

Effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomized, double-blind, cross-over trial

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Summary

Background Maternal avoidance of egg intake has been recommended to treat egg allergy in breastfed infants.

Objective To determine if the concentration of ovalbumin (OVA) in human milk is directly related to the quantity and form of egg consumed by breastfeeding mothers.

Methods Randomized, blinded, cross-over, intervention trial. Breastfeeding women ($n = 41$) attended four clinic days between 11 and 14 weeks of lactation and on each day were randomly allocated to receive a test breakfast, identical except for the egg content (no egg, one raw egg, half a cooked egg or one cooked egg). Breast milk samples were collected at two hourly intervals for 8 h and their OVA concentration measured by ELISA.

Results There was a direct, dose-response between the amount of cooked egg ingested and the peak OVA concentration (no egg 0.05 ng/mL [95% confidence interval (CI), 0.01–0.11], half a cooked egg 2.24 ng/mL [95% CI, 0.57–3.91], one cooked egg 3.16 ng/mL [95% CI, 1.41–4.91], $n = 41$, $P < 0.05$) as well as the total OVA excretion (no egg 0.18 ng/mL/h [95% CI, 0.04–0.39], half a cooked egg 4.93 ng/mL/h [95% CI, 1.40–8.46], one cooked egg 9.14 ng/mL/h [95% CI, 4.25–14.03], $n = 41$, $P < 0.05$). The peak concentration and total OVA excretion in response to one raw egg did not differ from ingesting half a cooked egg. There was no detectable OVA in the breast milk of 24% (10/41) women up to 8 h after any egg challenge.

Conclusion OVA was detected in the breast milk of lactating women up to 8 h after a controlled intake of egg. A dose-response correlation was indicated. As excretion of OVA in human milk appears to be a normal phenomenon, further studies need to determine the threshold of OVA excretion that leads to symptoms in egg-allergic breastfed infants.

Keywords egg, human milk, maternal antigen avoidance diet, ovalbumin, randomised controlled trial
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Introduction

Food allergy symptoms of eczema, colic, diarrhoea and vomiting have been observed in approximately one in every 200 exclusively breastfed infants [1]. The current consensus regarding the treatment of breastfed infants with food allergy is to trial a period of maternal dietary restriction [2, 3]. This recommendation assumes that food proteins ingested by women are absorbed and excreted antigenically active into breast milk. Previous studies have documented the presence of food proteins in human milk, including ovalbumin (OVA) [4, 5], bovine B-lactoglobulin [1, 4, 6–8], gliadin [9] and peanut proteins [10]. These studies have shown that even after the ingestion of the same challenge dose of a particular food, the frequency and concentration of detected food protein in breast milk is highly variable between women. Apart from the

natural biological variation that would be expected between individual women, some of this variability may be because of the influence of other factors including the stage of lactation, maternal atopy status or usual dietary intake of these food proteins. Furthermore, as cooking generally denatures protein, it is not known what effect the ingestion of raw vs. cooked foods has on the concentration of food proteins in human milk.

As a first step to further understanding the way food proteins are excreted into human milk, we undertook a randomized, cross-over trial in breastfeeding women at the same stage of lactation to determine whether the dietary dose of cooked egg influences OVA content in human milk and whether cooked vs. raw egg ingestion alters OVA content in human milk. A secondary aim was to determine whether maternal atopy status and usual dietary egg intake influenced the OVA content in human milk.

Egg was chosen as the food challenge because it can be restricted in the diet without nutritionally compromising lactating women and is one of the most common food allergens. Egg white is known to contain six major proteins,

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these being OVA, ovomucoid, ovotransferrin, ovomacroglobulin, ovomucin and lysozyme, with OVA contributing the largest portion (greater than 50%). There may be differences in absorption and excretion into human milk between these egg proteins. Hoffman [11] discusses the difficulty in inducing antibody production in rabbits to ovomucoid, resulting in techniques to measure OVA rather than ovomucoid in biological samples. Thus, we, along with the other previous studies in this field [4, 5], investigated the presence of dietary egg protein in breast milk, focusing on OVA.

Materials and methods

Participants

Breastfeeding mothers of term (gestational age at birth >37 weeks) singleton, newborn infants were approached in the postnatal ward at Women's and Children's Hospital (WCH) prior to discharge. Women with known egg allergy, or with any other food allergies (e.g. wheat), which were contraindicated by our clinic day menu, were excluded. The trial was explained to the mother and verbal consent was obtained by telephone at 8 weeks post-partum. At 8 weeks, if the women were continuing to exclusively breastfeed they were invited to participate in the trial.

Study design and treatments

Written informed consent was obtained prior to an appointment at 10 weeks post-partum according to the protocol approved by the Human Research Ethics Committee, WCH. At this appointment, mothers were instructed on how to follow an egg-free diet, socio-demographic information (age, parity, smoking habits and education level) was collected and a diet history was taken to estimate usual egg intake.

Each woman attended a clinic day at 11, 12, 13 and 14 weeks of lactation. Prior to each clinic day, the women followed an egg-free diet for 48 h and their compliance was assessed with detailed dietary intake records. Women attended the clinic after an overnight fast. They were supplied with a challenge breakfast and then an egg-free diet for the following 8 h. Prior to and at 2, 4, 6 and 8 h after ingestion of the test breakfast, 20 mL breast milk samples were collected into sterile pots, either manually or using an electronic breast pump (Ameda Hollister Inc., Libertyville, IL, USA), for analysis of the OVA concentration. The mothers breastfed their infants as per their usual feeding practice during each clinic day.

The test breakfasts were identical (blueberry muffin and banana milkshake) with the exception of the egg content. Prior to commencement of the study, recipes were developed to produce test breakfasts indistinguishable in taste and appearance to aid blinding. A standard muffin contains 1/6 of an egg, but for our trial we required each muffin to contain either 1/2 an egg or one whole egg. The test breakfasts were egg-free muffin and egg-free milkshake, muffin containing half a cooked egg (27.5 g) and egg-free milkshake, muffin containing one cooked egg (55 g) and egg-free milkshake or egg-free muffin and milkshake containing one raw egg (55 g). All milkshakes contained fresh banana and banana flavour-

ing to mask the raw egg. All muffins were cooked at 200 °C for 20 min.

Randomization and blinding

Each woman was given a study number that corresponded with a random allocation of four sequentially numbered sealed envelopes that described the test breakfast for each clinic day. The randomization schedule and the sealed envelopes were prepared by a consultant not involved in the day-to-day trial management. The envelopes were opened on the clinic day by the food service staff who prepared the required breakfast from standard recipes. This ensured that the researcher who both collected and analysed the breast milk samples remained blinded to the amount of egg consumed until all laboratory analyses were completed.

Assessments

Breast milk analyses

Ovalbumin concentration in human milk The breast milk samples were centrifuged (Sigma Laboratory Centrifuge 4K15, Osterode am Harz, Germany) at 2000 *g* for 20 min at 4 °C on the day of collection. The aqueous supernatant was frozen at -20 °C until analysis using a sandwich ELISA method. Flat bottom, polystyrene, high-binding 96-well assay plates (3590 Costar, Corning Incorporated, Corning, NY, USA) were coated with 100 µL per well of rabbit anti-OVA polyclonal antibody (Rockland, Gilbertsville, PA, USA) diluted at 1 in 2000 in 0.05 mol/L carbonate-bicarbonate buffer at pH 9.6 (Sigma-Aldrich, Saint Louis, MO, USA) and incubated overnight at 4 °C. The wells were washed three times with phosphate-buffered saline (PBS) and blocked with 300 µL per well of 1% fetal calf serum (FCS) in PBS, and incubated for 30 min at 37 °C and then 30 min at room temperature. Each well was washed three times with PBS. A standard curve for this ELISA method used breast milk (from women after 48 h on an egg-free diet) spiked with known quantities of OVA (chicken egg albumin grade V, Sigma-Aldrich) with a concentration range of 0–25 ng/mL. These breast milk standards, along with the breast milk samples collected from the participating women, were added in triplicate at 100 µL per well and incubated overnight at 4 °C. After washing each well three times with PBS+0.05% Tween 20, 100 µL per well of horseradish peroxidase-conjugated rabbit anti-OVA polyclonal antibody (Rockland), diluted at 1 in 10 000 with 0.1% FCS+1% polyethylene glycol 6000+PBS with 0.05% Tween 20, was added and incubated for 2 h at room temperature, 2 h at 4 °C, and then 1 h at room temperature. The wells were washed five times with PBS+0.05% Tween 20 and 150 µL per well of the substrate *o*-phenylenediamine dihydrochloride (SIGMA FAST OPD tablets, Sigma-Aldrich) solution was added and the colour reaction allowed to proceed in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 µL per well of 0.5 mol/L H₂SO₄ and read on an automatic microplate reader (Opsys MR, Dynex Technologies Inc., Chantilly, VA, USA) at optical density wavelength of 490 nm. The assay sensitivity for breast milk OVA detection was 0.1 ng/mL. The mean intra-assay coefficient

of variation (CV) for the quality control sample was 7.3% and the inter-assay CV was 12.1%.

Total protein content To reduce the influence of variation in total volume of breast milk produced by each woman over the 8 h of breast milk sample collection, the total protein content of the aqueous supernatant of each breast milk sample was analysed using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) based on the Bradford dye-binding procedure [12]. Bovine serum albumin was used as the protein standard. The OVA concentration (ng/mL) of each breast milk sample was then expressed relative to the total protein concentration (mg/mL) of that sample to give the OVA per protein content (ng OVA/mg protein).

Maternal atopy status Atopy status was assessed through the administration of an allergy questionnaire [13] at 10 weeks post-partum and by conducting skin prick testing to six allergens with a control and histamine challenge. An atopic person was defined as one who had at least one atopic disorder (asthma, eczema or hayfever) identified from the allergy questionnaire and a positive skin prick test (SPT) to at least one common environmental allergen [14]. A positive SPT was defined as the mean of two perpendicular weal diameters of 3 mm or greater in size than the mean weal of the negative control site at 20 min. All the skin prick testing was performed by the same researcher. The allergens tested were whole egg (AUST R32582) (1:20 w/v), standardized grass pollen perennial ryegrass (strength 10 000 BAU/mL), *Alternaria tenuis* (AUST R32618) (1:10 w/v), standardized cat hair (strength 10 000 BAU/mL), standardized mite (*Dermatophagoides farinae*) (strength 30 000 AU/mL) (AUST R32429), standardized mite (*D. pteronyssinus*) (strength 30 000 AU/mL) (AUST R32421), negative control containing 50% (v/v) glycerin as per all six allergens above and histamine (10 mg/mL) (Stallergenes, Antony, France). All allergens (except histamine) and negative control were supplied by Hollister-Stier (Spokane, WA, USA).

Sample size and statistical methods

In the absence of previously published data, we based our sample size estimate on being able to detect a difference of 0.5SD between groups with 80% power and an α -value of 0.01, to allow for multiple comparisons. Based on this estimate, 50 women, with complete paired data, were required. We also planned an independent review of the sample size estimate once we had data from 25 women. This review indicated that the proportion of women with OVA detected in their breast milk after the ingestion of no egg was 2/25, one raw egg was 9/25 and one cooked egg was 12/25. From these data, the sample size estimate was revised to include 40 women with complete paired data. This allowed us to detect an increase in the proportion of women with OVA in their breast milk from 8% (baseline) to 40% with 80% power and an α of 0.01.

The breast milk OVA concentration (ng/mL) of each woman in response to each test breakfast was plotted against time. The resulting curve was used to determine the peak OVA concentration (ng/mL) and total OVA excretion (ng/mL/h) by calculating the area under the curve as described by Matthews et al. [15]. This process was also repeated after the

breast milk OVA concentration had been corrected for the total protein content of each sample.

The effect of different test breakfasts on breast milk peak OVA concentration, total OVA excretion, peak OVA per protein content and total OVA excretion per protein content was assessed by Wilcoxon's signed ranks tests (as the data were not normally distributed). Pearson's χ^2 tests were used to determine the association between the frequency of OVA detection after ingesting the different test breakfasts and maternal atopy status. Spearman's rank order correlations were used to determine whether breast milk peak OVA or total OVA excretion were correlated with usual daily egg intake. All statistical computations were completed with SPSS for WINDOWS version 10.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

Results

Participation

A total of 585 women were screened for eligibility to participate in the trial between September 2001 and August 2002. Ninety-two women did not meet the inclusion criteria (the majority had ceased breastfeeding by 10 weeks post-partum), 16 women could not be re-contacted after the initial screening and 428 women declined to participate. The main reasons were because the mother was too busy with other children ($n = 141$), four visits to the hospital were too many ($n = 63$), too far to travel back to the hospital ($n = 58$) and the 8 h clinic days were too long ($n = 51$). Forty-nine women agreed to participate with 41 women successfully completing all aspects of the trial. Eight women withdrew from the study, six women were unable to attend the clinic days because of family illnesses and two women withdrew after having difficulty with the breast milk expression on their first clinic day.

Maternal characteristics, dietary compliance and maternal atopy status

Mean age of the women who completed the trial was 30.6 years and 83% had completed secondary education (Table 1). Most women (80%) were compliant with the egg-free diet prior to each test day. Only one woman consumed two scrambled eggs for breakfast 48 h prior to one of the test days. In all other cases, small quantities of egg were consumed as an ingredient in mayonnaise, ice cream, cake, biscuits and confectionery.

Table 1. Characteristics of the women completing the trial

Variable	$n = 41$
Age (years)	30.6 $\pm$ 5.7*
Parity (no.)	1.5 $\pm$ 0.8*
Completed secondary education	34 (83%)†
Smoking	2 (5%)†
Usual daily egg intake (eggs/day)	0.47 $\pm$ 0.36*
Atopic women‡	18 (44%)†

*Mean $\pm$ SD.

† n (%).

‡History of at least one allergic disease plus a positive skin prick test to at least one allergen.

Of the women who completed the trial, 18/41 (44%) were classified as atopic. The allergy questionnaire revealed 27% of the women had a history of asthma, 39% hayfever and 12% eczema. For skin prick testing, 68% of the women had a positive result to at least one allergen (39% rye grass pollen, 15% *Alternaria* mould, 17% cat, 20% house dust mite (HDM) (farinae), 41% HDM (pteronyssinus)). None of the women had a positive SPT to egg.

The eight women who withdrew were younger (25.6 vs. 30.6 years, $P < 0.05$, independent samples t -test) and were less likely to have completed secondary education (3/8, 38% vs. 34/41, 83%, $P < 0.05$, Pearson χ^2 test) compared with women who completed the trial.

Ovalbumin in human milk

OVA was detected in the breast milk of 3/41 (7%) women after consuming the no egg breakfast. OVA detection frequency increased to 19/41 (46%) after ingesting the half a cooked egg breakfast and further increased to 28/41 (68%) after consuming the one cooked egg breakfast (Table 2). Ten women (24%) had no OVA detected in their breast milk on all four clinic days.

Combined plots of all 41 women for OVA concentration in human milk as a function of time post-test breakfast are illustrated in Fig. 1. A direct, dose response was demonstrated between the amount of cooked egg ingested and the peak OVA concentration (no egg mean 0.05 ng/mL [95% confidence interval (CI), 0.01–0.11], half a cooked egg mean 2.24 ng/mL [95% CI, 0.57–3.91], one cooked egg mean 3.16 ng/mL [95% CI, 1.41–4.91], $P < 0.05$) and between the amount of cooked egg ingested and the total OVA excretion (no egg mean 0.18 ng/mL/h [95% CI, 0.04–0.39], half a cooked egg mean 4.93 ng/mL/h [95% CI, 1.40–8.46], one cooked egg mean 9.14 ng/mL/h [95% CI, 4.25–14.03], $P < 0.05$) (Table 2). The peak concentration and total OVA excretion in response to one raw egg did not differ from the half a cooked egg breakfast. Correcting for total protein content of each breast milk sample did not alter the pattern of results in response to each of the test breakfasts (Table 2).

Mean usual daily egg intake of the participating women was 0.47 ± 0.36 eggs/day. No correlations were found between each woman's usual daily egg intake and her breast milk peak OVA or total OVA excretion after each test breakfast (Spearman's ρ , $P = 0.46$ – 0.97). There was also no relationship between the frequency of OVA detection in breast milk and maternal atopy status (Pearson's χ^2 test, $P = 0.78$).

Discussion

Our trial represents a systematic study aimed at understanding the relationship between egg consumption and the appearance of OVA in human milk. The trial was the largest of its kind, and was blinded and well controlled. In an attempt to minimize inter-individual variation, each woman was her own control and the egg challenges were all conducted at the same stage of lactation. The careful study design has resulted in a number of key findings. There was a direct dose relationship between the concentration of cooked egg in the test meal and the OVA detected in breast milk, the response was greater to cooked egg compared with raw egg, and approximately one-quarter of the women had no OVA detected in their breast milk within 8 h of any egg challenge.

The consistency observed in response to increasing concentrations of cooked egg in the diet is particularly noteworthy. The increasing quantity of cooked egg in the challenge breakfasts resulted in increasing proportions of women with detectable OVA in their breast milk, an increasing peak OVA concentration as well as increasing total OVA excretion. These data clearly suggest that the quantity of cooked egg in the diet is directly related to the amount of egg protein that appears in breast milk. These observations were made in healthy women with no known metabolic or gastrointestinal illness and no known egg allergy, indicating that a proportion of egg protein finds its way into human milk despite the processes of digestion. Whether the OVA detected remains in its native form could not be ascertained from our trial, but previous studies involving raw egg challenges have demonstrated that the OVA detected in human milk matches native OVA [4]. Perhaps the more clinically relevant question is whether the OVA detected in breast milk retains its allergenicity. Matsumura et al. [16] described a series of case reports involving breastfed infants with eczema, where the infant's eczema resolved after elimination of eggs in the maternal diet and returned on maternal dietary egg challenge. However, the maternal egg challenges in these cases varied in type of egg-containing food (mayonnaise, hard boiled egg); there was no standard dose of egg eaten for the maternal challenge nor was breast milk OVA measured.

Previous studies that have measured OVA concentration in human milk have only done so in response to a raw egg challenge [4, 5]. Our results, using an ELISA detection method, are comparable with these previous studies that used

Table 2. OVA detection in human milk after four different dietary interventions

OVA detection	No egg ($n = 41$)	One raw egg ($n = 41$)	Half a cooked egg ($n = 41$)	One cooked egg ($n = 41$)
Frequency*	3 (7%) ^a	22 (54%) ^b	19 (46%) ^b	28 (68%) ^c
Peak concentration†				
ng/mL	0.05 [0.00] (–0.01–0.11) ^a	1.08 [0.28] (0.43–1.74) ^b	2.24 [0.00] (0.57–3.91) ^b	3.16 [1.18] (1.41–4.91) ^c
ng/mg protein	0.01 [0.00] (0.00–0.02) ^a	0.14 [0.03] (0.06–0.22) ^b	0.29 [0.00] (0.08–0.51) ^b	0.40 [0.16] (0.18–0.61) ^c
Total excretion†				
ng/mL/h	0.18 [0.00] (–0.04–0.39) ^a	2.38 [0.41] (0.90–3.85) ^b	4.93 [0.00] (1.40–8.46) ^b	9.14 [3.46] (4.25–14.03) ^c
ng/mg protein/h	0.03 [0.00] (–0.01–0.06) ^a	0.30 [0.05] (0.12–0.49) ^b	0.64 [0.00] (0.19–1.10) ^b	1.16 [0.39] (0.54–1.78) ^c

*[n (%)], ^{a,b,c} values with different superscripts indicate significant differences ($P < 0.05$) by Pearson χ^2 test.

†Mean [median] (95% CI), ^{a,b,c} values with different superscripts indicate significant differences ($P < 0.05$) by Wilcoxon's signed rank test.

CI, confidence interval; OVA, ovalbumin.

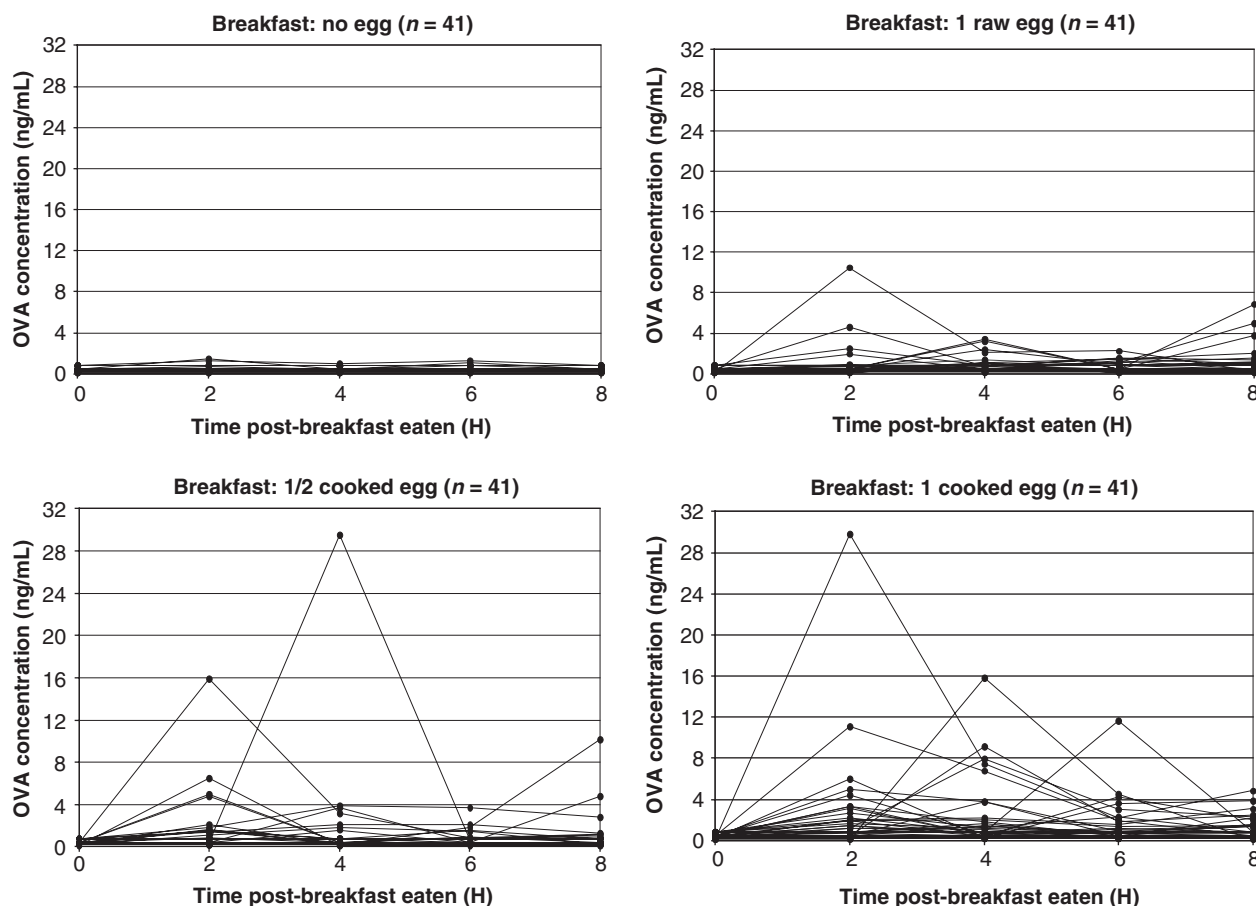


Fig. 1. Ovalbumin (OVA) excretion in human milk as a function of time post-test breakfast.

a radioimmunoassay detection method [4, 5]. In all, 22/41 (54%) of women in our trial had OVA detected in their milk after one raw egg compared with 13/22 (59%) and 14/19 (74%) in earlier reports [4, 5], and OVA peak concentrations of those women with detectable OVA after raw egg ingestion ranged from 0.17 to 10.48 ng/mL in our trial compared with 0.20 to 6.17 ng/mL [4, 5]. Despite this similarity, we observed a consistently greater response of OVA in human milk after one cooked egg compared with one raw egg. Anecdotal evidence suggests that raw egg ingestion may be more allergenic than cooked egg and this can be supported by case reports of egg-allergic children [17] who tolerated one cooked (hard boiled) egg on dietary challenge but later adversely reacted to ingestion of raw egg of unknown quantity. In our trial, we focused on measuring the concentration of OVA in human milk after the extremes of raw vs. well-cooked egg and our trial was not designed to assess human milk OVA allergenicity. In a recent study, Evenepoel et al. [18] determined that human small intestine digestion and absorption of cooked egg was 91% compared with 51% for raw egg, suggesting that a greater proportion of egg protein from cooked eggs enters the mother's circulation, and this may explain the greater concentration of OVA in human milk in response to cooked rather than raw egg.

The majority of previous studies that analysed breast milk samples after maternal ingestion of a specific challenge dose

of food protein found that the peak appearance in breast milk occurred between 1 and 4 h after ingestion of the challenge [4, 5, 9, 10]. Only one study [6] showed the peak appearance between 4 and 24 h (median of 8 h). Thus, in this study, we collected breast milk samples at two hourly intervals up to 8 h post-dietary challenge. Interestingly, 24% of the women had no OVA detected in their breast milk on any of the 4 clinic days, suggesting that some women either have a delayed excretion or may not excrete OVA in their breast milk, at least when challenged with one whole egg. Further studies with breast milk collections over a longer period of time (24–48 h) are needed to determine whether dietary restrictions in up to one-quarter of women may be unwarranted. However, the practicalities of such a study would probably limit both participation and compliance.

A high proportion (44%) of the women who participated in this trial were atopic. We suspect that their history of allergic disease may have motivated involvement in this time-consuming study for mothers of young infants. Despite this apparent selectivity towards atopic women, there was no relationship between OVA detection in human milk and maternal atopy status. Family history of atopy is regarded to be the strongest predictor for allergy development in infants [19]. Previous studies [6, 8, 10] were also unable to find any evidence that maternal atopy status influences the passage of food proteins into human milk.

The total dietary avoidance of a food protein can be difficult to achieve, may be nutritionally compromising, time consuming and socially restrictive, and many mothers question the need for total vs. partial avoidance of a food protein. This study has provided important information about the frequencies and concentrations of OVA in human milk after the ingestion of raw vs. cooked egg and after an increasing dose of cooked egg consumption. As none of the infants in this study had known egg allergy, further studies need to examine whether the results from this study are reproducible in a group of lactating women whose infants have an egg allergy. To improve the quality of dietary advice to breastfeeding mothers, future studies also need to determine the threshold of OVA excretion in human milk that leads to symptoms in egg-allergic breastfed infants.

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All authors participated in designing the study and interpreting the data. D. J. P. was responsible for recruitment, data collection, laboratory method development and data analysis, under the supervision of M. M. and M. S. G. D. J. P. wrote the paper with contributions from M. M. and M. S. G.

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