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Prospective investigation on the transfer of Ara h 2, the most potent peanut allergen, in human breast milk

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Running Title: Detection of Ara h 2 in human breast milk

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ABSTRACT

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Prospective investigation on the transfer of Ara h 2, the most potent peanut allergen, in human breast milk

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Background: Peanut allergy is one of the most severe food allergies. Whether breast feeding induces tolerance to peanuts or on the contrary, predisposes at risk-babies to occult allergic sensitization to peanuts is still a matter of discussion. We sought to investigate the transfer of the most potent peanut allergen Ara h 2 into human breast milk in a German breast milk study and to shed light on the time kinetics of Ara h 2 appearance.

Methods: We recruited 32 lactating, non-peanut allergic women, and collected breast milk samples at different time points after consumption of 100 g dry roasted peanuts. Breast milk samples were investigated for Ara h 2 with different immunological methods: by 2D immunoblotting with a patient's serum, by affinity enrichment using a monoclonal antibody against Ara h 2 followed by LC-MS/MS based detection and by a competitive inhibition ELISA for the detection of Ara h 2 and its digestion resistant peptides (DRP-Ara h 2).

Results: In a qualitative analysis Ara h 2 could be identified in a breast milk sample by 2D immunoblot by means of a patient's serum and furthermore by immunoaffinity enrichment followed by LC-MS/MS analysis. In a semi-quantitative analysis Ara h 2 and its digestion-resistant peptides were detected in the breast milk of 9 out of 32 subjects. Evidence suggests that Ara h 2 is excreted individually either rapidly (after 1 h, 2 h, 3 h or 4 h) or delayed (after 8 h or 12 h) and in different concentrations.

Conclusions: Time and concentration of secreted Ara h 2 in breast milk appears to be individually regulated. The identification of Ara h 2 in breast milk is the prerequisite for the investigation of its sensitizing or tolerogenic properties.

Key words: Allergen transfer, Ara h 2, digestion-resistant peptides of Ara h 2, ELISA, human breast milk, immunoblot analysis, immunoaffinity enrichment

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Abbreviations:

Ara h: *Arachis hypogaea*

BLQ: Below the Limit of Quantification

DRP-Ara h 2: Digestion-resistant peptides of Ara h 2

ELISA: Enzyme Linked Immunosorbent Assay

HRP: horse radish peroxidase

mab: monoclonal antibody

n: natural

2D: 2 dimensional

TTBS: Tris buffered saline with Tween 20

INTRODUCTION

Peanut allergy is one of the most severe and life-threatening food allergies. Approximately 1.4 to 3% of children suffer from peanut allergy in Western countries. During the last 10 years a 2-fold increase in the prevalence of diagnosed peanut allergy has been observed (1-3). Peanut allergy also affects more and more individuals in Germany (4). It is also the leading cause of anaphylactic fatalities in children and adolescents as documented by the anaphylaxis registry of the German speaking countries (5). Peanut allergens are known to possess high allergenic potency, and as little as 100 µg peanut protein can elicit allergic reactions in peanut allergic patients (6).

The allergens of peanuts have been studied intensively for more than 20 years with much focus placed on investigating the biochemical basis for the severity of peanut allergy. Sixteen allergens have been identified and listed in the World Health Organisation/International Union of Immunological Societies (WHO/IUIS) allergen database termed as (*Arachis hypogaea*) Ara h 1 to Ara h 17.

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Ara h 2 has gained the highest attention as it is regarded as the most potent peanut allergen and is described as a predictor for severe allergic reactions (7-9).

In the 1980s, it was suggested that peanut allergy develops due to the presence of peanut allergens in breast milk transferred from the maternal diet during lactation. But despite the restrictive recommendations of the American Academy of Paediatrics and the UK's Committee on Toxicology for mothers of at risk-infants to avoid eating peanuts during breast-feeding and to delay the introduction of peanut until 3 years of age, the prevalence of peanut allergy continues to increase rapidly (3,10).

An important milestone on this issue has been a study of Du Toit et al. in 2008 demonstrating that early oral intake of peanuts induces tolerance (11). In their study, Israeli Jewish children, typically given a peanut snack at 6 months of age, were one-tenth as likely to develop peanut allergy in comparison to Jewish children in the UK who avoided peanuts during childhood due to a public health recommendation. Du Toit et al. could confirm these data in a prospective, randomized trial within the Learning Early about Peanut Allergy (LEAP) study that early introduction of peanuts before 11 months of age decreases the risk of peanut allergy up to 70 to 80% (12). In their study the influence on early exposure to peanuts via breast milk during lactation has not been discussed.

As shown in numerous publications, breast milk as such is not hypoallergenic. Several food allergens have been identified in breast milk such as the cow's milk allergen β -lactoglobulin (13), the major egg allergens, ovalbumin and ovomucoid (14,15), and gliadin (16,17). Vadas and his coworkers (18) presented preliminary data in their brief report which included 23 healthy, lactating women in Canada. In their study peanut protein was identified in 11 of 23 subjects (48%) at concentrations ranging from 120 to 430 ng/mL within 1 to 3 hours in 10 subjects and delayed within 6 hours in one subject after oral ingestion of 50 g of dry roasted peanuts.

We initiated a breast milk study in Germany in 2004 and have recruited the largest population of lactating women that has been investigated prospectively thus far (n=32) for the secretion of peanut proteins into breast milk after consuming 100 g of dry roasted peanuts.

The aim of our study was to investigate whether the most potent peanut allergen Ara h 2 is transferred into breast milk and to get evidence at what time point after ingestion and in which concentration. The present paper describes the identification of the allergen - or peptides thereof - and the respective tool of detection via 2D immunoblot analysis, immunoaffinity enrichment strategy by LC-MS/MS and by a semi-quantitative analysis via a specific competitive inhibition ELISA. This allows us to design follow-up investigations with regard to breast milk as being a sensitization route or a vector that could be used to induce tolerance to peanuts very early in life.

MATERIALS and METHODS

Study population

The study was approved by the Ethics Committee of Hamburg and Schleswig-Holstein, Germany (approval numbers OB-018/04 and 08-222). Breast milk samples were collected from 32 non-peanut allergic lactating women who were included in the study in response to a contact to the Children's Hospital Altona, Hamburg, the breast feeding center in Bad Segeberg and posted notices in the Research Center Borstel. The major inclusion criterion was that the women consumed peanuts in their normal diet (albeit due to nutrition habits not in high doses), the major exclusion criterion was allergy to peanut to avoid probable complications. After avoiding consumption of legumes 24 h before breast milk collection the participants collected approximately 20 mL breast milk at time point t=0. Afterwards they consumed 100 g of dry roasted peanuts. The administered amount of 100 g peanuts was estimated with respect to the dose that is likely to be consumed as a snack and that may allow us to determine any positive values either in ELISA or in immunoblot analysis. Breast milk samples from subjects 1 to 15 were taken 1 hour (approximately 20 mL) after peanut

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ingestion according to the peak concentrations analyzed in the Canadian study of Vadas et al. (18). In the course of our study we modified the study design and collected breast milk samples at further intervals eventually collecting 0, 1, 2, 3, 4, 8 and 12 hours after peanut ingestion (see Table 1). The children were breast-fed during the study so breast feeding was not regulated throughout the breast milk sampling. Breast milk samples no. 1, 5 and 24 came from one woman providing samples in three different lactation periods. Woman no. 20 provided two sets of breast milk samples within one lactation period (termed as no. 20 and 20.1). Breast milk samples no. 34 and 35 were collected from one woman within one lactation period after ingestion of 100 g peanut butter (termed as no. 34) and of 100 g roasted peanuts (termed as no. 35).

Written informed consent was obtained from the subjects included in this study.

Human serum and monoclonal antibody

A serum sample was collected from a male peanut-allergic patient after giving informed consent. The patient had a convincing history of severe adverse reactions after peanut ingestion. Specific IgE to peanut and Ara h 2 was determined by CAP-FEIA (ImmunoCAP, ThermoFisher Scientific, Freiburg, Germany) revealing a high concentration of specific IgE against peanut extract (CAP 75.9 kU/L) and Ara h 2 (CAP 51 kU/L). Oral challenge tests with peanut were not performed as the patient refused a challenge with foods to which he had severe allergic reactions. The study was approved by the local ethics committee (approval No. 10-126).

The monoclonal antibody (mab; termed 1-2-1) against nAra h 2 was generated by BioGenes GmbH (Berlin, Germany) after immunization with natural Ara h 2 (nAra h 2 from Indoor Biotechnologies Ltd, Cardiff, Wales, and in house isolated nAra h 2 respectively (detailed in the Supporting Information). The immunization of the animals was performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. Due to German animal protection law the animal

experiments were registered as animal experiment projects at the LaGeSo Berlin (Landesamt für Gesundheit und Soziales), Division of Veterinary Department (reference number: H0161/09).

2D-PAGE, Westernblot and Westernblot inhibition

Prior to analysis the breast milk samples were centrifuged at 8,000 x g for 15 min. Two-dimensional electrophoresis was performed according to Görg et al. (19) and after electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by semi-dry blotting (20) for 45 min (detailed in the Supporting Information). Immunoblot inhibition experiment with breast milk sample no. 20 b was performed by preincubating patient's serum with 500 ng nAra h 2 (Indoor Biotechnologies) at room temperature for 2 h. Bound antibodies were detected by a horse radish peroxidase (HRP)-conjugated antibody, mouse anti-human IgE (Fc)-HRP (Southern Biotech, Birmingham, Alabama, US), in a 1:5,000 dilution in TTBS for 2 h. Immunostaining was performed by means of chemiluminescent western blot detection using Clarity Western ECL Substrate (Biorad, Hercules, CA, US). The detection of stained 2D blots was performed by using the ChemiDoc MP System (Biorad, Hercules).

Immobilization of Monoclonal Antibody on Paramagnetic Beads

The monoclonal antibody 1-2-1 against Ara h 2 was immobilized and crosslinked to Protein G Dynabeads (Life Technologies GmbH, Darmstadt, Germany) as described in the manufacturer's protocol. Briefly, the antibody (60 µg) was adjusted to 1000 µL with PBS for 100 µL Protein G beads.

Antibody-Based Enrichment Procedure and Elution

The affinity enrichment procedure followed by tryptic digestion was performed as described by Kull et al. (21) with slight modifications. Briefly, 500 µL of breast milk sample 20 a and 20 b, respectively, were incubated for 2 h with 8 µL anti-Ara h 2 beads under rotation. The

supernatant was discarded, and the beads were washed twice with 1 mL of PBS/T, twice with 1 mL of PBS and two more times with 1 mL of water. Elution was carried out with 25 μ L of 0.1% TFA in H₂O for 5 min. The supernatant was transferred into a new tube.

Reduction, Alkylation and Tryptic Digest

For neutralization 7 μ L of 400 mM NH₄HCO₃ were added. Afterwards, for the reduction of the disulfide bonds samples were incubated with 1.5 μ L of 1,4 dithiotreitol (400 mM) for 10 min at 95 °C. After cooling down to room temperature, thiol groups were alkylated by adding 3 μ L of 500 mM iodoacetamide and incubating for 30 min at 37°C. Afterwards, 5 μ L of 20 μ g/mL trypsin solution were added and incubated overnight at 37°C.

LC-MS/MS Analysis

LC-ESI MS analysis was performed on an Ultimate 3000 uHPLC system, equipped with an Acclaim PepMap RSLC C18-column (75 μ m x 50 cm, 2 μ m, 100 Å, Dionex), coupled online to a QExactive Plus mass spectrometer (Thermo, Bremen, Germany) via a nanospray flex ion source with a 10 μ m SilicaTip emitter (New Objective, Woburn, MA).

After injection, the analytes were washed for 2 min on a PepMap C18 guard column (300 μ m x 10 mm, Dionex) with 2% ACN, 0.05% TFA at a flow rate of 30 μ L/min.

Immunoprecipitated samples were separated by Ion pairing reversed-phase (IP-RP) HPLC using a 150 min water- acetonitrile gradient from 95% eluent A (water with 0.05 % FA) to 90% eluent B (80% ACN, 0.04% FA) at a flow rate of 300 nL/min, followed by a sharp increase to 90% B in 8 min and isocratic elution for 10 min; afterwards, the column was equilibrated with 95% A for 15 min.

The mass spectrometer was operated with Xcalibur software (v3.0.63). The spray voltage was set to 2.4 kV at capillary temperature of 250°C. MS conditions were: AGC target 3×10^6 ; maximum inject time 100 ms. Full scans of 300 – 2,000 m/z range were acquired in positive ion mode, with a resolution of 70,000 and calibrated by lock mass of the polysiloxane contaminant peak at m/z 445.120024. Parameters of data dependent MS² acquisition were

as follows: AGC target 1×10^5 ; maximum inject time 50 ms. Spectra were acquired with a resolution of 7,500. Charge states of 1 and over 8 were generally excluded from acquisition and dynamic exclusion time was set to 30 s.

Sequence Information

Proteome Discoverer Software (v1.4.0.288) was used for database search of raw files. Spectra were matched against a database of UniProt entries for Human (145,758 entries, May 2015), Peanut (1,120 entries, May 2015) and common contaminants (cRAP, 116 entries). Mascot (v 2.2.07), SequestHT and MS Amanda (v1.4.4.1909) search algorithms were used to annotate spectra with a fragment mass accuracy of 0.05 Da. Parent-mass accuracy was set to 5 ppm with trypsin specified as protease, allowing for two missed cleavages. Carbamidomethylation of cysteine was set as static modifications, oxidation of methionine as variable modifications.

Percolator (v 2.04) was used for posterior error calculation combining the results of the database searches restricted by q-value to $\text{FDR} \leq 0.01$. Single peptide identifications were excluded and protein groups were assigned using strict parsimony rules.

Competitive Inhibition ELISA for Detection of Ara h 2 and its Digestion-Resistant Peptides (DRP)

Digestion-resistant peptides of Ara h 2 (DRP-Ara h 2) were prepared as described by Koppelman et al. (22). Briefly, intact native Ara h 2 was purified from defatted, light roasted peanut flour. The purified Ara h 2 was then digested with trypsin for 20 minutes. Digestion-resistant peptides of Ara h 2 were then isolated and purified using hydrophobic interaction and size exclusion chromatography. These peptides were used to develop rabbit IgG antisera directed against the DRP-Ara h 2. The DRP and the rabbit IgG antibodies were used as reagents in the developed competitive inhibition ELISA in human breast milk

(detailed in the Supporting Information). The limit of quantification (LOQ) of the competitive inhibition ELISA was determined to be 40 ng Ara h 2 and its DRP/mL of breast milk.

RESULTS

Recruitment and characterization of healthy lactating mothers with peanut consumption on a regular basis

32 healthy lactating women (age range 26-42) from Northern Germany were enrolled who collected breast milk samples before and after ingestion of 100 g dry roasted peanuts. The age of the women, the time point of lactation while the sample was collected, atopy status and the consumption of peanuts during lactation were recorded in a questionnaire and the data summarized in Table 1.

Identification of Ara h 2 in breast milk by 2D immunoblot analysis

Ara h 2 was identified in breast milk in a 2D immunoblot by a patient's serum followed by sensitive chemiluminescent western blot detection. To determine immunoreactivity sample no. 20 was chosen due to sufficient breast milk material given by the mother before (20 a) and after (20 b; 2h after oral exposure to peanuts) ingestion of peanuts and a patient's serum with a high concentration of specific IgE against peanut extract (CAP 75.9 kU/L) and Ara h 2 (CAP 51 kU/L). In Fig. 1A the patient's serum recognized Ara h 2 in breast milk sample 20 b as four to six IgE-reactive spots of 20 to 26 kDa in a pI range of approximately 5. For IgE immunoblot inhibition experiment the serum was preincubated with 500 ng of nAra h 2 before being tested on a 2D immunoblot with breast milk sample 20 b. IgE binding to Ara h 2 was inhibited (Fig. 1B). The breast milk sample 20 a (before ingestion of peanuts) served as negative control (Fig. 1C) showing no IgE reactivity to Ara h 2. By use of 77.5 ng nAra h 2 spiked into breast milk sample 20 a the immunoreactive Ara h 2 spots were clearly detectable as at least 6 spots of 20 to 26 kDa in a pI range of approximately 5. Besides, several protein spots at 34 kDa, 72 kDa and 100 kDa were depicted (as breast milk proteins) within the tested pI range of 3 to 10 in Fig. 1 A-C.

Detection of Ara h 2 in breast milk by immunoaffinity enrichment and LC-MS/MS

The unambiguous detection of Ara h 2 was performed by means of a combination of immunoaffinity enrichment and LC-MS/MS analysis. The selected mab 1-2-1 showed reactivity against Ara h 2 but also against Ara h 1, Ara h 3 and Ara h 6 in immunoblot analysis (data not shown).

For immunoaffinity enrichment breast milk sample 20 b was incubated with anti-Ara h 2 beads and the identity of captured Ara h 2 was confirmed by means of LC-MS/MS (Table S1 Supporting Information). The corresponding peptide fragments generated after trypsin digestion identified not only Ara h 2 but also Ara h 1, Ara h 3 and Ara h 6 in the sample.

In Fig. 2 the identified tryptic fragments corresponding to the respective sequences of Ara h 2 (four isoforms) are printed in bold.

In contrast, neither Ara h 2 nor other peanut allergens were identified in the breast milk sample 20 a before peanut ingestion (data not shown).

Quantification of Ara h 2 in breast milk by a competitive inhibition ELISA

For the quantification of Ara h 2 we analysed the samples by means of a competitive inhibition ELISA as a screening tool with a rabbit polyclonal IgG antibody directed against the digestion-resistant portion of Ara h 2 (DRP-Ara h 2). This polyclonal antibody also shows cross-reactivity against intact Ara h 2. The results of the breast milk samples of our cohort analysed in the competitive inhibition ELISA are shown in Table 2. In 9 of 32 subjects peanut allergen had been transferred into breast milk, the concentration ranging between 46 and 2602 ng Ara h 2 and its DRP/mL breast milk. The time points of appearance and the concentrations differed interindividually between the lactating women. Ara h 2 and its DRP appeared within 1, 2, 3, 4 hours or even delayed after 8 or 12 hours after ingestion. One lactating mother secreted Ara h 2 and its DRP in two different lactation periods in concentrations that differed 20-fold (no. 1 and 5). Another woman showed a transfer of Ara h 2 and its peptides in concentrations that differed 4-fold after collecting breast milk samples twice within the same lactation period (no. 20 and 20.1).

DISCUSSION

In this study we could show that Ara h 2, the most potent peanut allergen, is transferred into breast milk, presumably at different time points (between 1 and 12 hours, interindividually). First, Ara h 2 was identified in one breast milk sample (sample 20 b) after immunostaining by means of chemiluminescent western blot detection in combination with a patient's serum. The inhibition of IgE binding to Ara h 2 by preincubating the patient's serum with nAra h 2 and the lack of Ara h 2 detection in the breast milk sample 20 a before ingestion of peanuts strengthens the immunological identification of the allergen within the breast milk matrix. The protein spots that are detectable apart from the Ara h 2 spot pattern (Fig. 1 A-C) presumably belong to high abundant breast milk proteins due to their molecular masses like caseins and lactoferrin.

The unambiguous identification of Ara h 2 could be obtained in the next step by the combination of affinity enrichment using a monoclonal antibody against Ara h 2 and a LC-MS/MS based detection. The mab that was selected for the enrichment procedure recognizes Ara h 2 and showed a cross-reactivity against the peanut allergens Ara h 1, Ara h 3, and Ara h 6, in an immunoblot analysis with the purified peanut allergens as target (data not shown). This observation might explain the detection of Ara h 1, Ara h 3, and Ara h 6 (besides the Ara h 2), in the breast milk sample 20 b by LC-MS/MS. One reason for the cross-reactivity of the anti-Ara h 2 antibody might be similar structural motifs on the molecules as described for the 2S albumin Ara h 2 and the unrelated cupins Ara h 1 and Ara h 3 (23).

To study the concentrations of Ara h 2 after ingestion we initially collected breast milk at time point 0 h and at 1 h after non-peanut allergic lactating mothers had consumed 100 g of dry roasted peanuts (subjects 1-15; Table 1). In order to investigate the time points of secreted Ara h 2 in more detail, we modified the study design and added further intervals, finally succeeding in collecting breast milk samples after 0, 1, 2, 4, 8 and 12 hours after peanut ingestion. Thus, we have prospectively recruited 32 lactating German women, the largest study population of this phenotype studied thus far.

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For the analysis of the recruited samples we succeeded in detecting the clinically relevant peanut allergen Ara h 2 in the complex breast milk matrix by means of a competitive inhibition ELISA relying on a rabbit IgG antiserum which is directed against the digestion-resistant portion of Ara h 2. Sensitive detection of DRP-Ara h 2 has been established in the matrix breast milk (24). Due to cross-reactivity we were able to identify both the secreted Ara h 2 allergen and its digestion resistant peptides in breast milk. By this means, we were able to show that Ara h 2 was transferred into breast milk in 9 of the investigated 32 subjects. On the basis of the time points of sampling (Table 1) the concentrations of Ara h 2 and its peptides ranged from 46 to 2602 ng Ara h 2 and DRP-Ara h 2 per mL breast milk, and evidence suggests that the time points of appearance after ingestion differed among the lactating women. Interestingly, Ara h 2 and its peptides appeared either after a short cut-transfer within 1, 2, 3, 4 hours or delayed after 8 or 12 hours following ingestion within the analysed samples. Their concentrations differed in samples of different lactation periods (no. 1 and 5) and even within one lactation period (no. 20 and 20.1) suggesting an intraindividually high variability of secreted Ara h 2 and its fragments. The variability of secreted food allergens have also been shown in other studies and are in accordance with our analysis (14,17,25,26). Taking into account that breast milk samples were not provided at all sampling intervals (0, 1, 2, 3, 4, 8, 12h) right from the beginning of the study we cannot fully state but assume that there is no correlation between the age, the time point of lactation when sampling, the atopy status, the consumption of a defined amount of peanuts and the amount of secreted Ara h 2 in our breast milk samples.

Apart from the Canadian breast milk study there is to our knowledge only one recent publication from April 2014 reporting data on the transfer of Ara h 6 into human breast milk. This was studied in only two non-atopic peanut tolerant mothers from France before and at different time points after consumption of 30 g of peanuts (27). Vadas et al. (18) demonstrated peak appearance of peanut proteins in concentrations from 120 to 430 ng/mL within 1 to 3 hours after ingesting 50 g of dry roasted peanuts. The proteins were rapidly cleared from breast milk and were described to be secreted without degradation. In the

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French study immunologically active Ara h 6 was detected within 10 minutes after peanut consumption, with peak concentrations only in the pg/mL range within the first hour after ingestion but long lasting excretion even 26 h after ingestion. In our study, however, in a population of 32 volunteers Ara h 2 appeared to either pass rapidly into the circulation after 1 or 2 hours, presumably following absorption via the oral mucosa as described by Dirks et al. (28), or delayed after 8 to 12 hours. Dirks et al. (28), for the first time, studied the mucosal absorption of allergenic peanut proteins into the circulation and a probable local uptake from the oral cavity. In their study they included six individuals who chewed 10 g of raw peanuts and spit out the peanuts without swallowing any of the material. Their serum was investigating in a histamine release measuring IgE cross-linking. The authors described a significant increase in histamine release already after 10 minutes, thereby providing evidence for a preintestinal uptake of peanut allergens.

However, different absorption kinetics depending on the complexity and composition of meals ingested before peanut consumption in our study group are probably responsible for this observation, too.

So far, it is not fully known how the Ara h 2 structures survive digestive enzymes and are secreted into breast milk, namely as intact proteins or degraded as peptides. Our findings are an interesting result not only as conclusive demonstration of the presence of Ara h 2 in breast milk sample but also in the context of the likely secretion of Ara h 2 in its undegraded form 2 h after peanut ingestion. Our work is ongoing whether the allergens or their fragments act as sensitizing or tolerogenic agents in the immune system of the breast-fed babies and under what conditions it will be the one or the other effect.

In conclusion, Ara h 2 could be conclusively identified in one breast milk sample via immunoblot analysis by means of a patient's serum and by immunoaffinity purification followed LC-MS/MS.

We were able to recruit a unique study population of lactating women that has been studied prospectively thus far and to demonstrate the transfer of Ara h 2, the marker allergen for severe peanut allergy, into breast milk after the consumption of 100 g of peanuts. The

competitive inhibition ELISA proved to be a suitable tool for the identification of intact Ara h 2 into breast milk on the one hand and the transfer of peptides thereof after gastro-intestinal allergen processing on the other hand.

Thus, we have now access to molecular tools to further investigate if and how Ara h 2 is processed *in vivo* in more detail and to determine if single peanut allergens and peptides thereof act as sensitizing or tolerogenic agents.

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Conflicts of interest

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1A-D. 2D immunoblot of breast milk sample 20. Separation was performed under reducing conditions. Detection antibody: patient's serum (chemiluminescent western blot detection). The breast milk concentration was 600 μ g. A: 2D immunoblot of breast milk sample 20 b (2 h after peanut ingestion), B: 2D immunoblot inhibition of (immobilized) nAra h 2 in breast milk sample 20 b with patient's serum by 500 ng of nAra h 2 as inhibitor, C: 2D immunoblot of breast milk sample 20 a (before peanut ingestion), D: 2D immunoblot of breast milk sample 20 a (before peanut ingestion) spiked with 77.5 ng nAra h 2.

Figure 2. Sequence alignment of four isoforms of Ara h 2 (UniProt accession number Q6PSU2). Identified sequences of Ara h 2 by means of LC-MS/MS analysis are highlighted in bold, the signal peptide is underlined.

TABLE LEGENDS

Table 1. Synopsis of data of lactating mothers included in the German breast milk study.

Table 2. Detection of Ara h 2 and its Digestion-resistant peptides (DRP-Ara h 2) by a Competitive Inhibition ELISA.

Table 1. Synopsis of data¹⁾ of lactating mothers included in the German breast milk study

Subject's ID	Age	Lactation time point (months)	Atopic status	Sensitizations		Peanut consumption (per month)	Time Points of Collection						
				Inhalant allergens	Foods		0 h	1h	2 h	3 h	4 h	8 h	12h
1	32	6.5	no	no	no	50 g	0 h	1 h	x	x	x	x	x
2	31	7	no	no	no	data not available	0 h	1 h	x	x	x	x	x
3	38	9	no	no	no	400 g	0 h	1 h	x	x	x	x	x
4	39	data not available	AD	no	no	<50 g	0 h	1 h	x	x	x	x	x
5	34	9.5	no	no	no	50 g	0 h	1 h	x	x	x	x	x
6	39	4.5	P, FA	TP, HDM	hz	100 g	0 h	1 h	x	x	x	x	x
7	39	8	PS	no	t, p	150 g	0 h	1 h	x	x	x	x	x
8	27	6	no	no	no	few	0 h	1 h	x	x	x	x	x
9	34	10.5	P, ND, FA	B, G, BH	ci	1000 g	0 h	1 h	x	x	x	x	x
10	32	2.5	AD	no	no	few	0 h	1 h	x	x	x	x	x
11	28	3	P	G	no	few	0 h	1 h	x	x	x	x	x
12	28	data not available	P, A	G	no	few	0 h	1 h	x	x	x	x	x
13	31	6	no	no	no	few	0 h	1 h	x	x	x	x	x
14	27	data not available	P	G	no	250g	0 h	1 h	x	x	x	x	x
15	42	12	P, A	G	no	65 g	0 h	1 h	x	x	x	x	x
16	32	6	no	no	no	400 g	0 h	1 h	x	3 h	x	x	x
17	36	3.5	no	no	no	few	0 h	x	2 h	x	4 h	x	x
18	29	6	lactose intolerance	no	no	400 g	0 h	x	2 h	x	4 h	x	x
19	37	4	no	no	no	200 g	0 h	x	2 h	x	4 h	x	x
20 and 20.1	32	5	allergy to antibiotic	no	no	150 g	0 h	x	2 h	x	4 h	x	x
21	29	data not available	no	no	no	few	0 h	x	2 h	x	4 h	x	x
22	32	3	contact dermatitis	no	no	200 g	0 h	x	2 h	x	4 h	x	x
23	26	6.5	no	no	no	few	0 h	x	2 h	x	4 h	x	x
24	36	5	AD	no	no	50 g	0 h	x	x	x	4 h	8 h	12 h
25	35	7	no	no	no	25 g	0 h	x	x	x	4 h	8 h	12 h
26	38	7.5	no	no	no	few	0 h	x	x	x	4 h	8 h	12 h
27	27	4	no	no	no	<200 g	0 h	x	x	x	4 h	8 h	12 h
28	36	8	no	no	no	250 g	0 h	x	x	x	4 h	8 h	12 h
29	30	4.5	P, A, FA	G, HDM, C		200 g	0 h	x	x	x	4 h	8 h	12 h
30	33	2.5	no	no	no	100 g	0 h	x	x	x	4 h	8 h	12 h
31	30	6.5	no	no	no	1000 g	0 h	x	x	x	4 h	8 h	12 h
32	27	3	P	G, TP, C	no	few	0 h	x	x	x	4 h	8 h	12 h
33	37	9.5	no	no	no	few	0 h	x	x	x	4 h	8 h	12 h
34	42	14	P, A	B, G, HDM, C	c, hz, ci	few	0 h	1 h	2 h	x	4 h	8 h	12 h
35	42	14	P, A	B, G, HDM, C	c, hz, ci	few	0 h	1 h	2 h	x	4 h	8 h	12 h

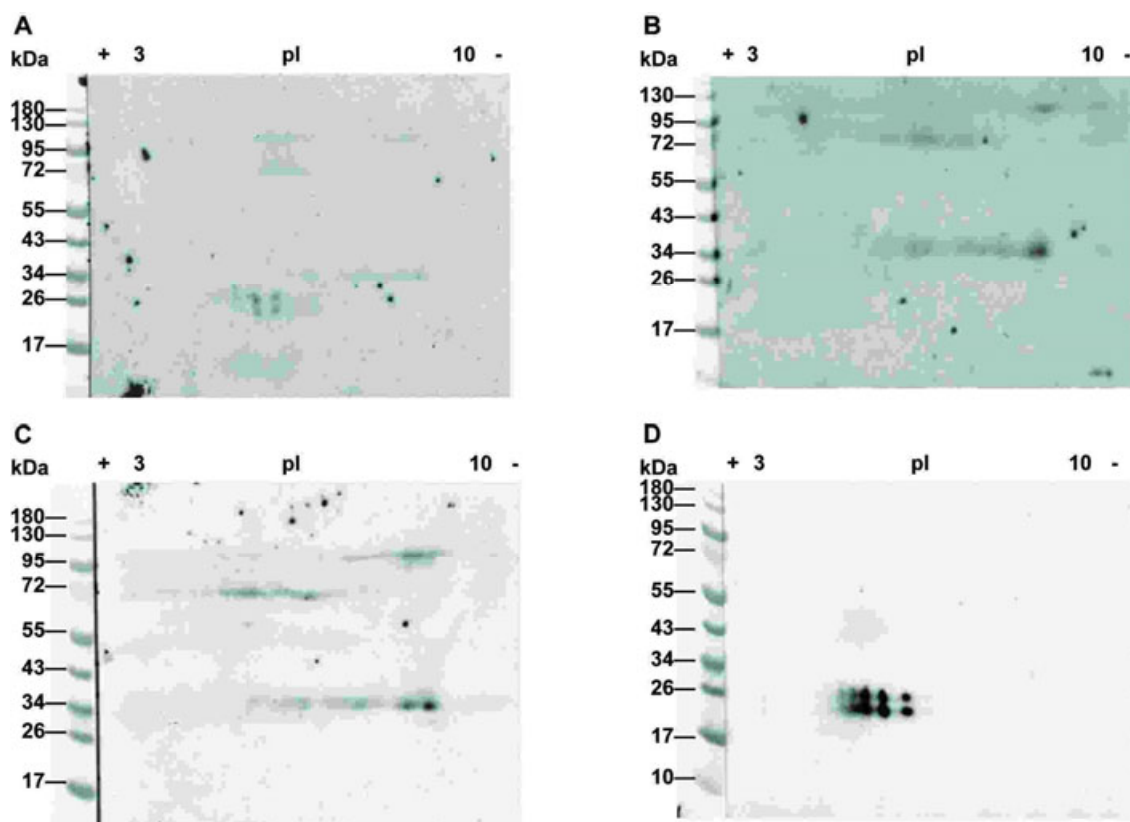
¹⁾ : by questionnaire; AD; atopic dermatitis. P: pollinosis, FA: food allergy, A: asthma, PS: pseudoallergy; B: birch; TP: tree pollen, G, grass; C, cat dander; HDM, house dust mite, BH: buckhorn; c, celery; hz: hazelnut; ci: citrus fruits, t: tomato; p: pepper; x: sample not collected. Subjects 1, 5 and 24; 20 and 20.1; 34 and 35 respectively are identical. Breast milk samples no. 1, 5, 24 were collected in different lactation periods of one woman, samples no.

20 and 20.1 in the same lactation period of one woman. Samples no. 34 and 35 were provided from one woman after ingestion of 100 g peanut butter (no. 34) and of 100 g roasted peanuts (no. 35).

Table 2. Detection of Ara h 2 and its Digestion-resistant peptides (DRP-Ara h 2) by a Competitive Inhibition ELISA

	Subject's ID	Collection period						
		0 h	1 h	2 h	3 h	4 h	8 h	12 h
Collection points: t=0h, t=1h	1	< BLQ	2602	no sample collected	no sample collected	no sample collected	no sample collected	no sample collected
	5	< BLQ	126	no sample collected	no sample collected	no sample collected	no sample collected	no sample collected
	15	< BLQ	73	no sample collected	no sample collected	no sample collected	no sample collected	no sample collected
	Breast milk sample of subjects 2, 3, 4, 6-14 were tested <BLQ.							
Collection points: t=0h, t=1h, t=3h	16	< BLQ	< BLQ	no sample collected	249	no sample collected	no sample collected	no sample collected
Collection points: t=0h, t=2h, t=4 h	17	< BLQ	no sample collected	397	no sample collected	< BLQ	no sample collected	no sample collected
	20	< BLQ	no sample collected	75	no sample collected	< BLQ	no sample collected	no sample collected
	20.1	< BLQ	no sample collected	315	no sample collected	< BLQ	no sample collected	no sample collected
	21	< BLQ	no sample collected	46	no sample collected	< BLQ	no sample collected	no sample collected
	Breast milk samples of subjects 18, 19, 22, 23 were tested <BLQ.							
Collection points: t=0h, t=4h, t=8h, t=12h	25	< BLQ	no sample collected	no sample collected	no sample collected	118	< BLQ	< BLQ
	27	< BLQ	no sample collected	no sample collected	no sample collected	< BLQ	88	240
	32	< BLQ	no sample collected	no sample collected	no sample collected	< BLQ	< BLQ	90
	Breast milk samples of subjects 24, 26, 28-31, 33 were tested <BLQ.							
Collection points: t=0h, t=1h, t=2h, t=4h, t=8h, t=12h	Breast milk samples of subjects 34 and 35 were tested <BLQ.							

Detection of Ara h 2 and its digestion-resistant peptides (DRP) in ng/mL breast milk; BLQ: Below the limit of quantification. Subjects 1, 5 and 24; 20 and 20.1; 34 and 35 respectively are identical.



Q6PSU2 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY
 Q6PSU2-3 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY
 Q6PSU2-2 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY
 Q6PSU2-4 MAKLTILVALALFLLAAHASARQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSY

Q6PSU2 GRDPYSPSQDPYSPSQDPDRRDPYSPSPYDRRGAGSSQHQRCCNELNEFENNQRCMCEA
 Q6PSU2-3 GRDPYSPSQDPYSPSQDPDRRDPYSPSPYDRRGAGSSQHQRCCNELNEFENNQRCMCEA
 Q6PSU2-2 GRDPYSPSQDP-----YSPSPYDRRGAGSSQHQRCCNELNEFENNQRCMCEA
 Q6PSU2-4 GRDPYSPSQDP-----YSPSPYDRRGAGSSQHQRCCNELNEFENNQRCMCEA

Q6PSU2 LQQIMENQSDRLQGRQEQQFKRELRNLPPQCCGLRAPQRCDFEVESGGRDRY
 Q6PSU2-3 LQQIMENQSDRLQGRQEQQFKRELRNLPPQCCGLRAPQRCDFEVESGGRD--
 Q6PSU2-2 LQQIMENQSDRLQGRQEQQFKRELRNLPPQCCGLRAPQRCDFEVESGGRDRY
 Q6PSU2-4 LQQIMENQSDRLQGRQEQQFKRELRNLPPQCCGLRAPQRCDFEVESGGRD--

SUPPORTING INFORMATION

Materials

Natural Ara h 2

Natural Ara h 2 was isolated from ground peanuts (Seeberger Riesen, Seeberger, Ulm, Germany) which were subjected to a lipophilic extraction using chloroform and methanol at pH 11 as described previously (1). The pellet was then extracted with a Tris/HCl buffer, pH 2.0, followed by two purification procedures, namely by hydrophobic interaction chromatography (HIC) and cation exchange chromatography (Source S, GE Healthcare).

2D-PAGE

An immobilized Novex IPG Zoom strip (pH gradient 3-10) (Invitrogen) was used as described by Görg et al. (2). An amount of 600 µg of breast milk from patient no. 20 a and 20 b, respectively, were loaded on the gel strip. The proteins were separated at 2 mA and 2 W with increasing voltage at 200 V for 20 min, 450 V for 15 min, 750 V for 17 min and 2000 V for 1 h. SDS-PAGE was performed in the second dimension by running a 12% acrylamide gradient Bis-Tris gel (Invitrogen). Molecular masses and pIs were estimated by running PageRuler Prestained Protein Ladder (10-170 kDa, Invitrogen) and IEF Marker 3-10, Liquid Mix (Serva, Heidelberg, Germany). After electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by semi-dry blotting (3) for 45 min. Membranes were blocked with Blocking Buffer (Sigma Aldrich, Munich, Germany) for 2 h. The patient's serum was diluted 1:20 in TTBS (100 mM Tris, 100 mM NaCl, 2.5 mM MgCl₂, 0.05% Tween 20) and incubated overnight.

Competitive Inhibition ELISA for Detection of Ara h 2 and its Digestion-Resistant Peptides (DRP)

Digestion-resistant peptides of Ara h 2 (DRP-Ara h 2) were prepared as described by Koppelman et al. (4). Maxisorp™ microtiter plates (Nalge Nunc International, Rochester, NY) were coated with 50 µl/well of DRP-Ara h 2 (500 ng/ml) in coating buffer (0.015 M

Na₂CO₃, 0.035 M NaHCO₃, and 0.02 % NaN₃, pH 9.6) and incubated overnight at 4°C. DRP-Ara h 2 standards were developed by serially diluting DRP-Ara h 2 1:3 (v/v) in negative control milk sample to obtain a standard curve ranging from 0 – 33,000 ng DRP-Ara h 2/ml. The spiked milk standards were centrifuged at 13,000 x g for 10 minutes to remove particulate matter and cellular debris. Breast milk samples of the cohort were also centrifuged at 13,000 x g for 10 minutes prior to analysis. Spiked standard curve samples and breast milk samples were incubated for 2 hours at 37°C with polyclonal rabbit IgG antisera directed against DRP-Ara h 2 (1:1000 dilution of rabbit anti-DRP-Ara h 2 polyclonal antisera in conjugate buffer containing 0.1% BSA in 0.025 M PBS, pH 7.4). Standard curve points and breast milk samples were then incubated for 2 hours at 37°C in the DRP-Ara h 2 coated microtiter plates. After subsequent washing, 100 µl/well of goat anti-rabbit IgG conjugated to alkaline phosphatase (Immunopure®, Pierce Protein Research, Thermo Fisher Scientific Inc., Waltham, MA) diluted 1:2500 in conjugate buffer was added to each well and incubated for 1 hour at 37°C. Plates were then washed 4 times with wash buffer, followed by the addition of 100 µl/well of *p*-nitrophenyl phosphate substrate (Sigma Fast™ tablets, Sigma-Aldrich, St. Louis, MO). The enzymatic reaction was allowed to proceed for 30 minutes at room temperature and was stopped with the addition of 100 µl/well of 1.0 M NaOH. Absorbance values at 405 nm were measured immediately after stopping the reaction using a BioTek ELx808 plate reader (BioTek Instruments, Inc., Winooski, VT). GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA) was used to generate standard curves utilizing the non-linear regression function. The limit of quantification of the competitive inhibition ELISA was determined to be 40 ng Ara h 2 and its DRP/mL of breast milk. Quantification of Ara h 2 and its DRP in breast milk was achieved by comparing the optical density of each sample to that of the standard curve.

During the development of the competitive inhibition ELISA, the specificity of the rabbit anti-DRP-Ara h 2 polyclonal antisera was evaluated to determine any potential cross-reactivity with commonly consumed foods. Eighty-eight foods including legumes, tree nuts, cereal

grains, vegetables, fruits, spices, and cow's milk were evaluated. No cross-reactivity was observed with these foods (data not shown).

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Table S1 Supporting Information. Peanut allergens detected in breast milk sample 20 b identified by LC-MS/MS (mab 1-2-1).

Table S1 Supporting Information. Peanut allergens detected in breast milk sample 20 b identified by LC-MS/MS (mab 1-2-1)

Allergen IUIS name	Protein name	Accession no.	MW [kDa]	pI	Sequence coverage [%]	MS/MS confirmation			
						Peptides	Modification	Experimental mass [M+H] ⁺ [Da]	Theoretical mass [M+H] ⁺ [Da]
Ara h 1	Conarachin (Fragment)	Q6PSU3	66.5	7.17	3.28 [3.42]	LEYDPR		792.389	792.389
						DLAFPGSGEQVEK		1376.670	1376.669
Ara h 2	Isoform 3 of Conglutin-7; Isoform 4 of Conglutinin-7	Q6PSU2-3	19.8	5.96	42.35 [48.32]	CCNELNEFENNQR	C1(CAM); C2(CAM)	1726.691	1726.691
		Q6PSU2-4	18.4	6.33	45.57 [52.55]	CDLEVESGGR	C1(CAM)	1121.489	1121.489
						CDLEVESGGRD	C1(CAM)	1236.516	1236.516
						ANLRPCEQHLMQK	C6(CAM)	1624.805	1624.805
						NLPQQCGLR	C6(CAM)	1085.553	1085.552
						CMCEALQQIMENQSDR	C1(CAM); C3(CAM)	2012.831	2012.829
						QQWELQGDR		1159.551	1159.549
						QQWELQGDRR		1315.651	1315.650
Ara h 3	Gly1	Q9FZ11	60.4	5.64	32.51 [33.86]	RPFYSNAPQEIFIQQGR		2051.047	2051.046
						VYDEELQEGHVLVVPQNFAVAGK		2541.299	2541.298
						QQPEENACQFQR	C8(CAM)	1534.671	1534.670
						SQSDNFEYVAFK		1434.653	1434.654
						SPDIYNPQAGSLK		1389.701	1389.701
						TANDLNLLILR		1255.738	1255.737
						WLGLSAEYGNLYR		1541.778	1541.775
						LESEGGYIETWNPNNQEFECAGVALSR	C20(CAM)	3070.389	3070.385
						GYFGLIFPGCPSTYEPAQQGR	C10(CAM)	2474.140	2474.145
						QIVQNLR		870.516	870.516
						QIVQNLRGENESEEEGAIVTVK		2442.248	2442.247
						GENESEEEGAIVTVK		1590.748	1590.749
	Arachin 6	A1DZF0	60.3	5.54	32.51 [33.86]	RPFYSNAPQEIFIQQGR		2051.047	2051.046
						VYDEELQEGHVLVVPQNFAVAGK		2541.299	2541.298
						QQPEENACQFQR	C8(CAM)	1534.671	1534.670
						QIVQNLR		870.516	870.516
						SQSENFYVAFK		1448.669	1448.669

						TANELNLLILR		1269.751	1269.753
						SPDIYNPQAGSLK		1389.701	1389.701
						WLGLSAEYGNLYR		1541.778	1541.775
						IESEGGYIETWNPNNQEFECAGVALSR	C20(CAM)	3070.389	3070.385
						GENESEEQGAIVTVK		1589.765	1589.765
						GYFGLIFPGCPSTYEPAQQGR	C10(CAM)	2474.140	2474.145
	Arachin Ahy-4	Q5I6T2	60.7	5.48	32.39 [33.66]	RPFYSNAPQEIFIQQGR		2051.047	2051.046
						VYDEELQEGHVLVVPQNFAVAGK		2541.299	2541.298
						QQPEENACQFQR	C8(CAM)	1534.671	1534.670
						QIVQNLR		870.516	870.516
						SQSDNFEYVAFK		1434.653	1434.654
						SPDIYNPQAGSLK		1389.701	1389.701
						TANDLNLLILR		1255.737	1255.737
						GENESEEQGAIVTVR		1618.756	1618.755
						WLGLSAEYGNLYR		1541.778	1541.775
						IESEGGYIETWNPNNQEFECAGVALSR	C20(CAM)	3070.389	3070.385
						QIVQNLRGENESEEQGAIVTVR		2470.256	2470.253
						GYFGLIFPGCPSTYEPAQQGR	C10(CAM)	2474.140	2474.145
	Arachin Ahy-2	Q647H3	61.5	5.73	32.03 [33.23]	VYDEELQEGHVLVVPQNFAVAGK		2541.299	2541.298
						RPFYSNAPQEIFIQQGR		2051.047	2051.046
						LESEGGYIETWNPNNQEFECAGVALSR	C20(CAM)	3070.389	3070.385
						SQSDNFEYVAFK		1434.653	1434.654
						TANDLNLLILR		1255.738	1255.737
						QQPEENACQFQR	C8(CAM)	1534.671	1534.670
						GENESDEQGAIVTVR		1603.759	1603.756
						SPDIYNPQAGSLK		1389.701	1389.701
						QILQNLRGENESDEQGAIVTVR		2469.269	2469.269
						WLGLSAEYGNLYR		1541.778	1541.775
						GYFGLIFPGCPSTYEPAQQGR	C10(CAM)	2474.140	2474.145
	Ara h 6	Conglutin	Q647G9	16.9	15.17 [17.74]	CDLDVSGGR	C1(CAM)	978.431	978.431
						CDLDVSGGRC	C1(CAM); C10(CAM)	1138.462	1138.462
						ELMNLPQQCNFR	C9(CAM)	1549.723	1549.725

CAM: Carbamidomethyl

[] Sequence coverage calculated without signal peptide