IMPROVEMENT OF *IN VITRO* FOOD ALLERGY DIAGNOSTICS BY IDENTIFYING UNIQUE ANTIBODY TRAITS

Anna Ehlers

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Improvement of *in vitro* food allergy diagnostics by identifying unique antibody traits
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Improvement of *in vitro* food allergy diagnostics by identifying unique antibody traits

Verbetering van *in vitro* diagnostiek naar voedselallergie door het identificeren van unieke antilichaam kenmerken
(met een samenvatting in het Nederlands)

Optimierung der *in vitro* Nahrungsmittelallergie-Diagnostik durch die Identifizierung von einzigartigen Antikörper-Merkmalen
(mit einer Zusammenfassung in deutscher Sprache)

Proefschrift

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Anna Maria Ehlers

geboren op 11 december 1990
te Itzehoe, Duitsland

Promotor

Prof. dr. A.C. Knulst

Copromotor

Dr. H.G. Otten

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General introduction

Food allergy

Food allergy is generally defined as an IgE-mediated immunological disorder leading to reactions against normally harmless proteins. Although allergenic proteins can be present in virtually any food, the major foods responsible for most food allergic reactions are peanut, tree nuts, cow's milk, hen's egg and (shell)fish[1]. The major causative food, however, varies regarding geographical location and age[2,3]. While cow's milk and hen's egg are responsible for most food allergic reactions in children, peanut and tree nut allergies are more common in adults[4]. Regardless of the causative food, subjects can experience symptoms ranging from mild oral itching to even, in rare cases, lethal anaphylaxis. Despite the risk of unexpected severe allergic reactions, allergen immunotherapy is still largely under investigation. The only treatment options are elimination diets and prescription of epinephrine auto-injectors[5,6]. To prevent unnecessary food restrictions, highly accurate diagnostic strategies, preferably minimally invasive, are required.

Food allergy diagnostics

In daily practice, food allergy diagnostics is comprised of careful evaluation of patient's medical history, complementary *in vitro* (measuring specific IgE (sIgE)) and/or *in vivo* (skin prick test) measurements, and double-blind placebo-controlled food challenges (DBPCFC) as the gold standard. DBPCFCs are characterised by gradual administration of increasing food doses to patients with suspected food allergy in comparison to placebo administration[7]. Those DBPCFCs, however, are burdensome for patients, costly and require dedicated hospital facilities accompanied by trained personnel. Hence, it is very desirable to reduce the number of required food challenges in the future. Since sIgE measurements are partly hampered by a lack of sensitivity (non-detectable clinically relevant sensitisation $\rightarrow$ „false-negative“) or specificity (detection of clinically irrelevant sensitisation $\rightarrow$ „false-positive“), it is currently not advised to replace food challenges by sIgE measurements in cases of uncertainty about the severity and/or the causative culprit food[8–10]. These obstacles indicate the strong need to develop improved platforms for sIgE measurements or novel minimally invasive *in vitro* diagnostic strategies.

Specific IgE measurements in food allergy diagnostics

Most commonly, sIgE levels are measured against a crude extract of the suspected food. Such extracts consist of allergenic and non-allergenic proteins solubilised in an appropriate buffer, which could lead to the potential lack of the responsible allergen in such extracts[11]. Moreover, clinically irrelevant sensitisation might be measured due to the presence of less relevant allergens next to more relevant ones. A promising approach to overcome these limitations was the introduction of component-resolved diagnostics (CRD). CRD is characterised by sIgE measurement against a single full-length allergenic component instead of measuring sIgE against whole food extracts[12]. In peanut allergy, the replacement of crude peanut extract by the major peanut allergen Ara h 2, belonging to the 2S albumin family, increased the specificity of sIgE measurements from 59% to approximately 84% using $\geq$ 0.35 kU/l as a cut-off level[13–17]. Moreover, CRD allows not only complexity reduction but also enables sIgE measurements against allergens absent in commercially available food extracts such as hydrophobic proteins. Hydrophobic proteins such as the oil-body associated oleosins have been described for peanut as a potential marker for (severe) peanut allergy[18]. Such proteins have also been described as potential allergens in hazelnut and sesame seed[19,20]. Despite such improvements by CRD, sIgE measurements are still partly hampered by false-negative and false-positive test outcomes that contradict clinical diagnosis[21].
We hypothesise that

- false-negative test outcomes might be prevented by identifying the missing single allergenic component for CRD
- false-positive test outcomes might be prevented by exploring differences in specific antibody repertoires between allergic and sensitised but tolerant patients regarding epitope (antibody's binding sites) recognition pattern, affinity or blocking ability of non-IgE antibodies to prevent sIgE binding.

Antibody repertoire in allergy

Generally, antibodies are comprised of two heavy and two corresponding light chains, which form the characteristic Y-shape and they are stabilised by disulphide bridges. The heavy and the light chain are divided into a variable and a constant region[22]. While the constant region defines the isotype (e.g. IgE) and therefore the effector function, the variable region is responsible for antigen binding[23] (Figure 1a). The variable region of the heavy chain is formed by recombination of variable (V), diversity (D) and joining (J) gene segments during maturation of naïve B cells (Figure 1b) The variable region of the light chain, on the other hand, is formed by the same mechanism but lacks the D gene segment[24]. Recombination of those genes results in variable regions characterised by four frame work regions (FR) and three complementarity-determining regions (CDR). Correct folding of the antibody allows the formation of an antigen-binding site (paratope) consisting of all three CDRs of which CDR3 is largely determining antibody's specificity[25]. V(D)J gene recombination forms the basis for the high diversity of antibodies binding virtually any antigen. This diversity is even enlarged by somatic hypermutations preferably introduced within CDRs during B cell maturation in the germinal centre accompanied by antigen presentation[26]. Potential differences in HCDR3 sequences or VH gene usage by allergic versus sensitised but tolerant individuals can be defined by analysing the repertoire of V(D)J sequences encoding allergen specific antibodies.

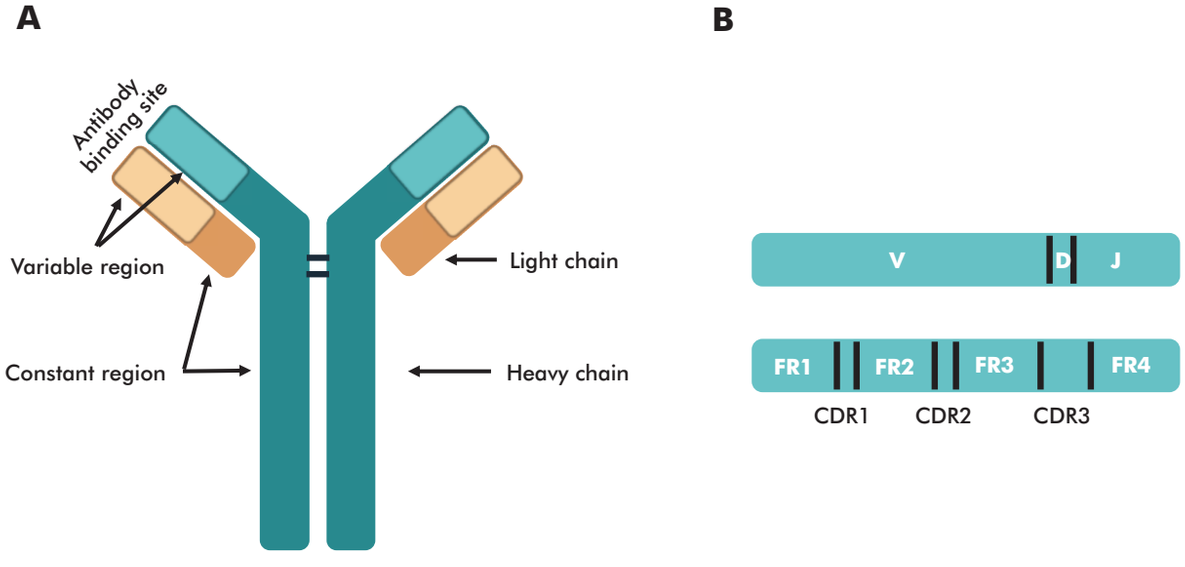

Figure 1: General antibody structure
A *Antibodies are comprised of two heavy and light chain consisting of a constant and a variable part;* ***B*** *variable regions are recombined by V, D and J gene segments resulting in four frame work regions (FR) and 3 complementarity-determining regions*

Thesis outline

The overall aimed of the present thesis is to define new and accurate testing strategies to prevent future discrepancies between *in vitro* and clinical diagnoses. All approaches used within this thesis are summarised in Figure 2.
Part 1 focusses on the prevention of „false-negative“ test outcomes resulting from non-detectable clinically relevant sensitisation in food allergic patients. We aimed to overcome this limitation by

- the identification of novel allergenic components from macadamia nut for stratifying macadamia nut allergic patients into patients experiencing rather mild versus severe symptoms (**chapter 2**)
- examining the diagnostic value of sesame oleosins in order to diagnose sesame allergic patients without detectable specific sIgE using conventional sIgE testing (**chapter 3**)

Part 2 focusses on the prevention of „false-positive“ test outcomes resulting from detectable clinically irrelevant sensitisation in sensitised but tolerant subjects. We aimed to overcome this limitation by defining differences in allergen specific antibody repertoires in allergic and sensitised but tolerant patients. To gain insights on these antibody repertoires, we performed

- linear epitope mapping:
  - of the hen’s egg components Gal d 1 and 3 in order to evaluate the potential of sIgE binding to these epitopes in discriminating between hen’s egg allergic and tolerant adults both sensitised to the full-length proteins (**chapter 4**)
  - of Ara h 7 isoforms, upon comparing them with Ara h 2 and 6 regarding their ability to induce degranulation, in order to elucidate which epitopes may explain observed divergent potencies in inducing degranulation (**chapter 5** and **chapter 6**).

and

- analysis of peanut 2S albumin (Ara h 2 and 6) specific B cells and their corresponding antibodies on single cell level:
  - In **chapter 7**, we evaluated the advantages of specific monoclonal antibodies (mAbs) compared to patient sera in order to define epitopes associated with allergy or tolerance and how to implement this knowledge into state-of-the-art CRD.

– In **chapter 8**, we compared two different methods to generate human mAbs from peripheral blood including single cell sequencing combined with heterologous expression and B cell immortalisation by Epstein-Barr Virus followed by limiting dilution.

– In **chapter 9**, we evaluated peanut 2S albumin-binding B cells from peanut allergic and peanut sensitised but tolerant subjects regarding their genetic features as VH family gene usage and HCDR3 sequence motifs. These insights may provide the basis for the development of novel *in vitro* diagnostic strategies.

Chapter 10 provides an overall discussion of the main findings in the context of the current literature.

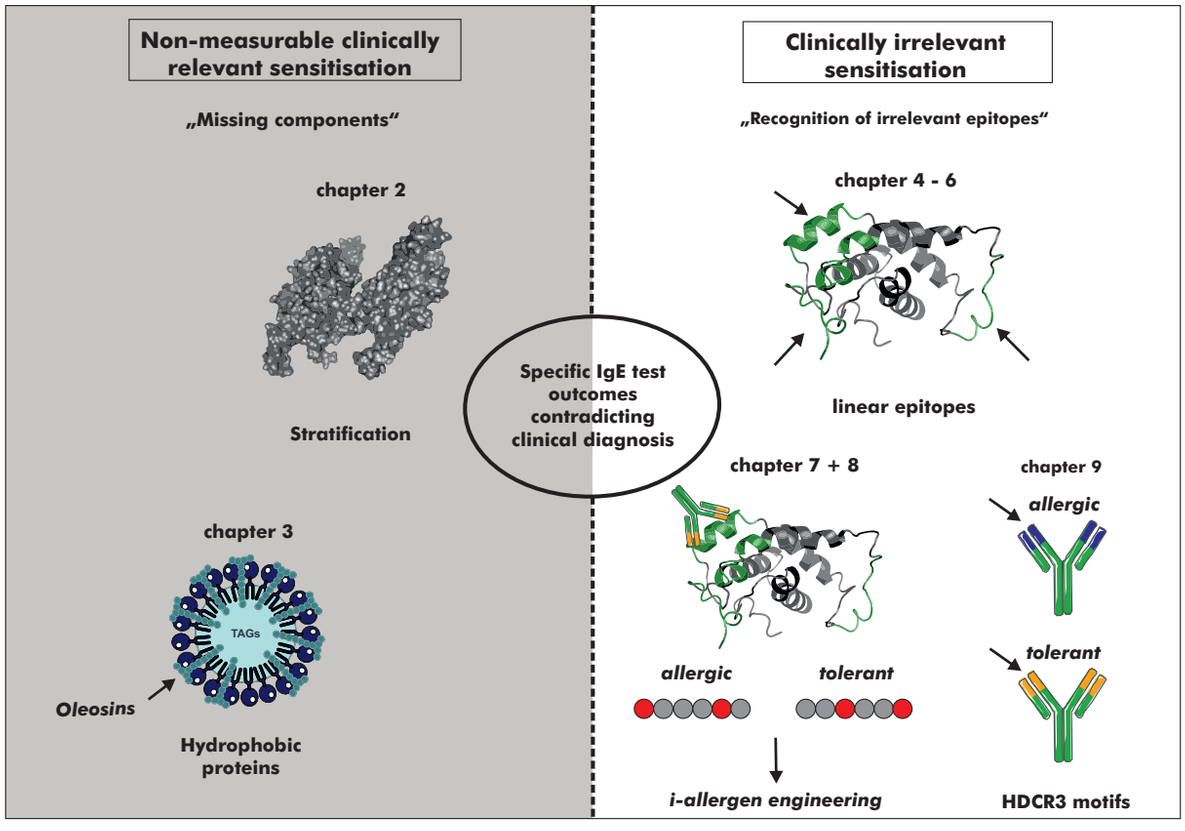

Figure 2: Overview on approaches to overcome „false-negative“ and „false-positive“ test outcomes contradicting clinical diagnosis

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Part I

Preventing „false-negative“ sIgE testing: non-detectable clinically relevant sensitisation


2

IgE-binding to vicilin-like antimicrobial peptides is associated with systemic reactions to macadamia nut

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Anna M. Ehlers[1,2], Stefanie Rohwer[3], Henny G. Otten[1], Bettina Brix[3], Thuy-My Le[1,2], Waltraud Suer[3], André C. Knulst[1,2]

[1]Center for Translational Immunology, University Medical Center Utrecht, Utrecht University, Netherlands
[2]Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Netherlands
[3]Euroimmun AG, Lübeck, Germany

Abstract

Background

Macadamia nut can induce fatal allergic reactions and changes in dietary habits will raise their consumption in industrialised countries. So far, diagnosis of macadamia nut allergy by sIgE solely relies on the macadamia nut extract, but single components are lacking.

Methods

Macadamia nut proteins recognised by IgE from 2 macadamia nut extract positive sera were identified by mass spectrometry (vicilin-like antimicrobial peptides: VLAP). Sensitisation to macadamia nut extract and heterologously expressed isoform VLAP-2-3 was evaluated in 82 nut allergic (NA) and 27 nut tolerant (NT) patients (no tree nut allergy reported) comprehending 10 macadamia nut allergic (MA) and 18 explicitly reported macadamia nut tolerant patients (MT), using line blots. Co-sensitisation to additional VLAP isoforms and other vicilins was evaluated in 8 MA, 12 MT and 14 NA patients sensitised to VLAP-2-3. Functional properties were determined by indirect basophil activation.

Results

Even though proteins recognised by IgE were identified as VLAP-2-1, 2-2 and 2-3, only peptides specifically belonging to VLAP-2-3 were detected by mass spectrometry. The macadamia nut extract was recognised by 33% of NA patients (27/82) including 3 MA patients and 26% of NT patients (7/27, 3 MT). Similarly, 29% of NA (24/82) patients showed partly strong sIgE-binding to VLAP-2-3 including 3 MA patients with systemic reactions to macadamia nut. Contrary, VLAP-2-3 was recognised by only 2 NT (1 MT) patients (7%) with very low sIgE titres. Simultaneous recognition of the isoforms VLAP-2-1 and 2-2 was observed in all patients positive for VLAP-2-3 with partly reduced sIgE titres in 59% of these patients. Additionally, all three VLAP isoforms were able to repeatedly induce basophil activation upon sensitisation with one MA serum.

Conclusion

VLAP proteins are the first described macadamia nut components with serological and functional allergenic properties and they are associated with systemic reactions to macadamia nut.

Introduction

Tree nut allergies are classified as allergy against edible fruits or seeds growing on woody plants including almonds, Brazil nut, cashew nut, hazelnut, macadamia nut, pecan, pine nuts, pistachio nuts and walnuts. Probable tree nut allergy, defined as self-reported allergy accompanied by doctor's diagnosis and/or sensitisation to the specific tree nut, was evaluated in a systematic review to range from 0.05 to 4.9% in children from different regions and was mostly caused by almonds (UK), cashew nut (US), hazelnut (Europe) or walnut (UK, US)[1]. The relative contribution of macadamia nut allergy ranged from 0.8 to 9% in clinical studies[2,3]. In the Western World, a raise in the number of macadamia nut allergies is expected due to steadily growing demand on healthy food and their increasing use in pastry and confectionery[4].

In several case reports, ingestion of macadamia nut was able to trigger fatal allergic reactions including anaphylaxis[5–8]. Although part of macadamia allergic (MA) patients showed false-negative sIgE against macadamia nut extract[9–11], proteins with molecular masses of 12, 17, 40, 45 and 50 kDa were recognised by IgE in western blot analyses[7,8]. Successful inhibition experiments with hazelnut extract or the walnut components Jug r 1 and Jug r 4 pinpointed towards 2S albumins or 11S globulins as potential allergens in macadamia nut[6,12]. Another seed storage protein family with known allergenic properties are vicilins[13,14] and a 666 amino acid large vicilin precursor has been described for macadamia nut[15]. Post-translational processing of this precursor results in highly abundant antimicrobial peptides with a molecular mass of 8.1 kDa and a isoelectric point (pI) of 10.1[16,17]. Generally, vicilins have been described as marker for severe, non-pollen related walnut and hazelnut allergy[18–20].

Characterisation of single components from macadamia nut may improve the diagnosis and stratification of macadamia nut allergic patients in the future. Hence, we screened macadamia nut extract for proteins being recognised by IgE and characterised vicilin-like antimicrobial peptides (VLAP) 2-1, 2-2 and 2-3 as first single components in nut allergic patients including macadamia nut allergic ones. We further addressed co-sensitisation to vicilins from tree nuts, seeds and legumes and the ability of VLAP isoforms to induce degranulation.

Methods

Patient selection

For the serological characterisation of VLAP from *macadamia integrifolia*, we aimed for sera from MA patients. However, macadamia nut is not a very common cause of food allergy yet, resulting in a small group of MA patients (n=10). A small group involves the risk that minor allergens, potentially recognised in only 10 to 20% of the patients, may stay undetected[21]. Hence, our population was enlarged with 72 nut allergic (NA) patients (in total n=82), potentially exhibiting sIgE against macadamia nut allergens and theoretically resulting in at least 8 patients recognising VLAP (82 patients · 0.1 = 8 patients). Patients (n=27) without known history of a tree nut allergy served as a nut tolerant (NT) reference group. It was not required that tolerance was explicitly reported for all tree nuts, resulting in 18 patients with explicitly reported macadamia nut tolerance.

For the recruitment, we retrospectively screened patients who visited the outpatient clinic of the University Medical Center (UMC) Utrecht between 2008 and 2018 and were diagnosed with a nut allergy (almonds, Brazil nut, cashew nut, hazelnut, macadamia nut, pistachio and/or walnut) or with tolerance to tree nuts. Diagnoses were confirmed by either double-blind placebo-controlled food challenge (DBPCFC) or suggestive history by a trained physician. Suggestive history was defined as typical IgE-mediated symptoms like oral allergy syndrome (OAS), skin reactions, gastrointestinal, respiratory or cardiovascular symptoms and an onset within 2 hours upon ingestion. Severity of the reported symptoms in MA patients was graded by Sampsons' classification[22].

A subpopulation with sufficient material for further analyses was comprised of 8 MA, 12 macadamia nut tolerant (MT) and 14 NA patients sensitised to VLAP-2-3. Ethical approval (number 18-428) was acquired from the biobank committee of the UMC Utrecht and detailed patient characteristics are given in Table 1.

Table 1: *Patient characteristics*

	Nut allergic	Nut tolerant
Number [n]	82	27
Macadamia nut allergy	10	NA
Macadamia nut tolerance	3	15
Not explicitly reported	69	12
Age (median [IQR])	28 [18-62]	33 [20-55]
Sex female [n, %]	57 [70%]	20 [74%]
Food challenge[A] [n, %]	13 [16%]	13 [48%]

Macadamia nut allergic patients

Patient	Reported symptoms	Severity[A]
MA-1	swelling lips, OAS[B], vomiting	Grade 3
MA-2	itching palate, swelling throat	Grade 3
MA-3	OAS	Grade 1
MA-4	OAS	Grade 1
MA-5	OAS	Grade 1
MA-6	OAS	Grade 1
MA-7	OAS, swelling throat, hoarseness, dyspnoea	Grade 4
MA-8	OAS, vomiting, dyspnoea	Grade 3
MA-9	OAS, angio-oedema, dyspnoea	Grade 4
MA-10	OAS, dyspnoea	Grade 4

[A] Severity score based on Sampson score[22]

[B] OAS = oral allergy syndrome

Sera for the identification of proteins recognised by IgE

Proteins of the macadamia nut extract recognised by IgE were identified with 2 macadamia nut sensitised samples from AbBaltis Reagents Ltd, Dublin, Ireland and Aalto Bio Reagents Ltd, Dublin, Ireland.

Protein extraction from macadamia nut

Roasted and salted commercially purchased macadamia nut kernels were ground and subsequently threefold defatted with acetone. Defatted and dried macadamia nut debris was resuspended in phosphate buffer saline (PBS, 50 mM sodium phosphate, 150 mM NaCl, pH 7.4) and incubated at 4 °C for 3 hours in an incubation shaker, followed by filtration and centrifugation (3.300 x g at 4 °C, 30 min). The pellet, achieved by 100% ammonium sulfate precipitation, was resuspended in PBS and subsequently dialysed against PBS.

Preparative gel electrophoresis

Crude macadamia extract was precipitated with cooled acetone and incubated at -20 °C for 2 hours. For isoelectric focusing (IEF), the resulted pellet (20.000 x g at 4°C for 30 min) was resuspended in IEF buffer (2.5% sulfobetaine 3-10, 4 M urea, 2 M thiourea, 0.5% (v/v) Carrier Ampholyte pH 3-10, 40 mM DTT and 0.003% bromophenol blue) and an IPG-strip (ReadyStrip IPG Strips 7 cm pH 3 to 10, linear pH gradient, Bio-Rad) was rehydrated with this sample overnight at room temperature. Proteins were separated in the first dimension by isoelectric focusing using a PROTEAN i12 IEF System (final voltage: 4000 V for 15.000 Vh, Bio-Rad) and in the second dimension on the basis of their apparent molecular mass by polyacrylamide gel electrophoresis upon reduction and alkylation with 15 mM DTT and 135 mM IAA. Proteins were either stained with colloidal blue-silver staining[23] or transferred to a nitrocellulose membrane. Successful transfer was confirmed by reversible Ponceau S staining.

Mass spectrometry

Proteins recognised by IgE and separated by 2D gel electrophoresis were identified by mass spectrometry as described previously[24]. Briefly, destained proteins were digested in-gel by trypsin for 3 hours at 37 °C and generated peptides, mixed with α-cyano-4-hydroxycinnamic acid, were spotted onto a MTP Anchor Chip 384 TF target (Bruker, Daltonics, Bremen, Germany). Spotted peptides were measured by matrix-assisted laser desorption/ionisation-time of flight/TOF mass spectrometry (MALDI-TOF/TOF) using an Autoflex III smartbeam TOF/TOF200 System (Bruker,Daltonics, Bremen, Germany) together with the flexControl 3.04 software. For peptide mass fingerprinting (PMF), MS spectra were acquired in a positive ion reflector mode with 6000 shots ranging from 600 to 4.000 Da. Spectra were calibrated with external commercially available Peptide Calibration Standard II processed with the flexAnalysis 3.04 software and BioTools 3.2 was used to evaluate the resulting peak lists. Database search was performed with the MASCOT search engine MASCOT Server 2.3 (Matrix Science, London, U.K.) using the NCBI database (2013/12/09) including 111 proteins from *macadamia integrifolia* and the search parameters were set to mass tolerance of 80 ppm, acceptance of one missed trypsin cleavage site, carbamidomethylation of cysteine residues as fixed modification, and oxidation of methionine residues as variable modification. Protein hits with $p < 0.05$ were assigned as significant and the hits were confirmed by MS/MS measurements. For these measurements, two to five peptides of each identified protein were selected with the WARP feedback mechanism of BioTools for MS/MS and parent and fragment masses were recorded with 400 and 1,000 shots, respectively. MS/MS spectra were processed and analysed in the same way as the PMF spectra with a fragment mass tolerance of 0.7 Da. All measurements were performed in duplicates.

Heterologous expression and purification of recombinant allergens

VLAP-2-1, 2-2, 2-3, the C-terminal VLAP-2-3 fragment (aa 178-625) and vicilins from seeds, tree nuts and legumes (Ses i 3: Q9AUD0, Ana o 1: Q8L5L5, Pis v 3: B4X640, Jug r 2: Q9SEW4, Cor a 11: Q8S4P9, Ara h 1: P43238, Gly m 5.01: O22120, Gly m 5.02: Q9FZP9, Gly m 5.03: P25974, Pin k 2: V9VGU0) were heterologously expressed as fusion proteins containing a N-terminal-His (6x)-tag in *E. coli* as described previously[25]. Purification of heterologously expressed proteins was performed under denaturing conditions using an immobilized metal ion chromatography.

IgE binding capacity

Specific IgE binding to macadamia nut proteins from the crude extract was evaluated by western blot analyses. Shortly, nitrocellulose membrane was blocked with working strength universal buffer (WSUB, Article number ZD1100, EUROIMMUN AG, Germany) supplemented with 5% milk powder and upon extensive washing, sera diluted 1:10 in WSUB were applied for 1 hour at room temperature. Specific IgE binding was detected with α-human IgE antibodies coupled with alkaline phosphatase and visualisation was provided by applying nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate substrate for 10 min.
Crude macadamia extract, heterologously expressed VLAP isoforms and vicilins from seeds, tree nuts and legumes were coated on a line blot by EUROIMMUN AG, Germany. Evaluation of sIgE binding was performed as described above with the following modifications. Sera were diluted 1:11 in WSUB and applied overnight at room temperature. Specific IgE levels were evaluated as EUROLINE (EL)-intensities (RU) using the EUROLineScan 3.1 software.

Indirect basophil activation test

The indirect basophil activation test (BAT) was performed as previously described with minor modifications[26]. Briefly, isolated PBMCs were treated with lactic acid buffer (13.4 mM lactate, 140 mM NaCl, and 5 mM KCl, pH 3.9) for 2 min at room temperature to strip receptor-bound IgE. The reaction was stopped by applying neutralisation buffer to the cells (20 mM HEPES buffer, pH 7.4, 132 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4, 5.5 mM glucose, 0.5% HSA) and washing them twice with the same buffer. For re-loading IgE, individual serum samples, diluted in neutralisation buffer (1:2) supplemented with 10 µg/ml heparin and 8 mM EDTA, were applied to the cells and incubated for 90 min at 37 °C. Re-loaded cells were allowed to rest overnight in RPMI supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1 ng/ml

IL-3 at 37 °C and 5% CO2. Rested cells were stimulated with the macadamia nut extract, VLAP-2-1, 2-2, 2-3 and the C-terminal fragment of VLAP 2-3 diluted in RPMI + 1 ng/ml IL-3 for 30 min at 37 °C (range from 1 µg/ml to 1 ng/ml). The following samples were used as controls: RPMI + 1 ng/ml IL-3, 100 ng/ml C5a (R and D Systems), 1 µg/ml α-human IgE (Vector Laboratories), 10 µM fMLP and a serum from peanut allergic patients showing CD63 upregulation upon stimulation with Ara h 2. Stimulated cells were stained with CD123-FITC (Biolegend), CD63-PE (Monosan), CD203c-APC (Sony) and CD193-PerCP-Cy5.5 (Biolegend). Basophils were defined as $CD203c^+$, $CD123^+$ $CD193^+$ and basophil activation was calculated as the percentage of $CD63^+$ basophils. Threshold for basophil degranulation was set to 5% $CD63^+$ cells. All measurements were performed in duplicates.

Results

Vicilin-like antimicrobial peptides from *macadamia integrifolia* are recognized by IgE

Particularly proteins with a molecular mass between 53 and 67 kDa and a pI between 6.3 and 8.7 (Figure 1a) were strongly bound by sIgE from 2 macadamia nut sensitised sera (Serum 1: 18 RU VLAP-2-3 [**A**]; Serum 2: 114 RU VLAP-2-3 [**B**]). As shown in Table 2, these proteins (spots 20-28, Figure 1b and c) were identified as vicilin-like antimicrobial peptides (VLAP) 2-1 (Q9SPL5), 2-2 (Q9SPL4) and 2-3 (Q9SPL3) whereof VLAP-2-3 showed the highest probability scores in MS analyses (Suppl. Table S1) due to the detection of peptides solely specific for VLAP-2-3. Although full-length VLAPs possess molecular masses of 74.6 kDa (2-3) and 79.4 kDa (2-1 and 2-2), their dominant spots were 7 to 15 kDa smaller and no peptide belonging to the N-terminus was detected in MS/MS experiments. Due to this observation, we chose to express VLAP 2-1, 2-2 and 2-3 as full-length variants including a modified VLAP 2-3 variant lacking the N-terminus (aa 178-625).

Moreover, proteins with a molecular mass between 20 and 25 kDa and a pI between 6.5 and 7.9 (spots 42-44, 47, 48) were also strongly bound by sIgE in western blot analyses (Figure 1a). Despite high quality MS spectra with a great range of detected masses and high peak intensities, these proteins were not identified by aligning the spectra to the NCBI database, probably due to the limited number of proteins (n=111) described and listed for *macadamia integrifolia*.

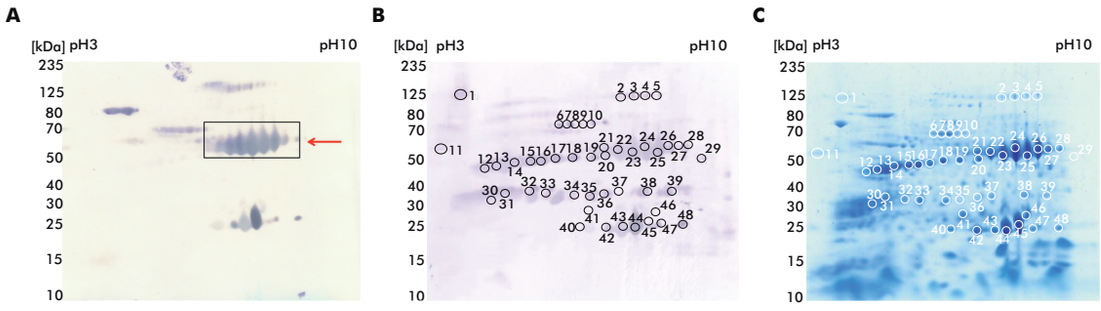

Figure 1: Identification of macadamia nut proteins recognised by sIgE
Macadamia nut proteins recognised by IgE were identified by 2D gel electrophoresis (4-20% SDS-PAGE) under reducing conditions, western blot analysis and subsequent mass spectrometry; ***A*** *and* ***B*** *2D western blot incubated with two different sera with sIgE against macadamia nut;* ***C*** *2D SDS-PAGE corresponding to* ***B*** *stained with colloidal Commassie stain. Analysed proteins are indicated with black circles and consecutive numbering*

Table 2: *Identified proteins by MS analysis*

Protein (accession number)	Spots
Vicilin-like antimicrobial peptides 2-3 (gi/75207035)	1, 2, 3, 4, 5, 11, 20-28, 41, 46
Vicilin-like antimicrobial peptides 2-2 (gi/75266171)	1, 3, 4, 11, 21-28, 41, 46
Vicilin-like antimicrobial peptides 2-1 (gi/75207036)	1, 3, 4, 11, 21-28, 41, 46

VLAP-2-3 may have the potential to distinguish between clinically relevant and irrelevant sensitisation to macadamia nut extract

As VLAP-2-3 was the dominant isoform detected in MS analyses, its serological recognition was evaluated in 82 NA and 27 NT patients comprehending 10 MA and 18 MT patients, in comparison with the macadamia nut extract (Figure 2a). The macadamia nut extract was recognised by 33% (27/82) NA and 26% (7/27) NT patients including 3 MA (30%, 3/10) and 3 MT (17%, 3/18) patients. A comparable number of NA patients (24/82, 29%), including the same 3 MA patients, showed sIgE-binding to VLAP-2-3. The percentage of NT patients, however, was lowered to only 7% (2/27 NT, 1/18 MT) with almost negligible sIgE titres. Whilst 8 NA patients with increased sIgE titres to the extract did not recognise VLAP-2-3 (range: 4-31 RU), 5 NA patients without detectable sIgE against the extract recognised VLAP-2-3 with mostly slightly increased sIgE titres (range: 3-25 RU). Taken together, VLAP-2-3 may have the potential to discriminate between macadamia nut allergic and tolerant patients as clinically irrelevant sensitisation to macadamia nut extract was not explained by sIgE binding to the component VLAP-2-3 in nut allergic patients.

Specific IgE against VLAP-2-3 was mainly present in macadamia nut allergic patients with severe symptoms

MA patients (3/10), recognising both the macadamia nut extract and VLAP-2-3, showed increased sIgE titres against VLAP-2-3 (23 to 105 RU) compared with the extract (17 to 70 RU). Reversed sensitisation patterns were observed for the 3 MT patients positive for macadamia nut extract (3 to 18 RU) whose sIgE titres to VLAP-2-3 were lower compared with the extract. Their sIgE titres against VLAP-2-3 were either very low (5 RU) or even undetectable. While all 3 MA patients positive for VLAP-2-3 experienced moderate to severe symptoms upon ingestion, the other 7 MA patients without sIgE against the extract or VLAP-2-3 suffered from rather mild to moderate symptoms except MA-7 (Table 1). Most of these patients (6/7) showed sensitisation to Bet v 1 and had a history of a pollen-related oral food allergy. The remaining MA patient showed sensitisation to Bet v 2, the profilin from birch pollen, which has also been associated with food sensitisation and oral allergy[27].

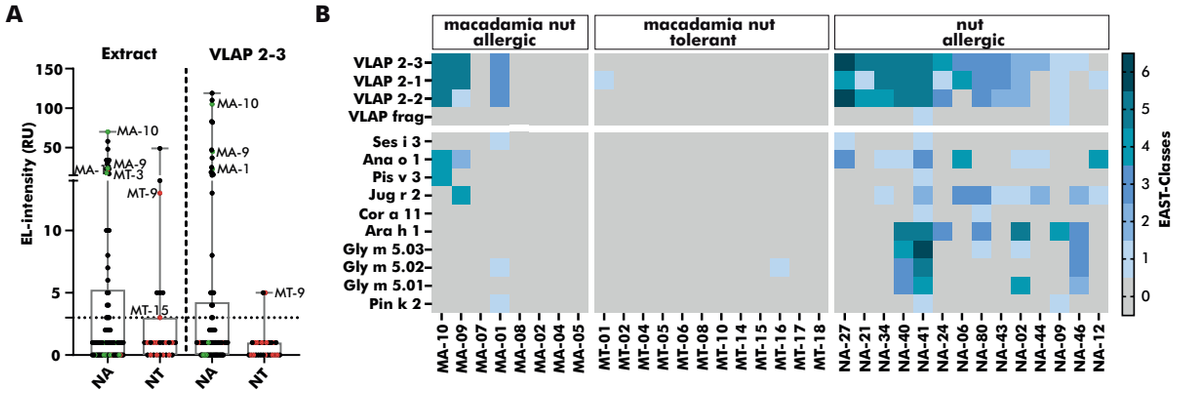

Figure 2: Sensitisation to macadamia nut, VLAP and vicilins from different sources
A *Sensitisation to macadamia nut extract and VLAP-2-3 was evaluated in 82 nut allergic (NA) and 27 nut tolerant (NT) patients including 10 macadamia nut allergic (MA) and 18 macadamia nut tolerant (MT) patients. Specific IgE titres from MA patients are indicated in green and sIgE titres from MT patients are shown in red;* ***B*** *Sensitisation to VLAP isoforms, a C-terminal VLAP-2-3 fragment (aa 178-625) and vicilins from seeds, tree nuts and legumes was evaluated in 8 MA, 12 MT and 14 NA patients sensitised to VLAP 2-3. MA patients are sorted by severity of their reaction to macadamia nut. Specific IgE levels are expressed as EAST classes; Cl. 0 = 0 to 2 RU, Cl. 1 = 3 to 6 RU, Cl. 2 = 7 to 15 RU, Cl. 3 = 16 to 30 RU, Cl. 4 = 31 to 50 RU, Cl. 5 = 51 to 100 RU, Cl. 6 = >100 RU*

N-terminal substitutions may explain divergent recognition of VLAP isoforms

Recognition of the different VLAP isoforms and the C-terminal fragment (aa 178-625) of VLAP-2-3, generated based on the lack of detected N-terminal peptides in MS analysis, was studied in a subgroup of 8 MA, 12 MT and 14 additional NA patients (Figure 2b). This subgroup was formed on both suggestive history to macadamia nut and sensiti-

sation to VLAP-2-3. Overall, patients recognising VLAP-2-3 were mostly also positive for the isoforms VLAP-2-1 and 2-2 but their sIgE binding to VLAP-2-1 and 2-2 was reduced in 59% of the patients. Lower or almost negligible sIgE binding was observed for the C-terminal fragment of VLAP-2-3, pointing towards relevant IgE binding sites and amino acid substitutions on the N-terminus not included in the sequence of the C-terminal VLAP-2-3 fragment. An amino acid sequence alignment in Figure 3 showed 13 amino acid substitutions on the N-terminus (aa 1-177 of VLAP-2-3) which were always consistent between two of the isoforms. In total, we observed 3 substitutions for VLAP-2-1, 4 substitutions for VLAP-2-2 and 6 substitutions for VLAP-2-3.

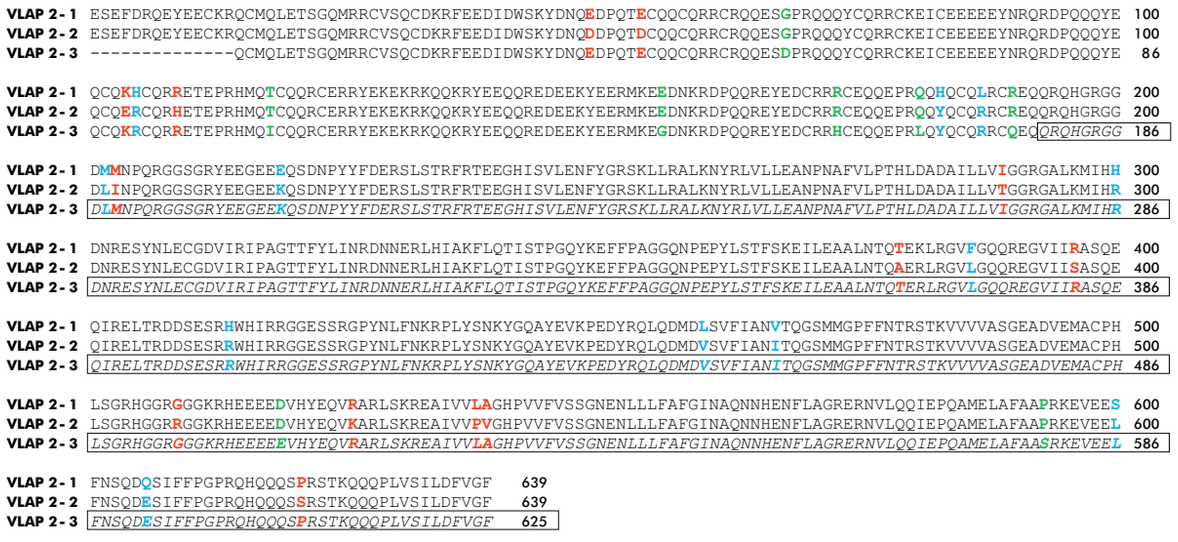

Figure 3: Amino acid sequence alignment of macadamia nut VLAP isoforms
Amino acid sequence alignment of the different VLAP isoforms with highlighted amino acid substitutions; Cyan: VLAP-2-1 ≠ VLAP-2-2 and VLAP-2-3; Red: VLAP-2-2 ≠ VLAP-2-1 and VLAP-2-3 Green: VLAP-2-3 ≠ VLAP-2-1 and VLAP-2-2; the C-terminal VLAP-2-3 fragment is indicated with a black box

Co-recognised vicilins were often associated with a respective nut allergy

Co-sensitisation to vicilins from seeds, tree nuts and legumes was determined in the same subpopulation. Recognition of VLAP isoforms was accompanied by sIgE binding to Ana o 1 (cashew nut) and Jug r 2 (walnut) in 59% of the patients sensitised to VLAP (Figure 2b). Regarding MA patients, Ana o 1 was co-recognised in 2 and Jug r 2 in 1 out of 3 MA patients. The recognition of additional vicilins was mostly but not exclusively associated with a corresponding food allergy (Supplementary, Online Repository). In contrast, hazelnut allergy was not at all associated with recognising Cor a 11 and peanut allergy was only partly associated with Ara h 1 recognition.

Vicilin-like antimicrobial peptides can induce degranulation

Besides serological characterisation of VLAP-2-3 and its isoforms, their ability to induce degranulation was evaluated by indirect BAT (Figure 4). Patient MA-10 (NA-29) showed dose-dependent BAT reactivity upon stimulation with all three VLAP isoforms starting from 10 ng/ml and reaching a plateau at around 100 ng/ml for VLAP-2-2 and 2-3 and at 1000 ng/ml for VLAP-2-1. Consistent with sensitisation data, stimulation with the C-terminal VLAP-2-3 fragment did not result in any upregulation of CD63 surface expression at all considered concentrations. This dose-dependent BAT reactivity was confirmed by repeating the experiment under the same conditions on a second day with freshly obtained basophils (Figure 4a). These basophils, however, showed a more flat-angel reactivity curve and the plateau was reached at tenfold higher concentrations. In comparison, stimulation with native macadamia nut extract resulted in a stronger CD63 upregulation than stimulation with recombinant VLAP components. To ensure that no activation was caused by remaining donor IgE, basophils were stimulated upon stripping with the different VLAP isoforms and macadamia nut extract leading to no CD63 upregulation (Figure 4b). As internal positive control, stripped basophils were loaded with serum from a peanut allergic patients sensitised to Ara h 2 (Figure 4c) and stimulation with Ara h 2 resulted in a dose-dependent upregulation of CD63. Overall, VLAP 2-3 and its isoforms are generally capable of inducing degranulation.

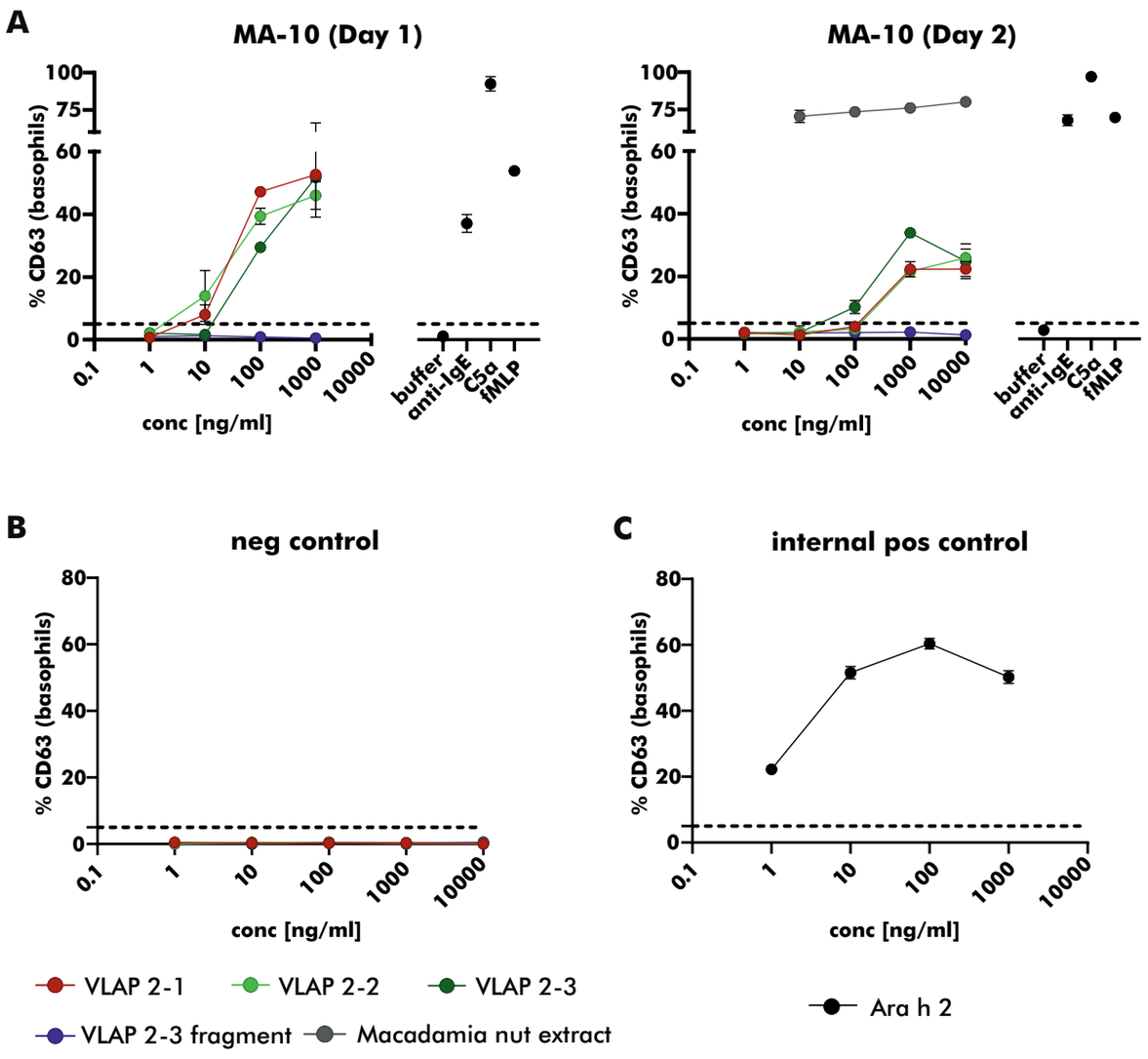

Figure 4: Indirect basophil activation by macadamia nut VLAP isoforms
Indirect basophil activation test to evaluate the potential of VLAP isoforms and the C-terminal VLAP-2-3 to induce degranulation
***A** Dose-response curves upon stimulation with VLAP isoforms, C-terminal VLAP 2-3 fragment and macadamia nut extract on two different days with freshly obtained basophils; **[B]** Dose-response curves of stripped basophils with remaining donor IgE stimulated under the same conditions as **A** (negative control); **C** Dose-response curve of basophils loaded with sera from a peanut allergic patient sensitised to Ara h 2 upon stimulation with Ara h 2*

Discussion

To date, the diagnosis of macadamia nut allergy by sIgE solely relies on the macadamia nut extract and single components are lacking. In the present study, macadamia nut proteins recognised by sIgE were identified as VLAPs with serological recognition in 33% of NA and 30% of MA patients and the ability to induce basophil activation. While MA patients recognised VLAPs with highly increased sIgE titres (23 to 105 RU), MT patients recognised VLAPs with almost negligible sIgE titres, indicating the potential of VLAPs to discriminate between MA and MT patients positive for the macadamia nut extract. Moreover, MA patients sensitised to VLAPs experienced moderate to severe symptoms upon ingestion, highlighting the potential of VLAPs as potential markers for systemic reactions to macadamia nut.

VLAP-2-3 and its isoforms, belonging to the vicilin family, were identified as macadamia nut proteins predominately recognised by sIgE from MA patients with moderate to severe symptoms. Accordingly, the vicilin from walnut, Jug r 2, has been described as a potential marker for severe allergic reactions in patients without pollen-related sensitisation[18,19]. Moreover, sensitisation to the vicilin from hazelnut, Cor a 11, was observed in children with severe hazelnut allergies, while Cor a 11 was scarcely recognised by adults with OAS upon hazelnut ingestion[20]. Specific IgE binding to cross-reactive carbohydrate determinants (CCDs) has been excluded by the use of recombinant allergens in those studies. Overall, vicilins, including the one from macadamia nut, appear to be markers for severe, potentially non-pollen related, allergies to tree nuts.

In contrast, MA patients, primarily suffering from mild symptoms upon ingestion, recognised neither macadamia nut extract nor VLAP proteins and most of these patients (6/7) were known for their Bet v 1-related oral food allergies. Since PR-10 proteins are underrepresented in aqueous extracts of several foods like hazelnut, a Bet v 1-homologue might also be underrepresented in the macadamia nut extract, theoretically explaining the lack of detected sIgE in those patients[28,29]. Moreover, no sIgE binding to VLAP-2-3 by patients with rather mild to moderate symptoms supports the potential of VLAP proteins to stratify MA patients into patients with rather mild and patients with rather severe symptoms upon macadamia nut ingestion.

While NA patients sensitised to VLAP 2-3 showed also sIgE-binding to the isoforms VLAP-2-1 and 2-2, nearly no recognition was observed for the C-terminal VLAP-2-3 fragment (aa 178-625). Accordingly, basophil activation was repeatedly achieved by stimulation with VLAP full-length proteins but not with the C-terminal VLAP-2-3 fragment using one serum, suggesting the location of critical epitopes on the N-terminus of VLAP. Contrary, no peptides belonging to the N-terminus were detected by mass spectrometry, potentially due to a mixture of degradation products present at the same molecular mass. Compared with literature, shared linear epitopes between VLAP and

Jug r 2 as well as structural epitopes of different vicilins (Ara h 1, Jug r 2, Cor a 11, Ana o 1) were predicted to be mainly located on the C-terminus[14,17], suggesting only co-sensitisation but not cross-reactivity between VLAP and vicilins from other tree nuts. This hypothesis is supported by negligible inhibition of sIgE-binding to macadamia nut extract with Jug r 2 in a study by Teuber and colleagues[12].

Additional macadamia nut proteins recognised by sIgE but not identified by MS analysis were detected at molecular masses of around 25, 35 and 45 kDa and a corresponding pI of 7.3 to 9.1, 4.1 to 8.9 and 4.2 to 6.1, respectively. These characteristics might correspond to either 2S albumins (25 kDa) or 11S globulins (25 kDa: basic subunit and 35 kDa: acidic subunit) described as allergens for several other tree nut and legume allergies[30–34]. Accordingly, previous inhibition experiments showed reduction in sIgE binding to the macadamia nut extract upon pre-incubation with Jug r 1 (2S albumin) and Jug r 4 (11S globulin), supporting the hypothetical presence of cross-reactive 2S albumins and 11S globulins in the macadamia nut extract[12]. The presence would explain IgE binding to the macadamia nut extract without recognition of VLAP-2-3 in 8 NA patients. The identification of the remaining proteins recognised by sIgE might be additionally beneficial for component-resolved diagnostics in macadamia nut allergy.

Overall, VLAPs are the first described single components from macadamia nut with serological and functional allergenic properties. These components appear to be supportive in identifying patients with systemic reactions to macadamia nut.

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Informed consent statement

This study was carried out in accordance with the University Medical Centre Utrecht, Biobank Regulations, which are in compliance with the applicable national and international laws and regulations. These regulations permit the use of ‘residual material from diagnostic testing’ for research, unless the patient objects (Article 8, ‘no objection’ procedure). None of the included patients objected the use of their serum. The protocol was approved by the Biobank Research Ethics Committee of the University Medical Centre Utrecht under the protocol number 18-428.

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Supplementary

Detailed description of individual patients characteristics are available at the Online Repository of the Journal Clinical and Translational Allergy (doi: 10.1186/s13601-020-00364-5).

Table S1: *Enclosed proteins detected by 2D gel electrophoresis and mass spectrometry analysis The macadamia nut extract was separated by IEF and SDS-PAGE. Spots corresponding to sIgE binding were analysed by mass spectrometry using peptide mass fingerprinting. The table shows the identified proteins in this fraction and MS/MS results are displayed in italic*

Spot	Protein (Acession number)	Cut-Off	Protein score	Seq coverage [%]	Molecular mass [kDa]
1	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*42*	149/*55*	31/*1*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*42*	*77*/*55*	16/*1*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	/*42*	/*55*	*1*	79.4
2	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74	113	24	74.6
3	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*40*	98/*18*	22/*1*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	*40*	*18*	*1*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	*40*	*18*	*1*	79.4
4	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*42*	191/*113*	35/*4*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*42*	110/*113*	24/*3*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*42*	80/*113*	21/*3*	79.4
5	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74	146	32	74.6
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*42*	176/*57*	37/*1*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*42*	99/*57*	24/*1*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*42*	99/*57*	24/*1*	79.4
12	-	-	-	-	-
13	-	-	-	-	-

Spot	Protein (Acession number)	Cut-Off	Protein score	Seq coverage [%]	Molecular mass [kDa]
14	-	-	-	-	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74	91	29	74.6
21	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*44*	236/*148*	41/*4*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*44*	164/*148*	33/*3*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*44*	94/*148*	27/*3*	79.4
22	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*44*	348/*156*	43/*3*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*44*	233/*156*	34/*3*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74	131	30	79.4
23	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*45*	366/*20*	44/*1*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*45*	245/*20*	37/*1*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74	166	31	79.4
24	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*44*	414/*145*	48/*3*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*44*	265/*145*	37/*3*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74	170	32	79.4
25	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*44*	409/*93*	45/*2*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74	259	32	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74	170	30	79.4
26	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*44*	382/*83*	43/*2*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74	276	34	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*44*	186/*83*	30/*2*	79.4

Spot	Protein (Acession number)	Cut-Off	Protein score	Seq coverage [%]	Molecular mass [kDa]
27	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74	289	41	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74	201	31	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*45*	148/*151*	31/*5*	79.4
28	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74	165	37	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74	119	28	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*44*	131/*192*	33/*8*	79.4
29	-	-	-	-	-
30	-	-	-	-	-
31	-	-	-	-	-
32	-	-	-	-	-
33	-	-	-	-	-
34	-	-	-	-	-
35	-	-	-	-	-
36	-	-	-	-	-
37	-	-	-	-	-
38	-	-	-	-	-
39	-	-	-	-	-
40	-	-	-	-	-
41	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*45*	190/*139*	34/*3*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*45*	116/*139*	27/*3*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74/*45*	117/*139*	29/*3*	79.4
42	-	-	-	-	-
43	-	-	-	-	-
46	Vicilin-like antimicrobial peptides 2-3 (gil75207035)	74/*44*	180/*30*	32/*1*	74.6
	Vicilin-like antimicrobial peptides 2-2 (gil75266171)	74/*44*	113/*30*	22/*1*	79.4
	Vicilin-like antimicrobial peptides 2-1 (gil75207036)	74	95	22	79.4
47	-	-	-	-	-
48	-	-	-	-	-


3

Sesame oleosins are minor allergens

Clin Transl Allergy 2019;9:32.

Anna M. Ehlers[1,2], Madlen Rossnagel[3], Bettina Brix[3], Mark A. Blankestijn[1,2], Thuy-My Le[1,2], Waltraud Suer[3], Henny G. Otten[1], André C. Knulst[1,2]

[1]Laboratory of Translational Immunology, Utrecht University, University Medical Center Utrecht, Utrecht, The Netherlands

[2]Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[3]EUROIMMUN, Lübeck, Germany

Abstract

Background

In daily practice, one-third of sesame allergic patients, confirmed by clinical history or food challenge, do not show any detectable specific IgE using current diagnostics. Currently used sesame extracts are water-based and therefore lacking hydrophobic proteins like oleosins. Oleosins, the stabilizer of lipid droplets in plants, are described as allergens in sesame, peanut, and hazelnut. In this study, we examined the role of oleosins in sesame allergy and their potential cross-reactivity between sesame and (pea)nuts.

Methods

Specific IgE and IgG sensitisation to native and heterologously expressed sesame components and oleosins from other nuts, free of seed storage proteins, was assessed by line blot and sera from 17 sesame allergic patients without detectable specific IgE sensitisation to sesame extract, and compared to 18 sesame allergic and 13 tolerant patients with specific IgE sensitisation to sesame extract.

Results

Sesame allergic patients without sensitisation showed no specific IgE to the tested sesame oleosins or components. Low levels of specific IgE to sesame oleosins were detected in 17% of sesame allergic and 15% of tolerant patients with sIgE sensitisation. Oleosins were recognised by serum IgG from multiple patients confirming immune reactivity and excluding technical issues leading to lack of specific IgE-binding to oleosins.

Conclusion

Sesame oleosins are minor allergens and appear to have no additonal value in diagnosing sesame allergy in adults based on sIgE and sIgG detection. There is a high need for additional diagnostic tools in those patients to minimise the number of required food challenges.

To the editor:

Diagnosis of sesame allergy by measuring specific IgE (sIgE) is based on extracts or the major allergen: 2S albumin Ses i 1. However, this leads to false negative results in around 30% of sesame allergic patients, a high frequency compared to other food allergies[1]. To overcome this obstacle, we evaluated the role of oleosins in sesame allergy. Oleosins are oil-body stabilising proteins and might be lacking in water-based extracts due to its hydrophobicity[2].

Adult sesame allergic and tolerant but sensitised patients who visited the outpatient clinic of the University Medical Centre Utrecht, The Netherlands, were retrospectively selected and allergy (n=35) or tolerance (n=13) was confirmed by food challenge or an experienced physician diagnosis. Sesame allergic patients were subdivided into patients without and with detectable sIgE (ImmunoCAP sesame extract $\geq$0.35 kU/L; without n=17; with n=18). Patient characteristics are described in Table 1. Sera with sIgE against native and heterologously expressed oleosins from different nuts and seeds acted as positive controls. Ethical approval was acquired from the biobank committee of the University Medical Centre Utrecht, number 18-428.
Known sesame components and oleosins (from sesame, walnut, hazelnut, peanut and soy) with and without the hydrophobic domain based on the TMHMM model (prediction of transmembrane helices being not available for antibody binding) were heterologously expressed[3]. For comparisons to the native form, oil-body associated proteins (OAPs) were isolated from sesame, walnut and pecan nut using a modified previously described method with an additional hydrophobic interaction chromatography (HIC) instead of a preparative gel electrophoresis to separate traces of seed storage proteins with similar molecular masses[4]. To examine the absence of seed storage proteins, enclosed proteins were identified by mass spectrometry and detected by western blot with α-human IgE-alkaline phosphatase (AP). Sensitisation to these components was deeply investigated by measuring sIgE and sIgG levels using line blots (EUROLINE, EUROIMMUN, Luebeck, Germany) according to manufacturer's instructions. IgG subtypes were examined using an ELISA coated with different heterologously expressed oleosins (Ses i 4, Ses i Oleosin, Jug r Oleosin-1, Jug r Oleosin-2) and detected with α-human IgG1-4 AP-conjugates. Detailed description of the methods is listed in the Method section.

Overall, the median age was 37 and allergic subjects suffered from typical symptoms being in line with an IgE-mediated hypersensitivity (characteristics are shown in Table 1). Gastro-intestinal symptoms were always accompanied by OAS or skin reactions (Suppl. Figure S1) and the allergic reactions took place within several minutes to half an hour after ingestion (data from 8/35), supporting the diagnosis 'food allergy'.

Table 1: *Patient demography of included sesame allergic and tolerant patients; Allergy was defined by food challenge or an experienced physician*

	Group 1a[A]	Group 1b[A]	Group 2[A]	p-value
Age [median (range)]	53 (29-85)	35 (27-51)	32 (27-58)	0.001
Sex female	14 (82%)	10 (56%)	10 (77%)	0.1865
Food challenge	8 (47%)	1 (6%)	1 (8%)	0.0034
Total IgE (kU/l)	295 (39 - >5000)	4470 (1010 - >5000)	>5000 (518 - >5000)	<0.0001
Symptoms[B]				
Mild (Müller 0)	6 (35%)	6 (33%)	N/A	
Moderate (Müller 1+2)	6 (35%)	6 (33%)	N/A	0.9693
Severe (Müller 3+4)	5 (29%)	6 (33%)	N/A	
Sensitisation[C] (n, median, range)				
Sesame extract ImmunoCAP	17 (0, 0-0.32 kU/l)	18 (4.5, 0.5-75 kU/l)	13 (3.9, 0.4-48 kU/l)	0.5338
Ses i 1 ISAC	6 (0 ISU)	10 (2.9, 0-22.5 ISU)	3 (0, 0-6 ISU)	0.4336

[A] G1a: sesame allergic patients without detectable sIgE sensitisation; G1b: sesame allergic patients with sIgE sensitisation; G2: sesame tolerant patients with sIgE sensitisation

[B] Symptom distribution of each group is shown in the Supplementary

[C] ImmunoCAP and ISAC data were compared between group 1b and 2 since group 1a was selected by lacking sIgE sensitisation; CAP > 0.35 kU/L was considered as positive

The sesame OAPs fraction (pool of HIC-fractions 4 and 5) used for sIgE measurements was free of seed storage protein traces as confirmed by western blot analysis with sera positive for these proteins from the study cohort (Figure 1a). Before performing a HIC, these sera reacted with proteins around 15 kDa, similar molecular masses as oleosins. After the additional purification step, only the positive control (PC) still recognised proteins at this height. Additionally, no seed storage proteins but sesame oleosins were detected by mass spectrometry (Suppl. Figure S2 and Suppl. Table S1 to S3).

Specific IgE-binding to sesame oleosins with levels above the detection limit (EAST-class 1-2) was detected in 17% of sensitised allergic and 15% of tolerant patients. However, none of the non-sensitised sesame allergic patients showed sIgE-binding neither to sesame oleosins nor to any other sesame component, except one serum showing low sIgE level to the 11S globulin Ses i 7 (G1a_6). Control sera, selected for sIgE binding to sesame (PC) or peanut oleosins (C1-3), showed sIgE binding to oleosins from different sources, confirming the binding capacity of heterologously expressed and native oleosins. Specific IgE-binding to oleosins in sensitised allergic patients was accompanied by recognising other sesame components, especially Ses i 1 and 2 whilst in tolerant patients was not (Figure 1b and c). Patients showing IgE reactivity

to heterologously expressed sesame oleosins were co-sensitised to heterologously expressed oleosins from walnut, hazelnut, and peanut, indicating potential cross-reactivity (G1b_15, G1b_17). All oleosins expressed as full-length variants were recognised at least by one serum, except Cor a 12. In case of Cor a 13, the full-length and the hp-variant of Cor a 13 were recognised in parallel (G1b_17, PC).

Although oleosins were infrequently recognised by sIgE, they were bound by serum IgG from multiple patients across all groups confirming immune reactivity and excluding technical issues leading to lack of specific IgE binding. Depletion of IgG by protein G columns in these sera did not result in sIgE binding (Suppl. Figure S3), excluding competition between IgG and IgE. Specific IgG was mainly detected for Ses i 4, Ses i Ol, Jug r Ol-1 and Jug r Ol-2 and significantly increased in sesame allergic patients without detectable sIgE sensitisation compared with the other groups (Ses i 4: G1a vs G1b, p=0.0014; Ses i Ol: G1a vs G2, p=0.03; Jug r Ol-2: G1a vs G1b, p=0.0049). The most prevalent subtype recognising oleosins was IgG1, while IgG2, IgG3 and IgG4 levels were dependent on the oleosin of interest (Suppl. Figure S4 and S5).

Up to 30% of sesame allergic patients, including those suffering from severe reactions, cannot be diagnosed by commercially available diagnostic tests[1,5]. Launched aqueous sesame extracts do not contain hydrophobic proteins like oleosins. We demonstrated that oleosins were clearly recognised in 17% of sensitised sesame allergic patients but did not have any additional diagnostic value compared to sesame extract and Ses i 1, especially in patients without detectable sIgE sensitisation. Contrary to our findings, 90% recognition in sesame allergic patients (31% without sIgE to sesame extract) was reported previously[6]. Moreover, OAPs reactivity was detected in 36% of hazelnut allergic patients and therefore considered as relevant diagnostic markers, particularly in patients without detectable sIgE[7]. This discrepancy might be explained by the absence of seed storage proteins or fragments thereof[8] in our native preparation accomplished by HIC, which was confirmed by western blot analysis and mass spectrometry.
Oleosins were recognised by sIgE levels slightly above the detection limit (EAST-class 1-2). This is in line with a hazelnut study across Europe showing a prevalence of 20% for the hazelnut oleosin Cor a 12, but low sIgE titre up to 1 kU/l (= EAST-class 2)[9]. In our test system, even lower sIgE titres might be explained by competition between different oleosins coated on the same line blot.
In conclusion, sesame oleosins are minor allergens and appear to have no additional value in diagnosing sesame allergy in adults based on sIgE and sIgG detection. We propose a prospective study to evaluate the diagnostic value of direct basophil activation tests due to the high need for additional diagnostic tools in those patients.

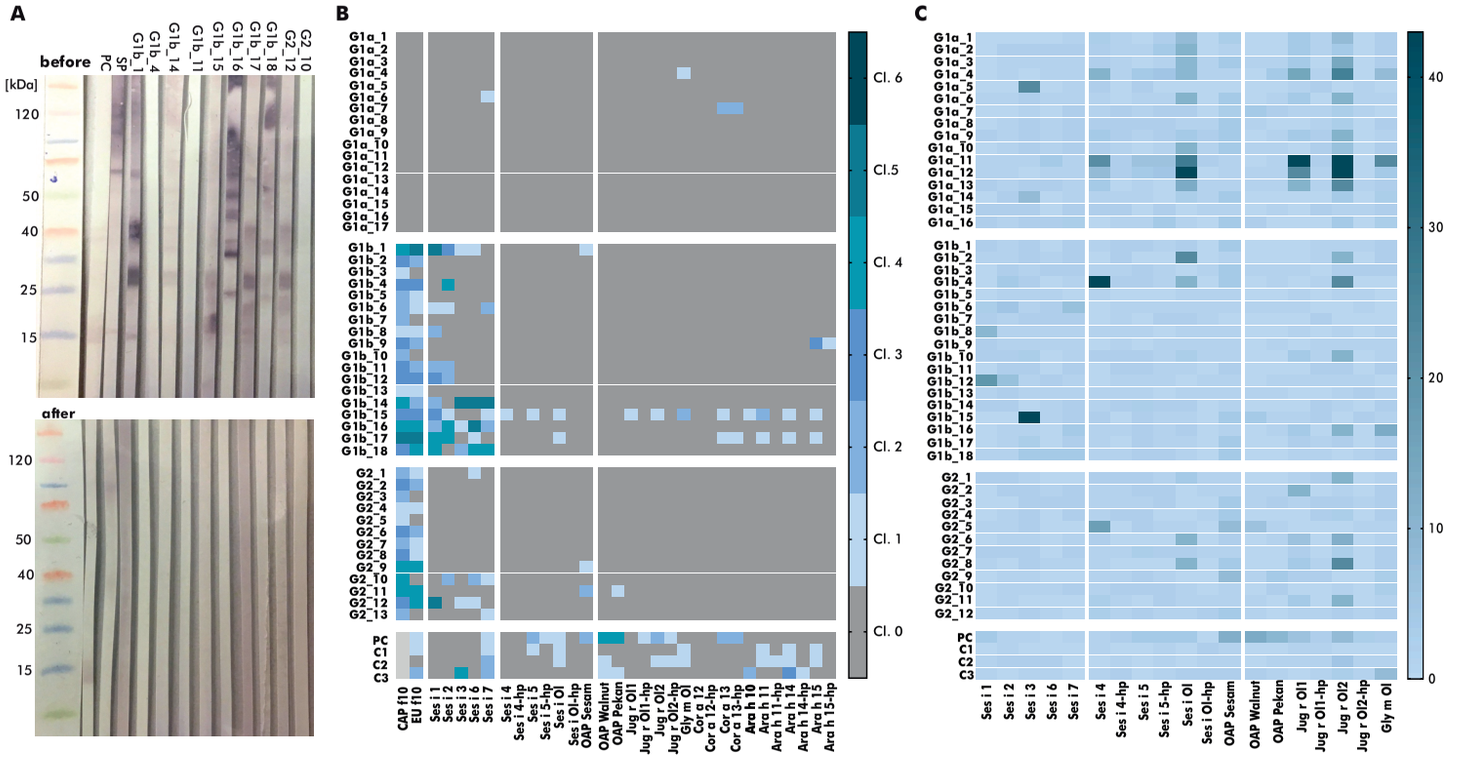

Figure 1: Western Blot analysis and sIgE and sIgG sensitisation pattern

A *Western-Blot analysis of an intermediate purification step (before HIC) and of the final OAPs fraction (after HIC) with sera containing sIgE to sesame seed storage proteins and oleosins. Samples were separated by SDS-PAGE using a 4-12% Bis-Tris gel and blotted onto a nitrocellulose membrane. Bound IgE were detected by α-human IgE antibodies conjugate labelled with alkaline phosphatase; PC is a oleosin-positive serum, SP is a sesame seed storage positive serum not used in this study, sera G1b_1, 4, 14, 11, 15-18, G2_12 and 10 are sera with specific IgE to sesame seed storage proteins and partly with slightly elevated sIgE levels to sesame oleosins;* ***B*** *IgE sensitisation pattern to sesame extract from ImmunoCAP and EUROLINE, sesame components and oleosins from sesame, walnut, soy, hazelnut and peanut expressed as EAST (Enzyme-Allergo-Sorbent Test classification)-classes and sorted by severity of symptoms to sesame, light grey: not determined;* ***C*** *IgG-sensitisation pattern to sesame components and oleosins from sesame, walnut, pecan and soy expressed as EUROLINE (EL)-intensities and sorted by severity of symptoms to sesame; G1a: sesame allergic patients without detectable sIgE sensitisation; G1b: sesame allergic patients with sIgE sensitisation; G2: sesame tolerant patients with sIgE sensitisation; PC: positive control selected by sIgE to sesame oleosins; C1-3: positive controls selected by sIgE to peanut oleosins*

Methods

Patient selection

Sesame allergic and tolerant patients who visited the outpatient clinic of the University Medical Centre Utrecht, The Netherlands, between 2012 and 2017 were retrospectively selected. The selected patients were first divided into one of two groups regarding allergy (n=35) or tolerance (n=13) confirmed by food challenge or an experienced physician. Allergic patients were subdivided into patients without (n=17) and with (n=18) detectable sIgE sensitisation (ImmunoCAP sesame extract $\geq$0.35 kU/L). Tolerant patients without detectable sIgE sensitisation were excluded. Positive controls were sera with sIgE against native and heterologously expressed oleosins from different nuts and seeds. Ethical approval was acquired from the biobank committee of the University Medical Centre Utrecht under the number 18-428.

Purification of oil-body associated proteins

Oil-body associated proteins (OAPs) were isolated from sesame, walnut and pecan nut as described previously with the following modifications[2,4,7]. Sesame seeds were ground 1:2 w/v in 50 mM NaH_2PO_4 buffer pH 7.4 containing 1 mM EDTA, 10 mM KCl, 2 mM DTT and 20% w/v sucrose (grinding buffer) using a blender. The ground sesame seeds were filtered over a gauze and layered 1:1 v/v with flotation buffer (50 mM NaH_2PO_4, 1 mM EDTA, 10 mM KCl, 2 mM DTT, pH 7.4). The gradient was centrifuged for 30 min at 4 °C using a swing-bucket rotor at 16.000 x g. After centrifugation, a white fat pad was formed on top of the gradient. After resuspension of the fat pad in urea buffer (50 mM NaH_2PO_4, 9 M urea, pH 7.4), the suspension was incubated for 15 min at room temperature, layered 1:1 v/v with 50 mM NaH_2PO_4 buffer pH 7.4 and centrifuged under the same conditions. This step was repeated twice, and the resulting fat pad was resuspended in grinding buffer containing 0.1% Tween-20 and layered 1:1 v/v with 50 mM NaH_2PO_4 buffer pH 7.4. After centrifugation, the fat pad was resuspended in grinding buffer containing 2 M NaCl and layered with flotation buffer containing 2 M NaCl. After resuspension of the fat pad in grinding buffer, proteins were precipitated with a fourfold volume of ice-cold acetone and washed twice. After air-drying, the pellet was solubilised in a detergent-containing buffer and dialyzed against a 50 mM Tris-HCl buffer, pH 7.5. To separate traces of seed storage proteins, the protein solution was applied on a hydrophobic interaction chromatography column according to manufacturer's instructions.

Heterologous expression and purification of recombinant allergens

Sesame components (Ses i 1, 2, 3, 6 and 7) and oleosins (sesame, walnut, hazelnut and peanut) were expressed as fusion proteins with N-terminal His(6x) in *E. coli* as previously described[10,11]. The hp-variants of oleosins were expressed without the hydrophobic part determined by using the TMHMM model[3]. All heterologous expressed proteins were purified by immobilised metal ion chromatography under denaturing conditions. Full-length oleosins were dialyzed against citrate buffer pH 5.5 for applying on the line blot.

Mass spectrometry

Enclosed proteins of the OAPs fractions from sesame, walnut and pecan nut were identified by mass spectrometry. Reduced (0.02 M DTT) and alkylated (0.06 M iodoacetamide) proteins were separated in a 4-12% polyacrylamide gel stained with colloidal Coomassie. After excising and achromatising, these proteins were digested in-gel by trypsin for 3 hours at 37 °C. The resulting peptides were subsequently extracted and spotted with α-cyano-4-hydroxycinnamic acid onto a MTP Anchor Chip 384 TF target (Bruker, Billerica, Massachusetts, US). Spotted peptides were measured by matrix-assisted laser desorption/ionisation-time of flight/TOF mass spectrometry (MALDI-TOF /TOF) using an Autoflex III smartbeam TOF/TOF200 System (Bruker, Billerica, Massachusetts, US) combined with the flexControl 3.4 software. MS spectra for peptide mass fingerprinting (PMF) were acquired in a positive ion reflector mode with 6000 shots ranging from 600 to 4.000 Da. For spectra calibration, external commercially available Peptide Calibration Standard II was processed with flexAnalysis 3.4 and the resulting peak lists were examined with BioTools 3.2. MS spectra for protein identification were analysed by submitting them to the MASCOT search engine MASCOT Server 2.3 (Matrix Science, London, U.K.) searching against the NCBI database (2016/12/19) including 33.467 proteins from *Sesamum indicum*, 56.036 proteins from *Juglans regia* and 278 proteins from *Carya illinoinensis*. Search parameters were set as follows: mass tolerance of 80 ppm, acceptance of one missed trypsin cleavage site, carbamidomethylation of cysteine residues as fixed modification, and oxidation of methionine residues as variable modification. For evaluation of protein hits, significance was defined as $p < 0.05$. PMF hits were confirmed by selecting two to five peptides of each identified protein with the WARP feedback mechanism of BioTools for MS/MS measurements. Recording of parent and fragment masses were performed with 400 and 1,000 shots, respectively. Resulted spectra were processed and analysed as described above with a fragment mass tolerance of 0.7 Da. These measurements were performed twice with different preparations in duplicates.

Line Blot

Sensitisation was defined by measuring sIgE and sIgG levels using a line blot (EUROLINE, EUROIMMUN, Luebeck, Germany) according manufacturer's instructions. For IgE detection, line blots were incubated overnight with 1 ml diluted serum (1:11 in universal buffer), some of them IgG-depleted (Protein G columns, GE Healthcare, Buckinghamshire, Great Britain), at room temperature on a rocket shaker. For detection of sIgG levels, 0.51 ml diluted serum (1:51 in universal buffer) was incubated for one hour at room temperature. Bound serum antibodies were detected with α-human IgE or IgG alkaline phosphatase-coupled conjugate and visualised by adding nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate substrate. The binding of antibodies was evaluated using the EUROLINEScan software. For IgE measurement, band intensities were reported as intensity levels or classes, leaned on the Enzyme-Allergo-Sorbent Test (EAST) classification[12]. EUROLINE intensities (EL-intensities) of 3 or greater were considered as positive, corresponding to classes from 1 to 6. For IgG measurement, band intensities smaller than 8 were considered as negative. These measurements were performed once.

Total IgE levels

Total IgE levels were determined using the ELISA technique (EUROIMMUN, Lübeck, Germany). In brief, 100 µl of diluted serum (1:10 in universal buffer) were applied per well and incubated for 30 min at room temperature. Bound IgE was detected by applying α-IgE horse radish peroxidase-coupled conjugate and visualised by adding tetramethylbenzidine. Optical intensities (OD at 450 nm) were evaluated with a calibration curve and converted into IU/ml. These measurements were performed once in duplicates.

IgG subtype determination

Microtiter plates were coated with heterologously expressed full-length oleosins (9 µg/mL in PBS, pH 7.5) over night at 4 °C. After blocking, 100 µl of diluted serum (1:25 in blocking buffer) was applied in duplicates and allowed to react for 1 hour at room temperature on an orbital shaker. Bound antibodies were detected using α-human IgG1-4 AP-conjugates and stained with p-Nitrophenyl Phosphate Disodium salt (Thermofisher Scientific, Waltham, MA, USA). The OD was read at 405 nm using an automated spectrophotometer (iMark, Bio-Rad, Hercules, CA, USA). OD values of each IgG subtype measured for sera previously considered as positive (line blot) were divided by the OD value of the negative control. These measurements were performed twice in duplicates.

Data analysis

The baseline data were statistically analysed using one-way ANOVA or Mann-Whitney-U-test for continuous data and Fisher's exact test for categorical data. For comparison of sIgG levels between the patient groups, Kruskal–Wallis and Dunn's post-hoc tests were used. Statistical evaluation was performed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and SPSS Statistics 21 (IBM Corporation, Armonk, NY, USA). P values ≤ 0.05 were considered as statistically significant.

Informed consent statement

This study was carried out in accordance with the University Medical Centre Utrecht, Biobank Regulations, which are in compliance with the applicable national and international laws and regulations. These regulations permit the use of 'residual material from diagnostic testing' for research, unless the patient objects (Article 8, 'no objection' procedure). None of the included patients objected the use of their serum. The protocol was approved by the Biobank Research Ethics Committee of the University Medical Centre Utrecht under the protocol number 18-428.

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Supplementary

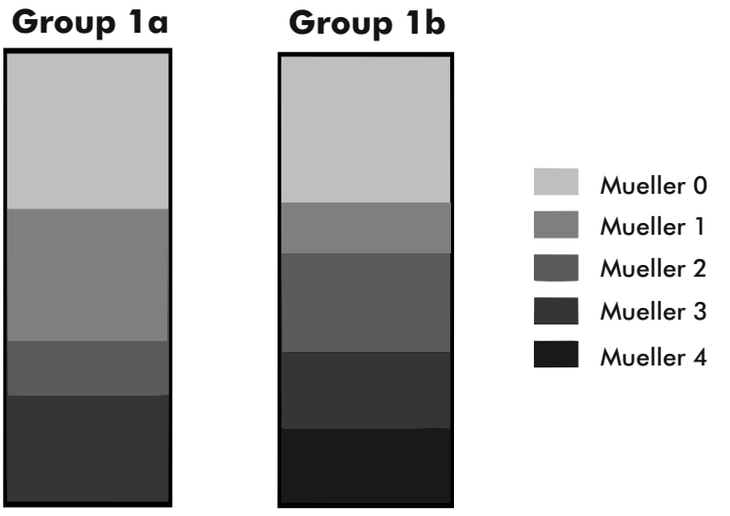


Figure S1: Distinct symptom distribution between sesame allergic patients with and without detectable sIgE sensitisation
Sesame allergic patients without detectable sIgE sensitisation (G1a) showed more often skin related reactions (Mueller 1) compared to patients with sensitisation (G1b). The other way around, patients of G1b showed more often gastro-intestinal symptoms (Mueller 2) and cardiovascular reactions (Mueller 4)

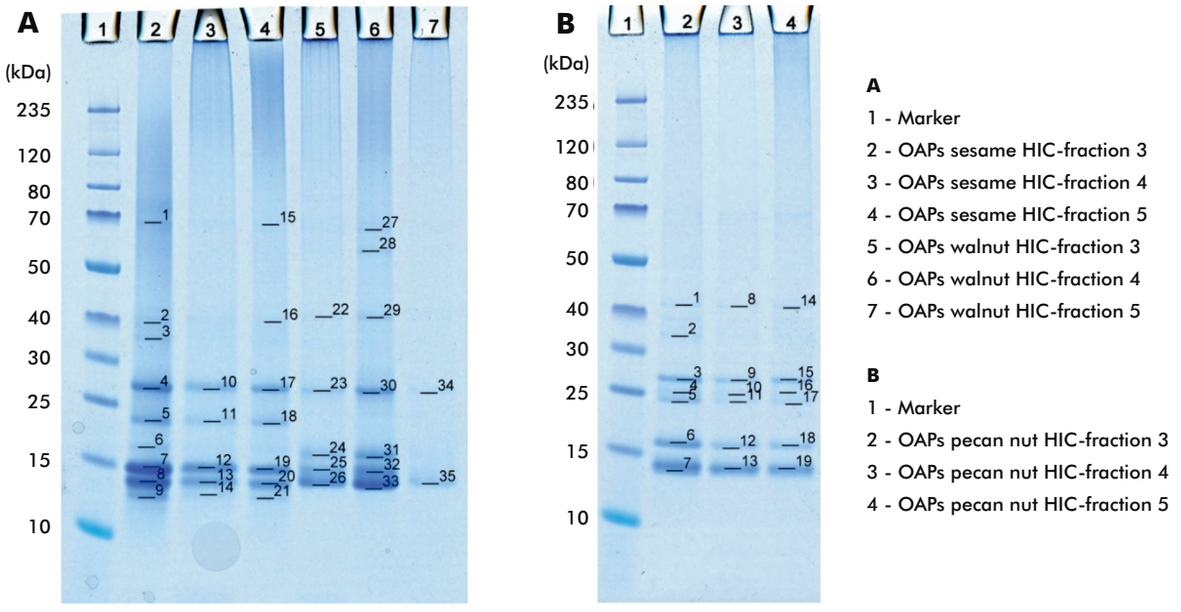


Figure S2: Gel images for mass spectrometry analyses of OAPs fractions
Gel images of elution fraction of OAPs from sesame, walnut and pecan nut after hydrophobic interaction chromatography used for mass spectrometry analyses

Table S1: *The native sesame OAPs fractions were analysed by mass spectrometry using peptide mass fingerprint. The table shows the identified proteins in these fractions, italic: MS/MS analysis*

Band	Protein (Accession Number)	Cut-Off	Protein Score	Sequence Coverage [%]	Molecular Mass [kDa]
Sesame OAPs: HIC-Elution-Fraction 3					
1	uncharacterised protein LOC105177100 (NP_001292915.1)	*49*	*45*	*10*	17.4
	15 kDa oleosin (AAD42942.1)	*49*	*23*	*6*	15.2
	Predicted: oleosin 1-like (XP_011076526.1)	*49*	*23*	*4*	24.0
2	11-beta-hydroxysteroid dehydrogenase-like 5 (NP_001291330.1)	79/*50*	165/*55*	54/*4*	41.3
3	11-beta-hydroxysteroid dehydrogenase-1B-like (NP_001291322.1)	79/*50*	123/*31*	50/*2*	39.7
	11-beta-hydroxysteroid dehydrogenase-like 5 (NP_001291330.1)	79/*50*	87/*20*	41/*4*	41.3
4	peroxygenase (NP_001291323.1)	79/*50*	76/*115*	40/*23*	27.8
5	PREDICTED: legumin-B-like (XP_011083024)	79/*50*	93/*59*	35/*8*	52.1
	peroxygenase (NP_001291323.1)	79/*50*	80/*81*	32/*10*	27.8
	11S globulin precursor isoform 4 (ABB60055.1)	79/*50*	80/*59*	32/*8*	53.0
6	PREDICTED: legumin-B-like (XP_011083024)	79	72	33	52.1
	11S globulin precursor isoform 4 (ABB60055.1)	79	60	28	53.0
	15 kDa oleosin (AAD42942.1)	*50*	*22*	*6*	15.2
	PREDICTED: oleosin 1-like (XP_011076526.1)	*50*	*22*	*4*	24.0
7	15 kDa oleosin (AAD42942.1)	*50*	*61*	*6*	15.2
	PREDICTED: oleosin 1-like (XP_011076526.1)	*50*	*61*	*4*	24.0

Band	Protein (Accession Number)	Cut-Off	Protein Score	Sequence Coverage [%]	Molecular Mass [kDa]
8	uncharacterised protein LOC105177100 (NP_001292915.1)	*51*	*43*	*10*	17.4
9	uncharacterised protein LOC105177100 (NP_001292915.1)	49	40	10	17.4
	15 kDa oleosin (AAD42942.1)	49	35	6	15.2
	PREDICTED: oleosin 1-like (XP_011076526.1)	49	35	4	24.0
	PREDICTED: oleosin 16.4 kDa (XP_011097414.1)	49	20	10	17.4
Sesame OAPs: HIC-Elution-Fraction 4					
10	Peroxygenase (NP_001291323.1)	79/*49*	108/*27*	41/*8*	27.8
11	Peroxygenase (NP_001291323.1)	*51*	*81*	*10*	27.8
12	uncharacterised protein LOC105177100 (NP_001292915.1)	79	57	39	17.4
	15 kDa oleosin (AAD42942.1)	*50*	*47*	*6*	15.2
	PREDICTED: oleosin 1-like (XP_011076526.1)	*50*	*47*	*4*	24.0
13	uncharacterised protein LOC105177100 (NP_001292915.1)	*51*	*24*	*10*	17.4
	PREDICTED: oleosin 1-like (XP_011081346.1)	*51*	*22*	*7*	14.8
	15 kDa oleosin (AAD42942.1)	*51*	*21*	*6*	15.2
	PREDICTED: oleosin 1-like (XP_011076526.1)	*51*	*21*	*4*	24.0
14	PREDICTED: DDB1- and CUL4-associated factor 8 (XP_011089371.1)	79	58	22	54.1
	uncharacterised protein LOC105177100 (NP_001292915.1)	49	15	10	17.4

Band	Protein (Accession Number)	Cut-Off	Protein Score	Sequence Coverage [%]	Molecular Mass [kDa]
Sesame OAPs: HIC-Elution-Fraction 5					
15	-	-	-	-	-
16	11-beta-hydroxysteroid dehydrogenase-like-5 (NP_001291330.1)	79/*50*	190/*70*	53/*7*	41.3
17	Peroxygenase (NP_001291323.1)	79/*50*	119/*73*	53/*13*	27.8
18	Peroxygenase (NP_001291323.1)	79/*50*	80/*63*	*27*/*10*	27.8
19	uncharacterised protein LOC105177100 (NP_001292915.1)	*50*	*67*	*10*	17.4
	15 kDa oleosin (AAD42942.1)	*50*	*52*	*6*	15.2
	PREDICTED: oleosin 1-like (XP_011076526.1)	*50*	*52*	*4*	24.0
20	PREDICTED: oleosin 1-like (XP_011081346.1)	51	46	7	14.8
	uncharacterised protein LOC105177100 (NP_001292915.1)	51	43	10	17.4
21	PREDICTED: DDB1- and CUL4-associated factor 8 (XP_011089371.1)	79	91	36	54.1
	uncharacterised protein LOC105177100 (NP_001292915.1)	*49*	*17*	*10*	17.4

Table S2: *The native walnut OAPs fractions were analysed by mass spectrometry using peptide mass fingerprint. The table shows the identified proteins in these fractions.*

Band	Protein (Accession Number)	Cut-Off	Protein Score	Sequence Coverage [%]	Molecular Mass [kDa]
Walnut OAPs: HIC-Elution-Fraction 3					
22	-	-	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	-	-	-
26	Oleosin (AET74077.1)	79	40	38	14.7
Walnut OAPs: HIC-Elution-Fraction 4					
27	-	-	-	-	-
28	-	-	-	-	-
29	-	-	-	-	-
30	-	-	-	-	-
31	-	-	-	-	-
32	-	-	-	-	-
33	Oleosin (AET74077.1)	79	48	38	14.7
Walnut OAPs: HIC-Elution-Fraction 5					
34	-	-	-	-	-
16	Oleosin (AET74077.1)	79	53	35	14.7

Table S3: *The native pecan nut OAPs fractions were analysed by mass spectrometry using peptide mass fingerprint. The table shows the identified proteins in these fractions.*

Band	Protein (Accession Number)	Cut-Off	Protein Score	Sequence Coverage [%]	Molecular Mass [kDa]
Pecan nut OAPs: HIC-Elution-Fraction 3					
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
Pecan OAPs: HIC-Elution-Fraction 4					
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	-	-
11	-	-	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
Pecan nut OAPs: HIC-Elution-Fraction 5					
14	-	-	-	-	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-

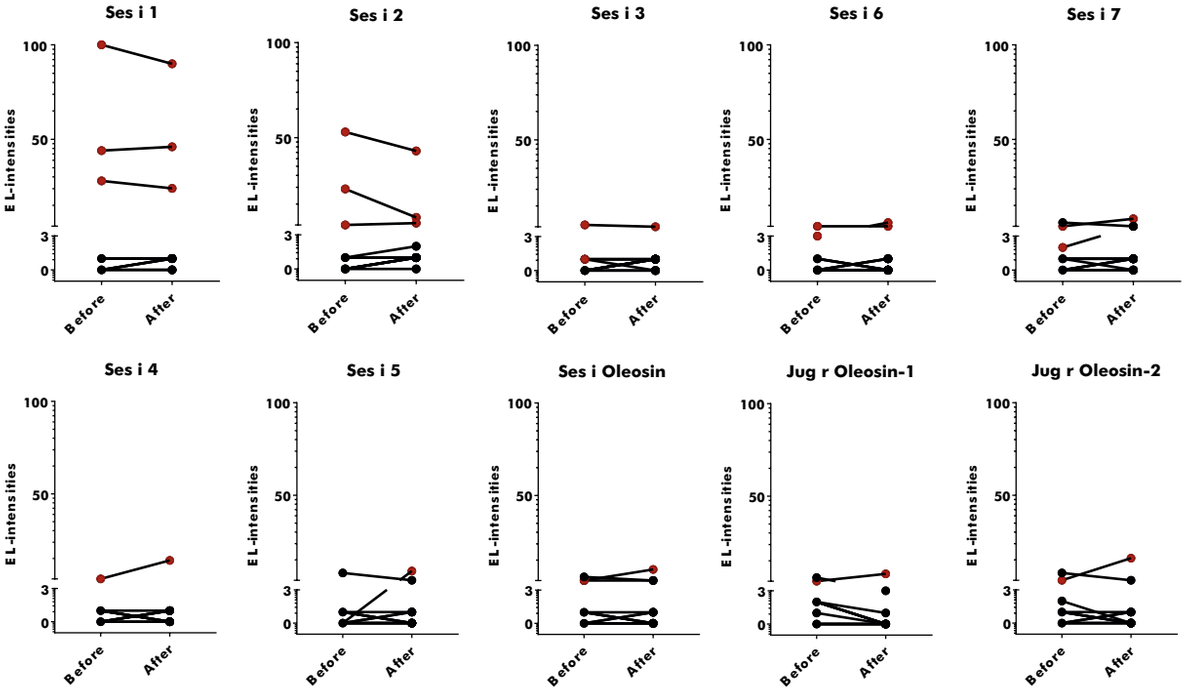


Figure S3: IgG depletion did not influence the recognition of sesame oleosins by sIgE
Sera with high sIgG levels to oleosins (G1a_4, G1a_12, G1a_13 and G2_8) showed no increase in sIgE levels to sesame components or oleosins while sera with low sIgG levels but IgE sensitisation to sesame (G1b_1, G1b_15, G1b_17, G2_9 and G2_11) showed a scarce decrease in sIgE levels after IgG depletion, confirming no depletion of IgE during IgG depletion. One serum positive for sesame oleosins, G1b_15, displayed an increase in sIgE levels to oleosins although no sIgG to these components were detected. Sera with sIgE levels greater than an EUROLINE (EL-) intensity of 3 were marked accordingly to their study group (green: group 1a – sesame allergic without sensitisation, red: groub 1b – sesame allergic with sensitisation, black: group 2 – sesame tolerant with sensitisation)

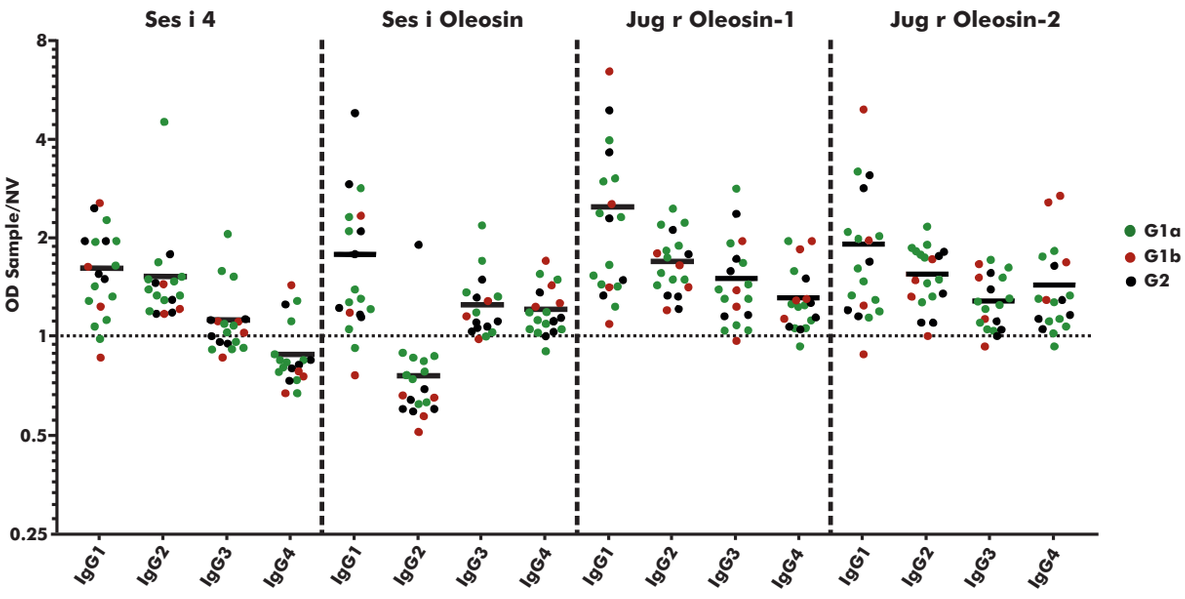


Figure S4: Subtype analysis of sera with elevated sIgG levels for Ses i 4, Ses i Ol, Jug r Ol-1 and Jug r Ol-2

Detection of the IgG subtype bound to Ses i 4, Ses i Oleosin, Jug r Oleosin-1 and Jug r Oleosin-2 from serum with specific IgG to oleosins (EUROLINE-intensities >8); Scatter-blot with the measured OD value ratios (sample/negative value [NV]) divided by subtype for each oleosin. Horizontal lines mark the mean value. Green: Group 1a - sesame allergic patients without detectable sIgE sensitisation; Red: Group 1b - sesame allergic patients with sIgE sensitisation; Black: Group 2 - sesame tolerant patients with sIgE sensitisation

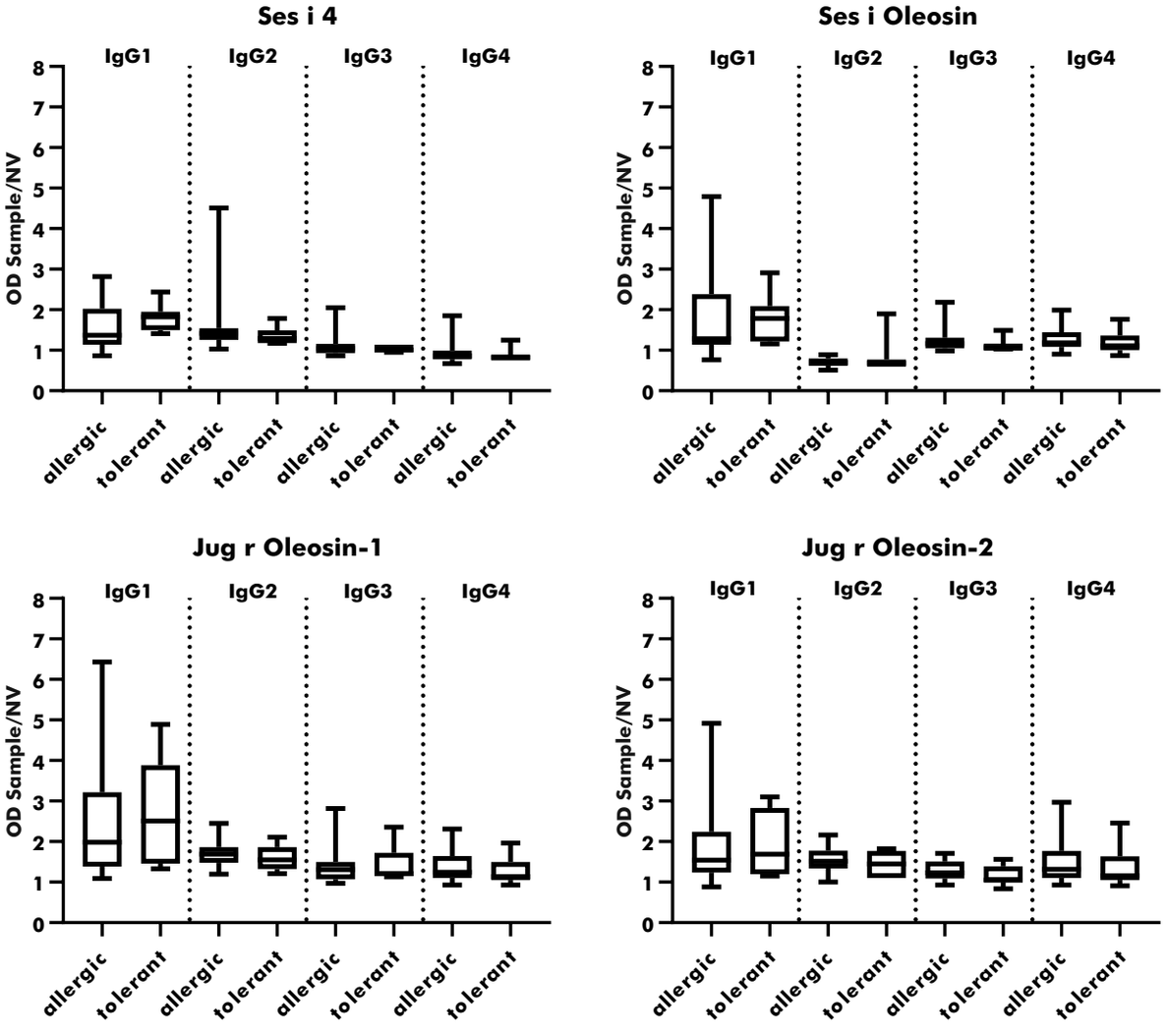


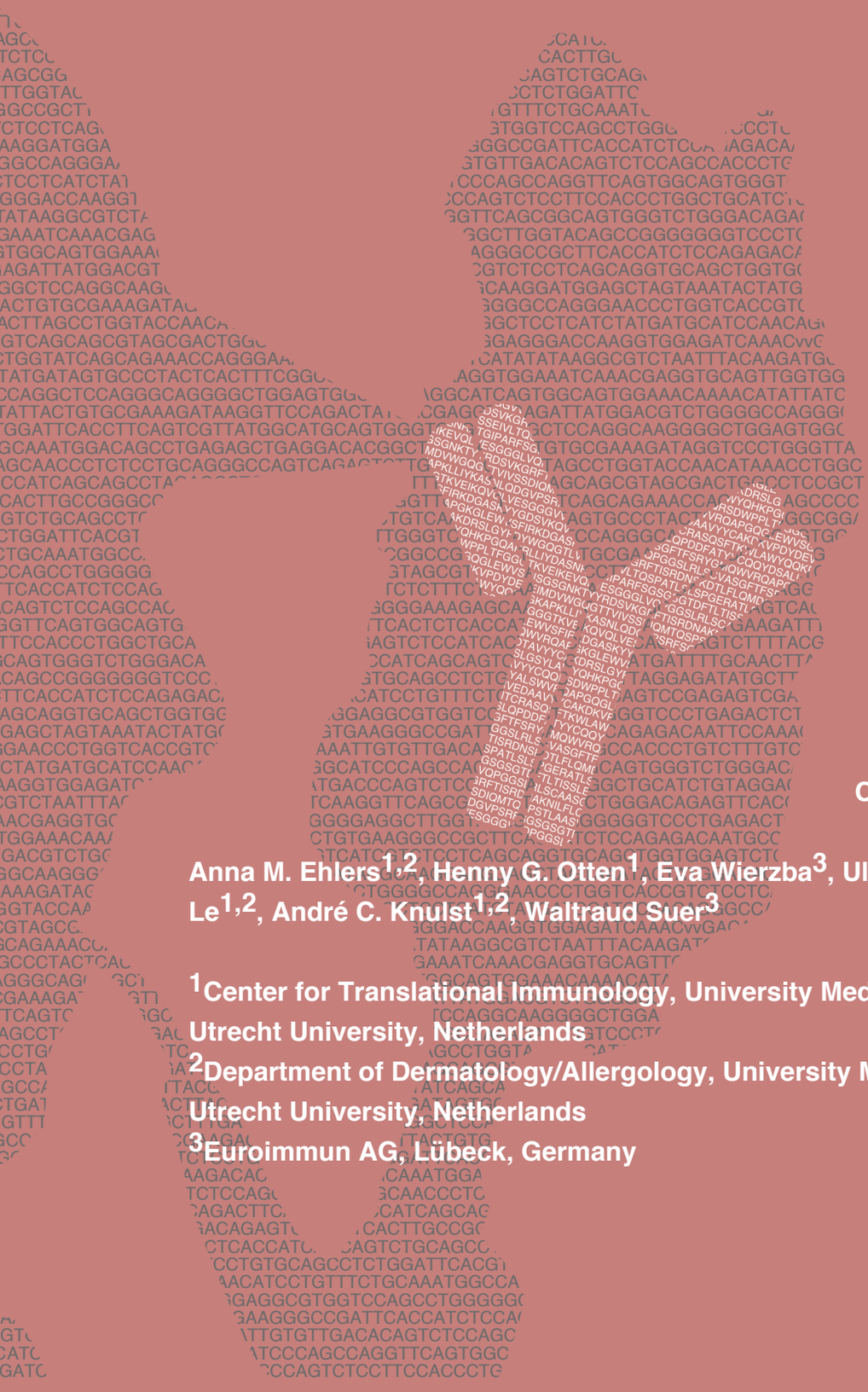
Figure S5: IgG subtype comparison between allergic and tolerant patients
Detection of the IgG subtype bound to Ses i 4, Ses i Oleosin, Jug r Oleosin-1 and Jug r Oleosin-2 from serum with specific IgG to oleosins (EUROLINE-intensities >8); the data were separated by allergic and tolerant patients, IgG subtype and protein (oleosin)

Part II

Preventing „false-positive“ sIgE testing: clinically irrelevant sensitisation


4

Detection of specific IgE against linear epitopes from Gal d 1 has additional value in diagnosing hen's egg allergy in adults



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Anna M. Ehlers[1,2], Henny G. Otten[1], Eva Wierzba[3], Ulrike Flügge[3], Thuy-My Le[1,2], André C. Knulst[1,2], Waltraud Suer[3]

[1]Center for Translational Immunology, University Medical Center Utrecht, Utrecht University, Netherlands

[2]Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Netherlands

[3]Euroimmun AG, Lübeck, Germany

Abstract

Background

Although hen's egg allergy is more prevalent in children, up to 0.6% of adults from different European countries suffers from a persistent or newly-onset hen's egg allergy, making accurate diagnosis in adults necessary. However, sensitisation to hen's egg extracts, components and linear epitopes are solely studied in children.

Methods

Hen's egg allergic (n=16) and tolerant (n=20) adults were selected by sensitisation towards recombinant components rGal d 1 and/or 3. Sensitisation profiles towards egg white and yolk extract and the native components Gal d 1, 2, 3 and 4 were respectively evaluated with the ImmunoCAP or the EUROLINE system. Characterisation of linear epitopes was performed with a peptide microarray containing 15mer peptides representing the entire sequence of mature Gal d 1 and 3.

Results

Overall, sIgE titres against hen's egg extracts and single components overlapped largely between allergic and tolerant adults. Although the median sIgE/sIgG4 ratio to Gal d 1 was increased in allergic adults, the range was comparable between both groups. Clinically relevant sensitisation to Gal d 1 was confirmed by sIgE binding to the linear epitopes aa 30-41, aa 39-50 or aa 84-95 in 6/13 allergic adults, mainly suffering from objective symptoms. In comparison, these epitopes were recognized by 1/15 tolerant patient. Only a few linear epitopes were detected for Gal d 3, suggesting a greater importance of conformational epitopes for the recognition of Gal d 3.

Conclusion and Clinical Relevance

Specific IgE binding to linear epitopes of Gal d 1 is highly specific in identifying hen's egg allergic adults with objective symptoms.

Introduction

Hen's egg is known as a major cause of food allergic reactions in children and this allergy is often outgrown by the age of five. Nevertheless, the prevalence of hen's egg allergy in adults average 0.02 to 0.6% across European countries[1,2]. Although hen's egg allergy in adulthood is predominantly a persistent allergy developed in childhood, it can also be newly developed later in life[3]. In a study conducted in the US, 29% of all hen's egg allergic adults suffered from an adult-onset allergy[4].

Diagnosis of hen's egg allergy is comprised of anamnesis, skin prick test, measuring sIgE and food challenges. Food challenges are the gold standard, but they are burdensome, expensive and require dedicated hospital facilities and personnel. To avoid or replace food challenges, intensive research has been performed to improve the diagnostic value of sIgE measurements in hen's egg allergic children. In a systematic review, an evaluation of sIgE measurements towards egg white extract in children ranging from infants to adolescents showed an overall sensitivity of 0.93, but only a specificity of 0.49[5].

Component-resolved diagnostics improved the accuracy of sIgE measurement in several food allergies[6]. Major allergenic components of hen's egg white, which are responsible for most of its allergenicity, are ovomucoid (Gal d 1), a thermo-stable allergen, ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4). Ovomucoid, the major allergen of egg white, is by far the most studied component in relation to hen's egg allergy in children and although ovomucoid is classified as a prognostic marker for persistent hen's egg allergy, its superior role compared to egg white extract has been debated[7].

Patients' sera contain polyclonal IgE antibodies recognising a broad range of epitopes comprised of either sequential residues of the amino acid sequence (linear) or amino acids closely located upon folding (conformational). Epitope mapping approaches aim to identify clinically relevant epitopes which are undetectable by measuring sIgE against extracts or full-length single components. In hen's egg allergy, linear epitope mapping of Gal d 1 identified epitopes (aa 1-10, aa 11-20, aa 47-56 and aa 113-122) exclusively recognised by children with persistent hen's egg allergy[8]. Comparable allergenic parts (aa 1-10, aa 11-20 and aa 47-56) were described as immunodominant linear epitopes by several other studies[9–11].

So far, the impact of sIgE titres to hen's egg components and sIgE binding to their linear epitopes on discriminating between clinically relevant and irrelevant sensitisation is poorly studied in hen's egg allergic and tolerant adults. To this end, we evaluated sensitisation patterns and sIgE titres to hen's egg components (Gal d 1, 2, 3 and 4) in allergic and tolerant, but sensitised adults. Since Gal d 1 is known as the most important single component for diagnosing hen's egg allergy in children, recognition of linear

epitopes derived from Gal d 1 was evaluated by peptide chip analysis. Since the role of Gal d 2 is controversially discussed[7], we decided to additionally map the linear epitopes of Gal d 3, another major egg white allergen of which little information is known so far.

Methods

Patient selection

Patients (n=35) sensitised to at least one of the recombinant hen's egg components rGal d 1 and rGal d 3, examined in 121 patients with hen's egg related sensitisation (SPT, ImmunoCAP, ISAC) by Western Blot, were retrospectively selected from patients who visited the Dermatology/Allergology outpatient clinic of the University Medical Center (UMC) Utrecht between 2008 and 2018. These patients were divided into (a) hen's egg allergic (n=16) and (b) hen's egg tolerant (n=20) patients based on either double-blind placebo-controlled food challenge (DBPCFC) with heated egg or convincing history confirmed by a trained physician (challenged: 44% allergic group, 29% tolerant group). Convincing history was defined as immediate symptoms including oral allergy syndrome, skin reactions, gastrointestinal, respiratory, or cardiovascular symptoms and an onset within 2 hours after ingestion. Gastrointestinal symptoms had to be combined with at least one additional immediate type symptom.
For the epitope discovery, sera from 13 allergic (8 suffering from objective symptoms) and 15 tolerant patients were applied on the peptide chip. Ethical approval (number 18-428) was acquired from the biobank committee of the UMC Utrecht.

Heterologous expression of hen's egg components

The mature hen's egg components Gal d 1 (accession number: P01005) and Gal d 3 (accession number: P02789) were heterologously expressed as fusion proteins with N-terminal-His (6x)-tag in *E. coli* and purified as previously described[12,13]. All heterologously expressed proteins were purified by immobilised metal ion chromatography under denaturing conditions. Purified rGal d 1 and 3 were separated by gel electrophoresis and blotted onto a nitrocellulose membrane.

Determination of sIgE and sIgG4 sensitisation

Sensitisation to egg white and yolk extract was determined using the commercially available ImmunoCAP system and sIgE and sIgG4 sensitisation to the native components Gal d 1, 2, 3 and 4 was measured using the EUROLINE immunoblot strip

"Paediatrics' 1" (DP 3812-1601-1 E, EUROIMMUN AG, Germany) according to manufacturer's instructions. Briefly, the immunoblots were manually incubated overnight at room temperature with serum diluted 1:11 (IgE) or 1:51 (IgG4) in working strength universal buffer (WSUB). After extensive washing with WSUB, bound IgE and IgG4 antibodies were detected with α-human IgE or IgG4-conjugate coupled with alkaline phosphatase. Upon another extensive washing step, visualisation was provided by applying nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate substrate for ten minutes and specific IgE levels were evaluated as EUROLINE (EL)-intensities and expressed as response units. Specific IgE binding to heterologously expressed hen's egg components were determined under the same conditions.

Microarray design

A microarray with synthetic 15mer peptides, comprising the sequence of the mature Gal d 1 (accession number: P01005) and Gal d 3 (accession number: P02789) (offset=3 due to limited space), was commercially obtained (PEPperPRINT). The peptide length of 15 amino acids was in accordance with the experience of PEPperPRINT to provide sufficient sensitivity without significant formation of secondary structures. All peptides were printed in triplicates with a linker consisting of 2 ß-alanine and one aspartic acid. This linker was chosen to circumvent the binding of negatively charged fluorescent dyes to positively charged amino acids which are close to the array surface.

Microarray design

The microarray incubation was performed as previously described[14]. Briefly, patient sera were diluted 1:4 in WSUB and incubated overnight. For detecting bound specific IgE and IgG4, a biotinylated α-IgE antibody (clone MHE-18 1:5000, BioLegend) and simultaneously a biotinylated α-human IgG4 coupled with Neutravidin DyLight 680 (clone HP6025, 1:5000, Southern Biotech) were applied on the microarray and incubated for 60 min at room temperature. Bound biotinylated human α-IgE antibodies were visualised by adding Neutravidin DyLight 800 (1:5000, Thermo Fisher) for 60 min at room temperature. After extensive washing and drying, the microarray slides were scanned at a wavelength of 700 nm for IgG4 and 800 nm for IgE (intensity: 8.5) and the focus was set to 0.8 mm and the resolution to 21 µm.

Microarray evaluation

For data evaluation, the fluorescent signals for each peptide were obtained using the Pepslide Analyzer Software (SICASYS) with the fixed-spot adjustment and the logarithmic signal-to-noise ratios (S) were computed according to the following quotation:

$$S_i = \log_2 \cdot \frac{Total\ fluorescene\ (Peptide)}{Background\ Flourescence\ (Peptide)}$$

For normalisation, the S-values were compared to the S-values of blank spots, resulting in z-scores defined as:

$$Z_i = \frac{S_i - Median\ (SBlank)}{Median\ Deviation\ (SBlank)}$$

Epitopes were defined as recognition of 2-4 contiguous peptides with a median z-score $\geq$3.0 and the amino acid residues were counted based on the amino acid sequence without signal peptide.

Determination of surfaced exposed epitopes

Surface exposed residues of an epitope were determined by submitting the 3D structure (Gal d 3, PDB ID: 1OVT) to the http://curie.utmb.edu/getarea.html interface[15]. Under the conditions (default settings) as radius of the water probe set to 1.4 and no gradient in calculations, the algorithm calculates the probability of each residue to be solvent accessible. For an epitope, at least 25% of its residues must have a greater probability than 50% to be solvent accessible for calling this epitope „surface-exposed“. The definition was confirmed by mapping the linear epitopes onto the 3D structure of Gal d 3 (pdb: 1OVT) using PyMol 1.3 (Schrödinger, Inc, US). The corresponding images are shown in Suppl. Figure S1.

Statistical analyses

Statistical differences between hen’s egg allergic and tolerant adults regarding their sensitisation profiles were evaluated with the non-parametric Mann-Whitney-U-test and visualised by GraphPad Prism 8.3. For peptides and epitopes derived from Gal d 1, their recognition by IgE was evaluated by principle component analyses in R. Heat maps were generated in R using the ‘ComplexHeatmap’ package[16].

Results

Patient characteristics

Patients sensitised to the recombinant components rGal d 1 and/ or rGal d 3 were divided into (a) allergic (75% female) and (b) tolerant patients (52% female) based on food challenge outcome or convincing history. Patients with subjective symptoms were more often diagnosed by a food challenge (4/6: 67%) compared with patients with objective symptoms (3/7: 43%), reducing at least the risk of misclassification. Allergic patients showed a median age of 25 and were overall younger than the tolerant patients with a median age of 28, although the age range was comparable (p=0.63). Even though the majority of patients were co-sensitised to nGal d 1 and nGal d 3, one allergic and one tolerant patient were mono-sensitised to nGal d 1 and one tolerant patient was mono-sensitised to nGal d 3. Interestingly, up to 94% of all included patients, irrespective of allergy or tolerance, suffered from atopic dermatitis. Besides, more than 60% of all included patients experienced symptoms related to allergic asthma (allergic 63%, tolerant 75%) and allergic rhinitis (allergic 81%, tolerant 60%). All characteristics are shown in Table 1.

Table 1: *Patient characteristics and sensitisation data*

	Allergic	Tolerant
Number [n]	16	19
Age (median [IQR])	25 [19-65]	28 [19-70]
Sex female [n, %]	12 [75%]	11 [58%]
Food challenge[A] [n, %]	7 [44%]	5 [26%}
Symptoms [n, %]		
Subjective	6 [37%]	N/A
Objective	10 [63%]	N/A
No symptoms	N/A	19 [100%]
Sensitisation [median, range]		
ImmunoCAP egg white	7.0 kU/l [0.6-77 kU/l]	4.6 kU/l [0.5-55 kU/l]
ImmunoCAP egg yolk	0.9 kU/l [0-36 kU/l]	3.3 kU/l [0-19.4 kU/l]
EUROLINE Gal d 1[B]	67 RU [20-110 RU]	33 RU [0-105 RU]
EUROLINE Gal d 2[B]	81 RU [28-113 RU]	55 RU [6-115 RU]
EUROLINE Gal d 3[B]	44 RU [0-85 RU]	43 RU [0-117 RU]
EUROLINE Gal d 4[B]	0 RU [0-30 RU]	1 RU [0-28 RU]
Co-morbidities [n, %]		
Atopic dermatitis	15 [94%]	18 [95%]
Allergic asthma	10 [63%]	15 [79%]
Allergic rhinitis	13 [81%]	12 [63%]

[A] food challenges with heated hen's egg

[B] EUROLINE intensities expressed as response units (RU): < 3 = EAST-class 0; 3-6 = EAST-class 1; 7-15 = EAST-class 2; 16-30 = EAST-class 3; 31-50 = EAST-class 4; 51-100 = EAST-class 5; >100 = EAST-class 6

sIgE levels towards hen's egg extracts overlapped largely between allergic and tolerant but sensitised patients

All patients of this study were included based on their sensitisation to at least one heterologously expressed hen's egg component (rGal d 1 and/or 3) and hence, detectable sensitisation to hen's egg extracts was detected in all tested patients. Specific IgE titres towards egg white and yolk extract overlapped greatly between allergic and tolerant adults, resulting in low specificity even at increased cut-off levels (0.53 at 5 kU/l). Overall, tolerant patients tend to have lower sIgE titres to egg white extract than allergic patients (median 4.6 kU/l vs 7.0 kU/l). On the other hand, sIgE levels to yolk extract were even higher in tolerant patients (median 3.3 kU/l) compared with allergic patients (median 0.9 kU/l), suggesting greater relevance of hen's egg white proteins compared to yolk-derived ones (Figure 1a). No statistically significant differences were observed for egg white and egg yolk extract.

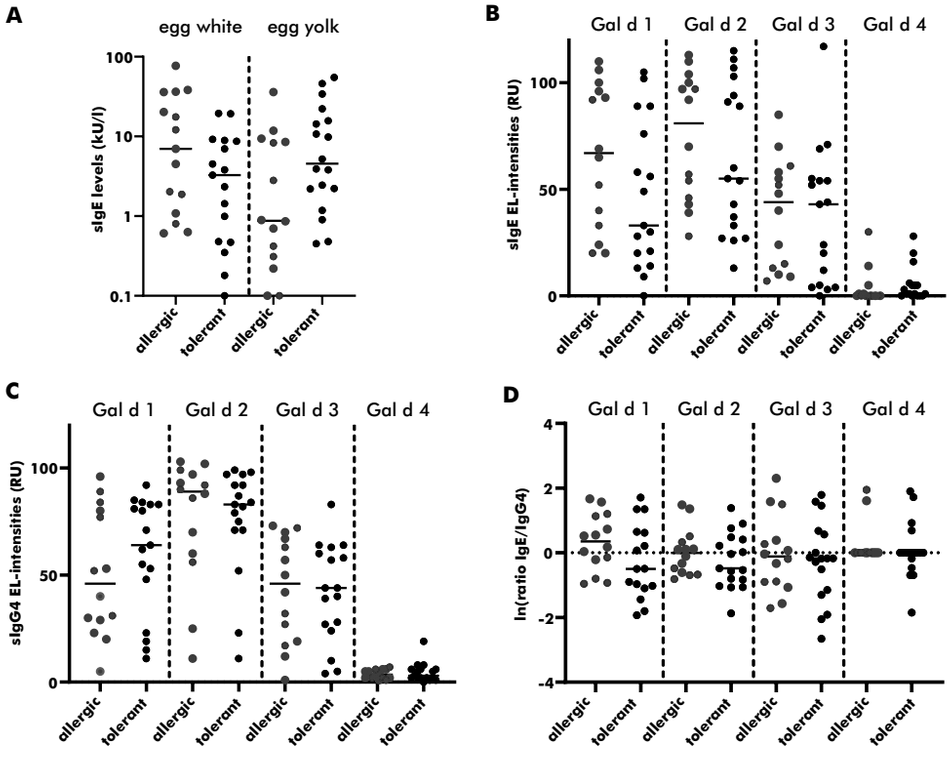

Figure 1: Sensitisation profiles of tolerant and allergic patients to hen´s egg extracts and components
A *sIgE levels to egg white and egg yolk extract measured with the ImmunoCAP system (kU/l) split by allergic and tolerant patients; represented with the median;* ***B*** *and* ***C*** *sIgE and sIgG4 levels to native hen's egg components Gal d 1, 2, 3 and 4 measured with the EUROLINE-immunoblot (EL-intensities, RU) split by allergic and tolerant patients; represented with the median;* ***D*** *Log-transformed sIgE/IgG4 ratios to native hen's egg components Gal d 1, 2, 3 and 4 resulting from the measures shown in* ***B*** *and* ***C****; represented with the median*

Gal d 1 sIgE/sIgG4 ratios were higher in allergic patients

As sIgE levels towards hen's egg white extract were on average higher in allergic than in tolerant patients, we next analysed the relevance of antibodies against native components present in egg white. Although allergic and tolerant patients showed similar sensitisation patterns towards nGal d 1, 2, 3 and 4 (Suppl. Figure S2), the tolerant group showed a decreased median of 33 EL-intensities towards nGal d 1 whilst allergic patients showed a median of 67 EL-intensities (Figure 1b). On the other hand, the tolerant group showed an increased median of sIgG4 levels against nGal d 1 (tolerant: 71 EL-intensities, allergic: 46 EL-intensities), resulting in a decreased median of sIgE/sIgG4 ratios for nGal d 1 (median:0.5 vs 0.35, Figure 1b to d) in tolerant compared to allergic adults. The range, however, was comparable between both groups. Regarding nGal d 2, 3 and 4, also no statistically significant differences were observed.

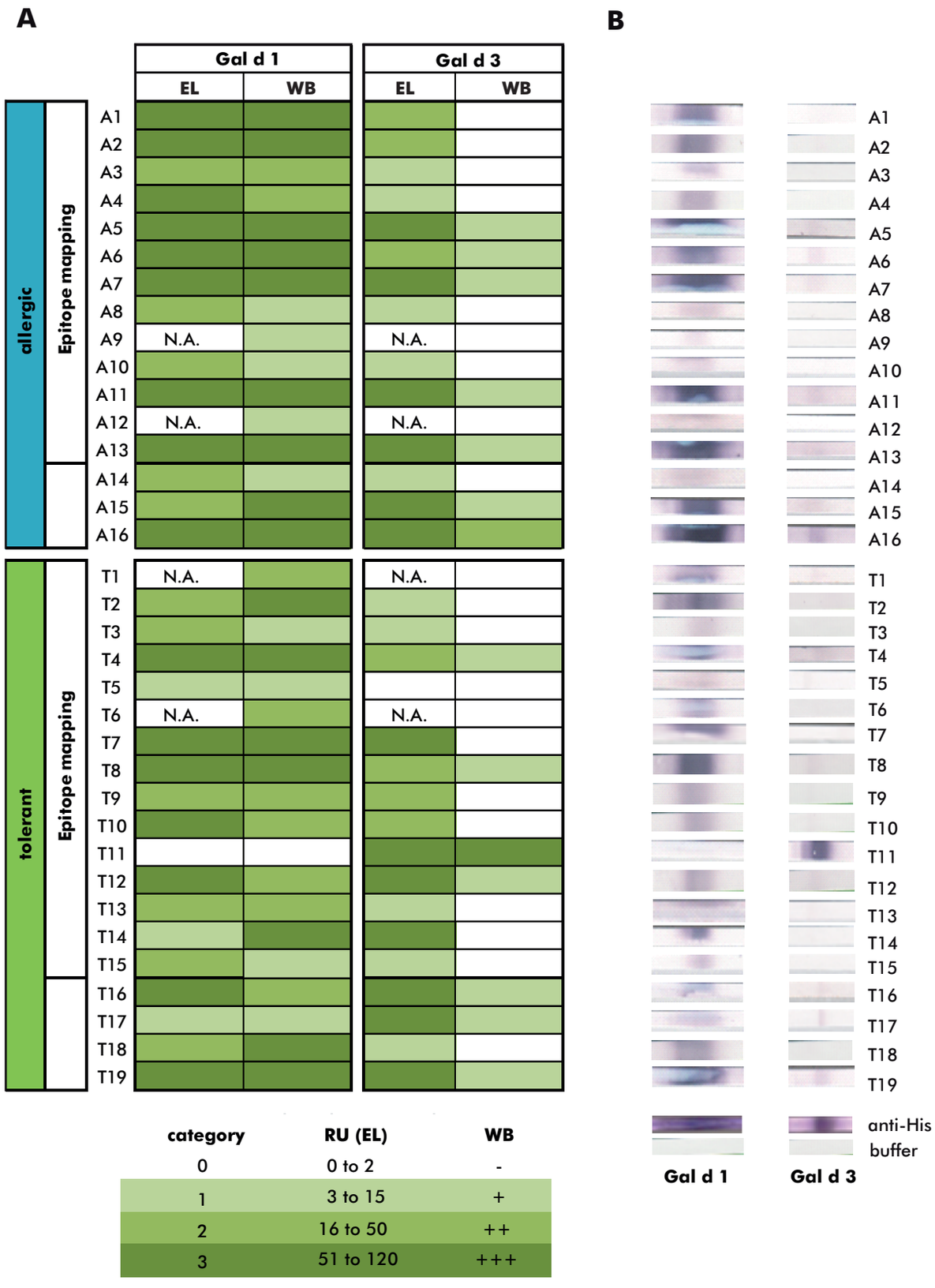

Figure 2: Linearization effects sIgE binding to Gal d 3
A *Comparison of IgE-binding capacity between native (EL) and linearised Gal d 1 and Gal d 3 (WB); the number of bound IgE is divided into categories defined as low (light green), moderate (green) and high (dark green);* ***B*** *original WB showing sIgE-binding to the recombinant components rGal d 1 and rGal d 3*

Binding of sIgE to Gal d 3 was greatly reduced upon linearisation

Although nGal d 3 was recognised by lower sIgE titres compared with nGal d 1 and nGal d 2, nGal d 3 was recognised by all allergic patients and 93% of tolerant patients. As shown in Figure 2, the binding capacity of nGal d 3 was greatly reduced upon linearisation (western blot (WB)) of the heterologously expressed rGal d 3 compared to the native component. For instance, patient A6 reacted strongly to the native form of nGal d 3 while no binding to the linearized form was observed, indicating the importance of conformational epitopes for the recognition of Gal d 3 by IgE. In contrast, the binding capacity of rGal d 1 was hardly affected by linearisation, pointing to the relevance of linear epitopes in recognising Gal d 1.

Linear epitope recognition of Gal d 1 confirms clinically relevant sensitisation in allergic patients with objective symptoms

As linearisation of rGal d 1 only marginally affected IgE binding, we next analysed linear epitope recognition with a peptide microarray. By means of principle component analysis with normalized values (z-scores) for each peptide, one allergic patient was separated from the remaining patients within the first dimension and four allergic patients were separated within the second dimension. This effect was even more dominant by repeating the principle component analysis with the mean z-scores of consensus sequences (epitopes) (Suppl. Figure S3). Principle component 1 (dimension 1) was dominantly driven by epitope aa 45-56 (97%) while principle component 2 was mainly driven by aa 30-41 (49%) and aa 84-95 (47%), having the greatest impact on discrimination. Accordingly to the principle component analysis, 6/13 allergic (46%) – 4 suffered from objective symptoms – and 1/15 (7%) tolerant patient recognised at least one of the epitopes aa 30-41, aa 39-50 and aa 84-95 as shown in Figure 3a, indicating the potential of these epitopes to confirm clinically relevant sensitisation to Gal d 1. Even though these findings have to be validated in a larger cohort, the recognition of these epitopes were highly specific (0.93) compared to sIgE titres to egg white extract (0.53 at 5 kU/l) or sIgE/sIgG4 ratios for Gal d 1 (0.63 at a ratio of 1).

Allergic patients who recognised aa 30-41 combined with aa 84-95, experienced respiratory symptoms (n=2) or severe OAS (n=1). The epitope aa 45-56, known to be exclusively recognised by children with persistent hen's egg allergy[8], was recognised by 5 allergic (38%) but also by 4 tolerant patients (27%). Moreover, most of the epitopes recognised by IgE were simultaneously recognised by IgG4 derived from the same individual. However, 3 allergic patients (23%), all suffering from mild subjective symptoms as manifested by food challenges with heated hen's egg, did not recognise any epitope by either IgE or IgG4, suggesting a higher relevance of conformational epitopes in recognising Gal d 1 by patients with mild symptoms.

Most patients with objective symptoms also recognised linear epitopes of Gal d 3

Additionally, a linear epitope mapping was also performed for Gal d 3. Regarding Gal d 3, only a small number of different epitopes (n=11) were recognised by IgE in relation to its molecular mass (78 kDa) as already indicated by the reduction in sIgE binding upon linearisation. In total, only nine different epitopes were recognised by six allergic patients while two epitopes were recognised by one tolerant patient (Figure 3b). These epitopes bound by IgE were mostly located on the surface of Gal d 3 and therefore easily accessible for antibody binding. Surface-exposed epitopes were defined as possessing $\geq$3 residues ($\geq$25%) facing the outside of the three-dimensional structure (indicated with red stars in Figure 3b). Three of the allergic patients and one tolerant recognised at least one surface-exposed epitope by sIgE. These three patients, all suffering from objective symptoms, were the same patients who recognised at least one of the linear epitopes, which confirmed clinically relevant sensitisation to Gal d 1. In contrast, IgG4 antibodies bound to a larger number (n=30) of epitopes, although most of them were just recognised by IgG4 from one to two individual patients except for the epitopes aa 396-407 and aa 483-494 not located on the protein surface. Taken together, sIgE binding to linear epitopes of Gal d 3 has no additional value compared to linear epitope recognition of Gal d 1.

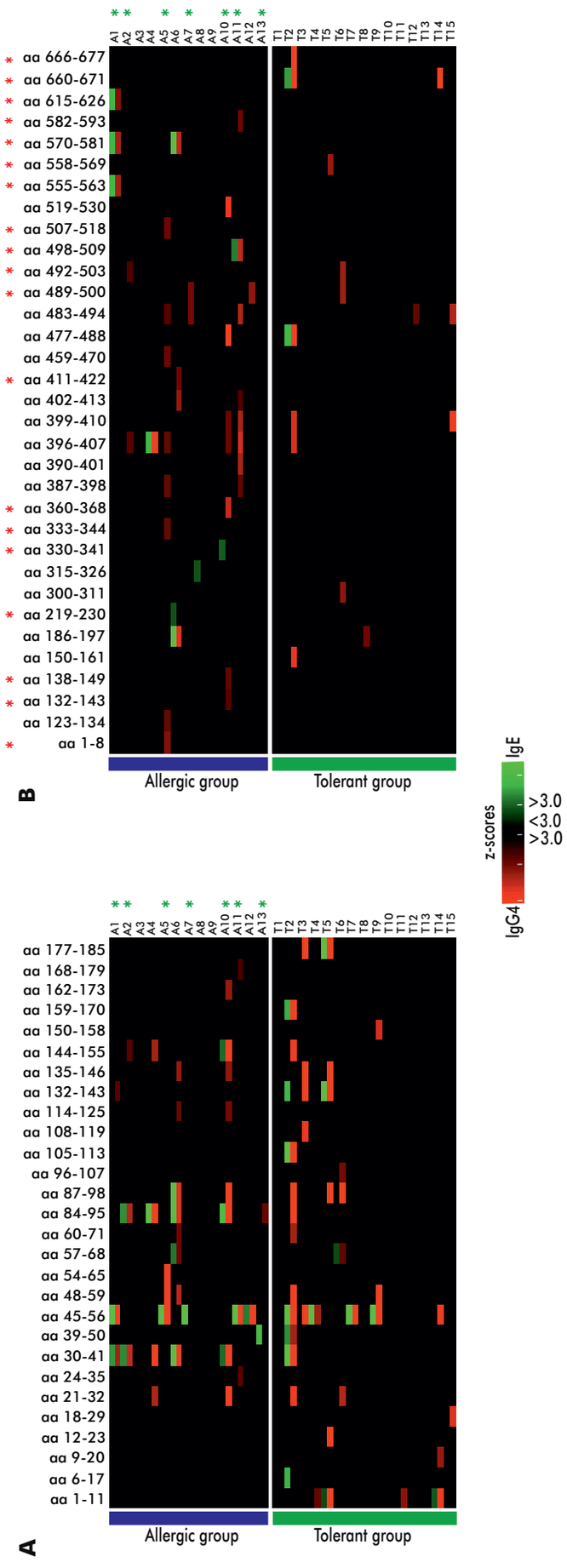

Figure 3: Epitope recognition pattern of Gal d 1 and 3
*Heatmap of **A** Gal d 1 and **B** Gal d 3 showing the IgE (green) and IgG4 (red) epitope recognition pattern of 13 hen's egg allergic and 15 hen's egg tolerant adults expressed as mean z-scores. Epitopes recognised by IgE and IgG4 from the same individual are shown in rows underneath each other. Surface-exposed epitopes of Gal d 3 are indicated with a red star and patients suffering from objective symptoms are highlighted with a green star.*

Discussion

So far, sensitisation to hen's egg extracts, components and linear epitopes is solely studied in children, although persistent and newly-onset hen's egg allergy do appear in adults with a prevalence of 0.02 to 0.6% across European countries[1,2]. In the present study, we showed great overlap in sIgE levels to hen's egg extracts or single components between allergic and tolerant, but sensitised adults. Clinically relevant sensitisation to Gal d 1 was confirmed by sIgE binding to the linear epitopes aa 30-41, aa 39-50 or aa 84-95 in 6 out of 13 hen's egg allergic adults, mainly suffering from objective symptoms. In contrast, patients with mild subjective symptoms showed no binding to linear epitopes of Gal d 1.

This is, to our knowledge, the first study focussing on sensitisation patterns in hen's egg allergic and tolerant adults. While largely overlapping sIgE titres to egg white extract were not able to clearly discriminate between allergy and tolerance in adults, the definition of clinically relevant cut-off levels appeared to be supportive in diagnosing raw or heated hen's egg allergy in children[5,17–19]. Despite lacking the complete information about heated egg tolerance in our study population, a similar tendency of higher sIgE titres to egg white extract was observed in allergic (median: 7.0 kU/l) compared with tolerant adults (median: 4.6 kU/l). Although our cohort selection based on sensitisation towards at least one heterologously expressed hen's egg component (rGal d 1 and/or rGal d 3) resulted in a certain selection bias, overlapping sIgE titres to Gal d 1 (up to 10 kU/l) and therefore to egg white extract, containing Gal d 1 as major allergen, were also described in hen's egg allergic and tolerant children[20].

While sIgE binding to Gal d 3 strongly decreased upon linearisation (SDS-PAGE under reducing conditions and western blotting), the sIgE binding to Gal d 1 was only slightly altered, pointing to the importance of linear epitopes for the recognition of Gal d 1. Nevertheless, 23% of the allergic (subjective symptoms) and 53% of the tolerant patients who showed sIgE binding to the linearised form of Gal d 1 did not show any IgE binding towards linear epitopes on the microarray, suggesting incomplete linearisation of Gal d 1 potentially due to reduced accessibility of disulphide bridges by reducing agents[21]. Incomplete linearisation and the lack of linear epitope recognition in a part of the patients pinpoint to the importance of conformational epitopes for the recognition of Gal d 1 in those patients. A similar observation was made by Martínez-Botas and colleagues where 34% of hen's egg allergic children strongly positive to Gal d 1 did not recognise any linear epitope by IgE[10], suggesting exclusive recognition of conformational epitopes in a subpopulation of hen's egg allergic patients.

The epitopes aa 30-41, aa 39-50 and aa 84-95 from Gal d 1 were mostly recognised by allergic patients (6/13 allergic vs 1/15 tolerant patients) who suffered from objective symptoms (4/7) upon hen's egg ingestion, confirming clinically relevant sensitisation to

Gal d 1 despite overlapping sIgE titres between allergic and tolerant adults. Although these epitopes were described independently in different studies with hen's egg allergic children[9,11,22], they did not belong to the so-called "informative" epitopes (aa 1-10, aa 11-20, aa 47-56 and aa 113–122) which showed great potential to predict persistent hen's egg allergy in children[8]. The epitope aa 47-56, however, was recognised by 38% of allergic vs 27% of tolerant adults (overall 32% of all patients) in the present study, suggesting divergent IgE specificities in adulthood compared to childhood. These differences may also be related to study design and inclusion criteria.

Notably, epitopes recognised by IgE were often simultaneously bound by IgG4 from the same individual irrespectively of their status, suggesting a clonal relationship between the IgE and IgG4 antibodies. A clonal relationship would imply that IgE producing B cells partially originate from $IgG4^+$ B cells or that some IgE^+ and $IgG4^+$ B cells share the same origin. Clonal analysis of allergic patients showed a predominately clonal relationship between $IgG1^+$ and IgE^+ B cells, suggesting that $IgG1^+$ B cells might be the shared origin[23]. Compared to children, this potential clonal relationship between IgE and IgG4 appeared to be less dominant, since IgE-binding overlapped only partially with the binding characteristics of IgG4[10]. This discrepancy between children and adults regarding potential clonal relationship between IgE and $IgG4^+$ B cells might indicate the alteration of the origin for IgE^+ and $IgG4^+$ B cells over time. However, more research is needed to confirm this hypothesis.

As a step forward, the promising results of the present study should be validated in a prospective cohort exclusively diagnosed by food challenge, minimising the risk of misclassification. Moreover, inclusion based on suspicion of egg allergy provides a broader population with more distinct sensitisation patterns such as mono-sensitisation to Gal d 2.

In conclusion, sIgE-binding to linear epitope of Gal d 1 (aa 30-41, aa 39-50 or aa 84-95) is highly specific to identify hen's egg allergic adults, mainly suffering from objective symptoms and may improve sIgE diagnostics as an additional tool to conventional testing using egg extracts and single allergen components.

Acknowledgments

We would like to thank B. Brix and M. Klinge for critical and fruitful discussions. Line blots and corresponding reagents were kindly provided by EUROIMMUN AG, Lübeck, Germany. This study was funded by a grant from the European Regional Development Fund of the European Union (TBI-V-1-098-E).

Informed consent statement

This study was carried out in accordance with the University Medical Center Utrecht, Biobank Regulations, which are in compliance with the applicable national and international laws and regulations. These regulations permit the use of 'residual material from diagnostic testing' for research, unless the patient objects (Article 8, 'no objection' procedure). None of the included patients objected the use of their serum. The protocol was approved by the Biobank Research Ethics Committee of the University Medical Center Utrecht under the protocol number 18-428.

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Supplementary

Detailed description of individual patients characteristics are available at the Online Repository of the Journal Clinical and Experimental Allergy (doi: 10.1111/cea.13730).

Table S1: *Identified epitopes of Gal d 1 in comparison to the literature*

Peptide	Residues[A]	IgE	IgG4	Publication IgE[B]
AEVDCSRFPNA	1-11	X	X	1,2,*4*
SRFPNATDKEGK	6-17	X		1,5
PNATDKEGKDVL	9-20	X		1,*4*,5
TDKEGKDVLVCN	12-23	X		2
DVLVCNKDLRPI	18-29	X		
VCNKDLRPICGT	21-32	X		
KDLRPICGTDGV	24-35	X		
CGTDGVTYTNDC	30-41	X	X	2,3,4
NDCLLCAYSIEF	39-50	X	X	3,4
AYSIEFGTNISK	45-56	X	X	1,*4*,5
IEFGTNISKEHD	48-59	X		
ISKEHDGECKET	54-65	X		2,3
EHDGECKETVPM	57-68	X	X	2,4
GECKETVPMNCS	60-71	X		
CSSYAN	71-75			3
DGKVMVLCNRA	80-90			3
MVLCNRAFNPVC	84-95	X	X	1,4
CNRAFNPVCGTD	87-98	X		
GTDGVTYDNECL	96-107	X		3,5
ECLLCAHKV	105-113	X	X	2,4
LCAHKVEQGASV	108-119	X		
EQGASVDKRHDG	114-125	X		*4*
KRHDGGCRKELAAV	121-134			2,3
AAVSVDCSEYPK	132-143	X	X	4
SVDCSEYPKPDC	135-146	X		
PDCTAEDRPLCG	144-155	X	X	
DRPLCGSDN	150-158	X		4
KTYGNKCNFCNA	159-170	X	X	3
GNKCNFCNAVVE	162-173	X		3
CNAVVESNGTLT	168-179	X		
TLTLSHFGK	177-185	X	X	1,3

[A] without signal sequence

[B] 1: Cooke *et al.* 1997; 2: Holen *et al.* 2001; 3: Mine and Zhang 2002; 4: Järvinen *et al.* 2007, italic = informative; 5: Martínez-Botas *et al.* 2013

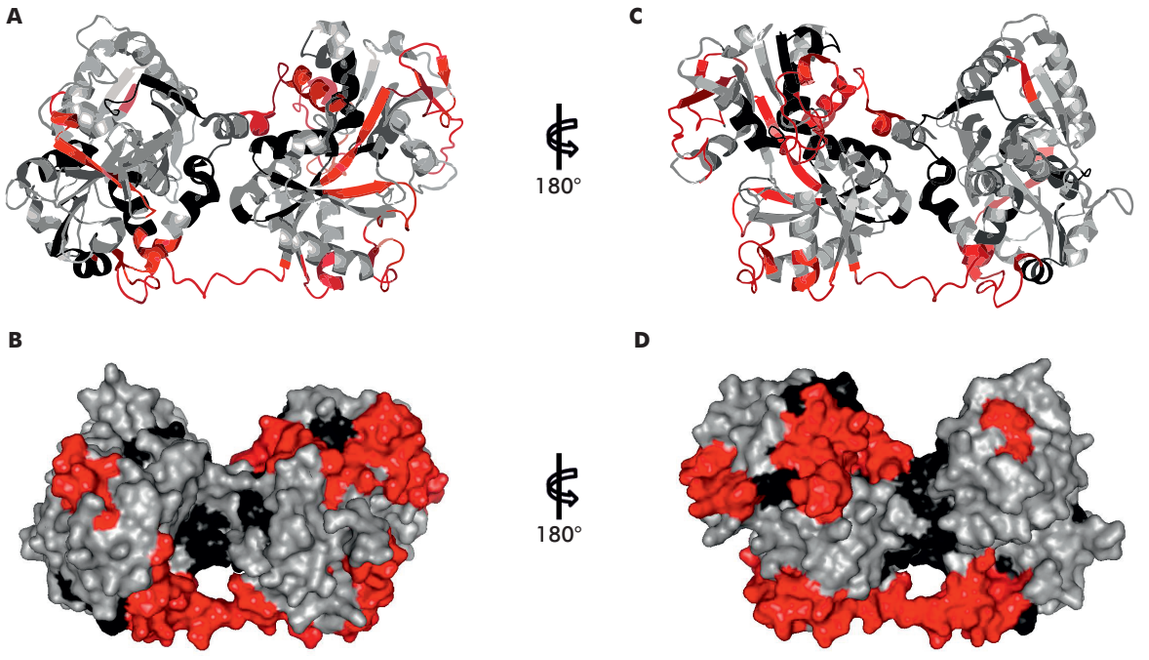

Figure S1: Mapping of linear epitopes onto the 3D structure of Gal d 3
The as surface-exposed (red) and non-surface exposed (black) defined epitopes were mapped onto the 3D structure of Gal d 3 (pdb: 1OVT); ***A*** *Cartoon view highlighting the dominant location of as surface-exposed defined epitopes in the loops of Gal d 3;* ***B*** *View on the solvent-accessible surface of Gal d 3;* ***C*** *Cartoon view as in A but turned for 180°;* ***D*** *View on the solvent-accessible surface of Gal d 3 as in B but turned for 180°*

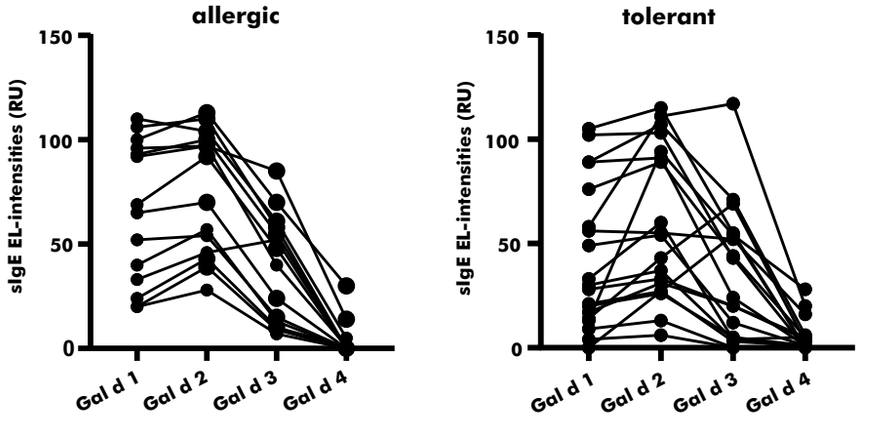

Figure S2: Patients' sensitisation profiles
Sensitisation profiles of hen's egg allergic and tolerant patients to the native hen's egg components Gal d 1, Gal d 2, Gal d 3 and Gal d 4 are separately shown.

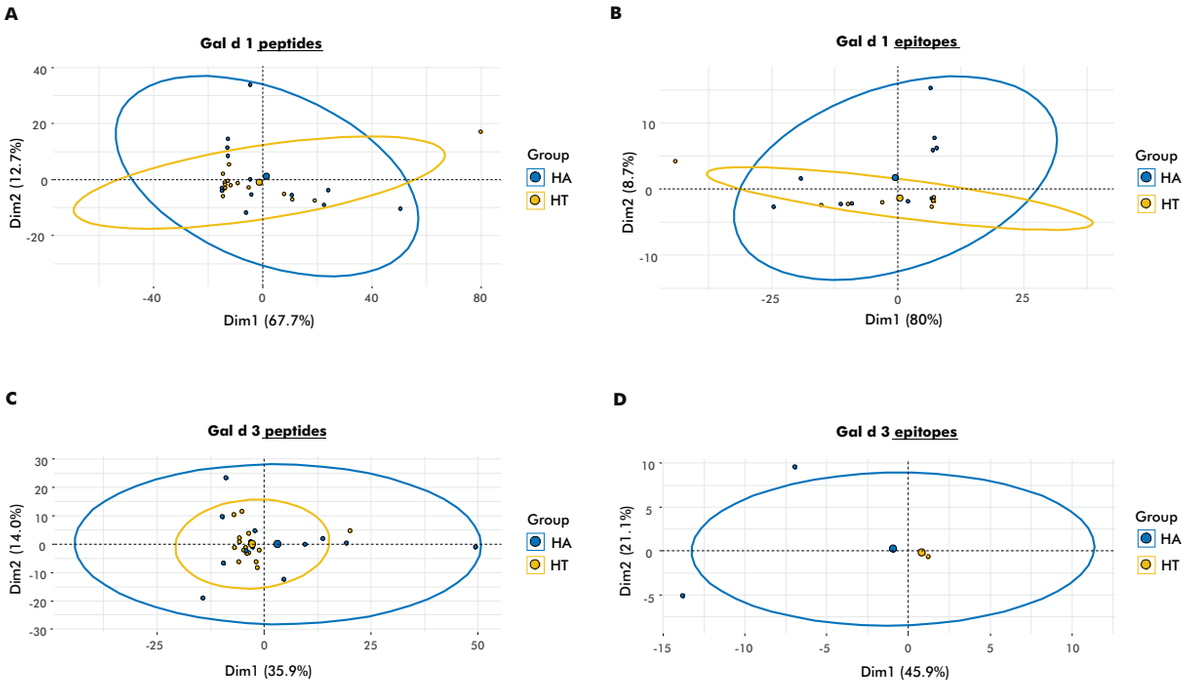

Figure S3: Principle component analysis

A *Principle component analysis of peptides derived from Gal d 1 recognised by IgE and* ***B*** *principle component analysis of epitopes derived from Gal d 1 recognised by IgE;* ***C*** *Principle component analysis of peptides derived from Gal d 3 recognised by IgE and* ***D*** *principle component analysis of epitopes derived from Gal d 3 recognised by IgE*


5

2S protein Ara h 7.0201 has unique epitopes compared to other Ara h 7 isoforms and is comparable to 2S proteins Ara h 2 and 6 in basophil degranulation capacity

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Simone M. Hayen[1,2], Anna M.Ehlers[1,2], Constance F. den Hartog Jager[1], Johan Garssen[3,4], Edward F. Knol[1,2], André C.Knulst[1,2], Waltraud Suer[5], Linette E. M. Willemsen[3], Henny G. Otten[2]

[1]Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[2]Laboratory of Translational Immunology, Utrecht University, University Medical Center Utrecht, Utrecht, The Netherlands

[3]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands

[4]Nutricia Research, Immunology, Utrecht, The Netherlands

[5]EUROIMMUN, Lübeck, German

Abstract

Background

Screening for specific IgE against 2S albumin proteins Ara h 2 and 6 has a good positive predictive value in diagnosing peanut allergy. From the third 2S albumin member Ara h 7, three isoforms have been identified. Their allergenicity has not been elucidated.

Objective

This study investigated the allergenicity of Ara h 7 isoforms compared to Ara h 2 and 6.

Methods

Sensitization of 15 DBPCFC-confirmed peanut allergic patients to recombinant Ara h 2.0201, 6.01 and isoforms of recombinant Ara h 7 was determined by IgE immunoblotting strips. A basophil activation test (BAT) was performed in nine patients to determine IgE-crosslinking capacities of the allergens. Sensitivity to the allergens was tested in five patients that were sensitized to at least one Ara h 7 isoform, by a concentration range in the BAT. 3D-prediction models and sequence alignments were used to visualize differences between isoforms and to predict allergenic epitope regions.

Results

Sensitization to Ara h 7.0201 was most frequent (80%) and showed to be equally potent as Ara h 2.0201 and 6.01 in inducing basophil degranulation. Sensitization to Ara h 7.0201 together with Ara h 2.0201 and/or 6.01 was observed, indicating the presence of unique epitopes compared to the other two isoforms. Differences between the three Ara h 7 isoforms were observed in C-terminal cysteine residues, pepsin and trypsin cleavage sites and three single amino acid substitutions.

Conclusion and Clinical relevance

The majority of peanut-allergic patients are sensitized to isoform Ara h 7.0201, which is functionally as active as Ara h 2.0201 and 6.01. Unique epitopes are most likely located in the C-terminus or an allergenic loop region which is a known allergenic epitope region for Ara h 2.0201 and 6.01. Due to its unique epitopes and allergenicity, it is an interesting candidate to improve the diagnostic accuracy for peanut allergy.

Introduction

It is estimated that approximately 11.4-13.1% of children (0-17 years) and 3.2-5.1% of adults (>18 years) in European countries are sensitized against at least one food allergen, based on detection of specific IgE in serum[1]. Most food allergies are IgE-mediated, and symptoms develop within minutes to a few hours after ingestion of the specific allergen. Among food allergies, peanut allergens are most frequent in eliciting a fatal food reaction, and it is estimated that 0.6% of the total population is affected by peanut allergy[2]. By determining specific IgE to recombinant or purified peanut proteins rather than crude peanut extract, component-resolved diagnostics (CRD) has proven to be a useful tool to improve diagnostic accuracy for peanut allergy[3]. However, the functionality of several potentially relevant allergens remains unknown, since their capacity to induce effector cell degranulation has never been tested in patient samples before.

To date, seventeen peanut allergens (Ara h 1-17) are known and most of them have been sequenced and cloned[3–6]. Previous research indicated that screening for specific IgE against 2S albumins Ara h 2 and 6 is to date most effective in diagnosing peanut allergy, since the majority of peanut-allergic patients have specific IgE against these allergens[6–9]. Ara h 2 and 6 are both proteins belonging to the conglutin family, and Ara h 6 has approximately 53% sequence identity with Ara h 2[5,6].

Besides determining specific IgE against Ara h 2 and 6 for diagnosing peanut allergy, a third 2S protein Ara h 7 is currently gaining attention as predictor for peanut allergy[10]. Although Ara h 7 makes up only 0.5% of total peanut protein[3], it is a storage protein and therefore considered an allergen with a potential strong diagnostic value[11]. In comparison, the abundance of Ara h 2 ranges between 5.9–9.3% of total peanut protein and for Ara h 6, this is 4-14%[3,12]. By phage display technology, isoforms Ara h 7.0101 and Ara h 7.0201 were previously identified as allergens, but only Ara h 7.0201 was identified in peanut-extract[5]. Ara h 7.0101 shares 42% sequence identity with Ara h 2 and 45% with Ara h 6, whereas Ara h 7.0201 shares 44% sequence identity with Ara h 2 and 52% with Ara h 6[5,13]. Besides these two isoforms of Ara h 7, a third isoform labelled Ara h 7.0301 shares 70% sequence identity with Ara h 7, and was also identified in peanut-extract[5,14]. Previous research indicated the presence of unique epitopes on these Ara h 7 isoforms, and therefore they may be relevant in peanut allergy diagnosis[10].

Since the functionality of Ara h 7 has not yet been studied extensively, the aim of this study was to identify whether the capacity of Ara h 7 to induce basophil degranulation using whole blood from peanut-allergic patients is similar to Ara h 2 and 6. In addition, the allergen recognition pattern of the three isoforms of Ara h 7 by peanut allergic patients was studied. Furthermore, in relation to their functionality, the amino acid sequence and a 3D-prediction protein model were used to predict epitopes or regions of Ara h 7 that are important in inducing basophil degranulation and allergenicity.

Materials and Methods

Study design and study population

Assessment of sensitization to 2S peanut allergens was performed with residual plasma of fifteen peanut-allergic patients that visited the outpatient clinic of Dermatology/Allergology at the University Medical Center Utrecht in 2015-2017 for clinical research. Table 1 shows data on gender, age and historical data on SPT, subjective and objective doses determined by DBPCFC and Müller score. Of these fifteen patients, nine random patients that were scheduled for visiting the UMC for clinical research were able to donate blood for the functional basophil activation test. Five random patients from the complete cohort that were sensitized to at least one Ara h 7 isoform were recruited for the concentration range BAT. Inclusion criteria consisted of a type I allergic reaction to peanut, confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC). Use of prednisone, other immunosuppressants or pregnancy were exclusion criteria. Informed consent was obtained of all patients prior to the study. The study was reviewed and approved by the Ethics Committee of the University Medical Center Utrecht (NL51606.041.15).

Expression and purification of crude peanut extract and recombinant allergens

Crude peanut extract (CPE) was obtained by blending raw peanuts, followed by extraction at room temperature with Tris/NaCL buffer (20 mM Tris, 150 mM NaCL, pH 7.2). Supernatant was filtered twice and diluted in 1x PBS to the appropriate concentration. Recombinant peanut allergens Ara h 2.0201 (Acc.no. Q6PSU2), Ara h 6.01 (Acc.no Q647G9), Ara h 7.0101 (Acc.no. Q9SQH1), Ara h 7.0201 (Acc.no. B4XID4) and Ara h 7.0301 (Acc. No. Q647G8) were provided by Euroimmun and produced as described previously[10,15].

Immunoblot

Immunoblots (Euroline, Euroimmun, Lübeck, Germany) were used to determine sensitization of fifteen patients to isoforms of recombinant peanut proteins of Ara h 2.0201, Ara h 6.01, Ara h 7.0101, Ara h 7.0201 and Ara h 7.0301. Immunoblots and reagents were kindly provided by Euroimmun and experiments were performed according to the manufacturer's instructions. In short, the Euroline strips were incubated on a rocking shaker overnight at RT with 100 µL of 1:11 diluted patient plasma in universal buffer. Binding of antibodies was visualized with an enzyme-labelled α-human IgE antibody in combination with substrate nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate. The results were evaluated with EuroLineScan software. The intensity of the bands was measured and an intensity level of 3 or higher was considered positive (arbitrary units). Line blot intensity values of 2 and lower were considered negative.

Basophil activation test

Whole heparinized blood was obtained from 9 out of 15 peanut-allergic patients and a BAT was performed. Blood samples were stimulated for 30 minutes with increasing concentrations or 1000 ng/mL of crude peanut extract (CPE), or separate recombinant peanut allergens in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 1 ng/mL IL-3 (R and D Systems). Control samples for the basophil activation test were rVP40 (recombinant VP40, control protein) and buffer. Leukocytes were stained with an antibody cocktail of CD45-PO (Life Technologies), CD123-FITC (Biolegend), HLA-DR-PB (Biolegend), CD63-PE (Monosan), CD41-PE-Cy7 (Beckman coulter), CD203c (Biolegend). Basophils were defined as $CD45^+$, $CD203c^+$, $CD123^+$, and $HLA\text{-}DR^-$,$CD41^-$, and degranulation was quantified by determining the surface expression of CD63. Results were expressed as percentage of CD63 bright basophils. A threshold above 5% degranulation was considered positive.

3D protein models and distance mapping

3D protein models were created with Protein Homology/analogY Recognition Engine (PHYRE)[16]. Further graphics and analyses such as distance mapping were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco[17].

Data analysis and statistics

Correlation between percentage degranulation of basophils and intensity of the Euroline strips was determined with Spearman's correlation coefficient, since the data were not normally distributed. GraphPad Prism 7 (GraphPad Software, USA) was used for statistical testing and visualizing data.

Table 1: *Patient characteristics.*
Sex, age, Skin Prick Test (SPT), results of DBPCFC and Müller score per peanut-allergic subject

Patient	Sex (M/F)	Age (y)	SPT peanut (mm[A])	Subjective ED (mg[B])	Objective ED (mg)[B]	Müller score[C]
N01	F	41	3+	10	-	2
N02	M	37	3+	0.1	300	4
N03	M	45	4+	100	-	2
N04	F	50	4+	10	10	3
N05	F	35	4+	0.1	-	4
N06	F	27	4+	4	40	2
N07	M	42	5+	not known	300	3
N08	M	24	4+	100	>30000	1
N09	F	24	3+	not known	>30000	3
N10	F	18	4+	300	1000	3
N11	F	32	4+	10	3000	2
N12	M	27	5+	0.1	1000	3
N13	M	25	3+	10	-	2
N14	F	26	4+	0.1	100	3
N15	F	34	4+	40	12000	2

[A] Skin prick test (mm), a diameter of 3 mm (3+) was considered positive

[B] Subjective and objective effective dose (ED) during DBPCFC indicated in mg.

[C] Müller score 0: Symptoms oral cavity, 1: Symptoms of the skin and mucous membranes, 2: Gastrointestinal symptoms, 3: Respiratory symptoms, 4: Cardiovascular symptoms.

Results

Peanut-allergic patients can be sensitized to Ara h 2.0201, Ara h 6.01 and all isoforms of Ara h 7

In fifteen peanut-allergic patients, sensitization to Ara h 2.0201, 6.01 and the three isoforms of Ara h 7 was established by means of the immunoblot strips (Figure 1a). Sensitization to Ara h 2.0201 was most abundant; 14 out of 15 patients were sensitized to this allergen, followed by sensitization to Ara h 6.01 and 7.0201, which were both recognized by 12 patients (80%). Two patients were mono-sensitized to Ara h 2.0201 (N01, N09), while also co-sensitization for multiple allergens occurred. 40% of the patients were sensitized to all allergens, while one patient (N15) recognized all allergens except

Ara h 2.0201 (Figure 1b). Ara h 7.0201 sensitization in combination with Ara h 2.0201 and/or Ara h 6.01 was found for three patients (N03, N04, N11), while sensitization to Ara h 7.0101 or Ara h 7.0301 was never observed in the absence of Ara h 7.0201.

A

		Line blot intensity (Arbitrary Units)				
Patient	CAP peanut (kU/L)	Ara h 2.0201	Ara h 6.01	Ara h 7.0101	Ara h 7.0201	Ara h 7.0301
N01	1.7	4	2	2	2	2
N02	44	67	71	5	3	0
N03	1.8	3	11	0	7	0
N04	12	4	0	2	5	1
N05	85	18	31	10	34	5
N06	12.8	3	62	8	3	1
N07	42.7	57	58	26	74	25
N08	1.9	4	5	1	2	0
N09	1.0	3	2	2	2	0
N10	>100	167	81	56	115	106
N11	n.d.	12	26	1	5	1
N12	66	74	96	6	106	12
N13	11.2	29	4	3	40	4
N14	9.7	50	61	8	54	4
N15	1.6	2	17	8	12	3
		14/15 (93%)	12/15 (80%)	9/15 (60%)	12/15 (80%)	7/15 (47%)

B

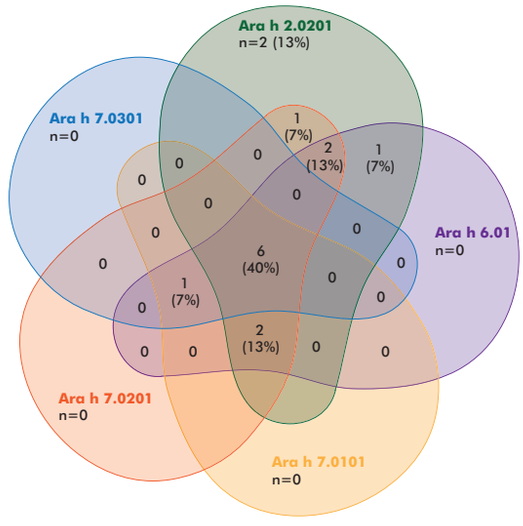

Figure 1: Co-sensitization to peanut allergens of fifteen peanut-allergic patients
A *Specific IgE (kU/L) against peanut determined by ImmunoCAP are indicated per patient in the left column. Sensitization to allergens Ara h 2.0201, 6.0101, 7.0101, 7.0201 and 7.0301 by lineblot are shown as intensity values (arbitrary units) with intensity values > 3 considered positive.*; ***B*** *Venn diagram illustrating co-sensitization to peanut allergens Ara h 2.0201, 6.0101, 7.0101, 7.0201 and 7.0301 based on intensity level Euroline strips (n=15).*

All Ara h 7 isoforms can induce basophil degranulation, and their line blot intensity levels correlate with basophil degranulation

Basophil degranulation experiments were performed for nine peanut-allergic patients, with the optimal allergen concentration of 1000 ng/mL (Figure 2a). All allergens were able to induce basophil degranulation. Of the three Ara h 7 isoforms, Ara h 7.0201 was most effective, since it induced basophil degranulation in six patients, followed by Ara h 7.0301 which induced basophil degranulation in two patients. Ara h 7.0101 was able to induce degranulation in only one patient. Besides basophil degranulation, the correlation between the intensity levels of the Euroline strips, which were directly related to levels of specific IgE, and the percentage of basophil degranulation was determined (Figure 2a). A significant correlation ($p < 0.05$), $r = 0.8$-0.9 was observed between the lineblot intensity and basophil degranulation upon Ara h 2.0201, 6.01 or 7.0201 exposure (Figure 2b).

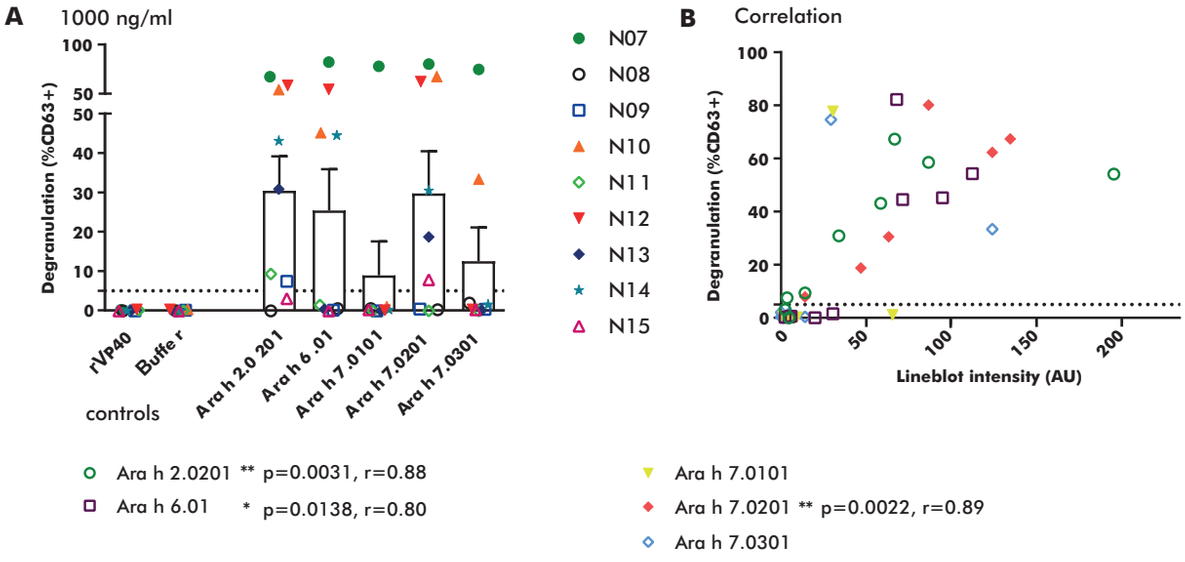

Figure 2: Functionality Ara h 7 isoforms in basophil activation test
A *BAT assay in peanut-allergic patients at an allergen concentration of 1000 ng/mL. Degranulation is indicated as percentage of CD63$^+$ cells (n=9).*; ***B*** *Spearman correlation between degranulation in the BAT assay versus the intensity levels of the lineblot strips (n=9),* * $p < 0.05$, ** $p < 0.01$.

Isoforms Ara h 7.0201 and Ara h 7.0301 can induce basophil degranulation at least as well as Ara h 2.0201 and 6.01

To compare the ability to induce basophil degranulation at low allergen concentrations, a concentration range of allergens was used in the BAT in whole blood of five patients that were sensitized against at least one isoform of Ara h 7. Althouh patient N06 was sensitized to Ara h 2.0201, 6.01, 7.0101 and 7.0201, basophil degranulation was only detected upon exposure to CPE and Ara h 6.01 (Figure 3a), which was probably related to relatively low intensity levels of sensitization for Ara h 2.0201 and 7. In comparison, N10 and N14 showed already high basophil degranulation upon low concentrations (0.3 ng/mL) of isoforms Ara h 7.0201 and Ara h 7.0301 (Figure 3c and e), while basophils of other patients degranulated around 10 ng/mL of Ara h 2, 6 and 7. In the other two patients (N07, N12) the maximal degranulation upon exposure to the Ara h 7 isoforms was comparable to Ara h 2.0201 and 6.01. These data indicate that isoform Ara h 7.0201 is at least as effective as recombinant Ara h 2.0201 and Ara h 6.01 in terms of inducing basophil degranulation.

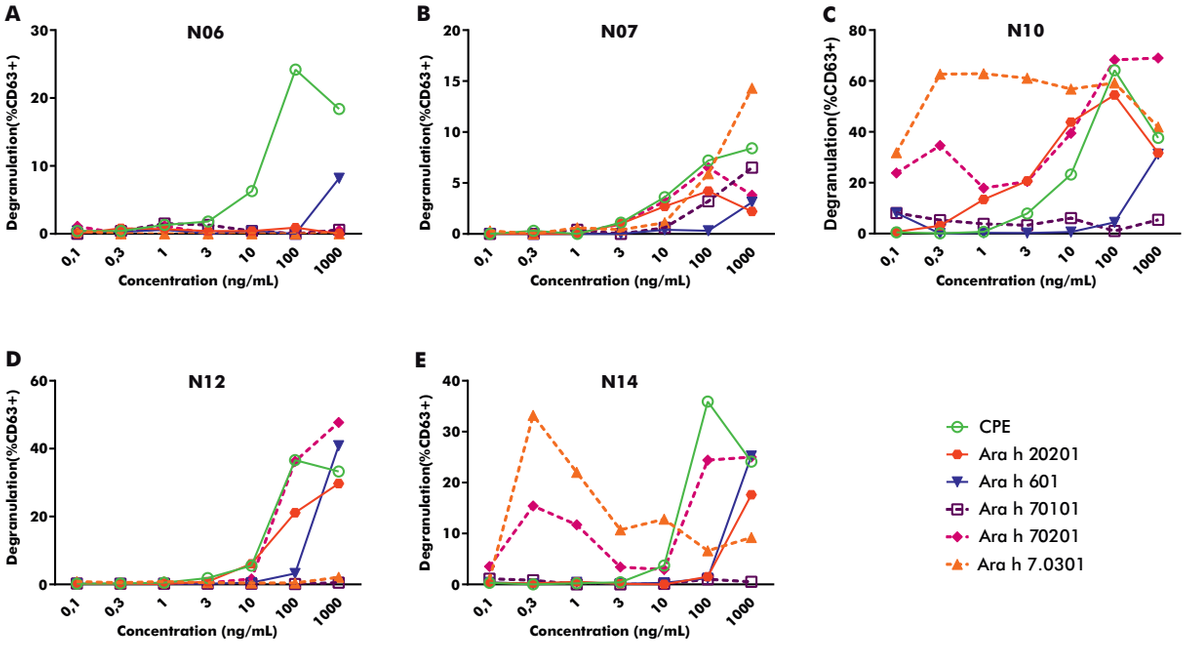

Figure 3: Degranulation of basophils after exposure to different allergen concentration
A-E *Basophil activation of 5 peanut allergic patients in a BAT assay with an allergen concentration range (CPE, Ara h 2.0201, 6.0101, 7.0101, 7.0201 and 7.0301).*

Sequential differences in sequence alignment of Ara h 7 isoforms compared to Ara h 2.0201 and 6.01

A sequence alignment between Ara h 2.0201, 6.01 and the three Ara h 7 isoforms was performed to explain the difference in efficacy of Ara h 7.0201 to induce basophil degranulation in more patients compared to the other two Ara h 7 isoforms (Figure 4a). Known linear epitopes of Ara h 2 and 6 recognized by allergic patients are highlighted in color[18]. Of the three isoforms, Ara h 7.0201 showed most sequence similarity with Ara h 2.0201 and 6.01 in the C-terminal regions that are known to carry allergenic linear epitopes of Ara h 2 and 6 (orange underlined sequence)[18]. Moreover, Ara h 7.0201 is the only isoform containing 8 cysteine residues (underlined C-residues), similar to the conserved cysteine pattern of Ara h 2.0201 and 6.01, whereas Ara h 7.0101 and 7.0301 only contain 6 cysteine residues. Cysteine residues play an important role in the folding and stability of proteins[11]. Furthermore, Ara h 7.0201 differed in three amino acid positions from both other isoforms (Figure 4b, arrows). These differences influence polarity, hydrophobicity, charge, and trypsin cleavage sites (blue residues). In addition, more differences in trypsin (blue) and pepsin (red) cleavage sites were observed in the C-terminus (highlighted end) between the three different isoforms, which play an important role in the enzymatic digestion and thus can influence stability and allergenicity of proteins (Figure 4b).

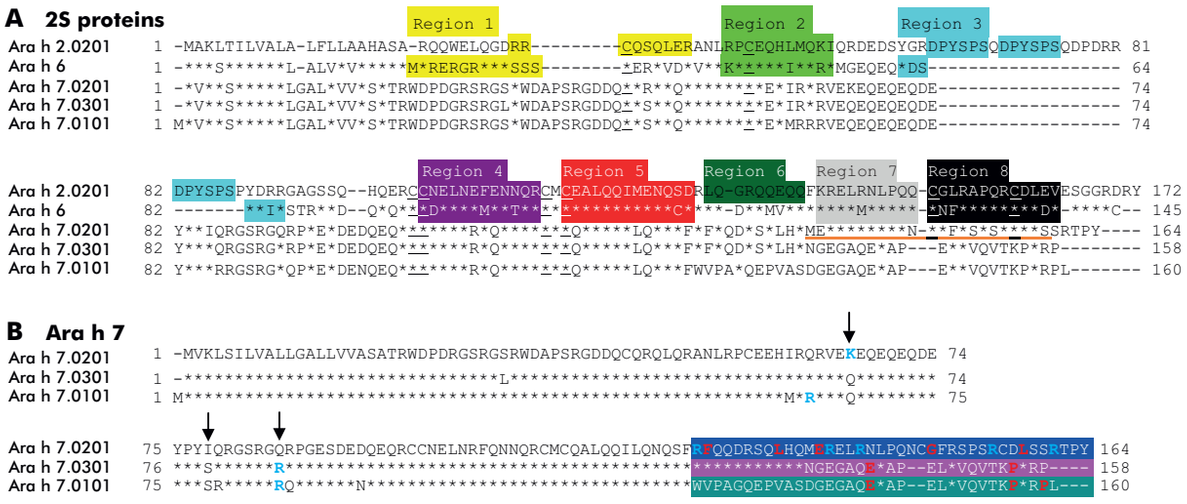

Figure 4: Sequence alignment of Ara h 2.0201, 6.0101, 7.0201, 7.0101 and 7.0301
A *Known allergenic epitopes of Ara h 2.0201 and 6 are color highlighted and explained below. Cysteine residues are underlined. Stars indicate similarities compared to Ara h 2.0201. C-terminal similarity of Ara h 7.0201 compared to Ara h 2.0201 and 6 is underlined in orange.*; ***B*** *Sequence alignment of Ara h 7.0201, 7.0101 and 7.0301. Similarities compared to Ara h 7.0201 are indicated with stars. Arrows indicate differences in amino acid sequence of Ara h 7.0201 to both other isoforms. Blue residues indicate trypsin cleavage sites and red residues pepsin cleavage sites. The C-terminus is highlighted at the end.*

3D-structural differences between Ara h 7 isoforms related to known allergenic epitope sites of Ara h 2.0201 and 6.01

In contrast to Ara h 2 and 6, of which crystal structures have been described[2,19], no crystal structure is available for the isoforms of Ara h 7 and isoforms Ara h 2.0201 and 6.01. In addition to the sequence alignment, predictive protein 3D-models of all isoforms were therefore created with PHYRE and UCSF Chimera (Figure 5). Known allergenic linear epitopes of Ara h 2.0201 and 6.01 are highlighted in the same colors as displayed in Figure 4 (Figure 5a and b)[18]. Figure 5 c-e shows the predicted 3D models of the Ara h 7 isoforms. The sequence alignment indicated that most differences were located in the C-terminus. In the 3D-models, a main structural difference with Ara h 7.0101 was observed in this C-terminus (turqoise), and some smaller differences were observed between Ara h 7.0201 and 7.0301 in this region (pink vs blue). The three amino acids that differ between these three isoforms (light blue) were all located in a loop which is a known epitope region for Ara h 2.0201 and 6.01 (region 3). Since these changes in amino acids can influence hydrophobicity, polarity and charge, an amino acid distance analysis was performed (UCSF Chimera, Suppl. Figure S1). Indeed, mainly in the loop region (green circle), differences in distance between amino acid residues were observed (Suppl. Figure S1).

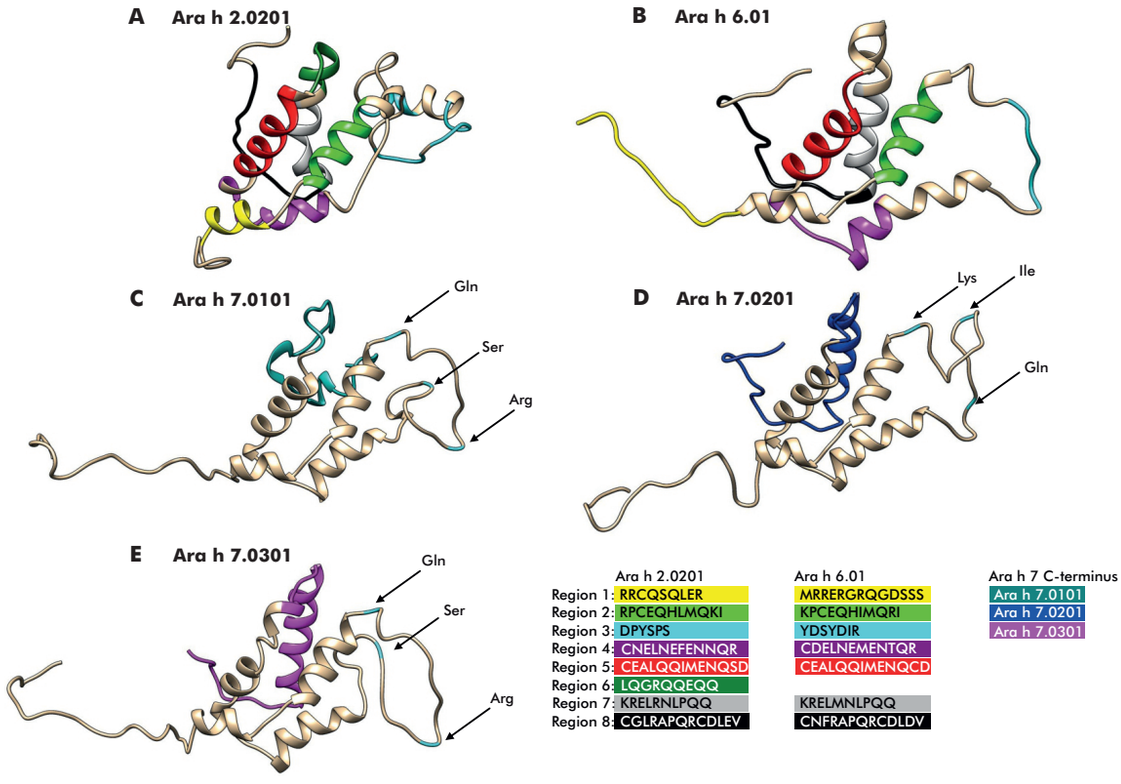

Figure 5: 3D protein prediction models of Ara h 2.0201, 6.0101 and 7
A-B *3D-model of Ara h 2.0201 and 6, known allergenic epitopes are indicated in color according to the color-scheme of Figure 4.;* ***C-E*** *3D prediction models of the three Ara h 7 isoforms. Turquoise (Ara h 7.0101), blue (Ara h 7.0201) or pink (Ara h 7.0301) indicates the C-terminus, light blue residues indicated with arrows indicate the main differences between Ara h 7.0201 and the other two isoforms in loop region 3.*

Discussion

Ara h 2 and 6 have proven to be two of the most informative peanut-allergens in the diagnosis of peanut allergy, since most patients have specific IgE against one or both allergens[7]. In addition, the current study showed that 80% of the 15 peanut-allergic patients studied, were sensitized to one or multiple isoforms of a third recombinant 2S albumin member Ara h 7, mostly in combination with recombinant Ara h 2.0201 or 6.01. This is probably explained by the sequence identity between these three isoforms and Ara h 2.0201 and 6.01[2,5,6,8,9]. Ara h 7.0201 showed the highest sensitization frequency amongst peanut-allergic patients (80%), which was comparable to sensitization to Ara h 2.0201 and 6.01 (93 and 80%, respectively).

To the best of our knowledge, this is the first time that the functionality of recombinant Ara h 7 isoforms was tested, rather than only determining specific IgE binding in patient samples. Although the BAT assay can be a variable assay, grouped results indicate that Ara h 7.0201 was overall able to induce basophil degranulation comparable to Ara h 2.0201 and 6.01. In two independent patients, Ara h 7.0201 and 7.0301 were able

to induce basophil degranulation at relatively low concentrations of allergen comparable to CPE and Ara h 2.0201 and 6.01, suggesting that these specific Ara h 7 isoforms can be recognized by sensitized individuals and increase efficacy in stimulating basophil degranulation. While this could not be directly related to sensitization levels of the line blot strips, it indicates that some patients can react to low concentrations of Ara h 7. Although Ara h 7 represents only 0.5% of peanut protein content, in contrast to 4–14% for Ara h 2 and 6[3,12], this allergen has the potency to induce responses at low concentrations. Sensitization to isoform Ara h 7.0101 was observed in 60% of peanut allergic patients, although biologic activity was observed in only one patient. Ara h 7.0101 was identified with phage display technology, but could not be retrieved in peanut extract[5], which is most likely the explanation for this reduced biologic activity. Cross-reactivity between the three isoforms may explain the observed sensitization for this isoform.

A limitation of this study is the lack of native Ara h 7 which is not commercially available and difficult to isolate due to its low abundance. Hence, all experiments were performed with recombinant proteins. Native Ara h 2 and 6 have been shown to induce basophil degranulation at lower concentrations of allergen than those of the recombinant proteins used in this study[9]. Nevertheless, the recombinant Ara h 7 proved to be able to induce basophil degranulation in some patients at already low concentration, indicating that it might even be more reactive in crude peanut extract.

A limitation of the BAT assay is that the response of patients to specific allergens can be significantly variable and does not always follow the typical bell-shaped dose-response curve[20] as e.g. observed for patient N10 and N14. The BAT tests performed in this study were only performed once for each patient. Nevertheless, Ara h 7.0201 appeared to be the most promising Ara h 7 isoform in optimizing peanut allergy diagnosis, as it possessed a similar sensitization profile and efficacy in basophil degranulation compared to recombinant Ara h 2.0201 and 6.01. Moreover, Ara h 7.0201 contains potentially unique epitopes compared to the other two Ara h 7 isoforms, since patients were more often sensitized to this particular isoform and its recognition was partly exclusively accompanied by Ara h 2.0201 and 6.01 (N03, N04 and N11). Sensitization to one of the other two isoforms in combination with Ara h 2.0201 and/or 6.01 was not observed. Although Ara h 7.0201 contains cross-reactive epitopes with Ara h 2.0201 and 6.01, a previous study indicated that mono-sensitization against Ara h 7.0201 was observed in two out of fifteen patients[10]. This suggests that Ara h 7.0201 indeed contains one or more epitopes not present on the other Ara h 7 isoforms or Ara h 2.0201 and 6.01. The similarity of Ara h 7.0201 in sensitization and basophil degranulation with Ara h 2.0201 and 6.01 is most likely related to the C-terminus of Ara h 7.0201, as it fits into the conserved cysteine conglutin family pattern of at least eight cysteine residues[5]. These C-terminal cysteine residues are important for protein stability and determine the IgE binding of allergens[21]. By 3D protein modeling, the three main differences in

amino-acid sequence of the Ara h 7 isoforms were visualized in a loop region that is a known allergenic epitope for Ara h 2.0201 and 6.01. Due to these amino acid substitutions, small changes in distances between amino acids occur, which could contribute to enhanced exposure of an epitope. Combining these two findings, it is expected that the unique epitopes of Ara h 7.0201 are located in either the C-terminus or in this loop region 3. Therefore, differences in enzymatic digestion by pepsin and trypsin may influence the allergenicity of Ara h 7 isoforms. Taken together previous data[10] and the data presented in this paper, we hypothesize that determining specific IgE to Ara h 7.0201 can be of additional value in peanut allergy diagnosis. Ara h 7.0201 contains unique epitopes and is functionally as active as Ara h 2.0201 and 6.01 in inducing basophil degranulation. In addition, in some patients Ara h 7 can already provoke basophil degranulation at low concentrations. Due to cross-reactivity between Ara h 2.0201, 6.01 and 7, the latter one could have a potential strong diagnostic value.

Acknowledgements

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Supplementary

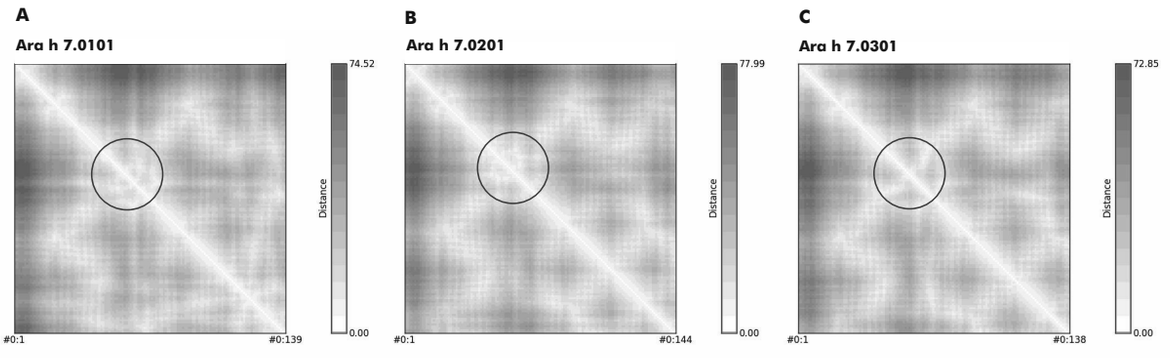
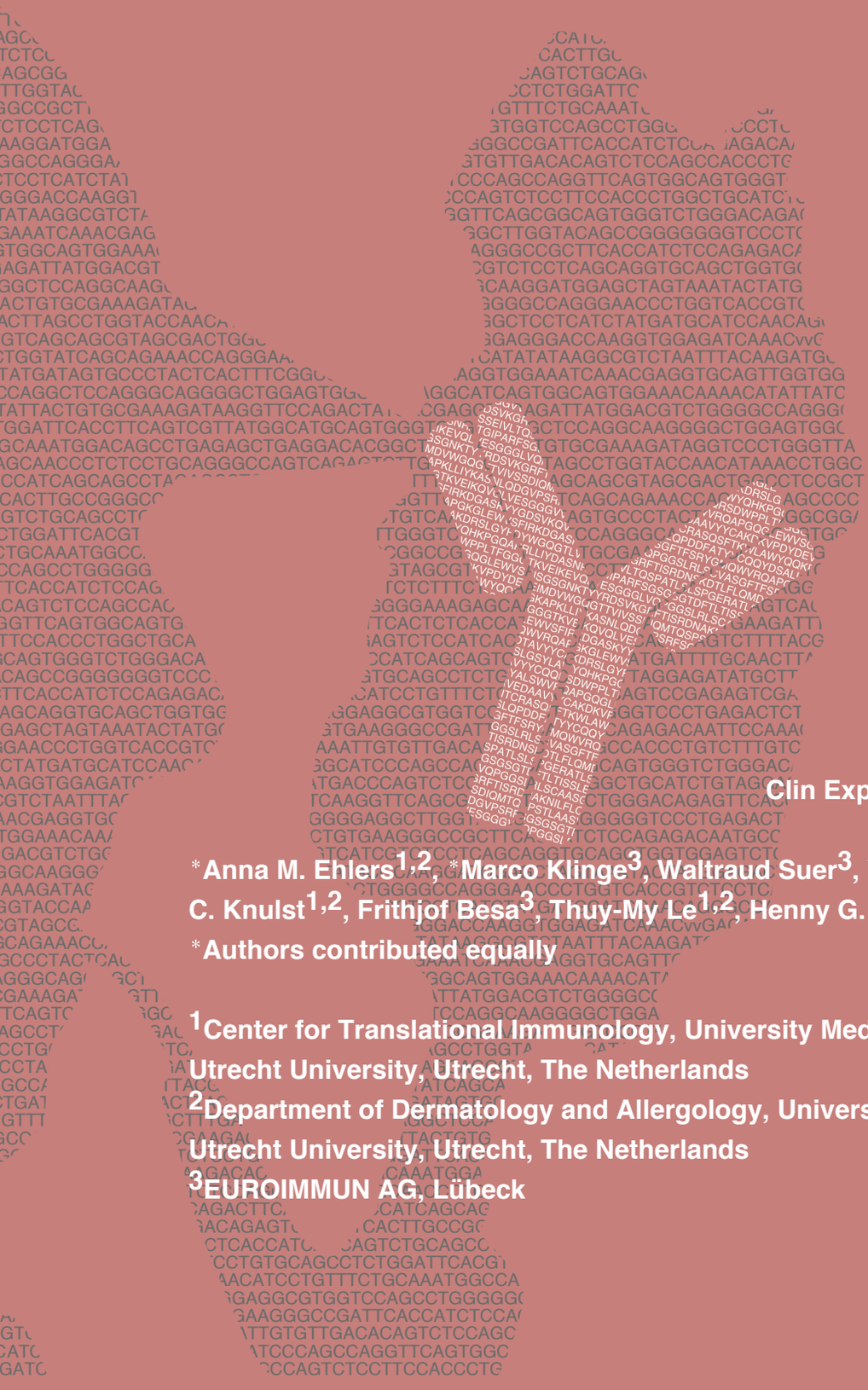


Figure S1: Residue distance map
*Distance maps of **A** Ara h 7.0101, **B** 0201 and **C** 0301. The distance of each amino acid is related to every other amino acid. The distances between the residues are indicated according to the color scale (Å), where white indicates that amino acid residues are in close proximity.*


6

Ara h 7 isoforms share many linear epitopes: Are 3D epitopes crucial to elucidate divergent abilities?



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*Anna M. Ehlers[1,2], *Marco Klinge[3], Waltraud Suer[3], Yvonne Weimann[3], André C. Knulst[1,2], Frithjof Besa[3], Thuy-My Le[1,2], Henny G. Otten[1]

*Authors contributed equally

[1]Center for Translational Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[2]Department of Dermatology and Allergology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[3]EUROIMMUN AG, Lübeck

Abstract

Background

The peanut allergens Ara h 2, 6 and 7 are potent allergens and can trigger severe reactions. Ara h 7 consists of three isoforms differing in their ability to induce basophil degranulation, whereas the ability of Ara h 7.0201 is comparable to Ara h 2 and 6 as shown in previous literature. Thus, we sought to identify linear epitopes of Ara h 7.0101, 7.0201 and 7.0301 recognised by IgE and IgG4 from patients sensitised to Ara h 7 and to investigate their potential to elucidate divergent abilities of the Ara h 7 isoforms in inducing basophil activation.

Methods

Linear epitopes recognised by IgE and IgG4 were mapped by peptide microarray analysis containing 15mer peptides of Ara h 2.0201, 6, 7.0101, 7.0201 and 7.0301 and 39 peanut allergic patients sensitised to Ara h 7 (discovery). For validation, 20mer peptides containing the minimal epitope and surrounding amino acids were incubated with 25 sensitised patients and 10 controls (validation).

Results

Three out of 14 linear epitopes were unique for each isoform (Ara h 7.0101: aa 97-109; Ara h 7.0201: aa 122-133; Ara h 7.0301: aa 65-74) but scarcely recognised by IgE. The main linear IgE epitope (aa 51-57) located in the long flexible loop of all Ara h 7 isoforms was bound by antibodies from 31% of the patients (discovery and validation cohort). Regarding IgG4, 55% of the patients recognised an epitope present on all isoforms (aa 55-65) whereas epitope aa 129-137, only present on Ara h 7.0101/.0301, was recognised by 38% of the patients. Recognition was highly individual, although 20% of the patients recognised any linear epitope neither by IgE nor by IgG4 despite a low mean z-score of ≥ 1.7. Remarkably, only 50% of the patients recognised one or more epitopes by IgE.

Conclusion and Clinical Relevance

Ara h 7 isoforms share many linear epitopes being easily accessible for antibody binding. Unique epitopes, essential to elucidate divergent potencies, were scarcely recognised, suggesting a crucial involvement of conformational epitopes.

Introduction

Peanut allergy is one of the most prevalent food allergies worldwide and is often triggered by Ara h 8, a birch pollen-related PR-10 protein, or seed storage proteins such as Ara h 2 and 6 (2S albumins) in Western and Central Europe. While sensitisation to Ara h 8 often results in mild reactions such as oral allergy syndrome, Ara h 2 and 6 can induce severe reactions, including anaphylaxis[1,2]. Ara h 7 is the third member of the 2S albumin family, but it is far less abundant in peanut than Ara h 2 and 6 and shares a sequence homology of 51% and 61%, respectively[3].

For diagnosing peanut allergy, IgE antibodies against Ara h 2 and 6 are known to have a very high positive predictive value[4,5]. Recently, IgE binding to Ara h 7 showed a discriminative ability comparable to Ara h 2 and 6 and this 2S albumin was as potent as Ara h 2 and 6 in inducing basophil degranulation[6]. For Ara h 7, three different isoforms – Ara h 7.0101, 7.0201, 7.0301 – have been described whilst Ara h 7.0101 has only be detected on cDNA level but not on protein level[7]. Ara h 7.0201 was the most potent isoform to induce degranulation. IgE binding to this allergen were not fully inhibited by Ara h 7.0101, although IgE-binding to Ara h 7.0101 was completely inhibited by Ara h 7.0201. These data suggest the presence of unique epitopes on Ara h 7.0201[8]. By amino acid sequence comparisons, these unique epitopes might be located on the distinctive C-terminus or created by amino acid substitutions within the flexible loops sensitive to pepsin or trypsin digestion[6].

The aim of the study was to define linear epitopes of Ara h 7 isoforms. To this end, we performed a linear epitope mapping using the peptide microarray technique and applying sera from allergic and tolerant patients sensitised to Ara h 7.

Methods

Patient selection

For identifying linear epitopes, sera with specific IgE to peanut extract (ImmunoCAP) were screened by EUROLINE (EL, Euroimmun AG, Lübeck, Germany) for sIgE binding to heterologously expressed Ara h 7.0201. Overall, 39 sera with sIgE levels ≥16 intensity units (EL) for Ara h 7.0201 were applied for the peptide microarray analysis, and peanut allergy was confirmed by food challenge according to the international consensus protocol or experienced physician diagnosis (discovery cohort)[9]. The cut-off level was chosen to guarantee the detection of a broad epitope spectrum. In the next phase, the identified epitopes were validated by 25 DBPCFC-confirmed peanut allergic (n=22) or tolerant (n=3) patients with sIgE levels ≥3 intensity units for Ara h 7.0201 (validation cohort)[8,9]. As control, 10 sera without sIgE to Ara h 7.0201 were used. In Table 1 the comparison between CAP-classes, concentrations and Euroline intensity units are shown[10]. Ethical approval was acquired from the biobank committee of the University Medical Center Utrecht, number 18-428.

Table 1: *EUROLINE intensities*

EAST-class	0	1	2	3	4	5	6
EL-intensity level	0-2	3-6	7-15	16-30	31-50	51-100	>100
ImmunoCAP [kU/L]	< 0.35	0.35 - < 0.7	0.7 - < 3.5	3.5 - < 17.5	17.5 - < 50	50 - < 100	≥100

Peptide chip design

For the discovery phase, a peptide microarray with overlapping 15mer peptides was commercially obtained (PEPperPRINT), comprising the sequences of Ara h 7.0101, 7.0201, 7.0301 (offset: 1), 2.0101, and 6.0101 (offset: 2). According to the experiences of PEPperPRINT, 15 mer peptides have the optimal length to provide sufficient sensitivity without significant induction of secondary structures. All peptides were printed as triplicates with a 3 amino acids linker (2x ß-alanine and 1x aspartic acid) to prevent binding of negatively charged fluorescent dyes to positively charged amino acids close to the array surface. For validating the discovered minimal epitopes, a new microarray layout was applied. For each epitope, a 20mer peptide was designed containing the minimal epitope and surrounding amino acids, replenished with glycine and serine amino acids.

Microarray incubation

All dilutions and washing steps were performed in working strength universal buffer (EUROIMMUN, purchase order number ZW1100). Patient samples were diluted 1:4 and incubated on the microarrays at 4 °C overnight. For the detection of bound IgE and IgG4 antibodies, the arrays were incubated with biotinylated α-IgE antibody (clone MHE-18 1:5000, BioLegend) and biotin α-human IgG4 coupled with Neutravidin DyLight 680 (clone HP6025, 1:5000, Southern Biotech) for one hour at room temperature. After washing, the arrays were incubated for one hour at room temperature with fluorescent Neutravidin DyLight 800 (Thermo Fisher), diluted 1:5000 for IgE detection. After washing with dipping buffer (1 mM Tris-HCl pH 7.4), the peptide microarray slides were dried and scanned with a Licor Odyssey Imager at a wavelength of 800 nm (intensity: 10). Image focus was set to 0.8 mm and an image resolution of 21 µm was chosen.

Microarray evaluation

Fluorescent signals were extracted using Pepslide Analyzer Software (SICASYS) and exported to CSV files. For data evaluation, logarithmic signal-noise-ratios (S) were calculated for each peptide according to:

$$S_i = \log_2 \cdot \frac{Total\ fluorescene\ (Peptide)}{Background\ Flourescence\ (Peptide)}$$

These S-values were normalized against the S-values of blank spots on the array, resulting in a z-score defined as:

$$Z_i = \frac{S_i - Median\ (SBlank)}{Mean\ Deviation\ (SBlank)}$$

Significance levels of positive peptide binding were defined based on z-scores as followed: $z > 1.7$ ($p < 0.05$;*); $z > 2.4$ ($p < 0.01$;**); $z > 3.0$ ($p < 0.001$;***); $z > 4.0$ ($p < 0.0001$). Peptides were only considered if the coefficient of variation for the triplicate of each peptide was lower than 50%. Recognised peptides were defined as epitopes if 3-5 contiguous peptides with a mean z-score $\geq$1.7 were detected.

Determination of sIgE and sIgG4 sensitisation

Specific IgE and IgG4 sensitisation to the full-length protein Ara h 7 was assessed by line blots (EUROIMMUN, Luebeck, Germany) according to manufacturer's instructions. Briefly, sera were applied (1:11 for IgE, 1:51 for IgG4) overnight and after washing three times with universal buffer, bound IgE were detected by an α-human IgE and α-human IgG4-antibody labelled with alkaline phosphatase. Visualisation was provided by adding nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate substrate for ten minutes after washing three times with universal buffer.

Modelling 3D structure of Ara h 7.0201

3D structure of Ara h 7.0201 was assessed by Modeller software[11]. Since the amino acid sequences of 2S albumins differ, homology modelling with multiple input sequences and crystal or NMR structures was chosen (Ara h 2 reference 3OB4, Ara h 6 reference 1W2Q, Ric c 3 reference 1PSY, sunflower 2S albumin reference 1S6D, rapeseed 2S albumin reference 1SM7). Five resulting models were evaluated by DOPE score and the model with the lowest score was selected (DOPE score = -11083.618).

Model assessment

To evaluate the 3D model, the DOPE score per residue was assessed. DOPE scores > -0.3 indicate levels of relatively high energy, pointing towards structural errors. Additionally, the model was applied to the ModFOLD6 server[12,13] calculating a residue error plot, a global model quality score (0.5348) and a p-value ($p < 0.0001$) (Figure S1). Overall, the structure was of high quality, apart from the flexible loops which are also experimentally difficult to assess.

Data analysis

The baseline data were statistically analysed using one-way ANOVA or Mann-Whitney-U-test for continuous data and Fisher's exact test for categorical data. Statistical evaluation was performed with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and SPSS Statistics 21 (IBM Corporation, Armonk, NY, USA). P values ≥ 0.05 were considered as statistically significant.

Results

Unique linear IgE-epitopes of Ara h 7 isoforms were marginally recognised

To define unique epitopes of Ara h 7 isoforms, we mapped linear epitopes of these proteins by peptide chip analysis using patients' sera with IgE levels for Ara h 7 $\geq$16 intensity units (corresponding to ImmunoCAP-class > 2). Patient characteristics are shown in Table 2. Overall, 14 different linear amino acid sequences (A-L) were bound by IgE (green) or IgG4 (red) as shown in Figure 1a. Epitope codes are listed in Table 3. Epitope E affiliating to all isoforms was recognised by most patients with a frequency of 31% for IgE and 5% for IgG4 in the discovery cohort. Whilst epitope E was predominately recognised by IgE, epitope F and L were mainly bound by IgG4 (61.5% and 54%). Contrary, the unique epitope G (Ara h 7.0301) showed an IgE recognition frequency of only 2.5% and epitope I (Ara h 7.0101) was not recognised by IgE at all. These unique epitopes were present in the core of the 3D structure of the proteins and theoretically, they would be only accessible after enzymatic digestion by pepsin or trypsin. In contrast, the unique epitope K (Ara h 7.0201) is located on the flexible C-terminus and was recognised by IgE from 10% of the included patients. However, this epitope was only recognised by IgG4 (16%) in the validation cohort (Figure 1b). Overall, epitope E was the main IgE epitope in the discovery and validation cohort.

Recognition patterns of linear epitopes were highly individual

All patients showed individual linear epitope recognition patterns as shown in Figure 1a and b. Two patients (D-06 and D-35) recognised up to four different epitopes by IgE in the discovery cohort. Occasionally, epitopes, particularly epitope E and F, were bound simultaneously by patients' IgE and IgG4 antibodies (yellow). Moreover, epitopes bound by IgG4 were located in the neighbourhood of an epitope recognised by IgE in eight patients. For example, epitope E was bound by IgE and epitopes D and F were bound by IgG4 in patient D-04. Certainly, half of the patients did not show any sIgE binding to linear peptides whilst all of them recognised the complete allergens, and ten of them showed at least IgG4-binding. This suggests the importance of conformational epitopes to elucidate strong IgE-binding to the full-length protein. Importance of conformational epitopes was supported by IgE-binding to even fewer linear epitopes in the validation cohort.

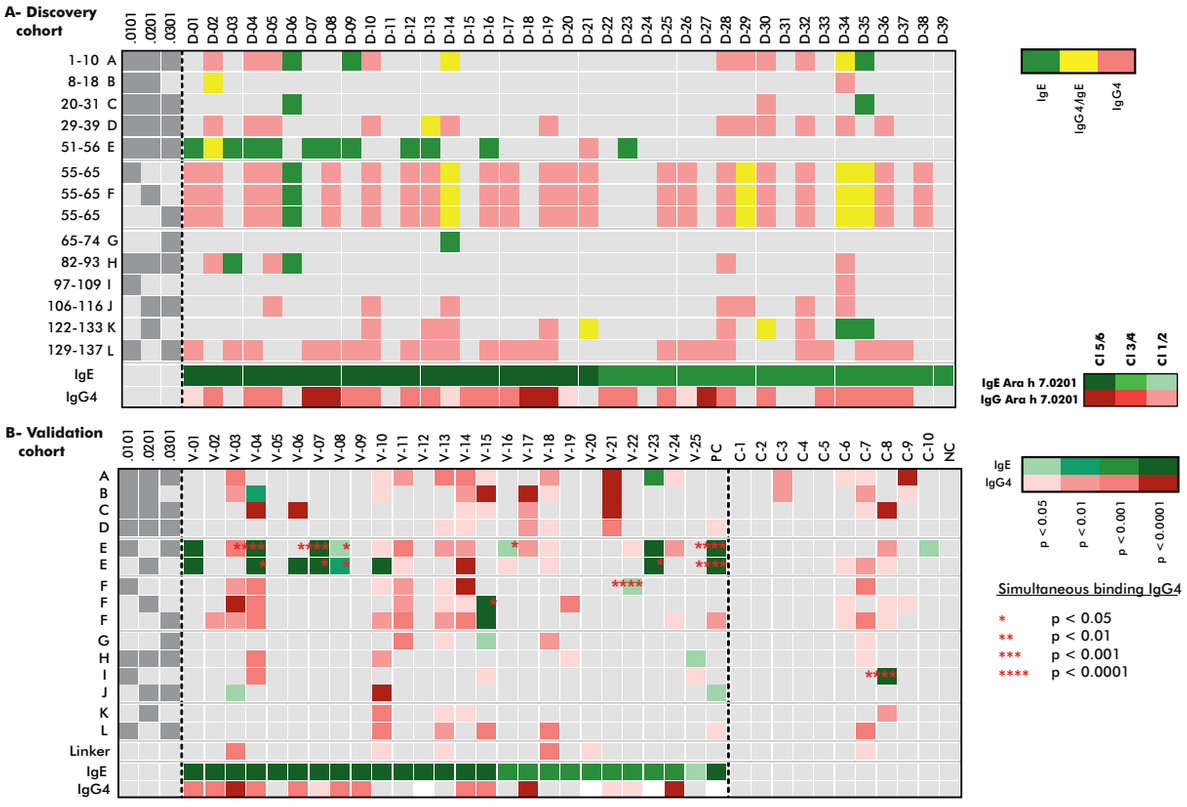

Figure 1: Individual linear epitope recognition

Individual epitope recognition patterns for IgE (green), for IgG4 (red), and for co-recognition by IgE and IgG4 (yellow); on the left-hand side, dark grey dots indicate the respective isoform(s) for each epitope. The relative IgE and IgG4 sensitisation to Ara h 7 is indicated with different gradation of green (IgE) and red (IgG4) in the bottom of each heatmap. ***A*** *Discovery cohort selected by sIgE levels ≥ 16 intensity units for Ara h 7.0201, the numbers on the left-hand side indicate the different minimal epitopes identified;* ***B*** *Validation cohort selected by sIgE levels ≥ 3 intensity units for Ara h 7.0201 and controls without sIgE to Ara h 7.0201; for validation purposes, peptides containing the minimal epitope and surrounding amino acids were used resulting occasionally in more than one peptide containing the same minimal epitope (e. g. epitope E). Epitopes recognised by IgE and IgG4 are highlighted based on their significance level (* is p < 0.05, ** is p < 0.01, *** is p < 0.001, **** is p < 0.0001)*

Table 2: *Patient characteristics and sensitisation data*

	Discovery cohort	Validation cohort	Control group	p-value
Number [n]	39	25	10	
Age (median [IQR])	25 [18-54]	23 [18-38]	39 [20-66]	0.001[D]
Sex female [n, %]	21 (54%)	8 (32%)	7 (70%)	0.084
Food challenge [n, %]	6 (15%)	25 (100%)	10 (100%)	<.0001[E]
Symptoms [n, %]				
Objective	25 (64%)	14 (56%)	N/A	
Subjective	13 (33%)	7 (28%)	N/A	0.341
No symptoms	N/A	3 (12%)	8 (80%)[A]	
Sensitisation [median (range)]				
ImmunoCAP peanut extract	19 (1.2-73 kU/l)	11.2 (0.62-100 kU/l)	0.4 (0-9.35 kU/l) [B]	0.378[F]
EUROLINE Ara h 2[C]	30 (1-98 EL-int.)	71 (1- >100 EL-int.)	<3 (0-2 EL-int.)	0.008[F]
EUROLINE Ara h 6[C]	58 (4- >100 EL-int.)	56 (2- >100 EL-int.)	<3 (0-1 EL-int.)	0.992[F]
EUROLINE Ara h 7[C]	61 (16- >100 EL-int.)	98 (4- >100 EL-int.)	< 3 (0-1 EL-int.)	0.084[F]

[A] Two provocations were inconclusive

[B] Data from n=6

[C] EUROLINE intensities (EL-int.): < 3 = EAST-class 0; 3-6 = EAST-class 1; 7-15 = EAST-class 2; 16-30 = EAST-class 3; 31-50 = EAST-class 4; 51-100 = EAST-class 5; >100 = EAST-class 6

[D] Significant difference between discovery or validation cohort and control group (age)

[E] Significant difference between discovery cohort and validation cohort or control group (food challenge)

[F] p-value discovery vs validation

Similar epitope recognition results in divergent ability to induce basophil degranulation

In a previous study, the ability to induce basophil degranulation was studied for all Ara h 7 isoforms[6]. Patients D-23, V-01 and D-13 correspond to the patients N07, N12 and N14, respectively, in that study. Even though IgE antibodies from patients D-23 and V-01 recognised only epitope E, patient D-23 with high IgE titres to all full-length proteins showed overall low degranulation, whereas patient V-01, also having high IgE titre, showed strong basophil degranulation after stimulation with Ara h 7.0201. Patient D-13 had a more diverse sIgE recognition profile (epitopes E+D which are present on all Ara h 7 isoforms) and degranulation was induced by Ara h 7.0201 and 7.0301, although sIgE levels to Ara h 7.0301 were low. These data indicate that IgE recognition patterns of peptides alone cannot elucidate the divergent ability in inducing basophil degranulation.

Amino acid replacements do not necessarily influence binding and recognition

A complete overview of identified linear epitopes is shown in Figure 2. Even though epitope F showed amino acid differences between all isoforms, these differences did not result in divergent minimal epitope recognition within the discovery cohort (Figure 1a). This observation suggested the relevance of residues 55-57 (Tyr-Pro-Tyr) and 61-63 (Gly-Ser-Arg) for antibody binding. Surprisingly, antibody binding of epitope F was not influenced by polar amino acids as glutamine and arginine (aa 59, aa 65). However, surrounding amino acids of epitope F, as considered in the peptide design for the validation cohort, had a great impact on antibody binding (aa 66; V-02, V-10, V-15, V-19, V-22, V-24). Moreover, surrounding residues of epitope E differing between Ara h 7.0101/.0301 and 7.0201 (Lys46Gln and Ile58Ser) resulted in divergent antibody binding of the minimal epitope (Figure 1b). IgE antibodies of two patients (V-06 and V-10) only recognised the sequence of Ara h 7.0201 and IgG4 antibodies of six patients only the sequence of Ara h 7.0101/.0301. This indicates an oligoclonal antibody repertoire recognising the same minimal epitope.

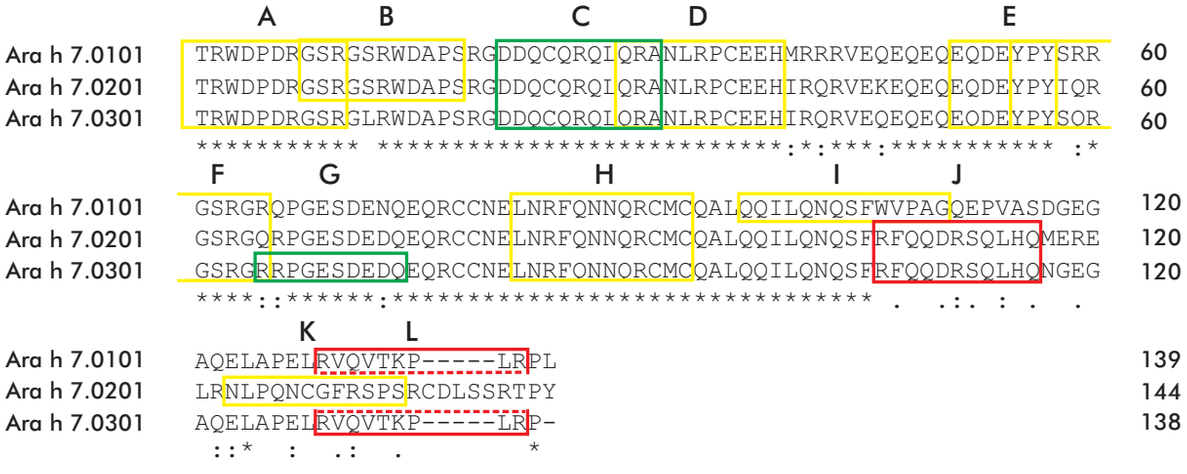

Figure 2: Linear epitopes of Ara h 7 isoforms
Identified linear epitopes highlighted on the amino acid sequences of Ara h 7.0101, .0201 and .0301, green: exclusively recognised by IgE; yellow: recognised by IgE and/or IgG4; red: exclusively recognised by IgG4

Table 3: *Linear epitopes of Ara h 7 isoforms*

Epitope	Residues[A]	aa sequence	Isoform	Specificity
A	1-9	TRWDPDRGSR	all	IgE/IgG4
B	8-18	GSRGSRW-DAPS	.0101, .0201	IgE/IgG4
C	20-31	DDQCQRQIQRA	all	IgE/IgG4
D	29-39	QRANLRPCEEH	all	IgE/IgG4
E	51-57	EQDEYPY	all	IgE/IgG4
	55-65	YPYSRRGSRGR	.0101	IgE/IgG4
F	55-65	YPYIQRGSRGQ	.0201	IgE/IgG4
	55-65	YPYSQRGSRGR	.0301	IgE/IgG4
G	65-74	RRPGESDEDQ	.0301	IgE
H	82-93	LNRFQNNQR-CMC	all	IgE/IgG4
I	97-109	QQILQNQS-FWVPA	.0101	IgG4
J	106-116	RFQQDRSQLHQ	.0201, .0301	IgG4
K	122-133	NLPQNCGFR-SPS	.0201	IgE/IgG4
L	129-137	RVQVTKPLR	.0101, .0301	IgG4

[A] without signal sequence

Linear epitopes recognised by IgE are often located on flexible loops

The location of epitopes was shown by applying epitope recognition patterns of four individual patients on the 3D model of Ara h 7.0201 (Figure 3). Epitopes A, B, E, F, K were situated within the flexible loops of the model and epitopes D, H, J within a combination of a flexible loop and an α-helix. Epitopes A, B, F and K contained multiple theoretical cutting sites for trypsin and pepsin whilst epitope E contained only cutting sites in the beginning and in the end of its amino acid sequence. Cutting sites of epitope E were altered by substitutions of surrounding amino acids. A cutting site for trypsin was introduced on Ara h 7.0201 (aa 46) and a cutting site for pepsin on Ara h 7.0101/.0301 (aa 56). Contrary, unique linear epitopes of Ara h 7.0101 and .0301 were located in the core, not accessible without enzymatic digestion. Due to less disulphide bridges, these isoforms are probably less resistant against enzymatic digestion, making these amino acid sequences more easily accessible for Ara h 7.0101/.0301 than for Ara h 7.0201.

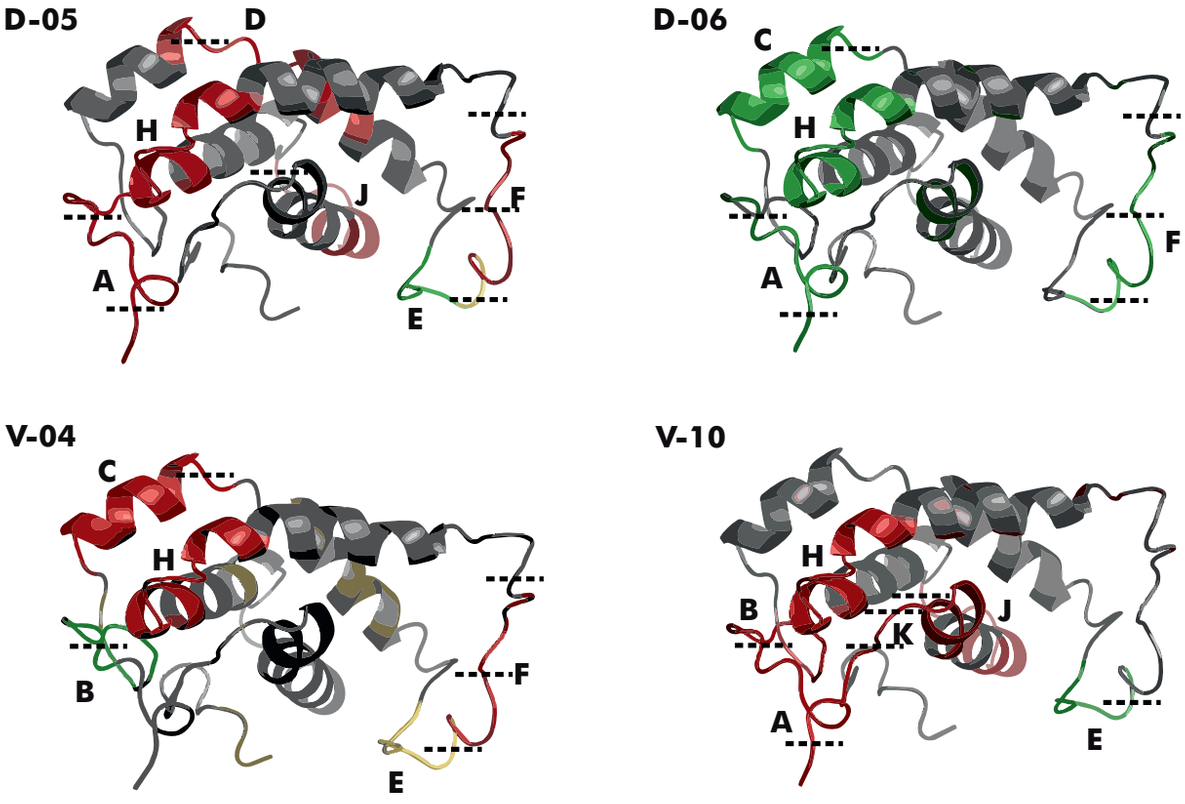

Figure 3: Linear epitopes mapped onto the 3D structure of Ara h 7.0201
Divergent epitope recognition patterns of four selected patients (D-05, D-06, V-04 and V-10) were mapped on the 3D model of Ara h 7.0201 and theoretical cutting site were marked with black dash lines. Epitopes recognised by IgE are highlighted in green and epitopes recognised by IgG4 are highlighted in red.

Discussion

Divergent abilities of Ara h 7 isoforms in inducing basophil degranulation suggested the presence of unique epitopes for Ara h 7.0201 in comparison with the other isoforms. This hypothesis was supported by inhibition assays showing complete inhibition of IgE binding to Ara h 7.0101 by Ara h 7.0201, although IgE binding to Ara h 7.0201 was not fully inhibited by Ara h 7.0101[6,8]. These observations can partially be elucidated by the newly mapped linear epitopes in this study. Epitope E was predominately recognised by IgE in 31% of the patients whilst epitope F was immunodominant regarding IgG4 binding (55%). Nevertheless, unique epitopes of all isoforms were scarcely recognised and linear epitopes in general were infrequently recognised despite the relatively low threshold of a mean z-score of $\geq$1.7. Since the absence of epitope recognition by IgE was not associated with lower sIgE levels to Ara h 7 (Figure 1), lack of sensitivity can be excluded. Thus, our results suggest the importance of conformational epitopes and to lesser extent the importance of IgE antibodies with deviant affinities to understand divergent abilities of the Ara h 7 isoforms in inducing basophil degranulation.

The main linear IgE epitope (E), located in the long flexible loop, was recognised by only 32.5% of the patients. Other epitopes were detected by even less patients. Limited IgE binding to linear epitopes has also been observed for Ara h 6 and to a fewer extent for Ara h 2. For Ara h 6, seven linear IgE epitopes have been identified whereof the main epitope (aa 97-106 without signal sequence) was recognised by only 30% of the patients, comparable to our data for Ara h 7 (31% for epitope E). Regarding Ara h 2, linear epitopes were recognised by a greater number of patients, varying from 75% to 100% of the included patients depending on the study considered (aa 10-18 and aa 42-60 without signal sequence)[14,15]. Contrary to the other two 2S albumins, Ara h 2 is endowed with a very long flexible loop containing the main epitope and five proline residues (cf. 0 for Ara h 6 and 1 for Ara h 7). Proline residues can be modified by Maillard reaction in presence of reducing sugars occurring during the roasting process. This modification has been shown to increase the allergenicity of Ara h 2 and underlined the importance of proline residues[16,17]. However, deletion of flexible loops containing the main linear epitopes diminished the sIgE binding only on an individual basis, showing the additive of conformational epitopes to elucidate IgE binding to Ara h 2[16,18].

Recognition of linear Ara h 7 epitopes by individual patients was highly distinct, stretching from multiple to none linear epitopes recognised by IgE. Individual recognition patterns were also observed for Ara h 2 in previously conducted studies[19,20]. Occasionally, in the present study epitope recognition by IgE was accompanied by IgG4 binding for the same epitope. Additionally, IgG4 binding was observed in the neighbourhood of an epitope bound by IgE. Consistently with our results, overlapping IgE and IgG4 epitopes for Ara h 1, 2, 3, 6, 8, and 9 were described previously in partly severely reacting peanut allergic patients[21]. Moreover, amino acids important for antibody binding were consistent for IgE and IgG4 binding which is in line with our observation for epitope F[20]. Contrary, amino acid replacements in the surrounding area of epitope E influenced the binding of IgE and IgG4 antibodies. While aa 46 was important for IgG4 binding in six patients, aa 58 was important for IgE binding in two patients, suggesting an oligo- or polyclonal response towards one epitope and limited clonal relation between IgE and IgG4 antibodies, at least in some patients. Overall, IgE and IgG4 can bind the same epitope, also simultaneously, although critical amino acids can differ for IgE and IgG4 antibodies.

Linear epitopes were mostly located in the flexible loops of the 3D protein model. Due to their loci, these epitopes are accessible for antibody binding, but also for enzymatic degradation. Mainly epitope E was located on the longest flexible loop of the Ara h 7 isoforms. This is in line with the main epitopes of Ara h 2 and 6 showing similar loci. However, unique epitopes of Ara h 7.0101 and 7.0301 were found in the α-helices, making them only accessible after enzymatic digestion.

To validate the applied technique, we additionally mapped linear epitopes of Ara h 2

and 6. Compared with previous literature, the same main epitopes were found, although two epitopes of Ara h 2 and two of Ara h 6 were not detected (Suppl. Table S1). However, comparing previous literature among each other, the epitopes described were only partly detected by other studies[14,15,21].

The identification of conformational epitopes might help elucidating the divergent potencies of the Ara h 7 isoforms. Since detecting and characterising conformational epitopes is more sophisticated than the identification of linear epitopes, especially with polyclonal serum, human monoclonal antibodies might be a suitable tool for the identification of conformational epitopes. Moreover, human monoclonal antibodies directed to one specific epitope can also be characterised for their exact affinity[22].

In conclusion, recognition of the 14 new mapped linear epitopes belonging to all three Ara h 7 isoforms was highly individual, and epitopes predominately bound by IgG4 varied from epitopes bound by IgE. These recognition patterns scarcely elucidated the divergent potency of Ara h 7 isoforms, indicating the importance of conformational epitopes for the recognition of Ara h 7.

Informed consent statement

This study was carried out in accordance with the University Medical Center Utrecht, Biobank Regulations, which are in compliance with the applicable national and international laws and regulations. These regulations permit the use of 'residual material from diagnostic testing' for research, unless the patient objects (Article 8, 'no objection' procedure). None of the included patients objected the use of their serum. The protocol was approved by the Biobank Research Ethics Committee of the University Medical Center Utrecht under the protocol number 18-428.

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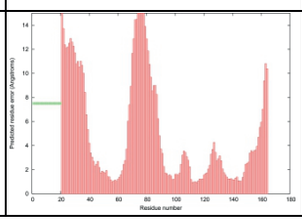
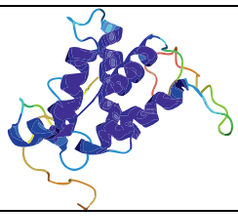
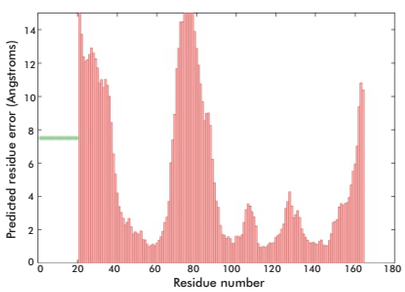
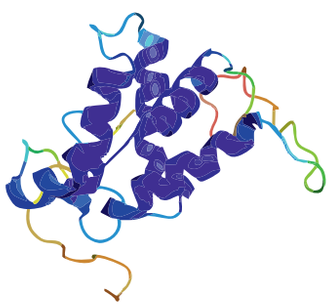
Supplementary

Graphical ModFOLD6 results for Ara h 7.0201				
Model name	**Confidence and p -value**	**Global model quality score**	**Residue error plot**	**3D view of residue error**
Ara h 7.0201	**CERT: 4.792E -4**	0.4604		

A



B



C

p-value cut-off	Confidence	Description
p < 0.001	CERT	Less than a 1/1000 chance that the model is incorrect.
p < 0.01	HIGH	Less than a 1/100 chance that the model is incorrect.
p < 0.05	MEDIUM	Less than a 1/20 chance that the model is incorrect.
p < 0.1	LOW	Less than a 1/10 chance that the model is incorrect.
p >0.1	POOR	Likely to be a poor model with little or no similarity to the native structure

Figure S1: Quality assessment of the 3D structure of Ara h 7.0201
*The quality of the Ara h 7.0201 3D model was assessed by ModFOLD6 webserver (https://www.reading.ac.uk/bioinf/ModFOLD/); **A** Errorresidue plot showing the predicted residue error; the loop regionshave a high predicted residue error whilst α-helices have a lowpredicted residue error. In general, loop regionsare hard to predict and to measure since loops are very flexible; **B** Predicted 3D model ofArah7.0201 with highlighted residues based on B-factors; **C** Table overviewof quality scores used bythe ModFOLD6 webserver*

Table S1: *Linear epitopes of Ara h 7 isoforms*
italic: residues only present in previous described epitopes

Protein	Peptide	Residues[A]	Publication[B]	Identified?	Specificity
Ara h 2.0201	*HASA*RQQWEL	1-5	1	x (overlapped)	IgE/IgG4
	QWELQGDR	3-10	1		
	DRRCQSQLER	9-18	1, 2, 3	x	IgG4
	LRPCEG(Q)HLMQ	21-30	1, 3	x	IgG4
	KIQRDEDS	31-38	1		
	PEQHLMQ KIQRDEDSY	25-30/31-39	2	x	IgG4
	RDPYSP	41-47/ 60-65	1, 2, 3	x	IgE/IgG4
	SQDPYSPS	47-54	1	x	IgE/IgG4
	DPYSPSPYDRR	61-71	2, 3	x	IgE/IgG4
	RRGAGSSQHQ	70-79	2	x	IgE
	CNELNEFENNQR	83-94	2, 3	x	IgE
	CEALQQIMENQSD	97-109	1, 3		
	QQIMENQ	101-107	1		
	LQGRQQ*EQQ*	111-119	1, 3	x	IgG4
	KRELRNL*PQQ*	121-130	1, 2, 3	x	IgE/IgG4
	*CGLRAP*QRCDLEVE	137-144	1, 3	x	IgE
	EVESGGRDR	142-150	2		
Ara h 6	MRRERGRGGDSSS	1-13	3	x	IgE/IgG4
	SSCERQVDRVNLK	11-24	new	x	IgG4
	KPCEQHIMQRI	24-34	3	x	IgG4
	GEQEQ	36-40	new	x	IgE
	YDSYDIR	41-47	3	x	IgE/IgG4
	CDELNEMENTQR	59-70	3		
	QQQRCCDELNE	54-64	new	x	IgG4
	CEALQQIMENQCD	73-85	3	x	IgE
	KRELRMLPQQ	97-106	3	x	IgE/IgG4
	CNFRAPQRCDLDV	107-119	3		
	RCDLDVSGGRC	114-124	new	x	IgE/IgG4

[A] without signal sequence

[B] 1: Stanley *et al.* 1997; 2: Shreffler *et al.* 2005; 3: Ostu *et al.* 2015


7

Can alternative epitope mapping approaches increase the impact of B-cell epitopes in food allergy diagnostics?

Clin Exp Allergy 2019;49:17–26

Anna M. Ehlers[1,2], Mark A. Blankestijn[1,2], André C. Knulst[1,2], Marco Klinge[3], Henny G. Otten[1]

[1]Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[2]Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

[3]EUROIMMUN AG, Lübeck, Germany

Abstract

In vitro allergy diagnostics are currently based on detection of specific IgE binding on intact allergens or a mixture thereof. This approach has drawbacks as it may yield false negative and/or false positive results. Thus, we review the impact of known B-cell epitopes of food allergens to predict transience or persistence, tolerance or allergy and the severity of an allergic reaction and to examine new epitope mapping strategies meant to improve serum-based allergy diagnostics. Recent epitope mapping approaches have been worthwhile in epitope identification and may increase the specificity of allergy diagnostics by using epitopes predominately recognised by allergic patients in some cases. However, these approaches did not lead to discrimination between clinically relevant and irrelevant epitopes so far, since the polyclonal serum IgE binding epitope spectrum seems to be too individual, independent of the disease status of the patients. New epitope mapping strategies are necessary to overcome these obstacles. The use of patient-derived monoclonal antibodies instead of patient sera for functional characterisation of clinically relevant and irrelevant epitope combinations, distinguished by their ability to induce degranulation, might be a promising approach to gain more insight into the allergic reaction and to improve serum-based allergy diagnostics.

Introduction

Food allergy is currently diagnosed by careful history, food challenges, skin prick test (SPT), and measurement of specific IgE (sIgE). The double-blind placebo-controlled food challenge (DBPCFC) is the gold standard, but it is a costly and burdensome procedure. Both SPT and sIgE measurement, using entire foods or single allergenic components, are hampered by false positive test outcomes[1]. This might be related to the presence of both clinically relevant and irrelevant antibodies as it has been shown for serum-based diagnostics of anti-neutrophilic cytoplasmic antibody-associated vasculitis[2]. Serum-based measurement might be improved by defining the epitope specificity, affinity, critical amino acids and antibody isotypes relevant for allergy.
Extensive research has been carried out, especially for peanut[3–10] and cow's milk[11–16], to identify the IgE- and IgG4-epitopes of food allergens. Linear epitopes, composed of a continuous amino acid sequence, have been detected by screening patient sera on sequential overlapping peptide libraries or allergen fragments. Conformational epitopes, composed of sterically closed amino acids upon folding, were characterised by phage display technique or mass spectrometry partly in combination with B-cell epitope prediction web tools or software, although the use of these techniques still has to be proven in future studies[6,17–21].
So far, it is impossible to discriminate between clinically relevant and irrelevant epitopes or combinations using current approaches, and to use these differences as diagnostic or prognostic markers. This review will discuss current knowledge, based on a relatively small number of studies investigating linear as well as conformational epitopes and comparing allergic and tolerant patients, and will propose alternative approaches for epitope mapping, focussing on epitope specificity and how this might impact serum-based allergy diagnostics.

Requirements for effector cell degranulation by FcεRI cross-linking

The major requirement for degranulation is the cross-linking of at least two FcεRI receptors. Cross-linking will only be feasible if two FcεεRI receptors are spaced apart by 50 to 240 Å. This range has been defined by using artifical allergens and hence might be somewhat smaller or larger for native allergens[22–24]. Consequently, the distance of two functional IgE-epitopes within one combination is restricted to the required distance of two FcεRI receptors. As an example, possible IgE epitope combinations of Ara h 2.0201, based on a 3D model built with the SWISS model web portal, are shown in Figure 1[25–29]. Residue distances greater than 35 Å, calculated with Chimera UCSF[30], were considered as a functional epitope combinations, highlighted on the 3D structure using Schrödinger Release 2018-1 (Maestro, Schrödinger, LLC, New York,

NY, 2018). Additionally, at least one epitope of the combination must be recognised with high affinity since the cross-linking has to take place for at least 100 seconds. Moreover, at least 1000 cross-links of FcεRI receptors on the surface of one effector cell have to take place[31–33]. If all these requirements for FcεRI crosslinking are met, the extent of degranulation is regulated by sIgE concentration, affinity, the ratio of allergen sIgE-antibodies compared to total IgE, and the specificity and number of epitopes recognised[34]. These requirements suggest that certain epitope/antibody combinations are only present in allergic and not in tolerant patients. However, basophils from 10-20% of the general population do not respond at all due to low expression of syk and/or SHIP-1 resulting in the inhibition of intracellular signalling[35–38]. Thus, different expression levels might regulate the extent of degranulation.

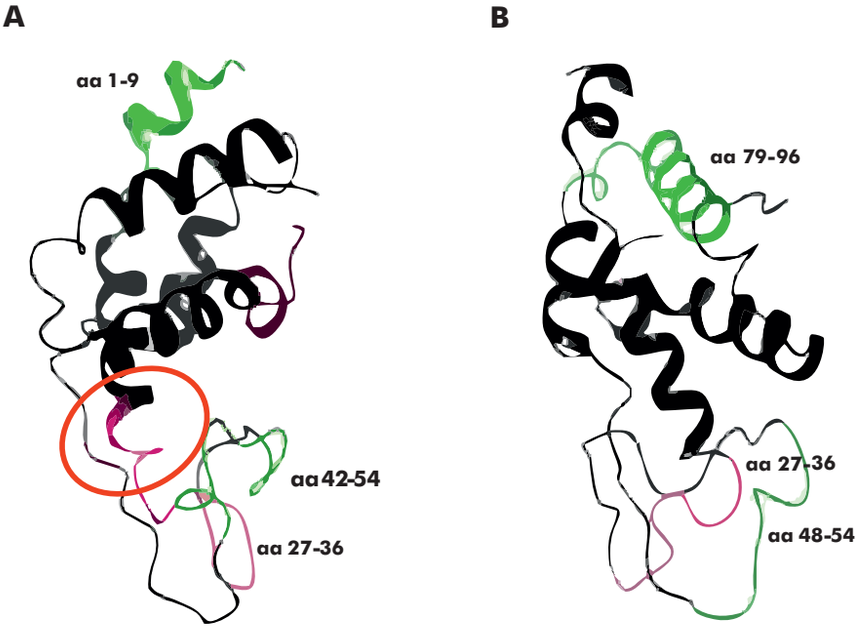


Figure 1: Determination of potentially relevant epitope combinations of the major peanut allergen Ara h 2 *The 3D structure of Ara h 2 was obtained by Swiss-Model and residues distances were determined by creating a residue distance map. Since the greatest distance was smaller than 50 Å, distances between 40 and 45 Å were considered as distances more likely to form relevant epitope combinations (highlighted in green). Distances between 35 and 40 Å were considered as possible epitope combinations but less likely (highlighted in pink). Predicted conformational epitopes by identified mimotopes are surrounded by red circles.;* ***A*** *One possible relevant epitope combination (40-45 Å) regarding the used model is between the described epitope aa 1-9 and the epitope aa 42-54 highlighted in green. Additionally, a combination with the same epitope aa 1-9 and the epitope aa 27-36 highlighted in pink is less likely (35-40 Å).;* ***B*** *Another possible relevant epitope combination (40-45 Å) regarding the used model is between the described epitope aa 79-96 and the epitope aa 48-54 highlighted in green. Additionally, a combination with the same epitope aa 79-96 and the epitope aa 27-36 highlighted in pink is less likely (35-40 Å).*

Food allergens as antigens: Influence of processing and digestion

Food allergens are modified by processing during industrial manufacturing or food preparation[39]. After processing, a fraction of these antigens will enter the buccal mucosa unaltered and the remainder will be reprocessed by human digestion before entering the tissue and the blood stream[40–42].

They can be modified during industrial manufacturing, which can lead to aggregation or modification of polysaccharide structures or appropriate amino acid residues. Certainly, only a portion of the same allergen is affected. One example is the roasting of peanuts, which usually leads, by Maillard reaction, to the modification of amino acids, especially the hydroxylation of prolines. In the study of Bernard *et al.*[43,44], the modification of the proline residue located in the DPYSP^{OH}S motif of Ara h 2 (aa 49-54 and 61-66 for Ara h 2.0201 and aa 49-54 for Ara h 2.0101) caused an increase in allergenicity. The same increase in allergenicity has been observed for the peanut allergen Ara h 1[43]. Processing effects vary, so for example allergenicity of the hen's egg allergen ovalbumin is decreased upon heat treatment, as shown by patients reacting to raw hen's egg, but not to cooked ones[45]. Modification by heating has also been observed for cow's milk, with allergens α- and ß-lactalbumin tending to aggregate upon heat treatment while caseins stay unaffected. However, testing *in vitro* processed cow's milk allergens showed inconsistent results between *in vitro* degranulation and the clinical history of the patients, often showing tolerance to heated milk[46]. Such observations of allergenicity changes require further research, to define allergen features that lead to increase or decrease. Knowledge of these features might help to predict the potential allergenicity of proteins even though different conditions of one processing method will have a great impact.

After ingestion, a part of the allergen enters the buccal mucosa and subsequently the bloodstream without being digested; however, as demonstrated in studies with peanut, gastric processing seems to further enhance the uptake and degranulation[40,42]. The influence of digestion on food allergens has been estimated in several studies and is dependent on the allergen structure. Stable proteins, including Ara h 2 and ovalbumin, remain unaffected by low pH and proteolysis[47] whereas Ara h 1 and 3, more labile proteins, are fragmented by pepsin. Upon entering the gut, peptides derived from digestion tend to aggregate due to the basic pH in the gut[48–50], which may lead either to shielding of previously accessible epitopes or to the development of new, presumably conformational epitopes. In short, industrial manufacturing, in combination with intestinal processing as well as matrix effects (not discussed here), influences the allergenicity of food proteins potentially by changing epitope profiles even though aggregation can also affect the solubility of the allergen. Precipitated and non-soluble allergen can falsely pretend no IgE binding in *in vitro* studies.

Identification of linear and conformational food allergen B-cell epitopes

Linear epitopes of several food allergens have been identified, mostly by overlapping peptide libraries, allergen fragments or phage display peptide libraries[9,12,15,51,52]. These approaches were partly coupled with B-cell epitope prediction software or webtools like ABCPred, BepiPred 1.0, and DNAStar Protean[21,53]. Moreover, in the studies of Zheng *et al.* and Chen *et al.*, the predicted B-cell epitopes were experimentally confirmed with high accordance[17,54]. However, no discrimination can be made between epitopes recognised by IgE, IgG and IgA probably important in the allergy context. So far, prediction models cannot be used without experimental data to result in more accurate diagnosis or immunotherapy since current prediction models do not consider the special requirements of the allergic reaction described above. In comparison to linear epitopes, the detection of conformational epitopes requires more sophisticated techniques, like X-ray crystallography, mass spectrometry or phage display libraries[55]. These phage display libraries can consist of peptides deriving from the allergen of interest or of random peptides. By using random libraries, peptides recognised by allergen specific antibodies have been shown to consist of sequences mimicking a continuous or discontinuous epitope of the allergen; such peptides are called mimotopes and can be mapped on the 3D structure of the allergen by predictive webtools like EpiSearch[10,56]. A large drawback of these approaches is their failure to detect alterations in allergens caused by post-translational modifications or processing and specific antibodies can only be detected against single epitopes and never in combination with others. Additionally, the assignment of mimotopes to surface patches of an allergen is solely based on *in silico* approaches and thus hampers the reliability of the outcome. Although mimotope mapping has been performed for a few allergens with conclusive functional results[10,57,58], it still has to be in context with inhibition and mutation studies using the full-length protein. The general limitation is the requirement of a high-resolution structure for the allergen of interest, constraining a broad application of this approach. Admittedly, mass spectrometry can be used to investigate the influence of post-translational modification using native proteins, and X-ray crystallography to detect epitope combinations. Co-crystallization studies have been performed with murine monoclonal IgG antibodies being able to reduce binding of human polyclonal IgE[59–62]. Continuatively, co-crystallization has been carried out using monoclonal IgE antibodies generated by combinatorial heavy and light chain libraries of allergic patients. However, it has not been proven weather these antibodies also occur naturally[63,64]. Information from these studies can help in understanding the features being responsible for allergenicity, and in defining critical amino acids more precisely. This knowledge can support the creation of more accurate serum-based diagnostics by modifying critical amino acids recognised

by clinically non-relevant IgE-antibodies. Moreover, it will give the opportunity to develop hypoallergenic variants for immunotherapy and better (IgE-) epitope prediction tools[65]. However, the co-crystallization of polyclonal serum antibodies bound to the allergen of interest is an almost insuperable bottleneck, making X-ray crystallography a more theoretical approach for conformational epitope mapping. These obstacles might be overcome by human-derived monoclonal IgE-antibodies.

Discrimination between persistence and transience by means of IgE binding epitopes

Most cow's milk allergic children outgrow their allergy by 3-4 years of age, although 15% remain allergic. In comparison, HEA arises later in childhood and 34% of these children will retain a persistent allergy[66]. Persistence has been studied through analysis of the epitope recognition pattern in patient sera. In CMA, persistent allergy was clearly associated with multiple IgE binding epitopes on αS1-, αS2-, κ-casein, α- and ß-lactalbumin as these were not recognised by IgE antibodies of children with transient allergy. However, the recognised epitopes do not coincide in different studies[12,15,67–69]. In HEA, four linear IgE binding epitopes of ovomucoid have been associated with persistent allergy since they were not recognised by IgE antibodies of transient allergic children[70,71]. In short, these data favour the use of epitopes to predict persistence and to discriminate between transience and persistence more precisely.

Discrimination between allergy and tolerance by means of IgE binding epitopes

For allergies less likely to be outgrown, identification of epitopes/antibodies discriminating between tolerance and allergy is essential to avoid unnecessary food elimination therapies. In cases of peanut allergy, tolerant patients appeared to recognise the same IgE binding epitope spectrum on Ara h 2 (linear epitopes) as allergic patients, although individual allergic patients recognised a higher number of different epitopes with higher IgE titres[72]. By means of the two key marker epitopes Ara h 2_10 (aa 28-42) and Ara h 2_18 (aa 52-66), allergic patients were correctly diagnosed with a sensitivity of 70% and a specificity of 60%. Sensitivity and specificity rose to respectively 90% and 95% by adding the IgE binding epitopes Ara h 1_16 (aa 46-60) and Ara h 3_140 (aa 418-432), which were recognised by few allergic patients[72]. In comparison, the sensitivity and specificity of intact Ara h 2 were defined as respectively 60-100% and 60-96%, showing no advantage using these two key marker epitopes[73]. Meanwhile, the conformational IgE binding epitope pattern of Gly m 4 showed no correlation with clinical reactivity at all[74]. The impact of linear or conformational epitopes remains restricted in

terms of ruling out tolerance or confirming allergy, as tolerant patients recognise the same epitope spectrum, although less frequently. In summary, to date, no clear discrimination between allergic and tolerant patients is feasible based on the detection of epitopes.

Severity prediction of an allergic reaction by means of IgE binding epitopes

Several studies have been performed to predict the severity of an allergic reaction by using explicit or multiple IgE binding epitopes of one or multiple allergenic components. To assess whether the severity of allergic reactions towards peanut is related to the type and number of epitopes recognised, patients with different symptoms (ranging from mild to severe and a sIgE level greater than 14 kU/L) have been studied[9]. Patients suffering from severe symptoms recognised fewer linear epitopes than patients with mild symptoms, suggesting that specific epitopes may be more relevant than the quantity of epitopes recognised. This may relate to the requirements for effector cell degranulation or suggest a greater relevance for conformational epitopes. However, sIgE binding to conformational B-cell epitopes did not contribute to severe symptoms as assessed by mimotopes mapped to the surface of Ara h 2 and 6[10]. In CMA, the difference in IgE binding epitope recognition pattern was analysed by including patients allergic to all forms of milk, patients tolerating heated milk, and patients with an outgrown allergy. Patients reactive to all forms of milk had a more diverse IgE and IgG4 epitope recognition pattern, comparable to a study of Sackesen *et al.* in which patients with persistent CMA reacted to processed milk and patient with transient tolerated processed milk[75]. The IgE binding pattern of patients non-reactive to heated milk was comparable to the recognition pattern of patients who had outgrown their allergy, although the IgG4 binding pattern was comparable to patients being allergic to all forms of milk. Patients tolerating heated milk had the lowest sIgG4 level and possessed low affinity IgE antibodies as did the tolerant group. In contrast, when investigated using a competition assay, the allergic group exhibited low and high affinity antibodies[76]. Low IgG4 levels in heated milk tolerating patients suggest a direct class switch from μ to ε without a mature germinal centre and thus, less somatic hypermutations and affinity maturation. High affinity antibodies might be generated by sequential class switching from μ to γ4 and to a lesser extent by a subsequent class switch to ε[77]. In several studies, auxiliary sIgG4 levels were similar between tolerant and allergic patients[78,79]. In conclusion, severity prediction can be made regarding the tolerance of processed or non-processed food since transient patients do tolerate processed food and patients with persistence CMA do not, but they recognise specific epitopes not recognised by transient patients. Thus, a more specified dietary advice might be given based on recognised epitopes.

However, the prediction of symptom severity is not yet feasible and due to a range of host-related factors will probably remain difficult[80,81]. Maximum release and the sensitivity of mast cells and basophils are dependent on intrinsic factors such as the regular exposure to an antigen that influences the production of a histamine-releasing factor interacting with surface-bound IgE. This interaction might lead to a higher extent of degranulation and/or spontaneously release without antigen exposure[82]. Additionally, different miRNAs have been implicated in up- or downregulation of genes involved in key signalling of mast cells (inhibition by miR-155 and miR-223; enhancement by miR-142-3p and miR-221)[83–86]. Moreover, polymorphism of mediator degrading enzymes can influence host-related mediator tolerance thresholds by increasing or decreasing enzyme levels or metabolic capacity. Polymorphism of the histamine degrading enzyme N-methyltransferase was associated with a higher risk of asthma and patients showing SNP His645Asp on the diamine oxidase (DAO) metabolising histamines were prone to develop an allergic reaction at lower sIgE levels[87–89]. Further, the polymorphism of platelet-activating factor acetyl hydrolase Thr198 and Val397 leads to lower substrate affinity prolonging the activity of platelet-activating factor[90], correlated positively with the severity of systemic reactions and anaphylaxis[91,92]. Finally, mast cell priming might be stimulated by medicines like beta-blockers and ACE inhibitors, increasing the severity of an allergic reaction[93].

Impact of IgG binding epitopes in allergy prediction

Investigations of the impact of epitopes in allergy prognostic and diagnostic have been mostly based on IgE binding epitopes, although antibodies of a different isotypes, mainly IgG4, seem to play a role in tolerance induction or retaining tolerance. In several studies, IgG4 increases coincided with decreases in sIgE, after outgrowing allergy or successful allergen immunotherapy (AIT)[78,79,94]. No statistically significant association with IgG4 epitopes comparable to IgE epitope pattern was found regarding persistence and transience, or severity of symptoms for the investigated food allergens peanut and cow's milk[6,12,72,76,95,96]. Moreover, conflictive results were obtained regarding the overlap between IgE and IgG4 binding patterns. Caubet *et al.* 2017 have been shown that transience is associated with similar IgE and IgG4 binding pattern while Savilahti *et al.* 2010 found similar IgE- and IgG4 binding pattern in patients with transient and persistent CMA[69,97]. In contrast, ratios of IgE to IgG4 could, in some studies, distinguish between peanut tolerant and allergic patients in children and adults[98,99], supporting the hypothesis that specific IgG4 can block IgE binding to definite epitopes. In a study with peanut sensitised children divided by tolerance and allergy, mast cell (LAD2 cells) and basophil degranulation induced by sera from peanut allergic children was inhibited by pre-incubation of the allergen with sera from peanut tolerant children prior to stimula-

tion. However, degranulation was only partially restored using IgG4-depleted sera[99]. These results can have several explanations:

- sterical hindrance of pre-bound IgE-antibodies from peanut tolerant children
- blocking of IgE binding from allergic children by pre-bound specific IgG4
- the binding of antigen by specific IgG bound by the FcγRIIb (CD32b) receptor on the surface of basophils or mast cells inhibiting degranulation by co-aggregation of the FcεRI and FcγRIIb[100]
- or a mixture thereof

For further investigation, the stimulation allergen can be pre-incubated with different well-characterised (epitope specificity, affinity) monoclonal IgG and IgE antibodies from peanut tolerant patients or a mixture thereof instead of polyclonal sera.

Impact of IgE and IgG binding epitopes in food allergy immunotherapy monitoring

In contrast to allergy prediction, the impact of IgG, especially IgG4, binding epitopes in AIT monitoring has been investigated more intensively. In general, patients, irrespective of the AIT outcome, showed an increase in allergen specific IgG4 levels as well as a temporary increase in IL-10 secreting FoxP3 positive Treg cells controlling a class-switch to IgG4 and a decrease in sIgE levels[101–104]. In a peanut AIT trial, newly formed IgG4-antibodies had specificities partly identical to identified IgE binding epitopes. Simultaneously, the total sIgE decreased, although, as also shown in CMA AIT trials, new IgE specificities were developed[94]. Additionally, patients who discontinued AIT due to adverse reactions showed an increase in quantities and affinity of epitope sIgE paired with a greater diversity of recognised IgE and IgG4 binding epitopes with little overlap[105]. Based on IgE binding pattern prior starting AIT, a prediction model for safety (=number of adverse reaction while AIT) and efficacy (= time required to achieve desensitization) was developed. The model included two sets of 16 IgE binding regions of caseins to be associated with safety and efficacy[106].
In further milk and peanut AIT trials, the induction of a hyporesponsive state of basophils was observed. This state was additionally applicable for hen's egg sensitised patients in the population of peanut allergic patients[107,108]. A possible explanation is the IgG epitope specific co-aggregation of the inhibitory FcγRIIb (CD23b) and FcεRI receptor potentially inhibits the PI3k pathway by SHIP-1 activation and subsequently loss of syk expression[109]. This might also explain the risk reduction in high risk infants to develop a peanut allergy by early and regular introduction of peanut consumption as

shown in the LEAP and LEAP-On study. Infants in the consumption group, compared to the avoidance group, showed higher specific IgG4 levels and IgG4/IgE ratios[110,111]. IgG4 binding epitopes do not seem to have a high impact in allergy prediction but do seem to be useful in food AIT monitoring. However, further investigation on IgG- and IgE binding epitope pattern in patients with a positive and negative outcome must be performed.

Limitation of these approaches

For most IgE binding epitopes, a limited number of studies were performed to pinpoint epitopes to be used as diagnostic markers. These attempts were only partly successful as allergy and tolerance still cannot be predicted by using IgE B-cell epitopes. A fundamental limitation is related to the use of polyclonal sera containing a mixture of antibodies with multiple isotypes recognising diagnostically relevant and irrelevant epitopes. This heterogeneous antibody repertoire in serum was confirmed by mutagenesis studies showing multiple amino acids to be critical for one epitope in CMA[112,113]. Another essential limitation is the restricted focus on IgE binding epitopes since the role of IgG or IgA binding epitopes is almost unknown. Thus, for improving serum-based food allergy diagnostics, human monoclonal antibodies instead of polyclonal patient sera might help to define clinically relevant epitopes and to investigate the role of antibodies with different isotypes, as explained below.

Different epitope mapping approaches may increase the contribution of allergen specific B-cell epitopes

To overcome the limitations described in the previous paragraphs, requirements for degranulation should be considered. Since cross-linking of at least two FcεRI receptors is crucial, an allergic subject must feature IgE antibodies recognising two epitopes with a distinct distance on the cell surface, of which at least one with high affinity[22]. In contrast, two IgE antibodies, with low affinity recognising a relevant epitope combination, or one IgE antibody recognising one epitope of a relevant epitope combination may occur in a tolerant patient without causing degranulation. Current IgE serum-based diagnostics cannot discriminate between high- and low-affinity antibodies, impeding the discrimination between relevant and irrelevant epitopes or combinations. Instead of polyclonal sera, a more distinct characterisation of specific epitopes might be feasible by using human monoclonal antibodies. This approach may also give precise information about epitope specificity and affinity (illustrated in Figure 2). Furthermore, co-crystallization of an epitope-specific monoclonal antibody or a ScFv with an allergen of interest is significantly more likely to be successful compared to the use of polyclonal sera, which

offers a chance to detect authentic conformational epitopes.

Since IgE producing B cells in blood are scarce, the first studies with IgE monoclonal antibodies were performed with murine allergen-specific antibodies, originally IgG but subsequently recombinantly expressed as IgE antibodies[34]. In addition, human monoclonal IgE antibodies were obtained by construction of phage display ScFv hybrid libraries with allergic donor-derived epsilon heavy chain and synthetic variable regions for the light chain[114,115]. However, these approaches did not address the whole allergen specific antibody repertoire of one subject. In studies by Hoh *et al.* and Patil *et al.*[116,117], a part of the antibody repertoire was analysed by single cell sequencing of the specific B-cell receptor. The lack of sequenced receptors derived from IgE B cells can be explained by the small number of PBMCs used in these studies (1 to $10 \cdot 10^6$ cells/experiment) whereas the frequency of Ara h 1 or 2 specific B-cells was determined to be around 0.01% of the B cell fraction in allergic patients[117]. Due to this low frequency, we recommend the use of a large volume of blood derived from allergic or tolerant donors to increase the number of circulating allergen specific B cells. These B cells can be single cell sorted and expanded *in vitro*, or the mRNA can be isolated directly[118]. Moreover, specific B cell clones can be obtained by immortalisation and limited dilution, but this approach may cause *in vitro* class switching or lead to selection bias due to the expansion of the fittest clones. Additionally, we propose, in contrast to Hecker *et al.*[115], the isolation of all allergen specific B-cells irrespective of the isotype produced as the role of different allergen-specific isotypes remains unclear. Finally, we recommend the inclusion of tolerant and allergic patients sensitised to the same allergen for the generation of specific monoclonal antibodies. Monoclonal antibody repertoires within these two groups may allow the discovery of differences in epitope specificity and affinity. To ensure the capture of B cells producing antibodies with comparable affinity and epitope specificity as the polyclonal serum repertoire, the plasma of the patients should be saved and analysed for their epitope recognition spectrum and affinity. In addition, competition assays with monoclonal antibodies and serum antibodies can be performed to detect differences in affinity.

Potentially, these findings can contribute not only to improvement of food allergy diagnostics but also monitoring of allergy immunotherapy. This epitope mapping strategy will be restricted by the absence of short and long-living plasma cells in the blood as well as the still discussed presence of IgE^+ memory B cells[77,119–122]. If re-stimulated IgG memory B cells are the source of class-switched IgE producing cells, instead of IgE memory B cells, the sequence of IgG B cell receptors can be recombinantly expressed as IgE antibodies and functionally characterised as IgG and IgE antibodies. Based on this hypothesis by Aalberse and Platts-Mills 2004, IgE^+B cells class-switched from IgG^+ memory cells are not able to survive in the germinal centre where affinity maturation takes place and the variable region of the IgE^+ B cell receptor does not undergo

further somatic hypermutations[77]. Another aspect to take into account is the expression of the low affinity FcεRII (CD23) on non-allergen specific translational B cells in the blood stream[123,124]. The cells can bind allergen specific IgE antibodies leading to the selection of B cells irrelevant for allergy. Non-specific antibodies can be excluded through a specificity check, such as performing an ELISA coated with the allergen of interest.

Another approach to enlarging the impact of B cell epitopes in food allergy diagnostics might be the development of improved computational epitope prediction. To exceed the abilities of already available prediction software or webtools for B cell epitopes, computational approaches for predicting allergenic functionality must meet, as described above, the special requirements for degranulation, for example the defined distance between two epitopes. Before using these distances as a basis for prediction, they should be estimated by natural and not, as now, by artificial allergens. Furthermore, new algorithms must consider the potential oligomerization of allergenic proteins, since this can increase the number of possible epitope combinations for triggering effector cell degranulation.

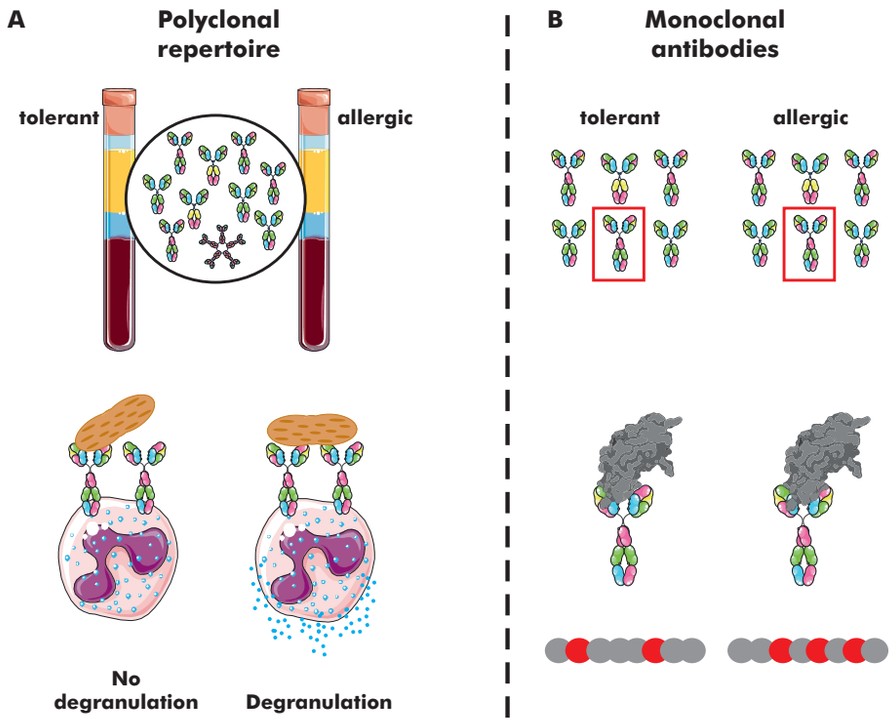


Figure 2: Defining differences in epitope binding pattern of tolerant but sensitised and allergic patients are hampered by similar appearing polyclonal antibody repertoire (Parts of the graphic from Servier Medical Art by Servier)
A *The polyclonal repertoires in sera of tolerant but sensitised and allergic patients seem to be similar but only antibodies out of the repertoire from allergic patients are able to induce an allergic reaction;* ***B*** *The use of monoclonal antibodies enables a more extensive characterisation of individual antibodies responsible for the allergic reaction. Tolerant as well as allergic patient can possess antibodies with similar epitope specificities but with different affinity.*

Conclusion

The use of monoclonal antibodies might be a powerful tool to define the allergen specific antibody repertoire of tolerant and allergic patients more precisely in terms of epitope specificity, affinity and feasible epitope combinations. This will produce more knowledge about the reasons some sensitised patients can tolerate an allergen with no symptoms while others will experience a (severe) allergic reaction. Differences in antibody repertoires can lead to improved sIgE measurement by modifying critical amino acids recognised by clinically irrelevant antibodies and thus, preventing false-positive test results and avoiding burdensome food challenges. In addition, the characterisation of allergen specific antibody repertoires during immunotherapy may lead to a better understanding of the underlying mechanism.

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Comparison of two strategies to generate specific human monoclonal antibodies: Which method to choose for which purpose?

submitted

Anna M. Ehlers[1,2], Constance F. den Hartog Jager[1,2], Tineke Kardol-Hoefnagel[1], Miriam M. D. K. Katsburg[1], André C. Knulst[1,2], Henny G. Otten[1]

[1]Center for Translational Immunology, University Medical Center Utrecht, Utrecht University, Netherlands

[2]Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Netherlands

Abstract

Human monoclonal antibodies (mAbs) are valuable tools to link genetic information with functional features and to provide a platform for conformational epitope mapping. Strategies to generate human mAbs from peripheral blood have been described and used in several studies including single cell sequencing of antigen-binding B cells and the establishment of antigen-specific monoclonal Epstein-Barr Virus (EBV) immortalised lymphoblastoid cell lines (LCLs). However, direct comparisons of these two strategies are scarce. Hence, we sought to set up these two strategies in our laboratory using peanut 2S albumins (allergens) and the autoantigen ARHGDIB as antigen targets to directly compare these strategies regarding costs, time expenditure, recovery, throughput and complexity. Regarding single cell sequencing, up to 50% of corresponding V(D)J gene transcripts were successfully amplified of which 54% were successfully cloned into expression vectors used for heterologous expression. Seventy-five percent of heterologously expressed mAbs showed specific binding to peanut 2S albumins resulting in an overall recovery of around 25%. In comparison, the establishment of monoclonal EBV-LCLs showed a lower overall recovery of around 16%. Heterologous expression of a mAb carrying the same variable region as its native counterpart showed comparable concentration-dependent binding abilities. By directly comparing those two strategies, single cell sequencing allows a broad examination of antigen-binding mAbs in a moderate-throughput manner, while the establishment of monoclonal EBV-LCLs is a powerful tool to select a small number of highly reactive mAbs restricted to certain B cell subpopulations. Overall, both strategies, initially set-up for peanut 2S albumins, are suitable to obtain human mAbs and they are easily transferrable to other target antigens as shown for ARHGDIB.

Introduction

Antibody diversity enables the adaptive immune system to generate a humoral response against virtually any antigen. Gene recombination of variable (V), diversity (D) and joining (J) gene segments for the heavy chain and V and J gene segments for the corresponding light chain results in a wide variety of antibodies with distinct specificities. The diversity is even enlarged by imprecision during the V(D)J gene rearrangement process[1,2]. The introduction of somatic hypermutations is an additional tool to increase diversity but more importantly to strengthen antibody's affinity against the respective target[3].

Disease-related specific antibody repertoires are often studied by comparing specific B cell subpopulations between patients and healthy donors using next generation sequencing[4–7]. This powerful approach, however, does not provide any information about antigen reactivity, affinity and functionality. For this reason, several studies included the generation of human monoclonal antibodies (mAbs) in order to assess their functionality and to map their characteristics to their genetic features[8,9]. Besides identifying genetic features associated with health or disease, human mAbs can support the mapping of conformational epitopes formed by closely located amino acids upon folding[9]. While linear epitopes, comprised of sequential amino acids, can easily be mapped by e.g. peptide microarrays, the mapping of conformational epitopes requires more sophisticated techniques such as mass spectrometry, nuclear magnetic resonance spectroscopy and/or mutation libraries[10]. Since these techniques can hardly be executed with patient serum containing polyclonal antibodies, human mAbs are powerful tools to overcome this obstacle. Moreover, data obtained with mAbs derived from humans are thought to be more easily translatable to clinical research compared with data obtained with e.g. mouse-derived mAbs[9,11].

The first human mAbs were obtained in the early 90's by phage display technology using single chain or Fab fragment libraries[12,13]. Nearly simultaneously, transgenic animals consisting of human immunoglobulin genes provided an additional tool[14]. These approaches, however, are artificial and cannot represent a complete human antibody repertoire, emphasising the advantage of mAbs generated from human peripheral blood or tissues. The first strategy to generate human mAbs from peripheral blood included the establishment of immortalised B cell lines by Epstein-Barr Virus (EBV) infection followed by limiting dilution cloning[15,16]. More recently, the immortalisation by EBV was partly replaced by BCl-6 overexpression mimicking a germinal centre status accompanied by constant antibody secretion[17]. The most recent technique, however, is single cell sequencing with subsequent heterologous antibody expression[18].

This study provides a detailed description and comparison of two different strategies to generate human mAbs including single cell sequencing of antigen-binding B cells

(Method 1) and the establishment of monoclonal EBV-immortalised B cell lines (Method 2). Both methods were used to generate human mAbs against peanut 2S albumins, major allergens in peanut allergy, and anti-Rho guanosine diphosphate dissociation inhibitor 2 (RhoGDI2, alternative 'ARHGDIB'), a non-HLA target potentially involved in graft failure upon kidney transplantation[19]. Method 1 is especially suitable for a broad examination of antigen-binding mAbs due to a less selective process compared with Method 2. Method 2, however, is a powerful tool to select a small number of highly reactive mAbs potentially applicable in the development of treatment strategies. A schematic overview of both methods is shown in Figure 1.

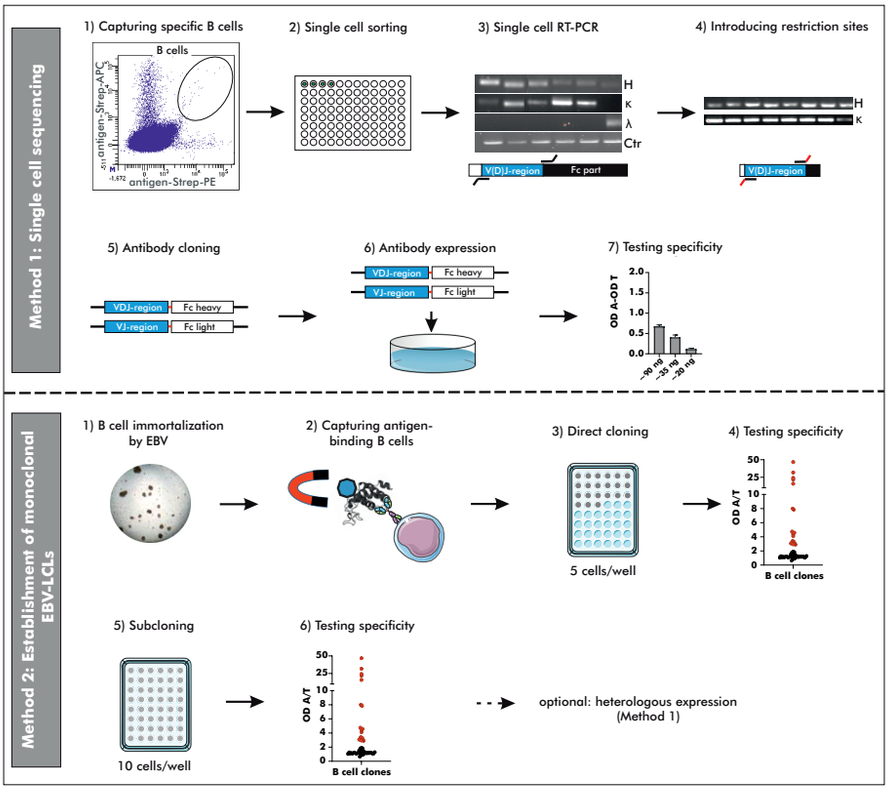

Figure 1: Schematic overview on two different methods to generate human mAbs
Method 1 „Single cell sequencing" *B cells for single cell sorting were captured by double-positive antigen-tetramer staining (1+2). Upon transcription of total mRNA into cDNA, the V(D)J gene transcripts of the heavy and corresponding light chain were amplified by reverse transcriptase PCR using multiplex primers (3). The V(D)J gene usage was determined by Sanger sequencing and respective specific simplex primers were used to introduce restriction sites for subsequent cloning (4). Amplified V(D)J gene transcripts were cloned into commercially available pFUSEss-IgH vectors (Invivogen) carrying either the heavy or light chain backbone (5). Vectors with correctly incorporated V(D)J gene transcripts were used for transient mammalian cell transfection (6) and the specificity was examined upon heterologous expression using a direct ELISA (7).*
Method 2 „Establishment of monoclonal EBV-LCLs" *Antigen-binding B cells were captured from B cells immortalised by Epstein-Barr Virus (EBV) using antigen-coupled magnetic beads (1+2). Based on theoretical frequency of antigen-binding B cells, cells were directly cloned by seeding 5 cells/well on top of irradiated PBMCs as feeder cells (3). After 4 weeks of culturing, the supernatants were checked for antibodies binding specifically to the antigen of interest (4). B cells with supernatant containing specific antibodies are seeded for an additional round of cloning (5) and the resulting supernatant was screened for specific antibodies (6) after additional 4 weeks of culturing. Monoclonality was checked by Sanger sequencing and the mAbs can be heterologously expressed as described for Method 1.*

Methods

Method 1: „Single cell sequencing of antigen-binding B cells“

Antigen-tetramer formation

The isolated peanut 2S albumin fraction or heterologously expressed ARHGDIB were treated with an excess of biotin in accordance with manufacturer's instructions. The excess of biotin resulted on average in four biotin molecules per one molecule protein (EZ-Link^{TM} Sulfo-NHS-Biotin, ThermoFisher Scientific)[20]. Antigen-tetramers were subsequently formed by separately adding streptavidin-PE or streptavidin-APC to the biotinylated 2S albumin fraction or ARHGDIB in a molecular ratio of 1:1 (streptavidin: protein)[21,22].

Single-cell sorting of antigen specific B cells

Blood was drawn into heparin-coated tubes and freshly processed within 24 hours. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation and B cells were, subsequently, negatively enriched from the PBMCs fraction by magnetic beads (B cell isolation kit II, Miltenyi Biotech). Enriched B cells were stained for antigen specificity with CD45-PO (1:40, Life Technologies), CD3-PB (1:160, BD Pharming), CD19-FITC (1:20, BD Pharming), CD14-PE-Cy7 (1:800, ITK Diagnostics BV), CD16- PE-Cy7 (1:1000, BD Pharming) and antigen-tetramers (1:5000 (= 75 pg 2S albumins[23])[1]) , 1:500 (= 1.1 ng ARHGDIB[23])[2]). Stained B cells were gated for CD45^{+}, CD3^{-}, CD19^{+}, antigen tetramers (double positive)$^{+}$ and CD14/16^{-} and single cell sorted into 96 wells plates supplemented with 14 mM DTT and 11.2 U RNAse inhibitor in a total volume of 4 µl 0.5x PBS. Plates containing single-sorted B cells were immediately put on dry ice. These plates were stored at -80 °C until further processing. While PBMCs from a donor with specific B cells in previous experiments served as positive control, B cells stained with biotin were used as negative control (Ctr 1). The gating was based on the respective negative control 1. An additional negative control (Ctr 2) was added to evaluate the optimal dilution for the antigen-tetramers. This control consisted of a pre-incubation step with non-biotinylated antigen followed by the normal staining protocol described above.

[1] $\rightarrow$ 30 $\cdot$ 10^6 antigen-tetramers sufficient for approximately 150-300 resting B cells
[2] $\rightarrow$ 112 $\cdot$ 10^6 antigen-tetramers sufficient for approximately 500-1000 resting B cells

Gene amplification of B cell receptors

V(D)J gene transcripts amplification was performed as previous described with minor modifications[18,21,24]. While keeping the frozen B cells on dry ice, they were supplemented with 1.4% NP-40, 3 U RNAse inhibitor and 7 µM hexamer primers reaching a total volume of 7.5 µl. For primer annealing, this mixture was subsequently incubated for one minute at 68 °C and cDNA transcription was performed in accordance with manufacturer's instruction (SuperScript III, ThermoFisher Scientific).The resulting cDNA was subsequently used as template for the amplification of the heavy chain gene transcript and its corresponding light chain gene transcript. Amplification was accomplished with 1.25 U hot-start DNA polymerase (AmpliTaq Gold, ThermoFisher Scientific), 2.5 mM $MgCl_2$, 1 mM dNTP mix and 40 nM multiplex primers (Suppl. Table S1 - S3) for 50 cycles. The annealing temperature was set to 62, 60 and 58 °C for heavy, κ and λ light chain, respectively.

Sequence analysis of B cell receptors

Amplified V(D)J gene transcripts were purified by adding 0.5 µl Exo RI (0.01 U, Bioke) and 1 µl FAST-AP (1 U, ThermoFisher Scientific) and incubating this mixture for 30 min at 37 °C followed by 20 min at 80 °C. Purified heavy chain gene transcripts were Sanger sequenced using 200 nM multiplex reverse or framework (FR)1 forward primers. Light chain gene transcripts were sequenced using 200 nM of the respective reverse primers. To check the quality of the sequences, they were evaluated using Chromas Lite 2.6.5. Double peaks, potentially resulting from errors in the beginning of the amplification reaction, were aligned to their germline and corrected if plausible. Quality-checked gene sequences were saved as FASTA files and subsequently used for automatic germline alignment using the IgBLAST web interface (reference: IMGT database)[25]. The resulting output was written into a SQLite database using R.

Cloning of V(D)J gene transcripts

To ensure the cloning of the entire V(D)J gene transcript without the introduction of additional amino acids, restriction sites were introduced with specific V gene forward and J gene reverse primers. This introduction was achieved using 0.5 U Phusion high-fidelity DNA polymerase (New England BioLabs (NEB)) in presence of 1x reaction buffer, 2.5 mM $MgCl_2$, 1 mM dNTP mix and 400 nM respective forward and reverse primer. The annealing temperature of 62, 60 and 58 °C for heavy, κ and λ light chain, respectively and the amplification reaction was performed for 30 cycles. Gene products, purified accordingly to manufacturer's instructions (NucleoSpin gel and PCR

clean up, Macherey Nagel), were digested with 1 U of the respective restriction enzymes Eco RI and NhE I (heavy chain, NEB), BsWi (κ light chain, NEB) or Avr II (λ light chain, NEB). Before cloning the digests into human IgH (IgE, IgG1, IgG4) and IgL (κ, λ) expression vectors[3], they were purified as described above and phosphorylated with 1 U T4-polynucleotide kinase (PNK, NEB) for 60 min at 37 °C followed by 20 min at 65 °C. To prevent self-ligation, 1 µg of digested and gel-purified parent vector (1 U respective restriction enzyme) was dephosphorylated with 4 U FAST-AP (ThermoFisher Scientific) for 10 min at 37 °C followed by 10 min at 70 °C. Dephosphorylated vectors were mixed with digests in a molecular ratio of 5:1 and incubated with 1 U T4 ligase (NEB) for 60 min at room temperature. The ligated vector was transformed into competent *E. coli* Top10 cells by incubating the mixture for 45 sec at 42 °C (heat shock). Overnight grown colonies were screened for incorporated V(D)J gene transcripts by PCR using 400 nM forward primers binding to the respective FR2 region and 400 nM reverse primers suitable for the constant part of the respective vector (Suppl. Table S1 - S3) in presence of 2.5 U AmpliTaq polymerase (ThermoFisher Scientific), 2.5 mM $MgCl_2$ and 1 mM dNTP mix. The annealing temperature was set to 63, 62 and 61 °C for heavy, κ and λ light chain, respectively and the amplification cycle was repeated for 30 times. Positive clones were grown overnight in 3 ml LB medium containing either zeocin or blasticidin. Purified vectors (NucleoSpin Plasmid EasyPure, Macherey Nagel) were Sanger sequenced. The correctness was verified by aligning the vector sequence to the first sequence result.

Heterologous expression of monoclonal antibodies

For heterologous expression of human mAbs, human embryonic kidney (HEK) 293F cells were cultured in FreeStyle 293 expression medium (ThermoFisher Scientific) using 125 ml shaking culture flasks. Exponentially growing cells at a confluence of 80% and viability of 90% were transiently transfected with VH and VL expression vectors in a ratio of 2:3 (total 0.5 µg plasmid DNA per $1 \cdot 10^6$ cells) using 150 mm^2 culture plates and 293fectin (2 µl/µg plasmid DNA, ThermoFisher Scientific). To ensure a sufficient intake, the expression vectors were supplemented with 0.5 µg pAdvantage plasmid (Promega). The supernatant was harvested three days upon transfection and stored at -20 °C for further analyses.

[3]hEF1-HTLV promotor, IL-2 signal sequence, multiple cloning site upstream the constant regions and zeocin (IgH) or blasticidin (IgL) resistance genes for selection (pFUSEss vector series, Invivogen)

Method 2: „Establishment of monoclonal EBV-LCLs“

Immortialisation of enriched B cells by Epstein-Barr-Virus

B cells, isolated from heparin blood as described for Method 1, were immortalised with EBV in presence of the TLR9 agonist CpG 2006 and the immunosuppressive Cyclosporine A to establish lymphoblastoid cell lines (LCLs)[15]. In detail, 1 ml of EBV-containing supernatant, obtained from growing B-95.8 cells, was added to $5 \cdot 10^6$ pelleted B cells and incubated for 60 min at 37 °C and 5% CO2. Upon washing with 1 ml PBS, infected B cells were resuspended in 3 ml RPMI-1640 supplemented with 20% fetal calf serum (FCS), 2.5 µg/ml CpG 2006, 1 µg/ml Cyclosporine A and 1% Penicillin-Streptomycin (Pen/Strep). The suspension was cultured at 37 °C and 5% CO2 until visible clusters were formed and the medium changed its colour from red to yellow due to acidification.

Isolation of antigen-binding B cells

Antigen-binding B cells were isolated from LCLs with an excess of antigen-coupled NHS-activated magnetic beads (ThermoFisher Scientific). The antigen was coupled to the beads in accordance with manufacturer's instruction. For the isolation, LCLs ($10 \cdot 10^6$ cells) were pelleted and cooled on ice for 60 min. The pellet was subsequently resuspended in 100 µl PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA accompanied by 30 µl of antigen-coupled beads and cooled on ice for another 10 min. This suspension was applied on a magnetic column (MACS Cell Separation Columns, Miltenyi Biotec B.V.) and the separation was achieved in accordance with manufacturer's instruction. The elution fraction was collected as antigen-binding B cells and was used to generate antigen-binding monoclonal LCLs.

Direct cloning

The number of isolated antigen-binding LCLs was estimated based on the frequency of antigen-binding B cells determined by flow cytometry analysis (around 0.01% of the B cells - Method 1). The elution fraction of the magnetic separation was mixed with $1 \cdot 10^6$/ml irradiated PBMCs (35 Gy) suspended in RPMI-1640 containing 20% FCS and 1% Pen/Strep to achieve a concentration of 25 antigen-binding LCLs/ml. 200 µl of this mixture was transferred to one well of a flat-bottom 96 wells plate (= 5 cells/well). The cells were allowed to grow for 4 weeks without re-feeding and the plate was tilted after the first week of culturing to keep the cells in close contact after the feeder cells died off. The plate was straightened again after an additional week of culturing to avoid too close contact between steadily proliferating cells[16].

Generation monoclonal LCLs by limiting dilution cloning

LCLs with supernatant containing antibodies specific to the antigen of interest were used for a second round of cloning to generate monoclonal LCLs. To this end, positive LCLs from the direct cloning step were counted and diluted to 50 cells/ml in RPMI-1640 supplemented with 20% FCS, 1% Pen/Strep and $1 \cdot 10^6$/ml irradiated PBMCs (35 Gy). This mixture was seeded in a volume of 200 µl/well in a flat-bottom 96 wells plate (10 cells/well) and incubated for 4 weeks as described above. For defining the optimal seeding density, a range from 0.3 to 10 cells/well were used. LCLs secreting antibodies specific for the antigen of interest were transferred to a 5 mL round bottom polystyrene test tube and cultured until a visible pellet was observed and the medium colour changed from red to yellow. For further expansion, the LCLs were first transferred to a 25 cm^2 culture flask and subsequently to a 75 cm^2 culture flask. Expanded LCLs secreting specific mAbs were frozen at -80 °C and the supernatant containing mAbs was stored at -20 °C. Monoclonality was checked by Sanger Sequencing as described for Method 1.

RNA extraction

Total RNA was isolated accordingly to manufacturer's instruction (RNA-Bee, BioConnect). Briefly, pelleted LCLs were homogenized in 1 ml RNA-Bee and the RNA was separated from the genomic DNA by adding 200 µl of chloroform and spinning for 15 min at 12.000 x g and 4 °C. The colourless phase was transferred to 500 µl ice-cold isopropanol and incubated for 10 min on ice to precipitate RNA. The precipitate was washed with 75% ethanol and the resulted pellet was resolved in RNAse free water for 15 min at 55 °C. Extracted RNA was either stored at -80 °C or immediately used.

cDNA transcription and gene transcript amplification

RNA was transcripted into cDNA in accordance with manufacturer's instructions (Transcriptor First Strand cDNA Synthesis Kit, Roche). Briefly, 2 µl of random hexamer nucleotides (5 µM) were mixed with 8 µl of RNA template (1 µg) and the mixture was incubated for 3 min at 85 °C. Upon cooling down, 10 µl cDNA reaction mixture containing 1X RT-buffer, 5 mM $MgCl_2$, 1 mM dNTP mix, 5 U RNA inhibitor, 1.5 U avian myeloblastosis virus (AMV)-RT and 10 mM gelatine was added and incubated for 90 min at 42 °C. The reaction was stopped by inactivating the AMV-RT for 3 min at 85 °C. cDNA was either stored at -20 °C or immediately used for V(D)J gene transcript amplification as described for Method 1. Contrary to Method 1, the primer concentrations were adjusted to 400 nM instead of 40 nM.

Examination of specific antigen-binding

Specific binding to peanut 2S albumins

Specificity of native and heterologously produced mAbs to peanut 2S albumins was tested using a direct ELISA. Briefly, plates were coated by applying either 0.3 µg/well Ara h 2 and 6[26] or transferrin (negative control) overnight at room temperature. On the following day, the plate was blocked with PBS supplemented with 1% BSA and 0.1% Tween-20 (blocking buffer) for 60 min at room temperature. Subsequently, supernatants were applied upon 1:2 dilution in blocking buffer (EBV LCLs) or in serial dilution (1 to 10 µg/ml) (heterologously expressed mAbs) 60 min at room temperature under continuous shaking. Bound antibodies from EBV-LCLs were detected with α-human kappa (1:10.000, SouthernBiotech) and α-human lambda antibodies coupled with horse radish peroxidase (1:5000, SouthernBiotech) for 60 min at room temperature under continuous shaking. Bound heterologously expressed mAbs, on the other hand, were detected by either goat α-human IgE (1:5000, KPL) or goat α-human IgG (1:2000, Jackson ImmunoResearch) antibodies coupled with horse-radish peroxidase under the same conditions. Visualisation was provided by adding tetramethylbenzidine (TMB) for 15 min in the dark and the optical density (OD) was measured at 450 nm. Native Abs were considered for further cloning or analysis when the sample OD was at least two times higher than the OD of the negative control. Heterologously expressed mAbs were defined as specific if the sample OD was, upon subtraction of the negative control OD value, at least 1.5 times greater than the OD obtained with culture medium. mAbs with OD values above 1 at a concentration of 10 µg/ml were considered as strong binders, mAbs with OD values between 0.07 and 1 at all concentration steps were considered as moderate binders and mAbs with increased OD values at the highest concentration of 10 µg/ml, but no detectable OD at the lowest concentration of 1 µg/ml, were considered as weak binders.

Specific binding to ARHGDIB

Antibody specificity to ARHGDIB was evaluated using ARHGDIB-coupled microspheres diluted in PBS supplemented with 0.1% BSA (wash buffer)[20]. IgG-coupled microspheres served as positive control whilst empty and transferrin-coupled microspheres served as negative controls. All incubation steps were performed in the dark, at room temperature and with continuous shaking.

For each antibody to be tested, 1500 microspheres consisting of 4 colours, each individually coated, were incubated overnight with 50 µl undiluted HEK293 (Method 1) or EBV-LCLs supernatant (Method 2) containing the respective mAb. Upon washing with a Bio-Plex Pro Wash station (Bio-Rad), bound mAbs were detected with either an

1-step (Method 1) or a 2-step procedure (Method 2). For the 1-step procedure, 50 µl of 1:50 diluted PE-conjugated goat-α human IgG antibody (Jackson ImmunoResearch) was added and incubated for 30 min. For the 2-step procedure, 50 µl of goat α-human kappa (1:100, Southern Biotech) and goat α-human lambda antibody (1:32, Southern Biotech) was added and incubated for 30 min. For the second step, 50 µl of 1:100 diluted PE-conjugated donkey α-goat IgG antibody was added and incubated for additional 30 min. For the readout of both procedures, 50 µl of washing buffer was added and the median fluorescence intensities (MFI) were measured on a Luminex 200 flow analyser (Luminex Corp) (50 counts, 75 µl sample volume, 90 sec time out).

Recovery rate and cost calculation

The recovery rate of specific human mAbs was calculated for both methods as followed:
Method 1:

$$\text{recovery} = 100\% \cdot \text{amplification efficiency} \cdot \text{cloning efficiency} \cdot \text{portion specific mAbs}$$
$$\text{recovery} = 100\% \cdot 0.5 \cdot 0.54 \cdot 0.75$$
$$\text{recovery} = 20.3\%$$

Method 2:

$$\text{recovery} = \frac{n({}^{s}_{dc}wells) \cdot efficiency_{dc} \cdot n\frac{mAbs}{{}^{s}_{dc}wells} \cdot efficiency_{mc}}{n(antigen{-}binding\ B\ cell_{theo})} \cdot 100\%$$
$$\text{recovery} = \frac{200 \cdot 0.8 \cdot 2 \cdot 0.55}{1000} \cdot 100\%$$
$$\text{recovery} = 17.6\%$$

$n({}^{s}_{dc}\text{wells})$ = seeded wells for direct cloning based on theoretically antigen-binding B cells
efficiency_{dc} = efficiency of direct cloning (approximate 2S albumins and ARHGDIB)
efficiency_{mc} = efficiency of the second round of cloning
$\frac{mAbs}{{}^{s}_{dc}wells}$ = approximate number of mAbs obtained from one seeded well for direct cloning

Costs for the generation of one single human mAb was calculated by adding up the expenses and correcting it for the recovery rate of the respective strategy. Personal costs were included based on an average salary of a research technician and the approximate working hours needed to produce one single mAb.

Results

Method 1: „Single cell sequencing“

Double tetramer-staining reduced the selection of non-specific CD19$^+$ B cells

Antigen-binding B cells for subsequent single cell V(D)J gene transcript amplification were detected by flow cytometry using antigen-tetramers formed with fluorophore - labelled streptavidin. For the development of an optimal staining protocol, enriched B cells, derived from the same blood bank donor, were stained with 2S albumin-tetramers accompanied by either a single (APC) or two distinct fluorophores (APC and PE). As shown in Figure 2a, B cells single-positive for 2S albumin-tetramer binding accounted for 0.1% of the total CD19$^+$ B cell fraction. Both controls - staining with biotin without the antigen (Ctr 1) and blocking with unlabelled antigen (Ctr 2) - showed, however, a comparable percentage of 2S albumin-binding B cells (Ctr 1: 0.092%; Ctr 2: 0.097%). The subtraction of Ctr 2 resulted in a final percentage of 0.008% 2S albumin-binding B cells. In comparison, the fraction of double-positive 2S albumin-binding B cells was already reduced to a percentage of 0.004% without any background staining, indicating the potential of double antigen-tetramer staining for identifying bona fide antigen-binding B cells. For validation purposes, double-tetramer staining was used for the detection of 2S albumin (Figure 2b) or ARHGDIB-binding B cells (Figure 2c) in 6 independent blood bank donors. 2S albumin-binding B cells ranged from 0.002 to 0.007% whilst ARHGDIB-binding B cells ranged from 0.005 to 0.015%, indicating a good reproducibility of the developed staining protocol.

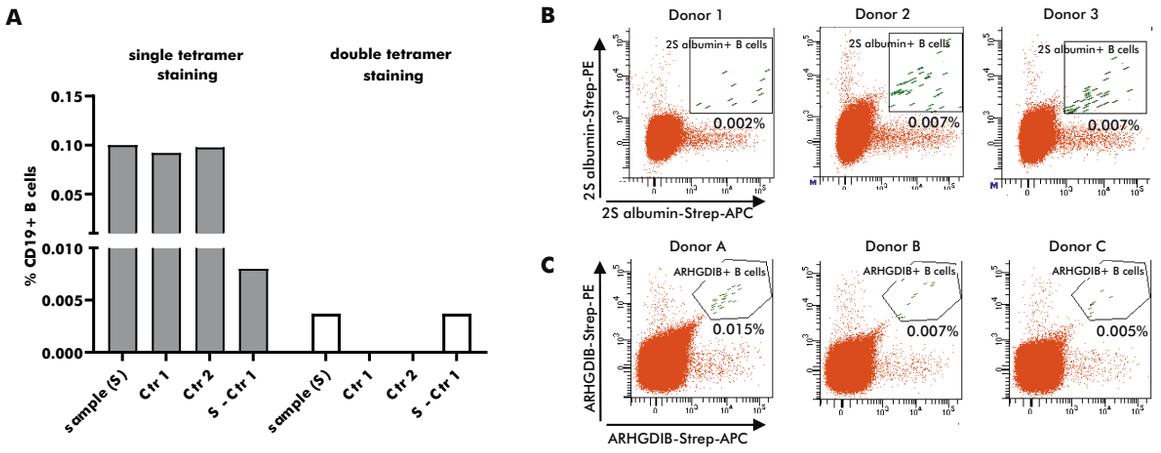

Figure 2: Selection of antigen-binding B cells using antigen-tetramers
A *Percentage of 2S albumin-binding B cells in relation to the total CD19$^+$ B cell fraction using single or double-tetramer staining. Control 1 (Ctr 1) represents the staining with only biotin and Control 2 (Ctr 2) is executed by pre-blocking with unlabelled 2S albumin prior normal staining procedure. The final percentage is calculated by subtracting Ctr 2 from the sample.;* ***B*** *and* ***C*** *FACS plots representing antigen-binding B cells in relation to the total B cell population of independent donors. 2S albumin-binding B cells are shown in* ***B*** *and ARHGDIB-binding B cells in* ***C****.*

V(D)J gene transcript amplification efficiency is donor-dependent

Amplification efficiency upon single cell sorting of 2S albumin-binding B cells was evaluated in 6 independent blood bank donors. Amplification efficiency of the heavy chain VDJ gene transcript from 24 to 50 individual B cells ranged from 29 to 63% whilst the percentage of successfully amplified V(D)J gene transcripts from the heavy and corresponding light chain was reduced and ranged from 17 to 50%. Although a low amplification efficiency was shown for two donors, most of the donors (4/6) showed sufficient amplification efficiencies of approximately 50%[4]. Cloning of successfully amplified heavy and corresponding light chain gene transcripts resulted into an overall cloning efficiency of 54% which may be increased by ordering the not successfully cloned gene sequences commercially[5]. Individual cloning efficiencies were estimated to 76% for the heavy chain gene transcripts and 71% for the light chain gene transcripts (78% kappa and 31% lambda).

[4]Note: We observed higher amplification efficiencies when primer aliquots were stored in 50 mM Tris-HCl buffer supplemented with 2 mM EDTA instead of RNAse free water.

[5]Note: We observed successful cloning of commercially obtained gene sequences while the cloning of the original gene sequences failed.

Antibodies derived from double-positive tetramer-binding B cells are mostly specific

The specificity of human mAbs, generated by single cell sequencing, can only be examined upon heterologous expression in the end of the workflow. Overall, 32 heterologously expressed mAbs from 10 different donor carried the variable region of 2S albumin-binding B cells and 1 mAb carried the variable region of an ARHGDIB-binding B cell and their concentrations varied between 0.1 and 10 µg/ml. Binding to 2S albumins was observed in 75% (24/32) of all heterologously expressed mAbs. Based on their binding abilities, examined by comparing their achieved OD values at different concentrations, they were roughly categorised into weak (n=13), moderate (n=8) and strong (n=1) binders (Figure 3a and 3b). This variability in target binding indicates that our selection strategy was not restricted to only strongly binding B cells and implicates no selection bias regarding distinct affinities. Our staining protocol can also be adjusted to different antigen targets as the mAb generated from an ARHGDIB-binding B cell showed strong binding to ARHGDIB-coupled microspheres with an ARHGDIB/transferrin ratio greater than 2 (Figure 3c).

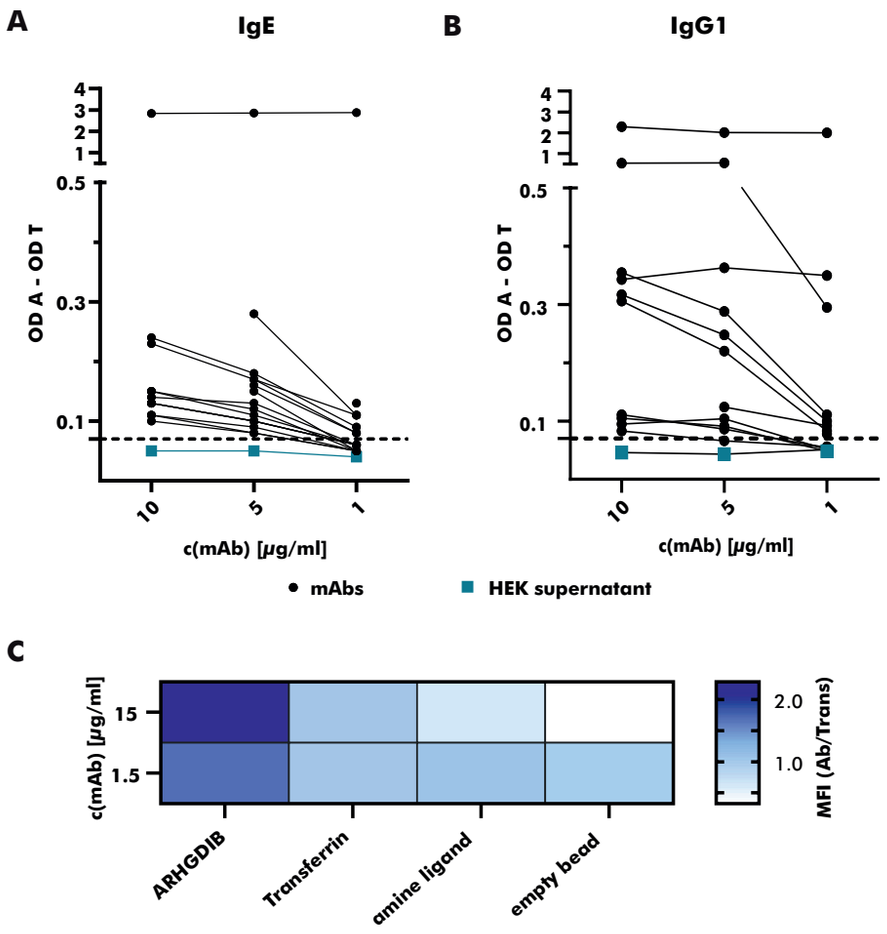

Figure 3: Specificity of heterologously expressed mAbs (Method 1)
A *The binding of serial-diluted (1 to 10 µg/ml) human mAbs (IgE) towards peanut 2S albumins was measured using α-human IgE-HRP as detection antibody. Measured OD values were corrected by subtracting the OD value measured for the control antigen (transferrin) and compared to the OD value obtained with HEK supernatant not containing any antibodies (turquoise). The threshold was set to an OD value 1.5 times greater than the OD value obtained with HEK supernatant;* ***B*** *The binding of serial-diluted (1 to 10 µg/ml) human mAbs (IgG1) towards peanut 2S albumins was measured using α-human IgG1-HRP as detection antibody. The measured OD value was corrected as described for* ***A****.;* ***C*** *The specificity of the mAb derived from an ARHGDIB-binding B cell was examined using ARHGDIB-coupled microspheres. The median fluorescence intensity (MFI) towards ARHGDIB was evaluated in relation to the MFI towards transferrin as control antigen.*

Method 2: „Establishment of monoclonal EBV-LCLs“

LCLs isolated with antigen-coupled beads are mostly antigen-specific

Native Abs can be produced by *in vitro* culturing of primary B cells secreting polyclonal Abs upon activation. Since *in vitro* culturing of primary B cells is challenging[27], especially in the absence of a particular antigen, we chose for the establishment of LCLs by EBV immortalisation. Immortalised LCLs were selected for their binding to either peanut 2S albumins or ARHGDIB. As shown in Figure 4a, almost all polyclonal EBV-LCLs (98.8%) obtained from the first round of cloning secreted antibodies specifically binding to 2S albumins (2S albumin/transferrin ratio $\geq$2) compared to the supernatant of irradiated PBMCs (Ctr). This large number of positive EBV-LCLs (100%) was confirmed by selecting for 2S albumin-binding EBV-LCLs using a second blood bank donor. Although the number of positive clones was reduced for ARHGDIB-binding B cells (Donor 1: 67%, Donor 2: 56%), a representative number of EBV-LCLs was selected for a second round of cloning to achieve monoclonality (Figure 4b).

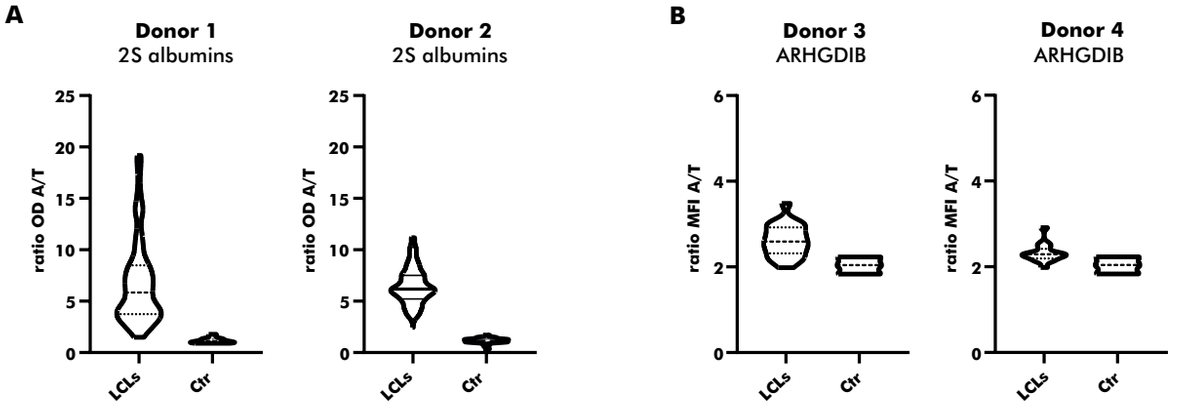

Figure 4: Direct cloning of peanut 2S albumin and ARHGDIB-binding EBV-LCLs
A *Antibodies secreted by directly cloned EBV-LCLs were screened for their binding to peanut 2S albumins using a direct ELISA. EBV-LCLs secreting antibodies with an OD ratio (2S albumin/transferrin)* $\geq$*2 were selected for an additional round of cloning.;* ***B*** *Antibodies secreted by directly cloned EBV-LCLs were screened for their binding to ARHGDIB using antigen-coupled microspheres. EBV-LCLs secreting antibodies with a MFI ratio (ARHGDIB/transferrin)* $\geq$*2 were selected for an additional round of cloning.*

Optimal seeding density to achieve monoclonal LCL clones

LCLs positive for antigen-binding were used for an additional round of cloning to achieve LCLs secreting monoclonal instead of polyclonal Abs. EBV-LCLs from the first blood bank donor were used to determine the optimal seeding density. Even though a seeding density of 0.3 cells/well results theoretically in the highest probability of obtaining monoclonal LCLs, only a small number of wells contained LCLs showing proliferation. By increasing the seeding density, the number of LCLs secreting Abs binding to 2S

albumins rose in accordance with the number of LCLs seeded per well. Despite a high seeding density of 10 cells/well, 55% of sequenced EBV-LCLs (6/11) showed monoclonality, leading to a compromise between a high number of proliferating EBV-LCLs and a reasonable rate of achieved monoclonal EBV-LCLs (Figure 5a). Seeding 2S albumin-binding EBV-LCLs from the second blood bank donor at a density of 10 cells/well resulted in an approximate recovery of 2 EBV-LCLs with specific binding to 2S albumins per seeded plate (Figure 5b). A comparable recovery was achieved for ARHGDIB-binding EBV-LCLs from another two independent blood bank donors (Figure 5c).

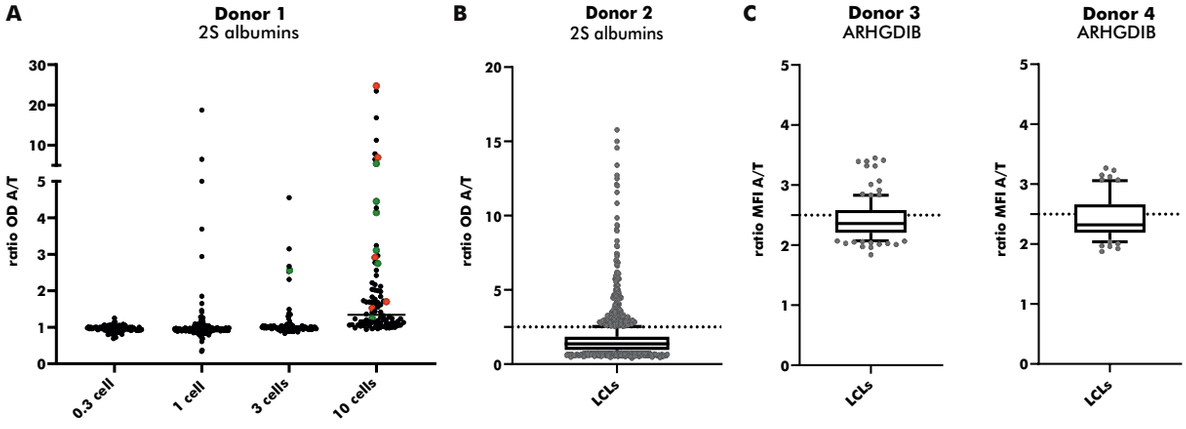

Figure 5: Subcloning of 2S albumin and ARHGDIB-binding EBV-LCLs
A *Determination of the optimal seeding density for the second round of EBV-LCLs cloning. EBV-LCLs with specific binding to peanut 2S albumins (ratio 2S albumins/transferrin ≥2) were considered for expansion. The V(D)J gene transcript of these EBV-LCLs were Sanger sequenced to check for monoclonality. Monoclonal EBV-LCLs are highlighted in green and polyclonal EBV-LCLs are highlighted in red.* ***B*** *Antibodies secreted by subcloned EBV-LCLs were screened for their binding to peanut 2S albumins. EBV-LCLs with a ratio (2S albumin/transferrin) ≥ 2 were considered for expansion.;* ***C*** *Antibodies secreted by subcloned EBV-LCLs were screened for their binding to ARHGDIB. EBV-LCLs with a ratio (ARHGDIB/transferrin) ≥ 2.5 were considered for expansion.*

Heterologously expressed mAbs showed comparable binding to 2S albumins

To validate heterologous expression of mAbs secreted by established EBV-LCLs, one pair of heavy and corresponding light chain variable regions was selected from the pool of 2S albumin-specific monoclonal EBV-LCLs. Upon heterologous expression with a vector containing the IgG1 backbone, native and heterologously expressed counterparts were applied on a direct ELISA in a serial dilution as shown in Figure 6. The measured OD values were comparable between the native IgM mAb and the heterologously expressed IgG1 mAb (light chain detection), indicating that mAbs from subcloned EBV-LCLs can also be heterologously expressed with comparable binding abilities.

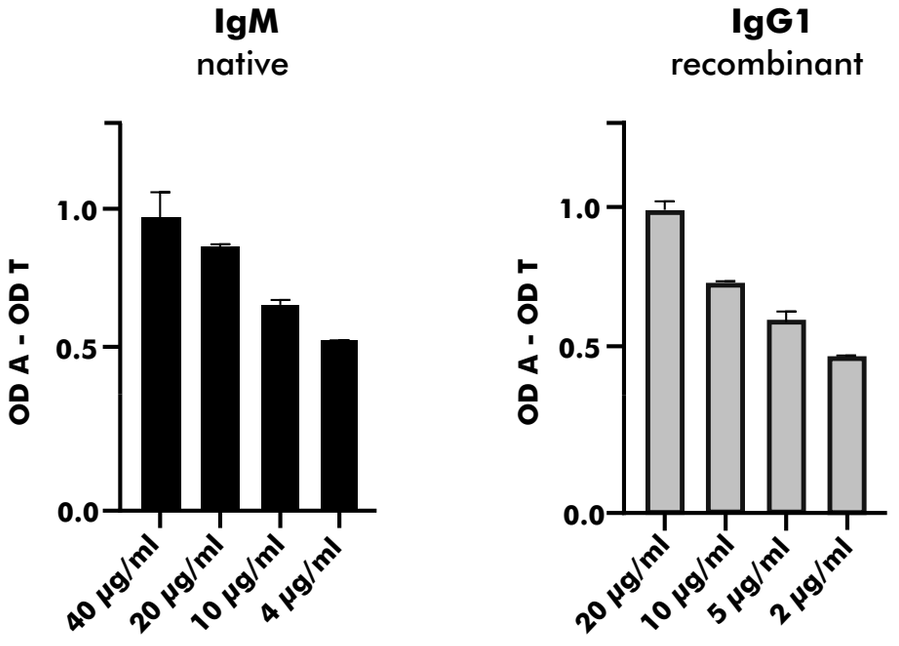

Figure 6: Heterologous expression of a human mAb with specific binding to peanut 2S albumins
A mAb carrying the same variable region as native antibodies obtained from a monoclonal EBV-LCL was heterologously expressed with a vector containing the IgG1 backbone. Its binding ability expressed as OD values (corrected for binding to transferrin) was compared to the binding ability of its native counterpart.

Single cell sequencing allows higher throughput

Both methods described within this chapter are characterised by several up- and downsides influencing the choice which method to use. A comparison regarding costs, expenditure of time, recovery, throughput and complexity is shown in Table 1.

Table 1: *Comparison of both methods to generate human mAbs*

	Method 1: „Single cell“	Method 2: „EBV-LCLS“
Costs[A]	650-750€	450€
Time	3 weeks	12 weeks
Adaptation	sorting strategy: 3 weeks	no special adaptation needed
Recovery	20-29%[B]	18%[C]
Throughput	+++	+
Compexity	+++	+++

[A] Costs per one single human mAb

[B] Cloning efficiency of 54% to 76% (might be achieved by ordering gene sequences to increase cloning efficiency)

[C] The number of subcloned B cells is reduced by reaching the capacity threshold. This has been reflected with the limited throughput.

Without considering any further characterisation, the generation of one single human mAb can add up to 750€. The establishment of monoclonal EBV-LCLs, however, is generally less expensive (400 - 500€) compared twith the generation by single sequencing (650 - 750€) resulting from expensive reagents required for single cell amplification. On the other hand, the workflow of single cell sequencing shortens the required time from 12 weeks including long culturing periods to only 3 weeks. Even though both methods showed comparable recovery rates in our experiments, the establishment of monoclonal EBV-LCLs reaches easily a capacity threshold limiting the overall throughput and leading to random selection of clones to proceed with. Hence, the establishment of monoclonal EBV-LCLs is a suitable tool to generate a limited number of human mAbs, especially due to the ability to screen for binding, functionality and neutralisation capacity throughout the workflow. Single cell sequencing is, however, a more suitable tool for a broader examination of antigen-specific B cells, since a much higher throughput can be achieved. Unfortunately, specificity and functionality can only be examined in the end of the workflow. Both methods are extremely laborious and highly complex.

Discussion

Studying human-derived mAbs offers the possibility to link genetic information to functional features, making them indispensable in modern molecular biology research. In this study, we directly compared two distinct strategies to generate (highly) specific mAbs from peripheral blood of human donors, *i.e.*: single cell sequencing and the establishment of monoclonal EBV-LCLs. While single cell sequencing is a suitable tool to generate a large panel of mAbs with distinct binding features, the establishment of monoclonal EBV-LCLs provides the possibility to screen for specificity and functionality throughout the workflow. Both strategies, initially set-up for mAbs specifically directed against peanut 2S albumins, were easily adaptable to other antigen targets as shown for ARHGDIB.

Corresponding V(D)J gene transcripts were successfully amplified from up to 50% of all single cell sorted 2S albumin-binding B cells. The overall recovery of 2S albumin-specific human mAbs, however, was reduced to around 20% when cloning efficiency (54%) and proportion of mAbs with proven specificity (75%) was taken into consideration. The cloning efficiency may be increased by ordering gene sequences without cloning success commercially. Overall, our amplification efficiency (up to 50%) corresponds to the work of Tiller and colleagues who described an amplification efficiency of up to 60%[18]. Moreover, a comparable overall recovery of 27% has been shown for single cell sequencing of antigen-specific B cells from guinea pigs[28]. Increased amplification efficiencies of 90 to even 100% have been described for performing a com-

parable amplification protocol in triplicates[29]. However, such an approach will simultaneously increase the probability of amplification errors, potentially hampering gene analysis and antibody cloning.

Regarding the establishment of antigen-specific monoclonal EBV-LCLs, the overall recovery was estimated to around 18%. This is in accordance with cloning efficiencies of around 15% observed for limiting dilution approaches upon EBV immortalisation[16]. However, we lack the information of what proportion of antigen-specific B cells were initially immortalised by EBV. To overcome this limitation, Fraussen and colleagues described in their protocol the immortalisation of 50 cells/well upon sorting the desired B cell subpopulation[30]. However, we were not able to immortalise such small numbers of B cells successfully in our laboratory.

Both strategies are characterised by their own strengths and limitations. Single cell sequencing, on the one hand, enables the execution in a moderate-throughput manner and the selection of antigen-specific B cells is not restricted to certain subpopulations as EBV immortalisation is restricted to CpG-activated memory B cells[15]. These advantages make the single cell sequencing platform a suitable tool for a broad examination of antigen-binding B cells and their corresponding mAbs. The establishment of monoclonal EBV-LCLs, on the other hand, provides continuous screening for specific binding and functionality throughout the workflow, resulting in an easy selection of mAbs with high affinity towards their targets and making this strategy a powerful tool in therapeutic research. Moreover, this strategy enables the comparison of heterologously expressed mAbs with their natural counterparts, allowing the identification of potential structure alterations by post-translational modifications during heterologous expression[31,32]. An additional advantage of generating human mAbs by establishing monoclonal EBV-LCLs is their limited need for expensive reagents.

In conclusion, both strategies - single cell sequencing and establishment of monoclonal EBV-LCLs - are able to generate (highly) antigen-specific human mAbs and they are easily adaptable to other target antigens. The recommended method to choose is dependent on the research question to explore as both strategies have their own strengths and limitations.

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Supplementary

Table S1: *Primers for heavy chain gene transcripts, restriction site underlined*

Primer	Sequence
Heavy chain primer forward	
Single cell RT-PCR	
5' L-Vh1	ACAGGTGCCCACTCCCAGGTGCAG
5' L-Vh3	AAGGTGTCCAGTGTGARGTGCAG
5' L-Vh4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5' L-Vh5	CAAGGAGTCTGTTCCGAGGTGCAG
Introduction restriction sites	
5' EcoRI_VH1	ATATTGAATTCGCAGGTGCAGCTGGTGCAG
5' EcoRI_VH1/5	ATATTGAATTCGGAGGTGCAGCTGGTGCAG
5' EcoRI_VH1-18	ATATTGAATTCGCAGGTTCAGCTGGTGCAG
5' EcoRI_VH1-24	ATATTGAATTCGCAGGTCCAGCTGGTACAG
5' EcoRI_VH3	ATATTGAATTCGGAGGTGCAGCTGGTGGAG
5' EcoRI_VH3-23	ATATTGAATTCGGAGGTGCAGCTGTTGGAG
5' EcoRI_VH3-33	ATATTGAATTCGCAGGTGCAGCTGGTGGAG
5' EcoRI_VH3-9	ATATTGAATTCGGAAGTGCAGCTGGTGGAG
5' EcoRI_VH4	ATATTGAATTCGCAGGTGCAGCTGCAGGAG
5' EcoRI_VH4-34	ATATTGAATTCGCAGGTGCAGCTACAGCAGTG
5' EcoRI_VH4-39	ATATTGAATTCGCAGCTGCAGCTGCAGGAG
5' EcoRI_VH6-1	ATATTGAATTCGCAGGTACAGCTGCAGCAG
Sanger sequencing	
5' Vh1-FR1_(1-2)	GGCCTCAGTGAAGGTCTCCTGCAAG
5' Vh2-FR1_(2-5)	GTCTGGTCCTACGCTGGTGAAACCC
5' Vh3-FR1_(3-7)	CTGGGGGGTCCCTGAGACTCTCCTG
5' Vh4-FR1_(4-4)	CTTCGGAGACCCTGTCCCTCACCTG
5' Vh5-FR1_(5-51)	CGGGGAGTCTCTGAAGATCTCCTGT
5' Vh6-FR1_(6-1)	TCGCAGACCCTCTCACTCACCTGTG
Colony screening	
5' Vh1-FR2_(1-2)	CTGGGTGCGACAGGCCCCTGGACAA
5' Vh2-FR2_(2-5)	TGGATCCGTCAGCCCCCAGGGAAGG
5' Vh3-FR2_(3-7)	GGTCCGCCAGGCTCCAGGGAA
5' Vh4-FR2_(4-4)	TGGATCCGCCAGCCCCCAGGGAAGG
5' Vh5-FR2_(5-51)	GGGTGCGCCAGATGCCCGGGAAAGG
5' Vh6-FR2_(6-1)	TGGATCAGGCAGTCCCCATCGAGAG
5' Vh7-FR2_(7)	TTGGGTGCGACAGGCCCCTGGACAA
Heavy chain primer reverse	
Single cell RT-PCR	
3' CH1_IgA^{A}	AGCCCTGGACCAGGCA

3' CH1_IgE[A,B]	GAAGACGGATGGGCTCTGT
3' CH1_IgG[A,B]	GGAAGGTGTGCACGCCGCTG
3' CH1_IgM[A]	GGGAATTCTCACAGGAGACG

Introduction restriction sites

3' NheI_JH1/2/4/5	ATGCTAGCTGAGGAGACGGTGACCAG
3' NheI_JH3	ATGCTAGCTGAAGAGACGGTGACCATTG
3' NheI_JH6	ATGCTAGCTGAGGAGACGGTGACCGTG

Table S2: *Primers for kappa light chain gene transcripts, restriction site underlined*

Primer	Sequence
Kappa light chain primer forward	
Single cell RT-PCR	
5' L-Vκ1/2	ATGAGGSTCCCYGCTCAGCTGGTGG
5' L-Vκ3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5' L-Vκ4	ATTTCTCTGTTGCTCTGGATCTCTG
Introduction restriction sites	
5' EcoRI_Vκ1-5	ATATTGAATTCAGACATCCAGATGACCCAGTC
5' EcoRI_Vκ1-9	ATATTGAATTCAGACATCCAGTTGACCCAGTCT
5' EcoRI_Vκ1D-43	ATATTGAATTCAGCCATCCGGATGACCCAGTC
5' EcoRI_Vκ2-24	ATATTGAATTCAGATATTGTGATGACCCAGAC
5' EcoRI_Vκ2-28	ATATTGAATTCAGATATTGTGATGACTCAGTC
5' EcoRI_Vκ2-30	ATATTGAATTCAGATGTTGTGATGACTCAGTC
5' EcoRI_Vκ3-11	ATATTGAATTCAGAAATTGTGTTGACACAGTC
5' EcoRI_Vκ3-15	ATATTGAATTCAGAAATAGTGATGACGCAGTC
5' EcoRI_Vκ3-20	ATATTGAATTCAGAAATTGTGTTGACGCAGTCT
5' EcoRI_Vκ4-1	ATATTGAATTCAGACATCGTGATGACCCAGTC
Colony screening	
5' V1κf/6	TCAAGGTTCAGCGGCAGTGGATCTG
5' Vκ2f	GGCCTCCATCTCCTGCAGGTCTAGTC
5' Vκ3f	CCAGGCTCCTCATCTATGATGCATCC
5' Vκ4_int	CAACTGCAAGTCCAGCCAGAGTGTTTT
5' Vκ5_int	CCTGCAAAGCCAGCCAAGACATTGAT
5' Vκ7_int	GACCGATTTCACCCTCACAATTAATCC
Kappa light chain primer reverse	
Single cell RT-PCR	
3' Cκ 494[A,B]	GTGCTGTCCTTGCTGTCCTGCT
Introduction restriction sites	
3' BsiWI_Jκ1/4	ATCGTACGTTTGATYTCCACCTTGGTC
3' BsiWI_Jκ2	ATCGTACGTTTGATCTCCAGCTTGGTC
3' BsiWI_Jκ3	ATCGTACGTTTGATATCCACTTTGGTC
3' BsiWI_Jκ5	ATCGTACGTTTAATCTCCAGTCGTGTC

[A] Sanger Sequencing

[B] Colony screening

Table S3: *Primers for lambda light chain gene transcripts, restriction site underlined*

Primer	Sequence
Lambda light chain primer forward	
Single cell RT-PCR	
5' L-Vλ1	GGTCCTGGGCCCAGTCTGTGCTG
5' L-Vλ2	GGTCCTGGGCCCAGTCTGCCCTG
5' L-Vλ3	GCTCTGTGACCTCCTATGAGCTG
5' L-Vλ4/5	GGTCTCTCTCSCAGCYGTTGCTG
5' L-Vλ6	GTTCTTGGGCCAATTTTATGCT
5' L-Vλ7	GGTCCAATTCYCAGGCTGTGGTG
5' L-Vλ8	GAGTGGATTCTCAGACTGTGGTG
Introduction restriction sites	
5' EcoRI_Vλ1	ATATTGAATTCGCAGTCTGTGCTGACKCAG
5' EcoRI_Vλ2	ATATTGAATTCGCAGTCTGCCCTGACTCAG
5' EcoRI_Vλ3	ATATTGAATTCGTCCTATGAGCTGACWCAG
5' EcoRI_Vλ4/5	ATATTGAATTCGCAGCYTGTGCTGACTCA
5' EcoRI_Vλ6	ATATTGAATTCGAATTTTATGCTGACTCAG
5' EcoRI_Vλ7/8	ATATTGAATTCGCAGRCTGTGGTGACYCAG
Colony screening	
5' Vλ1/2_int	ATTCTCTGGCTCCAAGTCTGGC
5' Vλ3_int	GGATCCCTGAGCGATTCTCTGG
Lambda light chain primer reverse	
Single cell RT-PCR	
3' Cλ [A,B]	CACCAGTGTGGCCTTGTTGGCTTG
Introduction restriction sites	
3' AvrII_Jλ1	ATTCCTAGGACGGTGACCTTGGT
3' AvrII_Jλ2/3	ATTCCTAGGACGGTCAGCTTGGT
3' AvrII_Jλ6	ATTCCTAGGACGGTCACCTTGGT
3' AvrII_Jλ7-1	ATTCCTAGGACGGTCAGCTGGGT
3' AvrII_Jλ7-2	ATTCCTAGGGCGGTCAGCTGGGT

[A] Sanger Sequencing

[B] Colony screening


9

Distinction between peanut allergy and tolerance by characterization of B-cell receptor repertoires

submitted

Anna M. Ehlers[1,2], Constance F. den Hartog Jager[1,2], André C. Knulst[1,2], Henny G. Otten[1]

[1]Center for Translational Immunology, University Medical Center Utrecht, Utrecht University, Netherlands

[2] Department of Dermatology/Allergology, University Medical Center Utrecht, Utrecht University, Netherlands

Abstract

Specific IgE against a peanut 2S albumin (Ara h 2 or 6) is the best predictor of clinically relevant peanut sensitization. However, sIgE levels of peanut allergic and those of peanut sensitized but tolerant patients partly overlap, highlighting the need for improved diagnostics to prevent unnecessary food restrictions. By single-cell sequencing of 2S albumin-binding B cells, we explored differences in V(D)J gene transcripts encoding for 2S albumin-specific monoclonal antibodies from 6 peanut allergic, 6 peanut sensitized but tolerant , and 5 non-atopic donors. In the allergic donors, 89% of heavy chain gene transcripts consisted of VH3 family genes, compared with only 54% in sensitized but tolerant and 63% of non-atopic donors. Additionally, certain HCDR3 sequence motifs were associated with allergy or tolerance upon hierarchical clustering of their Levenshtein distances. These sequence motifs may support the diagnosis of patients with suspected peanut allergy and sensitization to Ara h 2 and/or 6.

Introduction

Food allergy affects around 2.5% of the European population[1]. Symptoms of food allergic reactions can range from mild oral itching to life-threatening anaphylaxis. Even though anxiety for unexpected allergic reactions can have a great negative impact on patients' quality of life, elimination diets and prescription of epinephrine auto-injectors are still the main treatment options in daily practice. To ensure precise dietary advices, there is a strong need for accurate, preferably minimally invasive, diagnostic strategies. Current food allergy diagnostics comprise careful history, skin prick tests, measuring specific IgE (sIgE) and double-blind placebo-controlled food challenges (DBPCFC) as the gold standard. However, DBPCFCs are burdensome for the patient, costly and require dedicated hospital facilities[2,3]. On the other hand, current technologies to measure sIgE detect both clinically relevant and irrelevant sensitization, potentially leading to incorrect diagnosis and consequently unnecessary food restrictions[4,5]. Clinically relevant peanut sensitization is associated with sIgE against the major, partly cross-reactive, peanut allergens belonging to the 2S albumin family, Ara h 2 and 6. In previous studies in adults, 100% positive predictive values for sIgE against Ara h 2 and 6 were found using sIgE positivity thresholds of respectively 1.75 kU/l and 1.8 kU/l. Specific IgE levels below these thresholds, however, overlapped between allergic and tolerant subjects, preventing precise diagnosis[6,7].

The occurrence of clinically irrelevant sensitization to Ara h 2 and 6 might be explained by differences in peanut (Ara h 2 and 6) specific antibody repertoires. These differences may include the presence of non-IgE antibodies blocking the binding of clinically relevant epitopes by sIgE of tolerant patients. Moreover, differences may also be based on antibody affinity and/or epitope recognition patterns. So far, no clear differences between allergic and tolerant subjects were observed by epitope mapping approaches[8]. These evaluations may have been hampered by the use of patient sera consisting of polyclonal IgE antibodies. Sera of allergic subjects usually contain mixtures of antibodies recognizing both clinically relevant and irrelevant epitopes. On the other hand, sera from tolerant subjects might contain antibodies recognizing clinically relevant epitopes with insufficient affinity for successful FcεRI receptor crosslinking, as well as antibodies recognizing clinically irrelevant epitopes[9]. Hence, deep analysis of monoclonal antibodies (mAbs) from specific B cells may provide more insights into differences in specific antibody repertoires between allergic and tolerant subjects.

To this end, we analyzed gene sequences encoding the variable region of peanut 2S albumin specific mAbs from 6 allergic and 6 sensitized but tolerant adults. In particular, sequence motifs of the HCDR3 region, the most important region for recognizing antigens[10], were hierarchically clustered. Clustering of HCDR3 regions resulted in four motifs exclusively present in allergic donors and three motifs associated with tolerance.

Methods

Patient selection

Blood samples (100 ml) were drawn from allergic (n=6) and tolerant (n=6) adults sensitized to Ara h 2 and/or 6 (ImmunoCAP $\geq$0.1 kU/l[11]). Their diagnoses were confirmed by DBPCFCs or in the case of two tolerant subjects, by convincing history of tolerated peanut ingestion, as evaluated by a trained physician. Tolerant patients consumed at least small amounts of peanuts $\leq$2 weeks before study inclusion. Five non-atopic donors (total IgE $\leq$100 IU/ml) served as reference. The study, conducted in accordance with the declaration of Helsinki, was ethically approved (number 17-945) by the medical ethical committee of the University Medical Center Utrecht and informed consent was given by all participants.

Isolation of peanut 2S albumins

Roasted, salted, and ground peanuts were soaked in 50 mM sodium citrate buffer (pH 5.5) and incubated overnight at 4 °C. The filtrate was applied onto a cation ion exchange column (Capto S, GE Healthcare) and the pooled fraction, containing proteins with molecular masses between 10 and 25 kDa, was separated by size exclusion (Superdex 75 pg, GE Healthcare). Enclosed proteins were analyzed by mass spectrometry, as described previously[12], and by inhibition assays.
Inhibition assays were performed with a concentration range of 0-100 µg/ml peanut 2S albumin fraction and the commercially available EUROLINE (EL) DPA-Dx Peanut 1 strip (EUROIMMUN AG) including the seed storage proteins Ara h 2, 6 and 7. Briefly, 1:11 in washing buffer diluted sera were pre-incubated with the respective concentration of the peanut 2S albumin fraction for 30 min at room temperature. Pre-incubated sera were applied onto EL strips and incubated overnight at room temperature. Bound IgE antibodies were detected by applying α-human IgE conjugate coupled with alkaline phosphatase for 60 min at room temperature. Visualization was achieved by applying nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate substrate for 10 min and specific IgE levels were evaluated as EUROLINE (EL)-intensities. Inhibition was calculated as the percentage of remaining EL-intensities compared to the EL-intensities without pre-incubation with the peanut 2S albumin fraction. Results of the MS analysis and inhibition experiments are shown in the Suppl. Table S1 and Figure S1.

Allergen-tetramer formation

An excess of biotin was added to the isolated peanut 2S albumin fraction resulting on average in four biotin molecules per one molecule protein (EZ-Link^{TM} Sulfo-NHS-

Biotin, ThermoFisher Scientific). Allergen-tetramers were formed by separately adding streptavidin-R-phycoerythrin or streptavidin-Allophycocyanin (ThermoFisher Scientific) to the biotinylated 2S albumin fraction in a molecular ratio of 1:1 (streptavidin: protein)[13,14].

Single-cell sorting of allergen specific B cells

Blood drawn into heparin-coated tubes was freshly processed within 24 hours and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. B cells were negatively enriched from the PBMCs fraction by magnetic beads (B cell isolation kit II, Miltenyi Biotec) and they were stained for allergen specificity with CD45-Pacific Orange (1:40, ThermoFisher Scientific), CD3-Pacific Blue (1:160, BD Biosciences), CD19-Fluorescein isothiocyanate (1:20, BD Biosciences), CD14-R-phycoerythrin/cyanine dye 7 (1:800, Sony Biotechnology), CD16-R-phycoerythrin/ cyanine dye 7 (1:1000, BD Biosciences) and allergen-tetramers (75 pg $\rightarrow 30 \cdot 10^6$ antigen-tetramers sufficient for approximately 150-300 resting B cells[15]). Cells gated for $CD45^+$, $CD3^-$, $CD19^+$, allergen tetramers (double positive)$^+$ and $CD14/16^-$ were single cell sorted into 96 well plates supplemented with 14 mM DTT and 11.2 U RNAse inhibitor (N8080119, ThermoFisher Scientific) in 0.5x PBS. Sorted cells were immediately put on dry ice and stored at -80 °C until further processing. PBMCs from a donor who showed specific B cells in previous experiments served as positive control and staining with only biotin served as negative control. The gating was based on the negative control (example FACS plot is shown in the Suppl. Figure S2).

Gene amplification of B cell receptors

B cell receptors were amplified as previously described with minor modifications[13,16,17]. Briefly, frozen B cells were put on dry ice, supplemented with 1.4% NP-40, 3 U RNAse inhibitor (N8080119, ThermoFisher Scientific) and 7 µM hexamer primers and subsequently incubated for one minute at 68 °C for primer annealing. cDNA transcription was performed accordingly to manufacturer's instruction (SuperScript III, ThermoFisher Scientific) and cDNA was used as a template for amplifying the variable gene transcript of the heavy and the corresponding light chain. Amplification was performed with 1.25 U hot-start DNA polymerase (AmpliTaq Gold, ThermoFisher Scientific) and 40 nmol multiplex primers for 50 cycles and the annealing temperature was set to 62, 60 and 58 °C for heavy, κ and λ light chain, respectively.

Sequence analysis of B cell receptors

Enzyme purified heavy chain amplification products were sequenced (Sanger sequencing, Macrogen) with either multiplex reverse primers or framework (FR) 1 forward primers (isotype determination) while the light chain amplification products were all sequenced with the respective reverse primers. All sequences were checked for their quality using Chromas Lite 2.6.5, and double peaks, potentially resulting from amplification errors in the beginning of the amplification reaction, were aligned and corrected for the germline if plausible. Quality-checked gene sequences, saved as FASTA files, were used for automatic germline alignment using the IgBLAST web interface (reference: IMGT database)[18] and the output was automatically written into a SQLite database using R, version 3.6.3. For further evaluation, sequences were considered if the sequence was valid and declared as 'productive'.

Cloning of gene products from single cells

Restriction sites were introduced with specific V gene forward primers and J gene reverse primers, ensuring that the entire variable region is cloned without any additional amino acids, using 0.5 U Phusion high-fidelity DNA polymerase (NEB). The amplification was performed for 30 cycles with an annealing temperature of 62, 60 and 58 °C for heavy, κ and λ, respectively, and purified gene products were digested with the respective restriction enzymes Eco RI and NhE I (heavy chain), BsWi (κ light chain) or Avr II (λ light chain). Before cloning these digests into human IgH (IgE, IgG1, IgG4) and IgL (κ, λ) expression vectors (pFUSEss vector series, Invivogen), they were purified and phosphorylated (T4-PNK, NEB). All vectors contained an hEF1-HTLV promotor, an IL-2 signal sequence, a multiple cloning site upstream the constant regions and zeocin (IgH) or blasticidin (IgL) resistance genes for selection. To prevent self-ligation, the digested parent vector was dephosphorylated (parent vector, FAST-AP, ThermoFisher Scientific) before the digests were added in a molecular ratio of 5:1 (T4 ligase, NEB). Heat-shock transformation was performed by adding the ligated vector to competent *E. coli* Top10 cells and incubating them for 45 sec at 42 °C. Grown colonies were screened for correctly incorporated variable regions by PCR, using forward primers binding to the respective FR2 region and reverse primers suitable for the constant part of the vector. Positive clones were Sanger sequenced and the correctness was checked by aligning the vector sequence with the first sequence result.

Heterologous expression of monoclonal antibodies

Human embryonic kidney (HEK) 293F cells were cultured in FreeStyle 293 expression medium (ThermoFisher Scientific) using 125 ml shaking culture flasks. Exponentially

growing cells (80% confluence, 90% viability) were transiently transfected with VH and VL expression vectors in a ratio of 2:3 (total 0.5 µg plasmid DNA per $1 \cdot 10^6$ cells) in 150 mm culture plates using 293fectin (2 µl/µg plasmid DNA, ThermoFisher Scientific). Three days upon transfection, the supernatant was harvested and stored at -20 °C for further analyses.

Determination allergen specificity

Specificity of heterologously expressed mAbs was tested using a direct ELISA. Coating was performed overnight at room temperature with either 0.3 µg/well Ara h 2/6[19] or transferrin (negative control). Upon blocking (PBS + 1% BSA + 0.1% Tween-20), serial-diluted thawed (1x) supernatant was applied for one hour at room temperature and bound mAbs were detected by goat α-human IgE (1:5000, KPL) or α-human IgG (1:2000, Jackson ImmunoResearch) antibodies coupled with horse-radish peroxidase. Visualization was provided by adding tetramethylbenzidine (TMB) for 15 min in the dark and the OD was measured at 450 nm. The cut-off levels were determined for IgE and IgG1 mAbs individually and were defined as the blank value (HEK293F cells supernatant) plus three times its standard deviation. A mAb was defined as specific when the sample had an OD greater than the set cut-off levels upon subtraction of the negative control (transferrin) OD value. The negative control OD value was defined as the OD value of the mAb upon subtraction of its blank value.

Specificity of patient serum and inhibition experiments

ELISA to measure serum sIgA and inhibition experiments were performed as described in the previous paragraph with the following modifications:

1. Patients' serum samples were 1:10 diluted in blocking buffer before application (duplicates, same sample)
2. Detection of sIgA binding by goat α-human IgA (1:2000, Southern Biotech) coupled with horse-radish peroxidase and incubation for 60 min at room temperature
3. Determination of optimal dilution for sIgE inhibition experiments by applying serum samples in a dilution series (1:10, 1:20, 1:50, 1:100); optimal serum dilution for inhibition experiments was defined as the first dilution showing lowered OD values in relation to the dilution yielding the maximum value or maintaining a plateau phase
4. Additional incubation step prior to serum application with the respective mAb in a concentration-dependent manner (1 to 100 ng/ml) and incubation for 60 min at room temperature

Indirect BAT

Functionality of 2S albumin-specific mAbs were evaluated by indirect basophil activation test (BAT)[20]. Briefly, isolated PBMCs were treated with 13.4 mM lactic acid buffer to strip off receptor-bound IgE. Receptors were re-loaded with human mAbs expressed as IgE, supplemented with 10 µg/ml heparin and 8 mM EDTA and incubated for 90 min at 37 °C. After extensive washing, re-loaded cells were cultured overnight in RPMI supplemented with 10% fetal calf serum and 1 ng/ml IL-3 at 37 °C. Dose-dependent stimulation was performed with a mixture of native Ara h 2/6 and 1 ng/ml IL-3 (1 µg/ml to 5 ng/ml). RPMI + 1 g/ml IL-3, 100 ng/ml C5a (R and D Systems, 2037-C5), 1 µg/ml α-human IgE (Vector Laboratories, BA-3040-.5) and 10 µM fMLP (Sigma Aldrich, F3506) served as negative and positive controls, respectively. Stimulated cells were stained for IL-3 activated basophils with CD123-Fluorescein isothiocyanate (1:400, BioLegend), CD63-R-phycoerythrin (1:800, Sanbio B.V.), CD203c-Allophycocyanin (1:160, Sony Biotechnology) and CD193-eridinin-Chlorophyll-Protein/Cyanine5.5 (1:160, Sony Biotechnology) and basophils were defined as $CD203c^+$ $CD123^+$ $CD193^+$. Basophil activation was calculated as the percentage of $CD63^+$ basophils and the threshold was set to 5% $CD63^+$ cells. All measurements were performed in duplicates and the gating strategy is shown in the Suppl. Figure S2.

Data analyses

Comparisons of continuous data between allergic and tolerant patients were statistically analyzed with the two-sided non-parametric Mann-Whitney-U-test. Correction for multiple testing was obtained by Bonferroni correction. Comparison of continuous data between allergic, tolerant and non-atopic donors was statistically evaluated using the non-parametric Kruskal-Wallis test combined with Dunn's multiple testing correction. Categorical comparisons were performed with the Chi Square test. Descriptive analysis consisted of isotype distribution, mutational status and VH usage. Moreover, related clones were defined as sequences resulted from identical VH and JH genes and HCDR3 length and related clones were aligned using ClustalW2[21]. Sequence motifs were identified by calculating Levenshtein distances (R package 'factoextra') of HCDR3 regions combined with hierarchical clustering (default unsupervised clustering in R). HCDR3 sequences with five or fewer differences were defined as one motif. Physical properties of the HCDR3 regions were calculated using the BRepertoire interface[22]. For the latter analysis, non-redundant HCDR3 amino acid sequences were used.

Results

IgE levels overlapped between allergic and tolerant donors

In order to study differences in antibody repertoires between peanut allergic and peanut sensitized (IgE) but tolerant patients, blood was drawn from peanut allergic (n=6, age: 27-41) and tolerant (n=6, age: 27-63) donors sensitized to Ara h 2 and/or 6 ($\geq$0.1 kU/l ImmunoCAP). Specific IgE levels to Ara h 2 ranged from 1.0 to 72 kU/l in the allergic and from 0 to 1.7 kU/l in the tolerant group. Specific IgE levels to Ara h 6 were lower compared to sIgE levels to Ara h 2 and ranged from 0 to 13 kU/l in the allergic and from 0 to 0.9 kU/l in the tolerant group, suggesting a more important role of Ara h 2. Mono-sensitization to either Ara h 2 or 6 was detected in both groups, although it occurred more often within the tolerant group (allergic: Pt 01, tolerant: Pt 02, 03, 07) as shown in Table 1.

Table 1: *Patient characteristics*

Patient	Sex	Age	Last reaction	Severity (prov)	Ara h 2 [kU/l]	Ara h 6 [kU/l]	Total IgE [kU/l]
Allergic group							
Pt 05	f	34	3 years	severe	1.0	1.4	625
Pt 09	f	29	1 year	moderate	2.4	5.3	807
Pt 01	f	39	1.5 years	moderate	3.3	0	14.5
Pt 04	m	27	7 years	moderate	8.2	1.7	417
Pt 10	f	27	3 years	severe	23	7.9	252
Pt 06	m	41	2 years	moderate	72	13.1	83.3
Tolerant group							
Pt 02	m	45	N/A	N/A	0	0.9	14.2
Pt 07	f	29	N/A	N/A	0.2	0	250
Pt 08	m	36	N/A	N/A	0.6	0.2	98.3
Pt 11	m	27	N/A	N/A	1.3	0.8	>1000
Pt 03	m	63	N/A	N/A	1.7	0	208
Pt 12	f	31	N/A	N/A	2.3	0.3	>1000

Frequency of peanut 2S albumin-binding B cells was significantly higher in allergic donors

As a first step, the frequencies of 2S albumin-binding B cells were compared between peanut allergic patients, peanut sensitized but tolerant patients, and non-atopic controls. 2S albumin-binding B cells, double-positive for allergen-tetramer staining, were defined as putatively specific, and their frequency was expressed as percentage of total B cells acquired from the respective sample. The frequency ranged from 0.0004% to 0.0164% across all included donors. While the frequency in allergic patients (median: 0.01%, 95% CI: 0.005 – 0.164) was only slightly increased compared to the tolerant patients (median: 0.006%, 95% CI: 0.0016 – 0.014), the frequency was significantly elevated compared with non-atopic controls (median: 0.002%, 95% CI: 0.0004 – 0.004, p=0.008 (**)). Those 2S albumin-binding B cells were single-cell sorted and served as source for the amplification of the heavy and corresponding light chain gene transcripts. No correlation was found between the frequency of 2S albumin-binding B cells and the number of 2S albumin-binding B cells from which the heavy and the corresponding light chain gene transcripts were successfully amplified and sequenced. A high number of V(D)J gene transcripts were successfully amplified from the B cells of patients 4 and 6 – both allergic – while none or only one V(D)J gene transcript was successfully amplified from B cells of patient 2 (tolerant) and 9 (allergic), respectively. For both of these patients, only a small number of 2S albumin-binding B cells were sorted (Pt 2: 8, Pt 9: 12), which may exclude amplification failure as a reason for obtaining such a low number of successfully amplified gene transcripts. Frequencies, number of sorted 2S albumin-binding B cells and successfully amplified corresponding V(D)J gene transcripts are shown in Figure 1[1]. Taken together, those successfully sequenced gene transcripts - 280 heavy chain gene transcripts, 221 light chain gene transcripts – provide a good basis for further analyses.

[1] Sorted B cells/amplified corresponding V(D)J gene transcripts - allergic: Pt 1 (32/49), Pt 4 (63/192), Pt 5 (8/96), Pt 6 (61/192), Pt 9 (1/12), Pt 10 (19/96); tolerant: Pt 2 (0/8), Pt 3 (7/108), Pt 7 (13/40), Pt 8 (7/36), Pt 11 (36/144), Pt 12 (17/72); non-atopic: NA-1 (21/50), NA-2 (14/32), NA-3 (7/36), NA-4 (8/48), NA-5 (12/24)

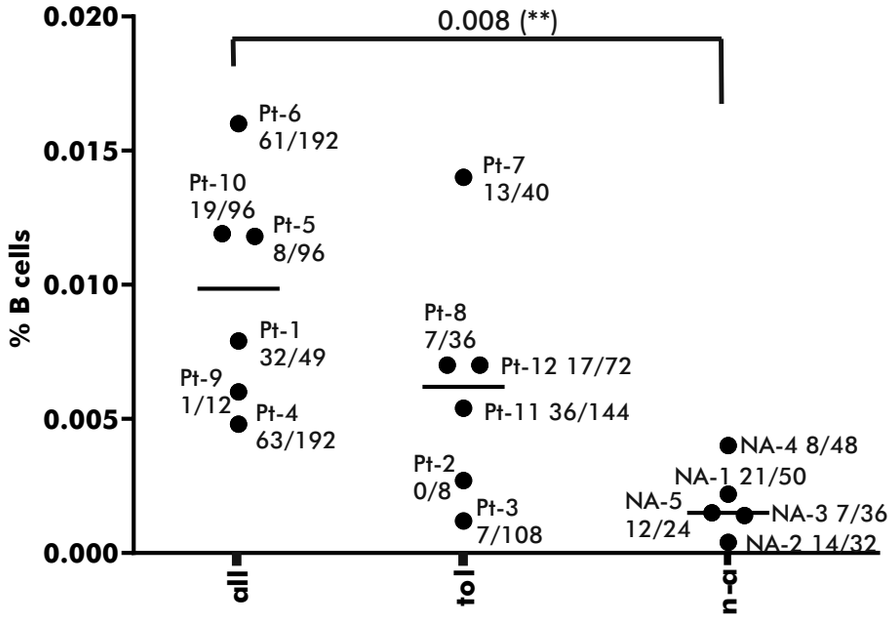

Figure 1: Frequency of 2S albumin-binding B cells
Frequency of 2S albumin-binding B cells, double-positive for allergen-tetramer (PE and APC) staining, expressed as percentage of the total B cell number acquired from the respective sample, indicated with the median. Each frequency is labelled with the respective patient number and the number of successfully amplified corresponding heavy and light V(D)J gene transcripts in relation to sorted B cells. Statistical significance was proven with the Kruskal-Wallis test and corrected for multiple testing using the Dunn's multiple comparison test (adjusted p-value); all = allergic, tol = tolerant, n-a = non-atopic

2S albumin-binding B cells show specific binding to Ara h 2 and 6

In order to verify the specificity of identified 2S albumin-binding B cells, 42 different sets of heavy and corresponding light chain gene transcripts were heterologously expressed as IgE or IgG1 mAbs. The majority of these mAbs (81%, 34/42), mostly derived from allergic and tolerant patients, showed specific binding to Ara h 2 and 6 at 10 µg/ml or lower in a concentration-dependent manner (Figure 2a). These heterologously expressed mAbs originated from 2S albumin-binding IgM^+ (41%, 14/34), $IgG1^+$ (3%, 1/34), $IgG2^+$ (23%, 8/34), IgE^+ (3%, 1/34), $IgA1^+$ (18%, 6/34) and $IgA2^+$ (9%, 3/34) B cells. While the two mAbs with relatively high optical density (OD) values originated from $IgG1^+$ and $IgA1^+$ B cells, mAbs with relatively low OD values originated predominately from IgM^+ B cells. About half of the specific mAbs (58%, 11/19) tested for separate binding to Ara h 2 or 6 recognized both allergens, and had comparable OD values. Eight mAbs exclusively recognized either Ara h 2 or 6 (21%, 4/19 Ara h 2 and 21%, 4/19 Ara h 6) (Figure 2b). The remaining antibodies without specific binding to Ara h 2 or 6 at 10 µg/ml originated predominately from IgM+ B cells of non-atopic controls (88%, 7/8). These data confirm the specificity of most selected 2S albumin-binding B cells and support the relevance of the gene analyses described in the following paragraphs.

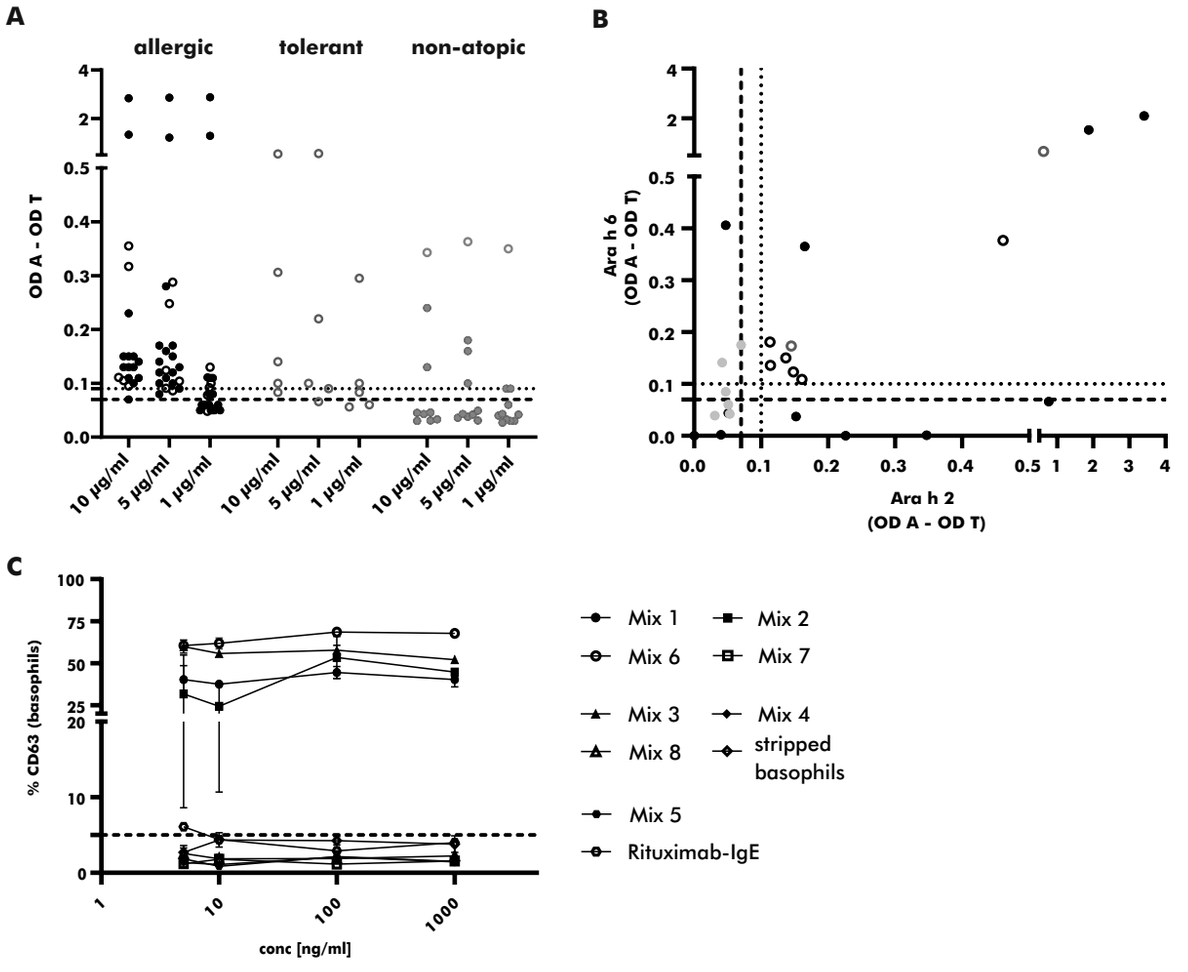

Figure 2: Specificity and functionality of heterologously expressed mAbs
A *Concentration-dependent (1 to 10 µg/ml) binding of heterologously expressed mAbs (n = 42) to Ara h 2 and 6 defined as OD values corrected for their binding to transferrin;* ***B*** *Binding of heterologously expressed mAbs to Ara h 2 and 6 (n = 24) at 10 µg/ml defined as OD values corrected for their binding to transferrin; filled dots indicate mAbs expressed as IgE and open dots mAbs expressed as IgG1, bold dash line is the cut-off for IgE mAbs and non-bold dash line the cut-off for IgG1 antibodies; black = allergic, grey = tolerant, light grey = non-atopic;* ***C*** *Upregulation of CD63 upon loading of human basophils with mAb mixture and subsequent stimulation with Ara h 2 and 6 in a concentration-dependent manner (5 to 1000 ng/ml); the dots indicate the mean of duplicate measurements and their range; Mix 1 = Pt 6-2, Mix 2 = Pt 6-2 + Pt 6-17, Mix 3 = Pt 6-2 + Pt 3-2 + Pt 6-11, Mix 4 = Pt 6-17, Pt 3-2, Pt 6-11, Mix 5 = Pt 1-32, Pt 6-11, Pt 6-6, Pt 6-8, Mix 6 = NA 1-10 + Pt 4-28 + Pt 4-34 + Pt 6-2, Mix 7 = Pt NA 1-10 + Pt 4-28 + Pt 4-34, Mix 8 = Pt 1-32 + Pt 6-15 + Pt 6-21, stripped basophils before loading, Rituximab expressed as IgE; dash line: threshold level set to 5%*

Selected 2S albumin-specific mAbs can induce degranulation

As IgE B cell memory is potentially stored in $IgG1^+$ memory B cells and to a lesser extent in B cells belonging to another isotype, the functionality of mAbs originated from non-IgE 2S albumin-binding B cells were tested for their functionality[23–25]. To determine mAb functionality, stripped human basophils were loaded with mixtures of randomly selected mAbs (expressed as IgE) and subsequently stimulated with Ara h 2 and 6 (Figure 2c and Suppl. Figure S4). Stimulated basophils showed individual CD63 upregulation

upon stimulation when loaded with mixtures containing the mAb Pt 6-2 (Mix 1, 2, 3 and 6). Such responses were already achieved at low concentrations (5 ng/ml Ara h 2/6). Moreover, the overall CD63 upregulation induced by Mix 3 and 6 was even higher than the CD63 upregulation induced by the α-IgE control at all allergen concentrations, pointing to an additive effect by using mAb Pt 6-2 (Mix 1) in combination with additional mAbs. The functionality of mAb Pt 6-2 and the observed additive effect confirm the selection of specific and functional B cells with the FACS sorting strategy presented in this study.

Allergic and tolerant donors possess more class-switched 2S albumin-binding B cells than non-atopic controls

All successfully sequenced heavy chain gene transcripts were initially analyzed for isotype distribution, as class-switching is mostly accompanied by somatic hypermutation maturation upon antigen challenge. Overall, IgM was the most dominant isotype across all groups. Notably, its prevalence was much lower in allergic (47%) and tolerant (53%) donors than in the non-atopic reference group (94%), indicating more antigen-challenged and matured specific B cells originated from allergic and tolerant patients. Moreover, the prevalence of IgA2-expressing B cells was larger increased in the tolerant group (20%), while a comparable distribution was observed in allergic and non-atopic donors (2%), as shown in Figure 3a. This finding suggests potential protection by specific IgA in tolerant patients, as IgA is generally able to prevent mucosal antigen crossing[26]. Regarding IgE, only a small number of V(D)J gene transcripts derived from IgE^+ B cells were successfully amplified (allergic: 1; tolerant: 2), possibly due to the extreme low abundance (0.002 to 1%) of IgE^+ B cells within the circulation[27]. Even though slightly higher proportions $IgG3^+$ (around 1%) and $IgG4^+$ B cells (around 0.75%) are known[28,29], no V(D)J gene transcripts derived from $IgG3^+$ and $IgG4^+$ B cells were amplified. Overall, the individual isotype distribution varied for each patient, e.g. 2S albumin-binding B cells of the allergic patient 4 were predominantly IgM^+ B cells, while those of patient 1 were predominantly IgG^+ B cells with a shift to class-switched B cells in peanut allergic and tolerant patients. This indicates that the 2S albumin-binding B cells from allergic and tolerant patients are more often matured and antigen-challenged B cells compared with the non-atopic control group.

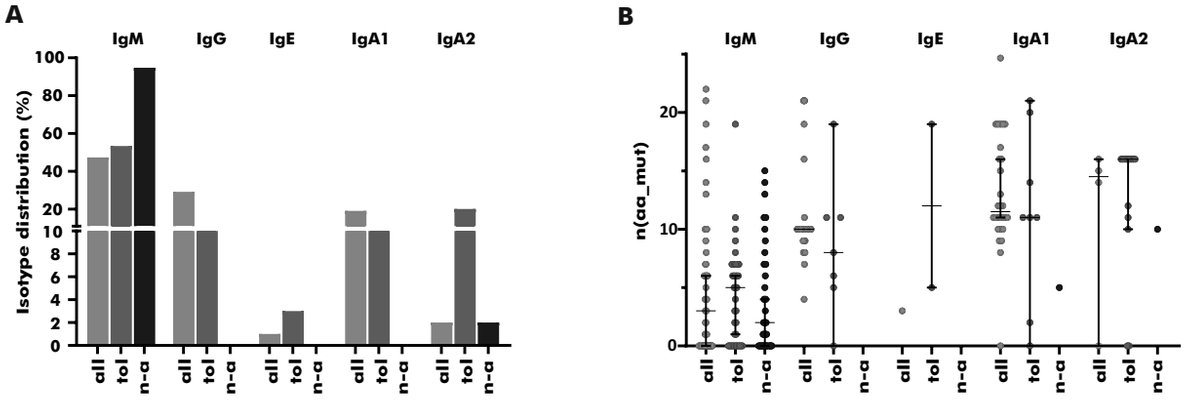

Figure 3: Descriptive gene analysis
*Descriptive gene lineage analysis of successfully sequenced heavy chain gene transcripts defined as productive (allergic n = 151; tolerant n = 68; non-atopic, n = 51), irrespectively of successfully sequenced corresponding light chain gene transcript;**A** Isotype distribution split for allergic (all), tolerant (tol) and non-atopic (n-a) donors; **B** Numbers of non-silent mutations expressed with the median (95% CI) and split for allergic (all), tolerant (tol) and non-atopic (n-a) donors*

IgM$^+$ B cells from allergic donors partly show strong maturation by somatic hypermutations

The introduction of non-silent (mutations on amino acid level) somatic hypermutations can increase target specificity and affinity during B cell maturation. Hence, non-silent mutations are an indication of antigen-challenged and matured B cells. As shown in Figure 3b, the median number of non-silent mutations was increased in VDJ heavy chain gene transcripts of class-switched B cells and was the highest for IgA1$^+$ and IgA2$^+$ B cells (IgA1: 5-11 mutations, IgA2: 10-16 mutations). The number of non-silent mutations in VDJ gene transcripts of IgM expressing B cells, on the other hand, ranged from 0 to more than 10 in all study groups. Of note, a small number of IgM expressing B cells, mostly originating from the allergic group, consisted of highly mutated heavy chain gene transcripts with mutation numbers comparable to the other isotypes, pointing to potential maturation of 2S albumin-binding IgM$^+$ B cells by antigen challenge.

VH3 family genes are predominately used in gene transcripts of allergic donors

While the variable region of the heavy chain gene transcript consists of recombined V, D and J genes, the light chain lacks the D gene component. The V gene accounts for most nucleotides of the variable region and contributes, besides D and J genes, to the CDR3 region. Hence, the V gene lineage of the heavy chain can greatly affect B cell receptor specificity and affinity. In line with B cell repertoires described in literature[30], VH3 family genes were observed to dominate across all groups, with a shift to higher proportions (89%, mainly IGHV3-30, VH3-23 and VH3-72) in allergic donors (X^2 (2, n = 183) = 23.67, p < .0001). This significant difference was still present upon neglecting redundant sequences from the same donor, excluding a bias by proliferation of certain 2S albumin-binding B cells. In turn, IGHV4 genes accounted for a larger part of incorporated VH genes in tolerant (21%) donors than in allergic ones (7%) (Table 2). Overall, VH family gene usage differs between allergic and tolerant patients sensitized to Ara h 2/6, supporting the hypothesis of differences in allergen specific antibody repertoires between allergic and tolerant patients.

Table 2: *Comparison of the VH family gene usage between the present study, healthy controls and Ara h 2-related datasets (OIT trails)(transcripts from allergic, n = 120; tolerant, n = 63; non-atopic, n = 52)*

VH family	Ghraichy[A 31]	Goldstein[32]	Patil[13]	Hoh[16]	Allergic	Tolerant	Non-atopic
VH1 [%]	5-18	15-18	23	18	2	21	3.8
VH2 [%]	2	4	-	-	-	-	-
VH3 [%]	40-65	46-50	62	60	89[B]	54	63
VH4 [%]	25-35	22-25	15	15	6.3	21	29
VH5 [%]	1-2	5-7	-	-	1.3	2.9	3.8
VH7 [%]	0.5-1	0.05-1	-	-	1.3	-	-

[A] Age group between 25-40 years used for comparison, most closely related to the average age of the study population; data for different subsets were combined

[B] dominant usage of VH3 family genes in all individuals

IgE$^+$ B cell is largely clonally related to an IgG1+ B cell in allergic patients

In total, three VDJ gene transcripts were successfully amplified from IgE$^+$ B cells, representing the smallest isotype group when disregarding IgG3 and IgG4. Where two VDJ gene transcripts of IgE$^+$ B cells derived from one tolerant donor were not closely related to another amplified VDJ gene transcript derived from the same donor, the VDJ gene transcript of one IgE$^+$ B cell originating from an allergic donor (Pt 10) was nearly identical to the gene transcript of an IgG1$^+$ B cell derived from an unrelated allergic donor (Pt 04). These two sequences differed only in one silent mutation within the FR4 region and one non-silent mutation within the FR2 region, pointing towards clonal relationship between IgE$^+$ and IgG1$^+$ B cells and conserved clones between unrelated donors (Figure 4a). This finding supports the hypothesis that the IgE memory is stored in IgG1$^+$ B cells and to lesser extent in B cells of another isotype[24,25].

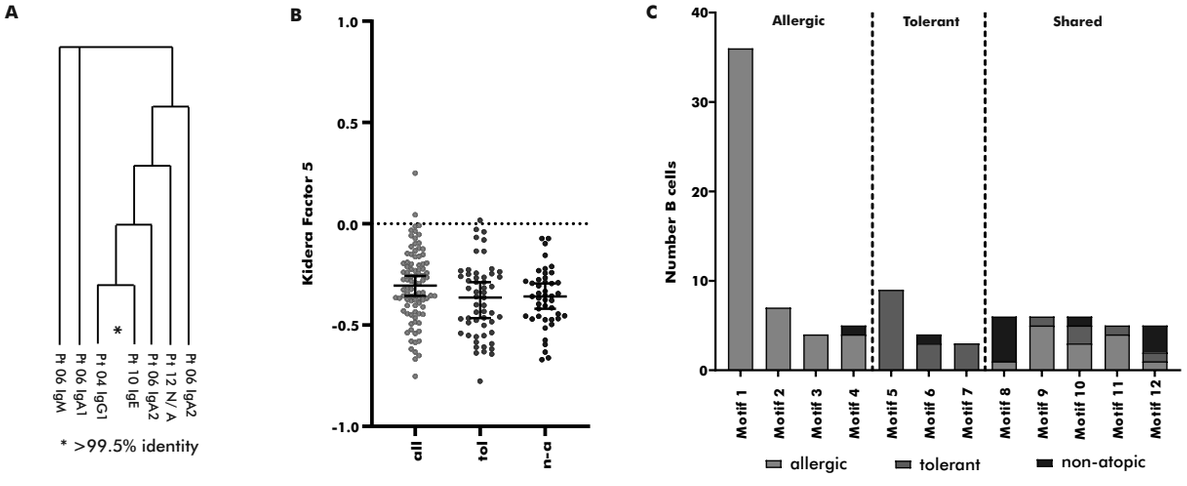

Figure 4: IgE-related clonotypes and motif analysis of HCDR3 regions
A *Clonotypes were defined as identical V gene, J gene and HCDR3 length and related clones were aligned using ClustalW2. The IgE mAb of Pt 10 was highly related to an IgG1 antibody of Pt 4.* ***B*** *Tendency of non-redundant HCDR3 regions (allergic n = 88; tolerant n = 55; non-atopic, n = 48) to form double-bended structures on amino acid level expressed as Kidera factor 5 in allergic (all), tolerant (tol) and non-atopic (n-a) donors (median and 95% CI). Correction for multiple measurements (Bonferroni) resulted in no statistically significant difference. More physicochemical properties of the HCDR3 region are shown in the Suppl. Figure S5 and S6.* ***C*** *HCDR3 sequence motifs (allergic n = 151; tolerant n = 68; non-atopic, n = 51) were analyzed by Levenshtein distances and hierarchical clustering (detailed description in the method section) and sequences with distances $\geq$5 were grouped into one motif. More detailed description of the motifs is presented in Table 3*

HCDR3 sequence motifs are related to allergy or tolerance

As HCDR3 regions have a great impact on antigen binding, their characteristics were more deeply analyzed. Detailed analysis of physiochemical properties, such as amino acid distribution, aliphatic index, Boman index and the 10 Kidera factors, are shown in Suppl. Figure S5 and S6[22] . The Kidera factor 5, expressing the preference to form double-bended structures, was significantly higher for the HCDR3 region of allergic donors compared with that of tolerant donors ($p = 0.0226$, non-significant upon Bonferroni correction, Figure 4b). Nevertheless, the mean Boman index, describing the theoretical ability to bind proteins, was comparable between HCDR3 regions of allergic, tolerant, and non-atopic donors. For deeper analysis, HCDR3 sequence motifs associated with peanut allergy and/or tolerance, were evaluated by Levenshtein distances ($\geq$5 differences defined as replacement, deletion or insertion) and hierarchical clustering. Levenshtein distances were favored over clonal relationship analysis because of individual genetic variation regarding haplotype diversity, single nucleotide polymorphisms (SNPs), gene copy number and preferred gene lineage[33]. For this analysis, all successfully amplified and sequenced heavy chain gene transcripts were used irrespectively of successfully sequenced corresponding light chain gene transcripts. Overall, four unique HCDR3 sequence motifs were associated with peanut allergy and three with peanut tolerance (Figure 4c, Table 3). The most dominant motif was 'CARDSSALEIYNRFDPW' (motif 1), which was derived from 36 different B cells belonging to three different allergic donors (Pt 1 (n=32), Pt 5 (n=3), Pt 6 (n=1)) including 32 nearly identical B cells of Pt 1 (monoclonal proliferation). This motif was formed by VH3-30, DH3-3 and JH5 genes. Besides motifs exclusively related to either allergy or tolerance, five motifs were shared between allergic, tolerant, and non-atopic donors. It must be mentioned that one motif (CARNVFDGYWLVYW) associated with tolerance was only found in Pt 11 and no motif was shared between all allergic or tolerant donors. Heterologously expressed mAbs corresponding to these motifs showed specific binding to Ara h 2 or 6, supporting the relevance of the identified motifs and their potential role in diagnosing patients with suspected peanut allergy.

Table 3: *HCDR3 sequence motifs associated with peanut allergy or tolerance*
bold: nucleotide difference, expressed and tested antibody, italic: mAbs from tolerant patients

Motif	HCDR3 sequence	Antibodies	Isotype	Specificity
Allergy				
1	CARDSSALEIYNRFDPW	Pt 1-1 - **Pt 1-32^{S}**, Pt 5-3, Pt 5-4, **Pt 5-8^{S}**, **Pt 6-43^{S}**	IgG2	Ara h 2 + 6
2	CVKDRQQYSSRWLDSW	Pt 5-2, Pt 5-5, **Pt 6-32^{S}**, Pt 6-42, Pt 6-45, Pt 6-47, Pt 6-48	IgA1	Ara h 2+ 6
3	CASMDILAANTHFGMDVW	**Pt 6-17^{S}**, Pt 6-37, **Pt 6-52^{S}**, **Pt 10-19^{S}**	IgG2	Ara h 2 + 6
	CARG**LV**GA**NF**YYYMDVW	**Pt 4-10^{S}**, Pt 4-41, Pt 4-61	IgM	Ara h 2
4	CARG**RS**GA**TY**YYYMDVW	**NA 4-5^{S}**	IgM	Ara h 2 + 6
	CARG**RA**G**PSY**YYYMDVW	Pt 4-4	IgM	
Tolerance				
5	CARNVFDGYWLVYW	*Pt 11-3, Pt 11-4, **Pt 11-14^{S}**, Pt 11-15, Pt 11-23, Pt 11-24, Pt 11-25, Pt 11-26*	IgA2	Ara h 2 + 6
6	CAREGHYSSQFDYW	*Pt 8-3, Pt 8-4, Pt 8-5*, NA 2-3	IgM	n. e.
7	C**A**RD**Y**G**G**YPHAAF**D**IW	*Pt 11-11*	IgM	n. e.
	C**T**RD**T**G**T**YPHAAF**N**IW	**Pt 3-1, Pt 3-5^{S}**	IgM, IgG2	Ara h 2 + 6
Shared				
8	CTRPYRAFNWA**I**GHW	NA 1-15	IgM	n. e.
	CTRPYRAFNWA**T**GHW	NA 1-17, **NA 5-2^{S}** (only Ara h 2), **NA 5-5^{S}**, NA 5-11, Pt 6-19	IgM, IgA1	Ara h 2 + 6
9	CARVSSSWHTEYW	*Pt 3-7*, Pt 6-33, **Pt 6-46^{S}**, Pt 6-53, Pt 6-60, Pt 6-61	IgA1	Ara h 2 + 6
10	CAR**GIIDK**YGMDVW	Pt 6-38, *Pt 7-6*, Pt 10-13, Pt 10-14	IgG2	n. e.
	CAR**E YY**YGMDVW	NA 4-1	IgM	n. e.
	CART **LG**YGMDVW	*Pt 11-16*	IgM	n. e.
11	C**T**RGA**VS**YTRHFQ**F**W	**Pt 4-20^{S}**	IgM	Ara h 2
	C**V**RGA**LA**YTRHFQ**Y**W	**Pt 4-34^{S}**	IgM	
	C**V**RGA**MS**NTRHFQ**Y**W	**Pt 4-28^{S}**	IgM	Ara h 2/6^{A}
	C**A**RGA**MS**YTRHFQ**Y**W	Pt 4-8, Pt 4-40, **Pt 4-63^{S}**, *Pt 8-6*	IgM	Ara h 2/6^{A}
12	CA**KAY**GSGSY**L**FDYW	*Pt 7-4*, **NA 1-10^{S}**	IgM	Ara h 2 + 6
	CA**RGG**GSGSY**T**FDYW	**Pt 10-6^{S}**, NA 1-4, NA 1-13	IgM	Ara h 2 + 6

^{S} specificity to Ara h 2/6 proven
^{A} only specificity against mixture of Ara h 2 and 6

Discussion

Specific IgE levels to Ara h 2 and 6 between 0.1 and 1.8 kU/l overlap between peanut allergic and tolerant subjects[6,7], risking inaccurate diagnosis, and therefore indicating the need for new diagnostic strategies. In the present study, we observed a preferential usage of VH3 family genes in peanut 2S albumin-specific B cells from peanut allergic patients. Additionally, we identified 2S albumin binding B cells carrying HCDR3 sequence motifs either related to peanut allergy or tolerance. This finding may lead towards new diagnostic strategies able to discriminate between allergy and tolerance in sensitized patients with suspected peanut allergy.

Despite a large number of amplified heavy chain VDJ gene transcripts (n = 280), only three of them belonged to IgE class-switched B cells. This low abundance may be potentially explained by dominant class-switching to IgE in the tissue[23], low BCR surface expression of plasmablast-like IgE^+ B cells[34–36] and the extreme low abundance of IgE^+ B cells within the circulation[27]. Nevertheless, the relevance of the B cells sorted within the present study is supported by the close relationship between one heavy chain gene transcript from an IgE^+ B cell and a gene transcript from an $IgG1^+$ B cell derived from an unrelated peanut allergic donor. Additionally, some generated mAbs were able to slightly inhibit patient-derived serum IgE-binding to Ara h 2 and 6 (Suppl. Figure S3). Correspondingly, there is evidence that human IgE^+ B cells are predominately plasmablasts or plasma cells generated by sequential class-switching from $IgG1^+$ B cells (γ switch region remnants) and that the humoral IgE memory is contained in $IgG1^+$ memory B cells[25]. Further evidence for this theory was obtained by clonal relationship analyses, since IgE^+ B cells were dominantly clonally related to $IgG1^+$ B cells, but also, to a lesser extent, to B cells of other isotypes[23,24,34]. In contrast, IgE memory in mice has been shown to be at least partly embedded in IgE^+ memory B cells[37].

By contrast to the low number of IgE^+ B cells, IgM^+ B cells represented the largest isotype group, as detected in respectively 47, 53 and 94% of allergic, tolerant and non-atopic donors. Additionally, IgM^+ B cells from non-atopic donors, all specific for Ara h 2 and/or 6, shared HCDR3 motifs (motif 4, 8, 10) with IgM^+, $IgG2^+$ and $IgA1^+$ B cells from allergic donors. Clonal relationships between $IgA/G/M^+$ and IgE^+ B cells have been described for B cells derived from gut tissues by Hoh and colleagues[23,38]. Combining the findings from the present study with the finding of Hoh and colleagues leads to the suggestion that non-atopic donors can potentially possess non-IgE antibodies with required specificity or affinity to theoretically induce an allergic reaction.

Moreover, tolerant donors showed a higher proportion of IgA^+ B cells and tended to have higher specific IgA serum levels than allergic donors (Suppl. Figure S3). Allergen-specific IgA serum levels have been shown to be increased in peanut allergic subjects undergoing oral or sublingual immunotherapy compared with their baseline levels.

Moreover, salivary IgA levels have been closely associated with the degree of tolerance induction confirmed by DBPCFCs, pointing to a protective role of specific IgA against mucosal allergen absorption[26,39]. Such a protective role is supported by increased intestinal permeability in the absence of IgA in mice[40]. Taken together, these findings suggest a potential protective effect of allergen-specific IgA in the mucosa of sensitized but tolerant patients.

Regarding gene lineage, VH3 family genes were significantly more often used ($p < .0001$) in heavy chain gene transcripts of peanut 2S albumin-specific B cells from allergic donors (89%) than in those from tolerant (54%) and non-atopic donors (63%). Previous studies on VH gene usage of heavy chain gene transcripts in healthy donors showed VH3 family gene usage in 40 to 65% of the B cells, which was comparable to the usage in the tolerant and non-atopic groups in our study (Table 2)[31,32]. These findings suggest a shift towards VH3 family gene usage in 2S albumin specific B cells from allergic donors. Contrary to our findings, other datasets of Ara h 2 specific B cells did not observe a similar shift in VH family gene usage[13,16]. These datasets, however, were generated from patients undergoing peanut oral immunotherapy with a focus on different time points during immunotherapy. This fact hampers the comparison between the present and previous Ara h 2-related datasets. Moreover, conflicting results regarding preferred VH gene usage were described for different food and respiratory allergies, with a shift to VH3 usage for α-alpha Gal antibodies and α-grass pollen Phl p 6 and 11 antibodies[41,42], indicating that the preferred VH family gene usage may be allergen dependent. Moreover, the preferred usage might depend on the status of allergy or tolerance and can potentially be used for diagnostic purposes upon validation in a larger patient cohort. A validation study will also provide information about the number of detected 2S albumin-binding B cells is sufficient for a diagnostic workflow.

Besides differences in VH family gene usage, certain HCDR3 sequence motifs were associated with either peanut allergy or tolerance and appear to have the ability to discriminate between those two groups. The main HCDR3 sequence motif 'CARDSSALEIYNRFDPW' was associated with peanut allergy and derived from recombined VH3-30, DH3-3 and JH5 genes. Comparably, VH3-30*18 was incorporated in the VDJ gene transcript of clonally related IgE+ B cells specific for Ara h 2 in the study of Croote and colleagues[34]. Additionally, a highly similar HCDR3 region (CAREGYESSGFDYW) to motif 6 (CAREGHYSSQFDYW), associated with tolerance, has been described for peanut allergic subjects undergoing oral immunotherapy[16]. Oral immunotherapy may shape the antibody repertoire towards repertoires present in tolerant subjects. These comparisons support the potential of HCDR3 motifs in diagnosing peanut allergy and tolerance.

In conclusion, the dominant usage of VH3 family genes and the identification of HCDR3 sequence motifs related to either peanut allergy or tolerance may potentially lead to the

development of new diagnostic strategies for subjects with suspected peanut allergy and sensitization to Ara h 2 and/or 6. Validation of these HCDR3 sequence motifs in a larger patient cohort may be achieved using next-generated sequencing approaches, potentially combined with the sorting strategy of 2S albumin-binding B cells presented in this study. Next-generation sequencing allows high-throughput and can be more easily implemented in diagnostic routine[43,44].

Acknowledgments

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Supplementary

All DNA sequences are available at the NCBI genbank under the accession numbers MW271045 - MW271525.

Table S1: *Mass spectrometry analysis of the 2S albumin fraction from roasted peanut*
italic: MS/MS analysis

No.	Protein (Accession Number)	Cut-Off	Protein Score	Sequence Coverage [%]	Molecular Mass [kDa]
1	Ara h 2.01 allergen (ACN62248.1)	80/*51*	49/*39*	32/*16*	19.0
2	Ara h 2.01 allergen (ACN62248.1)	80/*51*	93/*56*	51/*16*	19.0
3	Ara h 6 (Q647G9.1) 80/*48*	103/*70*	71/*17*	17.5	
	Ara h 7.0301 (AAU21496.1)	80	64	37	18.8
4	Ara h 6 (Q647G9.1)	80/*48*	54/*43*	39/*8*	17.5
5	Ara h 6 (Q647G9.1)	*49*	*14*	*8*	*17.5*

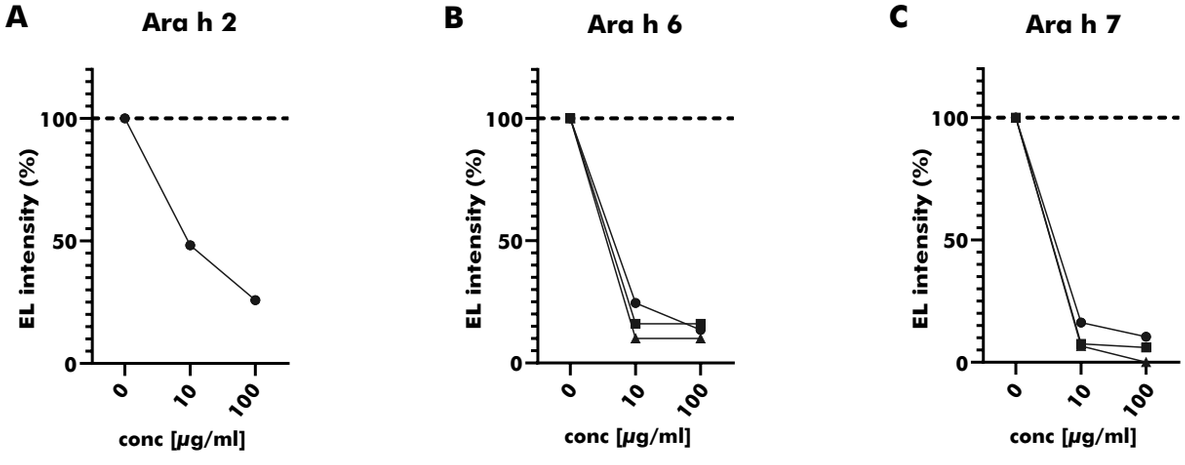

Figure S1: Inhibition of the peanut 2S albumin fraction with Ara h 2, 6 and 7
*Inhibition of serum binding to **A** Ara h 2, **B** Ara h 6 and **C** Ara h 7 in concentration-dependent manner (0-100 µg/ml peanut 2S albumin fraction) with the isolated peanut 2S albumin fraction confirming the present of these allergens in the 2S albumin fraction. Dots: serum with increased sIgE titers for Ara h 2, 6 and 7, Squares: serum with increased sIgE titers to Ara h 6 and 7, Triangle: serum with increased sIgE titers to Ara h 6 and 7*

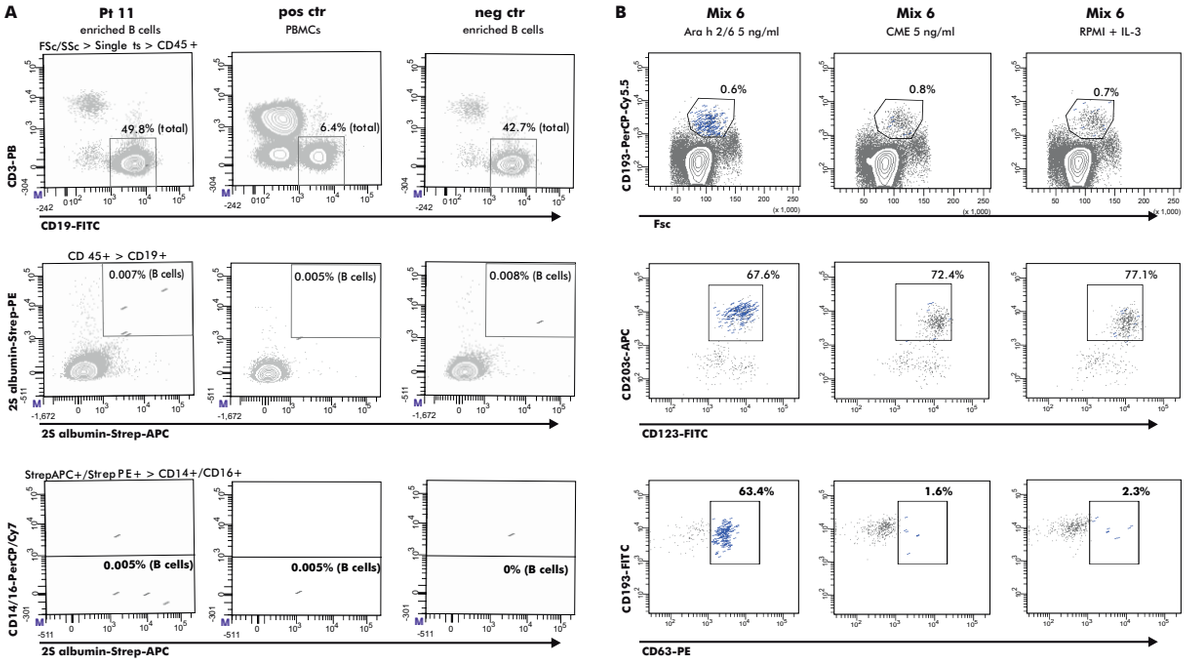

Figure S2: FACS gating strategies for the selection of 2S albumin-specific B cells and indirect basophils activation test

A *Selection of 2S albumin-binding B cells – the lowest gate panel was used for sorting;* ***B*** *Indirect basophil activation test; CME = cow's milk extract (control)*

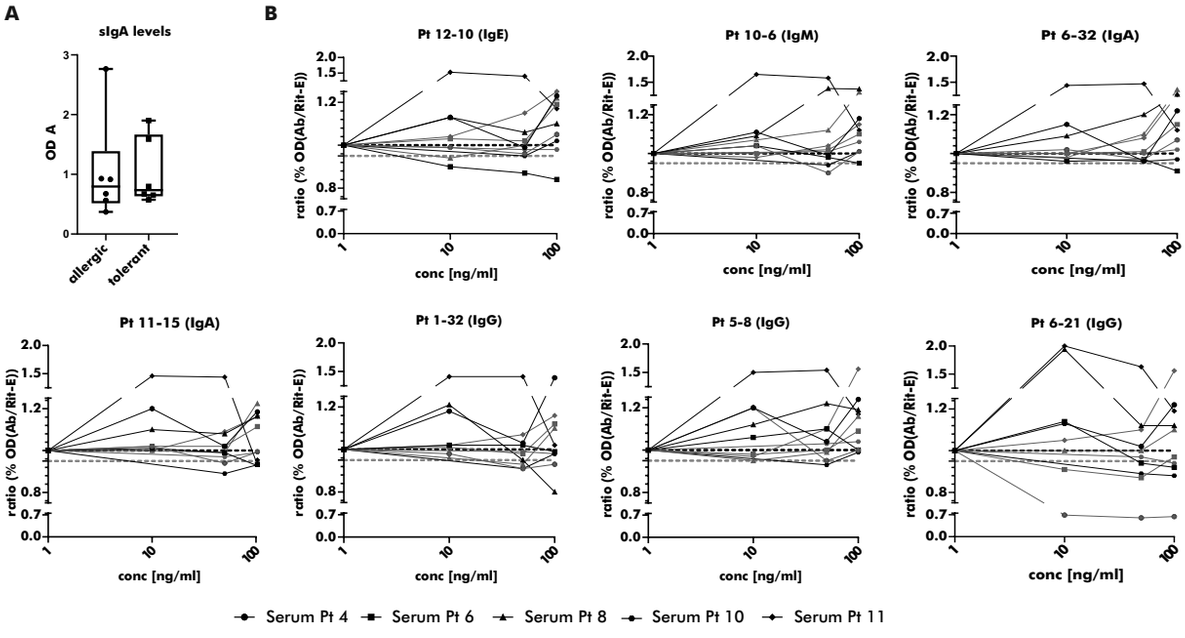

Figure S3: Binding of specific IgA to peanut 2S albumins and inhibition of sIgE binding to 2S albumins by 2S albumin-specific mAbs

A *Specific IgA levels against Ara h 2 and 6 were measured in patients' serum samples, each dot is the mean of duplicate measurements, box plots shows the interquartile range of measured sIgA levels;* ***B*** *Inhibition of sIgE binding from patients' serum samples by pre-incubation with 7 distinct 2S albumin-specific mAbs expressed as IgG1 in a dose-dependent manner (1 to 100 ng/ml, mean of duplicates, the original isotype is displayed in brackets); inhibition is the remaining OD signal of the serum without pre-incubation and is displayed as the ratio of the inhibition by the respective mAb and Rituximab (negative control) expressed as IgG1; the critical range of 0 to 5% inhibition is highlighted by two dash lines*

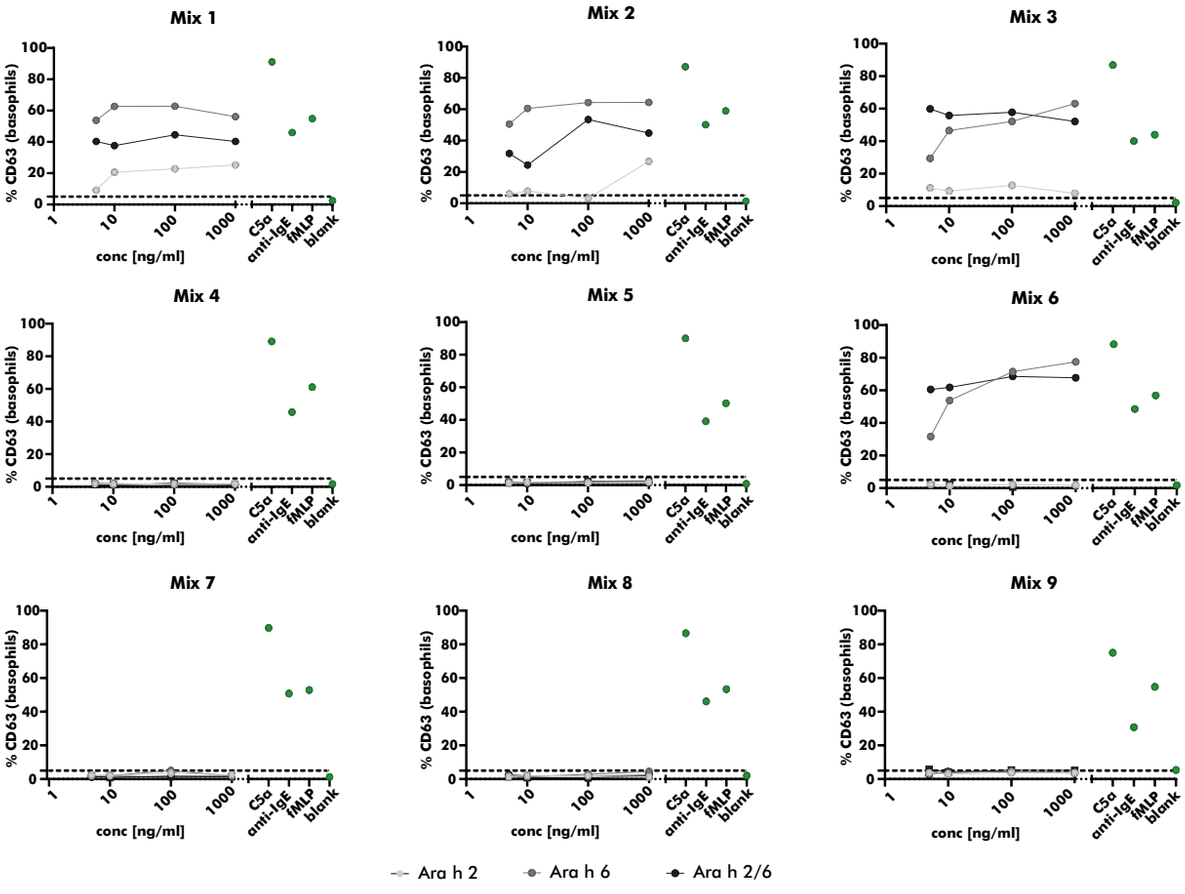

Figure S4: Indirect basophil activation tests with 2S albumin-specific mAbs including all controls
Upregulation of CD63 upon loading of human basophils with mAb mixture and subsequent stimulation with Ara h 2 and 6 in a concentration-dependent manner (5 to 1000 ng/ml); the dots indicate the mean of duplicate measurements; Mix 1 = Pt 6-2, Mix 2 = Pt 6-2 + Pt 6-17, Mix 3 = Pt 6-2 + Pt 3-2 + Pt 6-11, Mix 4 = Pt 6-17, Pt 3-2, Pt 6-11, Mix 5 = Pt 1-32, Pt 6-11, Pt 6-6, Pt 6-8, Mix 6 = NA 1-10 + Pt 4-28 + Pt 4-34 + Pt 6-2, Mix 7 = Pt NA 1-10 + Pt 4-28 + Pt 4-34, Mix 8 = Pt 1-32 + Pt 6-15 + Pt 6-21, stripped basophils before loading, Rituximab expressed as IgE; dash line: threshold level set to 5%

Physical properties of the HCDR3 regions were calculated using the BRepertoire web server 1. For this analysis, only non-redundant HCDR3 amino acid sequences (allergic n = 88; tolerant n = 55; non-atopic, n = 48) were used.

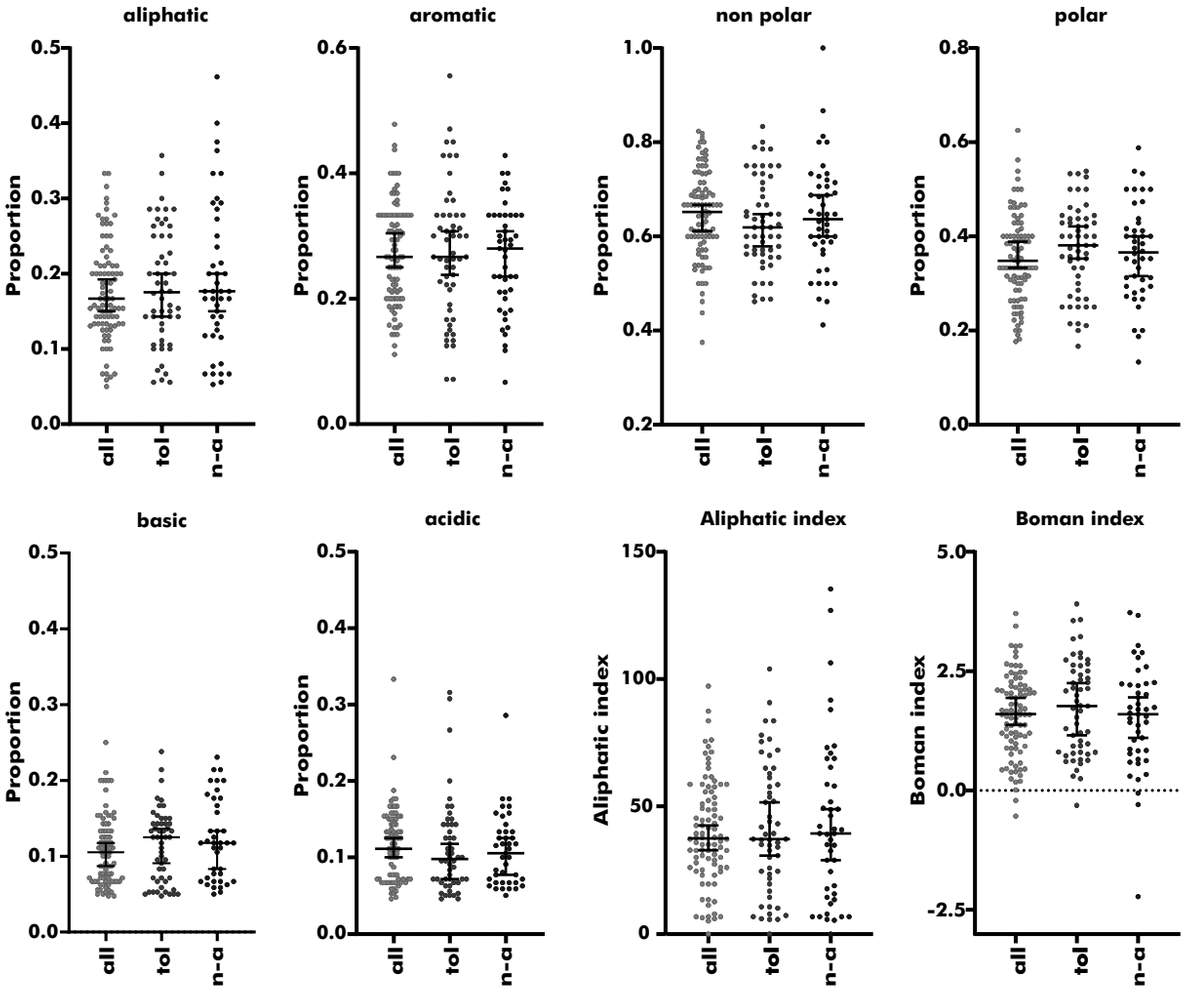

Figure S5: Proportion of amino acids within HCDR3 regions
Proportion of amino acids are categorized into different groups (aliphatic, aromatic, non-polar, polar, basic and acidic); calculation of the aliphatic index and the Boman index; Boman indices $\geq$2.48 indicate high probability for protein-protein interactions, all sample sets show the median with the 95% confidence interval; all = allergic, tol = tolerant, n-a = non-atopic

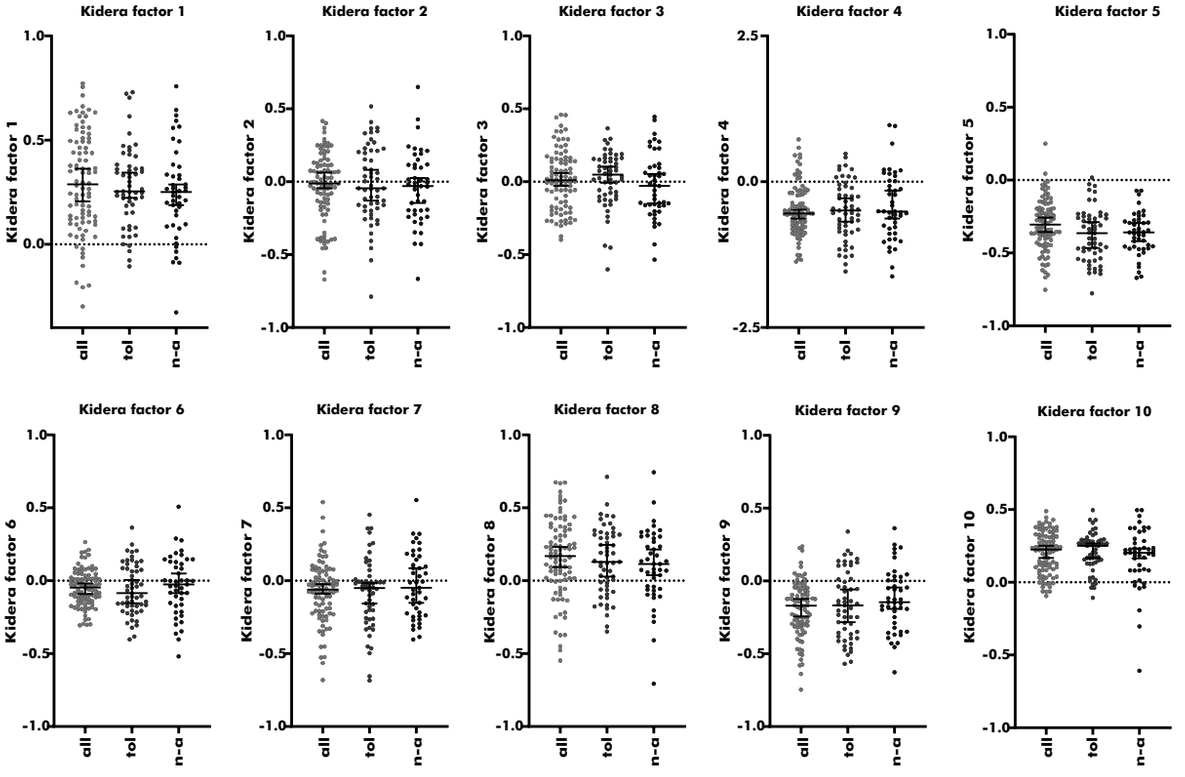

Figure S6: Properties of HCDR3 regions expressed as Kidera factors (KF) 1-10
KF1: Helix/bend preference, KF2: side chain size, KF3: extended structure preference, KF4: Hydrophobicity, KF5: double-bend preference , KF6: partial specific volume, KF7: flat extended preference, KF8: occurrence in alpha region, KF9: pK-C, KF10: surrounding hydrophobicity, all sample sets show the median with the 95% confidence interval; all = allergic, tol = tolerant, n-a = non-atopic


10

General discussion

General discussion

Food allergy is an IgE-mediated, multifaceted clinical manifestation with symptoms reaching from mild oral itching to generalised life-threatening conditions like anaphylaxis. Even though food allergies can have a great impact on patients' quality of life, the cure of food allergies remains an unsolved issue. In daily practice, the only commonly used treatment options are elimination diets although the clinical implementation of oral immunotherapies is starting to take place[1]. To give precise dietary advice, to ensure a healthy and balanced diet and to prevent unnecessary food avoidance, there is a need for accurate, preferably minimally invasive, diagnostic strategies. Food allergy diagnosis is generally based on careful history and complementary *in vivo* (SPT) or *in vitro* (sIgE) diagnostics. However, food allergy diagnosis by burdensome and costly oral food challenges requiring dedicated hospital facilities is still the gold standard. Numbers of those food challenges may be reduced by the development of highly accurate *in vitro* diagnostics.

Instead of measuring sIgE levels against a whole food allergen extract containing many different allergenic and non-allergenic proteins, component-resolved diagnostics (CRD) provide the possibility to measure sIgE levels against individual allergenic components in a simplex or multiplex manner[2]. Specific IgE binding against single components such as peanut Ara h 2, hazelnut Cor a 14 or cashew nut Ana o 3, all belonging to the 2S albumin family, has been shown to be useful in stratifying patient's risk for (severe) allergic reactions[3–6]. However, sIgE measurements to certain foods/allergens are still hampered by lacking sufficient sensitivity due to non-detectable clinically relevant sensitisation („false-negative") or specificity by detecting clinically irrelevant sensitisation („false-negative") (Figure 1, indicated by the text highlighted in red).

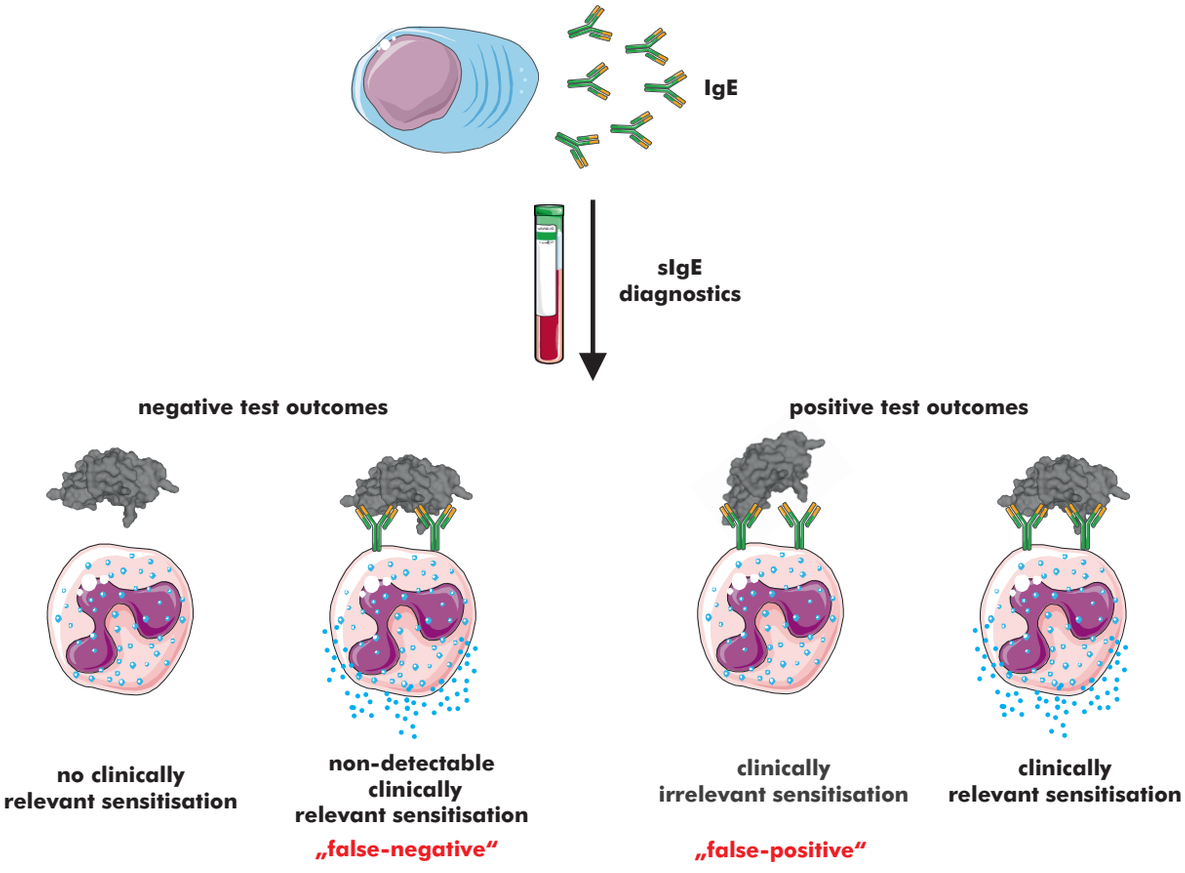

Figure 1: Current drawbacks of sIgE measurements

The research presented in this thesis focused on the development of possible strategies to overcome the present disadvantages of sIgE measurements in order to improve their clinical application in the future.

Part 1: Preventing („false-negative“) sIgE testing: non-detectable clinically relevant sensitisation

A proportion of allergic patients, ranging from 18% for peanut to 30% for sesame seed[7–9], are characterised by non-detectable clinically relevant sensitisation (referred to as „false-negative“) using conventional sIgE diagnostics. Possible explanations for those false-negative test outcomes and potential solutions (a to c) are summarised in Figure 2:

A The allergenic component recognised by clinically relevant sIgE is currently unknown

→ Identification of novel allergenic components potentially belonging to a protein family with known allergenic potential in order to stratify patients at risk[4,10] (**chapter 2**)

→ Studying sIgE binding to hydrophobic proteins (e.g. oleosins) present in the lipid phase of the respective food source but potentially absent in commercially available, aqueous-based food extracts[11,12] (**chapter 3**)

B Incorrect protein folding or incorrect post-translational modifications prevent binding of clinically relevant sIgE

→ Disruption of protein folding during manufacturing may be prevented by optimised expression systems and purification strategies[13]

→ Co-factors such as lipids may be essential for the correct folding of the protein by e.g. binding to the hydrophobic cavity of novel or already known allergenic components (**future directive chapter 3**)[14]

→ Simulation of food processing or gastrointestinal ingestion *in vitro* may change protein folding in such a way that it more closely mimics the *in vivo* situation[15,16]

C Clinically relevant sensitisation cannot be serologically measured despite surface-bound sIgE on mast cells and basophils; this may be caused by 1) sIgE levels under the detection limit[17] or 2) high sIgG levels saturating the binding sites and hence, preventing binding by sIgE

→ performing basophil activation test *ex vivo*

D The underlying mechanism of the allergic reaction is non-IgE mediated characterised by late-onset and exclusive gastrointestinal manifestations – this explanation is less likely because patients with false-negative outcomes are mostly characterised by early onset and gastrointestinal manifestations are accompanied by additional immediate symptoms such as respiratory or cardiovascular indications[18,19]

E Allergic symptoms are related to IgG-mediated anaphylaxis triggered by macrophages or neutrophils – this explanation is less likely because its existence has so far only been proven in mice. Additionally, at least 100fold higher antigen-doses are required compared to IgE-mediated reactions which can be hardly be achieved by systemically absorption of food antigens[20–22]

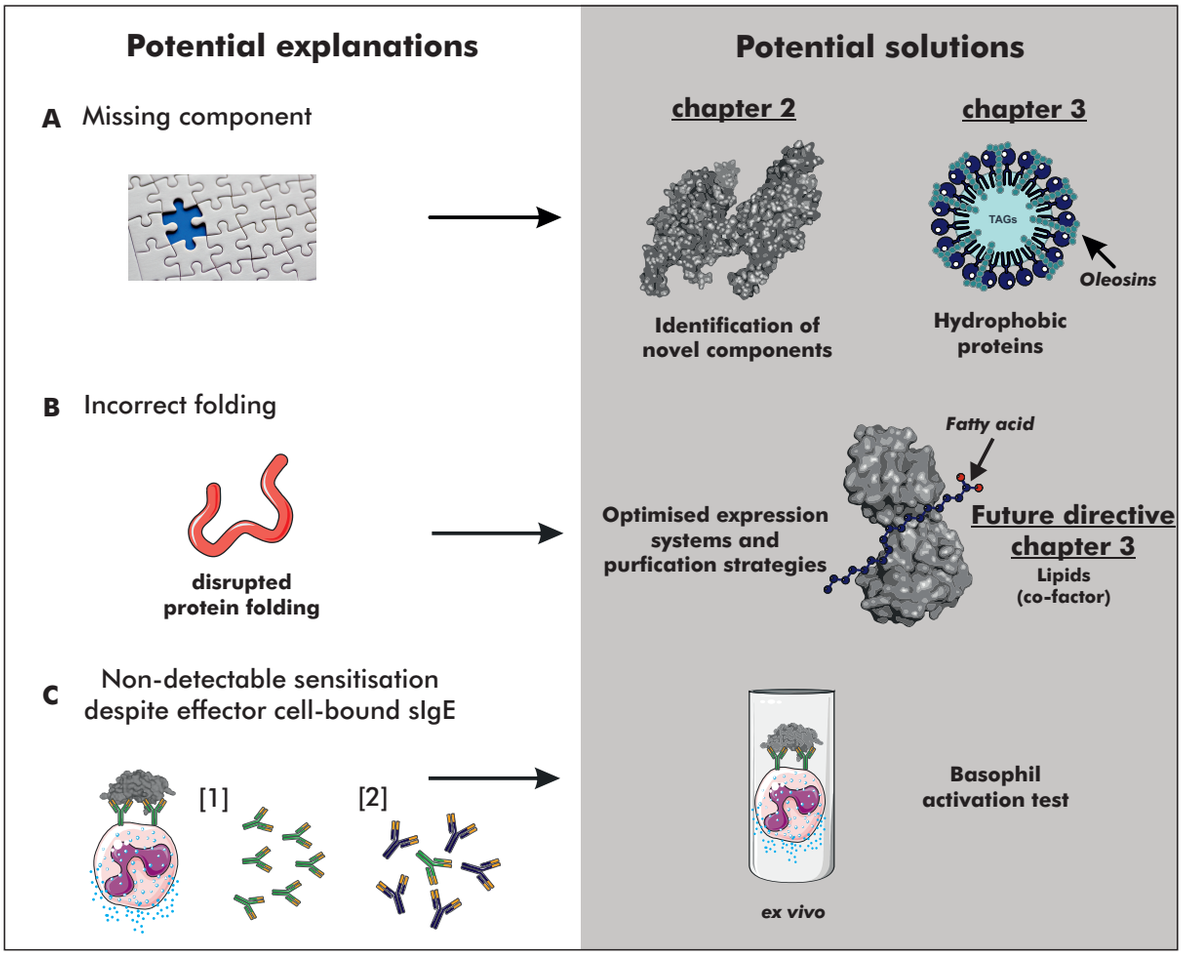

Figure 2: Summary of potential explanations (left side) of „false-negative" sIgE testing in allergic subjects and their potential solutions (right side)

Seed storage proteins are important single components for sIgE diagnostics

Specific IgE binding to seed storage proteins including 2S albumins, 11S and 7S globulins (vicilins) is known for its association with clinically relevant sensitisation and the risk for severe allergic reactions to seeds, legumes, and tree nuts. The diagnostic value of sIgE measurements against seed storage proteins has been extensively studied for peanut and to a lesser extent for tree nuts such as walnut and hazelnut[23]. The peanut 2S albumins Ara h 2 (AUC: 0.85 in adults; 0.94 in children) and 6 (AUC: 0.85 in adults; 0.98 in children) are known as highly valuable single components for the prediction of peanut allergy in Northern Europe[9,24,25]. Besides 2S albumins, sIgE binding to vicilins, especially recombinant (excluding the presence of cross-reactive carbohydrate determinants (CCDs)) Jug r 2 (walnut), has also been associated with clinically relevant sensitisation, although the evidence is less strong than the evidence for 2S albumins. Moreover, specific IgE binding to the walnut vicilin Jug r 6 has been more often accompanied by rather mild reactions. This latter vicilin showed greater cross-reactivity to vicilins from other tree nuts and legumes[10,26–28]. In **chapter 2**, vicilins from macadamia nut, an allergen source with increasing relevance, have been identified as the first single allergenic components for CRD in macadamia nut allergy. Specific IgE binding to these vicilins was only detected in patients experiencing moderate to severe symptoms upon ingestion. Next to vicilins, we hypothesise the discovery and characterisation of additional single allergenic components from macadamia nut. These components might be 2S albumins and 11S globulins as Jug r 1 (2S albumin) and Jug r 4 (11S globulin) partly inhibited sIgE binding to macadamia nut extract[29]. In comparison to other tree nuts and peanut, a 2S albumin from macadamia nut might serve as an additional marker for clinically relevant sensitisation to macadamia nut or even replace our newly identified vicilin markers if these components are implemented in CRD in the future. However, we expect that the vicilin from macadamia nut will be a marker for macadamia nut allergy in a subpopulation of macadamia nut allergic patients. This hypothesis is supported by the potential of vicilins to induce even severe allergic reactions, although vicilins are more often responsible for cross-reactivity between legumes or tree nuts and rather mild food allergies[26,30,31].

Lipophilic allergens in component-resolved diagnostics

Even though a large number of allergic patients shows sIgE binding against known single allergenic components, there is still a remaining subpopulation without detectable sensitisation. This subpopulation ranges from 18% for peanut to 30% for sesame seed[7–9]. Undetectable sensitisation in those patients may be explained by an IgE re-

sponse against lipophilic proteins absent in commercially available food extracts. The lack of lipophilic proteins such as oil-body associated proteins (OAPs) in food extracts is caused by defatting steps prior extract preparation[32]. OAPs such as oleosins from peanut, hazelnut and sesame seed have been recognised by sIgE from allergic patients with partly false-negative sIgE testing, suggesting an important role of these proteins for CRD[7,11,12]. Hence, in **chapter 3** we studied the diagnostic value of sIgE binding to sesame oleosins in sesame allergic patients with negative testing to aqueous extract or known hydrophilic single components (e.g. 2S albumin Ses i 1)[7]. Contrary to our exspectations, sIgE binding to native and heterologously expressed sesame oleosins was completely absent in sesame allergic patients with false-negative testing. Additionally, sesame oleosins were recognised with rather low sIgE levels by only 15% of allergic and tolerant patients with detectable sensitisation. In contrast to previous studies, we developed an improved purification protocol for OAPs including hydrophobic interaction chromatography as a last purification step. This protocol possesses the strength to separate even traces of seed storage proteins from the OAPs fraction as shown by mass spectrometry and western blot analyses. This improved purification strategy of native OAPs and heterologous expression of oleosins offered the possibility to exclusively study the role of oleosins in sIgE diagnostics. The use of hydrophobic interaction chromatography, however, could have disrupted lipids potentially essential for recognition of OAPs by sIgE. Lipids may support correct and natural folding of OAPs by stabilising their long hydrophobic core[14]. Moreover, lipids might also play a critical role in the recognition of known allergenic components by sIgE in a subset of allergic patients[33]. Currently, the exact role of hydrophobic proteins is not completely understood. Future research should focus on their recognition in the context of lipid availability while ensuring the absence of other known allergenic components. Overall, we hypothesise that lipids are important for the recognition by sIgE of the responsible allergens in patients without detectable sensitisation using conventional testing.

Future directive: Lipids may contribute to the recognition of single allergenic components

The interaction between lipids and allergens is an emerging field of research and lipids are involved in several processes including the activation of the innate immune system[34]. In the context of sensitisation, lipids can be presented to invariant natural killer T cells (NKT cells) by the non-polymorphic MHC class 1 molecule CD1 expressed on several antigen-presenting cells. Although lipids, such as LPS binding to different toll-like receptors (TLRs), generally induce a Th1 response, small amounts of LPS binding to TLR4 in presence of the respective inhalant allergen have been able to induce a Th2 response[14]. This special involvement in the sensitisation process has also been

shown for polar lipids from Brazil Nut as these were essential for inducing an IgE and IgG response to the recombinant 2S albumin Ber e 1 in mice. Moreover, lipids from cypress pollen induced proliferation of human derived NKT cells *ex vivo*. Notably, these lipids were also recognised by sIgE from cypress pollen allergic patients, indicating a direct involvement in recognition by sIgE[35,36]. On the protein level itself, lipids can protect allergens from digestion in the gastrointestinal tract or function as an allergen carrier through the epithelium as hypothesised for oleosins[37]. This protective role of lipids suggests a combined presentation of allergens and lipids to immune cells triggering an IgE response. Nevertheless, lipids can also have a direct effect on allergens and their allergenic properties. A large panel of allergens is able to bind lipids via their hydrophobic cavities closely located to their surface or via electrostatic/hydrophobic interactions[14,38,39]. These interactions between lipids and proteins can greatly influence the folding of the protein. Hence, new conformational epitopes are potentially formed or previous covered linear epitopes become accessible. Conformational changes by lipid binding has been most intensively studied for non-specific lipid transfer proteins (nsLTPs), known as markers for severe allergic reactions in the Mediterranean area. Their binding by sIgE and their ability to induce basophil degranulation were enhanced in presence of oleic acid. This effect was mainly pronounced for the peach nsLTP Pru p 3 and to a lesser extent for the nsLTPs from walnut (Jug r 3) and hazelnut (Cor a 8)[26,33,40]. These data suggest that it might be beneficial to study the impact of lipids on allergen recognition by IgE for other known lipid-binding allergens including (sesame) oleosins. Preliminary (unpublished) experiments of our research group with the lipid phase of peanut showed sIgE binding by sera from peanut allergic patients without detectable sIgE sensitisation to peanut (18%) using conventional testing[9]. We therefore assume that recombinant allergen-lipid complexes formed *in vitro* with the responsible lipid ligands may offer improved clinical application of CRD in the future. However, the fact that lipid-protein complexes might not be as easy to handle needs to be taken into consideration. This may require the development of an adjusted platform for testing these complexes *in vitro* compared to the platforms currently commercially available.

Part 2: Preventing „false-positive“ sIgE testing: clinically irrelevant sensitisation

The detection of clinically irrelevant sensitisation to single components associated with (severe) allergic reactions (referred to as „false-positive“) complicates the interpretation of positive sIgE testing in approximately 10% of sensitised but tolerant patients[41,42]. This may lead to inaccurate diagnosis and consequently dietary advices accompanied by unnecessary food avoidance.

False-positive sIgE testing implicates the lack of degranulation induction upon allergen exposure *in vivo*[1] despite bound sIgE on the surface of effector cells via high affinity FcεRI receptors. This phenomenon might be explained by certain antibody-related requirements identified for degranulation induction (Figure 3, left side). IgE-mediated degranulation can only be induced by cross-linking of at least two, but preferably more, FcεRI receptors and consequently by the recognition of at least two different epitopes on the surface of multivalent allergens[43,44]. The required spacing between these epitopes has been estimated to a distance between 5 and 24 nm (50-240 Å) using artificial antigens. A larger spacing also appeared to be beneficial for the natural allergen Phl p 7 **[a]**[45,46]. However, approximately one third of all food allergens, including 2S albumins and PR-10 proteins, are even smaller than 10 nm and thus the requested spacing may only be achieved by aggregation events[47]. Additionally, at least one of the IgE antibodies has to bind with high affinity to its binding site[46]. Notably, the required affinity is reduced for recognition of multivalent allergens due to increasing avidity **[b]**[45,48]. Besides requirements for sufficient cross-linking, blocking antibodies (IgG or IgA) can hinder the binding of clinically relevant epitopes by surface-bound IgE **[c]**[22,49,50]. Moreover, cross-linking of Fc εRI receptors with inhibitory FcγRIIb receptors, loaded with allergen-specific IgG, can lead to attenuated degranulation. This attenuation is achieved by inhibitory interaction of phosphorylated SHIP-1 (Src homology 2 containing inositol 5’ polyphosphatase 1) with the FcεRI signalling cascade **[d]**[51]. The latter mode of action has only been confirmed for human basophils, while human skin mast cells lack this inhibitory receptor[52,53].

Another potential explanation of false-positive sIgE testing is related to distinct glycosylation patterns of IgE antibodies (Fc part or variable region) between allergic and sensitised but tolerant patients **[e]**. Glycosylation of variable regions is achieved by the introduction of N- or O-glycosylation sites during somatic hypermutation maturation (SHM). These glycosylations, mostly located in CDR regions, can affect antibody’s

[1] Remark: Positive SPT or BAT results contradicting clinical diagnosis might be explained by high allergen concentrations not reflecting the concentration present *in vivo* upon ingestion, potentially triggering non-specific stimulation (in **chapter 9**, we observed non-specific stimulation of basophils loaded with peanut 2S albumin-specific mAbs upon stimulation with high concentration (1 µg/ml) of cow’s milk extract). Concentration threshold might be patient dependent.

affinity to its target[54,55]. On the other hand, distinct glycosylation patterns of the Fc part have been shown to influence the binding of IgG antibodies to Fcγ receptors in an antigen-specific manner by altering affinities of their interactions. While sialylation of IgG antibodies has been associated with anti-inflammatory traits, sialylation of IgE antibodies was more likely in peanut allergic subjects than in non-atopic controls[56–59]. Notably, impaired sialylation of IgG was induced by persistent antigen exposure[60]. This may explain why continuous antigen (e.g. peanut) exposure can have protective effects on developing a clinically relevant allergy by sensitised but tolerant children[61]. However, it is poorly understood how the information on antigen-specific glycosylation patterns is stored in the respective B cells.

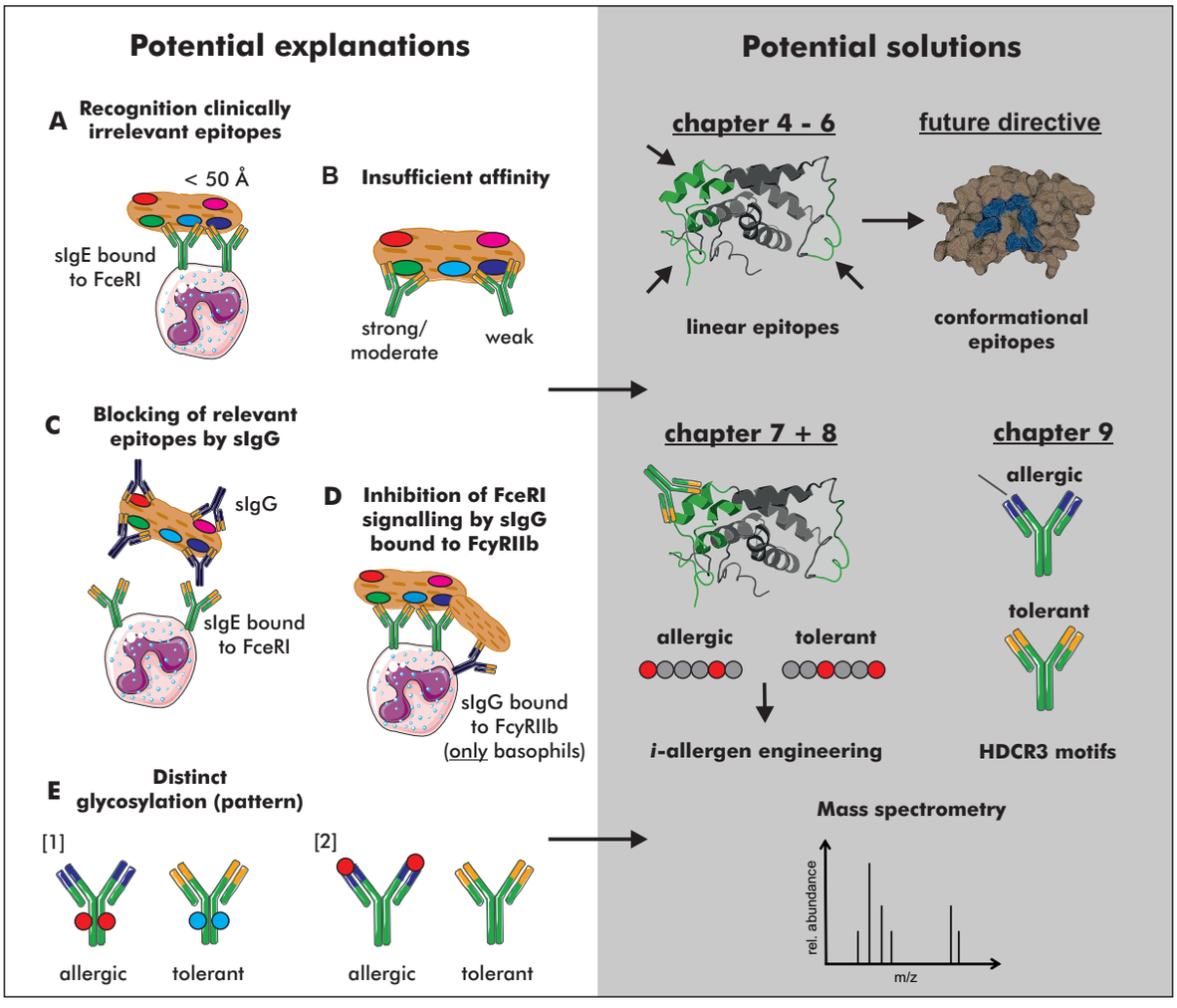

Figure 3: Summary of potential explanations (left side) of „false-positive" sIgE testing in sensitised but tolerant subjects and their potential solutions (right side); clinically relevant epitopes are highlighted in green and blue

These potential explanations support the hypothesis of differences in specific antibody repertoires; one supporting the development of allergic reactions in allergic patients and one lacking this ability in sensitised but tolerant patients. To detect such differ-

ences, the focus has to be changed from sIgE binding to entire (single) components towards specific epitopes. Potential strategies to address distinct antibody repertoires are shown on the right side of Figure 3:

- Studying linear epitope recognition patterns using polyclonal patient sera (**chapter 4-6**)
- Combining linear epitope recognition patterns with the recognition of conformational epitopes to complete the picture (future directive)
- Polyclonal sera comprise a complex antibody mixture preventing high resolution epitope mapping ($\rightarrow$ epitope dissimilarities on amino acid level); high resolution epitope mapping can be achieved by using antigen-specific human monoclonal antibodies (mAbs) (**chapter 7 and 8**)
- High resolution epitope mapping may support the engineering of single components (*i*-allergens) recognised by sIgE from allergic but not from sensitised but tolerant patients (**chapter 7 and 8**)
- Evaluating differences of specific antibody repertoires on antibody level: Sequences of the variable regions, especially of the HCDR3 region, may have unique features such as amino acid motifs or increased numbers of glycosylation sites associated with either allergy or tolerance (**chapter 9**)
- Evaluating differences in glycosylation patterns using mass spectrometry analysis or evaluating gene/protein expression levels of enzymes involved in antibody glycosylation of antigen-specific B cells

The focus on antibody-related explanations may not entirely unravel the complex picture of why sIgE sensitisation is not always attended by clinical symptoms. Polymorphisms in promotor regions, epigenetics or miRNA control mechanisms have great influence on gene and protein expression[62,63]. Individual gene/protein expression levels may increase or decrease the susceptibility of effector cells to extrinsic stimuli like antigen exposure. Moreover, these changes may alter the production of pre-formed mediators and/or *de novo* formed mediators released during degranulation. On the other hand, the susceptibility of the surrounding environment towards released mediators may influence the occurrence and severity of clinical symptoms, e.g. levels of PAF-AH (platelet activation factor-acetyl hydrolase, an enzyme degrading the pro-inflammatory mediator PAF) have been inversely correlated with levels of PAF. Subsequently, levels of PAF-AH were also associated with less severe allergic reactions[64,65]. All these latter explanations, however, are not able to elucidate the reason why polysensitisation can be partly clinically relevant and partly irrelevant in the same patient.

Impact of sIgE binding to linear epitopes on diagnosing food allergies

Linear epitopes can be directly exposed on the surface of allergens or they can become accessible upon food processing or proteolysis in the gastrointestinal tract, similarly important for food allergy sensitisation and effector cell stimulation. In the last decades, linear epitopes derived from several allergens such as peanut Ara h 2 and hen's egg Gal d 1 have been intensively studied regarding their recognition by sIgE. Overall, broad recognition of several linear epitopes (degree of IgE clonality) seems to be associated with allergy and partly with severity of clinical symptoms upon exposure[66]. Defined sets of linear epitopes, however, have only be described for a limited number of food allergens. Recognition of these sets have mainly been described as prognostic markers for *e.g.* persistence of cow's milk and hen's egg allergy in children[67–69]. In **chapter 4**, we identified three potential peptide markers for the discrimination between hen's egg allergic and tolerant adults similarly sensitised to the major hen's egg allergen Gal d 1. Specific IgE binding to at least one of these three epitopes largely increased the specificity compared to sIgE binding against the full-length allergen and may therefore serve as an additional diagnostic tool in diagnosing hen's egg allergic adults in the future. Comparably, sIgE binding to peptide markers of Ara h 2 has been shown to slightly increase diagnostic accuracy in peanut sensitised children compared to sIgE binding to full-length Ara h 2[70]. In contrast, sIgE binding to linear epitopes of Ara h 7 isoforms, which are also 2S albumins like Ara h 2 and 6, provided no basis for elucidating their divergent potencies to induce degranulation (**chapter 5** and **6**). This observation emphasises the importance of conformational epitopes in recognising Ara h 7. Similarly, recognition of full-length Ara h 6 and to a lesser extent of Ara h 2 were not completely explained by sIgE binding to linear epitopes[71–73]. The greater importance of linear epitopes for recognising Ara h 2 compared to Ara h 6 and 7 may be explained by an extremely long loop carrying important linear epitopes already accessible without disrupting its 3D structure[74]. Unlike linear epitopes, conformational epitopes are formed upon correct folding of the allergen, suggesting that correctly folded food allergens are presented to the immune system during sensitisation and effector cell stimulation. This presentation might be dependent on lasting allergen stability due to a high number of disulphide bridges. Moreover, such presentation may also be achieved by intact allergen crossing over the buccal mucosa which additionally may explain the early onset of many food allergic reactions[75]. Overall, the nature of epitopes (linear vs conformational) being important for recognition by sIgE appears to be allergen-specific, as their importance already appears to differ for the three peanut 2S albumins. Additionally, each food allergen shows a distinct susceptibility to processing and digestion, potentially masking epitopes or making other epitopes accessible. We therefore suggest

additionally considering sIgE binding to conformational epitopes as well as to linear epitopes, although it has been suggested that especially linear epitopes are important in food allergy due to food processing and digestion.

Clinical application of sIgE binding to epitopes in food allergy diagnosis

As mentioned in the previous paragraph, allergy has been mostly associated with a broad recognition of linear epitopes. Even though broad recognition patterns increase the probability of binding epitope combinations with sufficient spacing, assessment of such panels would require microarrays covering the entire sequence of an allergen[66]. Moreover, no cut-off levels (= minimal number of recognised linear epitopes) have yet been defined. Such cut-off levels would facilitate clinician's diagnosis when using sIgE binding to linear epitopes as a diagnostic tool. Defined sets of linear epitopes, however, provide a larger clinical applicability. These sets are not only easier to interpret, they are also easier to implement in already commercially available diagnostic platforms. For clinical implementation, we still lack clinical studies on technological applicability, diagnostic accuracy, additional diagnostic value and cost comparison to currently available conventional testing[66]. Specific IgE binding to conformational epitopes as a diagnostic tool, however, will require the development of more sophisticated *in vitro* platforms than required for linear epitopes since the 3D structure of the epitopes has to be conserved. A potential solution might be the translation of conformational epitopes into linear peptides mimicking the structure of the conformational epitope[76,77].

High resolution epitope mapping with human monoclonal antibodies to define clinically relevant epitopes

Normally, epitope mapping in food allergy research is performed with polyclonal patient sera. Even though polyclonal patient sera are easily accessible for *in vitro* research, they contain a complex mixture of distinct antibodies. This mixture may hamper the definition of single clinically relevant epitopes for *in vitro* diagnostics. As discussed in our review (**chapter 7**), specific antibodies present in sera of sensitised but tolerant patients can theoretically recognise clinically relevant epitopes with lower affinity than required for sufficient cross-linking. Antibodies in sera from allergic patients, on the other hand, may recognise clinically irrelevant epitopes as well as the ones responsible for successful cross-linking. Moreover, blocking antibodies (IgG or IgA) may be present in sera of sensitised but tolerant patients preventing sIgE binding to clinically relevant epitopes. To overcome these limitations, we proposed the use/generation of antigen-specific human mAbs derived from allergic and sensitised but tolerant patients. This

tool provides a less complex matrix and offers the possibility to perform high resolution epitope mapping on amino acid level - same amino acid sequence but distinct critical amino acids. Additional advantages of human mAbs are the possibility to connect genetic features with specific allergen binding and their use for conformational epitope mapping requiring sophisticated techniques not compatible with complex polyclonal patient sera[78].

In **chapter 8**, we set up and compared two pipelines for the generation of human mAbs derived from peripheral blood. One strategy entailed B cell immortalisation by EBV and limited dilution, while the other strategy was characterised by single cell sequencing and heterologous antibody expression in mammalian cells. The major advantage of single cell sequencing is the broad examination of antigen-binding mAbs due to a less selective process compared to EBV immortalisation combined with limited dilution. However, both techniques solely rely on BCR expression of antigen-specific B cells for selection. Hence, plasma blasts and IgE+ B cells with limited surface expression are hard to detect and plasma cells are nearly completely excluded from selection[79,80]. In vaccine research, plasma cells were selected 3-5 days after vaccination without staining for antigen binding. Most of the plasma cells were specific for the target antigen, since the boost greatly enlarged the plasma cell compartment for plasma cells recognising one specific antigen[81–83]. This interesting approach is, unfortunately, limited in allergy research, as the last time point of stimulation is hard to define (unknown ingestion). Moreover, the choice of the antigen for B cell selection is especially critical in food allergy research. The antigen is presented in a mixture of intact and partly digested conditions. Additionally, food processing may have influenced the folding of the antigen presented to the immune system. Overall, human mAbs are a great step forward towards defining clinically relevant epitopes and will provide new insights on antibodies produced by allergic and sensitised but tolerant patients.

Allergen engineering to prevent the binding of clinically irrelevant sIgE

Human mAbs generated for defining differences in antibody repertoires of allergic and tolerant patients sensitised to Ara h 2 and 6 (**chapter 9**) may be beneficial for high resolution epitope mapping as discussed in the previous paragraph. By combining the exact binding sites of these antibodies with their potency to induce degranulation, epitopes can be categorised into clinically relevant and irrelevant ones. This knowledge offers the possibility to develop engineered/improved allergens for sIgE diagnostics (Figure 4). Improved allergens may be achieved by site-directed mutations within clinically irrelevant epitopes to prevent sIgE binding from sensitised but tolerant patients. Moreover, exact site-directed mutations within clinically relevant epitopes may also prevent

sIgE binding from tolerant patients as only critical amino acids may differ. The use of such engineered/improved allergens (*i*-allergens) in CRD may dramatically reduce the measurement of clinically irrelevant sensitisation, facilitating the extrapolation of *in vitro* diagnostics to relevant dietary restrictions in the future.

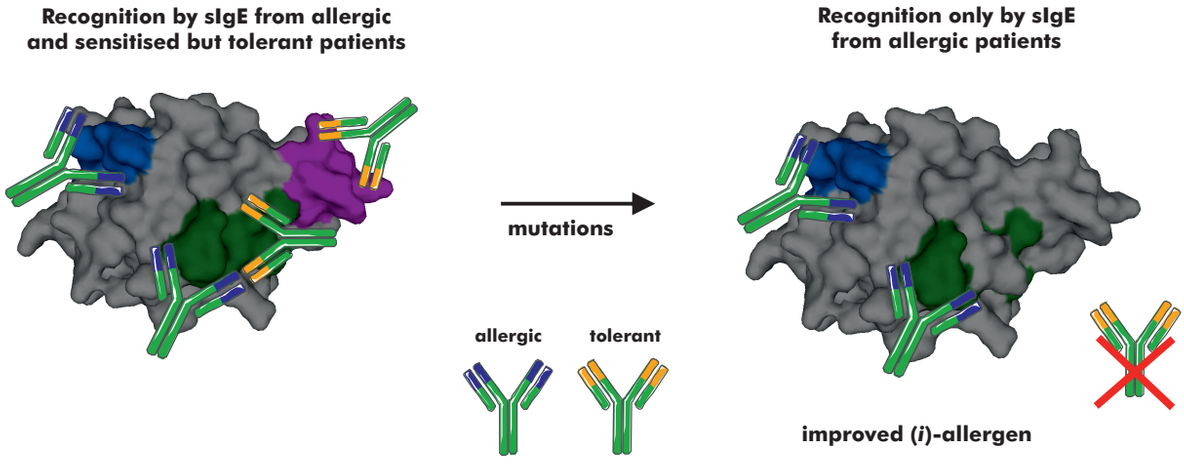

Figure 4: Site-directed mutations of clinically irrelevant epitopes can support the development of improved (*i*)-allergens by preventing sIgE binding from sensitised but tolerant patients; clinically relevant epitopes are highlighted in green and blue; antibodies from allergic patients possess a blue variable region and antibodies from tolerant patients possess a yellow variable region

Genetic fingerprints as a step towards a new diagnostic strategy

One major advantage of human mAbs is the straightforward combination of genetic features with antigen specificity, providing a powerful platform to study genetic features associated with health or disease. In **chapter 9**, HCDR3 region motifs of human mAbs directed against the peanut allergens Ara h 2 and 6 were partly associated with allergy or tolerance despite overlapping sIgE levels against these components. This finding presents a first step towards the development of novel, non-sIgE based *in vitro* diagnostic strategies to discriminate between allergy and tolerance in case of clinically irrelevant sensitisation. A new diagnostic strategy may combine our selection procedure for Ara h 2 and 6 specific B cells from peripheral blood (FACS) with high-throughput next generation sequencing. High-throughput next generation sequencing offers a less laborious platform than Sanger sequencing of single-sorted cells. Comparable approaches are already used for cancer diagnostics[84].

Future perspectives of food allergy *in-vitro* diagnostics: Preventing „individualised diagnostics“

The nature of each allergen potentially responsible for sensitisation and subsequently for the allergic reaction is unique. Hence, each allergen requires individual strategies to diagnose their associated food allergies. The *in vitro* diagnostics of animal-related allergies such as hen's egg have already shown promising results using epitope-based approaches. This approach, however, seems to be more limited for plant-derived allergens such as peanut, since no specific sets of epitopes could be defined. For peanut with its major allergens Ara h 2 and 6, classical CRD is already a highly valuable tool to identify patients at risk. However, tolerant patients with increased sIgE levels to Ara h 2 and inconclusive history may experience diet limitations accompanied by a negative impact on their quality of life. In this case, the development of engineered allergens (*i*-allergens) will offer the chance to minimise these clinically irrelevant test outcomes. Despite potentially higher costs, an alternative to *i*-allergens for CRD, in case of development failure, may be the analysis of HCDR3 regions derived from antibodies recognising Ara h 2 and 6 (Figure 5). Overall, the way to improve the diagnostics for a certain allergen has to be adjusted to its nature, and the best approach will differ between allergens.

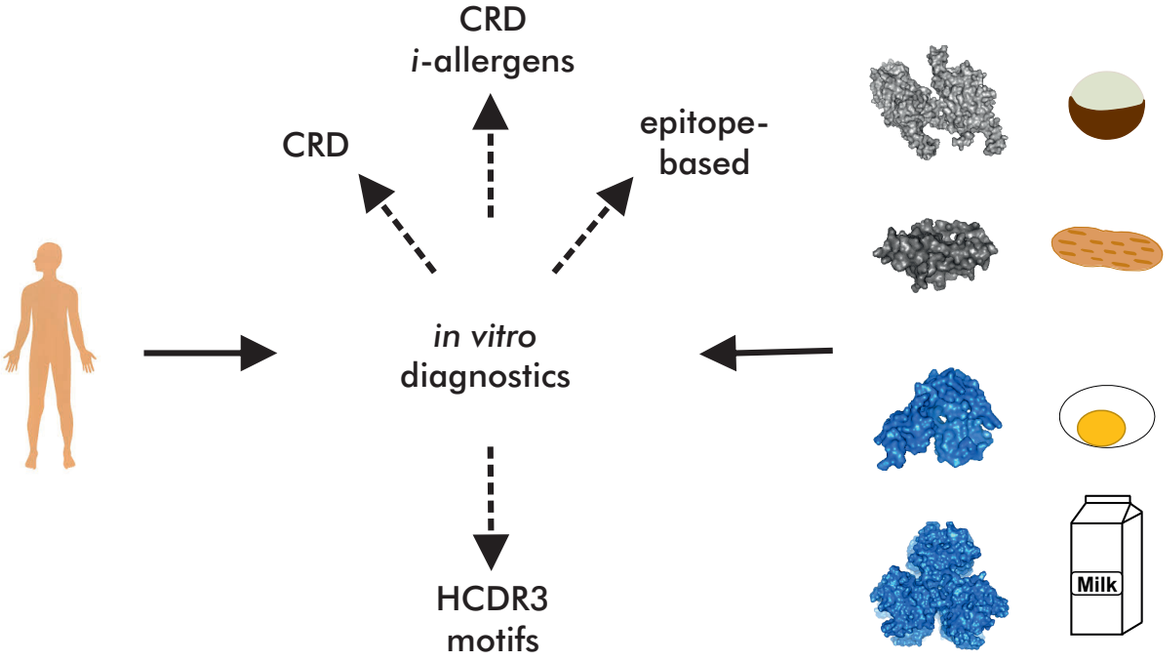

Figure 5: Individualised *in vitro* diagnostics strategies for unique allergens, indicating that each allergen needs the development of its own optimised *in vitro* diagnostic strategy

The benefit of developing the best *in vitro* diagnostic strategy for each individual allergen should be estimated before starting a long-lasting and costly development. Regarding Bet v 1-related food allergies, it might be highly interesting to predict the food sources which can or cannot be eaten. However, the rather mild character of these allergies provide the possibility to safely test the respective food sources at home. Nevertheless, sensitisation to Gly m 4, the PR-10 protein from soy, is partly associated with even severe reactions, indicating the benefit of developing an epitope-based or *i*-allergen approach for this particular PR-10 protein[85]. Moreover, accurate diagnostic strategies are primarily required for allergens often hidden in a large panel of foods while allergens like kiwi are more easily avoidable.

Despite the promising future perspectives of these *in vitro* diagnostic strategies, they are limited in predicting the severity of an allergic reaction. To achieve allergy combined with severity prediction, the individualised approaches discussed in the thesis should be combined with diagnostics focussing on severity prediction. However, these tools are not yet developed and may be based on epigenetic features, biomarkers related to effector cells (gene expression, miRNAs) or glycosylation patterns of IgE antibodies[56,86,87].

Concluding remarks

Overall, the results presented in this thesis provide a basis for developing *in vitro* diagnostic strategies preventing „false-negative“ and „false-positive“ sIgE testing. The exact development strategy is dependent on the unique nature of the allergen, *e.g.* „false-positive“ results to Ara h 2 and 6 may be prevented by analysing HCDR3 patterns while sIgE binding to linear epitopes of Gal d 1 appear to be promising for discriminating between hen’s egg allergy and tolerance in adults. Overall, individually improved *in vitro* diagnostics on allergen level may potentially reduce the required number of burdensome food challenges in the future.

Main findings of the presented thesis

Preventing „false-negative“ sIgE testing: non-detectable clinically relevant sensitisation

- Specific IgE binding to macadamia nut vicilins may support the stratification of macadamia nut allergic patients experiencing moderate to severe symptoms (**chapter 2**)
- Highly purified native and heterologously expressed sesame oleosins were not recognised in sesame allergic adults with false-negative sIgE testing. Hence, sIgE binding to sesame oleosins has no additional value in diagnosing sesame allergic adults (**chapter 3**)
- Our purification strategy of native oleosins, potentially disrupting lipids, pinpoints to a potential role of lipids in allergen recognition. The evaluation of this role might be a future directive of research to minimise the number „false-negative“ test outcomes

Preventing „false-positive“ sIgE testing: non-detectable clinically relevant sensitisation

- Specific IgE measurement against linear epitopes of Gal d 1 (aa 30-41, aa 39-50, aa 84-95) supports the discrimination between hen’s egg allergic and tolerant adults similarly sensitised to full-length Gal d 1. Hen’s egg allergic adults recognising these epitopes suffered mainly from objective symptoms (**chapter 4**).
- The isoform Ara h 7.0201 has comparable potency to induce degranulation compared to the other peanut 2S albumins Ara h 2 and 6, while the other isoforms Ara h 7.0101 and Ara h 7.0301 showed lower potencies (**chapter 5**). Lower potency of the latter isoforms was not explained by linear epitope recognition patterns, leading to the assumption that conformational epitopes play a critical role in this context (**chapter 6**)
- We assume that the use of specific human mAbs supports the identification of clinically irrelevant epitopes and can be used for conformational epitope mapping requiring sophisticated techniques (**chapter 7** and **8**)
- Certain HCDR3 region motifs of peanut 2S albumin-specific mAbs were either associated with peanut allergy or tolerance. This observation provides a first step towards the development of a novel diagnostic strategy for discriminating between allergy and tolerance on antibody level (**chapter 8** and **9**)

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11

Nederlandse samenvatting

Nederlandse samenvatting

De incidentie van voedselallergieën groeit wereldwijd nog flink. In Europa heeft gemiddeld drie procent van de bevolking last van een voedselallergie. Bij een voedsel allergische reactie reageert ons immuunsysteem op ongevaarlijke eiwitten die in onze voeding zitten. Deze eiwitten, ook wel allergenen genoemd, die door ons immuunsysteem als gevaarlijk beschouwd worden, komen in Europa vooral voor in pinda, hazelnoot, kippenei, koemelk, fruit (perzik, appel), vis en schaaldieren. Door de herkenning van deze allergenen door ons immuunsysteem, worden afweerstoffen, zo genaamde antilichamen, aangemaakt die tot verschillende subgroepen behoren - bij allergische patiënten worden daarnaast nog antilichamen van het type E (IgE) aangemaakt. IgE dat een bepaald allergeen herkent, wordt specifiek IgE genoemd.

Diagnose van een voedselallergie

De diagnose van voedselallergie kan opgesplitst worden in vier verschillende onderdelen: vraaggesprek (anamnese), huidtest, bloedonderzoek (**Box 1**) en de dubbelblinde voedselprovocatie (**Box 2**). Bij binnenkomst van een patiënt met verdenking op voedselallergie wordt allereerst gevraagd naar het optreden van klachten veroorzaakt door bepaalde voedingsmiddelen en hoe snel deze klachten ontstaan. Na het vraaggesprek volgt meestal een huidtest, de zo genaamde skin prik test. Bij de skin prik test wordt een druppel met het extract van het verdachte voedingsmiddel op de huid aangebracht, waarna de huid wordt aangeprikt door de druppel heen. Indien er galbulten op de huid ontstaan, is dat een bewijs voor aanwezigheid van IgE antilichamen tegen het betreffende voedingsmiddel. Als de anamnese en resultaten van de skin prik test uiteen lopen, wordt aanvullend bloedonderzoek uitgevoerd. Daarbij wordt het bloed onderzocht op aanwezigheid van specifiek IgE tegen het verdachte voedingsmiddel of een bepaald voedingseiwit/allergeen. Helaas kunnen zowel de skin prick test als het bloedonderzoek resultaten opleveren die in tegen scheid staan met de uitslag van een dubbelblinde provocatie, waarbij kleine hoeveelheden van het verdachte voedingsmiddel worden gegeven (**Box 2**):

1. De test toont geen specifieke IgE antilichamen, terwijl er wel sprake is van een bevestigde voedselallergie (fout-negatieve test uitslag)

2. De test toont specifieke IgE antilichamen, terwijl er geen sprake is van een bevestigde voedselallergie (fout-positieve test uitslag)

Box 1: Bloedonderzoek – Bepalen van specifiek IgE
Als IgE antilichamen tegen een bepaald eiwit/allergeen gericht zijn, noemen we het specifiek IgE. Specifiek IgE dat in het bloed van een patiënt zit, kan met een bloedtest aangetoond worden. Bij zo'n bloedtest wordt patiënten serum - de vloeistof die over blijft naar het stollen van bloed - aan het allergeen blot gesteld en vervolgens gemeten hoeveel IgE antilichamen het allergeen binden. Specifiek IgE antilichamen kunnen worden gemeten tegen een hele mix van allergenen afkomstig van een voedingsbron (extract) of tegen een bepaald voedingseiwit/allergeen (component). In de daagse routine worden zulke testen van bedrijven aangeleverd.

Box 2: Duppelblinde voedselprovocatie
Bij een voedselprovocatie neemt de patiënt het verdachte voedingsmiddel in, te beginnen met een hele kleine hoeveelheid, die verstopt zit in bijv. een stukje koek. Tijdens de voedselprovocatie wordt de dosis in kleine stappen verhoogd, waarbij tussen iedere dosering tenminste 30 minuten gewacht wordt om te zien of er klachten ontstaan. Ter controle wordt op de ene dag een koek met het verdachte voedingsmiddel gegeven en op de andere dag een koek zonder het voedingsmiddel (placebo). Hoewel deze methode als gouden standaard beschouwd wordt, zitten er ook nadelen aan. Zo kan een voedselprovocatie alleen in een gespecialiseerde unit in het ziekenhuis plaatsvinden, wat duur is en getraind personeel vereist, maar ook kan de patiënt onaangename klachten ervaren.

Het doel van dit proefschrift was om de diagnose van een voedselallergie op basis van bloedtesten te verbeteren en daarmee het aantal benodigde, belastende voedselprovocaties te verminderen.

1. Fout-negatieve test uitslagen

Een deel van de voedsel allergische patiënten – 18% van de pinda- en 30% van de sesamzaad allergische patiënten - heeft geen aantoonbaar specifiek IgE in het bloed. Een verklaring voor het optreden van fout-negatieve test uitslagen is de afwezigheid van het allergeen waartegen de patiënt specifiek IgE heeft aangemaakt, in de huidige door bedrijven geleverde bloedtesten.

In **hoofdstuk 2** hebben we nog niet geïdentificeerde allergenen van macadamia noten bestudeerd. Momenteel is slechts een klein aantal patiënten met een voedselallergie allergisch voor macadamia noten. Er wordt echter verwacht dat deze aantallen gaan stijgen, aangezien de consumptie van macadamia noten flink groeit. In ons onderzoek hebben we een nieuw allergeen kunnen aantonen, behorend tot een groep eiwitten genaamd vicilinen. Eiwitten behorend tot de viciline groep zijn ook voor andere voedingsmiddelen (bijv. pinda) als allergenen bekend. Dit nieuwe allergeen wordt vooral herkend door patiënten met matig tot ernstige klachten en kan daarom belangrijk zijn bij het identificeren van allergische patiënten met een verhoogd risico op ernstige klachten.

De huidige bloedtesten die door bedrijven kunnen worden geleverd, bevatten vooral allergenen die in water oplosbaar zijn. Daarentegen zijn eiwitten die niet in water, maar in vet oplosbaar zijn meestal afwezig. Er zijn echter wel in vet oplosbare eiwitten, ook wel oleosines genoemd, die als allergenen bekend zijn. Specifiek IgE tegen deze allergenen kunnen niet met behulp van de huidige bloedtesten aangetoond worden. Om de rol van deze eiwitten beter in kaart te brengen, hebben we in **hoofdstuk 3** oleosines uit sesamzaad en hun binding door specifiek IgE onderzocht. In tegenstelling tot onze verwachting konden we bijna geen specifiek IgE tegen sesam oleosines aantonen. Indien er wel specifiek IgE tegen sesam oleosines aantoonbaar was, hadden deze patiënten ook aantoonbaar specifiek IgE tegen bekende water oplosbare allergenen. Deze resultaten wijzen erop dat sesam oleosines geen bijdrage kunnen leveren aan verbetering van de huidige bloedtesten voor sesamzaad.

2. Fout-positieve test uitslagen

Het aantal fout-positieve test uitslagen is afhankelijk van het allergeen waartegen specifiek IgE bepaald wordt. Fout-positieve specifiek IgE metingen tegen pinda extract - meestal gebruikt in de huidige bloedtesten - komen vaker voor (rond 30-35%) dan fout-positieve specifiek IgE metingen tegen het stabiele pinda allergeen Ara h 2 (**Box 3**). Hoewel specifiek IgE tegen Ara h 2 in ongeveer 90% van patiënten een pinda allergie aantoont, kan ongeveer 10% van de patiënten met specifiek IgE tegen Ara h 2 pinda eten zonder klachten te ontwikkelen. Als de diagnose uitsluitend gebaseerd zou zijn op deze test, zou dit tot een verkeerde diagnose en onnodige dieet restricties leiden. Het optreden van fout-positieve test uitslagen kan mogelijk deels verklaard worden door verschillen tussen IgE antilichamen van allergische en tolerante patiënten. Hierbij hebben we vooral gekeken naar verschillen waar de IgE antilichaam precies aan het allergeen kan binden. In **hoofdstuk 4** en **6** hebben we naar verschillende IgE herkenningspatronen gekeken - de herkenning van lineaire epitopen (**Box 4**).

Box 3: Benaming van allergenen
De benaming van allergenen bestaat uit de Latijnse naam van de bron en een doorlopend getal. Hier een voorbeeld voor de benaming van een allergenen afkomstig van pinda:
*Latijnse naam: **Ara**chis **h**ypogaea → **Ara h 2***

Box 4: Lineaire en conformationele epitopen
Als een antilichaam aan een allergeen bindt dan vindt dat op een specifieke plek van het eiwit plaats. Deze specifieke bindingslocaties van antilichamen op allergenen worden epitopen genoemd. Ze bestaan uit keten van kleine bouw blokken: aminozuren. Zij kunnen in twee verschillende categorieën ingedeeld worden:

1. *Lineaire epitopen: deze bestaan uit aminozuren die in de keten op elkaar volgen*
2. *Conformationele epitopen: De lineaire keten van aminozuren komt meestal gevouwen voor, zodat aminozuren dicht bij elkaar in de buurt kunnen komen zonder in de lineaire keten naast elkaar te zitten. Als een antilichaam aminozuren herkent die door de gevouwen structuur bij elkaar in de buurt komen, noemen we dit een driedimentionaal of conformationeel epitoop.*

Hoofdstuk 4 was gericht op de epitopen van de hoofd allergenen van kippenei, met als doel om fout-positieve testen voor kippenallergie bij volwassenen te voorkomen. Kippenei allergie komt vaak voor bij kinderen en deze ‚groeien er meestal overheen'. Echter komt kippenei allergie in mindere mate ook voor bij volwassenen en ontstaat deels ook pas op volwassen leeftijd. Uit ons onderzoek is gebleken dat een aantal volwassenen niet allergisch is voor kippenei, ondanks verhoogd specifiek IgE tegen kippenei extract en de hoofdallergenen Gal d 1 en 3. Dit is een aanwijzing daarvoor dat meten van specifiek IgE tegen het complete allergeen Gal d 1 en 3 niet gebruikt kan worden om een kippenallergie bij volwassenen te voorspellen hoewel specifiek IgE tegen Gal d 1 wel aanwijzing is voor een kippenei allergie bij kinderen. Door het bestuderen van de IgE herkenningspatronen konden we drie lineaire epitopen van Gal d 1 identificeren die met voorrang herkend worden door specifiek IgE van kippenei allergische patiënten met vooral ernstige klachten. Daarmee kan diagnostiek gericht op deze drie epitopen bijdragen aan het verminderen van rond 90% fout-positieve testuitslagen bij kippenei allergie diagnostiek van volwassenen.

Naast Ara h 2 bevat pinda nog twee allergenen uit dezelfde eiwitfamilie: Ara h 6 en 7. Door het bepalen van specifiek IgE tegen deze allergenen kan een pinda allergie net zo goed voorspelt worden. Ara h 7 is erbij bijzonder omdat drie verschillende Ara h 7 isovormen (**Box 5**) in pinda voorkomen. Door in het laboratorium te bestuderen in hoeverre deze isovormen de cellen die voor de allergische reactie verantwoordelijk zijn, kunnen activeren, konden we in **hoofdstuk 5** laten zien dat isovorm 2 de grootste potentie daarvoor heeft. Deze potentie is vergelijkbaar met de potenties van Ara h 2 en 6.

Naar aanleiding van de resultaten in **hoofdstuk 5** hebben we in **hoofdstuk 6** naar een verklaring gezocht waarom de isovorm 2 een grotere potentie heeft dan de andere Ara h 7 isovormen. Deze vraag hebben we net als in **hoofdstuk 4** geprobeerd te beantwoorden door het bestuderen van de specifiek IgE binding aan lineaire epi-

topen. Dit verklaarde het verschillende gedrag van de Ara h 7 isovormen echter niet. Daarbij viel op dat er over het algemeen minder lineaire epitopen door specifiek IgE herkend werden dan verwacht. Specifiek IgE van sommige patiënten herkende zelfs niet één lineaire epitoop. Deze resultaten suggereren dat conformationele epitopen bij de herkenning van Ara h 7 isovormen waarschijnlijk een grotere rol spelen dan lineaire epitopen. Dit wijst erop dat het niet voldoende is om lineaire epitopen te bestuderen en dat in de toekomst meer onderzoek naar conformationele epitopen gedaan moet worden.

Box 5: Isovormen
*In pinda komt het pinda allergeen Ara h 7 in drie verschillende vormen voor. Ieder vorm bestaat uit bijna dezelfde bouw blokken maar wijst wel verschillen in vergelijking met de andere vormen op. Deze soort gelijke eiwitten noemen we isovormen en de isovormen van Ara h 7 dragen de volgende namen: Ara h 7.0**1**01, Ara h 7.0**2**01 en Ara h 7.0**3**01.*

Voor de studies naar de herkenning van lineaire epitopen in **hoofdstuk 4** en **6** hebben we patiënten sera gebruikt die een mix van IgE antilichamen bevatten die verschillende epitopen herkennen. In **hoofdstuk 7** hebben we geconstateerd dat door het gebruik van patiënten sera mogelijk relevante epitopen niet duidelijk of zelfs helemaal niet geïdentificeerd kunnen worden. Dit kan veroorzaakt worden doordat:

- serum van allergische patiënten zowel IgE antilichamen kan bevatten die epitopen herkennen die voor het optreden van een allergische reactie relevant zijn, als IgE antilichamen die epitopen herkennen die niet van belang zijn. Dit bemoeilijkt de identificatie van relevante epitopen.
- serum van tolerante patiënten antilichamen kan bevatten die ogenschijnlijk relevante epitopen herkennen. Deze epitopen zijn echter toch niet relevant, omdat het binden door het antilichaam waarschijnlijk niet sterk genoeg is om de cellen die voor een allergische reactie van belang zijn, te activeren.

Een mogelijke oplossing is om deze antilichamen afzonderlijk van elkaar te bekijken. Dit hebben we gedaan door de extractie van DNA uit een B cel die één specifiek antilichaam aanmaakt. Deze DNA-informatie kan vervolgens gebruikt worden om het specifieke antilichaam dat deze B cel normaalgesproken maakt te produceren in het laboratorium. Als deze in het laboratorium geproduceerde antilichamen allemaal de DNA informatie uit één B cel bevatten, worden ze monoklonaal genoemd.

In **hoofdstuk 8** hebben we op basis van de literatuur twee methodes ontwikkeld om monoklonale antilichamen in het laboratorium te produceren:

1. Door witte bloedcellen die antilichamen maken (de B cellen) te infecteren met het Epstein-Barr virus, waardoor ze onsterfelijk gemaakt worden. Deze B cellen kunnen vervolgens zo opgegroeid worden dat één kweek slechts dochters van één

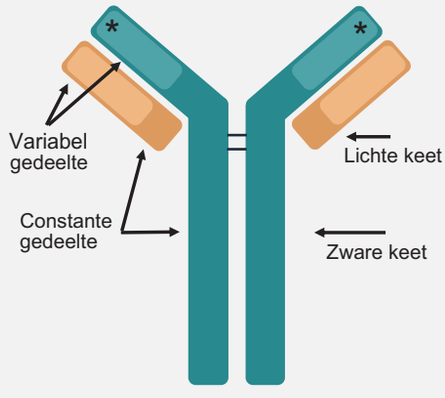
bepaalde B cel bevat. Als de B cellen in deze kweek antilichamen aanmaken, kunnen deze een type antilichamen aanmaken (monoclonale antilichamen) die voor verder onderzoek gebruikt worden. Deze monoklonale antilichamen kunnen dan op basis van hun binding aan een specifiek allergeen zoals het pinda allergeen Ara h 2 geselecteerd worden.

2. B cellen bezitten een receptor op hun oppervlakte die dezelfde DNA-informatie, dus dezelfde specificiteit, bezit als de antilichamen die door deze B cellen gemaakt worden. Daardoor kunnen we B cellen op basis van hun specificiteit selecteren. Door één B cel te selecteren weten we dat de DNA-informatie van deze B cel gebruikt is voor het maken van die betreffende monoklonale antistof.

Aan beide methodes zitten voor- en nadelen. De eerste methode heeft als nadeel dat hij gebruikt kan worden om slechts een klein aantal verschillende monoklonale antilichamen te produceren, terwijl het voordeel is dat deze methode goedkoper is dan de tweede methode. De tweede methode heeft echter als voordeel dat hij voor een bredere selectie van monoklonale antilichamen gebruikt kan worden. Voor ons patiënten studie in **hoofdstuk 9** hebben ervoor gekozen methode 2 te gebruiken. De opbouw van een antilichaam staat in **Box 6** beschreven.

Box 6: Antilichaam opbouw

Een antilichaam bestaat uit twee zware en twee lichte ketens. Deze ketens omvatten telkens een constant gedeelte en een variabel gedeelte. Het variabel gedeelte bepaalt welk eiwit/allergeen herkent wordt. Het zware gedeelte is anderzijds ervoor verantwoordelijk op welke manier de lichaam op de herkenning van een eiwit gaat reageren.



Het variabel gedeelte bestaat uit drie verschillende genen: V, D en J genen. Tijdens de ontwikkeling van een B cel worden deze genen willekeurig uit een pool (65 V genen, 27 D genen, 6 J genen) geselecteerd en met elkaar gecombineerd. Dit leidt tot heel veel mogelijke combinaties die het herkennen van bijna oneindig verschillende eiwitten waarborgt. Voor het herkennen van een eiwit is de regio tussen het einde van het V gen en het begin van het J gen - naar vertaling in aminozuren - meest belangrijk.

Om na te gaan of met behulp van deze monoklonale antilichamen de diagnostiek kan worden verbeterd, hebben we in **hoofdstuk 9** B cellen van pinda allergische en pinda tolerante patiënten verkregen die allemaal specifiek IgE hadden tegen de pinda allergenen Ara h 2 en 6. Deze B cellen werden gebruikt om monoklonale antilichamen in het laboratorium te produceren. Door het bestuderen van hun DNA-informatie konden we belangrijke verschillen tussen pinda allergische en tolerante patiënten aantonen:

- Het variabel gedeelte van antilichamen afkomstig van allergische patiënten werd veel vaker door één bepaalde groep V genen gevormd.
- De regio die naar het vertalen in aminozuren het meest belangrijk is voor het binden aan het allergeen, heeft deels een unieke genetische opbouw als de antilichaam afkomstig is van een pinda allergische patiënt.

Deze resultaten wijzen erop dat er DNA-patronen bestaan die onderscheid kunnen maken tussen pinda allergische en pinda tolerante patiënten. Als er een eenvoudigere techniek ontwikkeld zou worden voor methode 2, kunnen bovenstaande resultaten bijdragen aan de ontwikkeling van een bloedtest die een beter onderscheidt maakt tussen pinda allergische en niet pinda allergische patiënten.

De resultaten van dit proefschrift vormen een solide basis voor de ontwikkeling van verbeterde of nieuwe bloedtesten die fout-negatieve en fout-positieve test uitslagen kunnen verminderen of zelfs voorkomen. Afhankelijk van het allergeen bestaan er verschillende mogelijkheden om dit doel te bereiken: bijvoorbeeld de bepaling van specifiek IgE tegen lineaire epitopen voor het kippenei allergeen Gal d 1 of het evalueren van antilichaam DNA-patronen voor de pinda allergenen Ara h 2 en 6. Ongeacht de strategie die uiteindelijk voor een bepaald allergeen gebruikt wordt/moet worden, worden bloedtesten betrouwbaarder en kunnen zo het aantal vereiste voedselprovocaties in de toekomst verminderen. Daarnaast zijn de herkenning van het nieuwe macadamia noot allergeen en ook van de lineaire epitopen van Gal d 1 waarschijnlijk in staat om mensen met een verhoogd risico op een ernstige reactie te voorspellen.


12

Zusammenfassung in deutscher Sprache

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Nahrungsmittelallergien nehmen weltweit stetig zu. In Europa leiden durchschnittlich drei Prozent der Bevölkerung an einer Nahrungsmittelallergie. Dabei reagiert das menschliche Immunsystem auf sonst harmlose Eiweiße (Proteine), die in Nahrungsmitteln enthalten sein können. Diese als gefährlich eingestuften Proteine, auch Allergene genannt, kommen in Europa hauptsächlich in Erdnüssen, Haselnüssen, Hühnereiern, Kuhmilch, Früchten, Fisch und Krustentieren vor. Bei der Erkennung dieser Allergene durch das menschliche Immunsystem werden Antikörper verschiedener Untergruppen gegen das jeweilige Allergen hergestellt – bei Allergikern wird zusätzlich auch die Untergruppe der sogenannten Antikörper vom Typ E (IgE) hergestellt. IgE, das ein bestimmtes Allergen erkennt, wird spezifisches IgE genannt.

Diagnose einer Nahrungsmittelallergie

Die Diagnose einer Nahrungsmittelallergie kann in vier Bereiche eingeteilt werden: Befragung (Anamnese), Hauttest, Bluttest (**Box 1**) und doppelblinde Nahrungsmittelprovokation (**Box 2**). Ein Patient mit Verdacht auf eine Nahrungsmittelallergie wird bei dem ersten Besuch bei einem Allergologen zu den Beschwerden, die durch bestimmte Lebensmittel verursacht werden, befragt. Hierbei wird zusätzlich ergründet, wie schnell diese Beschwerden auftreten. Nach der Befragung wird in der Regel ein Hauttest, der sogenannte Skin Prick Test, durchgeführt. Bei diesem wird ein Tropfen eines Extrakts, das aus dem vermuteten Lebensmittel gewonnen wird, auf die Haut aufgetragen. Anschließend wird die Haut durch den Tropfen hindurch angestochen. Das Auftreten von Hautschwellungen ist ein Hinweis auf das Vorhandensein von IgE-Antikörpern gegen das betreffende Lebensmittel. Im Falle einer Diskrepanz zwischen Anamnese und Testergebnis werden zusätzliche Bluttests durchgeführt. Dabei wird das Blut auf spezifisches IgE gegen das vermutete Lebensmittel oder ein bestimmtes Lebensmittelprotein/Allergen untersucht. Der Skin Prick Test als auch der Bluttest können zu Ergebnissen führen, die dem Ergebnis einer doppelblinden Nahrungsmittelprovokation, bei der kleine Portionen des verdachten Lebensmittels verabreicht werden, widersprechen (**Box 2**):

1. Es können mit dem Bluttest keine spezifischen IgE-Antikörper nachgewiesen werden, obwohl eine bestätigte Nahrungsmittelallergie vorliegt (falsch-negatives Testergebnis)

2. Es können mit dem Bluttest spezifische IgE-Antikörper nachgewiesen werden, obwohl keine bestätigte Nahrungsmittelallergie vorliegt (falsch-positives Testergebnis).

Box 1: Bluttest - Bestimmung von spezifischem IgE
IgE-Antikörper, welche gegen ein bestimmtes Protein/Allergen gerichtet sind, werden als spezifische IgE bezeichnet. Spezifisches IgE, das sich im Blut eines Patienten befindet, kann mit einem Bluttest nachgewiesen werden. Hierbei wird das Patientenserum - die Flüssigkeit, die bei der Blutgerinnung zurückbleibt - mit dem Allergen zusammengebracht und anschließend gemessen, wie viele IgE-Antikörper an das Allergen gebunden haben. Die Bestimmung des spezifischen IgEs kann mit einer Mischung aus mehreren Allergenen, die aus derselben Nahrungsquelle stammen (Extrakt) oder mit einem bestimmten Nahrungsmittelprotein/Allergen (Komponente) durchgeführt werden. In der täglichen Routine werden Tests verwendet, die kommerziell hergestellt sind.

Box 2: Doppelblinde Nahrungsmittelprovokation
Bei einer Nahrungsmittelprovokation nimmt der Patient das vermeintlich allergieauslösende Lebensmittel zu sich, wobei mit einer sehr kleinen Menge begonnen wird. Diese kann beispielsweise in einem Stück Kuchen versteckt sein. Während der Provokation wird die Dosis in kleinen Schritten erhöht, wobei zwischen jeder Dosis mindestens 30 Minuten gewartet wird, um festzustellen, ob Beschwerden auftreten. Zur Kontrolle wird an einem Tag ein Kuchen, der das vermeintliche Nahrungsmittel enthält, verabreicht und an einem weiteren Tag ein Kuchen ohne das verdächtige Nahrungsmittel (Placebo). Obwohl diese Methode als Goldstandard angesehen wird, weist sie auch weitreichende Nachteile auf. Beispielsweise kann eine Nahrungsmittelprovokation nur auf einer dafür spezialisierten Station im Krankenhaus durchgeführt werden. Dies führt zu hohen Kosten und erfordert geschultes Personal. Auch können hierbei unangenehme Beschwerden als auch lebensbedrohliche Symptome auftreten

Ziel dieser Arbeit war die Verbesserung der Diagnose von Nahrungsmittallergien mit Hilfe von Bluttests, um die Anzahl der erforderlichen und belastenden Nahrungsmittelprovokationen zu verringern.

1. Falsch-negative Testergebnisse

Einige Patienten mit einer Nahrungsmittelallergie - 18% der Erdnuss- und 30% der Sesamsamen-Allergiker - haben kein nachweisbares spezifisches IgE im Blut. Eine Erklärung für das Auftreten falsch-negativer Testergebnisse ist das Fehlen des betreffenden Allergens in den Bluttests, die kommerziell hergestellt werden.

In **Kapitel 2** wurde die Macadamianuss auf noch nicht bekannte Allergene hin untersucht. Bislang ist nur eine kleine Anzahl von Nahrungsmittelallergikern allergisch gegen Macadamianüsse. Es wird jedoch erwartet, dass die Zahl der Macadamianuss-Allergiker wachsen wird, da der Konsum von Macadamianüssen kontinuierlich zunimmt. Im Verlauf der Arbeit wurde ein neues Macadamia-Allergen nachgewiesen, das zu einer Gruppe von Proteinen gehört, die Viciline genannt werden. Proteine der Vicilin-Gruppe sind auch als Allergene für andere Nahrungsmittel (z. B. Erdnüsse) bekannt. Diese neuen Allergene werden hauptsächlich von Patienten mit mittelschweren bis schweren Beschwerden erkannt und können daher wichtig sein, um allergische Patienten mit

einem erhöhten Risiko auf schwerwiegende Verläufe zu identifizieren.

Bluttests, die momentan kommerziell erhältlich sind, beinhalten hauptsächlich wasserlösliche Allergene. Im Gegensatz dazu fehlen Proteine, die nicht wasser-, sondern fettlöslich sind. Es gibt jedoch fettlösliche Proteine, z.B. sogenannte Oleosine, die bereits als Allergene identifiziert sind. Spezifisches IgE gegen diese Allergene kann daher nicht mit den derzeit zur Verfügung stehenden Bluttests nachgewiesen werden. In **Kapitel 3** wurden Oleosine aus Sesamsamen auf ihre Bindung durch spezifisches IgE hin untersucht, um das Potenzial dieser Proteine für die Allergiediagnostik besser zu verstehen. Entgegen der Erwartungen konnte kaum spezifisches IgE gegen Oleosine aus Sesamsamen nachgewiesen werden. War spezifisches IgE gegen diese Oleosine nachweisbar, so hatten diese Patienten auch nachweisbares spezifisches IgE gegen bekannte wasserlösliche Allergene. Diese Ergebnisse zeigen, dass Oleosine aus Sesamsamen nicht dazu beitragen können, die aktuellen Bluttests für Sesam-Allergiker zu verbessern.

2. Falsch-positive Testergebnisse

Die Anzahl der falsch-positiven Testergebnisse hängt von dem Allergen ab, gegen das das spezifische IgE bestimmt wird. Falsch-positive spezifische IgE-Messungen gegen Erdnussextrakt - meistens in momentan erhältlichen Bluttests verwendet - sind häufiger (etwa 30-35%) als falsch-positive spezifische IgE-Messungen gegen das stabile Erdnussallergen Ara h 2 (**Box 3**). Obwohl spezifisches IgE gegen Ara h 2 bei etwa 90% der Patienten auf eine Erdnuss-Allergie hinweist, können etwa 10% der Patienten mit spezifischem IgE gegen Ara h 2 Erdnüsse essen, ohne Beschwerden zu entwickeln. Würde die Diagnose ausschließlich auf diesem Test basieren, würde dies zu einer Fehldiagnose und unnötigen diätetischen Einschränkungen führen. Das Auftreten falsch-positiver Testergebnisse kann möglicherweise durch Unterschiede bei IgE-Antikörpern zwischen allergischen und toleranten Patienten erklärt werden. In der vorliegenden Arbeit wurde bezüglich möglicher Unterschiede betrachtet, auf welche Weise IgE-Antikörper an Allergene binden. In den **Kapiteln 4** und **6** haben wir verschiedene Erkennungsmuster von spezifischen IgE untersucht, insbesondere die Erkennung von linearen Epitopen (**Box 4**).

Box 3: Namensgebung von Allergenen
Die Namen der Allergene bestehen aus dem lateinischen Namen der Quelle und einer fortlaufenden Nummer. Hier ein Beispiel für den Namen eines Allergens aus Erdnuss:
*Lateinischer Name: **Ara**chis **h**ypogaea → **Ara h 2***

Box 4: Lineare und konformationelle Epitope
Wenn ein Antikörper an ein Allergen bindet, findet dies an einer bestimmten Stelle des Proteins statt. Diese spezifischen Bindungsstellen von Antikörpern werden Epitope genannt. Sie bestehen aus einer Kette kleiner Bausteine, den sogenannten Aminosäuren und können in zwei verschiedene Kategorien unterteilt werden:

1. *Lineare Epitope bestehen aus Aminosäuren, die in der Kette aufeinander folgen.*
2. *Konformationelle Epitope: Die lineare Kette, die aus Aminosäuren besteht, kann so gefaltet sein, dass Aminosäuren nahe beieinander liegen, ohne dass sie in der linearen Kette nebeneinander vorkommen. Wenn ein Antikörper Aminosäuren erkennt, die in der linearen Kette weiter voneinander entfernt sind und die aufgrund der gefalteten Struktur nun nahe beieinander liegen, wird dies als dreidimensionales oder konformationelles Epitop bezeichnet.*

In **Kapitel 4** wurden Epitope der Hauptallergene von Hühnereiern untersucht, um zukünftig falsch-positive Testergebnisse in Bezug auf Hühnerei-Allergien bei Erwachsenen zu vermeiden. Eine Hühnerei-Allergie tritt bei Kindern häufig auf und wächst sich normalerweise aus. Zuweilen kann eine fortdauernde Hühnerei-Allergie aus dem Kindesalter jedoch auch bei Erwachsenen auftreten oder sie entwickelt sich erst im Erwachsenenalter. Es konnte gezeigt werden, dass eine Reihe von Erwachsenen trotz eines erhöhten spezifischen IgE gegen Hühnereiextrakt und gegen die Hauptallergene Gal d 1 und 3 nicht allergisch auf Hühnerei reagieren. Dies ist ein Hinweis darauf, dass das spezifische IgE gegen das Allergen Gal d 1 und 3 nicht verwendet werden kann, um eine Hühnerei-Allergie bei Erwachsenen vorherzusagen, obwohl spezifisches IgE gegen Gal d 1 auf eine solche bei Kindern hinweist. Durch die nähere Betrachtung der IgE-Erkennungsmuster konnten drei lineare Epitope von Gal d 1 identifiziert werden, die vorrangig von spezifischem IgE bei Hühnerei-Allergikern mit vornehmlich schweren Beschwerden erkannt werden. Dies bedeutet, dass eine Diagnostik, die auf spezifisches IgE gegen diese drei Epitope basiert, zu einer Verringerung der falsch-positiven Testergebnisse bei der Diagnose von Hühnerei-Allergien im Erwachsenenalter um etwa 90% beitragen kann.

Zusätzlich zu Ara h 2 besitzt die Erdnuss noch zwei Allergene aus derselben Proteinfamilie (2S Albumine): Ara h 6 und 7. Durch die Bestimmung des spezifischen IgEs gegen diese Allergene kann eine Erdnuss-Allergie ebenso zuverlässig vorhergesagt werden. Die Erdnuss besitzt drei verschiedene Ara h 7-Isoformen (**Box 5**). Es wurde untersucht, inwieweit diese Isoformen Zellen aktivieren können, die für die allergische Reaktion verantwortlich sind. Es zeigte sich in **Kapitel 5**, dass die Isoform 2 das größte Potenzial aufzeigt, vergleichbar mit den Potentialen von Ara h 2 und 6.

Basierend auf den Ergebnissen in **Kapitel 5** wurde in **Kapitel 6** untersucht, warum die Isoform 2 ein größeres allergenes Potenzial im Vergleich zu den anderen Ara h 7-

Isoformen besitzt. In Analogie zu **Kapitel 4** wurde hierzu die Bindung von spezifischen IgE an lineare Epitope analysiert. Jedoch konnten nicht die unterschiedlichen Potenziale der Ara h 7-Isoformen erklärt werden. Auffällig war, dass weniger lineare Epitope von spezifischem IgE erkannt wurden als erwartet. Es zeigte sich sogar, dass spezifisches IgE einiger Patienten keine linearen Epitope erkannte. Diese Ergebnisse legen nahe, dass konformationelle Epitope bei der Erkennung von Ara h 7-Isoformen eine größere Rolle spielen als lineare Epitope. In Zukunft sollten daher neben den linearen Epitopen auch konformationelle Epitope eine größere Beachtung finden.

Box 5: Isoformen
*In Erdnüssen kommt das Erdnussallergen Ara h 7 in drei verschiedenen Formen vor. Jede Form besteht aus fast den gleichen Bausteinen, weist jedoch gewisse Unterschiede zu den anderen Formen auf. Diese ähnlichen Proteine werden als Isoformen bezeichnet; die Isoformen von Ara h 7 besitzen die Namen Ara h 7.0**1**01, Ara h 7.0**2**01 und Ara h 7.0**3**01.*

Für die Untersuchungen zur Erkennung von linearen Epitopen in den **Kapiteln 4** und **6** wurden Patientenseren verwendet. Patientenseren enthalten für gewöhnlich eine Mischung von IgE-Antikörpern. Diese IgE-Antikörper können eine Reihe von verschiedenen Epitopen erkennen. In **Kapitel 7** wurde die Theorie aufgestellt, dass bei der Verwendung von Patientenseren potenziell relevante Epitope nicht eindeutig oder gar nicht identifiziert werden können. Dies kann folgendermaßen erklärt werden:

- Das Serum von allergischen Patienten kann sowohl IgE-Antikörper enthalten, die für die Auslösung einer allergischen Reaktion relevante Epitope erkennen, als auch IgE-Antikörper, die nicht relevante Epitope erkennen. Diese Mischung erschwert die Identifizierung der relevanten Epitope.
- Das Serum toleranter Patienten kann Antikörper enthalten, die scheinbar relevante Epitope erkennen. Diese Epitope sind jedoch eigentlich irrelevant, da die Bindung durch den Antikörper wahrscheinlich nicht stark genug ist, um Zellen, die für eine allergische Reaktion verantwortlich sind, zu aktivieren.

Eine mögliche Lösung besteht darin, diese Antikörper separat zu betrachten. Dazu kann DNA aus einer B-Zelle - die Produktionsstelle von identischen Antikörpern - extrahiert werden. Diese DNA-Information kann dann verwendet werden, um den spezifischen Antikörper, den die B-Zelle normalerweise im Körper bildet, im Labor herzustellen. Wenn Antikörper alle dieselbe definierte DNA-Information einer B-Zelle tragen, werden sie als monoklonal bezeichnet.

In **Kapitel 8** wurden basierend auf der Literatur zwei Methoden entwickelt, um monoklonale Antikörper herzustellen:

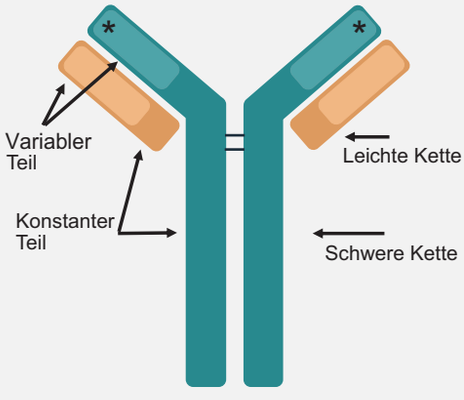
1. B-Zellen können durch die Infektion mit dem Epstein-Barr-Virus unsterblich gemacht werden. Diese B-Zellen können danach im Labor so gezüchtet werden, dass Kulturen aus identischen Kopien einer bestimmten B-Zelle bestehen. Diese identischen B-Zellen können anhand ihrer gebildeten monoklonalen Antikörper anschließend so ausgewählt werden, dass diese ein Protein von Interesse erkennen - in unserem Falle das Erdnuss-Allergen Ara h 2.

2. B-Zellen besitzen auf ihrer Außenhülle einen Rezeptor, der identisch zu dem Antikörper ist, der durch die B-Zelle hergestellt wird. Das bedeutet, dass dieser Rezeptor die gleiche DNA-Information trägt und das gleiche Bindungsverhalten wie die von diesen B-Zellen hergestellten Antikörper aufweist. Durch Auswahl dieser bestimmten B-Zelle konnte gezeigt werden, dass die DNA-Informationen aus dieser B-Zelle verwendet werden können, um damit monoklonale Antikörper herzustellen.

Beide Methoden besitzen Vor- und Nachteile. Das erste Verfahren hat den Nachteil, dass damit nur eine begrenzte Anzahl verschiedener monoklonaler Antikörper hergestellt werden kann. Dennoch ist dieses Verfahren kostengünstiger im Vergleich zum zweiten Verfahren, das den Vorteil hat, für eine größere Auswahl monoklonaler Antikörper verwendet werden zu können. Für die Patientenstudie in **Kapitel 9** wurde Methode 2 verwendet. Der Aufbau eines Antikörpers ist in **Box 6** dargestellt.

Box 6: Aufbau eines Antikörpers

Ein Antikörper besteht aus zwei schweren und zwei leichten Ketten. Diese Ketten umfassen jeweils einen konstanten und einen variablen Teil. Der variable Teil bestimmt, welches Protein/Allergen erkannt wird. Der schwere Teil hingegen ist dafür verantwortlich, wie der Körper auf die Erkennung eines Proteins reagiert.



Der variable Teil besteht aus verschiedenen Abschnitten, der durch 3 Gene codiert wird: V-, D- und J-Genen. Während der Entwicklung einer B-Zelle werden diese Gene zufällig aus einem Pool ausgewählt (65 V-Gene, 27 D-Gene, 6 J-Gene) und miteinander kombiniert. Dies führt zu vielen möglichen Kombinationen, die die Erkennung von nahezu unendlich vielen, unterschiedlichen Proteinen gewährleisten. Für die Proteinerkennung ist der in Aminosäuren übersetzte Bereich zwischen dem Ende des V-Gens und dem Beginn des J-Gens am wichtigsten.

In **Kapitel 9** wurden B-Zellen aus dem Blut von Patienten mit Erdnuss-Allergie und solchen mit Erdnuss-Toleranz isoliert, die spezifisches IgE gegen die Erdnuss-Allergene

Ara h 2 und 6 produzierten. Diese B-Zellen wurden verwendet, um monoklonale Antikörper herzustellen und um zu untersuchen, ob mit Hilfe dieser monoklonalen Antikörper die Diagnostik von Erdnuss-Allergien verbessert werden kann. In Analysen der DNA-Informationen aus diesen B-Zellen wurden Unterschiede zwischen erdnussallergischen und -toleranten Patienten festgestellt:

- Der variable Teil der Antikörper von Allergikern besitzt viel häufiger V-Gene (**Box 6**), die einer bestimmten Gruppe angehören.
- Die Region, die nach der Übersetzung in Aminosäuren für die Bindung an das Allergen am wichtigsten ist, weist teilweise einzigartige DNA-Informationen auf, wenn der Antikörper von einem Erdnuss-Allergiker stammt.

Diese Ergebnisse zeigen, dass DNA-Muster existieren, die sich zwischen Erdnuss-Allergikern und toleranten Patienten, die Erdnüsse essen können, unterscheiden. Wenn eine Methode, die technisch einfacher ist als die hier verwendete entwickelt würde, könnten die zuvor genannten Ergebnisse zur Entwicklung eines Bluttests beitragen, der besser zwischen Patienten mit und ohne Erdnuss-Allergie unterscheidet.

Die Ergebnisse dieser Arbeit bieten eine hochwertige Grundlage für die Entwicklung verbesserter und neuer Bluttests, mit denen falsch-negative und falsch-positive Testergebnisse reduziert oder sogar verhindert werden können. Je nach Allergen gibt es verschiedene Möglichkeiten, um dieses Ziel zu erreichen wie zum Beispiel die Bestimmung von spezifischem IgE gegen lineare Epitope für das Hühnerei-Allergen Gal d 1 oder die Auswertung von Antikörper-DNA-Mustern für die Erdnuss-Allergene Ara h 2 und 6. Unabhängig von der Strategie, die letztendlich für ein bestimmtes Allergen angewendet wird bzw. werden sollte, können Bluttest zuverlässiger werden. Dies kann die Anzahl der erforderlichen Nahrungsmittelprovokationen in der Zukunft verringern. Darüber hinaus ist es wahrscheinlich, dass durch die Bestimmung von spezifischen IgE gegen das Macadamianuss-Allergen sowie gegen die linearen Epitope von Gal d 1 Patienten mit einem erhöhten Risiko auf eine schwere allergische Reaktion identifiziert werden können.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Appendices

Abbreviations

aa	amino acid
AIT	Allergen immunotherapy
AMV	Avian myeloblastosis virus
AP	Alkaline phosphatase
AUC	Area under the curve
BAT	Basophil activation test
CCDs	Cross-reactive carbohydrate determinants
CDR	Complementarity-determining region
CMA	Cow's milk allergy
CME	Cow's milk extract
CRD	Component resolved diagnostics
CPE	Crude peanut extract
DAO	Diamine oxidase
DBPCFC	Double-blind placebo-controlled food challenge
DOPE	Discrete Optimized Protein Energy
EBV	Epstein Barr Virus
EL	EUROLINE
FCS	Fetal calf serum
FR	Frame work
HEA	Hen's egg allergy
HEK	Human embryonic kidney cells
HIC	Hydrophobic interaction chromatography
i-allergen	improved allergen
IEF	Isoelectric focussing
IMGT	International ImMunoGeneTics information system
IPG strip	Immobilised pH gradient strip
IQR	Interquartile range
LCLs	Lymphoblastoid Cell Lines
mAb	monoclonal antibody
MA	Macadamia nut allergic
MFI	Mean fluorescence intensity

MT	Macadamia nut tolerant
NA	Nut allergic
NKT cells	Natural killer T cells
nsLTP	non-specific Lipid transfer protein
NT	Nut tolerant
OAPs	Oil-body associated proteins
OAS	Oral allergy syndrome
OD	Optical density
PAF	Platelet-activating factor
PAF-AH	Platelet-activating factor acetylhydrolase
PBMCs	Peripheral blood mononuclear cells
PC	Positive control
PHYRE	Protein Homology/AnalogY Recognition Engine
pI	isoelectric point
PMF	Peptide mass fingerprinting
PNK	Polynucleotide Kinase
RU	Response unit
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHIP-1	Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1
SHM	Somatic hypermutation
sIgE	specific IgE
sIgG	specific IgG
SNP	Single nucleotide polymorphism
SPT	Skin prick test
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TMHMM	Transmembrane helix prediction hidden Markov model
UMCU	University Medical Center Utrecht
VDJ	recombined variable, diversity, joining genes
VLAP	Vicilin-like anti-microbial peptides
WB	Western Blot

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Curriculum vitae

Anna Ehlers was born on the 11^{th} of December 1990 in Itzehoe, Germany. She graduated from High School in 2009 at the Alexander-van-Humboldt-College in Neumünster. In the same year, she started the bachelor program 'Chemical and Environmental Engineering' at the University of Applied Sciences in Lübeck. After graduating with her bachelor thesis entitled 'Substantial utilization of cacao pod husks' in 2013, she started the Master program 'Biochemical Engineering' at the University of Applied Sciences in Lübeck. She graduated cum laude in 2015 after completing her master thesis entitled 'Method development for the isolation of oil body-associated proteins and their characterisation', which she conducted at EUROIMMUN AG in Lübeck. Directly after her graduation, she started as a scientist at EUROIMMUN AG working on the isolation and production of native and recombinant proteins for *in vitro* allergy diagnostics.
In October 2016, Anna moved to the Netherlands and started as a PhD student at the University Medical Center Utrecht under the supervision of Prof. dr. André Knulst and dr. Henny Otten. During her PhD, she evaluated different possibilities to improve *in vitro* food allergy diagnostics in order to prevent test outcomes contradicting clinical diagnosis. The results are presented and discussed in this thesis.