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IgE Cross-Reactivity of Cashew Nut Allergens

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Keywords

Cashew nut · Tree nut allergy · IgE cross-reactivity · Food allergy · Allergenicity · Immunoblotting

Abstract

Background: Allergic sensitisation towards cashew nut often happens without a clear history of eating cashew nut. IgE cross-reactivity between cashew and pistachio nut is well described; however, the ability of cashew nut-specific IgE to cross-react to common tree nut species and other Anacardiaceae, like mango, pink peppercorn, or sumac is largely unknown. ***Objectives:*** Cashew nut allergic individuals may cross-react to foods that are phylogenetically related to cashew. We aimed to determine IgE cross-sensitisation and cross-reactivity profiles in cashew nut-sensitised subjects, towards botanically related proteins of other Anacardiaceae family members and related tree nut species. ***Method:*** Sera from children with a suspected cashew nut allergy (n = 56) were assessed for IgE sensitisation to common tree nuts, mango, pink peppercorn, and sumac using dot blot technique. Allergen cross-reactivity patterns between Anacardiaceae species were subsequently examined by SDS-PAGE and immunoblot inhibition, and IgE-reactive allergens were identified by LC-MS/MS. ***Results:*** From the 56 subjects analysed, 36 were positive on dot blot for cashew nut (63%). Of these, 50% were mono-sensitised to cashew nuts, 19% were co-sensitised to Anacardiaceae species, and 31% were co-sensitised to tree nuts. Subjects co-sensitised to Anacardiaceae species displayed a different allergen recognition pattern than subjects sensitised to common tree nuts. In pink

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S.B.-N. and M.R. contributed equally to this paper. N.W.d.J. and H.J.W. share senior authorship.

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peppercorn, putative albumin- and legumin-type seed storage proteins were found to cross-react with serum of cashew nut-sensitised subjects in vitro. In addition, a putative luminal binding protein was identified, which, among others, may be involved in cross-reactivity between several Anacardiaceae species. ***Conclusions:*** Results demonstrate the in vitro presence of IgE cross-sensitisation in children towards multiple Anacardiaceae species. In this study, putative novel allergens were identified in cashew, pistachio, and pink peppercorn, which may pose factors that underlie the observed cross-sensitivity to these species. The clinical relevance of this widespread cross-sensitisation is unknown.

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Introduction

Cashew nut allergy has been recognised as a severe tree nut allergy amongst (Dutch) children and young adults [1–3], and its prevalence seems to be increasing [4, 5]. Often, young children suffer from a cashew nut allergy without a clear history of cashew nut consumption [3]. This raises the question whether cashew nut allergy manifests from a primary sensitisation or is caused by cross-sensitisation to botanically related or unrelated foods.

Cashew belongs to the family of Anacardiaceae, categorised under the taxonomic class of Magnoliopsida to which most common tree nuts belong, as depicted in Figure 1. Several studies have shown that a tree nut-allergic patient has considerable chance of being sensitised (86%) [6] or allergic to multiple tree nuts (35–37% based on clinical history [7, 8] and 14–47% based on food challenges [6, 9]). The underlying reason is thought to be the major sequential and structural homology between the highly abundant seed storage proteins (glycinins, vicilins, and 2S albumins) and to a lesser extent the defence-related proteins (nsLTP, chitinases, and PR-10 proteins, e.g. Bet v 1 homologues) and pan allergens (profilin and hevein-related proteins) present in tree nuts and other botanically related foods [10, 11].

Cross-sensitisation between cashew and other tree nuts, such as hazelnut and walnut has been reported at IgE level [12–15] as well as at T-cell level [16, 17] where mostly Ana o 1 and Ana o 2 acted as cross-reacting allergens [16]. Amongst Anacardiaceae members, allergic cross-reactivity between pistachio and cashew nut is well recognised [12, 15, 18–21] and avoidance of both nuts is advised in case of a confirmed cashew nut allergy [22]. The strong phylogenetic relationship between cashew and pistachio nut is reflected by the high amino acid similarity and conserved three-dimensional regions between the cashew nut and pistachio seed storage allergens Ana o 1/Pis v 3 (7S vicilin), Ana o 2/Pis v 2 (11S legumin), and Ana o 3/Pis v 1 (2S albumin) with a similarity of 78, 80, and 70%, respectively [15, 20, 23].

Mango, pink peppercorn (often included in peppercorn blends and seasoning mixes), and the Middle Eastern spice sumac are also phylogenetically classified as Anacardiaceae. Recent case reports describing the incidence of cashew nut-allergic patients experiencing anaphylaxis after consumption of pink peppercorn or sumac emphasise the potential risk of cross-reactivity among different members of the Anacardiaceae family [24, 25]. Mango has shown to be an important cross-reacting food for patients suffering from the "celery-mugwort-spice syndrome" and "latex-fruit syndrome" [26], partly caused by the Bet v 1 and 2-like type allergens [27–30]. However, mango-cashew nut cross-sensitisation seems to be of less clinical relevance as only few cases have been reported of mango allergic individuals co-sensitised to pistachio [31] or cashew apple fruit [32]. Although such findings suggest the presence of potentially cross-sensitising and cross-reacting proteins between different members of the Anacardiaceae, no (cross-reactive) allergenic proteins for pink peppercorn, mango, or sumac have been identified as yet. Moreover, widespread cross-sensitisation in patients to these related allergens without prior consumption, makes identification of the primary sensitising agent extremely difficult.

Therefore, the aim of the present study was (1) to visualise co-sensitisation patterns (i.e., presence of specific IgE antibodies (sIgE) towards mango, pink peppercorn, sumac, and related tree nuts) in serum of children suspected of a cashew nut allergy, and (2) to examine the allergenic cross-reactivity of cashew nut proteins present in pistachio, mango, and pink peppercorn by means of immunoblot inhibition assays in order to study the associated IgE binding affinity of cashew nut allergens towards multiple anacardiaceous species.

Materials and Methods

Materials and Reagents

Patient Serum

In total, 176 patients with a suspected cashew nut allergy (sensitised in combination with either a positive history or never eaten before) participated in the multi-centre prospective study "Improvement of Diagnostic mEthods for ALlergy assessment" with cashew allergy in children as a showcase (IDEAL study) with trial

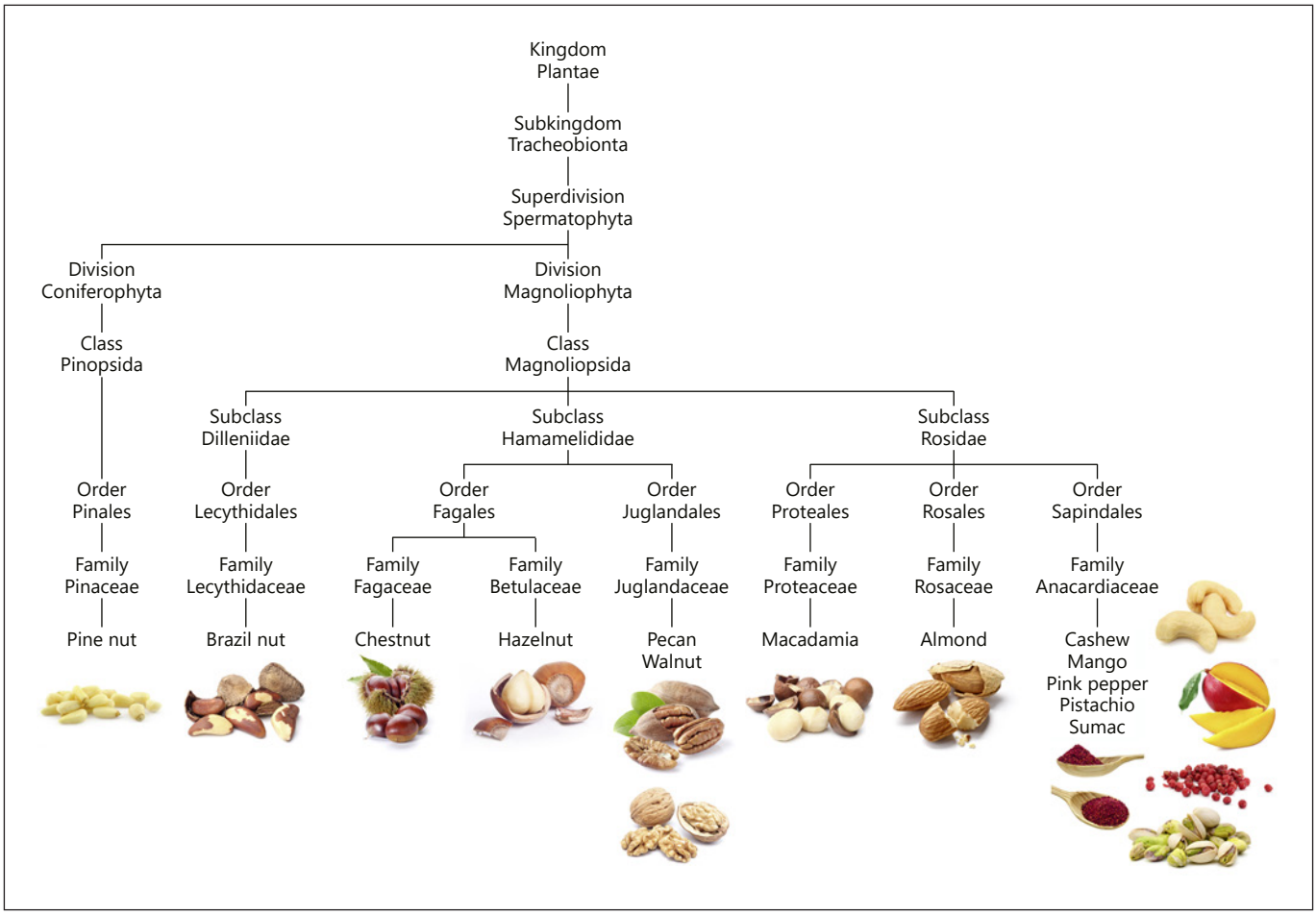


Fig. 1. Phylogenetic classification of Anacardiaceae family members in accordance to other tree nuts: almond (*Prunus dulcis*), Brazil nut (*Bertholletia excelsa*), cashew (*Anacardium accidentale*), chestnut (genus *Castanea*), hazelnut (genus *Corylus*), macademia (genus *Macademia*), mango (genus *Magnifera*), pecan (*Carya illinoinensis*), pine nut (*Pinus koraiensis*), pink peppercorn (*Schinus terebinthifolia/molle*), pistachio (*Pistacia vera*), sumac (genus *Rhus*), and walnut (*Juglans regia*). Taxonomic data were obtained from plants.usda.gov. Photos are from Shutterstock.

number NTR3572. A subset of 56 sera from children (between 2 and 17 years old) included in the study at Erasmus MC Rotterdam, with sufficient serum for further research analysis, were selected for additional investigations. Patient medical profiles, including results from Siemens IMMULITE 2000 XPi Immunoassay serum IgE measurements, skin prick tests (SPTs), and cashew nut-focused double-blind placebo-controlled food challenges (DBPCFCs) were gathered from the existing published IDEAL database [3].

Nuts, Consumables, and Reagents

For this study, members of the Anacardiaceae family (cashew nut, pistachio, mango, pink peppercorn, and sumac) and nuts from other families (pine nut, Brazil nut, chestnut, hazelnut, pecan nut, walnut, macadamia, and almond) were investigated (Fig. 1). All nuts, except pine nuts and macadamia nuts, were purchased raw in shell, to avoid allergen cross-contamination that might otherwise occur during the retail phase. Raw pine nuts (Take One, Rotterdam, the Netherlands) and dry roasted macadamia nuts (Horizon Natuurvoeding BV, IJsselstein, the Netherlands) were purchased as peeled nuts. Cashew nut, pistachio, and walnut as well as ground sumac (Nergiz Grossmarkt GmbH, Gronau, Germany) were kindly provided by Intersnack BV (Doetinchem, The Netherlands). Pink peppercorn kernels were from Fuchs Gewürze GmbH (Dissen, Germany). Mango fruit and all other nuts were purchased at the local supermarket. Consumables, chemicals, and reagents, except where stated otherwise, were obtained from Sigma Aldrich (St. Louis, USA).

Dot Blot Immunoassays

Total Protein Extraction

Depending on the size and availability of shelled nuts, 3–30 nuts were cut in small pieces using a single-use cutting board and knife and mixed to obtain a representative sample batch for each type of nut. In case of mango, the peel and flesh of the fruit were cut into little pieces followed by immediate acetone extraction (1: 2.5 w/v) at 4 °C for 2 h while stirring in order to deplete excess

Table 1. Post hoc analysis (i.e., analysis criteria that were not specified before seeing the data) used to classify patient sera into sensitization groups I–IV according to dot blot spot intensity results (Fig. 3)

Group	Particulars
I	Positive for cashew; positive for ≥1 other member within the Anacardiaceae family; positive for ≤1 nut outside the family of Anacardiaceae
II	Positive for cashew, but negative for other members of the Anacardiaceae family, positive for ≤2 nuts outside the family of Anacardiaceae
III	Positive for cashew and ≥1 other member within the Anacardiaceae family; positive for ≥2 nuts outside the family of Anacardiaceae
IV	Negative for cashew

amounts of pectin. After filtration (Whatman 595 1/2, Dassel, Germany), the acetone extraction was repeated, and the mango pieces were subsequently dried overnight and stored at 4 °C. Dried berries were used in its entirety (pink peppercorn) or powdered (sumac).

Of each nut, seed, and fruit sample, two protein extracts were prepared: a denatured extract in urea/phosphate buffer and a non-denatured extract in Tris buffer. The urea/phosphate extracts were prepared by homogenising 0.5 g of sample in 10 mL of buffer (20 mM sodium phosphate pH 7; 1 mM NaCl; 8 M urea) as described by Burks et al. [33] using an Ultra-Turrax (IKA, Staufen, Germany) and incubating o/n at 4 °C under continued stirring. Protein extracts were obtained by centrifugation and stored at 4 °C until further use. The Tris extracts were prepared by homogenising 1 g of sample in 10 mL Tris buffer (20 mM Tris pH 7.6; 150 mM NaCl; 1 mM EDTA) [34] using the same procedure as described for the urea/phosphate buffer. The same extraction procedures were applied for pink peppercorn and sumac, except that 2.5 and 5 g of sample was used per urea/phosphate or Tris buffer, respectively. In case of mango, 5 g of the acetone-extracted peel and flesh was used per extraction buffer.

In between extractions, the Ultra-Turrax dispersing element was disassembled, and parts were incubated for 15 min in 1 M NaOH followed by a rinsing step in distilled water to clean the in- and outside from any residual protein to avoid allergen carry-over between extractions.

Protein Quantification

Protein concentration of each extract was determined by Bradford assay (Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturers' instructions. To ensure equal spotting on dot blot, the concentration of each protein fraction as determined by Bradford was verified by colloidal gold staining of 0.5 µL droplets (500 ng/L) spotted in duplicate on 0.2-µm Protran BA 83 nitrocellulose membranes (Whatman, Dassel, Germany) placed on a polyester backbone (GL Precision, San Jose, CA, USA). Densitometric analyses were performed using a Universal Hood III and Image Lab 4.1 software (both Bio-Rad, Hercules, CA, USA) and concentrations were adjusted when necessary.

Dot Blot Assay

To obtain a representative protein extract, equal amounts of the urea/phosphate fraction and Tris fraction were mixed to a final concentration of 500 ng/µL. Subsequently, 250 ng was spotted in duplicate on a 35 × 6 mm (L × W) square strip of 0.2-µm nitrocellulose membrane placed on a polyester backbone. Each strip was then dried for 1 h at 37 °C and stored at room temperature in the dark for up to 1 week. Per patient, one strip was used to analyse the IgE reactivity to the different nuts, seeds, and mango protein fractions using the dot blot technique as described earlier [35]. A maximum of 10 patients' sera were screened per handling, every time taking along an antibody background control strip incubated with TBS buffer instead of patient serum. Spot intensities after 5 min of staining were analysed using a Universal Hood III and Image Lab 4.1 software. Non-specific antibody staining as measured on the control strips were subtracted from the patient serum strips per spot per screening batch.

$$\text{Spot intensity} = \text{mean}\,(\text{spot1}_{\text{serum}} - \text{spot}_{\text{control}}, \text{spot2}_{\text{serum}} - \text{spot}_{\text{control}})$$

IgE Sensitisation towards Cross-Reactive Carbohydrate Determinants

Cashew total protein extract (Tris:urea/phosphate; 1:1), bromelain from pineapple stem (B5144), and ascorbate oxidase from *Cucurbita* sp. (A0157) were spotted in duplicate and incubated with TBS or serum pool of patient group III (group description is clarified in Table 1) as described above. Serum of patient group I was not evaluated for CCD sensitisation due to limitation in serum quantity.

Western Blot Immunoassays

Patient Selection

Patient groups I and III (Table 1) were chosen for further selective investigations, as these groups showed specifically in vitro co-sensitisation to multiple Anacardiaceae species. As some of the serum samples were low in volume, consequently, only a part of the sera per group could be used for further investigations, and the number of immunoblotting experiments that could be performed was limited even when sera were pooled.

SDS PAGE and Western Blotting

SDS PAGE (denatured and reduced) and Western blotting of cashew nut, pistachio, mango, and pink peppercorn protein fractions were performed as described by Reitsma et al. [36]. Sumac extracts smeared heavily on SDS PAGE (data not shown) and were therefore excluded from further immunoblot experiments. Tris and urea/phosphate extracted protein fractions mixed 1:1 (w/w; 15 µg in total per lane), were separated by SDS-PAGE on NuPage 10% BIS/TRIS gels according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and either stained by Simply Blue safe stain (Thermo Fisher Scientific Inc., Rockford, IL, USA) or transferred to a 0.2-µm nitrocellulose membrane (LKB, Bromma, Sweden).

For Western blotting, membranes were either incubated in sera selected from patient group I (6 out of 7 were used: patient No. 27, 30, 39, 49, 55, and 62; pooled equal in volumes) or sera selected from patient group III (6 out of 11 sera were used: patient No. 5, 15, 53, 54, 58, and 63; pooled equal in volumes). Membranes incubated in TBS buffer without serum were used as an antibody back-

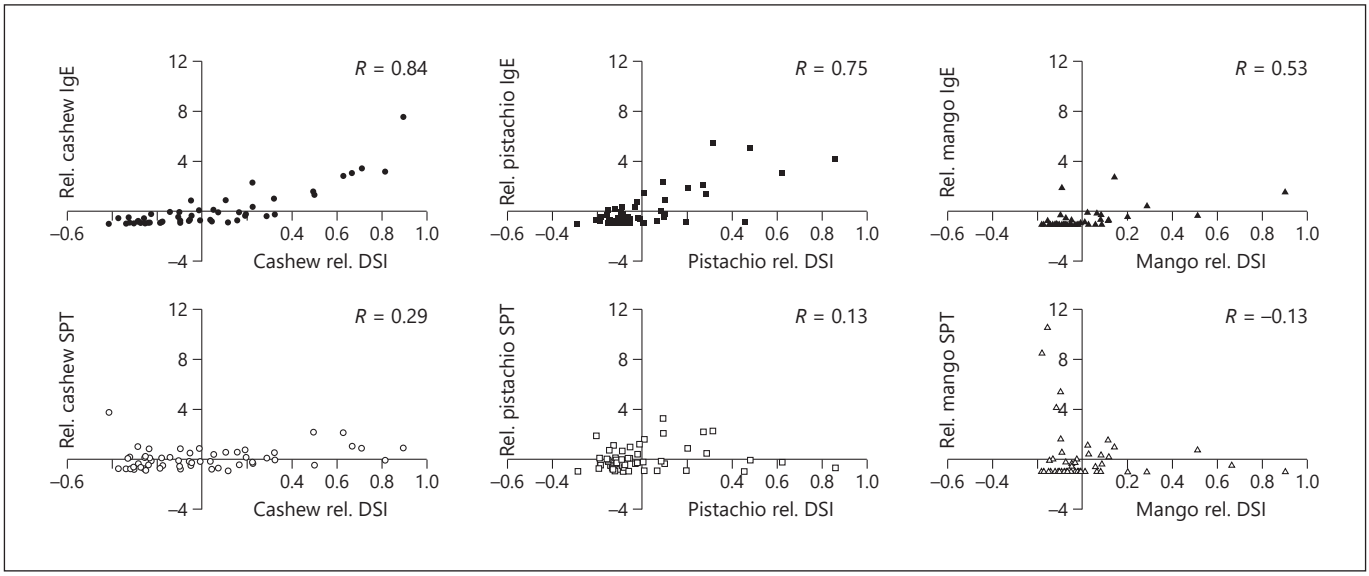


Fig. 2. Correlations between dot blot sIgE measurements and clinical sIgE (IMMULITE) and SPT measurements for cashew, pistachio, and mango in all patients. Clinical sIgE/sSPT data are displayed as relative IgE/SPT on the *y* axis. Relative dot blot sIgE (DSI) is displayed on the *x* axis. The correlation coefficient R, the degree of linear correlation between the two variables X and Y, is indicated for each plot.

ground control. Immunolocalisation of ribulose and luminal binding protein (BiP) was performed as described above, using a rabbit anti-RuBisCo polyclonal antibody (MBS715138; 1:2,000) from *Spinacia oleracea* (MyBioSource Inc., San Diego, CA, USA) and a rabbit anti-luminal BiP (BiP2; AS09 481; 1:2,000) polyclonal antibody from *Arabidopsis thaliana* (Agrisera AB, Vännas, Sweden) according to the manufacturer's instructions. An alkaline phosphatase-conjugated goat anti-rabbit polyclonal secondary antibody (A3687; 1:20,000) and NBT/BCIP staining were used for visualisation.

Western blot inhibition assays were performed as described above, except that the serum pools used were preincubated with 1 mg/ml cashew protein (Tris and urea/phosphate fractions 1:1) for 2.5 h at room temperature prior to incubation with nitrocellulose membrane. Blots were stained for 7 min (Western blots) or 20 min (inhibition blots).

Protein Identification

IgE reactive protein bands as visualised by Western blotting were excised from corresponding Simply Blue safe stained SDS PAGE gels. Protein identification by LC-MS/MS was performed as described by Reitsma et al. [36] with the following minor adjustments: the 5 most intense peaks with charge state 2–4 in the full MS scans were fragmented in a HCD collision cell with a normalised collision energy of 28%. Further, the lower MS2 mass was set to 140 with automatic maximum and a mass resolution of 17,500 (at m/z 200).

LC-MS/MS data acquired by the Q-Exactive were processed using ProteomeDiscoverer software 1.4 (Thermo Scientific). The obtained fragmentation spectra were searched against a protein database using Sequest HT with precursor mass tolerance of 10 ppm and fragment mass tolerance of 20 mDa. The database, downloaded on February 2, 2015, from the NCBI, contained all available protein sequences known for: Anacardiaceae (containing cashew nut family species), *Arachis hypogaea* (peanut), *Bertholletia* (containing Brazil nut species), *Carya illinoinensis* (pecan), *Castanea* (containing chestnut species), *Corylus* (containing hazelnut species), *Corylus avellana* (European hazelnut), *Juglans* (containing walnut species), *Macadamia* (containing macadamia nut species), *Mangifera* (containing mango species), *Pinaceae pinus* (pine nut), *Prunus dulcis* (almond), and the order of Sapindales.

Raw LC-MS/MS processing data were pre-screened, removing unlikely protein matches such as human keratin, peptides showing a poor peak pattern, as well as intense protein bands retrieving low numbers of matched peptides. Final results are presented in Table 3. As only the 5 most intense mass peaks were used for LC-MS/MS analysis, we prioritised high abundant proteins over lower abundant proteins of comparable size present in the excised bands.

Statistics

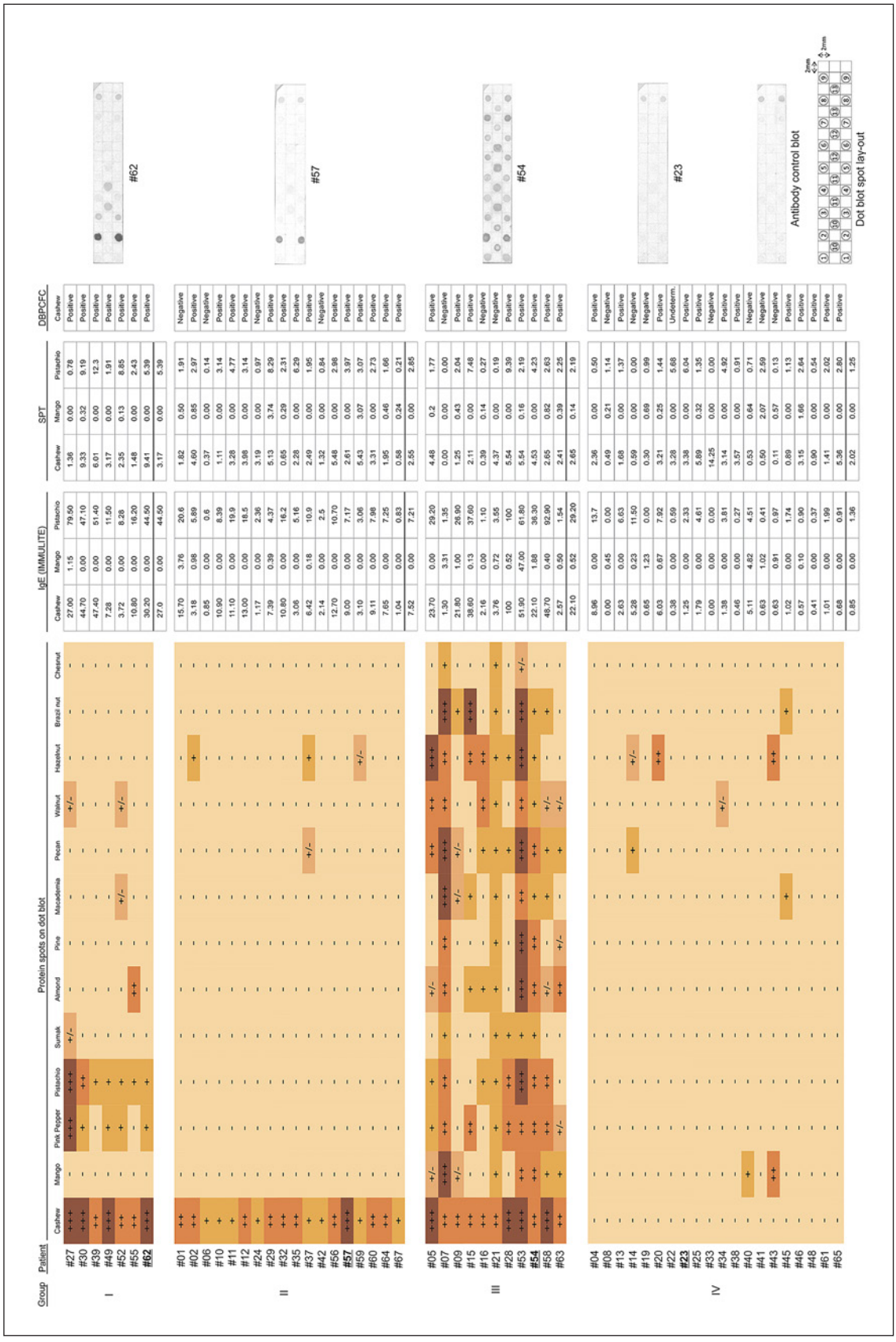
Correlation coefficients (R) between dot blot sIgE, IMMULITE sIgE, and SPT results were calculated by Excel using the Pearson correlation formula:

$$\rho_{x,y} = \mathrm{Cov}\,(X,Y)/(\sigma_x \bullet \sigma_y)$$

The standard variation of medians (σ) was calculated by multiplying the median absolute deviation (MAD) by the normal median distribution factor 1.483 in Excel using the formula:

$$\sigma = 1.483\ \mathrm{MAD}$$

Significance between group medians was evaluated by the Kruskal-Wallis one-way analysis of variance test using Genstat 18th edition. Groups with a χ^2 probability (*p* value) below 0.05 were considered to not have equal medians.



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Results

IgE Sensitisation Profiles of Patient Sera

Fifty-six children with a suspected cashew nut allergy (e.g., sensitised to cashew either in IgE and/or SPT [3], who have participated in the IDEAL study, were without pre-knowledge of DBPCFC outcome assessed for IgE sensitisation to other members of the Anacardiaceae family (pistachio, mango, pink peppercorn, and sumac) and members of the tree nut family (almond, Brazil nut, chestnut, hazelnut, macadamia, pecan, pine nut, and walnut) using dot blot immunoassays to evaluate sensitisation profiles. When comparing both types of sIgE binding measurements for the Anacardiaceae species, high correlations between dot blot and IMMULITE sIgE were seen for both cashew nut (R = 0.84) and pistachio (R = 0.75) but not for mango (Fig. 2). In contrast, no significant correlation was observed between dot blot sIgE and positive SPT results (R = 0.29 and R = 0.13 for cashew nut and pistachio, respectively).

Interestingly, based on relative dot blot spot intensities of IgE-reactive protein spots and post hoc analysis of sIgE binding patterns, we were able to classify patients into four different groups according to their sensitisation profiles (Fig. 3): group I, patients that showed co-sensitisation profiles towards only Anacardiaceae species; group II, patient reacting to proteins extracted from cashew nuts but not to proteins from other Anacardiaceae; group III, patients that reacted to several different tree nuts and to Anacardiaceae species; and in group IV, patients that did not respond to cashew nut protein on dot blot. Details of the post hoc analysis are specified in Table 1.

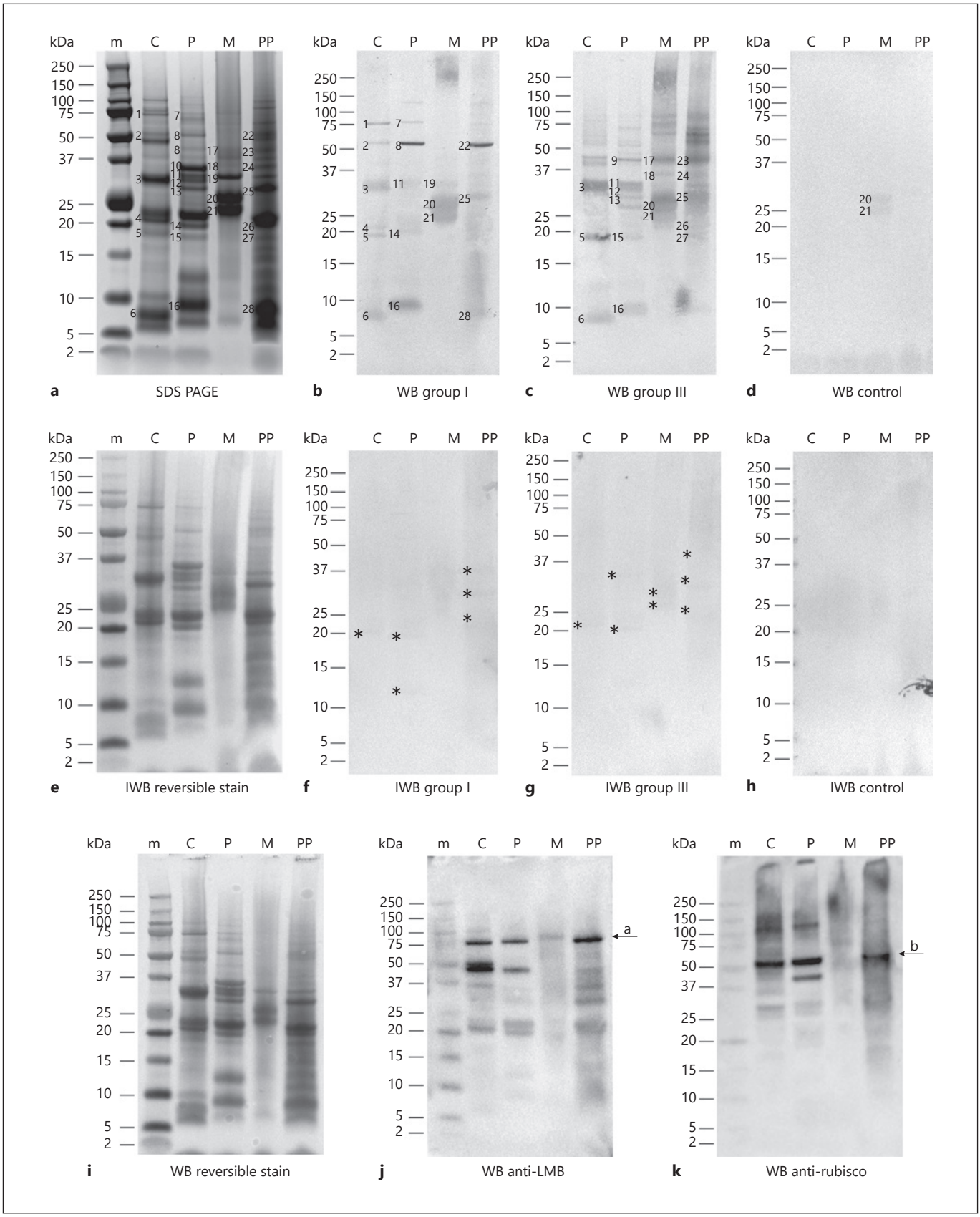
All 7 patients displaying a group I profile showed a clinically relevant cashew nut sensitisation (positive DBPCFC) as specified in Table 2. Group II contained 18 members. Within these 18 patients, 4 patients (22%) displayed a clinically non-relevant cashew nut sensitisation based on a negative DBPCFC test outcome. Eleven patients showed sensitisation against almost all protein fractions tested (group III members). Three patients (28%)

Table 2. Characteristics of patients classified into groups I–IV according to dot blot [3]

Group	I	II	III	IV
Patients, *n*	7 (13%)	18 (32%)	11 (20%)	20 (36%)
Male gender	5 (71%)	7 (39%)	7 (64%)	10 (50%)
Mean age, years	7.7 (3–13)	7.9 (2–13)	8.2 (3–14)	9.3 (2–17)
Atopic disease symptoms				
Asthma	3 (43%)	94 (25%)	7 (65%)	8 (40%)
Eczema	4 (57%)	9 (50%)	7 (64%)	13 (65%)
Hay fever	2 (29%)	5 (28%)	7 (64%)	10 (50%)
Atopy	5 (71%)	11 (61%)	11 (100%)	14 (70%)
Median sIgE cashew nut, kU/L (σ)*	27.0 (26.2)[a]	7.5 (6.5)[b]	22.1 (29.0)[a]	0.9 (0.7)[c]
Median SPT cashew nut HEP (σ)	3.2 (2.7)[a]	2.6 (2.0)[a]	2.7 (2.6)[a]	2.0 (2.1)[a]
Outcome of DBPCFC test				
Positive	7 (100%)	14 (78%)	8 (72%)	12 (60%)
Negative	0	4 (22%)	3 (28%)	7 (35%)
Undetermined	0	0	0	1 (5%)

sIgE, specific IgE; SPT, skin prick test; DBPCFC, double-blind placebo-controlled food challenge; HEP, histamine equivalent prick index area; *, as measured by Immulite; σ, standard deviation of the medians. Superscript letters indicate significant differences between group medians, $p < 0.05$.

Fig. 3. Relative dot blot spot intensities in relation to corresponding IgE (Siemens IMMULITE), SPT, and DBPCFC data per patient and classification into groups (I–IV). Per group, the median IgE and SPT values are indicated below the lines. Patient numbers correspond to the last two digits in the patient ID series 1110001 until 1110079 in the IDEAL study by Van der Valk et al. [3]. Colours were used to differentiate in spot intensity (–, +/–, and + until +++): light orange <1.27; salmon 1.27–1.3; yellow/orange 1.3–1.5; orange 1.5–2; brown >2. Examples of a dot blot for each patient group are shown to the right. An antibody control blot shows any non-specific background staining used for data normalisation. The dot blot spot layout indicates the location of each protein sample on the nitrocellulose membranes: (1) walnut, (2) cashew, (3) pistachio, (4) sumac, (5) macadamia, (6) almond, (7) Brazil nut, (8) pecan, (9) chestnut, (10) mango, (11) pink peppercorn, (12) pine nut, (13) hazelnut.



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within this group tested negative in DBPCFCs. All group III children suffer from atopy and disease symptoms as asthma and hay fever which are twice as frequent within this patient group in comparison to group I, which might be reflected in the dot blot sensitisation profile (sensitisation towards multiple botanically semi-related foods). Twenty patients (group IV) showed no sIgE-binding activity to cashew nut extract on dot blot. As specified by van der Valk et al. [3], 7 patients of this group were also negative in the DBPCFC with cashew nut, and for 1 patient the DBPCFC-outcome was undetermined. Group IV also showed the lowest median sensitisation grade in IMMULITE sIgE for cashew nut (0.9 kU/L) and SPT (2.0 HEP index area). In contrast, median cashew nut sIgE as measured by IMMULITE was highest in group I (27.0 kU/L) and group III (22.1 kU/L) patients.

Based on the results above, we hypothesise that cashew nut allergic individuals might have a high chance of being co-sensitised to other nuts, seeds, or fruits and that differences in sensitisation profiles can be visualised by dot blot immunoassays.

Group-Specific Allergen Profiles Visualised by Western Blotting

Next, we aimed to identify the putative allergens underlying the cross-sensitisation profiles of patient groups I and III. Group-specific allergen profiles were visualised by Western blotting using pooled serum from patient groups I and III separately, as depicted in Figure 4. Because of the limited amounts of patient serum, the immunoblot analyses were focused on the specific Anacardiaceae family members (cashew, pistachio, mango, and pink peppercorn).

Fig. 4. SDS PAGE (**a**), Western blots (**b–d**, **i–k**), and Western inhibition blots (**e–h**) of cashew (C), pistachio (P), mango (M), and pink peppercorn (PP) protein fractions. **a** SDS PAGE Coomassie staining. **b** Western blot using a serum pool of patient group I. **c** Western blot using a serum pool of patient group III. **d** Western blot control using TBS. **e** Reversible staining after nitrocellulose transfer. **f** Western blot using patient group I serum inhibited with cashew protein extract. **g** Western blot using patient group III serum inhibited with cashew protein extract. **h** Western blot control using TBS inhibited with cashew protein extract. **i** Reversible staining after nitrocellulose transfer, **j** Western blot using an anti-luminal binding protein antibody. **k** Western blot using an anti-rubisco antibody. Numbers correspond to excised bands used for LC-MS/MS protein identification as depicted in Table 2. **f**, **g** Asterisks indicate protein bands still faintly visible on the inhibition Western blots. **j**, **k** Arrows in Western blots point out the luminal binding protein bands (arrow a) and rubisco protein bands (arrow b).

In both groups, patients showed IgE co-sensitisation to protein extracts from pistachio, mango, and pink peppercorn (Fig. 4a–d). An antibody control blot revealed that only some unspecific background binding occurred to the mango protein fraction (Fig. 4d). Interestingly, group I and III patients displayed contrasting IgE sensitisation patterns. As expected from results observed by dot blot, group III patients showed IgE sensitisation to many different bands in all protein fractions while group I patients only to a few protein bands. Protein bands representing 11S globulins, albeit recognised differently by each patient group, were identified in cashew nut (Ana o 2, excised bands 2–5), pistachio (Pis v 2 and Pis v 5, excised bands 11, 12, 14, and 15), and pink peppercorn (excised bands 23, 25, and 26). The 7S vicilin allergen Ana o 1 in cashew nut was not identified in any of the blots, which was already noted in earlier research by Reitsma et al. [36] using serum from the IDEAL patient cohort. Pis v 3, the 7S vicilin allergen in pistachio was however identified in excised bands 9. Also, the 2S albumins, cashew nut allergen Ana o 3 and Pis v 1 in pistachio, represented in bands 6 and 16 respectively, were recognised by both patient groups.

In addition to the already known cashew nut seed storage allergens, putative novel cross-reactive Anacardiaceae allergens were identified. Protein bands of ca. 54 and 73 kDa (excised bands 2, 8, 22 and 1, 7, respectively) specifically visualised by serum of group I patients in all nut/seed protein fractions, were tentatively identified as ribulose-1,5-bisphosphate carboxylase oxygenase and luminal BiP, respectively (Table 3). The observed location and identity of these IgE reactive proteins on SDS PAGE were confirmed using specific antibodies (Fig. 4i–k). In pink peppercorn, a putative 2S albumin allergen ca. 8 kDa in size was identified in excised band 28.

Although only minor IgE reactivity towards mango was observed on dot blot (Fig. 3), a clear reactivity on Western blot was observed by both group I and III towards several chitinases and β-1,3-glucanases (excised bands 17–21) present in the mango protein fraction. Some non-specific binding towards the chitinase bands 20 and 21 was observed in the control blot of which the exact cause is unclear. Nevertheless, the corresponding bands in Figure 4c, d were clearly higher in intensity, indicating additional IgE-specific binding activity.

Immunoblot Inhibition by Cashew Nut Protein

The in vitro sIgE cross-reactivity to allergen extracts from the *Anacardiaceae* family in both serum pools from patient groups I and III was determined by pre-incubat-

Table 3. Identification of IgE-reactive proteins in excised bands using LC-MS/MS

Band No.	Matching protein	Western blot analysis		LC-MS/MS analysis				
		serum pool No.	mass, kDa	accession	E-score	calc. mass, kDa	uniq. pept., *n*	sequence coverage, %
Cashew nut total protein extract								
1	Luminal binding protein (*Ca*)	1	73	CAC14168	$7.5e^{-9}$	73.6	25	35
2	Ribulose partial (*Ao*)	1	54	AAS79700	$2.5e^{-9}$	51.6	3	42
	11S globulin Ana o 2 (*Ao*)			AAN76862	$1.3e^{-9}$	52.0	34	78
3	11S globulin Ana o 2 (*Ao*)	1/3	32	AAN76862	$3.3e^{-10}$	30 LS	43	86
4	11S globulin Ana o 2 (*Ao*)	1	21	AAN76862	$6.5e^{-10}$	21 SS	43	77
5	11S globulin Ana o 2 (*Ao*)	1/3	19	AAN76862	$1.5e^{-9}$	21 SS	29	66
	11S globulin Pis v 2.0201 (*Pv*)			ABG73110	$2.3e^{-9}$	17–20 SS [15]	4	23
6	2S albumin Ana o 3 (*Ao*)	1/3	8	AAL91665	$6.0e^{-9}$	8 LS	10	40
Pistachio total protein extract								
7	Luminal binding protein (*Ca*)	1	76	CAC14168	$1.1e^{-9}$	73.6	18	25
8	Ribulose partial (*Pc*)	1	54	CBI68284	$1.5e^{-10}$	50.8	36	66
9	7S vicilin, partical, Pis v 3 (*Pv*)	3	41	ABO36677	$4.4e^{-9}$	59.8 (45 on SDS) [14]	37	58
10	7S vicilin, partical, Pis v 3 (*Pv*)	3	34	ABO36677	$6.5e^{-10}$	59.8 (38 on SDS) [14]	28	44
11	11S globulin Pis v 5.0101 (*Pv*)	3	32	ACB55490	$3.3e^{-10}$	30–40 LS [15]	39	87
	11S globulin Pis v 2.0201 (*Pv*)			ABG73110	$3.6e^{-9}$	30–40 LS [15]	19	72
12	11S globulin Pis v 2.0101 (*Pv*)	3	29	ABG73109	$1.8e^{-10}$	30–40 LS [15]	27	73
	11S globulin Pis v 5.0101 (*Pv*)			ACB55490	$1.9e^{-10}$	30–40 LS [15]	33	82
13	Unknown	3	26	–	–	–	–	–
14	11S globulin Pis v 2.0201 (*Pv*)	1	19	ABG73110	$6.4e^{-10}$	17–20 SS [15]	24	76
	11S globulin Pis v 5.0101 (*Pv*)			ACB55490	$4.6e^{-9}$	30–40 SS [15]	19	58
15	11S globulin Pis v 2.0201 (*Pv*)	3	18	ABG73110	$5.0e^{-9}$	17–20 SS [15]	23	73
	11S globulin Pis v 5.0101 (*Pv*)			ACB55490	$1.6e^{-9}$	13–24 SS [15]	22	53
16	2S albumin Pis v 1 (*Pv*)	1/3	9	ABG73108	$2.2e^{-10}$	17.3/(7 on SDS) [37]	18	50
Mango total protein extract								
17	Chitinase partial (*Mi*)	3	42	ACD69683	$9.8e^{-8}$	20, 25.5, 46, 50, 75 [38]	10	49
18	Chitinase partial (*Mi*)	3	35	ACD69683	$1.1e^{-9}$	See band 17	12	65
	β-1,3-glucanase (*Mi*)			ABD16200	$1.8e^{-9}$	19.5, 33-36, 42-46 [39]	10	64
19	β-1,3-glucanase (*Mi*)	1/?	33	ABD16200	$7.8e^{-10}$	See band 18	13	65
	Chitinase partial (*Mi*)			ACD69683	$1.3e^{-9}$	See band 17	12	65
20	Chitinase partial (*Mi*)	1/3	27	ACD69683	$5.7e^{-10}$	See band 17	20	87
21	Chitinase partial (*Mi*)	1/3	25	ACD69683	$1.5e^{-9}$	See band 17	12	53
	β-1,3-glucanase (*Mi*)			ABD16200	$2.7e^{-8}$	See band 18	9	60
Pink pepper total protein extract								
22	Ribulose (La)	1/?	52	AEB65826	$1.4e^{-9}$	51.2	5	51
23	11S globulin Pis v 2.0201 (*Pv*)	3	42	ABG73110	$4.6e^{-8}$	30–40 LS [15]	8	43
	11S globulin Pis v 5.0101 (*Pv*)			ACB55490	$5.7e^{-8}$	30–40 LS [15]	4	12
24	Unknown	3	35	–	–	–	–	–
25	Hypothetical protein partial (*Pt*)	3	29	AEW08142	$3.9e^{-8}$	16.3	1	52
	11S globulin Pis v 2.0201 (*Pv*)			ABG73110	$3.7e^{-8}$	30–40 LS [15]	3	27
26	11S globulin Pis v 2.0201 (*Pv*)	3	19	ABG73110	$2.8e^{-10}$	17–20 SS [15]	12	47
	11S globulin Pis v 5.0101 (*Pv*)			ACB55490	$1.3e^{-10}$	13–24 SS [15]	5	14
27	ADP ribosylation factor (*Ah*)	3	17	AEV66152	$7.4e^{-8}$	20.7	10	62
28	2S albumin Ana o 3 (*Ao*)	1/?	8	AAL91665	$1.0e^{-8}$	8 LS	4	27

Binomial species nomenclature abbreviations: *Ao*, *Anacardium occidentale*; *Ah*, *Arachis hypogaea*; *Ca*, *Corylus avellana*; *La*, *Loxostylis alata*; *Mi*, *Mangifera indica*; *Pt*, *Pinus* taeda; *Pc*, *Pistacia chinensis*; and *Pv*, *Pistacia vera*. LS, large subunit; SS, small subunit; uniq. pept., number of unique peptides.

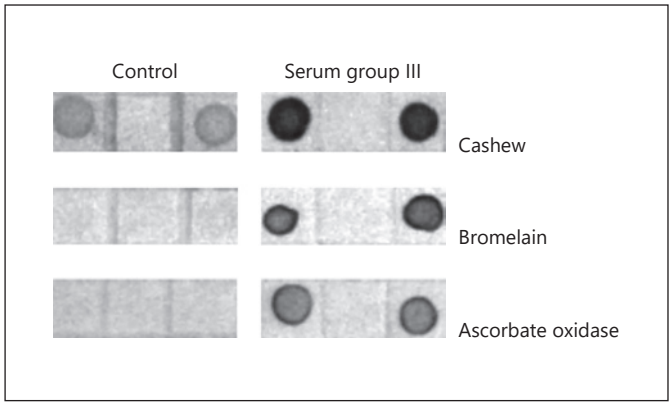

Fig. 5. CCD sensitisation of patient group III. Dot blot immunostainings of cashew nut, bromelain, and ascorbate oxidase spotted in duplicate and incubated with TBS (control) or serum pool of patient group III.

ing the serum pools with cashew nut protein extract prior to immunoblotting. Cashew nut protein was able to inhibit IgE immunostaining almost completely in all fractions, including the mango fraction (Fig. 4e–h). This finding suggests that cashew nut is most likely the primary sensitiser in these patients.

Sensitisation to CCDs

Complete immunoblot inhibition of the mango IgE-reactive chitinase and β-1,3-glucanase bands by cashew nut extract was rather unexpected as for cashew nut, these types of proteins have not been shown to be allergenic. IgE cross-reactivity between non-homologous and non-related allergens, such as observed for cashew nut and mango, can in some cases be explained by antigenic cross-reactive carbohydrate determinants (CCD) on glycoproteins which can affect in vitro allergy diagnosis. Patients within group III displayed IgE reactivity to bromelain and ascorbate oxidase (Fig. 5), two well-known CCD-containing glycoproteins. This might partly explain the extensive immunoblot inhibition results observed in Figure 4f, g.

Discussion

In this study, we demonstrated different IgE sensitisation profiles in the sera of 56 children with a suspected cashew nut allergy towards Anacardiaceae members and common tree nut species using dot blot immunoassays. Some patients (12.5%) in this subpopulation, with cashew protein-binding sIgE as shown on dot blot, demonstrated negative DBPCFCs [3] as depicted in Figure 3. This suggests a clinically non-relevant IgE sensitisation to cashew nut protein. Also, patients with a positive DBPCFC but negative dot blot reactivity were observed (21.4%; IgE sensitisation profile IV, for details see next paragraph). As IMMULITE read-outs confirmed the presence of cashew-sIgE in all of these patients, the protein extractability for some of the cashew nut allergens might not have been optimal in the Tris and urea/phosphate buffers used in our study, or the applied dot blot technique was insufficiently sensitive. Possibly, the choice of raw cashew nuts in this study in contrast to the use of roasted cashew nuts in the original IDEAL study explains some of the discrepancies. One might speculate that heat-labile allergens are not picked up by a DBPCFC using cashew-containing muffins. On the other hand, the generation of possible neoallergens [40, 41] or novel IgE-binding epitopes (as observed in roasted peanut) [42–44] as a result of the Maillard reaction during the heating process of cashews might provoke allergic symptoms in certain patients, while proteins from raw nuts might not. However, as cashews are usually consumed blanched or roasted, the chance that some patients are primarily susceptible to raw cashew nuts, is very small.

Based on the dot blot spot intensity profiles, four different IgE sensitisation profiles (I–IV) could be distinguished, and patients were grouped accordingly. To our knowledge, this is the first study showing that specific sensitisation profiles can be identified using this immunoblot technique. Notably, 19% of patients tested (classified as group III patients) displayed IgE sensitisation towards almost all protein fractions tested. For children, it is not unusual to be sensitised to multiple nut species such as in these group III patients, where not all sensitisations necessarily result in clinical symptoms [45]. The low sensitisation profiles for some of the patients in the negative dot blot group IV support the reasoning that the dot blot detection limit might not be ideal for minimal IgE quantification. Overall, the dot blot data suggest that sensitisation to cashew nut is not always correlated with a general sensitisation to multiple members of the Anacardiaceae family as only half of patients displaying sIgE to cashew nut protein were co-sensitised towards either pistachio, mango, pink peppercorn, or sumac (group I and III vs. group II). There is a possibility that the sensitisation profiles of the tested patients might slightly differ when testing processed nuts. However, we expect that a mono-sensitisation for cashew nut (group II) will be distinguishable from a multi-sensitisation profile (group I and group III

patients) regardless of whether proteins are extracted from raw or processed nuts.

Whether the observed co-sensitisation in patients has been the result of independent sensitisation to multiple foods versus true cross-reacting proteins was further investigated using Western blotting for the Anacardiaceae species in group I and group III patients. Patients within group I merely showed IgE sensitisation to allergenic 2S albumins and/or 11S globulins in cashew nut, pistachio, and pink peppercorn, but not to any of the 7S vicilin allergens. The absence of vicilin-sIgE in these patients could explain the observed low co-sensitisation to other tree nuts, as Ana o 1 is deemed to be the responsible cross-reactive factor between different tree nuts [12–16]. Surprisingly, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) in cashew nut, pistachio and pink peppercorn protein fractions was specifically detectable by this patient group I. Additionally, for these same patients peptide homologs of the cross-reactive luminal BiP from *C. avellana* pollen [46] were recovered from the ca. 73–76 kDa IgE-reactive protein bands in cashew and pistachio nut. Rubisco has been suggested as an allergen in spinach, tomato, and cannabis [47, 48], and additional putative BiP allergens have been identified in cannabis seed [48] and chickpea [49]. The clinical relevance of IgE-reactive rubisco and/or BiPs proteins for a cashew or pistachio nut allergy, also in relation to cross-reactivity towards tree nuts and stability during heat processing, has yet to be elucidated.

Multiple 11S globulin bands in cashew nut (Ana o 2), pistachio (Pis v 2/5), and pink peppercorn were recognised by group III patients as well as 2S albumins (Ana o 3, Pis v 1) and a 7S vicilin (Pis v 1). The diversity of such IgE reactivity might relate to the multiple tree nut sensitisation profiles seen on dot blot for this patient group. Cross-reactivity between inhalant and food allergens likely plays an important role in this multi-food sensitised group and most likely accounts for the observed extensive cross-sensitisation patterns.

Despite the non-reactivity of group I patients to mango protein on dot blot, tentative chitinases and β-1,3-glucanases from mango [50], both pathogenesis-related proteins found to be allergenic in multiple fruits and seeds [51, 52], were recognised by both patient groups on Western blot. Such differences between results might be due to differences in methodology used between the dot blot and Western blot techniques. However, part of the observed chitinase IgE reactivity was slightly biased by weak unspecific antibody-binding activity as concluded from the WB control. Although mango can cause severe anaphylactic reactions [30, 53, 54], immediate or delayed type manifesting hypersensitivity reactions to mango are distinctly rare [50] and most patients within our study had negative SPT results to this fruit. Furthermore, in a follow-up study using a small subset of the IDEAL patient cohort [21], 18 of 29 patients sensitised to cashew and pistachio nut, already consumed mango without symptoms, while the remaining 11 responded negative in an open food challenge with mango. Thus, despite observed IgE cross reactivity with cashew nut in our Western blots, both allergen types are seemingly not clinically relevant.

Inhibition Western blotting revealed considerable, and patient group-independent, cross-reactivity between cashew and pistachio nut, mango, and pink peppercorn. Cross-reactivity between cashew nut, pistachio, and pink peppercorn was expected because of the high cross-reactive nature of seed storage proteins. However, for the mango IgE-reactive bands, this was rather unexpected as cashew is not known to contain any allergenic chitinases or β-1,3-glucanases. In addition, also several high molecular weight bands were detected in the mango sample for both patient groups which were absent on the inhibition Western blot, suggesting cross-reactivity. Unfortunately, we were unable to identify these bands by LC-MS/MS. Individual bands were indistinguishable and could not be excised from the SDS gel. CCD sensitisation in patients as visualised for group III patients (Fig. 5) might explain the observed mango-cashew co-sensitisation as approximately one-fifth of patients with an allergy seem to develop antibodies against CCDs with low clinical significance [55]. In the inhibition Western blot for group I patients, an additional band ca. 13 kDa in size was noticed, which was not detected in the normal immunoblot. Possibly, IgE antibodies were prevented from binding to this low allergenic band by blocking factors present in the serum pool that were eliminated in the inhibition experiment. Based on the observation that cashew protein was able to completely inhibit IgE-binding to proteins from related species, we conclude that, in the patient group studied, cashew nut must be the primary sensitising agent.

To conclude, our results show that a large proportion of patients with a cashew nut allergy are IgE sensitised to multiple other Anacardiaceae species and/or tree nut species. Using immunoblotting, we have identified putative cross-reactive allergens and/or allergens underlying cashew sensitisation in young children. These putative novel allergens, which were identified in cashew nut, pistachio, and pink peppercorn justify further prospective studies to determine and understand their clinical rele-

vance and to develop effective immunotherapy strategies to treat or prevent cashew nut sensitisation in young children.

Furthermore, cashew nut-allergic children with co-sensitisation to pink peppercorn and sumac spice should precautionary exclude these foods from the diet to decrease potential risks of unwanted allergic reactions. In the absence of protocols for DBPCFs with pink peppercorn and sumac spice, the clinical relevance of these sensitisations remains unclear.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Acquisition and design of the experimental work was done by S.B.-N, HJW, and N.W.d.J. S.B.-N. and M.R. designed and performed most of the immunoblot experiments. LC-MS/MS and data analysis was performed by J.H.G.C. and T.A.H.P.A. J.P.M.v.d.V. performed the ImmunoCAP and SPT experiments and interpretation of results. All named authors substantially contributed to drafting and revising the article and approved the final version to be published.

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