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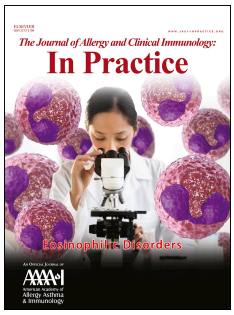

Basophil activation test reduces oral food challenges to nuts and sesame

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Basophil activation test reduces oral food challenges to nuts and sesame

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Word count: 3472

Tables: 4 tables

Figures: 4 figures

Online repository: 2 tables and 1 figure

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Abstract

Background: Nut allergic patients are often IgE sensitized to other nuts/seeds and need multiple oral food challenges (OFC) before the safe nuts can be introduced in the diet. However, OFC are time-consuming and risky procedures.

Objective: to assess the utility of the basophil activation test (BAT) to predict the allergic status and reduce the need for OFC in children with one or more nut or seed allergies.

Methods: Participants in the Pronuts study recruited at the Geneva and the London centers were tested on the BAT to hazelnut, cashew nut, sesame, almond and peanut, Ara h 1, Ara h 2, Ara h 6, using FlowCAST, a commercially available BAT kit, and flow cytometry.

Results: The BAT to hazelnut, cashew nut, sesame, almond and peanut discriminated between allergic and non-allergic children, to the respective nut or seed. The optimal allergen concentration and their optimal, positive and negative cut-offs were identified for BAT and the other tests, for each nut and seed. Using BAT as a second step in the diagnostic process, following equivocal skin prick test and IgE to extracts and components, reduced the number of total OFCs by 5-15% and of positive OFC by 33-75% (except for hazelnut) with 0% false-negatives and a diagnostic accuracy of 96-100%.

Conclusion: The BAT proved to be a useful diagnostic tool, used in a stepwise approach, to predict the allergic status and reduce the number of OFC in the Pronuts study patients with at least one nut allergy willing to consume selected nuts.

Abstract word count: 249 words

Highlights box:

1. ***What is already known about this topic?***

The introduction of nuts and seeds in the diet of children with one or more nut allergies is safe and feasible; however, due to polysensitization, this often requires multiple oral food challenges (OFC).

2. ***What does this article add to our knowledge?***

The basophil activation test (BAT), when used following skin prick and specific IgE testing, can reduce the number of OFCs, particularly positive OFCs, maintaining very high diagnostic accuracy.

3. How does this study impact current management guidelines?

In children with one or more nut allergies, needing OFC to clarify the allergic status to other nuts, a positive BAT confirms allergy whreas a negative BAT requires OFC before recommending nut consumption or avoidance.

Keywords:

Food allergy, basophil activation test, tree nuts, sesame seed, peanut, skin prick test, specific IgE, diagnosis, severity, threshold dose

Abbreviations:

BAT, basophil activation test

OFC, oral food challenge

ROC, receiver operator curve

SPT, skin prick testing

sIgE, specific IgE

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Introduction:

IgE sensitization to tree nuts and seeds is common in children with peanut and other nut and seed allergies and does not necessarily translate into clinical reactivity[1, 2]. Tree nut and seed allergies can lead to not only dietary but also social restrictions and significant anxiety associated with the fear of developing potentially severe allergic reactions unexpectedly[3, 4]. This has wider implications in the lives of children and their families and can significantly impact on their quality of life[3-5]. A significant proportion of children allergic to one or more nuts or seeds are able to tolerate other nut(s)[2]. In motivated families, interested and able to consume selected nuts whilst avoiding others, the allergic status to individual nuts and seeds can be verified and selective consumption of the nuts to which there is proven tolerance can be encouraged[6-8]. This should be accompanied by comprehensive information about potential risks, namely cross-contamination and misidentification of nuts, and the need to continue regular consumption of the safe nuts at home[9]. The Pronuts study recently demonstrated that introduction of nuts and seeds in the diet of children with one or more nut allergies is safe and feasible[2].

Fear of co-allergy in children allergic to one or more nuts frequently leads to blanket advice to avoid all nuts. Concerns regarding potential allergy to nuts also arise also when managing children with other food allergies, with family history of nut allergies and/or with underlying atopic conditions. The demonstration of sensitisation to nuts on SPT or sIgE testing can heighten such concerns. While non-sensitized children without a history of reaction are often recommended to introduce the nuts in the diet at home, sensitized children might have to undergo oral food challenge (OFC) and often multiple OFCs in order to allow safe consumption of nuts and seeds that children are not allergic to[2]. Given the risk and resources involved in the performance of OFC, it would be beneficial to have a diagnostic approach that could reduce the number of children requiring OFC and allow proactive introduction of safe nuts in the diet.

The basophil activation test (BAT) is a flow cytometry-based test which assesses the expression of activation markers, namely CD63, on the surface of blood basophils following stimulation with allergen or controls[10]. We previously demonstrated that the BAT to peanut had 97% diagnostic accuracy and could

reduce the number of children requiring an OFC by about 67%[11]. We have further validated the diagnostic utility of BAT in a large prospectively independent study of well-characterised patients[12]. Considering the high specificity of BAT and the practicalities involved in its performance (e.g. BAT requires fresh blood and flow cytometry), we have proposed that the BAT could be used as a second-step in the diagnosis of food allergy, in patients for whom the combination of the clinical history with SPT or IgE testing could not lead the clinician to a definite diagnosis[13, 14].

In this sub-study of the PRONUTS study, we aimed to assess the utility of BAT, using a commercially available kit, to diagnose nut and seed allergies in patients with at least one nut or seed allergy and the impact of BAT on the number of OFC required to reach an accurate diagnosis and enable the clinician to provide appropriate advice on avoidance or consumption of nuts or seeds. We hypothesized that BAT had high diagnostic accuracy and allowed reduction in the number of OFCs required, thus leading to a more accurate and safe approach to diagnosing tree nut and seed allergies.

Methods:

The Pronuts study

The Pronuts study (NCT01744990 in Clinicaltrials.gov) was a prospective multicentre study, with recruitment undertaken between 2012 and 2015, which aimed to assess safety and feasibility of introducing nuts in the diet of children with at least a single nut allergy. The method is described extensively elsewhere. Briefly, children aged between 6 months and 16 years in specialised Pediatric Allergy centres in London, Geneva and Valencia were invited to participate. At the core of the recruitment was the confirmation of the diagnosis of allergy to at least one nut, including peanut, sesame and tree nuts. Diagnosis of allergy was confirmed by positive OFC or convincing history of IgE-mediated allergic reaction to the culprit nut in the previous 12 months and SPT and sIgE greater or equal to the 95% positive predicting value for the respective nut or seed allergy (e.g. 8 mm on SPT and 15 KU/L on

specific IgE to peanut[11, 15]). Exclusion criteria were uncontrolled asthma, chronic urticaria, chronic systemic disease, daily antihistamine or oral allergy syndrome only to the index nut, history of life-threatening anaphylaxis as defined by documented desaturation <89%, >20% drop systolic in blood pressure or admission to a paediatric intensive care unit (other cases of anaphylaxis were admissible). Ethical approval was obtained at each site, namely UK (14/LO/0066), Geneva (CER 12-020PS) and Valencia (2012/0108), and written informed consent was obtained from all participants.

Study procedures

Children screened for entry into the study underwent clinical assessment, skin prick testing, blood collection for sIgE testing and BAT and oral food challenges. For each nut/seed, 3 groups of patients were defined based on the allergic status (allergic vs non-allergic) and on the presence of allergen-specific IgE: sensitized allergic, sensitized non-allergic and non-sensitized non-allergic. The clinical information, SPT and OFC results were not available to the performers of sIgE or BAT. Clinical information and SPT results were available to the team performing OFC. As this sub-study focuses on the utility of the BAT to peanut, sesame, cashew, hazelnut and almond and the BAT was performed only at the London and Geneva sites, the analyses presented here are limited to data acquired at these two study sites and for the aforementioned nuts and seeds.

Skin prick testing and specific IgE measurements

Skin prick testing was performed using plastic lancets Stallerpoint® and commercial allergen extracts for peanut, hazelnut, cashew and almond (Stallergenes, France) and tahini paste (Meridian Foods, UK) for sesame. Maximum wheal diameter was recorded after 15 minutes.

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Serum sIgE levels to allergen extracts (cashew nut, sesame, hazelnut, almond and peanut) and to individual allergens (Ara h 1/2/3/8/9, Cor a 1/8/9/14 and Ana o 3) were measured using ImmunoCAP (Thermofisher, Uppsala, Sweden).

Basophil activation test

BAT was performed to hazelnut, cashew nut, sesame, almond and peanut extracts and peanut components Ara h 1, Ara h 2, Ara h 6, using stimulants (CAST® allergens, Basel, Switzerland) and reagents provided in the Flow CAST® kit (BÜHLMANN, Basel, Switzerland) and following the manufacturer's instructions. A schematic figure of the BAT procedure has been included in a previous publication[13]. Briefly, blood was collected in an EDTA-containing Vacutainer tube and mixed gently. Stimulation and lysing buffers were pre-warmed to room temperature. Allergens were diluted following the allergen-dilution scheme shown in Table E1. Equal volume (50µL) of stimulant and whole blood and 100µL of stimulation buffer were added to 5 mL tubes and mixed gently. Staining reagent (20µL) containing anti-CCR3-PE and anti-CD63-FITC was added subsequently. All tubes were mixed, covered and incubated at 37°C for 25 minutes in an incubator, after which 2 mL of lysing reagent was added and each tube vortexed gently and incubated for 10 minutes at room temperature in the dark. After centrifugation at 500xg for 5 min, supernatants were decanted gently and pellets resuspended and kept at 4°C until analyses. Flow cytometry was performed at each site in a FACS CantoII with FACSDiva software (BD Biosciences, San Jose, Calif) and data were analyzed using FlowJo software (version 7.6.5; TreeStar, Ashland, Ore) by an investigator who was blinded to the clinical features of the participants. Basophils were gated as SSClow/CCR3+ and activation was expressed as %CD63+ basophils. corrected for the spontaneous basophil activation (i.e. subtracted the %CD63+ basophils in the unstimulated condition). All the flow cytometry data were analysed by the same researcher at the London center who was blind to all the clinical features. Reagents for BAT were provided by BÜHLMANN under agreements with King's College London and Geneva University Hospitals.

Oral food challenges

OFC were unblinded and performed following the PRACTALL guidelines reaching a cumulative dose of 4.43g of nut protein for patients of 36 months of age or older and 3.43g for younger children. Allergic reactions were treated according to the local hospital guidelines. Children with positive OFC were recommended to avoid the nut strictly in the diet and provided with an emergency treatment plan, whilst children with negative OFC were recommended to consume the nut regularly in the diet.

Statistical analyses

Qualitative variables were reported as number and percentage and compared using Chi-squared test. Chi squared test was also used to compare all categorical variables. Quantitative variables were reported as median and interquartile range and compared using Mann-Whitney and Kruskall-Wallis tests for two or more than two groups, respectively. Receiver operator curve (ROC) analyses were used to assess the discriminative ability of tests between allergic and non-allergic subjects. Optimal concentration of allergen for the BAT was determined based on the largest area under the ROC curve. Comparison of ROC curves was made by DeLong Test included in SAS ROCCONTRAST Statement[16]. Optimal, negative and positive cut-offs were determined based on Youden index, 95% negative predictive value and 95% positive predictive value. Cut-offs generated based on this dataset were used to determine the equivocal cases when assessing the diagnostic work-up in two steps. Seven (7.8%) subjects had non-responder basophils and were excluded from the ROC curve analyses as were subjects without result for the other tests as only subjects with complete datasets could be included. Demographic and clinical characteristics of these 7 patients did not differ from the rest of the population (Table E2). In the real-life assessment of BAT used as a second step in the diagnostic process, subjects with non-responder basophils were included. For all tests, including BAT, results at or above the 95% positive predictive value (PPV) cut-off

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were considered positive; results below the 95% NPV were considered negative and the results between cut-offs were considered equivocal. The impact in the number of OFC was calculated as if all patients had undergone OFC with the outcome of OFC based on the allergic status (Figure 1). SAS 9.4 was used for all analysis, a p value <0.05 was considered statistically significant.

Results:

Study population

Ninety two children were assessed for possible allergy to cashew, hazelnut, almond, peanut and sesame seed at the London and Geneva centres and ninety (98%) were tested on the BAT to all five foods. The consort diagram in Figure 1 shows the definition and outcome of reference standard and the outcome of the BAT for each nut or seed. Demographic, clinical and immunologic characteristics of the studied population is reported in Table I. The prevalence of co-sensitizations and co-allergies to different nuts was previously published for the whole Pronuts study cohort[2]. Overall, the most common allergy in the cohort studied here was peanut allergy followed by cashew nut, hazelnut, sesame seed and almond allergies. Cashew nut allergy was more common in Geneva but the prevalence of atopic co-morbidities, namely eczema, asthma and allergic rhinitis, was similar across centres. Children seen in London were slightly younger and showed a higher proportion of activated basophils in response to peanut, Ara h 2 and the IgE-mediated positive control anti-FcεRI (but not the non-IgE-mediated positive control fMLP) compared to children seen in Geneva.

Basophil activation test discriminated peanut, tree nut and seed allergic from non-allergic children

The BAT to hazelnut, cashew nut, sesame, almond, peanut, Ara h 1, Ara h 2, Ara h 6 showed a higher proportion of activated basophils in allergic compared to non-allergic subjects (Figure 2 and Table II) ($p<0.001$ in the vast majority of allergen concentrations). Ara h 2 on the BAT performed better than Ara h

6, Ara h 1 or peanut extract. For each nut, an optimal allergen concentration was identified based on the largest area under the ROC curve built for the discrimination between allergy and tolerance (Figure E1). Optimal concentrations of allergen tested were 22.73 ng/mL for peanut, 45.45 ng/mL for Ara h 1, 24.55 ng/mL for Ara h 2, 0.91 ng/mL for Ara h 6, 4.545 ng/mL for hazelnut, 22.73 ng/mL for cashew, 113.64 ng/mL for almond and 113.64 ng/mL for sesame.

Based on ROC curve analyses, cut-offs were generated for BAT to each nut or seed, including the optimal cut-off (i.e. best balance between sensitivity and specificity determined by the Youden index), negative cut-off (i.e. closest to the 95% NPV) and positive cut-off (i.e. closest to the 95% PPV). The sensitivity, specificity, PPV and NPV for each cut-off are indicated in Table III. Although not statistically significant except for cashew, the area under the ROC curve for BAT was larger than the ones for the other available tests in the diagnosis of sesame and almond, similar for hazelnut and lower for peanut and cashew nut allergies (Figure 3).

For BAT to peanut components, we also looked at the diagnostic performance in patients who were sensitized to the respective components and these were generally superior than the performance of the same tests in the whole population (Figure E2).

Basophil activation test as a second step in the diagnostic work-up reduces the number of oral food challenges

Given the high specificity of the BAT, which complements the high sensitivity of SPT and sIgE, and the practicalities involved in the performance of BAT, which requires fresh blood processed soon after collection and flow cytometry, we had proposed, in a previous study[11], that BAT would be most useful as a second step in the diagnostic work-up for peanut allergy, done in patients with equivocal results for SPT and sIgE to clarify the allergic status. Patients with positive BAT would have confirmed peanut allergy and patients with a BAT result below the positive cut-off (i.e. negative or intermediate BAT) or non-

responder basophils would need an OFC. We assessed the impact of this approach in the number of OFC not only to peanut but also to the other nuts and seeds assessed on the BAT. Table E3 The cut-offs indicates positive, optimal and negative cut-offs for SPT, sIgE to whole extract and components with the respective sensitivity, specificity and predictive values. Patients with results greater or equal to the 95% PPV cut-off were considered allergic, patients with results lower than the 95% NPV cut-off were considered not allergic and the patients with any combination of the two or with results that fell between the 95% PPV and 95% NPV cut-offs were considered equivocal. See Figure E3 for a graphical representation of the cut-offs and allergic status to cashew nut.

The diagnostic accuracy and resulting number of OFC following this approach (i.e. a first step consisting of SPT and sIgE and a second step consisting of BAT) are represented in Table IV for participants with equivocal combination of SPT, sIgE to extracts and sIgE to individual allergens or components. Figure 4 shows similar figures for SPT followed by BAT, sIgE to extracts followed by BAT and sIgE to components followed by BAT. Globally, the approach of using BAT as a second step in the diagnostic work-up for nut and seed allergies had a 97-100% accuracy with 0% false-negatives and ensured a 5-15% reduction in the number of OFC, except for BAT to hazelnut. The reduction in positive OFC seen with this approach ranged between 50 and 75% for the same nuts, thus sparing children from experiencing uncomfortable and potentially severe allergic reactions.

Discussion:

Avoiding nuts and seeds can have a significant negative impact on the quality of life and mental health of allergic children and their families. The majority of children with nut or seed allergies can tolerate other nuts in their diet and motivated and informed families can be recommended selective nut eating, whilst avoiding the culprit nuts to which the child is allergic. The Pronuts study confirmed that introduction of nuts/seeds in the diet of children with one or more nut allergies is feasible and safe[2]; however, this may require multiple OFC given that IgE sensitization to multiple nuts and seeds is common in nut allergic.

OFC can be stressful for patients and families and can potentially cause allergic reactions of unpredictable severity. The BAT has shown to have high specificity to diagnose peanut allergy in previous studies, and can be used as a second-step in the diagnostic work-up of food allergy[11]. We applied this concept to participants in the Pronuts study, who had one or more nut/seed allergies, and were being assessed for possible allergy to the other nuts and sesame. We found that the diagnostic performance of BAT and the other tests varied between nuts/seed but generally BAT distinguished well between allergic and non-allergic children, among children with one or more allergies to nuts or sesame. Although not statistically significant except for cashew, the area under the ROC curve for BAT was larger than the ones of the other available tests in the diagnosis of sesame and almond, similar for hazelnut and lower for peanut and cashew nut allergies (Figure 3). BAT to Ara h 2 was better than BAT to peanut, Ara h 1 or Ara h 6. The performance of BAT to peanut components was even better when only children sensitized to that specific allergen, further supporting the use of BAT as a second lines test when IgE sensitization could not support a definitive diagnosis. When applied as a second step in the diagnostic work-up, BAT had 96-100% diagnostic accuracy and allowed a reduction in OFC, particularly of positive OFC (except for hazelnut), thus rendering the diagnosis of food allergy at the same time accurate, safe and more comfortable for children with suspected nut/seed allergies.

Doing BAT only in patients with an equivocal diagnosis following clinical history, SPT and sIgE and doing OFC in patients with negative or equivocal BAT result, i.e. between positive and negative cut-offs or non-responder basophils, allowed a reduction in patients experiencing allergic reactions during OFC. This reduction varied between 50 and 75% in the whole population of patients tested and between 33 and 50% for the subgroup of patients who underwent OFC as part of the Pronuts study protocol; except for hazelnut allergy, for which BAT did not make a difference in the number of OFC, probably because its diagnostic performance was very similar to that of the other tests. These high percentages of reduction in OFC relate, however, to small event numbers and therefore may have lower impact in terms of patient numbers, depending on the scale on which BAT is applied in clinical pratice. Adopting the same approach

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of doing BAT as a second step, following only SPT or only sIgE, also enabled a reduction in OFC and particularly of positive OFC. Generally, doing SPT and BAT was better than doing sIgE and BAT (except for sesame), enabling the greatest reduction in OFC; however, these approaches with fewer tests resulted in a small proportion of false-positives and false-negatives. The false-negatives are the most concerning as they can result in accidental reactions in the community, which are potentially severe. Performing all tests reduced the false-negatives to zero but often led to more OFC overall. From a practical point of view, it is important to note that we collected blood for BAT immediately after SPT in the majority of patients and that the same sequence was followed in previous studies[11, 12]. Although blood for BAT should not be collected after in vivo procedures with a significant risk of systemic allergic reactions, such as intradermal tests and provocation tests, SPT to foods did not seem to affect BAT performance. Tahini was used for sesame SPT as this contains fat and lipophilic allergens that are often not represented in defatted allergen extracts. A recent study demonstrated that using both extract and tahini paste leads to a better combination of sensitivity and specificity, with the extract providing higher specificity and tahini providing higher sensitivity[17].

The overlap in BAT results between allergic and non-allergic subjects was smaller for sesame, reflecting the superior diagnostic accuracy of BAT to sesame compared to BAT to peanut or tree nuts. The performance of BAT to peanut in absolute terms was not as good as previously reported by us[11]. Differences in the BAT methodology between the two studies are likely to have accounted for this discrepancy, as the patient population is similar, particularly in the London site, and the performance of the other tests namely SPT and Ara h 2-sIgE are comparable in both studies. Different methods for performing the BAT have been described and the methodology adopted can have an impact on the results, from the laboratory procedure to flow cytometry and data analyses[14, 18, 19]. Aspects of the methodology to consider are: the markers chosen to identify the basophil population, the fluorochromes used, the allergen extract preparations, the allergen concentration selected and the anticoagulant used for blood collection. EDTA chelates calcium and therefore prevents the calcium influx into the basophils required for

degranulation[20], which has advantages for stabilisation of samples before testing but requires addition of calcium at the time of the BAT experiment in a given concentration, which may or may not correspond to the physiological concentration of individual patients. These are some of the aspects to consider if a methodological study is to be performed; however, only a head-to-head comparison of both BAT methods would allow us to confirm this. The BAT performance for hazelnut and cashew reported in the Nutcracker study was apparently better[21]; however, differences in the patient population may have contributed to this as in the Nutcracker study only patients who had no history of reaction to the nut were challenged and thus it is possible that more highly allergic (who were not challenged) patients with higher results for BAT were included, allowing a better discrimination between allergic and non-allergic subjects.

We found that the performance of BAT to Ara h 2 was superior to that of BAT to peanut extract, Ara h 1 or Ara h 6. This reflects the superior diagnostic discriminative ability of Ara h 2 compared to the other allergen preparations, particularly compared to peanut extract and Ara h 1, as previously shown for serologic tests[11, 22]. We have demonstrated the dominance of Ara h 2 also over Ara h 6 in a recently published study using IgE binding and inhibition assays and cellular effector assays[23]. In our previous BAT to peanut study[11], that we have recently validated using the same BAT methodology in a very large population[24], we did not perform BAT to Ara h 2, but it would be challenging to have improved the diagnostic utility of BAT to peanut in our previous study, which had sensitivity and specificity already above 95%. The disadvantage of using a single allergen in the BAT, as opposed to the whole extract, is that some allergic patients may not be sensitized to that individual allergen, potentially resulting in a false-negative test. On the contrary, the BAT may become more specific, as may have been the case if we had performed BAT to Cor a 14 alongside hazelnut in the present study, given that BAT to hazelnut had quite a few false-positives, possibly due to sensitization to PR-10 proteins secondary to tree pollen allergy.

The Pronuts study constitutes a discovery cohort and our findings need to be validated in an independent cohort. The cut-offs generated are likely to be suited to population with a similar (high) prevalence of nut allergies, as expected in patients seen in a specialized Allergy clinic. Once validated, this approach could be very useful for clinicians evaluating polysensitized children with suspected peanut, tree nut and sesame seed allergies. Attention should be given to extrapolate these cut-offs only to populations that are similar to the Pronuts study population.

In summary, BAT can potentially be very helpful in the management of children with one or more nut allergies to identify the safe nuts that can be introduced in the diet. As BAT is very specific in confirming nut and seed allergies, BAT may reduce the number of patients that experience allergic reactions during OFC thus improving the safety profile of this procedure and opening up room for other indications for OFC, namely educational and psychotherapeutic purposes. In the future, external validation of our findings in independent cohorts and standardization of the methodology so that their reliable and consistent application can be broadened and used to improve the care of a larger number of children with suspected food allergies.

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Tables and figure legends:

Table I. Demographic and clinical characteristics of participants in this sub-study of the Pronuts study.

Clinical characteristics	Study population (n=90)	GB (n=49)	GE (n=41)	p value
Age (years)	5.1 (3- 9)	4.4 (2- 8)	5.8 (4- 10)	**0.031**
Gender, male - n (%)	54.4% (49/ 90)	55.1% (27/ 49)	53.7% (22/ 41)	0.891
Atopic eczema - n (%)	61.1% (55/ 90)	63.3% (31/ 49)	58.5% (24/ 41)	0.647
Allergic rhinitis - n (%)	46.7% (42/ 90)	42.9% (21/ 49)	51.2% (21/ 41)	0.428
Asthma - n (%)	32.2% (29/ 90)	24.5% (12/ 49)	41.5% (17/ 41)	0.086
Other food allergy - n (%)	41.1% (37/ 90)	44.9% (22/ 49)	36.6% (15/ 41)	0.425
Nut and seed allergies – n (%)				
Hazelnut allergy	32.2% (29/ 90)	30.6% (15/ 49)	34.1% (14/ 41)	0.721
Cashew nut allergy	41.1% (37/ 90)	28.6% (14/ 49)	56.1% (23/ 41)	**0.008**
Sesame seed allergy	13.3% (12/ 90)	14.3% (7/ 49)	12.2% (5/ 41)	0.771
Almond allergy	3.3% (3/ 90)	2.0% (1/ 49)	4.9% (2/ 41)	0.455
Peanut allergy	57.8% (52/ 90)	63.3% (31/ 49)	51.2% (21/ 41)	0.249

Median (IQR) for quantitative variables. GB, Great Britain site; GE, Geneva site.

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Table II. Immunological characteristics of allergic and non-allergic subjects (n=83). Median and inter-quartile range are represented. Subjects with non-responder basophils were excluded.

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	Non-allergic	Allergic	p value	AUC ROC (95% CI)		
Hazelnut allergy	**N=57**	**N=26**				
SPT weal diameter (mm)	0.0 (0- 4)	10.0 (6- 14)	**<0.001**	0.8721	0.7984	0.9458
Specific IgE (KU/L)						
Hazelnut	0.64 (0.1- 3.8)	6.45 (2.5- 18.5)	**<0.001**	0.7763	0.6764	0.8762
Cor a 1	0.01 (0.0- 2.1)	0.57 (0.0- 10.7)	**0.026**	0.6495	0.5205	0.7785
Cor a 8	0.01 (0.0- 0.0)	0.02 (0.0- 0.1)	0.058	0.6082	0.4784	0.7380
Cor a 9	0.13 (0.0- 0.9)	4.20 (0.3- 8.8)	**<0.001**	0.7390	0.6173	0.8608
Cor a 14	0.02 (0.0- 0.1)	3.27 (0.3- 16.0)	**<0.001**	0.8659	0.7717	0.9600
Basophil activation test (%CD63+ Basophils)						
Hazelnut 113.64 ng/ml	0.0 (0- 1)	7.7 (0- 36)	**<0.001**	0.7510	0.6301	0.8719
Hazelnut 22.73 ng/ml	0.0 (0- 0)	19.8 (8- 52)	**<0.001**	0.8556	0.7558	0.9554
Hazelnut 4.545 ng/ml	0.0 (0- 0)	9.6 (1- 31)	**<0.001**	0.8691	0.7831	0.9551
Hazelnut 0.9091 ng/ml	0.0 (0- 0)	3.2 (0- 26)	**<0.001**	0.8424	0.7519	0.9330
Cashew nut allergy	**N=48**	**N=35**				
SPT weal diameter (mm)	0.0 (0- 2)	12.0 (9- 15)	**<0.001**	0.9762	0.9422	1.0000
Specific IgE to cashew (KU/L)	0.19 (0.0- 0.7)	4.15 (1.1- 10.8)	**<0.001**	0.8867	0.8148	0.9587
Specific IgE to Ana o 3 (KU/L)	0.01 (0.0- 0.1)	3.89 (0.9- 10.7)	**<0.001**	0.9737	0.9417	1.0000
Basophil activation test (%CD63+ Basophils)						
Cashew 113.64 ng/ml	0.0 (0- 1)	12. (2- 45)	**<0.001**	0.8673	0.7798	0.9548
Cashew 22.73 ng/ml	0.0 (0- 1)	14.4 (3- 51)	**<0.001**	0.8750	0.7939	0.9561
Cashew 4.545 ng/ml	0.0 (0- 1)	13.3 (1- 34)	**<0.001**	0.8452	0.7577	0.9328
Cashew 0.9091 ng/ml	0.0 (0- 1)	1.9 (0- 17)	**0.001**	0.7036	0.5892	0.8180
Almond allergy	**N=79**	**N=3**				
SPT weal diameter (mm)	0.0 (0- 2)	8.0 (3- 12)	**0.005**	0.8945	0.7028	1.0000
Specific IgE to almond (KU/L)	0.20 (0.1- 1.3)	1.64 (1.5- 2.8)	0.065	0.8143	0.7250	0.9037
Basophil activation test (%CD63+ Basophils)						
Almond 113.64 ng/ml	0.1 (0- 1)	14.5 (1- 38)	**0.013**	0.9125	0.7895	1.0000
Almond 22.73 ng/ml	0.2 (0- 1)	8.6 (0- 44)	0.085	0.7833	0.4402	1.0000
Almond 4.545 ng/ml	0.1 (0- 1)	15.3 (1- 17)	**0.018**	0.8833	0.6981	1.0000
Almond 0.9091 ng/ml	0.0 (0- 1)	0.1 (0- 22)	0.396	0.6292	0.2427	1.0000
Sesame seed allergy	**N=71**	**N=12**				
SPT weal diameter (mm)	0.0 (0- 1)	12.5 (8- 21)	**<0.001**	0.9137	0.7969	1.0000
Specific IgE to sesame (KU/L)	0.30 (0.1- 2.3)	3.10 (1.6- 29.1)	**<0.001**	0.8173	0.7140	0.9205
Basophil activation test (%CD63+ Basophils)						
Sesame 113.64 ng/ml	0.0 (0- 0)	27.7 (11- 79)	**<0.001**	0.9337	0.8109	1.0000

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Sesame 22.73 ng/ml	0.1 (0- 1)	26.6 (1- 48)	**<0.001**	0.8504	0.7004	1.0000
Sesame 4.545 ng/ml	0.0 (0- 1)	2.7 (0- 16)	**0.003**	0.7359	0.5552	0.9166
Sesame 0.9091 ng/ml	0.0 (0- 1)	0.3 (0- 3)	0.306	0.5874	0.3961	0.7788
Peanut allergy	**N=35**	**N=48**				
SPT weal diameter (mm)	0.0 (0- 3)	10.5 (9- 15)	**<0.001**	0.9314	0.8734	0.9893
Specific IgE (KU/L)						
Peanut	0.35 (0.1- 2.1)	14.60 (3.4- 50.9)	**<0.001**	0.8984	0.8328	0.9639
Ara h 1	0.01 (0.0- 0.1)	0.72 (0.0- 11.9)	**<0.001**	0.7696	0.6686	0.8706
Ara h 2	0.01 (0.0-0.1)	10.80 (1.6-33.6)	**<0.001**	0.9536	0.9033	1.0000
Ara h 3	0.03 (0.0- 0.1)	0.13 (0.0- 1.6)	**0.028**	0.6222	0.4993	0.7451
Ara h 8	0.03 (0.0- 1.0)	0.01 (0.0- 1.3)	0.406	0.5585	0.4352	0.6817
Ara h 9	0.01 (0.0- 0.1)	0.01 (0.0- 0.0)	0.155	0.3768	0.2588	0.4948
Basophil activation test (%CD63+ Basophils)						
Peanut 22.73 ng/ml	0.3 (0- 1)	37.3 (10- 68)	**<0.001**	0.8655	0.7862	0.9447
Peanut 4.55 ng/ml	0.0 (0- 0)	28.7 (2- 53)	**<0.001**	0.8595	0.7810	0.9381
Peanut 0.909 ng/ml	0.0 (0- 0)	6.1 (0- 24)	**<0.001**	0.7595	0.6621	0.8570
Ara h 1 22.724 ng/ml	0.0 (0- 1)	24.0 (0- 50)	**<0.001**	0.7753	0.6780	0.8726
Ara h 1 4.545 ng/ml	0.0 (0- 1)	8.6 (0- 36)	**<0.001**	0.7762	0.6807	0.8717
Ara h 1 0.9091 ng/ml	0.0 (0- 0)	1.0 (0- 11)	**<0.001**	0.7173	0.6119	0.8226
Ara h 2 4.55 ng/ml	0.0 (0- 0)	20.5 (3- 53)	**<0.001**	0.8696	0.7891	0.9502
Ara h 2 0.91 ng/ml	0.0 (0- 0)	20.7 (5- 47)	**<0.001**	0.8524	0.7686	0.9362
Ara h 2 0.182 ng/ml	0.0 (0- 1)	17.7 (2- 50)	**<0.001**	0.8256	0.7376	0.9136
Ara h 6 4.55 ng/ml	0.0 (0- 0)	27.1 (1- 67)	**<0.001**	0.8250	0.7373	0.9127
Ara h 6 0.91 ng/ml	0.0 (0- 0)	14.0 (0- 48)	**<0.001**	0.8295	0.7459	0.9130
Ara h 6 0.182 ng/ml	0.0 (0- 0)	2.1 (0- 61)	**<0.001**	0.7137	0.6084	0.8190

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Table III. Cut-offs for the basophil activation test to different nuts and their diagnostic performance (n=83, non-responders were excluded).

Allergen	Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
BAT to Hazelnut	60.98	15.38 (4.4-34.9)	100.00 (93.7-100)	100.00 (39.8-100)	72.15 (60.9-81.7)
	0.924	80.77 (60.7-93.5)	87.72 (76.3-94.9)	75 (55.1-89.3)	90.91 (80.0-97.0)
	0.13	92.31(74.9-99.1)	66.67 (52.9-78.6)	55.81 (39.9-70.9)	95.00 (83.1-99.4)
BAT to Cashew	25.21	42.86 (26.3-60.7)	100 (92.6-100)	100 (78.2-100)	70.6 (58.3-81.0)
	1.79	82.86 (66.4-93.4)	87.5 (74.8-95.3)	82.86 (66.4-93.4)	87.5 (74.8-95.3)
	0.36	88.57 (73.3-96.8)	70.83(55.9-83.1)	68.89 (53.4-81.8)	89.5 (75.2-97.1)
BAT to Sesame	16.11	66.67 (34.9-90.1)	100 (94.9-100)	100 (63.1-100)	94.67 (86.9-98.5)
	8.15	91.67 (61.5-99.8)	98.59 (92.4-100)	91.67 (61.5-99.8)	98.59 (92.4-100)
	14.26	75 (42.8-94.5)	98.59 (92.4-100)	90 (55.6-99.8)	95.89 (88.5-99.1)
BAT to Almond	37.57	33.33 (0.8-90.6)	100 (95.5-100)	100 (2.5-100)	97.56 (91.5-99.7)
	0.825	100 (29.2-100)	80 (69.6-88.1)	15.79 (3.4-39.6)	100 (94.4-100)
	18.63	33.33 (0.8-90.6)	93.75 (86.0-97.9)	16.67 (0.4-64.1)	97.4 (90.9-99.7)
BAT to Peanut	42.11	45.83 (31.4-60.8)	97.14 (85.1-99.9)	95.65 (78.1-99.9)	56.67 (43.2-69.4)
	4.717	81.25 (67.4-91.1)	85.7 (69.7-95.2)	88.64 (75.4-96.2)	76.92 (60.7-88.9)
	0.124	93.75 (82.8-98.7)	37.14 (21.5-55.1)	67.16 (54.6-78.2)	81.25 (54.4-96.0)
BAT to Ara h 1	16.02	39.58 (25.8-54.7)	97.14 (85.1-99.9)	95.00 (75.1-99.9)	53.97 (40.9-66.6)
	0.82	64.58 (49.5-77.8)	85.71 (69.7-95.2)	86.11 (70.5-95.3)	63.83 (48.5-77.3)
	0.005	79.17 (65.0-89.5)	60.00 (42.1-76.1)	73.08 (59.0-84.4)	67.74 (48.6-83.3)
BAT to Ara h 2	2.264	79.17 (65.0-89.5)	94.29 (80.8-99.3)	95 (83.1-99.4)	76.74 (61.4-88.2)
	0.57	83.33 (69.8-92.5)	91.43 (76.9-98.2)	93.02 (80.9-98.5)	80 (64.4-91.0)
	0.375	85.42 (72.2-93.9)	85.71 (69.7-95.2)	89.13 (76.4-96.4)	81.08 (64.8-92.0)

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BAT to Ara h 6	26.71	43.75 (29.5-58.8)	97.14 (85.1-99.9)	95.45 (77.2-99.9)	55.74 (42.5-68.5)
	0.96	72.92 (58.2-84.7)	88.57 (73.3-96.8)	89.74 (75.8-97.1)	70.45 (54.8-83.2)
	0.325	79.17 (65.0-89.5)	74.29 (56.7-87.5)	80.85 (66.7-90.9)	72.22 (54.8-85.8)

Footnote: Optimal concentrations of allergen were 22.73 ng/ml for peanut, 45.45 ng/ml for Ara h 1, 4.55 ng/ml for Ara h 2, 0.91 ng/ml for Ara h 6, 4.545 ng/ml for hazelnut, 22.73 ng/ml for cashew, 113.64 ng/ml for almond and 113.64 ng/ml for sesame.

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	Outcome of history SPT, sIgE to extracts and components			Misdiagnosis		Outcome of BAT		Outcome of OFC and misdiagnosis	Correct diagnosis - % total patients	Nr BATs required - % total patients	Total OFCs – with BAT (without BAT) and %reduction	Positive OFCs with BAT (without BAT) and %reduction
Cashew nut allergy	NA	**29**	*13*	**FN=0**	*FN=0*							
	Equivocal	**27**	*17*			NR or intermediate	**8** *6*	**2** *1* **CA**	**99%** *98%*	**31%** *29%*	**11%** *6%*	**75%** *50%*
						Negative	**16** *10*	**FN=0** *0*	**87/88** *57/58*	**27** *17*	**24 (27)** *16 (17)*	**2 (8)** *1(2)*
						Positive	**3** *1*	**FP=0** *0*				
	Allergic	**32**	*28*	**FP=1**	*FP=1*							
Sesame seed allergy	NA	**50**	*14*	**FN=0**	*FN=0*							
	Equivocal	**35**	*13*			NR or intermediate	**4** *2*	**1** *1* **SA**	**99%** *100%*	**39%** *45%*	**11%** *15%*	**50%** *50%*
						Negative	**27** *8*	**FN=3** *2*	**88/89** *29/29*	**35** *13*	**31 (35)** *11 (13)*	**4 (8)** *3(6)*
						Positive	**4** *2*	**FP=1** *0*				
	Allergic	**4**	*2*	**FP=0**	*FP=0*							
Almond allergy	NA	**69**	*40*	**FN=0**	*FN=0*							
	Equivocal	**19**	*17*			NR or intermediate	**4** *4*	***All NA***	**100%** *100%*	**21%** *29%*	**5%** *6%*	**75%** *50%*
						Negative	**14** *12*	**FN=1** *1*	**89/89** *58/58*	**19** *17*	**18(19)** 16 (17)	**1 (4)** *1(2)*
						Positive	**1** *1*	**FP=0** *0*				
	Allergic	**1**	*1*	**FP=0**	*FP=0*							
Hazelnut allergy	NA	**17**	*7*	**FN=0**	*FN=0*							
	Equivocal	**59**	*38*			NR or intermediate	**32** *22*	**15** *12* **HA**	**98%** *100%*	**67%** *70%*	**0%** *0%*	**0%** *0%*
						Negative	**27** *16*	**FN=1** *1*	**86/88** *54/54*	**59** *38*	**59 (59)** *38 (38)*	**15 (15)** *13(13)*
						Positive	**0** *0*	**FP=0** *0*				
	Allergic	**12**	*9*	**FP=0**	*FP=1*							
Peanut allergy	NA	**9**	*2*	**FN=0**	*FN=0*							
	Equivocal	**34**	*17*			NR or intermediate	**7** *3*	***All NA***	**97%** *96%*	**39%** *36%*	**15%** *12%*	**60%** *33%*

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						Negative	**22**	12	**FN=2**	*2*	**85/88**	*45/47*	**34**	*17*	**29 (34)**	*15*	**2 (5)**	*2(3)*
															(17)			
						Positive	**5**	*2*	**FP=2**	*1*								
Allergic	45	28	**FP=1**	*FP=1*														

Table IV. Testing the proposed approach to using the basophil activation test to diagnose nut and sesame seed allergies – numbers in bold indicate the results for the whole population and numbers in italic refer to the subgroup who were actually challenged to the individual nuts as part of the Pronuts study. Allergic patients had results at or above the 95% positive predictive value (PPV) cut-off or a combination of above the 95% negative predictive value (NPV) and above the 95% PPV; non-allergics had below the 95% NPV for all tests; and equivocal were the remaining cases.

(subjects with results for all tests were included, including subjects with non-responder basophils: n=88 for hazelnut, n=88 for peanut, n=88 for cashew, n=89 for almond, n=89 for sesame). FN, false negative; FP, false positive; NR, non-responder; HA, hazelnut allergic; CA, cashew nut allergic; SA, sesame seed allergic.

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Figure legends

Figure 1. Consort diagram.

Footnote: Hx, clinical history; OFC, oral food challenge; SPT skin prick test; BAT, basophil activation test. BAT allergen stimulation used in this diagram were: 4.545 ng/ml hazelnut extract, 22.73 ng/ml cashew nut extract, 113.64 ng/ml sesame extract, 113.64 ng/ml for almond and 4.55 ng/ml Ara h 2, all CAST allergens.

Figure 2. Basophil activation to optimal concentration of nut or sesame extract in allergic (in red), sensitized non-allergic (in green) and non-sensitized non-allergic children (in brown). n=83 (7 participants with non-responder basophils were excluded).

A. Hazelnut;
B. Cashew
C. Sesame
D. Almond
E. Peanut
F. Ara h 1
G. Ara h 2
H. Ara h 6

Figure 3. Receiver operating characteristic (ROC) curve for different tests for the various nut allergies.

A. Hazelnut allergy (p=0.230 for comparison of areas under the ROC curves)
B. Cashew nut allergy (p=0.007 for comparison of areas under the ROC curves)
C. Sesame seed allergy (p=0.215 for comparison of areas under the ROC curves)
D. Almond allergy (p=0.232 for comparison of areas under the ROC curves)
E. Peanut allergy (p=0.094 for comparison of areas under the ROC curves)

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Figure 4. Impact of the basophil activation test as a second step in the diagnostic work-up following a first step consisting of SPT, specific IgE to the extract and specific IgE to the best component (Ara h 2 for peanut, Cor a 14 for hazelnut and Ana o 3 for cashew nut), SPT only, specific IgE only or specific IgE to the best component only.

A. Peanut
B. Cashew nut
C. Sesame seed

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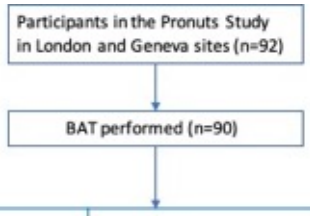

Nut Seed	HAZELNUT	CASHEW NUT	SESAME	ALMOND	PEANUT
Reference Standard	OFC (n=55) - Allergic (n=23) - Non-allergic (n=32) Hx + SPT/IgE (n=35) - Allergic (n=6) - Non-allergic (n=29)	OFC (n=64) - Allergic (n=30) - Non-allergic (n=34) Hx + SPT/IgE (n=26) - Allergic (n=7) - Non-allergic (n=19)	OFC (n=29) - Allergic (n=8) - Non-allergic (n=21) Hx + SPT/IgE (n=61) - Allergic (n=4) - Non-allergic (n=57)	OFC (n=58) - Allergic (n=3) - Non-allergic (n=55) Hx + SPT/IgE (n=32) - Allergic (n=0) - Non-allergic (n=32)	OFC (n=49) - Allergic (n=32) - Non-allergic (n=17) Hx + SPT/IgE (n=41) - Allergic (n=20) - Non-allergic (n=21)
Basophil activation test (BAT)	BAT POSITIVE (n=4) - Allergic (n=4) - Non-allergic (n=0)	BAT POSITIVE (n=15) - Allergic (n=15) - Non-allergic (n=0)	BAT POSITIVE (n=8) - Allergic (n=8) - Non-allergic (n=0)	BAT POSITIVE (n=1) - Allergic (n=1) - Non-allergic (n=0)	BAT POSITIVE (n=40) - Allergic (n=38) - Non-allergic (n=2)
	BAT NEGATIVE (n=40) - Allergic (n=2) - Non-allergic (n=38)	BAT NEGATIVE (n=38) - Allergic (n=4) - Non-allergic (n=34)	BAT NEGATIVE (n=73) - Allergic (n=3) - Non-allergic (n=70)	BAT NEGATIVE (n=77) - Allergic (n=2) - Non-allergic (n=75)	BAT NEGATIVE (n=37) - Allergic (n=7) - Non-allergic (n=30)
	BAT INTERMEDIATE (n=39) - Allergic (n=20) - Non-allergic (n=19)	BAT INTERMEDIATE (n=30) - Allergic (n=16) - Non-allergic (n=14)	BAT INTERMEDIATE (n=2) - Allergic (n=1) - Non-allergic (n=1)	BAT INTERMEDIATE (n=5) - Allergic (n=0) - Non-allergic (n=5)	BAT INTERMEDIATE (n=6) - Allergic (n=3) - Non-allergic (n=3)
	BAT NON-RESPONDER (N=7) - Allergic (n=3) - Non-allergic (n=4)	BAT NON-RESPONDER (N=7) - Allergic (n=2) - Non-allergic (n=5)	BAT NON-RESPONDER (N=7) - Allergic (n=0) - Non-allergic (n=7)	BAT NON-RESPONDER (N=7) - Allergic (n=0) - Non-allergic (n=7)	BAT NON-RESPONDER (N=7) - Allergic (n=4) - Non-allergic (n=3)

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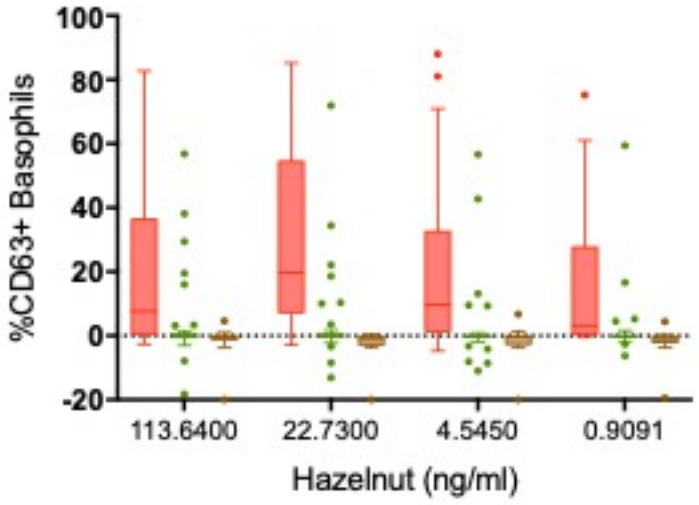

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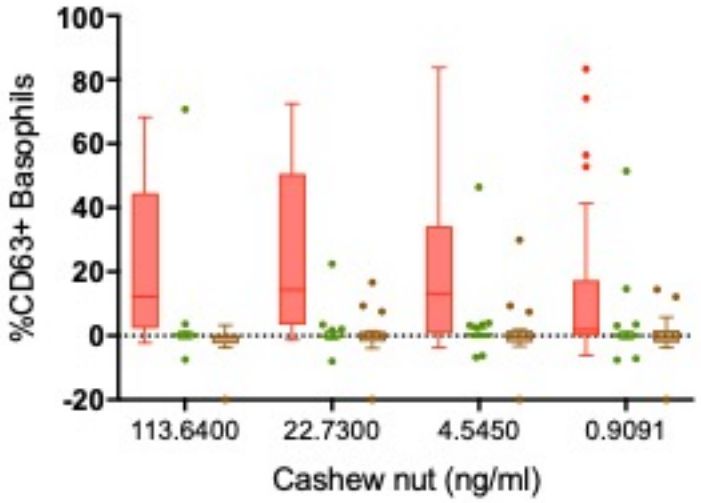

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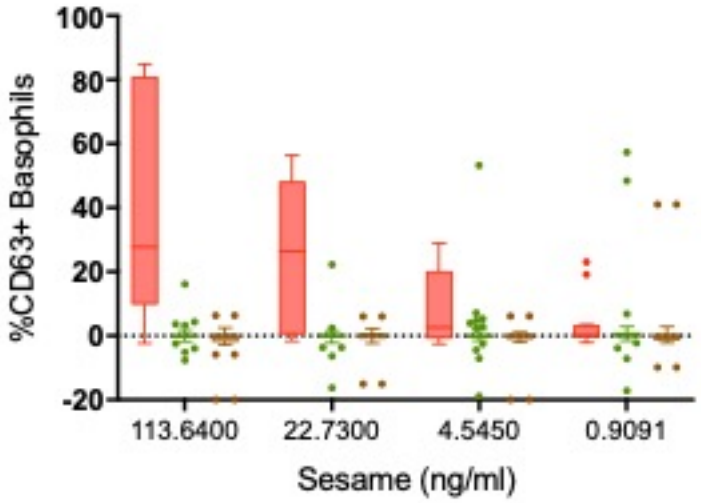

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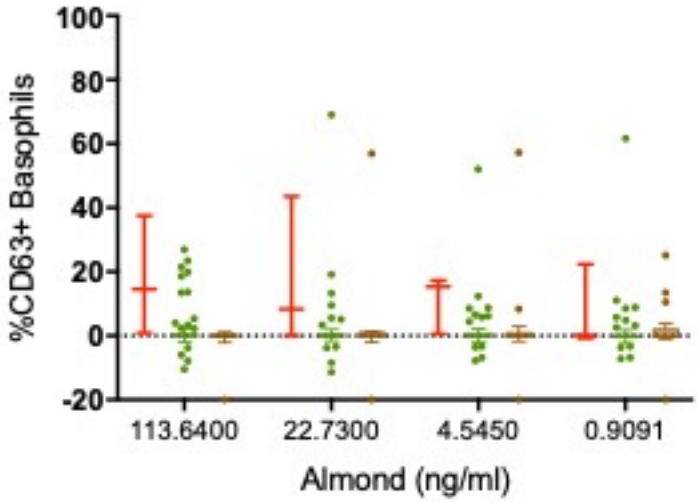

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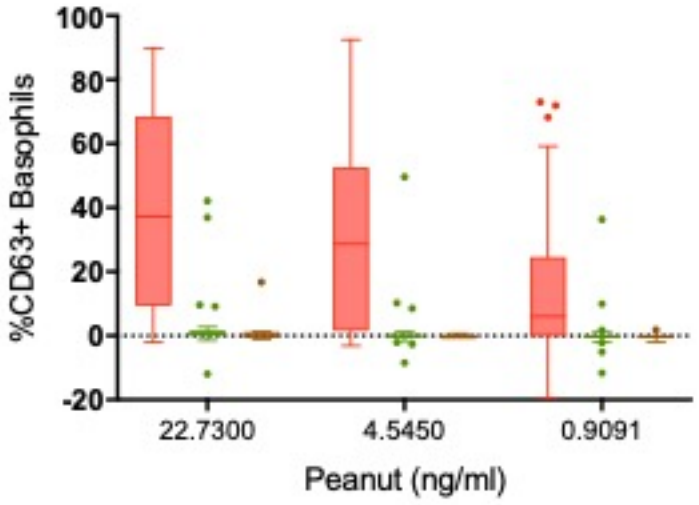

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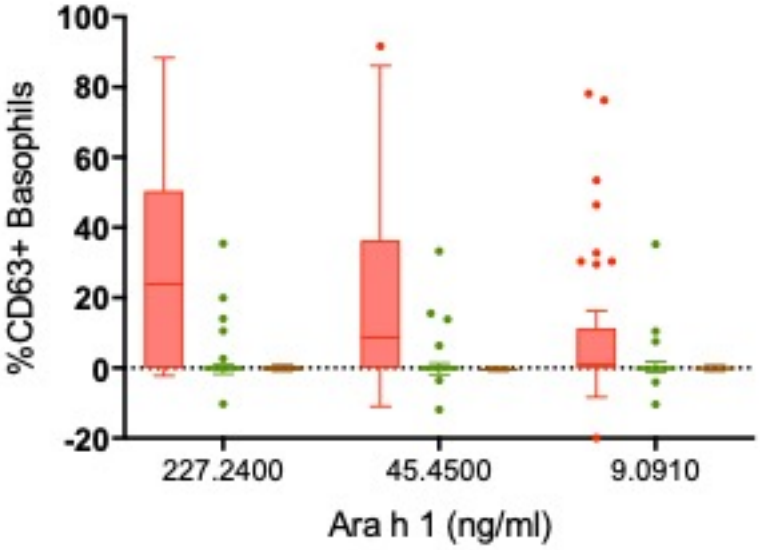

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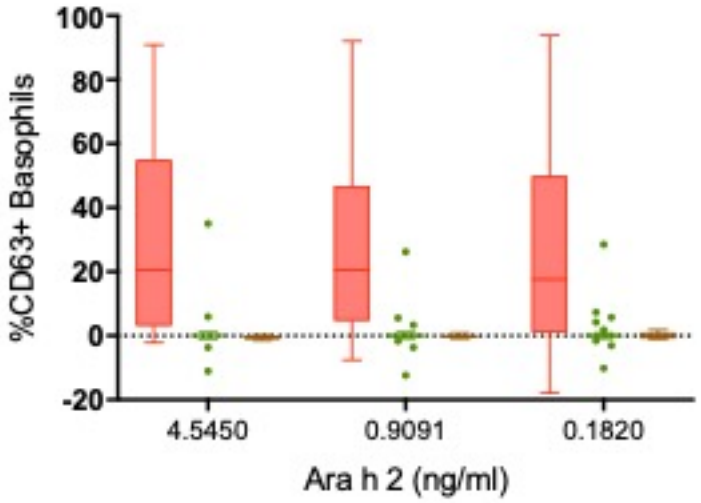

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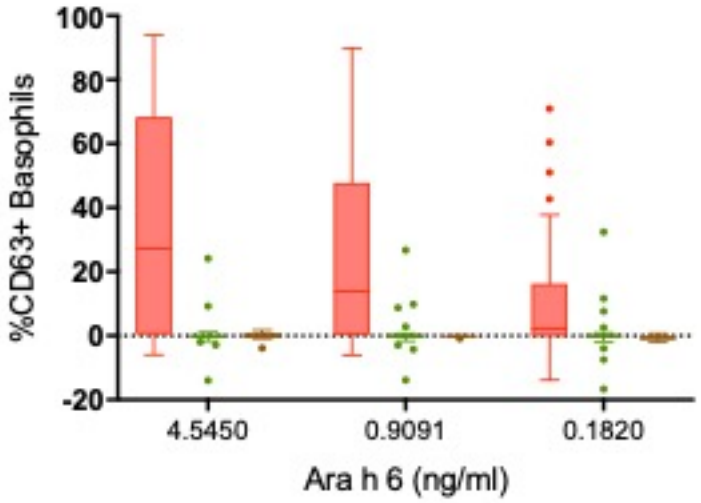

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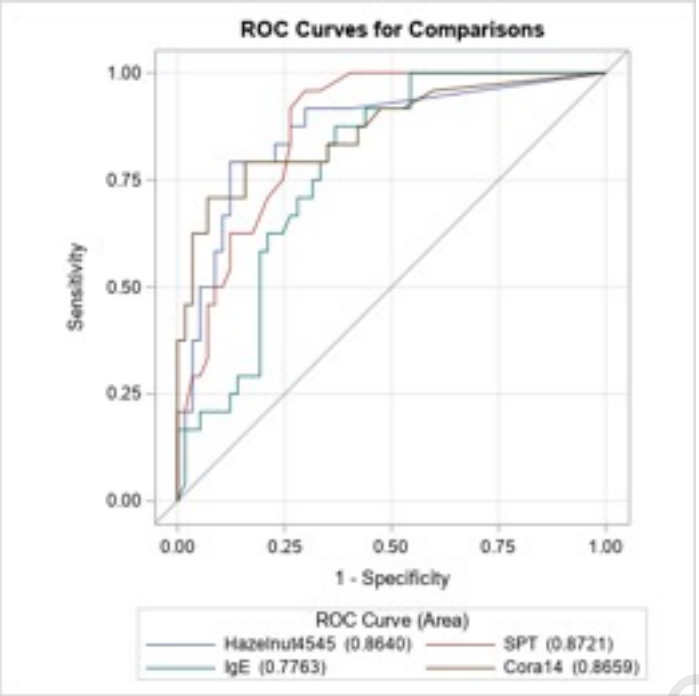

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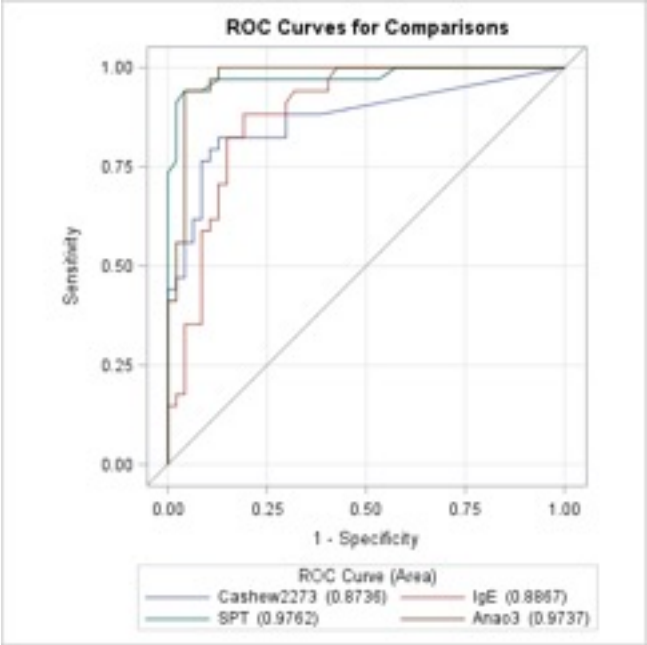

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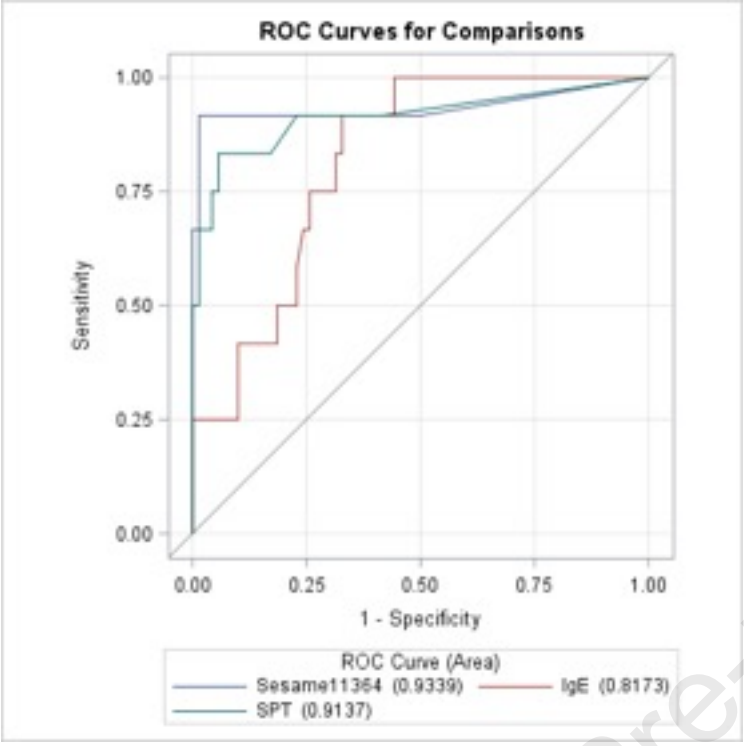

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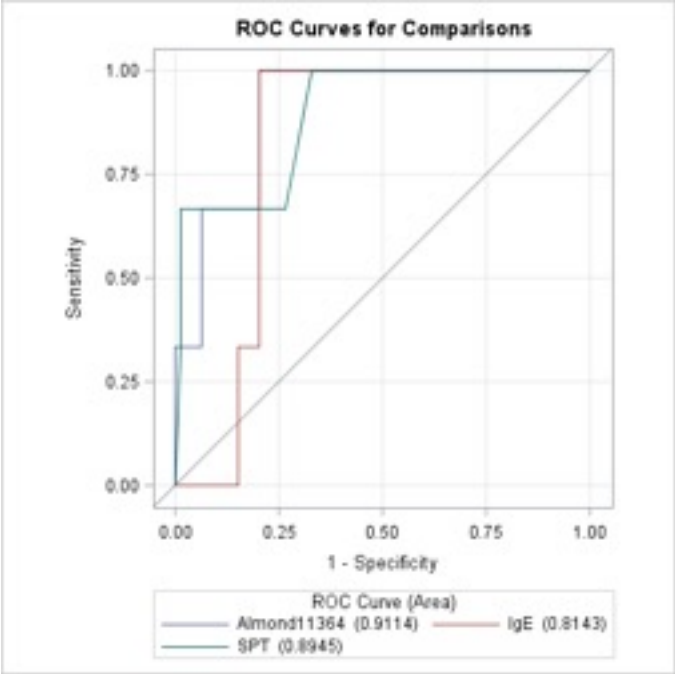

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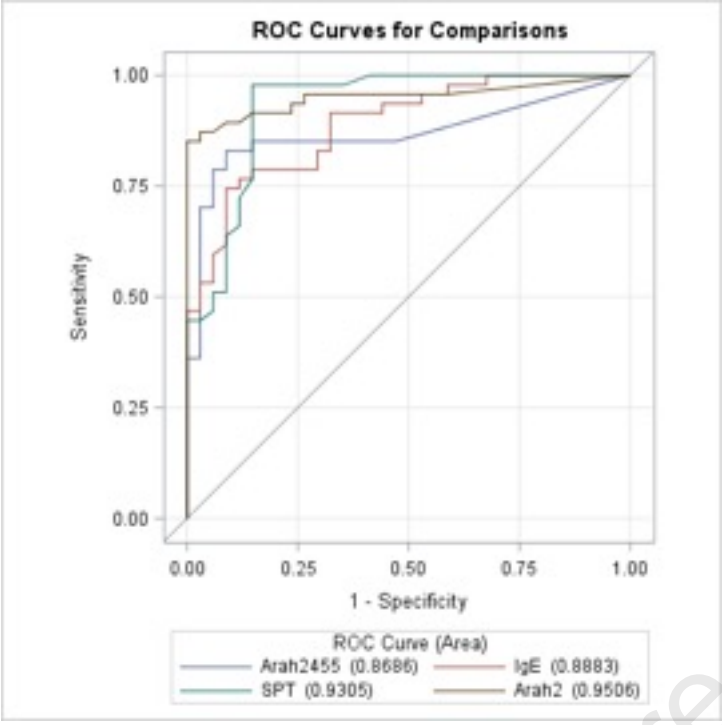

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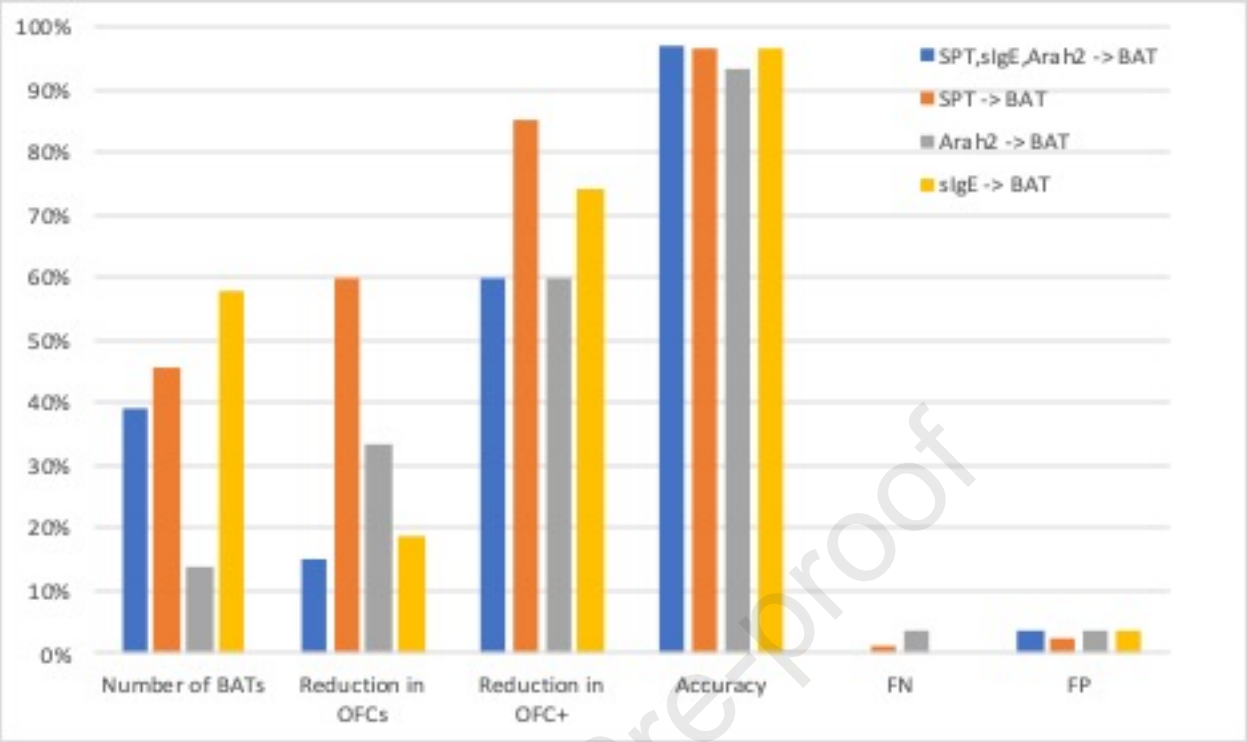

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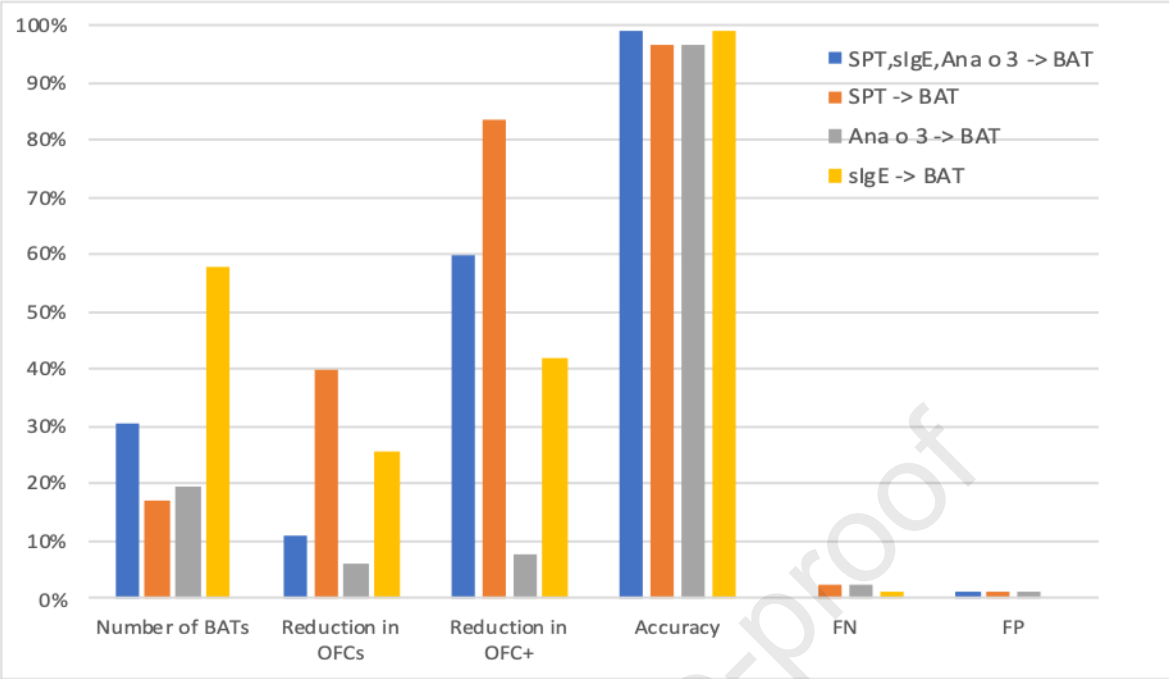

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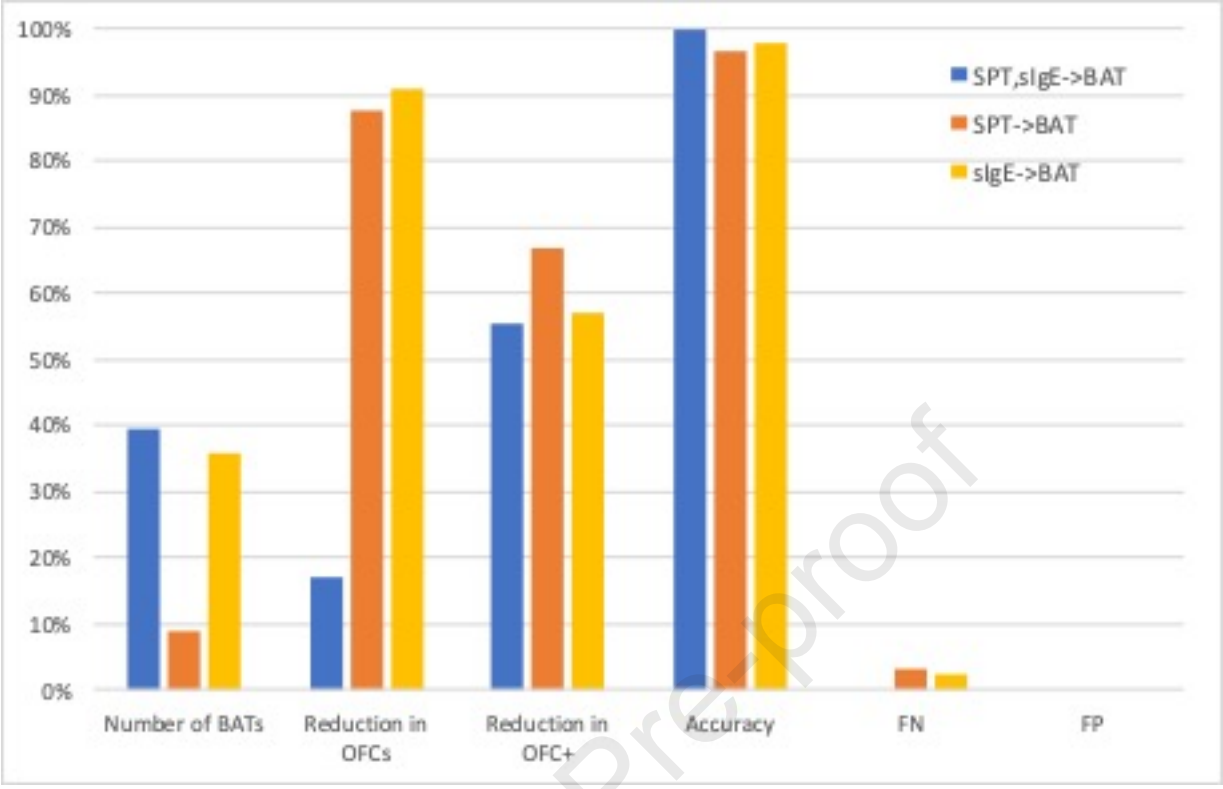

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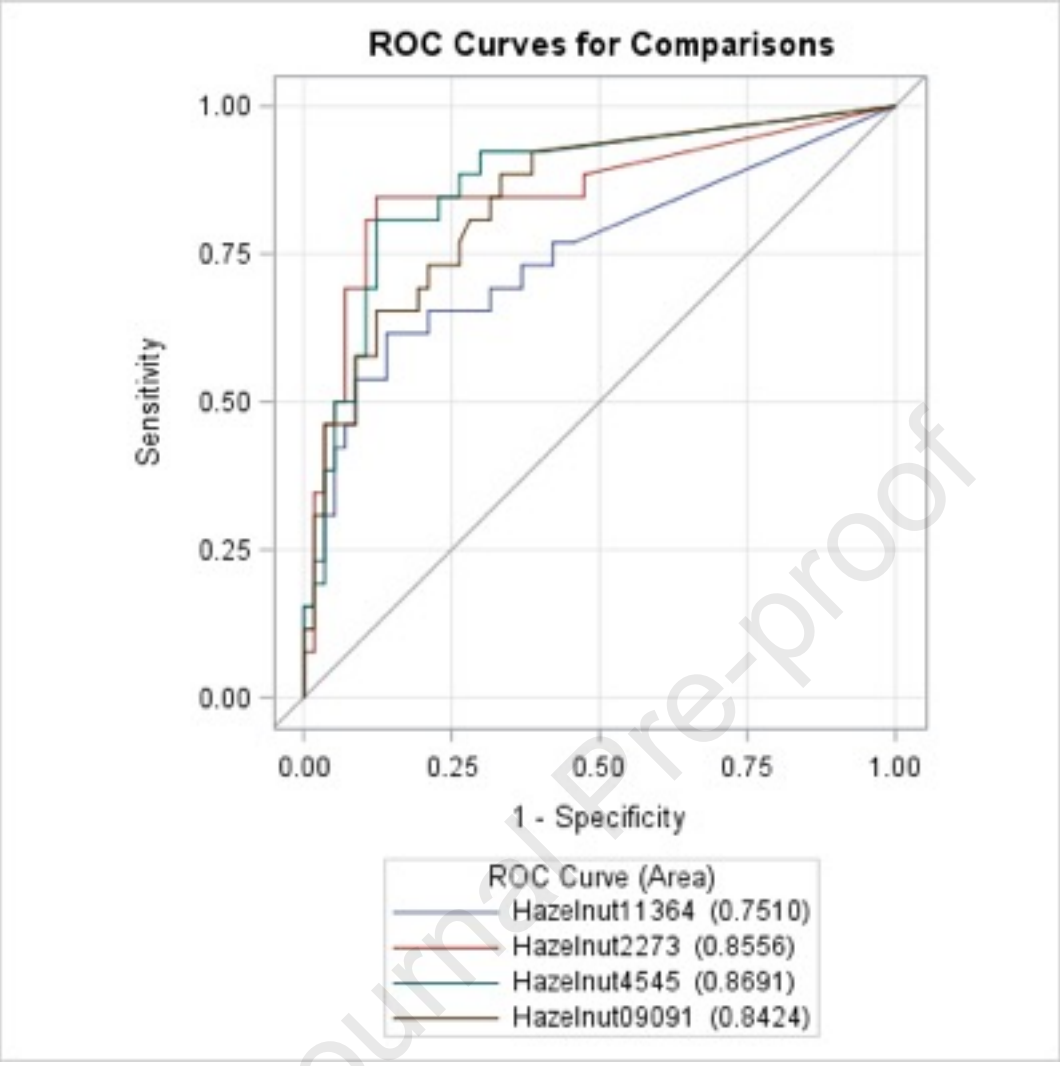

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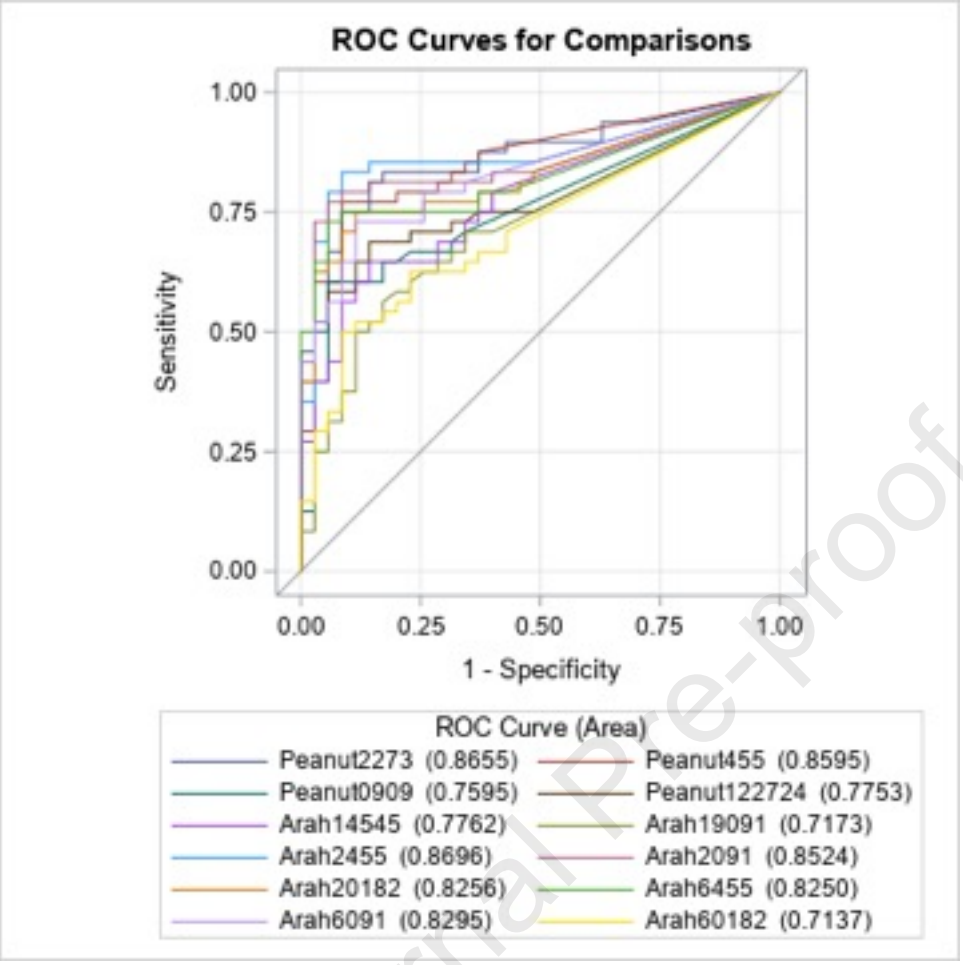

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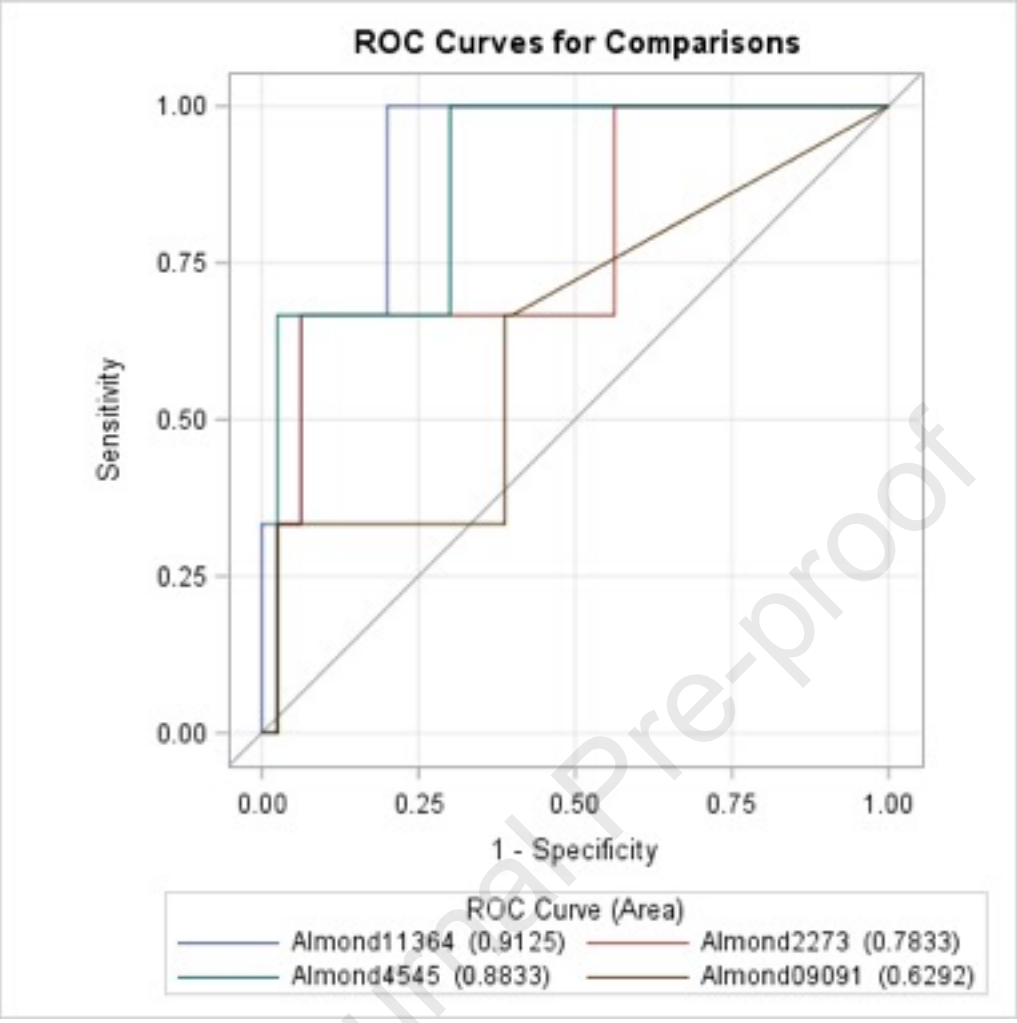

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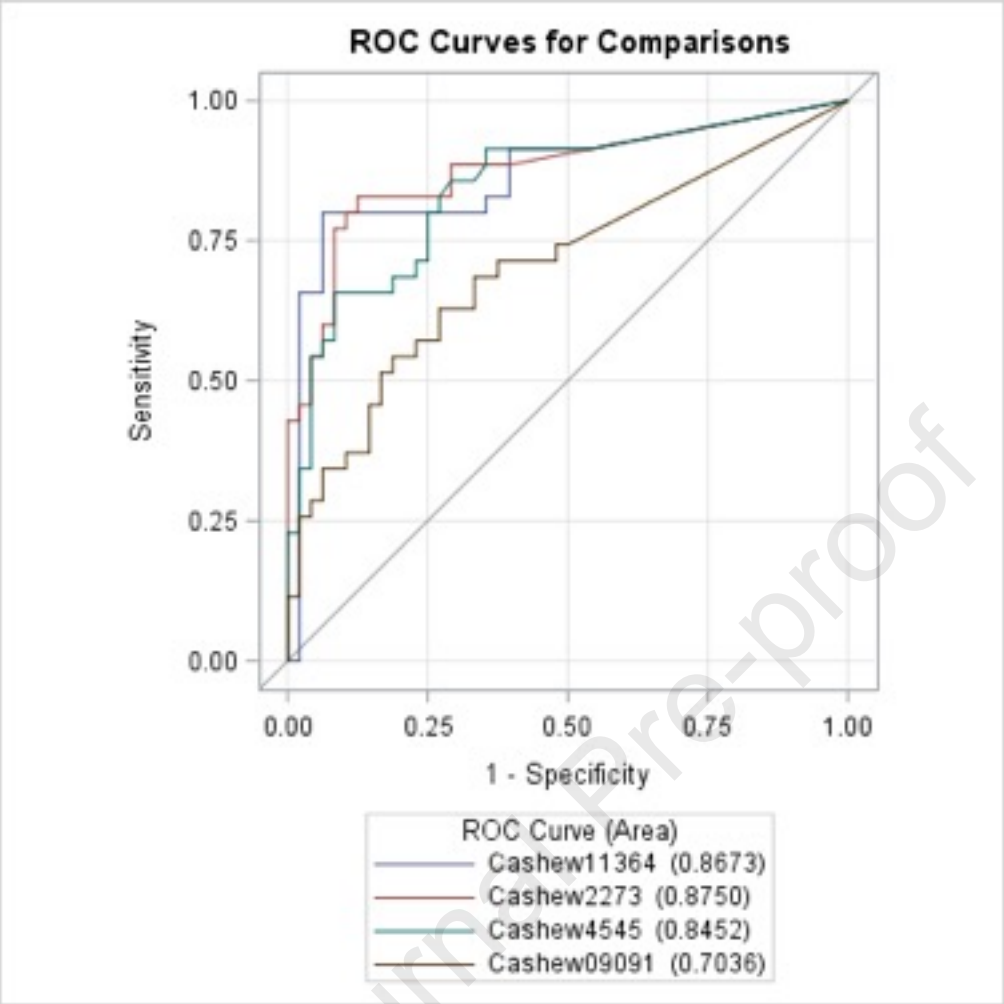

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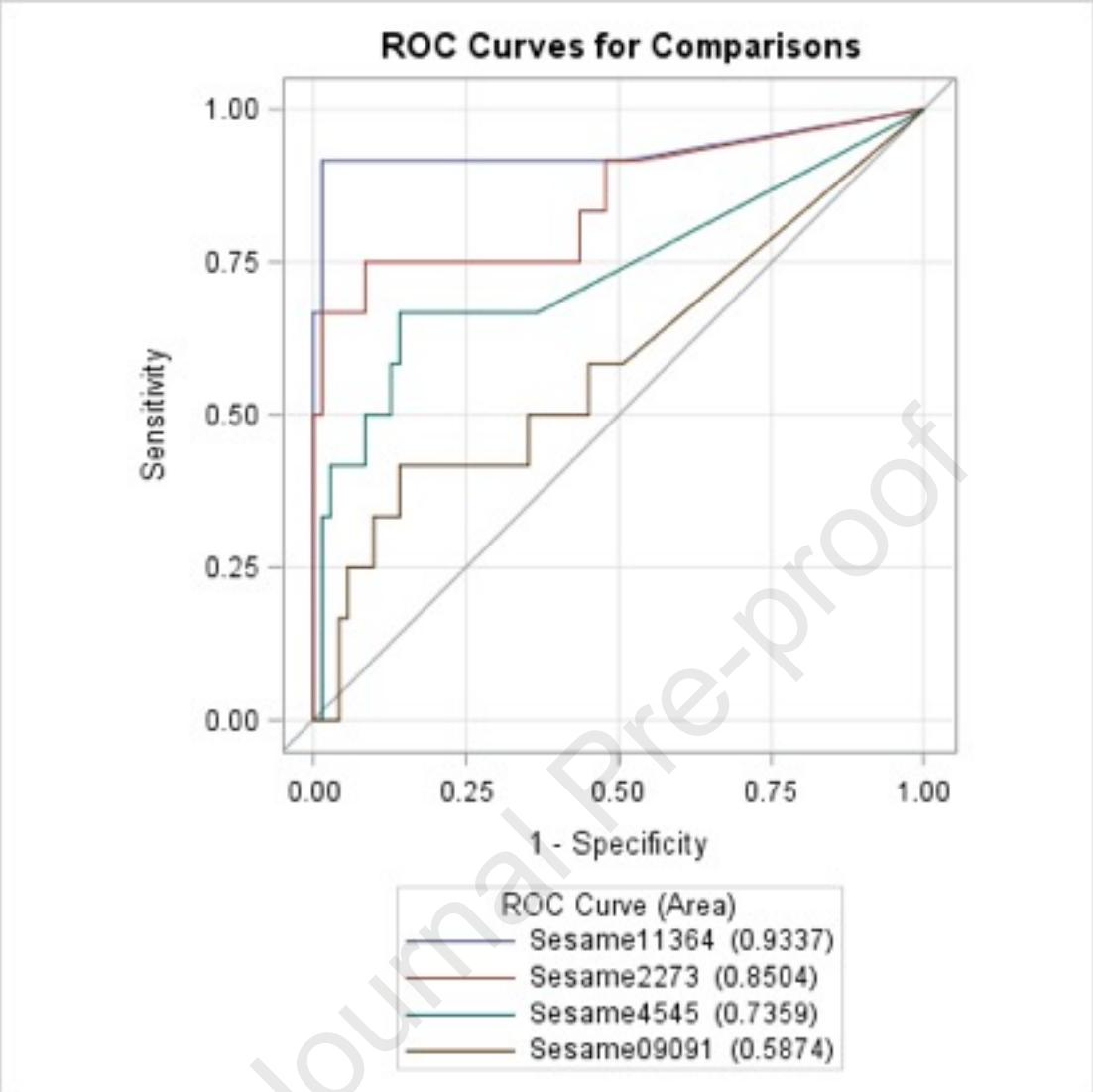

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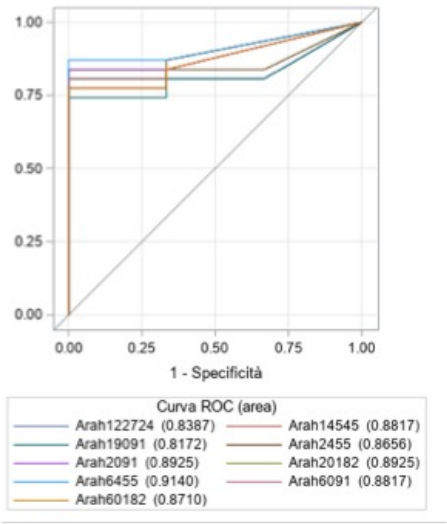

ROC	Mann-Whitney			
	Area	SE	CI 95%	
Arah122724	0.8387	0.0682	0.7050	0.9724
Arah14545	0.8817	0.0685	0.7474	1.0000
Arah19091	0.8172	0.0762	0.6679	0.9665
Arah2455	0.8656	0.0621	0.7439	0.9873
Arah2091	0.8925	0.0522	0.7901	0.9948
Arah20182	0.8925	0.0601	0.7746	1.0000
Arah6455	0.9140	0.0461	0.8236	1.0000
Arah6091	0.8817	0.0588	0.7664	0.9970
Arah60182	0.8710	0.0664	0.7407	1.0000

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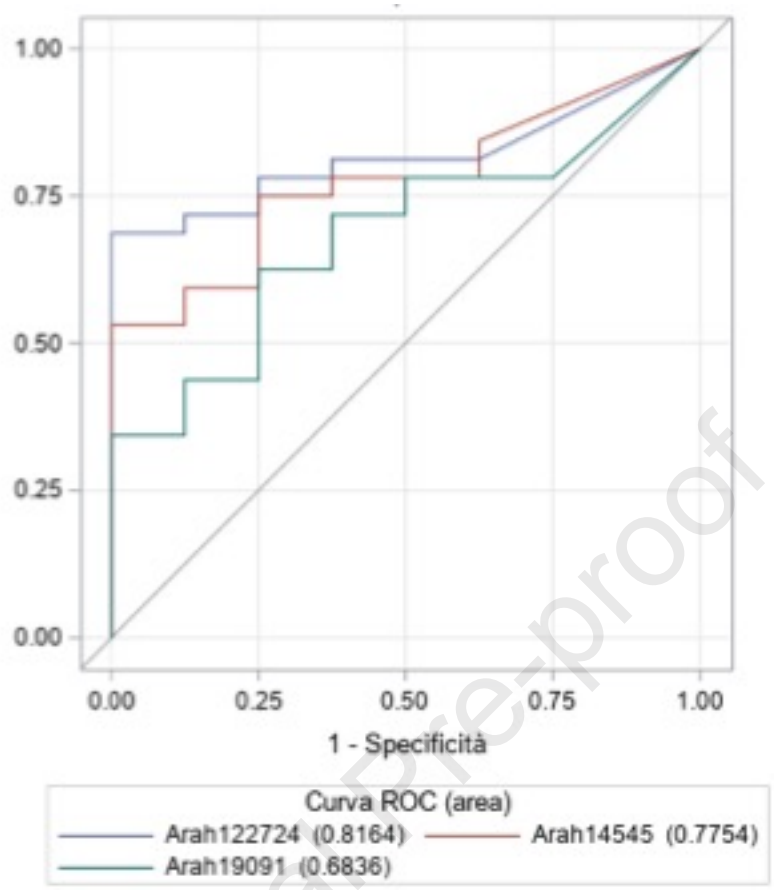

ROC	Mann-Whitney			
	Area	SE	CI 95%	
Arah122724	0.8164	0.0641	0.6907	0.9421
Arah14545	0.7754	0.0761	0.6262	0.9245
Arah19091	0.6836	0.0929	0.5016	0.8656

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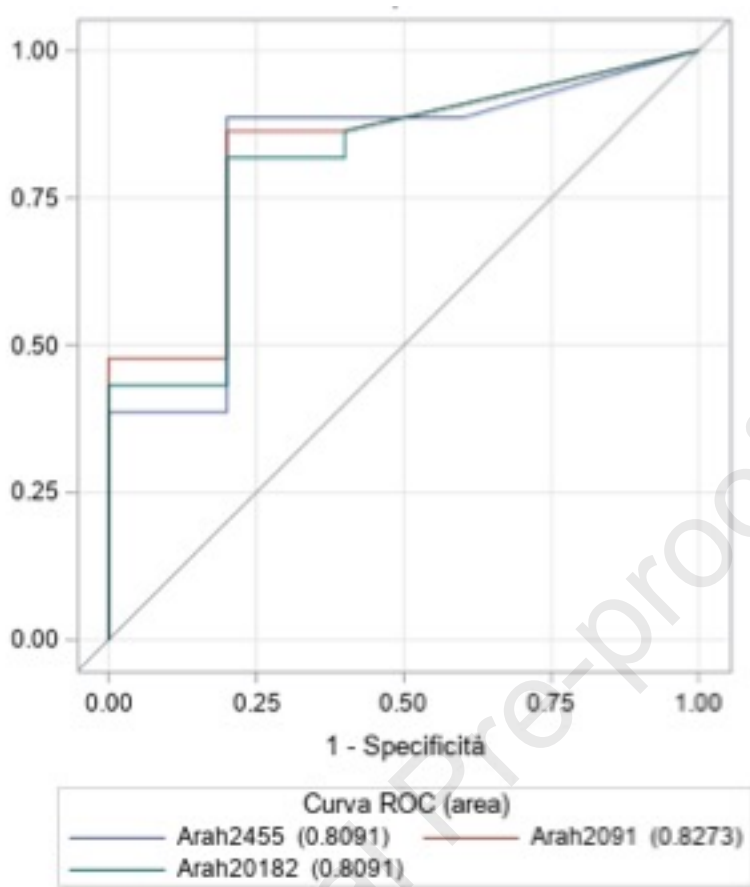

ROC	Mann-Whitney			
	Area	SE	CI 95%	
Arah2455	0.8091	0.1124	0.5887	1.0000
Arah2091	0.8273	0.0951	0.6408	1.0000
Arah20182	0.8091	0.1030	0.6072	1.0000

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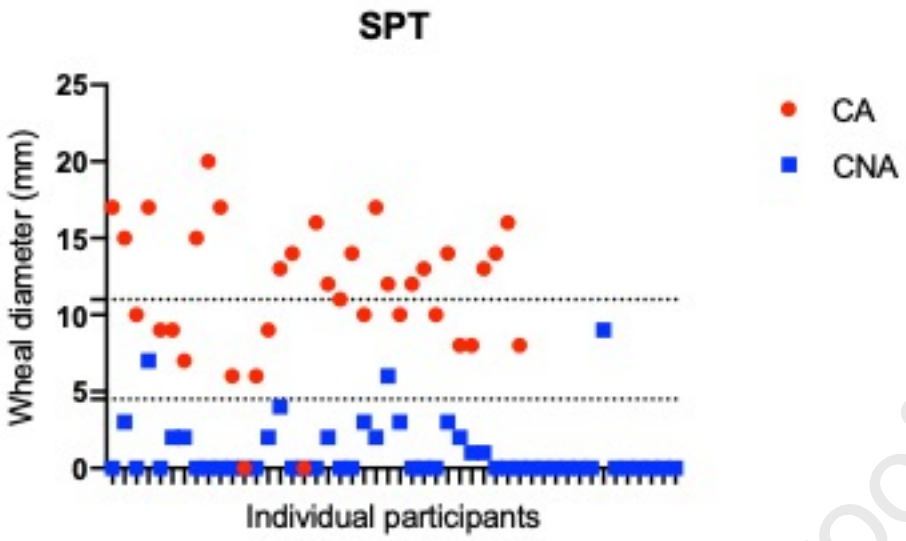

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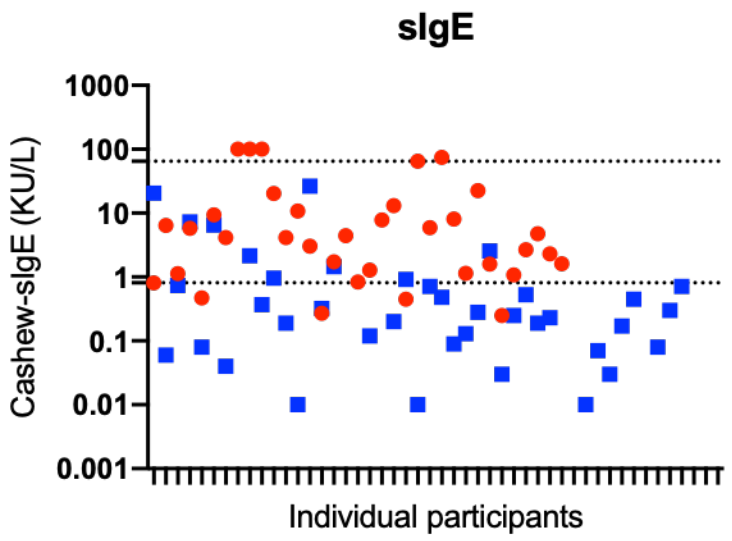

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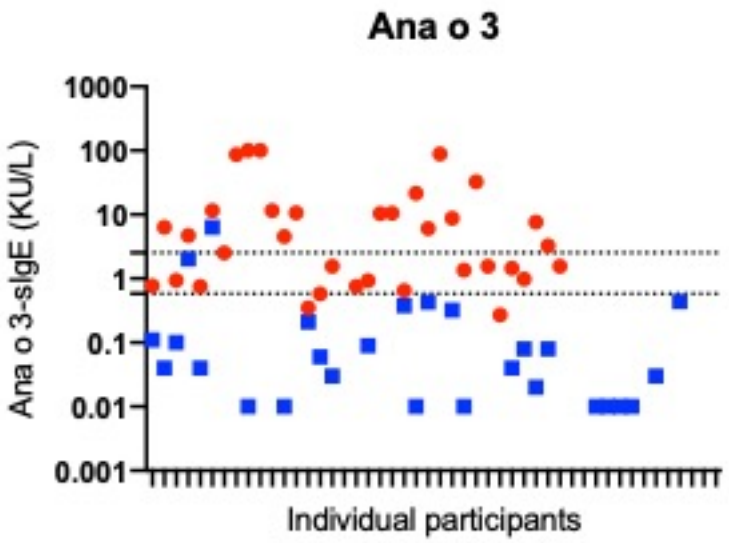

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