J. R. Metcalfe, N. D'Vaz, M. Makrides, M. S. Gold, P. Quinn, C. E. West, R. Loh, S. L. Prescott and D. J. Palmer

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Elevated IL-5 and IL-13 responses to egg proteins predate the introduction of egg in solid foods in infants with eczema.

Short title: Egg IL-5 and IL-13 responses in infants with eczema.

Jessica R. Metcalfe[1], Nina D'Vaz [1,2], Maria Makrides [3,4,5], Michael S. Gold [5,6], Patrick Quinn[6], Christina E. West[1,7], Richard Loh[8], Susan L. Prescott [1,2,8], Debra J. Palmer [1,3]

Affiliations

[1] School of Paediatrics and Child Health, The University of Western Australia

[2] Telethon KIDS Institute, The University of Western Australia

[3] Women's & Children's Health Research Institute

[4] Healthy Mothers, Babies and Children, South Australian Health and Medical Research Institute

[5] School of Paediatrics and Reproductive Health, University of Adelaide

[6] Children, Youth and Women's Health Service

[7] Department of Clinical Sciences, Pediatrics, Umeå University

[8] Department of Immunology, Princess Margaret Hospital

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Corresponding Author:

Dr Debra Palmer

Email: debbie.palmer@uwa.edu.au

Phone: +618 9340 8834

Postal address: School of Paediatrics and Child Health, University of Western Australia, PO Box D184, Princess Margaret Hospital, Perth WA 6001, Australia.

Key words

Allergy prevention; cytokines; eczema; egg allergy; egg protein; infancy.

Abbreviations

CON - conalbumin

LYS - lysozyme

OM - ovomucoid

OVA - ovalbumin

PBMC - peripheral blood mononuclear cells

RCT - randomised controlled trial

RPMI - Roswell Park Memorial Institute

SCORAD - Scoring Atopic Dermatitis

Abstract

Background: Egg allergy is a leading cause of food allergy in young infants, however little is known about early allergen specific T cell responses which predate the presentation of egg allergy, and if these are altered by early egg exposure.

Objective: To investigate the early T cell responses to multiple egg proteins in relation to

patterns of egg exposure and subsequent IgE-mediated egg allergy.

Methods: Egg-specific T cell cytokine responses (IL-5, IL-13, IL-10, IFNγ and TNFα) to ovomucoid (OM), ovalbumin (OVA), conalbumin (CON) and lysozyme (LYS) were measured in infants with eczema at 4 months of age (n=40), before randomisation to receive 'early egg' or a placebo as part of a randomised controlled trial (Australian New Zealand Clinical Trials Registry number 12609000415202), and at 12 months of age (n=58), when IgE-mediated egg allergy was assessed by skin prick test and food challenge.

Results: In 4 month old infants, who had not directly ingested egg, those who subsequently developed egg allergy already had significantly higher Th2 cytokine responses to multiple egg allergens, particularly elevated IL-13 responses to OVA (P=0.004), OM (P=0.012) and LYS (P=0.003), and elevated IL-5 to the same antigens (P=0.031, 0.04 and 0.003 respectively). IL-13 responses (to OVA and LYS) and IL-5 responses (to LYS) at 4 months significantly predicted egg allergy at 12 months. All responses significantly declined with age in the egg allergic infants, and this did not appear to be modified by 'early' introduction of egg.

Conclusions & Clinical Relevance: Elevated egg-specific Th2 cytokine responses were established prior to egg ingestion at 4 months and were not significantly altered by introduction of egg. Th2 responses at 4 months of age predicted egg allergy at 12 months, suggesting that this could be used as a biomarker to select infants for early prevention and management strategies.

Introduction

Hen's egg is one of the most common food allergens to induce T helper 2 (Th2) allergic immune responses in young infants[1-3]. IgE-mediated allergic reactions can occur early in infancy[4], often on first ingestion of egg in solid foods[4-6]. Our previous study indicated

that as many as one third of infants with eczema may have evidence of sensitisation and IgE-mediated symptoms on ingestion of egg at 4 months of age[4]. This suggests much earlier dysregulation of allergen-specific T-cell responses, and that these may be established and consolidated even before 4 months of age in some children, particularly children with eczema. In addition to genetic predisposition, this increase risk has been attributed to increased sensitisation risk through impaired cutaneous barrier function[7]. However, this is highly variable and not all infants with severe eczema develop egg or other food allergies. Given the risk of reactivity in this group, there is a recognised need to further characterise the preceeding immunological events leading to egg sensitisation, as this may help define pathways to sensitisation, facilitate early identification of children likely to react, and direct potential strategies for preventive interventions in the future.

As an important prelude, this study investigated the egg allergen specific T-cell responses in this high risk group of children, with moderate to severe eczema, prior to their presentation with egg allergy. While most previous studies of egg-sensitised patients have focused on responses to ovalbmumin (OVA) as the most abundant protein in hen's egg[8-10], this study provided the opportunity to examine early responses to a broader range of hen's egg proteins including ovomucoid (OM, Gal d 1), ovalbumin (OVA, Gal d 2), conalbumin (CON, Gal d 3), and lysozyme (LYS, Gal d 4), which are also capable of inducing the production of specific-IgE[11].

For the first time we investigated how early patterns of T cell responsiveness to this wider range of hen's egg allergens at four months of age predicted subsequent IgE-mediated egg allergy. Additionally we determined whether earlier introduction of egg in solid foods modified the subsequent egg-specific cytokine responses at 12 months of age.

Materials and methods

Subjects

The study population comprised a subset of infants who participated in a randomized controlled trial (RCT) investigating the effects of early, regular egg consumption on the development of IgE-mediated egg allergy (Australian New Zealand Clinical Trials Registry number 12609000415202). This study was approved by the Princess Margaret Hospital Human Research Ethics Committee (approval number 1635/EP), and written parental consent was obtained from all the participants. Full details of the RCT have been previously published[4]. Briefly, infants with moderate to severe eczema determined using a standardized Scoring Atopic Dermatitis (SCORAD)[12] score of $\geq$ 15 and no known ingestion of egg in solid foods were recruited at 4 months of age. The infants were randomized to receive either one teaspoon of pasteurized raw whole egg powder (intervention group) or rice powder (control group) daily from 4 to 8 months of age. At 8 months of age, cooked egg was introduced to both the intervention and control group infants after a medically observed introduction of hard-boiled egg. The primary outcome was IgE-mediated egg allergy at 12 months of age defined by a medically observed allergic reaction to a pasteurized raw egg challenge and a positive skin prick test to egg[4]. The subset of RCT participants included in this study was determined by availability of sufficient blood volume collected for cell culture analysis.

Blood collection and processing

Blood samples were collected at 4 months of age prior to any ingestion of the study powder, and again at 12 months of age on the day of a skin prick test and egg challenge. Peripheral blood was collected by venipuncture into lithium-heparinized tubes and processed within 4 hours. Heparinized whole blood was pelleted by centrifugation, and plasma was collected and stored at -80°C. Where blood volume allowed, cells were separated using density

centrifugation (Lymphoprep™) method. Peripheral blood mononuclear cells (PBMC's) were isolated, washed using Roswell Park Memorial Institute (RPMI) media (Gibco Life Technology, Grand Island, NY, USA) and stored in RPMI (49%), heat-inactivated fetal calf serum (43.5%) and dimethylsulphate (7.5%). Cells were stored in 1 ml aliquots at a concentration of no more than 15 x 10^6 cells/ml, transferred to a CoolCell® and immediately stored at -80℃ for a standardized controlled-rate of -1℃/minute cell freezing. Within 24 hours of freezing, PBMC's were transferred to liquid nitrogen for long-term storage.

Mononuclear cell culture

PBMC cell culture was conducted using the methods as per detailed previously [13,14]. Briefly, cryopreserved mononuclear cells were thawed and transferred to RPMI culture media. Cells were counted, viability tested using try-pan blue (Gibco Life Technology, Grand Island, NY, USA) and transferred to AIM V (Gibco Life Technology) tissue culture media with 2-mercaptoethanol (ME) (Sigma-Aldrich Co, NSW Australia) at a concentration of 2 x 10^6 cells/ml. Hen's egg allergens: a) ovalbumin (OVA 100μg/ml), b) ovomucoid (OVM 1mg/ml), c) conalbumin (CON 200μg/ml) and d) lysozyme (LYS 500μg/ml), all purchased from Sigma-Aldrich Co, NSW Australia. These concentrations were identified as optimal for in vitro T cell stimulation in preliminary titration experiments. As OVA is routinely used to stimulate PBMC's at a concentration of 100ug/ml, this was used as the starting concentration for the other egg allergens. The concentration was deemed optimal when responses were consistent in known egg-allergic infants, whilst maintaining minimal responses in unaffected infants. A mitogen phytohaemagglutinin (PHA) was used as a positive control, to ensure that PBMC's were responding suitably to stimulation. Non-stimulated negative controls were also included for each infant. Lymphocytes were cultured for 48 hours in 5% CO_2 incubators at 37°C before supernatants were collected and stored at -20°C for batch cytokine analysis. The

number of stimulations varied between individuals, and was determined by the number of available mononuclear cells.

Cytokine measurements – Luminex Xmap multiplex

Cytokines in once-thawed lymphocyte culture supernatants were quantified using Luminex Xmap multiplex technology (Luminex Corp, Austin, TX, USA) using an in-house method previously described[8]. Primary and secondary antibodies for cytokines IL-5, IL-10, IL-13, IFNγ and TNFα were purchased from BD Biosciences (North Ryde, Australia). Standards for IL-5, IL-10 and IFNγ were purchased from BD Bioscience, and IL-13 and TNFα standards were purchased from R&D Systems (Minneapolis, USA). Quality controls were run on each plate. The lower detection limit of the assay was 3 pg/ml and the upper limit varied between 10000-30000 pg/ml. Samples that were below detection limit were assigned the value of the lowest detection (3 pg/ml). The cytokine levels were shown as the difference between the stimulated cells and control cells, which were not stimulated.

Clinical outcomes and allergy assessments

Throughout this study, an allergic reaction was defined as at least 3 concurrent non-contact urticaria persisting for at least 5 minutes and/or generalized skin erythema, and/or vomiting, and/or anaphylaxis within 2 hours of allergen exposure[4]. All infants (including those that reacted to the study powder at 4 months of age) underwent an allergy assessment at 12 months of age, including a SCORAD assessment, skin prick testing, blood sample collection and egg challenge[4]. The presence of IgE-mediated egg allergy at 12 months of age was defined as a positive allergic reaction during a medically supervised raw egg challenge and evidence of sensitization (positive skin prick test) to egg, or medical advice not to proceed with the challenge due to a previous serious allergic reaction to egg.

Statistical analysis

Differences in means for parametric data were compared using T-tests. Non-parametric data were analyzed between groups using Mann-Whitney *U*-tests. Chi square tests were used for comparisons of categorical data between groups. Where possible non-parametric data were log transformed to achieve a normal distribution for the remaining statistics. Binary logistic regression was used to calculate prediction of allergic outcomes. Paired *t*-tests were used to quantify changes over time. All statistics were performed using SPSS v20 (IBM), and figures were generated using Prism v 6 (GraphPad Software Inc.).

Results

Study Population

This study included 68 infants from the clinical trial who had blood samples available for cytokine analysis, as illustrated in Figure 1. The baseline characteristics of this subset, shown in Table 1, are representative of the total 86 participants in the RCT. Cytokine responses were measured in 40 infants at 4 months of age (n=22 'early egg' intervention group, n= 18 'delayed egg' rice control group) and 58 infants at 12 months of age (n=33 'early egg' intervention group, n=25 'delayed egg' rice control group). For 30 infants (n=15 'early egg' group, n=15 'delayed egg' group) cytokine data was available at both time points. We compared T cell responses in infants according to egg reactivity (at both 4 and 12 months) and according to the study intervention.

Baseline cytokine responses at 4 months of age, and comparison of responses in 4-month old egg-reactors and non-reactors.

Prior to the intervention, there were no differences in cytokine responses for IL-5 or IL-13 between the 'early egg' (intervention) and 'delayed egg' (control) group in response to any of

the egg allergens: OVA, OM, CON or LYS at 4 months of age (Table 2). There were also no differences between the groups for IL-10, IFNγ or TNFα responses as summarized in supplementary table 1 available in the online repository.

A total of 15/49 (31%) infants in the 'early egg' intervention group had a confirmed allergic reaction to the pasteurized raw egg powder at study enrolment. Cytokine response data was available for 5 infants who reacted to the egg study powder and 17 infants who tolerated the egg powder. In those infants who reacted to the egg powder, egg-specific induced Th2 cytokines were significantly higher: IL-13 (OVA, OM and LYS), and IL-5 (OVA and CON) than infants who tolerated the egg powder (Figure 2). There was also a significantly higher production of IFNγ to lysozyme in the egg powder reactors (p=0.011), than in the non-reactors (data in supplementary table 2). There were no other differences in IFNγ, IL-10 or TNFα between infants who reacted and those who tolerated the egg powder at 4 months of age, data as summarized in supplementary table 2 available in the online repository. PHA stimulation was used to assess viability in all samples, and the level of cytokines produced did not differ significantly between groups, with age or phenotype (results not shown).

Effect of the dietary intervention on cytokine responses at 12 months of age

Egg-specific Th2 cytokines IL-5 and IL-13 responses at 12 months of age did not differ according to the intervention groups (as shown in Table 3). No differences between the groups were also found for IL-10, IFNγ or TNFα responses, as summarized in supplementary table 3 available in the online repository. .

Relationship between early cytokine responses (at 4 months) and subsequent IgE-mediated egg allergy at 12 months of age

A total of 35 infants (n=12 with IgE-mediated egg allergy) had cytokine responses measured at 4 months of age and egg allergy assessed at 12 months of age. Elevated IL-5 and IL-13 responses to egg allergens (OVA, OM, LYS) at 4 months of age were associated with IgE-mediated egg allergy at 12 months of age (Figure 3). There were no associations between IL-10, TNFα and IFNγ responses with IgE-mediated egg allergy, data not shown.

In 30 infants (n=11 with IgE-mediated egg allergy) with both 4 and 12 month cytokine data as well as clinical egg allergy status data at 12 months, IgE-mediated egg allergy was predicted by 4 month of age OVA IL-13 (β =1.1; 95%CI 1.09-8.7; P=0.034), LYS IL-13 (β =1.2; 95%CI 1.2-9.3; P= 0.03) and LYS IL-5 (β =0.8; 95%CI 1.2-4.3; P=0.014).

Changes in cytokine responses with age.

In 30 infants (n=11 with IgE-mediated egg allergy) with cytokine data available at both time points, infants with IgE-mediated egg allergy at 12 months of age showed a striking decrease in induced Th2 cytokine responses between 4 and 12 months of age (Figure 4). Whilst all egg allergens followed the same pattern, IL-13 decreased significantly in response to stimulation with OM and LYS (P=0.007 and P=0.019 respectively) and IL-5 in response to LYS (P=0.012). Infants who tolerated egg at 12 months of age, did not show any significant decrease in IL-5 and IL-13 responses between 4 and 12 months (Figure 4).

Relationship between IgE-mediated egg allergy and cytokine responses at 12 months At 12 months of age, 58 infants had T cell responses measured (n=25 with IgE-mediated egg allergy) and clinical egg allergy status data assessed at the same time point. At this age, only

cytokine responses to LYS (IL-5 (P=0.035), IL-13 (P=0.034)) were significantly higher in infants with IgE-mediated egg allergy (Figure 3). There were no significant differences for any other egg allergen for cytokines IL-5 and IL-13 (Figure 3). Again there were no differences for IL-10, IFNγ or TNFα for any of the egg allergens, data as summarized in supplementary table 4 available in the online repository. At 12 months of age, LYS IL-13 and IL-5 predicted egg allergy status at that time point (β =0.4; 95%CI 1.0-2.4; P= 0.039) and (β =0.3; 95%CI 1.0-1.8; P= 0.05) respectively.

Relationship between eczema (SCORAD) assessments and cytokine responses

Eczema (SCORAD) assessments and cytokine data were available in 40 infants at 4 months and 58 infants at 12 months of age. There was no association between IL-5 or IL-13 responses to any of the egg proteins and eczema severity on the day of assessment for either time point. The data collected on topical steroid use was not sufficient to accurately assess in relation to cytokine production.

Egg specific IgG4 levels and cytokine responses

Egg-specific IgG4 levels were significantly higher in the infants who received the egg powder from 4 months of age[4]. However, production of cytokines, including egg-specific regulatory cytokine IL-10, was not correlated with the level of egg-specific IgG4 measured at 12 months of age.

Discussion

This is the first study to report patterns of infant PBMC responses to a comprehensive array of egg proteins in relation to patterns of egg exposure and subsequent egg allergy. We have confirmed strong early Th2 responses to multiple egg proteins (OVA, OM, CON, LYS) in a

high proportion of infants with eczema by 4 months of age, prior to the introduction of egg in solid foods. Moreover, IL-5 and IL-13 responses at this age predicted the development of challenge-proven egg allergy later in infancy.

These findings clearly demonstrate that immunological events leading to egg sensitisation are commonly initiated prior to the introduction of egg in solid foods, particularly in this high-risk phenotype. This highlights the need to understand other potential mechanisms and routes of sensitisation, during lactation or even in utero. Egg proteins are known to cross the placenta[15], and have been detected in breast milk [16], providing potential avenues of exposure. Transcutaneous exposure also may be a particularly important route of exposure in children with moderate to severe eczema[7]. In addition to impaired skin barrier function, children with eczema also show evidence of increased gut mucosal permeability[17,18], which may provide an additional mechanism in dysregulation of mucosal responses and the development of food allergy. On the other hand, many children without eczema still develop food allergy, and it will be important to repeat these studies in egg allergic children without eczema.

Another interesting finding in this study, was that the intervention with early regular oral exposure to egg from 4 months of age was not associated with any significant effects on egg-specific IL-5, IL-10, IL-13, IFNγ or TNFα cytokine responses. This could be because Th2 cytokine production was already well established in many infants by 4 months of age prior to the intervention. It is also recognized that the development of oral tolerance is not necessarily associated with the reduction in allergen specific IgE or underlying Th2 responses, as noted in studies of oral immunotherapy[19]. Other immunological parameters, such as allergen specific IgG4, are more consistently associated with oral tolerance. Indeed,

we have previously noted that this intervention was associated with significantly higher egg-specific IgG4 levels at 12 months of age compared to the 'delayed egg' control group[4]. However, while this suggests that the early, regular introduction of egg did influence underlying tolerance-associated cellular mechanisms, we did not see any continuous effects on the production of cytokines such as IL-10 and IFNγ, which have been associated with tolerance in other studies[20]. Whilst egg allergic infants did produce significantly higher levels of IFNγ in response to lysozyme at four months of age, this was a stand-alone result in only five infants, and it is therefore not possible to draw any conclusions about early egg specific regulatory responses. It is possible that the intervention induced changes in regulatory T-cell function, but for logistic reasons and small sample volumes, it was not possible to examine this.

The dynamics of the T cell responses were also of significant interest in this population. It is notable that both IL-5 and IL-13 Th2 responses were more pronounced at 4 months and waned with age, even in children who had IgE-mediated reactions at 12 months of age on challenge. By 12 months, only LYS IL-5 and IL-13 remained significantly elevated in egg allergic children. In particular, IL-13 responses to other egg allergens (OVA, OM, CON) were comparable to the non-allergic children, despite continued clinical reactivity. Although LYS makes up a small percentage of total egg protein (3.5%)[21], up to 35% of egg allergic patients have been shown to produce LYS specific-IgE[22]. Thus, LYS seems to be inducing more sustained egg-specific inflammatory responses, with higher and more persistent production of IL-5 and IL-13 than the other egg proteins.

In conclusion, we have demonstrated that four egg proteins (OVA, OM, CON, LYS) are capable of inducing Th2 cytokine responses associated with the presence of IgE-mediated

egg allergy. Additionally we have shown that these egg-induced immune responses at 4 months of age predicted egg allergy outcomes at 12 months of age. These results suggest that early egg-specific T cell responses may have a long-lasting effect in egg allergy development pathways. With egg allergy now one of the most common food allergies affecting children in early childhood[3], this study is demonstrating a need for further investigation of the influence of egg protein exposures in early life, prior to the introduction of solid foods, on the development of egg-specific Th2 cytokine responses.

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Conflict of interest: The authors have no conflicts of interests to declare.

Figure legends

Figure 1: Flow diagram of trial participants and blood samples collected**.**

Figure 2: Differences in Th2 cytokine levels for infants randomised into the 'early egg' intervention group (n=22) who either reacted to the egg powder (n=5, light boxes) or tolerated the egg powder (n=17, dark boxes) at 4 months of age. IL-5 levels (a) and IL-13 (b) are shown as median with 10-90th percentile. *($P<0.05$).

Figure 3: IL-5 and IL-13 responses to egg allergens (a) at 4 months of age (n=35, 12 infants with subsequent egg allergy at 12 months of age) and (b) at 12 months of age (n=58, 25 infants with egg-allergy). Categorised based on infants who at 12 months of age had IgE-mediated egg allergy (light) or infants who tolerated egg (dark). Data shown represents median with 10-90th percentile. *($P<0.05$).

Figure 4: Changes in IL-5 and IL-13 cytokine responses from 4 months to 12 months of age. Cytokine responses in infants with (a) IgE mediated egg allergy and (b) infants who tolerated egg. Graphs are shown as median with interquartile range.

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Table 1: Baseline characteristics of study participants.

Characteristic	***Present Study (n=68)***	***Original RCT (n=86)***
Maternal age at birth (years) *	32.7 (5.0)	32.5 (4.7)
Maternal Caucasian race ^	52 (76.5%)	68 (79.1%)
Caesarean-section birth ^	24 (35.3%)	28 (32.6%)
1st degree relative history of allergic disease ^	61 (89.7%)	79 (91.9%)
Infant male sex ^	46 (67.6%)	57 (66.3%)
Age of onset of eczema (months) *	1.8 (1.0)	1.8 (1.0)
Eczema severity (objective SCORAD score) &	12.6 (4.6-25.8)	15.2 (7.2-26.53)
Use of prescription steroid cream ^	54 (79.4%)	68 (79.1%)
Ever breastfed ^	67 (98.5%)	85 (98.8%)
Breastfed at randomisation ^	54 (79.4%)	71 (82.6%)
Smoking in the household ^	8 (11.8%)	11 (12.8%)

*Values are *mean (standard deviation), ^ numbers (percentages) or & median (Inter Quartile Range).*

Table 2: Baseline Cytokine Responses at 4 months of age. *IL-13 and IL-5 responses (pg/ml) per group prior to the introduction of egg in solid foods.*

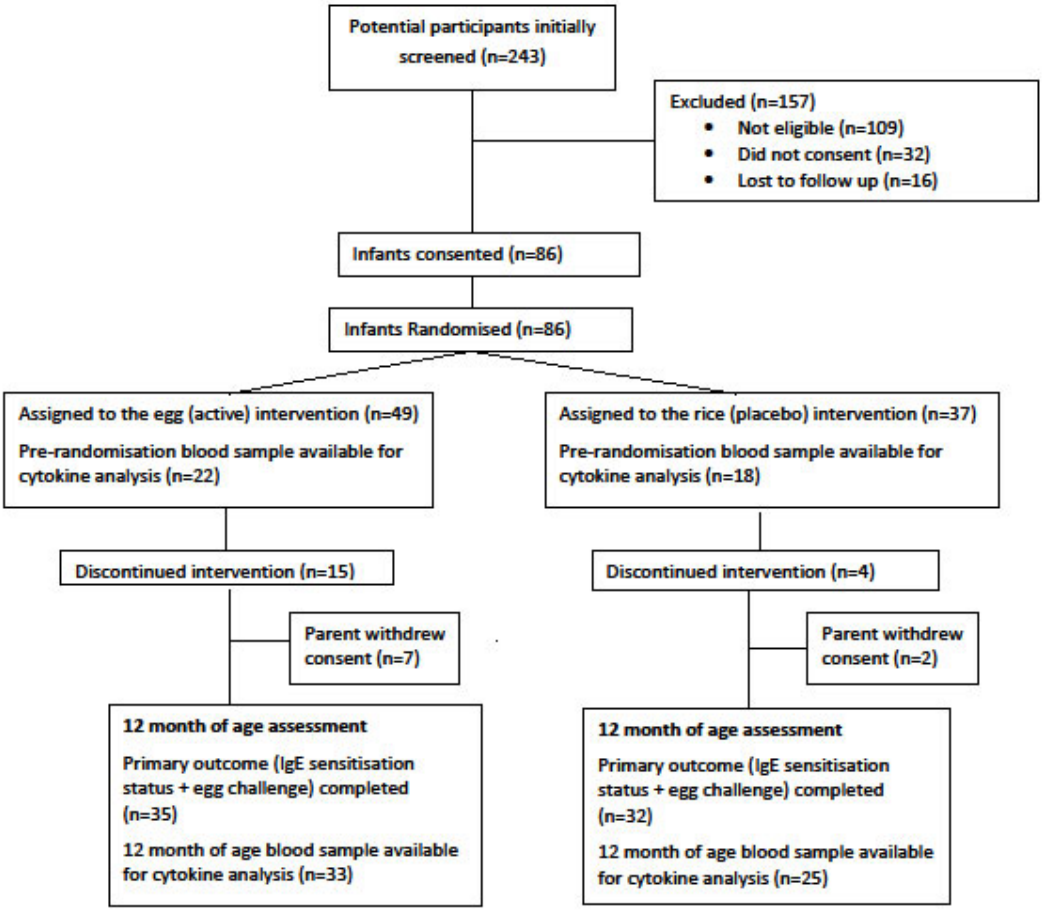
Cytokine	***Egg protein***	***'Early Egg' intervention (n=22)***	***'Delayed Egg' Control (n=18)***	***P value***
IL-13*	OVA	215.9 (118.0-511.3)	202.4 (109.4-556.1)	0.819
	OM	226.1 (110.5-578.0)	220.3 (54.7-399.0)	0.299
	CON	152.3 (75.6-395.2)	73.1 (28.5-513.1)	0.585
	LYS	198.6 (99.2-748.6)	395.8 (161.2-676.5)	0.528
IL-5*	OVA	7.33 (1.4-51.0)	14.3 (1.0-40.6)	0.717
	OM	10.6 (1.3-43.6)	2.42 (1.0-25.5)	0.286
	CON	2.42 (0.9-13.3)	2.42 (1.0-16.2)	0.644
	LYS	15.4 (1.7-60.9)	22.0 (5.8-98.8)	0.377

** median (Inter Quartile Range)*

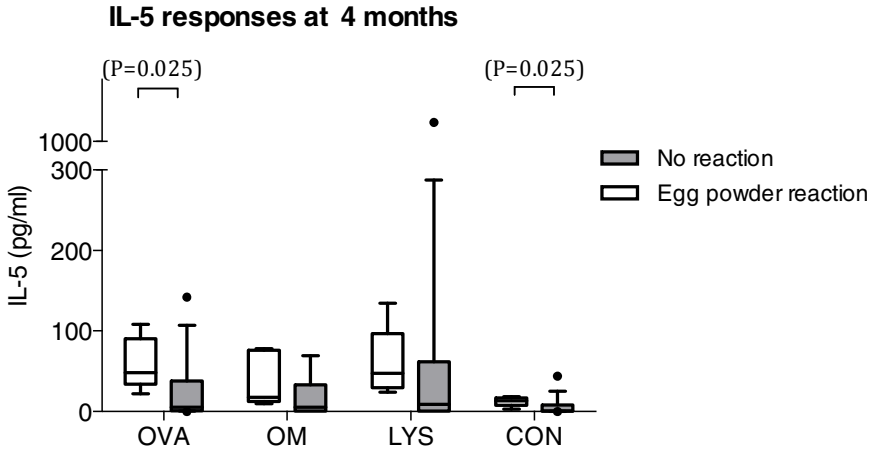
Table 3: ***Cytokine responses at 12 months of age.*** *IL-13 and IL-5 responses (pg/ml) per group.*

Cytokine	***Egg protein***	***'Early Egg' intervention (n=33)***	***'Delayed Egg' Control (n=25)***	***P value***
IL-13*	OVA	172.6 (94.3-422.2)	109.7 (58.7-279.0)	0.151
	OM	112.8 (34.9-208.8)	52.6 (21.4-120.1)	0.215
	CON	126.6 (44.5-204.7)	91.3 (40.8-207.3)	0.580
	LYS	98.6 (52.2-342.8)	90.0 (36.6-230.7)	0.733
IL-5*	OVA	14.2 (1.0-47.8)	3.4 (1.0-19.4)	0.410
	OM	3.1 (1.0-10.6)	1.0 (1.0-5.24)	0.311
	CON	1.0 (1.0-3.4)	1.0 (1.0-1.2)	0.494
	LYS	2.2 (1.0-24.5)	2.7 (1.0-15.0)	0.985

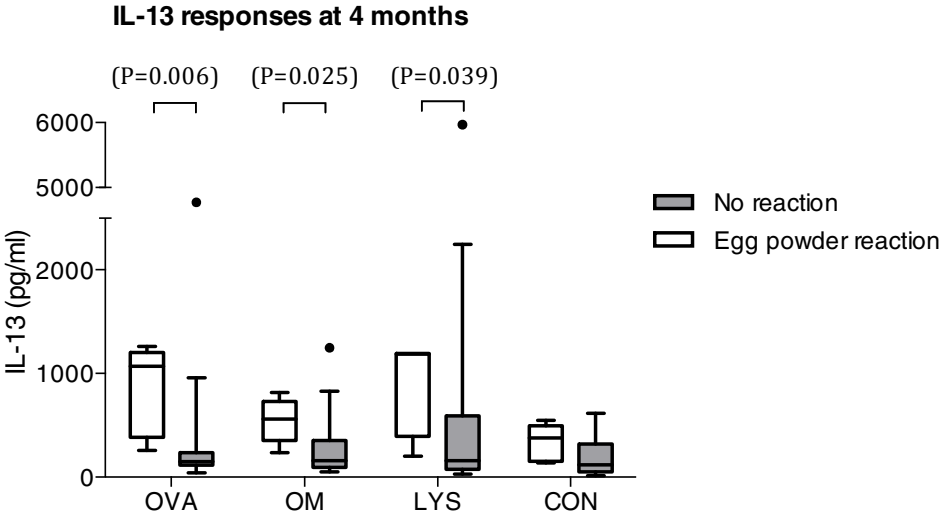
* *median (Inter Quartile Range)*



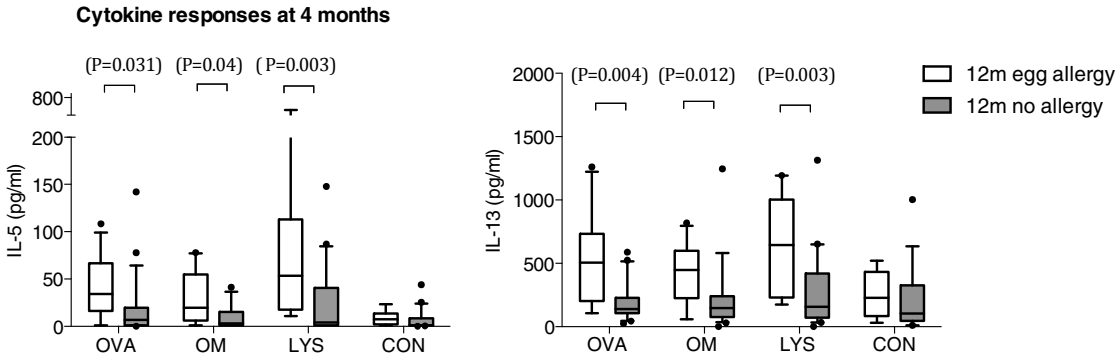
(a)



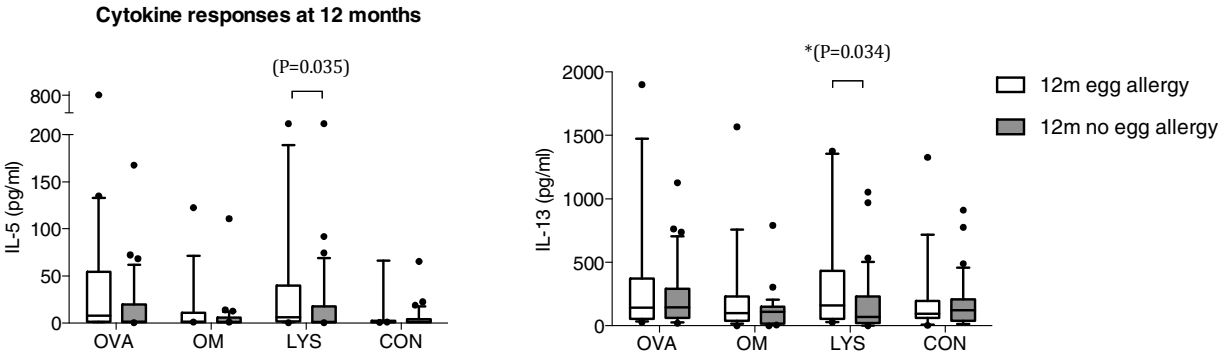
(b)



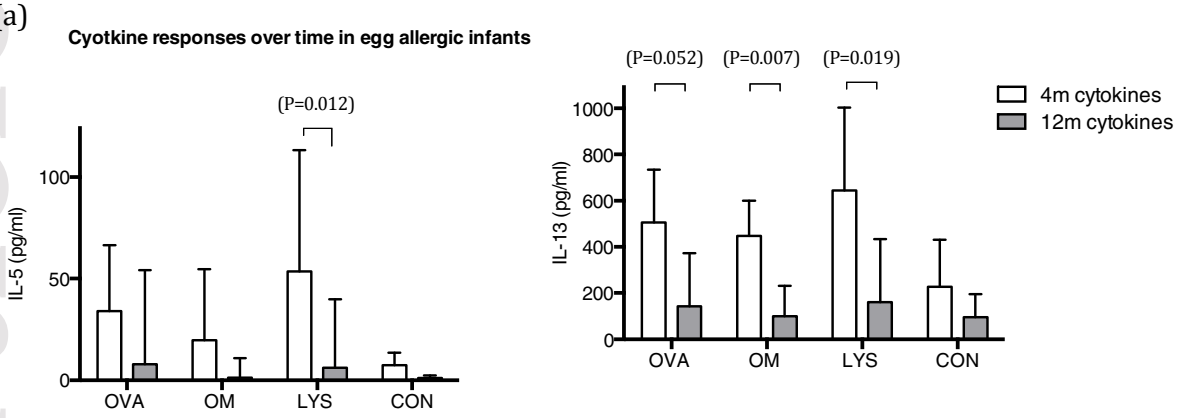
(a)



(b)



(a)



(b)