


Reactivity to allergenic food contaminants: A study on products on the market

Alessandro Fiocchi¹  | Linda Monaci² | Elisabetta De Angelis² |
Veronica Calandrelli¹ | Lamia Dahdah¹ | Rocco Valluzzi¹ | Sara Urbani¹ |
Carmen Mazzuca¹ | Stefania Arasi¹ | Arianna Cafarotti¹ | Carla Riccardi¹ |
Maria Cristina Artesani¹ | Lorenza Putignani^{3,4} | Valentina Pecora¹ |
Valeria Marzano³ | Vincenzo Fierro¹

¹Allergy Dpt, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

²Institute of Sciences of Food Production, CNR-ISPA, Bari, Italy

³Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

⁴Unit of Human Microbiome, Department of Diagnostics and Laboratory Medicine, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

Correspondence

Alessandro Fiocchi, Pediatric Hospital Bambino Gesù, Piazza S. Onofrio 4, 00169, Rome, Italy.

Email: alessandro.fiocchi@allegriallergia.net

Funding information

Soremartec Italia S.R.L., Alba, Italy, Grant/Award Number: n.a.; Allegria ONLUS, Milan, Italy, Grant/Award Number: n.a.

Abstract

Background: The frequency and severity of reactions in food-allergic consumers exposed to unintentional food allergen contamination during production is unknown. To warn allergic consumers, it has been suggested for pre-packaged foods to be precautionary labelled when the food allergen contamination may exceed the amount to which 1%–5% of the population could react (ED01–ED05). ED01 for hazelnut and milk have been estimated at 0.1 and 0.2 mg, respectively, by the Voluntary Incidental Trace Allergen Labelling (VITAL) initiative. The respective reference doses recommended by the FAO/WHO Codex consultation are 3 and 2 mg. We evaluated the reactivity to potential traces of milk and hazelnut allergens in allergen-free pre-packaged products by children affected by severe allergies to milk and hazelnuts.

Methods: Oral Food Challenges with commercially available hazelnut-free wafer biscuits and milk-free chocolate pralines were administered to patients with severe food allergies to hazelnut and cow's milk, respectively. Contamination levels of milk or hazelnut allergens were measured using chromatographic separation interfaced with triple quadrupole mass spectrometry.

Results: No hazelnut allergic patient showed allergic reactions to exposure to biscuits, nor any milk allergic patient displayed allergic reactions to the dark chocolate praline. While no hazelnut trace was detected in biscuits, the praline was found to be contaminated by milk at concentrations ranging between 8 and 35 mg total protein/kg food. In our dose model, these amounts exceeded 1.5–10 times the VITAL ED01 and reached the threshold suggested by the FAO/WHO Codex consultation.

Conclusions: Upon the consumption of food products available on the market, many patients with severe food allergies tolerate significantly higher doses of allergen

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2023 The Authors. Clinical and Translational Allergy published by John Wiley & Sons Ltd on behalf of European Academy of Allergy and Clinical Immunology.

than reference doses indicated in the VITAL system used for precautionary allergen labelling. These doses support the safety of the FAO/WHO recommended reference doses.

KEYWORDS

allergen toxicity, food allergy, hazelnut allergy, milk allergy, precautionary allergen labelling

1 | INTRODUCTION

Food trace reactions have been reported in 18% of children¹ and in 32% of adults with food allergy.² In such patients, tree nuts, cow's milk, and peanuts are the most common suspected contaminating allergens. In a significant number of countries, if such foods are contained as an ingredient in a pre-packaged product they are required to be reported on the label as allergens by law.^{3,4} If the same allergen is not an ingredient of the pre-packaged product, but may contaminate it, many manufacturers choose to communicate potential allergen presence using Precautionary Allergen Labelling (PAL). Precautionary Allergen Labelling is an unregulated, voluntary practice aimed to protect consumers suffering from IgE-mediated food allergy from unexpected reactions that could also be potentially dangerous.

As PAL poses several problems to allergic consumers,⁵⁻⁸ the international authorities together with the scientific community are attempting to discipline this matter. In 2019, the FAO/WHO Codex Committee on Food Labelling asked for scientific advice aimed to develop guidance on the use of PAL. The commission set up in response to this appeal proposed risk management and mitigation strategies. The Codex Expert consultation indicated as the most suitable strategy to establish health-based guidance values for PAL the use of food allergen thresholds based on a dose/probabilistic hazard assessment.⁹ This choice substantially adopted the approach proposed by the Allergen Bureau's Voluntary Incidental Trace Allergen Labelling (VITAL) Programme,¹⁰ reducing the number of priority allergens. Elaborating on the concept of a minimum amount able to determine allergic reactions, VITAL defined the 'eliciting doses' (EDs) for specific food allergens, indicating the dose (mg) predicted to provoke reactions in a defined proportion of the allergic population.¹¹⁻¹³ The VITAL EDs were derived from the dose distribution of individual minimum EDs, based on the results of the Oral Food Challenges (OFCs) performed for diagnostic purposes and published in the scientific literature.¹⁴ The Codex expert committee proposed thresholds based on the published ED05 values (the dose predicted to provoke reactions in 5% of the at-risk allergic population)¹¹ as the most appropriate reference doses to guarantee the transparency of information to the consumer and safeguard his safety.¹⁵

A decisional process for PAL based on EDs for specific food allergens makes sense, as (I) the probability to have a reaction depends on the EDs,¹⁶ (II) the EDs depend on the type of food,¹⁷ (III) OIT (Oral Immunotherapy) studies indicate that increasing the ED reduces the

risk of an allergic reaction from accidental exposure,¹⁸ and (IV) the use of anti-IgE reduces the likelihood and the severity of the reactions by increasing the threshold doses for different foods.¹⁹ However, up to now, translating these indications into practical applications has proven difficult.¹⁴ Among other difficulties, VITAL has not been adopted on a large scale due to the difficulties in verifying it with the current analytic capabilities.²⁰ On the other hand, the suspicion hovers that the VITAL thresholds may be overconservative, because the source data of the project refer to populations of patients allergic to foods *tout court*, and not to populations of severe food allergic.²¹ It has been observed that no fatal reactions have ever occurred after exposure to allergen concentration of less than 5 mg of protein for any allergen, although allergic—but not life-threatening—reactions can occur at such levels.²² From this observation, the European GA2LEN network proposed a potential conservative threshold/risk management cut-off of five parts per million (ppm) or 0.5 mg referred to 100 g of food size. Such proposed threshold far exceeds the VITAL thresholds for many of the allergenic foods considered, and has been criticized as inaccurate.²³

Under this uncertainty frame, studies are lacking aiming at defining the frequency and severity of reactions in severe food-allergic patients exposed to doses below the FAO/WHO reference dose, the ED01 threshold set by VITAL, the GA2LEN threshold, or the recent reference dose of 0.3 mg (ED01) recommended for total milk proteins for risk assessment and management purposes.¹³

The present study is an attempt to contribute to fill this lack of knowledge. In a 'real life' setting, we aimed to verify the reactivity to potential traces of milk and hazelnut allergens in milk and hazelnut-free products in a group of children affected by severe allergies to milk and hazelnuts.

2 | METHODS

2.1 | Study products and clinical outcomes

The study was designed to evaluate whether patients with severe food allergy might experience clinical reactions to two commercially available pre-packaged foods: hazelnut-free cocoa and milk wafer biscuits with milk and cocoa fillings and a milk-free chocolate confectionery praline. Such cookies are labelled as "may contain hazelnut traces" and "may contain milk traces", respectively. Aiming at evaluating any relationship existing between the potential doses of food allergens traces contained in the studied products and expected

reactions that are likely to occur in case of cross-contamination by allergens, we carried out in parallel a clinical and chemical evaluation. Mass spectrometric analysis was carried out to quantify potential traces of hazelnut and milk detected in the biscuit and in the dark chocolate praline, respectively, and the potential reactivity of the contaminated products was evaluated by the OFC test.

2.2 | Population

We admitted to the study milk/hazelnut protein allergic children aged between 2 and 18 years with history of anaphylaxis, defined as a systemic food hypersensitivity reaction, rapid in onset and potentially fatal at any time in the past.²⁴ Participants must have received a prescription of epinephrine, whether they had used it or not. They had to be sensitized to hazelnut or milk by both skin test (cut-off greater than 3 mm wheal²⁵) and specific IgE dosage for milk or hazelnut (ImmunoCAP Thermo Fisher, Uppsala, Sweden, cut-off >0.35 kUI/L). Patients also had to show IgE positivity for at least one of the major molecular allergens: for CMA, Bos d 4, Bos d 5, and/or Bos d 8; for hazelnut allergy sufferers, Cor a 8, Cor a 9, and/or Cor a 14 (ImmunoCAP, cut-off >0.35 kUI/L). None of them was allowed to consume products containing traces of the respective food allergens.

The patients underwent confirmatory seven steps of OFC using pasteurized low-fat milk or raw chopped hazelnut. Specifically, for milk, we set the lowest dose to 3.43 mg of protein and the total amount of protein to 4955.8 mg, corresponding to 0.1 and 144.4 mL, respectively. For hazelnut, we set the lowest dose to 13.8 mg and the total amount of protein at 4222.8 mg of proteins (about 0.1 and 30.6 g, corresponding to 20 hazelnuts) (Table 1). We derived the protein content of the foods from the U.S. Department of Agriculture.²⁶ The doses were administered 20 min apart until objective symptoms appeared.^{27,28} We stopped the administration when objective symptoms appeared, according to the scheme shown in Table SI. The reactive symptoms classified patients into five groups, from subjective (nausea, abdominal pain, pruritus, oral allergy syndrome) to systemic reactions (Table SII).²⁹

TABLE 1 Equivalent amounts of milk and hazelnut used in diagnostic Oral Food Challenges (OFCs).

| Step dose | Raw chopped hazelnut | Hazelnut protein | Pasteurized cow's milk | Cow's milk protein |
|------------------------|----------------------|-------------------|------------------------|--------------------|
| 1 | 150 mg | 20.7 mg | 0.1 mL | 3.43 mg |
| 2 | 390 mg | 51.06 mg | 0.3 mL | 10.29 mg |
| 3 | 500 mg | 69 mg | 1 mL | 34.32 mg |
| 4 | 1000 mg | 138 mg | 3 mL | 102.96 mg |
| 5 | 2000 mg | 276 mg | 10 mL | 343.2 mg |
| 6 | 4000 mg | 552 mg | 30 mL | 1029.6 mg |
| 7 | 8000 mg | 1104 mg | 100 mL | 3432 mg |
| Cumulative dose | 16.040 g | 2192.13 mg | 144.4 ml | 4955.80 mg |

In order to include clinically relevant patients for our query, admission to the next phase of the protocol was limited to patients who reacted to the first, second, or third dose of OFC, that is, those allergic to up to 1 gm hazelnut or up to 1.4 mL of milk.

Children, or their parents or legal guardian, were required to sign a statement of informed consent that met the privacy requirements including those of the GDPR. The local ethics committee gave ethical approval to the protocol (Resolution #851, July 30th, 2018).

2.3 | Procedures

Two groups of participants were evaluated, with (a) hazelnut allergy or (b) CMA. Group (a) was challenged with different batches of wafer biscuits with milk and chocolate filling, group (b) was exposed to different batches of dark chocolate pralines.

In group (a), we performed a SPT (Skin Prick Test) with fresh hazelnut, baked products containing hazelnut and the filled biscuit.²⁴ After that, OFCs were administered in seven increasing doses. The first six doses were the same for each age group, the seventh varied according to patient's age. Each dose was administered every 20 min for a duration of 140 min (Table 2). Patients were monitored for 2 h after the administration of the last dose.

For group (b) patients, the SPTs with the following products were performed: commercially available cow's milk, alpha-lactalbumin, betalactoglobulin, casein (Lofarma, Milano, Italy), fresh pasteurized cow's milk, baked products containing milk, and shells of dark chocolate pralines. In this group, OFCs were performed on four portions of chocolate for a total weight of 9 gr of product for each chocolate; OFC was refracted in 6 doses, administered every 20 min, for a total test duration of 100 min (Table 2). Similar to the biscuit protocol, the last dose (i.e. the sixth) changed according to patient's age.

In both groups, the amount of the last dose was commensurate with the age of the children (Table 2). The patients in group (a) assumed a total number of biscuits oscillating between 50.8 gms under 5 years and 76.4 gms over 14 years. Those in group (b) assumed a quantity fluctuating between 35.4 gms for children under 5 years and 62.4 gms for children above 14.

| Step dose | Group (a)—filled biscuits | Group (b)—dark chocolate pralines |
|-----------|---|---|
| 1 | 0.4 gr | 0.56 gr |
| 2 | 0.8 gr | 1.125 gr |
| 3 | 1.6 gr | 2.25 gr |
| 4 | 3.2 gr | 4.5 gr |
| 5 | 6.4 gr | 9 gr |
| 6 | 12.8 gr | 18.0 gr for subjects <5 yo or 27.0 gr for subjects 6–9 yo |
| 7 | 25.6 gr for subjects <5 yo or 35.6 gr for subjects 6–9 yo or 45.6 gr for subjects 10–13 or 51.2 gr for subjects 14–18 yo | or 36.0 gr for subjects 10–13 or 45.0 gr for subjects 14–18 yo |

TABLE 2 Oral Food Challenge (OFC) doses with study products.

2.4 | Analytical protocol for the determination of milk allergens in dark chocolate pralines

The analysis for the determination of contamination levels of milk allergens used a method based on chromatographic separation interfaced with triple quadrupole mass spectrometry (LC-MS/MS).

2.4.1 | Dark chocolate pralines milling

For the extraction and analysis of milk allergens in dark chocolate-based samples, the analytical method based on LC-MS/MS detection developed within the ThRAII project^{30,31} and validated in house was followed. Briefly, chocolate products were grinded using a laboratory blender and the final mixture was filtered through a 1 mm cut-off sieve. The obtained powder was stored in the fridge until its use.

2.4.2 | Protein extraction, digestion and peptides marker selection

For protein extraction, 2 g of chocolate was extracted with 20 mL of Tris-HCl 200 mM pH 9.2 and 5M urea and subjected to purification as detailed elsewhere.³² An aliquot was then digested with trypsin and the resulting peptide pool purified on SPE StrataX columns (1cc, 30 mg, #8B-S100-TAK, Phenomenex Torrance, California, USA) before the final injection into the HPLC-MS apparatus. The identification of the milk allergenic ingredient in the different samples was achieved by the detection of two main peptides: FFVAPFPEVFGK peptide (FFV, m/z 692.9) belonging to the alpha-S1 casein protein and NAVPITPTLNR peptide (NAV, m/z 598.3) released from alpha-S2 casein tryptic digestion, along with the respective transitions monitored in the Multiple Reaction Monitoring (MRM) analyses, namely 920.5/991.5/676.4/823.4 for FFV and 911.5/285.2/701.4/600.3 for NAV. In tab S III, any relevant information about milk peptide markers was reported. Samples were injected in duplicate into the LC/MS system.

2.4.3 | Calibration line

For the quantification of the milk ingredient in the dark chocolate samples, a calibration line was constructed by adding calculated amounts of the synthetic peptide FFVAPFPEVFGK (m/z 692.9) to the digest of a milk-free chocolate sample after purification on SPE columns.

A four specific calibration line was constructed by relating the peak area detected for each transition to the respective concentration expressed in fmol/ μ L. The quantification of the milk in the individual samples was carried out using the equation of the transition line showing the best sensitivity, that is, the transition 692.9/920.4 by interpolation of the peak areas detected for the transition 920.4 in the different samples.

In order to quantify the milk protein concentration in the analysed product, the FFV peptide concentration (fmol/ μ L) was converted into micrograms of milk protein by the previously reported correction and conversion factors.³²

Twenty-nine samples marked as “Choco_B_01” to “Choco_B_29” were analysed in replicate.

2.5 | Analytical protocol for the determination of hazelnut allergens in filled biscuits

Snack samples marked as “Biscuits_01” to “Biscuits_30” were processed and analysed by the HPLC/QqQ-MS according to the method currently described with the aim to detect any trace of hazelnut proteins.

2.5.1 | Filled biscuits milling

The products were firstly grinded by using a laboratory blender, in particular, three iterative cycles of pulsed blending at decreasing speed were applied in order to minimize the sample heating (pulses: 5sON/5sOFF + 5sON/5sOFF, speed 16,000 rpm I cycle, 14,000 rpm II cycle, 13,000 rpm III cycle). The obtained flour was

sieved through a 2 mm mesh and then stored at -20°C until its use. The procedure was equally applied to all snack samples. Independent blades and containers were used for each sample to avoid cross-contamination.

2.5.2 | Preparation of isotopically labelled synthetic peptides standard solution

An explorative study tailored to confirm the presence/absence of hazelnut allergenic ingredient before quantification was accomplished by using isotopically labelled synthetic peptides (heavy peptides) purchased from Thermo Fisher Scientific (Bremen, Germany). Specifically, isotopic labelling was produced on the terminal Lysine (K) or Arginine (R) of the amino acid sequence to obtain a shift mass with respect to the unlabelled peptides. Heavy peptides (AQUA Basic) were singly provided in lyophilized form to be then re-suspended in 5% (v/v) acetonitrile/water to get the final concentration of 6250 fmol/ μL . Peptide solutions were then divided into different aliquots and stored at -20°C until use. Heavy peptide amino acidic sequences were chosen on the basis of the marker peptides identified within the ThRAII project^{30,31} for tracing hazelnut in food-processed samples.

2.5.3 | Protein extraction, in solution digestion and peptide mixture purification

After grinding, samples were submitted for protein extraction/purification. Specifically, protein extraction was performed as already reported in our previous paper.³³ Before purification, an aliquot of isotopically labelled synthetic peptides was added to the peptide mixture in order to obtain the final concentration of 25 fmol/ μL .

Thirty samples from 12 different lots, marked as "Biscuits_01" to "Biscuits_30", were finally injected in replicate in LC-MS/MS equipment.

2.5.4 | Peptides for the identification of hazelnut proteins

Table SIV reports the hazelnut peptides chosen as markers for tracing this allergen in the biscuit samples. Specifically, two main peptides were selected for identifying hazelnut in the samples, namely: ADIYTEQVGR peptide (ADI, m/z 576.3) and ALPDDVLA-NAFQISR peptide (ALP, m/z 815.4) both belonging to the 11S Seed Storage Globulin. The respective transitions monitored in the MRM analyses were 852.4/689.4/588.3/693.3 for ADI and 906.5/835.4/1019.6/1445.7 for ALP. In tab S IV, any relevant information about hazelnut peptide markers is reported. Samples were injected in duplicate into the LC/MS system.

2.6 | LC-MS analysis for milk and hazelnut allergen detection

For LC-MS analyses, a system consisting of an LX50 UHPLC pump provided with an autosampler and an ESI interface connected to a QSight 220 triple mass spectrometer (PerkinElmer, Inc., Waltham, MA USA) was used. Peptide separation was accomplished on a PerkinElmer Brownlee SPP ES-C18 (2.1×150 mm; $2.7 \mu\text{m}$; 160 \AA) (PerkinElmer Inc., Waltham, MA, USA) at a flow rate of 300 $\mu\text{L}/\text{min}$, using a binary gradient composed by $\text{H}_2\text{O} + 0.1\%$ formic acid (solvent A) and Acetonitrile + 0.1% formic acid (solvent B). The gradient elution programme was 0–3 isocratic steps at 10% B, then a linear increase from 10% to 35% B in 25 min; step change to 90% B then isocratic for 15 min; step change to 10% B then isocratic for 17 min for column conditioning. For each sample 18 μL was loaded. Mass spectrometer analysis was performed in duplicates in MRM by setting the resolution to "Unit" option (0.7 ± 0.1 amu) for both Q1 and Q3 events. MS conditions were set as reported elsewhere.³³ Instrumental control and data processing were obtained using the Simplicity - 3Q v. 1.6.5 (PerkinElmer Inc., Waltham, MA, USA) commercial software.

3 | RESULTS

3.1 | Clinical data

Between September 1st, 2018, and October 31st, 2022, 207 patients underwent milk and 111 hazelnut OFCs. Six of these procedures, or 1.88%, were DBPCFC. Thirty-one patients were reported with possible allergic reactions to both milk and hazelnut.

Sixty-seven (32.4%) of the patients who completed the OFC with milk, and sixty-five (58.6%) of those with suspected hazelnut allergy gave a positive result (Table 3). The proportion of positive tests was significantly higher for hazelnut (chi-square = 8.0511, $p = 0.004$). Forty-two (31.8%) of the positive patients had been reported with a severe systemic reaction at clinical history, according to the established clinical criteria,²⁵ without difference among groups (a) and (b).

Sixty patients, 38 males and 22 females (mean age 8.76 years, range 2.21–17.85), satisfying the inclusion criteria and reacting to low doses of cow's milk (30) or hazelnut (30), agreed to participate in the study (Table 3). Fifteen of them (20%; 10 in group (a) and 5 in group (b)) reported reactions to suspected allergen(s) not mentioned as ingredient or warning on the label at their clinical history. Only one patient from each of the two groups developed objective symptoms on the first diagnostic OFC dose. Twenty-eight of them developed at OFC urticarial eruptions and were classified in Group 2, 22 developed Group 3 reactions, and 10 patients developed Group 4 systemic reactions (five with urticaria and dyspnoea, two with urticaria and mild hypotension, one with emesis and dyspnoea, one with emesis with dyspnoea, and one with urticaria dyspnoea and emesis). Eight out of 10 of Group 4 reactions occurred with hazelnut ingestion.

| | Milk | Hazelnut | Total |
|------------------------------------|--------------------|--------------------|--------------------|
| Age (years), mean (min, max) | 9.02 (2.21, 17.85) | 8.19 (2.47, 17.43) | 8.76 (2.21, 17.85) |
| Male, n (%) | 20 (69) | 18 (60) | 38 (63.3) |
| Respiratory allergy, n (%) | 18 (62) | 24 (80) | 42 (71.2) |
| Diagnostic challenges performed, n | 29 | 30 | 59 |
| Open, n (%) | 143 (97.9) | 141 (99.3) | 284 (98.6) |
| DBPCFC, n (%) | 1 (3.4) | 0 | 1 (1.7) |
| First dose responders, n (%) | 1 (3.4) | 1 (3.3) | 2 (3.4) |
| Second dose responders, n (%) | 7 (24.1) | 13 (43.3) | 20 (33.9) |
| Third dose responders, n (%) | 21 (72.4) | 16 (53.3) | 37 (62.7) |

TABLE 3 Demographic characteristics of the 59 patients included in the study.

As a girl allergic to milk turned 18 before she could undergo the experimental OFCs and had to be excluded from the evaluation, we report here the data of 59 patients.

In group (a), while all the SPTs with hazelnut were positive (wheat diameter 5–28 mm), the SPTs with the filled biscuits returned negative. In group (b), the SPTs returned positive with the following proportions: alpha-lactalbumin 41.4% (12/29); betalactoglobulin, 48.3% (14/29); casein, 93.1% (27/29); dark chocolate pralines, 3.4% (1/29). In this group, the range of wheat diameters was between 3 and 25 mm.

At specific IgE determination, patients in group (a) were found to be positive for hazelnut molecular allergens in the following proportions: Cor a 8, 50% (15/30); Cor a 9, 73% (22/30); Cor a 14, 66.6% (20/30). Those with CMA (group b) were positive for Bos d 4 in 44.8% of cases (13/29), Bos d 5 in 55.2% of cases (16/29), and Bos d 8 in 96.5% of cases (28/29).

In study product OFCs, the possible food allergen contamination did not show allergic reactions to the exposure to the biscuits, nor has any milk allergic patient shown allergic reactions to the dark chocolate. Thus, the possible food allergen contamination in the tested products, if any, did not reach clinically significant levels.

3.2 | Determination of the presence of hazelnut proteins in the filled biscuits

The MRM analysis of both marker peptides revealed that no hazelnut trace was present in any sample analysed (Biscuits 1–30). Comparing the chromatogram plots produced by LC-MS/MS analysis of each sample by monitoring each single transition derived from ADI and ALP precursors in unlabelled and labelled forms, no peak areas corresponding to hazelnut peptides were revealed or, in any case, they were found below the signal produced by the presumable minimum detectable concentration. An example of the chromatogram peaks produced by LC-MS/MS analysis of the Biscuits-03 sample referred to a selected transition of ALP and ADI unlabelled/labelled peptides is reported in Figure 1, plots A and B, respectively.

3.3 | Determination of the presence of milk proteins in dark chocolate pralines

Table 4 shows the concentration values of milk proteins estimated on the quantitative analysis obtained by LC-MS, after the application of the conversion factors and correction to the dark chocolate samples analysed. In this case, the samples ($n = 29$) were actually found to be contaminated by small quantities of milk, ranging between 8 and 35 mg total milk protein/kg food. In light of this finding, our patients assumed during OFC doses of milk proteins ranging from 0.20 to 2.18 mg, as indicated in Table 5.

4 | DISCUSSION

The aim of this study was to verify whether, in a population of individuals severely allergic to milk or hazelnut, exposure to foods potentially contaminated by the allergen could cause significant reactions. Unlike all other studies evaluating low-dose reactivity, its design was not a retrospective evaluation of the thresholds of foods in the diagnostic OFCs, but a prospective exposure to potentially contaminated foods. As it was conceived without a predetermined contamination of the tested foods, this was a real-life study.

The tested products proved to be safe on this highly selected population. As none of the 59 children had reactions, the potential contaminating allergens did not reach clinically relevant levels.

For group (a), the absence of a measurable hazelnut contamination does not allow us to draw different conclusions from those already reached for milk and egg.²⁶ In fact, for hazelnut, the absence of traces deemed quantifiable by mass spectrometry reassures about the safety of exposure to these filled biscuits.

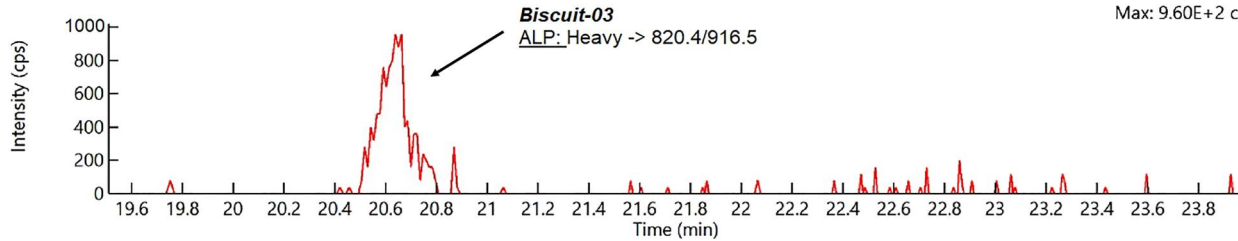
Conversely, the chocolate administered in OFCs to group (b) exhibited actual milk contamination, ranging between 8 and 35 mg total protein/kg food. These concentrations exceed by 1.5–6 times the threshold of 5 mg total milk protein/kg food proposed by GA2LEN for the voluntary declaration of traces of food allergens in processed foods.²¹ The NOAEL for our patients was found at

2022-09-07: KinderCardsFER_03-HeavyCor625fmol_rep1_194709

EIC +MRM 820.40/916.50 (4 pairs) EV: 30 V CC: -31 V Exp "ALP_Heavy_820_MRM" Cora9 ALP 815.3

Number of Scans: 467

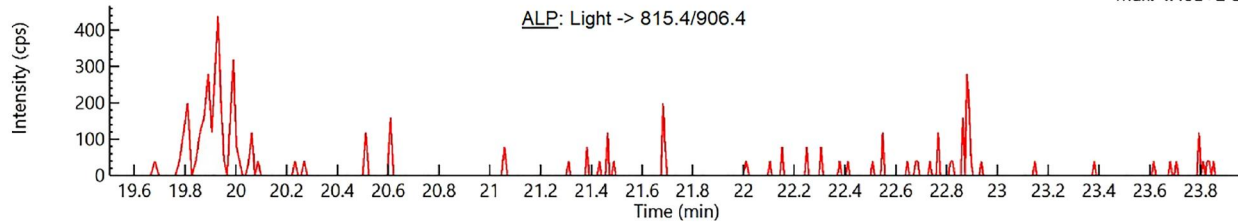
Max: 9.60E+2 cps



EIC +MRM 815.40/906.40 (4 pairs) EV: 30 V CC: -31 V Exp "ALP-815-MRM" Cora9 ALP 815.3

Number of Scans: 467

Max: 4.40E+2 cps

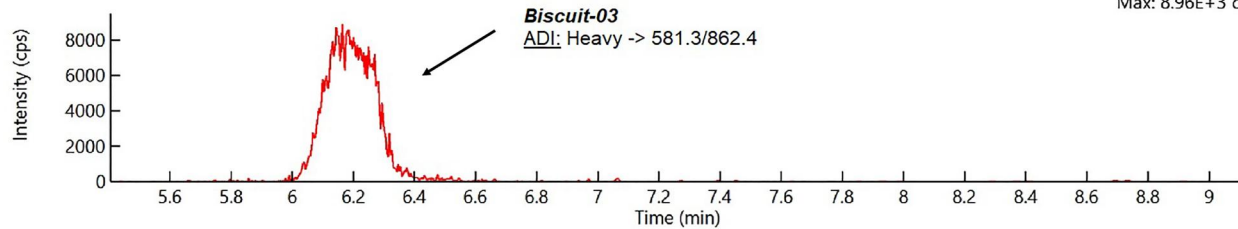


2022-09-07: KinderCardsFER_03-HeavyCor625fmol_rep1_194709

EIC +MRM 581.30/862.40 (4 pairs) EV: 20 V CC: -24 V Exp "ADI_Heavy_581_MRM" Cora9 ADI 576.1

Number of Scans: 465

Max: 8.96E+3 cps



EIC +MRM 576.40/852.30 (4 pairs) EV: 20 V CC: -24 V Exp "ADI-576-MRM" Cora9 ADI 576.1

Number of Scans: 465

Max: 1.60E+2 cps

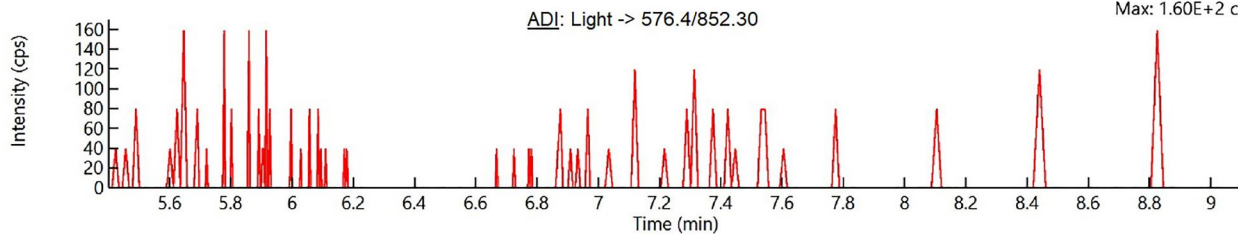


FIGURE 1 Comparison between Multiple Reaction Monitoring (MRM) signals produced by analysing the Biscuits-03 sample and monitoring the transition 815.4/906.4 m/z for ALP (plot A) and 576.4/852.3 for ADI (plot B) peptides in labelled (heavy) and unlabelled (light) forms.

amounts up to 10 times the VITAL ED01¹¹ and reached the FAO/WHO threshold. In this study, the exposure to such amount of milk traces in chocolate pralines was not detrimental to the health of children with severe CMA. As chocolates and bonbons, and especially milk in dark chocolate products, are among the most frequently reported causes of reactions to trace foods,² our finding is reassuring about the specific safety of the product. More in general, it raises questions about the real opportunity to apply precautionary labelling to similar pre-packaged foods.

In fact, the precautionary thresholds calculated from the VITAL collaboration are based on retrospective studies of tests performed for diagnostic purposes. Such OFCs are not specifically designed to evaluate thresholds; their results may vary with several factors, including the challenge technique, the age of patients and the case-mix of individual centres. It is reasonable to expect that centres serving patients with less severe food allergies may have a higher reactivity threshold, whereas third-level centres could have lower thresholds. Nevertheless, in a third-level centre such as the Bambino

TABLE 4 Milk protein concentration assessed in dark chocolate praline samples by LC-MS after application of appropriate conversion factors.

| Sample | Marker concentration (fmol peptide/ μ l extract) | Milk protein concentration ^a corrected by the conversion and pre-concentration factor (mg total milk protein/kg food) |
|------------------|--|--|
| Choco_B01_030322 | 273 | 21 |
| Choco_B02_030322 | 293 | 23 |
| Choco_B03_080322 | 365 | 28 |
| Choco_B04_080322 | 320 | 24 |
| Choco_B05_080322 | 327 | 25 |
| Choco_B06_080322 | 303 | 23 |
| Choco_B07_080322 | 331 | 25 |
| Choco_B08_030322 | 451 | 35 |
| Choco_B09_030322 | 390 | 30 |
| Choco_B10_090322 | 286 | 22 |
| Choco_B11_090322 | 168 | 13 |
| Choco_B12_090322 | 199 | 15 |
| Choco_B13_090322 | 222 | 17 |
| Choco_B14_040322 | 230 | 18 |
| Choco_B15_040322 | 142 | 11 |
| Choco_B16_090322 | 257 | 20 |
| Choco_B17_150322 | 177 | 13 |
| Choco_B18_150322 | 206 | 16 |
| Choco_B19_150322 | 202 | 16 |
| Choco_B20_150322 | 231 | 18 |
| Choco_B21_040322 | 107 | 8 |
| Choco_B22_150322 | 179 | 14 |
| Choco_B23_150322 | 149 | 11 |
| Choco_B24_150322 | 123 | 9 |
| Choco_B25_150322 | 161 | 12 |
| Choco_B26_150322 | 162 | 12 |
| Choco_B27_150322 | 140 | 11 |
| Choco_B28_150322 | 160 | 12 |
| Choco_B29_150322 | 160 | 12 |

^aMilk concentrations were rounded up.

Gesù Paediatric Hospital, we recently published milk thresholds of 0.3 mg of total protein.¹⁷ In a similar setting, the discrete ED01 for milk was found at 1.1 mg.³³ Such values exceed the threshold for milk by 1.5 and 5.5 times, respectively.

Thus, it is not impossible to hypothesize that the VITAL thresholds are over-conservative. This hypothesis is supported by the conclusions of the FAO/WHO expert consultation that considered

TABLE 5 Amount of milk protein by group (b) (dark chocolate pralines) consumed during experimental Oral Food Challenge (OFC) in different age groups.

| Patient age, years | Chocolate dose | Milk protein min, μ g | Milk protein max, μ g |
|--------------------|----------------|---------------------------|---------------------------|
| <5 | 35.2 gr | 281.6 | 1232 |
| 6–9 | 44.2 gr | 353.6 | 1547 |
| 10–13 | 53.2 gr | 425.6 | 1862 |
| 14–18 | 62.2 gr | 497.6 | 2177 |

ED05-values the appropriate basis for establishing health-based guidance values (reference doses). The results of our study support the safety of the ED05-based reference dose for milk as recommended by the FAO/WHO expert consultation.⁹

European, U.S., Australian, and Canadian regulations require food business operators to implement controls to manage allergens and ensure they are properly declared, but the use of PAL is voluntary. In none of these countries have the regulatory authorities suggested quantitative thresholds below which a PAL is not necessary. This may encourage the food producers to be overcautious, as they are responsible for any accidents caused by contamination of undeclared food allergens. Should our results be confirmed, a re-thinking of this legislation could be possible. However, to consolidate these data, it will be necessary to prospectively evaluate the reaction of populations of severely allergic patients to foods contaminated with tiny, increasing doses of food allergens. This method, widely used to evaluate the thresholds of disparate biological phenomena,^{34–36} could identify 'no-observed-adverse-effect-concentrations (NOAECs)' of food allergens and might provide additional proof of the validity of ED-distributions^{11,12} and the safety of the ED05-based reference doses recommended by the FAO/WHO expert consultation.⁹

The results of this study allow ancillary considerations.

First, we used an unprecedented reaction severity rating, including a history of anaphylactic reactions, the prescription of epinephrine, confirmed sensitization, and sensitization to allergens considered highly hazardous.³⁷ To the best of our knowledge, such stringent inclusion criteria have not been used in previous studies. We believe that this type of patient can be further exploited in future studies to investigate reactivity to allergens contaminating food. The recently published definition of Severe Food Allergy³⁸ will help identify patients who require an absolute exclusion of foods containing traces.

Second, a significant proportion of patients undergoing OFCs in paediatric allergy service manifests low-dose reactions. In our series, this proportion is different for cow's milk (32%) and hazelnut (58%). While observation coincides with the concept that nut reactions can occur at extremely low thresholds,¹⁶ the severity of the reactions was not different in the two groups.

Third, the percentage of patients allergic to Cor a 14 and Cor a 9 is a relevant in group (a), while sensitization to Bos d 8 is almost

universal in group (b). Many studies accomplished on hazelnut allergy indicated the association between sensitization to the structural proteins Cor a 9 (legumin) and Cor a 14 (2S-albumin) and the occurrence of anaphylactic reactions.³⁸⁻⁴¹ Likewise, Bos d 8 (casein) has been since a long time indicated as a key molecular allergen for CMA.^{42,43}

The major limitation of this study was its design as a real-life study. This choice was made deliberately in an attempt to make data available to the scientific community resulting from the ordinary exposure to which patients with severe food allergies are subject. However, given the absolute absence of hazelnut contamination in the wafers, in one of its arms the results could be compared with the current reactivity thresholds. Another possible limitation is that our sample - although made up of patients who reacted to low doses - cannot fail to include patients who reacted to trigger doses of up to 70 mg of protein. While this value is certainly higher than the trace values, this is the only way to characterize a low-dose reactive patient population. This threshold does not exclude that our patients were reactive to traces, and they had reported a frequency of reactions to foods not present as ingredients comparable to that of other reported populations.^{1,2} Perhaps the major limitation, however, is our limited size of population of food-allergic children screened, and the fact that this is made of milk-allergic children only. This does not allow the study to be elevated to a universal standard.

In conclusion, our data may yield the hypothesis that reaction thresholds to allergens contaminating food products, at least in the case of milk, may be higher than estimated so far. To be confirmed, they will need to be replicated on severely allergic individuals exposed to minute, known, and increasing amounts of allergenic contaminants aiming to identify NOAELs. Only by following this approach would it be possible to establish a real scale of danger referred to the amount of the contaminating allergen in a specific food. In a context where the practice of PAL is widely extended, reducing the possibilities of food choices of allergic consumers, this approach could lead research to go against the tide.

AUTHOR CONTRIBUTIONS

Alessandro Fiocchi: Conceptualization (lead); Data curation (supporting); Formal analysis (supporting); Funding acquisition (lead); Investigation (supporting); Methodology (lead); Project administration (lead); Resources (supporting); Supervision (lead); Validation (equal); Writing - original draft (lead). **Linda Monaci:** Data curation (lead); Investigation (equal); Writing - original draft (equal). **Veronica Calandrelli:** Data curation (equal); Investigation (equal). **Elisabetta De Angelis:** Investigation (equal). **Lamia Dahdah:** Investigation (equal); Writing - review & editing (equal). **Rocco Valluzzi:** Data curation (equal); Formal analysis (equal); Methodology (equal); Writing - review & editing (equal). **Sara Urbani:** Investigation (equal). **Carmen Mazzuca:** Data curation (equal); Investigation (equal). **Stefania Arasi:** Data curation (equal); Visualization (equal); Writing - review & editing (equal). **Arianna Cafarotti:** Investigation (equal). **Carla Riccardi:** Investigation (equal). **Maria Cristina Artesani:**

Investigation (equal). **Lorenza Putignani:** Investigation (equal). **Valentina Pecora:** Writing - review & editing (equal). **Valeria Marzano:** Investigation (equal). **Vincenzo Fierro:** Conceptualization (equal); Investigation (equal); Project administration (equal); Writing - review & editing (equal).

ACKNOWLEDGEMENTS

The author acknowledges Dr. Filippo Brandi, PhD, President of Allegria ONLUS. This study would not have been possible without the commitment of Dr. Roberto Menta, Dr. Ileana Manera, Dr. Ginevra Rosso of Soremartec. Funded by Soremartec Italia S.R.L., Alba (CN), Italy, and Allegria ONLUS, Milan, Italy.

Open access funding provided by BIBLIOSAN.

CONFLICT OF INTEREST STATEMENT

Alessandro Fiocchi: Danone SA, Aimmune, Hipp GmbH, Abbott SA, Novartis, Ferrero, Sanofi. Linda Monaci: Barilla. Veronica Calandrelli: none. Elisabetta De Angelis: none. Lamia Dahdah: none. Rocco Valluzzi: none. Sara Urbani: none. Carmen Mazzuca: none. Stefania Arasi: Novartis, DBV, Aimmune. Arianna Cafarotti: none. Carla Riccardi: none. Maria Cristina Artesani: none. Lorenza Putignani: none. Valentina Pecora: none. Valeria Marzano: none. Vincenzo Fierro: none.

DATA AVAILABILITY STATEMENT

Source data available upon request.

ORCID

Alessandro Fiocchi  <https://orcid.org/0000-0002-2549-0523>

REFERENCES

- Graham F, Benhamou AH, Liu YJ, Caubet JC, Eigenmann PA. Real-life evaluation of tolerance to foods with precautionary allergen labeling in children with IgE-mediated food allergy. *Allergy*. 2023; 78(9):2558-2561. accepted. <https://doi.org/10.1111/all.15821>
- Blom WM, Michelsen-Huisman AD, van Os-Medendorp H, et al. Accidental food allergy reactions: products and undeclared ingredients. *J Allergy Clin Immunol*. 2018;142(3):865-875. <https://doi.org/10.1016/j.jaci.2018.04.041>
- Baker R. The global status of food allergen labeling laws. *Calif West Law Rev*. 2018;54(2). Article 4. Available at: Accessed March 21, 2023. <https://scholarlycommons.law.cwsl.edu/cwlr/vol54/iss2/4>
- Fiocchi A, Riso D, DunnGalvin A, et al. Food labelling issues for severe food allergic patients. *World Allergy Organ J*. 2021;14(10):100598. <https://doi.org/10.1016/j.waojou.2021.100598>
- Dinardo G, Fierro V, Del Giudice MM, Urbani S, Fiocchi A. Food-labelling issues for severe food-allergic consumers. *Curr Opin Allergy Clin Immunol*. 2023;23(3):233-238. <https://doi.org/10.1097/aci.0000000000000902>
- Allen KJ, Turner PJ, Pawankar R, et al. Precautionary labelling of foods for allergen content: are we ready for a global framework? *World Allergy Organ J*. 2014;7:10. <https://doi.org/10.1186/1939-4551-7-10>
- La Vieille S, Hourihane JO, Baumert JL. Precautionary allergen labelling: what advice is available for health care professionals, allergists, and allergic consumers? *J Allergy Clin Immunol Pract*. 2023; 11(4):977-985.

8. Scurlock AM, Brown E, Davis CM. Food insecurity in children and adults with food allergies. *Ann Allergy Asthma Immunol.* 2022;129(4):424-429. <https://doi.org/10.1016/j.anaai.2022.08.012>
9. <https://www.who.int/publications/i/item/9789240065420>, accessed May 22, 2023
10. Taylor SB, Christensen G, Grinter K, Sherlock R, Warren L. The allergen Bureau VITAL program. *J AOAC Int.* 2018;101(1):77-82. <https://doi.org/10.5740/jaoacint.17-0392>
11. Houben GF, Baumert JL, Blom WM, et al. Full range of population Eliciting Dose values for 14 priority allergenic foods and recommendations for use in risk characterization. *Food Chem Toxicol.* 2020;146:111831. <https://doi.org/10.1016/j.fct.2020.111831>
12. Remington BC, Westerhout J, Meima MY, et al. Updated population minimal eliciting dose distributions for use in risk assessment of 14 priority food allergens. *Food Chem Toxicol.* 2020;139:111259. <https://doi.org/10.1016/j.fct.2020.111259>
13. Blom WM, Westerhout J, Baumert JL, et al. Updated full range of Eliciting Dose values for Cow's milk for use in food allergen risk assessment. *Food Chem Toxicol.* 2022;168:113381. <https://doi.org/10.1016/j.fct.2022.113381>
14. Madsen CB, van den Dungen MW, Cochrane S, et al. Can we define a level of protection for allergic consumers that everyone can accept? *Regul Toxicol Pharmacol.* 2020;117:104751. <https://doi.org/10.1016/j.yrtph.2020.104751>
15. Turner PJ, Patel N, Ballmer-Weber BK, et al. Peanut can Be used as a reference allergen for hazard characterization in food allergen risk management: a rapid evidence assessment and meta-analysis. *J Allergy Clin Immunol Pract.* 2022;10(1):59-70. <https://doi.org/10.1016/j.jaip.2021.08.008>
16. Ballmer-Weber BK, Fernandez-Rivas M, Beyer K, et al. How much is too much? Threshold dose distributions for 5 food allergens. *J Allergy Clin Immunol.* 2015;135(4):964-971. <https://doi.org/10.1016/j.jaci.2014.10.047>
17. Valluzzi RL, Riccardi C, Arasi S, et al. Cow's milk and egg protein threshold dose distributions in children tolerant to beef, baked milk, and baked egg. *Allergy.* 2022;77(10):3052-3060. <https://doi.org/10.1111/all.15397>
18. Remington BC, Krone T, Koppelman SJ. Quantitative risk reduction through peanut immunotherapy: safety benefits of an increased threshold in Europe. *Pediatr Allergy Immunol.* 2018;29(7):762-772. <https://doi.org/10.1111/pai.12961>
19. Fiocchi A, Artesani MC, Riccardi C, et al. Impact of omalizumab on food allergy in patients treated for asthma: a real-life study. *J Allergy Clin Immunol Pract.* 2019;7(6):1901-1909. <https://doi.org/10.1016/j.jaip.2019.01.023>
20. Linders YFM, Lentz LR, Blom WM, et al. Precautionary allergen labeling: current communication problems and potential for future improvements. *Food Control.* 2023;147:109561. <https://doi.org/10.1016/j.foodcont.2022.109561>
21. Arasi S, Nurmatov U, Dunn-Galvin A, et al. Consensus on Definition of Food Allergy SEverity (DEFASE) an integrated mixed methods systematic review. *World Allergy Organ J.* 2021;14(3):100503. <https://doi.org/10.1016/j.waojou.2020.100503>
22. Zuberbier T, Dörr T, Aberer W, et al. Proposal of 0.5 mg of protein/100 g of processed food as threshold for voluntary declaration of food allergen traces in processed food-A first step in an initiative to better inform patients and avoid fatal allergic reactions: a GA²LEN position paper. *Allergy.* 2022;77(6):1736-1750. <https://doi.org/10.1111/all.15167>
23. Turner PJ, Baumert JL, Beyer K, et al. EAACI Taskforce on Food allergen thresholds. "Too high, too low": the complexities of using thresholds in isolation to inform precautionary allergen ("may contain") labels. *Allergy.* 2022;77(6):1661-1666. <https://doi.org/10.1111/all.15202>
24. Cardona V, Ansoategui IJ, Ebisawa M, et al. World allergy organization anaphylaxis guidance 2020. *World Allergy Organ J.* 2020;13(10):100472. <https://doi.org/10.1016/j.waojou.2020.100472>
25. Fierro V, Marzano V, Monaci L, et al. Threshold of reactivity and tolerance to precautionary allergen-labelled biscuits of baked milk- and egg-allergic children. *Nutrients.* 2021;13(12):4540. <https://doi.org/10.3390/nu13124540>
26. <https://fdc.nal.usda.gov/index.html>, accessed March 23, 2023
27. Nowak-Wegrzyn A, Assa'ad AH, Bahna SL, et al. Adverse reactions to food committee of American academy of allergy, asthma & immunology. Work group report: oral food challenge testing. *J Allergy Clin Immunol.* 2009;123(6 Suppl 1):S365-S383. <https://doi.org/10.1016/j.jaci.2009.03.042>
28. Keil T, McBride D, Grimshaw K, et al. The multinational birth cohort of EuroPrevall: background, aims and methods. *Allergy.* 2010;65(4):482-490. <https://doi.org/10.1111/j.1398-9995.2009.02171.x>
29. Sampson HA. Anaphylaxis and emergency treatment. *Pediatrics.* 2003;111(Suppl ment_3):1601-1608. <https://doi.org/10.1542/peds.111.s3.1601>
30. Huet AC, Paulus M, Henrotin J, et al. Development of incurred chocolate bars and broth powder with six fully characterised food allergens as test materials for food allergen analysis. *Anal Bioanal Chem.* 2022;414(8):2553-2570. <https://doi.org/10.1007/s00216-022-03912-z>
31. Henrotin J, Pilolli R, Huet AC, et al. Optimization of a sample preparation workflow based on UHPLC-MS/MS method for multi-allergen detection in chocolate: an outcome of the ThRAIL project. *Food Control.* 2023;143:109256. <https://doi.org/10.1016/j.foodcont.2022.109256>
32. Monaci L, De Angelis E, Guagnano R, et al. Validation of a MS based proteomics method for milk and egg quantification in cookies at the lowest VITAL levels: an alternative to the use of precautionary labeling. *Foods.* 2020;9(10):1489. <https://doi.org/10.3390/foods9101489>
33. Katz Y, Melchior M, Elizur A, Nachshon L, Remington BC, Leshno M. Informing risk management for patients with cow's milk allergy based on threshold dose distributions using low-dose food challenges. *J Allergy Clin Immunol Pract.* 2023;11(4):1116-1122.
34. Özay H, Çetin AÇ, Ecevit MC. Determination of retronasal olfactory threshold values. *Laryngoscope.* 2021;131(7):1608-1614. <https://doi.org/10.1002/lary.29395>
35. Lovell DP. Dose-response and threshold-mediated mechanisms in mutagenesis: statistical models and study design. *Mutat Res.* 2000;464(1):87-95. [https://doi.org/10.1016/s1383-5718\(99\)00169-2](https://doi.org/10.1016/s1383-5718(99)00169-2)
36. Pauluhn J. Development of a respiratory sensitization/elicitation protocol of toluene diisocyanate (TDI) in Brown Norway rats to derive an elicitation-based occupational exposure level. *Toxicology.* 2014;319:10-22. <https://doi.org/10.1016/j.tox.2014.02.006>
37. Arasi S, Nurmatov U, Dunn-Galvin A, et al. WAO consensus on Definition of food allergy SEverity (DEFASE). *World Allergy Organ J.* 2023;16(3):100753. <https://doi.org/10.1016/j.waojou.2023.100753>
38. Uotila R, Röntynen P, Pelkonen AS, Voutilainen H, Kaarina Kukkonen A, Mäkelä MJ. For hazelnut allergy, component testing of Cor a 9 and Cor a 14 is relevant also in birch-endemic areas. *Allergy.* 2020;75(11):2977-2980. <https://doi.org/10.1111/all.14430>
39. Borres MP, Sato S, Ebisawa M. Recent advances in diagnosing and managing nut allergies with focus on hazelnuts, walnuts, and cashew nuts. *World Allergy Organ J.* 2022;15(4):100641. <https://doi.org/10.1016/j.waojou.2022.100641>
40. Caffarelli C, Mastroianni C, Santoro A, Criscione M, Procaccianti M. Component-resolved diagnosis of hazelnut allergy in children. *Nutrients.* 2021;13(2):640. <https://doi.org/10.3390/nu13020640>

41. Dreskin SC, Koppelman SJ, Andorf S, et al. The importance of the 2S albumins for allergenicity and cross-reactivity of peanuts, tree nuts, and sesame seeds. *J Allergy Clin Immunol*. 2021;147(4):1154-1163. <https://doi.org/10.1016/j.jaci.2020.11.004>
42. Beyer K, Jarvinen KM, Bardina L, et al. IgE-binding peptides coupled to a commercial matrix as a diagnostic instrument for persistent cow's milk allergy. *J Allergy Clin Immunol*. 2005;116(3):704-705. <https://doi.org/10.1016/j.jaci.2005.05.007>
43. Ayats-Vidal R, Valdesoiro-Navarrete L, García-González M, Asensio-De la Cruz O, Larramona-Carrera H, Bosque-García M. Predictors of a positive oral food challenge to cow's milk in children sensitized to cow's milk. *Allergol Immunopathol*. 2020;48(6):568-575. <https://doi.org/10.1016/j.aller.2020.03.007>

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Fiocchi A, Monaci L, De Angelis E, et al. Reactivity to allergenic food contaminants: a study on products on the market. *Clin Transl Allergy*. 2023:e12301. <https://doi.org/10.1002/ctt2.12301>