Circulating Ara h 6 as a marker of peanut protein absorption in tolerant and allergic humans following ingestion of peanut-containing foods

Hervé Bernard, Paul J Turner, Sandrine Ah-leung, Monica Ruiz-garcia, Elizabeth Naomi Clare Mills, Karine Adel-Patient

Received: 13 December 2019 | Revised: 30 March 2020 | Accepted: 29 May 2020

DOI: 10.1111/cea.13706

ORIGINAL ARTICLE

Allergens

WILEY

Circulating Ara h 6 as a marker of peanut protein absorption in tolerant and allergic humans following ingestion of peanut-containing foods

Hervé Bernard[1] | Paul J. Turner[2] | Sandrine Ah-Leung[1] | Monica Ruiz-Garcia[2] | Elizabeth Naomi Clare Mills[3] | Karine Adel-Patient[1]

[1]CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS)/ Service de Pharmacologie et d'Immunoanalyse, Université Paris-Saclay, Gif-sur-Yvette, France

[2]Section of Inflammation, Repair & Development, National Heart & Lung Institute, Imperial College London, London, UK

[3]Division of Infection, Immunity and Respiratory Medicine, School of Biological Sciences and Manchester Institute of Biotechnology, Manchester Academic Health Sciences Centre, University of Manchester, Manchester, UK

Correspondence
Hervé Bernard, CEA, INRAE, DMTS/SPI, Laboratoire d'Immuno-Allergie Alimentaire, Université Paris-Saclay, Bat 136, CEA de Saclay, F-91191 Gif-sur-Yvette, France.
Email: herve.bernard-inra@cea.fr

Funding information
This research is supported by the following grants: the European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement no. 312147 (Integrated Approaches to Food Allergen and Allergy Risk Management [iFAAM]); MRC Clinician Scientist award to PJT (reference MR/K010468/1); UK Food Standards Agency, INRAE. The clinical challenges received additional support through the NIHR Biomedical Research Centre based at Imperial College Healthcare NHS Trust, Royal Brompton Hospitals NHS Foundation Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of the NHS, NIHR, or the Department of Health.

Abstract

Background: Bioaccessibility of food allergens may be a key determinant of allergic reactions.

Objective: To develop a protocol allowing the detection of the major peanut allergen, Ara h 6, in the bloodstream following ingestion of low amounts of peanut and to compare Ara h 6 bioaccessibility by food matrix. We further assessed for differences in absorption in healthy versus peanut-allergic volunteers.

Methods: A blood pretreatment combining acidic shock and thermal treatment was developed. This protocol was then applied to blood samples collected from human volunteers (n = 6, healthy controls; n = 14, peanut-allergic patients) at various time-points following ingestion of increasing levels of peanut incurred in different food matrices (cookies, peanut butter and chocolate dessert). Immunodetection was performed using an in-house immunoassay.

Results: An original pretreatment protocol was optimized, resulting in irreversible dissociation of human antibodies-Ara h 6 immune complex, thus rendering Ara h 6 accessible for its immunodetection. Ara h 6 was detected in samples from all volunteers following ingestion of 300-1000 mg peanut protein, although variations in the kinetics of passage were observed between individuals and matrices. Interestingly, in peanut-allergic subjects, Ara h 6 could be detected following ingestion of lower doses and at higher concentrations than in non-allergic volunteers.

Bernard and Turner are joint first authors and contributed equally to this work.

Conclusions and Clinical Relevance: The kinetics and intensity of Ara h 6 passage in bloodstream depend on both individual and food matrix. Peanut-allergic patients appear to demonstrate higher absorption rate, the clinical significance of which warrants further evaluation.

KEYWORDS
allergy, Ara h 6, bioaccessibility, food matrix, peanut

1 | INTRODUCTION

Allergen exposure in IgE-mediated food allergy can cause clinical reactions within minutes, including anaphylaxis. The release, digestion and subsequent absorption of allergen from a food matrix in the mouth and gastrointestinal tract are likely to be a determinant of allergic reactions. Although the resistance of some food proteins to gastroduodenal digestion impacts on their ability to trigger a systemic response,[1] little is known as to how uptake in the gut affects both symptoms and kinetics of an ensuing allergic reaction.

Absorption of non-digested dietary antigens and their passage into the bloodstream is a normal physiological process. Dietary proteins have been detected in human fluids and compartments in healthy volunteers, reflecting their bioaccessibility.[2] Peanut proteins were detected in serum using a cell-based assay, following both ingestion but also isolated chewing (without ingestion) in non-allergic volunteers, demonstrating the existence of an oral phase to allergen absorption across the buccal mucosa.[3] Food proteins have also been detected at low levels in placental tissue[4-6] and in breastmilk.[7-11] The transfer of food antigens may be enhanced in diseases where intestinal barrier integrity is impaired, such as coeliac disease[12] and in subjects with underlying food allergy,[13-16] although data are limited. Intestinal permeability is not predictive of food allergy,[17] but may play a role in specific conditions such as wheat-dependent exercise-induced anaphylaxis.[13] Whether modified gut permeability might be a contributory factor or consequence of food allergy beyond exercise-induced anaphylaxis to wheat is unclear. However, the assessment of passage of allergens into the bloodstream could yield insights into the pathogenesis of IgE-mediated food allergy and provide information on factors which contribute to symptom severity.[18]

The passage of food allergens into the blood may be affected by the form in which they are ingested, which in turn reflects the impact of food processing and of the food matrix on physicochemical properties of allergens. This can be due to the chemical modification, aggregation, intermolecular interactions and/or denaturation of the allergen itself, as well as interactions of the allergen with matrix components.[19] The inclusion of egg or cow's milk into a baked matrix does not seem to impact on the dose of allergen needed to elicit objective symptoms in allergic patients,[20,21] although fat-rich matrices can impact on both the kinetics of symptoms and potentially their severity, at least for peanut.[22] Higher fat contents may prolong gastric residence time, delaying the onset of symptoms and potentially allowing more allergen to be consumed.[23] The protein content of the food matrix may also affect allergen absorption.[24]

Ara h 6, a peanut 2S albumin, is a small and compact protein which is highly resistant to gastrointestinal digestion and is able to cross the intestinal epithelial barrier in a Caco-2 cell model.[25-27] It is a major allergenic determinant of peanut allergy,[28,29] which is the most common cause of food-related anaphylaxis in Europe.[30,31] Ara h 6 is therefore a good candidate to evaluate transfer of peanut allergen into the blood compartment. Indeed, a recent study detected low levels of Ara h 6 (but not Ara h 2) in the sera of some but not all healthy volunteers following ingestion of high amounts of peanut (at least 25 g peanut protein, equivalent to >100 peanuts).[32] Allergen detection in blood is difficult, because the allergens may be present at very low concentrations and be modified during digestion, impairing detection by antibody-dependent assays. In addition, immunoassays are subject to interference from the interaction of food proteins or fragments derived thereof with other plasma constituents, such as allergen-specific antibodies: JanssenDuijghuijsen et al recently described the significant interference of IgG antibodies on the immunodetection of Ara h 6 following ingestion of peanut by healthy volunteers.[33]

We therefore aimed to develop a simple blood pretreatment to minimize interference from serum proteins such as specific antibodies, irrespective of their isotype and concentration. We combined this approach with an in-house immunoassay previously used for Ara h 6 detection in breastmilk.[10] Using this approach, we assessed the absorption and kinetics of Ara h 6 passage into the blood following ingestion of low amounts of peanut incurred in different food matrices. We also evaluated whether uptake kinetics might differ between peanut-allergic and non-allergic individuals.

2 | MATERIALS AND METHODS

2.1 | Biological material

For the development and optimization of the pretreatment protocol and associated immunoassay, plasma from peanut-allergic patients was purchased from PlasmaLab International (Everett, WA, USA). Following assay optimization, serum samples were collected from healthy volunteers and peanut-allergic patients as described below.

2.2 | Analysis of specific humoral response to Ara h 6 in plasma/serum

Enzyme immunometric assays were performed in 96-well microtiter plates (Immunoplate Maxisorb®, Nunc) using AutoPlate Washer and Microfill dispenser equipment from BioTek instruments (Avantec). Specific antibodies against Ara h 6 purified from roasted peanut[34] were analysed by direct enzyme allergosorbant test on allergen-coated plates, as previously described.[35,36]

2.3 | Food matrices used for feeding trials and food challenges

The challenge matrices are described in Tables S1-S3. Roasted peanuts were purchased from KP nuts; peanut butter from Kraft Foods. Defatted light roasted peanut flour (Golden Peanut Company; 12% fat) was incurred at different levels into a water-continuous dessert base matrix adapted from that developed within the EuroPrevall project (EU-funded FP6) for double-blinded, placebo-controlled food challenges (DBPCFC) and hydrated prior to use.[37,38] The same peanut flour was also included in a baked cookie matrix. The protein content of the defatted peanut flour was determined by Kjeldahl total nitrogen employing a conversion factor of 5.4, while the manufacturer's specified protein content was used for roasted peanut and peanut butter.

2.4 | Human subjects and ethics

Subjects were recruited at Imperial College (London, UK). Informed consent was obtained from all volunteers, and the study was approved by the NHS Health Research Authority (reference 15/LO/0286).

2.5 | Healthy volunteer study

Volunteers (n = 6) were fasted for at least four hours prior to study and had avoided peanut consumption for at least 7 days prior. An intravenous cannula was sited and baseline samples taken, following which volunteers ingested increasing amounts of peanut proteins (100-3000 mg peanut protein) incurred in one of four different matrices (water-continuous dessert matrix, cookie, peanut butter and as roasted peanuts). The different matrices were tested by each volunteer on separate occasions, at least one week apart, and blood samples collected as shown in Figure S1. Due to the excessive amount of water-continuous dessert matrix required to provide a 3000 mg dose, the final dose was instead substituted with a 10 g portion of roasted peanuts (~2.5 g peanut protein) to serve as a positive control. Blood samples were allowed to clot at room temperature for 20 minutes, centrifuged and serum aliquoted and snap-frozen at −80°C.

2.6 | Oral food challenges in peanut-allergic individuals

Peanut-allergic subjects (n = 14; Table 1) were recruited to a randomized crossover study (TRACE Peanut study, described elsewhere)[39] and had undergone initial double-blind, placebo-controlled food challenge (DBPCFC) to peanut to confirm their allergic status. Participants then underwent further DBPCFC conducted according international consensus criteria (PRACTALL).[40] We used an identical protocol to that used in the TRACE Peanut study.[39] In brief, DBPCFC was performed on two separate days, at least 14 days apart. On each day, subjects were fasted from at least 4 hours prior to challenge. A cannula was sited and baseline blood sample collected. Subjects then received increasing doses, every 30 minutes, of peanut protein (or placebo) at the following doses: 3 μg, 30 μg, 300 μg, 3 mg, 30 mg, 100 mg, 300 mg and 1000 mg (incurred in the same water-continuous dessert matrix used above) until stopping criteria were met.[39] The order of DBPCFC challenges was determined by a computer-generated randomization table. Serum samples were collected at 30 and 120 minutes after occurrence of objective allergic reaction and processed as above.

2.7 | Production and characterization of monoclonal antibody for detection of Ara h 6

Anti-Ara h6 monoclonal antibodies (mAbs) were generated in mice using a classical fusion procedure,[41] and Ara h 6 purified from roasted peanut as immunogen. The antibody-secreting cells (hybridomas) were screened by analysing cell supernatants on microtiter plates coated with goat anti-mouse antibodies (Jackson ImmunoResearch, Europe Ltd.) and using acetylcholinesterase (AChE)-labelled Ara h 6 or Ara h 2 as tracers. Positive hybridoma cells were cloned and further expanded to obtained mAbs. mAbs were first screened for

TABLE 1 Baseline demographics of peanut-allergic volunteers

	Peanut-allergic volunteers (n = 14) expressed as median and (range) values
Age (y)	26 (18-40)
Gender (% male)	50%
SPT to peanut (mm)	10 (5-23)
IgE to peanut (kUA/L)	15.7 (0.6-111)
IgE to Ara h 1 (kUA/L)	0.71 (<0.1-26)
IgE to Ara h 2 (kUA/L)	8.7 (0.2-74)
IgE to Ara h 3 (kUA/L)	<0.1 (<0.1-15)
IgE to Ara h 6 (kUA/L)	4.8 (0.6-48)
IgG1 to Ara h 6 (ng/mL)	900 (280-3380)
IgG4 to Ara h6 (ng/mL)	240 (<10-820)
Eliciting dose at challenge (mg peanut protein)	133 (33-1433)
Anaphylaxis at challenge	21%

reactivity towards Ara h 6 and Ara h 2, and their specificity fully characterized using mutated Ara h 6, proteolysed isoforms of Ara h 6 and chimeric 2S-albumins, as previously described for human IgE-binding studies.[34,42,43] Anti-Ara h 6 mAbs produced have been involved in various studies.[44-46] The two mAbs selected for the current study recognized proteolysed forms of Ara h 6 and were directed against C-terminal and N-terminal part of Ara h 6, respectively.

2.8 | Ara h 6 quantification in human plasma/serum samples

Anti-Ara h 6 mAb was immobilized on 96-well microtiter plates (Immunoplate Maxisorp®, Nunc) at 5 μg/mL in 50 mM phosphate buffer, pH 7.4, for 24 h at 4°C. Plates were then washed and saturated for at least 4 h at room temperature using EIA buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin free of protease and immunoglobulins (Sigma-Aldrich), 0.15 M NaCl, 0.01% sodium azide, pH 7.4). Diluted plasma/serum samples (1/3 to 1/5) or internal standards (see below) were added to each well (50 μL) and incubated for 2 hours at room temperature under agitation. Wells were then washed and 50 μL of the complementary biotinylated anti-Ara h 6 mAb (antibody:biotin molar ratio 1:30, EZ-link® NHS-PEG4-biotin, Thermo Scientific) were dispatched to each well at a concentration of 100 ng/mL in EIA buffer containing mouse IgG from non-immune serum (25 μg/mL) in order to suppress interference due to heterophilic antibodies. After a further 2 hours incubation at room temperature, wells were washed and 50 μL of AChE-labelled streptavidin were dispensed for 20 minutes at room temperature. After washing, Ellman's reagent was used as enzyme substrate and absorbance was measured at 414 nm using automatic reader plates. $Mean_{blank}$ and SD_{blank} were estimated by measuring 8 to 10 replicates of signals obtained with EIA buffer. The limit of detection (LoD, $mean_{blank} + 3xSD_{blank}$) and of quantification (LoQ, $mean_{blank} + 10^{*}SD_{blank}$) of Ara h 6 were 0.006 and 0.008 ng/mL, respectively. No cross-reactivity was observed with other peanut proteins, including Ara h 2 (Table S4).

To quantify Ara h 6 in samples from the volunteers/allergic patients after the different feeding trials or DBPCFC, Whole Peanut Protein Extract (WPPE) was prepared from each of the peanut source incurred in matrices (ie roasted peanut, peanut flour or peanut butter) and used as a standard for Ara h 6 quantification. One gram of roasted peanut, peanut flour or peanut butter was resuspended in 20 mL of 0.3% sodium borate pH 9.0 including 0.9% n-Octyl-β-D-glucopyranoside, 10 mM EDTA and a protease inhibitor cocktail (4-(2-aminoethyl) benzenesulphonyl fluoride, leupeptin, pepstatin A, chymostatin, benzamidine). Suspension was further homogenized using an Ultra-Turrax® Tube drive (IKA®-Werke) with individual dispersing vessel (DT-50). After stirring for 2 hours at 40°C, the suspension was centrifuged at 3000 *g* for 20 minutes at 4°C. The supernatant (S1) was collected, and the pellet suspended in 4 M guanidinium chloride. After stirring for 1 hour at 40°C, the soluble fraction was separated by centrifugation and a second supernatant (S2) collected. The two supernatants were separately dialysed against 20 mM phosphate pH 7.4 buffer. Protein content was quantified using bicinchoninic acid (BCA) assay and the two supernatants characterized by SDS-PAGE analysis and finally pooled before aliquoting and storing at −80°C. Protein recovery was at least 75%. Ara h 6 content in the different WPPEs were assessed by the sandwich ELISA assay described in this work, using Ara h 6 purified from roasted peanut as a reference.[34] Whole peanut protein extracted from roasted peanut, peanut flour or butter contained 3.8%, 4% and 4.4% of Ara h 6, respectively.

2.9 | Treatment of blood samples

Two hundred microlitre aliquots of plasma or serum were mixed with 120 μL of 10 mM phosphate buffer pH 7.4. Then, 80 μL of various buffers (1 M sodium citrate pH 3.0, 1 M sodium acetate pH 4.0, 1 M sodium acetate pH 5.0, 1 M potassium phosphate pH7.4) was added to a final concentration of 0.2 M and final pH ranging from 3 to 7.4 (acidic shock), with or without an additional heating step (30 minutes at 60°C). Addition of acetonitrile was also tested. Samples were then centrifuged (10 000 *g*, 10 min, +20°C) and 200 μL of supernatants collected and pH carefully adjusted to 7-7.5 with 2 M NaOH. A solution of 0.2 M phosphate buffer pH 7.4, 0.3% bovine serum albumin free of protease and immunoglobulins, 0.45 M NaCl was then added to adjust final volume at 300 μL before Ara h 6 quantification.

2.10 | Statistical analysis

All statistical analyses were performed using Prism for Windows (GraphPad Software). For non-allergic volunteers, paired non-parametric test was performed, using Wilcoxon test to compare values during kinetics. Absorption of Ara h 6 in allergic and non-allergic volunteers at any given dose/time-point were compared using Mann-Whitney *U* test between groups and Friedman's test within the same group. In all cases, differences between groups were regarded as significant when $P < .05$.

3 | RESULTS

3.1 | Endogenous antibodies interfere with Ara h 6 detection in plasma/serum

We first evaluated immunodetection of Ara h 6 in blood samples from healthy volunteers collected prior to peanut ingestion and then spiked with WPPE (0-10 ng/mL, corresponding to 0-0.4 ng/mL of Ara h 6). Ara h 6 was poorly detected in spiked serum (Figure S2A), and detection was not enhanced when using a mix of mAbs with various specificities, both as capture and revelation antibodies, in the Ara h 6 immunoassays (Figure S2B). Similar experiment performed on individually spiked sera from 4 other healthy volunteers showed comparable results, with a maximum recovery of 50% depending on

the serum tested. These results suggested that factors present in serum were interfering with Ara h 6 detection.

Ara h 6 recovery was also poor (around 10%) when analysing pooled plasma from peanut-allergic patients spiked with 2.5-10 ng/ml of WPPE (corresponding to 0.1-0.4 ng/ml of Ara h 6) (Figure S3). Ara h 6 detection was improved (recovery of 30%) if the plasma had been depleted of IgE, and was increased up to ~70% when upstream partial depletion of immunoglobulins was performed using protein A (removing mainly IgGs), demonstrating that antibodies (such as IgG and IgE) present in plasma were causing significant interference with Ara h 6 immunodetection (Figure S3). Moreover, Ara h 6 was poorly detected in the flowthrough fraction when plasma was spiked prior to protein A depletion, indicating that Ara h 6 was complexed to immunoglobulins retained on protein A.

Accordingly, Ara h 6-specific IgE, IgG1, IgG2 and IgG4 were detected at significant levels in blood samples from healthy volunteers and peanut-allergic patients (Table S5). Sera from healthy volunteers contained less anti-Ara h 6 IgGs compared to peanut-allergic patients, and no detectable IgE to Ara h 6. Allergic patients exhibited various levels of IgE, IgG1 and IgG4 specific to Ara h 6 (0.6-48 kUA/L, 280-3380 ng/mL and <10-820 ng/mL respectively).

3.2 | Development of a pretreatment protocol to suppress interference due to endogenous anti-Ara h 6 antibodies

We then tested various pretreatment protocols on spiked samples with the aim to irreversibly dissociate endogenous plasma protein-Ara h 6 complexes, thus liberating Ara h 6 for immunodetection. Tested conditions relied on acidic shock, with or without additional heat treatment. We utilized commercially-sourced pooled plasma from peanut-allergic patients due to its anti-Ara h 6 IgE and IgGs levels and the availability of sufficient volume for protocol development. We observed that Ara h 6 recognition by mAb was not drastically modified by the tested treatments, by assaying Ara h 6 in buffer spiked with 20 ng/mL WPPE (0.8 ng/mL of Ara h 6) and then treated (Figure 1, empty grey bars). No background signal intensity, assessed on non-spiked buffer, was observed after the different treatments. Ara h 6 immunodetection in spiked buffer was only slightly decreased after acidic shock, and additional heating of sample (30 minutes, 60°C) after acidic shock did not further decrease recovery (Figure 1 grey bars, conditions C-IV to CVIII).

A similar experiment was then performed using pooled plasma from peanut-allergic patients spiked with 20 ng/mL WPPE. We confirmed that dilution of spiked plasma in phosphate buffer led to a very low recovery of Ara h 6 below 5% (Figure 1, red bar, condition C-0). Recovery remained very low (≈10%) when performing an acidic shock (pH ranging from 5 to 3). Additional heating after acidic shock resulted in formation of a precipitate in the plasma (which was removed by centrifugation); final recovery of Ara h 6 in the supernatant was greatly enhanced (red bars, conditions C-V to C-VII). Highest recovery was obtained when acidic shock (0.2 M citrate buffer pH3.0) and heat treatment (60°C, 30min) were performed simultaneously, with more than 90% of Ara h 6 recovered (condition C-VIII), which was equivalent to that observed in spiked buffer under the same conditions. This strategy was selected as the optimal pretreatment for further evaluation.

Buffer or pooled plasma from peanut-allergic patients was then spiked with increasing concentrations of peanut proteins, ranging

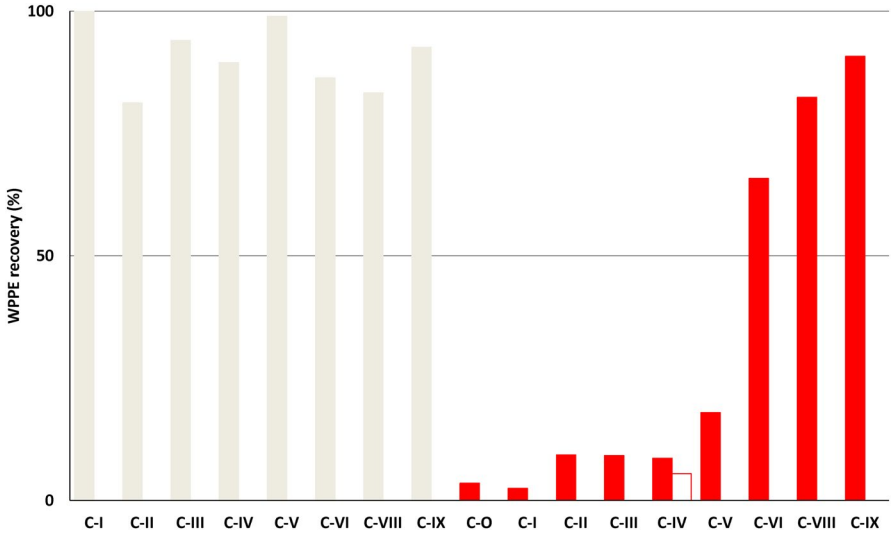

FIGURE 1 Assessment of the impact of various sample treatments on final recovery of Ara h 6 in buffer or in plasma previously spiked or not with 20 ng/mL of whole peanut protein extract (WPPE). Beige full bars: buffer spiked with 20 ng/mL of WPPE. Red full bars: plasma from peanut-allergic patients spiked with 20 ng/mL of WPPE. Open bars: Non-specific binding observed in non-spiked buffer or plasma submitted to the same treatments. Samples treatments: C-0: phosphate pH 7.4; C-I: acetate pH 5.0; C-II: acetate pH 4.0; C-III: acetate pH 4.0 + ACN; C-IV: phosphate pH 7.4 + 60°C for 30 min; C-V: acetate pH 5.0 + 60°C for 30 min; C-VI: acetate pH 4.0 + 60°C for 30 min; C-VII: citrate pH 3.0 + 60°C for 30 min; C-VIII: simultaneous citrate pH 3.0 and 60°C for 30 min

from 0.16 to 20 ng/mL (corresponding to 0.007-0.80 ng/mL of Ara h 6), and then treated (or not) using our optimal pretreatment. Whereas Ara h 6 was poorly detected in spiked sera without treatment, even at higher concentrations, recovery was high in treated plasma (Figure S4). Curves obtained in treated plasma were equivalent to those obtained in buffer. The limit of detection for Ara h 6 in spiked and treated sera was 0.2 ng/mL of equivalent peanut protein, close to that obtained in buffer.

Finally, spiking experiments were performed on individual serum samples from healthy volunteers (n = 6) and allergic patients (n = 14) collected before the feeding trials. Each sample was spiked (or not) with 20 ng/mL WPPE (corresponding to 0.8 ng/mL of Ara h 6). Ara h 6 detection was then performed on both spiked and unspiked sera, with or without the acidic shock/heat pretreatment. No significant signal was observed in non-spiked basal serum (with our without treatment). In untreated samples, 22%-50% of Ara h 6 was recovered in sera from healthy volunteers, but <5% in spiked samples from peanut-allergic patients (Figure 2). However, pretreatment resulted in Ara h 6 recovery of 88%-99% and 72%-98% in individual samples from healthy volunteers and allergic patients, respectively (Figure 2).

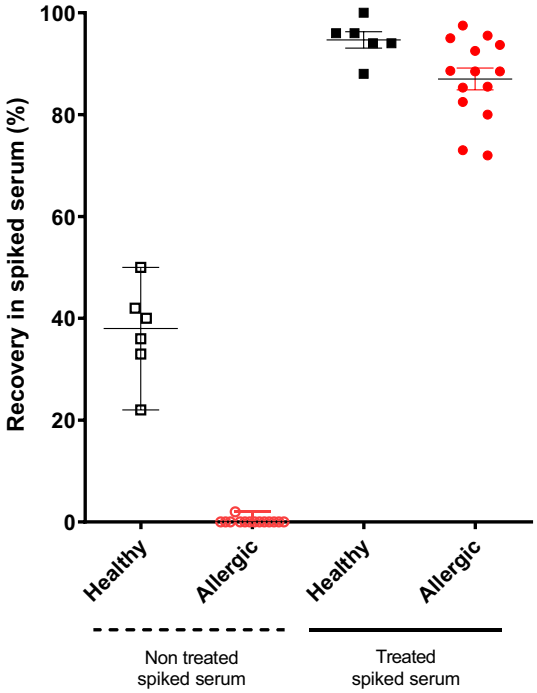

FIGURE 2 Recovery of Ara h 6 in serum from 6 healthy (black square) and 14 allergic volunteers (red circle) spiked with 20 ng/mL of WPPE. Aliquots of spiked serum were untreated (open symbols) or treated (closed symbols) using simultaneous acidic shock (citrate, pH 3) and heating (60°C, 30 minutes) and diluted to 1/3 before Ara h 6 detection

3.3 | Detection of Ara h 6 in blood from healthy volunteers following roasted peanut kernel ingestion

Ara h 6 was then assayed in serum samples from six healthy volunteers (median age 28 years, 50% male) collected following ingestion of increasing quantities of peanut as the roasted kernel (protocol shown in Figure S1) and then subjected to pretreatment.

Ingestion of 100 mg peanut protein led to significant detection of Ara h 6 in four of six healthy volunteers, 30 min post-ingestion (Figure 3). Ara h 6 was detected in all the 6 volunteers following ingestion of 300 mg peanut protein, and its concentration in serum increased proportionately with the amount of peanut consumed. Thirty minutes following ingestion of 3000 mg peanut protein, 0.12-0.36 ng/mL (mean 0.20 ng/mL) of Ara h 6 was detected, corresponding to an equivalent of 3-9 ng/mL of WPPE (mean 5 ng/mL). Assuming a circulating blood volume of ~5 L in volunteers, this equates to a recovery of ~0.001% of peanut protein consumed. Slight variability in both Ara h 6 levels and kinetics of absorption were observed between healthy volunteers: Ara h 6 in serum increased progressively in volunteers A and E over 2 hours, while in volunteers C and F, peak levels were observed at 30 minutes after ingestion.

3.4 | Impact of the food matrix on Ara h 6 bioaccessibility

We then compared the absorption kinetics of Ara h 6 in the same healthy volunteers after ingestion of peanut incurred in different matrices, that is peanut kernel, cookies, water-continuous dessert base and peanut buffer. Ara h 6 was detected in most of the volunteers 30 minutes after ingestion of 300 mg of peanut protein, irrespective of the matrix (Figure 4). However, there were differences between the matrices tested: Ara h 6 was detected in all samples from 30 minutes following ingestion of 1000 mg peanut protein when incorporated into a cookie or peanut butter, but only after 120 minutes with the water-continuous dessert base. Interestingly, Ara h 6 concentrations in serum were highest following ingestion of peanut as the roasted kernel. We did not otherwise observe statistical differences in absorption/detection kinetics between the different matrices, although these data are limited by the small sample size and inter-individual variability.

3.5 | Absorption of Ara h 6 in peanut-allergic compared to healthy volunteers

Finally, we compared minimum ingestion levels of peanut needed to result in detectable Ara h 6 in treated samples, in 8 healthy (six of whom were included in the data presented above) and 14 peanut-allergic subjects, the latter who had undergone repeat DBPCFC to peanut using the same water-continuous dessert base as that eaten by non-allergic volunteers. In allergic subjects, Ara h 6 was detected following consumption amounts as low as 30 mg peanut protein ($P < .05$, Friedman's test) in contrast to healthy volunteers ingesting the same discrete dose of peanut protein (Figure 5). No Ara h 6 was detected at placebo challenge.

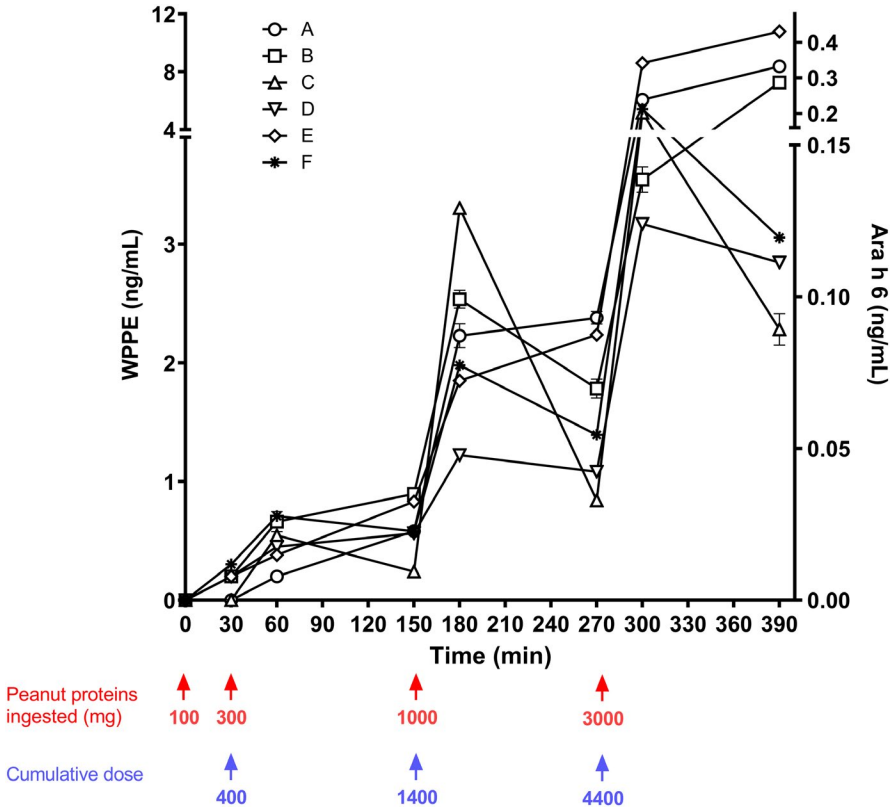

FIGURE 3 Kinetics of Ara h 6 bioaccessibility assessed in six healthy volunteers who ingested increasing amounts of peanut proteins as the roasted kernel. Ara h 6 immunoassay was performed on individual plasma collected at various time-points (Figure S1). Samples were treated using simultaneous acidic shock (citrate, pH 3) and heating (60°C, 30 minutes). Results are expressed as ng/mL of whole peanut protein extract (Left *Y*-axis) and as ng/mL of Ara h 6 (Right *Y*-axis)

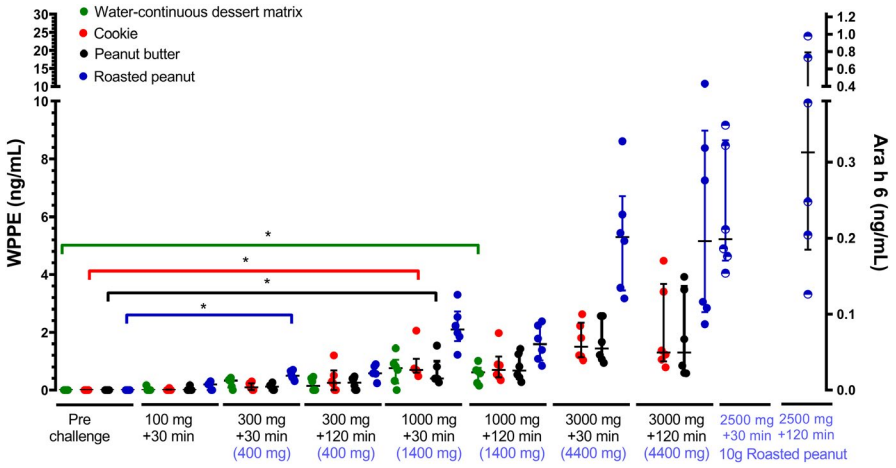

FIGURE 4 Ara h 6 detection expressed as ng/mL of WPPE (Left *Y*-axis) and as ng/mL of Ara h 6 (Right *Y*-axis) in treated serum from the six healthy volunteers who ingested increasing amount of peanut proteins incorporated in various food matrices and as roasted peanut kernels. *$P < .05$

4 | DISCUSSION

Immunoassays generally remain the method of choice in detecting food allergens in complex food matrices due to their high sensitivity, specificity and simplicity, and have also been applied to the detection of peanut allergens such as Ara h 6 in human biological samples.[10,11,32,33] However, endogenous proteins, particularly in the blood, can cause significant interference, either by inducing a non-specific signal (eg heterophilic antibodies) or limiting the detection of the target molecule. Immunoassays (including Ara h 6 ELISA) have been used to evaluate peanut absorption in healthy individuals following consumption of high amounts (100 g) of roasted peanut or peanut flour, but Ara h 6 was undetectable in 33%-40% of volunteers. [32,33] Using spiking experiments in combination with immunoglobulin G depletion, JanssenDuijghuijsen et al concluded that allergen-specific IgG/IgG_4 in the blood of healthy volunteers interfered with the detection of Ara h 6 following peanut consumption.[33] Our data are consistent with this and further suggest that Ara h 6-specific IgG (IgG1 and IgG4) but also IgE antibodies may interfere or even totally inhibit Ara h 6 immunodetection, even with a highly sensitive immunoassay. We further demonstrated through spiking experiments that the recovery of Ara h 6 is dramatically lower in serum from peanut-allergic patients compared to non-allergic individuals who presented with lower concentrations of Ara h 6-specific IgG and no IgE.

We therefore developed a specific pretreatment to induce irreversible dissociation of human Ig-Ara h 6 complexes, thus liberating Ara h 6 for detection. Such a treatment had to be compatible

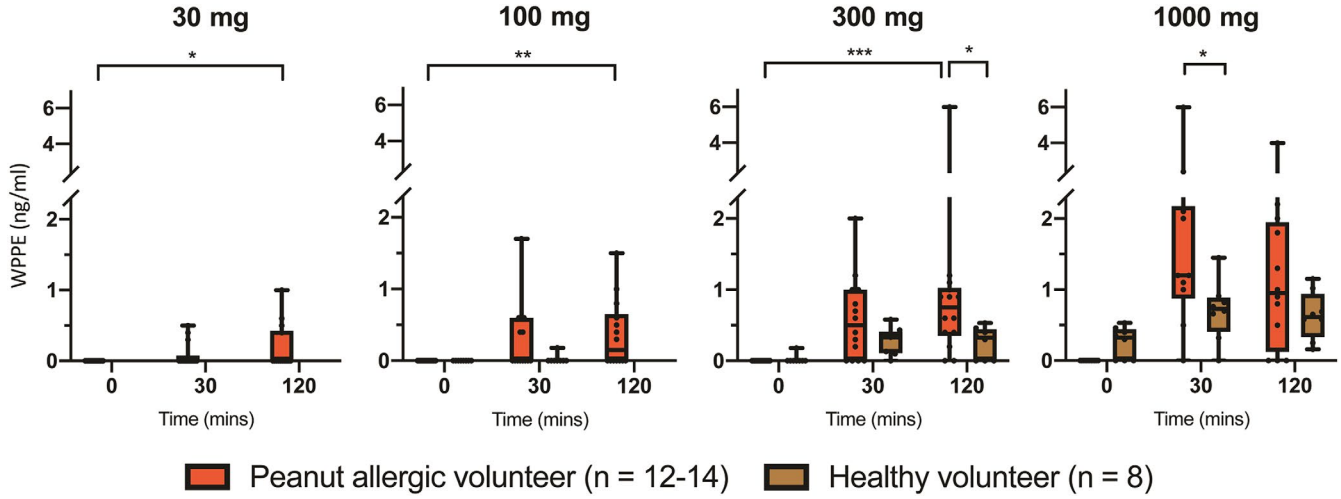

FIGURE 5 Ara h 6 detection (expressed in equivalents of WPPE) in treated serum samples from 8 healthy volunteers versus 14 allergic volunteers prior to, and following ingestion of discrete doses of peanut protein in a water-continuous dessert base. Samples were collected 30 and 120 minutes after consumption in healthy volunteers, and 30 and 120 minutes following objective reaction to the same amount of peanut protein consumed in allergic individuals. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$

with our immunoassay conditions and not cause structural changes that would prevent recognition of Ara h 6 by the mAb used in the immunoassay. Acidic shock is classically used to disrupt the binding of antigens to antibodies during immunopurification, but the acidic conditions tested were insufficient, probably because they did not irreversibly denature antibodies and prevent further re-association of endogenous antibody to the liberated Ara h 6. Various physical and chemical methods have been previously used to fractionate serum proteins. For example, heating plasma to 60°C at pH 5 induced denaturation and precipitation of proteins other than albumin.[47] Many studies have described the impact of temperature and/or pH on denaturation, unfolding and aggregation states of immunoglobulins,[48-50] thus reducing their capacity to bind antigen. Since Ara h 6 has been shown to be a highly structured thermostable soluble protein,[51] we applied various combinations of acidic and heat pretreatment to dissociate Ara h 6-Ig complexes and then denature the dissociated antibodies. The resulting procedure, combining simultaneous acidic shock at pH 3 and heating at 60°C, allowed almost complete recovery of Ara h 6, irrespective of the concentrations and isotype of endogenous antibodies. Although the procedure may have led to slight structural modifications of Ara h 6, it allowed further recognition of Ara h 6 without compromising sensitivity.

With this strategy, we were successful in detecting Ara h 6 in serum samples collected from both healthy and allergic volunteers following ingestion of much lower amounts of peanut (<1 g) than that previously reported in the literature. Actually, we were able to detect Ara h 6 following consumption of 300 mg peanut protein (equivalent to ~1½ peanuts) by all healthy volunteers, with Ara h 6 detection increasing in a dose-dependent manner. JanssenDuijghuijsen et al estimated that their assay was able to detect 0.0001% of Ara h 6 intake, following consumption of 100 g peanut (~25 g peanut protein).[33] Applying a similar methodology to our data, we were able to measure 0.12-0.36 ng/mL of Ara h 6 (3-9 ng/mL of WPPE) in serum from volunteers, 30 minutes following ingestion of 120 mg of Ara h 6 (3000 mg of peanut proteins from roasted kernels). This equates to ~0.001% of estimated intake, that is ~10× more recovery than that described by JanssenDuijghuijsen et al.

Of note, significantly lower levels of ingestion (just 30 mg peanut protein) were required in peanut-allergic individuals to detect peanut protein in patient sera. In addition, higher levels of Ara h 6 were detected in sera from peanut-allergic individuals compared to healthy controls, for the same dose of peanut exposure in the same matrix (the feeding protocols could not be identical, due to the need to utilize a low dose, incremental challenge protocol for allergic volunteers to ensure patient safety). These data imply that peanut-allergic individuals have different absorption kinetics from non-allergic individuals, potentially due to antibody-mediated facilitated absorption, something that has also been demonstrated via the low affinity-IgE receptor in a laboratory model.[52] Whether these differences contribute towards the development of clinical allergy needs further investigation.

The kinetics of Ara h 6 passage into the bloodstream observed in human healthy volunteers are consistent with previous work on absorption of allergenic proteins in non-allergic human volunteers who ingested peanut,[3] although inter-individual variability was evident, with some volunteers showing a constant and progressive increase of Ara h 6 concentrations in plasma from ingestion to 120 minutes post-ingestion, while others achieved peak levels within 30 minutes. Other factors can also modify the bioaccessibility of food proteins. Food processing may alter structure of allergens, depending on their physicochemical properties. Moreover, the composition of the food matrix and its caloric value alters the digestive process, modifying digestive secretions and other factors such as gastric residence time. These will all impact on apparent allergen bioaccessibility. It is difficult to determine which might be the predominant factors in explaining the differences in Ara h 6 kinetics we observed. In healthy

volunteers, the bioaccessibility of Ara h 6 was greatest when peanut was ingested as the roasted kernel. Thus, incorporating peanut in complex food matrix or using defatted peanut flour is likely to affect Ara h 6 passage into bloodstream. The fat content of the food matrix can affect onset and severity of symptoms experienced in peanut-allergic individuals,[22,23] which may be due to reduced IgE-binding capacity to peanut proteins for food matrices with higher fat content[22,53] and/or delayed gastric emptying.[23]

In conclusion, we have developed a method which allows for the evaluation of peanut allergen passage into the blood following ingestion of small amounts of peanut protein in human subjects, something which hitherto has not been possible in allergic individuals due to the small levels of allergen exposure needed to trigger reactions. Our study underlines the variability of absorption kinetics between volunteers—and in particular, allergic versus non-allergic subjects—and the impact of the food matrix on bioaccessibility. Further studies are needed to assess how other factors, such as medication, alcohol and exercise, may further impact upon this, and the extent to which levels of bioaccessible allergen may determine clinical reactions.

ACKNOWLEDGEMENTS

We thank Professor Stephen Durham, Dr Robert Boyle and Dr Isabel Skypala for their clinical support. We also thank all the volunteers who provided samples for the analyses presented in this report, including allergic volunteers recruited through the TRACE Peanut study (funded by the UK Food Standards Agency). We are grateful to the TRACE study investigators (CI A Clark) and the Food Standards Agency for their support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

CLINICAL TRIAL REGISTRATION

The study was approved by the UK NHS Health Research Authority (reference 15/LO/0286).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Hervé Bernard https://orcid.org/0000-0003-3971-1021
Paul J. Turner https://orcid.org/0000-0001-9862-5161
Karine Adel-Patient https://orcid.org/0000-0002-2242-0626

REFERENCES

1. Moreno FJ. Gastrointestinal digestion of food allergens: effect on their allergenicity. *Biomed Pharmacother Biomedecine Pharmacother*. 2007;61:50-60.
2. Husby S. Dietary antigens: uptake and humoral immunity in man. *APMIS Suppl*. 1988;1:1-40.
3. Dirks CG, Pedersen MH, Platzer MH, Bindslev-Jensen C, Skov PS, Poulsen LK. Does absorption across the buccal mucosa explain early onset of food-induced allergic systemic reactions? *J Allergy Clin Immunol*. 2005;115:1321-1323.
4. Edelbauer M, Loibichler C, Nentwich I, Gerstmayr M, Urbanek R, Szépfalusi Z. Maternally delivered nutritive allergens in cord blood and in placental tissue of term and preterm neonates. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2004;34:189-193.
5. Loibichler C, Pichler J, Gerstmayr M, et al. Materno-fetal passage of nutritive and inhalant allergens across placentas of term and preterm deliveries perfused in vitro. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2002;32:1546-1551.
6. Vance GHS, Lewis SA, Grimshaw KEC, et al. Exposure of the fetus and infant to hens' egg ovalbumin via the placenta and breast milk in relation to maternal intake of dietary egg. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2005;35:1318-1326.
7. Lovegrove JA, Hampton SM, Morgan JB. The immunological and long-term atopic outcome of infants born to women following a milk-free diet during late pregnancy and lactation: a pilot study. *Br J Nutr*. 1994;71:223-238.
8. Fukushima Y, Kawata Y, Onda T, Kitagawa M. Consumption of cow milk and egg by lactating women and the presence of beta-lactoglobulin and ovalbumin in breast milk. *Am J Clin Nutr*. 1997;65:30-35.
9. Palmer DJ, Gold MS, Makrides M. Effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomized, double-blind, cross-over trial. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2005;35:173-178.
10. Bernard H, Ah-Leung S, Drumare M-F, et al. Peanut allergens are rapidly transferred in human breast milk and can prevent sensitization in mice. *Allergy*. 2014;69:888-897.
11. Schocker F, Scharf A, Kull S, Jappe U. 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. *Int Arch Allergy Immunol*. 2017;174:17-25.
12. Husby S, Foged N, Høst A, Svehag SE. Passage of dietary antigens into the blood of children with coeliac disease. Quantification and size distribution of absorbed antigens. *Gut*. 1987;28:1062-1072.
13. Matsuo H, Morimoto K, Akaki T, et al. Exercise and aspirin increase levels of circulating gliadin peptides in patients with wheat-dependent exercise-induced anaphylaxis. *Clin Exp Allergy J Br Soc Allergy Clin Immunol*. 2005;35:461-466.
14. Ventura MT, Polimeno L, Amoruso AC, et al. Intestinal permeability in patients with adverse reactions to food. *Dig Liver Dis*. 2006;38:732-736.
15. Järvinen KM, Konstantinou GN, Pilapil M, et al. Intestinal permeability in children with food allergy on specific elimination diets. *Pediatr Allergy Immunol*. 2013;24:589-595.
16. König J, Wells J, Cani PD, et al. Human intestinal barrier function in health and disease. *Clin Transl Gastroenterol*. 2016;7:e196.
17. Perrier C, Corthésy B. Gut permeability and food allergies. *Clin Exp Allergy*. 2011;41:20-28.
18. Turner PJ, Baumert JL, Beyer K, et al. Can we identify patients at risk of life-threatening allergic reactions to food? *Allergy*. 2016;71:1241-1255.
19. Teuber SS. Hypothesis: the protein body effect and other aspects of food matrix effects. *Ann N Y Acad Sci*. 2002;964:111-116.
20. Remington BC, Westerhout J, Campbell DE, Turner PJ. Minimal impact of extensive heating of hen's egg and cow's milk in a food matrix on threshold dose-distribution curves. *Allergy*. 2017;72:1816-1819.
21. Libbers L, Flokstra-de Blok BMJ, Vlieg-Boerstra BJ, et al. No matrix effect in double-blind, placebo-controlled egg challenges in egg allergic children. *Clin Exp Allergy*. 2013;43:1067-1070.
22. Grimshaw KEC, King RM, Nordlee JA, Hefle SL, Warner JO, Hourihane JOB. Presentation of allergen in different food preparations affects the nature of the allergic reaction–a case series. *Clin Exp Allergy*. 2003;33:1581-1585.

23. Mackie A, Knulst A, Le T-M, et al. High fat food increases gastric residence and thus thresholds for objective symptoms in allergic patients. *Mol Nutr Food Res*. 2012;56:1708-1714.
24. Schulten V, Lauer I, Scheurer S, Thalhammer T, Bohle B. A food matrix reduces digestion and absorption of food allergens in vivo. *Mol Nutr Food Res*. 2011;55:1484-1491.
25. Lehmann K, Schweimer K, Reese G, et al. Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions. *Biochem J*. 2006;395:463-472.
26. 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.
27. Price DB, Ackland ML, Burks W, Knight MI, Suphioglu C. Peanut allergens alter intestinal barrier permeability and tight junction localisation in Caco-2 cell cultures. *Cell Physiol Biochem*. 2014;33:1758-1777.
28. Blanc F, Adel-Patient K, Drumare M-F, Paty E, Wal J-M, Bernard H. Capacity of purified peanut allergens to induce degranulation in a functional in vitro assay: Ara h 2 and Ara h 6 are the most efficient elicitors. *Clin Exp Allergy*. 2009;39:1277-1285.
29. Klemans RJB, Knol EF, Bruijnzeel-Koomen CAFM, Knulst AC. The diagnostic accuracy of specific IgE to Ara h 6 in adults is as good as Ara h 2. *Allergy*. 2014;69:1112-1114.
30. Worm M, Moneret-Vautrin A, Scherer K, et al. First European data from the network of severe allergic reactions (NORA). *Allergy*. 2014;69:1397-1404.
31. Turner PJ, Gowland MH, Sharma V, et al. Increase in anaphylaxis-related hospitalizations but no increase in fatalities: an analysis of United Kingdom national anaphylaxis data, 1992–2012. *J Allergy Clin Immunol*. 2015;135:956-963.e1.
32. Mose AP, Mortz E, Skov PS, et al. The quest for ingested peanut protein in human serum. *Allergy*. 2020;75:1721-1729.
33. JanssenDuijghuijsen LM, Wichers HJ, van Norren K, et al. Detection of peanut allergen in human blood after consumption of peanuts is skewed by endogenous immunoglobulins. *J Immunol Methods*. 2017;440:52-57.
34. Bernard H, Mondoulet L, Drumare MF, et al. Identification of a new natural Ara h 6 isoform and of its proteolytic product as major allergens in peanut. *J Agric Food Chem*. 2007;55:9663-9669.
35. Bernard H, Paty E, Mondoulet L, et al. Serological characteristics of peanut allergy in children. *Allergy*. 2003;58:1285-1292.
36. Adel-Patient K, Lezmi G, Castelli FA, et al. Deep analysis of immune response and metabolic signature in children with food protein induced enterocolitis to cow's milk. *Clin Transl Allergy*. 2018;8:38.
37. Cochrane SA, Salt LJ, Wantling E, et al. Development of a standardized low-dose double-blind placebo-controlled challenge vehicle for the EuroPrevall project. *Allergy*. 2012;67:107-113.
38. Fernández-Rivas M, Barreales L, Mackie AR, et al. The EuroPrevall outpatient clinic study on food allergy: background and methodology. *Allergy*. 2015;70:576-584.
39. Dua S, Ruiz-Garcia M, Bond S, et al. Effect of sleep deprivation and exercise on reaction threshold in adults with peanut allergy: a randomized controlled study. *J Allergy Clin Immunol*. 2019;144:1584-1594.e2.
40. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, et al. Standardizing double-blind, placebo-controlled oral food challenges: American Academy of Allergy, Asthma & Immunology-European Academy of Allergy and Clinical Immunology PRACTALL consensus report. *J Allergy Clin Immunol*. 2012;130:1260-1274.
41. Negroni L, Bernard H, Clement G, et al. Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. *J Immunol Methods*. 1998;220:25-37.
42. Hazebrouck S, Guillon B, Drumare M-F, Paty E, Wal J-M, Bernard H. Trypsin resistance of the major peanut allergen Ara h 6 and allergenicity of the digestion products are abolished after selective disruption of disulfide bonds. *Mol Nutr Food Res*. 2012;56:548-557.
43. Hazebrouck S, Guillon B, Paty E, Adel-Patient K, Bernard H. Conformational IgE-binding epitopes of major peanut allergen Ara h 2 revealed with chimeric 2S-albumins. *J Allergy Clin Immunol*. 2018;141:AB179.
44. Clement G, Boquet D, Mondoulet L, Lamourette P, Bernard H, Wal JM. Expression in *Escherichia coli* and disulfide bridge mapping of PSC33, an allergenic 2S albumin from peanut. *Protein Expr Purif*. 2005;44:110-120.
45. Bernard H, Drumare M-F, Guillon B, Paty E, Scheinmann P, Wal J-M. Immunochemical characterisation of structure and allergenicity of peanut 2S albumins using different formats of immunoassays. *Anal Bioanal Chem*. 2009;395:139-146.
46. Guillon B, Bernard H, Drumare M-F, Hazebrouck S, Adel-Patient K. Heat processing of peanut seed enhances the sensitization potential of the major peanut allergen Ara h 6. *Mol Nutr Food Res*. 2016;60:2722-2735.
47. Denizli A. Plasma fractionation: conventional and chromatographic methods for albumin purification. *JBiolChem*. 2011;39:315-341.
48. Akazawa-Ogawa Y, Nagai H, Hagihara Y. Heat denaturation of the antibody, a multi-domain protein. *Biophys Rev*. 2018;10:255-258.
49. Wu H, Truncali K, Ritchie J, et al. Weak protein interactions and pH- and temperature-dependent aggregation of human Fc1. *mAbs*. 2015;7:1072-1083.
50. Vermeer AW, Norde W. The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. *Biophys J*. 2000;78:394-404.
51. Suhr M, Wicklein D, Lepp U, Becker W-M. 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.
52. Li H, Nowak-Wegrzyn A, Charlop-Powers Z, et al. Transcytosis of IgE-antigen complexes by CD23a in human intestinal epithelial cells and its role in food allergy. *Gastroenterology*. 2006;131:47-58.
53. van Odijk J, Ahlstedt S, Bengtsson U, Borres MP, Hulthén L. Double-blind placebo-controlled challenges for peanut allergy the efficiency of blinding procedures and the allergenic activity of peanut availability in the recipes. *Allergy*. 2005;60:602-605.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Bernard H, Turner PJ, Ah-Leung S, Ruiz-Garcia M, Clare Mills EN, Adel-Patient K. Circulating Ara h 6 as a marker of peanut protein absorption in tolerant and allergic humans following ingestion of peanut-containing foods. *Clin Exp Allergy*. 2020;50:1093–1102. https://doi.org/10.1111/cea.13706