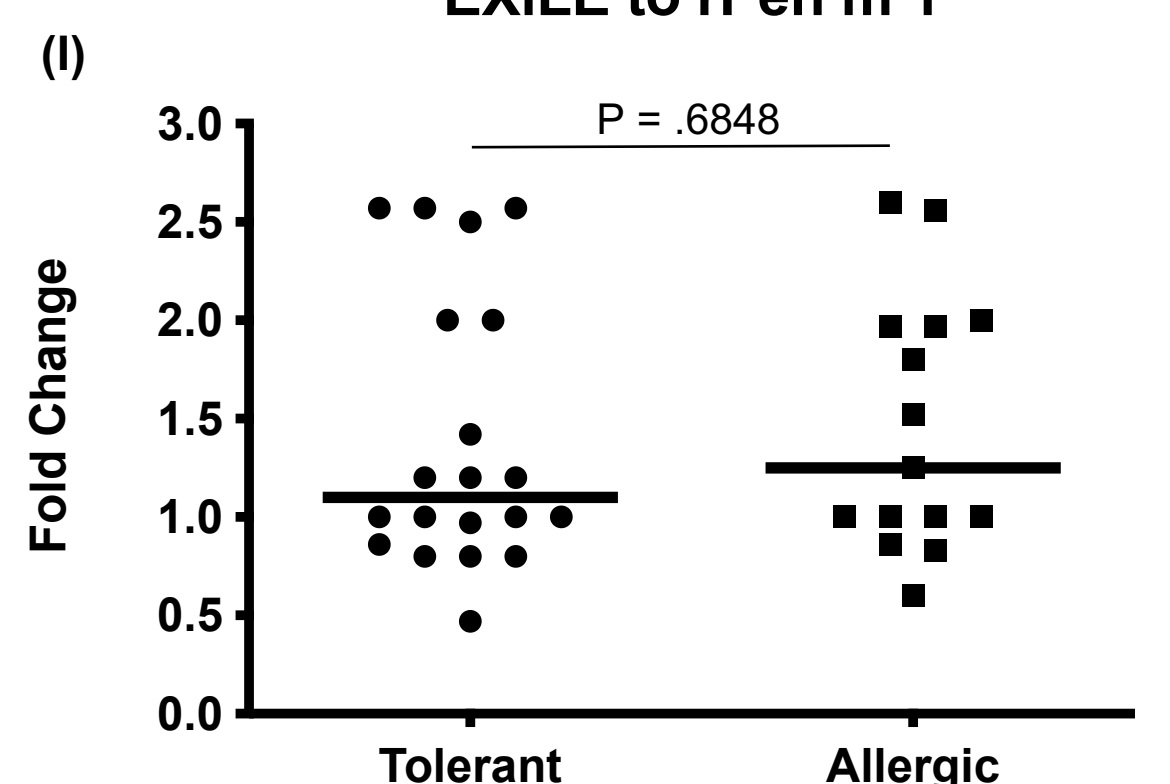
(G)

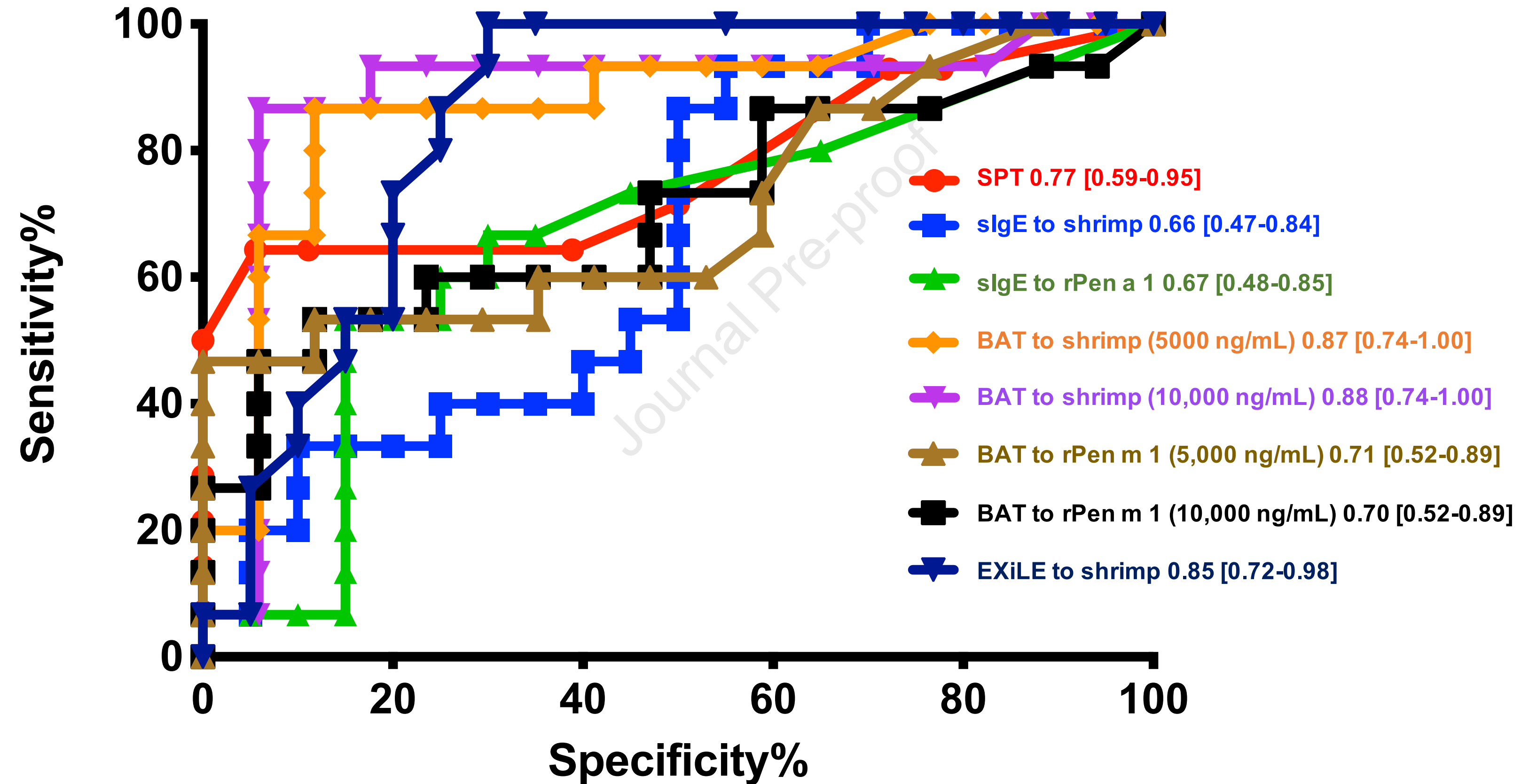


(H)

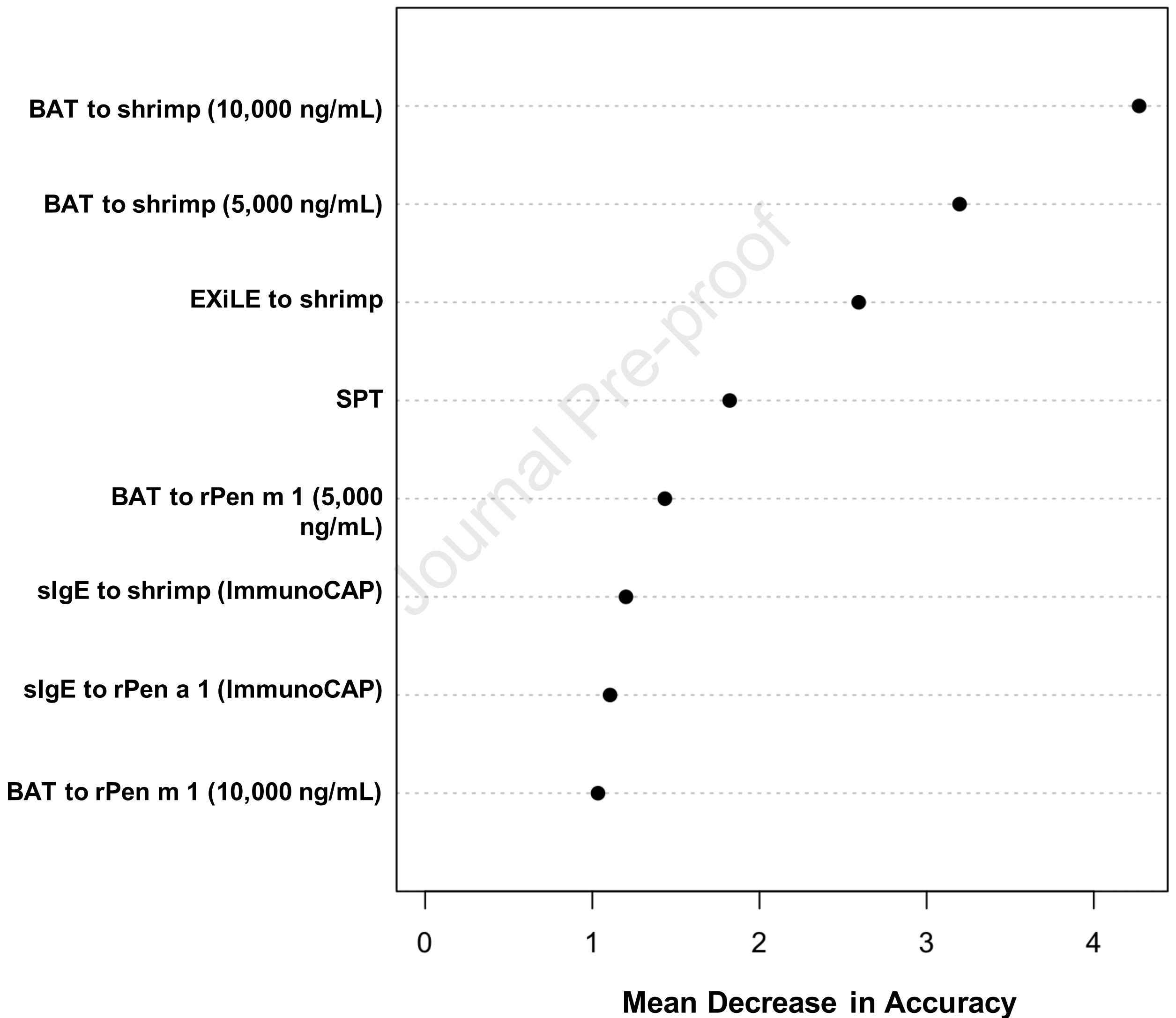


(I)

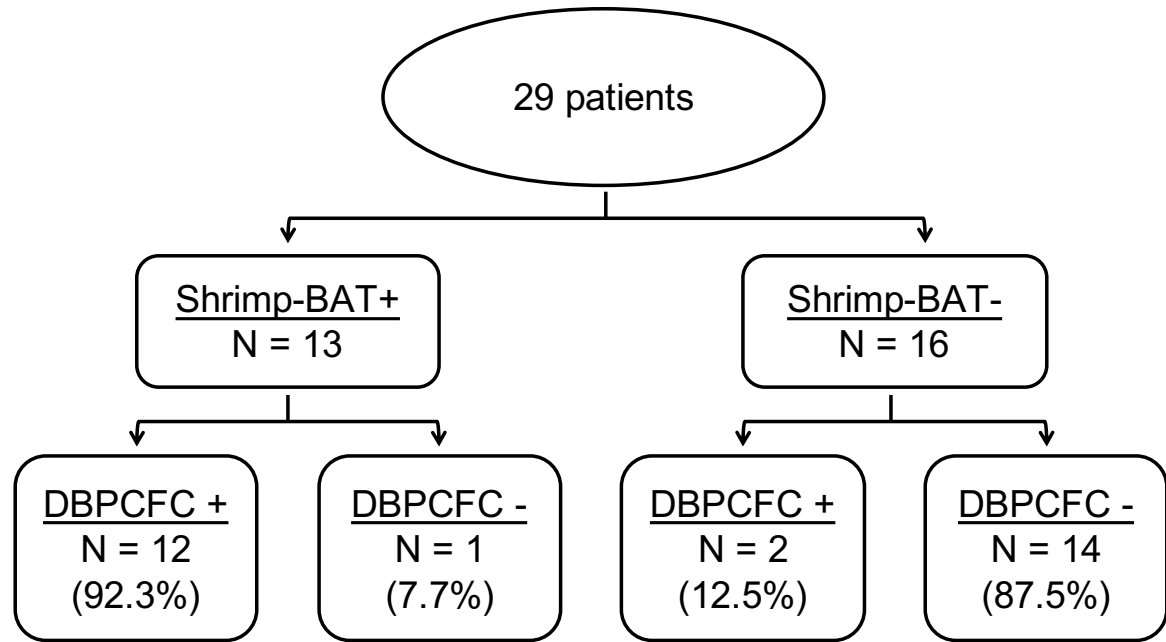
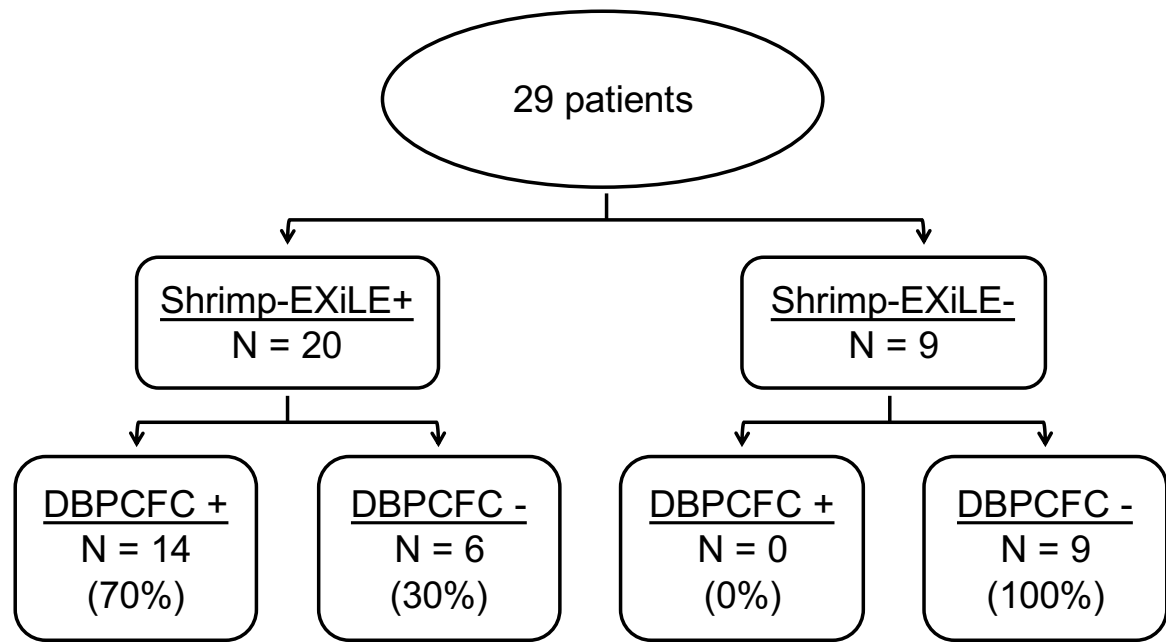
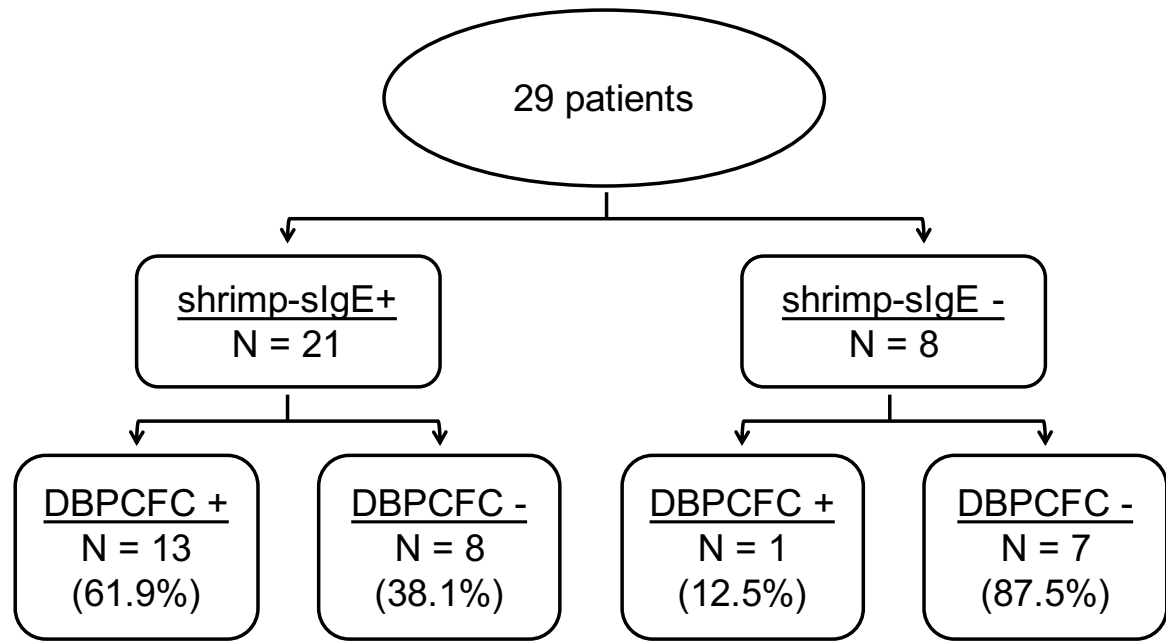
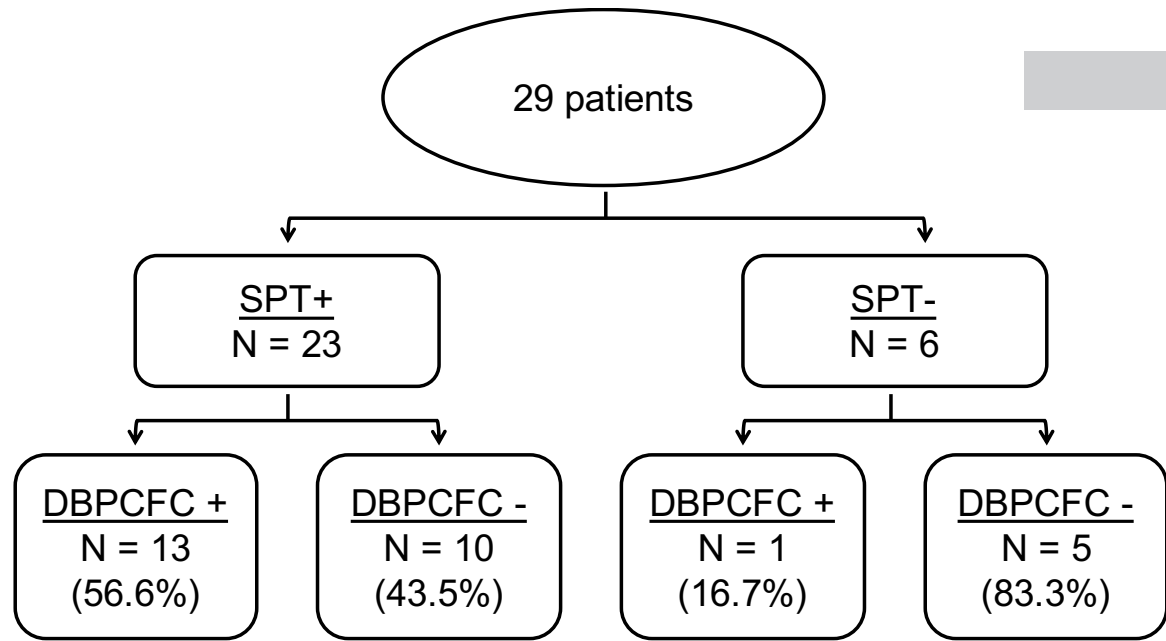




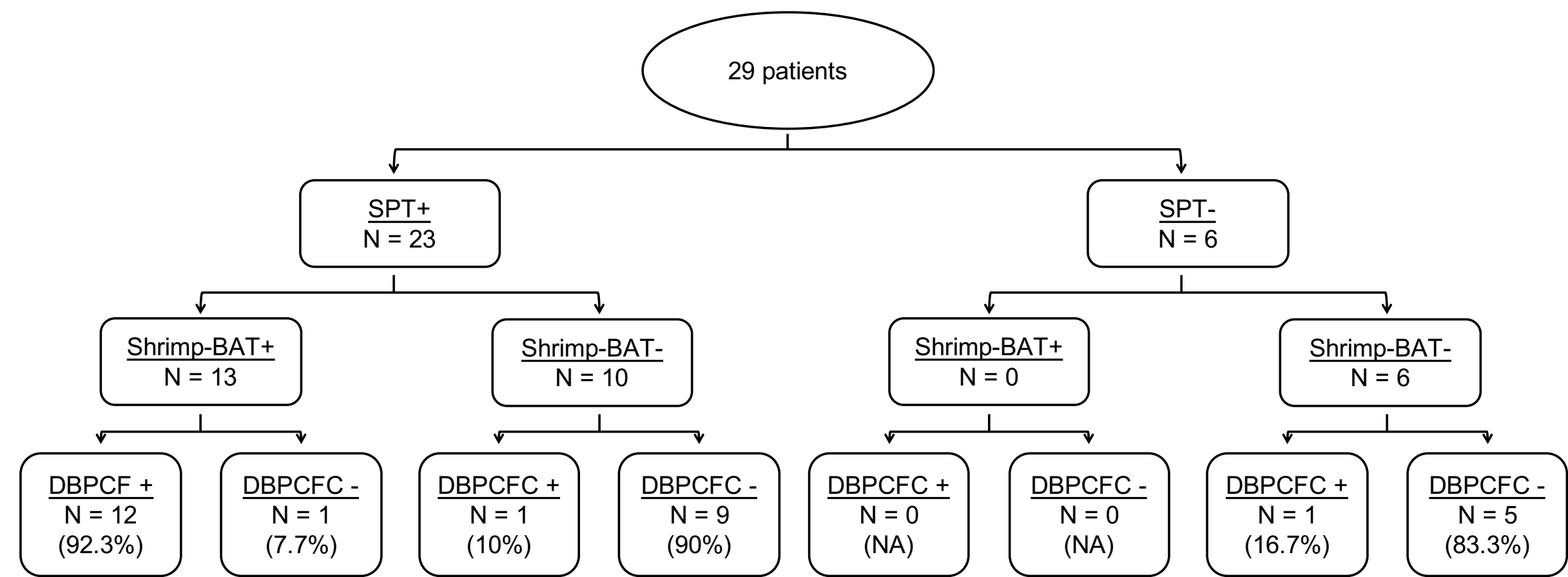
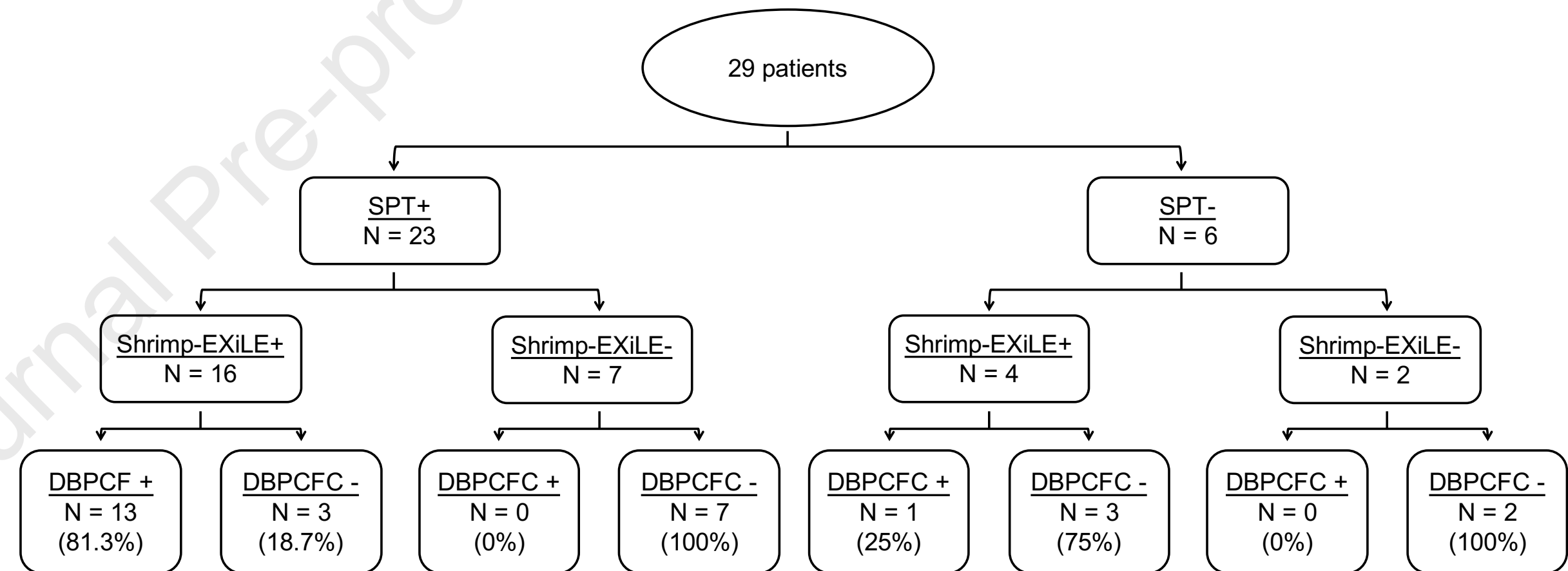
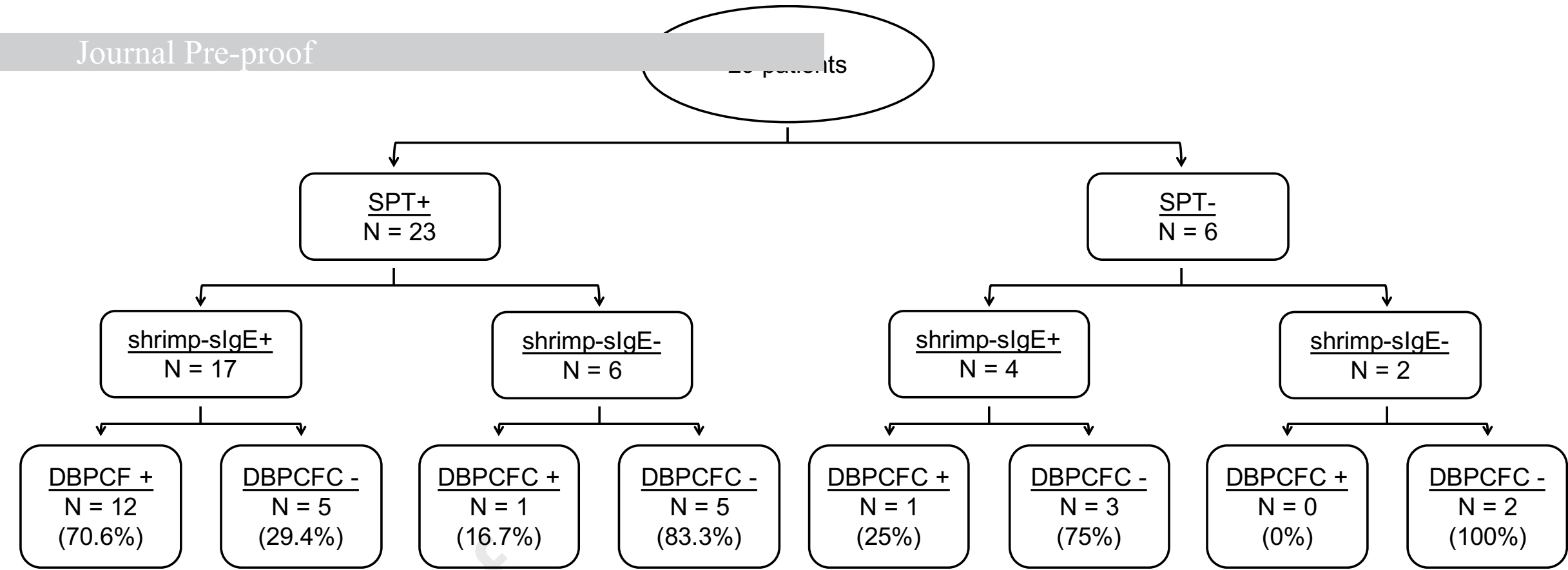
Variable Importance Plot



(A)



(B)



Placebo



Active



(A)

