Cracking the cashew nut:

Strategies to identify novel allergens

Shanna Bastiaan-Net

Propositions

1. The cashew nut is literally and metaphorically a tough nut to crack (this thesis).

2. As minor allergens can still have a profound impact on the quality of life of allergic individuals they should not be downplayed (this thesis).

3. There is a trade-off between the high publication output requirements in science and long-term and high-risk research.

4. Experimental research leads are like a bouquet of flowers, most begin to wilt one by one and just occasionally some set roots.

5. The phrase “Let medicine be thy food and let food be thy medicine” by Hippocrates, could be translated to: ‘If food be thy allergy, let this food then be thy medicine’.

6. Covid-19 pandemic changed the opinion on, and possibilities of, working from home.

7. Pushing yourself deliberately beyond your comfort zone, allows you to grow as a person as well as in science.

Propositions belonging to the thesis, entitled

Cracking the cashew nut: Strategies to identify novel allergens

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Wageningen, 28 October 2020

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Thesis

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Table of Contents

Chapter 1 **7**
General introduction

Chapter 2 **27**
Overview of *in vivo* and *ex vivo* endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins?

53
Chapter 3
IgE cross-reactivity of cashew nut allergens

Chapter 4 **75**
IgE cross-reactivity measurement of cashew nut, hazelnut and peanut using a novel IMMULITE inhibition method

Chapter 5 **91**
Identification and *in silico* bioinformatics analysis of PR10 proteins in cashew nut.

Chapter 6 **115**
Cashew nut seed storage proteins are comprised of multigene families and include three Ana o 3 isotypes

Chapter 7 **143**
General discussion

Appendix
Summary 175
Acknowledgements 179
About the author 185

Chapter 1

General introduction

1. General introduction

1.1 Introduction to the thesis

Food allergy is defined as an aberrant, immune-mediated adverse reaction occurring on exposure to food proteins[1,2], which can manifest in nausea, diarrhoea, eczema or even in death when not appropriately treated. The incidence, prevalence and severity of food allergy has been increasing in the last decades, particularly in westernised countries[1,3,4]. Currently, food allergy has an estimated prevalence of 3-10% in developed countries[5-7], and is thus considered an important health issue. Tree nuts (for example cashew nut), and other food sources like milk, egg, fish, crustacean shellfish, peanut, soybean and wheat are considered as the major eight allergenic foods in the EU and USA and as such need to be declared as labelled ingredients on food packages to protect allergic consumers from unnecessary risks (https://www.fda.gov/). Within the EU, mandatory ingredient information should also include celery, mustard, sesame seeds, lupin, molluscs and sulphites, according the Annex II to Regulation (EU) No. 1169/2011 and No. 78/2014 amendment.

With increasing world prosperity, the demand for high-quality food products and a wider range of choice of (foreign) food products will increase. The introduction of novel food products, irrespective of the region of origin, will inevitably increase the risk of introducing new food allergies in the future. For instance, before the introduction of kiwi trees in 1962 in the EU and USA, practically no one had heard of a kiwi allergy. Nearly 20 years later, in 1981, the first case study of an adverse reaction to kiwifruit appeared[8], while currently the estimated prevalence of kiwifruit sensitization in Spain is 1.8% of the general population[9]. How such, in principle, harmless proteins become allergenic and are able to cause food allergies is not fully understood. Moreover, not all allergenic proteins present in each (major) allergenic food source have been identified as yet. To halt the increasing prevalence of food allergy and improve medical treatments, a better understanding of the pathophysiological processes underlying food allergy sensitisation is necessary. Therefore, studies that monitor and characterise novel allergenic proteins in food sources are pivotal. In addition, increased knowledge on the immunogenic properties of allergenic proteins may help to answer the question why some proteins become allergenic[10-13]. Such studies will aid to judge whether a novel food product or source might form a risk in the future and are thus essential to protect consumers from unexpected danger.

This introductory chapter first explains what a food allergy is and what causes it. Also discussed are the proteins that are specifically involved in plant-mediated food allergies. This is followed by an overview of different techniques that are employed to identify and characterise allergens. In this study, cashew nut has been used as a model food to search for novel putative allergenic proteins. Therefore, a brief

overview of the allergenicity of cashew nut is presented and this chapter will end with a concise overview of the thesis.

1.2 Food allergy

Food allergy is generally defined as an abnormal, immune-mediated adverse reaction occurring reproducibly on exposure to foods or more precisely, towards food proteins[1,2]. Also food additives, like some colours, preservatives, flavourings or antioxidants can be implicated in food allergic reactions[14,15]. The development of a food allergy (this dissertation focuses only on the common type 1 reaction) consists of two phases, namely the sensitisation phase and the elicitation phase (Figure 1).

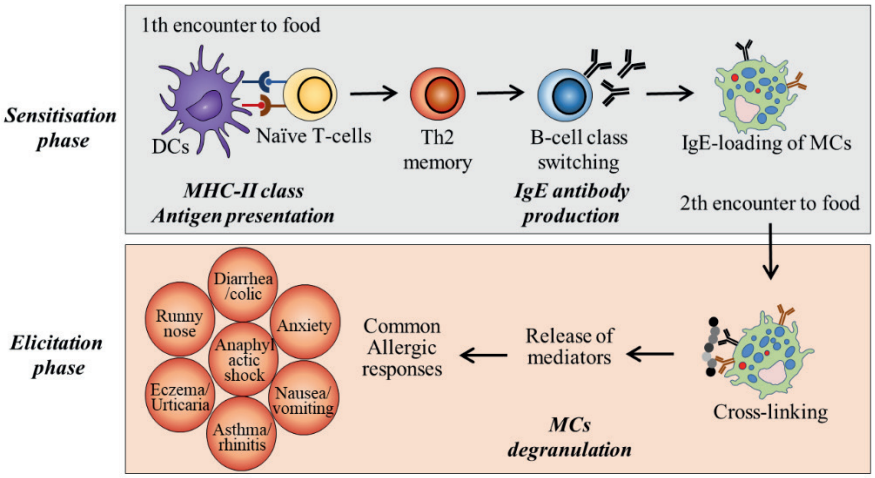


Figure 1. Simplified mechanism of the IgE-associated food allergic sensitisation and elicitation reaction. DCs: dendritic cells, MCs: mast cells. Figure adapted/based on Valenta et al[1].

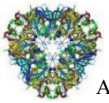
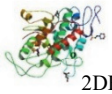
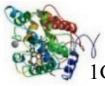
Absorbed small protein peptides, initially released from ingested foods by digestive enzymes and taken up in the gastrointestinal track, will be processed by antigen presenting cells in the gut lumen, such as dendritic cells (DCs) or macrophages[16]. Subsequent presentation of processed peptides to naive antigen specific T helper cells (Th) via the major histocompatibility complex class II molecules (MHC-II) will either lead to the development of oral tolerance or to food allergic sensitisation. In case of sensitisation, the Th cells differentiate into interleukin-producing (IL-4, IL-5, Il-10 and IL-13) effector Th2 cells, causing class switching of B-cells into IgE antibody-secreting plasma cells[17-19]. Released antigen-specific IgE (sIgE) directly binds to the high affinity FcεRI receptor present on tissue mast cells (MCs) and blood basophils. Upon a second encounter with the same type of protein, IgE-peptide recognition results in degranulation of MCs via allergen-induced IgE cross-linking and release of inflammatory mediators which result in the elicitation of food allergy-associated symptoms[1,2,20,21]. Moreover, after a primary elicitation, the allergic immune response is invigorated with repeated exposure to the allergenic food, intensifying the severity of the allergic response[1].

1.3 Plant food allergens

Proteins responsible for the initiation of the allergic response can be classified into families and superfamilies, based on the synopsis of allergology and protein evolution[22]. According to the Allfam database, a total of 1042 allergens have been described of which 959 allergens have been assigned to 151 different protein families (Statistics from 2017-03-07; accessed 2020-05-09). Considering the 16,306 protein families currently classified, allergens are only distributed over a very restricted number of protein families according to similarities in their biochemical functions, primary structure and immunological cross-reactivity[22-24]. Food allergens can be distinguished as plant, animal, fungal or insect-derived, depending on the source of foods they originate from. As cashew nut is used as a model food in this thesis, the focus area only includes the families of plant proteins. Common plant food allergen families, their function and characteristics have been listed in Table 1.

Table 1. Plant food allergen families commonly found in nuts and/or seeds with their features, function and characteristics (reviewed by Valenta et al[1], Hauser et al[23], www.allergome.org)

Superfamily	Family	Group	Features	Function
Prolamin High proline and glutamine content; eight conserved cysteine residues; rich in α-helices; predominantly heat stable and resistant to gastrointestinal digestion.	2S Albumins  Ber e 1	Major seed storage protein	Heterodimeric; 4-9kDa; four conserved disulphide bonds	Nitrogen and sulphur donor; antifungal activity; seed germination
	Cereal α-amylase/trypsin inhibitors  1HSS	Hydrolases	Eight-stranded α/β barrel structure	Interference of starch conversion; plant defence (anti-insect)
	nsLTPs (also included in the PR-family as PR-14)  Cor a 8	Lipid transfer proteins	Unique to flowering plants; 7-9kDa; four conserved α-helices and disulphide bonds forming a hydrophobic tunnel.	Phospholipid interaction; plant defence (anti-fungal, anti-bacteria)
	Cereal prolamines	Major seed storage protein	Sulphur rich; intrachain disulphide bonds	Seed germination.
Cupin Conserved consensus sequence motifs and one or two β-barrel cupin core domains	7S (vicilin-like globulins)  Ara h 1	Major seed storage protein	Homotrimeric; 150-190kDa with 40-80kDa subunits; disk-shape structure	Seed germination.
	11S (legumin-like globulins)	Major seed storage protein	Hexameric; disulphide linked 30-40kDa acidic	Seed germination.

	 Ara h 3		polypeptide to 20kDa basic polypeptide;	
	Cysteine protease C1 family	protease		
	Germins  1FI2	Carbohydrate isomerases/ epimerases	Includes only 1 allergen to date	
Pathogenis- related protein (PR)* Unrelated protein families (17); induced upon environmental stress, pathogen infection and antibiotic/chemical stimuli; in general stable at low pH and resistant to proteolysis and pH- or heat-induced denaturation (except PR-10).	PR-3  2DKV	Class I chitinases	N-terminal Hevein-like chitin binding domain (latex-fruit-syndrome)	Hydrolysis of chitin polymers; plant defence (anti-fungal, anti-insect)
	PR-5  Act d 2	Thaumatin-like proteins	Anti-parallel β-sheets; eight conserved disulfide bonds; 20kDa	Osmotic stress; anti-fungal activity
	PR-9  1GZA	Peroxidases	Heme-group	H_2O_2-dependant oxidation; plant defence
	PR-10  Ara h 8	Bet v 1 -related	Conserved α-β fold solvent-accessible cavity; Bet v 1 domain (pollen-fruit syndrome)	Steroid/fatty acids/ cytokine carrier; membrane binding;
Enzymes and protease inhibitors** Enzymes or protease inhibitors that based on characteristics cannot be classified in above mentioned superfamilies.	Thioredoxins	Protease		General disulphide oxido-reductase
	β-amylases	Trypsin inhibitors		Hydrolysis of starch-type polysaccharides
	Papain-like Cysteine proteases	Protease/protease inhibitor	25-28kDa, three conserved disulphide bonds; α-helix domain and β-barrel	
	kunitz-type protease inhibitors	Protease/protease inhibitor	Two conserved disulphide bridges; 16-20kDa	Inhibition of proteolysis
Others	Profilins  Ara h 5	Actin-binding proteins	12-15kDa; typical anti-parallel β-sheets structure;	Cell motility; actin microfilament polymerisation; cytokinesis; cell elongation

				(pollen tubes and root hairs)
	Oleosins	Oil body proteins	16-24kDa; Conserved 70aa hydrophobic core; hydrophilic N- and C-terminal;	Stabilisation of triacyl-glycerol oil bodies; lipid storage and seed germination
	Expansins  Phl p 1	Ripening-related proteins	Cysteine-rich?; 28kDa	Fruit/pollen ripening
	Chlorophyll-binding proteins	In seeds?		Light-receptor capturing and energy excitation
	Luminal binding protein			
	Manganese superoxide dismutase			
	Seed specific biotinylated proteins			
	60S acidic ribosomal binding proteins			

* PR-1 family was not included as no members have been identified yet in seeds/nuts; ** Isoflavone reductases, glycoside hydrolases, patatins, subtilisin-like serine proteases, berberine bridge enzymes, cystatins, cyclophilins and phenylcoumaran benzylic ether reductases were not included in this table as no seed allergens have been classified in these families yet. nsLTP: non-specific lipid transfer protein; TLPs: thaumatin-like proteins. Chrystal structures were obtained from the RCSB PDB database (www.rcsb.org).

Next to classification into protein families, plant-derived food allergens can be further distinguished as class I or class II, according to the features of clinical manifestation they manifest. Class I allergens are primary sensitizers whose sensitisation route predominantly occurs via the skin or gastrointestinal tract and are usually responsible for eliciting moderate to severe reactions. A class II allergy on the other hand, is initiated by sensitisation to non-food airborne-allergens in the respiratory tract, and cross-reactive plant-derived proteins act as elicitors of the allergic immune response[23,25-30]. For the latter, allergic symptoms are usually mild and transient, and typically limited to the oropharynx (or middle throat area)[31], although systemic reactions may occasionally occur[32,33]. The Bet v 1-like, profilin and some members of the nsLTP allergen family are typical examples of class II plant food allergens[31,34-37]. The percentage of allergic individuals that elicit an allergic reaction response to a protein of an allergenic food defines whether a protein is called a major (>50%) or minor (<50%) allergen. This division in class I or II allergens is thus not linked to the severity of elicited allergic symptoms, but is

subject to geographical influences and classification can therefore vary from region to region[23]. For instance, the class I allergen Pru p 3 (nsLTP from peach) is considered a major allergen in Mediterranean areas while in central Europe it is regarded a minor allergen. To complicate allergen classification, Pru p 3 has also been shown to be a cross-reactive elicitor in mugwort pollen-LTP allergy, which would actually define Pru p 3 as a class II allergen[38]!

Classification of allergenic proteins in families, based on structure and functionality, will also significantly facilitate the prediction of putative primary and/or secondary (cross-reactive) novel allergic sensitizers in newly introduced food sources. In addition, structural/functional comparison between allergic and non-allergenic members of well-defined protein families might help to understand the biggest question in allergology: "what makes an allergen an allergen?"[22].

1.4 Strategies to identify allergens

The allergenicity of food, proteins or residual peptides can be assessed by the combined use of several immunological assays which determine the IgE-binding and elicitation capacity before and after industrial processing[10]. Aalberse et al[12] stated that a 'complete' allergen should possess three distinct molecular properties: (1) able to bind IgE antibodies, (2) to elicit an allergic reaction and (3) to sensitise an

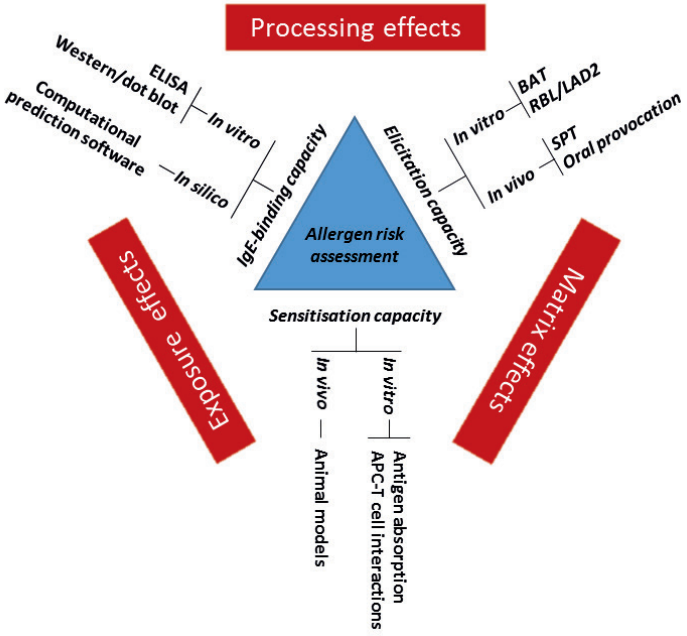


Figure 2. Techniques used in allergen risk assessment and factors that can influence the allergenicity of proteins. APC: antigen presenting cell; BAT: basophil activation test; LAD2: laboratory of allergic diseases 2 cells; RBL: rat basophilic leukaemia cells; SPT: skin prick test.

individual. Thus class II allergens, that are typically cross-reactive with pollen or latex allergens[39,40], are considered 'innocuous' or 'incomplete' allergens since they lack any sensitising abilities in contrast to class I allergens, which are always 'complete' allergens[10]. Since no single characteristic of a dietary protein is sufficient in predicting its allergenic potential, it is recommended to apply multiple techniques to support the risk assessment process of a novel protein source[10]. Techniques that can be applied to define a protein as allergenic, as based on the above proposed three prerequisite immunological molecular characteristics, are presented in Figure 2.

The first prerequisite of a protein, to possess IgE-binding capacity, can be assessed using immunoblotting (1D or 2D western blot/dot blot) and/or enzyme linked immunosorbent assays (ELISA) provided that IgE-antibodies against the target protein are available. Western blots provide qualitative information (e.g. size and presence of quaternary structures), while ELISA allows for quantification of the IgE-binding capacity[11]. When serum IgE is unavailable, such as in the case of a novel food protein, *in silico* predictive risk assessments can be performed to assess whether the protein sequence represents an existing allergen or whether it has potential to cross-react with an existing allergen. Primary, secondary and tertiary structure predictions or analytical analyses of the target protein contribute to the classification in protein families and provide insight into expected post-translational modifications as well as expected stability after food processing and gastrointestinal digestion[11]. However, such predictions will ultimately need experimental verification and *in silico* methodologies cannot predict whether a novel protein has sensitisation capacity[41].

Once IgE-binding efficacy has been demonstrated, the next step involves identifying epitope regions. Epitope binding regions, the amino-acid sequence an IgE antibody binds to, can be conformational or linear. Linear epitope regions, which are a stretch of consecutive amino acids, can be rather easily studied using synthesised peptides that span the entire allergen sequence and partially overlap, using a dot blot or peptide micro-array format (Figure 3)[42,43]. Conformational epitopes are much more challenging to identify, as they are formed by protein folding which brings distantly located amino acids into spatial proximity with each other[44]. Techniques to study conformational epitopes are less available and require more complicated methods. X-ray crystallography of an allergen-antibody complex is the most precise strategy to identify conformational epitopes but requires sophisticated algorithms and skilled technical expertise[44]. Nowadays, several alternative techniques have been developed to study conformational epitopes which have been summarized by Breiteneder et al[44]. Techniques that do not require the use of isotope-labelling, are e.g. the mimotope phage peptide display technology[45], epitope grafting[44] and hydrogen deuterium exchange mass spectrometry (HDX-MS)[46] (Figure 3).

Secondly, for a protein to possess allergenicity, it must not only have the capacity to bind IgE but also to initiate IgE-crosslinking (Figure 1). *In vitro/ex vivo* mediated IgE cross-linking can be studied using cellular mediator release assays[47], which include the

humanized rat basophilic leukaemia (RBL) cell lines RBL-2H3[48-51] (expressing the α-subunit of the human FcεRI receptor) and RBL-SX38[52,53] (expressing the α, β and γ chains of the human FcεRI receptor), the human mast cell line LAD2[54], and the basophil activation test (BAT)[55,56]. The BAT assay, which is based on isolated primary blood basophils from allergic or non-allergic individuals, may be performed directly or indirectly via loading of stripped basophils. The advantages of using a cell line over the

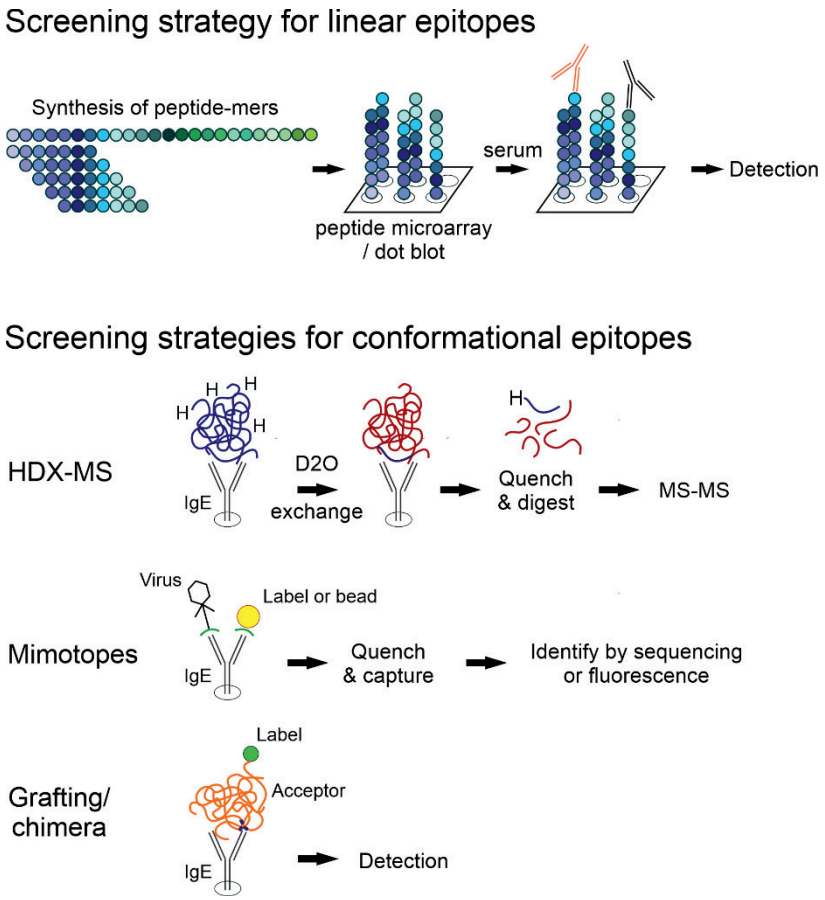


Figure 3. Technologies to identify linear and conformational IgE-binding epitopes. HDX-MS makes use of amide hydrogen/deuterium exchange upon protein-protein interaction. Accessible hydrogen molecules on the allergen which are not bound by IgE are replaced by deuterium. After proteolysis, protein fragments are subjected to mass spectrometry (MS). Protein fragments that were protected by the antibody contain hydrogen and can be distinguished by their lower molecular weight as compared to the deuterated fragments[46]. Mimotopes are randomly designed peptides (based on amino acid sequence of allergen using algorithm predictions) that mimic natural epitopes[45]. In case the protein crystal structure is determined, crystallography grafting techniques can best be applied to design chimeras. This technique makes use of homologous proteins with low to no IgE binding, where individual IgE epitopes are grafted on the surface area. Homologous stretches (for linear epitopes) or spatially adjacent amino acids (for conformational epitopes) in the recipient model are replaced for residues of the allergen to be studied. This chimera can subsequently be used in IgE-binding assays[44].

BAT assay includes elimination of time limitations in performing the assay within 48h and, in case of the direct BAT assay, no requirement for IgE-dissociation[47]. *In vivo* IgE-mediated mast cell degranulation can be validated by skin tests, such as the skin-prick test, "prick-to-prick" test or an intradermal test, while delayed-type allergic reactions can be diagnosed by atopy patch tests[1]. Clinical relevance of allergic elicitation should be demonstrated by provocation tests like an open food challenge (OFC) or a single or double-blind placebo-controlled food challenge (DBPCFC) test[1,57,58].

Lastly, demonstrating sensitization capacity is still the most difficult of the three prerequisites for a protein to be truly allergenic. Despite the many efforts lately performed to summarise the current knowledge of allergic sensitisation and established prediction models, the scientific community agrees/recognises that the current mechanistic understanding of factors involved in food protein sensitisation is still incomplete[59-63]. Despite their limitations, current developed epithelial transport models can provide insight into the absorption and transport of proteins and protein-induced barrier disruption and immune activation[59,62]. For instance, monitoring the cytokines TSLP, IL-33, and IL-25, that may be released by epithelial cells upon allergen-induced barrier disruption, can help to interpret inflammatory mechanisms leading to sensitization, as these cytokines promote an overall Th2 environment (see Figure 1)[64-67]. *In vitro* dendritic cell activation assays for the determination of naive T-cell priming and Th1/Th2 polarisation upon antigen/allergen uptake, -processing and -presentation by DCs are comprehensively summarized by Humeniuk et al[17]. Important in these models is to take purity and endotoxin contaminations of the test compound into account as these will reduce the threshold for T-cell activation[68]. On the other hand, the use of animal models that are specifically geared to discovering immunological mechanisms behind sensitisation creates the possibility to integrate cells of the innate and adaptive immune system in all their complexity[69,70]. One should be aware however that the allergen dose and purity, frequency of immunisations and route of administration (intradermal/intragastric/intraperitoneal) during sensitisation and challenge with or without the use of adjuvants, apart from the animal model used[10,71], may greatly influence the risk assessment outcome[72,73]. Inclusion of the food matrix in both *in vitro* and *in vivo* experimental models may also be crucial for the immunostimulatory activity of some pure proteins, as was shown for peanut allergens[74], which seem to possess little intrinsic immune-stimulating capacity when applied in pure form, in contrast to whole peanut extract.

The intrinsic properties of a protein, such as structure, solubility and stability, epithelial transportation and immunological status may be influenced by food manufacturing and processing techniques[75]. Allergen risk assessment studies must take these factors into account by testing the target protein in the form that a person can come into contact with[76]. The route of protein exposure (e.g. respiratory, skin or oral) would for instance define the necessity of a simulated gastrointestinal digestion procedure prior to exposure assays and define which epithelial models

would be applicable to use for transport studies and immunological response assays[59,62]. In addition, food matrix properties can influence gastrointestinal digestibility, as mentioned by Bøgh et al[10] and Pekar et al[77], while interactions of allergens with matrix-originating ligands might be able to dampen certain food processing effects[22].

1.5 Cashew nut as a model

The tropical perennial cashew tree (*Anacardium occidentale* L.), belonging to the botanical family of the Anacardiaceae is likely to be native to South America, but nowadays also grown in North America, Asia, Africa and Australia (for review see Dendena et al[78]). The cashew nut is a kidney-shaped fruit drupe, growing externally from the cashew nut apple, the accessory- or pseudofruit of the cashew tree (Figure 4). The seed kernel is surrounded by a double shell; the reddish-brown peel or testa, and a though leathery outer shell containing the so called cashew nut shell liquid[79].

Figure 4. Cashew nut growing below the cashew nut apple (left) and deshelled nuts with and without peel (right). Photos are obtained from Shutterstock.

Harvested nuts are traditionally sun-dried before processing to bring down the moisture content from 25 to approx. 7-8%[80,81]. Next, nuts are roasted or steam-cooked to increase the brittleness of the shell and loosening of the kernel. Removal of the shell is performed by cracking or cutting after which seeds are oven-heated or roasted to remove the peel. Seed kernels are then graded by size, colour and quality and subsequently packed to extend their shelf life and for easy transportation[78,82]. With a wide-world production of 789,050 metric tons in 2017/18, cashew nut is the third most produced nut, after almond and walnut (1,240,425 and 871,849 metric tons respectively (International Nuts and Dried Fruit Council (INC) statistical yearbook 2017/2018). Counted over a decade, this is a 32% raise in production. Being a transit country, the Netherlands imported over 50 metric tons of shelled cashews in 2016 and with an average of 1015 g consumption per capita, including industrial consumption, the Netherlands was the leading European country of cashew nuts consumption in 2016. This high consumption level might explain the relatively high prevalence and severity of cashew nut allergy in the Netherlands. Le et al[83] estimated that 20% of Dutch tree nut allergic adults suffered from an allergy to cashew nut. Severe symptoms like anaphylaxis are common for a cashew nut allergy and can even be more prevalent compared to symptoms caused by peanuts[84-86]. Associated to this, the minimal dose of cashew nut protein eliciting objective allergic symptoms in 5% of patients (ED_{05}) as tested in a

Dutch cohort, was 1 mg[86]. This equals just 3-5 mg of cashew nut kernel when taking into account the nut's protein content of 19%-36%[87-88].

Up till now, only three cashew nut allergenic proteins have been identified and to some extent characterised for stability and immunogenicity[89-91], which have been designated Ana o 1, Ana o 2 and Ana o 3 as reviewed by Mendes et al[92] and van der Valk et al[93] (Table 2). The major food allergen Ana o 1, a 7S vicilin-like protein of 62-63 kDa, was identified in 2002 using cDNA library immunoscreening[94]. Two isoforms exist, Ana o 1.0101 and Ana o 1.0102 which differ in only a single nucleotide. Multiple linear epitopes have been identified of which 3 appear to be immuno-dominant[94]. Epitope-stretches participating in conformational epitopes have also been suggested[95].

The major allergenic globulin protein in cashew is Ana o 2, an 11S legumin protein of 52 kDa[96]. Monomeric Ana o 2 consists of a light chain of *ca*. 21 kDa and a 30-33 kDa heavy chain polypeptide which can dimerize[91,96,97]. Only one isoform has been cloned and in which one conformational epitope[98] and multiple linear epitopes have been identified[96], of which some seem to be part of predicted conformational epitopes[99].

Table 2. The cashew nut allergens. Table modified from Mendes et al[92].

Allergen	Isoforms	MW* (kDa)	Length (aa)	Nucleotide (NCBI)	Protein (NCBI)	Protein (Uniprot)
Ana o 1	Ana o 1.0101	62.8	538	AF395894	AAM73730	Q8L5L5
	Ana o 1.0102	61.6	536	AF395893	AAM73729	Q8L5L6
Ana o 2	Ana o 2.0101	52.0	457	AF453947	AAN76862	Q8GZP6
Ana o 3	Ana o 3.0101	16.3	138	AY081853	AAL91665	Q8H2B8

* Based on coding sequence

In 2005, the 2S albumin allergen Ana o 3 was cloned[100]. Mature Ana o 3 (14 kDa) is composed of a small (3-4 kDa) and a large (8-10 kDa) subunit linked and stabilised by two inter-chain and intra-chain disulphide bonds[100,101]. The existence of allelic variances (isoallergens) and isoforms has been suggested[91,100], although only one sequence (Ana o 3.0101) has been elucidated thus far. Epitope mapping revealed the large subunit of Ana o 3 to be the most IgE-reactive[91]. For the diagnosis of cashew nut allergy, IgE-binding specificity against the identified major seed storage allergens Ana o 1, 2 and 3 have found to be of clinical predictive value[102,103]. Although albumins (45.6%) and globulins (42.4%) form the predominant proteins in cashew nut[104], it is well established that multiple allergenic protein families exist in nuts and seeds (Table 1) which makes it very likely that also cashew nut would harbour additional allergens that are not taken into account for clinical allergy diagnostics and treatment strategies today.

2. Aims and order of thesis chapters

The limited knowledge of immunogenic proteins present in cashew nut that underlie the elicitation and severity of allergic symptoms in a cashew nut allergy, makes cashew nut a suitable model food source to identify and isolate novel allergens. The aim of this thesis is to apply different bio-molecular characterisation techniques to identify putative novel allergens in cashew nut and to characterise their immunological capacity and relevance for cashew nut allergy diagnostics.

In **Chapter 2** of this thesis, a review is presented discussing the applicability of *in vivo* murine-type food allergy models to assess the sensitisation and elicitation capacity of food proteins. The experimental **Chapters 3 and 4** describe the use of immuno-inhibition techniques to assess cross-sensitisation and cross-reactivity profiles in cashew nut allergic patients which are indicative for the existence of novel allergens in cashew nut. By using SDS-PAGE-based immunoblot inhibition in chapter 3, putative novel allergens were identified in cashew and related Anacardiaceae species (pistachio and pink peppercorn) which presented IgE cross-reactive activity *in vitro*. In chapter 4, the IMMULITE coated-beads technology of Siemens Healthcare GmbH was applied to assess specific cross-sensitisation patterns of cashew nut allergic children to peanut and hazelnut. For accurate diagnostics and medical treatment purposes, once a patient is diagnosed with a cashew nut allergy it is important to diagnose precisely which allergens and epitope regions are causing the observed symptoms in order to select the most effective treatment. As the currently known allergen repertoire of cashew nut is likely incomplete (as concluded from chapter 3 and 4), we used next generation sequencing to create an RNAseq cDNA library which allows identification of putative allergenic proteins based on sequence homology. **Chapter 5** describes the identification of PR10-like proteins in cashew nut that, based on *in silico* risk analysis are predicted to be cross-reactive in birch-pollen driven allergies. In **Chapter 6** we elucidate the sequence of novel Ana o 3 isotypes and how to obtain pure fractions for each of the isotypes for subsequent IgE-binding capacity testing. Also, a first step has been taken in comparing the epitope regions between the different isotypes to predict their allergenicity *in vivo*. The results and implications of previous chapters are discussed in **Chapter 7** and new findings, theories and future perspectives for molecular diagnosis and prevention of cashew nut allergy are provided.

References

1. Valenta R, Hochwallner H, Linhart B, Pahr S. Food allergies: the basics. Gastroenterology. 2015;148(6):1120-31.e4.
2. Tordesillas L, Berin MC, Sampson HA. Immunology of Food Allergy Immunity. 2017;47(1):32-50.
3. Tang ML, Mullins RJ. Food allergy: is prevalence increasing? Intern Med J. 2017;47(3):256-261.
4. Yue D, Ciccolini A, Avilla E, Waserman S. Food allergy and anaphylaxis. J Asthma Allergy. 2018; 11:111–120.
5. Nwaru BI, Hickstein L, Panesar SS, Roberts G, Muraro A, Sheikh A; EAACI Food Allergy and Anaphylaxis Guidelines Group. Prevalence of common food allergies in Europe: a systematic review and meta-analysis. Allergy. 2014;69(8):992-1007.
6. Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. J Allergy Clin Immunol. 2014;133(2):291–307. e295
7. Savage J, Johns CB. Food allergy: epidemiology and natural history. Immunol Allergy Clin North Am. 2015;35(1):45-59.
8. Fine AJ. Hypersensitivity reaction to kiwi fruit (Chinese gooseberry, Actinidia chinensis). J Allergy Clin Immunol. 1981;68(3):235-7.
9. Mills EN, Mackie AR, Burney P, Beyer K, Frewer L, Madsen C, Botjes E, Crevel RW, van Ree R. The prevalence, cost and basis of food allergy across Europe. Allergy. 2007;62:717–722.
10. Bøgh KL, Madsen CB. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? Crit Rev Food Sci Nutr. 2016;56(9):1545-67.
11. Mazzucchelli G, Holzhauser T, Cirkovic Velickovic T, Diaz-Perales A, Molina E, Roncada P, Rodrigues P, Verhoeckx K, Hoffmann-Sommergruber K. Current (Food) Allergenic Risk Assessment: Is It Fit for Novel Foods? Status Quo and Identification of Gaps. Mol Nutr Food Res. 2018;62(1).
12. Aalberse RC. Structural biology of allergens. J Allergy Clin Immunol 2000; 106:228-238.
13. Koppelman SJ, Luykx DM, de Jongh HH, Veldhuizen WJ. Physicochemical characterization of allergens: quantity, identity, purity, aggregation and conformation. Arb Paul Ehrlich Inst Bundesinstitut Impfstoffe Biomed Arzneim Langen Hess. 2009;96:39-53; Diskussion 53-4.
14. Smith E. Food allergy and intolerance: an international chemical safety perspective. Environ Toxicol Pharmacol. 1997;4(1-2):3-7.
15. Ring J, Krämer U, Schäfer T, Behrendt H. Why are allergies increasing? Curr Opin Immunol. 2001;13(6):701-8.
16. Humeniuk P, Dubiela P, Hoffmann-Sommergruber K. Dendritic Cells and Their Role in Allergy: Uptake, Proteolytic Processing and Presentation of Allergens. Int J Mol Sci. 2017;18(7):1491.
17. Vercelli D, Geha RS. Regulation of isotype switching. Curr Opin Immunol. 1992;4(6):794-797.
18. Romagnani S. The Th1/Th2 paradigm. Immunol Today. 1997;18(6):263-6.
19. Muraille E, Leo O. Revisiting the Th1/Th2 paradigm. Scand J Immunol. 1998;47(1):1-9.
20. Blank U, Rivera J. The ins and outs of IgE-dependent mast-cell exocytosis. Trends in Immunol. 2004;25:266-273.
21. Satitsuksanoa P, Jansen K, Głobińska A, Van de Veen W, Akdis M. Regulatory Immune Mechanisms in Tolerance to Food Allergy. Front Immunol. 2018;9:2939.
22. Breiteneder H, Mills EN. Molecular properties of food allergens. J Allergy Clin Immunol. 2005;115(1):14-23; quiz 24.
23. Hauser M, Egger M, Wallner M, Wopfner N, Schmidt G, Ferreira F. Molecular Properties of Plant Food Allergens: A Current Classification into Protein Families. Open Immunol J. 2008;1:1-12.
24. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol. 2008;121: 847-52.
25. Valenta R, Kraft D. Type 1 allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. J Allergy Clin Immunol. 1996;97:893–895.
26. Crespo JF, Rodriguez J. Food allergy in adulthood. Allergy. 2003;58:98-113.
27. Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Colombo G, Crivellaro M, De Carli M, Della Torre E, Della Torre F, Heffler E, Lodi Rizzini F, Longo R, Manzotti G, Marcotulli M, Melchiorre A, Minale P, Morandi P, Moreni B, Moschella A, Murzilli F, Nebiolo F, Poppa M, Randazzo S, Rossi G, Senna GE. Causes of food-induced anaphylaxis in Italian adults: a multi-centre study. Int Arch Allergy Immunol. 2009;150(3):271-7.
28. Han Y, Kim J, Ahn K. Food allergy. Korean J Pediatr. 2012;55:153–158.

29. Harrer A, Egger M, Gadermaier G, Erler A, Hauser M, Ferreira F, Himly M. Characterization of plant food allergens: an overview on physicochemical and immunological techniques. Mol Nutr Food Res. 2010;54(1):93-112.
30. Egawa G. Pathomechanism of 'skin-originated' allergic diseases. Immunol Med. 2018;41(4):170-176.
31. Price A, Ramachandran S, Smith GP, Stevenson ML, Pomeranz MK, Cohen DE. Oral allergy syndrome (pollen-food allergy syndrome). Dermatitis. 2015;26(2):78-88.
32. van Zuuren EJ, Terreehorst I, Tupker RA, Hiemstra PS, Akkerdaas JH. Anaphylaxis after consuming soy products in patients with birch pollinosis. Allergy. 2010;65(10):1348-9.
33. Ando K, Watanabe D, Tamada Y, Matsumoto Y. Oral allergy syndrome with severe anaphylaxis induced by pistachio. Int J Dermatol 2011;50(5):632Y633.
34. Kleine-Tebbe J, Herold DA. Cross-reactive allergen clusters in pollen-associated food allergy. Hautarzt. 2003;54:130.
35. García BE, Lombardero M, Echechipía S, Olaguibel JM, Díaz-Perales A, Sánchez-Monge R, Barber D, Salcedo G, Tabar AI. Respiratory allergy to peach leaves and lipid transfer proteins. Clin Exp Allergy. 2004;34:291-295.
36. Borghesan F, Mistrello G, Roncarolo D, Amato S, Plebani M, Asero R. Respiratory allergy to lipid transfer protein. Int Arch Allergy Immunol. 2008;147:161-165.
37. Barber D, de la Torre F, Lombardero M, Antépara I, Colas C, Dávila I, Tabar AI, Vidal C, Villalba M, Salcedo G, Rodríguez R. Component-resolved diagnosis of pollen allergy based on skin testing with profilin, polcalcin and lipid transfer protein pan-allergens. Clin Exp Allergy. 2009;39:1764-1773.
38. Lombardero M, Garcia-Selles FJ, Polo F, et al. Prevalence of sensitization to Artemisia allergens Art v 1, Art v 3 and Art v 60 kDa. Cross-reactivity among Art v 3 and other relevant lipid-transfer protein allergens. Clin Exp Allergy. 2004;34(9):1415-21.
39. van Ree R. Clinical importance of non-specific lipid transfer proteins as food allergens. Biochem Soc Trans. 2002;30:910–913.
40. Kondo Y, Urisu A. Oral allergy syndrome. Allergol Int. 2009;58(4):485-91.
41. Hayes M, Rougé P, Barre A, Herouet-Guicheney C, Roggen EL. In silico tools for exploring potential human allergy to proteins. Drug Discov Today: Disease Models. 2015;17-18:3-11.
42. Palacin A, Gomez-Casado C, Rivas LA, et al. Graph based study of allergen cross-reactivity of plant lipid transfer proteins (LTPs) using microarray in a multicenter study. PLoS One. 2012;7(12):e50799.
43. Palacin A, Rivas LA, Gomez-Casado C, et al. The involvement of thaumatin-like proteins in plant food cross-reactivity: a multicenter study using a specific protein microarray. PLoS One. 2012;7(9):e44088.
44. Breiteneder H. Mapping of conformational IgE epitopes of food allergens. Allergy. 2018;73(11):2107-2109.
45. Chen X, Dreskin SC. Application of phage peptide display technology for the study of food allergen epitopes. Mol Nutr Food Res. 2017;61(6).
46. Zhang Q, Yang J, Bautista J, Badithe A, Olson W, Liu Y. Epitope Mapping by HDX-MS Elucidates the Surface Coverage of Antigens Associated with High Blocking Efficiency of Antibodies to Birch Pollen Allergen. Anal Chem. 2018;90(19):11315-11323.
47. Rashid A, Sadroddiny E, Ye HT, Vratimos A, Sabban S, Carey E, Helm B. Review: Diagnostic and therapeutic applications of rat basophilic leukemia cells. Mol Immunol. 2012;52(3-4):224-8.
48. Barsumian EL, Isersky C, Petrino MG, Siraganian RP. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. Eur J Immunol. 1981;11(4):317-23.
49. Gilfillan AM, Kado-Fong H, Wiggan GA, Hakimi J, Kent U, Kochan JP. Conservation of signal transduction mechanisms via the human Fc epsilon RI alpha after transfection into a rat mast cell line, RBL 2H3. J Immunol. 1992;149(7):2445-51.
50. Wilson AP, Pullar CE, Camp AM, Helm BA. Human IgE mediates stimulus secretion coupling in rat basophilic leukemia cells transfected with the alpha chain of the human high-affinity receptor. Eur J Immunol. 1993;23(1):240-4.
51. Vogel L, Luttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. Allergy. 2005;60:1021-1028.
52. Wiegand TW, Williams PB, Dreskin SC, Jouvin MH, Kinet JP, Tasset D. High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. J Immunol. 1996;157:221-30.

53. Blanc F, Adel-Patient K, Drumare MF, Paty E, Wal JM, Bernard H. Capacity of purified peanut allergens to induce degranulation in a functional in vitro assay: Ara h 2 and Ara h 6 are the most efficient elicitors. Clin Exp Allergy. 2009;39:1277-1285.
54. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, Rao VK, Metcalfe DD. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res. 2003;27(8):677-82.
55. McGowan EC, Saini S. Update on the performance and application of basophil activation tests. Curr Allergy Asthma Rep. 2013;13(1):101-9
56. Santos AF, Lack G. Basophil activation test: food challenge in a test tube or specialist research tool? Clin Transl Allergy. 2016;6:10.
57. Gellerstedt M, Bengtsson U, Niggemann B. Methodological issues in the diagnostic work-up of food allergy: a real challenge. J Investig Allergol Clin Immunol. 2007;17(6):350-6.
58. Zuidmeer L, Goldhahn K, Rona RJ, Gislason D, Madsen C, Summers C, Sodergren E, Dahlstrom J, Lindner T, Sigurdardottir ST, McBride D, Keil T. The prevalence of plant food allergies: a systematic review. J Allergy Clin Immunol. 2008;121(5):1210-1218.e4.
59. Cubells-Baeza N, Verhoeckx KCM, Larre C, Denery-Papini S, Gavrovic-Jankulovic M, Diaz Perales A. Applicability of Epithelial Models in Protein Permeability/Transport Studies and Food Allergy. Drug Discov Today: Disease Models. 2015;17-18:13-21.
60. van Bilsen JHM, Sienkiewicz-Szłapka E, Lozano-Ojalvo D, Willemsen LEM, Antunes CM, Molina E, Smit JJ, Wróblewska B, Wichers HJ, Knol EF, Ladics GS, Pieters RHH, Denery-Papini S, Vissers YM, Bavaro SL, Larré C, Verhoeckx KCM, Roggen EL. Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins. Clin Transl Allergy. 2017;7:13.
61. Bøgh KL, van Bilsen J, Głogowski R, López-Expósito I, Bouchaud G, Blanchard C, Bodinier M, Smit J, Pieters R, Bastiaan-Net S, de Wit N, Untersmayr E, Adel-Patient K, Knippels L, Epstein MM, Noti M, Nygaard UC, Kimber I, Verhoeckx K, O'Mahony L. Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. Clin Transl Allergy. 2016;6:21.
62. Gavrovic-Jankulovic M, Willemsen LEM. Epithelial models to study food allergen induced barrier disruption and immuneactivation. Drug Disc Today: Dis Models. 2016; DDMOD-426.
63. Noti M. New perspectives on the initiation of allergic immune responses at barrier sites. Curr Opin Immunol. 2018;54:130-136.
64. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt Rd R, Bazan F, Kastelein RA, Liu YJ. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol. 2002;3(7):673-80.
65. Mjösberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, Fokkens WJ, Cupedo T, Spits H. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol. 2011;12(11):1055-62.
66. Licona-Limón P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. Nat Immunol. 2013;14(6):536-42.
67. Halim TY, Steer CA, Mathä L, Gold MJ, Martinez-Gonzalez I, McNagny KM, McKenzie AN, Takei F. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. Immunity. 2014;40(3):425-35.
68. Velickovic TC, Jankov RM. Design and modifications of allergens for improving specific immunotherapy. Inflamm Allergy Drug Targets. 2008;7(4):270-8.
69. Van Gramberg JL, de Veer M, O'Hehir RE, Meeusen EN, Bischof RJ. Use of animal models to investigate major allergens associated with food allergy. J Allergy (Cairo). 2013;2013:635695.
70. Smit JJ, Noti M, O'Mahony L. The use of animal models to discover immunological mechanisms underpinning sensitization to food allergens. Drug Discov Today: Dis Models. 2015;17-18:63-69.
71. Ladics GS, Fry J, Goodman R, Herouet-Guicheney C, Hoffmann-Sommergruber K, Madsen CB, Penninks A, Pomés A, Roggen EL, Smit J, Wal JM. Allergic sensitization: screening methods. Clin Transl Allergy. 2014;4(1):13.
72. Liu T, Navarro S, Lopata AL. Current advances of murine models for food allergy. Mol Immunol 2016;70:104-17.
73. Arámburo-Galvez JG, Sotelo-Cruz N, Flores-Mendoza LK, Gracia-Valenzuela MH, Chiquete-Elizalde FIR, Espinoza-Alderete JG, Trejo-Martínez H, Canizalez-Román VA, Ontiveros N,

Cabrera-Chávez F. Assessment of the Sensitizing Potential of Proteins in BALB/c Mice: Comparison of Three Protocols of Intraperitoneal Sensitization. Nutrients. 2018;10(7).
74. van Wijk F, Nierkens S, Hassing I, Feijen M, Koppelman SJ, de Jong GA, Pieters R, Knippels LM. The effect of the food matrix on in vivo immune responses to purified peanut allergens. Toxicol Sci. 2005;86:333-341.
75. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, Herouet-Guicheney C, Holzhauser T, Shimojo R, van der Bolt N, Wichers H, Kimber I. Food processing and allergenicity. Food Chem Toxicol. 2015;80:223-240.
76. Mazzucchelli G, Holzhauser T, Cirkovic Velickovic T, Diaz-Perales A, Molina E, Roncada P, Rodrigues P, Verhoeckx K, Hoffmann-Sommergruber K. Current (Food) Allergenic Risk Assessment: Is It Fit for Novel Foods? Status Quo and Identification of Gaps. Mol Nutr Food Res. 2018;62(1):1700278.
77. Pekar J, Ret D, Untersmayr E. Stability of allergens. Mol Immunol. 2018;100:14-20.
78. Dendena B, Corsi S. Cashew, from seed to market: a review. Agron Sustain Dev 2014;34:753-772.
79. Tyman JHP, Morris LJ. The composition of cashewnut-shell liquid (CNSL) and the detection of a novel phenolic ingredient. J Chromatogr A. 1967;27:287–288.
80. Asogwa EU, Hammed LA, Ndubuaku TCN. Integrated production and protection practices of cashew (Anacardium occidentale) in Nigeria. Afr J Biotechnol. 2008;7:25.
81. Mohod A, Jain S, Powar AG. Energy option for small scale cashew nut processing in India. Energy Res J. 2010;1(1):47.
82. Fitzpatrick J (2011) Cashew nut processing equipment study-summary. African Cashew initiative.http://www.africancashewalliance.com/sites/default/files/documents/equipment_study_ab_pdf_final_13_9_2011.pdf.
83. Le TM, Lindner TM, Pasmans SG, Guikers CL, van Hoffen E, Bruijnzeel-Koomen CA, Knulst AC. Reported food allergy to peanut, tree nuts and fruit: comparison of clinical manifestations, prescription of medication and impact on daily life. Allergy. 2008;63(7):910-6
84. Davoren M, Peake J. Cashew nut allergy is associated with a high risk of anaphylaxis. Arch Dis Childhood. 2005;90:1084-1085.
85. Clark AT, Angnostou K, Ean PW. Cashew nut causes more severe reactions than peanut: case matched comparison in 141 children. Allergy. 2007;62:913-916.
86. van der Valk JP, Gerth van Wijk R, Dubois AE, de Groot H, Reitsma M, Vlieg-Boerstra B, Savelkoul HF, Wichers HJ, de Jong NW. Multicentre Double-Blind Placebo-Controlled Food Challenge Study in Children Sensitised to Cashew Nut. PLoS One. 2016;11(3):e0151055.
87. Venkatachalam M, Sathe SK. Chemical composition of selected edible nut seeds. J Agric Food Chem. 2006;54(13):4705-14.
88. Aremu MO, Olonisakin A, Bako DA, Madu PC. Compositional studies and physicochemical characteristics of cashew nut (Anarcadium occidentale) flour. Pak J Nutr. 2006;5(4):328–333.
89. Teuber SS, Sathe SK, Peterson WR, Roux KH. Characterization of the soluble allergenic proteins of cashew nut (Anacardium occidentale L.). J Agric Food Chem. 2002;50:6543–6549.
90. Venkatachalam M, Monaghan EK, Kshirsagar HH, Robotham JM, O'Donnell SE, Gerber MS, Roux KH, Sathe SK. Effects of processing on immunoreactivity of cashew nut (Anacardium occidentale L.) seed flour proteins. J Agric Food Chem. 2008;56(19):8998-9005.
91. Reitsma M, Bastiaan-Net S, Sforza S1, van der Valk JP2, van Gerth van Wijk R2, Savelkoul HF, de Jong NW2, Wichers HJ. Purification and Characterization of Anacardium occidentale (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3. J Agric Food Chem. 2016;64(5):1191-201.
92. Mendes C, Costa J, Vicente AA, Oliveira MB, Mafra I. Cashew Nut Allergy: Clinical Relevance and Allergen Characterisation. Clin Rev Allergy Immunol. 2019;57(1):1-22.
93. van der Valk JP, Dubois AE, Gerth van Wijk R, Wichers HJ, de Jong NW. Systematic review on cashew nut allergy. Allergy. 2014;69(6):692-8.
94. Wang F, Robotham JM, Teuber SS, Tawde P, Sathe SK, Roux KH. Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family. J Allergy Clin Immunol. 2002;110(1):160-6.
95. Guan X, Noble KA, Tao Y, Roux KH, Sathe SK, Young NL, Marshall AG. Epitope mapping of 7S cashew antigen in complex with antibody by solution-phase H/D exchange monitored by FT-ICR mass spectrometry. J Mass Spectrom. 2015;50(6):812-9.
96. Wang F, Robotham JM, Teuber SS, Sathe SK, Roux KH. Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family. Int Arch Allergy Immunol. 2003;132:27-39.
97. Teuber SS, Sathe SK, Peterson WR, Roux KH. Characterization of the soluble allergenic proteins of cashew nut (Anacardium occidentale L.). J Agric Food Chem. 2002;50(22):6543-9.

98. Robotham JM, Xia LX,Willison LN, Teuber SS, Sathe SK, Roux KH. Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope. Mol Immunol. 2010;47:1830–1838.
99. Barre A, Jacquet G, Sordet C, Culerrier R, Rougé P. Homology modelling and conformational analysis of IgEbinding epitopes of Ara h 3 and other legumin allergens with a cupin fold from tree nuts. Mol Immunol. 2007;44:3243–3255.
100. Robotham JM, Wang FW, Vest VM, Teuber SS, Sathe SK, Beyer K, Roux KH. Ana o 3, an important cashew nut (Anacardium occidentale L.) allergen of the 2S albumin family. J Allergy Clin Immunol. 2005;115(6):1284-90.
101. Shewry PR, Napier JA, Tatham AS. Seed storage proteins-structures and biosynthesis. Plant Cell. 1995;7:945–956.
102. Lange L, Lasota L, Finger A, Vlajnic D, Büsing S, Meister J, Broekaert I, Pfannenstiel C, Friedrichs F, Price M, Trendelenburg V, Niggemann B, Beyer K. Ana o 3-specific IgE is a good predictor for clinically relevant cashew allergy in children. Allergy. 2017;72(4):598-603.
103. van der Valk JP, Gerth van Wijk R, Vergouwe Y, Steyerberg EW, Reitsma M, Wichers HJ, Savelkoul HF, Vlieg-Boerstra B, de Groot H, Dubois AE, de Jong NW. sIgE Ana o 1, 2 and 3 accurately distinguish tolerant from allergic children sensitized to cashew nuts. Clin Exp Allergy. 2017;47(1):113-120.
104. Sathe SK. Solubilization and electrophoretic characterization of cashew nut (Anacardium occidentale) proteins. Food Chem. 1994;51:319-324.

Chapter 2

Overview of *in vivo* and *ex vivo* endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins?

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Abstract

Significant efforts are necessary to introduce new dietary protein sources to feed a growing world population while maintaining food supply chain sustainability. Such a sustainable protein transition includes the use of highly modified proteins from side streams or the introduction of new protein sources that may lead to increased clinically relevant allergic sensitization. With food allergy being a major health problem of increasing concern, understanding the potential allergenicity of new or modified proteins is crucial to ensure public health protection. The best predictive risk assessment methods currently relied on are *in vivo* models, making the choice of endpoint parameters a key element in evaluating the sensitizing capacity of novel proteins. Here, we provide a comprehensive overview of the most frequently used *in vivo* and *ex vivo* endpoints in murine food allergy models, addressing their strengths and limitations for assessing sensitization risks. For optimal lab-to-lab reproducibility and reliable use of predictive tests for protein risk assessment, it is important that researchers maintain and apply the same relevant parameters and procedures. Thus, there is an urgent need for a consensus on key food allergy parameters to be applied in future food allergy research in synergy between both knowledge institutes and clinicians.

Keywords: food allergy, animal models, biomarkers, prevention

1. Introduction

A variety of *in vitro* and *in vivo* models have been developed that address the factors and mechanisms involved in the sensitization to food proteins[1-4]. Currently, approaches are being developed using protein chemistry and *in vitro* and *in silico* methods to characterize food proteins and derivatives that arise during product processing and reformulation, which may explain why certain food proteins induce sensitization of the immune system, while others are tolerated[5,6]. However, elucidating the mechanisms underlying allergen sensitization is a complex, multidimensional problem that often requires a wide range of additional *in vivo* and *ex vivo* experimentation[5], as a wide range of molecules, tissues and cells play a role in the mechanisms underlying food allergen sensitization[1]. For instance, epithelial release of thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-25 and IL-33 upon local epithelial stress support type 2 helper T (Th2) cell pathology by attracting IL-4 secreting lymphoid cells, basophils, and invariant natural killer T (iNKT) cells[7]. Il-4 promotes surface expression of Th2-costimulatory molecule OX40 ligand on dendritic cells (DCs)[8] and cytokine secretion by Th2 lymphoid cells (ILC2s), which further augments DC activity and suppresses allergen-specific regulatory T (Treg) cells[9,10]. This complexity, as depicted in Figure 1, illustrates the need for experimental food allergy models that integrate such complex cell-tissue communication to assess the sensitization potential of new protein sources.

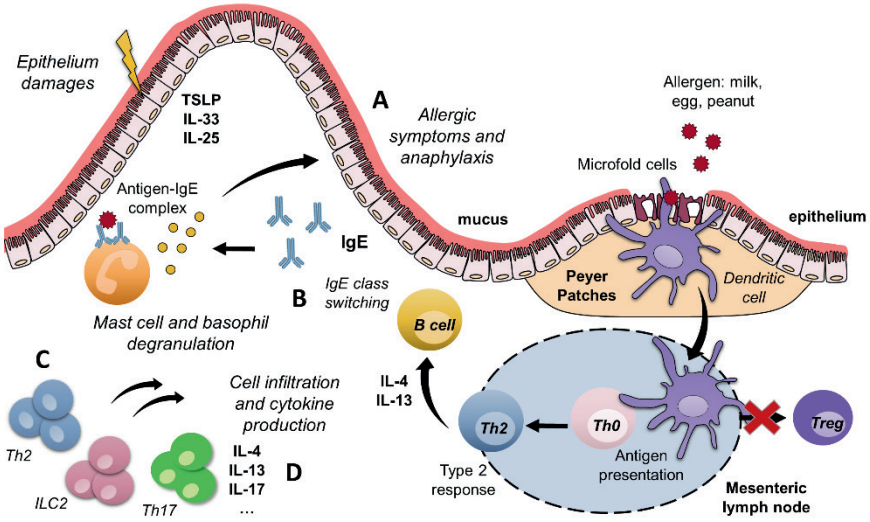

Figure 1. Immune mechanisms of food allergy and its associated principal measured endpoints. (A) Assessment of allergic symptoms (body temperature) after allergen challenge, (B) Evaluation of immunoglobulin (IgE) in serum, (C) Phenotyping of T cells population, (D) Cytokine production in response to allergen restimulation (ex vivo assay).

Murine food allergy models, even though they have their limitations, are currently the best predictive models available to evaluate the food-sensitizing capacity of new food proteins before introducing them into the human diet. Although researchers aim to reduce the use of experimental animals to address the 3R principle that guide animal experimentation to replace (alternative model), reduce (minimize number of animals) and refine (minimize animal pain and enhance animal welfare), there is a lack of replacement models such as *in silico* prediction models, *in vitro* primary cell assays, or tissue explants assays that are able to characterize and predict the human responses to food proteins.

In the past, numerous experimental food allergy models have been developed to assess food allergenicity. However, interlaboratory differences in the models used with respect to sensitization and elicitation route, choice of adjuvant, clinical signs, genetic background of the animals, housing conditions and microbiomes composition and metabolic activity in the different vivaria often make it difficult to draw generalized conclusions[5]. It is important to note that almost all models (except genetic models) require adjuvants to trigger sensitization. Therefore, the choice of the adjuvants together with the exposure route are crucial points to consider. In addition, there are numerous *in vivo*, *ex vivo* and *in vitro* parameters evaluated for the assessment of food allergy. Figure 2 illustrates the types of *in vivo* (inside a living organism) or *ex vivo* (outside an organism) methodology and endpoints used in experimental murine models of food allergy. However, there is a need to establish a list of reliable, validated and effective endpoint parameters to guide researchers working with animal models of food allergy. In this review, we describe a selective list of the most commonly used experimental applied endpoints in food allergies with a focus on milk, egg and peanut allergens and critically evaluate their applicability for evaluating sensitization potency. Each endpoint was selected and critically described with strengths and limitation based on consortium experience and occurrence in literature.

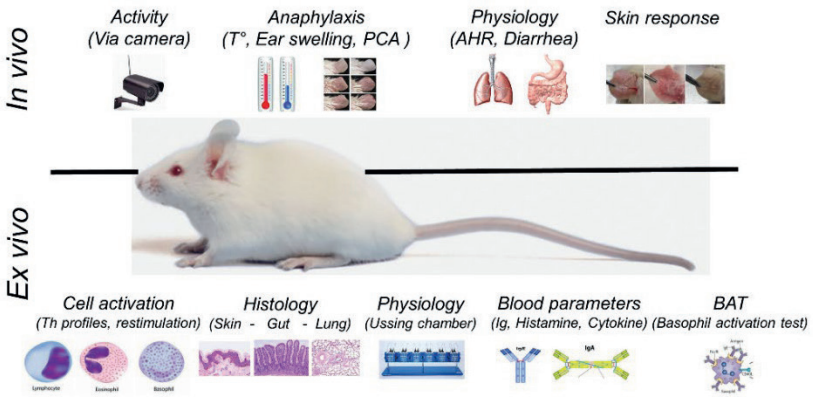

Figure 2. In vivo and ex vivo methodological endpoints used in murine food allergy models.

2. Endpoints

2.1 Measurement of body temperature

In murine-type models of food allergy to milk, eggs and peanuts, a drop in the core body temperature is often observed after repetitive allergen challenge. This change in body temperature is an indicator of anaphylaxis (Table 1). Temperature is measured before and 30 min to 1h after allergen challenge, but this parameter can also be monitored over time[11,12]. Animals sensitized to a given food matrix or protein may display a significant reduction in body temperature (0.5 to 10°C)[3,4] compared to that of naive animals. For an adequate level of sensitivity, 5 to 16 animals per group should have their temperatures measured using a rectally inserted thermal probe[13], but it is also possible to measure changes over time for individual animals using an electronic ID transponder implanted subcutaneously[14,15]. To refine, improve and objectify the currently applied manual monitoring methods, an automatic imaging method has been developed[16]. It involves a noninvasive measurement of the whole-body surface temperature paired with assessment of activity (see also supplementary section about activity/behavior via camera). Anaphylaxis imaging has been used in three *in vivo* allergy mouse models for i) milk allergy, ii) egg allergy and iii) peanut allergy in proof-of-principle experiments and suggests that imaging technology represents a reliable noninvasive method for objective monitoring of small animals during anaphylaxis over time. This method can be useful for monitoring diseases associated with changes in both body temperature and physical behavior.

Strengths:

- *The measurement of core body temperature is a cost-effective, reliable assessment of the allergic reaction*
- *Therapeutic or preventative strategies for the reduction of allergic reactions can be easily evaluated*
- *Can be used to evaluate the severity of allergic shock and differences between allergens subjected to physical transformations (i.e. native versus processed)*

Limitations:

- *The occurrence of anaphylaxis is dependent on the mouse strain used: Balbc or C3H mice are prone to develop anaphylaxis whereas C57BL/6 or A/J mice necessitate stringent exposure protocols to achieve sensitization*
- *The clinical score may be biased as a consequence of the laboratory environment, stress level, animal strain and technical experimenter*
- *A decrease in temperature is only observed after a food/allergen challenge after a previous sensitization event; this endpoint therefore contains no predictive value for the sensitization potential of a food protein*

Table 1. Table of studies measuring body temperature in allergic reactions

Mouse/rat model	Allergen	Number of animals	δT°	Therapeutic or preventive strategy	Conditions of measure	System of measurement	Ref.
C3H/HeOuJ	Whey	N=6-12	4°C	Prevention with omega-3 long chain polyunsaturated fatty acids	1 h after challenge	Implantable electronic ID transponder	15
C3H/HeOuJ	OVA	N=4-6	5°C	Prevention with prebiotics: scGOS/lcFOS/pAOS	30 min after challenge	Implantable electronic ID transponder	14
BALB/c	Beta-lact	N=10	1.7°C	Prevention with ratios of omega-6 and omega-3 fatty acids	Before, 30 and 45 min after challenge	Rectal probe	42
BALB/c	Peanut, egg, milk	N=3-5	3°C	Anaphylaxis imaging	Monitoring after challenge	Imaging method for whole-body surface temperature	16
C3H/HeN	Whey	N=12-15	4°C	Microbiota composition and allergy protection	Before and 45 minutes after challenge	Rectal probe	43
BN	OVA	N=8	3°C	To develop an effective and rapid model of FA in Brown Norway rats	60, 90 and 120 min after challenge	Rectal probe	44
BALB/c	BLG	N=5	5°C	Nitration process of allergen	Before and 15 and 30 min after i.v.	Rectal probe	45
BALB/c	OVA	N=10	0.5°C	Microbiota composition and allergy protection	Before and 5 and 10 min after i.v. OVA challenge	Rectal probe	46
BALB/c	OVA	N=5	1.5°C	Anti-acid medication for risk of food allergy	Before and 15 min after challenge	Rectal probe	47
BALB/c	OVA	N=10	None	Heat process on allergen	30 min after challenge	Rectal probe	48
C3H/HeOuJ	Whey	N= 10	2 to 10°C	Hydrolyze process on allergen	Measurement of temperature over time: 0, 15, 30, 60, 120 minutes after challenge	A programmable temperature transponder implanted subcutaneously	11
C3H/HeOuJ	Caseinate	N=6	5°C	Transglutaminase cross-linked caseinate process	Measurement of temperature over time: 0, 15, 30, 60, after challenge	A programmable temperature transponder implanted subcutaneously	12
BALB/cTac	OVA	N=5-10	5°C	Microbiota signature in allergy	Measurement every 5 minutes: 5 to 60 minutes	Rectal probe coupled to a Physitemp Thermalert Model TH-5	49

Table 1. Table of studies measuring body temperature in allergic reactions (continued)

Mouse/rat model	Allergen	Number of animals	δT°	Therapeutic or preventive strategy	Conditions of measure	System of measurement	Ref.
BALB/c	OVA	N=10	1°	To develop models of food allergy and oral tolerance	30 min after challenge	Rectal probe	50
C57BL/6	Peanut	N=4-10	2°C	Commensal bacteria for allergy protection	After challenge	Rectal probe	51
C3H/HeJ and BALB/c	Peanut	N=5	3 to 5°C	Skin sensitization study	30 min after challenge	Rectal thermometer (WPI Instruments)	52
C3H/HeJ and BALB/c	OVA or peanut	N=5	4°C	Epicutaneous immunotherapy	30 min after challenge	Rectal thermometer (WPI Instruments)	32
C3H/HeJ	BLG	N=10	3°C	Pasteurization process on milk allergen	After challenge	Rectal probe	33

Technical recommendations:

- *Using a rectal probe, mice or rats must be acclimated to the experimental room at least one hour before starting the temperature measurements to obtain stable values*
- *The rectal temperature must be evaluated 10 min to 1.5 hours after the challenge*
- *The animal temperature can be registered over time using a programmable temperature transponder implanted subcutaneously*

2.2 Evaluation of immunoglobulins in serum

While in vivo measurements are essential to assess the elicitation of an allergic response, they do not provide insight into de novo allergen sensitization. Therefore, blood, tissue or organs must be collected and further analyzed by ex vivo methods. Serum immunoglobulin (Ig) content is the most common parameter measured when evaluating sensitization to food allergens in animal models, followed by fecal IgA (see supplementary section), as antibody responses are considered a direct indicator of allergen sensitization together with mast cell and basophil degranulation.

IgE is the most common Ig isotype measured when evaluating the allergenicity of food proteins and is regularly quantified in parallel with IgG1 (Table 2). Total and antigen-specific Ig levels can be analyzed, where the latter is a measure of how dosing with a given food or protein influences the overall level of IgE or IgG. Serum-specific IgE and IgG can be quantified by a series of different ex vivo methods, where ELISAs are the most commonly applied, followed by immunoblotting methods and mediator release assays (Figure 3). Whereas specific IgG in general is measured by means of an indirect ELISA[17], specific IgE is most often measured by antibody-capture ELISA[18]. In fact, IgE is the least abundant Ig isotype in serum (with an approximate amount of only one IgE for every 50,000 IgGs[19]), making it difficult for IgE to compete for binding to proteins coated on ELISA plates. Other methods of measuring specific IgE include enzyme allergosorbent test (EAST) immunoblotting[20]. When measuring specific IgEs by means of in-house-developed antibody-capture ELISAs, there is a need for coupling the protein of interest to a molecule against which labeled secondary Igs are commercially available, as secondary Igs for direct binding to the proteins of interest can rarely be purchased. Molecules coupled to the protein of interest are most often digoxigenin (DIG)[17] or biotin[21], with the additional advantage that they serve as signal amplifiers (Figure 3).

Not only is the total level of specific Igs of interest in evaluating the sensitization response in animal models, the increase in affinity between Igs and the allergen is also important. Studies have shown that the binding strength between specific IgEs

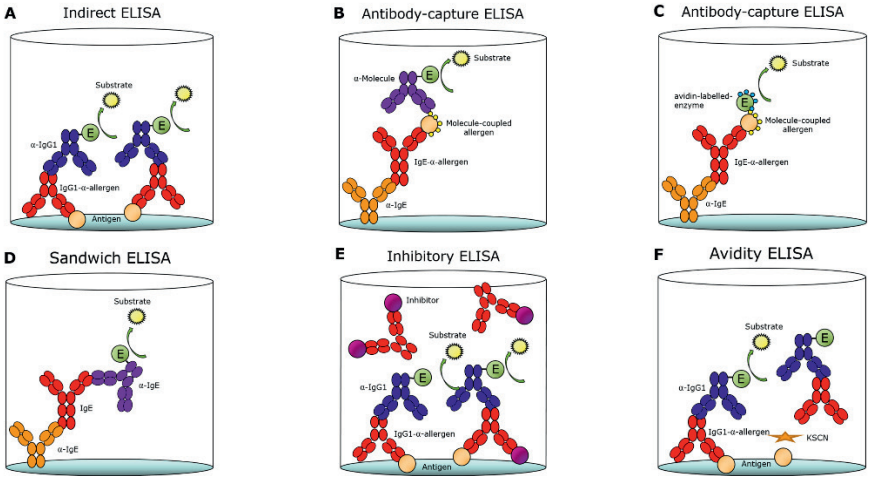

Figure 3. ELISA methods. Antibodies (Abs) can be evaluated by means of different ELISA.

and the corresponding allergens is of great importance for the induction of a degranulation response and thereby the severity of the allergic disease[22,23]. The avidity can be measured by means of simple potassium thiocyanate (KSCN) ELISAs which have shown that no general relationship exists between the level and avidity of specific Igs[24,25], though a correlation may be observed during a multiple antigen exposure immune response. This method, although not very sensitive, is based on the ratio of the areas derived from the curves obtained by plotting the OD and log of the sera dilution in the ELISA experiment with and without thiocyanate treatment. Where measures of specific IgE only allow for evaluation of sensitization, they provide no indication of the biological relevance of the IgEs present in the serum and thereby the clinical relevance of the food allergy model.

To provide insights into the biological relevance of secreted IgEs, functional tests should be performed, such as the in vivo temperature drop, a skin prick test (SPT) or evaluation of challenge-derived symptoms. Further, ex vivo mediator release tests such as the rat basophilic leukemia (RBL) assay and basophil activation test (BAT) enable an evaluation of the biological relevance of the IgE raised in food allergy animal models (see supplementary section for description and opinion about mediator release assays and additional passive cutaneous anaphylaxis (PCA) and active cutaneous anaphylaxis (ACA) models).

Strengths:

- *Specific IgE antibody analysis is the most trustworthy measure of sensitization*
- *Measures of specific IgE antibodies are often used to evaluate not only sensitization but also the potential severity of the allergic reaction after a second encounter*

Table 2. Studies using measurements of Igs from serum.

Mouse/rat model	Allergen	Sensitization and challenge	Ig measured in serum	Aim of the study	Ref
BALB/c	OVA	I.g. + CT followed by i.g. challenge	IgG1, IgG2a: indirect ELISA IgE: Ab-capture ELISA	To elucidate the class of bioactive polyphenols that exhibit a beneficial anti-allergic effect and to assess whether the protective effect matches the in vivo bioavailable metabolite concentrations.	21
BALB/c	OVA,	I.p. followed by i.g.	IgG1, IgG2a, IgA: indirect ELISA	To investigate how thermal processing influences the ability of ovalbumin (OVA) to induce allergic symptoms and immune responses in a mouse model of food allergy.	48
C57BL/6J	OVA	Oral (by feeding) + s.c. + alum followed by i.d. (ear test) challenge	IgG1: indirect ELISA IgE: Ab-capture ELISA	To investigate the potential of hydrolyzed egg and whole egg to induce tolerance by the oral route (i.e., by feeding).	53
BN	OVA	I.p. + alum + tBp followed by i.g. challenge	IgG1, IgG2a, IgG2b, IgA: indirect ELISA IgE: Ab-capture ELISA	To develop an effective and rapid model of food allergy with only one i.p. injection of the allergen with alum together with toxin from Bordetella pertussis (tBp) to promote IgE production and two weeks later the i.g. administration of allergens.	44
BALB/c	WPC, WPH or BLG	I.p. with BLG + alum followed by oral (solution in drinking bottle) challenge with WPC and WPH	IgE: Ab-capture ELISA	To develop an experimental murine model of food allergy to the cow's protein ß-lactoglobulin (BLG) that mimics the main clinical characteristics of human disease as well as to examine the allergenic and immunological properties of extensively hydrolyzed whey proteins.	54
BALB/c	BLG	I.p. + alum or FCA or FIA	IgG1, IgG2a, IgE: indirect ELISA	To study the sensitizing capacity of BLG and the influence of the use of adjuvant.	55
BALB/c	Whole milk	I.g. +/- CT followed by i.g. challenge	IgG1, IgG2a: indirect ELISA IgE: EAST (enzyme allergosorbent test)	To map epitopes of the major soybean allergen Gly m 5 that are corecognized by casein-specific antibodies and to identify a peptide responsible for the cross-reactivity.	20
C3H HeOuJ	WPC, ALA, BLG	I.g. + CT	IgG1, IgE: indirect ELISA	To test a panel of high and low allergenic proteins.	56
C3H HeOuJ PF	WPC or WPH	I.g. + CT followed by i.g. + i.d. challenge	IgG1, IgE: indirect ELISA	To validate a mouse model for cow's milk allergy to assess the potential allergenicity of hydrolyzed cow's milk-based infant formulas.	11

Table 2. Studies using measurements of Igs from serum (continued).

Mouse/rat model	Allergen	Sensitization and challenge	Ig measured in serum	Aim of the study	Ref
BN	Ara h 1	I.p.	IgG1, IgG2a: indirect ELISA IgE: Ab-capture ELISA	To investigate the ability of digested protein - Ara h 1 to sensitize.	17
BN	WPH, BLG	I.p. +/- alum	IgG2a, IgE: indirect ELISA IgG1: inhibitory ELISA	To provide a thorough analysis of the immunogenicity and allergenicity of hydrolyzed cow's milk proteins for use in infant formulas.	57
BALB/c	Extract	I.c. + CpG + CT or + non/CpG + CT followed by i.g. + CT challenge	IgG1, IgG2a, IgE, IgA: indirect ELISA	To evaluate the effect of the application peanut extract (PE) alone or mixed with CT and unmethylated sequences (CpG) as adjuvant on the intact skin.	58
BALB/cJ	Roasted extract or Ara h 1	I.n. or e.c. followed by i.g. +/- CT challenge	IgG1: indirect ELISA IgE: Ab-capture ELISA	To assess the impact of repeated short-term epicutaneous (e.c.) applications on intact skin or after repeated intranasal (i.n). administration of food allergens from roasted peanut.	59
BALB/c	Ara h 1, Ara h 2, Ara h 3, Ara h 6 and Ara h 6 with no S-S bridges	I.p. + alum	IgG1, IgE: indirect ELISA (protein G for IgG removal)	To investigate the impact of heat processing of peanut seed on the sensitization to native Ara h 6.	60
C3H/HeJ	Extract	I.g. + CT followed by i.p. challenge	IgG1, IgG2a: indirect ELISA IgE, IgA: Ab-capture ELISA	To reveal the immune responses that are induced against peanuts allergens during sensitization, including the very early responses.	61
C3H/HeJ	Whole peanut	I.g. + CT followed by i.g. challenge	IgE: indirect ELISA	To develop a murine model of IgE-mediated peanut allergy that closely mimics human peanut allergy.	62
BN	Ara h 1	I.p.	IgG1, IgG2a: indirect ELISA and inhibitory ELISA for IgG1 IgE: Ab-capture ELISA Total IgE: sandwich ELISA	To study the sensitizing capacity of four different 7S proteins and to determine whether related proteins would induce similar sensitization when removed from their 'normal' matrix.	63
BN	Ara h 1	I.p.	IgG1, IgG2a: indirect ELISA IgE: Ab-capture ELISA	To investigate the ability of digested protein - Ara h 1 to sensitize.	17

- *Measurements of antibodies can be performed without the use of advanced equipment such as a cytometer or robotics*

Limitations:

- *Assays often need to be developed in-house, restricting the possibilities for comparison between labs*
- *IgE only accounts for a fraction of all serum antibodies, requiring more advanced ELISAs for analysis of specific IgE*
- *IgE levels do not predict the clinical severity of a food allergy model, and other ex vitro experiments are needed to further address this parameter*
- *Measures with optical density (OD) as the unit only allow for one serum dilution*

Technical recommendations:

- *Antibody-capture ELISAs should be used for the measurement of specific IgE*
- *Other antibody parameters in addition to the amount of total and specific antibodies are relevant and should be measured, such as clonality and avidity*
- *Measures of total and specific antibodies should always be expressed as titer values or as concentrations with no upper or lower limit for dilutions*
- *Serum depleted of IgG using protein G columns before use in indirect ELISAs needs to be considered*

2.3 Phenotyping of T cell populations

Assessment of serum Ig levels provides important information about the sensitization phase but does not allow for quantification of immune cell responses, including cellular infiltration to sites of allergic inflammation. The phenotyping of innate (*e.g.*, macrophages, eosinophils, basophils, neutrophils, dendritic cells) and adaptive (B and T cells) responses is indispensable for assessing the mechanisms of allergic sensitization (Table 3). Immune cells are generally isolated from organs, including the mesenteric lymph nodes, spleen, lung, skin or intestine, and analyzed by flow cytometry. Typically, allergic inflammation is characterized by a predominantly type 2 immune response and secretion of the canonical type 2 cytokines IL-4, IL-5, IL-9 and IL-13 by innate immune cells (*e.g.*, eosinophils, basophils, mast cells (MCs), type 2 innate lymphoid cells and polarized Th2 cells)[18,19]. Indeed, in mice specifically expressing the ovalbumin-T cell receptor, sensitization to ovalbumin in their diet induced the expansion of IL-4-producing $CD4^+$ T cells in mesenteric lymph nodes, the spleen and Peyer's patches[26]. Importantly, adoptive transfer of antigen-specific CD4+ T cells derived from mesenteric lymph nodes of OVA-sensitized mice is sufficient to transfer allergen-induced diarrhea to naïve recipients. The recipient mice also display an upregulation of the Th2-related chemokines CCL17 and CCL22 in the small intestine[27]. In addition to polarized Th2 responses, the proportion of other common T cell subtypes, such as Th1 and Th17 that are characterized by the production of IFN-γ and IL-17, respectively, can also be elevated in lymphoid

organs of allergic mice. In contrast, expansion and/or the regulatory capacity of $CD25^+$ $Foxp3^+$ T cells associated with tolerance are often compromised in many food allergy models[28]. Additionally, other T cell subtypes can be involved in food allergy pathogenesis. The recently discovered Th9 subset and associated IL-9 secretion were found to be involved in food allergy and especially in peanut allergies[29]. IL-9 is mainly responsible for the production of IL-4 by Th2 cells to promote mucosal mast cell accumulation and secretion of mucus and chemokines by epithelial cells to sustain allergic inflammation[30]. To a lesser extent, γδT cells found in the intestinal epithelium and in the lamina propria were also shown to be involved in food allergy. These cells are involved in blocking the induction of tolerance and modulating inflammatory responses[31].

Strengths:

- *Precise mechanistic insights into the cellular response in isolated organs and tissues support the sensitizing potential of food proteins when combined with additional readouts*
- *Precise determination of the T cell profile by using specific markers of the T cell population*
- *Quantitative evaluation of the infiltrating cell population by flow cytometry*

Limitations:

- *Analysis of cell populations without the contribution of neighboring cell tissue (loss of microenvironment)*
- *Isolation of immune cells from tissues relies on enzymatic digestion protocols and may thus alter phenotypical and functional properties of the cells of interest*
- *Difficulty with the separation of minor subpopulations*
- *Sacrifice of the animal is required for organ and tissue sampling*
- *Need for sophisticated equipment such as FACS*
- *Type 2 immune response-associated mucus production in tissues makes cell isolation difficult and can create bias in cell phenotyping and frequencies*

Technical recommendations:

- *Remove fat and store organs, tissues and cells at 4°C to avoid uncontrolled cell death or degradation of surface markers*
- *Perform flow cytometry and culturing the same day as the animal sacrifice*
- *Phenotyping of T cells can be achieved by intracellular cytokine/transcription factor staining using flow cytometry*

2.4 Cytokine production in response to allergen restimulation

The logical follow-up to analysis of infiltration/expansion of innate and adaptive immune cells in the tissues and organs is the evaluation of cytokine secretion. This evaluation comes directly from serum or from lymphatic tissue cells restimulated *ex vivo*. Food allergen stimulation of only lymphatic tissue cells, or in coculture

Table 3. Studies using immune infiltrate as readout of allergic inflammation to egg, milk and peanut proteins

Mouse/rat Model	Allergen	Allergen sensitization	Allergen challenge	Immune infiltrate	Method	Therapeutic/preventive strategy	Ref
C57BL/6 or BALB/c	OVA, crude peanut extract	Skin	intragastric	Mast cells, eosinophils	Flow cytometry for intestinal eosinophils /mast cells, chloroacetate esterase staining for mast cells in jejunum, H&E staining	Anti-TSLP treatment or basophil depletion limits food allergen sensitization and the development of intestinal food allergy	64
C57BL/6	OVA	Skin	intragastric	Mast cells, eosinophils	Flow cytometry for intestinal eosinophils /mast cells, chloroacetate esterase staining for mast cells in jejunum, H&E staining	Targeting basophil-derived IL-4 reduces food allergen sensitization and limits intestinal food allergy	7
BALB/c	Raw or roasted peanut extracts	Skin	intragastric	Eosinophils	Flow cytometry for eosinophils in the small intestinal lamina propria; H&E staining jejunum	N.A.	65
BALB/c	OVA	Skin	intragastric	Eosinophils	Flow cytometry for peripheral eosinophils; H&E staining jejunum	Basophil depletion attenuates intestinal allergy; CD4 T cell depletion limits TSLP-mediated intestinal food allergy	66
BALB/c	OVA	Systemic	intragastric	Mast cells, CD4 T cells	H&E staining jejunum, chloroacetate esterase staining of mast cells in the jejunum; flow cytometry of CD4+ T cells in the small intestinal lamina propria	Treatment with mast cell stabilizing cromolyn sodium protects against food allergen sensitization	67
BALB/c	OVA	Skin	intragastric	Mast cells	Chloroacetate esterase staining of connective tissue mast cells in the jejunum	Targeting of IgE responses prevented intestinal mast cell expansion and anaphylaxis	68
various	OVA	Intestine	intragastric	Allergen-specific Tregs that acquire Th2 mast cells	Flow cytometry of small intestinal Foxp3+ Tregs; chloroacetate esterase staining of connective tissue mast cells in the jejunum	N.A.	69
BALB/c	OVA; whole peanut extract	Intestine	intragastric	Mast cells; eosinophils	H&E staining jejunum; pinacyanol erythrosine staining to determine mast cell numbers and granulation status in the jejunum	N.A.	70

Table 3. Studies using immune infiltrate as readout of allergic inflammation to egg, milk and peanut proteins (continued).

Mouse/rat Model	Allergen	Allergen sensitization	Allergen challenge	Immune infiltrate	Method	Therapeutic/preventive strategy	Ref
BALB/c	OVA	Skin	intragastric	Mast cells	Chloroacetate esterase staining of mast cells in the jejunum	ST2 blockade attenuates food-induced anaphylaxis	71
C3H/HeJ; BALB/c	OVA; ground peanut	Skin; systemic	intragastric	Lap+ Tregs	Flow cytometry for Lap+ Tregs in the lamina propria	Clinical protection induced by epicutaneous immunotherapy (EPIT)	72
BALB/c	BLG	Intestine	intragastric	Lamina propria lymphocytes	ELISPOT IL-12, IL-17 producing lymphocytes from the intestinal lamina propria	Blocking TSLP signaling prevents food allergy	73

with dendritic cells, allows for the immunophenotyping of the immune cell populations specific for the exposed food antigen or matrix. To confirm allergen specificity, splenocytes, mesenteric lymph node cells or lamina propria cells isolated from sensitized and/or challenged mice are restimulated with corresponding allergenic proteins or peptides. After culture for up to 5 days, cytokines associated with the inflammatory response (IL-4, IL-5, IL-13, IL-17, and IFN-γ) and the regulatory response (IL-10 and TGF-β) are analyzed in the supernatants by ELISA[26-30] or a multiplex system. The cytokine production indicates whether T cells were primed toward the challenged food proteins and distinguishes Th1 or Th2 cell type responses. The prototypical type 2 cytokines include IL-4, IL-5 and IL-13. While IL-4 is critical for the polarization of Th2 cells and IgE class-switching in B cells[30], IL-5 promotes the activation, proliferation and survival of eosinophils, and IL-13 induces mucus production from goblet cells. Additional assays may be used including proteomics and gene expression profiling by PCR or microarray technology, that provide mechanistic insights and potential drug targets.

Strengths:

- *Precise assessment of the allergen specificity by restimulating cells with the same allergen used in the animal model*
- *Class determination of the T cell response by evaluation of cytokine production in the supernatant of sorted T cells*
- *Higher production of cytokines can be obtained after proliferation and restimulation with the antigen than by direct measurement in serum*

Limitations:

- *Restimulation with allergens can activate nonspecific T cells due to certain cross-reactivity*
- *Difficult to obtain a level above the sensitivity threshold with cells isolated from naïve mice*
- *Some mechanistic endpoints are not equally important in animals and humans*

Technical recommendations:

- *For allergen presentation, presorted T cells need to be co-cultured with dendritic cells*
- *MHC peptide – tetramers can be used to sort specific T cells and have better assessment of allergen specificity*
- *Need for positive (polyclonal anti-CD3/anti-CD28) and negative control (non-allergen) stimuli to ensure proper T cell responsiveness*
- *Endotoxin levels within the allergen extract need to be controlled to prevent bias in restimulation responses*
- *Ideally, when using gene expression sequencing data, this method should be confirmed with at least one other technology (e.g. flow cytometry)*

- *As cells and mediators associated with immune responses change rapidly, longitudinal assessments of mechanistic endpoints will be more informative than single time point assessments. The timing of the measurements will depend on the research question, e.g., sensitization mechanisms versus mechanisms of acute allergic responses following (re)challenge*

2.5 Future analysis of food allergy models

To date, the methods to study intestinal pathophysiology are *in vitro* culture systems with cell lines or explanted mucosa grown in monolayers[35,36], intestinal organoid cultures[37,38] and ''gut-on-a-chip'' devices[39,40]. These technologies have offered many insights into gut physiology, but they lack cellular complexity, architecture, and immune and inflammatory responses that are crucial for a comprehensive understanding of underlying disease mechanisms and pathways. Alternatively, *in vivo* animal models provide the intact organ in the context of the vascular supply, systemic mediators and circulating cells. However, *in vivo* experiments may be hampered by technical difficulties, including interindividual variability and maintenance of constant and reproducible experimental conditions[5].

To address the limitations of *in vitro* and *in vivo* models of gut disease, Yissachar *et al*[41] developed a chamber unit for culturing 12- to 14-day-old mouse colon or small intestine segments under highly controlled conditions. Of particular interest is that the chamber unit has two paired inputs and outputs that allow for controlled introduction of molecules or microbes into the lumen while simultaneously introducing continuous replenishment of medium to support tissue viability. The tissue remains intact, and the overall structure with epithelial cell layers is preserved for at least 24 hours, making this method suitable for studying epithelial transport of food allergens and their effect on epithelial integrity. However, other measurements are currently difficult due to the very short time that such tissue explants can be maintained. Furthermore, the enteric nervous system structure is maintained, and immune cells are detected as they found in healthy intestinal biopsies. It is possible to envisage the use of this type of *ex vivo* chamber unit in food allergy research by using intestinal fragments from naive, sensitized and allergic animals to introduce a variety of food proteins. It is thus possible to further elucidate pathways involved in luminal physiology and antigen uptake and presentation and make comparisons between known allergenic and non-allergenic proteins. This approach may lead to novel insights into new proteins and cross-reactive proteins and to the development of a predictive model for food allergy.

Additional studies related to the survival and growth of anaerobic and aerobic microbiota revealed that the *ex vivo* colonization of cultured tissue with selected microbes may be possible. Indeed, changes in the composition and metabolic activity of gut microbes can influence all aspects of innate and adaptive immune

processes within the mucosa (see also supplementary section for stool consistency as a readout in food allergy assessment). Thus, focusing on the effect of diverse microbiota profiles and specific bacteria on immunological responses upon the introduction of allergenic proteins may lead to novel mechanisms, therapeutic targets or predictive models. However, intra- and inter-laboratory variability in microbiome composition and metabolic activity after birth as a result of the breeding environment is also a major underlying cause for conflicting results between experiments. This variability must be taken into account beforehand in the experimental design of an animal trial[5]. It is also noteworthy to consider the possible development of highly controlled chamber units for food allergy research used in combination with *in vivo* models to provide a new powerful strategy for studying mechanisms in the intestine.

Strengths:

- *The tissue structure, cellular components and neural system are highly preserved*
- *The model provides the possibility to study immediate responses generated after the introduction of different molecules and microbes*

Limitations:

- *Only short-term responses can be evaluated due to changes that can occur in the tissue over time*
- *Currently, only intestinal segments from 12- to 14-day-old mice have been tested*
- *Tissue preparation and assembly require specific skills*

3. Conclusion

The recent broadening of our knowledge of food allergy pathogenesis and development of murine food allergy models has enabled us to model the allergic elicitation reaction as well as the preceding sensitization events and observe relevant symptoms with different food proteins (milk, egg, and peanut). The principal endpoint parameters described in this review are critical parameters that should be evaluated in a correct manner so that they may be powerful in the different rodent models.

Characterizing a food allergy model using temperature, level of Igs, phenotyping of the cell infiltrate and cytokine production gives an overview of the reaction while providing us insight into the degree of sensitizing capacity of the allergen used. Nevertheless, even though the *in vivo* measurements and the *ex vivo* experiments provide us with many answers about the immune response and the sensitization phase, we still do not have a complete overview of the immune mechanisms

behind each reaction. There is still a strong need to better define the allergic reaction to predict the clinical outcomes of sensitization to novel food proteins. Although the current available models are suitable for studying the pathophysiology of food allergy, they still cannot predict the magnitude of the allergic potential of a particular allergen. Discovering and highlighting the molecules and cells involved in both sensitization and elicitation are necessary to improve risk assessment models and to facilitate the introduction of novel protein sources into our diet with a low risk of allergic sensitization.

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Conflicts of interest
Dr. Blanchard reports other from Nestec, outside the submitted work; Dr. O'Mahony reports personal fees from AHL, grants from GSK, outside the submitted work; The other authors declare no conflict of interest.

Supplementary information
See for supplemental endpoints, Tables and references Doi:10.1111/all.13943 (open access).

References

1. Smit JJ, Noti M, O'Mahony L. The use of animal models to discover immunological mechanisms underpinning sensitization to food allergens. *Drug Discov Today Dis Model* 2015;17–18:63–69.
2. Hussain M, Noti M. Experimental food allergy models to study the role of innate immune cells as initiators of allergen-specific Th2 immune responses. *Drug Discov Today Dis Model* 2015;17–18:55–62.
3. Gavrovic-Jankulovic M, Willemsen LEM. Epithelial models to study food allergen-induced barrier disruption and immune activation. *Drug Discov Today Dis Model* 2015;17–18:29–36.
4. Cubells-Baeza N, Verhoeckx KCM, Larre C, Denery-Papini S, Gavrovic-Jankulovic M, Diaz Perales A. Applicability of epithelial models in protein permeability/transport studies and food allergy. *Drug Discov Today Dis Model* 2015;17–18:13–21.
5. Bøgh KL, van Bilsen J, Głogowski R, López-Expósito I, Bouchaud G, Blanchard C et al. Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. *Clin Transl Allergy* 2016;6:21.
6. Mazzucchelli G, Holzhauser T, Cirkovic Velickovic T, Diaz-Perales A, Molina E, Roncada P et al. Current (Food) Allergenic Risk Assessment: Is It Fit for Novel Foods? Status Quo and Identification of Gaps. *Mol Nutr Food Res* 2018;62:1700278.
7. Hussain M, Borcard L, Walsh KP, Pena Rodriguez M, Mueller C, Kim BS et al. Basophil-derived IL-4 promotes epicutaneous antigen sensitization concomitant with the development of food allergy. *J Allergy Clin Immunol* 2018;141:223–234.e5.
8. Blazquez AB, Berin MC. Gastrointestinal Dendritic Cells Promote Th2 Skewing via OX40L. *J Immunol* 2008;180:4441–4450.
9. Noval Rivas M, Burton OT, Oettgen HC, Chatila T. IL-4 production by group 2 innate lymphoid cells promotes food allergy by blocking regulatory T-cell function. *J Allergy Clin Immunol* 2016;138:801–811.e9.
10. Khodoun M V., Tomar S, Tocker JE, Wang YH, Finkelman FD. Prevention of food allergy development and suppression of established food allergy by neutralization of thymic stromal lymphopoietin, IL-25, and IL-33. *J Allergy Clin Immunol* 2018;141:171–179.e1.
11. van Esch BCAM, van Bilsen JHM, Jeurink PV, Garssen J, Penninks AH, Smit JJ et al. Interlaboratory evaluation of a cow's milk allergy mouse model to assess the allergenicity of hydrolysed cow's milk based infant formulas. *Toxicol Lett* 2013;220:95–102.
12. van Esch BCAM, Gros-van Hest M, Westerbeek H, Garssen J. Sensitizing capacity and allergenicity of enzymatically cross-linked sodium caseinate in comparison to sodium caseinate in a mouse model for cow's milk allergy. *Toxicol Lett* 2013;218:50–55.
13. Li X-M, Srivastava K, Huleatt JW, Bottomly K, Burks AW, Sampson HA. Engineered Recombinant Peanut Protein and Heat-Killed Listeria monocytogenes Coadministration Protects Against Peanut-Induced Anaphylaxis in a Murine Model. *J Immunol* 2003;170:3289–3295.
14. Hogenkamp A, Knippels LMJ, Garssen J, Esch BCAM Van. Supplementation of Mice with Specific Nondigestible Oligosaccharides during Pregnancy or Lactation Leads to Diminished Sensitization and Allergy in the Female. *J of Nutrition* 2015;145:996-1002
15. van den Elsen LWJ, Meulenbroek LAPM, van Esch BCAM, Hofman GA, Boon L, Garssen J et al. CD25+ regulatory T cells transfer n-3 long chain polyunsaturated fatty acids-induced tolerance in mice allergic to cow's milk protein. *Allergy* 2013;68:1562–1570.
16. Manzano-Szalai K, Pali-Schöll I, Krishnamurthy D, Stremnitzer C, Flaschberger I, Jensen-Jarolim E. Anaphylaxis Imaging: Non-Invasive Measurement of Surface Body Temperature and Physical Activity in Small Animals. *PLoS One* 2016;11 e0150819
17. Bøgh KL, Kroghsbo S, Dahl L, Rigby NM, Barkholt V, Mills ENC et al. Digested Ara h 1 has sensitizing capacity in Brown Norway rats. *Clin Exp Allergy* 2009;39:1611–1621.
18. Madsen JL, Kroghsbo S, Madsen CB, Pozdnyakova I, Barkholt V, Bøgh KL. The impact of structural integrity and route of administration on the antibody specificity against three cow's milk allergens - a study in Brown Norway rats. *Clin Transl Allergy* 2014;4:25.
19. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* 2008;8:205–217.
20. Curciarello R, Smaldini PL, Candreva AM, González V, Parisi G, Cauerhff A et al. Targeting a Cross-Reactive Gly m 5 Soy Peptide as Responsible for Hypersensitivity Reactions in a Milk Allergy Mouse Model. *PLoS One* 2014;9:e82341.
21. Singh A, Demont A, Actis-Goretta L, Holvoet S, Lévêques A, Lepage M et al. Identification of epicatechin as one of the key bioactive constituents of polyphenol-enriched extracts that

demonstrate an anti-allergic effect in a murine model of food allergy. *Br J Nutr* 2014;112:358–368.
22. El-Khouly F, Lewis SA, Pons L, Burks AW, Hourihane JO. IgG and IgE avidity characteristics of peanut allergic individuals. *Pediatr Allergy Immunol* 2007;18:607–613.
23. Wang J, Lin J, Bardina L, Goldis M, Nowak-Węgrzyn A, Shreffler WG et al. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *J Allergy Clin Immunol* 2010;125:695–702.e6.
24. Pullen GR, Fitzgerald MG HC. Antibody avidity determination by ELISA using thiocyanate elution. - PubMed - NCBI. J Immunol Methods. 1986;:83–90.
25. Lew AM, Anders RF, Edwards SJ, Langford CJ. Comparison of antibody avidity and titre elicited by peptide as a protein conjugate or as expressed in vaccinia. *Immunology* 1988;65:311.
26. Nakajima-Adachi H, Kikuchi A, Fujimura Y, Shibahara K, Makino T, Goseki-Sone M et al. Peyer's Patches and Mesenteric Lymph Nodes Cooperatively Promote Enteropathy in a Mouse Model of Food Allergy. *PLoS One* 2014;9:e107492.
27. Knight AK, Bele A, Zhang S, Mayer L, Sampson HA, Berin MC et al. CD4 T cells activated in the mesenteric lymph node mediate gastrointestinal food allergy in mice. 2007;:1234–1243.
28. Jonathan H. DeLong, Kelly A. Hetherington, Erik Wambre, Eddie A. James, David Robinson and WWK. Ara h 1-Reactive T Cells in Peanut Allergic Individuals. *J allergy Clin Immunol* 2011;127:1211–1219.
29. Brough HA, Cousins DJ, Munteanu A, Wong YF, Sudra A, Makinson K et al. IL-9 is a key component of memory THcell peanut-specific responses from children with peanut allergy. *J Allergy Clin Immunol* 2014;134:1329–1338.e10.
30. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E et al. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. *J Allergy Clin Immunol* 2011;127:701-21.e1-70.
31. Frossard CP, Asigbetse KE, Burger D, Eigenmann PA. Gut T cell receptor-γδ + intraepithelial lymphocytes are activated selectively by cholera toxin to break oral tolerance in mice. *Clin Exp Immunol* 2015;180:118–130.
32. Tordesillas L, Goswami R, Benedé S, Grishina G, Dunkin D, Järvinen KM et al. Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J Clin Invest* 2014;124:4965–4975.
33. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy* 2008;63:882–890.
34. Saidova A, Hershkop AM, Ponce M, Eiwegger T. Allergen-Specific T Cells in IgE-Mediated Food Allergy. *Arch Immunol Ther Exp (Warsz)* 2018;66:161–170.
35. Haller D, Bode C, Hammes WP, Pfeifer AM, Schiffrin EJ, Blum S. Non-pathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut* 2000;47:79–87.
36. Tsilingiri K, Barbosa T, Penna G, Caprioli F, Sonzogni A, Viale G et al. Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model. *Gut* 2012;61:1007–1015.
37. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009;459:262–265.
38. Clevers H. Modeling Development and Disease with Organoids. *Cell* 2016;165:1586–1597.
39. Kim HJ, Li H, Collins JJ, Ingber DE. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc Natl Acad Sci* 2016;113:E7–E15.
40. Kim HJ, Huh D, Hamilton G, Ingber DE. Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab Chip* 2012;12:2165.
41. Yissachar N, Zhou Y, Ung L, Lai NY, Mohan JF, Ehrlicher A et al. An Intestinal Organ Culture System Uncovers a Role for the Nervous System in Microbe-Immune Crosstalk. *Cell* 2017;168:1135–1148.e12.
42. Thang CL, Boye JI, Shi HN, Zhao X. Effects of supplementing different ratios of omega-3 and omega-6 fatty acids in western-style diets on cow's milk protein allergy in a mouse model. *Mol Nutr Food Res* 2013;57:2029–2038.
43. Rodriguez B, Prioult G, Hacini-Rachinel F, Moine D, Bruttin A, Ngom-Bru C et al. Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell

response. *FEMS Microbiol Ecol* 2012;79:192–202.
44. Abril-Gil M, Garcia-Just A, Pérez-Cano FJ, Franch À, Castell M. Development and Characterization of an Effective Food Allergy Model in Brown Norway Rats. *PLoS One* 2015;10:e0125314.
45. Diesner SC, Schultz C, Ackaert C, Oostingh GJ, Ondracek A, Stremnitzer C et al. Nitration of β-Lactoglobulin but Not of Ovomucoid Enhances Anaphylactic Responses in Food Allergic Mice. *PLoS One* 2015;10:e0126279.
46. Diesner SC, Bergmayr C, Pfitzner B, Assmann V, Krishnamurthy D, Starkl P et al. A distinct microbiota composition is associated with protection from food allergy in an oral mouse immunization model. *Clin Immunol* 2016;173:10–18.
47. Diesner SC, Knittelfelder R, Krishnamurthy D, Pali-Schöll I, Gajdzik L, Jensen-Jarolim E et al. Dose-dependent food allergy induction against ovalbumin under acid-suppression: A murine food allergy model. *Immunol Lett* 2008;121:45–51.
48. Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, Srutkova D et al. Heat-Induced Structural Changes Affect OVA-Antigen Processing and Reduce Allergic Response in Mouse Model of Food Allergy. *PLoS One* 2012;7:e37156.
49. Noval Rivas M, Burton OT, Wise P, Zhang Y, Hobson SA, Garcia Lloret M et al. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. *J Allergy Clin Immunol* 2013;131:201–212.
50. Perrier C, Corthésy B. Gut permeability and food allergies. *Clin Exp Allergy* 2011;41:20–28.
51. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK et al. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A* 2014;111:13145–13150.
52. Tordesillas L, Rahman AH, Hartmann BM, Sampson HA, Berin MC. Mass cytometry profiling the response of basophils and the complete peripheral blood compartment to peanut. *J Allergy Clin Immunol* 2016;138:1741–1744.e9.
53. Hacini-Rachinel F, Vissers YM, Doucet-Ladevéze R, Blanchard C, Demont A, Perrot M et al. Low-Allergenic Hydrolyzed Egg Induces Oral Tolerance in Mice. *Int Arch Allergy Immunol* 2014;164:64–73.
54. Gomes-Santos AC, Fonseca RC, Lemos L, Reis DS, Moreira TG, Souza AL et al. Hydrolyzed whey protein prevents the development of food allergy to ??-lactoglobulin in sensitized mice. *Cell Immunol* 2015;298:47–53.
55. Adel-Patient K, Nahori M-A, Proust B, Lapa e Silva JR, Creminon C, Wal J-M et al. Elicitation of the allergic reaction in beta-lactoglobulin-sensitized Balb/c mice: biochemical and clinical manifestations differ according to the structure of the allergen used for challenge. *Clin Exp Allergy* 2003;33:376–385.
56. Smit J, Zeeuw-Brouwer M de, van Roest M, de Jong G, van Bilsen J. Evaluation of the sensitizing potential of food proteins using two mouse models. *Toxicol Lett* 2016;262:62–69.
57. Bøgh KL, Barkholt V, Madsen CB. Characterization of the Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates - A Study in Brown Norway Rats. *Scand J Immunol* 2015;81:274–283.
58. Adel-Patient K, Ah-Leung S, Bernard H, Durieux-Alexandrenne C, Créminon C, Wal J-M. Oral Sensitization to Peanut Is Highly Enhanced by Application of Peanut Extracts to Intact Skin, but Is Prevented when CpG and Cholera Toxin Are Added. *Int Arch Allergy Immunol* 2007;143:10–20.
59. Wavrin S, Bernard H, Wal JM, Adel-Patient K. Cutaneous or respiratory exposures to peanut allergens in mice and their impacts on subsequent oral exposure. *Int Arch Allergy Immunol* 2014;164:189–199.
60. Guillon B, Bernard H, Drumare MF, Hazebrouck S, Adel-Patient K. Heat processing of peanut seed enhances the sensitization potential of the major peanut allergen Ara h 6. *Mol Nutr Food Res* 2016;60:2722–2735.
61. van Wijk F, Hartgring S, Koppelman SJ, Pieters R, Knippels LMJ. Mixed antibody and T cell responses to peanut and the peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 in an oral sensitization model. *Clin Exp Allergy* 2004;34:1422–1428.
62. Li X-M, Serebrisky D, Lee S-Y, Huang C-K, Bardina L, Schofield BH et al. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000;106:150–158.
63. Kroghsbo S, Bøgh KL, Rigby NM, Mills ENC, Rogers A, Madsen CB. Sensitization with 7S Globulins from Peanut, Hazelnut, Soy or Pea Induces IgE with Different Biological Activities Which Are Modified by Soy Tolerance. *Int Arch Allergy Immunol* 2011;155:212–224.
64. Noti M, Kim BS, Siracusa MC, Rak GD, Kubo M, Moghaddam AE et al. Exposure to food

allergens through inflamed skin promotes intestinal food allergy through the thymic stromal lymphopoietin-basophil axis. *J Allergy Clin Immunol* 2014;133:1390–1399, 1399.e1-6.
65. Moghaddam AE, Hillson WR, Noti M, Gartlan KH, Johnson S, Thomas B et al. Dry roasting enhances peanut-induced allergic sensitization across mucosal and cutaneous routes in mice. *J Allergy Clin Immunol* 2014;134:1453–1456.
66. Han H, Thelen TD, Comeau MR, Ziegler SF. Thymic stromal lymphopoietin-mediated epicutaneous inflammation promotes acute diarrhea and anaphylaxis. *J Clin Invest* 2014;124:5442–5452.
67. Forbes EE, Groschwitz K, Abonia JP, Brandt EB, Cohen E, Blanchard C et al. IL-9– and mast cell–mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J Exp Med* 2008;205:897–913.
68. Bartnikas LM, Gurish MF, Burton OT, Leisten S, Janssen E, Oettgen HC et al. Epicutaneous sensitization results in IgE-dependent intestinal mast cell expansion and food-induced anaphylaxis. *J Allergy Clin Immunol* 2013;131:451–460.e6.
69. Noval Rivas M, Burton OT, Wise P, Charbonnier L-M, Georgiev P, Oettgen HC et al. Regulatory T Cell Reprogramming toward a Th2-Cell-like Lineage Impairs Oral Tolerance and Promotes Food Allergy. *Immunity* 2015;42:512–523.
70. Ganeshan K, Neilsen C V., Hadsaitong A, Schleimer RP, Luo X, Bryce PJ. Impairing oral tolerance promotes allergy and anaphylaxis: A new murine food allergy model. *J Allergy Clin Immunol* 2009;123:231–238.e4.
71. Galand C, Leyva-Castillo JM, Yoon J, Han A, Lee MS, McKenzie ANJ et al. IL-33 promotes food anaphylaxis in epicutaneously sensitized mice by targeting mast cells. *J Allergy Clin Immunol* 2016;138:1356–1366.
72. Tordesillas L, Mondoulet L, Blazquez AB, Benhamou P-H, Sampson HA, Berin MC. Epicutaneous immunotherapy induces gastrointestinal LAP + regulatory T cells and prevents food-induced anaphylaxis. *J Allergy Clin Immunol* 2017;139:189–201.e4.
73. Frossard CP, Zimmerli SC, Rincon Garriz JM, Eigenmann PA. Food allergy in mice is modulated through the thymic stromal lymphopoietin pathway. *Clin Transl Allergy* 2015;6:2.
74. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA et al. Mast cells are required for experimental oral allergen–induced diarrhea. *J Clin Invest* 2003;112:1666–1677.
75. Wang J-H, Fan S-W, Zhu W-Y. Development of Gut Microbiota in a Mouse Model of Ovalbumin-induced Allergic Diarrhea under Sub-barrier System. *Asian-Australasian J Anim Sci* 2013;26:545–551.
76. Yang B, Xiao L, Liu S, Liu X, Luo Y, Ji Q et al. Exploration of the effect of probiotics supplementation on intestinal microbiota of food allergic mice. *Am J Transl Res* 2017;9:376–385.
77. Kraneveld AD, Sagar S, Garssen J, Folkerts G. The two faces of mast cells in food allergy and allergic asthma: The possible concept of Yin Yang. *Biochim Biophys Acta - Mol Basis Dis* 2012;1822:93–99.
78. Dahlin JS, Hallgren J. Mast cell progenitors: Origin, development and migration to tissues. *Mol Immunol* 2015;63:9–17.
79. Tshori S, Nechushtan H. Mast cell transcription factors—Regulators of cell fate and phenotype. *Biochim Biophys Acta - Mol Basis Dis* 2012;1822:42–48.
80. Kunii J, Takahashi K, Kasakura K, Tsuda M, Nakano K, Hosono A et al. Commensal bacteria promote migration of mast cells into the intestine. *Immunobiology* 2011;216:692–697.
81. Yang B, Yang C, Wang P, Li J, Huang H, Ji Q et al. Food Allergen-Induced Mast Cell Degranulation is Dependent on PI3K-Mediated Reactive Oxygen Species Production and Upregulation of Store-Operated Calcium Channel Subunits. *Scand J Immunol* 2013;78:35–43.
82. Santos AF, Lack G. Basophil activation test: food challenge in a test tube or specialist research tool? *Clin Transl Allergy* 2016;6:10.
83. McGowan EC, Saini S. Update on the Performance and Application of Basophil Activation Tests. *Curr Allergy Asthma Rep* 2013;13:101–109.
84. Torrero MN, Larson D, Hübner MP, Mitre E. CD200R surface expression as a marker of murine basophil activation. *Clin Exp Allergy* 2009;39:361–369.
85. Bakocevic N, Claser C, Yoshikawa S, Jones LA, Chew S, Goh CC et al. CD41 is a reliable identification and activation marker for murine basophils in the steady state and during helminth and malarial infections. *Eur J Immunol* 2014;44:1823–1834.
86. Iwamoto H, Matsubara T, Nakazato Y, Namba K, Takeda Y. Decreased expression of CD200R3 on mouse basophils as a novel marker for IgG1-mediated anaphylaxis. *Immunity, Inflamm Dis* 2015;3:280–288.

87. Bridts CH, Sabato V, Mertens C, Hagendorens MM, De Clerck LS, Ebo DG. Flow Cytometric Allergy Diagnosis: Basophil Activation Techniques. In: *Methods in molecular biology (Clifton, N.J.)*. 2014:1192: 147–159.
88. Perrier C, Thierry A-C, Mercenier A, Corthésy B. Allergen-specific antibody and cytokine responses, mast cell reactivity and intestinal permeability upon oral challenge of sensitized and tolerized mice. *Clin Exp Allergy* 2009;40:153–162.
89. de Theije CGM, Wu J, Koelink PJ, Korte-Bouws GAH, Borre Y, Kas MJH et al. Autistic-like behavioural and neurochemical changes in a mouse model of food allergy. *Behav Brain Res* 2014;261:265–274.
90. Smaldini P, Curciarello R, Candreva A, Rey MA, Fossati CA, Petruccelli S et al. In vivo Evidence of Cross-Reactivity between Cow's Milk and Soybean Proteins in a Mouse Model of Food Allergy. *Int Arch Allergy Immunol* 2012;158:335–346.
91. Zhou C, Ludmila T, Sun N, Wang C, Pu Q, Huang K et al. BALB/c mice can be used to evaluate allergenicity of different food protein extracts. *Food Agric Immunol* 2016;27:589–603.
92. Srivastava KD, Siefert A, Fahmy TM, Caplan MJ, Li X-M, Sampson HA. Investigation of peanut oral immunotherapy with CpG/peanut nanoparticles in a murine model of peanut allergy. *J Allergy Clin Immunol* 2016;138:536–543.e4.
93. Negaoui H, El Mecherfi KE, Tadjer SA, Grar H, Kheroua O, Saidi D. Bovine lactoferrin allergenicity as studied in murine model of allergy. *Food Agric Immunol* 2016;27:711–723.
94. Kucuk ZY, Strait R, Khodoun M V., Mahler A, Hogan S, Finkelman FD. Induction and suppression of allergic diarrhea and systemic anaphylaxis in a murine model of food allergy. *J Allergy Clin Immunol* 2012;129:1343–1348.
95. Kinney SRM, Carlson L, Ser-Dolansky J, Thompson C, Shah S, Gambrah A et al. Curcumin Ingestion Inhibits Mastocytosis and Suppresses Intestinal Anaphylaxis in a Murine Model of Food Allergy. *PLoS One* 2015;10:e0132467.
96. Herouet-Guicheney C, Aldemir H, Bars R, de Barbeyrac D, Kennel P, Rouquié D et al. Inter-laboratory comparisons of assessment of the allergenic potential of proteins in mice. *J Appl Toxicol* 2009;29:141–148.
97. Dearman RJ, Skinner RA, Herouet C, Labay K, Debruyne E, Kimber I. Induction of IgE antibody responses by protein allergens: inter-laboratory comparisons. *Food Chem Toxicol* 2003;41:1509–1516.
98. Dearman RJ, Kimber I. Assessment of Protein Allergenicity Following Systemic Exposure in Mice: Relationship with Immunogenicity. *Toxicology* 2009;262:1.
99. Arakawa T, Deguchi T, Sakazaki F, Ogino H, Okuno T, Ueno H. Supplementary seleno-L-methionine suppresses active cutaneous anaphylaxis reaction. *Biol Pharm Bull* 2013;36:1969–1974.
100. Park J-H, Choi T-S. Splenocyte proliferation and anaphylaxis induced by BSA challenge in a D-galactose-induced aging mouse model. *Cent Eur J Immunol* 2016;3:324–327.
101. Ogino H, Sakazaki F, Okuno T, Arakawa T, Ueno H. Oxidized dietary oils enhance immediate- and/or delayed-type allergic reactions in BALB/c mice. *Allergol Int* 2015;64:66–72.
102. Inagaki N, Miura T, Nagai H, Koda A. Active cutaneous anaphylaxis (ACA) in the mouse ear. *Jpn J Pharmacol* 1992;59:201–208.
103. Makabe-Kobayashi Y, Hori Y, Adachi T, Ishigaki-Suzuki S, Kikuchi Y, Kagaya Y et al. The control effect of histamine on body temperature and respiratory function in IgE-dependent systemic anaphylaxis. *J Allergy Clin Immunol* 2002;110:298–303.
104. Balbino B, Sibilano R, Starkl P, Marichal T, Gaudenzio N, Karasuyama H et al. Pathways of immediate hypothermia and leukocyte infiltration in an adjuvant-free mouse model of anaphylaxis. *J Allergy Clin Immunol* 2017;139:584–596.e10.
105. Smit JJ, Willemsen K, Hassing I, Fiechter D, Storm G, van Bloois L et al. Contribution of Classic and Alternative Effector Pathways in Peanut-Induced Anaphylactic Responses. *PLoS One* 2011;6:e28917.
106. Wang Z, Mascarenhas N, Eckmann L, Miyamoto Y, Sun X, Kawakami T et al. Skin microbiome promotes mast cell maturation by triggering stem cell factor production in keratinocytes. *J Allergy Clin Immunol* 2017;139:1205–1216.e6.
107. Vaali K, Puumalainen TJ, Lehto M, Wolff H, Rita H, Alenius H et al. Murine model of food allergy after epicutaneous sensitization: Role of mucosal mast cell protease-1. *Scand J Gastroenterol* 2006;41:1405–1413.
108. Schwarzer M, Hermanova P, Srutkova D, Golias J, Hudcovic T, Sinkora M et al. Germ-free mice exhibit mast cells with impaired functionality and gut homing and do not develop food allergy. *bioRxiv* 2018;10:394213.

109. Huang J, Zhong Y, Cai W, Zhang H, Tang W, Chen B. The effects of probiotics supplementation timing on an ovalbumin-sensitized rat model. *FEMS Immunol Med Microbiol* 2010;60:132–141.

Chapter 3

IgE cross-reactivity of cashew nut allergens

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Abstract

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. 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. 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. 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 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. 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 wide spread cross-sensitisation is unknown.

Keywords: Cashew nut, tree nut allergy, IgE cross-reactivity, food allergy, Allergenicity, Immunoblotting

1. Introduction

Cashew nut allergy has been recognized 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 recognized[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 Anacardiacea. Recent case reports describing the incidence of cashew nut allergic patients experiencing anaphylaxis after consumption of pink peppercorn or sumac emphasize 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, wide spread cross-sensitization 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 visualize 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.

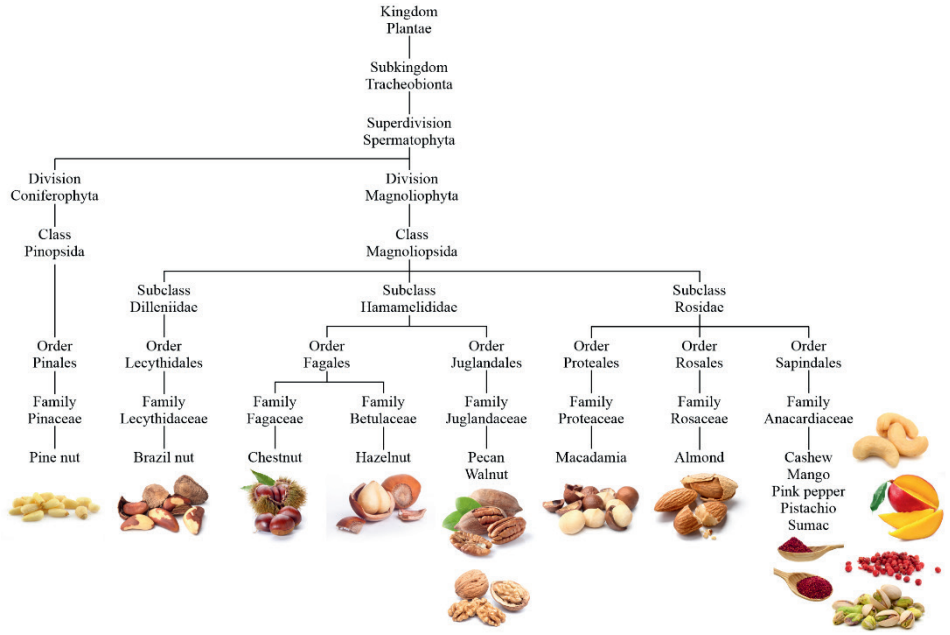


Figure 1. Phylogenetic classification of Anacardiaceae family members in accordance to other tree nuts: almond (*Prunus dulcis*), Brazil nut (*Bertholletia excelsa*), cashew (*Anacardium accidentale*), chestnut (genus Castanea), hazelnut (genus Corylus), macademia (genus Macademia), Mango (genus Magnifera), 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.

2. Material and Methods

2.1 Materials and reagents

2.1.1 Patient serum. In total, 176 patients with a suspected cashew nut allergy (sensitized 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 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].

2.1.2 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 when stated otherwise, were obtained from Sigma Aldrich (St. Louis, USA).

2.2 Dot-blot immunoassays

2.2.1 Total protein extraction. Depending on the size and availability of shelled nuts, 3 to 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 2hrs while stirring in order to deplete excess amounts of pectin. After filtration (Whatman 595 ½, 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 homogenizing 0.5 g of sample in 10 ml of buffer (20 mM Sodium Phosphate pH7; 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 homogenizing 1 g of sample in 10 ml Tris buffer (20mM Tris pH7.6; 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 g 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.

2.2.2 Protein quantification. Protein concentration of each extract was determined by Bradford assay (Thermo Fisher Scientific, Inc. Rockford, IL, USA) according to 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 duplo* on 0.2-µm Protran BA 83 nitrocellulose membranes (Whatman, Dassel, Germany) placed on a polyester backbone (GL Precision, San Jose, 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.

2.2.3 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 duplo* on a 35mm x 6mm (LxW) square strip of 0.2-µm nitrocellulose membrane placed on a polyester backbone. Each strip was then dried for 1 hr at 37°C and stored at RT in the dark for up to one 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.
Spot intensity = mean ($spot1_{serum}$-$spot_{control}$, $spot2_{serum}$-$spot_{control}$).

2.2.4 IgE sensitisation towards Cross-reactive Carbohydrate Determinants (CCDs). Cashew total protein extract (Tris:urea/phosphate; 1:1), bromelain from pineapple

stem (B5144) and ascorbate oxidase from *Cucurbita* sp. (A0157) were spotted *in duplo* 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.

2.3 Western blot immunoassays

2.3.1 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 were limited even when sera were pooled.

2.3.2 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, 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 # 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 # 5, 15, 53, 54, 58, and 63; pooled equal in volumes). Membranes incubated in TBS buffer without serum were used as an antibody background control. Immunolocalization of ribulose and luminal binding protein has been 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 binding protein (BiP2; AS09 481; 1:2,000) polyclonal antibody from *Arabidopsis thaliana* (Agrisera AB, Vännas, Sweden) according to 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 pre-incubated with 1 mg/ml cashew protein (Tris and urea/phosphate fractions 1:1) for 2.5 hours at RT prior to incubation with nitrocellulose membrane. Blots were stained for 7 min (western blots) or 20 min (inhibition blots).

2.3.3 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 normalized 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 2nd 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.

2.4 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 Chi-square probability (p-value) below 0.05 were considered to not have equal medians.

3. Results

3.1 IgE-sensitisation profiles of patient sera

Fifty-six children with a suspected cashew nut allergy (e.g. sensitized 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 (Figure 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).

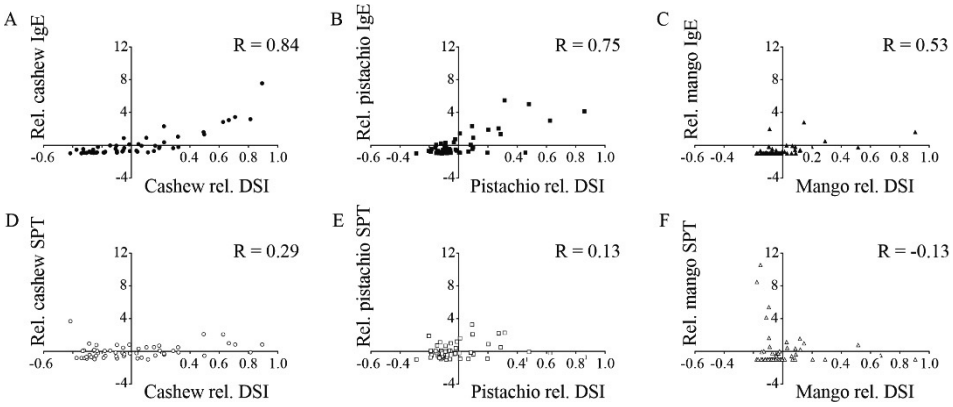


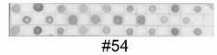
Figure 2. Correlations between dot blot sIgE measurements and clinical sIgE (IMMULITE) and SPT measurements for cashew, pistachio and mango in all patients. Clinical sIgE/sSPT data is displayed as relative IgE/SPT on the y-axis. Relative dot blot sIgE (DSI) is displayed on the x-axis. The correlation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

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 in four different groups according to their sensitisation profiles (Figure 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.

Group	Patient	Protein spots on dot blot													IgE (IMMULITE)			SPT			DBPCFC
		Cashew	Mango	Pink Pepper	Pistachio	Sumak	Almond	Pine	Macademia	Pecan	Walnut	Hazelnut	Brazil nut	Chesnut	Cashew	Mango	Pistachio	Cashew	Mango	Pistachio	Cashew
I	#27	+++	-	+++	+++	+/-	-	-	-	-	+/-	-	-	-	27.00	1.15	79.50	1.36	0.00	0.78	Positive
	#30	+++	-	+	++	-	-	-	-	-	-	-	-	-	44.70	0.00	47.10	9.33	0.32	9.19	Positive
	#39	++	-	-	+	-	-	-	-	-	-	-	-	-	47.40	0.00	51.40	6.01	0.00	12.3	Positive
	#49	+++	-	+	+	-	-	-	-	-	-	-	-	-	7.28	0.00	11.50	3.17	0.00	1.91	Positive
	#52	++	-	+	+	-	-	-	+/-	-	+/-	-	-	-	3.72	0.00	8.28	2.35	0.13	8.85	Positive
	#55	++	-	-	+	-	++	-	-	-	-	-	-	-	10.80	0.00	16.20	1.48	0.00	2.43	Positive
	#62	+++	-	+	+	-	-	-	-	-	-	-	-	-	30.20	0.00	44.50	9.41	0.00	5.39	Positive
															27.0	0.00	44.50	3.17	0.00	5.39	
II	#01	++	-	-	-	-	-	-	-	-	-	-	-	-	15.70	3.76	20.6	1.82	0.50	1.91	Negative
	#02	++	-	-	-	-	-	-	-	-	-	+	-	-	3.18	0.98	5.89	4.60	0.85	2.97	Positive
	#06	+	-	-	-	-	-	-	-	-	-	-	-	-	0.85	0.00	0.3	0.37	0.00	0.14	Negative
	#10	+	-	-	-	-	-	-	-	-	-	-	-	-	10.90	0.00	8.39	1.11	0.00	3.14	Positive
	#11	+	-	-	-	-	-	-	-	-	-	-	-	-	11.10	0.00	19.9	3.28	0.00	4.77	Positive
	#12	++	-	-	-	-	-	-	-	-	-	-	-	-	13.00	0.00	18.5	3.98	0.00	3.14	Positive
	#24	+	-	-	-	-	-	-	-	-	-	-	-	-	1.17	0.00	2.36	3.19	0.00	0.97	Negative
	#29	++	-	-	-	-	-	-	-	-	-	-	-	-	7.39	0.39	4.37	5.13	3.74	8.29	Positive
	#32	++	-	-	-	-	-	-	-	-	-	-	-	-	10.80	0.00	16.2	0.65	0.29	2.31	Positive
	#35	++	-	-	-	-	-	-	-	-	-	-	-	-	3.06	0.00	5.16	2.28	0.00	6.29	Positive
	#37	+	-	-	-	-	-	-	-	+/-	-	+	-	-	6.42	0.18	10.9	2.49	0.00	1.95	Positive
	#42	+	-	-	-	-	-	-	-	-	-	-	-	-	2.14	0.00	2.5	1.32	0.00	0.84	Negative
	#56	++	-	-	-	-	-	-	-	-	-	-	-	-	12.70	0.00	10.70	5.48	0.00	2.98	Positive
	#57	+++	-	-	-	-	-	-	-	-	-	-	-	-	9.00	0.00	7.17	2.61	0.00	3.97	Positive
	#59	+	-	-	-	-	-	-	-	-	-	+/-	-	-	3.10	0.00	3.06	5.43	3.07	3.07	Positive
	#60	++	-	-	-	-	-	-	-	-	-	-	-	-	9.11	0.00	7.98	3.31	0.00	2.73	Positive
	#64	++	-	-	-	-	-	-	-	-	-	-	-	-	7.65	0.00	7.25	1.95	0.46	1.66	Positive
	#67	+	-	-	-	-	-	-	-	-	-	-	-	-	1.04	0.00	0.83	0.58	0.24	0.21	Positive
															7.52	0.00	7.21	2.55	0.00	2.85	
III	#05	+++	+/-	+	+	-	+/-	-	-	++	++	+++	-	-	23.70	0.00	29.20	4.48	0.2	1.77	Positive
	#07	++	+++	++	++	+	++	++	+++	+++	++	++	+++	+	1.30	3.31	1.35	0.00	0.00	0.00	Negative
	#09	++	+/-	-	-	-	-	-	+/-	+/-	-	-	+	-	21.80	1.00	26.90	1.25	0.43	2.04	Positive
	#15	++	-	++	-	-	+	-	+	-	-	++	+++	-	38.60	0.13	37.60	2.11	0.00	7.48	Positive
	#16	++	-	-	+	-	+	-	-	+	++	++	-	-	2.16	0.00	1.10	0.39	0.14	0.27	Negative
	#21	++	+	+	+	+	+	+	+	+	+	+	+	+	3.76	0.72	3.55	4.37	0.00	0.19	Negative
	#28	+++	-	++	++	+	-	-	-	+	-	+	-	-	100	0.52	100	5.54	0.00	9.39	Positive
	#53	+++	++	++	+++	+	+++	+++	++	+++	++	+++	+++	+/-	51.90	47.00	61.80	5.54	0.16	2.19	Positive
	#54	++	++	++	++	+	++	++	+	++	+	+	+	-	22.10	1.88	36.30	4.53	0.00	4.23	Positive
	#58	+++	+	++	++	-	+/-	-	+	+	+/-	-	+	-	48.70	0.40	92.90	2.65	0.82	2.63	Positive
	#63	++	+	+/-	-	-	++	+/-	-	+	+/-	-	-	-	2.57	0.50	1.54	2.41	0.39	2.25	Positive
															22.10	0.52	29.20	2.65	0.14	2.19	
IV	#04	-	-	-	-	-	-	-	-	-	-	-	-	-	8.96	0.00	13.7	2.36	0.00	0.50	Positive
	#08	-	-	-	-	-	-	-	-	-	-	-	-	-	0.00	0.45	0.00	0.49	0.21	1.14	Negative
	#13	-	-	-	-	-	-	-	-	-	-	-	-	-	2.63	0.00	6.63	1.68	0.00	1.37	Positive
	#14	-	-	-	-	-	-	-	-	+	-	+/-	-	-	5.28	0.23	11.50	0.59	0.00	0.00	Negative
	#19	-	-	-	-	-	-	-	-	-	-	-	-	-	0.65	1.23	0.00	0.30	0.69	0.99	Negative
	#20	-	-	-	-	-	-	-	-	-	-	++	-	-	6.03	0.67	7.92	3.21	0.25	1.44	Positive
	#22	-	-	-	-	-	-	-	-	-	-	-	-	-	0.38	0.00	0.59	3.28	0.00	5.68	Undeterm.
	#23	-	-	-	-	-	-	-	-	-	-	-	-	-	1.25	0.00	2.33	3.38	0.00	6.04	Positive
	#25	-	-	-	-	-	-	-	-	-	-	-	-	-	1.79	0.00	4.61	5.89	0.32	1.35	Positive
	#33	-	-	-	-	-	-	-	-	-	-	-	-	-	0.00	0.00	0.00	14.25	0.00	0.00	Negative
	#34	-	-	-	-	-	-	-	-	-	+/-	-	-	-	1.38	0.00	3.81	3.14	0.00	4.92	Positive
	#38	-	-	-	-	-	-	-	-	-	-	-	-	-	0.46	0.00	0.27	3.57	0.00	0.91	Positive
	#40	-	+	-	-	-	-	-	-	-	-	-	-	-	5.11	4.82	4.51	0.53	0.64	0.71	Negative
	#41	-	-	-	-	-	-	-	-	-	-	-	-	-	0.63	1.02	0.41	0.50	2.07	2.59	Negative
	#43	-	++	-	-	-	-	-	-	-	-	++	-	-	0.63	0.91	0.97	0.11	0.57	0.13	Negative
	#45	-	-	-	-	-	-	-	+	-	-	-	+	-	1.02	0.00	1.74	0.89	0.00	1.13	Positive
	#46	-	-	-	-	-	-	-	-	-	-	-	-	-	0.57	0.10	0.90	3.15	1.66	2.64	Positive
	#48	-	-	-	-	-	-	-	-	-	-	-	-	-	0.41	0.00	0.37	0.90	0.00	0.54	Positive
	#61	-	-	-	-	-	-	-	-	-	-	-	-	-	1.01	0.00	1.99	1.41	0.00	2.02	Positive
	#65	-	-	-	-	-	-	-	-	-	-	-	-	-	0.68	0.00	0.91	5.36	0.00	2.80	Positive
															0.85	0.00	1.36	2.02	0.00	1.25	

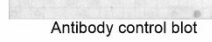
#62

#57

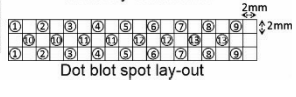


#54

#23



Antibody control blot



Dot blot spot lay-out

Figure 3. Relative dot blot spot intensities in relation to corresponding IgE (Siemens IMMULITE), SPT and DBPCFC data per patient and classification into groups (I to IV). Per group, the median IgE and SPT values are indicated below the lines. Patient numbers corresponds to the last two digits in the patient ID series 1110001 till 1110079 in the IDEAL study by Van der Valk et al[3]. Colours were used to differentiate in spot intensity (-, -/+ and + till +++): 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 lay-out 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.

All seven 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, four 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%) 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], seven patients of this group were also negative in the DBPCFC with cashew nut and for one patient the DBPCFC-outcome was undetermined. Group IV also showed the lowest median sensitization 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.

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 (Figure 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.

Based on the results above we hypothesize 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 visualized by dot blot immunoassays.

Table 2. Patient characteristics of dot blot classified groups I to IV[3].

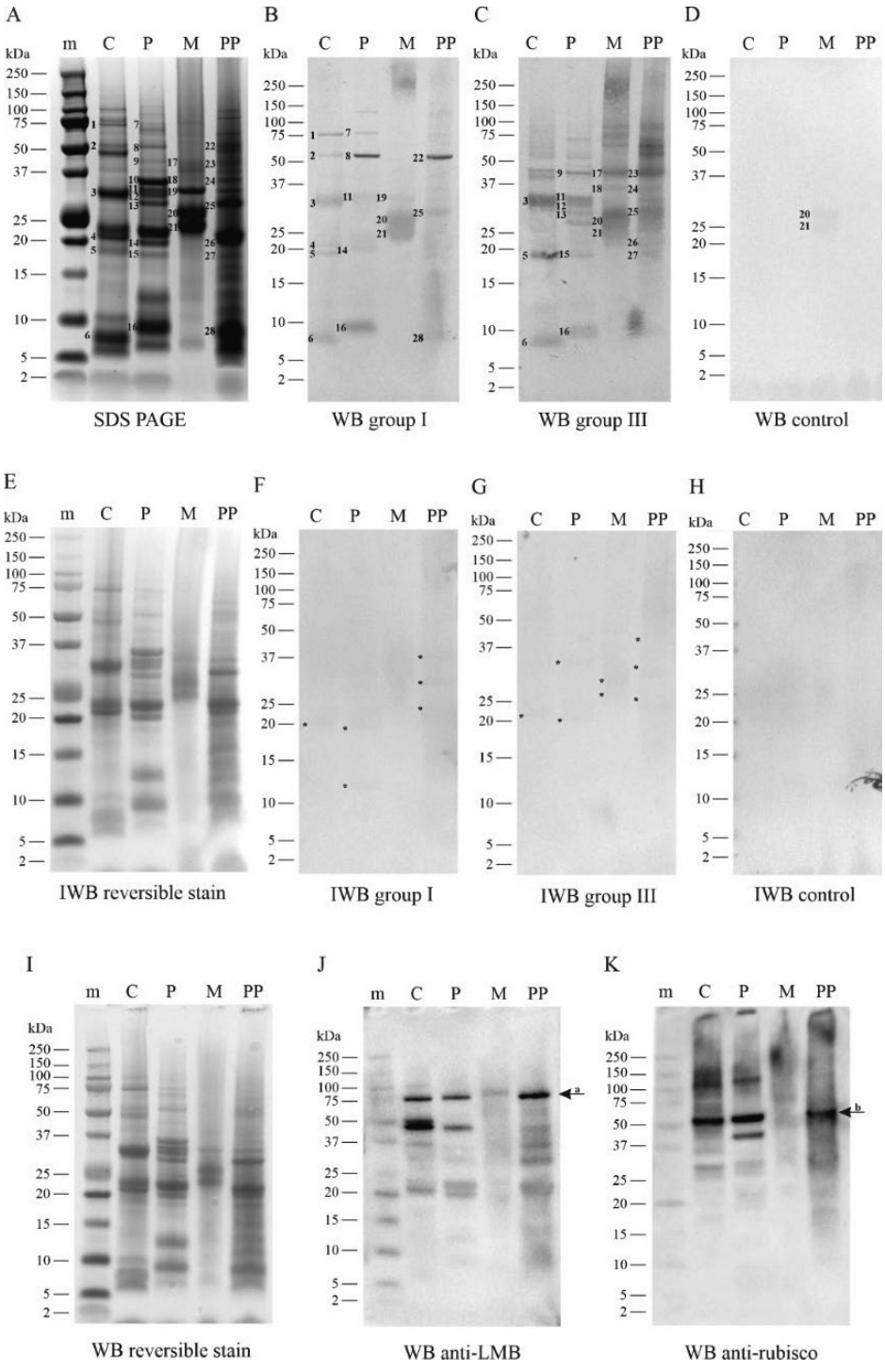
Group	**I**		**II**		**III**		**IV**	
Number of patients	**7**	**(13%)**	**18**	**(32%)**	**11**	**(20%)**	**20**	**(36%)**
Male gender	5	(71%)	7	(39%)	7	(64%)	10	(50%)
Mean age, years	7.7	(3-13)	7.9	(2-13)	8.2	(3-14)	9.3	(2-17)
Atopic disease symptoms								
Asthma	3	(43%)	94	(25%)	7	(65%)	8	(40%)
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[a]	(26.2)	7.5[b]	(6.5)	22.1[a]	(29.0)	0.9[c]	(0.7)
Median SPT cashew nut HEP (σ)	3.2[a]	(2.7)	2.6[a]	(2.0)	2.7[a]	(2.6)	2.0[a]	(2.1)
Outcome 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; a-c indicate significant differences between group medians $p<0.05$.

3.2 Group-specific allergen profiles visualized 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 visualized 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).

In both groups, patients showed IgE co-sensitisation to protein extracts from pistachio, mango and pink peppercorn (Figure 4A-D). An antibody control blot revealed that only some unspecific background binding occurred to the mango protein fraction (Figure 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 recognized differently by each patient group, were identified in cashew nut (Ana o 2, excised bands 2 to 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 recognized by both patients groups.


A
B
C
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m
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250
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75
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37
25
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15
10
5
2
SDS PAGE
WB group I
WB group III
WB control
E
F
G
H
IWB reversible stain
IWB group I
IWB group III
IWB control
I
J
K
WB reversible stain
WB anti-LMB
WB anti-rubisco
a
b

Figure 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. * indicate protein bands still faintly visible on the inhibition western blots (F-G). Arrows in western blots J-K point out the luminal binding protein bands (arrow a) and rubisco protein bands (arrow b).

In addition to the already known cashew nut seed storage allergens, putative novel cross-reactive *Anacardeaceae* allergens were identified. Protein bands of ca. 54 kDa and 73 kDa (excised bands 2, 8, 22 and 1, 7 respectively) specifically visualized by serum of group I patients in all nut/seed protein fractions, were tentatively identified as ribulose-1,5-bisphosphate carboxylase oxygenase and luminal binding protein respectively (Table 3). The observed location and identity of these IgE reactive proteins on SDS PAGE were confirmed using specific antibodies (Figure 4I-K). In pink peppercorn, a putative 2S albumin allergen of ca. 8 kDa in size was identified in excised band 28. Although only minor IgE reactivity towards mango was observed on dot blot (Figure 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 and 4D were clearly higher in intensity, indicating additional IgE-specific binding activity.

3.2 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-incubating 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 (Figure 4E-H). This finding suggests that cashew nut is most likely the primary sensitizer in these patients.

3.3 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

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

		Western blot analysis		LC-MS/MS analysis				
Band no.	Matching protein	Serum pool no.	Mass (kDa)	Accession	E-score	Calc. Mass (kDa)	No. uniq. peptides	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 parial (*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%

diagnosis. Patients within group III displayed IgE-reactivity to bromelain and ascorbate oxidase (Figure 5), two well-known CCD-containing glycoproteins. This might partly explain the extensive immunoblot inhibition results observed in Figure 4F and G.

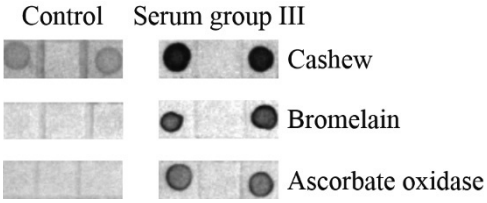

Fig. 5. CCD sensitisation of patient group III. Dot blot immunostainings of cashew nut, bromelain and ascorbate oxidase spotted in duplo and incubated with TBS (control) or serum pool of patient group III.

4. Discussion

In this study, we demonstrated different IgE sensitisation profiles in serum 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 to 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 sensitized 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 versus 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 binding protein (BiP) from *Corylus 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 before 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), in pistachio (Pis v 2/5) and pink peppercorn were recognized 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 sensitized group and most likely account for the observed extensive cross- sensitization 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 (PR)-proteins found to be allergenic in multiple fruits and seeds[51,52], were recognized 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 sensitized 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, suggestion 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 within patients as visualized for group III patients (Figure 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 of 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 relevance and to develop effective immunotherapy strategies to treat or prevent cashew nut sensitisation in young children.
Furthermore, cashew nut allergic children with co- sensitization 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 DBPCF's with pink peppercorn and sumac spice, the clinical relevance of these sensitizations remains unclear.

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Conflicts of interest
The authors have no conflicts of interest to declare.

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References

1. de Silva IL, Mehr SS, Tey D, Tang ML. Paediatric anaphylaxis: a 5 year retrospective review. Allergy 2008;63:1071-1076.
2. Vetander M, Helander D, Flodström C, Ostblom E, Alfvén T, Ly DH, Hedlin G, Lilja G, Nilsson C, Wickman M. Anaphylaxis and reactions to foods in children--a population-based case study of emergency department visits. Clin Exp Allergy 2012; 42:568-577.
3. van der Valk JPM, Gerth van Wijk R, Dubois AE, de Groot H, Reitsma M, Vlieg-Boerstra B, Savelkoul HF, Wichers HJ, de Jong NW. Multicentre Double-Blind Placebo-Controlled Food Challenge Study in Children Sensitised to Cashew Nut. PLoS One 2016;11:e0151055
4. Sicherer SH, Furlong TJ, Muñoz-Furlong A, Burks AW, Sampson HA. A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. J Allergy Clin Immunol 2001;108:128-132.
5. Johnson J, Malinovschi A, Alving K, Lidholm J, Borres MP, Nordvall L. Ten-year review reveals changing trends and severity of allergic reactions to nuts and other foods. Acta paediatr 2014;103:862-867.
6. Clark AT, Ewan PW. The development and progression of allergy to multiple nuts at different ages. Pediatr Allergy Immunol 2005;16:507-11.
7. Ewan PW. Clinical study of peanut and nut allergy in 62 consecutive patients: new features and associations. BMJ 1996;312:1074-1078.
8. Sicherer SH, Burks AW, Sampson HA. Clinical features of acute allergic reactions to peanut and tree nuts in children. Pediatrics 1998;102:e6.
9. Bock SA, Atkins FM. The natural history of peanut allergy. J Allergy Clin Immunol 1989;83:900-904.
10. Willison LN, Sathe SK, Roux KH. Production and analysis of recombinant tree nut allergens. Methods 2014;66:34-43.
11. Polk BI, Dinakarpandian D, Nanda M, Barnes C, Dinakar C. Association of tree nut and coconut sensitizations. Ann Allergy Asthma Immunol 2016;117:412-416.
12. Goetz DW, Whisman BA, Goetz AD. Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis, and human specific igE serologic surveys. Ann Allergy Asthma Immunol 2005;95:45-52.
13. Barre A, Sordet C, Culerrier R, Rancé F, Didier A, Rougé P. Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. Mol Immunol 2008;45:1231-1240.
14. Willison LN, Tawde P, Robotham JM, Penney RM, Teuber SS, Sathe SK, Roux KH. Pistachio vicilin, Pis v 3, is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen, Ana o 1. Clin Exp Allergy 2008;38:1229-38.
15. Noorbakhsh R, Mortazavi SA, Sankian M, Shahidi F, Tehrani M, Azad FJ, Behmanesh F, Varasteh A. Pistachio allergy-prevalence and in vitro cross-reactivity with other nuts. Allergol Int 2011;60:425-32.
16. Kulis M, Pons L, Burks AW. In vivo and T cell cross-reactivity between walnut, cashew and peanut. Int Arch Allergy Immunol. 2009;148:109-117.
17. Archila LD, Chow IT, McGinty JW, Renand A, Jeong D, Robinson D, Farrington ML, Kwok WW. Ana o 1 and Ana o 2 cashew allergens share cross-reactive CD4(+) T cell epitopes with other tree nuts. Clin Exp Allergy 2016;46:871-883.
18. Parra FM, Cuevas M, Lezaun A, Alonso MD, Beristain AM, Losada E. Pistachio nut hypersensitivity: identification of pistachio nut allergens. Clin Exp Allergy 1993;23:996-1001.
19. Rancé F, Bidat E, Bourrier T, Sabouraud D. Cashew allergy: observations of 42 children without associated peanut allergy. Allergy 2003;58:1311-1314.
20. Hasegawa M, Inomata N, Yamazaki H, Morita A, Kirino M, Ikezawa Z. Clinical features of four cases with cashew nut allergy and cross-reactivity between cashew nut and pistachio. Allergol Int 2009;58:209-215.
21. van der Valk JPM, Bouche RE, Gerth van Wijk R, de Groot H, Wichers HJ, Dubois AEJ, de Jong NW. Low percentage of clinically relevant pistachio nut and mango co-sensitisation in cashew nut sensitised children. Clin Transl Allergy. 2017;7:8.
22. van der Valk JPM, Dubois AEJ, Gerth van Wijk R, Wichers H., de Jong NW. Systematic review on cashew nut allergy. Allergy 2014;69:692-698.
23. Rougé P, Thibau F, Bourrier T, Saggio B, Culerrier R, Rancé F, Barre A. Signification Clinique des allergens croissants de la noix de Cajou (*Anacardium occidentale*). Revue francaise dállergologie 2011;51:31-35.

24. Kim JMD, Minikes NMD. 422 A Rare Case of Food-induced Anaphylaxis to Pink Peppercorns World Allergy Organ J 2012; 5(Suppl 2): S152.
25. Che CT, Douglas L, Liem J. Case reports of peanut-fenugreek and cashew-sumac cross-reactivity. J Allergy Clin Immunol Pract. 2017;5:510-511.
26. Paschke A, Kinder H, Zunker K, Wigotzki M, Steinhart H, Wessbecher R, Vieluf I. Characterization of cross-reacting allergens in mango fruit. Allergy 2001;56:237–242
27. Vargas Correa JB, Sánchez Solís L, Farfán Ale JA, Noguchi H, Moguel Baños MT, Vargas de la Peña MI. Allergological study of pollen of mango (Magnifera indica) and cross reactivity with pollen of piru (Schinus molle). Rev Alerg 1991;38:134–138
28. André F, André C, Colin L, Cacaraci F, Cavagna S. Role of new allergens and of allergen consumption in the increased incidence of food sensitizations in France. Toxicology 1994;93:77-83
29. Wellhausen A, Schöning B, Petersen A, Vieths S. IgE binding to a new cross-reactive structure: a 35 kDa protein in birch pollen, exotic fruit and other plant foods. Z Ernahrungswiss 1996;35:348–355.
30. Duque S, Fernandez-Pellon L, Rodriguez F. Mango allergy in a latex-sensitized patient. Allergy 1999;54:1004-1005.
31. Renner R, Hipler C, Treudler R, Harth W, Süss A, Simon JC. Identification of a 27 kDa protein in patients with anaphylactic reactions to mango. J Investig Allergol Clin Immunol 2008;18:476-481.
32. Hegde VL, Venkatesh YP. Anaphylaxis following ingestion of mango fruit. J Investig Allergol Clin Immunol 2007;17:341-4.
33. Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T. Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. J. Allergy Clin. Immunol 1991;88:172-179.
34. Gagnon C, Poysa V, Cober ER, Gleddie S. Soybean Allergens Affecting North American Patients Identified by 2D Gels and Mass Spectrometry. Food Anal. Methods 2010;3:363–374.
35. van Broekhoven S, Bastiaan-Net S, de Jong NW, Wichers HJ. Influence of processing and in vitro digestion on the allergic cross-reactivity of three mealworm species. Food Chem 2016;196:1075-1083.
36. Reitsma M, Bastiaan-Net S, Sforza S, van der Valk JP, Gerth van Wijk R, Savelkoul HF, de Jong NW, Wichers HJ. Purification and Characterization of Anacardium occidentale (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3. J Agric Food Chem. 2016;64:1191-201.
37. Ahn K, Bardina L, Grishina G, Beyer K, Sampson HA. Identification of two pistachio allergens, Pis v 1 and Pis v 2, belonging to the 2S albumin and 11S globulin family. Clin Exp Allergy 2009;39:926-934.
38. Diaz-Perales A, Collada C, Blanco C, Sanchez-Monge R, Carrillo T, Aragoncillo C, Salcedo G. Cross-reactions in the latex-fruit syndrome: A relevant role of chitinases but not of complex asparagine-linked glycans. J Allergy Clin Immunol 1999;104:681-687.
39. Palomares O, Villalba M, Quiralte J, Polo F, Rodriguez R. 1,3-beta-glucanases as candidates in latex-pollen-vegetable food cross-reactivity. Clin Exp Allergy 2005;35:345-351.
40. Malanin K, Lundberg M, Johansson SG. Anaphylactic reaction caused by neoallergens in heated pecan nut. Allergy 1995;50:988-991.
41. Berrens L. Neoallergens in heated pecan nut: products of Maillard-type degradation? Allergy 1996;51:277-278.
42. Beyer K, Morrow E, Li XM, Bardina L, Bannon GA, Burks AW, Sampson HA. Effects of cooking methods on peanut allergenicity. J Allergy Clin Immunol 2001;107:1077-1081.
43. Maleki SJ, Chung SY, Champagne ET, Raufman JP. The effects of roasting on the allergenic properties of peanut proteins. J Allergy Clin Immunol 2000;106:763-768.
44. Mondoulet L, Paty E, Drumare MF, Ah-Leung S, Scheinmann P, Willemot RM, Wal JM, Bernard H. Influence of thermal processing on the allergenicity of peanut proteins. J Agric Food Chem 2005;53:4547-4553.
45. Flinterman AE, Hoekstra MO, Meijer Y, van Ree R, Akkerdaas JH, Bruijnzeel-Koomen CA, Knulst AC, Pasmans SG. Clinical reactivity to hazelnut in children: association with sensitization to birch pollen or nuts? J Allergy Clin Immunol 2006;118:1186-1189.
46. Gruehn S, Suphioglu C, O'Hehir RE, Volkmann D. Molecular cloning and characterization of hazel pollen protein (70 kD) as a luminal binding protein (BiP): a novel cross-reactive plant allergen. Int Arch Allergy Immunol 2003;131:91-100.
47. Foti C, Damiani E, Zambonin CG, Cassano N, Nettis E, Ferrannini A, Calvano CD, Aresta A, Romita P, Aloia AM, Vena GA. Urticaria and angioedema to rubisco allergen in spinach and

tomato. Ann Allergy Asthma Immunol 2012;108:60-61.
48. Nayak AP, Green BJ, Sussman G, Berlin N, Lata H, Chandra S, ElSohly MA, Hettick JM, Beezhold DH. Characterization of Cannabis sativa allergens. Ann Allergy Asthma Immunol 2013;111:32-37.
49. Bar-El Dadon S, Pascual CY, Eshel D, Teper-Bamnolker P, Ibáñez MD, Reifen R. Vicilin and the basic subunit of legumin are putative chickpea allergens. Food Chem 2013;138:13-18.
50. Fasoli E, Righetti, PG. The peel and pulp of mango fruit: a proteomic samba. Biochim Biophys Acta 2013;1834:2539-2545.
51. Aleksic I, Popovic M, Dimitrijevic R, Andjelkovic U, Vassilopoulou E, Sinaniotis A, Atanaskovic-Markovic M, Lindner B, Petersen A, Papadopoulos NG, Gavrovic-Jankulovic M. Molecular and immunological characterization of Mus a 5 allergen from banana fruit. Mol Nutr Food Res 2012;56:446-453.
52. Volpicella M, Leoni C, Fanizza I, Placido A., Pastorello EA, Ceci LR. Overview of plant chitinases identified as food allergens. J. Agric. Food Chem 2014;62:5734-5742.
53. Miell J, Papouchado M, Marshall AJ. Anaphylactic reaction after eating a mango. BMJ 1988; 297: 1639–1640.
54. Weinstein S, Bassiri-Tehrani S, Cohen DE. Allergic contact dermatitis to mango flesh. Int J Dermatol 2004;43:195-196.
55. Jin C, Hantusch B, Hemmer W, Stadlmann J, Altmann F. Affinity of IgE and IgG against cross-reactive carbohydrate determinants on plant and insect glycoproteins. J Allergy Clin Immunol 2008;121:185-190.

Chapter 4

IgE cross-reactivity measurement of cashew nut, hazelnut and peanut using a novel IMMULITE inhibition method

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Abstract

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. 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. 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. 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

1. 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 van der 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% (n=14) indicated to also suffer from clinical symptoms upon ingestion of all three seeds/nuts (cashew nut, hazelnut and peanut). Although cross-sensitisation seems less likely due to low level of botanical relations[7], structural identity 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. Five of the 14 multi-allergic children in the IDEAL cohort could be selected for further research on co- and/or cross sensitization patterns to specific allergen components.

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.

2. Material and Methods

2.1 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 (Trial number NTR3572)[11], 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), peanut (f13) and birch pollen (t3)[6].

2.2 SPT measurements

SPTs against whole nut extracts were performed with cashew nut, hazelnut and peanut, a positive control (histamine 10mg/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 2000g, and the supernatant was passed through a 0.22-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].

2.3 sIgE inhibition study

For the IgE-based inhibition tests with cashew nut, hazelnut, peanut 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 beads, 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 adjustor.

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 μg/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 Bollen et al[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 singly by pre-incubating sera with inhibitory allergen preparations mixed 1:1 for 1 hour at room temperature before

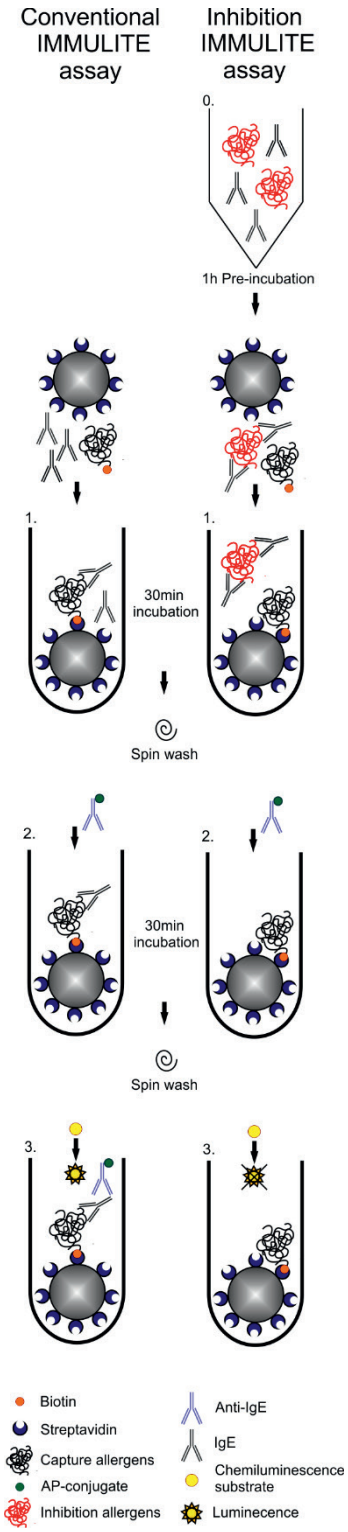

Figure 1. IMMULITE® inhibition methodology. (0) Serum sIgE is pre-incubated with or without an inhibition protein extract; (1) Serum and biotinylated capture allergens are incubated with streptavidin-coated beads; (2) AP-conjugated anti-IgE antibodies is added to the reaction mix; (3) Addition of AP-specific substrate results in luminescence that can be quantified. AP: alkaline phosphatase.

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 = (serum pre-incubated with PBS - serum pre-incubated with inhibiter)/ serum pre-incubated with PBS) x 100%

2.4 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 ImmunCAP ISAC kit (Thermo Fisher Scientific; Waltham, MA, USA). An assay for Cor a 11 was not commercially available. Antibody levels above 0.35kU/L as obtained by IMMULITE and ImmunoCAP 250 were considered positive.

3. Results

3.1 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 and/or co -reactivity 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.

3.2 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%).

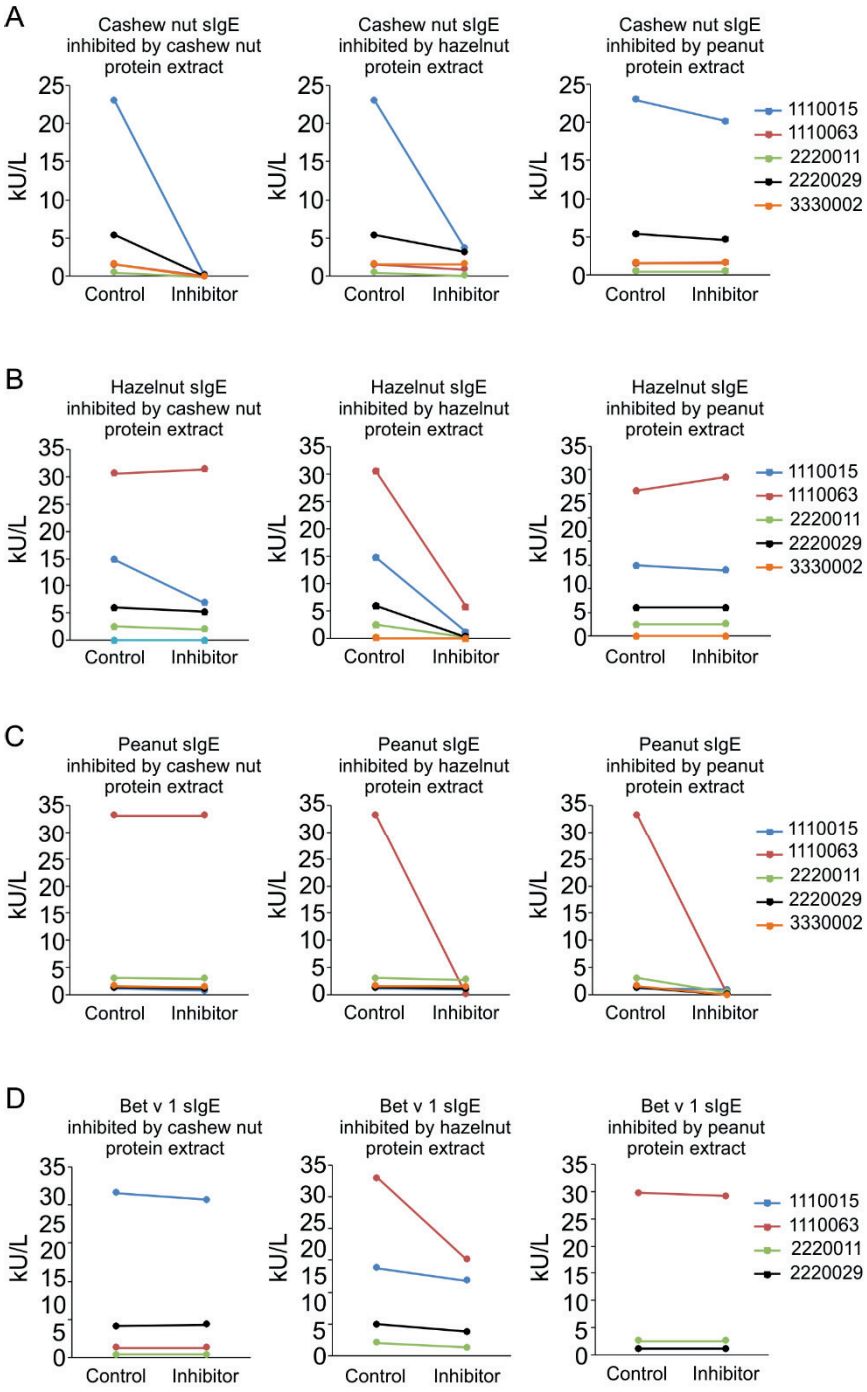

Figure 2. IMMULITE sIgE inhibitions by a total cashew nut, hazelnut or peanut protein extract. (A) Inhibition of cashew nut sIgE (f202); (B) Inhibition of hazelnut sIgE (f17); (C) Inhibition of peanut sIgE (f13); (D) Inhibition of Bet v 1 sIgE (a89).

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.

3.3 Allergen-sIgE diagnosis

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

We hypothesize that the relatively strong cashew nut/hazelnut inhibition observed in patient #1110015 and #2220029 might 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 a 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, a PR10-related hazelnut/peanut cross-reactivity might possibly explain the observed inhibition of

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

	Cashew nut			Hazelnut			Peanut			Birch pollen		
Patient	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	1.0	>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 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.

	Cashew nut sIgE			Hazelnut sIgE			Peanut sIgE				Birch pollen sIgE
Patient	nAna o 1 (7S)	nAna o 2 (11S)	nAna o 3 (2S)	rCor a 1 (PR10)	nCor a 9 (11S)	rCor a 14 (2S)	rAra h 1 (7S)	rAra h 2 (2S)	rAra h 3 (11S)	rAra h 8 (PR10)	rBet v 1 (PR10)
1110015	2.4	34.0	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	0.5	13.0	Neg	5.7	Neg	15.6	61.3
2220029	1.9	10.9	16.0	6.8	2.1	Neg	Neg	3.0	Neg	2.47	15.8
3330002	1.3	5.0	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

2S: 2S albumin

7S: 7S vicilin

11S: 11S globulin

PR10: pathogenesis related protein 10

Neg: Negative

hazelnut-sIgE by cashew nut (41.2%) and peanut (31.4%) extract. Although the positive 2S albumin sensitisation to cashew nut (Ana o 3), hazelnut (Cor a 14) and peanut (Ara h 2) indicates possible cross-reactivity, no hazelnut nor cashew nut-sIgE inhibition with peanut extract was observed for patient #2220011. This suggests that co-recognition of 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.

3.4 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

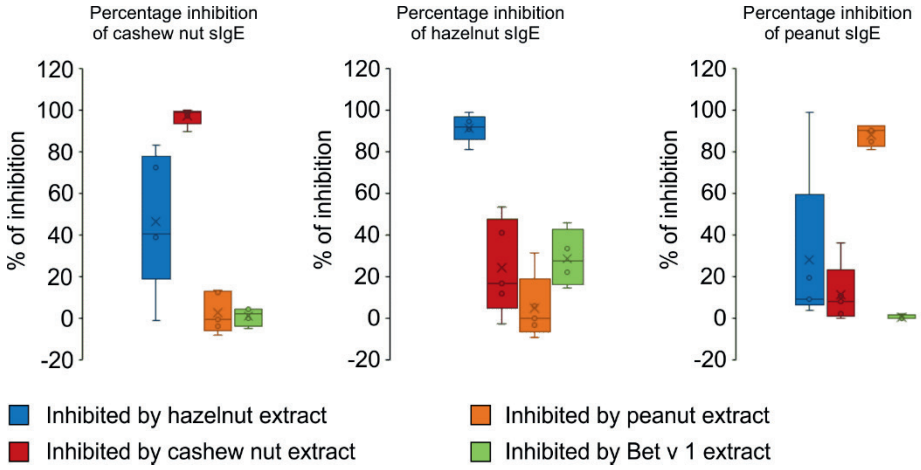

Figure 3. Overview of the mean inhibition rates in percentages.

2D. 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.

4. 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, a strong sIgE cross-reactivity was observed between hazelnut and cashew nut protein extracts, which might possibly have been 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, a 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. Perhaps, the Ara h 8-specific IgE antibodies in these patients recognize predominantly conformational epitopes that are destroyed upon heating of peanut. Although PR10 proteins are heat sensitive, Ara h 8 has been suggested as major allergen in patients with a combined birch pollen and peanut allergy[22,23]. 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 inhibiter of hazelnut-sIgE.

A 2S albumin sensitisation for peanut is commonly associated with severe systemic reactions[24], 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[16,25] and IgE-cross reactivity of peanut specific albumins occurs primarily between its isotypes rather than with tree nut 2S albumins[24,26]. For instance, peanut did not display cross-reactivity with the 2S albumin Jug r 1 from walnut[27] nor with 2S albumins from Brazil nut[28], 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 de Leon et al[29], in which no inhibition of peanut-sIgE by cashew nut was observed, although cross-reactive allergen reactivity existed between hazelnut and peanut. De Leon et al[29] applied immobilised peanut extract in their inhibition ELISAs while in the IMMULITE® technique protein conformation during inhibition is preserved which possibly explains the contrasts observed in inhibition efficiency. 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[30] and Flinterman et al[31], 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[32,33]. However, as none of the patients showed a 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. A limitation in our current study is the use of two different specific IgE measurement methods, the ImmunoCAP and the ISAC, due to low serum availability. Both methods were compared earlier[34,35] and detection rates for ISAC and ImmunoCAP were comparable: 65% and 71% respectively in patients with nut allergy. Although the detection rates apparently only slightly differ, we cannot rule out that this is of influence on our results.

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 ImmunCAP technique[36]. Although reproducibility of the new method was not tested, 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[36] or the commonly applied immunoblot or enzyme-linked immunosorbent assay (ELISA) inhibition tests is that inhibition of biotinylated allergens and detection is conducted in liquid form, before conjugation to streptavidin-coated beads takes place, 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. In addition, available serum levels limited the amount of specific allergen-inhibitions that could be performed. Globulin-specific inhibitions with Ana o 2 and Cor a 9 in particular could have contributed significantly to the understanding of sensitisation factors in our study population.

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.
Thus, future validation experiments should be performed using larger patient cohorts to compare results obtained using the IMMULITE® inhibition technology with currently applied inhibition ELISA or inhibition ImmunoCAP technologies as well as to further validate its reproducibility and applicability in allergy diagnostics.

5. 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[37]. 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[3]. We have demonstrated that the IMMULITE® technique can indeed be applied to evaluate IgE cross-reactivity between protein extracts and between specific allergens

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Conflicts of 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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References

1. Sicherer SH, Muñoz-Furlong A, Godbold JH, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. J Allergy Clin Immunol 2010;125:1322-6.
2. Johnson J, Malinovschi A, Alving K, Lidholm J, Borres MP, Nordvall L. Ten-year review reveals changing trends and severity of allergic reactions to nuts and other foods. Acta Paediatr 2014;103:862–7.
3. Weinberger T, Sicherer S. Current perspectives on tree nut allergy: a review. J Asthma Allergy 2018;11:41-51.
4. Goetz DW, Whisman BA, Goetz AD. Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis, and human specific igE serologic surveys. Ann Allergy Asthma Immunol 2005;95:45–52.
5. Clark AT, Ewan PW. The development and progression of allergy to multiple nuts at different ages. Pediatr Allergy Immunol 2005;16:507-11.
6. van der Valk JP, Gerth van Wijk R, Dubois AE, de Groot H, Reitsma M, Vlieg-Boerstra B, et al. Multicentre Double-Blind Placebo-Controlled Food Challenge Study in Children Sensitised to Cashew Nut. PLoS One 2016;11:e0151055.
7. Bastiaan-Net S, Reitsma M, Cordewener JHG, van der Valk JPM, America TAHP, Dubois AEJ, et al. IgE Cross-Reactivity of Cashew Nut Allergens. Int Arch Allergy Immunol 2019;178:19-32.
8. Popescu FD. Cross-reactivity between aeroallergens and food allergens. World J Methodol 2015;5:31-50.
9. Smeekens JM, Bagley K, Kulis M. Tree nut allergies: Allergen homology, cross-reactivity, and implications for therapy. Clin Exp Allergy 2018;48:762-772.
10. Chan ES, Greenhawt MJ, Fleischer DM, Caubet JC. Managing Cross-Reactivity in Those with Peanut Allergy. Allergy Clin Immunol Pract 2019;7:381-6.
11. van der Valk JP, Gerth van Wijk R, Hoorn E, Groenendijk L, Groenendijk IM, de Jong NW. Measurement and interpretation of skin prick test results. Clin Transl Allergy 2016;6:8.
12. de Groot H, de Jong NW, Vuijk MH, Gerth van Wijk R. Birch pollinosis and atopy caused by apple, peach, and hazelnut; comparison of three extraction procedures with two apple strains. Allergy 1996;51:712–18.
13. Bollen MA, Garcia A, Cordewener JH, Wichers HJ, Helsper JP, Savelkoul HF, van Boekel MA. Purification and characterization of natural Bet v 1 from birch pollen and related allergens from carrot and celery. Mol Nutr Food Res 2007;51:1527-36.
14. van der Valk JP, Gerth van Wijk R, Vergouwe Y, Steyerberg EW, Reitsma M, Wichers HJ, et al. sIgE Ana o 1, 2 and 3 accurately distinguish tolerant from allergic children sensitized to cashew nuts. Clin Exp Allergy 2017;47:113-20.
15. Geiselhart S, Hoffmann-Sommergruber K, Bublin M. Tree nut allergens. Mol Immunol 2018;100:71-81.
16. Aalberse RC. Structural biology of allergens. J Allergy Clin Immunol 2000;106:228-38.
17. Mueller GA, Maleki SJ, Pedersen LC. The molecular basis of peanut allergy. Curr Allergy Asthma Rep 2014;14:429.
18. Wallowitz ML, Teuber S, Beyer K, Sampson HA, Roux KH, Sathe SK, et al. Cross-reactivity of walnut, cashew, and hazelnut legumin proteins in tree nut allergic patients. J Allergy Clin Immunol 2004;113:524.
19. Wallowitz M, Peterson WR, Uratsu S, Comstock SS, Dandekar AM, Teuber SS. Jug r 4, a legumin group food allergen from walnut (Juglans regia Cv. Chandler). J Agric Food Chem 2006;54:8369-75.
20. Lange L, Lasota L, Finger A, Vlajnic D, Büsing S, Meister J, et al. Ana o 3-specific IgE is a good predictor for clinically relevant cashew allergy in children. Allergy 2017;72:598-603.
21. Eller E, Mortz CG, Bindslev-Jensen C. Cor a 14 is the superior serological marker for hazelnut allergy in children, independent of concomitant peanut allergy. Allergy 2016;71:556-62.
22. Mittag D, Akkerdaas J, Ballmer-Weber BK, Vogel L, Wensing M, Becker W-M, Koppelman SJ, Knulst AC, Helbling A, Hefle SL, Van Ree R, Vieths S. Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. J Allergy Clin Immunol 2004;114:1410–1417.
23. Hurlburt BK, Offermann LR, McBride JK, Majorek KA, Maleki SJ, Chruszcz M. Structure and function of the peanut panallergen Ara h 8. J Biol Chem 2013;288(52):36890-901.
24. Moreno FJ. and Alfonso Clemente A. 2S Albumin Storage Proteins: What Makes them Food Allergens? Open Biochem J 2008;2:16–28.

25. Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H, Mills EN. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. J Allergy Clin Immunol 2005;115:163-70.
26. Lehmann K, Schweimer K, Reese G, Randow S, Suhr M, Becker WM, Vieths S, Rösch P. Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions. Biochem J 2006;395:463-72.
27. Sordet C, Culerrier R, Granier C, Rancé F, Didier A, Barre A, Rougé P. Expression of Jug r 1, the 2S albumin allergen from walnut (Juglans regia), as a correctly folded and functional recombinant protein. Peptides 2009;30:1213-21.
28. Clemente A, Chambers SJ, Lodi F, Nicoletti C, Brett GM. Use of the indirect competitive ELISA for the detection of Brazil nut in food products. Food Control 2004;15:65-9.
29. de Leon MP, Glaspole IN, Drew AC, Rolland JM, O'Hehir RE, Suphioglu C. Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. Clin Exp Allergy 2003;33:1273-80.
30. Costa J, Mafra I, Carrapatoso I, Oliveira MB. Hazelnut Allergens: Molecular Characterization, Detection, and Clinical Relevance. Crit Rev Food Sci Nutr 2016;56:2579-605.
31. Flinterman AE, Akkerdaas JH, Knulst AC, van Ree R, Pasmans SG. Hazelnut allergy: from pollen-associated mild allergy to severe anaphylactic reactions. Curr Opin Allergy Clin Immunol 2008;8:261-5.
32. Zuidmeer L, van Ree R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. Curr Opin Allergy Clin Immunol 2007;7:269-73.
33. Ruiz-García M, García Del Potro M, Fernández-Nieto M, Barber D, Jimeno-Nogales L, Sastre J. Profilin: a relevant aeroallergen? J Allergy Clin Immunol 2011;128:416-8.
34. Williams P, Önell A, Baldracchini F, Hui V, Jolles S, El-Shanawany T. Evaluation of a novel automated allergy microarray platform compared with three other allergy test methods. Clin Exp Immunol 2016;184:1-10.
35. Griffiths RLM, El-Shanawany T, Jolles SRA, Selwood C, Heaps AG, Carne EM, Williams PE. Comparison of the Performance of Skin Prick, ImmunoCAP, and ISAC Tests in the Diagnosis of Patients with Allergy. Int Arch Allergy Immunol 2017;172:215-223.
36. Schmidt-Hieltjes Y, Teodorowicz M, Jansen A, den Hartog G, Elfvering-Berendsen L, de Jong NW, Savelkoul HF, Ruinemans-Koerts J. An alternative inhibition method for determining cross-reactive allergens. Clin Chem Lab Med 2017;55:248-53.
37. Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C, Hofmaier S, et al. EAACI Molecular Allergology User's Guide. Pediatr Allergy Immunol 2016;27:1-250.

Chapter 5

Identification and *in silico* bioinformatics analysis of PR10 proteins in cashew nut.

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Abstract

Proteins from cashew nut can elicit mild to severe allergic reactions. Three allergenic proteins have already been identified, and it is expected that additional allergens are present in cashew nut. PATHOGENESIS-RELATED PROTEIN 10 (PR10) allergens from pollen have been found to elicit similar allergic reactions as those from nuts and seeds. Therefore, we investigated the presence of PR10 genes in cashew nut. Using RNA-seq analysis, we were able to identify several PR10-like transcripts in cashew nut and clone six putative PR10 genes. In addition, PR10 protein expression in raw cashew nuts was confirmed by immunoblotting and LC-MS/MS analyses. An *in silico* allergenicity assessment suggested that all identified cashew PR10 proteins are potentially allergenic and may represent three different isoallergens.

Keywords: *Anacardium occidentale*, cashew nut, oral allergy syndrome (OAS), PR10, Bet v 1-like, RNA-seq, *in silico* allergenicity analysis.

1. Introduction

The cashew tree (*Anacardium occidentale* L.) is a tropical perennial tree native to South America[1]. In the harvest season of 2017/2018, cashew nut production reached near 790,000 metric tons (on kernel basis), with Western Africa as lead producer representing 43% of the world share (International Nut and Dried Fruit Council; https://www.nutfruit.org/). Cashew nuts are appreciated for their taste and nutritional properties (such as high lipid and essential amino acids content, and rich in minerals like potassium, magnesium and calcium)[2-4]. In addition, they are suggested to have positive health effects, as consumption of the cashew nut kernel has been linked to reduction of cholesterol levels and coronary heart disease risks[1,5-6]. Some cashew nut proteins however, may induce adverse reactions in tree nut allergic individuals, with symptoms ranging from mild (like nausea, diarrhea, eczema, asthma) to severe reactions[7] which are associated with a high risk of anaphylaxis[8]. Three allergens have been identified and characterized in cashew nut; Ana o 1 and Ana o 2 from the cupin family and Ana o 3 belonging to the albumin family[9-10]. Importantly, the pathophysiology of cashew nut allergic responses of some patients indicates mild oropharyngeal symptoms (i.e. symptoms in the middle throat area, including the oral cavity)[11-14] that match the oral allergy syndrome (OAS): oral tingling or itching (pruritus) with or without swelling of the lips, oral mucosa and throat (angioedema)[13,15]. According to studies of Li et al[11] and Hasegawa et al[13], between 100% and 75% of respectively studied patients 'cohorts showed OAS associated to cashew nut consumption. Also 64% of patients in a cohort of 176 children manifested typical OAS during a cashew nut food challenge test[14]. Proteins typically responsible for OAS include proteases, α-amylase inhibitors, peroxidases, profilins, seed-storage proteins, pathogenesis related proteins (PRs), thiol proteases and lectins in vegetables[16-20].

Bet v 1 from birch pollen is a main elicitor of pollen allergy symptoms and the first identified allergenic member of the family 10 of pathogenesis-related proteins (PR10)[21]. Bet v 1 cross-reactive homologues that act as elicitors of a food-mediated OAS allergic immune response have been found in various fruits, vegetables, nuts (hazelnut, walnut, almond and peanut) and seeds[20-23]. For instance, Ara h 8, the Bet v 1-homolog in peanut, is most likely responsible for the cross-reactivity observed between birch and peanut and its associated OAS symptoms[24], while the PR10 protein Jug r 5 is evidently associated with the manifestation of a birch pollen-associated walnut allergy[25].

Despite the fact that cashew nut allergy is often accompanied by symptoms consistent with OAS associated with a PR10-allergen hypersensitivity, no information is available on the presence of cross-reactive PR10 genes in cashew nuts. Therefore, we employed an RNA-seq analysis to identify PR10-like transcripts in cashew nut. Subsequent cloning and sequence analysis enabled us to

identify multiple PR10 genes in cashew nut and allowed us to perform an *in silico* prediction analysis for allergenic potency of the identified putative cashew PR10 proteins.

2. Material and methods

2.1 Sample preparation and RNA isolation

Technical details about sample preparation before RNA isolation, the RNA-seq transcriptome profiling and the RNA-seq data analysis and BLAST analyses specifications can be found in the supplementary data.

2.2 Cloning of PR10-like sequences

PR10-like sequences were amplified from cashew nut RNA using contig specific primers (supplemental Table S1). First, extracted RNA was converted by Oligo(dT)20 primers included in the iScript Select cDNA Synthesis Kit after which PR10-like sequences were amplified by contig-specific primers using the MT platinum SuperFi DNA proofreading polymerase kit according to manufacturer's instructions. Amplified PCR products were A-tailed and sub-cloned into the plasmid pGEM-T easy for sequencing (BaseClear B.V.; Leiden, The Netherlands). A minimum of four clones per construct were subjected to sequence verification. Cloned PR10-like sequences have been deposited into the NCBI GenBank database with the following accession numbers: MN258363 (#25355-15), MN258364 (#25514-14), MN258365 (#25514-15), MN258366 (#18220-11), MN258367 (#18220-12) and MN258368 (#18220-25).

2.3 Property analysis

2.3.1 Sequence alignments. A phylogenetic tree based on the deduced protein sequences of the cashew nut PR10-like genes and PR10 allergens from nuts and legumes was created in the Clustal Omega program of UniProt (https://www.uniprot.org/align/). Protein sequence alignments were conducted in ClustalW 1.7 (http://www.ch.embnet.org/software/ClustalW.html). Pairwise sequences identity and similarity were calculated via SIAS (http://imed.med.ucm.es/Tools/sias.html).

2.3.2 Co- and post-transcriptional modifications. The intra-domain feature scan in PROSITE database (https://prosite.expasy.org/) was used to predict putative phosphorylation sites, N-myristoylation sites and N-glycosylation sites in the deduced protein sequences of PR10-like cashew proteins. The Simple Modular Architecture ResearchTool (SMART, http://smart.embl-heidelberg.de/) was used for the PFAM domain search[61].

2.3.3 Structural modelling. For structure predictions, alignments of the deduced protein sequences of each of the cloned cashew PR10 proteins, the major birch pollen allergen Bet v 1.0101 (PDB-id: 4bkd and 1bv1) and the major cherry allergen Pru av 1.0101 (PruAV1; PDB-id:1E09) were created. The structure 1E09 was used as modelling template. For prediction of tertiary structure, structural modelling was performed using the Modeller program (version 9.16)[62]. Two-hundred comparative models were generated for each sequence, after which the models with lowest corresponding DOPE scores were selected for image generation using Pymol (version 1.4). Secondary structure prediction was performed as described by Offermann et al[63] using ClustalW and ESPrit3.0 (http://espript.ibcp.fr/ESPript/ESPript/) to extract and visualize sequence alignments.

2.4 Detection of PR10 protein in cashew nut by Western blot

Protein extract was prepared from fresh milled raw cashew nuts as described by Wangorsch et al[25] and its concentration was determined by Bradford according to manufacturer's instructions. SDS-PAGE protein separation was carried out on NuPAGE 1 mm 10 % Bis-Tris gels (Novex by Life Technologies) under non-reducing conditions by loading 10-100 µg of denatured cashew protein in NuPAGE LDS sample buffer alongside a Precision Plus Protein Dual Xtra molecular weight marker (Bio-Rad Laboratories Inc., California, USA). Gels were either stained with Bio-Safe™ Coomassie Stain (Bio-Rad Laboratories Inc., California, USA) or subjected to western blotting as previously described[64]. Blotting was carried out using specific Bet v 1 (BETVIA, rabbit polyclonal antibody, orb51330; dilution 1:1000; Biorbyt, Cambridge, UK) and Ara h 8 (rabbit polyclonal antibody, PA-AH8, dilution 1:1000; Indoor Biotechnologies, Cardiff, UK) antibodies alongside 10 µg of a native Bet v 1 and recombinant Ara h 8 positive control (NA-BV1-1 and RP-AH8 respectively; Indoor Biotechnologies, Cardiff, UK). Imaging and analysis were performed using a Universal Hood III and Image Lab 4.1. software (Bio-Rad Laboratories Inc., California, USA).

2.5 LC-MS/MS protein identification

2.5.1 Sample preparation. Of each protein sample, 100 µg was suspended in 100 µL 2% (w/v) SDS in 20 mM DTT. Suspensions were sonicated for 10 minutes followed by incubation at 60°C for 30 minutes. After cooling to room temperature Iodoacetamide was added from a 0.5 M stock to a final concentration of 50 mM, and suspensions were incubated in the dark for 30 minutes. From each suspension 50 µg of protein, according to the Bradford analysis carried out on the original protein extract, was used for trypsin (1:10) digestion according to the S-Trap™ Micro Spin Colum Digestion Protocol from ProtiFi (Huntington, NY, USA). After digestion, peptides were eluted with 50% acetonitrile in 0.1% formic acid. Eluates were dried by Speedvac and subsequently dissolved in 40 µL 2% acetonitrile in 0.1% formic acid.

Two different processing methods were carried out in a repeat experiment. One aliquot was incubated with addition of 1% RapiGest (Waters Corporation, Milford, MA, USA) in Tris/HCl pH7.4 and 1 µg of Trypsin (1:50; Promega Gold Sequencing grade). After overnight digestion at 37°C, peptides were acidified with 1% TFA (trifluoric acid) and the digest was centrifuged at 16000 rpm. The supernatant was loaded onto an OASIS HLB SPE microcolumn (Waters Corporation, Milford, MA, USA), washed twice with 100 µL 2% acetonitrile in 0.1% formic acid and eluted with 50 µL 50% acetonitrile in 0.1% formic acid. Another 50 µg aliquot was again processed according to the S-Trap™ Micro Spin Colum Digestion Protocol from ProtiFi (Huntington, NY, USA). Eluates were dried and dissolved as described above.

2.5.2 LC-MS/MS. The first set of peptide eluates were injected onto a nanoAcquity UPLC (Waters Corporation, Milford, MA, USA), trapped onto a Symmetry C18 2 cm x 180 µm trap column. Using a 60-minute gradient from 4 to 16 to 30% and final to 85% acetonitrile in 0.1% formic acid, peptides were separated on an analytical charged surface hybrid CSH column, 15 cm x 75 µm, 1.8µm particle size at 50°C at a flow rate of 400 nL per minute. Column effluent was on-line connected to a QexactivePlus using a nanoFlex electrospray.

For the independent replicate experiment (RapiGest and S-trap digests) peptide eluates were loaded onto an Easy-nLCII (ThermoFisher Scientific, Waltham, MA, USA) equipped with a PepSep trap column 2 cm x 100 µm and separation column 8 cm x 75 µm, 3 µm particle size at 24°C at a flow rate of 200 nL per minute. Elution was a 24-minute gradient from 10 to 30 to 45% and final to 85% acetonitrile in 0.1% formic acid. Column effluent was on-line connected to a QexactivePlus using a nanoFlex electrospray (ThermoFisher Scientific, Waltham, USA).

In both experiments, MS acquisition was performed using a DDA method with alternating MS1 scan at resolution 70000 profile mode, AGC target 3e6, maxIT 50 ms, scan range 500-1400 m/z, and subsequently 10 MS2 scans centroid mode, resolution 17.500 AGC target 5e4, maxIT100 ms, with isolation window 1,6 m/z at NCE=28 on with preferred peptide match ions of charges 2, 3 or 4 and a dynamic exclusion window of 30 seconds.

2.5.3 Data processing. LC-MS/MS spectra were processed using MetaMorpheus version 0.0.295[65] for the first sample set. Peptide identification was performed using a protein sequence database composed of all PR10 RNA-seq contig sequences including additional identified allelic variants, plus 111 proteins from Anacardium taxon A171928 as present in UniProt database (on Dec 2017), plus a set of frequent contaminant proteins (e.g. trypsin, keratins, BSA, etc.). The combined search database contained 12 non-decoy protein entries including 490 contaminant sequences. The following search settings were used: protease =

trypsin; maximum missed cleavages = 2; minimum peptide length = 4; maximum peptide length = unspecified; initiator methionine behaviour = Variable; fixed modifications = Carbamidomethyl on C, Carbamidomethyl on U; variable modifications = Oxidation on M; max mods per peptide = 2; max modification isoforms = 24; precursor mass tolerance = ±5 PPM; product mass tolerance = ±20 PPM; report PSM ambiguity = True. A minimum of 2 peptides were required for protein identification.

The two samples belonging to the replicate experiment were processed using MaxQuant (version 1.6.5.)[66] using the same protein sequence database and a set of contaminant proteins as default in MaxQuant. Search parameters included a minimum peptide length of 6, fixed modifications = Carbamidomethyl on C, variable modifications = Oxidation on M. A minimum of 1 peptide per protein was accepted at PSM FDR 1% and Protein FDR 1% . For visualization and evaluation purposes an example msms.txt result file from MaxQuant for each of the detected cashew nut PR10 contigs was loaded into the software Skyline[67], together with the raw files. Identified peptides peaks were integrated in MS profiles, and the peptide spectra matches were exported as presented in Figure S1.

Ion intensity and PEP scores for peptides identified in each of the two LC-MS/MS experiments are visualised in Table S4A. iBAQ scores for Ana o 3.0101 and each of the PR10 contigs in cashew nut as detected by MaxQuant protein identification analysis are listed in Table S4B for semi label-free quantification. Ana o 3.0101 was chosen for this comparison as the protein mass of this 2S albumin is close to the protein mass of the PR10 proteins.

2.6 Assessment for potential allergenicity

2.6.1 80-aa sliding window, 6-mer and 8-mer component analysis. The 6-mer and 8-mer component analysis was performed by assessing the deduced amino acid sequence of cashew PR10-like proteins using the online available software tools SDAP and AllergenOnline v12, respectively[68-69]. Both software tools also assessed the 80-aa sliding window alignment.

2.6.2 Analysis of Allergenicity. The computational predictive tools AllerTOPv.2 and AllergenFPv.1.0 were applied to predict protein allergenicity and cross-reactivity. The AllerTOPv.2 and AllergenFP are alignment-free allergen prediction models based on various amino acid descriptors, taking into account residue hydrophobicity, size, abundance and α-helix and β-strand forming propensities[70-71].

2.6.3 Prediction of B- and T-cell epitopes. MHC subtype A1 T-cell epitopes were predicted using the NetCTL-1.2 online prediction tool (http:// www.cbs.dtu.dk/services/NetCTL/) applying a threshold of 0.75[72]. The structure

based tools Ellipro (http://tools.iedb.org/ellipro/)[73], BPAP (http://imed.med.ucm.es/Tools/antigenic.pl) and BepiPred 1.0 with threshold 0.35 (http://www.cbs.dtu.dk/services/BepiPred-1.0/)[74] were used for the prediction of B-cell epitopes.

3. Results

3.1 Identification of putative cashew nut PR10-like genes by transcriptome analysis

Next-generation sequencing of RNA extracted from cashew nut resulted in an RNA-seq library of 65,599,531 trimmed reads with an average length of 112.3 basepairs (bp). A summary of statistics after sequencing is presented in Table 1. Genome alignment of reads for transcript assembly was not possible due to the lack of an existing reference genome database for cashew nut. Therefore, we used a *de novo* transcriptome assembly approach which generated a BLAST library consisting of 53,114 contigs with a minimum and maximum contig length of 126 and 12,132 bp, respectively. Fifty percent of the entire assembly is contained in contigs ≥ than 804 bp.

Table 1. Summary of statistics of the RNA-seq library and *de novo* transcriptome assembly.

	Count (no.)	Average length (bp)	Total bases (bp)
Reads	65,599,550	112.33	7,368,725,189
Matched reads[a]	58,971,799	112.27	6,620,625,613
Non matched reads[b]	6,627,751	112.87	748,099,576
Reads in pairs[c]	55,271,842	124.93	
Broken paired reads	3,699,957	125.31	
Contigs	53,114	599	31,860,598

a. Number of reads that showed an overlap with each other
b. Number of reads that contained unique transcript sequence
c. Reads that have been sequenced from both ends

Next, we used a BLAST query in the cashew nut transcriptome to identify putative PR10 proteins. Since PR10 protein sequences (derived from nut/seed) are not available for members within the cashew family (family of Anacardiaceae), we used the nut-derived PR10 allergen Pru du 1 from almond from the phylogenetically related Rosaceae family[26]. This BLAST search identified nine contigs within the cashew RNA-seq paired reads dataset, that shared 32-55% sequence identity with Pru du 1 isoforms (Table 2). Sequence alignment revealed that only 3 of the 9 contigs identified contained a complete open reading frame (ORF) sequence. These were contig #18220, #25355 and #25514, whose

Table 2. Identified contigs using the PR10 allergen Pru du 1 from almond as BLAST query, ranked according to total score value. Putative PR10 amino acid sequences corresponding to each contig were aligned to Pru du 1 using Clustal W (1.7) multiple sequence alignment for comparison reasons.

Contig mapping				BLAST results			
Contig No.	Consensus length (bp)	No. of reads	Average coverage	Total Score	Min. E-value	Max Identity (%)	Identity with
Contig #25355	529	34	7.26	368-423	5.15^{-44}-2.33^{-52}	49.69-54.72	Pru du 1.01 t/m Pru du 1.06
Contig #25514	626	76	13.88	322-353	6.47^{-37}-4.01^{-41}	40.52-43.23	Pru du 1.01 t/m Pru du 1.06
Contig #18220	711	277	43.52	344-376	8.04^{-40}-1.74^{-44}	44.87-50.00	Pru du 1.01 t/m Pru du 1.06
Contig #16127	421	539	142.77	195-229	5.21^{-19}-6.24^{-24}	39.39-41.84	Pru du 1.01 t/m Pru du 1.06
Contig #18221	236	28	11.80	139-171	2.79^{-11}-4.57^{-16}	37.66-55.36	Pru du 1.01 t/m Pru du 1.06
Contig #16128	456	315	74.49	106-164	2.39^{-06}-1.55^{-14}	36.36-48.98	Pru du 1.01 t/m Pru du 1.06
Contig #25317	718	234	35.74	104-113	2.39^{-06}-8.83^{-07}	36.36-39.39	Pru du 1.01 t/m Pru du 1.06
Contig #4938	732	3835	578.90	104	4.84^{-06}	31.88	Pru du 1.01
Contig #25513	429	54	13.93	93	7.37^{-05}	38.89	Pru du 1.01

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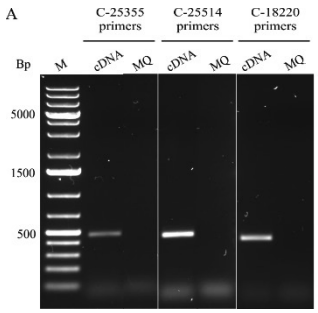
Pru du 1.01      MGVFTYESEFTSEIPPELFKAFVLDADNLVPKIAPQAIKHSELESGDGPGTIKKITFGSGSQ-YGYVKHKIDSIDKEN----HSYSYTLTEGDALGSNLEKISYETKLVASPSGSSIIKSTSHIHTKGDVEIKEEH-V-KAGKEKASNLFKLIETYLKGHPDAYN
Contig #25355    MGVTTFTESFSSPVPARRLFKAFVLDFDNLLPKLMPQVFKNIETIEGDGGPGTIKKLNISEGGE-VKYLKHRIDALDKEK---LIYNYTIIEGDAM-DKIESYSYEIKVEVSPDGGCKGTTVNKYYPKTGILEEEK-I-KEARAKAMGLYKVVEGYLLANPDAY-
Contig #25514    MAVITDQLEVACTLPADKMFKGFVLDADDVFPKVMPQAIKSAELIISGDGGAGSIRKVCVLSDDK-LMYMKHKVDFLDKEN---LVFCYTIFEGDFLSKEEKVVVEMTKWESGPDGSSIFKATAKFYIVPGFEGAENF-I-MTEKSKAIGMIKAVEAHLKAN-----
Contig #18220    MGFACGEFEIESVLPAAKMFQASVLDADQLFPKIMSQAIKSAELLQGDGAGSIRKVELVESD----SYMKHKVDALDKET---FVYNYTIFEGDTLTDKEEKIVVEMTKWESTPAGSIFKSSVKFYTLPSEVPGES-LLNKSKEKATAMVKAVEAYLQAN-----
Contig #16127    ------------------------------------------------GSS-IGYVKHRIDMIDKEN---YMCKYTFIDSDFLMDKLEYITVEVKFEGHGHRGCVGRITSEFKPKEGIEIKIDI-I-ELGKDRSIGMYEVVEAYLLAHPNAY-
Contig #18221    MGFATGEEVATALPAAKMFQGAVLDADQLFPKIMSQAIKSAELLEGDGGAGSIRK-----------------------------------------------------------------------------------------
Contig #16128    ----------FVAQVTPAKMFKALILDSHNICPKLMPSIIKTMEYIEGNGEVGSIKQINFTEG-----------------------------Q--NSY-----------------------GSFL-----------LYVNSQH-L-SLDKR-----
Contig #25317    ------------------------LELGSLVDKLLPDVLGKVFEVIEGDGGVGTIKLTFPPGTPGAGYMKEIFKTIDEEK-----------------------------------------------------RVKETE-M-IEG-----------
Contig #4938     ----------------------MFR------NDQIMSKINPEMLAHAEYIQGDGSPGSIRLFKLGHAVQ--NYVKESTQKIEKVSIGRSVTYRVVEGE-----------------------------------------------------------
Contig #25513    ------------------------------------------------------------------------------------------------------------TKWESSPAGSIIFKSTVKFYTLPSEVPAENLV-NTAKGKTTVMVKAVEAYLQAN-----
```

sequences were subsequently used for cloning. The ORF in contig #25355 showed the highest sequence identity to Pru du 1.

To confirm the presence of the identified putative *PR10* ORFs in cashew nuts we used PCR-based cloning using contig-specific primers (supplementary Table 1). Sequence analysis of amplified full-length ORFs (Figure 1A) confirmed the *PR10-like* gene sequences that were predicted by the *de novo* transcript assembly. In addition, one or more genetic variants for two of the *PR10-like* ORFs were identified which differed slightly in length and sequence. These multiple allelic variants were found in *PR10* contig #25514 (clones #14 and 15) and *PR10* contig #18220 (clones #11, 12 and 25) (Figure 1B). The deduced proteins of the identified variants ranged in length between 154-159 amino acids (aa) and the molecular weights (Mw) were predicted to be in range of 16.9-17.8 kDa while pI values ranged from 4.7 to 5.0, as observed for other PR10 proteins[27].

Sequence comparisons between the isolated clones and the assembled RNA-seq contigs showed a high level of sequence similarity. For example, clone #25355-15 showed 99% aa-homology with contig #25355 while clones #25514-14 and -15 are 100% and 98% homologous to contig #25514, respectively. Clones representing contig #18220 showed 99% (#18220-11), 100% (#18220-12) and 99% (#18220-25) homology with the original contig ORF sequence. Thus, in this study the RNA-seq approach proved to be an accurate and powerful approach to identify the presence and genetic variants of *PR10-like* sequences.

A



B

Identified cashew *PR10-like* clones and deduced protein characteristics

Clone no.	Gene length (bp)	Protein lenght (aa)	Mw (kDa)	pI
#25355-15	477	159	17.8	5.0
#25514-14	465	155	17.2	4.7
#25514-15	462	154	17.0	4.7
#18220-11	462	154	16.9	4.9
#18220-12	462	154	16.9	4.9
#18220-25	462	154	16.9	4.9

Figure 1. Cloning of cashew PR10-like genes. (A) PCR amplification of PR10-like genes identified in contigs #25355, #18220 and #25514; (B) Characteristics of the identified cashew PR10-like clones and their different variants. bp; base pairs; aa: amino acids; kDa: kilo Dalton; pI: isoelectric point.

3.2 Bioinformatics analysis of the putative PR10-like proteins of cashew

To further verify that the putative PR10 proteins identified in cashew are indeed related to PATHOGENESIS-RELATED proteins belonging to the PR10 family, a general NCBI-BLAST was performed using their deduced aa sequence as query (FASTA search). As shown in Table S2, the top 5 BLAST results corresponded to

other PR10 proteins and all putative cashew PR10 proteins display a high identity to the PR10 proteins Pru av 1 and Pru ar 1 from cherry and apricot, respectively. Moreover, all identified clones contain the Prosite PS00451 'pathogenesis-related proteins Bet v I family signature' G-x(2)-[LIVMF]-x(4)-E-x(2,3)-[CSTAENV]-x(8,9)-[GNDS]-[GS](2)-[CS]-x(2)-[KT]-x(4)-[FY] (for cellular localization, membrane-protein and protein–protein interactions) as well as the PFAM Bet v 1 domain (PF00407).

Next, the putative cashew PR10 protein sequences and Pru du 1 were aligned to the PR10 reference protein Bet v 1 from birch pollen and their predicted co- and post-translational modification sites were analysed (Figure 2 and Table S3). All identified sequences contain the Bet v 1 characteristic common feature of a glycine-rich P-loop motif (GxGGxGxxK)[28-29], although variants of clones #25514 and #18220 contain an additional arginine before the lysine in the P-loop region (GxGGxGxxxK). The structural P-loop element facilitates nucleotide-binding interactions in some proteins[28]. Clone #25355-15 shows a similar deduced aa-sequence length as Pru du 1 and Bet v 1, while the other cashew PR10-like proteins are five aa shorter at the C-terminal end.

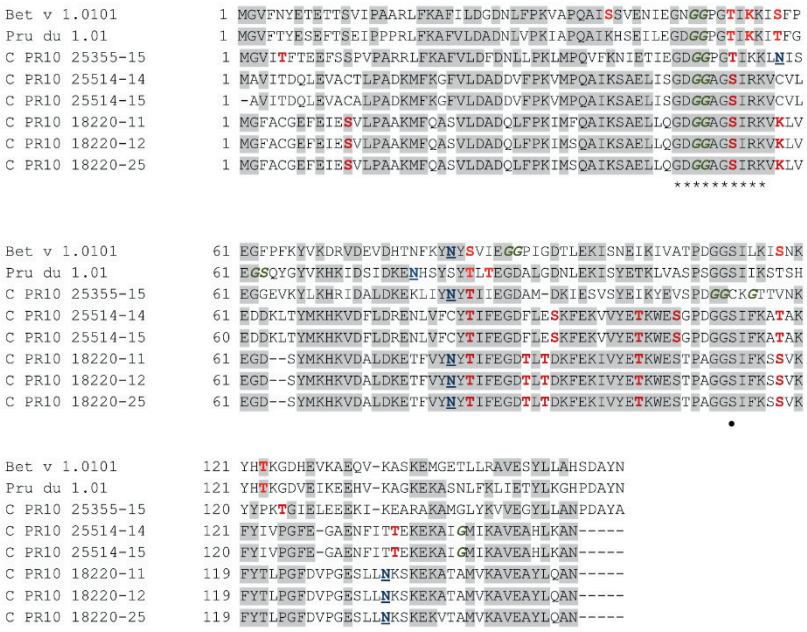


Figure 2. Clustal alignment of the cashew PR10-like proteins, Bet v 1.0101 (P15494) from birch pollen and Pru du 1.0101 (ACE80939.1) from almond. Cashew nut AA-regions that are identical to the PR10 proteins of birch and/or almond are shaded in grey. Putative phosphorylation sites are indicated in bolt red, putative N-myristoylation sites are indicated in bold italic green and predicted N-glycosylations sites are blue underlined. Stars underneath the alignment mark the p-loop region in Bet v 1.0101. The • indicates Ser112 essential for IgE cross-reactivity between Bet v 1 and Mal d 1.

All clones contain putative co-translational myristoylation sites, allowing for membrane targeting and protein-protein and protein-lipid interactions[30], and post-translational phosphorylation sites which may greatly define the structural conformation of a protein, its signalling pathways and metabolism[31-32]. Compared to a single predicted N-glycosylation site in Bet v 1 and Pru du 1, two N-glycosylation sites were predicted for clones #18220 and #25355, while these sites are lacking in clones #25514-14 and -15.

A similarity and identity analysis of the deduced amino acids between the PR10-like proteins from cashew and various tree nuts and legumes is shown in Figure 3A. The cashew PR10-like proteins show the highest sequence identity with PR10 allergens from almond, chestnut and hazelnut (36-53%) as compared to leguminous PR10 allergens Ara h 8 and Gly m 4 (31-43%). Cluster analysis

A

Similarity / Identity	Bet v 1.0101	#25355-15	#25514-14	#25514-15	#18220-11	#18220-12	#18220-25	Ara h 8.0101	Ara h 8.0201	Cas s 1.0101	Cor a 1.0401	Cor a 1.0402	Cor a 1.0403	Cor a 1.0404	Gly m 4.0101	Jug r 5.0101	Pru du 1.01	Pru du 1.02	Pru du 1.03	Pru du 1.04	Pru du 1.05	Pru du 1.06A	Pru du 1.06B
Bet v 1.0101	100	58	45	45	50	50	50	61	57	67	75	74	74	75	60	74	71	71	69	72	73	70	71
#25355-15	47	100	51	50	52	52	52	57	54	61	60	60	60	60	57	66	64	66	63	61	66	66	67
#25514-14	35	43	100	99	72	72	71	47	47	50	48	48	48	49	44	50	50	51	50	50	50	52	53
#25514-15	35	42	99	100	71	71	71	45	47	50	48	48	47	48	43	49	49	50	50	50	49	52	52
#18220-11	41	42	62	62	100	99	99	49	50	53	50	49	50	50	45	54	52	52	55	57	56	55	55
#18220-12	41	42	62	62	99	100	99	49	50	53	50	49	50	50	45	54	52	52	55	57	56	55	55
#18220-25	41	42	62	61	99	99	100	49	50	53	50	48	49	49	45	53	51	52	54	56	55	54	55
Ara h 8.0101	46	41	32	31	37	37	37	100	68	61	65	65	64	65	79	68	62	63	65	63	63	65	65
Ara h 8.0201	41	43	33	33	36	36	36	54	100	60	58	58	58	58	63	65	59	60	60	58	60	60	61
Cas s 1.0101	58	53	42	42	44	44	43	47	48	100	68	68	68	68	61	79	75	78	76	74	75	73	74
Cor a 1.0401	68	49	42	42	45	45	44	54	46	60	100	97	98	99	66	74	71	72	70	70	72	72	73
Cor a 1.0402	66	48	41	41	44	44	43	54	45	60	97	100	99	97	66	74	71	73	71	70	72	72	73
Cor a 1.0403	66	48	41	41	44	44	44	54	45	60	98	99	100	96	65	73	71	73	71	70	71	71	72
Cor a 1.0404	67	48	42	42	44	44	44	54	45	59	99	97	96	100	66	74	71	73	71	70	72	72	73
Gly m 4.0101	46	42	32	32	34	34	34	71	50	50	58	56	56	58	100	67	63	63	62	63	63	62	63
Jug r 5.0101	66	53	41	40	45	45	44	54	50	70	68	68	68	68	54	100	83	82	81	83	81	81	82
Pru du 1.01	58	50	40	40	45	45	44	50	48	64	64	64	64	63	53	74	100	87	86	84	83	81	82
Pru du 1.02	57	53	40	40	44	44	43	50	47	65	63	63	63	63	51	73	77	100	93	83	86	84	85
Pru du 1.03	57	50	39	39	45	45	44	54	44	63	62	63	63	62	51	74	78	86	100	83	85	84	85
Pru du 1.04	60	48	37	36	45	45	45	50	45	64	62	62	62	62	52	77	77	75	75	100	83	84	84
Pru du 1.05	61	53	41	41	49	49	49	52	47	63	63	62	62	62	53	73	76	75	78	75	100	90	89
Pru du 1.06A	56	50	39	38	46	46	45	50	44	61	61	60	60	60	51	71	74	73	76	76	84	100	99
Pru du 1.06B	57	50	39	39	46	46	45	52	44	62	61	61	61	61	53	73	74	75	78	76	83	97	100

B

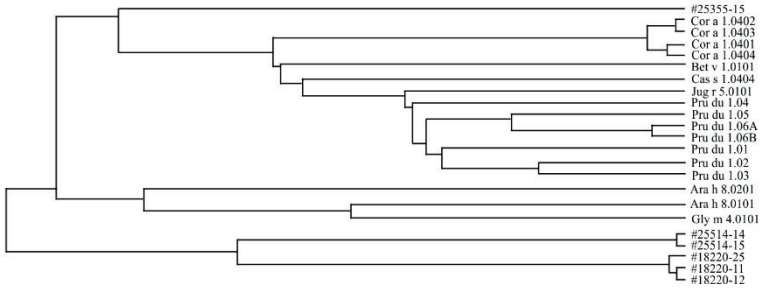


Figure 3. Similarity and identity analysis (A) and phylogenetic clustering (B) of cashew PR10 proteins, Bet v 1 from birch pollen (Bet v 1A; 4bkd-1bv1) and the well-studied PR10 allergens from almond, chestnut, hazelnut, peanut, soybean and walnut. Pru du 1.01 (ACE80939.1), Pru du 1.02 (ACE80941.1), Pru du 1.03 (ACE80943.1), Pru du 1.04 (ACE80945.1), Pru du 1.05 (ACE80947.1), Pru.du.1.06A (ACE80951.1) and Pru du 1.06B (ACE80949.1) from Almond; Ara h 8.0101 (AAQ91847.1) and Ara h

8.0201 (ABP97433.1) from Peanut; Cas s 1.0101 (ACJ23861.1) from Sweet chestnut; Cor a 1.0401 (AAD48405.1), Cor a 1.0402 (AAG40329.1), Cor a 1.0403 (AAG40330.1) and Cor a 1.0404 (AAG40331.1) from European hazelnut; Gly m 4.0101 (CAA42646.1) from Soybean; Jug r 5.0101 (APD76154.1) from English walnut; Bet v 1 (Bet v 1A; 4bkd-1bv1) from birch pollen.

visualised a similar trend in phylogenetic relationships as the similarity and identity analysis (Figure 3B). The sequence identities to Bet v 1 are in the expected range of 35-47%[27] where a low aa-identity does not exclude the ability to cross-react with Bet v 1- specific IgE antibodies, as *in vitro* demonstrated for Dau c 1 (PR10 from carrot) which displays only 38% sequence identity with Bet v 1[33].

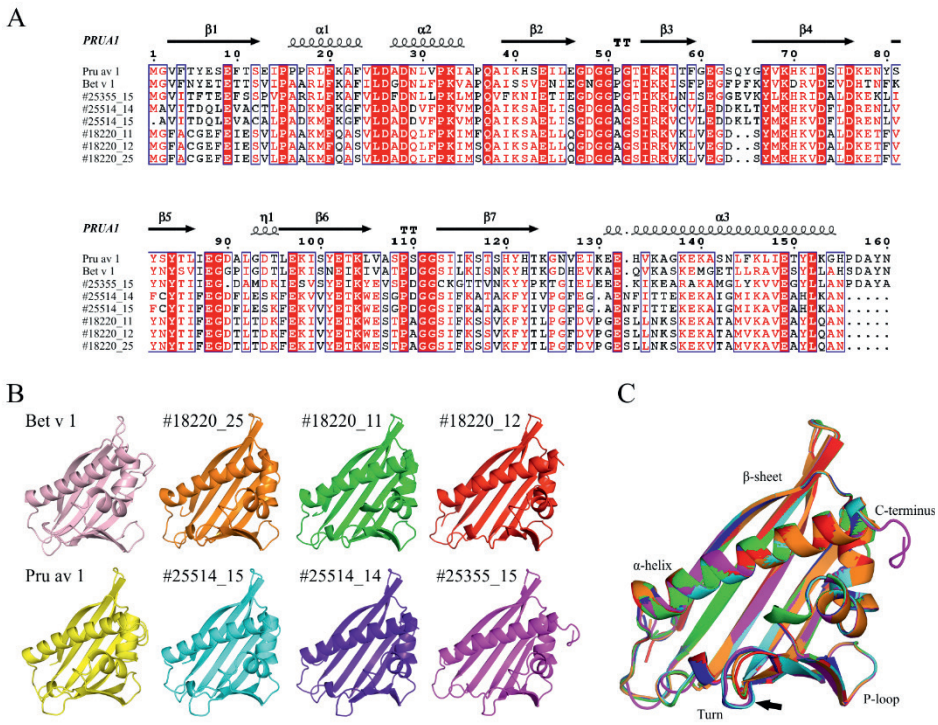


Figure 4. Structural modelling of the putative cashew PR10-like proteins using the PRUA1 NMR structure as template. (A) ClustalW alignment of the cashew PR10-like proteins and PR10 allergens from Bet v 1 and Pru av 1using the software Esprit. The α-helices, β-sheets and turns (TT) of Pru av 1 (PRUA1) are indicated above the alignment; (B) Structural modelling of tertiary structure using the program Modeller and Pymol; (C) Superimposed view of models generated for #25355-15, #25514-14, #25514-15, #18220-11, #18220-12 and #18220-25. The arrow indicates a difference in the predicted turn area.

Based on the deduced protein sequence of the identified *PR10-like* clones, a prediction was made of the structural features of the cashew PR10-like proteins. Since the protein crystal structure for Pru du 1 is lacking, we used the NMR structure of the major cherry allergen from *Prunus avium*, Pru av 1 (PruAV1; PDB code 1E09), as template as all cashew PR10 clones displayed a high sequence identity to Pru av 1 (42%-52%; see supplementary Table S2). Structural modelling

(Figure 4) shows that the predicted cashew PR10-like protein structures are highly similar to the Bet v 1A[29] and Pru av 1[34] crystal structures. All displayed the characteristic basket-like hydrophobic cavity formed by two V-shaped short α-helices wrapped around a long C-terminal α-helix and a folded seven-stranded antiparallel β-sheet[33]. Some small differences in α-helix bending could be observed as well as the length of the turn around residue 65, which is shorter in the structures of the #18220 proteins (indicated by an arrow).

The NCBI BLAST results as well as the other bioinformatics analyses, including the high similarity between the predicted cashew PR10-like protein conformational structures and the crystal structure of Bet v 1, strongly suggest that the identified *PR10* genes in cashew nut indeed belong to the family of *PR10* genes.

3.3 Presence of PR10 proteins in cashew nut extract

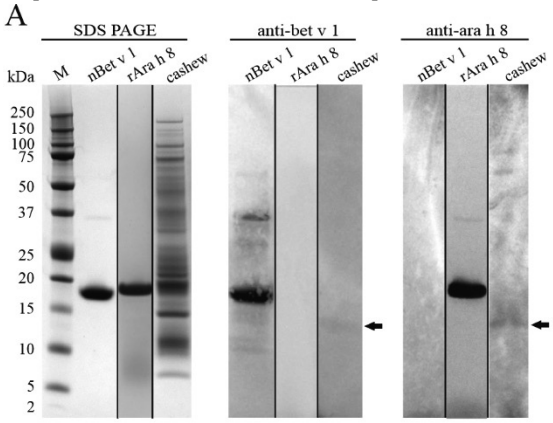
The presence of *PR10* RNA in cashew nuts does not mean that the corresponding proteins are also present. Two approaches have been applied to demonstrate the presence of PR10 proteins in cashew nut: immunoblotting using commercial IgG antibodies against Bet v 1 and Ara h 8 (PR10 protein from peanut[35]), and LC-MS/MS peptide identification using the identified cashew *PR10* RNA-seq contig sequences as well as the cloned *PR10* gene variances as database-query (Figure 5). Both anti-Bet v 1 and anti-Ara h 8 antibodies showed some binding affinity to a cashew nut protein, resulting in a very faint band of around 13-14kDa in size (Figure 5A). The polyclonal antibodies used seem to be highly selective based on the positive control results, which could explain their weak binding to cashew nut protein. Based on the deduced aa-sequence, the expected size of cashew PR10 proteins would lay between 16.9 and 17.8kDa, as also visible for native Bet v 1. Detection of a slightly smaller protein in the cashew nut protein extract could indicate potential proteolytic hydrolysis during the extraction procedure. The fact that PR10-like protein peptides, corresponding to RNA-seq contigs #4938, #25355 and #25514, were identified in the cashew nut protein extract by LC-MS/MS, confirms that *PR10* genes are indeed expressed in cashew nut although likely much less than Ana o 3 (Figure 5B and Table S4A and B).

3.4 *In silico* analysis of potential allergenicity

As PR10 proteins from fruits, vegetables and nuts are commonly associated with a birch pollen related allergy[18], we performed several *in silico* prediction analyses using online available software tools to examine the potential allergenicity of identified cashew PR10 proteins (see supplementary Tables S5-S7), for which the results are summarised in Table 3. First, the Food and Agriculture Organisation/World Health Organisation (FAO/WHO) CODEX Alimentarius guidelines (2001) were assessed. These state that a sequence is potentially allergenic if it either has an identity of at least 6 contiguous amino acids OR ≥35% sequence identity over an alignment length window of ≥80 aa when compared to

known allergens[36]. The allergenicity prediction criteria were assessed using the software tools AllergenOnline and SDAP, as listed in Tables S5A and B respectively. In particular, clone #25355 was predicted to contain multiple 6-mers and even 8-

A



B

Pr10 contig 4938
MFR|NDQIMSK|INPEMLAHAEYIQGDGSPGSLR|LFK|LGPAVQNYVK|ESTQK|IEK|VEIGR|SVTYR|VVEGE

PR10 contig 25355
MGVITFTEEFSSPVPAR|R|LFK|AFVLDFDNLLPK|LMPQVFK|NIETIEGDGGPGTIK|K|LNISEGGEVK|YLK|HR|
IDALDK|EK|LIYNYTIIEGDAMDK|IESVSYEIK|YEVSPDGGCK|GTTVNK|YYPK|TGIELEEEK|IK|EARAK|A
MGLYK|VVEGYLLANPDAYA

PR10 contig 25514
MAVITDQLEVACTLPADK|MFK|GFVLDADDVFPK|VMPQAIK|SAELISGDGGAGSIR|K|VCVLEDDK|LTYMK|
HKVDFLDR|ENLVFCYTIFEGDFLESK|FEK|VVYETK|WESGPDGGSIFK|ATAK|FYIVPGFEGAENFITTEK|EKAIG
MIK|AVEAHLK|AN

Figure 5. Identification of PR10 proteins in cashew nut total protein extract. (A) SDS PAGE gel electrophoresis and western blot of the positive controls nBet v 1 and rAra h 8 and raw cashew nut extract using anti-bet v 1 and anti-ara h 8 specific antibodies. The arrow points towards a positive band in cashew nut extract; (B) LC-MS/MS peptide identification in raw cashew nut extract after trypsin digestion. Identified peptides in contigs 4938, 25355 and 25514 are underlined. Trypsin cleavage sites are indicated by the symbol |. Sequence coverage for contig 4938 was 47%, 12% for contig 25355 while for contig 25514, sequence coverage was 34%.

mers peptide sequences identical to peptides in existing allergens. In addition, each of the cashew PR10 proteins showed 179 hits in the 80-mers sliding window alignment analyses. According to the FOA/WHO guidelines, all identified cashew PR10-like proteins would be labelled as potential allergens (Table 3).

Furthermore, we used the web-based computational system AllergenFP and AllerTOPv.2. The AllerTOPv.2 program predicted that all cashew PR10 proteins are possible allergens and to be cross-reactive with IgE antibodies recognising homologous allergens (Table S6). The AllergenFP prediction indicted that 4 out of the 6 PR10 proteins of cashew nut are potentially allergenic. In this case PR10

#25514 clones 14 and 15 were not ranked as potential allergens and these small differences are likely due to the use of different computational methods.

Table 3. Summary of performed *in silico* allergenicity prediction analyses using several online prediction servers.

Software	Link	Prediction
SDAP	http://fermi.utmb.edu/	For each cashew PR10-like protein, multiple 6-mers and 80-mers sliding windows have been identified suggesting cross-reacting characteristics.
AllergenOnline	http://www.allergenonline.org/	Multiple 8-mer hits for #1-15 and one to two hits for #2-14/15 and #3-11/12/25. All showed 179 hits of 80-mers sliding windows suggesting cross-reacting characteristics.
AllerTOPv.2	http://www.ddg-pharmfac.net/AllerTOP/	All cashew PR10-like proteins, except clones #25514-14 and-15 , are predicted to be probably allergenic with nearest allergen matches being Bet v 1-like allergens.
AllergenFPv.1.0	http://www.ddg-pharmfac.net/AllergenFP/	All cashew PR10-like proteins are predicted to be probably allergenic with nearest allergen matches being Bet v 1-like allergens.
BepiPred 1.0	http://www.cbs.dtu.dk/services/BepiPred-1.0/	Each cashew PR10-like protein contains several predicted linear B-cell epitopes
BPAP	http://imed.med.ucm.es/Tools/antigenic.pl	Each cashew PR10-like protein contains several predicted linear B-cell epitopes
ElliPro	http://tools.iedb.org/ellipro/	Multiple continuous as well as discontinues B-cell epitopes have been predicted for each cashew PR10-like protein
NetCTL-1.2	http://www.cbs.dtu.dk/services/NetCTL/	3 to 6 MHC-class ligands and 146-151 T-cell epitope peptides have been predicted using the cashew PR10-like proteins as query.

When a protein is predicted to be allergenic or to be cross-reactive, it should contain antigenic epitope regions that allow for binding to secreted antibodies or antigen-specific cell membrane receptors[37]. Antigenic B-cell epitopes, the aa-region that is recognised by an IgE-antibody, can be linear (continuous, *ca*. 10%) or conformational (partial continuous or discontinuous, *ca*. 90%). T-cell epitopes on the other hand (the aa-region presented on antigen-presenting cells (APC) by the major histocompatibility complex (MHC) molecules) are commonly continuous. Using epitope prediction software tools, several continuous and discontinuous B-cell epitopes were predicted for each of the cashew PR10-like protein clones identified (Table S7). In addition, MHC-class peptides and T-cell epitopes have been predicted.

Predicted B-cell epitopes where annotated on the structural model of PR10 #25355-15 to evaluate the prediction value of the three software tools used (Figure 6). ElliPro 1.0 predicts almost all epitopes in the flexible regions (i.e. links between the structural elements) which are generally the most antigenic[38].

The epitope region ENIEGNGGPG recognised by Bet v 1-specific IgE antibodies within the p-loop region (E43-G52) is predicted in each cashew *PR10-like* clone (underlined in Table S7) with 80%, 60% and 50% identical amino acids in #25355, #25514 and #18220, respectively. Whether two or more amino substitutions in this epitope region might affect the level of Bet v 1-specific IgE cross-reactivity will have to be determined. Also, amino acid S112 shown to be crucial for IgE binding of Mal d 1 and Pru av 1 and cross-reactivity with Bet v 1[39-40] is present in the sequence of both #25514 and #18220 (Figure 2).

Thus, we employed a range of analyses (AllergenOnline, SDAP, NetCTL-1.2, BPAP, BepiPred, AllergenFP and AllerTOPv.2) and the results combined show that the identified PR10 proteins from cashew nut are possibly allergenic and may indeed cross-react with Bet v 1-specific IgE antibodies.

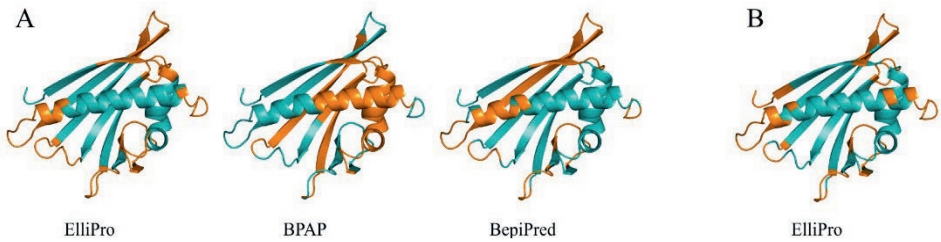


Figure 6. Predicted epitopes for #25355-15 as indicated on the modelled tertiary structure. (A) Continuous epitopes predicted by the software tools ElliPro, BPAP and BepiPred 1.0; (B) Discontinuous epitopes predicted by ElliPro 1.0.

4. Discussion

Cashew nut is solely consumed after proper shelling and roasting, which significantly improves the sensory characteristics (smell, flavour, texture, taste) and eliminates the risks associated with traces of irritating substances derived from the shell (anacardic acid, cardanol and cardol)[1,41]. In general, PR10 family proteins are considered heat-labile and their allergenicity is destroyed or strongly reduced upon heating, at least in fruits and vegetables (reviewed by Fernandes et al[42]). However, Ara h 8 and Gly m 4, the Bet v 1-allergenic homologs from peanut and soy respectively, have shown to be thermally resistant to some extent and able to provoke clinical responses even after heat treatment[43,44]. Similarly, roasted hazelnuts can still provoke allergic reactions in Cor a 1-monosensitised individuals[45]. Thus, since medically relevant OAS complaints, consistent with a PR10 sensitisation, are often reported in a patient's anamnesis after consumption of cashew nut, although consumed in processed form, suggests that clinically reactive PR10 proteins may still be present in the kernel. This was the underlying reason for demonstrating the presence of PR10 proteins in cashew nut in this study.

Using RNA-seq transcriptome profiling and sequence specific cloning, we were able to identify 3 different isotypes of PR10 proteins in cashew nut with several allelic variances. Sequence identity analyses and structural modelling confirmed their identity as Bet v 1 homologous proteins belonging to the PR10 protein family. Six partial ORFs identified in the RNA-seq contig BLAST point out the presence of various other isotypes or isoforms of *PR10-like* sequences in cashew nut, which might be elongated and extracted using Rapid amplification of cDNA ends (RACE) techniques in the future. In addition to the presence of PR10 mRNA, two independent LC-MS/MS analysis experiments and immunoblotting assays indicated the presence of PR10 protein in cashew nut as well. Using LC-MS/MS, we were able to detect 3 PR10-coding contigs out of 9 contigs identified. Possibly, trypsin inhibitors limiting the efficiency of the LC-MS/MS sample preparations might have been present in our protein extract[46], which could be one of the reasons why peptides of only 3 contigs were traced back. Another reason might be a possible low concentration of some of the PR10 contigs in our extract. When comparing the protein iBAQ scores of the detected PR10 contigs with the score for Ana o 3.0101, which has more or less the same protein mass, the PR10 proteins are presumably at 99 times (for #25355) to 2970 times (for #25514) a lower concentration (Table S4B). However, proper protein quantification using spiked standards in multiple biological replicates should confirm this.

The existence of multigene *PR10* copies in cashew nut is in line with findings for the *PR10* gene Gly m 4 for which multiple copies exist in the soybean genome[47].

Chromosome studies in cashew nut populations[48,49] suggest an overall diploidic genotype but does not rule out the existence of polyploid species. However, it is also likely that seeds pooled for the RNA extraction procedure originated from different trees and thus represent different genotypes.

To assess the possible allergenicity of the cashew PR10 proteins, a preliminary *in silico* -prediction analysis was performed. The presence of multiple 6-mers, 8-mers and 80-mers sliding window peptides with cross-reacting characteristics, the potential allergenicity predictions by the online software tools AllerTOPv.2 and Allergenv1.0 as well as the presence of various predicted B-cell epitopes has led us to conclude that the identified cashew PR10 proteins should be considered as potential allergens that are predicted to exhibit IgE cross-reactivity with Bet v 1. Thus, cashew PR10 proteins might have been the causative agents for observed OAS symptoms in cashew allergic patients in earlier studies[11-14] or even be responsible for more severe symptoms. Severe cases of OAS aggravating to systemic reactions, have been observed in allergic reactions to peanut and pistachio[17,44,50] estimated that around 5% of OAS patients have symptoms progressing to systemic responses including nausea, vomiting, abdominal pain, upper respiratory obstruction or anaphylaxis.

Most importantly, clinical relevance of identified PR10 proteins in processed cashew nuts still needs to be demonstrated through IgE-immunoassays (e.g. basophil activation test (BAT), skin prick test (SPT) and/or ELISAs) to actually identify these proteins as real allergens. It might be however, that not all of the *PR10-like* genes present in cashew nut are clinically relevant and thus their individual and possibly their combined allergenicity should be quantified. Expression levels of the different PR10 isoforms and isoallergens might even fluctuate per genus, origin or per season, depending on climate and environmental or geographical factors/influences[1]. Thus, influence of variation in exposure levels should be taken into account in future risk assessments as well as tolerance thresholds per isoallergen. However, cashew nut-provoked OAS symptoms should be carefully interpreted especially when symptoms emerge at low doses of cashew nut exposure. Oral allergy symptoms are frequently reported by peanut allergic individuals, especially when exposed to very low doses between 100µg- 5 mg of peanut protein[51]. This implies that seed storage proteins, which are commonly seen as major allergens causing severe allergic reactions, can also provoke subjective reactions (oral itching) and mild objective reactions (lip swelling) that correspond to OAS symptoms associated with a PR10 sensitization. Besides, OAS symptoms might also be caused by other PR-family members, such as non-specific lipid transfer proteins (nsLTPs; PR-14) or thaumatin-like proteins (TLPs; PR-5), or by proteins belonging to the profilin family[18]. Current investigations are ongoing to investigate whether such allergen family members are also expressed in cashew nut.

Lastly, the mechanism behind how some seed/nut PR10 proteins retain their allergenicity after heating is still an intriguing question. Seeds are plant organs that usually have a low water content and that have several protective adaptations to cope with dehydration which protects cellular integrity and stabilizes proteins, RNA and DNA. Further, seeds contain high levels of storage compounds, like sugar, fat and proteins. In this sense, seeds are different from fruit and vegetable tissues and the seed matrix can play a role in the protection of PR10 allergenic proteins from thermal destruction. Interestingly, this protection from thermal destruction has been observed in fat/oil-rich leguminous seeds (peanut and soy) and nuts (hazelnut)[35,45,52,53]. The total fat content in cashew nut is high as well and accounts for 48.3% of the total weight[54], which is comparable to the lipid content reported for peanut (40-50%)[55]. In addition, PR10 stability has also been linked to binding to their ligands. The characteristic structure of Bet v 1 and its homologues, comprising of seven-stranded β-sheets flanked by three α-helices forming a central basket-like hydrophobic cavity[34], allows binding of a variety of lipophilic ligands[56]. Like Bet v 1[57], Ara h 8 is hypothesised to bind flavonoids (quercitin, apigenin and daidzein), and lipid sterols[24,53,55]. This ligand binding provided increased thermal proteolytic stability to the Bet v 1[58] and Ara h 8[43] structure. Thus, it seems possible that cashew nut PR10-like proteins may function as flavonoid or sterol carriers. Whether thermal degradation of cashew PR10 proteins is influenced by the seed matrix and its ligands, and thereby their allergenic cross-reactivity, remains an important issue to be investigated.

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Conflicts of interest
The authors declare no competing interest.

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Supplementary information
See for supplemental material and methods, Figures and Tables Doi: 10.1002/pro.3856 (open access).

References

1. Dendena B, Corsi F (2014) Cashew, from seed to market: a review. Agron Sustain Dev 34:753-772.
2. Aremu MO, Olonisakin A, Bako DA, Madu PC (2006) Compositional studies and physicochemical characteristics of cashew nut (Anarcadium occidentale) flour. Pak J Nutr 5(4):328–333.
3. Aremu MO, Ogunlade I, Olonisakin A (2007) Fatty acid and amino acid composition of protein concentrate from cashew nut (Anarcadium occidentale) grown in Nasarawa State,Nigeria. Pak J Nutr 6(5):419-423.
4. Ryan E, Galvin K, O'Connor TP, Maguire AR, O'Brien NM (2006) Fatty acid profile, tocopherol, squalene and phytosterol content of brazil, pecan, pine, pistachio and cashewnuts. Int J Food Sci Nutr 57(3-4): 219-228.
5. Hu FB, Manson JE, Willett WC (2001) Types of dietary fat and risk of coronary heart disease: a critical review. J Am Coll Nutr 20:5-19.
6. Mah E, Schulz JA, Kaden VN, Lawless AL, Rotor J, Mantilla LB, Liska DJ (2017) Cashew Consumption Reduces Total and LDL Cholesterol: A Randomized, Crossover, Controlled-Feeding Trial. Am J Clin Nutr 105:1070-1078.
7. Weinberger T, Sicherer S (2018) Current perspectives on tree nut allergy: a review. Current perspectives on tree nut allergy: a review. J Asthma Allergy 11:41-51.
8. Davoren M, Peake J (2005) Cashew nut allergy is associated with a high risk of anaphylaxis. Arch Dis Child 90(10):1084-5.
9. Mendes C, Costa J, Vicente AA, Oliveira MB, Mafra I (2019) Cashew Nut Allergy: Clinical Relevance and Allergen Characterisation. Clin Rev Allergy Immunol 57(1):1-22.
10. van der Valk JP, Gerth van Wijk R, Vergouwe Y, Steyerberg EW, Reitsma M, Wichers HJ, Savelkoul HF, Vlieg-Boerstra B, de Groot H, Dubois AE, de Jong NW (2017) sIgE Ana o 1, 2 and 3 accurately distinguish tolerant from allergic children sensitized to cashew nuts. Clin Exp Allergy 47(1):113-120.
11. Li H, Zhang H (1997) Hypersensitivity of cashew nut. Chin Med Sci J 12(3):189-92.
12. Rancé F, Bidat E, Bourrier T, Sabouraud D (2003) Cashew allergy: observations of 42 children without associated peanut allergy. Allergy 58(12):1311-4.
13. Hasegawa M, Inomata N, Yamazaki H, Morita A, Kirino M, Ikezawa Z (2009) Clinical features of four cases with cashew nut allergy and cross-reactivity between cashew nut and pistachio. Allergol Int 58(2):209-15.
14. van der Valk JP, Gerth van Wijk R, Dubois AE, de Groot H, Reitsma M, Vlieg-Boerstra B, Savelkoul HF, Wichers HJ, de Jong NW (2016) Multicentre Double-Blind Placebo-Controlled Food Challenge Study in Children Sensitised to Cashew Nut. PLoS One 11(3):e0151055.
15. Noorbakhsh R, Mortazavi SA, Sankian M, Shahidi F, Tehrani M, Azad FJ, Behmanesh F, Varasteh A (2011) Pistachio allergy-prevalence and in vitro cross-reactivity with other nuts. Allergol Int 60(4):425-32.
16. Valenta R, Kraft D (1996) Type 1 allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. J Allergy Clin Immunol 97(4):893Y895.
17. Ma S, Sicherer SH, Nowak-Wegrzyn A (2003) A survey on the management of pollen-food allergy syndrome in allergy practices. J Allergy Clin Immunol 112(4):784Y788.
18. Price A, Ramachandran S, Smith GP, Stevenson ML, Pomeranz MK, Cohen DE (2015) Oral allergy syndrome (pollen-food allergy syndrome). Dermatitis 26(2):78-88.
19. Breiteneder H, Radauer C (2004) A classification of plant food allergens. J Allergy Clin Immunol 113(5):821-830.
20. van Odijk J, Sjölander S, Brostedt P, Borres MP, Englund H (2017) High frequency of IgE sensitization towards kiwi seed storage proteins among peanut allergic individuals also reporting allergy to kiwi. Clin Mol Allergy 15:18.
21. Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr WR, Valent P, et al (1993) Purification and characterization of recombinant Bet v I, the major birch pollen allergen. Immunological equivalence to natural Bet v I. J Biol Chem 268(26):19574-80.
22. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H (2008) Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol 121(4):847-852.
23. Vieths S, Scheurer S, Ballmer-Weber B (2002) Current understanding of cross-reactivity of food allergens and pollen. Ann N Y Acad Sci 964:47-68.

24. Hurlburt BK, Offermann LR, McBride JK, Majorek KA, Maleki SJ, Chruszcz M (2013) Structure and function of the peanut panallergen Ara h 8. J Biol Chem 288(52):36890-901.
25. Wangorsch A, Jamin A, Lidholm J, Grani N, Lang C, Ballmer-Weber B, Vieths S, Scheurer S (2017) Identification and implication of an allergenic PR-10 protein from walnut in birch pollen associated walnut allergy. Mol. Nutr. Food Res 61(4):1-9.
26. Bastiaan-Net S, Reitsma M, Cordewener JHG, van der Valk JPM, America TAHP, Dubois AEJ, Gerth van Wijk R, Savelkoul HFJ, de Jong NW, Wichers HJ (2019) IgE Cross-Reactivity of Cashew Nut Allergens. Int Arch Allergy Immunol 178(1):19-32.
27. Midoro-Horiuti T, Brooks EG, Goldblum RM (2001) Pathogenesis-related proteins of plants as allergens. Ann Allergy Asthma Immunol 87(4):261-71.
28. Saraste M, Sibbald PR, Wittinghofer A (1990) The P-loop – a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci 15:430-434.
29. Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ, Schou C, Løwenstein H, Spangfort MD (1996) X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat Struct Biol 3(12):1040-5.
30. Farazi TA (2001) "The Biology and Enzymology of Protein N-Myristoylation". J Biol Chem 276(43):39501-39504.
31. Johnson LN, Barford D (1993) The effects of phosphorylation on the structure and function of proteins. Annu Rev Biophys Biomol Struct 22:199-232.
32. Cieśla J, Frączyk T, Rode W (2011) Phosphorylation of basic amino acid residues in proteins: important but easily missed. Acta Biochim Pol 58(2):137-147.
33. Hoffmann-Sommergruber K, O'Riordain G, Ahorn H, Ebner C, Laimer Da Camara Machado M, Pühringer H, Scheiner O, Breiteneder H (1999) Molecular characterization of Dau c 1, the Bet v 1 homologous protein from carrot and its cross-reactivity with Bet v 1 and Api g 1. Clin Exp Allergy 29(6):840-7.
34. Neudecker P, Schweimer K, Nerkamp J, Scheurer S, Vieths S, Sticht H, Rösch P (2001) Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. J Biol Chem 276(25):22756-63.
35. Mittag D, Akkerdaas J, Ballmer-Weber BK, Vogel L, Wensing M, Becker WM, Koppelman SJ, Knulst AC, Helbling A, Hefle SL, Van Ree R, Vieths S (2004) Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. J Allergy Clin Immunol 114(6):1410-7.
36. Ladics GS, Bannon GA, Silvanovich A, Cressman RF (2007) Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. Mol Nutr Food Res 51(8):985-98.
37. Yang X, Yu X. An introduction to epitope prediction methods and software (2009) Rev Med Virol 19(2):77-96.
38. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 12(2):231-42.
39. Scheurer S, Son DY, Boehm M, Karamloo F, Franke S, Hoffmann A, Haustein D, Vieths S (1999) Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen. Mol Immunol 36(3):155-67.
40. Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S (1999) Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. Eur J Nutr 38(4):201-15.
41. Tyman JHP (1977) Long-chain phenols : VIII. Quantitative analysis of the unsaturated constituents of phenolic lipids by thin-layer chromatography-mass spectrometry. J Chromatogr 136(2):289-300.
42. Fernandes H, Michalska K, Sikorski M, Jaskolski M (2013) Structural and functional aspects of PR-10 proteins. FEBS J 280(5):1169-99.
43. Petersen A, Rennert S, Kull S, Becker WM, Notbohm H, Goldmann T, Jappe U (2014) Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8. Biol Chem 395(2):239-50.
44. Mittag D, Vieths S, Vogel L, Becker WM, Rihs HP, Helbling A, Wüthrich B, Ballmer-Weber BK (2004) Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. J Allergy Clin Immunol 113(1):148-54.
45. Hansen KS, Ballmer-Weber BK, Lüttkopf D, Skov PS, Wüthrich B, Bindslev-Jensen C, Vieths S, Poulsen LK (2003) Roasted hazelnuts--allergenic activity evaluated by double-blind, placebo-

controlled food challenge. Allergy 58(2):132-8.
46. Mohan M, Kozhithodi S, Nayarisseri A, Elyas KK (2018) Screening, Purification and Characterization of Protease Inhibitor from Capsicum frutescens. Bioinformation 14(6):285-293.
47. Crowell DN, John ME, Russell D, Amasino RM (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol 18(3):459-66.
48. Aliyu OM, Awopetu JA (2007) Chromosome studies in Cashew (Anacardium occidentale L.). Afr J Biotechnol 6(2):131-136.
49. Archak S, Gaikwad AB, Gautam D, Rao EV, Swamy KR, Karihaloo JL (2003) Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (Anacardium occidentale L.) accessions of India. Genome 46(3):362-9.
50. Ando K, Watanabe D, Tamada Y Matsumoto Y. (2011) Oral allergy syndrome with severe anaphylaxis induced by pistachio. Int J Dermatol 50(5):632Y633.
51. Hourihane JB, Kilburn SA, Nordlee JA, Hefle SL, Taylor SL, Warner JO (1997) An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. J Allergy Clin Immunol 100(5):596-600.
52. Hansen KS, Ballmer-Weber BK, Lüttkopf D, Skov PS, Wüthrich B, Bindslev-Jensen C, Vieths S, Poulsen LK (2003) Roasted hazelnuts--allergenic activity evaluated by double-blind, placebo-controlled food challenge. Allergy 58(2):132-8.
53. Petersen A, Rennert S, Kull S, Becker WM, Notbohm H, Goldmann T, Jappe U (2014) Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8. Biol Chem 395(2):239-50.
54. Rico R, Bulló M, Salas-Salvadó J (2015) Nutritional composition of raw fresh cashew (Anacardium occidentale L.) kernels from different origin. Food Sci Nutr 4(2):329-38.
55. Riecken S, Lindner B, Petersen A, Jappe U, Becker WM 2008 Purification and characterization of natural Ara h 8, the Bet v 1 homologous allergen from peanut, provides a novel isoform. Biol Chem 389(4):415-423.
56. Sancho AI, Wangorsch A, Jensen BM, Watson A, Alexeev Y, Johnson PE, Mackie AR, Neubauer A, Reese G, Ballmer-Weber B, Hoffmann-Sommergruber K, Skov PS, Vieths S, Mills EN (2011) Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: impact on structure and allergenic activity. Mol Nutr Food Res 55(11):1690-9.
57. Seutter von Loetzen C, Hoffmann T, Hartl MJ, Schweimer K, Schwab W, Rösch P, Hartl-Spiegelhauer O (2014) Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. Biochem J 457(3):379-90.
58. Asam C, Batista AL, Moraes AH, de Paula VS, Almeida FC, Aglas L, Kitzmüller C, Bohle B, Ebner C, Ferreira F, Wallner M, Valente AP (2014) Bet v 1--a Trojan horse for small ligands boosting allergic sensitization? Clin Exp Allergy 44(8):1083-93.
59. Maia J, Dekkers BJW, Provart NJ, Ligterink W, Hilhorst HWM (2011) The Re-Establishment of Desiccation Tolerance in Germinated Arabidopsis thaliana Seeds and Its Associated Transcriptome. PLoS ONE 6(12): e29123.
60. Silvanovich A, Bannon G, McClain S (2009) The use of E-scores to determine the quality of protein alignments. Regul Toxicol Pharmacol 54(3):S26-31.
61. Ponting CP, Schultz J, Milpetz F, Bork P (1999) SMART: identification and annotation of domains from signalling and extracellular protein sequences. Nucleic Acids Res 27(1):229-32.
62. Eswar N, Webb B, Marti-Renom MA, Madhusudhan M, Eramian D, Shen M-y, Pieper U, Sali A (2006) Comparative protein structure modeling using Modeller. Curr Protoc Protein Sci 5:Unit 5.6.
63. Offermann L, Perdue M, He J, Hurlburt B, Maleki S, Chruszcz M (2015) Structural Biology of Peanut Allergens. J Contemp Immunol 2(1):1-26.
64. Reitsma M, Bastiaan-Net S, Sforza S, van der Valk JP, van Gerth van Wijk R, Savelkoul HF, de Jong NW, Wichers HJ (2016) Purification and Characterization of Anacardium occidentale (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3. J Agric Food Chem 64(5): 1191–201.
65. Solntsev SK, Shortreed MR, Frey BL, Smith LM (2018) Enhanced Global Post-translational Modification Discovery with MetaMorpheus. J Proteome Res 17(5):1844-1851.
66. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367-1372.
67. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL,

Liebler DC, MacCoss MJ (2010) "Skyline: an open source document editor for creating and analyzing targeted proteomics experiments". Bioinformatics 26(7):966-8.
68. Ivanciuc, O., Schein. C. H., and Braun, W (2003) SDAP: Database and Computational Tools for Allergenic Proteins. Nucleic Acids Res 31(1):359-362.
69. Goodman RE, Ebisawa M, Ferreira F, Sampson HA, van Ree R, Vieths S, Baumert JL, Bohle B, Lalithambika S, Wise J, Taylor SL (2016) AllergenOnline: A peer-reviewed, curated allergen database to assess novel food proteins for potential cross-reactivity. Mol. Nutr. Food Res 60(5):1183-1198.
70. Dimitrov I, Bangov I, Flower DR, Doytchinova I (2014) AllerTOP v.2--a server for in silico prediction of allergens. J Mol Model 20(6):2278.
71. Dimitrov I, Naneva L, Doytchinova I, Bangov I (2014) AllergenFP: allergenicity prediction by descriptor fingerprints. Bioinformatics 30(6):846-51.
72. Larsen MV, Lundegaard C, Lamberth K, Buus S, Lund O, Nielsen M (2007) Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. BMC Bioinformatics 8:424.
73. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, Sette A, Peters B (2008) ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics 9:514.
74. Larsen JE, Lund O, Nielsen M (2006) Improved method for predicting linear B-cell epitopes. Immunome Res 24(2):2.

Chapter 6

Cashew nut seed storage proteins are comprised of multigene families and include three Ana o 3 isotypes

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Abstract

Seed storage proteins in edible nuts are important food allergens, which can cause systemic allergic reactions and/or severe allergic reactions (anaphylaxis), especially the 2S storage albumins. Cashew nuts (*Anacardium occidentale*) may cause more severe allergic reactions than peanut and at very low threshold doses. Three seed storage proteins are identified in cashew nut that act as allergens, i.e. Ana o 1.0101/1.0102 (7S vicilin), Ana o 2.0101 (11S globulin) and Ana o 3.0101 (2S albumin). Since seed storage proteins often span a family of multiple genes, we aimed to investigate the presence of additional seed storage protein genes in cashew nuts using a bioinformatic approach, including RNA-seq data analysis, to identify potential allergenic seed storage proteins other than those already used in allergy diagnosis. This analysis, allowed us to identify several Ana o 1-like, Ana o 2-like and Ana o 3-like transcripts in cashew nut. We confirmed the expression and sequence of two novel Ana o 3 isotypes by gene cloning, designated #1903 and #2387, showing 71% and 84% protein homology with Ana o 3.0101, respectively. Epitope mapping suggest that these two novel Ana o 3 isotypes are potentially allergenic and it is thus advisable that future research focusing on cashew nut allergy take all 2S albumin types into account.

Keywords: Cashew nut, seed storage proteins, Ana o 1, Ana o 2, Ana o 3, 2S albumins, allergen isotypes.

1. Introduction

Seeds are the most important survival organ in seed shedding plants. To support germination and growth of the seedling, the seed contains a nutrient reservoir, which is stored in the embryo (mostly in the embryonic leaves or cotyledons) and/or in the storage tissue called the endosperm. These stored nutrients are rich in carbohydrates, oil and proteins. Due to their nutritional value seeds, like those from cereals and legumes, are an important food source for humans and it is estimated that 70% of our food comes directly from seeds[1]. The relative amounts of the storage compounds (carbohydrates, oil and proteins) are for a large part dependent on genetic factors, resulting in variations in protein storage that can range from 7% up to 37% of the seed's composition (for details on a set of important crop species, see Bewley et al[1]). The vast majority of proteins in seeds belong the so-called seed storage proteins (SSPs). Nearly a hundred years ago, Osborne[2] made a first classification of the major SSPs depending on their solubility in diluted salt solution into the superfamily of globulins (soluble) and prolamins (insoluble). Later, a further subdivision was made, according to their fractionation behaviour using sucrose density gradient centrifugation[3]. This resulted in a further division into 7S globulins (e.g. β-conglutin, vicilin, convicilin and vicilin-type), 7S basic globulins (e.g. γ-conglutin), 11S globulins (e.g. α-conglutin, legumin, legumin-like and glycinin) and, within the prolamin superfamily, the 2S albumins[4]. These SSPs are often encoded by multi-gene families.

Storage proteins appear to not solely function as nutrient reservoirs. There are indications that they also play an important role in the seed's defence. Antimicrobial activity has been assigned to some globulin-type of proteins[5,6], while 2S albumins are known for their antifungal activity[7-10]. In addition, a 2S albumin proprotein from Indian mustard was found to function as a trypsin inhibitor and in this way to function in insect resistance[11]. SSPs also play a role in seed longevity. SPPs protect the seed from oxidative stress during seed storage, thereby maintaining the functionalities of important proteins required for seed germination and seedling formation[12].

For some individuals, seed (and thus SSPs) consumption can lead to serious health problems as SSPs belong to two out of four protein superfamilies that contain most identified food allergens (i.e. prolamins and globulins, (>65%))[13], and (especially the 2S storage albumins) account for the majority of systemic allergic reactions (anaphylaxis cases)[14]. The with allergy related SSPs comprise multigene families. For example, peanut (*Arachis hypogaea*) expresses three isotypes (including multiple isoforms) of 2S albumins (Ara h 2, Ara h 6 and Ara h 7), while for soy (*Glycine max*) multiple 7S (Gly m5) and 11S (Gly m 6) globulin isoforms have been identified (http://www.allergen.org/). Protein isoforms are encoded by allelic variances or originate from the same gene as a result of alternative splicing events

or post-translational processing, while isotypes are encoded by different sets of genes[15]. Cashew nuts (*Anacardium occidentale*) may cause more severe allergic reactions than peanut[16] and at very low threshold doses[17-18]. The allergens considered important in cashew nut allergy include three SSPs, namely Ana o 1.0101 (7S vicilin), Ana o 2.0101 (11S globulin) and Ana o 3.0101 (2S albumin)[19-21]. Two allelic variants or isoforms are known for Ana o 1, designated as Ana o 1.0101 and Ana o 1.0102, which differ in just one amino acid (994 A/G)[19]. Furthermore, Robotham et al[21] hinted on the existence of three native Ana o 3 large-subunit isoforms. Reitsma et al[22] have found evidence in cashew nut protein extract for the existence of multiple iso-allergens or isoforms for Ana o 1, Ana o 2 as well as Ana o 3.

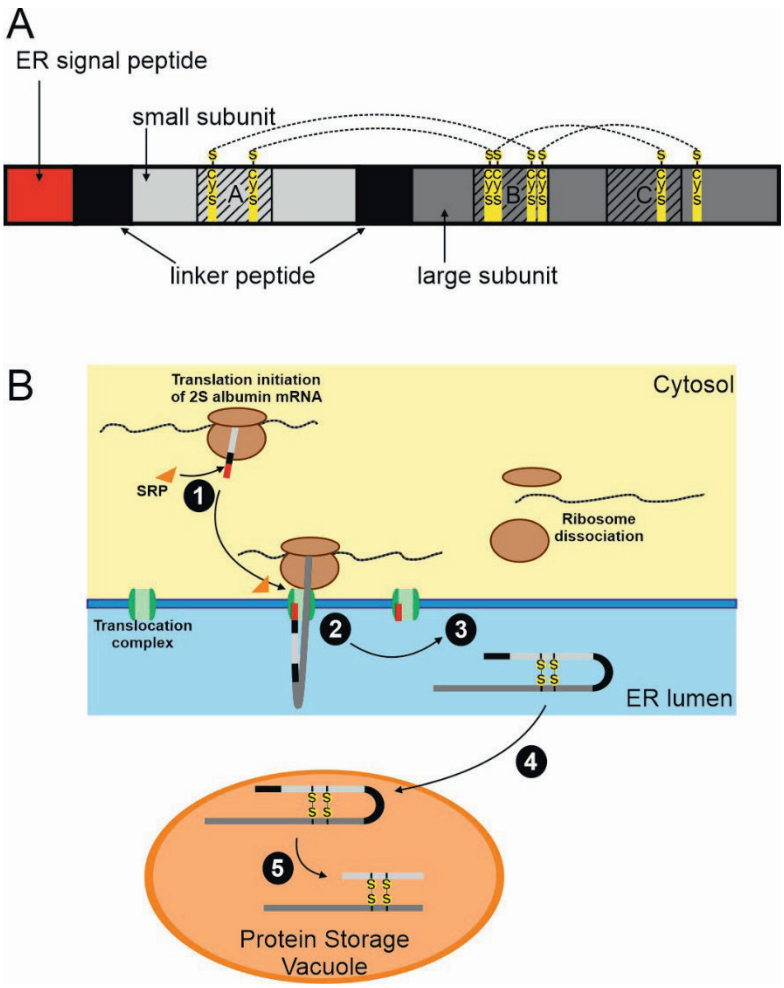

Figure 1. Schematic illustration of 2S albumin preproprotein structure and post-translational processing. (A) Domain structure of a typical 2S albumin preproprotein. The signal peptide and the linker peptides are removed during protein processing (see also in B). The 2S albumin small and large subunit contain

three conserved regions (shades areas) named A, B and C[28]. A total of eight cysteine amino acids (indicated in yellow) are conserved in 2S albumins, which contribute to protein folding and 3D structure through the formation of disulphide bridges (indicated by the dashed lines, according to Moreno and Clemente[29]); (B) Upon translation of the 2S albumin mRNA by ribosomes in the cytosol, the endoplasmic reticulum (ER) signal peptide is recognized by a small recognition particle (SRP, indicated by the orange triangle). This directs the 2S albumin-translating ribosome to a translocator complex in the ER membrane (1). Upon translation, the preproprotein precursor is transported to the ER lumen (2). There, the ER signal peptide is removed by peptidase cleavage at the signal peptidase cleavage site generating the proprotein[30] (3). Formation of disulphide bridges at conserved cysteine residues allows for structure formation and transport to the protein storage vacuole. There are at least four routes (including Golgi-dependent and Golgi-independent routes) known for seed storage proteins to the protein storage vacuole (PSV)[1] (4). In the PSV, the final 2S albumin heterodimer is formed by protein body endoproteases which remove linker peptides resulting in separation of the small subunit from the large subunit (5). Illustration was based on Heldt and Piechulla[31] and Moreno et al[29].

Storage proteins are generally translated as a larger preproprotein precursor which undergoes various post-translational processing steps before the final storage protein is formed, which is schematically illustrated for a typical 2S albumin in Figure 1. For instance, processing of the 16.3 kDa preproprotein precursor of Ana o 3.0101 is suggested to result in a ~11.8-12.8 kDa Ana o 3 heterodimer, consisting of ~3.7-4.5 kDa and 8.1-8.4 kDa subunits joined together by disulphide bridges[22]. However, alternative processing variants do exist. The sunflower 2S albumin SFA-8 is, in contrast to most other 2S albumins, not cleaved into a small and large subunit element but rather consists of a single polypeptide chain interlinked by disulphide bridging[23].

Since for each of the cashew seed storage protein types only one complete gene has been identified and cloned (Ana o 1.0102 is truncated at the N-terminal[19]), the aim of this study was to investigate whether additional genes could be identified in cashew nut coding for SSPs of the 2S albumin, 7S vicilin, and 11S globulin class. Using a next generation sequencing (NGS) database generated from cashew nut mRNA[24] (see Chapter 5), the presence of additional globulin and albumin genes was demonstrated. As plasma IgE against Ana o 3 is considered to be the most predictive marker for cashew nut allergy[25-27] and appears to be correlated with a high risk of severe anaphylaxis[14], the identified Ana o 3 isotypes were cloned and evaluated for their importance in cashew nut allergy diagnostics.

2. Material and methods

2.1 Samples & chemicals

Raw in-shell cashews nuts from Cambodia were kindly provided by Intersnack B.V. (Doetinchem, The Netherlands). Chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless stated otherwise. Primers were from Biolegio B.V. (Nijmegen, The Netherlands). MT platinum SuperFi DNA polymerase and the

pPIC9K vector were from Invitrogen (Carlsbad, CA, USA). The pGEM®-T easy vector system was purchased from Promega Benelux B.V. (Leiden, The Netherlands) while the iScript Select cDNA Synthesis Kit and Precision Plus Protein™ Dual Xtra marker was from Bio-Rad Laboratories B.V. (Veenendaal, The Netherlands). SDS-PAGE gel electrophoresis equipment, Pichia EasyComp™ kit and the Coomassie Bradford assay kit was from Thermo Fisher Scientific (Waltham, MA, USA). The ÄKTA pure chromatography system including columns were from GE Healthcare (Chicago, IL, USA) while the Amicon Ultra-15 centrifugal filters were purchased from Millipore (Darmstadt, Germany).

2.2 RNA-seq BLAST analyses

BLAST tblastn analyses were performed on the cashew nut RNA-seq paired assembly consensus database (SRA accession code PRJNA566328) in Gx CLC Genomics Workbench version 10.1.1 (Qiagen; Hilden, Germany) as described previously[24]. The seed storage allergen amino acid sequences of almond (*Prunus dulcis*), brazil nut (*Bertholletia excelsa*), cashew nut (*Anacardium occidentale*), hazelnut (*Corylus avellana*), lentil (*Lens culinaris*), peanut (*Arachis hypogeae*), pecan (*Carya illinoensis*), Chinese pine nut (*Pinus koraiensis*), pistachio (*Pistacia vera*), soybean (*Glycine max*) and walnut (*Juglans nigra* and *Juglans regia*) were used as query when available: Ana o 1.0101 (Q8L5L5), Ara h 1.0101 (P43238), Car i 2.0101 (B3STU4), Cor a 11.0101 (Q8S4P9), Gly m 5.0101-5.0301 (O22120, Q9FZP9, P25974), Jug n 2.0101 (Q7Y1C1), Jug r 2.0101 (Q9SEW4), Jug r 6.0101 (AOA2I4E5L6), Len c 1.0101 (Q84UI1), Pin k 2.0101 (V9VGUO), Pis s 1.0101 (Q702P1) and Pis v 3.0101 (B4X640) for 7S Vicilin screening; Ana o 2.0101 (Q8GZP6), Ara h 3.0101-3.0201 (O82580, Q9SQH7), Ber e 2.0101 (Q84ND2), Car i 4.0101 (B5KVH4), Cor a 9.0101 (Q8W1C2), Gly m 6.0101-6.0501 (P04776, P04405, P11828, P02858, P04347), Jug n 4.0101 (A0A1L6K371), Jug r 4.0101 (Q2TPW5), Pis v 2.0101-2.0201 (B7P073, B7P074), Pis v 5.0101 (B7SLJ1) and Pru du 6.0101-6.0201 (E3SH28, E3SH29) for 11S globulin screening; Ana 0 3.0101 (Q8H2B8), Ara h 2.0101 (Q6PSU2), Ara h 6.0101 (Q647G9), Ara h 7.0101 (Q9SQH1), Ber e 1.0101 (P04403), Car i 1.0101 (Q84XA9), Cor a 14.0101 (D0PWG2), Gly m 8.0101 (P19594), Jug r 1.0101 (P93198) and Pis v 1.0101 (B7P072) for 2S albumin screening. Selection of reliable homologs was based on a Maximal % identity score of 40% in relation to the Maximum score and E-value.

2.3 Protein extractions

Total protein extracted from blanched defatted cashew nuts using an ammonium bicarbonate buffer (pH7.9; 0.1M ammonium bicarbonate, 0.5M NaCl) or Urea buffer (20mM sodium phosphate buffer pH7.0, 1mM NaCl, 8M Urea) were obtained as described by Reitsma et al[22] and Bastiaan-Net et al[32], respectively.

A cashew nut protein fraction containing native Ana o 3 was obtained by ammonium sulphate precipitation and ultrafiltration as previously described[22]. Protein

concentrations were determined by the Bradford assay kit according to manufacturer's instructions using BSA (2-10μg/ml) as a standard.

2.4 SDS PAGE

Reduced and nonreduced denaturing SDS-PAGE gel electrophoresis was performed on precast NuPAGE 10% Bis-Tris gels as previously described[22]. As marker, the Precision Plus Protein™ Dual Xtra was used.

2.5 LC-MS/MS protein identification

Ana o 3 isotype specific peptides in total protein extracts (100 μg) as well as in excised bands from SDS PAGE, were identified using targeted LC-MS/MS as described previously by Bastiaan-Net et al[24].

2.6 Ion-exchange chromatography

The native Ana o 3 fraction was concentrated and equilibrated with Eluent A (20 mM Tris/HCl pH8, 1M NaCl; 0.2mm filtered) in five consecutive rounds using 3kDa Amicon Ultra-15 centrifugal filters. Isotypes purification via ion-exchange chromatography was performed by injecting the Ana o 3 fraction (2.19 mg) in pre-equilibrated Source15Q column (12x20cm) on a ÄKTA pure chromatography system. The column was equilibrated with eluent A (5ml) followed by 7 ml of 20 mM Tris/HCl (pH8, 0.2 mm filtered). Gradient elution was performed between eluent A en B (10 mM Tris/HCl pH8, 1M NaCl) using a ramp of 0% to 60% B for 27 min. Once per minute, eluted fractions were collected at a flowrate of 0.6ml/min.

2.7 Cloning of Ana o 3 isotype sequences

Extraction and sub-cloning of the Ana o 3 isotype genes from mRNA to pGEM®-T easy has been performed as previously described [24]. Ana o 3 isotype-specific primers were designed in the 5'- and 3'- untranslated regions (UTRs) for contig 1903 (forward 5'-TTCCATAATCCCCAACGG-'3; reverse 5'-GCATAATCATCTTCCACTCATC-'3) and contig 2387 (forward 5'-CATCATTCAAACACAAATATAATAAAC-'3; reverse 5'-ACTTCATCCACCAGTGC-'3). Four clones per construct were verified by sequencing (Baseclear B.V.; Leiden, the Netherlands) and their consensus sequence has been deposited into the NCBI GenBank database with the following accession numbers: MT182946 (Ana o 3-1903 #6), MT182947 (Ana o 3-2387 #4) and MT182948 (Ana o 3-2387 #6).

2.8 Tertiary structure prediction

Prediction of tertiary structures was performed as described before[24]. For structure predictions, the deduced protein sequences of the cloned 2S albumin precursors were used minus the first 34 residues. As template, the solution NMR structure PDB-ID 1sm7 (recombinant pronapin precursor) of *Brassica napus* was used as nearest available structure.

2.9 PEPperMAP® Epitope Mapping

Epitope microarray mapping on the deduced protein sequence was performed by PEPperPRINT GmbH (Heidelberg, Germany). For each Ana o 3 isotype, 15 aa linear peptides with a peptide-peptide overlap of 13 aa were printed *in duplo,* spanning the entire preproprotein precursor sequence. The resulting peptide microarray was subsequently incubated with plasma of a cashew allergic individual (Donor I.D. #26741-EW; Bleed #22733), purchased from PlasmaLab International (Everett, WA, USA). Signal intensities were provided as normalised means of *duplo* spots.

3. Results and discussion

3.1 BLAST screening for globulin and albumin-type of seed storage protein coding regions.

To search for cashew nut transcripts that show identity with previously identified allergenic seed storage proteins, a RNA-seq database analysis was applied. As previously shown for the identification of PR10-like transcripts in cashew nuts, this approach has proven to be very effective for the identification of protein isotypes[24] (see Chapter 5).

A BLAST screening using fourteen common 7S vicilin allergens as query taking an E-value of <E-20 as limit, resulted in a selection of eight contigs with homology to Ana o 1 (contigs #46, #591, #2164, #2904, #11773, #25774, #42843, #45295). Contigs #25774 and #45295 contained no open reading frame (ORF) while contigs #11773, and #42843 represented ORFs too small to contain a full length 7S vicilin gene (Table 1). For these reasons, these contigs were discarded from further analyses. Each of the remaining characterised contigs contain the Cupin 1 domain (pfam 00190/ InterPro IPR013096) which represents the conserved barrel domain of the cupin-like superfamily to which both 7S and 11S globulin seed storage proteins belong to[33].

Contig #46 has the highest no. of reads and corresponds to the Ana o 1.0101 allergen in cashew (an alignment of the deduced protein sequences is shown in Supplementary Figure S1). The other identified 7S vicilin-like contigs show only ~24% identity with Ana o 1.0101 and even cluster separately from the other well-known vicilin allergens (Figure 2A). Low homology between cupin-like genes within one species is not uncommon as seen for the common walnut allergens Jug r 2 and Jug r 6 which only share 37% aa homology.

Table 1. Selected 7S vicilin-homologous contigs, ranked according to contig # and presence of a representative ORF. ORF characteristics and BLAST results are only shown for the most likely 7S vicilin contigs homologues. Bp: number of base pears; Aa: number of amino acids; Mw: molecular weight; pI: protein isoelectric point.

Contig mapping			ORF characteristics				BLAST results				
Contig #	Consensus length (bp)	No. of reads	Bp	Aa	Mw (kDa)	pI	Max Score	E-value	Max Identity (%)	Highest identity with	Conserved domains
46	2031	648,778	1617	538	61.840	5.53	461-2398	$6.57E^{-50}$-0	30.34-100	Ana o 1.0101	One Cupin 1 domain
591	1568	43,627	1553 (fragment)	517	59.698	5.21	163-321	$2.02E^{-12}$-$4.3E^{-25}$	21.69-27.5	Pis v 3.0101	One Cupin 1 domain
2164	1750	5,748	1488	495	56.132	6.94	185-346	$2.36E^{-14}$-$7.95E^{-35}$	20.72-26.91	Pis v 3.0101	Two cupin 1 domains
2904	1583	2,785	15000	499	56.806	6.15	214-340	$3.32E^{-19}$-$2.79E^{-34}$	20.72-25.62	Pis v 3.0101	Two Cupin 1 domains
11773	964	411	306								
25774	294	27	No orf								
42843	814	43	424 (fragment)								
45295	425	20	No orf								

Table 2. Selected 11S legumin-homologous contigs, ranked according to contig # and presence of a representative ORF. ORF characteristics and BLAST results are only shown for the most likely 11S legumin contigs homologues. Bp: number of base pears; Aa: number of amino acids; Mw: molecular weight; pI: protein isoelectric point.

Contig mapping			ORF characteristics				BLAST results				
Contig #	Consensus length (bp)	No. of reads	Bp	Aa	Mw (kDa)	pI	Max Score	E-value	Max Identity (%)	Highest identity with	Conserved domains
40	2132	769,001	1398	465	52.814	6.16	454-2157	$5.12E^{-49}$-0	45.05-100	Ana o 2.0101	Cupin-like family domain
2693	1495	3,864	1071	356	38.237	7.17	250-360	$9.59E^{-23}$-$1.75E^{-37}$	22.00-29.58	Pru du 6.0101	Cupin-like family domain
11609	236	1,177	No ORF								
15483	430	2,948	No ORF								
20253	302	31	No ORF								
23259	236	10	No ORF								
49045	248	9	No ORF								

Table 3. ORF characteristics and BLAST results for selected 2S albumin-homologous contigs, ranked according to contig #. Bp: number of base pears; Aa: number of amino acids; Mw: molecular weight; pI: protein isoelectric point.

Contig mapping			ORF characteristics				BLAST results				
Contig #	Consensus length (bp)	No. of reads	Bp	Aa	Mw (kDa)	pI	Max Score	E-value	Max Identity (%)	Highest identity with	Conserved domains
1903	681	8,698	441	146	17.211	5.38	100-425	$1.15E^{-5}$-$2.01E^{-53}$	42.34-74.19	Pis v 1.0101	α-amylase binding domain
1904	658	7,959	417	138	16.335	5.49	105-378	$2.10E^{-06}$-$1.07E^{-46}$	41.51-100	Ana 0 3.0101	α-amylase binding domain
2387	730	27,040	417	138	16.262	5.42	117-369	$5.13E^{-08}$-$9.56E^{-45}$	44.76-82.05	Ana 0 3.0101	α-amylase binding domain

All vicilins of legume seeds are found to be highly heterogeneous and consist of many different subunits as a result of post-translational processing, gene duplication and/or mutations[34]. In accordance, Reitsma et al[22] suggested the existence of nine putative Ana o 1 isoforms, merely generated by different post-translation modifications of the same gene. Assuming all contigs identified are indeed 7S vicilin isotypes, additional modification and cleavage forms would have to exist to add up to nine Ana o 1 isoforms/isotypes.

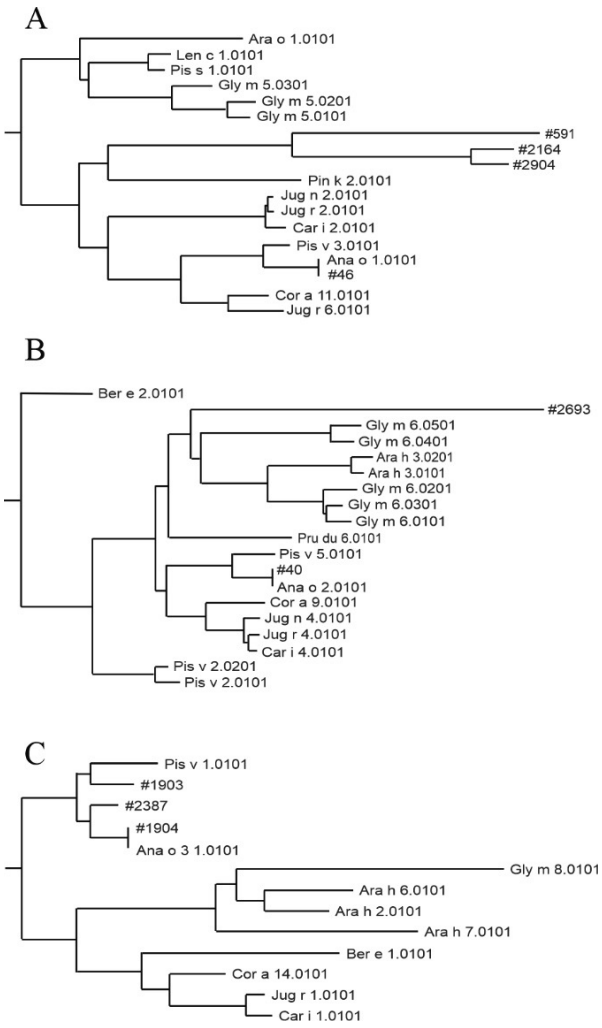

Figure 2. Phylogenetic trees for the SSP proteins identified in cashew nut. Phylogenetic tree construction using Neighbor-joining method and Jukes-Cantor as protein distance measure (average of 100 booth strapping); (A) Phylogenetic distribution of common 7S vicilin-type allergens and contigs #46, #591, #2164 and #2904; (B) Phylogenetic distribution of common 11S globulin-type allergens and contigs #40 and #2693; (C) Phylogenetic distribution of common 2S albumin-type allergens and contigs #1903, #1904 and #2387.

To investigate the number of 11S globulin genes in cashew nut, eighteen allergenic 11S seed storage protein sequences were used as BLAST query. Seven contigs with homology to legumin seed storage proteins, of which two displayed a complete ORF, were identified (Table 2). Contig #40 represents the Ana o 2.0101 gene whereas contig #2693 might represent a new Ana o 2.0101-isotype, although the ORF sequence is rather small for an 11S globulin-type protein (Figure S2). Clustering using Neighbor-joining analysis revealed that Contig #2693 clusters closer to the legumes than to tree nut-derived 11S globulins (Figure 2B). Although the sequence has still to be verified via 5'-UTR sub-cloning, the as yet unidentified N-terminus of Ana o 2.0101[19] has now been completed by the sequence in contig #40, which may promote future research on this gene.

A 2S albumin BLAST query using ten well known nut/seed allergen sequences resulted in the identification of three ORF-bearing contigs showing 71-100% amino acid identity with Ana o 3.0101 (Table 3). All contain the α-amylase binding domain (cd00261) present in 2S albumins. The ORF in contig #1904 is 100% identical to Ana o 3.0101 while ORF #1903 shows higher identity to the pistachio 2S albumin Pis v 1.0101 (Figure 2C and 3). The Anacardiaceae 2S albumins from cashew and pistachio cluster separately from the other tree nut- and legume-derived 2S albumin proteins.

Thus, next to Ana o 1.0101/Ana o 1.0102 and Ana o 2.0101, there are additional cupin-like genes existing in cashew nut representing 7S vicilin-like and 11S-legumin-like proteins that may, when translated in the seed as well, contribute to the sensitisation/elicitation events in cashew nut allergy. This, however, needs to be investigated in more detail in future research. In addition, two novel isotypes of Ana o 3 were identified. As serum IgE towards Ana o 3 in patients is considered an important marker for clinical manifestation of a cashew nut allergy[25-27], we focused our subsequent studies on this allergen and its homologs.

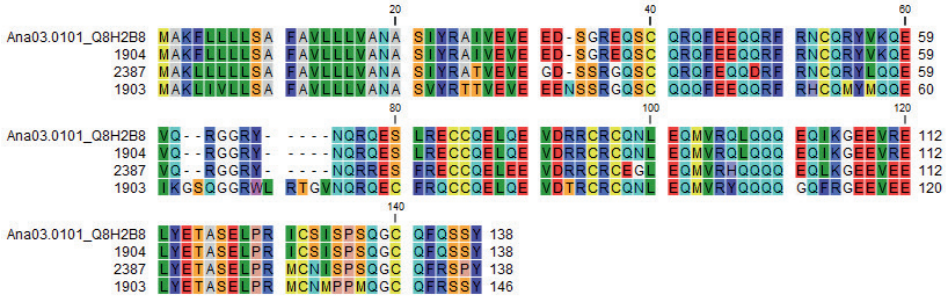

Figure 3. Aa sequence alignment for the 2S albumin-like cashew contigs and Ana o 3.0101.

3.2 Confirming the presence of Ana o 3 isotypes in cashew nut

First, we investigated if all three 2S albumin types are expressed and translated in mature cashew nut seeds originating from Cambodia. Cloning using contig-specific primers designed in the UTRs and subsequent sequence verification confirmed the expression and the sequence of both new isotypes (Figure 4A, B). Moreover, for isotype #2387, a single nucleotide transition base variant (C/T) at position 184 was obtained in colony 6, causing an Arg/Tryp amino acid substitution in position 62 (R62W, indicated by a square). This variation was also present in the RNA-seq database in 19% of the reads covering this nucleotide (1134 times A vs 5980 times G; complement strain). Secondly, targeted LC-MS/MS analysis indicated that all three isotype proteins were present in a cashew nut total protein extract (Figure 4C, see supplemental Table S1 for more details). A single variant specific peptide is

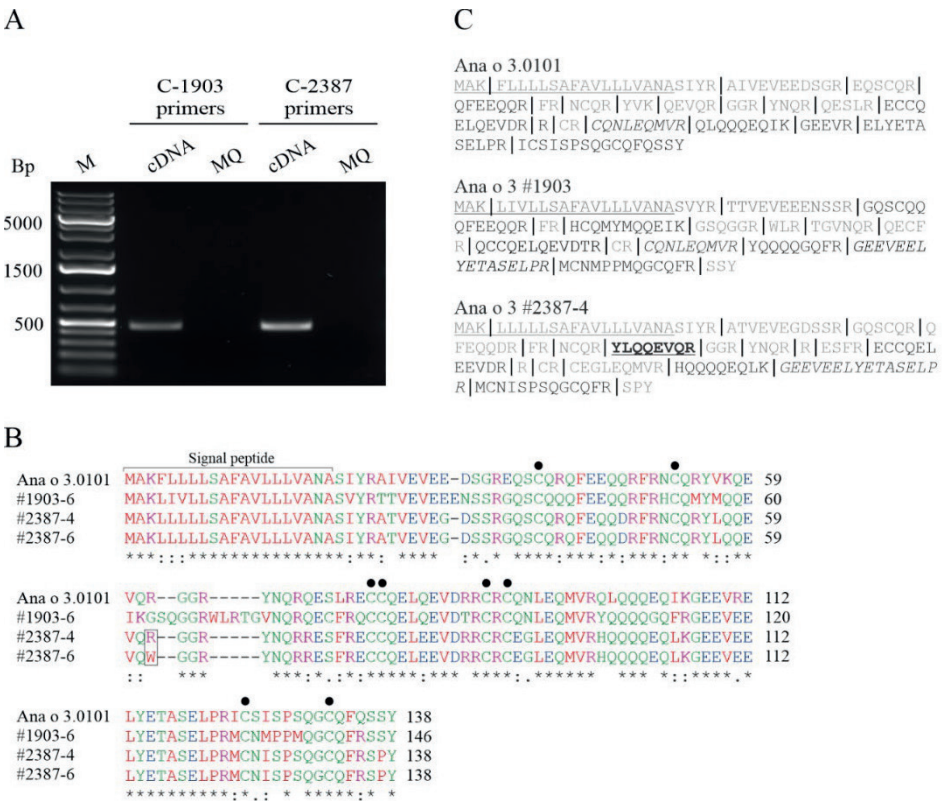

Figure 4. mRNA and protein expression of 2S albumins in cashew nut seeds. (A) PCR of Ana o 3 isotypes #1903 and #2387 using contig-specific UTR primers; (B) Clustal O (1.2.4) multiple sequence alignment of the 2S albumin genes as verified by sequencing; Bacterial colony numbers are indicated behind each isotype. Conserved cysteines are indicated by a black dot. The sequence variant for #2387 is marked by a square; (C) Peptide identification using targeted LC-MS/MS analysis in a total ammonium bicarbonate protein extract; The signal peptide is underlined, identified peptides are in black, undetected peptides are grey, isotype-nonspecific peptides are in Italic while the #2387 isoform-specific peptide is in Bold; Trypsin cleavage sites are indicated by |.

available to distinguish variants #2387-4 and #2387-6 of which only the variant specific peptide for #2387-4 has been detected.

3.3 Preproprotein characteristics

As seen for *Ricinus communis* (castor bean) 2S albumins[35], all three cashew nut 2S albumin preproprotein precursors are rich in glutamine (Gln or Q) with 16.4%, 13.8% and 15.9% for isotypes #1903, #2387 and Ana o 3.0101 respectively. The N-terminal signal peptide sequences (1-20aa) are highly conserved, hydrophobic (80% of aa have hydrophobic side chains) and contain the terminal signal peptidase cleavage site Ala-x-Ala[30] (Figure 4B). Members of the prolamin superfamily are primarily characterized by the presence of eight conserved cysteine residues (necessary for disulphide bridge formation), and the conserved cysteine motif as designated by Kreis et al[36], -Cys-Cys-(X9)-Cys-(X1)-Cys is indeed present in the putative large subunits of the cashew nut 2S albumins. Isotype #1903 has an additional Cys at residue 80.

In addition, structural modelling of the deduced amino acid sequence of the proprotein precursors revealed that all three isotypes share the typical alpha-helical structure and 3D conformation of 2S albumins (Figure 5). The Arg/Tryp amino acid substitution in #2387-6 could potentially modify the predicted tertiary structure for #2387 variant 6 because Tryp is a neutral amino acid bearing a hydrophobic indole ring[37], while Arg is a basic amino acid bearing a positively charged amine-containing side chain able to form hydrogen bonds[38]. However, our analysis suggests that the replacement of Arg by a Tryp does not influence the *in silico* predicted structure (Figure 5).

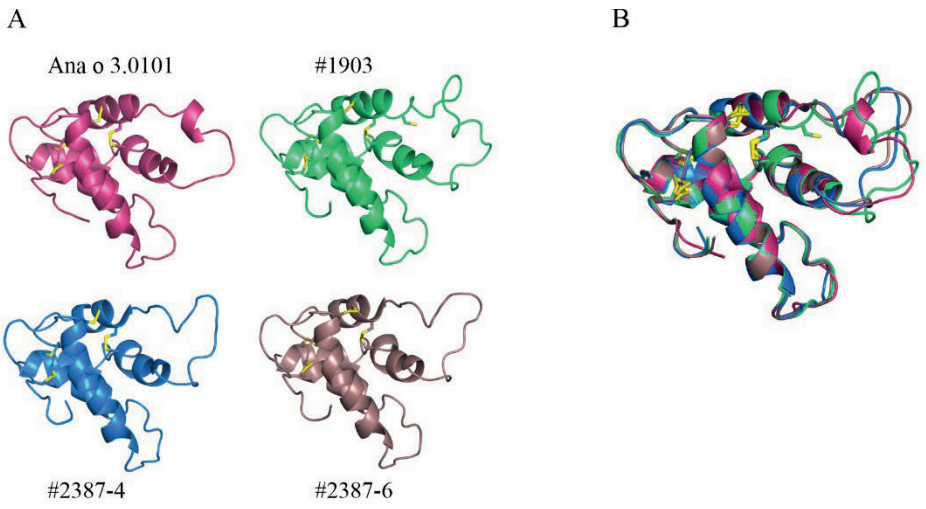

Figure 5. Structural modelling of the cashew nut 2S albumin isotype preproteins predicted using the template structure of Brassica napus PDB-ID 1sm7. Disulphide bridges are indicated in yellow. (A) Predicted tertiary structure of Ana 0 3.0101, #1903 and #2387 preproteins; (B) Superimposed view.

3.4 Putative allergenicity differences

Since Ana o 3.0101 is considered a major allergen, it is important to investigate whether isotype monosensitisation and IgE cross-reactivity should be considered in cashew nut allergy. The major and minor epitope regions as identified by Reitsma et al[22] in Ana o 3.0101 were compared with the deduced amino acid sequences of isotypes #1903 and #2387 (Figure 6). Based on the observed

A

Epitope region 49-69

Ana o 3.0101	aa49-69	FRNCQRYVKQEVQ--RGGRY-----NQR	
#1903	aa50-77	FR**H**CQ**M**Y**MQ**QE**IKGSQ**GGR**WLRTGV**NQR	46%
#2387	aa49-69	FRNCQRY**LQ**QEVQ--RGGRY-----NQR	90%

B

Epitope region 85-108

Ana o 3.0101	aa85-108	RRCRCQNLEQMVRQLQQQEQIKGE	
#1903	aa93-116	**T**RCRCQNLEQMVR**YQ**QQQ**G**Q**FR**GE	75%
#2387	aa85-108	RRCRC**EG**LEQMVR**HQ**QQQEQ**L**KGE	79%

C

Epitope region 121-135

Ana o 3.0101	aa121-135	PRICSISPSQGCQFQ	
#1903	aa129-143	PR**M**C**NMP**P**M**QGCQF**R**	60%
#2387	aa121-135	PR**M**C**N**ISPSQGCQF**R**	80%

D

2S albumin immuno-dominant regions in the hypervariable loop

Ana o 3.0101	aa103-114	EQIKGEEVRELY	
#1903	aa111-122	**G**Q**FR**GEEV**E**ELY	67%
#2387	aa103-114	EQ**L**KGEEV**E**ELY	83%
Jug r 1		**QGLR**GEE**ME**E**MV**	33%
Car i 1		E**G**IRGEE**ME**E**MV**	58%
Ric c 3		**G**Q**LH**GEE**SERVA**	33%
Ber e 1		**EM**Q**PR**GE**QMR**R**MM**	33%

Figure 6. Epitope regions of Ana o 3.0101 and their aa-sequence homology with isotypes #1903 and #2387. (A) Minor epitope region between amino acids 49 and 69 in the small subunit of Ana o 3.0101; (B) Major epitope region between amino acids 85-108 in the large subunit of Ana o 3.0101; (C) Minor epitope region between amino acids 121-135 in the large subunit of Ana o 3.0101; (D) 2S albumin immuno-dominant regions of Jug r 1, Car i 1, Ric c 3 and Ber e 1 aligned to Ana o 3.0101 and isotypes #1903 and #2387. Percentage similarity of the aa regions in #1903 and #2387 with Ana o 3.0101 are specified behind each sequence. Sequence differences with the Ana o 3.0101 region are in bold.

similarity, it is likely that a cashew allergic individual with antibodies against Ana o 3.0101 might also react to isotype #2387. Isotype #1903 shares less sequence similarity in the Ana o 3.0101 epitope regions, especially in the small subunit. Indeed, using a protein peptide microarray approach with plasma from one cashew allergic individual (n=1), less IgE-binding was observed in the epitope regions 93-116 and 121-135 of #1903 (Figure 7). Since Reitsma et al[22] designated the region 85-108 in Ana o 3.0101 as high IgE-binding, this suggests that little cross-reactivity between these isotypes may occur. On the other hand, while #1903 displays only 46% homology with Ana o 3.0101 in epitope region 50-77aa, IgE-binding can be observed specifically to the C-terminus of this region. IgE cross-reactivity could be an explanation for this, but it may also be that isotype-specific antibodies are produced. Mapping was performed on the Ana o 3 precursors, not taking putative differences in post-translational processing into account. As these are just preliminary results, equal allergenicity as well as cross-reactivity between the 3 isotypes can as yet not be excluded from these *in silico* approaches.

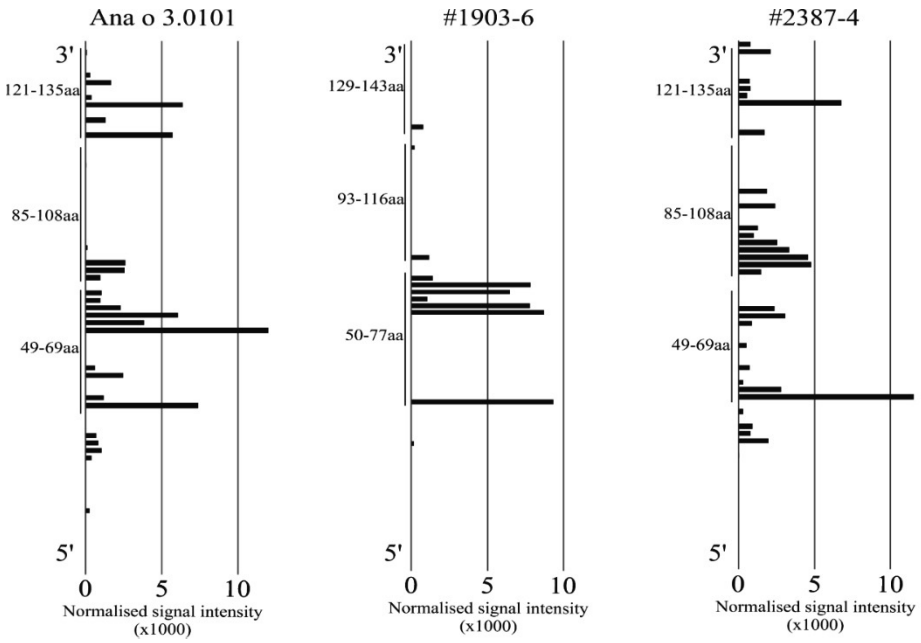

Figure 7. Epitope mapping using a protein peptide microarray approach.

For most 2S albumin allergens, the immuno-dominant region corresponds to the hypervariable loop that is highly exposed in the protein tertiary structure [39]. The immuno-dominant epitopes[40-41] of Jug r 1 (walnut; QGLRGEEMEEMV), Car i 1 (pecan nut; EGIRGEEMEEMV), Ber e 1 (brazil nut; EM(Q)PRGEQMRRMM) and Ric c 3 (castor bean; GQLHGEESERVA) were aligned to the Ana o 3 isotypes, that revealed

considerable homology in this loop region (Figure 6D). Both #1903 and #2387 isotypes share 50% of the amino acid sequence in this region with Car i 1, Jug r 1 and Ric c 3.

3.5 Purification of native Ana o 3 isotypes

For allergy diagnostics it is important to investigate whether all isotypes contribute to the elicitation of clinical symptoms and if each has the same potency to bind IgE. To be able to investigate this, it is preferable that the isotypes can be tested in pure form. We examined the 2S albumin-containing ammonium sulphate precipitate obtained by Reitsma et al[22] by chromatography, as we wanted to know whether this preparation technique results in a precipitate containing all isotypes or only Ana o 3.0101. The most relevant fractions were separated on SDS PAGE, which indicated that isotype separation using ion-exchange chromatography resulted in partly purified fractions (Figure 8).

SDS PAGE under non-reducing condition of elution fractions B5 till C2 suggest the presence of three different isotypes (Figure 8B).That is, B5 and B6 seem to contain a protein of ~11.4 kDa protein. A protein with a calculated size of ~11.0 kDa is present in B7 till B10, which gradually disappears in fractions B11 till C2 with increasing conductivity. A ~12.6 kDa isotype starts to elute from B9 on and is almost pure in C2. A faint band containing a ~8.7 kDa protein was present in B5-7 which likely represents a contamination with a non-albumin type of protein. It is not expected to be a degradation product of Ana o 3, as 2S albumins are usually very stable[29]. The 22.4 kDa band which gradually becomes stronger in fraction B8 till C2 could be a dimeric form of the Ana o 3 isotypes.

In the presence of reducing agent (2-mercaptoethanol), the small and large subunits of the Ana o 3 isotypes can be analysed (Figure 8C). Fractions B5-6 seem to contain a small subunit with an estimated size of ~5.7 kDa while in B7 till C2, the small subunit seems to be slightly smaller (~5.3 kDa). Although the SDS PAGE gel composition used allowed us to observe size differences it is not ideal to accurately assess such small differences. The large subunit visible in B5-9 is estimated to be ~6.6 kDa while the middle band in B12, C1 and C2 is of a size of ~7.4 kDa. The second large subunit present in B10-C2 is around ~9.1 kDa.

The ~11.4kDa protein in Figure 9B consists of a ~5.7 kDa small subunit and a ~6.6 kDa estimated large subunit. The ~11 kDa isotype in B7-9, contains a slightly smaller subunit (estimated at ~5.3 kDa) but an equally sized large subunit. The middle band present in B12, C1 and C2 is slightly shifting to an estimated size of ~7.4 kDa suggesting the existence of more than three isotypes/isoforms. The smaller subunit in C2 presumably forms, together with the ~9.1 kDa large subunit, the ~12.6 kDa isotype.

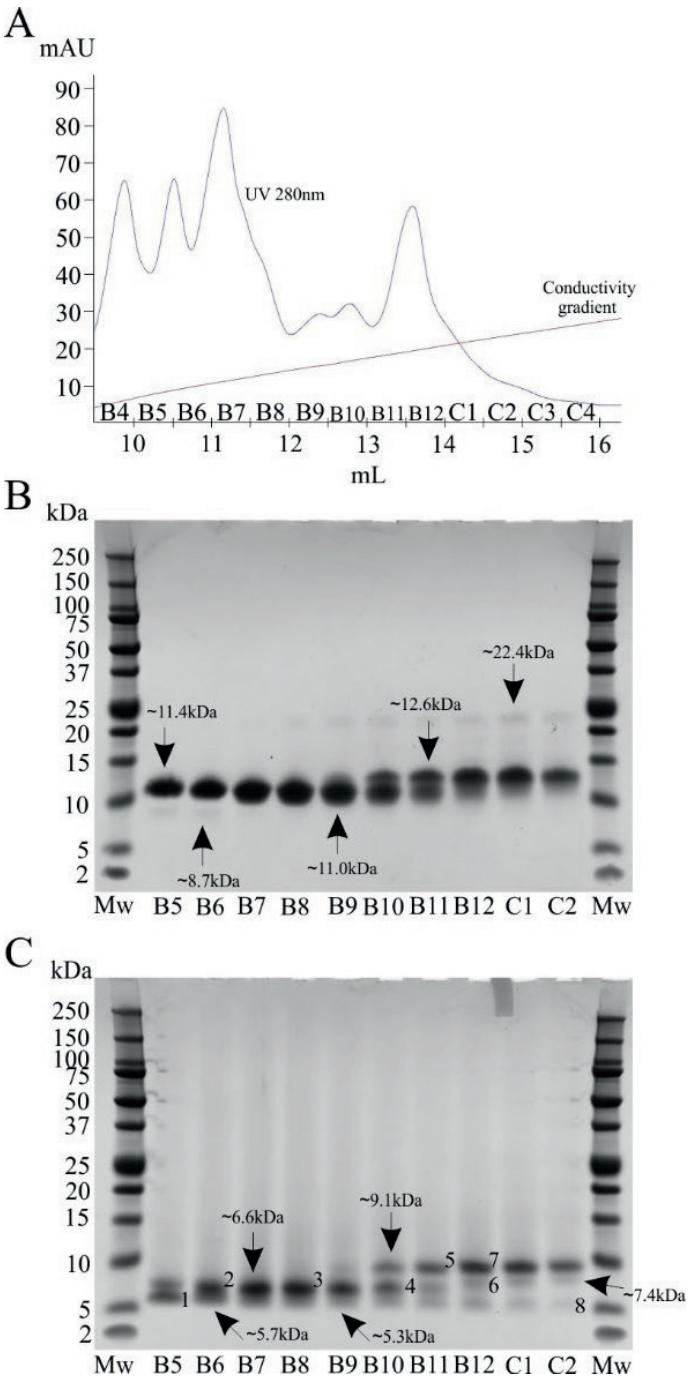

Figure 8. Ana o 3 isotype separation using ion-exchange chromatography. (A) UV280nm ion-exchange chromatogram for fractions B4 till C4; (B) Non-reducing SDS PAGE for fractions B5 till C2, including molecular weight estimations; (C) Reducing SDS PAGE for fractions B5 till C2. Bands that were excised for LC-MS/MS identification are numbered and correspond to Table 4.

Table 4. LC-MS/MS identification of in Figure 8 excised SDS PAGE gel bands. In grey: undetected peptides; Underlined: leader peptide; In black: detected peptides; Italic: isotype non-specific peptide; Bold: #2387 isoform-specific peptide.

Ana o 3 isotype	Peptides upon trypsin cleavage	Identified in
#1903	MAKLIVLLSAFAVLLLVANASVYR| TTVEVEEENSSR| GQSCQQQFEEQQR| FR| HCQMYMQQEIK| GSQGGR| WLR| TGVNQR| QECFR| QCCQELQEVDTR| CR| ***CQNLEQMVR***| YQQQQGQFR| GEEVEE LYETASELPR| **MCNMPPMQGCQFR**| SSY	SDS PAGE band 6, 8
#1904 (Ana o 3.0101)	MAKFLLLLSAFAVLLLVANASIYR| **AIVEVEEDSGR**| EQSCQR| QFEEQQR| FR| NCQR| **YVK**| QEVQR| GGR| YNQR| QESLR| **ECCQELQEVDR**| R| CR| ***CQNLEQMVR***| QLQQQEQIK| GEEVR| ELYETASELPR| ICSISPSQGCQFQSSY	SDS PAGE band 1, 2, 3, 4, 6
#2387-4	MAKLLLLLSAFAVLLLVANASIYR| ATVEVEGDSSR| GQSCQR| QFEQQDR| FR| NCQR| **YLQQEV** **QR**| GGRYNQR| R| **ESFR**| **ECCQELEEVDR**| R| CR| **CEGLEQMVR**| HQQQQEQLK| GEEVEELYETASEL PR| **MCNISPSQGCQFR**| SPY	SDS PAGE bands 5, 6, 7, 8
#2387-6	MAKLLLLLSAFAVLLLVANASIYR| ATVEVEGDSSR| GQSCQR| QFEQQDR| FR| NCQR| **YLQQEV** **QWGGR**| YNQR| R| **ESFR**| **ECCQELEEVDR**| R| CR| **CEGLEQMVR**| HQQQQEQLK| GEEVEELYETASEL PR| **MCNISPSQGCQFR**| SPY	SDS PAGE bands 5, 6, 7, 8

To confirm which isotype is present in each of the fractions, bands presumed to represent different isotypes where subjected to LC-MS/MS analysis (Table 4). Ana o 3.0101 peptides were predominately identified in excised bands 1, 2, 3 and 4 and can be obtained readily in pure form by pooling fractions B5-B8. Excised bands 6 (~7.4 kDa) and 8 (~5.3 kDa) contained respectively the large and small subunits of Ana o 3 isotype #1903 while isotype #2387 is clearly represented by the ~9.1 kDa bands 5 and 7. Even the R62W variant of #2387-6 was distinguished by its unique peptide YLQQEVQWGGR.

The expected protein precursor size (Mw), excluding the leader peptide is 15158.7 Da and 14195.4 Da for isotypes #1903 and #2387 respectively. However, the LC-MS/MS identifications show the exact opposite, since the large subunit runs higher in a reducing SDS-PAGE gel for #2387 than for #1903. This suggest a larger Mw for the final #2387 storage protein. Differences in structural intermolecular interactions under non-reducing conditions (i.e. disulphide bridges) as well as differences in SDS binding affinity may cause such unexpected differences in mass migration in SDS PAGE gels[22]. However, the unexpected isotype Mw differences are more likely to be caused by differences in linker peptide cleavages during post-translational processing of the precursor proproteins. 2S albumin precursors are considerably processed to produce the final storage protein (see also Figure 1), consisting of a small and large subunit that are associated by two inter-chain disulphide bonds, with two additional intra-chain disulphide bonds present within the large subunit[29]. Moreover, Reitsma et al[22] already showed that Ana o 3 precursor processing is subjected to both N- and C-terminal microheterogeneity. The putative GGRYNQ linker sequence (residues 63-68) removed by endoprotease activity between the small and large subunit in Ana o 3.0101[22] is present in isotype #2387 but absent in #1903, making it more likely that a larger intersubunit linker sequence is removed in #1903 during post-translational processing. Specific processing sites could possibly be verified in the future by N-terminal microsequencing each of the isotypes subunits, provided the N-terminals are not blocked by cyclization as has been observed in other studies[35,42].

4. Conclusion

In nuts and seeds, the SSPs which serve as nutrient reservoir for germination and seeding growth, are regarded major allergens. These include the cashew nut seed storage proteins Ana o 1, Ana o 2 and Ana o 3 which are regarded as major allergens and are the dominant causative agents of a cashew nut allergy. Using NGS combined with database searches, we have shown that these seed storage proteins in cashew nut are derived from multigene families. The acquired information enhances our

understanding on putatively important cashew nut allergens and complements the previous studies of Robotham et al[21] and Reitsma et al[22].

Protein isoforms can be generated by either alternative spicing events and/or post-translational modifications of a single gene, or by the expression of different genes. Cloning of NGS-predicted Ana o 3-like sequences confirmed the existence of three Ana o 3 isotypes in cashew nut. Previous research identified eight putative large subunits for Ana o 3 by 2D gel electrophoresis: 4 protein spots of ~10kDa and 4 spots of ~8kDa[22]. This suggests that additional isoforms might be generated from the three different Ana o 3 isotype proproteins, presumably by variations in post-translational processing.

At present, Ana o 3.0101 is officially recognised by the WHO/IUIS Allergen Nomenclature Sub-Committee (http:www.allergen.org). Based on the amino acid sequence homology of the 2S albumin isotypes identified in this study, Ana o 3 contig clone #1903 would be considered an isotype and may in the future be assigned as Ana o 3.02. Contig #2387 clones #4 and 6 would be considered allelic variances of the same gene and might be assigned the isoform names Ana o 3.0301 and Ana o 3.0302. However, official naming only takes place when allergenicity (i.e. IgE-binding capacity) has been proven for at least 5 to 10 cashew nut allergic individuals[43].

From the ion-exchange chromatography data we can conclude that it is nearly impossible to acquire all Ana o 3 isotypes in pure form. Thus, for future immunological response analyses, more sophisticated separation procedures should be applied or alternatively, isotypes should be generated recombinantly. To ensure proper post-translational processing and protein folding, recombinant expression in a eukaryotic organism (e.g. the yeast *Pichia pastoris*), should be considered in conjunction with adequate purification procedures before immunogenicity and allergenicity of the Ana o 3 isotypes could be investigated in more detail.

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Conflicts of interest
The authors declare to have no competing interest.

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Diagnostics, HAL allergy, Intersnack the Netherlands B.V., ALK-Abello B.V., and the Dutch Anaphylaxis Network.

References

1. Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H. Seeds: physiology of development, germination and dormancy. New York: Springer. 2013.
2. Osborne TB. The vegetable proteins. Monographs on Biochemistry. Pp. xiii+154. 2nd edition. London: Longmans green and Co. 1924.
3. Miflin BJ, Burgess SR, Shewry PR. The development of protein bodies in the storage tissue in seeds: subcellular separations of homogenates of barley, maize and wheat endosperms and of pea cotyledons. J Exp Botany 1981;32(126):199-219.
4. Foley RC, Gao LL, Spriggs A, Soo LY, Goggin DE, Smith PM, Atkins CA, Singh KB. Identification and characterisation of seed storage protein transcripts from *Lupinus angustifolius*. BMC Plant Biol 2011;11:59.
5. Marcus JP, Green JL, Goulter KC, Manners JM. A family of antimicrobial peptides is produced by processing of a 7S globulin protein in *Macadamia integrifolia* kernels. Plant J 1999;19(6):699-710.
6. Abdel-Shafi S, Al-Mohammadi AR, Osman A, Enan G, Abdel-Hameid S, Sitohy M. Characterization and Antibacterial Activity of 7S and 11S Globulins Isolated from Cowpea Seed Protein. Molecules. 2019;24(6):E1082.
7. Terras FR, Schoofs HM, De Bolle MF, Van Leuven F, Rees SB, Vanderleyden J, Cammue BP, Broekaert WF. Analysis of two novel classes of plant antifungal proteins from radish (Raphanus sativus L.) seeds. J Biol Chem 1992;267(22):15301-9.
8. Terras FR, Torrekens S, Van Leuven F, Osborn RW, Vanderleyden J, Cammue BP, Broekaert WF. A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species. FEBS Lett 1993;316(3):233-40.
9. Odintsova TI, Rogozhin EA, Sklyar IV, Musolyamov AK, Kudryavtsev AM, Pukhalsky VA, Smirnov AN, Grishin EV, Egorov TA. Antifungal activity of storage 2S albumins from seeds of the invasive weed dandelion Taraxacum officinale Wigg. Protein Pept Lett 2010;17(4):522-9.
10. Duan XH, Jiang R, Wen YJ, Bin JH. Some 2S albumin from peanut seeds exhibits inhibitory activity against Aspergillus flavus. Plant Physiol Biochem 2013;66:84-90.
11. Mandal S, Kundu P, Roy B, and Mandal RK. (2002) Precursor of the inactive 2S Seed Storage Protein from the Indian mustard Brassica juncea is a novel trypsin inhibitor. J Biolog Chem 2002;227(40):37161-37168.
12. Nguyen TP, Cueff G, Hegedus DD, Rajjou L and Bentsink L. A role for seed storage proteins in Arabidopsis seed longevity. J Exp Bot 2015;66:6399–6413.
13. Jenkins JA, Griffiths-Jones S, Shewry PR, Breiteneder H, Mills EN. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an in silico analysis. J Allergy Clin Immunol 2005;115(1):163-70.
14. Blazowski L, Majak P, Kurzawa R, Kuna P, Jerzynska J. Food allergy endotype with high risk of severe anaphylaxis in children-Monosensitization to cashew 2S albumin Ana o 3. Allergy 2019;74(10):1945-1955.
15. Schlüter H, Apweiler R, Holzhütter H, Jungblut PR. Finding one's way in proteomics: a protein species nomenclature. Chem Central J 2009;3:11.
16. Clark AT, Anagnostou K, Ewan PW. Cashew nut causes more severe reactions than peanut: case-matched comparison in 141 children. Allergy 2007;62(8):913-6.
17. Van der Valk JP, Gerth van Wijk R, Baumert JL, Nordlee JA, Vlieg-Boerstra BJ, de Groot H, Dubois AE, de Jong NW. Threshold dose distribution and eliciting dose of cashew nut allergy. Ann Allergy Asthma Immunol 2016;117(6):712-714.
18. Divaret-Chauveau A, Clerc-Urmès I, Renaudin JM, Cordebar V, Dumond P, Schweitzer C. Threshold dose distribution for cashew nut allergy in children. Clin Exp Allergy. 2018 Jul;48(7):902-904.
19. Wang F, Robotham JM, Teuber SS, Tawde P, Sathe SK, Roux KH. Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family. J Allergy Clin Immunol 2002;110(1):160-6.
20. Wang F, Robotham JM, Teuber SS, Sathe SK, Roux KH. Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family. Int Arch Allergy Immunol 2003;132(1):27-

39.
21. Robotham JM, Wang F, Seamon V, Teuber SS, Sathe SK, Sampson HA, Beyer K, Seavy M, Roux KH. Ana o 3, an important cashew nut (Anacardium occidentale L.) allergen of the 2S albumin family. J Allergy Clin Immunol 2005;115(6):1284-90.
22. Reitsma M, Bastiaan-Net S, Sforza S, van der Valk JP, van Gerth van Wijk R, Savelkoul HF, de Jong NW, Wichers HJ. Purification and Characterization of Anacardium occidentale (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3. J Agric Food Chem 2016;64(5):1191-201.
23. Pantoja-Uceda D, Shewry PR, Bruix M, Tatham AS, Santoro J, Rico M. Solution structure of a methionine-rich 2S albumin from sunflower seeds: relationship to its allergenic and emulsifying properties. Biochemistry 2004;43(22):6976-86.
24. Bastiaan-Net S, Pina-Pérez MC, Dekkers BJW, Westphal AH, America AHP, Ariëns RMC, de Jong NW, Wichers HJ, Mes JJ. Identification and in silico bioinformatics analysis of PR10 proteins in cashew nut. Protein Sci 2020.
25. Savvatianos S, Konstantinopoulos AP, Borgå Å, Stavroulakis G, Lidholm J, Borres MP, Manousakis E, Papadopoulos NG. Sensitization to cashew nut 2S albumin, Ana o 3, is highly predictive of cashew and pistachio allergy in Greek children. J Allergy Clin Immunol 2015;136(1):192-4.
26. Lange L, Lasota L, Finger A, Vlajnic D, Büsing S, Meister J, Broekaert I, Pfannenstiel C, Friedrichs F, Price M, Trendelenburg V, Niggemann B, Beyer K. Ana o 3-specific IgE is a good predictor for clinically relevant cashew allergy in children. Allergy 2017;72(4):598-603.
27. Van der Valk JP, Gerth van Wijk R1, Vergouwe Y, Steyerberg EW, Reitsma M, Wichers HJ, Savelkoul HF, Vlieg-Boerstra B, de Groot H, Dubois AE, de Jong NW. sIgE Ana o 1, 2 and 3 accurately distinguish tolerant from allergic children sensitized to cashew nuts. Clin Exp Allergy 2017;47(1):113-120.
28. Shewry PR, Napier JA, and Tatham AS. Seed storage proteins: structures and biosynthesis. Plant Cell 2002;7:945-956.
29. Moreno FJ, Clemente A. 2S Albumin Storage Proteins: What Makes them Food Allergens? Open Biochem J 2008;2:16-28.
30. Von Heijne G. How signal sequences maintain cleavage specificity. J Mol Biol 1984;173:243-251.
31. Heldt H-W and Piechulla B. Plant Biochemistry. 4th Edition. Amsterdam: Elsevier. 2011.
32. Bastiaan-Net S, Reitsma M, Cordewener JHG, van der Valk JPM, America TAHP, Dubois AEJ, Gerth van Wijk R, Savelkoul HFJ, de Jong NW, Wichers HJ. IgE Cross-Reactivity of Cashew Nut Allergens. Int Arch Allergy Immunol 2019;178(1):19-32.
33. Dunwell JM. Cupins: a new superfamily of functionally diverse proteins that include germins and plant storage proteins. Biotechnol Genet Eng Rev. 1998;15:1-32.
34. Ozias-Akins P and Breitener H. The functional biology of peanut allergens and possible links to their allergenicity. Allergy 2019;74:888–898.
35. Irwin SD, Keen JN, Findlay JB, Lord JM. The *Ricinus communis* 2S albumin precursor: a single preproprotein may be processed into two different heterodimeric storage proteins. Mol Gen Genet 1990;222(2-3):400-8.
36. Kreis M, Shewry PR, Ford BG, Ford J, Miflin BJ. Structure and evolution of seed storage proteins and their genes with particular reference to those of wheat, barley and rye. Oxford Surv Plant Mol Cell Biol 1985;2:253-317.
37. Palego L, Betti L, Rossi A, Giannaccini G. Tryptophan Biochemistry: Structural, Nutritional, Metabolic, and Medical Aspects in Humans. J Amino Acids. 2016; 2016: 8952520.
38. Kobko N and Dannenberg JJ. Cooperativity in Amide Hydrogen Bonding Chains. Relation between Energy, Position, and H-Bond Chain Length in Peptide and Protein Folding Models. J Phys Chem A 2003; 107(48):10389-10395.
39. Moreno FJ, Mellon FA, Wickham MSJ, Bottrill AR, Mills C. Stability of the Major Allergen Brazil Nut 2S Albumin (Ber E 1) to Physiologically Relevant in Vitro Gastrointestinal Digestion FEBS J 2005;272(2):341-52.
40. Alcocer MJ, Murtagh GJ, Wilson PB, Progias P, Lin J, Archer DB. The major human structural IgE epitope of the Brazil nut allergen Ber e 1: a chimaeric and protein microarray approach. J Mol

Biol 2004;343(3):759-69.
41. Barre A, Borges JP, Culerrier R, Rougé P. Homology modelling of the major peanut allergen Ara h 2 and surface mapping of IgE-binding epitopes. Immunol Lett 2005;100(2):153-8.
42. Sharief FS, Li SS. Amino Acid Sequence of Small and Large Subunits of Seed Storage Protein From Ricinus Communis. J Biol Chem 1982;257(24):14753-9.
43. Pomés A, Davies JM, Gadermaier G, Hilger C, Holzhaouser T, Lidholm J, Lopata AL, Mueller GA, Nandy A, Radauer C, Chan SK, Jappe U, Kleine-Tebbe J, Thomas WR, Chapman MD, van Hage M, van Ree R, Vieths S, Raulf M, Goodman RE. WHO/IUIS Allergen Nomenclature: Providing a common language. Mol Immunol 2018;100:3-13.

Supplementary information

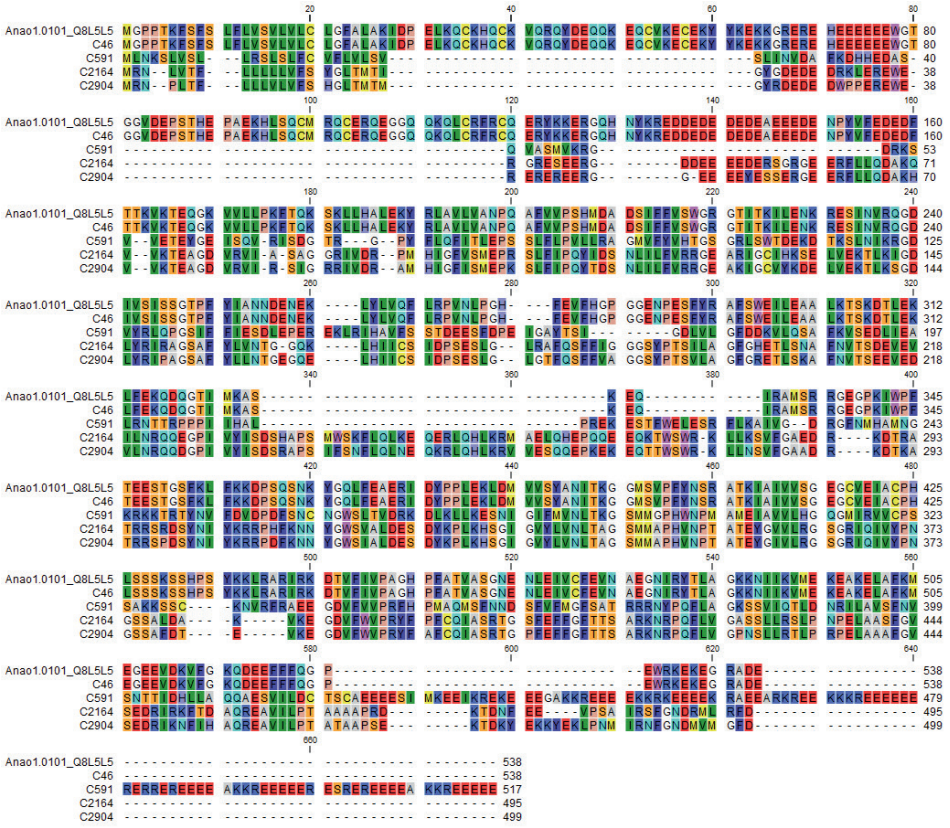

Figure S1. CLC bio sequence alignment for the deduced amino acid sequences of the 7S vicilin-like cashew contigs and Ana o 1.0101.

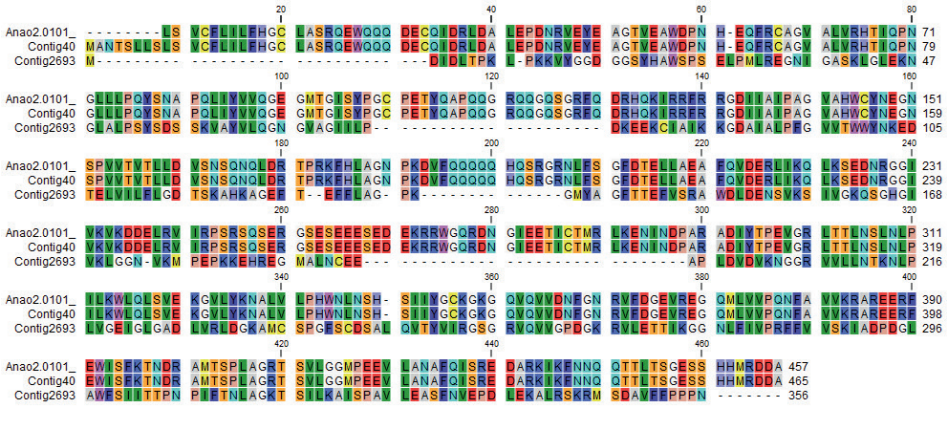

Figure S2. CLC bio sequence alignment for the deduced amino acid sequences of the 11S legumin-like cashew contigs and Ana o 2.0101.

Table S1. Ana o 3 isotype peptide identification by LC-MS/MS in total Ammonium bicarbonate protein extract. Isotype specific peptides are underlined.

Ana o 3 isotype	Variant	Peptide base sequence	Intensity
Ana o 3.0101		QFEEQQR	1.91E+05
		ECCQELQEVDR	5.06E+06
		ECCQELQEVDRR	8.37E+06
		CQNLEQMVR	2.22E+06
		QLQQQEQIK	1.85E+06
		QLQQQEQIKGEEVR	3.14E+08
		GEEVRELYETASELPR	9.89E+05
		ELYETASELPR	8.09E+08
		ICSISPSQGCQFQSSY	4.69E+05
#1903		GQSCQQQFEEQQR	2.04E+06
		HCQMYMQQEIK	4.49E+06
		QCCQELQEVDTR	5.41E+06
		CQNLEQMVR	2.22E+06
		YQQQQGQFR	4.36E+06
		GEEVEELYETASELPR	4.59E+06
#2387		HQQQQEQLKGEEVEELYETASELPR	5.75E+06
		MCNISPSQGCQFR	3.80E+05
	4	YLQQEVQR	1.82E+07
	6	YLQQEVQWGGR	ND

ND: not detected

Chapter 7

General discussion

1. Cracking cashew nut allergy

Around the late 1990s, a major increase in the prevalence of skin and food allergy was observed as opposed to the more respiratory allergic (asthmatic and allergic rhinitis) population that came up in the 1950s[1]. This was referred to as the "second allergic wave" or "second allergic march". Over the years, research has contributed to raising awareness of potential causes and several factors have been defined that may have influenced the clear rise in the development of food allergy[2-5], such as changes in lifestyle (stress, diet, hygiene), environmental factors (climate change, pollution), route of allergen exposure as well as dose and frequency, and disease impact (use of antibiotics, infectious diseases).

For the prevalence of cashew nut allergy, changes in "lifestyle" is perhaps the biggest influencer because, due to the import of exotic vegetables, fruits and nuts (see also **chapter 1**), our diet has changed drastically in the past decades and the amount of ready-to-eat meals and also highly processed products in the supermarket shelves have increased drastically in numbers. In addition, cultural traditions and globalization influenced what we eat and how we season our meals with herbs, spices and flavours. Very likely, this introduction of new and advanced food products has contributed to the general increase in prevalence of food allergies in the last few decades[5]. Nuts and legumes (peanut and soy) are among the most common allergenic foods. With the development of a global market for cashew nuts in the 1950s, its consumption steadily increased[6] and so did the prevalence of a cashew nut allergy in the last 15-20 years[7,8].

In this thesis entitled 'Cracking the cashew nut: strategies to identify and characterize novel allergens', we aimed to broaden the current knowledge on cashew nut allergens beyond those already known (Ana o 1, Ana o 2 and Ana o 3). Our knowledge of cashew nut proteins that can trigger an allergic reaction is currently very limited, especially compared to other nuts or seeds such as peanut, walnut and hazelnut where the allergen repertoire has been researched much more widely (Figure 1). Using immunoblot and immuno-inhibition techniques (**Chapter 3 and 4**) we evidenced that additional allergens must be present in cashew nuts, presumably represented by the allergen families commonly found in nuts and/or seeds (see **Chapter 1**). Using next generation sequencing, we created a genetic database that allowed us to identify additional 2S albumin and PR10 genes, that might represent novel allergens in cashew nut (**Chapter 5 and 6)**. Knowledge of newly identified cashew nut protein provides a basis for further research to extend clinical diagnostic tests and treatments currently available for cashew nut allergy.

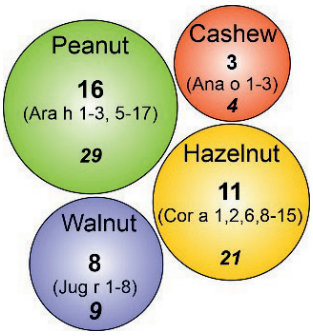

Figure 1. Allergen repertoire of peanut, walnut, hazelnut and cashew nut. The total number of identified allergens is indicated in bold. The total number of genetic variants representing the number of identified allergens is indicated in bold italic.

There is no treatment yet to cure an allergy to cashew nut. Avoidance strategies, such as food avoidance and development of immunotherapeutic strategies may help present-day patients with severe allergic symptoms, but are unlikely to stabilise or reduce the rising prevalence of food allergies[4]. It is therefore more sensible to focus research predominately on incidence prevention strategies, which requires knowledge of exactly how sensitization of cashew nut allergy occurs and which allergenic proteins are involved in this process.

The research questions and putative strategies to tackle these aspects are briefly discussed in the upcoming sections of this chapter. First, the different types of allergenic proteins that may be present in cashew nuts are discussed in more detail. Then, possible factors involved in the sensitization process of an allergy to cashew nuts are further elaborated. Finally, the future perspectives section provides strategies that should provide us with insight into which allergens are predominately involved in the sensitization process, and how to implement possible preventative measures as well as immunotherapy treatments to aid the quality of life of cashew nut allergic individuals in the near future.

2. The allergen repertoire

2.1 Putative cashew nut allergens

The first reports describing the characterization of soluble allergenic proteins in cashew nut appeared in 2002. Using western IgE-immunoblotting, Teuber et al[9] described the identification of dominant IgE-binding antigen peptides that, upon sequencing, showed high homology to legumin-group and 2S albumin proteins. Wang et al[10,11] cloned the first members of allergenic seed storage proteins in cashew nut. The 7S vicilin genes expressing Ana o 1.0101 and Ana o 1.0102 were cloned in 2002, followed by the 11S globulin gene Ana o 2.0101 in 2003. Just two years later, the Ana o 3.0101 (2S albumin) gene was sequenced[12]. Remarkably, no additional allergens in cashew nuts have been identified in the past 15 years. The

prevalence of cashew nut allergy has increased over the years and its symptoms can be severe[7,8,13,14], warranting further research. Due to the many studies devoted to the underlying causes of a peanut allergy[15,16], peanut can be considered as the model food in nut/seed allergen research. We now know that peanut harbours up to 16 types of allergens with several associated isotypes and isoforms (Figure 1)[17,18]. We have exploited this knowledge that multiple allergens have been identified in other seeds and nuts, to identify homologous proteins, which may contribute to cashew nut allergenicity.

Many of the first identified allergens from cashew nut have been picked up by bacterial cDNA library expression and subsequent immunoblotting with patients' sera to identify clones with IgE-binding affinity[10-12]. This was a fairly common method in the 2000s but is now fallen into disuse. Although it is successful for picking up allergens that are highly abundant in seeds, this technique is not suitable for identifying low abundance proteins. In addition, success depended on the size of the cDNA library and how many clones were screened. It also happened that cDNA clones were not entirely complete, such as with Ana o 2.0101, where the N terminus is missing[11]. In this thesis, we applied a next generation sequencing (NGS) approach, which nowadays is a cost-effective way to create an extensive transcriptome cDNA expression library. The advantage of this technology is that it can be stored indefinitely, is accessible worldwide for analysis, and the transcriptome library is much more complete then the conventional bacterial cDNA libraries.

In this work, RNA-seq analysis has proven to be a very effective approach to identify putative cashew nut allergen homologs. Based on our hypothesis in **Chapter 4** that a Bet v 1-related cross-reactivity may play a role in cashew nut allergy[19], we looked for PR10-type protein homologs in the cashew nut RNA-seq database. As a result, in **Chapter 5** we described the identification of multiple genes encoding PR10 proteins in cashew nut[20]. Since seed storage proteins are known to consist of a multigene family and are important IgE-markers in the diagnosis of cashew nut allergy[21-23], we next screened for the presence of additional globulin- and albumin-type of genes. The presence of two novel Ana o 3 isotypes has been elaborated on in **Chapter 6**. Also, several additional globulin-like genes are present in the RNA-seq database possibly coding for Ana o 1 and Ana o 2-like isotypes. In **Chapter 3**, we hypothesized the existence of a luminal binding protein (BiP) in cashew nut with IgE-binding properties. BiPs are considered minor allergens responsible for at least a part of the allergenic cross-reactivity between pollen and plant foods[24] and have thus far only been identified in hazelnut pollen (Cor a 10.0101) and chickpea kernels (Cic a 10)[24,25]. The cashew nut RNA-seq database holds many ORF-bearing contigs with homology to the BiP allergen Cor a 10.0101. Immunoassays on purified or cloned BiPs from cashew should reveal if this type of protein is indeed able to cross-link cashew nut sIgE and which

pollen allergen is putatively involved in the observed Anacardiaceous IgE-cross-reactivity in **Chapter 3**.

Table 1. Overview of allergens and allergen-homologs in cashew nut. [a]Preproprotein deduced amino acid sequence; [b]NCBI code; [c]Data not shown.

Superfamily	Family	Group	Name	Allergen	Mw (kDa)[a]	GenBank[b]	Reference
Prolamin	2S Albumins	Major seed storage protein	Ana o 3	Ana o 3.0101	16.335	AY081853	Robotham et al[12]
			Isotype 1903	---	17.211	MT182946	This thesis (Chapter 6)
			Isotype 2387	---	16.262	MT182947/ MT182948	This thesis (Chapter 6)
	nsLTPs	Lipid transfer proteins	Isotype 1070	---	12.321	MT328903	This thesis[c]
			Isotype 14530	---	11.781	MT328904	This thesis[c]
			Isotype 16979	---	11.913	MT328905	This thesis[c]
			Isotype 19300	---	11.721	MT328906	This thesis[c]
Cupin	7S (vicilin-like globulins)	Major seed storage protein	Ana o 1	Ana o 1.0101	61.841	AF395894	Wang et al[10]
				Ana o 1.0102	61.639	AF395893	Wang et al[10]
	11S (legumin-like globulins)	Major seed storage protein	Ana o 2	Ana o 2.0101	52.814	AF453947	Wang et al[11]
Pathogenis-related protein (PR)	PR-10	Bet v 1 -related	Isotype 18220	---	16.914 -16.974	MN258366/ MN258367/ MN258368	This thesis (Chapter 5)
			Isotype 25355	---	17.824	MN258363	This thesis (Chapter 5)
			Isotype 25514	---	17.160 -17.190	MN258364/ MN258365	This thesis (Chapter 5)
Others	Oleosins	Oil body proteins	Isotype 56	---	14.951	MT328907	This thesis[c]
			Isotype 62	---	15.676	MT328908	This thesis[c]
			Isotype 293	---	18.194 -18.234	MT328909/ MT328910	This thesis[c]
			Isotype 429	---	16.536	MT328911	This thesis[c]
			Isotype 595	---	19.079 -19.107	MT328913/ MT328914	This thesis[c]
			Isotype 2017	---	18.246	MT328912	This thesis[c]

In addition to the globulin and albumin major allergen families in seeds, non-specific lipid transfer proteins (nsLTPs) and oleosins are more likely to cause severe allergic anaphylaxis than other protein types[26]. nsLTPs are pathogenesis-related proteins of 9-10kDa that facilitate the movement of lipids between membranes while oleosins, structural proteins of 15-30kDa, prevent the coalescence of oil bodies (OBs) during seed maturation. Because of their lipophilic nature, oleosins are currently underrepresented in most diagnostic extracts[27] as the lipid fraction is removed by default to avoid background immune reactions in e.g. skin prick tests (SPTs). Now that clinicians and researchers are aware of this fact, oleosins have found to be allergenic in peanut[28], hazelnut[27,29] and sesame seed[30]. Using the same screening approach as described in **Chapter 5 and 6**, the sequence of four nsLTPs and six oleosin-type of genes have been confirmed by cloning and resequencing. Table 1 summarizes the current identified allergens and allergen-homologs in cashew nut to date, which covers the vast majority of superfamilies known to harbor major and minor protein allergens (**Chapter 1**). This list of genes and proteins provides a valuable basis for further research. Once these individual cashew nut proteins can be obtained in pure form, their intrinsic importance in cashew nut allergy can be explored.

1.2 Intrinsic allergenicity of putative cashew nut proteins

The development of an IgE-type food allergy consists of two phases namely the sensitisation phase, in which the immune system is skewed to a pro-allergic inflammation state, and the elicitation phase when an allergic response cascade is initiated upon re-exposure of the same allergenic food (**Chapter 1**). Proteins possess allergenicity if they have the capacity to bind and cross-link IgE-antibodies bound to membrane high affinity IgE receptors and via this activate immune cells (including basophilic granulocytes and mast cells) that elicit symptoms of an allergic reaction, hence their name allergens. The immunogenicity of an allergen is defined by how strong it stimulates a cellular immune response and how the intensity of this cellular response and the resulting formation of specific IgE antibodies eventually may correlate to provoked allergic symptoms[31]. The immunogenicity and allergenicity of cashews' seed storage proteins, Ana o 1, Ana o 2 and Ana o 3 has been well established by clinical cohort studies using complete nuts and/or crude protein extracts as well as by *in vitro* immuno-assay studies[7,8,14,32]. This work identified 15 novel, putative cashew nut allergens such as the PR10, nsLTP, oleosin and albumin isotype proteins (see Table 1). A critical future step is to determine the intrinsic immunogenicity and allergenicity of these identified proteins. In order to study their impact on IgE-sensitisation and cross-linking abilities in cashew nut allergic patients, it is crucial to acquire these proteins in a pure form, without contamination by other allergens or isoforms from the same allergen type. One way to establish this is to employ a recombinant protein production platform where the choice of most optimal production organism would depend on the necessary post-translational processes that the

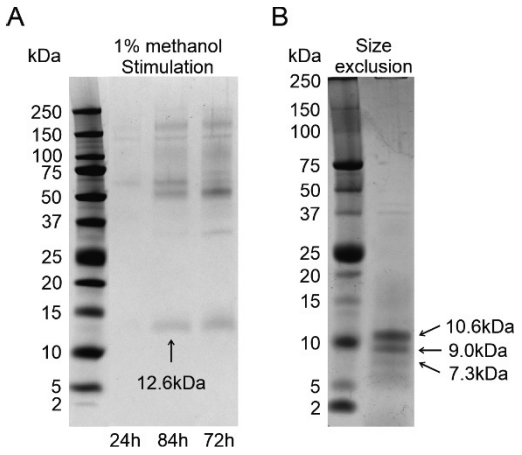

Figure 2. Recombinant expression of Ana o 3.0101 by P. pastoris strain GS115. A: Methanol induction stimulated the secretion of a ~12.6kDa band under reducing conditions, slightly higher than the expected ~11.4kDa Mw of nAna o 3.0101.; B: Purification of the 72h stimulation sample by size-exclusion chromatography suggests that the 12.6kDa band is also partially proteolytically cleaved into a ~10.6kDa, ~9.0kDa and ~7.3kDa band (reducing SDS PAGE).

native protein would normally undergo[33]. For instance, the 2S albumin allergen Ana o 3 undergoes proteolytic cleavage and disulphide bridge formation to acquire its functional structure (**Chapter 6**), while Ana o 1, the 7S vicilin in cashew nut, is a glycosylated protein[10]. The methylotrophic yeast *Pichia pastoris* has proven a suitable production host for recombinant expression and correct structural folding of 2S albumins, as shown by Alcocer et al[34] and Murtagh et al[35] for the 2S albumin proteins Ber e 1 (from Brazil nut) and SFA8 (sunflower). As a pilot experiment, we have sub-cloned the Ana o 3.0101 isotype precursor sequence (minus the leader peptide[36]) into the yeast expression-vector pPIC9K for recombinant expression by *P. pastoris*. Initial results indicated that methanol stimulation induced the secretion of a ~12.6 kDa protein under reducing conditions which, upon purification, appears to be at least partially proteolytically processed (Figure 2).

Carbohydrate groups attached to proteins, termed N-glycans, can provide non-clinically relevant IgE binding in immunoassays (so-called cross-reactive carbohydrate determinants (CCDs)), which can bias the interpretation of clinical relevancy of positive binding[37]. To overcome this, recombinant allergens are often produced in their non-glycosylated form in *E. coli*, since *E. coli* strains are not capable of glycosylation. However, glycans can be important cofactors for sensitization as they can direct proallergic inflammatory immune responses and thus potentially contribute to the immunogenicity of allergens[38]. *P. pastoris* yields protein-bound oligosaccharides that are in general of much shorter chain length than found for the yeast *Saccharomyces cerevisiae*[39]. However, most common yeast strains are known for their over-glycosylation. This was also observed by Reitsma et al, when producing rAna o 1.0101 in the yeast strain X33[40]. In addition, yeast

glycans are not exactly synthesized the same way as plant glycans. The importance of correct imitation of plant glycans has been demonstrated by Shreffler et al[41]. nAra h 1, the 7S vicilin from peanut, was shown to activate monocyte-derived dendritic cells (MDDCs) in a glycan-dependant manner, via internalisation by the CD209 (DC-SIGN) receptor. Activated MDDCs had the ability to direct T cell proliferation to IL-4 and IL-13 producing Th2 cells, which is a critical step in allergic sensitization. DC-SIGN has a high affinity for fucosylated glycans[41] while yeasts favours the production of β-mannose glycan structures[33]. Future alternative plant-based production systems to yeast hosts could be protoplasts[42], algae[43] or perhaps duckweed. The latter two are rather easy to grow on lab scale as well as in large production installations and can be genetically transformed[44,45]. The commercial large scale production is upcoming for algae and duckweed because of their high lipid and protein content respectively[46,47].

We have investigated the IgE-binding and IgE cross-reactive capacity of cashew nut proteins by several types of immunoassays and inhibition techniques (western blot/dot blot and the IMMULITE® technique; see **Chapters 3 and 4**). While these techniques are important as they provide rapid insight into IgE binding capacity of allergens, they do not demonstrate the clinical relevance of IgE binding, and thus no insight into the intrinsic allergenicity of the allergens studied. Thus, as stated by Tordesillas et al[48]: the use of patients-derived sera does not always guarantee proper discrimination between individuals that are only IgE-sensitized or truly allergic. Prediction of allergic elicitation responses can only be studied by assays comprising the IgE cross-linking phase (see **Chapter 1**), such as mediator release assays, SPT or oral challenges. Advisably, the static IgE immunoassays should always be followed up with cross-linking assays to verify the clinical importance of an IgE-binding protein in the allergic elicitation event. Although multiple *in vitro* and *in vivo* assays exist to measure the allergenicity of an allergen, none of these techniques can be used to accurately determine the sensitising potential of allergens (**Chapter 2**).

3. Cashew nut sensitisation

Allergic sensitisation in young children towards cashew nut happens often without a clear history of cashew nut consumption[14]. Since mango is botanically related to cashew nut and often used in toddler fruit snacks, we originally hypothesized that mango could be the primary sensitizer of cashew nut allergy at early age and that allergic symptoms elicited upon cashew nut consumption was the result of IgE cross-reactivity to allergen homologs (**Chapter 3**). This could not be scientifically substantiated as no IgE cross-reactivity was observed between cashew nut and mango proteins, although considerable cross-reactivity was observed between

cashew nut, pistachio and pink peppercorn proteins, which are foods botanically related to cashew nut as well. As the observed *in vitro* allergenic pistachio-cashew nut cross-reactivity represented only a low clinical relevance[49], we concluded that cashew nut itself must be the primary sensitizer in most cashew nut allergic patients (**Chapter 3)**. However, the exact biological mechanism and the initiation site where sensitisation by cashew nut allergens takes place, has not yet been elucidated. However, there are interesting experimental results in literature that provide important clues that are discussed further below.

3.1 Routes of sensitisation

From the IDEAL cohort study[14], it is evident that cashew nut sensitisation predominantly develops early in life. Food allergy in general is, especially in neonates and toddlers, often associated with atopic dermatitis (itchy, red, swollen, and cracked skin)[50]. It was generally accepted that a food allergy develops via the oral and/or respiratory sensitization route. However, more recently, there is growing evidence that the atopic dermatitis-associated skin barrier dysfunction is responsible for the predisposition for food allergic sensitisation, as reviewed by Zheng et al[51], which is a strong indication that food allergic sensitization can occur via the skin. This resulted in an important paradigm shift and it is now assumed that under normal conditions, oral consumption of proteins results in tolerance, while skin contact results in sensitisation[52,53]. This is referred to as the dual-allergen exposure hypothesis[54]. Especially individuals with a mutation in the filaggrin gene, involved in skin barrier keratinisation, are genetically predisposed to develop atopic dermatitis and thus, subsequently also food allergy[55-57]. How and via which initiation route cashew nut allergens enter the body and activate the immune system (sensitisation via the lungs, skin or intestine) is still unresolved (Figure 3).

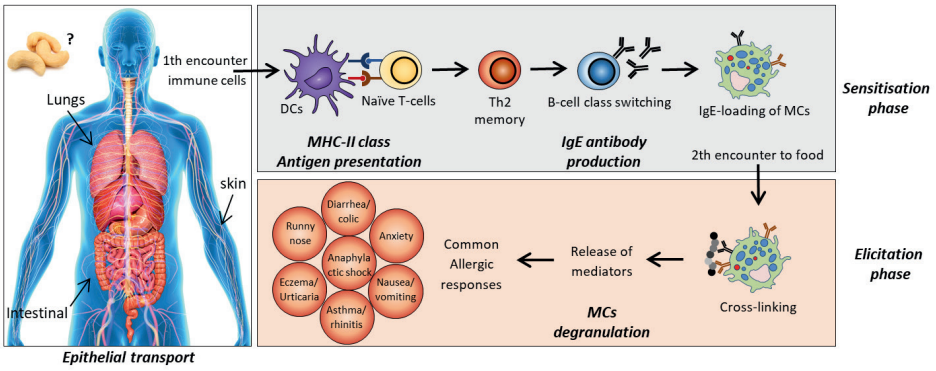

Figure 3. Putative sensitisation route of cashew nut allergy, depicting the epithelial transport phase, immune sensitisation phase and elicitation phase (chapter 1). How cashew nut allergens enter the body, through epithelial transport via the lungs (respiratory), skin (transdermal) or intestine (oral), and sensitise the immune system is still unresolved.

Cashew tree pollen has been reported as an allergen source and could be linked to aggravation of rhinitis (hay fever) symptoms during cashew flowering season[58]. Although it is likely that cashew tree pollen allergens may evoke cross-reactive symptoms towards homologs in cashew nut, this cannot be the mechanism behind a cashew nut sensitization for patients in Europe, as in the northern hemisphere there are no cashew tree plantations. Inhaling dust or flour from cashew nuts (like baker's asthma for wheat[59]) may explain some sensitizing events, but only for occupational and not for normal house-hold cases. On the contrary, the dual-allergen exposure hypothesis most likely explains the sensitisation mechanism underlaying cashew nut allergy, as hypothesized in a study by Luyt et al[60]. They found that the risk of sensitisation (defined as a SPT wheal size ≥3mm) and allergy (defined as SPT wheal size ≥8mm) for cashew and pistachio was higher in South Asian children than in Caucasian children in the area of Leicester-shire (UK), while no such difference in risk was observed for peanut sensitisation or allergy. By comparing ethnic groups living in the same geographical area, which excludes environmental factors, they could hypothesise that skin exposure in Asian households would be higher due to a greater amount of cashew and pistachio in their diet. To clarify, it is assumed that house-hold dust may contain high levels of cashew/pistachio protein when it is consumed frequently, thereby increasing the risk of early skin exposure and sensitisation when babies start to crawl, as has been shown in several peanut studies[61-63]. Earlier, a study among Israeli and UK Jewish children suggested that rather than avoidance, early introduction of peanut consumption would lead to oral tolerance[64], which they later confirmed with the LEAP study[65]. Many additional studies have since supported the hypothesis that peanut sensitization occurs primarily as a result of environmental skin (transdermal) exposure, as summarized by Foong et al[66]. Transdermal sensitization as alternative to the oral route has even been suggested for nsLTP-associated peach allergy. Large amounts of Pru p 3 has been found in peach fuzz, present on the skin of peaches when picked fresh from the tree which can cause peach-induced contact urticaria (swelling and reddening of the skin, similar to atopic dermatitis). It is speculated that this could explain the high prevalence of nsLTP sensitization in Mediterranean countries while virtually absent in northern European countries[67].

3.2 Adjuvants in the food matrix

Only a very small proportion of the total amount of proteins consumed are allergenic and belong to just a fraction of the protein families classified today[68,69]. Much effort has been put into answering the question "What makes a dietary protein an allergen?" Yet, there is no explanatory consensus and, despite years of research, it has proved to be an uncrackable nut[53,70-74]. Information is emerging that allergenicity cannot be attributed solely to the structural and physicochemical properties of the allergens themselves[67,75,76]. Perhaps we should ask ourselves the following question instead: "What is in the food matrix of cashew nut that allows

its dietary proteins to become sensitizers?" For instance, not all identified allergens have the capacity to be sensitizers, unless there is already an imbalance in the immune system (like a proallergic inflammatory response), evoked by nonallergenic components and substances in food matrix that act as adjuvants. Such an evoked lack of immunosuppression can result in food immune tolerance not being reached, hence resulting in food sensitisation[57,74,77]. For example, 2S albumins are present in almost all edible seeds and many are considered major class I allergens[68]. However, not all daily consumed seeds are classified as major allergenic foods. "Why then are not all seeds predominantly known to be major allergenic foods?", is an intriguing question that remains for now a mystery.

3.2.1 Matrix components as allergen stabilizers.

When testing (putative) allergens in purified form, it should be taken into account that food matrix effects are disregarded. In real life, the intestinal epithelial barrier and cells of the immune system do not encounter a single purified allergen but a conglomeration of (hydrolysed) proteins, lipids and carbohydrates, i.e. the food matrix. Some allergenic protein types have a transport function and are capable of binding such matrix elements, for instance the PR10-type proteins and nsLTPs. Especially lipid binding seems to be a common feature for many allergens[78]. The total fat percentage of cashew nuts has been estimated to be between ~40.4 and 66.2% of the total weight, depending on the origin of kernel production and the procedures used for deshelling and processing[79-83]. The majority of lipids in cashew nut seems to be comprised of unsaturated and saturated fatty acids (FA; ~79-80% and ~20-21% respectively) with oleic acid and linoleic acid as predominant FAs[80,82,83]. With these high lipid contents is likely that a proportion of cashew nut proteins are lipid-bound, especially linoleic and oleic acid as nsLTPs have been shown to form very stable complexes with these types of C10-C18 chain unsaturated FAs[84].

Protein-ligand interactions can lead to conformational changes which might increase an allergens' structural stability to for instance heat and/or gastro-intestinal digestion. Also, conformational changes can lead to less or more exposure of IgE epitopes on the proteins' surface. For instance, the major allergen of birch pollen, the PR10-type allergen Bet v 1 is highly allergenic, and it is suggested that its immunogenicity can be explained by its high binding affinity for hydrophobic ligands[85]. Ligand binding appears to stabilize the allergen and makes it more resistant to degradation, as the glycine rich P-loop (containing the major IgE epitope, **Chapter 5**) was hydrolysed more slowly when the ligand-binding tunnel was occupied by the phospholipid phosphatidylcholine, a major constituent of cell membranes involved in membrane-mediated cell signalling[86,87]. Increased thermal proteolytic stability was also observed for the PR10 allergen Ara h 8 in peanut when bound to flavonoids or lipid sterols[88,89]. We discussed the possibility that cashew nut PR10 proteins retain their conformational structures even after

nuts have been heat treated, possibly by binding to a stabilizing ligand, being it either a lipid or a flavonoid (**Chapter 5**). Bohle et al[90] found out that heated PR-allergens may lose the capacity to cross-link IgE, but can retain their ability to activate allergen-specific T-cells which may trigger atopic dermatitis, at which point a patient becomes predisposed for sensitisation to other cashew nut allergens.

Naturally, oleosins and nsLTPs are lipid-binding proteins. Oleosins stabilise oil bodies by interacting with phospholipids[91] while nsLTPs are involved in intracellular lipid trafficking[92]. NsLTPs are particularly heat stable and resistant against proteolytic enzymes[93], whether their ligand-binding site is occupied or not. However, in the presence of a reducing agent, the heat-stabilising effect of lipid binding became also for this type of allergen apparent[94]. In line with this, a slightly protective effect to gastric digestion was shown for grape LTPs mixed with phosphatidylcholine, although this did not appear to affect the allergenicity of the complex[95]. Dubiela et al[96] demonstrated that binding oleic acid increased the IgE-reactivity and basophil activation of the peach nsLTP Pru p 3 by conformational changes. However, phytosphingosine and not oleic acid seems to be the natural ligand of Pru p 3, as demonstrated by Cubells-Baeza and co-workers[97], leaving the discussion about the allergenicity of lipid-complexed nsLTPs controversial[76].

It has been suggested that, because of disulphide-bridge pairing in their CXC segment, 2S albumin proteins do not form an internal cavity able to bind a lipid molecule[98]. Rather, as shown for 2S albumins from sunflower, their high proportion of hydrophobic residues allows the formation of hydrophobic clusters on their surface[99], giving them the ability to form highly stable emulsions with oil in water mixtures[100]. However, structural analysis of Ber e 1, the 2S albumin of Brazil nut, suggested the presence of a hydrophobic binding pocket like seen for nsLTPs[101]. One year later, the proposed capacity to bind hydrophobic molecules, i.e. lipids, was confirmed using ANS (1-anilinonaphthalene-8-sulfonic acid) and bis-ANS (4,4-dianilino-1,1-binaphthyl-5,5 disulfonic acid) binding[75]. ANS and bis-ANS are fluorescent probes that dramatically increase in fluorescence levels when moving from a polar environment (water) to a hydrophobic environment such as to hydrophobic surface patches on proteins or their binding pockets[102]. The rather easy strategy of ANS and bis-ANS fluorescence shifting could be applied to verify lipid binding capacities of cashew nut proteins such as the Ana o 3 isotypes, nsLTPs as well as the PR10 isotypes. When applied, it is important to test relevant matrix components, i.e. components that naturally occur in cashew nut. The latter is not always applied in fundamental research, which sometimes makes the relevance of research results unclear.

3.2.2 Adjuvant effect of unbound matrix components

Some proteins are known as major allergens but fail the capacity to cause allergic sensitisation without extrinsic factors present within the food matrix[103]. It becomes more and more apparent that lipids, either from the food matrix itself or from microbial contaminations, are clearly one of the key players modulating the allergic sensitisation process[78]. As stated by Scheurer and Schülke[76], allergens and lipids are delivered either admixed in an unbound form or as a ligand-interacting complex to the immune system upon ingestion of food.

An interesting study was conducted by Dearman et al[103] to evaluate the effect of endogenous nut lipids on the sensitizing capacity of the 2S albumin Ber e 1 from Brazil nut. BALB/c mice were immunized by intraperitoneal injection with native Ber e 1 (nBer e 1) and with *P. pastoris*-produced rBer e 1. Contrary to the sensitizing capacity of nBer e 1, exposure to lipid-free allergen failed to induce detectible serum levels of IgG or IgE, while co-administration of a total lipid fraction and sterol-rich and polar-lipid fractions from brazil nut resulted in remarkable adjuvant effects on these antibody levels[75,103]. Similar adjuvant potential was observed for peanut lipids[104]. Dearman et al[103] suggested that the

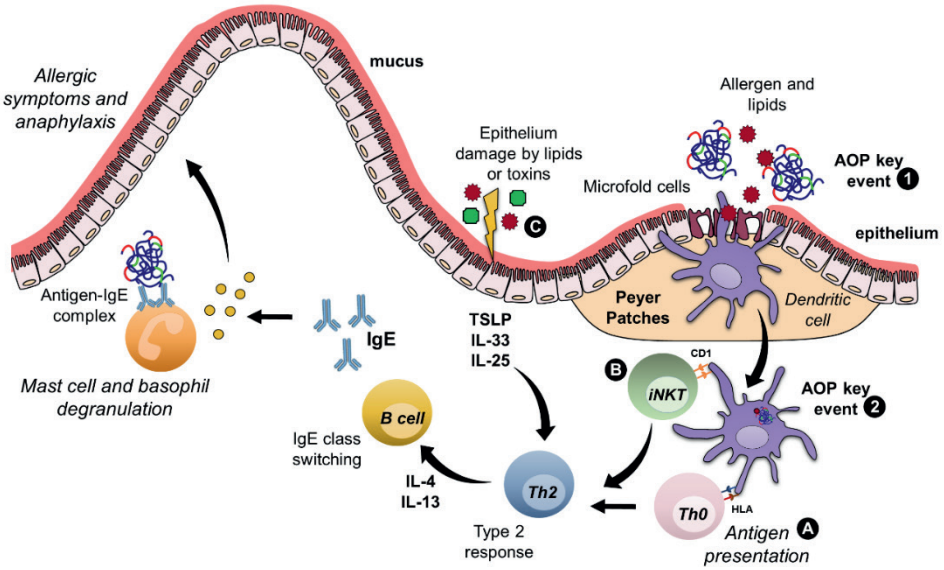

Figure 4. Putative sensitisation mechanism of cashew nut lipids-based antigens. A: Antigen presentation by the HLA complex leading to Th2 proliferation and B cell class switching to IgE-producing plasma cells; B: Lipids (depicted as red stars) might be presented directly to iNKT cells via the CD1 complex, skewing Th2 proliferation; C: Epithelial damage because of atopic dermatitis or by lipids or toxins can lead to the secretion of the cytokines IL-33, IL-25 and TSLP, which skew Th2 type inflammation. The adverse outcome pathway key event numbers 1 and 2 represent, respectively, epithelial antigen transport and T cell interaction with antigen presenting cells[111] (see section 4.2.2). Figure modified from Castan et al[112] and adapted to Del Moral et al[110].

impact of lipids should be at the sensitisation phase, rather than in the elicitation phase where antibody recognition takes place as the epitopes for nBer e 1 and rBer e 1 were similar. Therefore, it seems not unlikely that lipids from cashew nut may influence the efficacy of antigen presentation by antigen presenting cells (APCs; DC and macrophages) that process translocated proteins and peptides (Figure 4A). Such a mechanism has also been proposed by Mirotti et al[75] for the lipid adjuvant effect on nBer e 1 sensitization and hinted on by Tordesillas et al[105] for the increased sensitisation efficacy of lipid-bound Pru p 3. Lipoproteins and fatty acids have been shown to interact with toll like receptors (TLR) present on APCs, thereby possibly nudging their inflammation stage accordingly[106]. Alternatively, as seen for lipids from various pollen[107-109], endogenous cashew nut lipids can be presented directly as antigens to T-lymphocytes by the CD1 complex on APCs, stimulating Th2 proliferation and skewing indirect IgE production (Figure 4B). CD1 is a major histocompatibility complex (MHC) class-I-like molecule that can bind distinct lipid-based antigens. CD1 activation attracts T lymphocytes of the invariant natural killer T (iNKT) cell type that, when activated, produce large amounts of the cytokines IL-4 and IFN-γ that are implicated in the allergic sensitisation cascade[110].

According to Rico et al[82] cashew nuts contain up to fourteen types of fatty acids. With ~61% of the total fat content, oleic acid is the most abundant followed by linoleic, palmitic and stearic acids. The total sterol content in fat has been estimated to be ~200-286mg/100g by Rico et al[82] and Griffin & Dean[83] but there seem to be huge discrepancies in literature regarding total sterol contents. Phytosterols are stored in the lipid content of plant seed for future growth, as they play a key role in cell membrane functionality, cellular differentiation and proliferation[113]. The adjuvant-holding sterol-rich and polar-lipid fraction from Brazil nut in the study of Dearman et al[103] was rich in triglycerides, sterols and the phospholipids phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. Like in other nuts, phosphatidylcholine is also the predominant phospholipid in cashew nut, followed by phosphatidylserine and phosphatidylethanolamine. Exact levels can be dependent on the processing steps the nuts were exposed to, as heat treatment such as dry-roasting can decrease total phospholipid levels and relative ratios of specific isoform types[83,114]. This stressed the necessity to extract specific lipid-fractions from cashew nut and study their potential immunomodulatory effects on allergen sensitisation.

3.2.3 Adjuvant effect of microbial toxins

Next to food matrix adjuvants, common food contaminants such as toxins derived from bacteria, may impede oral tolerance and significantly impact the immune response provoked by allergens, i.e. the lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria, cholera toxin (CTX) from *Vibrio cholerae* and staphylococcal enterotoxin B (SEB) from *Staphylacoccus aureus*[115-117]. Transport of

LPS over the intestinal barrier and subsequent toll-like receptor (TLR)-mediated immune cell induction has been shown to increase IgE production[115,116]. However, the probability that cholera toxin would have acted as a cofactor in the underlaying cause of a cashew nut allergy is rather unlikely as in 2014, just 14 confirmed cases of cholera infection were reported to the European Centre for Disease Prevention and Control (ECDPC) agency [https://www.ecdc.europa.eu/en/publications-data/cholera-annual-epidemiological-report-2016-2014-data]. SEB on the other hand could be a possible cofactor in food allergic sensitisation. Although a weaker adjuvant, this toxin is produced by *S. aureus* bacteria strains that are frequently found in the upper respiratory tract and on the skin of eczematous patients[118]. Like with atopic dermatitis, severity of eczema has been associated with increased risk of food allergies[119] and the presence of these bacteria on the skin could therefore possibly explain for some cashew allergic patients why they were sensitive to skin sensitization.

4. Future prospects

From the topics discussed in this general discussion, it is clear that more knowledge is needed of the biochemical and immunological mechanisms and environmental factors underlying cashew nut sensitization. Important actuators of a cashew nut allergy should be elucidated, so that future research can ultimately be devoted to two main focus areas: 1] Preventing that allergic sensitization occurs in the first place and 2] treatment of an already manifested allergy.

4.1 Diagnostics

Until a treatment is available, it is important that the diagnosis of a cashew allergy is as comprehensive as possible. A highly unmet need is the ability to predict a clinical outcome of a food challenge, and especially the severity of allergic reactions, by measurements of blood serum markers, as this would drastically reduce costs and burden for the patient. Serum IgE towards the cashew nut allergen Ana o 3 seems to correlate with severe risk of anaphylaxis[120], however the models to predict severity of the allergic response in allergy diagnostics are far from complete. It is suggested that the interactions between platelets and basophils (a type of white blood cell involved in the allergic elicitation phase), via basophil-secreted platelet activating factor (PAF) upon IgE cross-linking, may contribute to anaphylaxis severity[121-123]. Circulating plasma PAF could thus potentially function as a marker for prediction of severity or measured in *ex vivo* studies when applying the BAT assay. Also, it seems that the number of epitopes (see **Chapter 1**) that are recognized by a patients can be informative for clinical severity, as shown in milk allergy[124]. Allergen epitope screening is not yet common practise in accustomed allergy diagnostics. On site and easy to interpret

microarray flow devices, containing crucial epitope regions (sequential and/or conformational) of the allergen repertoire from major allergenic foods (for instance tree nuts) could be developed to support the standard IgE measurements. Recognition of sequential or conformational epitopes can also sometimes reveal whether an allergy in childhood may be persistent or can be outgrown[123].

Another limitation of current diagnostics is the lack of purified allergens in food allergen tests. This is especially important to advice patients dietary avoidance and, in the future, to approach a personalised immunotherapy strategy (see also section 4.3). Current allergy component-resolved diagnostics (CRD), to detect specific IgE against individual purified native or recombinant allergens, relays primarily on two platforms, viz. the single and multiplex ImmunoCAP sIgE/ISAC® platforms of Thermo Fisher Scientific and the IMMULITE® sIgE platform of Siemens Healthineers AG. The ImmunoCAP ISAC® protein micro-array platform contains 112 solid-phase allergens while there are currently 183 single plex food allergen test combined available. Still, in the CRD study of Blazoski et al[120], where they aimed to identify food allergen components responsible for severe anaphylaxis in 237 children, 14.3% (34/237) of the systemic allergic reactions and anaphylaxis cases could not be explained by the available allergen diagnostics methods. Moreover, of the just 27 molecular allergen tests available, only Ana o 3 is represented as a single plex component assay for diagnosis of cashew nut allergy. In order to gain an accurate representation of the allergen repertoire in cashew nut and to study which allergens play a major role in the sensitization process, it is important that the molecular diagnostic tests for cashew nut allergy screening are expanded with at least all the (putative) allergen types identified in this thesis and possibly also their isotypes. Preferably, such a diagnostic multiplex platform for cashew nut allergy should also take into account possible cross-reactive allergens, especially in related Anacardiaceous species. Shortly after publication of **Chapter 3**, in which we describe the possibility of IgE cross-reactivity between cashew nut and pink peppercorn allergens, two additional case studies of adverse reactions to pink peppercorn consumption were reported[125]. This stresses the importance of informing patients of cross-reactivity risks for foods that are not quickly linked to a cashew nut allergy. An additional form of cashew nu allergy may arise when consumption of the cashew nut apple, the pseudofruit of the cashew tree (see **Chapter 1**), increases. Raw consumption is less appreciated due to the amount of tannins present conferring an astringent flavour, and is mainly restricted to South America[126]. Yet, cashew apple juice concentrate is increasingly used in vinegar, chutney, candies and jams and could thus represent a rising source of novel IgE-reactive cashew nut proteins[127], that are currently not represented in cashew nut allergy diagnostics.

4.2 Strategies to study immunological sensitization routes

Although the antigenicity of the major cashew nut allergens Ana o 1, Ana o 2 and Ana o 3 is well documented, their intrinsic immunogenicity and thus capacity to sensitize, is mechanistically hardly described. For instance, a mice model study by Parvataneni et al[128] confirmed that cashew nut itself holds innate immunogenic activity but it is still unclear how cashew nut allergens cross the epithelial barrier and, once absorbed, how they are recognized, internalized and processed for presentation to the immune system. It is believed that the intrinsic immunogenicity and antigenicity of a protein is influenced by various features, including size, digestion and heat stability, glycosylation status, enzyme activity, ligand complexation, number of IgE epitopes, etc.[103]. Not all these features have been studied in depth for each of the acknowledged cashew nut allergens and not at all for the novel putative allergenic cashew nut proteins described in this thesis.

4.2.1 In vivo/ex vivo strategies

A shortcoming of most clinical cashew nut studies described in literature is the absence of immunological studies focusing on sensitization mechanism and allergen presentation by immune cells, and the lack of predictive murine models (**Chapter 2**) forces researchers to develop and apply other techniques to gain more insight into this mechanism. Cashew nut allergic patients could be asked to participate in studies that aim at unravelling the route of sensitization. As elaborated (section 3), sensitisation of cashew nut allergy might occur via three routes, namely the lungs (airway), skin (transdermal) or gut (intestinal) route (see Figure 3). Simplified, upon exposure to antigens presented by antigen presenting immune cells (APCs), naive T lymphocytes (T cells) differentiate into antigen-specific memory T cells (Figure 4). These memory T cells are also primed to express tissue-specific cell adhesion molecules, also referred to as trafficking or homing receptors, that enable them to preferentially home to the initial site of immunizing tissue to initiate an organ-specific immune responses[129]. Thus, phenotyping of circulating antigen-specific T cell populations and their homing receptors in cashew nut allergic patients could reveal in which tissue sensitisation was initiated. Blood circulating cashew nut-responsive T cells can be identified and sampled from peripheral blood mononuclear cells (PBMCs) by gating on increased CD154 expression (CD40L) after short-term *ex-vivo* stimulation with whole cashew nut extract free from LPS[130]. CD40 ligand (CD40L) is expressed on activated T-cells. Further gating on CRTH2 and CD200R[131], both markers for Th2, can reveal whether the cashew reactive Th cells show a predominately Th2 profile. Next, these cashew-specific Th2 lymphocytes should be checked for both airway- (CCR4 [C-C motif chemokine receptor 4]), skin- (CCR10 [C-C motif chemokine receptor 10] or CLA [cutaneous lymphocyte-associated antigen]) and gut-homing (α4β7 integrin) molecules to evaluate the route of sensitisation in cashew allergic patient cohorts, like previously investigated for peanut allergic patients[130,132-134].

Further, it is also essential to investigate the role of lipids or lipid sensitization. This is shown by mapping the proportion of cashew reactive Th cells that have iNKT cell features (CD161+), which may indicate if cashew nut sensitisation, perhaps partly, acts via CD1-dependant lipid sensitisation (section 3.2.2). In line, stimulating PBMCs from cashew nut allergic individuals to purified lipid fractions of cashew nut with or without allergen co-administration can possibly elucidate the intrinsic sensitisation capacity and mechanism of each of the cashew nut allergens identified (Table 1). αGalCer, a derivative of a marine sponge can be used as a positive control in such experiments, as it strongly stimulates iNKT cells via specific CD1d binding[75]. In addition, the established mouse allergy model for cashew nuts, as described by Parvataneni et al[128] can be applied to identify food matrix-derived adjuvant factors in cashew nut sensitization, such as done for the role of extrinsic lipids in Brazil nut allergy[75,103] (see also section 3.2.1). Unfortunately, most current animal-based diagnostic *in vivo* food allergy models are not suitable for studying the immunogenicity triggers underlying the sensitization phase (reviewed in **Chapter 2**). Thus, for more in-depth mechanistic studies, there is a need for the development of predictive *in vitro/ ex vivo* sensitization models, aimed at the transdermal-, intestinal- and respiratory epithelial sensitization routes.

4.2.2 In vitro strategies

Van Bilsen and co-authors[111] established an adverse outcome pathway (AOP) to structure current available information on mechanisms and pathways evidenced to be involved in food allergen sensitization. According to this AOP, the first key event in antigen sensitisation is acquiring access to the underlying immune system via epithelial antigen transport, leaving in the middle whether this is via the lungs, skin or gastrointestinal tract. There is currently no established consensus on which epithelial model(s) should be used to study this first key event. Intestinal epithelial transport or sampling of intact (undigested) allergens or antigenic peptides occurs via the paracellular (between cells) or transcellular (through cells) route, partly depending on their solubility and aggregation state[124,135]. Thus, a consensus intestinal epithelial sensitisation model should at least be capable of these common protein absorption mechanisms. In addition, it is important that each type of epithelial model (lung/skin/gastrointestinal) is able to secrete one or all of the cytokines IL-33, IL-25 and TSLP upon stress experience. These specific cytokines are able to promote allergic inflammation (see **Chapter 2)** and co-determine T lymphocytes differentiation. The Caco-2 transwell model is currently the prominent model for intestinal absorption studies[137], but whether also the allergic inflammation reaction cascade can be measured in this model is not clear. Intestinal models consisting of primary cells, like intestinal stem cells-derived organoid cultures (**Chapter 2**) or the EpiIntestinal™ small intestinal microtissue model of MatTek Corporation[138], might be worth evaluating for both prerequisites, since they comprise all intestinal cell types and thus better represent an *in vivo* like

epithelial cell composition. Human keratinocytes have been successfully applied in skin sensitisation studies for peanut[104,139] and such cells could represent a first prediction model candidate for studying skin absorption and provoked stress responses of cashew nut allergens. Another vital key event described in the AOP is the interaction of APC with T cells[111]. A dendritic cell (DC)-T cell model using primary cells as developed by Hoppenbrouwers et al[140] could provide insights into HLA-driven antigen presentation while the iNTK reporter cell line model of Humeniuk et al[141] can be used to evaluate lipid-antigen interactions in cashew nut sensitization (Figure 4).

4.3 Treatment strategies

In the Netherlands, cashew nut allergy is especially prevalent in children, which suggest that sensitization predominately occurs at a young age[14]. Early introduction of peanut in infants that are at risk for developing allergies, has shown to be effective in primary prevention of peanut allergy[65]. If it appears that the sensitization mechanism behind a cashew nut allergy is similar to that of a peanut allergy (predominately via the skin, while oral exposure promotes tolerance), early introduction of cashew nut could be a first treatment regimen to be clinically tested to avoid sensitization in children. Once a cashew nut allergy has been established though, strict avoidance is currently the most given advice to circumvent unwanted allergic reactions. Even so, many severe allergic reactions happen unexpectedly due to incorrect or unclear product labelling or by allergen cross-contamination. For example, many cashew nut allergic patients are seriously at risk of reactions after accidental ingestion of traces in out-of-home situations such as in restaurants or at parties. Unsafe situations of this kind make that allergic individuals less likely participate in social events, which reduces their quality of life[142]. The lack of immunotherapy treatments for cashew nut allergy shows that research developments are lagging behind on those for peanut allergy. Allergen immunotherapy (AIT) is currently the only available medical intervention treatment that can reprogram the immune system from a sensitized phenotype to tolerance[143,144]. While AIT is already fairly implemented to treat respiratory and venom-related allergies in the US and emerging markets in Asia, AIT is still virtually unknown in Europe and many patients remain unaware of this treatment option[145]. Besides, treatment of food allergies by AIT has proven to remain challenging. At present, the only U.S. Food and Drug Administration (FDA) approved treatment for food allergy is a standardized oral immunotherapy (OIT) product for peanut allergy (https://www.fda.gov/).

An AIT treatment consists of different stages, the build-up phase, and the maintenance phase. The build-up phase focuses on desensitization. The tolerance threshold per patient is increased by incrementally increasing the allergen dose administered over a controlled period of time (Figure 5A). When the maximum dose is tolerated, the duration of the maintenance phase, in which the maximum

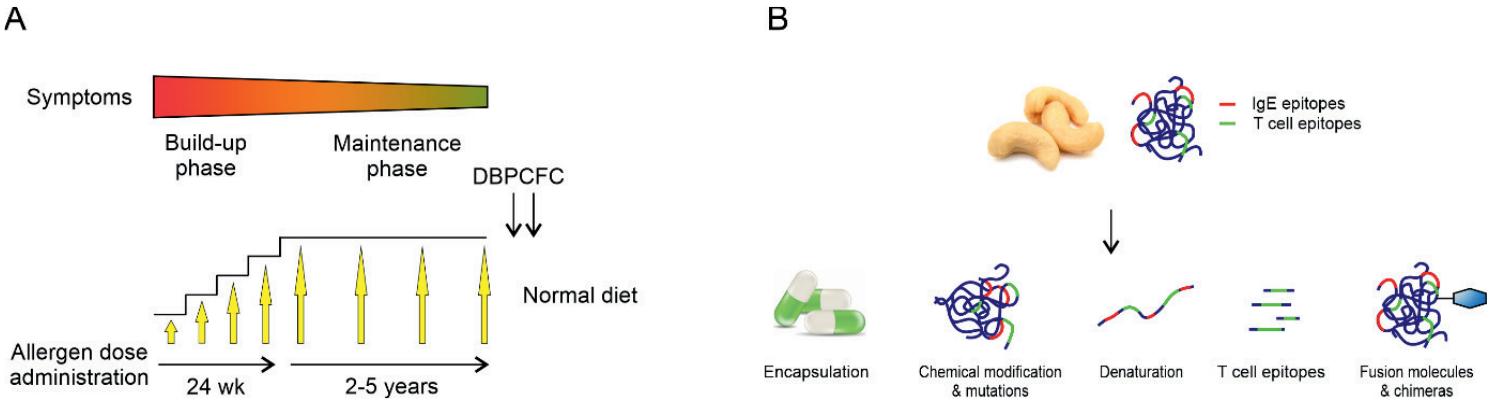

Figure 5. Immunotherapy outline and strategies. A: Schematic Overview of OIT. During or after the maintenance phase, the persistence of tolerance after OIT is tested, after which the participant can switch to a normal diet or relapses into continuing the maintenance dose. Dose starts usually with the lowest accepted threshold relatively safe and well tolerated in high-risk patients, building to a trace dose that is consistent with protection against accidental ingestion or cross-contamination, allowing for daily life protection[146]. Starting and maintenance dose for cashew nut OIT needs to be established. B: Strategies for putative cashew nut AIT vaccine products aimed at reduced IgE binding and/or increasing the accessibility of T-cell epitopes. Chemical modification results in allergoids with reduced IgE binding[144]. Denaturation may increase accessibility of T-cell epitopes and destroys conformational epitopes. T cell epitopes possessing modulatory functions can be synthesized and administered as a cocktail or as a single peptide while conjugation or fusion of allergens with immunomodulatory molecules can induce a tolerogenic immune responses[152]. Figure is a compilation from Wai et al[152], Valenta et al[153] and Fauquert et al[146].

dose is periodically administered, is determined by an oral food challenge[146]. The treatment dose can be applied orally (OIT; in case of food allergy AIT) as a paste or powder hidden in a vehicle food (to mask the taste) or, in the case of purified allergens, vaccines can be administered subcutaneously (SCIT; via skin injections) or sublingually (SLIT; tablet under the tongue)[145]. Kulis et al[147,148] have reported experimental cashew nut immunotherapy efficacy using protein extracts or pepsinized cashew nut proteins in a murine model of cashew allergy, but no cohort studies or clinical trials have yet been reported to treat cashew nut allergy. Most published food allergy-aimed OIT cohort studies were focused on desensitization of a peanut, egg or cow's milk allergy[149,150]. However, the sometimes low success rates and the commonly reported digestive symptoms (like abdominal pain, nausea and vomiting), oropharyngeal symptoms (swelling, itching in the upper throat area just behind the tongue) and the significant risk on side effects, including anaphylaxis and eosinophilic esophagitis (narrowing of the oesophagus, the muscular tube that connects the mouth and stomach[151]) affecting patient safety and compliance[145], have discouraged their implementation in routine clinical practice.

Thus, as stated by van der Kleij et al[154], a prerequisite for food allergy immunotherapy to become a standard in clinical allergy practices would be the development of AIT (vaccine) products with significantly fewer side effects. In the PITA study, Fauquert et al[146] used sealed capsules containing peanut flour instead of vehicle foods, to bypass the oral cavity and upper digestive tract, to limit adverse side effects involving the mouth and oesophagus. Besides encapsulation, other novel (experimental) AIT approaches aimed at increasing clinical efficacy and reducing adverse side effects are worth considering[145,155,156], most of which require knowledge of the immunodominant B- and T-cell epitopes of the major cashew allergens (Figure 5B). For example, peptides or food allergens can be engineered or chemically modified to lose their IgE cross-linking effectiveness, whilst retaining their ability to modulate allergen-specific T cells[145,156], as evident from peanut AIT studies[15,154]. Detailed knowledge of cashew nut immunodominant B- and T-cell epitopes is currently lacking. Linear IgE-epitope stretches in the major cashew allergens Ana o 1, Ana o 2 and Ana o 3 have been studied to some extent[10-12,36] (**Chapter 6**) but their sequences are not yet delineated to a functional length containing essential amino acids, nor are they classified for immunodominance. Key contributors to epitope-antibody binding interactions are suggested to imply 5 to 6 aa[157], while the described cashew nut allergen epitopes hold between 8 to 15 aa. Specific conformational epitopes for most cashew nut allergens have yet to be elucidated, as detailed knowledge of the allergen structure is required. Only one Ana o 2-specific conformational epitope has been described to date[158], so this is an area of research that requires attention (see chapter 1 for strategies). Cashew nut-specific T cell responses directed towards Ana o 1 and Ana o 2 were studied by Archila et al[159]. They identified multiple cashew nut unique peptides and suggested

that Ana o 1 and Ana o 2 share cross-reactive T cell epitopes with hazelnut and pistachio, but their clinical relevance in cashew peptide immunotherapy still needs to be determined[155].

Thus recapitulating, standardization of therapeutic extracts, allergoids or peptide mixtures, representing all relevant cashew nut allergens in reliable molecular composition and biologic effective units[160] would be the first prerequisite for setting up an AIT protocol for cashew nut allergic children.

References

1. Prescott S, Allen KJ. Food allergy: riding the second wave of the allergy epidemic. Pediatr. Allergy Immunol 2011;22:155-160.
2. Halken S. Prevention of food allergy. Environ Toxicol Pharmacol 1997;4(1-2):149-56.
3. Samartín S, Marcos A, Chandra RK. Food hypersensitivity. Nutrition Research 2001;21:473–97.
4. Tang MLK and Mullins RJ. Food allergy: is prevalence increasing? Intern Med J 2017;47(3):256-261.
5. Loh W and Tang MLK. The Epidemiology of Food Allergy in the Global Context. Int J Environ Res Public Health 2018;15(9): 2043.
6. Martin PJ, Topper CP, Bashiru RA, Boma F, De Waal D, Harries HC, Kasuga LJ, Katanila N, Kikoka LP, Lamboll R, Maddison AC, Majule AE, Masawe PA, Millanzi KJ, Nathaniels NQ, Shomari SH, Sijaona ME, Stathers T. Cashew nut production in Tanzania: Constraints and progress through integrated crop management. Crop Protection 1997;16(1):5-14.
7. McWilliam V, Koplin J, Lodge C, Tang M, Dharmage S, Allen K. The prevalence of tree nut allergy: a systematic review. Curr Allergy Asthma Rep 2015;15:1-13.
8. Mendes C, Costa J, Vicente AA, Oliveira MBPP, Mafra I. Cashew Nut Allergy: Clinical Relevance and Allergen Characterisation. Clin Rev Allergy Immunol 2019;57(1):1-22.
9. Teuber SS, Sathe SK, Peterson WR, Roux KH. Characterization of the soluble allergenic proteins of cashew nut (Anacardium occidentale L.). J Agric Food Chem 2002;50(22):6543-9.
10. Wang F, Robotham JM, Teuber SS, Tawde P, Sathe SK, Roux KH. Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family. J Allergy Clin Immunol 2002;110(1):160-6.
11. Wang F, Robotham JM, Teuber SS, Sathe SK, Roux KH. Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family. Int Arch Allergy Immunol 2003;132(1):27-39.
12. Robotham JM, Wang F, Seamon V, Teuber SS, Sathe SK, Sampson HA, Beyer K, Seavy M, Roux KH. Ana o 3, an important cashew nut (Anacardium occidentale L.) allergen of the 2S albumin family. J Allergy Clin Immunol 2005;115(6):1284-90.
13. Davoren M and Peake J. Cashew nut allergy is associated with a high risk of anaphylaxis. Arch Dis Child. 2005; 90(10):1084-1085.
14. van der Valk JPM, Gerth van Wijk R, Dubois AE, de Groot H, Reitsma M, Vlieg-Boerstra B, Savelkoul HF, Wichers HJ, de Jong NW. Multicentre Double-Blind Placebo-Controlled Food Challenge Study in Children Sensitised to Cashew Nut. PLoS One 2016;11:e0151055.
15. Commins SP, Kim EH, Orgel K, Kulis M. Peanut Allergy: New Developments and Clinical Implications. Curr Allergy Asthma Rep 2016;16(5):35.
16. Frith K, Katelaris CH. Current perspectives on peanut allergy. Intern Med J 2019;49(12):1480-1487.
17. Mueller GA, Maleki SJ, Pedersen LC. The molecular basis of peanut allergy. Curr Allergy Asthma Rep 2014;14(5):429.
18. Palladino C, Breiteneder H. Peanut allergens. Mol Immunol 2018;100:58-70.
19. Bastiaan-Net S, Batstra MR, Aazamy N, Savelkoul HFJ, van der Valk JPM, Gerth van Wijk R, Schreurs MWJ, Wichers HJ, de Jong NW. IgE Cross-Reactivity Measurement of Cashew Nut, Hazelnut and Peanut Using a Novel IMMULITE Inhibition Method. Clin Chem Lab Med 2020; Online ahead of print.
20. Bastiaan-Net S, Pina-Pérez MC, Dekkers BJW, Westphal AH, America AHP, Ariëns RMC, de Jong NW, Wichers HJ, Mes JJ. Identification and in Silico Bioinformatics Analysis of PR10 Proteins in Cashew Nut. Protein Sci 2020;29(7):1581-1595.
21. Savvatianos S, Konstantinopoulos AP, Borgå Å, Stavroulakis G, Lidholm J, Borres MP, Manousakis E, Papadopoulos NG. Sensitization to cashew nut 2S albumin, Ana o 3, is highly predictive of cashew and pistachio allergy in Greek children. J Allergy Clin Immunol 2015;136(1):192-194.
22. Lange L, Lasota L, Finger A, Vlajnic D, Büsing S, Meister J, Broekaert I , Pfannenstiel C, Friedrichs F, Price M, Trendelenburg V, Niggemann B, Beyer K. Ana o 3-specific IgE is a good

predictor for clinically relevant cashew allergy in children. Allergy 2017;72(4):598-603.
23. van der Valk JP, Gerth van Wijk R, Vergouwe Y, Steyerberg EW, Reitsma M, Wichers HJ, Savelkoul HF, Vlieg-Boerstra B, de Groot H, Dubois AE, de Jong NW. sIgE Ana o 1, 2 and 3 accurately distinguish tolerant from allergic children sensitized to cashew nuts. Clin Exp Allergy 2017;47(1):113-120.
24. Gruehn S, Suphioglu C, O'hehir RE, Volkmann D. Molecular Cloning and Characterization of Hazel Pollen Protein (70 kD) as a Luminal Binding Protein (BiP): A Novel Cross-Reactive Plant Allergen. Int Arch Allergy Immunol 2003;131(2):91-100.
25. Bar-El Dadon S, Pascual CY, Eshel D, Teper-Bamnolker P, Ibáñez MD, Reifen R. Vicilin and the basic subunit of legumin are putative chickpea allergens. Food Chem 2013;138(1):13-8.
26. Blazowski L, Majak P, Kurzawa R, Kuna P, Jerzynska J. Food Allergy Endotype With High Risk of Severe Anaphylaxis in children-Monosensitization to Cashew 2S Albumin Ana O 3. Allergy 2019;74(10):1945-1955.
27. Zuidmeer-Jongejan L, Fernández-Rivas M, Winter MGT, Akkerdaas JH, Summers C, Lebens A, Knulst AC, Schilte P, Briza P, Gadermaier G, van Ree R. Oil body-associated hazelnut allergens including oleosins are underrepresented in diagnostic extracts but associated with severe symptoms. Clin Transl Allergy 2014; 4:4.
28. Schwager C, Kull S, Krause S, Schocker F, Petersen A, Becker WM, Jappe U. Development of a novel strategy to isolate lipophilic allergens (oleosins) from peanuts. PLoS One. 2015; 10(4):e0123419.
29. Datema MR, Zuidmeer-Jongejan L, Asero R, Barreales L, Belohlavkova S, de Blay F, Bures P, Clausen M, Dubakiene R, Gislason D, Jedrzejczak-Czechowicz M, Kowalski ML, Knulst AC, Kralimarkova T, Le TM, Lovegrove A, Marsh J, Papadopoulos NG, Popov T, Del Prado N, Purohit A, Reese G, Reig I, Seneviratne SL, Sinaniotis A, Versteeg SA, Vieths S, Zwinderman AH, Mills C, Lidholm J, Hoffmann-Sommergruber K, Fernández-Rivas M, Ballmer-Weber B, van Ree R. Hazelnut allergy across Europe dissected molecularly: A EuroPrevall outpatient clinic survey. J Allergy Clin Immunol. 2015; 136(2):382-91.
30. Leduc V, Moneret-Vautrin DA, Tzen JT, Morisset M, Guerin L, Kanny G. Identification of oleosins as major allergens in sesame seed allergic patients. Allergy. 2006; 61(3):349-56.
31. Deak PE, Vrabel MR, Kiziltepe T, Bilgicer B. Determination of Crucial Immunogenic Epitopes in Major Peanut Allergy Protein, Ara h2, via Novel Nanoallergen Platform. Sci Rep 2017;7:3981.
32. van der Valk JP, Dubois AE, Gerth van Wijk R, Wichers HJ, de Jong NW. Systematic review on cashew nut allergy. Allergy 2014;69(6):692-8.
33. Kim H, Yoo SJ, Kang HA. Yeast synthetic biology for the production of recombinant therapeutic proteins. FEMS Yeast Res 2015;15(1):1-16.
34. Alcocer MJ, Murtagh GJ, Bailey K, Dumoulin M, Meseguer AS, Parker MJ, Archer DB. The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins. J Mol Biol 2002;324(1):165-75.
35. Murtagh GJ, Archer DB, Dumoulin M, Ridout S, Matthews S, Arshad SH, Alcocer MJ. In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. Clin Exp Allergy 2003;33(8):1147-52.
36. Reitsma M, Bastiaan-Net S, Sforza S, van der Valk JP, van Gerth van Wijk R, Savelkoul HF, de Jong NW, Wichers HJ. Purification and Characterization of Anacardium occidentale (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3. J Agric Food Chem. 2016;64(5):1191-201.
37. Altmann F. Coping with cross-reactive carbohydrate determinants in allergy diagnosis. Allergo J Int. 2016; 25(4): 98-105.
38. Schnaar RL. Glycans and glycan-binding proteins in immune regulation: A concise introduction to glycobiology for the allergist. J Allergy Clin Immunol 2015;135(3):609-15.
39. Bretthauer RK, Castellino FJ. Glycosylation of Pichia pastoris-derived proteins. Biotechnol Appl Biochem 1999;30(3):193-200.
40. Reitsma M, Bastiaan-Net S, van der Valk JPM, Savelkoul HFJ, de Jong NW, Wichers HJ. "Chapter 5: Pichia pastoris recombinantly expressed Anacardium accidentale (cashew nut) allergens Ana o 1, Ana o 2 and Ana o 3". In Reitsma M. The cashew allergens: a molecular and serological characterization. 2017;107-139.

41. Shreffler WG, Castro RR, Kucuk ZY, Charlop-Powers Z, Grishina G, Yoo S, Burks WA, Sampson HA. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. J Immunol 2006;177(6):3677-3685.
42. Davey MR, Anthony P, Power JB, Lowe KC. Plant protoplast technology: Current status. Acta Physiol Plant 2005;27:117–130.
43. Rasala BA, Mayfield SP. Photosynthetic biomanufacturing in green algae; production of recombinant proteins for industrial, nutritional, and medical uses. Photosynth Res 2015;123(3):227-39.
44. Yamamoto YT, Rajbhandari N, Lin X, Bergmann BA, Nishimura Y, Stomp A-M. Genetic transformation of duckweed Lemna gibba and Lemna minor. In Vitro Cell Dev Biol – Plant 2001;37:349–353.
45. Doron L, Segal N, Shapira M. Transgene Expression in Microalgae—From Tools to Applications. Front Plant Sci 2016;7:505.
46. Zhu LD, Li ZH, Hiltunen E. Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock. Biomed Res Int 2016;2016:8792548.
47. Appenroth KJ, Sree KS, Böhm V, Hammann S, Vetter W, Leiterer M, Jahreis G. Nutritional value of duckweeds (Lemnaceae) as human food. Food Chem 2017;217:266-273.
48. Tordesillas L, Berin MC, Sampson HA. Immunology of Food Allergy. Immunity 2017;47(1):32-50.
49. van der Valk JPM, El Bouche R, Gerth van Wijk R, de Groot H, Wichers HJ, Dubois AEJ, de Jong NW. Low Percentage of Clinically Relevant Pistachio Nut and Mango Co-Sensitisation in Cashew Nut Sensitised Children. Clin Transl Allergy 2017;7:8.
50. Carlsten C, Dimich-Ward H, Ferguson A, Watson W, Rousseau R, Dybuncio A, et al. Atopic dermatitis in a high-risk cohort: natural history, associated allergic outcomes, and risk factors. Ann Allergy Asthma Immunol 2013;110:24-8.
51. Zheng T, Yu J, Oh MH, Zhu Z. The atopic march: progression from atopic dermatitis to allergic rhinitis and asthma. Allergy Asthma Immunol Res 2011;3:67-73.
52. Asero R, Antonicelli L, Arena A, Bommarito L, Caruso B, Colombo G, Crivellaro M, De Carli M, Della Torre E, Della Torre F, Heffler E, Lodi Rizzini F, Longo R, Manzotti G, Marcotulli M, Melchiorre A, Minale P, Morandi P, Moreni B, Moschella A, Murzilli F, Nebiolo F, Poppa M, Randazzo S, Rossi G, Senna GE. Causes of food-induced anaphylaxis in Italian adults: a multi-centre study. Int Arch Allergy Immunol 2009;150(3):271-7.
53. Bøgh KL, Madsen CB. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? Crit Rev Food Sci Nutr 2016;56(9):1545-67.
54. West C. Introduction of Complementary Foods to Infants. Ann Nutr Metab 2017;70 Suppl 2:47-54.
55. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med 2011;365:1315-27.
56. McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol 2013;131(2):280-91.
57. Valenta R, Hochwallner H, Linhart B, Pahr S. Food allergies: the basics. Gastroenterology 2015;148(6):1120-31.e4.
58. Danella Figo D, De Amicis K, Neiva Santos de Aquino D, Pomiecinski F, Gadermaier G, Briza P, Eduardo Santos Galvão C, Bussador do Amaral J, de Oliveira Martins C, Fernandes Morato Castro F, Kalil J, Souza Santos K. Cashew Tree Pollen: An Unknown Source of IgE-Reactive Molecules. Int J Mol Sci 2019;20(10). pii: E2397.
59. Salvatori N, Reccardini F, Convento M, Purinan A, Colle R, De Carli S, Garzoni M, Lafiandra D, De Carli M. Asthma induced by inhalation of flour in adults with food allergy to wheat. Clin Exp Allergy 2008;38(8):1349-56.
60. Luyt DK, Vaughan D, Oyewole E, Stiefel G. Ethnic differences in prevalence of cashew nut, pistachio nut and almond allergy. Pediatr Allergy Immunol 2016;27(6):651-4.
61. Brough HA, Santos AF, Makinson K, Penagos M, Stephens AC, Douiri A, Fox AT, Du Toit G, Turcanu V, Lack G. Peanut protein in household dust is related to household peanut

consumption and is biologically active. J Allergy Clin Immunol 2013;132(3):630-638.
62. Brough HA, Simpson A, Makinson K, Hankinson J, Brown S, Douiri A, Belgrave DC, Penagos M, Stephens AC, McLean WH, Turcanu V, Nicolaou N, Custovic A, Lack G. Peanut allergy: effect of environmental peanut exposure in children with filaggrin loss-of-function mutations. J Allergy Clin Immunol 2014;134(4):867-875.e1.
63. Brough HA, Liu AH, Sicherer S, Makinson K, Douiri A, Brown SJ, Stephens AC, Irwin McLean WH, Turcanu V, Wood RA, Jones SM, Burks W, Dawson P, Stablein D, Sampson H, Lack G. Atopic dermatitis increases the effect of exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy. J Allergy Clin Immunol 2015;135(1):164-70.
64. Du Toit G, Katz Y, Sasieni P, Mesher D, Maleki SJ, Fisher HR, Fox AT, Turcanu V, Amir T, Zadik-Mnuhin G, Cohen A, Livne I, Lack G. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. J Allergy Clin Immunol 2008;122(5):984-91.
65. Du Toit G, Roberts G, Sayre PH, Bahnson HT, Radulovic S, Santos AF, Brough HA, Phippard D, Basting M, Feeney M, Turcanu V, Sever ML, Gomez Lorenzo M, Plaut M, Lack G; LEAP Study Team. Randomized trial of peanut consumption in infants at risk for peanut allergy. N Engl J Med 2015;372(9):803-13.
66. Foong RX, Brough H. The role of environmental exposure to peanut in the development of clinical allergy to peanut. Clin Exp Allergy 2017;47(10):1232-1238.
67. Asero R, Mistrello G, Amato S, Roncarolo D, Martinelli A, Zaccarini M. Peach fuzz contains large amounts of lipid transfer protein: is this the cause of the high prevalence of sensitization to LTP in Mediterranean countries? Eur Ann Allergy Clin Immunol 2006;38(4):118-21.
68. Hauser M, Egger M, Wallner M, Wopfner N, Schmidt G, Ferreira F. Molecular Properties of Plant Food Allergens: A Current Classification into Protein Families. Open Immunol J 2008;1:1-12.
69. Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. J Allergy Clin Immunol 2008;121: 847-52.
70. Lehrer SB, Horner WE, Reese G. Why are some proteins allergenic? Implications for biotechnology. Crit Rev Food Sci Nutr. 1996 Jul;36(6):553-64.
71. Huby RD, Dearman RJ, Kimber I. Why are some proteins allergens? Toxicol Sci 2000;55(2):235-46.
72. Bredehorst R, David K. What establishes a protein as an allergen? J Chromatogr B Biomed Sci Appl 2001;756(1-2):33-40.
73. Kimber I, Dearman RJ. Factors affecting the development of food allergy. Proc Nutr Soc 2002;61(4):435-9.
74. Breiteneder H, Mills EN. Molecular properties of food allergens. J Allergy Clin Immunol 2005;115(1):14-23; quiz 24.
75. Mirotti L, Florsheim E, Rundqvist L, Larsson G, Spinozzi F, Leite-de-Moraes M, Russo M, Alcocer M. Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. Allergy 2013;68(1):74-83.
76. Scheurer S, Schülke S. Interaction of Non-Specific Lipid-Transfer Proteins With Plant-Derived Lipids and Its Impact on Allergic Sensitization. Front Immunol 2018;9:1389.
77. Satitsuksanoa P, Jansen K, Głobińska A, Van de Veen W, Akdis M. Regulatory Immune Mechanisms in Tolerance to Food Allergy. Front Immunol 2018; 9: 2939.
78. Bublin M, Eiwegger T, Breiteneder H. Do lipids influence the allergic sensitization process? J Allergy Clin Immunol 2014;134(3):521-9.
79. Ryan E, Galvin K, O'Connor TP, Maguire AR, O'Brien NM. Fatty acid profile, tocopherol, squalene and phytosterol content of Brazil, pecan, pine, pistachio and cashew nuts. Int. J. Food Sci. Nutr 2006;57:219–228.
80. Venkatachalam M, Sathe SK. Chemical composition of selected edible nut seeds. J Agric Food Chem 2006;54(13):4705-14.
81. Trox J, Vadivel V, Vetter W, Stuetz W, Scherbaum V, Gola U, Nohr D, Biesalski HK. Bioactive compounds in cashew nut (Anacardium occidentale L.) kernels: effect of different shelling methods. J Agric Food Chem 2010;58(9):5341-6.

82. Rico R, Bulló M, Salas-Salvadó J. Nutritional composition of raw fresh cashew (Anacardium occidentale L.) kernels from different origin. Food Sci Nutr 2015;4(2):329-38.
83. Griffin LE and Dean LL. Nutrient Composition of Raw, Dry-Roasted, and Skin On Cashew nuts. J Food Research 2017;6(6):13-28.
84. Finkina EI, Melnikova DN, Bogdanov IV, Ovchinnikova TV. Lipid Transfer Proteins As Components of the Plant Innate Immune System: Structure, Functions, and Applications. Acta Naturae 2016;8(2):47-61.
85. Asam C, Batista AL, Moraes AH, de Paula VS, Almeida FCL, Aglas L, Kitzmüller C, Bohle B, Ebner C, Ferreira F, Wallner M, Valente AP. Bet v 1--a Trojan horse for small ligands boosting allergic sensitization?. Clin Exp Allergy 2014;44(8):1083-1093.
86. Jackowski S, Cronan JE, Rock CO. "Chapter 2: Lipid metabolism in procaryotes". In Vance DE, Vance J (eds.). Biochemistry of Lipids, Lipoproteins and Membranes. Elsevier 1991;80–81.
87. Sancho AI, Wangorsch A, Jensen BM, Watson A, Alexeev Y, Johnson PE, Mackie AR, Neubauer A, Reese G, Ballmer-Weber B, Hoffmann-Sommergruber K, Skov PS, Vieths S, Mills CEN. Responsiveness of the major birch allergen Bet v 1 scaffold to the gastric environment: impact on structure and allergenic activity. Mol Nutr Food Res 2011;55(11):1690-1699.
88. Hurlburt BK, Offermann LR, McBride JK, Majorek KA, Maleki SJ, Chruszcz M. Structure and function of the peanut panallergen Ara h 8. J Biol Chem 2013;288(52):36890-901.
89. Petersen A, Rennert S, Kull S, Becker WM, Notbohm H, Goldmann T, Jappe U. Roasting and lipid binding provide allergenic and proteolytic stability to the peanut allergen Ara h 8. Biol Chem 2014;395(2):239-50.
90. Bohle B, Zwölfer B, Heratizadeh A, Jahn-Schmid B, Dall Antonia Y, Alter M, Keller W, Zuidmeer L, van Ree R, Werfel T, Ebner C. Cooking birch pollen-related food: divergent consequences for IgE- and T cell-mediated reactivity in vitro and in vivo. J Allergy Clin Immunol 2006;118(1):242-249.
91. Tai SS, Chen MC, Peng CC, Tzen JT. Gene family of oleosin isoforms and their structural stabilization in sesame seed oil bodies. Biosci Biotechnol Biochem 2002;66(10):2146-53.
92. Blein JP, Coutos-Thévenot P, Marion D, Ponchet M. From elicitins to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. Trends Plant Sci 2002;7(7):293-6.
93. Zuidmeer LAPA, van Ree R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. Curr Opin Allergy Clin Immunol 2007;7(3):269-73.
94. Perrocheau L, Bakan B, Boivin P, Marion D. Stability of barley and malt lipid transfer protein 1 (LTP1) toward heating and reducing agents: relationships with the brewing process. J Agric Food Chem 2006 ;54(8):3108-13.
95. Vassilopoulou E, Rigby N, Moreno FJ, Zuidmeer L, Akkerdaas J, Tassios I, Papadopoulos NG, Saxoni-Papageorgiou P, van Ree R, Mills C. Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. J Allergy Clin Immunol 2006;118(2):473-80.
96. Dubiela P, Aina R, Polak D, Geiselhart S, Humeniuk P, Bohle B, Alessandri S, Del Conte R, Cantini F, Borowski T, Bublin M, Hoffmann-Sommergruber K. Enhanced Pru p 3 IgE-binding activity by selective free fatty acid-interaction. J Allergy Clin Immunol 2017;140(6):1728-1731.e10.
97. Cubells-Baeza N, Gómez-Casado C, Tordesillas L, Ramírez-Castillejo C, Garrido-Arandia M, González-Melendi P, Herrero M, Pacios LF, Díaz-Perales A. Identification of the ligand of Pru p 3, a peach LTP. Plant Mol Biol 2017;94(1-2):33-44.
98. Pantoja-Uceda D, Bruix M, Giménez-Gallego G, Rico M, Santoro J. Solution structure of RicC3, a 2S albumin storage protein from Ricinus communis. Biochemistry. 2003;42(47):13839-47.
99. Pantoja-Uceda D, Shewry PR, Bruix M, Tatham AS, Santoro J, Rico M. Solution structure of a methionine-rich 2S albumin from sunflower seeds: relationship to its allergenic and emulsifying properties. Biochemistry 2004;43(22):6976-86.
100. Guéguen J, Popineau Y, Anisimova IN, Fido RJ, Shewry PR, Tatham AS. Functionality of the 2S Albumin Seed Storage Proteins from Sunflower. J. Agric. Food Chem 1996;44(5):1184–1189.
101. Rundqvist L, Tengel T, Zdunek J, Björn E, Schleucher J, Alcocer MJ, Larsson G. Solution structure, copper binding and backbone dynamics of recombinant Ber e 1-the major allergen

from Brazil nut. PLoS One 2012;7(10):e46435.
102. Pastukhov AV, Ropson IJ. Fluorescent dyes as probes to study lipid-binding proteins. Proteins 2003;53(3):607-15.
103. Dearman RJ, Alcocer MJ, Kimber I. Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. Clin Exp Allergy. 2007 Apr;37(4):582-91.
104. Palladino C, Narzt MS, Bublin M, Schreiner M, Humeniuk P, Gschwandtner M, Hafner C, Hemmer W, Hoffmann-Sommergruber K, Mildner M, Palomares O, Gruber F, Breiteneder H. Peanut lipids display potential adjuvanticity by triggering a pro-inflammatory response in human keratinocytes. Allergy 2018;73(8):1746-1749.
105. Tordesillas L, Cubells-Baeza N, Gómez-Casado C, Berin C, Esteban V, Barcik W, O'Mahony L, Ramirez C, Pacios LF, Garrido-Arandia M, Díaz-Perales A. Mechanisms underlying induction of allergic sensitization by Pru p 3. Clin Exp Allergy 2017;47(11):1398-1408.
106. Weatherill AR, Lee JY, Zhao L, Lemay DG, Youn HS, Hwang DH. Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. J Immunol 2005;174(9):5390-7.
107. Agea E, Russano A, Bistoni O, Mannucci R, Nicoletti I, Corazzi L, Postle AD, De Libero G, Porcelli SA, Spinozzi F. Human CD1-restricted T cell recognition of lipids from pollens. J Exp Med 2005;202(2):295-308.
108. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S, de Benedictis FM, Spinozzi F. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted gamma delta T cells. J Allergy Clin Immunol 2006;117(5):1178-84.
109. Spinozzi F, Porcelli SA. Recognition of lipids from pollens by CD1-restricted T cells. Immunol Allergy Clin North Am 2007;27(1):79-92.
110. Del Moral MG, Martínez-Naves E2. The Role of Lipids in Development of Allergic Responses. Immune Netw 2017;17(3):133-143.
111. van Bilsen JHM, Sienkiewicz-Szłapka E, Lozano-Ojalvo D, et al. Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins. Clin Transl Allergy 2017;7:13.
112. Castan L, Bøgh KL, Maryniak NZ, Epstein MM, Kazemi S, O'Mahony L, Bodinier M, Smit JJ, van Bilsen JHM, Blanchard C, Głogowski R, Kozáková H, Schwarzer M, Noti M, de Wit N, Bouchaud G, Bastiaan-Net S. Overview of in vivo and ex vivo endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins? Allergy 2020;75(2):289-301.
113. Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi AM. Plant sterols: biosynthesis, biological function and their importance to human nutrition. J Sci Food Agric 2000;80:939-966.
114. Davis JP, Dean LL. Peanut composition, flavor and nutrition. In: Stalker, H. T., Wilson R. F. (Eds). Peanuts Genetics, Processing, and Utilization. San diego, CA, USA; Elsevier 2016;289-345.
115. Slater JE, Paupore EJ, Elwell MR, Truscott W. Lipopolysaccharide augments IgG and IgE responses of mice to the latex allergen Hev b 5. J Allergy Clin Immunol 1998;102(6 Pt 1):977-83.
116. Tulic MK, Holt PG, Sly PD. Modification of acute and late-phase allergic responses to ovalbumin with lipopolysaccharide. Int Arch Allergy Immunol 2002;129(2):119-28.
117. Aizpurua-Olaizola O, Sastre Torano J, Pukin A, Fu O, Boons GJ, de Jong GJ, Pieters RJ. Affinity capillary electrophoresis for the assessment of binding affinity of carbohydrate-based cholera toxin inhibitors. Electrophoresis 2018;39(2):344-347.
118. Monti G, Tonetto P, Mostert M, Oggero R. Staphylococcus aureus skin colonization in infants with atopic dermatitis. Dermatology 1996;193:83-87.
119. Martin PE, Eckert JK, Koplin JJ, Lowe AJ, Gurrin LC, Dharmage SC, Vuillermin P, Tang ML, Ponsonby AL, Matheson M, Hill DJ, Allen KJ, HealthNuts Study Investigators. Which infants with eczema are at risk of food allergy? Results from a population-based cohort. Clin. Exp. Allergy 2015;45:255-264.
120. Blazowski L, Majak P, Kurzawa R, Kuna P, Jerzynska J. Food Allergy Endotype With High Risk of Severe Anaphylaxis in children-Monosensitization to Cashew 2S Albumin Ana O 3. Allergy

2019;74(10):1945-1955.
121. Arias K, Baig M, Colangelo M, Chu D, Walker T, Goncharova S, Coyle A, Vadas P, Waserman S, Jordana M. Concurrent blockade of platelet-activating factor and histamine prevents life-threatening peanut-induced anaphylactic reactions. J. Allergy Clin. Immunol 2009;124:307-314.
122. Vadas P, Perelman B, Liss G. Platelet-activating factor, histamine, and tryptase levels in human anaphylaxis. J. Allergy Clin. Immunol 2013;131:144-149.
123. Tordesillas L, A.H. Rahman AH, Hartmann BM, Sampson HA, Berin MC. Mass cytometry profiling the response of basophils and the complete peripheral blood compartment to peanut. J. Allergy Clin. Immunol 2016;138:1741-1744.e9
124. Wang J, Lin J, Bardina L, et al. Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. J Allergy Clin Immunol 2010;125(3):695-702.e6.
125. Fong AT, Du Toit G, Versteeg SA, van Ree R. Pink Peppercorn: a cross-reactive risk for cashew- and pistachio-allergic patients. J Allergy Clin Immunol Pract 2018;S2213-2198(18)30837-7.
126. Dendena B, Corsi S. Cashew, from seed to market: a review. Agron Sustain Dev 2014;34:753–772.
127. Comstock SS, Robotham JM, Tawde P, Kshirsagar H, Sathe SK, Roux KH, Teuber SS. Immunoglobulin E-reactive proteins in cashew (Anacardium occidentale) apple juice concentrate. J Agric Food Chem 2008; 56(14):5977-82.
128. Parvataneni S, Gonipeta B, Tempelman RJ, Gangur V. Development of an adjuvant-free cashew nut allergy mouse model. Int Arch Allergy Immunol 2009;149(4):299-304.
129. Islam SA, Luster AD. T cell homing to epithelial barriers in allergic disease. Nat Med 2012;18(5):705-15.
130. Blom LH, Juel-Berg N, Larsen LF, Hansen KS, Poulsen LK. Circulating allergen-specific TH2 lymphocytes: CCR4+ rather than CLA+ is the predominant phenotype in peanut-allergic subjects. J Allergy Clin Immunol 2018;141(4):1498-1501.e5.
131. Blom LH, Martel BC, Larsen LF, Hansen CV, Christensen MP, Juel-Berg N, Litman T, Poulsen LK. The immunoglobulin superfamily member CD200R identifies cells involved in type 2 immune responses. Allergy 2017;72(7):1081-1090.
132. DeLong JH, Simpson KH, Wambre E, James EA, Robinson D, Kwok WW. Ara h 1-reactive T cells in individuals with peanut allergy. J Allergy Clin Immunol 2011;127(5):1211-8.e3.
133. Chan SM, Turcanu V, Stephens AC, Fox AT, Grieve AP, Lack G. Cutaneous lymphocyte antigen and α4β7 T-lymphocyte responses are associated with peanut allergy and tolerance in children. Allergy 2012;67(3):336-42.
134. Smeekens JM, Immormino RM, Balogh PA, Randell SH, Kulis MD, Moran TP. Indoor dust acts as an adjuvant to promote sensitization to peanut through the airway. Clin Exp Allergy 2019;49(11):1500-1511.
135. Price D, Ackland L, Suphioglu C. Nuts 'n' guts: transport of food allergens across the intestinal epithelium. Asia Pacific Allergy 2013;3(4):257-265.
136. Wang C-Y, Liu S, Xie X-N, Tan Z-R. Regulation profile of the intestinal peptide transporter 1 (PepT1). Drug Des Devel Ther 2017;11:3511–3517.
137. Angelis ID, Turco L. Caco-2 cells as a model for intestinal absorption. Curr Protoc Toxicol 2011;Chapter 20:Unit20.6.
138. Ayehunie S, Landry T, Stevens Z, Armento A, Hayden P, Klausner M. Human Primary Cell-Based Organotypic Microtissues for Modeling Small Intestinal Drug Absorption. Pharm Res 2018;35(4):72.
139. Tordesillas L, Goswami R, Benedé S, Grishina G, Dunkin D, Järvinen KM, Maleki SJ, Sampson HA, Berin MC. Skin Exposure Promotes a Th2-dependent Sensitization to Peanut Allergens. J Clin Invest 2014;124(11):4965-75.
140. Hoppenbrouwers T, Fogliano V, Garssen J, Pellegrini N, Willemsen LEM, Wichers HJ. Specific Polyunsaturated Fatty Acids Can Modulate in vitro Human moDC2s and Subsequent Th2 Cytokine Release. Front Immunol 2020;11:748.

141. Humeniuk P, Geiselhart S, Battin C, Webb T, Steinberger P, Paster W, Hoffmann-Sommergruber K. Generation of a Jurkat-based fluorescent reporter cell line to evaluate lipid antigen interaction with the human iNKT cell receptor. Sci Rep 2019;9:16382.
142. Flokstra-de Blok BM, Dubois AE, Vlieg-Boerstra BJ, Oude Elberink JN, Raat H, DunnGalvin A, Hourihane JO, Duiverman EJ. Health-related quality of life of food allergic patients: comparison with the general population and other diseases. Allergy 2010;65(2):238-44.
143. Bousquet J, Lockey R, Malling HJ, Alvarez-Cuesta E, Canonica GW, Chapman MD, Creticos PJ, Dayer JM, Durham SR, Demoly P, Goldstein RJ, Ishikawa T, Ito K, Kraft D, Lambert PH, Løwenstein H, Müller U, Norman PS, Reisman RE, Valenta R, Valovirta E, Yssel H. Allergen immunotherapy: therapeutic vaccines for allergic diseases. World Health Organization. American academy of Allergy, Asthma and Immunology. Ann Allergy Asthma Immunol 1998;81(5):401-5.
144. Jensen-Jarolim E, Bachmann MF, Bonini S, Jacobsen L, Jutel M, Klimek L, Mahler V, Mösges R, Moingeon P, O Hehir RE, Palomares O, Pfaar O, Renz H, Rhyner C, Roth-Walter F, Rudenko M, Savolainen J, Schmidt-Weber CB, Traidl-Hoffmann C, Kündig T. State-of-the-art in marketed adjuvants and formulations in Allergen Immunotherapy: A position paper of the European Academy of Allergy and Clinical Immunology (EAACI). Allergy 2020;75(4):746-760.
145. Hoffmann HJ, Valovirta E, Pfaar O, Moingeon P, Schmid JM, Skaarup SH, Cardell LO, Simonsen K, Larché M, Durham SR, Sørensen P. Novel approaches and perspectives in allergen immunotherapy. Allergy 2017;72(7):1022-1034.
146. Fauquert JL, Michaud E, Pereira B, Bernard L, Gourdon-Dubois N, Rouzaire PO, Rochette E, Merlin E, Evrard B; PITA Group. Peanut gastrointestinal delivery oral immunotherapy in adolescents: Results of the build-up phase of a randomized, double-blind, placebo-controlled trial (PITA study). Clin Exp Allergy 2018;48(7):862-874.
147. Kulis M, Li Y, Lane H, Pons L, Burks W. Single-tree nut immunotherapy attenuates allergic reactions in mice with hypersensitivity to multiple tree nuts. J Allergy Clin Immunol 2011;127(1):81-8.
148. Kulis M, Macqueen I, Li Y, Guo R, Zhong XP, Burks AW. Pepsinized cashew proteins are hypoallergenic and immunogenic and provide effective immunotherapy in mice with cashew allergy. J Allergy Clin Immunol 2012;130(3):716-23.
149. Parrish CP, Har D, Andrew Bird J. Current Status of Potential Therapies for IgE-Mediated Food Allergy. Curr Allergy Asthma Rep 2018;18(3):18.
150. Eiwegger T, Anagnostou K, Arasi S, et al. Conflicting verdicts on peanut oral immunotherapy from the Institute for Clinical and Economic Review and US Food and Drug Administration Advisory Committee: Where do we go from here? J Allergy Clin Immunol 2020;145(4):1153-1156. 1.
151. Katzka DA. Eosinophilic Esophagitis. Ann Intern Med 2020;172(9):ITC65-ITC80.
152. Wai CYY, Leung NYH, Leung PSC, Chu KH. Immunotherapy of Food Allergy: a Comprehensive Review. Clin Rev Allergy Immunol 2019;57(1):55-73.
153. Valenta R, Campana R, Focke-Tejkl M, Niederberger V. Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: Lessons from the past and novel mechanisms of action for the future. J Allergy Clin Immunol 2016;137(2):351-7.
154. van der Kleij HPM, Warmenhoven HJM, van Ree R, Versteeg SA, Pieters RHH, Dreskin SC, Knulst AC, van Hoffen E, Opstelten DJE, Koppelman SJ, Smit JJ. Chemically modified peanut extract shows increased safety while maintaining immunogenicity. Allergy 2019;74(5):986-995.
155. Pelgrim I, Savelkoul HFJ. "Chapter 17: Potency of T-Cell Epitope-Based Peptide Vaccines in Food Allergy Treatment". In: Mahmoudi M., Rezaei N. (eds) Nutrition and Immunity. Springer, Cham 2019:360-385.
156. Breiteneder H. Mapping of conformational IgE epitopes of food allergens. Allergy 2018;73(11):2107-2109.
157. Chen X, Negi SS, Liao S, Gao V, Braun W, Dreskin SC. Conformational IgE epitopes of peanut allergens Ara h 2 and Ara h 6. Clin Exp Allergy 2016;46(8):1120-1128.
158. Xia L, Willison LN, Porter L, Robotham JM, Teuber SS, Sathe SK, Roux KH. Mapping of a

conformational epitope on the cashew allergen Ana o 2: a discontinuous large subunit epitope dependent upon homologous or heterologous small subunit association. Mol Immunol 2010;47(9):1808-16.
159. Archila LD, Chow IT, McGinty JW, Renand A, Jeong D, Robinson D, Farrington ML, Kwok WW. Ana o 1 and Ana o 2 cashew allergens share cross-reactive CD4(+) T cell epitopes with other tree nuts. Clin Exp Allergy 2016;46(6):871-83.
160. Jeong KY, Hong CS, Lee JS, Park JW. Optimization of allergen standardization. Yonsei Med J. 2011;52(3):393-400.

Appendix

Summary

Summary

In this thesis entitled *'Cracking the cashew nut: strategies to identify and characterize novel allergens'*, we aimed to apply innovative strategies and technologies to identify and characterize putative allergenic proteins in cashew nut, to broaden the current knowledge on cashew nut allergens beyond those already known (Ana o 1, Ana o 2 and Ana o 3). Our knowledge of cashew nut proteins that can trigger an allergic reaction is currently very limited, especially compared to other nuts or seeds in which the allergen repertoire has been researched much more widely. Using several different strategies, we evidenced that additional allergens must be present in cashew nuts, which presumably contribute to the elicitation of allergic symptoms in cashew nut allergic patients. Knowledge of newly identified cashew nut proteins provides a basis for further research to extend clinical diagnostic tests and treatments currently available for cashew nut allergy.

Chapter 2 includes an opinion on the use of current *in vivo* and *ex vivo* endpoints in murine food allergy models and their suitability for evaluating the sensitizing capacity of protein concentrates and/or food products. An overview is given of the best predictive risk assessment methods and endpoint parameters currently relied on in *in vivo* food allergy models with a focus on milk, egg and peanut allergens, addressing their strengths and limitations for assessing sensitization risks. Findings indicated that, although the current available models are suitable for studying the pathophysiology of food allergy, they still couldn't predict the magnitude of the allergic potential of a particular allergen. Thus, there is still a strong need to better define the allergic reaction to predict the clinical outcomes of sensitization to novel food proteins. In addition, there is an urgent need for a consensus on key food allergy parameters to be applied in future food allergy research, to guarantee optimal lab-to-lab reproducibility and reliable use of predictive tests for protein risk assessment.

Cashew nut allergic individuals may develop cross-reactive responses to foods that are phylogenetically related to cashew nut. In **Chapter 3**, we therefore aimed to determine the IgE cross-sensitisation and cross-reactivity profiles in cashew nut sensitised subjects. Profiling was specifically aimed at botanically related proteins of common tree nut species and other Anacardiaceae family members like pistachio, mango, pink peppercorn or sumac. Half of cashew nut positive sera on dot blot were co- sensitised; 19% to solely Anacardiaceae species and 31% to tree nuts, which indicated that cross-sensitisation/cross-reactivity is widespread among cashew nut allergic individuals. Interestingly, subjects co-sensitised to Anacardiaceae species displayed a different allergen recognition pattern than subjects sensitised to common tree nuts. Putative underlying novel allergens were identified in cashew nut, pistachio and pink peppercorn, which demonstrated that indeed additional

allergens might exist in cashew nut that may pose factors underlying cashew nut allergic symptoms.

In line with these findings, we applied a novel IMMULITE®-based inhibition methodology in **Chapter 4**, to investigate the IgE cross-reactivity between cashew nut-, hazelnut- and peanut proteins in children that are multi-allergic to these foods. Observations indicated that hazelnut extract was a strong inhibitor of cashew nut sIgE while cashew nut extract was less able to inhibit hazelnut extract. In contrast, peanut extract showed the least inhibition potency. Importantly, there were 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, suggesting the existence of putative Bet v 1-like protein homologs in cashew nut.

Based on the strong indications that additional allergenic proteins may exist in cashew nut, cashew nut transcript profiling was conducted resulting in a RNA-seq database that can be used to screen for protein homologs of allergens identified in phylogenic related species. In **Chapter 5**, we applied this method to identify and characterize three PR10 proteins in cashew nut. The identification and partial characterization of two additional 2S albumin proteins, next to the major cashew nut 2S albumin Ana o 3.0101, are described in **Chapter 6**.

Finally, **Chapter 7** discusses the major findings of the different research chapters and pros and cons of the applied strategies. Additional putative cashew nut allergens are presented, identified using the RNAseq screening approach mentioned in chapter 5 and 6 which, although not yet characterized, likely contribute to the allergen repertoire of cashew nut. To conclude, future research opportunities are presented that could take our current knowledge of cashew nut allergy to a higher level.

Appendix

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However, I would like to address some personal words to thank my supervisors: Harry and Jurriaan, without your help, confidence and inexhaustible enthusiasm, I would never have started this PhD nor would I have completed it. Nicolette, thank you so much for teaching me the clinical aspects associated to food allergens and our many discussions about the various cashew nut allergic symptoms! Huub, you stimulated my critical thinking and without the help of the many CBI students, I would certainly not have been able to finish in 10 years' time! Thank you all for your efforts and guidance!

Most of all, I would like to thank my loving partner Bas for all his help, advice and patience, as well as my parents Emmy and Adriaan, for stimulating me to always push my boundaries and to never stop learning!

Thank you all!!

Appendix

About the author

About the author

Shanna Bastiaan-Net was born on October 31th 1978 in Kerkrade, The Netherlands. After completing middle school education at the Antonius Doctor College in Kerkrade, she started the study Plant Breeding and Crop Protection at Wageningen University in 1997. She graduated in 2001, in Molecular Crop Protection with a specialization in Plant Virology.

In 2002, she started as a research technician at Utrecht University, department Molecular Plant Physiology, where she worked for four years on how methylation influenced promoter-transcription factor binding using the model plant *Arabidopsis thaliana*.

In 2006, she started as a research scientist at Wageningen Food & Biobased Research (WFBR) in the department Postharvest Quality & Technology, where she developed diagnostic quality predicting tests for various fruits, vegetables and flowers to screen for storage disorders, disease infection, allergen content or customer quality. Later, she moved to the department Food, Health & Consumer Research, and specialized her research expertise towards food health and safety, developing *in vitro* assays to study nutrient perception (receptor-ligand interactions), gastrointestinal digestion and bioavailability (adsorption by intestinal epithelial cell models). In addition, she studies the bioactivity of food components, especially proteins, for their immunomodulating effects, as well as their safety aspects (i.e. allergenicity & toxicity). Next to performing research, she is project leader for small and large collaborations, performs acquisition, participates in European Research and Technology Networks (COST Actions "Imparas", "Infogest" and "Ungap") and is the contact person for the "Allergy Consortium Wageningen" (https://www.wur.nl/nl/Onderzoek-Resultaten/Projecten/Allergieconsortium .htm) and the research direction "Food digestion & Gut Health" within WFBR.

In 2010, Shanna started a PhD in addition to her job as research scientist, under supervision of Dr. Jurriaan Mes (WFBR), Dr. Nicolette de Jong (Erasmus MC, Internal Medicine, section Allergy & Clinical Immunology), Prof. Harry Wichers (WU Food Chemistry/WFBR) and Prof. Huub Savelkoul (WU Cell Biology & Immunology). Her study focused on the identification and characterization of putative allergenic proteins in cashew nut, and how they may contribute to the elicitation of allergic symptoms in cashew nut allergic patients, of which the results are written down in this thesis.

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List of publications

Jochems PGM, Keusters WR, America AHP, Rietveld PCS, **Bastiaan-Net S**, Ariëns RMC, Tomassen MMM, Lewis F, Li Y, Westphal KGC, Garssen J, Wichers HJ, van Bergenhenegouwen J, Masereeuw R. A combined microphysiological-computational omics approach in dietary protein evaluation. bioRxiv 2020.07.03.184689; preprint.

Liu Y, **Bastiaan-Net S**, Harry Wichers HJ. Current understanding of the structure and function of fungal immunomodulatory proteins. Front Nutr. - Food Chem. 2020; 7:132.

Bastiaan-Net S, Pina-Pérez MC, Dekkers BJW, Westphal AH, America AHP, Ariëns RMC, de Jong NW, Wichers HJ, Mes JJ. Identification and *in silico* bioinformatics analysis of PR10 proteins in cashew nut. Protein Sci. 2020;29(7):1581-1595.

Bastiaan-Net S, Batstra MR, Aazamy N, Savelkoul HFJ, van der Valk JPM, Gerth van Wijk R, Schreurs MWJ, Wichers HJ, de Jong NW. IgE cross-reactivity measurement of cashew nut, hazelnut and peanut using a novel IMMULITE inhibition method. Clin Chem Lab Med. 2020;Online ahead of print.

Castan L, Bøgh KL, Maryniak NZ, Epstein MM, Kazemi S, O'Mahony L, Bodinier M, Smit JJ, van Bilsen JHM, Blanchard C, Głogowski R, Kozáková H, Schwarzer M, Noti M, de Wit N, Bouchaud G, **Bastiaan-Net S**. Overview of in vivo and ex vivo endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins? Allergy. 2020;75(2):289-301.

Deng Y, Govers C, **Bastiaan-Net S**, van der Hulst N, Hettinga K, Wichers HJ. Hydrophobicity and aggregation, but not glycation, are key determinants for uptake of thermally processed β-lactoglobulin by THP-1 macrophages. Food Res Int. 2019;120:102-113.

Bastiaan-Net S, Reitsma M, Cordewener JHG, van der Valk JPM, America TAHP, Dubois AEJ, Gerth van Wijk R, Savelkoul HFJ, de Jong NW, Wichers HJ. IgE Cross-Reactivity of Cashew Nut Allergens. Int Arch Allergy Immunol. 2019;178(1):19-32.

Reitsma M, **Bastiaan-Net S**, Sijbrandij L, de Weert E, Sforza S, Gerth van Wijk R, Savelkoul HFJ, de Jong NW, Wichers HJ. Origin and Processing Methods Slightly Affect Allergenic Characteristics of Cashew Nuts (*Anacardium occidentale*). J Food Sci. 2018;83(4):1153-1164.

Bastiaan-Net S, van den Berg-Somhorst DBPM, Ariëns RMC, Paques M, Mes JJ. A novel functional screening assay to monitor sweet taste receptor activation *in vitro*. Flavour and Fragr J. 2018;33(2):173-183.

Bøgh KL, van Bilsen J, Głogowski R, López-Expósito I, Bouchaud G, Blanchard C, Bodinier M, Smit J, Pieters R, **Bastiaan-Net S**, de Wit N, Untersmayr E, Adel-Patient K, Knippels L, Epstein MM, Noti M, Nygaard UC, Kimber I, Verhoeckx K, O'Mahony L. Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. Clin Transl Allergy. 2016;6:21.

Reitsma M, **Bastiaan-Net S**, Sforza S, van der Valk JP, van Gerth van Wijk R, Savelkoul HF, de Jong NW, Wichers HJ. Purification and Characterization of *Anacardium occidentale* (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3. J Agric Food Chem. 2016 Feb 10;64(5):1191-201.

Bastiaan-Net S, van Broekhoven S, de Jong NW, Wichers HJ. Influence of processing and *in vitro* digestion on the allergic cross-reactivity of three mealworm species. Food Chem. 2016;196:1075-83.

Bastiaan-Net S, Chanput W, Hertz A, Zwittink RD, Mes JJ, Wichers HJ. Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (rFIPs). Int Immunopharmacol. 2013;15(1):167-75.

Weijn A, **Bastiaan-Net S**, Wichers HJ, Mes JJ. Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. Fungal Genet Biol. 2013;55:42-53.

van Meeteren U, Schouten R, Harkema H, **Bastiaan-Net S**, Woltering E. Predicting rose vase life in a supply chain. Acta Hortic. 2013;970:141-146.

Weijn A, Tomassen MMM, **Bastiaan-Net S**, Wigham MLI, Boer EPJ, Hendrix EAHJ, Baars JJP, Sonnenberg ASM, Wichers HJ, Mes JJ, A new method to apply and quantify bruising sensitivity of button mushrooms. LWT - Food Science and Technol. 2012;47: 308e314.

Vreeburg RA, **Bastiaan-Net S**, Mes JJ. Normalization genes for quantitative RT-PCR in differentiated Caco-2 cells used for food exposure studies. Food Funct. 2011;2(2):124-9.

Sulistyowati E, Mitter N, **Bastiaan-Net S**, Roossinck MJ, Ralf G. Dietzgen RG, Host range, symptom expression and RNA 3 sequence analyses of six Australian strains of Cucumber mosaic virus. Australas Plant Path, 2004;33:505–512.

Overview of completed training activities

Discipline specific courses	Year
International Mast cell and Basophil Meeting 1th (EMBRN-COST)	2012
International Mast cell and Basophil Meeting 3th (EMBRN-COST)	2014
Workshop The place of "omics" in the diagnostic lab (EAACI)[a]	2013
EuroBat meeting (EuroBat)	2014
International Symposium on Molecular Allergology 4th (EAACI-ISMA)	2010
International Symposium on Molecular Allergology 5th (EAACI-ISMA)	2013
International Symposium on Molecular Allergology 6th (EAACI-ISMA)[a]	2015
International Symposium on Molecular Allergology 8th (EAACI-ISMA)[a]	2019
International Conference on Food Digestion (INFOGEST)[a,b]	2014
Basic Flowcytometry Course (BD Biosciences)	2015
Workshop Allergen Immunotherapie (EAACI)	2016
EAACI-Online conference (EAACI)	2020

General courses and activities	Year
Scientific writing (WUR)	2011
"Mobilising your network in 2.5 hours" (Young AFSG)	2014
Commerciele Vaardigheden (Kenneth Smit training – WFBR)	2015
Writing a Grant Proposal (WUR)	2015
Getting things done (WFBR)	2016
Profile Dynamics (Hermonde – WFBR)	2016
Research Integrity & Ethics and Animal Science (WGS)	2017
Seminar Publish for Impact (WUR Library)	2017

Optional courses and activities	Year
Preparation of Research Proposal	2019
Themamiddag Allergenen - Wat kan wèl? (WA)[c]	2013
Themamiddag Allergenen - Wat kan wèl? (WA)[c]	2014
iFAAM consortium meetings[c]	2016-2017
COST-IMPARAS meetings[c]	2015-2018
Group WFBR/CBI expertise meetings[c]	2012-2020

Teaching obligations, students	Year
Master students, 6 months each, 4 students	2013, 2016, 2017, 2019
Bachelor students, 4 months each, 3 students	2015, 2016, 2018

Explanation of abbreviations
AFSG: Agrotechnology & Food Science Group
CBI: chair group Cell Biology & Immunology
COST: European Cooperation in Science and Technology organization
EAACI: European Academy of Allergy and Clinical Immunology organization
EMBRN: European Mast Cell and Basophil Research Network organization
EuroBat: The European Consortium on Application of Flow Cytometry in Allergy
iFAAM: European Union's Seventh Framework Program project on 'Integrated approaches to food allergen and allergy management'
IMPARAS: Cost Action network on 'Improving Allergy Risk Assessment Strategy for New Food Proteins'
INFOGEST: COST Action network on 'Improving health properties of food by sharing our knowledge on the digestive process'
ISMA: International Symposium on Molecular Allergology network
WA: Wageningen Academy
WFBR: Institute Food & Biobased Research
WGS: Wageningen Graduate Schools
WUR: Wageningen University & Research

[a] Poster presentation
[b] Organizing committee
[c] Oral presentation

Colophon

The work in this thesis was performed at Wageningen UR (WFBR). The research described in this thesis was supported by Technology Foundation STW (Grant number 11868), and (partly) financed by the Netherlands Organisation for Health Research and Development (ZonMw), via the program Enabling Technologies Hotels (Grand numbers 435000011 and 435003012). Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

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