

Exposure of the fetus and infant to hens' egg ovalbumin via the placenta and breast milk in relation to maternal intake of dietary egg

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Summary

Background Maternally derived allergens may be transferred to the developing infant during pregnancy and lactation. However, it is not known how manipulation of environmental allergen levels might impact on this early-life exposure.

Objective To measure dietary egg allergen (ovalbumin (OVA)) in gestation-associated environments, in relation to maternal dietary egg intake.

Method OVA was measured by allergen-specific ELISA in maternal blood collected throughout pregnancy, infant blood at birth (umbilical cord) and in breast milk at 3 months post-partum. Samples derived from pregnant women undergoing diagnostic amniocentesis at 16–18 weeks gestation who were not subject to any dietary intervention, and from pregnant women, with personal or partner atopy, randomized to complete dietary egg exclusion or an unmodified healthy diet before 20 weeks gestation as a primary allergy prevention strategy. Maternal dietary egg intake was monitored closely throughout the study period by diary record and serial measurement of OVA-specific immunoglobulin G concentration.

Results Circulating OVA was detected throughout pregnancy in 20% of women and correlated with both presence ($P < 0.001$) and concentration ($r = 0.754$, $P < 0.001$) of infant OVA at birth (umbilical cord). At 3 months post-partum OVA was detected in breast milk samples of 35% women, in higher concentrations than measured in blood. Blood and breast milk OVA were not related to maternal dietary intake or atopic pre-disposition.

Conclusions Rigorous dietary egg exclusion does not eliminate trans-placental and breast milk egg allergen passage. This early-life exposure could modulate developing immune responses.

Keywords atopy, breast milk, elimination diet, fetal blood, hens' egg ovalbumin

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Introduction

The nature of the primary encounters of the immune system with allergen is crucial in determining whether sensitivity occurs to that allergen, particularly in those with an atopic genotype. It is well recognized that post-natal environmental allergen exposure is central to the development of allergic responses [1, 2]. However, while exposure to maternally derived dietary allergen via breast milk has long been known to provoke food-associated allergic symptoms [3], the relevance of *in utero* allergen exposure for immunoglobulin (Ig)E sensitization or disease, at least in humans, remains a controversial issue [4–6].

The fetus is capable of making sophisticated immune responses, including the production of total and specific IgE [7, 8], and antigen-specific immune reactivity may commence from the second trimester of pregnancy [9, 10]. Also, while there is a strong hereditary component to the development of allergy, maternal atopic pre-disposition represents a particular risk for the infant [11–14]. Immunological factors in the pregnancy-associated environment of the atopic mother that might modulate developing immune responses include raised cord blood, amniotic fluid and breast milk IgE proportionate to circulating maternal levels [15–17] and distinctive alterations of cytokine balance [18–21]. Thus, it is conceivable that the nature of materno-fetal/neonatal allergen exposure, in association with an altered environment created by the atopic mother antenatally and in the immediate post-partum period, might drive T_H2-skewed immune responses on subsequent allergen exposure [22, 23].

Trans-placental passage of nutritive allergens, hen's egg ovalbumin (OVA) and cow's milk β -lactoglobulin (BLG),

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have been demonstrated using an *ex vivo* model of placental perfusion [24, 25]. Likewise, inhalant allergens, house-dust mite (HDM) Der p 1 and cat Fel d 1, have been detected in umbilical cord blood [26, 27]. However, to date, little is known about the relationship between external environmental allergen levels and those found in the pregnancy-associated environments of the mother and infant. This may impact on developing immune responses, and moreover, may provide insight as to the value of maternal allergen avoidance measures for primary allergy prevention. Accordingly, we sought to characterize the profile of maternally derived dietary egg allergen exposure during pregnancy and lactation, in relation to egg consumption and to maternal atopic pre-disposition.

Subjects and methods

The data were derived from two groups of pregnant women participating in allergy studies (Egg Avoidance Trial and Amniocentesis Study) in the Southampton Child Health Department.

Egg Avoidance Trial: overview

Pregnant women attending antenatal booking clinics in Southampton and surrounding areas, who had a personal or partner history of allergy, were invited to participate in a randomized controlled trial of egg avoidance during pregnancy and breastfeeding as a primary allergy prevention strategy. Parental allergy was confirmed during the initial recruitment visit in the Southampton Child Health Department by interview questionnaire [28] and positive skin prick testing (SPT) (weal size ≥ 2 mm, in the presence of appropriate responses to positive [histamine] and negative [saline] controls) to one or more of a panel of common dietary and inhalant allergens (HDM, cat, dog, timothy grass pollen, birch pollen, horse, alternaria, egg: all ALK, Abelló; Denmark). Women were randomized to either complete dietary egg exclusion or an unmodified healthy diet from 17 to 20 weeks' gestation until the end of lactation. Participants were excluded from the study if the pregnancy was complicated, if they had birds in the home, were egg allergic themselves or had ongoing dietary restrictions.

Dietary management At study recruitment, a research dietitian, dedicated to this project, gave all the women healthy eating advice and guided the intervention group with regard to following an egg-free diet (both overt and hidden forms of egg). A food diary kept during the week prior to recruitment was analysed for nutritional adequacy in general, and for eggs and egg products in particular, using 'CompEat 5' dietary analysis programme. All subjects were evaluated again at 24 and 32 weeks' gestation to ensure ongoing satisfactory nutritional balance and monitor overall egg intake by interview and analysis of a 7-day food diary completed during the preceding week. Known episodes of accidental egg ingestion were recorded prospectively by egg-avoiding women and the data collected at these visits.

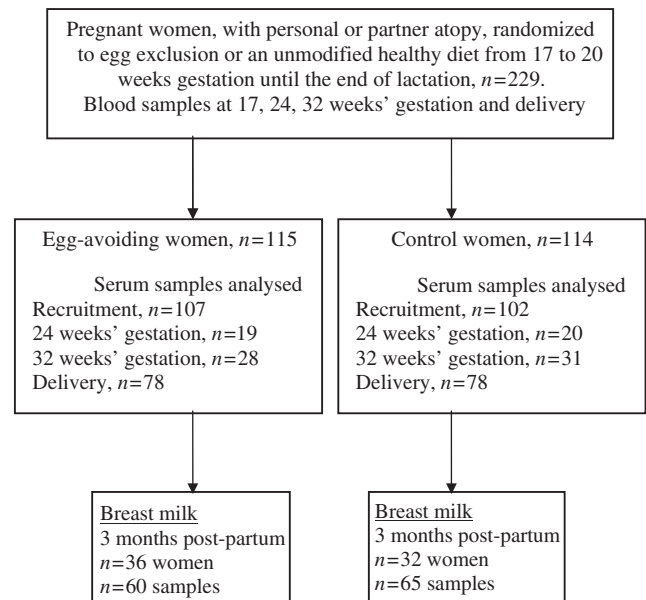


Fig. 1. Consort diagram outlining the samples considered in the current analyses, which derived from subjects participating in an Egg Avoidance Trial during pregnancy and breastfeeding as a primary allergy prevention strategy.

Egg Avoidance Trial study group

The current study related to those women participating in the Egg Avoidance Trial who donated blood and/or breast milk samples (Fig. 1).

Sera were prepared from maternal venous blood collected at study recruitment (17–20 weeks' gestation), 24 and 32 weeks' gestation and immediately following delivery of a healthy term (≥ 37 weeks') infant. Infant blood was also collected at birth by umbilical vein puncture. Breast milk samples were collected by manual expression at 3 months post-partum. These were subsequently centrifuged at 4000 g for 20 min at 4 °C to separate fat and cells from whey. Serum and breast milk whey samples were stored at -80 °C until use.

Blood samples at one or more time-points during pregnancy were analysed for 210 of the 229 (92%) women enrolled in the trial. Available samples at study recruitment ($n = 209$) and delivery ($n = 156$) were assayed for all subjects and a further subset of samples collected at 24 weeks' ($n = 39$) and 32 weeks' gestation ($n = 59$) were also selected for analysis. Data at all four time-points were available for 30 women (13 egg-avoiding women).

At 3 months post-partum, 68 women (38 egg-avoiding women) donated one or more breast milk samples ($n = 125$ samples).

Amniocentesis study

Archived plasma samples, prepared from maternal blood collected at 16–18 weeks' gestation ($n = 128$), were also available. These samples formed part of a prospective cohort study of antenatal maternal immune responses and the development of atopy. They derived from unselected pregnant women, with no dietary restrictions, undergoing

diagnostic amniocentesis at Princess Anne Hospital, Southampton and who proceeded with a healthy pregnancy.

Umbilical cord samples

Available cord samples were analysed for those infants whose matched maternal blood sample(s) (collected as part of the Egg Avoidance Trial or Amniocentesis Study) had detectable egg OVA at any time-point during pregnancy ($n = 27$). An equal number of infant cord samples were analysed where matched maternal blood samples were negative for detectable OVA during pregnancy.

Ethics

The women were recruited to the studies with informed consent. The projects were approved by Southampton and South West Hampshire and North and Mid Hampshire Local Research Ethics Committees (LREC numbers: 52/97, 021/A and 274/98).

Antigen detection

Concentrations of egg OVA in blood and breast milk whey were measured by in-house ELISA.

Briefly, microtitre plates (NUNC, Maxisorp, Roskilde, Sweden) were coated with goat IgG polyclonal anti-chicken egg OVA antibody (2.5 µg/mL; Cappel, ICN, USA) and incubated overnight at 4°C. After blocking non-specific binding with 3% bovine serum albumin (BSA), OVA standards (3.9 pg/mL–8 ng/mL; grade VII, Sigma Aldrich, Poole, UK), test samples (sera or plasma diluted 1 : 2 and 1 : 4; breast milk diluted 1 : 10), buffer without added serum/breast milk (blanks) and serum from a healthy, non-atopic, adult female volunteer self-selecting to exclude dietary egg vigilantly (negative control) were added to duplicate wells. After 2 h incubation at room temperature, capture antibody–OVA complexes were detected with rabbit IgG polyclonal anti-OVA antibody (0.5 µg/mL; Sigma Aldrich). The wells were then incubated with biotinylated goat anti-rabbit antibody (1 : 10 000; Dako, Ely, UK), followed by extravidin alkaline phosphatase (1 : 50 000; Sigma Aldrich). The substrate *p*-nitrophenyl phosphate (pNPP) (Sigma Aldrich) was applied and the absorbance read at 405 nm after stopping the reaction. The concentration of OVA in the samples was determined by comparison with the reference OVA standard curve. The limit of detection, defined as three standard deviations above the mean absorbances of the blank wells, was 15–30 pg/mL.

All samples from an individual subject were run on the same plate, on the same day. Samples from an equal number of egg-avoiding and control women were run on the same plate.

Detection assay validation

Assay specificity Assay specificity was confirmed by immunoblotting and inhibition ELISA experiments.

Immunoblotting Dot-Blot experiments were performed to compare binding of the anti-OVA antibodies with a range of unrelated antigens.

Human serum albumin (HSA), BSA, cow's milk BLG, egg ovomucoid, lysozyme and OVA (all Sigma Aldrich) were dotted (50 µL per spot) onto nitrocellulose paper (Hybond, Amersham Biosciences UK Ltd, Chalfont St Giles, UK) in a range of concentrations (100 ng/mL–100 µg/mL). Protein immobilization was confirmed by washing the membrane in Ponceau S solution (Sigma Aldrich) and unoccupied binding sites blocked with 3% BSA (Sigma Aldrich). Identical membrane strips were incubated with either rabbit IgG anti-OVA antibody (10 µg/mL; Sigma Aldrich), goat IgG anti-OVA antibody (1.25 µg/mL; Cappel, ICN) or appropriate species isotype controls (rabbit γ-globulin; 10 µg/mL; Perbio Science UK Ltd, Tattenhall, UK and goat γ-globulin; 1.25 µg/mL; R&D Systems Europe Ltd, Abingdon, UK). Antibody binding was visualized by incubating membrane strips with either goat anti-rabbit biotin (1 : 8000; Dako) or rabbit anti-goat biotin (1 : 20 000; Stratech Scientific Ltd, Soham, UK), followed by HRP-conjugated extravidin (1 : 2000; Sigma Aldrich) and application of diaminobenzidine [DAB] (Sigma Aldrich).

Detectable OVA in subject samples was characterized further by Western Blotting.

Selected blood and breast milk samples were separated using SDS-PAGE. Samples (10 µL per lane) were run under non-reducing conditions in 4–12% NuPAGE gel (Invitrogen, Paisley, UK). Reference markers of known molecular weight (MW) (SeeBlue Pre-Stained Standards, Invitrogen) and a positive OVA control (1 µg/mL; Sigma Aldrich) were run in the same gel. Following electrophoresis, the gel was transferred to nitrocellulose paper (Amersham), protein immobilization revealed by immersion in Ponceau S solution (Sigma Aldrich) and unoccupied binding sites blocked with 3% BSA (Sigma Aldrich). Identical membrane pairs were then incubated with either rabbit IgG anti-OVA antibody (10 µg/mL; Sigma Aldrich) or rabbit γ-globulin isotype control (10 µg/mL; Perbio Science UK Ltd), followed by HRP-conjugated donkey anti-rabbit (1 : 1000; Amersham). Antibody binding was visualized following application of enhanced chemiluminescence (ECL) reagents (0.125 mL/cm²; Amersham) and exposure to X-ray film (Hyperfilm, ECL, Amersham).

Inhibition enzyme-linked immunosorbent assays In a modified OVA detection assay, the rabbit IgG anti-OVA detector antibody was spiked with increasing doses of OVA or the unrelated allergen, BLG (2.6 pg/mL–125 µg/mL; both Sigma Aldrich) before adding to microtitre plate wells pre-incubated with OVA (200 ng/mL) and continuing the ELISA protocol, as previously. Control wells contained OVA, BLG or buffer without added allergen (blanks) and were incubated with unspiked rabbit IgG anti-OVA detector antibody.

The ability of the two spiking antigens to inhibit detection of the capture antibody–OVA complexes was compared by plotting assay absorbance (405 nm) against spiking antigen concentration.

Recovery and parallelism The degree to which the assay could detect antigen in the test sample (recovery) was quantified. Sera without detectable OVA ($n = 63$) were spiked with OVA (1 ng/mL; Sigma Aldrich) and the ELISA repeated. The concentration of spiking OVA that could be detected in these samples was determined by comparison of

sample absorbance (405 nm) with the reference OVA standard curve. OVA recovery was expressed as a percentage of the spiking dose.

Serial dilution of OVA-positive sera produced an assay absorbance profile mirroring that of the OVA reference standard, thereby confirming comparative behaviour of the antigen in the reference and test media (parallelism).

Serum ovalbumin immunoglobulin G measurement

Concentrations of egg OVA IgG were determined by ELISA in maternal and cord blood samples, as reported previously [29].

Briefly, microtitre plates (NUNC) were coated with 100 µg/mL OVA or BSA (as a control for non-specific binding) (both Sigma Aldrich) and incubated overnight at 4 °C. After blocking unoccupied binding sites (3% BSA), a reference serum pooled from non-food allergic, healthy women (diluted 1 : 50–1 : 6400), test serum samples (diluted 1 : 100) and buffer without added serum (blanks) were added to duplicate wells. Bound IgG was detected by HRP-conjugated rabbit anti-human IgG (1 : 6000; Dako). *O*-phenylenediamine hydrochloride (OPD) (Sigma Aldrich) substrate was added and the absorbance read at 492 nm after stopping the reaction. The absorbances on the BSA-coated plate were subtracted from those on the allergen-coated plates and the values expressed as arbitrary units (AU) by comparison with the reference serum standard curve.

Statistics

Samples with OVA measurements below the ELISA limit of detection were assigned a value of zero and included in the analyses.

Statistical comparisons of OVA presence and/or concentration involving the Egg Avoidance Trial categories (egg avoidance, controls) were made using both an intention to treat analysis, and with consideration of known subject cessation of the dietary exclusion measures (diet 'drop-out'). Exclusion of diet 'drop-outs' did not change statistical comparisons and consequently only the intention to treat analyses are reported.

Maternal OVA-specific IgG measurements were normally distributed following natural log transformation and thus the relationship between OVA recovery and IgG titre was analysed using the parametric test, Pearson's correlation coefficient. The remaining, non-normally distributed data were analysed using non-parametric tests. In comparing data from different groups, the Mann–Whitney *U*-test was applied. Spearman's rank r_s correlation coefficient was used to evaluate the relationships between maternal OVA concentrations during pregnancy, fetal concentrations at birth and dietary egg consumption. Associations and analyses of categorical data were made using χ^2 techniques, and if observed frequencies were less than 5, Fisher's exact test was used.

Statistical significance was considered when $P < 0.05$, and adjusted when conducting multiple comparisons. Calculations were carried out using SPSS for Windows software (version 11.5).

Results

Assay validation

Dot-Blot experiments confirmed no immunoreactivity of assay antibodies to the irrelevant antigens HSA, BSA, BLG, ovomucoid and lysozyme. Also, in the inhibition ELISA, spiking doses of OVA, but not the unrelated allergen, cow's milk BLG, were able to block detection of the capture antibody–antigen complex in a dose-dependent manner (data not shown).

Western blotting of subject blood and breast milk samples revealed specific anti-OVA antibody binding to protein of approximate MW 44 kDa (consistent with free OVA), but only in those samples with detectable OVA by ELISA (Fig. 2).

Recovery of a spiking dose (1 ng/mL) of OVA in ELISA-negative (no detectable OVA) maternal blood was inversely related to sample-specific IgG concentration ($r = -0.592$; $P < 0.001$) (Fig. 3).

Egg ovalbumin in maternal blood during pregnancy

Serum OVA was detectable at one or more time-points during pregnancy in blood samples collected from 41 of 210 (19.5%) women participating in the Egg Avoidance Trial (egg-avoiding and control women). Detection rates and OVA concentrations at each gestation time-point are shown in Table 1.

Detectable OVA at recruitment to the Egg Avoidance Trial (17 weeks' gestation) correlated with both detection (χ^2 , $P < 0.001$, all time-points) and OVA concentration at later gestation time-points (17 and 24 weeks $r = 0.816$; 17 and 32 weeks $r = 0.664$; 17 and 40 weeks $r = 0.774$; all $P < 0.001$).

At recruitment, serum OVA-specific IgG concentration was not significantly different between samples with, and without,

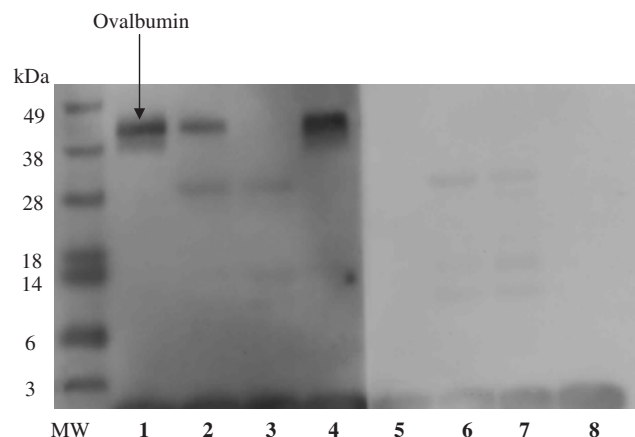
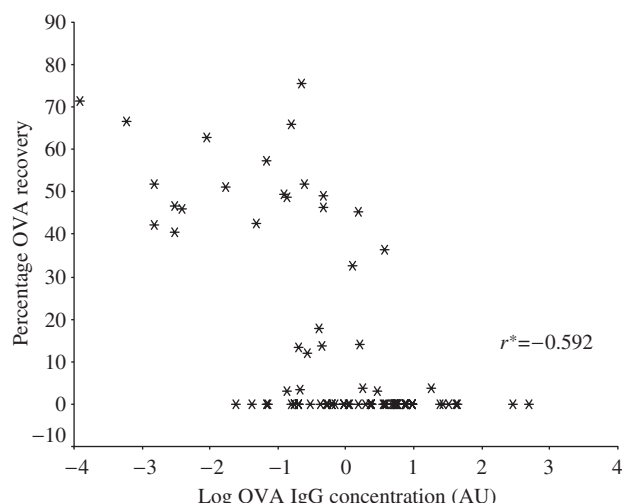


Fig. 2. Western blot for hen's egg ovalbumin (OVA) in breast milk and cord serum samples. Breast milk and cord serum samples deriving from subjects participating in the egg avoidance trial were Western blotted for OVA using enhanced chemiluminescence. Samples were probed either with rabbit immunoglobulin (Ig)G anti-OVA (lanes 1–4) or a rabbit IgG isotype control (lanes 5–8). The molecular weight markers (MW) are shown. Lanes contained OVA control samples (1 µg/mL) (lanes 1 and 5), breast milk with (lanes 2 and 6) or without (lanes 3 and 7) OVA, as detected by in-house ELISA, and an OVA-positive cord serum sample (lanes 4 and 8). Strong staining for OVA (MW 44 kDa) was found only in ELISA positive samples. Weak bands reflect non-specific binding, as indicated by their presence on the isotype control membrane.



*, Pearson's correlation coefficient ($P < 0.001$)

Fig. 3. Correlation of ovalbumin (OVA) recovery with sample OVA-specific immunoglobulin (IgG) concentration. The ability of the assay to detect all antigen in a test sample (recovery) was quantified as the percentage of a spiking dose of OVA (1 ng/mL) that could be detected in OVA-negative sera (endogenous OVA non-detectable). There was a significant negative correlation between recovery and sample-specific IgG concentration ($r = -0.592$, $P < 0.001$), indicating that specific IgG might inhibit antigen detection.

detectable OVA ($P = 0.122$). At term, women with detectable circulating OVA tended to have higher specific IgG levels ($P = 0.064$).

OVA was detectable at 16–18 weeks gestation in 26 of 128 (20.3%) archived plasma samples collected from unselected pregnant women (0.015–10.6 ng/mL, median 0.6 ng/mL) (Table 2).

Egg ovalbumin in maternal breast milk

At 3 months post-partum, OVA was detected in one or more breast milk samples from 24 of 68 (35%) women, in concentrations ranging 0.12–1258 ng/mL, median 0.70 ng/mL (Table 3).

Egg ovalbumin in fetal (umbilical cord) blood

OVA was detected in 19 of 54 selected cord samples. Cord blood was positive for OVA in 18 of 27 (67%) infants for whom one or more matched maternal blood samples were

Table 2. Table showing the frequency and concentration of detectable OVA in blood samples collected during pregnancy from women undergoing diagnostic amniocentesis at 16–18 weeks' gestation (Amniocentesis Study). Also, the frequency and concentration of detectable OVA in selected umbilical cord blood samples where matched maternal blood during pregnancy was either positive or negative for OVA

	Amniocentesis Study Maternal blood at 16–18 weeks' gestation	Cord blood
Number of samples	128	54
Number of OVA positive samples [%]	26 [20.3]	19
		18/27 [67] mat blood OVA + 1/27 [3.7] mat blood OVA –
Concentration range (ng/mL) (in positive samples)	0.015–10.6	0.05–5.67
Median (ng/mL)	0.60	0.31

OVA, ovalbumin.

also positive for OVA during pregnancy, irrespective of when detected during gestation. Only one of 27 (3.7%) cord samples was positive where matched maternal samples were negative for OVA (χ^2 , $P < 0.001$). Cord blood OVA concentration ranged between 0.05–5.67 ng/mL, median 0.31 ng/mL (Table 2) and correlated directly with maternal concentrations ($r = 0.754$, $P < 0.001$) (Fig. 4a).

Among cord samples deriving from Egg Avoidance Trial participants ($n = 43$), serum OVA IgG concentration was higher in samples with detectable OVA, compared with those without ($P = 0.018$) (Fig. 4b).

Ovalbumin presence during pregnancy and lactation in relation to egg intake and maternal atopic pre-disposition

Egg intake Among the Egg Avoidance Trial group, overall egg intake during pregnancy was significantly lower in egg-avoiding women (median 2.4 g, range 0.21–25.30 g) than in control women (median 41.7 g, range 2.97–149.16 g) ($P < 0.001$). Twenty-one women (21 of 115, 18%) abandoned the elimination diet during pregnancy (at mean gestation 25 weeks, range 18–33 weeks), of whom 14 gave blood or breast milk samples that were considered in the current analyses.

Egg-avoiding women were as likely to have detectable OVA in blood samples collected during pregnancy (including

Table 1. Table showing the frequency and concentration of detectable ovalbumin in blood samples collected during pregnancy (17, 24 and 32 weeks' gestation and at delivery) from women participating in a randomized controlled trial of egg avoidance as a primary allergy prevention study

Gestation (week number)	Number of positive serum samples [%]	Concentration range (ng/mL) [median]	Positive serum samples: egg-avoiders	Concentration range (ng/mL) [median]	Positive serum samples: control women	Concentration range (ng/mL) [median]
17 (Participant randomization)	33/209 [15.8]	0.06–17.4 [0.29]				
24	4/39 [10.2]	0.13–1.61 [0.34]	3/19	0.27–1.61 [0.40]	1/20	0.13
32	7/59 [11.8] (6/58)*	0.06–1.88 [0.25]	4/28 (3/26)*	0.25–1.88 [0.56]	3/31	0.06–0.21 [0.11]
40	22/156 [14.1] (21/144)*	0.02–14.58 [0.30]	10/78 (9/66)*	0.03–1.78 [0.28]	12/78	0.02–14.58 [0.34]

At each time point respective data for the individual study categories – egg avoiding and 'control' women – are also shown.

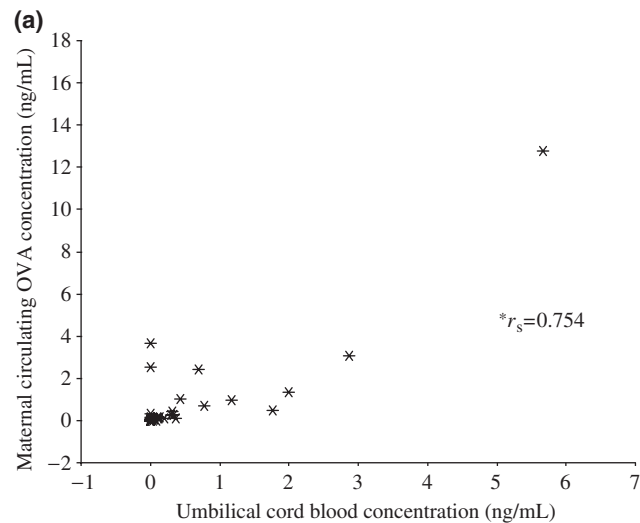
*Number in parentheses refers to numbers of egg-avoiding women adherent to elimination diet – i.e. diet 'drop-outs' excluded.

Table 3. Table showing the frequency and concentration of detectable OVA in breast milk samples collected at 3 months post-partum from women participating in a randomized controlled trial of egg avoidance as a primary allergy prevention study

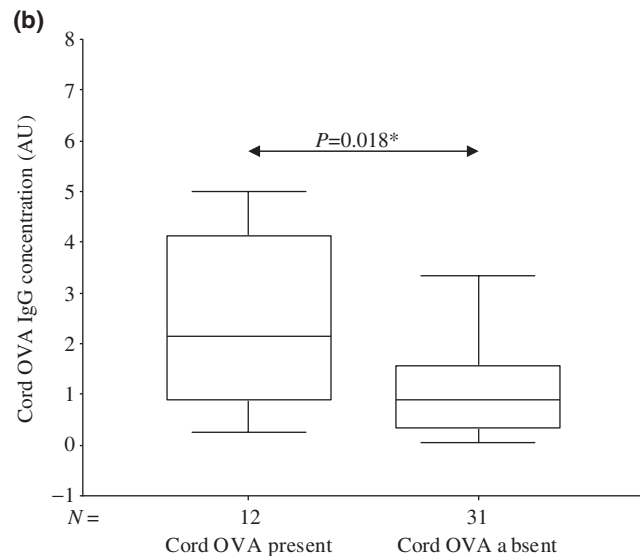
Breast milk (BM)	Total	Egg avoiders	Controls
Number of women with positive BM sample [%]	24/68 [35]	11/36 [30.5] (10/34)*	13/32 [40.6]
Number of OVA positive BM samples [%]	40/125 [32]	18/60 [30] (17/56)*	22/65 [33.8]
Concentration range (ng/mL) (in positive samples)	0.12–1258	0.12–170.9	0.14–1258
Median (ng/mL)	0.70	1.41	0.63

The respective data for the individual study categories – egg-avoiding and 'control' women – are also shown.

*Number in parentheses refers to numbers of egg-avoiding women adherent to elimination diet – ie, diet 'drop-outs' excluded.
OVA, ovalbumin.



*, Spearman's rank r_s correlation coefficient ($P < 0.001$)



*, Mann-Whitney U -Test

Fig. 4. (a) Correlation between concentrations of ovalbumin (OVA) in umbilical cord and maternal blood during pregnancy. There was a significant direct relationship between concentration of OVA found in maternal blood during pregnancy and infant blood at delivery. (b) Cord serum OVA immunoglobulin (IgG) concentration in relation to presence of detectable OVA. The diagram illustrates that umbilical cord samples (deriving from Egg Avoidance Trial participants) with detectable OVA had higher serum-specific IgG concentration than those without OVA. Serum OVA and specific IgG concentrations were measured by in-house ELISAs. The box-plot shows values in the interquartile range between the 25th and 75th percentiles. The central line is the median value and the whiskers extend to the lowest and highest values, excluding outliers.

recruitment), and breast milk samples collected at 3 months post-partum, as control women (pregnancy; $P = 0.968, 0.342, 0.698, 0.645$ at 17, 24, 32 and 40 weeks' gestation, respectively; lactation: $P = 0.386$). Also, concentrations of serum OVA did not differ significantly between egg-avoiding and control women ($P = 0.860, 0.240, 0.482, 0.621$ at 17, 24, 32 and 40 weeks' gestation, respectively), and at study recruitment and delivery were unrelated to the quantity of egg ingested, recorded prior to study commencement or during pregnancy (17 weeks $r = -0.03, P = 0.913$; term $r = -0.143, P = 0.787$).

Maternal atopy There was no association between maternal atopic pre-disposition and the presence or concentration of OVA in maternal blood at any time-point during pregnancy. Similarly, atopic and non-atopic women were as likely to have one or more OVA-containing breast milk samples at 3 months post-partum (18/48 atopic, 6/20 non-atopic women; $P = 0.555$). However, egg-avoiding, atopic women tended more frequently to have repeated positive breast milk OVA samples (16/42 samples) than if they were non-atopic (2/18 samples) ($P = 0.037$).

Discussion

Dietary exclusion measures during pregnancy and lactation have been considered carefully over the last two decades in studies of primary allergy prevention [30]. While no sustained allergic benefit of such interventions has been reported, and concerns for maternal and infant nutritional welfare have been raised, the relevance of maternal dietary manipulation for infant IgE sensitization and disease expression continues to be a pertinent question, as represented by the current UK government advice that pregnant women with a family history of allergy may wish to avoid eating peanuts and peanut-containing products during pregnancy and lactation [31]. Clearly, a greater understanding of the role of maternally derived allergen exposure in early life in the immunopathogenesis of allergic disease is essential in order to formulate appropriate intervention strategies. As a first step in this investigation, we have characterized how intake of dietary egg impacts on fetal/neonatal egg allergen exposure via the placenta and breast milk.

These data derive predominantly from subjects participating in a randomized controlled trial of egg avoidance, which incorporated comprehensive dietetic supervision and support. As a result, overall dietary compliance was excellent, though

not absolute, as 84% of egg-avoiding women reported at least one known dietary indiscretion during the intervention period. Moreover, the dietetic input facilitated prospective, serial recording of egg consumption throughout pregnancy and this detailed profile may not be feasible [5, 6], or accurate [32], for inhalant allergen exposure, which has been particularly challenged in relation to *in utero* immune responses [33].

Previously, a variety of food allergens have been detected in human breast milk and healthy adult sera, including cow's milk, egg, peanut and wheat proteins [34–42]. Indeed, a recent publication has reported the recovery of endogenous egg OVA and cow's milk BLG from placental tissue using a placental perfusion model, and quantities of OVA in term cord blood comparable with our data [43]. However, the present study is unique in describing a detailed, longitudinal picture of how maternal dietary intake, and in particular, dietary exclusion, throughout pregnancy interrelates with both circulating and breast milk allergen concentrations in a large series of samples derived from a birth cohort study, under normal physiological conditions.

This work has confirmed the presence of maternally derived dietary allergen in the fetal circulation. Thus, in conjunction with our earlier report of hen's egg OVA in amniotic fluid [10], it has demonstrated three routes of maternal allergen passage – trans-placentally, trans-amniotically and via breast milk – of which, breast milk predominates as a source of allergen dose. Furthermore, OVA in maternal blood, irrespective of the time-point detected during gestation, was significantly associated with the presence and concentration of OVA in infant blood at birth. This close relationship, and our previous data [10], together imply that the fetus may be exposed to dietary allergen from the second trimester of pregnancy by trans-placental and/or trans-amniotic routes.

OVA was detected in one or more blood samples from approximately 20% of pregnant women. Sample collection bore no relationship to the timing of the subjects' last meal and thus, as OVA detection has been shown to be optimal 2–3 h post-egg consumption [38], it is likely that our figures underestimate the true prevalence (frequency and dose) of fetal and neonatal allergen exposure. However, the key remaining questions are whether, and how, these parameters might impact on developing immune responses and infant clinical outcome.

The analysis revealed that strict adherence to an egg avoidance diet appeared to have no impact on the presence or concentration of OVA in serum and/or breast milk. There are a number of factors that might account for this paradox. Firstly, among this cohort, the vast majority of egg-avoiding women reported accidental ingestion of egg-containing foods on at least one occasion. It is quite possible that the number of such episodes may have been underestimated, or gone unrecognized. Secondly, the study protocol did not prohibit the women cooking eggs for other family members and this could have been a source of adventitious exposure, either via the gut or across the skin, particularly if the woman also had inflamed eczema [44–46]. At 6 months of age, serum OVA could be detected in 5.5% (six of 110) babies (median 0.19 ng/mL) (data not shown) and this finding supports a concept of inevitable environmental exposure, as the mothers participating in the trial were advised to delay the introduction of egg into infant weaning foods until after the 6-month clinical

review. Thirdly, as we have reported recently [29], egg-avoiding women had a significant fall in serum OVA-specific IgG concentration, reflecting their overall diminished egg consumption. As serum IgG also has a role in antigen elimination, these lower levels could permit the persistence of circulating, or placental stores of, free OVA [43, 47, 48].

Therefore, exposure to egg throughout pregnancy, even though infrequent and in small quantities, occurring in the context of reduced IgG-mediated antigen elimination, may explain the detection of OVA in the egg-avoiding group as a whole, and the possibility of enhanced detection in breast milk samples from atopic, egg-avoiding women who may, in addition, have increased antigen absorption [49]. Previous authors have found that the long-term pattern of milk ingestion determined BLG concentration in breast milk and their finding supports our current interpretation that the history of maternal OVA exposure over the prolonged study period may be revealed by the laboratory findings at each sampling time-point [37].

Overall, the important clinical implication of these data is that despite optimal attempts by a mother to exclude a food, this does not affect the probability that the fetus/newborn infant may be exposed to that allergen. Thus, this suggests that elimination diets during pregnancy and lactation, particularly if unsupported, may be a fruitless exercise.

In the course of ELISA development, it was revealed that recovery of a spiking dose of OVA was inversely related to sample-specific IgG concentration. While this might imply that the assay could detect only free allergen, in fact, serum-specific IgG concentration was not significantly different between samples with detectable OVA, and those without. Thus, it is possible that IgG might block epitope recognition by mechanisms which are subject-specific, and which depend on a complex interplay between concentration and antigen functional affinity [50]. Indeed, it is interesting that in this work detectable circulating OVA, both in maternal blood at delivery and in infant cord blood, appeared to be associated with higher specific IgG levels. As specific IgG concentration is a marker of dietary intake [51, 52], it is most likely that this correlation simply reflects subject exposure to greater quantities of egg. However, a functional association, particularly in relation to trans-placental OVA transport, should also be considered, as exogenous IgG was found to enhance materno-fetal allergen transfer in an *ex vivo* placenta model and cat Fel d 1 allergen has been identified in complex with IgG in up to 40% of cord samples [27]. In turn, complexing allergen with IgG could have repercussions for immune reactivity – either promoting immune priming by facilitating antigen focusing [53], or down-regulating immune responses via an immunoreceptor tyrosine-based inhibitory motif (ITIM) associated with the low-affinity IgG receptor, FcγRIIb (CD32b), present on B cells and T cells [54, 55]. Thus, the possible influence of maternal dietary practices on infant susceptibility to allergic sensitization through modulation of antigen form requires further consideration.

In summary, this study has demonstrated that the fetus and newborn infant may be exposed to maternally derived dietary allergen irrespective of maternal dietary exclusion practices. The nature of these initial encounters might have consequences for immune responses to post-natal allergen exposures.

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