

IgE Cross-Reactivity Measurement of Cashew Nut, Hazelnut and Peanuts using a Novel Immulite Inhibition Method

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Received date: September 19, 2019; Accepted Date: October 17, 2019; Published Date: October 24, 2019

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Abstract

Background: Tree nut allergic individuals are often sensitised towards multiple nuts and seeds. The underlying cause behind a multi-sensitisation for cashew nut, hazelnut, peanut and birch pollen is not always clear. We investigated whether IgE cross-reactivity between cashew nut-, hazelnut- and peanut proteins exists in children that are multi-allergic to these foods using a novel IMMULITE[®]-based inhibition methodology, and investigated which allergens might be responsible. In addition, we explored if an allergy to birch pollen might play a role in this co-sensitisation for cashew nut, hazelnut and peanut.

Methods: Serum of five children with a confirmed cashew nut allergy and suffering from allergic symptoms after eating peanut and hazelnut were subjected to inhibition immunoassays using the IMMULITE[®] 2000 Xpi. Serum specific IgE to seed storage allergens and pathogenesis related protein 10 (PR10) allergens were determined and used for molecular multicomponent allergen correlation analyses with observed clinical symptoms and obtained inhibition data.

Results: IgE cross-reactivity was observed in all patients. Hazelnut extract was a strong inhibitor of cashew nut sIgE (46.8%) while cashew nut extract was less able to inhibit hazelnut extract (22.8%). Peanut extract showed the least inhibition potency. Moreover, there are strong indications that a birch pollen sensitisation to Bet v 1 might play a role in the observed symptoms provoked upon ingestion of cashew nut and hazelnut.

Conclusion: By applying an adjusted working protocol, the IMMULITE[®] technology can be used to perform inhibition assays to determine the risk of sIgE cross-reactivity between very different food components.

Keywords: Cashew nut; IgE cross-reactivity; Allergy diagnostics; IMMULITE[®] technology; Hazelnut; Peanut

Abbreviations AP: Alkaline Phosphatase; CAP: ImmunoCAP IgE measurements; DBPCFC: Double-Blind Placebo-Controlled Food Challenge; ELISA: Enzyme-Linked Immunosorbent Assay; HEP: Histamine Equivalent Prick Index Area; IDEAL: Improvement of Diagnostic mEthods for ALlergy assessment; IgE: Immunoglobulin E antibody; IMM: IMMULITE[®] IgE measurements; ISAC: ImmunoCAP ISAC IgE measurements; kU/L: Kilo Units per Liter; N: Native; Neg: Negative; OAS: Oral Allergy Syndrome; PAAMOST: Precise Automated Area Measurement of Skin Test; PR10: Pathogenesis Related protein 10; R: Recombinant; sIgE: Specific IgE; SPT: Skin Prick Test; 2S: 2S Albumin; 7S: 7S Vicilin; 11S: 11S Globulin; w/v: Weight per volume

Introduction

Among food allergies, an allergy to tree nuts is relatively common affecting 0.05-7.3% of the population and its prevalence seems to be increasing, especially in children [1-3]. The majority of severe food allergy reactions as anaphylaxis are related to tree nut ingestions [4] and tree nut allergic individuals are often sensitised to multiple nuts and seeds [5]. Indeed, in the multi-centre Improvement of Diagnostic mEthods for ALlergy assessment (IDEAL) study of Valk et al. [6], co-sensitisation towards peanut and hazelnut was observed in more than 60% of Dutch cashew nut allergic (multi-sensitised) children of which 13% indicated to suffer from clinical symptoms upon ingestion of all three seeds/nuts (multi-allergic). Although cross-sensitisation seems less likely due to low level of botanical relations [7], homology between certain proteins like 2S albumins might be possible, and consequently may result in cross-reactive clinical symptoms. Cashew nut allergies cause predominantly severe reactions at very small exposure levels [6].

However, all except one child suffered from oral allergy syndrome (OAS)-related symptoms next to gastrointestinal complaints upon cashew nut ingestion and are IgE-sensitised to birch pollen.

Reported co-allergy and IgE cross-reactivity between major and minor allergens in hazelnut, peanut and birch pollen has been reviewed extensively [3,8-10]. However, an underlying cause that explains a multi-sensitisation to cashew nut, hazelnut, peanut and birch pollen has not been studied in detail. Thus, our aim in this study was to investigate whether IgE cross-reactivity between cashew nut, hazelnut and peanut proteins exists in children that are multi-allergic to these foods using a novel IMMULITE[®]-based inhibition methodology, and which allergens might be responsible for the observed IgE-cross-reactivity. In addition, we explored if an allergy to birch pollen might play a role in this co-sensitisation for cashew nut, hazelnut and peanut.

Material and Methods

Study design and subjects

Case histories including clinical symptoms after eating hazelnut and peanut were collected from the registered electronic patient files and questionnaires in the IDEAL-study, as well as the result of the double-blind placebo-controlled food challenge (DBPCFC) with cashew nut, Skin Prick Test (SPT) and IgE data specific for whole cashew nut (F220), hazelnut (f17), and peanut (f13) [6].

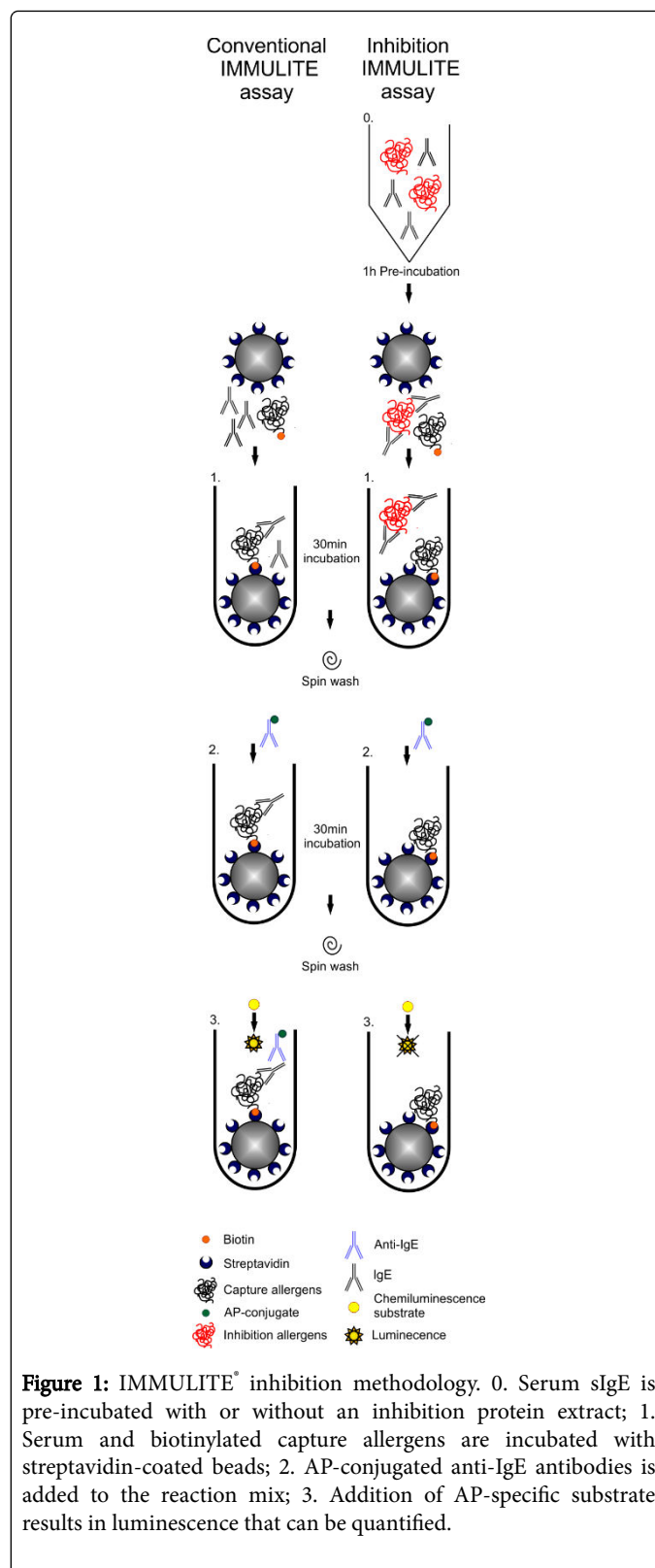
SPT measurements

SPTs against whole nut extracts were performed with cashew nut, hazelnut and peanut, a positive control (histamine 10 mg/ml; ALK-Abello, Nieuwegein, the Netherlands) in duplicate and PBS as a negative control. The Histamine Equivalent Prick (HEP)-index area was measured as described previously [11].

Protein extracts for SPTs were obtained from unsalted roasted cashew nut, and unsalted fresh hazelnut and peanuts (not roasted). Seeds were mechanically homogenized using a mortar and pestle, defatted by ether extraction and air-dried. A 10% (w/v) extract in PBS was centrifuged for 10 min at 2000 g, and the supernatant was passed through a 0.22- μ m filter. All extracts were stored in appropriate aliquots at -20°C until use in skin test. Before the skin tests the extracts were defrosted and mixed [12].

sIgE inhibition study

For the IgE-based inhibition tests with cashew nut, hazelnut and birch pollen, we developed a methodology for sIgE-inhibition testing on the fully automated IMMULITE[®] 200 XPI (see visual overview in Figure 1. This method is purely experimental without extensive validation and not performed before. For standard routine sIgE quantification, IMMULITE[®] makes use of an enzyme-enhanced chemiluminescent enzyme immunoassay. In short, a streptavidin-coated bead, biotinylated liquid allergen and a patient serum sample were mixed and incubated for 30 min. After a spin wash, an alkaline phosphatase-conjugated monoclonal antibody specific for human IgE (AP-IgE) is added and incubated for 30 minutes. After another spin-wash, presence of the AP-conjugate was measured by adding an AP-specific chemiluminescent substrate (phosphate ester of adamantyl dioxetane) which is converted to light. The intensity of the light produced is proportional to the amount of IgE present in the adjutor.



Allergens for the inhibition steps were prepared from a stock solution of nut/seed extract (5 mg/mL) that was provided by Siemens Healthcare diagnostics (Los Angeles, United States). For the whole food inhibition experiments, a 2% dilution in PBS (100 μ g/mL) of the

allergen stock of choice was used (cashew nut (f202), hazelnut (f17), peanut (f13)) while for the Bet v 1-specific inhibitions a concentration of 1.6 mg/mL (purified as described in [13]) in PBS was used. The nut/seed extracts were produced according to the same procedure as the extracts used in the normal IMMULITE® Xpi sIgE tests. Inhibition experiments were performed by pre-incubating sera with inhibitory allergen preparations mixed 1:1 for 1 hour at room temperature before proceeding with the normal IMMULITE® Xpi sIgE testing. Pre-incubations with PBS served as negative controls. The percentage of inhibition was calculated using the following formula:

$$\%inhibition = (\text{serum pre-incubated with PBS} - \text{serum pre-incubated with inhibitor}) / (\text{serum pre-incubated with PBS}) \times 100\%$$

Allergen sIgE measurements

Serum samples were analysed for sIgE antibodies against cashew nut specific allergens (Ana o 1,2,3) using the Siemens IMMULITE 2000 Xpi Immunoassay system (Siemens AG; Munich, Germany) [14]. Additional sIgE antibodies specific for nCor a 9 and rCor a 14 were determined using the ImmunoCAP 250 systems. Other sIgE measurements for hazelnut (rCor a 1), Birchpollen (rBet v 1), and

peanut (rAra h 1, rAra h 2, rAra h 3 and rAra h 8) were measured using the ImmunoCAP ISAC kit (Thermo Fisher Scientific; Waltham, MA, USA). An assay for Cor a 11 was not commercially available. Antibody levels above 0.35 kU/L as obtained by IMMULITE and ImmunoCAP 250 were considered positive.

Results

Clinical history

Of the 179 children included in the IDEAL study [6], 5 children with a confirmed DBPCFC-test against cashew nut plus a positive history of allergic symptoms after hazelnut and peanut ingestion were selected for this small follow-up study to investigate possible IgE cross-reactivity activity between cashew nut, hazelnut and peanut allergens. In addition to a clinically relevant food allergy, all children suffered from a birch pollen-related inhalation allergy. Baseline characteristics including SPT, whole food/pollen-sIgE and case history for cashew nut, hazelnut, peanut and birch pollen in the 5 selected patients from the IDEAL study can be found in Table 1.

Patient	Cashew nut			Hazelnut			Peanut			Birch pollen		
	Symptoms	SPT (HEP)	sIgE (kU/L)	Symptoms	SPT (HEP)	sIgE (kU/L)	Symptoms	SPT (HEP)	sIgE (kU/L)	Symptoms	SPT (HEP)	sIgE (kU/L)
1110015	Oral allergy, upper airway symptoms, nausea/stomach ache and vomiting	2,1	38,6	Oral allergy, redness in the mouth	4,4	26,7	Nausea/stomach ache/vomiting	5,9	2,8	Seasonal rhinitis, runny nose, sneezing, stuffy nose, asthma	1,3	88,9
1110063	Oral allergy, nausea/stomach ache and vomiting	2,4	2,6	Oral allergy, itch and irritation mouth/throat/lips	2,1	45,5	Redness and coughing	4,6	46,3	Asthma	0,9	>100
2220011	Oral allergy, nausea/stomach ache and vomiting	4,8	1,1	Nausea/stomach ache/vomiting	2,1	45,5	Nausea/stomach ache/vomiting	3,7	5,6	Seasonal rhinitis, runny nose, sneezing, stuffy nose	1	>100
2220029	Nausea/stomach ache and vomiting	3,3	9,3	Worsening of eczema	Neg	7,2	Upper airway symptoms	4,7	1,9	Seasonal rhinitis, runny nose, sneezing, stuffy nose	1,4	38,6
3330002	Oral allergy	2,9	3,5	Oral allergy, itch and irritation mouth/throat/lips	0,6	Neg	Upper airway symptoms	6,1	2,4	Seasonal rhinitis, stuffy nose	1,1	20,7

Table 1: Baseline characteristics including SPT, sIgE and Case history for cashew nut, hazelnut, peanut and birch pollen in the 5 selected patients.

Inhibition assays

To characterise possible cross-reactive allergens in the cashew nut allergic children, each serum sample was exposed to 6 inhibition tests using biotinylated cashew nut, hazelnut and peanut extract as detection allergen and non-biotinylated extracts as inhibitors. First, the inhibition of IgE that would be captured by cashew nut was investigated. As expected, inhibition of cashew nut-sIgE with cashew nut protein extract (= positive control) reached 90-99% (Figure 2). Hazelnut on the other hand, was able to inhibit cashew nut-sIgE detection in 4 of the 5 patients with a mean inhibition rate of 46.7%. Lowest mean inhibition of cashew nut sIgE was seen for peanut extract (2.6%).

Next, we attempted to inhibit hazelnut-sIgE binding. Cashew nut protein extract was able to inhibit hazelnut-sIgE detection in 4 of the 5 patients with a mean of 24.2% while peanut was able to inhibit hazelnut-sIgE only in patient #1110015 and #3330002 (mean inhibition rate 5.0%). The positive control extract (hazelnut) was again able to inhibit up to 99% of the hazelnut-sIgE.

Peanut-sIgE was inhibited more efficiently by hazelnut than with a cashew nut extract, especially in patient #1110063. These results indicate that IgE cross-reactivity between cashew nut and hazelnut clearly exists, but the role of peanut seems to be negligible.

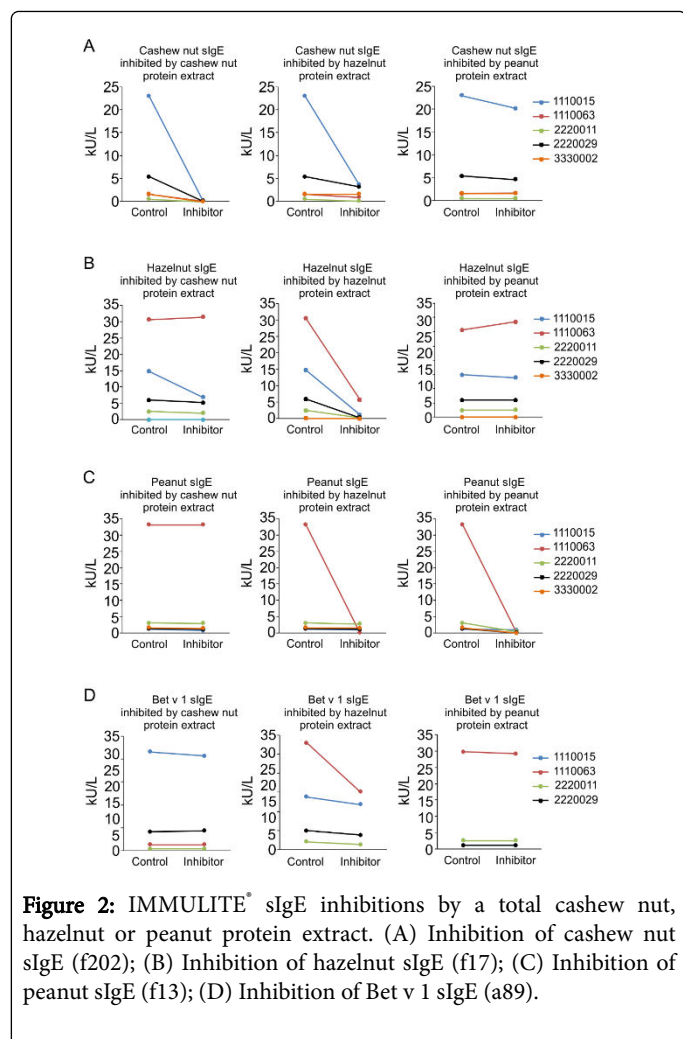


Figure 2: IMMULITE® sIgE inhibitions by a total cashew nut, hazelnut or peanut protein extract. (A) Inhibition of cashew nut sIgE (f202); (B) Inhibition of hazelnut sIgE (f17); (C) Inhibition of peanut sIgE (f13); (D) Inhibition of Bet v 1 sIgE (a89).

#1110015 and #2220029. Allergen cross-reactivity between nuts might be predominantly based on storage proteins [15]. In order to determine for each patient whether the albumin (2S) or globulin type (7S/11S) seed storage allergens might be involved in the observed whole food-sIgE inhibition activity, allergen-sIgE antibodies levels for cashew nut (Ana o 1, Ana o 2 and Ana o 3), hazelnut (Cor a 9 and Cor a 14) and peanut (Ara h 1, Ara h 2 and Ara h 3) were evaluated (Table 2). As all children suffered from a birch pollen inhalation allergy, also sIgE levels against the major birch pollen allergen Bet v 1 and their equivalents in hazelnut (Cor a 1) and peanut (Ara h 8) were measured.

The relatively strong cashew nut/hazelnut inhibition observed in patient #1110015 and #2220029 appears to be primarily caused by cross-reactivity between globulin allergens Ana o 2 and Cor a 9 rather than between 2S albumin allergens. Even though a mean inhibition rate of 12.8% was observed of cashew nut-sIgE by peanut extract, a peanut-related globulin sensitisation seems not to play a role in these two patients, as Ara h 1 and Ara h 3 sIgE were both negative. Possibly, a cross-reactivity between the albumin allergens Ana o 3/Ara h 2/Cor a 14 may explain the observed peanut inhibition activity.

Patient #1110063 hardly showed inhibition of cashew nut-sIgE with hazelnut and no inhibition of hazelnut-sIgE with cashew nut protein extract, even though the serum contains sIgE antibodies against the 2S and 11S storage protein allergens. On the other hand, peanut-sIgE in this serum was strongly inhibited by hazelnut protein extract. Also, this serum shows high sIgE levels for the Bet v 1-like allergens Cor a 1 and Ara h 8. This suggests that PR10-related hazelnut/peanut cross-reactivity might be a possible cause for the observed inhibition (although maybe not clinically relevant as no OAS is observed upon peanut ingestion).

The absence of cashew nut-sIgE inhibition by hazelnut or peanut was also observed for patient #3330002, indicating that cross-reactivity between the 2S albumins Ara h 2 and Ana o 3 is unlikely. Also for this patient, PR10-related hazelnut/peanut cross-reactivity might possibly explain the observed inhibition of hazelnut-sIgE by cashew nut (41.2%) and peanut (31.4%) extract.

Allergen-sIgE diagnosis

Hazelnut protein showed to be a strong inhibitor of IgE that also specifically binds to cashew nut protein, especially for patients

Patient	Cashew nut sIgE			Hazelnut sIgE			Peanut sIgE			Birch pollen sIgE	
	nAna o 1	nAna o 2	nAna o 3	rCor a 1	nCor a 9	rCor a 14	rAra h 1	rAra h 2	rAra h 3	rAra h 8	rBet v 1
	(7S)	(11S)	(2S)	(PR10)	(11S)	(2S)	(7S)	(2S)	(11S)	(PR10)	(PR10)
1110015	2.4	34	10.5	16.8	43.7	0.8	Neg	0.9	Neg	7.8	63.2
1110063	0.7	2.4	3.9	77.4	0.9	0.4	8.5	6.1	Neg	15.6	119.2
2220011	Neg	0,9	0.9	8	0.5	13	Neg	5.7	Neg	15.6	61.3
2220029	1.9	10.9	16	6.8	2.1	Neg	Neg	3	Neg	2.47	15.8
3330002	1.3	5	9.2	4.2	Neg	Neg	Neg	3.9	Neg	0.8	14.9
Analysed by	IMM	IMM	IMM	ISAC	CAP	CAP	ISAC	ISAC	ISAC	ISAC	ISAC

Table 2: sIgE (kU/L) levels of cashew nut, hazelnut, peanut and PR10 birch pollen allergens in the five selected sera, measured by ImmunoCAP (CAP), ImmunoCAP ISAC (ISAC) or IMMULITE® (IMM) methodology.

Although the positive 2S albumin sensitisation to cashew nut (Ana o 3), hazelnut (Cor a 14) and peanut (Ara h 2) does not indicate possible cross-reactivity, no hazelnut nor cashew nut-sIgE inhibition with peanut extract was observed for patient #2220011. This suggests that co-recognition of homologous allergens in cashew nut and hazelnut by peanut 2S albumin-sIgE is unlikely. The observed cashew nut/hazelnut inhibition in this patient (72.2% for cashew nut-sIgE and 16.7% for hazelnut-sIgE) could also be explained by 11S globulin-type of allergens.

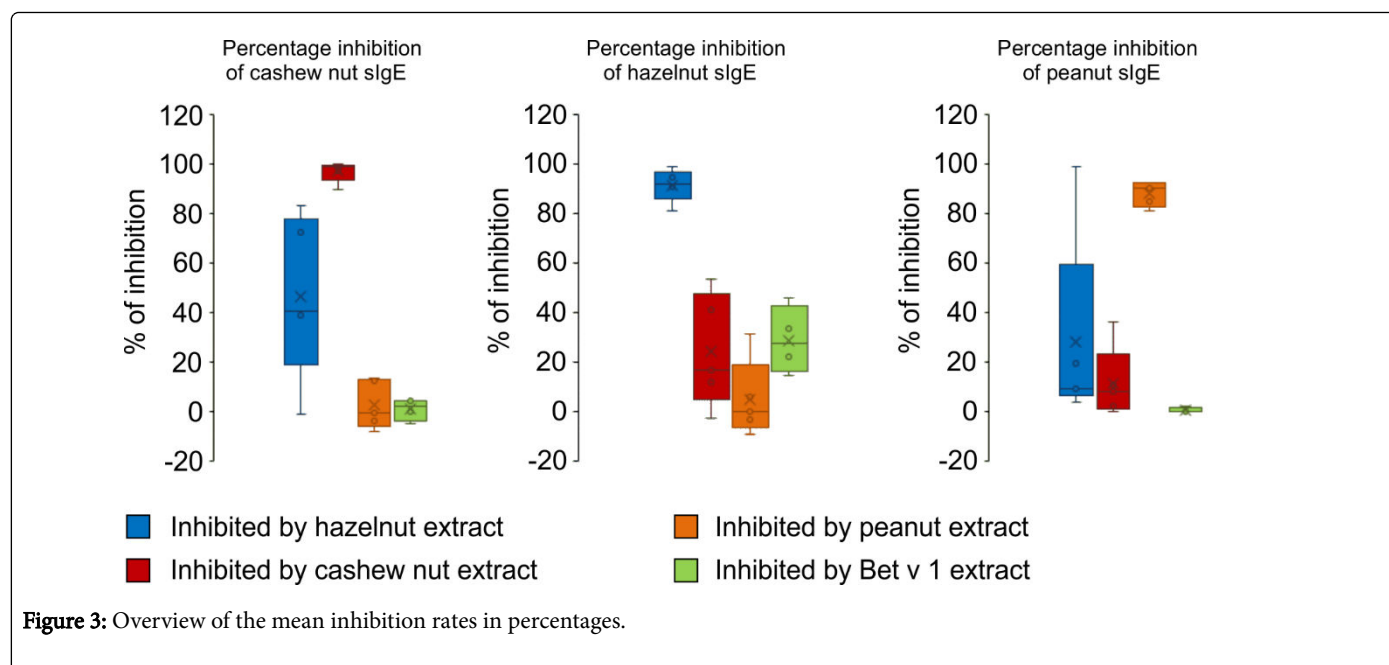
Overall, the observed allergen component analysis cannot fully explain all cashew nut/hazelnut/peanut sIgE-cross reactivity patterns in the individual patients' sera, suggesting the involvement of additional allergens in the inhibition reactions.

Bet v 1-specific IMMULITE® inhibitions

It was noticed that most patients, except #2220029, displayed mild oral allergy syndrome (OAS) symptoms after consumption of cashew

nut and hazelnut, next to the more severe gastrointestinal complaints. As all children are birch pollen-sensitised we speculated that the observed clinical symptoms as well as the measured IMMULITE® sIgE-inhibitions in some patients might be explained by a secondary (cross-reactive) reaction on Bet v 1-homologues in cashew nut, hazelnut and peanut. Therefore, an inhibition assay with nBet v 1 protein was performed on 4 of the 5 patients (for 3330002 not enough serum was left), as visualized in Figure 2.

Hazelnut-sIgE detection was inhibited in all patients with an average of 28.9% while cashew nut-sIgE was only reduced 4.17% in 2 of the 4 patients (#1110015 and #2220011). nBet v 1 hardly captured any peanut-sIgE, except in patient #1110063 (2.0%), which might be consistent with the lack of OAS symptoms in these patients upon peanut consumption. The Bet v 1 inhibition controls in each patient reached over 99% (data not shown). A summary of the mean inhibition rates in percentages are presented in Figure 3.



Discussion

IgE-cross-reactivity generally only occurs between proteins belonging to the same allergen family, mostly because of structural and sequential similarity [16,17]. In the studied population, only in patients #1110015 and #2220029, strong sIgE cross-reactivity was observed between hazelnut and cashew nut protein extracts, presumably caused by a specific 11S globulin sensitisation. IgE cross-reactivity between the globulin proteins Ana o 2 and Cor a 9 has been previously reported by Wallowitz et al. [18]. Also, *in vitro* cross-reactivity of cashew nut, hazelnut and peanut extract with the walnut 11S globulin Jug r 4 has been observed [19].

For patient #2220011, specific cashew nut/hazelnut globulin or albumin cross-reactivity could not be distinguished. For a cashew nut and hazelnut allergy, sensitisation towards the 2S albumins, Ana o 3 and Cor a 14, respectively, is considered a prediction marker for clinical allergy [14,20,21]. However, cross-reactivity between these albumins sharing only 43% amino acid identity is considered rare [16], although this requires further verification.

Peanut displayed the lowest inhibition potency in this study. Only one patient (#1110063) was positive for Ara h 1-sIgE while none of the patients studied were sensitised for the 11S-type globulins, although this could have been biased by the low sensitivity of the diagnostics method used (ISAC). A predominant 2S albumin sensitisation to peanut was detected, as well as a strong sensitisation to the birch pollen allergen Bet v 1 and its homolog Ara h 8. As none of the patients indicated OAS symptoms upon peanut ingestion, the Ara h 8 sensitisation in these patients seems to be clinically irrelevant, as also evident from the absence of a Bet v 1/peanut inhibition activity in 4 of the 5 patients. Unfortunately, a Bet v 1-inhibition test could not be performed for patient #3330002 due to serum limitations, while in this patient peanut extract was a particular strong inhibitor of hazelnut-sIgE.

A 2S albumin sensitisation for peanut is commonly associated with severe systemic reactions [22], while from the clinical history only mild upper airway symptoms are described for 3 of the 5 patients. In general, cross-reactivity between 2S albumins seems to be uncommon

due to their high amino acid sequence variability [16,23] and IgE-cross reactivity of peanut specific albumins occurs primarily between its isotypes rather than with tree nut 2S albumins [22,24]. For instance, peanut did not display cross-reactivity with the 2S albumin Jug r 1 from walnut [25] nor with 2S albumins from Brazil nut [26], which could explain the low peanut inhibition activity for these patients.

On the other hand, peanut-sIgE was inhibited on average 12.3 and 34.3% when pre-incubated with cashew nut or hazelnut extract, respectively. This contrasts a study of Leon et al. [27], in which no inhibition of peanut-sIgE by cashew nut was observed, although cross-reactive allergen reactivity existed between hazelnut and peanut. Why peanut-sIgE can be captured by hazelnut and cashew nut while peanut extract displays only weak inhibition potency cannot be explained from the allergen multicomponent analysis performed. Possibly, differences in the extract's relative allergen concentrations and/or measurement methods may have interfered in the observed varying degrees of inhibitory potency.

Hazelnut and cashew nut extracts were able to inhibit the detection of Bet v 1-sIgE in some of the patients (#1110015 and #2220011), suggesting that the OAS-related symptoms upon ingestion of hazelnut and cashew nut in these children could very well be caused by Bet v 1-related homologs in both tree nut extracts. A birch pollen/hazelnut cross-sensitisation is well-known as reviewed by Costa et al. [28] and Flinterman et al. [29], however evidence for a clinically relevant Bet v 1-related cross-reactivity with cashew nut is still lacking. Putative IgE-binding homologs of Bet v 1 (PR10) have been identified in cashew nut by our group (unpublished results) but, whether these allergens have cross-reactive potency manifesting in clinical reactions needs further investigation.

The symptoms upon cashew nut or hazelnut ingestion could also be caused by a non-PR10 related allergen sensitisation. Allergic reactions towards profilin or nsLTP proteins can also result in OAS symptoms [30,31]. However, as none of the patients showed an nsLTP or profilin sensitisation on the ISAC (results not shown), these allergens are most likely not involved in the clinical reactions of our 5 patients.

In this study, we have successfully demonstrated that the IMMULITE[®] technique can be used to perform IgE-inhibition assays, as previously also shown for the ImmunoCAP technique [32]. The specificity of the inhibition data measured using this method was demonstrated by the strong inhibition obtained by the positive controls. The advantage of this technique over the ImmunoCAP inhibition technique [33] or the commonly applied immunoblot or enzyme-linked immunosorbent assay (ELISA) inhibition tests is that inhibition and detection is conducted in liquid form meaning that the conformational properties of proteins are conserved, increasing physiological relevance. However, using this method, the minimal amount of serum needed per inhibition assay is still substantial (90 µl), meaning that no inhibition concentration curves could be performed because of serum availability limitations. This prevented us to acquire EC50 values (amount of protein extract needed to inhibited 50% of sIgE-binding), implying that the strength of inhibition or cross-reactive potency per protein extract could not be evaluated in this study.

From the inhibition data, we could not conclude which patients are primarily sensitised to cashew nut and secondary to hazelnut or vice versa. As only a small sub-population was tested the patients might be just co-sensitised and have a primary food allergy for cashew nut, hazelnut and birch pollen, and display no secondary food allergy. In

addition, we are not sure if the possible cross-reactivity observed in this study is caused by the major seed storage allergens, or minor allergens not yet identified in cashew nut.

Conclusion

Molecular diagnostic testing by measuring specific sIgE against individual allergen molecules or components using purified or recombinant allergens (CRD) provides detailed information on sensitization patterns to allergologists and enables a more accurate interpretation of allergic symptoms by distinguishing clinically relevant food protein sensitisation from non-relevant sensitisation that does not cause systemic reactions. Moreover, such a CRD analysis can broaden our understanding of which IgE cross-reactivity reactions between foods are to be expected in a patient group, which may guide dietary advice. We have demonstrated that the IMMULITE[®] technique can indeed be applied to evaluate IgE cross-reactivity between protein extracts and between specific allergens. This allowed us to evaluate whether clinical symptoms in children co-sensitised against cashew nut, hazelnut and peanut were possibly allergen type related and resulted from a primary reaction or from cross-reactivity to homologous allergens in each of the tested foods.

Acknowledgement

We kindly acknowledge Cees van Egeraat and Vincent Schaarman (Siemens healthcare Diagnostics Inc.) for their contributions to the IMMULITE[®] inhibition measurements.

Author contributions

SB, NS, HJW and NWDJ have given shape to the study design and interpretation of results. MRB has performed the IMMULITE[®] sIgE measurements as well as the inhibition tests. ImmunoCap and ISAC measurements were performed by MWJS. Patient clinical details were summarized by JPMvdV. RGVW, HFJS, HJW and NWDJ acquired the necessary funding and all authors have contributed to drafting and revising the article and approved the final version for publication.

Research funding

This project was funded by Technology Foundation STW (STW number 11868), the Dutch Food Allergy Foundation, Siemens Healthcare Diagnostics, HAL Allergy, Intersnack the Netherlands B.V., ALK-Abello B.V., and the Netherlands Anaphylaxis Network.

Competing interest

The funding organisations played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; nor in the decision to submit the report for publication.

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