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MR THANH D DANG (Orcid ID : 0000-0003-2010-5582)
DR RACHEL LOUISE PETERS (Orcid ID : 0000-0002-2411-6628)

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Ana o 3 sIgE testing increases the accuracy of cashew allergy diagnosis using a two-step model

Thanh D Dang, PhD[1,2,4], Rachel Peters, PhD[1,2,4], Melanie R Neeland, PhD[1,2], Tim Brettig, MD[1,2,4], Hayden Green, MD[1], Vicki McWilliam, MND[1,2,3,4], Mimi L. K. Tang, MD, PhD[1,2,3,4], Shyamali Dharmage, MD, PhD[1,2,4,5], Anne-Louise Ponsonby, PhD[1,4,5], Jennifer Koplin, PhD[1,2,4], Kirsten P Perrett, MD, PhD[1,2,3,4], for the HealthNuts investigators.

[1] Murdoch Children's Research Institute, Melbourne

[2] Department of Paediatrics, University of Melbourne,

[3] Department of Allergy and Immunology, Royal Children's Hospital

[4] Centre for Food & Allergy Research, Melbourne

[5] The Allergy and Lung Health Unit for Epidemiology and Biostatistics, University of Melbourne

[6] The Florey Institute of Neuroscience and Mental Health, the University of Melbourne

1-6 all in Parkville, VIC, Australia

Corresponding Author

Kirsten Perrett, MD, PhD

Murdoch Children's Research Institute,

Royal Children's Hospital,

Flemington Road, Parkville 3052

Victoria, Australia

Kirsten.perrett@mcri.edu.au

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ABSTRACT

Background: Measurement of cashew-specific IgE (sIgE) is often used to confirm sensitization but does not reliably diagnose clinical allergy. Ana o 3 is the dominant cashew allergen detected in 75-100% of patients with cashew allergy but not currently used in clinical practice.

Objectives: To determine if component-resolved diagnostics using specific IgE to the 2 S albumin from cashew, Ana o 3, improves the accuracy of diagnosing cashew allergy, thereby circumventing the need for an oral food challenge (OFC) in some patients.

Methods: A population-based sample of 5276 children was recruited at age 1 year and followed up at age 6 years. Children with positive cashew skin prick test at age 6 underwent an OFC to clarify allergy status. 47 children (mean age 5.02 ± 0.2) (33 cashew allergic and 14 cashew tolerant), had cashew sIgE and Ana o 3 sIgE quantified by ImmunoCAP System FEIA.

Results: A cut-off of >0.32kUA/L for Ana o 3 sIgE provided 95% specificity and 90% sensitivity, and correctly identified 90% of clinical cashew allergy. At the same specificity, the sensitivity for cashew sIgE (>8.5kUA/L) was only 26%. Sequential measurement of cashew sIgE followed by Ana o 3 sIgE diagnosed 90% of children with cashew allergy without the need for an OFC.

Conclusion: Ana o 3 sIgE testing provides higher diagnostic accuracy than cashew sIgE. Sequential measurement of cashew sIgE followed by Ana o 3 removed the need for a food

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challenge from 66% down to 12.8% (5-fold) of children compared with cashew sIgE testing alone.

Abstract word count: 245 words

Key message: This study is the first to show that using Ana o 3 specific IgE in a two-step model can predict cashew allergy with more accuracy than the current diagnostic blood test that rely on whole cashew specific IgE, thereby reducing the need for oral food challenges to clarify clinical allergy status.

Key words: Cashew allergy, diagnosis, diagnostic testing, tree nuts, Ana o 3, component resolved diagnostics, skin prick tests, ImmunoCAP, HealthNuts, IgE, oral food challenge

Abbreviations:

OFC: Oral Food challenge

ImmunoCAP FEIA: ImmunoCAP fluorescence enzyme immunoassay

SPT: Skin prick test

sIgE: Specific immunoglobulin E

PPV: Positive predictive value

ROC: Receiver operating characteristic

AUC: Area under curve

INTRODUCTION

Cashew nut has become an increasingly important food allergen and is one of the most common causes of food-induced anaphylaxis (1). Diagnosis is straightforward when there is an unequivocal history of clinical reaction to cashew ingestion(2). However, many patients can have more complicated histories, an oral food challenge (OFC) is required to confirm or exclude a diagnosis of cashew allergy(3). Although definitive, the OFC is time consuming, costly, and is associated with a risk of anaphylaxis. Skin prick test (SPT) and cashew-specific IgE (ImmunoCAP fluorescence enzyme immunoassay) blood test can be used to confirm cashew allergies, but not for the purposes of diagnosis because both have high sensitivity and low specificity for diagnosis of cashew allergy(4). Furthermore, these tests may be falsely positive due to cross-reactivity to other tree nuts (5, 6).

Component resolved diagnostics (CRD) is a new tool that has been shown to support the diagnostic pathway for some food allergens by avoiding OFCs (7-10). Ana o 3 is the 2S albumin seed storage protein of cashew, and like other proteins in the family, is a highly

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stable allergen (11). Two recent studies in Greece and Germany have found that Ana o 3 was highly predictive of cashew allergy with 93% sensitivity and 95% specificity but these findings need confirmation in other countries like Australia to confirm the possible regional differences in populations (12, 13).

The aim of our study is determine the use of Ana o 3 in the prediction of cashew allergy and develop models for Ana o 3 testing in the community and clinical using The HealthNuts cohort of clearly defined clinical phenotypes (14).

METHODS

Selection of subjects for IgE testing

The methods used in the HealthNuts study have been detailed previously (14). In brief, 11 to-15 month-old infants were recruited from 131 council-run immunization sessions across Melbourne, Australia, and were assessed for their food allergy status. Follow up methods at 4 and 6 years have been previously described (15), in brief, all participants were followed up via questionnaire (81.3% and 83% participation respectively at 4 and 6 years) capturing demographic details, history of food allergy and new food reactions, common allergen exposure information, history of asthma/wheeze and eczema. Included in the assessment is a SPT to a predetermined panel of 8 foods (milk, egg, peanut, wheat, sesame, cashew, almond and hazelnut). All those with a detectable cashew SPT weal were offered a cashew OFC unless they had a recent history of IgE mediated reaction to cashew and cashew SPT (Figure 1). Diagnosis of cashew allergy was defined as a positive food challenge or a clear-cut history of a recent reaction to cashew consistent with established OFC stopping criteria (14), combined with sensitisation to cashew extract. The protocol for cashew OFC were consistent with those of the Australian Society of Clinical Immunology and Allergy (ASCIA) using graded, incremental doses administered at 15- to 20-minute intervals with a top dose of 2 teaspoons of crushed cashew. All subjects with sufficient volume of plasma available for sIgE testing from the HealthNuts study were included in this study (Figure 1). If sample was available for the participant at both ages, the most recent sample from wave 3 (6 year time point) was selected for analysis.

Definitions

Cashew nut sensitisation: SPT ≥3mm (minus negative control) to cashew at clinical assessment

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OFC confirmed cashew nut allergy: Any of the following: (1) positive OFC and IgE sensitized (sIgE≥0.35kUA/l) at 4 or 6 years; (2) history of objective reaction in the past 12 months consistent with HealthNuts OFC stopping criteria following definite exposure to cashew nut and evidence of IgE sensitization at 6 years; or (3) positive OFC at age 4 years and SPT ≥8mm at 6 years of age (n=19).

Non-OFC cashew nut allergy: Any of the following: (1) SPT≥ 8mm at age 6 and one of the following, a) history of objective reaction >12 months ago consistent with HealthNuts OFC stopping criteria following definite exposure to the food of interest, or b) parent-report avoiding food due to allergy (n=14).

Cashew tolerant: Any of the following (1) negative OFC; (2) SPT 0-2mm; or (3) SPT 3-7mm and parent reported ingestion history (eaten >1 time since age 4) (n=14).

Cell separation and plasma collection and allergen-specific IgE analysis

Blood was collected into a sodium heparin tube (Sarstedt) after their respective 4yr or 6yr assessment. The blood was centrifugated off at 700g for 10 minutes within 2 hours after the blood was taken and the plasma was collected and frozen at -80^{o}C until use.

Allergen-sIgE was measured with the ImmunoCAP System FEIA (Phadia AB, Uppsala, Sweden). Plasma samples were analyzed for IgE to whole cashew and Ana o 3 (Phadia AB, Uppsala, Sweden).

Statistical Analysis

Data were analysed by generating the receiver operating characteristic (ROC) curve and both the analyses were performed using Graphpad Prism 6.02 software. The sensitivities and specificities were generated for a range of cut-offs for the ROC curve. The P value was reported for the curve, testing the null hypothesis that the area under the curve is equal to 0.50. We also quote estimated positive and negative likelihood ratios, as their interpretation is not dependent on the underlying disease prevalence or the pre-test probability of the individual, which potentially permits the reader to then transfer results to their own patients. A full discussion of the role of the likelihood ratio and interpretation of thresholds is given by Roberts and Lack[11]. The SPT, cashew sIgE, and Ana o 3sIgE had a skewed distribution and are reported as median and ranges. The proportions comparing the allergic and tolerant population were tested using the two proportion z-test to determine significance between the two groups. Significance was indicated by a p-value <0.05.

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Ethics

Ethics approval was obtained for the HealthNuts study from the Victorian State Government Office for Children (reference no. CDF/07/492), the Victorian State Government Department of Human Services (reference no. 10/07), and the Royal Children's Hospital Human Research Ethics Committee (reference no. 27047 & 32294A).

RESULTS

Clinical features of the study sample

A total of 47 children were selected from the HealthNuts cohort based on plasma availability and included in this study (Figure 1). 33 were cashew allergics, 19 confirmed with an OFC and 14 were included with a clear clinical reaction in the last 12 months together with a SPT≥8mm. Of the 14 cashew tolerant children, 4 were confirmed with a cashew OFC, 4 were sensitized tolerant and 6 were non-sensitized tolerant and were currently ingesting cashew in their diet. Clinical characteristics of selected cohort are outlined in Table 1 and Table 2. A sensitivity analysis comparing the two cashew allergic groups show the non-OFC group have higher cashew sIgE and SPT, and no differences in clinical characteristics (Table 2), and were combined for all subsequent analysis. A greater proportion of cashew allergic children had co-existing food allergies (72.7%) compared to the group of cashew tolerant children (33.3%), $p<0.05$, which was more likely to be a co-existing tree nut allergy (60.6%) compared to (0.67%).

Accuracy of diagnosing cashew allergy using SPT and cashew sIgE

Using previously defined threshold for diagnosing cashew allergy (SPT wheal ≥8 mm) (16), we assessed the utility of cashew SPTs and ImmunoCAP cashew sIgE measurements to diagnose cashew allergy in our cohort. 87.9% (n=29) had a SPT results of 8 mm or greater and could be given a diagnosis of cashew allergy; however, 12% (n=4) with SPT results of 3 to 7 mm would require an OFC to confirm the presence of allergy (Figure 3a). At a threshold of 8.5kUA/l for cashew sIgE, where 95% specificity was reached, only 26% with cashew allergy (n=9) could be given a diagnosis of cashew allergy, leaving 31 children with levels between 0.10 and 8.5 kUA/L and would require a OFC to confirm the presence of allergy (Figure 3a).

Ana o 3 ImmunoCAP testing

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To describe the accuracy of Ana o 3 sIgE testing, we report a number of Ana o 3 sIgE and cashew sIgE thresholds along with the sensitivities and specificities (Table 3). Ana o 3 sIgE level of >0.320 kUA/L provides 93.3% specificity and 90% sensitivity (95% CI, 73% to 95%), compared to cashew sIgE level of 8.54 kUA/L which provides a 95% specificity and a significantly lower sensitivity of 26% (95% CI, 13% to 44%; P <0.001; Table 3).

Compared with both SPTs and cashew sIgE measurements, measurement of Ana o 3 sIgE correctly identified more patients with true cashew allergy when cut offs for 93% specificity or a 95% PPV were applied. The mean Ana o 3 sIgE level for the 33 patients with cashew allergy was 6.86 (standard deviation 11.9) kUA/L compared with 0.193 (0.281) kUA/L in the cashew-sensitized subjects who did not have cashew allergy and 0.0246 (0.0377) in the non-sensitized, non-cashew allergic patients (p=0.001) (Table 2). The area under the curve for the cashew sIgE ROC curve is 0.83 (95% CI, 0.79-0.99) compared with an area under the curve of 0.98 (95% CI, 0.88-1.00) for Ana o 3, indicating that Ana o 3 performs significantly better than cashew sIgE (P < 0.027, Figure 2). We found the performance of Ana o 3 sIgE and cashew SPT are comparable (Supplementary Table 1), with the AUC for cashew SPT was 0.99 (95% CI, 0.89-1.00).

Diagnosing cashew allergy using a combination of previous methods and Ana o 3

We next investigated whether Ana o 3 could be used to sequentially to diagnose cashew allergy in patients who had SPT and sIgE below the cut-offs of 8mm and 8.5kUA/l respectively. Fig 3 represents the number of OFCs that would be required if the current thresholds for cashew sIgE measurements, SPTs, and Ana o 3 sIgE measurements were used to diagnose cashew allergy in the absence of any other tests. Of the 47 children included in this study, OFCs would be required to confirm the allergy status on 31 (66%) based on cashew sIgE, 8 (17%) based on cashew SPT, and 8 (17%) based on Ana o 3 measurements. Fig 4 shows the number of OFCs required when incorporating the two methods of SPT or cashew sIgE as the first line tests, together with Ana o 3 sIgE measurement as a second line of testing to help improve the accuracy of distinguishing patients with cashew allergy from those with cashew tolerance. In the first model, we report the results representative of a primary health care scenario involving only a blood test to diagnose cashew allergy. Of the 31 patients with cashew sIgE levels between 0.1 and 8.5 kUA/l successfully identified an additional 21 patients as cashew allergic, and 3 children as cashew tolerant with Ana o 3 sIgE testing (Figure 4a). Hence incorporating Ana o 3 testing in combination with cashew sIgE

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testing would reduce the number of OFCs needed by from 65.96% down to 12.76% (a fold change of 5.2). In the second model, Ana o 3 testing patients with cashew SPT between 3 and 8mm identified a further 6 patients allergic to cashew, reducing the number of OFCs required by 17.02% down to 4.26% (a fold change of 4) (Figure 4b).

DISCUSSION

This study reports the utility of Ana o 3 specific IgE testing in a cohort of with clearly defined clinical outcomes and developed testing models to correctly diagnose cashew allergy and reduce the number of OFCs. We found that Ana o 3 sIgE testing was more accurate in determining cashew allergy compared to cashew sIgE alone against the OFC. In addition, every allergic patient in our cohort was sensitized to Ana o 3, which means, on the other hand, that an undetectable level of specific IgE to Ana o 3 might be a good predictor of a negative challenge outcome.

The use of CRD for the improvement of food allergy diagnostics has been demonstrated for many foods, particularly with S2 albumin proteins such as Ara h 2 for peanut (7, 17). Our findings are similar to others which have reported Ana o 3 sIgE levels (12, 13, 18), indicating that Ana o 3 is consistently good for differentiating between allergic and tolerant paediatric patients. These studies including German, Japanese, Greek, and now Australian populations, consistently showing that Ana o 3 sIgE levels between 0.3-0.4kUA/l are highly sensitive and specific for cashew allergy (≥90%, and ≥95% respectively).

Although the performance of cashew SPT was comparable to Ana o 3 sIgE testing, SPT is usually performed in a specialist setting, with the patient waiting times are at present significant exceeding 12 months in many centres in Australia[14]. By comparison, blood testing for Ana o 3 and whole cashew sIgE can be easily be accessed in the community by primary and secondary healthcare professionals with access to diagnostic laboratories. Using our cohort, testing with cashew sIgE followed by Ana o 3 sIgE could substantially reduce the number of OFCs required to diagnose cashew allergy from 66% to 12%. Given that the cut-off of ≤0.1 kUA/l for Ana o 3 can identify 78.5% of cashew tolerant children whilst only having a 3% false negative rate, this would support the gradual introduction of cashew into the diet if the child has not already eaten the food. While the use of Ana o 3 in the diagnosis of cashew allergy in the community has significant advantages, in an allergy clinic setting, cashew SPT still provides a rapid and accurate method for determining cashew allergy, and

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Ana o 3 sIgE could be used as a subsequent test to reduce the number of patients requiring an OFC.

The strengths of this study include the cohort of clearly phenotyped cashew allergic and tolerant children through a SPT and an OFC. True population negative controls provided better evaluation of the performance of these tests as a screening tool for cashew allergy. This is the first study to present models on cashew SPT in combination with Ana o 3 sIgE and whole cashew sIgE on all subjects. The weakness of the current study is that not all children under went OFC as the gold standard in food allergy diagnostics. However, clinically relevant cashew allergy was determined very carefully focusing on clear objective reactions to cashew in the patients' history. Few studies have been presented on the pattern of concomitant food allergies in tree nut allergic patients (19) and our paediatric cohort.

In conclusion, our findings suggest that Ana o 3 sIgE testing used sequentially with cashew sIgE offers improved diagnostic accuracy for cashew allergy compared with whole cashew extract SPT or sIgE testing alone. This approach would be especially advantageous in settings where access to an allergy specialist and hence OFC is not readily available. The combined Ana o 3 and whole cashew sIgE testing approach substantially reduces the need for an OFC, which is expected to alleviate strain and demand on clinical allergy services.

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Table 1: Demographic and clinical characteristics, stratified by cashew allergy

	Cashew Tolerant (n=14)	**Cashew Allergic (n=33)**
Gender, male n (%)	8 (53.3)	20 (60.6)
Mean Age at OFC, yrs (SD)	4.6 (0.8)	5.2 (1.1)
Mean SPT, mm (SD)		
Cashew SPT	0.87(0.61)	13.2 (1.26)
Median sIgE, kUA/L (interquartile range)		
Cashew sIgE	0.13 (0.02-0.81)	2.09 (0.8-6.7)
Ana o 3 sIgE	0.03 (0-0.11)	2.45 (0.94-6.64)
Received Cashew OFC, n (%)		
Cashew OFC	3 (20)	19 (58)
Food Allergy Details, n (%)		
No other food allergies	10 (66.67)	9 (27.3)
Co-existing food allergy		
Peanut allergy	2 (13.3)	16 (48.5)

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Other tree nut €	1 (0.67)	20 (60.6)	
Allergy Sensitization	**Cashew Tolerant (n=14)**	**Cashew Sensitized Allergic (n=33)**	
		Cashew Allergic (n=19)	Probable Cashew Allergic (n=15)
Egg allergy	2 (13.3)	9 (27.3)	
Sesame allergy	2 (13.3)	5 (15.2)	
Food sensitisation details, n (%)			
Peanut 3-8mm	3 (20)	2 (6)	
Peanut>8mm	2 (13.3)	18 (54.5)	
Any tree nut 3-8mm #	4 (26.7)	5 (15.2)	
Any tree nut >8mm £	2 (13.3)	24 (72.6)	
Egg 3-8mm	1 (6.7)	8 (24.2)	
Egg >8mm	0	4 (12.1)	
Sesame 3-8mm	1 (6.7)	4 (12.1)	
Sesame >8mm	0	3 (9)	
Shellfish 3-8mm	0	0	
Shellfish >8mm	0	0	
Allergic disease history, n (%) ¶			
Current eczema	3 (20)	16 (48.5)	
Current asthma	3 (20)	22 (66.67)	
Current rhinitis	2 (13.3)	11 (33.33)	

€ Tree nut allergy: defined as a positive OFC or history of reaction and sensitized (≥3mm). Does not include those with SPT≥8mm defined as probable allergy in the HealthNuts (n=19). Individual tree nut allergy details - hazelnut=9, macadamia=2, pecan=2, pistachio=4, walnut=8

#Tree nut sensitisation 3-8mm: almond=18, hazelnut=30, macadamia=11, pecan=16, pistachio=72, walnut=44

£ Tree nut sensitisation >8mm: almond=3, hazelnut=15, macadamia=1, pecan=5, pistachio=21, walnut=15

¶ Current eczema, current asthma, current rhinitis=parent-report of doctor diagnosed eczema, asthma, rhinitis.

Table 2: Cashew SPT and sIgE stratified by cashew allergy

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Cashew SPT < 8mm, n (%)	14 (100%)	3 (16%)	0 (0%)
Mean Cashew SPT, mm (±SD)	0.87 (±0.61)	11.7 (±0.85)	15.6 (±1.89)
Cashew sIgE <8.5kUA/l, n (%)	14 (100%)	15 (79%)	10 (67%)
Mean Cashew sIgE kUA/l, (±SD)	0.90 (±0.56)	7.64 (±3.06)	15.0 (±5.86)
Median Cashew sIgE (kUA/l)	0.03 [0.02-0.96]	2.09 [0.24-44.4]	5.07 [0.1-79.4]
Ana o 3 sIgE < 0.32kUA/l, n (%)	14 (100%)	2 (11%)	2 (13%)
Mean Ana o 3 sIgE, kUA/l,(±SD)	0.11 (±0.06)	6.8 (±2.66)	14.3 (±5.32)
Median Ana o 3 sIgE (kUA/l)	1.88 [0.01-0.96]	2.45 [0-42.93]	6.72 [0.1-72.69]

346 A cut-off < 0.1kuA/l is classified as a negative result, and are considered non-sensitized.

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Table 3. Sensitivity and specificity of various cut-offs for Ana o 3 and cashew sIgE

Ana o 3-specitic IgE						Cashew-specific IgE					
Cashew allergics (n=19) versus Cashew sensitized tolerant (n=14)											
Cut-off (kUA/l)	**Sensitivity %**	**95% CI**	**Specificity %**	**95% CI**	**PLR***	**Cut-off (KUA/l)**	**Sensitivity %**	**95% CI**	**Specificity %**	**95% CI**	**PLR***
> 0.1046	95	76.39% to 99.74%	66.67	43.75% to 83.72%	2.85	> 0.1250	100	83.89% to 100.0%	42.11	23.14% to 63.72%	1.727
> 0.2135	95	76.39% to 99.74%	88.89	67.20% to 98.03%	8.55	> 0.3500	85	63.96% to 94.76%	63.16	41.04% to 80.85%	2.307
> 0.3399	90	69.90% to 98.22%	94.44	74.24% to 99.72%	16.2	> 1.055	65	43.29% to 81.88%	84.21	62.43% to 94.48%	4.117
> 0.6982	80	58.40% to 91.93%	94.44	74.24% to 99.72%	14.4	> 1.670	55	34.21% to 74.18%	84.21	62.43% to 94.48%	3.483
> 0.9071	75	53.13% to 88.81%	94.44	74.24% to 99.72%	13.5	> 6.695	25	11.19% to 46.87%	89.47	68.61% to 98.13%	2.375
> 0.9947	75	53.13% to 88.81%	100	82.41% to 100.0%	17.6	> 8.535	20	8.066% to 41.60%	94.74	75.36% to 99.73%	3.8
> 1.134	70	48.10% to 85.45%	100	82.41% to 100.0%	14.2	> 10.60	20	8.066% to 41.60%	100	83.18% to 100.0%	
All cashew allergics (n=33) versus Cashew sensitized tolerant (n=14)											
> 0.09909	100.0	89.42% to 100.0%	73.33	44.90% to 92.21%	3.750	> 0.3500	88.24	72.55% to 96.70%	63.16	38.36% to 83.71%	2.395
> 0.2135	93.94	79.77% to 99.26%	93.33	68.05% to 99.83%	14.09	> 0.8150	82.35	65.47% to 93.24%	73.68	48.80% to 90.85%	3.129
> 0.3272	90.91	75.67% to 98.08%	93.33	68.05% to 99.83%	13.64	> 1.025	76.47	58.83% to 89.25%	84.21	60.42% to 96.62%	4.843
> 0.6180	84.85	68.10% to 94.89%	93.33	68.05% to 99.83%	12.73	> 3.375	50.00	32.43% to 67.57%	89.47	66.86% to 98.70%	4.750

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> 0.9947	75.76	57.74% to 88.91%	100.0	78.20% to 100.0%	-	> 8.535	26.47	12.88% to 44.36%	94.74	73.97% to 99.87%	5.029
						> 9.320	26.47	12.88% to 44.36%	100.0	82.35% to 100.0%	-

* PLR is the positive likelihood ratio calculated by (sensitivity/(1-specificity)) and indicates the likelihood of having peanut allergy

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Figure Legends

Figure 1. Selection of subjects for Ana o 3 testing

Figure 2. ROC curves showing true positive rates (sensitivity) plotted against the false-positive rate (specificity) for different cut-off points of the quantified components of Ana o 3 (orange circles squares) and whole cashew extract (blue triangles). The points highlighted for Ana o 3 sIgE, cashew sIgE, cashew SPT indicate putative levels for determining 95% specificity (0.34kUA/l, 8.5kUA/l, and 8mm respectively) for cashew allergy. The area under curve is 0.986, 0.991, and 0.823 for Ana o 3 sIgE, cashew SPT, and cashew sIgE respectively.

Figure 3a-c. Comparison of various methods of diagnosing cashew allergy with Cashew sIgE (a), Cashew SPT (b) or Ana o 3 sIgE (c) followed by an oral food challenge. Patients from this study were examined using identified cut-offs for cashew sIgE and SPT to determine the stringency of each test. CA stands for cashew allergic and CT stands for cashew tolerant.

Figure 4a-b. Comparison of clinical scenarios for diagnosing cashew allergy using a 2-step model in a community setting (a), or an allergy clinic setting (b). Cashew sIgE or Cashew SPT was assessed as the first line test followed by Ana o 3 sIgE to help improve the diagnosis of cashew allergy when either cashew sIgE or cashew SPT tests results fall the respective cut-offs of either 0.35-8.5 kUA/l or 3-8mm. CA and CT denotes the number of cashew allergic and cashew tolerant children respectively that fall into the designated ranges.

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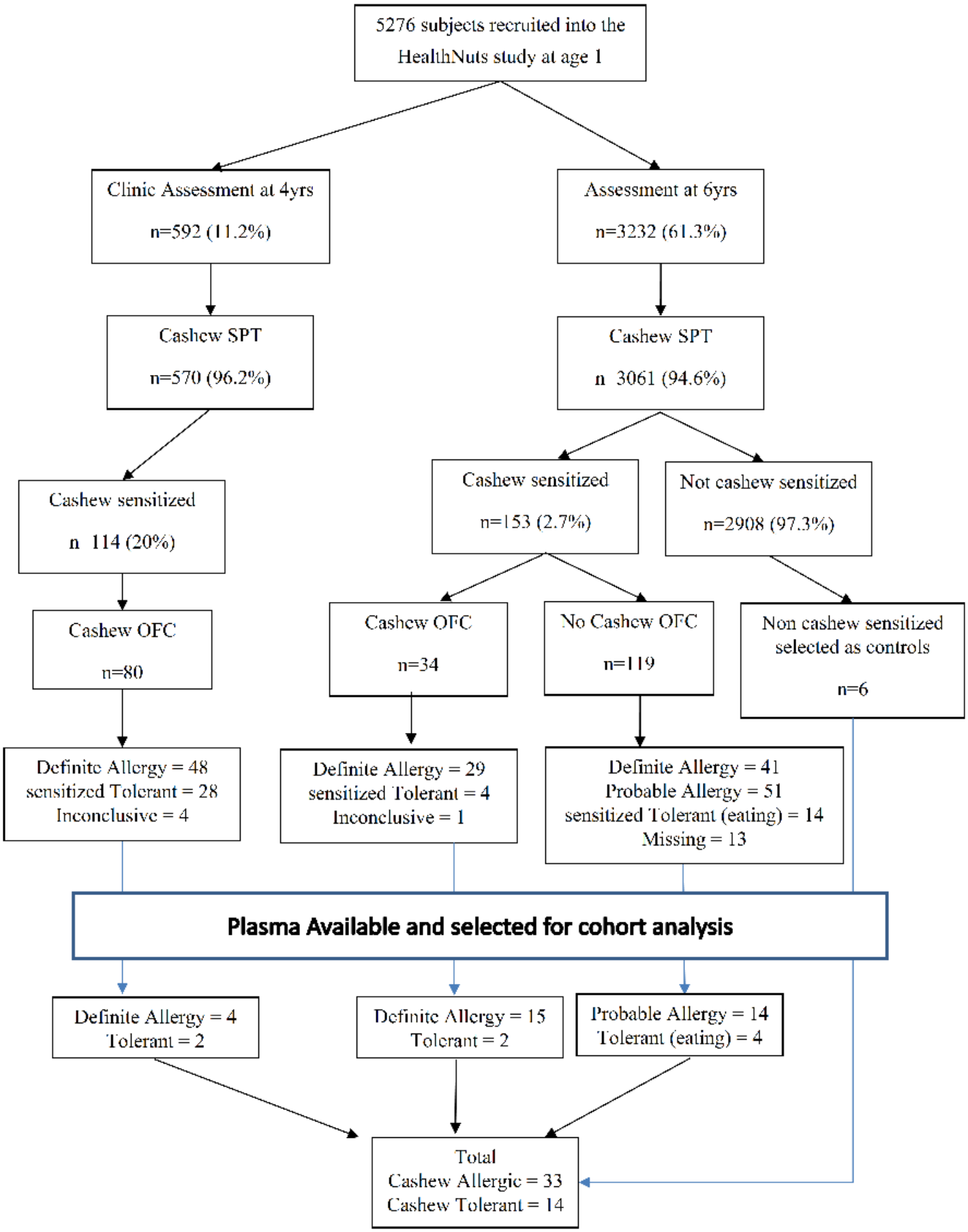

Figure 1. Selection of Cohort for Ana o 3 testing

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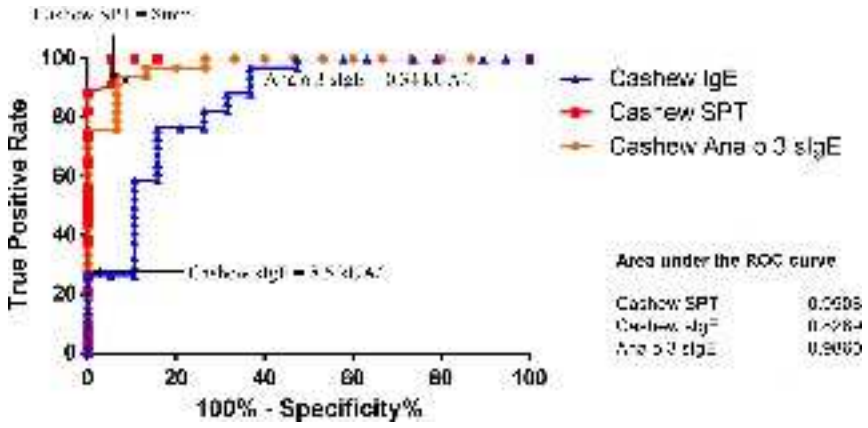

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Figure 3a-c. Comparison of various methods of diagnosing cashew allergy

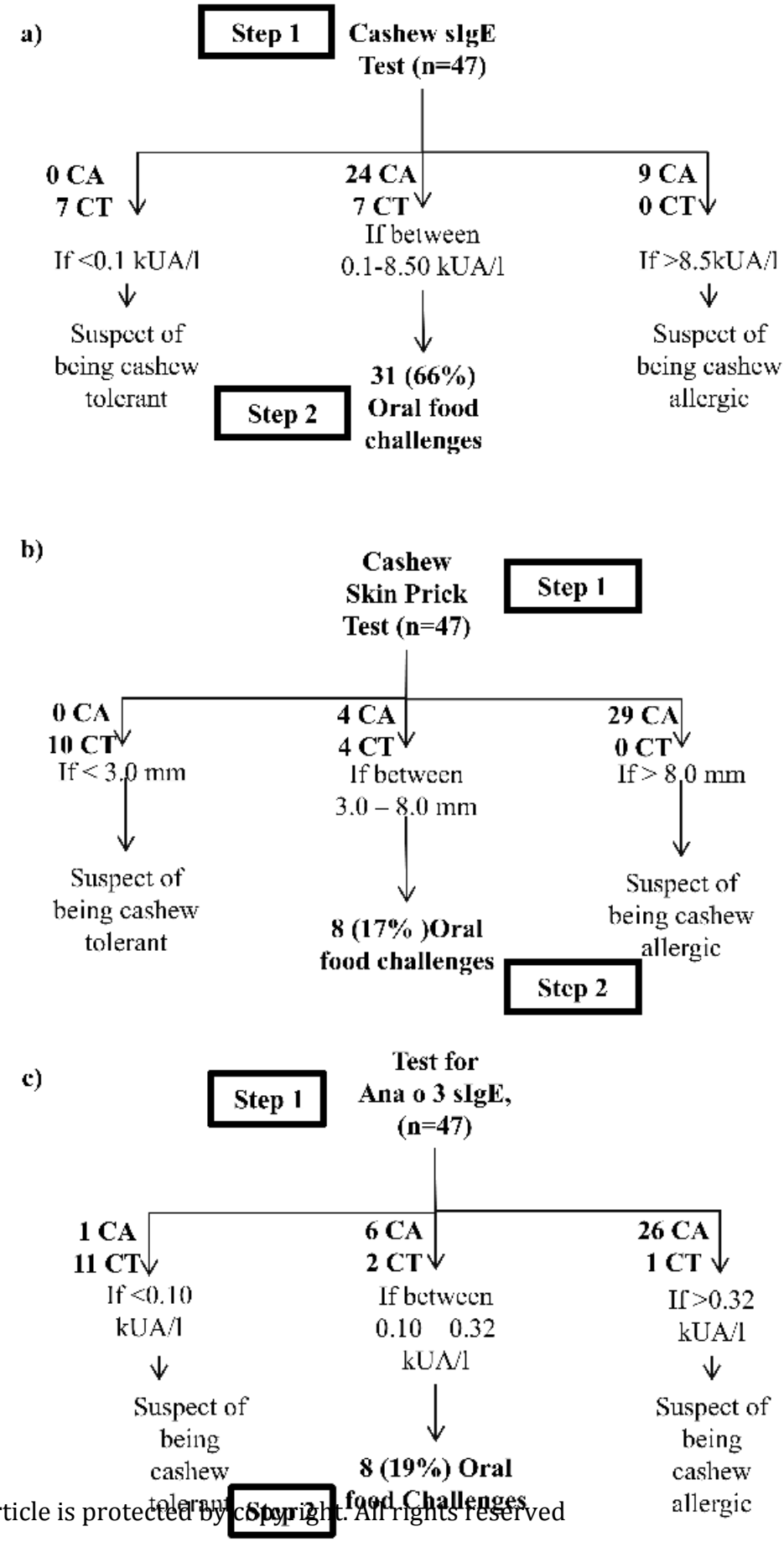

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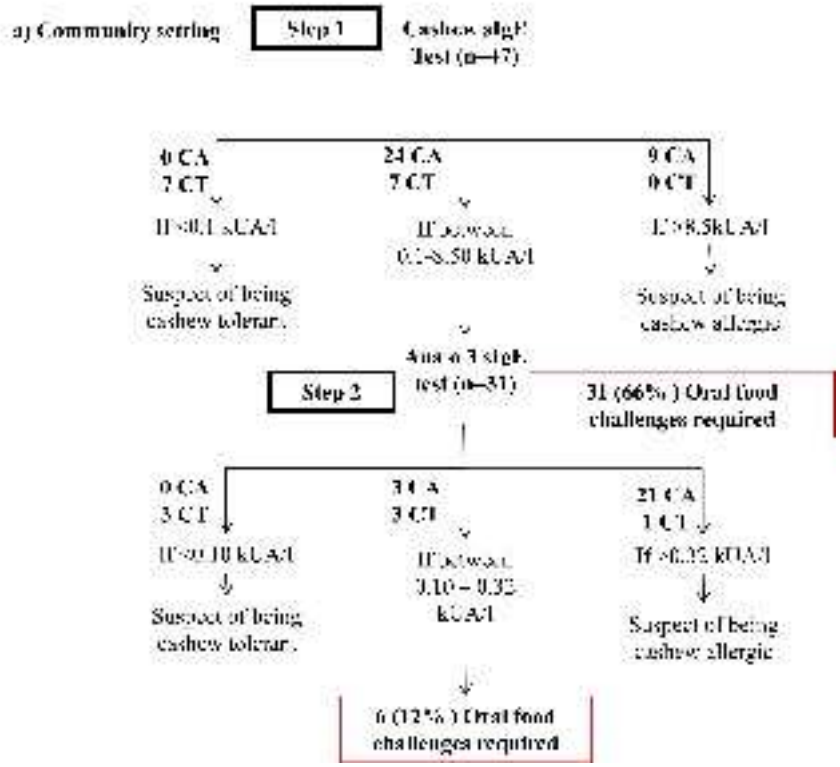
Figure 4a-b. Comparison of clinical scenarios for diagnosing cashew allergy



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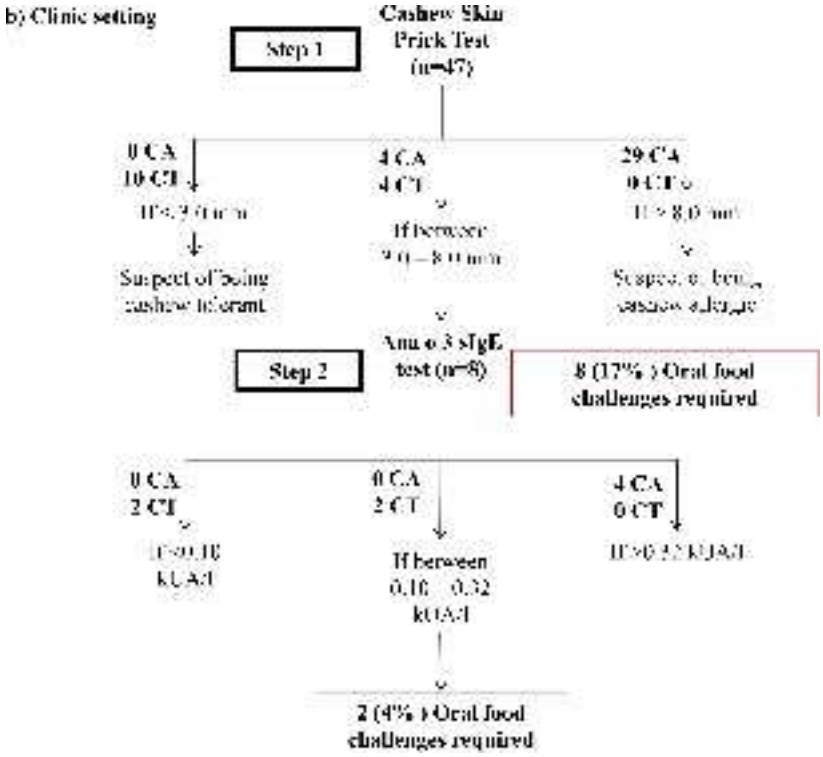

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