

Effects of maternal dietary egg intake during early lactation on human milk ovalbumin concentration: a randomized controlled trial

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6 **Effects of maternal dietary egg intake during early lactation on human milk**
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8 **ovalbumin concentration: a randomized controlled trial**
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12 Short title: Maternal egg intake and ovalbumin in human milk.
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Abstract

Background: There is limited understanding of how maternal diet affects breast milk food allergen concentrations, and whether exposure to allergens through this route influences the development of infant oral tolerance or sensitization.

Objective: To investigate how maternal dietary egg ingestion during early lactation influences egg protein (ovalbumin) levels detected in human breast milk.

Methods: In a randomized controlled trial, women were allocated to a dietary group for the first six weeks of lactation: high-egg diet (> 4 eggs per week), low-egg diet (1-3 eggs per week) or an egg-free diet. Breast milk samples were collected at two, four and six weeks of lactation for the measurement of ovalbumin. The permeability of the mammary epithelium was assessed by measuring the breast milk sodium: potassium ratio. Egg-specific IgE and IgG4 were measured in infant plasma at six weeks, and prior to the introduction of egg in solids at 16 weeks.

Results: Average maternal egg ingestion was associated with breast milk ovalbumin concentration. Specifically, for each additional egg ingested per week there was an average 25% increase in ovalbumin concentration (95% CI 5%–48%, $p=0.01$). Breast milk ovalbumin concentrations were significantly higher in the 'high-egg' group (> 4 eggs per week) compared with the 'egg-free' group ($p=0.04$). ~~However, but one third of women had no breast milk ovalbumin detected. there were no other between group differences.~~ No detectable associations were found between mammary epithelium permeability and breast milk ovalbumin concentrations. Infant plasma egg-specific IgG4 levels were also positively associated with maternal egg ingestion, with an average 22% (95% CI 3%-45%) increase in infant egg-specific IgG4 levels per additional egg consumed per week ($P=0.02$).

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Conclusions & Clinical Relevance: Increased maternal egg ingestion is associated with increased breast milk ovalbumin, and markers of immune tolerance in infants. These results highlight the potential for maternal diet to benefit infant oral tolerance development during lactation.

For Peer Review

Introduction

The unprecedented rise in food allergy has become a major public health issue [1] and reinforces the pressing need to define the early events leading to sensitization, and implement prevention strategies early in life. Our recent studies have shown that the processes leading to egg sensitization are already strongly established in many high risk infants in the first months of life, before they start solid foods [2]. Specifically, ~~that~~ a significant proportion of 4 month old infants with eczema already had established egg sensitization and prior to their 'first' introduction of egg in solid foods; ~~resulted~~ in clinical reactivity [2]. This implies that much earlier preventive interventions will ultimately be needed to promote the development of oral tolerance. Before this can be safely considered, it is essential to have a better understanding of the antecedent events that lead to the establishment of food allergy so early in infancy.

The initial route of food allergen exposure may be an important determinant of either sensitization or oral tolerance. Early oral exposure to allergens through the gut is critical for maintaining and reinforcing oral tolerance, and allergens secreted in breast milk are an important early source. In breastfed infants oral allergens are first encountered in the context of tolerogenic signals from maternal milk (including cytokines and allergen-antibody complexes) and variations in breast milk composition may influence the development of oral tolerance. Some animal models have demonstrated that allergens ingested in breast milk can induce oral tolerance [3, 4, 5], whereas a recent study found contrary results [6]. However so far oral tolerance induction via breast milk has not been shown in humans, even though Although common food allergen proteins have been detected in human breast milk; including peanut (arah h1 and arah h2) [3, 75], cow's milk (beta-lactoglobulin) [86-108], and

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6 egg protein (ovalbumin) [97-142], hence the role of these proteins in the development
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8 of oral tolerance in breast fed infants remains unknown.
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12 We have also previously demonstrated that the amount and type of maternal egg
13 ingestion influences human milk egg protein (ovalbumin, OVA) concentrations
14 within an eight hour period [120]. However, there is limited understanding of longer-
15 term influences of dietary patterns on either allergen secretion or impact on measures
16 of infant immune tolerance. There is evidence of variability in OVA concentrations
17 between women even after the consumption of the same amount and type of egg
18 [120], suggesting additional factors during digestion, absorption or excretion may
19 influence the amount of food protein present in breast milk. While, increased
20 permeability of the mammary epithelium has been correlated with an increased risk
21 for atopic disease in infants with allergic mothers [15] [Benn, 2004 #361], no
22 previous studies have examined whether mammary epithelium permeability affects
23 the amount of food protein detected in human breast milk.
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39 Before any new intervention trials to potentially optimize food allergen delivery
40 through breast milk can be considered, we firstly need a deeper understanding of
41 factors that may influence the passage of food proteins into human milk and effects
42 on infant immune tolerance. In this randomized controlled trial (RCT), we
43 investigated the ~~effect~~ accumulative effect of a longer duration (6 weeks) of following
44 a recommended amount of egg ingestion by breastfeeding mothers of a dietary
45 intervention during the first six weeks of lactation, to standardise maternal egg
46 ingestion, and assess this in relation to OVA detection in human breast milk and
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6 infant measures of sensitization (egg-specific IgE) or egg protein exposure~~tolerance~~
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8 (egg-specific IgG4) prior to commencement of solids foods. We also examined
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10 whether permeability of the mammary epithelium is related to OVA breast milk
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12 concentrations.
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For Peer Review

Materials and Methods

Study Design

Pregnant women planning to breastfeed were screened for a history of medically diagnosed allergic disease (asthma, eczema, hay-fever or IgE mediated food allergy). Women were excluded if they had an egg allergy, or if their infant was delivered before 36 weeks gestation. Written informed consent was obtained prior to study participation. Baseline data was collected, including family history of allergic disease, race, educational level, smoking in the household and dietary data on recent maternal and household egg intake. This study was approved by local ethical institutional review boards (Human Research Ethics Committees) at Princess Margaret Hospital (2060EP), St John of God Hospitals (#619): Murdoch, Mt. Lawley and Subiaco, and South Metropolitan Hospital (P/13/45): Kaleeya, in Western Australia. This RCT was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12613000643774).

Randomisation and allocation concealment

In late pregnancy (36-40 weeks gestation) each participating woman was assigned a unique study number and randomly allocated to one of three intervention groups. The dietary intervention required modifications to their usual diet so participants were not blinded to their group allocation. A researcher not involved in the clinic appointments prepared the randomization schedule in blocks of 15 and stratified by baseline maternal dietary egg intake. The stratum levels were defined as women who in the last week ate: a) more than 3 eggs, (b) between 1 and 3 eggs, and (c) less than 1 egg.

Dietary Intervention

To examine the effects of dietary modifications in the early postnatal period, participating women followed the dietary intervention for the first six weeks of lactation. Participants were randomly allocated to one of three dietary groups: high-egg diet (recommended consumption more than 4 eggs per week), low-egg diet (recommended consumption of 1-3 eggs per week) and an egg-free diet (avoidance of eggs and all egg containing foods). In both of the 'egg inclusion' groups (high-egg or low-egg), mothers included all forms of egg and egg containing foods towards their weekly target of egg ingestion (examples included hard boiled or fried egg, omelette, quiche, egg in baked goods, egg in meatballs).

Data collection and compliance

Participants used a diary card to record their weekly egg intake, or in the case of the egg-free group, to record any accidental ingestion of egg. To accurately track egg consumption for a variety of foods, participants were given a conversion table showing the amount of egg present in common egg containing foods. Average weekly egg ingestion from the diary card data was used to assess compliance and the dose-response relationship with OVA in breast milk, irrespective of group allocation. All women were contacted by telephone at two and four weeks of lactation and attended clinic appointments with study researchers at six weeks (end of intervention period) and sixteen weeks (prior to commencement of solids) of lactation. At each contact point, data was collected on compliance with the dietary intervention. Additional information was also collected about breastfeeding and formula feeding habits, any breastfeeding difficulties, maternal infections, and maternal antibiotic use.

Breast milk and blood sample collections

Breast milk samples (10 ml) were collected at two, four and six weeks of lactation. Women allocated to either the high-egg or low-egg intervention groups, were asked to consume the equivalent of one egg, between two and six hours prior to expressing their breast milk sample to optimize the detection of OVA [129]. Women allocated to the egg-free group were asked to continue to avoid eating any egg prior to breast milk sample collection. Infant blood (5 ml) was collected at six and sixteen weeks of lactation, and plasma was separated from whole blood for the measurement of egg-specific immunoglobulins. Both breast milk and blood samples were stored at -80 °C prior to analysis.

Detection of ovalbumin in ~~serum and~~ breast milk

OVA concentration was measured in skim breast milk (fat was separated from the breast milk by centrifugation at 4000 g for 20 min at 4 °C) using enzyme-linked immunosorbent assay (ELISA) kits (Alpha Diagnostic International, Texas, USA). The assay was conducted as per kit instructions with a detection range of 0.1 ng/ml to 4 ng/ml. All samples were run neat and in triplicate. Quality controls were provided with each kit, and an in house prepared control (OVA 2 ng/ml, Sigma Aldrich, NSW, Australia) was included on every plate. The commercial ELISA kit used for this study was checked for specificity using OVA spiked breast milk samples with a mean recovery rate of 81.3% +/- 6.2%. This was considered acceptable, as some blocking effects of the breast milk itself were expected. We also performed dilutions using OVA spiked breast milk, and a breast milk sample with known high levels of OVA (naturally excreted) and all performed as expected and did not suggest non-specific binding was occurring. The ELISA's were analyzed using a MultiSKAN ELISA plate

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6 reader (ThermoFisher Scientific, NSW Australia), at a wavelength of 450 nm.
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8 SkanIt™ 2.5.1 Research Edition software (ThermoFisher Scientific, NSW Australia)
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10 was used to interpret the results. If any samples were above the detection limit of
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12 4ng/ml, they were diluted and repeated to obtain fluorescence readings within range.
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14 Any samples that were below the lower limit of detection were assigned a value of
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16 one half the lowest limit of detection (0.05 ng/ml). All samples for each woman were
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18 run on the same plate. Additionally each plate contained a distribution of all three
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20 intervention groups (high-egg, low-egg and egg-free). The samples were coded using
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22 unidentifiable numbers to ensure the researcher remained blinded throughout the
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24 detection assays.

25 26 27 28 ***Mammary epithelium permeability assay***

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30 The sodium: potassium ratio in breast milk is associated with the permeability of the
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32 mammary epithelium. The concentrations of sodium and potassium in the breast milk
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34 samples were determined by the ion selective electrodes (C- 122 for sodium; C-123
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36 for potassium, Horiba, Japan). Calibration of the electrodes was conducted according
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38 to the manufacturer recommendations. For each measurement, the whole milk
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40 samples were thawed at 37°C for 1 hour. Prior to the measurement, the milk sample
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42 was then hand mixed for 15 seconds followed by 3 inversions. 300µl of the milk was
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44 pipetted onto the sensor pad of the electrode. The reading was taken when the results
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46 had stabilized for 15 seconds. After each measurement, the milk sample was removed
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48 from the pad and returned to the storage tube. The pad was then rinsed with double
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50 deionized water and wiped with Kim Wipes prior to the next measurement. All
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52 samples were analyzed at the same time in duplicate. The same procedures were
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54 applied to both electrodes. The sodium: potassium ratio was calculated in a 1:1 ratio.

Measurement of IgE and IgG4 in infant blood

Infant whole egg-specific IgE and egg white-specific IgG4 ~~plasma~~serum antibody concentrations were measured using the ImmunoCAP 250 system (Phadia AB, Uppsala, Sweden) at six and sixteen weeks of lactation. For specific IgE, the lower limit of detection was 0.1 kUA/L and for specific IgG4, the lower limit of detection was 0.07 mg of antibody/L.

Sample Size and Statistical Methods

The expected rate of detection of egg protein (OVA) in breast milk in women regularly consuming egg in their diet is at least 46%, compared to a detection rate of 6-7% in women following an egg-free diet during lactation. This is based on our previous RCTs of breast milk OVA detection [120, 134]. To detect an increase in egg protein content of breast milk from 7% to 46%, absolute increase of 39%, relative increase of 85%, (with 90% power, alpha-value 0.025 due to the three group design), we required 32 infants per group. To allow for any residual imbalance between the groups, even after randomisation, and to take into account of potential confounders (maternal age, dietary factors) in the analysis, we inflated our sample size by 20%, as well as a further 5% for loss to follow-up or withdrawal, hence the aim was to recruit a total of 120 women (40 per group) into the trial.

Maternal and infant baseline characteristics were summarized for each intervention (diet) group as means and standard deviations or medians and interquartile ranges (IQR) for continuous variables with symmetric and asymmetric distributions, respectively. Categorical variables were summarized as frequencies and percentages.

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6 Comparisons between intervention groups at baseline were performed using analysis
7 of variance (ANOVA), Kruskal-Wallis (non-parametric ANOVA) or Fisher's Exact
8 test as appropriate. Breast milk samples were assayed in triplicate at 2, 4 and 6 weeks
9 and summarised as individual median OVA concentrations. Median breast milk OVA
10 concentrations were log transformed prior to analysis using TOBIT regression, with a
11 lower limit of log(0.1) ng/ml, an upper limit of log(4.0) ng/ml and either dietary group
12 or average egg intake as the explanatory variable. Infant IgG4 concentrations at 6 and
13 16 weeks were summarized and analysed similarly using a lower limit of log(0.07)
14 mg of antibody/L. All analyses were performed in R software (version 3.2.1).
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Results

Recruitment commenced in August 2013 and the final assessment (at 16 weeks of lactation) appointments were completed in April 2015. A total of 2034 women were initially screened for eligibility, 1502 women did not meet eligibility criteria, with 1493 (99%) of these were ineligible due to no history of allergic disease. An additional 319 women who were eligible for the study did not give their consent to participate. In total, 120 women were randomised into this trial: high-egg (n= 40), low-egg (n=44), and egg-free (n=36) (Figure 1). There were no significant differences in baseline characteristics between the three intervention groups (Table 1). Ninety-two percent (111/120) of women attended the six week appointment, and eighty-five percent (102/120) were able to give a breast milk sample at six weeks of lactation to measure OVA concentration. At six weeks of lactation 105/112 (93.7%) and at 16 weeks of lactation 93/108 (86.1%) of the participants were still breastfeeding. Ninety percent (108/120 participants) attended the final appointment at 16 weeks of lactation. Three women randomized to the egg-free group withdrew prior to commencing the intervention because they were concerned about restricting their diet.

Intervention and dietary compliance

Overall 109/117 (93%) participants completed the full six-week intervention. Based on the diary card records of participant weekly egg intake, the median (IQR) amount of eggs eaten per week during the intervention for each group was: high-egg 5.0 (4.1-6.7), low-egg 2.4 (1.8-3.0), and egg-free 0.0 (0.0-0.25). Compliance in the high-egg group was 100%, with all 36/36 of the women complying with their prescribed intervention to consume more than four eggs per week for six weeks. 40/42 (95%) women in the low-egg group consumed on average the prescribed one to three eggs

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6 per week. Compliance in the egg-free group was lower with only 7/31 (23%) women
7 strictly adhering to an egg-free diet during the entire 6 week intervention period. This
8 improved over the six week intervention period, the median (IQR) eggs eaten per
9 week in week one was 0.00 (0.48) compared to by week six 0.00 (0.00) in the egg-
10 free group. Most cases of accidental egg exposure in the egg-free group were from
11 small quantities of egg found in common foods such as cake, mayonnaise, biscuits,
12 and noodles. At 16 weeks of lactation (10 weeks post-intervention), the mothers were
13 consuming a mean intake of 4.1 +/- 3.9 eggs per week.
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Breast milk ovalbumin content and dietary egg ingestion

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25 At the end of the intervention (six weeks of lactation), the women allocated to the
26 high-egg diet had significantly higher breast milk OVA levels than women eating an
27 egg-free diet (P=0.036). The median levels for each group are shown in Table 2.
28 There were no detectable differences between breast milk OVA levels of the low-egg
29 and the egg-free diet groups at two, four or six weeks (P=0.43, P=0.47 and P=0.42
30 respectively). Individual participant breast milk OVA concentrations at each time
31 point are shown in Figure 2. When breast milk OVA concentrations were examined
32 against actual maternal egg intake, the average number of eggs consumed per week
33 during the intervention was significantly associated with OVA concentration in breast
34 milk (log transformed) at six weeks ($\beta=0.22$, $SE(\beta)=0.09$, $P=0.01$). For each
35 additional egg ingested per week there was an average 25% increase in OVA
36 concentration (95% CI 5%–48%, $p=0.01$), conditional on detectable OVA levels
37 (Figure 3). There were no associations between average egg ingestion at two or four
38 weeks with OVA concentration in breast milk (P=0.83 and P=0.16 respectively).
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6 There were 92 participants with three consecutive breast milk samples available at
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8 two, four and six weeks of lactation. A total of 31/92 (33.7%) women did not have
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10 detectable OVA in their breast milk at any time point throughout the study. In the
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12 high-egg group, 8/30 (26.7%) women did not have any detectable OVA in any of
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14 their breast milk samples despite eating a minimum of four eggs per week. In the low-
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16 egg group 14/36 (38.9%) of women ~~in~~ did not have any detectable OVA in their
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18 breast milk. Despite following an egg-free diet, only 9/26 (34.6%) of women in the
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20 egg-free intervention group did not have any detectable OVA in their breast milk at
21
22 any time point throughout the intervention.
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24 25 26 *Mammary Epithelium Permeability*

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28 There were 95 women with measures of both mammary epithelium permeability and
29
30 breast milk OVA concentration. No detectable associations were found between
31
32 mammary epithelium permeability and breast milk OVA concentrations (unadjusted
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34 $P=0.14$, and adjusted for maternal dietary egg ingestion $P=0.18$).
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36 37 38 *Infant Egg-specific IgG4*

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40 Infant egg-specific IgG4 levels did not statistically differ between the three
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42 intervention groups at six weeks of lactation (Table 3). However there was a
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44 significant association between infant egg-specific IgG4 levels and actual average
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46 maternal egg ingestion ($P=0.02$). For each additional egg per week there was an
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48 average 22% increase in infant ~~serum~~ egg-specific IgG4 levels (95% CI 3%-45%) in
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50 those 60/82 (73%) infants with detectable IgG4 levels at six weeks of age (Figure 4).
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52 Ten weeks after the intervention period (at 16 weeks of lactation), the median egg-
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6 specific IgG4 levels were below the level of detection in infants (in all three groups)
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8 who were still breastfeeding (Table 3 [and Figure 5](#)).
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10 11 ***Infant Egg-specific IgE***

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13 At six weeks of age no infants in this study had detectable egg specific IgE (n=82).
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15 By 16 weeks of age 4/84 (4.8%) infants had detectable egg specific IgE. Only two
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17 infants (2.4%) had levels > 0.35 kUAL, both mothers of these infants were in the low-
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19 egg group. For the other two infants with low levels of detectable egg specific IgE
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21 (0.1-0.35 kUAL), one infant's mother was from the egg-free group and one from the
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23 high-egg group.
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Discussion

This is the first randomized controlled trial to investigate the effect of an early postnatal intervention to assess the effects of maternal egg ingestion on egg protein (OVA) concentrations in human milk. We have shown that higher dietary egg intakes of more than four eggs per week significantly increase the ovalbumin concentrations in human milk in the first six weeks of lactation. The average number of eggs **actually** consumed per week over the six week intervention period ~~also~~ correlated with the concentration of OVA in breast milk at six weeks of lactation. These relationships add to a previously observed short-term dose relationship between maternal egg ingestion on the concentrations of OVA in breast milk [120].

The underlying goal of the study was to explore the rationale that encouraging high intakes of the major food allergens in the maternal diet during breastfeeding could be a possible prevention strategy to reduce food allergy by modulating early oral tolerance to these foods, especially prior to the introduction of solid foods. Our finding that infant egg-specific IgG4 levels were positively associated with maternal egg ingestion during early lactation supports this hypothesis. This is also consistent with recent findings from a cohort study by Jarvinen et al [163] in which elimination of cow's milk from maternal diets in the first 3 months of life was associated with lower infant cow's milk-specific IgG4 levels. Furthermore they observed that lower cow's milk-specific IgG4 levels were also associated with cow's milk allergy confirmed by oral food challenge around 6 months of age [163]. We did however find that the waning of infant egg-specific IgG4 levels between six and sixteen weeks of age to a mean level below the level of assay quantification to be a point of interest. At 16 weeks, the mothers were consuming a mean intake of 4.1 eggs per week.

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6 however there was no longer an association between maternal egg ingestion and
7 infant egg-specific IgG4 levels. Although speculative, it is possible that as the infant
8 grows and increases in body size, the dose of egg protein in the breast milk also needs
9 to continue to increase in order to induce the production of IgG4. Additionally as the
10 frequency of breastfeeding usually decreases with infant age, infants may be exposed
11 to less OVA due to the transience of the protein through the breast milk.
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20 Ideally our maternal intervention period could have been extended to at least 4-6
21 months duration to fully assess the effects of maternal food allergen intake during
22 lactation on infant oral tolerance prior to the commencement of solid foods. For
23 pragmatic reasons we chose to initially test this concept in the immediate postnatal
24 period with a shorter intervention to reduce the participant burden. The initially lower
25 level of adherence and the higher rate of participant withdrawal from the egg-free
26 group does highlight the practical difficulty of food allergen avoidance diets.
27 However, the adherence in the egg-free dietary group did improve over time, and
28 compliance rates were high (95-100%) in both the low-egg and high-egg groups. It
29 was also interesting to note that given the average baseline maternal egg intake was 3-
30 4 eggs per week, that the trends in breast milk OVA concentrations over the
31 intervention period (as illustrated in Table 2) appear to be moving closer to what we
32 would expect to see from the three intervention groups, hence future trials should be
33 ideally designed with a longer intervention period.
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49 Consistent with our previous studies [129, 134], there were considerable variations
50 between women in the OVA levels detected in the breast milk samples - with 26-38%
51 of women in the egg ingestion intervention groups having undetectable breast milk
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6 levels of OVA at all time points. Four women who attributed to higher breast milk
7 levels of OVA in the egg avoidance group were known non-compliers, however they
8 did not account for all cases of OVA detected in breast milk samples of this group
9 avoiding egg. The detection of OVA in the avoidance group is again consistent with
10 our previous research [12, 13], where women had detection of OVA in their breast
11 milk samples despite being allocated to egg avoidance groups. Interestingly, there
12 were no associations found between breast milk OVA levels and measurements of
13 mammary epithelium permeability. ~~Further research is required to identify potential~~
14 ~~factors influencing these variations in food allergen passage into human milk.~~ Future
15 studies should also consider the development and use of methods to detect lower
16 concentrations of breast milk food allergens. We acknowledge that the measurement
17 of ovomucoid in the breast milk samples would have also been ideal, particularly with
18 its ability to continue its allergenic conformational structure after heating. However as
19 it only constitutes 11% of total egg white protein, compared to OVA comprising 57%,
20 and given the levels of OVA detected in breast milk are very low, we decided that
21 attempting to measure the ovomucoid concentrations in the breast milk samples in
22 this study would have resulted in the majority of samples with ovomucoid levels
23 below the assay detection sensitivity.

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43 The strengths of this trial include the frequent breast milk collections in early
44 lactation, with 77% of the participating women providing a breast milk sample at two,
45 four and six weeks of lactation. This study also has several novel aspects, including
46 the measurement of infant immunoglobulin levels twice prior to the commencement
47 of solid foods. The rate of infant egg sensitization at 16 weeks of age (2.4%) is
48 notably lower in this trial (in which only 13% of infants had eczema at 16 weeks)

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6 compared to our other recent trial in which *all* infants had eczema [2] and very high
7 rates of sensitization; 36% had egg-specific IgE >0.35 kU_A/L at 4 months of age *prior*
8 to first ingestion of egg in solid foods. This reflects the role of eczema as a major risk
9 factor in food sensitization, potentially the result of altered mucosal and cutaneous
10 barrier function, and putative transcutaneous sensitization [174, 185].
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18 **Conclusions**

19 This is the first randomized controlled trial to show that increasing maternal dietary
20 egg intake during early lactation, and associated higher breast milk egg protein
21 (OVA) concentrations, are associated with increased infant egg-specific IgG4 levels
22 at six weeks of age, especially when more than four eggs per week are eaten. This
23 suggests that maternal dietary allergens during lactation can modify measures of
24 infant immune tolerance to specific foods. This provides a platform for larger trials to
25 assess the impact of similar interventions (extended throughout lactation) to assess
26 clinical outcomes, such as challenge proven food allergy in later infancy and
27 childhood. However it must also be noted that in this trial and in our previous studies,
28 there were considerable variations between women in the OVA levels detected in the
29 breast milk samples and that approximately one third of women had undetectable
30 breast milk OVA levels. Hence further ~~Further~~ research is required to identify
31 potential factors influencing these variations in food allergen passage into human
32 milk.
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7
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16

17
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19
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21

22
23 Medela had no involvement in the design, analysis and interpretation of
24
25 the study nor did they have any input into the manuscript.
26

27
28 All other authors have no conflicts of interests to declare.
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6 **Figure legends**
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10 **Figure 1:** Flow diagram of trial participants.
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14 **Figure 2:** Individual participant breast milk ovalbumin (OVA) concentration (ng/ml)
15 per intervention group (egg-free, low-egg and high-egg) at two, four and six weeks of
16 lactation within the detection range of the assay. All values above the upper limit of
17 assay detection were assigned the value of 4.0 ng/ml in this figure.
18 ovalbumin (OVA) concentrations at two, four and six weeks of lactation.
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26 **Figure 3:** Average maternal egg intake per week and predicted breast milk ovalbumin
27 (OVA) concentration at six weeks of lactation. This figure is based on the Tobit
28 regression model of statistical analysis, reporting the predicted values, however the
29 levels below quantification are also taken into account. There is a line which
30 represents the lower limit of quantification.
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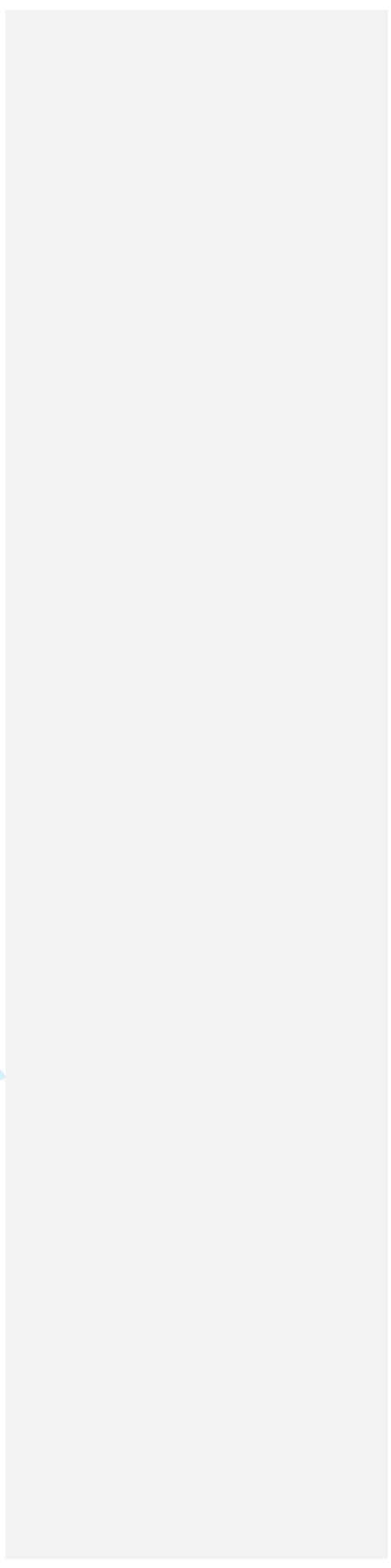
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38 **Figure 4:** Average maternal egg intake per week and predicted infant ~~serum~~-egg-
39 specific IgG4 levels at six weeks of lactation. This figure is based on the Tobit
40 regression model of statistical analysis, reporting the predicted values, however the
41 levels below quantification are also taken into account. There is a line which
42 represents the lower limit of quantification.
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49 **Figure 5:** Infant egg-specific IgG4 levels (mg antibody/L) per intervention group
50 (egg-free, low-egg and high-egg) at 6 weeks (6W) and 16 weeks (16W) of lactation
51 per intervention group.
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TABLE 1: Baseline characteristics per intervention group

	Egg Free (n=36)	Low Egg (n=44)	High Egg (n=40)	P-value
Maternal age at birth (y)*	33.1 (4.3)	33.1 (3.7)	33.0 (4.0)	.99
Maternal Caucasian race ^	29 (87.9%)	41 (93%)	37 (92%)	.27
Maternal completion of secondary school ^	32 (97%)	41 (93%)	37 (92%)	.79
Maternal smoking during pregnancy ^	1 (3%)	1 (2%)	0 (0%)	.74
Other smoking in the household ^	3 (9%)	4 (9%)	4 (10%)	1.0
Dog at home ^	15 (45%)	19 (44.2%)	21 (52%)	.72
Cat at home ^	10 (30%)	11 (25.6%)	11 (27%)	.90
Antibiotics during pregnancy ^	8 (24%)	13 (43.3%)	9 (23%)	.83
Paternal allergy ^	16 (49%)	28 (64%)	22 (55%)	.43
Total weekly household egg intake*	6.8 (3.9)	7.3 (4.9)	8.6 (4.2)	.20
Maternal Egg Intake *	3.1 (1.9)	3.4 (2.1)	3.9 (2.1)	.20
Parity greater than one	7 (21%)	6 (14%)	11 (27%)	.30
Infant birth weight (g)*	3452 (425)	3469 (394)	3494 (318)	.90
Delivery method via C-section ^	17 (53%)	19 (45%)	16 (43%)	.57
Infant male sex ^	21 (64%)	17 (39%)	20 (50 %)	.10
Gestational age at birth (weeks)*	39.5 (1.2)	39.5 (1.2)	39.7 (0.8)	.62

Legend: * mean (+/- SD), ^ frequency (percent)

Table 2: *Ovalbumin concentration of breast milk (ng/ml) per intervention group.*

Week of lactation	Egg free Median (IQR) n=33	Low egg Median (IQR) n=44	High egg Median (IQR) n=40	P-value (high egg - egg free)
2 weeks	0.14 (0.05-0.28) n=28 BLQ= 43% (12/28)	0.15 (0.05-0.68) n=40 BLQ= 45%(18/40)	0.05 (0.05-0.54) n=35 BLQ=51%(18/35)	0.73
4 weeks	0.05 (0.05-0.25) n=30 BLQ = 57% (17/30)	0.05 (0.05-0.48) n=39 BLQ =54% (21/39)	0.08 (0.05-0.65) n=36 BLQ=50% (18/36)	0.13
6 weeks	0.05 (0.05-0.20) n=27 BLQ=63%(17/27)	0.05 (0.05-0.41) n=39 BLQ=54%(21/39)	0.20 (0.05-0.96) n=33 BLQ=42%(14/33)	0.04

BLQ: Levels that were below the level of quantification for the assay (<0.1ng/ml)

Note: p-values were calculated using the TOBIT regression method

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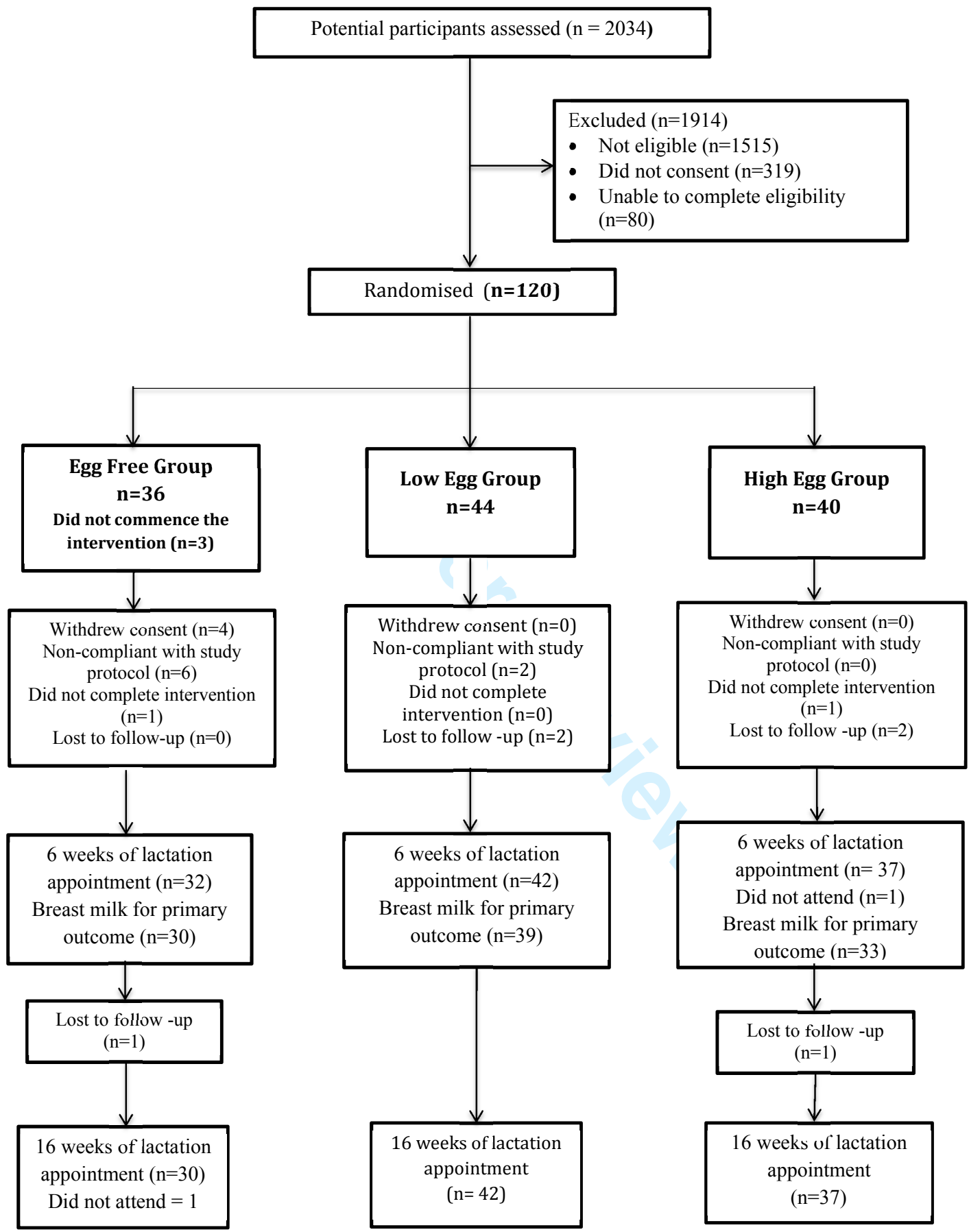
Table 3: Egg specific IgG4 levels (mg of antibody/L) per intervention group

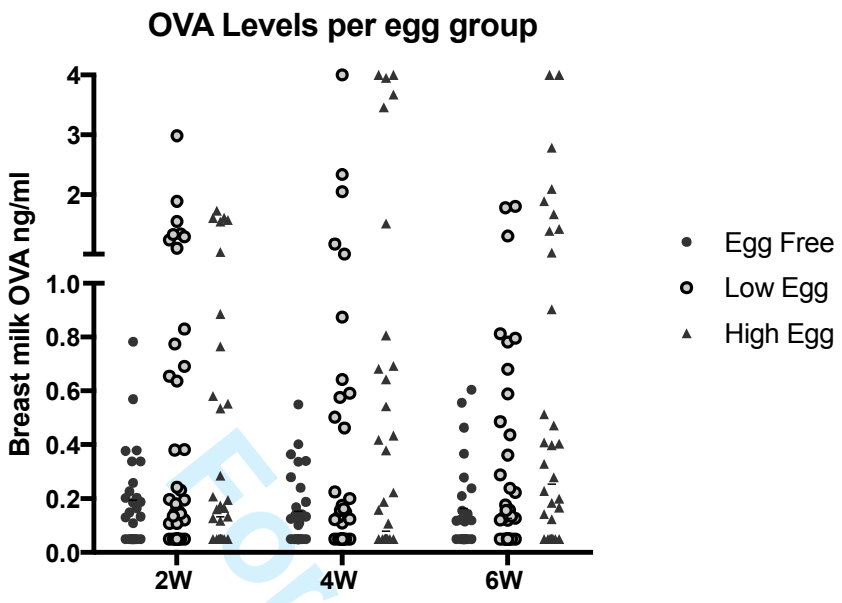
Week of lactation	Egg free Median (IQR)	Low egg Median (IQR)	High egg Median (IQR)
6 weeks	0.13 (0.04-0.92) n=26 BLQ: 38% (10/26)	0.41 (0.08-1.20) n=29 BLQ: 24% (7/29)	0.42 (0.08-1.10) n=27 BLQ: 18% (5/27)
16 weeks (post intervention)	0.04 (0.04-0.39) n=13 BLQ: 54% (7/13)	0.04 (0.04-0.14) n=32 BLQ: 53% (17/32)	0.04 (0.04-0.15) n=26 BLQ: 61% (16/26)

BLQ: Levels that were below the level of quantification for the assay (<0.07ng/ml)

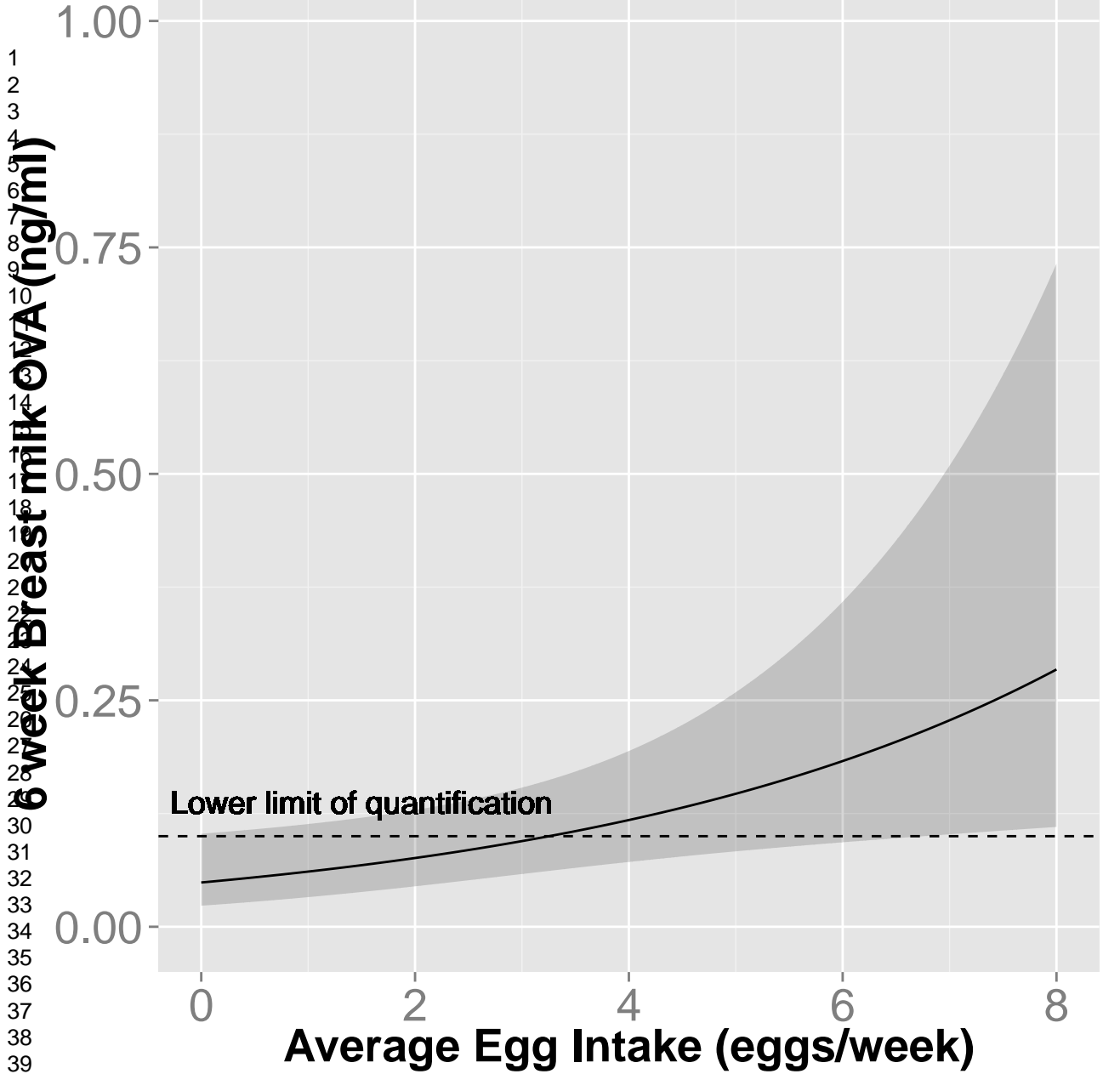
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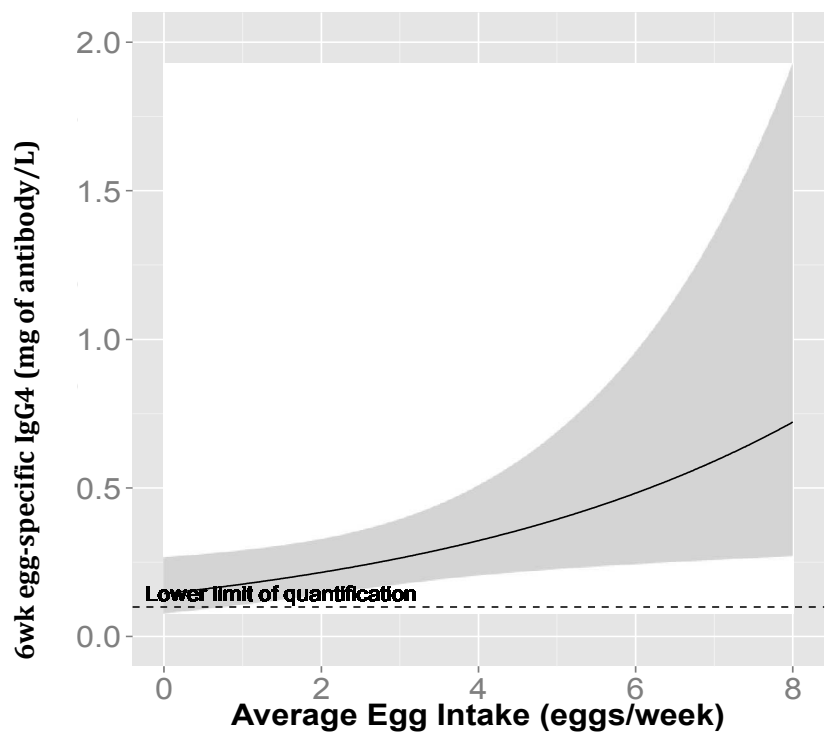




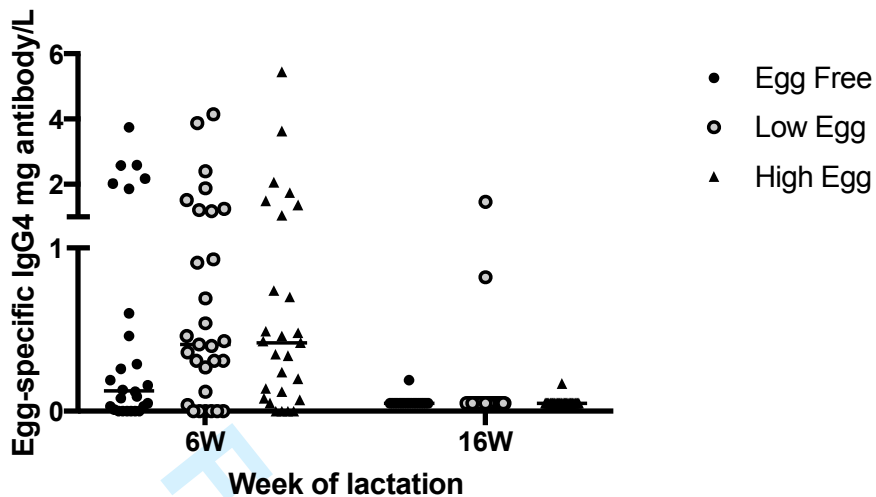
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