Detection of the Peanut Allergens Ara h 2 and Ara h 6 in Human Breast Milk: Development of 2 Sensitive and Specific Sandwich ELISA Assays

Frauke Schocker^a Alexandra Scharf^a Skadi Kull^a Uta Jappe^{a, b}

^aDivision of Clinical and Molecular Allergology, Research Center Borstel, Priority Research Area Asthma and Allergy, Airway Research Center North (ARCN), German Center for Lung Research (DZL), Borstel, and ^bInterdisciplinary Allergy Outpatient Clinic, Department of Internal Medicine, University of Lübeck, Lübeck, Germany

Keywords

Peanut allergy · Ara h 2 · Ara h 6 · Sandwich ELISA · Breast milk

Abstract

Background: Little is known about breast milk as a vehicle for tolerance development or sensitization to peanuts very early in life. Thus, well-characterized and highly sensitive detection systems for the reliable determination of peanut allergens in breast milk are mandatory. **Methods:** For the quantification of the marker allergens Ara h 2 and Ara h 6 in the low nanogram per milliliter range in breast milk samples of a German cohort, sensitive and highly specific sandwich ELISAs were optimized and validated. **Results:** The Ara h 2 ELISA revealed a limit of detection (LOD) of 1.3 ng Ara h 2/mL and a quantification range of 2.3–250 ng/mL, the Ara h 6 ELISA showed an LOD of 0.7 ng/mL and a working range of 1.1–14.4 ng/mL. The assays showed no relevant cross-reactivity against other potentially cross-reactive legume, seed, and tree nut extracts (<0.01%, except for Ara h 1 in the Ara h 2 ELISA <0.1%). Ara h 2 was detectable in breast milk samples from 14/40 (35%) of the participants in concentrations from 2.3 to 184 ng/mL, Ara h 6 appeared in 9/40 (22.5%) of the lactating

mothers between 1.1 and 9.7 ng/mL, and 1 highly positive sample with 79 ng/mL. Both allergens appeared at the same time points, but Ara h 6 in lower concentrations than Ara h 2. **Conclusions:** Sensitive and specific diagnostic tools for the determination of Ara h 2 and Ara h 6 in human breast milk were established. The kinetics of secreted Ara h 2 and Ara h 6 seem to be similar but with a difference in concentration. Follow-up investigations on their tolerogenic or sensitizing properties in breast milk become now accessible.

© 2017 S. Karger AG, Basel

Introduction

It is a well-known fact that breast milk is not allergen free, which is of considerable importance with regard to ongoing investigations on sensitization routes, the initiation of food allergy, the time points relevant for tolerance dynamics and, therefore, for preventive measures. In human breast milk the presence of β -lactoglobulin from cow's milk, ovalbumin and ovomucoid from eggs, and

F.S. und A.S. contributed equally to this work.

gliadin from wheat has been demonstrated in previous studies [1–5]. In terms of peanut allergy, recent data from the German Registry of Anaphylaxis revealed that peanut allergy is no longer a life-threatening problem of the USA only, but is of growing significance in German-speaking countries as well [6]. Very small children are primarily affected by severe peanut allergy and at risk of developing symptoms immediately at first consumption [7, 8]. Therefore, it is of increasing importance to focus on potential marker allergens to understand the mechanistic processes. Currently, the storage protein Ara h 2 is acknowledged as the most relevant marker allergen, associated with severe, sometimes fatal reactions [9–12]. Likewise, Ara h 6 is considered to predict peanut allergy with similar accuracy as both proteins belong to the 2S albumins with 59% sequence homology [13–15].

To date, statistics relating to peanut allergy caused by breastfeeding are lacking. However, recent studies suggest that early introduction to peanuts via breast milk may increase the chance for the development of tolerance to peanuts [16]. In the light of these observations quantification tools for Ara h 2 and Ara h 6 in breast milk are needed to evaluate whether small amounts of these peanut allergens may either lead to sensitization or train the immune system of the breastfed baby.

Vadas et al. [17] presented preliminary data on 23 healthy, lactating women in Canada and showed that whole peanut protein extract was detected in 11 of 23 subjects (48%) at a concentration ranging from 120 to 430 ng/mL within 1–3 h. Bernard et al. [18] investigated Ara h 6 in samples of 2 lactating women shortly after peanut consumption, with concentrations in the picogram/milliliter (pg/mL) range but long-lasting excretion up to 26 h after peanut ingestion. To the best of our knowledge, we have recruited the largest study population of lactating women in Germany since 2004 that has been assessed prospectively ($n = 40$) for the transfer of peanut proteins into breast milk after consuming 100 g of dry roasted peanuts after an otherwise legume-free diet. In our previous report from 2016 we were able to show by means of various immunoassays (2D immunoblotting, affinity enrichment followed by a LC/MS-MS-based detection and a competitive inhibition ELISA for the detection of Ara h 2 and its digestion-resistant peptides [DRP-Ara h 2]) that Ara h 2 is transferred into breast milk in relevant concentrations [19]. The aim of the present investigation was to develop a more sensitive and highly specific detection method for the quantification of Ara h 2 and simultaneously of another clinically relevant allergen, namely Ara h 6, in the complex lipid-rich breast milk matrix.

Materials and Methods

Antibody Production, Purification, and Labeling Ara h 2 ELISA

For the antibody screening, 6 in-house or commercially available polyclonal (pAb) and monoclonal antibodies (mAbs) were used. In terms of the in-house antibody production the antisera were raised against Ara h 2 or peanut extract. A polyclonal rabbit antiserum (pAb-E6/7) was raised against recombinant (r)Ara h 2, which reacted with peptides 6 and 7 of Ara h 2 (⁵⁹RDYSPSPQD-PYSPS⁷²) as described by Stanley et al. [20, 21]. A monospecific antiserum against the IgE-reactive epitope 3 of Ara h 2 (pAb-E3) was produced as previously described [20]. Two polyclonal antisera were raised against in-house peanut extract (pAb-PE) and against peanut proteins below 20 kDa (pAb-<20). The immunization of the animals was performed in strict accordance with the recommendations in Guide for Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the legal requirements of the state of Schleswig-Holstein, Germany.

An unlabeled and a biotinylated Ara h 2-specific mAb (MA-1C4 and BI-1C4) and an unlabeled Ara h 2-specific polyclonal antibody (pAb; PA-AH2) raised against natural Ara h 2 were purchased from INDOOR Biotechnologies Ltd (Cardiff, UK). Two of the Ara h 2-specific antibodies (pAb-E6/7 and BI-1C4) were used as detection antibodies in the antibody screening. The crude antiserum pAb-E6/7 was purified by affinity chromatography using Protein G HP Spin TrapTM columns from GE Healthcare (Frankfurt, Germany) following the manufacturer's instructions (detailed in the online suppl. material; for all online suppl. material, see www.karger.com/doi/10.1159/000479388). Purified pAb-E6/7 was biotinylated with EZ-Link Sulfo-NHS-Biotinylation Kit (PIERCE, Thermo Fisher, Darmstadt, Germany) following the manufacturer's protocol.

Ara h 6 ELISA

A polyclonal rabbit antiserum against rAra h 6 (pAb-Ara h 6) [13] and a commercially available mAb against Ara h 6 (MA-3B8; INDOOR Biotechnologies Ltd) were used for the antibody matching. For subsequent experiments, the antiserum against rAra h 6 was purified by affinity chromatography and biotinylated (as detailed in the online suppl. material).

Ara h 2 and Ara h 6 Standard. Natural Ara h 2 (NA-AH2-1) and Ara h 6 (NA-AH6-1) were purchased from INDOOR Biotechnologies Ltd and used for ELISA calibration.

Antibody Screening. For the Ara h 2 ELISA, the in-house (pAb-E6/7, pAb-E3, pAb-<20, pAb-PE) or commercially available (PA-AH2, MA-1C4) polyclonal and monoclonal antisera and Ara h 2 standard were used as reagents for the antibody screening. For the Ara h 6 ELISA the antiserum against rAra h 6 (pAb-Ara h 6) and mAb MA-3B8 were chosen. For the evaluation of the data of the antibody screening, the OD450 ratios (signal/blank ratios) of the positive (100 ng/mL Ara h 2 and Ara h 6, respectively) and negative controls (0 ng/mL Ara h 2 and Ara h 6) were calculated for each antibody combination (as detailed in the online suppl. material).

Breast Milk Samples. A German cohort of 40 lactating women without peanut allergy was established [19]. The study was approved by the Ethics Committee of Hamburg and Schleswig-Holstein, Germany (approval No. OB-018/04 and 08-222, respectively). Briefly, after a 24-h legume-free diet, breast milk samples were

collected before (as the basal sample) and after the ingestion of 100 g of dry roasted peanuts. Breast milk samples from subjects 1–15 were collected 1 h after peanut consumption. In the course of the study, breast milk samples were taken at further intervals. We gradually adapted the required collection time resulting in different times of sampling (in 1 subject at 1 and 3 h, in 7 subjects at 2 and 4 h, in 10 subjects at 4, 8, and 12 h, in 10 subjects at 1, 2, 4, 8, and 12 h, as detailed in online suppl. Table 1) [19]. Breast milk samples from subjects 1, 5, and 24 were obtained from 1 woman providing samples in 3 different lactation periods. Subjects 20 and 27 provided 3 sets of breast milk samples, and subject 21 provided 2 sets. Breast milk samples from subjects 34 and 35 were collected from 1 woman within 1 lactation period after the ingestion of 100 g of peanut butter (identified as No. 34) and of 100 g of roasted peanuts (identified as No. 35). Written informed consent was obtained from all of the subjects included in this study. The breast milk samples were frozen and stored until use. Before analysis, the samples were centrifuged at 8,000 g for 15 min to separate fat and cellular debris. The samples were diluted 10:1 (v/v; 90 µL sample + 10 µL sample buffer) with sample buffer (PBS, pH 7.4 containing 1% [v/v] Tween-20 and 30% [w/v] polyethylene glycol [PEG] 6000) for the Ara h 2 assay. For the Ara h 6 assay, the samples were diluted 1:2 (v/v; 50 µL sample + 50 µL sample buffer) with sample buffer (PBS, pH 7.4 containing 1% [v/v] Tween-20 and 6% [w/v] PEG 6000). PEG was used to improve the sensitivity of the assay, as shown by Salonen and Vaheri [22], who described the enhancing effect of PEG on the antibody-antigen binding in an ELISA. All samples from 1 subject were analyzed on the same plate and analyzed in triplicate. For spiking experiments and ELISA calibration, negative control breast milk was collected from women without the ingestion of peanuts during lactation.

Sandwich ELISA Procedure

Ara h 2 ELISA

Polystyrene microtiter plates (MaxiSorp F96, Nunc, Roskilde, Denmark) were coated with 100 µL/well of the Ara h 2-specific mAb MA-1C4 in carbonate-bicarbonate buffer (4 µg/mL, pH 9.6) overnight at 4°C. After rinsing with 250 µL/well washing solution (PBS, pH 7.4, containing 0.05% [v/v] Tween-20), the remaining binding sites were blocked with 200 µL/well of blocking solution (PBS pH 7.4 containing 0.05% [v/v] Tween-20 and 1% BSA) for 1 h at room temperature (RT). Ara h 2 standards were spiked using a serial dilution of NA-AH2-1 in negative control breast milk. After gently shaking (300 rpm) for 1 h at RT, the standards and samples were centrifuged for 15 min at 8,000 g to remove fat and cellular debris. Centrifuged standards and samples were diluted 10:1 (v/v; 90 µL sample + 10 µL sample buffer) in sample buffer and incubated on a shaker (300 rpm) for 15 min at RT. One hundred microliters of the standard curve samples (ranging from 2.5 to 250 ng/mL) and breast milk samples were incubated on the ELISA plates for 2 h at RT. The bound Ara h 2 was incubated with 100 µL/well of biotinylated pAb-E6/7 in blocking solution (2.5 µg/mL) for 2 h at RT, followed by 100 µL/well of POD-streptavidin conjugate (1:20,000 in blocking solution) for 1 h at RT. Subsequently, an enzymatic colorimetric reaction was started by adding 100 µL/well of substrate solution (TMB; Sigma-Aldrich). The reaction was stopped with 100 µL/well of 1 M H₂SO₄ after 10–15 min. Absorbance was read at 450 nm with 620 nm as the reference wavelength using a Tecan Infinite 200 microplate reader (Tecan, Männedorf, Switzerland).

Ara h 6 ELISA

The Ara h 6 ELISA was developed following the design of the Ara h 2 ELISA with the following modifications. The mAb MA-3B8 was coated at 2 µg/mL in carbonate-bicarbonate buffer (pH 9.6), the biotinylated pAb-Ara h 6 was used at a 1:5,000 dilution in blocking solution. Poly HRP-streptavidin conjugate (1:10,000 in blocking solution) was applied with a 1-h incubation at RT.

Evaluation and Statistics. For curve-fitting analysis and statistical analysis (Mann-Whitney U test) Prism graphics software 6.03 (GraphPad Prism Inc., San Diego, CA, USA) was used.

Validation of the ELISAs: Specificity, Limit of Detection and Limit of Quantification. Validation of the Ara h 2 and Ara h 6 sandwich ELISA was carried out following the recommendations of the European DIN EN 15633-1:2009 [23], DIN EN 15842:2010 [24], and the international ISO 11843-5:2008 [25] for food allergen ELISAs. The specificity of the assay was determined by cross-reactivity studies using extracts from legumes (yellow and blue lupine, soy bean), tree nuts (hazelnut, brazil nut, almond, cashew), seeds (pine nut), cereals (wheat), and different single food allergens. The food-stuffs were obtained from local stores in Schleswig-Holstein, Germany, and allergen extracts were prepared as previously described [26].

Single food allergens, including the peanut allergens (n)Ara h 1 (NA-AH1-1), (n)Ara h 2 (NA-AH2-1), (n)Ara h 6 (NA-AH6-1), and alpha-amylase inhibitor from wheat (A1520) were purchased from INDOOR Biotechnologies Ltd and Sigma-Aldrich, respectively. Prior to analysis, lyophilized extracts and alpha-amylase inhibitor from wheat were dissolved in PEG buffer (PBS, pH 7.4, containing 0.1% [v/v] Tween-20 and 3% [w/v] PEG 6000). Extracts and single allergens were diluted in PEG buffer (at a concentration of 20 µg/mL) and analyzed in the Ara h 2 ELISA with standard solutions of NA-AH2-1 ranging from 2.5 to 250 ng/mL in PEG buffer and in the Ara h 6 ELISA with standard solutions of NA-AH6-1 ranging from 0.16 to 160 ng/mL, respectively. Subsequent validation steps were performed in negative control breast milk. Calculated cross-reactivities referred to 100% of the Ara h 2 and Ara h 6 standards.

The precision of the Ara h 2 assay was assessed by using negative control breast milk containing an Ara h 2 concentration of 20 ng/mL after spiking with standard material ST-AH2-1. For the Ara h 6 ELISA, a spike and recovery experiment was performed likewise, applying a 20-ng/mL Ara h 6 standard.

A precision profile according to the respective ISO (ISO 11843-5:2008) [25] using 5 standard curves on 1 plate was performed to determine the limit of detection (LOD) and to define the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). The cut-off level of 30% coefficient of variation (CV) for LOD was taken from ISO, and 15% CV for the LLOQ and ULOQ were considered suitable for the intended use.

Results

Antibody Screening for the Sandwich ELISAs

Ara h 2 ELISA

In order to develop a sensitive and specific sandwich ELISA against Ara h 2, an antibody screening with 6 Ara h 2-specific or peanut-specific antibodies was per-

Fig. 1. a Antibody matching 1 for Ara h 2 ELISA with BI-1C4 as the detection antibody. BI-1C4, biotinylated Ara h 2-specific monoclonal mouse antibody (Indoor Biotechnologies); pAb-E6/7, polyclonal rabbit antiserum specific against epitopes 6 and 7 of rAra h 2; pAb-E3, polyclonal rabbit antiserum monospecific against epitope 3 of Ara h 2; pAb-PE, polyclonal rabbit antiserum raised against in-house peanut extract; pAb-<20, polyclonal rabbit antiserum raised against peanut proteins below 20 kDa. **b** Antibody matching 2 for Ara h 2 ELISA with pAb-E6/7 as the detection antibody. pAb-E6/7, biotinylated/unlabeled polyclonal rabbit antiserum specific against epitopes 6 and 7 of rAra h 2; pAb-E3, polyclonal rabbit antiserum monospecific against epitope 3 of Ara h 2; pAb-<20, polyclonal rabbit antiserum raised against peanut proteins below 20 kDa; PA-AH2, Ara h 2-specific polyclonal rabbit antiserum (Indoor Biotechnologies); MA-1C4, Ara h 2-specific monoclonal mouse antibody (Indoor Biotechnologies). **c** Antibody matching for Ara h 6 ELISA with pAb-Ara h 6 as the detection antibody. pAb-Ara h 6, polyclonal rabbit antiserum against rAra h 6; MA-3B8, Ara h 6-specific monoclonal mouse antibody (Indoor Biotechnologies).

formed. The highest OD450 ratios (signal/blank ratio) of the positive (100 ng/mL Ara h 2) and negative controls were obtained using the mAb MA-1C4 against Ara h 2 and the polyclonal rabbit antibody pAb-E6/7 against rAra h 2 (Fig. 1a, b). With MA-1C4 as the capture antibody and the biotinylated pAb-E6/7 as the detection antibody signal/blank ratios were considerably higher than vice versa (Fig. 1b). Consequently, this combination was selected to develop the ELISA.

Ara h 6 ELISA

For the Ara h 6 ELISA the mAb MA-3B8 and the pAb-Ara h 6 were matched, again showing the highest signal/blank ratios with the mAb as the capture antibody and

the polyclonal antiserum as the detection antibody (Fig. 1c).

Antibody Titration. The developed Ara h 2 ELISA was optimized by titrating capture antibody, biotinylated detection antibody, and enzyme conjugate (POD-streptavidin conjugate) against each other. Dilutions of 1:500 for capture antibody, 1:400 for detection antibody, and 1:20,000 (POD-streptavidin conjugate) were chosen, resulting in low background values and high sensitivity. In terms of the Ara h 6 ELISA, the capture antibody was diluted 1:1,000, the biotinylated detection antibody 1:5,000, and the poly-HRP-streptavidin conjugate was applied in a 1:10,000 dilution (data not shown).

Fig. 2. Precision profile of the Ara h 2 (**a**) and Ara h 6 (**b**) sandwich ELISA. Representative calibration curve (black line) with the precision profile (gray line). The cut-off level of 30% CV for LOD and 15% CV for LLOQ and ULOQ (measurement range) are shown as black dotted lines.

Specificity of the Sandwich ELISA

Ara h 2 ELISA

The specificity of the Ara h 2 ELISA was investigated with 12 food extracts and single allergens. No relevant cross-reactivity against Ara h 1 (<0.1%) and Ara h 6 as well as other potentially cross-reactive tested extracts from legume, seed, and tree nuts (all <0.01%) was found (online suppl. Table 2).

Ara h 6 ELISA

The Ara h 6 ELISA did not show any cross-reactivity for any of the tested single allergens and food extracts either (<0.01%; online suppl. Table 2).

Sensitivity

LOD and Limit of Quantification. To determine the LOD and the working range (LLOQ/ULOQ) of the ELISAs, precision profiles were performed according to ISO 11843-5:2008 [25]. The cut-off level for LOD and the working range (LLOQ/ULOQ) were defined with 30% CV and 15% CV, respectively. As shown in the precision profile (Fig. 2a), the calculated LOD of the Ara h 2 sandwich ELISA was determined as 1.3 ng/mL and the LLOQ as 2.3 ng/mL Ara h 2 standard in breast milk. The ULOQ was set to 250 ng/mL following the highest Ara h 2 standard. Therefore, the quantification range of the ELISA was between 2.3 and 250 ng/mL Ara h 2 in breast milk. The Ara h 6 ELISA revealed an LOD of 0.7 ng/mL; the working range (LLOQ/ULOQ) was determined between 1.1 and 14.4 ng/mL with a CV of 15% (Fig. 2b).

Detection of Ara h 2 and Ara h 6 in Human Breast Milk

In the course of our breast milk study, we modified our study design and collected samples at different time points after peanut ingestion (detailed in online suppl. Table 1). Therefore, the sample collection was not homogenous and samples at collection time points from 1 to 12 h were not available for every subject.

Basal samples and the collected breast milk samples from the available time points between 1 and 12 h after ingestion of 100 g of dry roasted peanuts were analyzed in the developed Ara h 2 and Ara h 6 sandwich ELISAs. The positive results of the investigated breast milk samples of 40 lactating women are depicted in online supplementary Table 1, showing the measured Ara h 2 and Ara h 6 concentration of each breast milk sample of the participants, and Figure 3a and b, comprising the Ara h 2 and Ara h 6 concentrations of the study group. Ara h 2 was detected in breast milk samples from 14/40 (35%) of the subjects, ranging from 2.3 to 184 ng/mL. In 11 of the subjects, Ara h 2 was already detectable within 1–4 h, while only 2 subjects showed an exclusive delayed transfer of Ara h 2 within 8–12 h after peanut consumption. As shown in Figure 3a, Ara h 2 concentrations were (slightly) not significantly higher after 1 h ($p = 0.051$), significantly higher after 2 h ($p = 0.002$), and after 4 h ($p = 0.029$), respectively, compared to the basal samples ($t = 0$) with a maximal Ara h 2 transfer in breast milk after 2 h.

Ara h 6 was detectable in breast milk samples of 9/40 (22.5%) of the subjects in concentrations between 1.1 and 79 ng/mL. In 6 subjects, Ara h 2 and Ara h 6 were simul-

Fig. 3. Ara h 2 and Ara h 6 concentrations in breast milk samples of the German study group measured by the developed Ara h 2 and Ara h 6 ELISA. **a** Concentration of Ara h 2 in human breast milk before and after different time points following peanut ingestion. **b** Concentration of Ara h 6 in human breast milk before and after

different time points following peanut ingestion. The Mann-Whitney U test was applied to compare the Ara h 2 and Ara 6 concentrations after different time points. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

taneously transferred at the same time points (subject No. 12, 14, 16, 20, 22, 23) within the quantification range. In 4 sample sets (No. 10, 18, 32, 36) Ara h 2 was detectable in the quantification range, whereas Ara h 6 was below the LLOQ, which is between the LOD and the LLOQ. Moreover, in subjects 11, 29, and 41, Ara h 2 could be measured within the quantification range of >2.3 ng/mL, whereas Ara h 6 was negative ($<LOD$). Vice versa, Ara h 6 concentrations greater than an LLOQ of 1.1 ng/mL could be measured (ID 21.1, 38, 39), while Ara h 2 was only detectable between LOD and LLOQ. Statistically, Ara h 6 appeared in the breast milk samples in a significantly higher concentration after 1 h ($p = 0.006$) and 2 h ($p = 0.001$) compared to the basal samples ($t = 0$). Again, the highest Ara h 6 values were analyzed after 2 h (Fig. 3b).

Discussion

In this study, 2 highly sensitive and specific sandwich ELISAs were developed in the matrix of breast milk detecting the most potent peanut allergens Ara h 2 and Ara h 6 in low concentrations down to 2.3 ng/mL and 1.1 ng/mL, respectively. In our recently published study, we were able to show the transfer of Ara h 2 into breast milk following the consumption of a defined amount of peanuts with different immunological methods. Based on a semiquantitative analysis via an inhibition ELISA,

Ara h 2 and its digestion-resistant peptides appeared individually excreted in different concentrations in breast milk [19]. However, here we were interested in an assay to detect Ara h 2 in a range below 40 ng/mL of breast milk as an analytical tool, and simultaneously to investigate the transfer of another important peanut allergen, namely Ara h 6, within the same study group by a sensitive detection method.

For the development and optimization of the sandwich ELISAs, an mAb against nAra h 2 (MA-1C4) and a biotin-conjugated rabbit pAb against Ara h 2 (pAb-E6/7) on the one hand and an mAb against nAra h 6 (MA-3B8) and a biotin-conjugated rabbit pAb against Ara h 6 (pAb-Ara h 6) on the other hand, were chosen as the catcher and detection antibodies. We were able to establish 2 ELISA systems for the reliable determination of Ara h 2 and Ara h 6 in the complex breast milk matrix without any relevant cross-reactivity against single allergens and extracts which have been selected for structural similarities, sequence identities, and a phylogenetic relationship. The included allergens and allergen sources for cross-reactivity studies were chosen according to their structure similarities with Ara h 2 and Ara h 6, which are the cross-reactive counterparts Ara h 6 and Ara h 2, respectively, the α -amylase-inhibitor [27], and the peanut allergen Ara h 1, which was described as being IgE cross-reactive because of high sequence similarity on surface-exposed loops [28]. However, none of the investigated allergens were

detected by the newly developed Ara h 2 and Ara h 6 sandwich ELISAs. The food allergen sources of almond, Brazil nut, lupine, and soybean were selected because of their high content of 2S albumins, which may be the cause of cross-reactivity. Furthermore, the lupine proteins α - and δ -conglutin were shown to be IgE cross-reactive [29]. These extracts were all negative in the Ara h 2 and Ara h 6 sandwich ELISAs.

By using a precision profile constructed according to ISO 11843-5:2008 [25], an LOD of 1.3 ng Ara h 2/mL breast milk and an LLOQ of 2.3 ng Ara h 2/mL breast milk were obtained. This means that the newly developed ELISA is superior in its sensitivity compared to the recently described inhibition ELISA detecting Ara h 2 and its digestion-resistant peptides with an LOD <40 ng/mL breast milk. In terms of the Ara h 6 ELISA, we also succeeded in establishing a very sensitive assay with an LOD of 0.7 ng Ara h 6/mL and an LLOQ of 1.1 ng Ara h 6/mL breast milk with a CV of 15%. Important factors that are responsible for the increased sensitivity of the Ara h 2 and Ara h 6 ELISAs are the optimal combination of the capture and detection antibody in a sandwich ELISA format and the signal amplification via the biotin/streptavidin detection system.

When comparing ELISA assays available for the detection of peanut proteins it becomes evident that they differ in their ability to recover peanut proteins in terms of their specificity, LOD, and quantification range, as summarized by Jayasena et al. [30]. Ara h 2 and Ara h 6 have been shown to be hardly recognized by these assays in contrast to Ara h 3, which was the most recognized allergen. Likewise, it has been described that the recovery of peanut allergens is strongly influenced by the matrix. Apart from our own study investigation on the passage of Ara h 2 into breast milk [19], to our knowledge there is only the French study on 2 lactating mothers that detected Ara h 6 concentrations in the pg/mL range [18]. However, to the best of our knowledge, we are the first group to study the ability of 2 single, clinically relevant peanut allergens to pass into human breast milk. Again, the transfer dynamics of the peanut allergens appear to be individually regulated, which is in accordance with food allergens ingested and excreted into breast milk following different frequencies and concentrations [summarized in 19]. Interestingly, both 2S albumins suggest a similar secretion behavior in its concentrations and time kinetics in most of the positive ELISA samples, meaning that a high Ara h 2 concentration in a breast milk sample corresponds to a relatively high Ara h 6 content, too, as measured by the altogether low concentrations of the Ara h 6 transfer into breast

milk. However, we were also able to see differences in that Ara h 2 could be measured within the quantification range of the assay, whereas Ara h 6 is either transferred in concentrations below the LLOQ or below the LOD of the Ara h 6 assay. Accordingly, when Ara h 6 was detectable within the quantification range, Ara h 2 could also be found to be present at very low concentrations (<LLOQ). Statistically, we could show that Ara h 2 was transferred at higher concentrations after 2 and 4 h in comparison to the Ara h 2 amount of the basal sample. Likewise, Ara h 6 was secreted in significantly higher concentrations after 1 and 2 h. With our data, we could show that the time points of appearance of both allergens correspond to each other. It was surprising that the Ara h 2 content in positive breast milk samples was higher than the Ara h 6 content of the same samples, often close to the LOD. According to the literature [summarized in 30], Ara h 2 and Ara h 6 are described to be present in approximately the same concentration of 6–9% in peanuts. Also, both allergens are described to survive digestion as relatively large digestion-resistant peptides [31] that can be taken up into the circulation. Therefore, one would expect that both 2S albumins are transferred in similar concentrations if following the same absorption mechanisms and secreted via the same pathways. On the molecular level, data assessing the interaction between peanut allergens and epithelial barriers are rare. Previous investigations using a Caco-2 human cell culture model of the intestinal epithelium showed that the major allergens Ara h 1 and Ara h 2, as well as Ara h 3 and Ara h 6, can cross via these cells [32]. Price et al. [33] recently specified the endocytic mechanisms by which Ara h 1 and Ara h 2 pass the Caco-2 cells.

However, no studies have been conducted on Ara h 2 in vivo which shed light on the passage of this allergen through the gastrointestinal tract, its appearance in the circulation, and transport to the body compartments including the mammary glands. In terms of Ara h 6, Bernard et al. [18] could show that Ara h 6 is excreted as free allergen and IgG-Ara h 6 and IgA-Ara h 6 immune complexes into breast milk after consuming 30 g of roasted peanuts. However, the concentrations of either free or complexed Ara h 6 in the pg/mL range up to 3.4 ng/mL come close to the concentrations we measured in our study group. JanssenDuijghuijsen et al. [34] showed that Ara h 6 appears in low concentrations (0.30 ± 0.16 ng/mL) in serum after the ingestion of 100 g of peanuts and that some individuals have IgG and IgG4, which inhibits the detection of this allergen in their ELISA system. In their study, they hypothesized that concentration ranges of food allergens in serum are detectable in lower concentrations than in breast

milk [34]. So far, according to our findings, evidence suggests that Ara h 6 is in fact secreted in higher concentrations than to be expected in serum. However, to assess this issue in more detail we would need serum samples of the included lactating women to compare the Ara h 6 concentration in the circulation and the breast milk samples, a methodological aspect that is an integral part of the subsequent investigation on the cohort.

The results of our study of Ara h 2 regarding its concentration in breast milk when compared to Ara h 6 are very important insofar as Ara h 2 has been shown to be a marker allergen for the severity of the clinical reaction to peanut. We therefore have not only chosen the relevant peanut allergen for our investigations, we are now able to investigate excretion dynamics and their impact on allergenicity.

In light of these interesting findings, subsequent studies within our cohort are under way to assess the passage of further peanut allergens with presumably different characteristics in their absorption into breast milk.

In the context of the allergenic potency of peanut allergens, Hourihane et al. [35] described that as little as 100 µg of peanut protein can elicit allergic reactions in peanut-allergic patients, but no precise information on threshold doses for single allergens exist, i.e., Ara h 2 or Ara h 6. However, the reliable detection of Ara h 2 and Ara h 6 at very low concentrations (ng/mL) in breast milk helps to address questions regarding their sensitizing or tolerogenic ability.

The development of the sensitive detection tools for Ara h 2 and Ara h 6 in breast milk are a prerequisite to performing follow-up investigations in terms of time kinetics and the potential transfer pathways of food allergens at low concentrations. Future studies can now be designed to further elucidate at which time points the presence of Ara h 2 and Ara h 6 in breast milk may be relevant for tolerance induction in the infant and, therefore, for the primary prevention of food allergy.

Acknowledgement

This paper represents in part the essential results of a master thesis performed by Alexandra Scharf, MSc. We thank Carolin Murawski for excellent and skillful technical assistance and the Research Center Borstel for financial support.

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

Part of this project was kindly supported by the German Research Foundation (DFG; SCHO 828/2-1). The work of A. Scharf was funded by the Research Center Borstel.

References

- Kilshaw PJ, Cant AJ: The passage of maternal dietary proteins into human breast milk. *Int Arch Allergy Appl Immunol* 1984;75:8–15.
- Fukushima Y, Kawata Y, Onda T, Kitagawa M: Consumption of cow's milk and egg by lactating women and the presence of beta-lactoglobulin and ovalbumin in breast milk. *Am J Clin Nutr* 1997;65:30–31.
- Cant A, Marsden RA, Kilshaw PJ: Egg and cow's milk hypersensitivity in exclusively breast fed infants with eczema, and detection of egg protein in breast milk. *Br Med J* 1985; 291:932–935.
- Troncone R, Scarcella A, Donatiello A, Cannataro P, Tarabuso A, Auricchio S: Passage of gliadin into human breast milk. *Acta Paediatr Scand* 1987;76:453–456.
- Chirido FG, Rumbo M, Añón MC, Fossati CA: Presence of high levels of non-degraded gliadin in breast milk from healthy mothers. *Scand J Gastroenterol* 1998;33:1186–1192.
- Worm M, Eckermann O, Dölle S, Aberer W, Beyer K, Hawranek T, Hompes S, Koehli A, Mahler V, Nemat K: Triggers and treatment of anaphylaxis: an analysis of 4,000 cases from Germany, Austria and Switzerland. *Dtsch Arztebl Int* 2014;111:367.
- Ewan PW: Clinical study of peanut and nut allergy in 62 consecutive patients: new features and associations. *BMJ* 1996;312:1074–1078.
- Hourihane JO, Dean TP, Warner JO: Peanut allergy in relation to heredity, maternal diet, and other atopic diseases: results of a questionnaire survey, skin prick testing and food challenges. *BMJ* 1996;313:518–521.
- Eller E, Bindslev-Jensen C: Clinical value of component-resolved diagnostics in peanut allergic patients. *Allergy* 2013;68:190–194.
- Klemans RJ, Otte D, Knol M, Knol E, Meijer Y, Gmelig-Meyling FH, Bruijnzeel-Koomen CA, Knulst AC, Pasmans SG: The diagnostic value of specific IgE to Ara h 2 to predict peanut allergy in children is comparable to a validated and updated diagnostic prediction model. *J Allergy Clin Immunol* 2013;131: 157–163.
- Dang TD, Tang M, Choo S, Licciardi PV, Koplin JJ, Martin PE, Tan T, Gurrin LC, Ponsonby AL, Tey D, Robinson M, Dharmage SC, Allen KJ: Increasing the accuracy of peanut allergy diagnosis by using Ara h 2. *J Allergy Clin Immunol* 2012;129:1056–1063.
- Martinet J, Couderc L, Renosi F, Bobée V, Marguet C, Boyer O: Diagnostic value of antigen-specific immunoglobulin E immunoassays against Ara h 2 and Ara h 8 peanut components in child food allergy. *Int Arch Allergy Immunol* 2016;169:216–222.

- 13 Suhr M, Wicklein D, Lepp U, Becker WM: Isolation and characterization of natural Ara h 6: evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat. *Mol Nutr Food Res* 2004;48:390–399.
- 14 Ackerbauer D, Bublin M, Radauer C, Varga E-M, Hafner C, Ebner C, Szépfalusi Z, Fröschl R, Hoffmann-Sommergruber K, Eiwegger T, Breiteneder H: Component-resolved IgE profiles in Austrian patients with a convincing history of peanut allergy. *Int Arch Allergy Immunol* 2015;166:13–24.
- 15 Koppelman SJ, de Jong GAH, Laaper-Ertmann M, Peeters KABM, Knulst AC, Hefle SL, Knol EF: Purification and immunoglobulin E-binding properties of peanut allergen Ara h 6: evidence for cross-reactivity with Ara h 2. *Clin Exp Allergy* 2005;35:490–497.
- 16 Young MC: Taking the leap earlier: the timing of tolerance. *Curr Opin Pediatr* 2015;27:736–740.
- 17 Vadas P, Wai Y, Burks W, Perelman B: Detection of peanut allergens in breast milk of lactating women. *JAMA* 2001;285:1746–1748.
- 18 Bernard H, Ah-Leung S, Drumare MF, Feraudet-Tarisse C, Verhasselt V, Wal JM, Crémillon C, Adel-Patient K: Peanut allergens are rapidly transferred in human breast milk and can prevent sensitization in mice. *Allergy* 2014;69:888–897.
- 19 Schocker F, Baumert J, Kull S, Petersen A, Becker WM, Jappe U: Prospective investigation on the transfer of Ara h 2, the most potent peanut allergen, in human breast milk of 32 volunteers. *Pediatr Allergy Immunol* 2016;27:348–355.
- 20 Gruber P, Suhr M, Frey A, Becker WM, Hoffmann T: Development of an epitope-specific analytical tool for the major peanut allergen Ara h 2 using a high-density multiple-antigenic peptide strategy. *Mol Nutr Food Res* 2004;48:449–458.
- 21 Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, Bannon GA: Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Arch Biochem Biophys* 1997;342:244–253.
- 22 Salonen EM, Vaheri A: Rapid solid-phase enzyme immunoassay for antibodies to viruses and other microbes: effects of polyethylene glycol. *J Immunol Methods* 1981;41:95–103.
- 23 British Standards Institution: Foodstuffs – Detection of Food Allergens by Immunological Methods – Part 1: General Considerations. London, BSI, 2009.
- 24 British Standards Institution: Detection of Food Allergens: General Considerations and Validation of Methods. London, BSI, 2010.
- 25 International Organization for Standardization: ISO 11843-5: Capability of Detection – Part 5: Methodology in the Linear and Non-Linear Calibration Cases. Geneva, ISO, 2008.
- 26 Schocker F, Lüttkopf D, Müller U, Thomas P, Vieths S, Becker WM: IgE binding to unique hazelnut allergens: identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions. *Eur J Nutr* 2000;39:172–180.
- 27 Mueller GA, Gosavi RA, Pomés A, Wünschmann S, Moon AF, London RE, Pedersen LC: Ara h 2: crystalstructure and IgE binding distinguish two subpopulations of peanut allergic patients by epitope diversity. *Allergy* 2011;66:878–885.
- 28 Bublin M, Kostadinova M, Radauer C, Hafner C, Szépfalusi Z, Varga EM, Maleki SJ, Hoffmann-Sommergruber K, Breiteneder H: IgE cross-reactivity between the major peanut allergen Ara h 2 and the nonhomologous allergens Ara h 1 and Ara h 3. *J Allergy Clin Immunol* 2013;132:118–124.
- 29 Dooper MM, Plassen C, Holden L, Lindvik H, Faeste CK: Immunoglobulin E cross-reactivity between lupine conglutins and peanut allergens in serum of lupine-allergic individuals. *J Invest Allergol Clin Immunol* 2009;19:283–291.
- 30 Jayasena S, Smits M, Fiechter D, de Jong A, Nordlee J, Baumert J, Taylor SL, Pieters RH, Koppelman SJ: Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. *J Agric Food Chem* 2015;63:1849–1855.
- 31 Koppelman SJ, Hefle SL, Taylor SL, de Jong GAH: Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. *Mol Nutr Food Res* 2010;54:1711–1721.
- 32 Price DB, Ackland ML, Burks W, Knight MI, Suphioglu C: Peanut allergens alter intestinal barrier permeability and tight junction localization in Caco-2 cell cultures. *Cell Physiol Biochem* 2014;33:1758–1777.
- 33 Price D, Ackland ML, Suphioglu C: Identifying epithelial endocytotic mechanisms of the peanut allergens Ara h 1 and Ara h 2. *Int Arch Allergy Immunol* 2017;172:106–115.
- 34 JanssenDuijghuijsen LM, Wichers HJ, van Norren K, Keijer J, Baumert JL, de Jong GA, Witkamp RF, Koppelman SJ: Detection of peanut allergen in human blood after consumption of peanuts is skewed by endogenous immunoglobulins. *Immunol Methods* 2017;440:52–57.
- 35 Hourihane JO, Kilburn SA, Nordlee JA, Hefle SL, Taylor SL, Warner JO: An evaluation of the sensitivity of subjects with peanut allergy to very low doses to peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol* 1997;100:596–600.