Early life egg allergen exposure in the primary prevention of egg allergy

Jessica Metcalfe BSc. (Molecular Biology)

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Statement of Candidate Contribution

The work presented in this thesis was conducted between August 2012 and June 2016 at the School of Paediatrics and Child Health, University of Western Australia, under the supervision of Professor Susan Prescott, Associate Professor Debbie Palmer, and Dr. Nina D’Vaz.

All of the experimental work, including participant recruitment, clinic appointments, data and blood collection, blood processing, data management, cell culture, cytokine analysis, breast milk ELISA analysis, plasma immunoglobulin analysis, and data analysis was performed by the author with the exceptions of those acknowledged below.

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Jessica Metcalfe contributed to the conception and design of the laboratory studies (70%), data collection (80%), analysis and interpretation of data (90%) and manuscript preparation (80%).

Jessica Metcalfe contributed to the conception and design of the study (50%), data and sample collection (90%), analysis and interpretation of data (90%) and manuscript preparation (80%).

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.
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Dedication

This thesis is dedicated to my number one, Leon, for reminding me that life is about pushing the boundaries of what you think you are capable of. Thank you for your love, humour, generosity and patience. Also to my family, mom, dad, and Dyl, who despite living on the other side of the world continue to be my greatest sources of support. My Australian family, Kerry, Peter and Shai thank you for adopting me, and welcoming me into your family. I have more gratitude and love than I can put down in words.
Abstract

**Background:** Allergic diseases are on the rise worldwide, and an alarming feature of this is the increase in severe food allergic reactions in very young children. Emerging insights into the development of oral tolerance via the gut associated lymphoid network enforces the need for oral allergen exposure early in life to initiate immunological processes leading to antigen-specific immune tolerance. With food-specific sensitisation occurring so early in infancy there is a need to optimise initial exposure to allergens in the postnatal period to promote the induction of tolerance.

**Aims:** The specific aims of this thesis are to 1) investigate egg-specific immune responses *with* the introduction of egg in solid foods, both immediately prior to any egg exposure and again at 12 months of age, and whether these responses are modified by early and regular introduction of egg in solid foods (Chapter 3); 2) investigate breast milk as a possible route for oral allergen exposure *prior* to the introduction of solid foods, by examining how maternal dietary egg ingestion influences egg protein (ovalbumin) levels of breast milk (Chapter 4), and how this exposure effects egg-specific immunoglobulin levels (IgE and IgG4) in the infant (Chapter 5).

**Methods:** Firstly, infants with eczema participating in a randomised controlled trial (the STAR study) designed to assess the effect of early (4 months) versus later (8 months) introduction of egg in solid foods, had blood samples taken alongside egg challenges and skin prick testing (n=68). Egg-specific immune responses (IL-5, IL-13, IL-10, INFg, TNF) to a panel of four egg allergens, ovalbumin (OVA), ovomucoid (OM), conalbumin (CON), and lysozyme (LYS) were examined both immediately prior to the introduction of egg in solid foods at 4 months of age (to ascertain whether there is egg-specific immune dysregulation prior to any oral exposure to whole egg) and again at 12 months of age. Egg-specific immune responses at both 4 and 12 months of age were examined against clinical reactivity with their first ingestion of egg, the
timing that egg in solid foods was introduced, and the diagnosis of IgE mediated egg allergy at 12 months of age (Chapter 3).

Next, to examine routes of egg allergen exposure during the immediate postnatal period, a randomised controlled trial was conducted (The QuEST Study). This trial was designed to assess the effect of maternal dietary egg ingestion on the concentration of egg protein in breast milk (Chapter 4). Women (n=120) with a history of allergic disease were allocated to a maternal dietary egg intervention: high-egg diet (> 4 eggs per week), low-egg diet (1-3 eggs per week) or an egg-free diet, for the first six weeks of lactation. Breast milk samples were collected at 2, 4 and 6 weeks of lactation for the measurement of egg protein (ovalbumin), using enzyme linked immuno-absorbent assay. To assess other factors that may influence the passage of egg protein from the diet into breast milk, breast permeability was measured via the sodium to potassium ratio in breast milk.

Infant blood was taken at the end of the intervention (6 weeks) and again before the introduction of solid foods (16 weeks of age) for the measurement of serum egg-specific IgE and IgG4, to assess whether maternal egg ingestion during lactation had any effect on infant immune markers (Chapter 5). Skin barrier function was measured in infants using the trans epidermal water loss (TEWL) method, to examine the possibility for transcutaneous sensitisation through a defective skin barrier using infant egg sensitisation (egg-specific IgE) at 16 weeks of age and any associations with eczema. Maternal skin barrier function was also measured using the TEWL method to investigate any associations with infant skin barrier function (Chapter 5).

**Results:** The STAR study which included infants with moderate to severe eczema (Chapter 3) revealed that 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
Furthermore 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 the ‘early’ introduction of egg. This study provides evidence that early life egg-specific immune dysregulation that predicts subsequent egg allergy may be established prior to the introduction of egg in solid foods in some infants, thus highlighting the need for intervention strategies to induce oral tolerance earlier in the postnatal period.

The QuEST Study allowed us to investigate breast milk as a pathway for early life allergen exposure, and demonstrated that maternal egg ingestion can be used to modify allergen exposure for breastfed infants (Chapter 4). Maternal dietary ingestion was positively associated with breast milk OVA concentration, where for each additional egg ingested per week there was an average 25% increase in OVA concentration (95% CI 5%–48%, p=0.01). Breast milk OVA concentrations were significantly higher in the ‘high-egg’ group (> 4 eggs per week) compared with the ‘egg-free’ group (p=0.04), however no other differences between groups were detected. Wide inter-woman variation in breast milk OVA levels despite ingesting the same amount of egg, and a proportion of women with no detectable levels of OVA in their breast milk at any time point, reveal the complexity of the passage of food proteins from the maternal diet into breast milk.

In Chapter 5, maternal dietary ingestion of egg was associated with infant egg-specific IgG4 levels. Each additional egg consumed per week resulted in an average 22% (95% CI 3%–45%) increase in infant egg-specific IgG4 levels (P=0.02). None of the infants had egg-specific IgE levels detectable at six weeks, and at 16 weeks of lactation only 2/84 (2.4%) had levels of IgE >0.35kU/L. Although speculative, these results suggest that oral exposure via breast milk is capable of inducing systemic immune responses in the infant resulting in the production of
IgG4, which has been associated with both allergen exposure and tolerogenic states in oral immunotherapy studies.

**Conclusions:** The studies presented in this thesis examine a critical period for immune development in the early postnatal period. The STAR study provides evidence that egg-specific Th2 cytokine responses established prior to the introduction of solid foods may have lasting effects on the development of egg allergy in infants with eczema. Examining pathways for allergen exposure prior to solid foods introduction, the QuEST study demonstrated that maternal dietary ingestion of egg during lactation was associated with breast milk egg protein levels and infant egg-specific IgG4 levels. This suggests allergen exposure for breastfed infants can be modified through maternal dietary interventions, which influence systemic allergen-specific responses in the neonate associated with tolerance induction. These findings lay the foundation for future studies to examine whether targeted interventions including maintenance of the skin barrier, in conjunction with maternal dietary ingestion of egg, may improve pathways leading to tolerance induction in infants at risk of developing allergic disease.
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2-ME – 2-mercaptoethanol
BEAT – Beating Egg Allergy Trial
BLG – beta lactoglobulin
BLQ: Below level of assay quantification
CON – conalbumin
DC – dendritic cell
EAT – Enquiring About Tolerance
ELISA – enzyme linked immunosorbent assay
GALT – gut associated lymphoid tissue
HEAP – Hens Egg Allergy Prevention
IFNγ – interferon gamma
IgE – Immunoglobulin E
IgG4 – Immunoglobulin G4
IL – interleukin
LEAP – Learning Early About Peanut allergy
LPS – lipopolysaccharide
LYS – lysozyme
MLN – mesenteric lymph node
OFC – oral food challenge
OM – ovomucoid
OVA – ovalbumin
PEAP – Prevention of peanut allergy by early consumption
PHA – phytohaemaglutinin
PBMC – peripheral blood mononuclear cells
QuEST – Questioning the role of Egg in lactation for the induction of Specific Tolerance
RAST – radioallergosorbent test
RCT – randomised controlled trial
RPMI – Roswell Park Memorial Institute
SCORAD – Scoring Atopic Dermatitis
SPT – skin prick test
STAR – Solids Timing for Allergy Reduction
STEP – Starting Timing for Egg Protein
TEWL – trans epidermal water loss
Th2 – T helper 2 cell
Treg – regulatory T cell
Chapter 1

The role of postnatal allergen exposure on food allergy development: a review of the literature
1.1 Food allergy in context

The rising prevalence of allergic disease is becoming a serious global health problem (1). An alarming feature of this is the escalation of potentially life threatening food-related anaphylactic reactions among very young children (2-7). The dramatic rise in food-related allergic reactions is part of a more general increase in a wide range of non-communicable diseases, including metabolic disease, obesity, heart disease, and autoimmune conditions (8). Although these diseases are phenotypically diverse, they have been attributed to many shared risk factors in modern lifestyles and urban environments, which have been linked with an increased risk of inflammation, beginning through effects early in immune development (9).

Of particular note, the rising prevalence of food allergy only became apparent relatively recently, lagging behind the rise in most other allergic disorders (10). Historically, the ‘allergy epidemic’ became most evident from the 1970’s with the escalation of asthma and respiratory allergies, however the rates of asthma have now plateaued in many higher income regions, and the current generation of children is instead experiencing an increasing prevalence of eczema and food allergy (10). Understanding the mechanisms driving the increasing prevalence of food allergy so early in life remains a key challenge in the development of allergy prevention strategies.

As one of the earliest manifestations of allergic disease, food allergy often predates other atopic disorders, and has a significant impact on the perceived quality of life (11). Early life food-sensitisation has been associated with an increased risk of wheeze asthma (pooled OR 2.9; 95% CI 2.0-4.0), eczema (pooled OR 2.7; 95% CI 1.7-4.4) and allergic rhinitis (pooled OR 3.1; 95% CI 1.9-4.9) from four to eight years of age (12). This sequential progression through multiple atopic disorders has been described as the “atopic march” (13), although the progression is not always consistent and mechanisms of association are not well understood. Nonetheless interventions targeted in early life could potentially also prevent the progression to other atopic
diseases, and a better understanding of the mechanisms leading to food sensitisation is fundamental in addressing these issues.

With food-specific sensitisation occurring so early in infancy (Section 1.5) there is a need to optimise initial exposure to allergens in the early postnatal period to promote the induction of oral tolerance (Section 1.10). This is likely an intricate balance between the environmental milieu (anti-inflammatory versus inflammatory signals), and the timing (in conjunction with the route) of initial allergen exposure during pregnancy, lactation, and solids foods introduction. While the in utero period likely plays a significant role in the development of food allergy, this thesis will focus on allergen exposure and tolerance induction in the postnatal period during lactation and infant diet. Particularly, in the shifting emphasis on the food allergen as a “friend” rather than “foe” in the prevention of food allergy and the development of oral tolerance.

1.2 Risk factors associated with food allergy

Multiple risk factors that modify the predisposition for food allergy have been identified, and are essential for highlighting high-risk populations, and strategies for targeted allergy prevention.

1.2.1 Eczema

Defects in the epithelial barrier, potentially as a route of transcutaneous allergen sensitisation, are a major risk factor for food allergy (14, 15). In Australia, infants with eczema have been found to have a five-fold greater risk of food allergy when compared to non-affected infants (16, 17). In other studies, the prevalence of food allergy in infants with eczema has varied between 20-81% (16-21), likely due to a range of factors including population characteristics, background environmental factors, eczema severity (17, 19) and the age of eczema onset (20). A study of over 2000 infants with eczema, demonstrated food sensitisation in 48.6% of infants, with the highest risk in infants who developed eczema in the first 3 months of life (21). Hen’s
egg allergy was the most common culprit in many of these studies (16, 17, 20, 21), however a recent study has also demonstrated up to 11 times greater risk of peanut allergy in children with eczema (16). Thus, understanding the pathogenesis of eczema associated skin barrier dysfunction in the development of food sensitisation and allergy remains of great interest, and further investigation is required.

As with other allergic conditions, eczema is the likely result of complex interactions between genetic, environmental, and immunological factors (22). Two groups of eczema related genes have been identified, 1) genes within a family of epithelium-related genes including filaggrin (FLG) and SPINK5, and 2) genes that are associated with immune function such as the production of IL-4, IL-5 and IL-13 (23). The single most replicated gene studied in infants with eczema is the ‘filament-aggregating protein’ gene (FLG) (24-26), which describes the proteins function of binding to keratin intermediate filaments, resulting in adequate skin barrier function (27). Loss of function in the FLG gene leads to dysfunction of the skin barrier and inadequate protection against environmental allergens, microbes, and other irritants, which increases the risk for transcutaneous sensitisation (Section 1.6.1) (27). It is notable that children with eczema may also have increased gut permeability (28, 29), allowing the potential passage of intact proteins across the gut barrier which may perpetuate immune responses to common food allergens (30). This suggests more generalised effects on epithelial barrier dysfunction, which could lead directly to altered mucosal immunity and a breakdown in oral tolerance (Section1.10). Thus, strategies to improve barrier integrity more generally may be important avenues in the prevention of food allergy.

In infants with eczema, risk factors for food allergy other than a dysfunctional skin barrier, include male sex and East Asian ancestry (16). Thus, while eczema clearly disposes to the development of food allergy, environmental factors that have systemic effects on immune modulation or genetics are likely to play an equally important role.
1.2.2 Genetic risk

Atopic heredity, namely a family history of allergic disease, is a well-recognised risk factor for food allergies. This was evident in early twin studies (31, 32), and more recently Sicherer et al reported higher rates of concordant peanut allergy in identical twins (64.3%) than among dizygotic twins (6.8%) (33). Similarly, infants born to atopic parents are more likely to have multiple allergic disorders (18.5%, 95% CI 15.0–22.5%) when compared to infants born to non-affected parents (6.3%, 95% CI 4.3–9.0%) (34). For food allergy alone, two or more allergic family members is strongly predictive of food allergy in the child (OR 1.8, 95% CI 1.5–2.3), whilst the effect is modest for infants born into families with only one allergic family member (OR 1.4, 95% CI 1.1–1.7) (34). Thus genetic predisposition is an important risk factor for the development of allergic disease.

Recent genetic studies suggest hundreds of genes may be implicated in food allergy. Perhaps the most significant genetic polymorphism identified in food allergy research is within the human leukocyte antigen (HLA) gene regions (35). Antigen presentation by HLA molecules, which are expressed in a range of immune cells (activated T cells, B cells and macrophages), play an important role in the development of antigen-specific immune responses associated with food allergy (36). Additionally genes associated with skin barrier function (Section 1.2.1), and immune function (37–39) have also been identified, which collectively present a genetic predisposition for the development of food allergy. While it’s clear that genetic heredity is an important determinant of allergic risk, the rapid increase in disease prevalence cannot be explained by germ-line genetics alone, although this is likely to be an important factor in individual susceptibility to environmental change.

The emerging field of epigenetics provides a mechanism for the intricate environment-gene interactions that lead to changes in gene expression, explaining how the changing environment can have lasting (and potentially heritable) effects on the regulation of our DNA expression (40). It is now recognised that epigenetic changes occur naturally in both the antenatal and early
postnatal periods as the immune system develops (41). Thus, critical disruptions of epigenetic mechanisms during this period could contribute to the dysregulated immune responses that underpin food allergy. Accordingly, epigenetic variants are emerging in association with food allergy. Of these, DNA methylation is one of the most studied mechanisms regulating gene expression. The aforementioned study by Hong also identified differentially methylated positions on four genes associated with peanut allergy at HLA genes to nearby SNP variants (c6orf10, HLA-DRB1, HLA-DRB5, HLA-DQB1) (35). Additionally another study identified differential methylation of DNA from CD4+ T cells in children with IgE mediated food allergy, which were preserved from birth to 12 months of age (42). This could have important implications on pro-inflammatory pathways leading to suboptimal T-cell function, which are present as early as birth (42). This new evidence that epigenetics has a role in food allergy development creates promise for targeted environmental interventions which could have lasting and potentially heritable effects.

1.2.3 Environmental factors

Multiple potential environmental factors have been linked to the development of food allergy, including latitude (influencing UV exposure and vitamin D levels), changes in microbial exposure, medications (antacids and proton pump inhibitors), exposure to pollutants and cigarette smoke, and decreased intake of certain dietary components (immunomodulatory nutrients and food allergens) (reviewed in (43, 44)). Some of the ‘modifiable’ risk factors are associated with urbanisation and many are implicated through recognised effects on either local (mucosal) or systemic immune modulation, although their precise role and relative influence remains unclear. Of these, diet has been one of the most significant changes with urbanisation, including more processed foods with altered nutrient profiles compared to traditional diets, and has important implications for overall health.

Diet has multiple and complex effects on the immune system, including well recognised influences through epigenetic modifications (45-47). While the influences of dietary patterns
are clearly complex, many specific nutrients have anti-inflammatory and immunomodulatory effects that have been associated with allergic disease including n-3 polyunsaturated fatty acids (48, 49), oligosaccharides (insoluble fibre) (50, 51), antioxidants (52), probiotics and fermented foods (53) and a variety of vitamins (54). Food diversity in the first year of life is also associated with decreased allergic disease, perhaps by providing a wide array of micro and macro nutrients required for optimal immune function (55, 56). Declining intake of these more ‘immunoprotective’ factors in western diets with more homogenized food sources, coupled with an increase in pro-inflammatory signals, may be providing less tolerogenic conditions during initial allergen encounter leading to food sensitisation.

1.3 Food allergens

As yet, it is still not clear why some proteins are more likely to result in specific allergen induced immune responses and sensitisation. IgE mediated food allergies are the most common type of food allergic reactions, where hen’s egg, cow’s milk, and peanut are the most frequently implicated food allergens in young children (57-59). It is also not clear why individual antigens result in differing natural history of food allergy, with varying prognoses. Peanut allergy tends to be more persistent with increased severity (60), where milk and hen’s egg allergies have historically been more transient. Recent reports from the EuroPrevall study suggest about half of infants will outgrow these allergies by 2 years of age (61, 62). Additionally with milk and egg proteins, extensively heated versions may be tolerated before raw or uncooked versions, and in some cases may accelerate the resolution of cow’s milk (63) and egg allergy (64). Despite this, the high prevalence and severe reactions caused by egg, peanut and milk early in infancy is a growing concern that needs to be addressed (2, 3). As differing clinical phenotypes of major allergens may be due to intrinsic features of the protein, there is growing interest in identifying individual proteins in each food source that may cause reactivity (65, 66).
1.3.1 Hen’s egg as the most common IgE mediated allergy in Australian infants

Hen’s egg allergy is one of the most prevalent IgE mediated food allergies in Australian children (57), and thus is the primary focus of this thesis. While some infants may outgrow their egg allergy, challenge proven egg allergy alone has been reported between 1.23-8.9% of the paediatric population, and is the most prevalent food allergy affecting infants (57, 59, 62, 67). With the ubiquitous nature of egg in our diet it is difficult to avoid completely, increasing the chance for accidental exposure. Investigating which proteins in egg are implicated in allergic reactions is essential to better understand the pathogenesis of egg allergy.

Multiple glycoproteins in egg white are capable of eliciting the production of IgE antibodies, with four major egg allergens identified: ovomucoid (OM, Gal d 1), ovalbumin (OVA, Gal d 2), conalbumin (CON, Gal d 3), and lysozyme (LYS, Gal d 4) (65). OM has been characterised as the most dominant protein causing severe reactions in even minute amounts (68). OVA is the most abundant protein making up 54% of total egg white protein (Figure 1.1), while LYS and CON are deemed less important in the existing framework of egg allergy phenotypes.

![Pie chart showing the percentage of total egg white protein](image)

Figure 1.1: Proteins in egg white capable of eliciting an allergic response. Shown as the % of total egg white protein
The allergic response dominance of OM may be due to its uniquely robust properties, as it is heat stable and also resistant to digestion with protease. Thus conformational IgE-binding epitopes remain intact even under extreme heat and exposure to digestive enzymes (69). Additionally four OM specific IgE-binding epitopes have been associated with persistent egg allergy phenotypes, rendering infants with OM sensitisation less likely to outgrow their allergy (70). Consequently while 80% of infants with an egg allergy tolerate baked and well cooked egg, infants with an ovomucoid allergy are not expected to tolerate any form of egg, are more likely to have severe reactions (71), and have an egg allergy which is more likely to persist into adulthood (70).

In contrast, the most abundant protein in egg white, OVA is a heat labile protein, allowing the IgE binding epitopes to be destroyed with cooking and digestion (69). As such, infants with OVA sensitisation are likely to tolerate well cooked and baked egg, but may still react to raw and undercooked egg. In the case of an OVA allergy, studies are investigating whether baked egg OIT is effective for the induction of oral tolerance in these children with mixed success (72, 73).

Most previous studies of investigating immune function of egg-sensitised patients have focused on responses to OVA as the most abundant protein in hen’s egg (49, 74, 75). All of these studies show that egg allergic patients produce elevated levels of inflammatory cytokines such as IL-5 and IL-13 in response to stimulation with OVA. With the recent notion that different phenotypes of egg allergy may be allergen specific (as discussed above), there is a gap in the literature in regards to cytokine responses to a broader range of egg proteins which are now implicated in egg allergy phenotypes.
1.4 Immune “dysfunction” in allergic infants

Cytokines are instrumental in orchestrating immune reactivity and tolerance, and normal adult immune function relies on a balanced ratio of Th1 and Th2 cytokine production, in order to tolerate harmless environmental proteins, while maintaining the ability to fight bacteria and pathogens. Allergic disease conversely has been associated with a ‘dysfunctional’ Th2 skewed cytokine profile (76).

During pregnancy the placental cells produce a Th2 dominant immune profile to protect the fetus from Th1 mediated cell rejection (77). Over the first year of life, there is a shift from a dominant Th2 profile remaining from foetal development, to a Th1 dominant response of a classic adult immune profile (78). Allergic infants are thought to have a reduced capacity to regulate Th2 responses early in life, and thus these inflammatory responses take over and a Th1 dominated immune response fails to develop (79). This imbalance promotes inflammation and is a part of the pathway that leads to allergic sensitisation (76). Thus upon oral exposure to food proteins, CD4+ T cells produce IL-4, IL-5, and IL-13 which activates B-cells and results in the production of allergen specific IgE (80).

In egg allergic children, Prescott et al (74) demonstrated an increased production of Th2 cytokines (IL-5, IL-13) after peripheral blood mononuclear cells (PBMC’s) were stimulated with ovalbumin and house dust mite, when compared to non-atopic individuals. Interestingly however, as children developed oral tolerance, regulatory cytokine (IFNg, IL-10) levels increased, and there was a marked decrease in the production of IL-5 (74), demonstrating that irregular immune patterns can be reversed back towards a normal phenotype.

Thus neonatal T-cell responses are shifted towards the suppression of cytotoxicity in the early postnatal period but are disadvantaged due to inexperience, making them susceptible to external manipulation (81). Over the first year of life, cell functionality is gained however allergic
infants appear to develop a dysregulated immune response, resulting in chronic inflammatory responses with exposure to some environmental allergens (78). Differences in allergen specific immune responses have been demonstrated at birth between infants who later developed atopy and those who did not (79), however allergen-specific immune responses in relation to the timing of oral allergen exposure has yet to be investigated. With the notion that oral allergen exposure drives oral tolerance induction, there is a need to investigate allergen specific immune responses in relation to patterns of food allergen exposure in infancy.

1.5 Infant sensitisation prior to the introduction of solid foods

There is increasing evidence of infant sensitisation to food allergens prior to introduction of solid foods, especially in infants with eczema. An initial study conducted by Monti et al (82) observed 27% (29/107) of infants with eczema were sensitised to egg (skin prick test (SPT)/radio-absorbent specific test (RAST) positive) prior to ingestion in solid foods. Despite careful exclusion of any infants who had ingested egg prior to enrolling in the study, the mean age for the SPT/RAST and oral egg challenge was 16 months in this study, increasing the chance of prior unintentional egg exposure due to the ubiquitous nature of egg in our diet.

Starting earlier in infancy, both the Solids Timing for Allergy Reduction (STAR) (83) and Learning Early About Peanut allergy (LEAP) (84) studies also demonstrated high rates of food-specific IgE sensitisation prior to oral exposure in solid foods (36% (egg) and 20% (peanut) respectively). Infants randomised to the intervention group in the STAR trial had their first taste of egg at 4 months of age, where 31% had a clinical reaction with the initial ingestion of egg in solids (83). In the LEAP study the mean age of peanut introduction was slightly older at 7.8 months, and the infants were screened based on their peanut SPT results at enrolment. Thus infants who had SPT > 4mm were excluded from the study, and did not introduce peanut into their diet. Neither the STAR study (83) or the study by Monti et al (82) screened infants for IgE sensitisation prior to enrolment, and as a result both of these studies had one infant with
anaphylactic symptoms after the ingestion of egg for the first time. Thus both authors iterate that caution should be exercised when introducing allergenic foods into the diet of infants with eczema, as there is a risk for serious allergic reactions.

More recently, other studies investigating the early introduction of egg have demonstrated higher than expected rates of egg sensitisation in infants prior to the introduction in solid foods, which are discussed in detail in Section 1.14. Two studies enrolled at risk infants with a family history of allergy, and saw egg-specific sensitisation in 5% (IgE >0.35) and 4% (SPT>2mm) respectively (85, 86). Another study of general population (normal risk) infants demonstrated a 5.7% rate of egg-specific sensitisation at 4-6 months of age prior to the introduction of solid foods (IgE>0.35) (87).

Diligent exclusion of infants who had any exposure to the questioned allergen prior to study enrolment in these studies demonstrates food-specific sensitivity prior to ingestion in solid foods, and indicates food allergen exposure earlier in the postnatal period may lead to the development of IgE sensitisation in some infants.

1.6 Pathways for allergen exposure in the postnatal period prior to the introduction of solid foods

Evidence that allergens can cross the placenta (88), into breast milk (Section 1.6.2) and through the epithelial barrier (89-91), all provide possible routes of allergen exposure prior to the introduction of solid foods. There is concern that exposure through ‘less tolerogenic’ routes, such as the skin is more likely to induce sensitisation, which is especially relevant for infants with eczema. Alternatively exposure to allergens via the oral route in breast milk may be favourable for the induction of oral tolerance. While allergen exposure in utero is likely to have important implications in the context of allergy development, this is beyond the scope of this thesis, and thus here I will focus on allergen exposure in the postnatal period.
1.6.1 Transcutaneous sensitisation

In the absence of oral exposure there is concern that exposure through ‘less tolerogenic’ routes, such as the skin is more likely to induce sensitisation. This hypothesis is known as ‘transcutaneous sensitisation’ and is especially relevant for infants with impaired skin barrier function (such as eczema) (92). This hypothesis was suggested when the use of ointments containing peanut oil significantly increased the risk of peanut sensitisation in infants with eczema (93). Loss of function mutation in the FLG gene has now also been associated with a higher incidence of food allergy in ten year old children (OR, 31.46; 95% CI, 2.86 to >100; P=0.005), and food sensitisation as young as four years old (OR, 4.23; 95% CI, 1.3-13.74; P=0.01) (14). This is consistent with evidence from animal models where even in the undamaged skin of mice, repeated topical exposure to peanut allergens led to peanut-specific sensitisation and anaphylaxis with rechallenge (94). A proposed mechanism for transcutaneous sensitisation is through the interaction of environmental proteins, such as food allergens, with the immune system via antigen presenting cells in the epidermis. This results in the production of Th2 cytokines, which then triggers the immune cascade leading to the activation of B cells and production of antigen-specific IgE (94, 95).

To further investigate this phenomenon, the trans epidermal water loss (TEWL) method is used as a non-invasive, and measurable parameter for skin barrier function (27). Thus far it is clear that an increase in TEWL is associated with increased damage in the skin barrier making it more permeable (90). In non-diseased individuals with full epidermal integrity there is minimal trans epidermal water loss (TEWL), providing sufficient protection against environmental microbes and allergens (91). Alternatively, increased TEWL in 2 day old infants predicts eczema at one year of age (96), has been associated with increased severity of eczema (97) and also with food sensitisation in 3 month old infants (91). This may explain why infants with eczema are significantly more likely to develop sensitisation to foods and pollens (12) and may contribute to the so called “atopic march”.
Whilst transcutaneous sensitisation is an interesting hypothesis with increasing evidence, it is plausible that there are other routes to the development of food allergy, such as via the gut, or transplacentally as some children without a history of eczema also develop food sensitisation and food allergy. The route of exposure, in conjunction with form, dose and timing of initial allergen exposure may all play a role as to whether sensitisation or tolerance induction is achieved.

1.6.2 Breast milk food allergens

The role of breast milk in the development of allergic disease is conflicting and has been long debated. This doesn’t necessarily mean allergy prevention through breastfeeding is not feasible, but more likely is a reflection of inadequate methodologies that do not capture the complexity of interactions involved. One of the primary downfalls in breastfeeding research is the inability to randomise due to ethical constraints. We therefore rely on results from observational studies to assess whether breastfeeding versus other modes of feeding (formula) influences the development of food allergy in infants. The second downfall of these studies is the retrospective study design used for most studies greatly increasing the risk for recall bias. Additionally definitions for breastfeeding (exclusive versus partial) and food allergy vary greatly between studies, and rely on parent reporting which may be biased by a participant’s belief of how they believe they should be feeding their infant. Thus the results from these studies must be interpreted with these limitations in mind.

Some breastfeeding and allergic disease study populations included high risk infants (at least a maternal history of atopy) (98-100), and others used general population birth cohorts including atopic and non-atopic families (101-106). Many of these studies relied on breastfeeding recall, some up to one year after birth (102, 103, 105) and another seven years later (106).
The gold standard for the diagnosis of food allergy is the use of an oral food challenge to any food suspected of causing allergic symptoms; this however was only done in one breastfeeding cohort study (101). Due to the time consuming and expensive nature of food challenges, they are not usually feasible in large epidemiological studies. As a result other studies have used various methods for determining the presence of food allergy such as food sensitisation (specific-IgE or SPT) (100, 102, 107), parent reported symptoms (103, 106) or doctor-diagnosed symptoms (98, 99, 104). With varying methods of assessing the presence of food allergy, the results are conflicting.

Several studies reported no difference in risk of food allergy for exclusively breastfed infants versus other modes of feeding (98, 102, 105), or the duration of exclusive breastfeeding (101). A German cohort, now conducted 15 years ago (100), reported an increased risk for hen’s egg sensitisation at one year of age (OR 4.9, 95% CI 1.2-20.4) in a subgroup analysis of infants with elevated IgE levels at birth who were exclusively breastfed > 5 months (however not in children with a history of family atopy or both a history of family atopy and cord blood IgE) and this effect was lost by 2 years of age. In contrast, two studies reported a protective effective of breastfeeding with infants who were not exclusively breastfed and at the highest risk for food allergy (104, 106). Kull et al (102) found that so long as there were no allergy symptoms while breastfeeding, there was a reduced risk of cow’s milk (OR 0.66, 95% CI 0.47-0.94) and wheat (OR 0.62, 95% CI 0.39-0.98) sensitisation in infants exclusively breastfed for > 4 months. With conflicting results there remains insufficient data available for the effects of breastfeeding on objective measures of food allergy at any age.

1.6.3 The detection of food protein in breast milk

Breast milk provides a “complex support system” for infants (108), providing not only nutrients and protection against infection (108), but also allergen exposure (Table 1.1), and immunoregulatory factors (Section 1.11). Increasing evidence is suggesting a role for breast milk in providing early oral allergen exposure for the promotion of oral tolerance.
Breast milk in biological composition is not hypoallergenic, and all major food allergens have been identified in human milk after maternal ingestion. Investigators have identified peanut (araH1 and araH2) (109), cow’s milk (beta lactoglobulin, BLG), (112-117) wheat (gliadin) (125, 126) and egg protein (ovalbumin, OVA) (88, 113, 116, 127-129) which are summarised in Table 1.1.

The concentration of food proteins in breast milk is classically measured after a maternal “challenge” of food, which includes the ingestion of a specified amount of the protein (Table 1.1). Periodic breast milk sampling follows this. In most past studies challenges were not blinded, with the exception of the two studies by Palmer et al (128, 129), which conducted double blind food challenges. A period of dietary allergen avoidance prior to the challenge was included in some cases (109, 113, 114, 123-125, 127-129), however in three cow’s milk studies there was no information regarding the avoidance of dairy foods prior to the challenge (112, 116, 120). In these instances it is unclear whether participants were ingesting dairy foods prior to the challenge, and potentially confounding the primary outcome.

The transient nature of food proteins in breast milk has been established by examining multiple samples expressed in intervals after maternal ingestion (109, 113, 120, 127-129). This generally includes a lag period, followed by an increase in allergen up to peak concentration, followed by a decrease back down to baseline. The exact pattern and timing of this kinetic appears to vary between women and also depends on the food allergen. For egg protein the peak concentration appears between 2-6 hours after ingestion (113, 127, 128), peanut was quicker with a peak concentration between 1-2 hours after ingestion (110) (109) and milk around 4 hours (113, 120). Peanut protein was detected as soon as 20 minutes after ingestion when peanut was consumed on an empty stomach, which was much sooner than peak concentration seen 1-2 hours after ingestion on a full stomach (110).
The studies summarised in Table 1.1 all report wide ranges in specific food protein concentrations in breast milk between women despite the ingestion of the same challenge amount of food protein. Additionally between studies with more than ten women, the number of participants with detectable levels of protein in their breast milk varied greatly (21%-95%). This large variation could be due to varying sampling schedules between studies or the sensitivity of the food protein detection assay. Palmer et al. highlighted a subset of women who after multiple samples following egg protein ingestion, fail to excrete ovalbumin (egg protein) in their breast milk at any time point throughout the study (128, 129). Delayed excretion of the food protein beyond the sampling schedule is possible; however, other complex biological factors involved in digestion, absorption or excretion could also influence the passage of food proteins from the maternal diet into breast milk.
Table 1.1: Measurement of food proteins in human milk

<table>
<thead>
<tr>
<th>Study</th>
<th>Allergen</th>
<th>Participants</th>
<th>Food challenge dose / intervention</th>
<th>Sample expressed (post ingestion)</th>
<th>Food protein concentration (range, ng/ml)</th>
<th>Allergen Detection frequency (% women)</th>
<th>Allergen detection method (sensitivity, ng/ml)</th>
<th>Peak allergen concentration</th>
<th>Association to maternal dietary ingestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilshaw and Cant, 1984(113)</td>
<td>Cow’s milk and egg</td>
<td>N=129 1 week to 12 months of lactation</td>
<td>1/2 pint cow’s milk &amp; one raw egg</td>
<td>Baseline &amp; 2, 4, 6 hrs</td>
<td>0.11-6.4 cow’s milk 0.26-6.17 egg</td>
<td>52% cow’s milk 59% egg</td>
<td>Radio-immunoassay (0.1)</td>
<td>4-6 hrs</td>
<td>Not reported</td>
</tr>
<tr>
<td>Cant et al, 1985(127)</td>
<td>Egg</td>
<td>N=19 &lt;6 months of lactation</td>
<td>One raw egg</td>
<td>Baseline &amp; 2, 4, 6 hrs</td>
<td>0.2-4.0</td>
<td>74%</td>
<td>Radio-immunoassay (0.1)</td>
<td>2-4 hrs</td>
<td>Not reported</td>
</tr>
<tr>
<td>Troncone et al, 1987(125)</td>
<td>Gluten</td>
<td>N=53 (n=6) 1 week to 5 months of lactation</td>
<td>20g gluten</td>
<td>2-4 hrs</td>
<td>5-95</td>
<td>68%</td>
<td>ELISA (5)</td>
<td>1 sample only (at 2-4 hours)</td>
<td>Not reported</td>
</tr>
<tr>
<td>Cavagni et al, 1988(114)</td>
<td>Cow’s milk and egg</td>
<td>N=13 Lactation stage not clear</td>
<td>1 cooked egg &amp; 100g cow’s milk</td>
<td>3-5 hrs</td>
<td>Results as positive or negative only</td>
<td>62%</td>
<td>Radio-immunoassay (0.01)</td>
<td>1 sample only</td>
<td>Not reported</td>
</tr>
<tr>
<td>Host et al, 1988(112)</td>
<td>Cow’s milk</td>
<td>N=19 Lactations stage not clear</td>
<td>500ml cow’s milk</td>
<td>4 hrs</td>
<td>0.5-45</td>
<td>21%</td>
<td>ELISA (0.3)</td>
<td>1 sample only</td>
<td>Not reported</td>
</tr>
<tr>
<td>Host et al, 1990(120)</td>
<td>Cow’s milk</td>
<td>N=20 1-2 weeks of lactation(atopic) &amp; 8-19 weeks (non-atopic)</td>
<td>500ml cow’s milk</td>
<td>baseline &amp; 4, 8, 12, 24 hrs</td>
<td>0.9-150</td>
<td>95%</td>
<td>ELISA (0.3)</td>
<td>4-24 hrs (median 8 hr)</td>
<td>Not reported</td>
</tr>
<tr>
<td>Sorva et al, 1994(123)</td>
<td>Cow’s milk</td>
<td>N=53 2-12 months of lactation</td>
<td>400ml cow’s milk</td>
<td>Baseline &amp; 1, 2 hrs</td>
<td>0.00-8.6? not clear</td>
<td>75%</td>
<td>ELISA (0.002)</td>
<td>Only 1 mother studied &gt;2hrs</td>
<td>Not reported</td>
</tr>
<tr>
<td>Study</td>
<td>Allergen</td>
<td>Participants</td>
<td>Food challenge dose / intervention</td>
<td>Sample expressed (post ingestion)</td>
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<td>Allergen Detection frequency (% women)</td>
<td>Allergen detection method (sensitivity, ng/ml)</td>
<td>Peak allergen concentration</td>
<td>Association to maternal dietary ingestion</td>
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<tr>
<td>Fukushima et al, 1997 (116)</td>
<td>Cow’s milk</td>
<td>N=24</td>
<td>4-10 months of lactation</td>
<td>200ml cow’s milk</td>
<td>1-3 hrs, 4-8 hrs, &amp; 9-15 hrs</td>
<td>Up to 16.5</td>
<td>63% ELISA (0.1)</td>
<td>Not reported</td>
<td>Yes</td>
</tr>
<tr>
<td>Jarvinene et al, 1999 (124)</td>
<td>Cow’s milk</td>
<td>N=26</td>
<td>2-9 months of lactation</td>
<td>100-400ml cow’s milk</td>
<td>baseline &amp; 1, 2, 3, 4 hrs</td>
<td>0.03-11.54</td>
<td>52% ELISA (not reported)</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Vadas et al, 2001 (109)</td>
<td>Peanut</td>
<td>N=23</td>
<td></td>
<td></td>
<td>Baseline &amp; 1, 2, 5, 4, 6, 8, 12 hrs</td>
<td>120-430</td>
<td>48% ELISA (not reported)</td>
<td>1-2 hrs</td>
<td>Not reported</td>
</tr>
<tr>
<td>Palmer et al, 2005 (128)</td>
<td>Egg (both cooked and raw)</td>
<td>N=49</td>
<td></td>
<td></td>
<td>Baseline &amp; 2, 4, 6, 8 hrs</td>
<td>0.1-30.0</td>
<td>76% ELISA (0.1)</td>
<td>2-6 hrs</td>
<td>Dose response effect No assoc. with usual egg intake</td>
</tr>
<tr>
<td>Bernard et al, 2014 (110, 117)</td>
<td>Peanut</td>
<td>N=2</td>
<td></td>
<td></td>
<td>0.1-3.4</td>
<td>100% ELISA (0.001)</td>
<td>1-2 hrs (only 2 mothers)</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Study</td>
<td>Allergen</td>
<td>Participants</td>
<td>Food challenge dose / intervention</td>
<td>Sample expressed (post ingestion)</td>
<td>Food protein concentration (range, ng/ml)</td>
<td>Allergen Detection frequency (% women)</td>
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<tr>
<td>Schocker et al 2016 (111)</td>
<td>Peanut (Arah h2)</td>
<td>N=32</td>
<td>100g dry roasted peanut</td>
<td>Baseline 1,2,3,4,8,12</td>
<td>46-2602</td>
<td>57% total samples, # of women unclear</td>
<td>ELISA</td>
<td>Not Reported</td>
<td>Not association</td>
</tr>
<tr>
<td>Bertino et al 1996 (117)</td>
<td>Cow’s milk</td>
<td>N=14</td>
<td>Unclear how diet was allocated</td>
<td>Intervention = 10 days</td>
<td>NA</td>
<td>0.2-304</td>
<td>ELISA</td>
<td>Not Reported</td>
<td>No association</td>
</tr>
<tr>
<td>Lovegrove et al 1996 (121)</td>
<td>Cow’s milk</td>
<td>N=22</td>
<td>RCT</td>
<td>No food challenge</td>
<td>NA</td>
<td>0.08-5.9</td>
<td>ELISA</td>
<td>Not Reported</td>
<td>No association</td>
</tr>
<tr>
<td>Vance et al 2005 (88)</td>
<td>Egg</td>
<td>N=68</td>
<td>RCT</td>
<td>No food challenge</td>
<td>NA</td>
<td>0.12-1258</td>
<td>ELISA (0.03)</td>
<td>Not reported</td>
<td>No association</td>
</tr>
<tr>
<td>Palmer et al 2008 (129)</td>
<td>Egg</td>
<td>N=32</td>
<td>RCT</td>
<td>Interventio = 3 weeks</td>
<td>Baseline &amp; 2,4,6 hrs</td>
<td>0.1-8.26</td>
<td>ELISA (0.1)</td>
<td>2-6 hrs</td>
<td>Yes</td>
</tr>
</tbody>
</table>

N= number of infants enrolled in study
NA= not applicable
1.7 Factors influencing food protein concentration in breast milk

1.7.1 Maternal dietary allergen ingestion

Breast milk composition is not static in nature and changes in response to pathogens, lactation stage, and maternal diet (108). The presence of food proteins in breast milk raises the question of whether patterns of maternal dietary ingestion influences breast milk allergen content, and thus has the ability to modulate allergen exposure for breastfed infants in early life.

Cohort studies

The relationship between varying patterns of maternal dietary ingestion and food protein concentration in human milk is still vague. Many of the early studies measuring food proteins in breast milk were conducted while mothers followed their standard diet, and thus no intervention or food challenges were conducted (115, 118, 119, 122, 126). In some instances data on maternal dietary ingestion was not collected, as the primary outcome was simply to confirm the presence of food allergens in human milk (115, 118, 126). Dietary data was collected in some cases (119, 122) but methods were not optimised for the association between diet and breast milk allergen concentration. Axelsson et al. reported no correlation between maternal diet and breast milk BLG concentration, however it was unclear whether they recorded the consumption of other dairy foods (hence all sources of cow’s milk protein) in the diet alongside cow’s milk, and thus actual BLG consumption cannot be ascertained (119). In another instance where baseline maternal dairy and cow’s milk ingestion data were reported, dietary data was not correlated with the concentration of BLG in breast milk (122).

In contrast, a prospective Japanese study (n=15) noted an association with long term patterns of cow’s milk ingestion and breast milk BLG concentration (116). Women who consumed the lowest amount of dairy foods throughout pregnancy had lower levels of BLG in their breast milk, despite all women ingesting ≥200ml cow’s milk daily for one week prior to (and including the day of) donating their breast milk sample (116). This would suggest that long-term patterns
of dairy food (cow’s milk protein) ingestion have a greater influence on the BLG concentration of breast milk than individual challenge doses, or short-term changes in dietary ingestion, however the number of participants in this study were low (n=15). At present there is insufficient evidence from cohort studies to adequately determine how maternal diet influences the allergen composition of breast milk.

**Intervention studies**

Intervention studies provide higher quality evidence than cohort studies. Four trials have been conducted which allocated mothers to allergen-specific dietary intervention groups and measured breast milk allergen concentration (Table 1.1). Cow’s milk has been investigated in two trials (117, 121), and both of these studies performed a small three-arm intervention. Bertino allocated participants (n=14) to a high cow’s milk diet, low cow’s milk diet or cow’s milk/dairy free diet during lactation(117). Lovegrove et al split atopic women into a cow’s milk elimination or standard diet, and then had a control group consisting of non-atopic women following a standard diet (n=38) from 36 weeks gestation and during lactation (121). Maternal dietary ingestion of cow’s milk protein was not associated with BLG concentration in breast milk in either study.

The study conducted by Bertino presented a number of limitations. Firstly, maternal compliance with the dietary intervention was not reported. Second, the timing of breast milk sample expression in relation to the maternal consumption of cow’s milk was not standardized, potentially introducing variability within the intervention groups. The largest fault with this study however was in the detection methodology for BLG, where the anti-beta-lactoglobulin antibody used in the ELISA was cross-reactive with human betacasein, human alpha-lactalbumin, and human lactoferrin proteins (117). As a result the concentration of BLG reported was not a true representation of breast milk allergen concentration.
Lovegrove introduced atopy as an additional factor of investigation, and reported that non-atopic women ingesting dairy foods had 26% lower BLG in their breast milk than atopic women. However similarly to other studies conducted in this era, they did not control the consumption of dairy around the breast milk sample, and with such small sample size this could account for any differences between groups (121).

Two randomised controlled trials have examined the effect of maternal dietary egg ingestion on breast milk ovalbumin concentration (88, 129) (Table 1.1). The intervention in both cases included an egg inclusion intervention group compared to a control egg avoidance group. Palmer et al conducted a double blind trial and provided a study muffin to be consumed daily during lactation for 21 days, which either included one egg or no egg (129). Vance randomised women to either an egg free diet or an unmodified standard diet from 24-32 weeks gestation through to 3 months postpartum.

The study by Vance was designed to assess egg ingestion in both pregnancy and lactation, and thus breast milk was only collected at one time point (3 months lactation) (88). The lack of standardisation of egg ingestion prior to the single breast milk sample resulted in only 35% of women in the egg inclusion group with detectable levels of OVA in their breast milk at three months. As a result this did not relate to dietary ingestion of egg. Thus it was concluded that women who were avoiding egg were just as likely to have OVA in their breast milk as women who were regularly ingesting egg. However multiple breast milk samples may have told a different story.

In contrast to the results from Vance, Palmer et al. demonstrated women ingesting one egg daily had consistently higher concentration of OVA in breast milk at all time points (day 3, 12, and 24), compared to women following an egg free diet (129). This was a well-conducted double blind randomised controlled trial, which standardised the collection of multiple samples of
breast milk (prior to muffin, then at 2, 4, and 6 hours post ingestion). As a result detection frequency was much higher in this study, and 75% of women who were ingesting egg had detectable levels of OVA in their breast milk. These findings are consistent with results from an earlier challenge study by Palmer et al, which showed a direct dose response relationship between the amount of maternal egg ingested and OVA concentration of human milk (128). Whilst these two studies highlight the short-term influences of egg ingestion on breast milk concentration, the effect of longer term patterns of egg ingestion are less clear.

The effect of long-term dietary patterns on breast milk food allergen concentration has important implications on any recommendations with regard to maternal dietary guidelines for allergy prevention. A mother’s usual dietary egg ingestion was not associated with breast milk OVA concentration in the challenge study conducted by Palmer et al, and egg consumption on the day (challenge dose) ultimately influenced the protein content of breast milk (128). The latter intervention study conducted by Palmer et al concluded that maternal ingestion of egg over a three week period results in higher levels of OVA in breast milk (129), however the dose in the study was twice the amount of egg the average Australian woman eats (0.47 +/- 0.36 eggs per day) and was all from a single source of egg exposure (daily muffin ingestion) (128). This makes it difficult to ascertain whether standard egg ingestion coming from a variety of foods in the maternal diet (including foods such as boiled or scrambled egg, quiche and cake), influences the OVA concentration of human milk. As mentioned previously, research on maternal cow’s milk ingestion suggests long-term dietary intake may have a greater influence than challenge doses or short term dietary changes (116). With increasing interest in oral tolerance induction for infants via breast milk derived food allergens, there is a need to better understand how long-term maternal diet modulates allergen exposure for breastfed infants in the postnatal period.

1.7.2 Breast permeability

Variations in detectable OVA concentrations between women even after the consumption of the same amount and type of egg in the diet (128), suggests additional factors during digestion,
absorption or excretion may influence the passage of food proteins into breast milk. Increased permeability of the breast epithelial has been correlated with an increased risk for atopic disease in infants with allergic mothers (130). In this study, infants born to atopic mothers with increased sodium(Na)/potassium(K) ratio (as a marker for breast permeability) were at a significantly greater risk for a positive skin prick test, and/or atopy (130). The underlying mechanism that drives permeability to influence atopy in the infant remains unclear.

Inflammation in the breast caused by infections such as mastitis leads to increased permeability of the breast mammary epithelia, and results in leakage of various components into breast milk (131). As thus it was proposed that atopic mother’s with naturally higher levels of inflammation, and also a predisposition to impaired skin barrier function (eczema), may also exhibit an impaired mammary epithelial barrier. However Benn and colleagues demonstrated that atopic women and non-atopic women do not have significantly different breast permeability (130). Instead it was the combination of maternal atopy and increased breast permeability that resulted in an elevated risk for infant allergy (130). A possible mechanism for this effect could be through the uncontrolled passage of food proteins into breast milk, however no previous studies have examined whether breast mammary epithelial permeability affects the amount of food protein in human breast milk.

1.8 Maternal elimination diets during lactation in the prevention of food allergy

There have been some previous trials that investigated the elimination of “allergenic” foods from the maternal diet during lactation for the prevention of infant allergic diseases. Eczema was the focus of most of these studies, however food sensitisation and food allergy were examined as a secondary outcomes in some studies, which are summarised in Table 1.2. Three studies were acceptably controlled, with a randomised controlled study design (132-134). Two studies used patient preference group allocation instead of randomisation (135, 136) and one study allocated individuals based on which town they lived in (137). The intervention arms in
these studies generally involved the maternal elimination of selected “allergenic” foods (cow’s milk, egg, nuts, fish, soya) from the diet during lactation, alongside a control group who simply followed their standard diet (detailed in Table 1.2). Herrmann et al (135) was the only study to use a three group intervention schedule (where the differing variable was the inclusion of dietary avoidance through the third trimester of pregnancy), and to include a minimum recommended consumption of cow’s milk and egg in the control group. The timing of the dietary intervention varied between studies, with some starting as early as the third trimester of pregnancy (132, 135), intervened for the full lactation period (132, 133, 135, 136) or just three months (137). Two studies also included delaying the introduction of allergenic foods in the infant diet (132, 133). Many of these studies do have long-term follow-up assessments now completed up to eight years (138, 139) and ten years of age (140).

The results are conflicting, and differing study protocols make it difficult to directly compare outcomes. Three studies reported no detectable effects of maternal dietary exclusion on food sensitisation in the infant at any time point (135, 136) (137). Of the randomised studies, two studies demonstrated lower rates of sensitisation in the dietary elimination group when compared to the controls (132, 133). In contrast Appelt et al. reported higher rates of sensitisation in the elimination group than the control group at two years of age (134). The study by Appelt et al (134), did not report any difference between the intervention and controls at one year of age, however at two years of age reported a lower rate of sensitisation to egg in the control group (p=0.03), and trends towards lower rates of sensitisation at seven years for both milk and egg in the control group (p=0.06 for both) compared to the elimination diet. Whilst this is a large study (n=497), trial quality and risk of bias are difficult to assess, because the information available is solely from a published abstract that provides no information on randomisation methods, participation retention, or method of analysis. Hence the validity of these results cannot be determined.
The Isle of Wight prevention study followed up infants at five time points post intervention: 1 year (133), 2 years (141), 4 years (142) and 8 years (138) and 18 years (Scott, 2012 #854) of age. In this study 58 women were allocated to the allergen elimination diet (dairy, egg, fish and nuts) for the entire duration of lactation. The primary results from this study presented ‘food intolerance’ as the outcome measure for food specific reactions (133). This was defined as a history of vomiting, diarrhoea, colic, or rash within 4 hours of ingestion of a recognised food allergen. At twelve months of age 7/58 (11%) of the infants in the control group were classified as ‘food intolerant’ compared to 2/58 (3%) in the elimination group, for both cow’s milk and egg (no statistical analysis reported). However the later publications used IgE mediated food allergy as the primary outcome and included SPT and oral food challenges. The 8 year follow-up (138) examined results from all time points to give an overall picture of sensitisation status during the full follow-up period. At eight years of age, the odds ratio for sensitisation to foods at any time for the intervention group, compared to the standard diet was 0.15 (0.03-0.80, P=0.10) after adjustment for confounders. Diagnosis of food allergy (IgE mediated) at any time over the eight years was lower in the elimination group (5/58, 8.6%), than the control group (16/62, 25.8%) (OR 0.29; 95% CI, 0.10-0.85, p=0.02). However this effect was lost after the repeated measurement analysis (OR 0.41; 95% CI, 0.11-1.53, p=0.18). By the 18 year follow up there was no longer any difference between groups for food sensitisation (Scott, 2012 #854).
### Table 1.2: Maternal elimination diets in the prevention of food allergy.

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Sample size (intervention, Control)</th>
<th>Maternal and infant intervention</th>
<th>Timing of intervention of (Age infant assessment)</th>
<th>Infant outcome measure</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hattevig, 1989 (137), Hattavig 1999 (140)</td>
<td>Non-randomised comparison Non-blinded examination</td>
<td>n=115, 6 withdrew I=65 and C=50</td>
<td>I: cow’s milk, fish, egg-free diet for mothers for first 3 months of lactation C: no maternal dietary restrictions</td>
<td>Lactation Solid foods (9 months) (10 years) (140)</td>
<td>SPT to egg, cow’s milk and fish + IgE to various foods at 10y</td>
<td>9 months= no difference in SPT sensitisation between I and C groups 10 y = no difference between groups for SPT sensitisation to foods, or IgE levels to foods included in maternal elimination diet.</td>
</tr>
<tr>
<td>Zeiger et al, 1989 (132), Zeiger 1992(143) Zeiger 1995</td>
<td>RCT</td>
<td>N=379, 91 withdrew I:103 and C:185 (completed)</td>
<td>I: cow’s milk, egg, peanut-free diet for mothers during last trimester and lactation + solids restrictions for infants C: no maternal dietary restrictions</td>
<td>Lactation Solid foods (1 year) (4 years) (7 years)</td>
<td>DBPCFC SPT to foods IgE to foods IgG to milk and egg</td>
<td>1 y = significantly lower rates of food allergy in the elimination group 4 y = no difference between groups for food allergy (after 2 y prevalence of food allergy dropped from 15% to 5% in the control group to balance out) 7 y = no difference between groups</td>
</tr>
<tr>
<td>Arshad, 1992 (133), Hide 1994 (141), Hide 1996, (142) Arshad 2007, Isle of Wright Study (138) (Scott, 2012 #854)</td>
<td>RCT Blinded examination</td>
<td>N=120 I=58 C=62</td>
<td>I: dairy, egg, nuts, fish, soya-free diet for mothers during lactation, and for the infants the same +wheat elimination in solids until 12 months. HDM avoidance C: no dietary restrictions for mother or infant</td>
<td>Lactation Solid foods (1,2,4, 8 years)</td>
<td>Food allergy = reported reaction to a food on 2 or more occasions.</td>
<td>No significant differences food allergy prevalence at 12 months. 50% lower prevalence of food allergy in intervention group across all time points when assessed at 7 years (did not reach statistical significance) 18 yrs no difference in food sensitisation between groups</td>
</tr>
<tr>
<td>Herrmann, 1996 (135)</td>
<td>Participant preference trial. Examination non-blinded</td>
<td>N=150 (I: 11:30, 12:33, 13:34, C: 41) 12 withdrew</td>
<td>I1: cow’s milk, egg, free diet for mothers last trimester of pregnancy and during lactation I2: cow’s milk, egg free diet for mothers for lactation only I3: mother’s consumed &gt;1000ml cow’s milk and 1 egg daily C: no maternal dietary restriction</td>
<td>Pregnancy Lactation (6 &amp; 12 months)</td>
<td>Specific IgE for cow’s milk &amp; egg</td>
<td>No differences for sensitisation rates at 12 months – incomplete sampling</td>
</tr>
<tr>
<td>Kilburn, 1998 (136)</td>
<td>Participants self-selected intervention group Examination blinded</td>
<td>n=111, 4 withdrew, I=13 and C=94</td>
<td>I: cow’s milk, egg, fish, nut-free diet for mothers during lactation C: no maternal dietary restrictions</td>
<td>Lactation (6,12 &amp; 18 months)</td>
<td>SPT to foods</td>
<td>No significant differences between groups for food sensitisation at any time</td>
</tr>
<tr>
<td>Appelt et al, 2004 (abstract only)</td>
<td>RCT</td>
<td>N=497 I:251 C:246</td>
<td>I: Cow’s milk, egg, nuts, fish-free diet for mother during third trimester and lactation.</td>
<td>Pregnancy Lactation (1,2, &amp; 7 years)</td>
<td>SPT to milk egg and peanut</td>
<td>No difference at 1y At 2y significantly lower egg sensitisation in control group and trend at 7y for lower sensitisation to egg and milk in control group</td>
</tr>
</tbody>
</table>
A large study by Zeiger et al (132) (n=379), reported significantly lower rates of food allergy (p<0.01) at one year of age in the dietary elimination group, assessed using SPT and double blind placebo controlled food challenge. However this study had a high withdrawal rate with 91/379 (24%) not completing the first assessment and at four and seven years of age (139, 143), this effect was no longer seen. Interestingly after two years of age the prevalence of food allergy in the control group dropped from 15% to 5%, which was equivalent to rates seen in the dietary elimination group. This suggests that if dietary elimination did have any effects, the natural course of food allergy and tolerance induction results in similar outcomes after two years of age.

To ultimately determine the influence of maternal elimination diets during lactation on infant food allergy outcomes, the gold standard would be to conduct oral food challenges at the end of the intervention. Oral food challenges however, were only conducted in one study (143) and only half of the participants agreed to undergo the challenge. Common markers for food sensitisation were used instead, such as SPT (132, 136-138), or food specific-IgE (135). Whilst these sensitisation measures are useful clinical tools in the diagnosis of an IgE mediated food allergy, food sensitisation without clinical reactivity is common. Thus studies that did not include oral food challenges are inconclusive in regards to food allergy prevention.

Interpreting the results from the two aforementioned RCT’s (143) (133) is complicated, as while they used oral food challenges as an outcome measure, they incorporated two dietary interventions: 1) maternal elimination diet and 2) the delayed introduction of allergenic foods in to the infant’s diet (dairy, wheat, soya and egg). Furthermore, the Isle of Wight study also included the reduction of house dust mite exposure in the intervention group (133). As a result these two studies were excluded from a recent meta-analysis titled “Maternal dietary antigen avoidance during pregnancy or lactation, or both, for preventing or treating atopic disease in the child”(144). As such it was concluded that there was no significant protective effect of maternal dietary allergen avoidance during lactation on the incidence of sensitisation at 1, 2 or 7 years of age (144). As emerging evidence is suggesting oral allergen exposure is required to induce
“normal” oral tolerance mechanisms in the gut, there is a pressing need for quality evidence based studies examining the role of breast milk in oral tolerance induction.

### 1.9 Oral Tolerance

Oral tolerance is an active immunological process, which requires oral allergen exposure to facilitate non-response (145). Thus, food allergy reflects a selective breakdown of this process. While it is unclear what the specific processes are that lead to oral tolerance induction in human infants, or how these pathways are disrupted in food allergy (146), experimental animal models provide considerable insight into these processes.

The gastrointestinal tract has evolved as the largest immunological organ in the body (147) with the gut-associated lymphoid tissue (GALT) organised into lymphoid centres (peyers patches and mesenteric lymph nodes (MLN)) and a range of cells residing in the epithelium and lamina propria. These local immune networks are constantly in contact with environmental proteins entering the gastrointestinal tract, together with commensal bacteria, and interact and inform systemic immune responses at other sites (147). The GALT is finely balanced to maintain gut homeostasis preventing excessive or inappropriate responses to harmless environmental proteins and commensal microbes, whilst ensuring sufficient protection against potential pathogens.

In this context, as generally harmless environmental proteins, the default response to food allergen exposure via the oral route is tolerance. This process is dependent on allergen ‘exposure’ to drive oral tolerance induction via the GALT (148, 149). Upon ingestion, a particular subset of dendritic cells (DC’s) found in the gut (CX3CR1+) captures antigenically intact protein using protrusions into the lumen (150). Antigen is then transferred to migratory CD103+ DC in the lamina propria, resulting in antigen presentation to T cells in the MLN, ultimately inducing the differentiation of T regulatory cells(150, 151 368). In this way an
orchestrated memory T-cell mediated suppression following oral allergen exposure is initiated in the GALT, resulting in the induction of oral tolerance. As these antigen-specific processes for oral tolerance are initiated very early in infancy, this period is also a particular window of risk for the failure of these processes and the development of food allergy.

### 1.10 Optimizing conditions for oral tolerance induction in the postnatal period

Molecules and cells involved in oral tolerance induction are continually being discovered, demonstrating that it is a complex and multi-layered process, which may occur before birth (in utero) or in the early postnatal period. With this in mind, there are important considerations for optimising postnatal tolerance induction in the clinical setting, which need to be addressed. Firstly, optimising initial oral allergen exposure, including the form, timing, dose and frequency of allergen exposure required. Secondly, optimising the environment during allergen encounter, more specifically in the gut milieu, including the presence of anti-inflammatory immune signals, immunomodulatory agents, gut maturation and the presence of commensal bacteria. Thus identifying optimal conditions for initial oral allergen exposure seems like a promising approach for the clinical application of oral tolerance induction.

#### 1.10.1 Allergen exposure

In animals, both the dose and timing of allergen feeding has been demonstrated to influence oral tolerance induction (152-155). In a study by Faria, continuous feeding of OVA resulted in suppression of both Th1 and Th2 type responses, and up regulation IL-10 and TGF-β, where low doses of OVA were more successful in inducing IL-10 and TGF-β responses than high doses (156). Thus frequent exposure to low doses of allergen favoured the induction of oral tolerance. Perhaps of equal significance the removal of food proteins from the diet results in the inability to induce oral tolerance, due to the lack of immune maturation (157). In human studies, the benefits of regular allergen exposure have been demonstrated in specific oral immunotherapy studies (158-161) and now also in promising results from allergy prevention.
studies (discussed in section 1.13). However, there are concerns as to the whether these studies induce lasting systemic non-responsiveness to the food once allergen exposure ceases, which is indicative of true oral tolerance induction (162, 163).

1.10.2 The gut milieu

The context in which food allergens are received via the GALT may be important in the induction of oral tolerance, in which immunomodulatory dietary factors may play a fundamental role. Diversity in the diet has recently been recognised as favourable in the context of allergic disease (55, 56), perhaps by providing access to a wider range of these immunomodulatory factors (such as Vitamin A & D), and a more diverse microbiome.

Vitamin A derived from the diet and metabolised into retinoic acid (RA), plays a key role in tolerance induction in the gut (164). RA leads to differentiation of FOXP3+ Tregs via CD103+ DC’s in the presence of transforming growth factor-beta (165). In neonatal mice vitamin A deficiency results in failure to initiate oral tolerance (166). This appears the result of a dual effect in which T-cell driven suppression facilitated by migratory DCs is not initiated, together with abnormal antigen transfer across the gut intestinal barrier (166). In this model, supplementation with vitamin A leads to re-establishment of oral tolerance and improvement of gut epithelial function (166). Vitamin D also influences DC and Treg function in the GALT by modulating signals between bacterial antigens in the gut, and Treg and DC populations in the lamina propria (160). Activation of vitamin D receptors promote CD4+ CD25+ FoxP3+ suppressor T cells that have been shown to stop the development of autoimmune disorders (167).

Both vitamin A and D play an integral role in maintaining the mucosal barrier by regulating tight junction proteins in the gut (166, 168). Impaired intestinal barrier function leads to “leaky gut” syndrome which allows the unregulated passage of environmental antigens and bacteria
across the intestinal barrier into the lamina propria, and is associated with a variety of conditions including inflammatory bowel disease, celiac disease, autoimmune conditions and allergy (Figure 1.2) (169). Antigenically intact food antigens in the lamina propria initiates an inflammatory immune response through production of cytokines and proteases (170). As a compounding effect, once an infant is sensitised to an antigen it is proposed that subsequent exposure further impairs intestinal barrier function in some infants perpetuating the disease (171). Thus in the absence of a healthy gut mucosal barrier oral tolerance induction may not be initiated.

Increasing research is emphasizing the importance of the microbiome in multiple facets of human health, including implications on immune function (172, 173). Declining microbial diversity in the gut is implicated in disrupted gut homeostasis and immune maturation resulting in the increase of allergy and other immune conditions (174, 175). For the neonate, microbiome colonization largely begins during the birthing process, and the infant’s early gut colonization relies greatly on which bacterial commensals are present in the maternal birth canal (depending on delivery method) and breast milk (176). This early colonization could have important lasting implications for allergy development in the infant.

In the context of food allergy, there is evidence to suggest that specific gut colonizing bacteria (Clostridia in particular) aids in preventing food sensitisation through the regulation of innate lymphoid function and improving the gut epithelia barrier (177). These bacterial commensals also work in favour of tolerance by promoting the production of IgA and FoxP3+ cells, which regulate inflammation induced by gut commensals and antigens in the gut lumen (177, 178).

1.11 Breast milk and oral tolerance induction

To optimise oral tolerance induction in the early postnatal period, infants may require allergen exposure in conjunction with breast milk immune factors (110, 179). Breast milk may also
provide an early source of vitamin A and D, as well as commensal bacteria during a period in which the neonate may be deficient. Thus early tolerance induction via breast milk is an attractive approach for the primary prevention of allergic disease, and animal studies have shown oral allergen exposure via breast milk to be protective against airway allergies such as asthma (180-182) and now also food reactivity (110, 179). As food proteins with IgE reactivity have now been identified in human milk (110), breast milk provides an opportunity to combine both the ideal environmental milieu, via protective immune factors and adequate gut barrier function, along with oral allergen exposure (Figure 1.2).

Antibodies in breast milk are key immunomodulators of neonatal immune responses. IgA is the primary antibody found in breast milk which modulates the mucosal immune system in two main ways 1) by binding to cells in peyer’s patches, allowing transport across the epithelial barrier, and 2) by the ingestion of sIgA by DC’s within the lamina propria, resulting in antigen presentation to Treg cells, initiating effector/regulatory pathways in the mucosal immune system (183). The importance of IgA has been demonstrated in human infants where low cow’s milk specific IgA levels in maternal milk have been associated with increased risk for cow’s milk allergy in the infant (184).

Experimental animal studies however are suggesting a role for maternal allergen–specific IgG in breast milk in tolerance induction, where breastfeeding by antigen-exposed mothers resulted in long-lasting protection from allergic airway inflammation (182, 185). Tolerance may be induced in this case via the formation of IgG-allergen complexes in the breast milk, which through uptake in the gut induces antigen-specific T regulatory cells (182), and by preventing Th2 skewed cytokine production (186). A recent study by Bernard (110), showed that neonatal mice fed human breast milk containing free peanut allergen and allergen-IgG complexes were significantly more likely to develop oral tolerance to peanut, when compared to mice fed breast milk with no peanut allergens. Additionally in the offspring exposed to human breast milk after maternal peanut consumption, peanut sensitisation did not result in a clinical response, and was
supported by a suppression in peanut specific immune responses, both Th2 (IgG1), and Th1(IgG2a). Thus free peanut allergen in combination with peanut-specific immune complexes was deemed beneficial for the development of oral tolerance.

Cytokine production in breast milk is heterogeneous between women, and the presence of maternal atopy is associated with increased levels of inflammatory cytokines IL-4, IL-5 and IL-13 when compared to non-atopic women (187). Despite this there is some evidence that children born to mothers with atopy benefit significantly from breastfeeding (188), and this could be due to the presence of anti-inflammatory cytokines. Transforming growth factor-β (TGF-β) is one of the key immunomodulatory cytokines in human milk (187). TGF-β in conjunction with interleukin-10 (IL-10) down regulates inflammatory cytokines and promotes the production of IgA, thus promoting oral tolerance induction (147). In the context of allergic disease, epidemiological studies have demonstrated associations between levels of TGF-β in breast milk and protection from eczema and wheeze in the infant (189, 190). In a mouse model, tolerance induction required neonatal exposure to breast milk containing a combination of TGF-β and allergen (181).
Figure 1.2: Proposed mechanism for tolerance induction in neonates. Breast milk provides dietary allergen in combination with immune factors which drive the development of tolerance when the gut barrier is adequately functioning. The controlled passage of allergen and sIgA across the epithelial barrier in the presence of retinoic acid (RA) results in the migration of DC’s to the MLN where Tregs are produced. When the gut barrier is compromised, or vital immunoregulatory factors are not present, oral tolerance is disrupted.
1.12 Allergen exposure in the prevention of food allergy: transforming guidelines

The original strategy for the prevention of allergy was the avoidance of potential allergens. This strategy was applied to both inhaled and food allergens and included avoidance in pregnancy, lactation and extended periods of early childhood. In the context of food allergy, from these early guidelines, researchers conducted intervention studies in the late 1980’s and 1990’s investigating the elimination of allergens from the maternal diet during pregnancy on food allergy outcomes in infants with conflicting and inconclusive results (Section 1.8). Despite this, from 2000, allergy prevention guidelines from the American Academy of Pediatrics (191) recommended that infants born into families with a history of allergic disease should avoid the major food allergens (peanut, egg, fish, wheat and dairy) whilst breastfeeding and may benefit from late introduction of eggs (after 2 years of age), peanuts, nuts and fish (after 3 years of age). As a consequence, there was a progressive trend towards allergen phobia. As a result, avoidance of allergenic foods while lactating and delayed introduction of allergenic foods into the weaning diet became commonplace, but despite this, food allergies have continued to rise (1).

In the decade that followed, observational studies began to indicate that this practice of delaying the introduction of allergenic foods (oats, wheat, dairy, fish and egg) into the infant diet beyond 6-10 months was associated with an increased risk for IgE sensitisation (192-194); and eczema (192-197). Collectively these observations lead to revisions to the allergy prevention recommendations in 2008 (198). Expert committees now agree there is no benefit to the avoidance of allergenic foods in pregnancy or lactation (198), or to delay the introduction of allergenic foods into the infant diet (198-200). After 2008, other observational studies have reinforced this stance (201, 202), including the Australian HealthNUTS study which demonstrated delayed introduction of hen’s egg beyond 10 months of age was associated with an increased risk for egg allergy, compared with earlier introduction at 4-6 months of age (adjusted OR, 1.6, 95% CI, 1.0-2.6) (202). However it was recognised that the level of evidence
in this area was generally weak, based largely on observational studies with methodological limitations.

Over the past few years several randomised control trials have been conducted to determine whether the age of introduction of food allergens into the infant diet reduces the risk of food allergy (Section 1.13). The results from these recent studies have lead to the release of the latest set of infant feeding guidelines in Australia which recommends that solids should be introduced “around 6 months but not before 4 months”, including the introduction of allergenic foods in the first year of life (Appendix H). Unfortunately research investigating the role of breast milk in food allergy development has not progressed since the last guidelines were issued, and thus is still based on the lack of evidence that avoidance of allergenic foods during lactation reduces the incidence food allergy.

1.13 Inducing tolerance using regular food allergen exposure for the prevention of food allergies

Table 1.3 summarises the RCTs, which have been investigating the role of regular introduction of allergenic foods (such as egg and peanut) during infancy for the prevention of food allergy, and also spells out all associated acronyms. These trials test the hypothesis that regular exposure to food allergens in infancy will induce oral tolerance, and reduce the risk of food allergy development. The majority of these RCT’s investigate the timing of introduction to one specific food allergen, either hen’s egg (STAR (83), STEP (85), BEAT (86), and HEAP (87) ) or peanut (LEAP (84)). The enquiring about tolerance (EAT) study in the United Kingdom (203) is unique as it investigated the intervention of exclusive breastfeeding until 3 months of age then sequential introduction of cow’s milk, egg, fish, wheat, sesame and peanut from 3 months of age compared to exclusive breastfeeding until around 6 months of age.
These RCTs have investigated whether the timing of introduction of food allergens in different ‘at risk’ populations of infants influences the development of food allergy. High-risk infants with eczema were targeted in STAR, LEAP and PEAP compared to intermediate risk (family history of allergic disease) infants in STEP and BEAT and general population in HEAP and EAT. Additionally some of the trials (BEAT, HEAP and LEAP) excluded infants based on positive skin prick test sensitisation prior to randomisation compared to other trials which did not use sensitisation as an exclusion criteria (STAR and STEP). All of these studies (except BEAT) included the gold standard method of oral food challenge for the diagnosis of an IgE mediated food allergy.

The early introduction of egg has been a large focus in both Australia and Germany however none of the available studies in varied populations demonstrate a benefit for the early introduction of egg to reduce egg allergy. Two studies (STAR (83) and STEP (85)) in infants at risk for allergy, both trend towards the early introduction of egg (around 6 months) opposed to later introduction (around 10 months). In these studies a lower proportion of infants in the early introduction group have confirmed egg allergy at 12 months of age (STAR early: 33% vs control: 51%; STEP early: 7.0% vs control 10.3%) however neither reach significance (P=0.11 and P=0.20 respectively). Contrary to STEP and STAR, The HEAP study included infants from the general population and showed an opposite trend, with 2.1% of infants in the early introduction group confirmed to have egg allergy compared to 0.6% in the control group (relative risk 3.3; 95% CI, 0.35-31.32; P=0.35) (87). Egg sensitisation (with or without the presence of clinical symptoms) was also examined in these studies with varied results. The STAR, STEP and HEAP studies show no significant difference in egg-specific IgE between groups at 12 months of age. Alternatively the BEAT study demonstrates a significant decrease in egg-specific IgE in the early introduction group compared to the control group (86).

Whilst the individual studies do not suggest a significant benefit for the early introduction of egg to reduce the risk for egg allergy, due to difficulties with recruitment many of these studies
did not reach their original target sample size (83, 85, 204). A recent meta-analysis examined all available evidence from published trials available and deemed there to be a reduced risk of egg allergy with the early introduction of egg (205). It should be noted that this meta analysis was published before one of the large egg studies (the STEP study(85)), however the results from STEP also trend towards the earlier introduction and thus would further substantiate the results found in this meta-analysis.

As discussed previously (in Section 1.6), high rates of egg-sensitisation at 4 months in the STAR study lead to a large number of adverse reactions 31% (15/49) to the egg intervention powder (including anaphylaxis). Other studies screened infants for egg sensitisation prior to enrolment including (LEAP(84), HEAP(87) and BEAT(86)), and also saw severe allergic reactions at study entry. In the HEAP study, there was 11 cases of anaphylaxis out of a total 23 infants who were challenged at study entry due to positive egg-specific IgE results. Despite the children in the HEAP study being low risk (general population versus infants with eczema) severe reactions were noted with first ingestion. This may be due to the pasteurized raw egg powder that was used in these studies, which is the most allergenic form of egg (206). It is possible there would have been a very different result had these studies used well-cooked or baked forms of egg which may be well tolerated in the first year of life (207).

More recently published is the LEAP study by Du Toit et al, (84) examining regular introduction of peanut in infants with eczema and/or egg allergy. This is a large scale, RCT that included 640 infants randomized between 4 and 11 months of age to either peanut ingestion or peanut avoidance, with the primary outcome being IgE mediated peanut allergy at 5 years of age. Unlike the STAR trial, this study had two arms separating infants based on peanut sensitisation status at randomisation, and thus included both the primary prevention of peanut allergy (non peanut-sensitised infants) and the secondary prevention of peanut allergy (peanut-sensitised infants, SPT <4mm). At 5 years of age, infants in the primary prevention group who were regularly ingesting peanuts (>3 times per week) had a significantly lower prevalence of
peanut allergy (1.9%) than infants in the avoidance group (13.7%) (P<0.001). The same was true for the infants in the secondary prevention arm, with 10.6% prevalence of peanut allergy in the consumption group and 35.3% in the avoidance group. These results suggest that regular peanut allergen exposure commencing in infancy will prevent peanut allergy in a significant proportion of children.

The EAT trial differed from both the STAR and LEAP studies by enrolling infants from the general population (n=1303) (203). Additionally the intervention did not include the administration of a study product, but instead feeding guidelines which either specified to start the introduction of allergenic foods (peanut, cooked egg, cow’s milk, sesame, whitefish and wheat), or to follow the standard advice in the United Kingdom of exclusive breast-feeding until 6 months of age, followed by the introduction of solid foods. In the EAT study there were no significant differences between the two groups, with 31/567 (5.6%) infants in the intervention group developing a food allergy compared to 39/595 (7.1%) in the standard introduction control group (P=0.32). The per protocol analysis did show a significant difference between the two intervention groups (P=0.01), however this result must be interpreted with caution as only 31.9% of those families allocated to the early introduction group actually adhered to the protocol. This highlights the potential difficulties in attempting to introduce multiple allergenic foods in young infants prior to six months of age (208).

Thus while the results from these three trials vary, collectively they favour the introduction of allergenic foods during infancy and there does not appear to be any benefit to delayed introduction. However, as there are some infants who are already sensitised to food allergens prior to introduction in solid foods, prevention strategies earlier in the antenatal and/or postnatal period may also be required.
Table 1.3: Randomised controlled trials on food allergen exposure in infancy to prevent food allergy

<table>
<thead>
<tr>
<th>Study Acronym</th>
<th>Full title</th>
<th>Study type (sample size)</th>
<th>Trial registration details</th>
<th>Population characteristics</th>
<th>Intervention</th>
<th>Comparison</th>
<th>Primary outcome</th>
</tr>
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<tbody>
<tr>
<td><strong>STAR (83)</strong> Adelaide &amp; Perth, (Australia)</td>
<td>Solids Timing for Allergy Reduction</td>
<td>Blinded, RCT (n=86) EGG</td>
<td>ACTRN 12609000415202 Date of trial registration 5/06/2009</td>
<td>High risk – infants with moderate to severe eczema</td>
<td>Daily consumption of raw egg powder from 4 months until 8 months of age. Cooked egg introduced from 8 months of age.</td>
<td>Daily consumption of placebo, rice powder from 4 months until 8 months of age. Cooked egg introduced from 8 months of age.</td>
<td>IgE mediated egg allergy at 12 months of age based on positive SPT and egg challenge</td>
</tr>
<tr>
<td><strong>STEP (Palmer, 2016 #833)</strong> Adelaide &amp; Perth (Australia)</td>
<td>Starting Timing for Egg Protein</td>
<td>Blinded, RCT (n=820) EGG</td>
<td>ACTRN 12610000388011 Date of trial registration 13/05/2010</td>
<td>Intermediate risk – maternal history of allergy + positive maternal SPT - no allergic disease in infant</td>
<td>Daily consumption of raw egg powder from 4-6.5 months until 10 months of age. Egg introduced from 10 months of age.</td>
<td>Daily consumption of placebo, image-matched rice powder from 4-6.5 months until 10 months of age. Egg introduced from 10 months of age.</td>
<td>IgE mediated egg allergy at 12 months of age based on positive SPT and egg challenge</td>
</tr>
<tr>
<td><strong>BEAT (Wei-Liang Tan, 2016 #834)</strong> Sydney (Australia)</td>
<td>Beating Egg Allergy Trial</td>
<td>Blinded, RCT (n=600) EGG</td>
<td>ACTRN 12611000535976 Date of trial registration 24/05/2011</td>
<td>Intermediate risk – first degree relative with atopy - no infant IgE sensitisation to egg at 4 m of age</td>
<td>Daily consumption of raw egg protein (350mg) from 4-6 m until 8 m of age, with egg free diet until 12 m then introduction of ‘normal unrestricted diet’ from 8 m.</td>
<td>Daily consumption of placebo rice powder from 4-6m until 12m of age, with egg free diet until 8 m then introduction of ‘normal unrestricted diet’ from 8 months of age.</td>
<td>IgE sensitisation to egg measured by SPT at 8 and 12 months of age.</td>
</tr>
<tr>
<td>(Japan)</td>
<td>Prevention of egg allergy in infants with atopic dermatitis</td>
<td>Blinded, RCT (n=200) EGG</td>
<td>JPRN-UMIN000008673 Date of trial registration 10/08/2012</td>
<td>High risk – infants with eczema</td>
<td>Daily consumption from 6 months of age of 50mg egg powder, then increased up to 250mg after 3 months.</td>
<td>Daily consumption from 6 months of age of placebo pumpkin powder.</td>
<td>Negative egg food challenge at one year old.</td>
</tr>
<tr>
<td>Study Acronym</td>
<td>Full title</td>
<td>Study type (sample size) &amp; Allergen</td>
<td>Trial registration details</td>
<td>Population characteristics</td>
<td>Intervention</td>
<td>Comparison</td>
<td>Primary outcome</td>
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<tr>
<td>EAT</td>
<td>Enquiring About Tolerance Study</td>
<td>(n=1303) cow’s milk, egg, wheat, sesame, fish and peanut</td>
<td>ISRCTN 14254740 Date of trial registration 31/03/2009</td>
<td>Normal risk – general population</td>
<td>Exclusive breastfeeding until 3 months of age then sequential introduction of cow’s milk, egg, wheat, sesame, fish and peanut from 3 months of age.</td>
<td>Exclusive breastfeeding until around 6 months of age and no early introduction of allergenic foods before 6 months of age.</td>
<td>Prevalence of IgE mediated food allergy to any of the 6 foods between 1 and 3 years of age, as defined by food challenge.</td>
</tr>
<tr>
<td>LEAP</td>
<td>Learning Early About Peanut allergy</td>
<td>RCT (n=640) PEANUT</td>
<td>NCT00329784 Date of trial registration 23/05/2006</td>
<td>High risk – infant with eczema and/or egg allergy</td>
<td>Peanut consumption in the form of an age appropriate peanut snack commencing from 4-11 months of age.</td>
<td>Peanut avoidance</td>
<td>Prevalence of clinically-defined peanut allergy at 5 years of age.</td>
</tr>
<tr>
<td>HEAP</td>
<td>Hens Egg Allergy Prevention Study</td>
<td>RCT (n=800) EGG</td>
<td>DRKS-ID: DRKS00005668 Date of trial Registration 28/01/2014(retrospective entry)</td>
<td>Normal risk – general population</td>
<td>2.5g raw egg protein 3 times a week starting from 4 (weaning solids) -12 months</td>
<td>Placebo (rice powder) 3 times a week from 4 (weaning solids) -12 months.</td>
<td>IgE mediated egg allergy at 12 months of age based on egg-specific IgE and double-blinded placebo-controlled food challenge.</td>
</tr>
<tr>
<td>PEAP</td>
<td>Prevention of peanut allergy by early consumption – a prospective study</td>
<td>Non-randomized self-allocated controlled trial (n=460) PEANUT</td>
<td>DRKS-ID DRKS00005487 Date of trial registration 25/03/2014 (retrospective entry)</td>
<td>High risk– eczema without peanut sensitisation</td>
<td>Peanut snack 3 times a week from 5-30 months</td>
<td>Peanut avoidance</td>
<td>IgE-mediated peanut allergy after one year of consumption or avoidance</td>
</tr>
</tbody>
</table>
1.14 Immunological changes with regular allergen exposure

Oral immunotherapy studies (OIT) provide a platform to examine underlying immunological processes involved with desensitisation to food allergens in humans. Most OIT studies have examined changes in specific antibody levels (IgE, IgG4) in response to regular allergen exposure. The response of IgE in response to OIT treatment is somewhat conflicting. Some studies observed a significant decrease in specific IgE levels after OIT (159, 161, 209, 210), however the same pattern has also been seen in control group children, or in children that developed natural tolerance on an elimination diet (161). Two studies also report no change in IgE levels in the OIT group from baseline, or when compared to the control group (211, 212). In contrast, allergen-specific IgG4 has been a useful marker for the development of immune desensitisation, without necessarily any sustained decline in IgE levels (213).

Changes in allergen-specific IgG4 are more consistent across OIT trials, with most showing significantly increased levels after OIT (158, 159, 212). This is consistent with the notion that high dose antigen exposure results in the production of IgG4. Skripak et al (212) saw an increase in the median cow’s milk specific IgG4 of 767% in the treatment group compared to no change in the placebo group. Perhaps of greater significance Burks et al (158) established that IgG4 levels were not only associated with allergen exposure but were also significantly higher in participants who were desensitised to egg than those children who reacted at all challenges (P<0.02). The ratio of allergen specific IgE to IgG4 has also been investigated in egg allergy as the best predictor for clinical reactivity, and has been shown to be more effective than either IgE or IgG4 alone (214, 215), possibly due to their opposing roles.

The role of IgG4 in the prevention of allergy is still under investigation. However, results from both the STAR (83) and LEAP (84) studies demonstrate significantly elevated levels of allergen-specific IgG4 in the infants who were regularly ingesting the allergen compared to the allergen avoidance groups. In the STAR study (83), the intervention group ate egg daily from
four to eight months resulting in significantly higher IgG4 levels at eight months, but this effect remained significant at twelve months (P<0.001 at both time points) despite infants in both groups introducing egg into the diet at 8 months. Although speculative, this suggests that the later introduction of egg at eight months was not as effective at inducing egg-specific IgG4 as egg introduced at 4 months, however further research is needed for conclusive evidence. The highly allergenic raw egg powder that was administered as the intervention from 4-8 months, in contrast to the baked and well-cooked egg introduced at 8 months of age, may also have contributed to this effect. While IgE is a long known marker for IgE mediated food allergy, IgG4 is providing a hypothesis which could explain discrepancies between allergen sensitisation and clinical food allergy, and thus immune mechanisms underlying oral tolerance remains an area of interest.

The mechanistic role of IgG4 in tolerance induction is still vague, however is produced in response to IL-10 (216-219), secreted by regulatory B cells (220). Santos demonstrated the protective effect of IgG4 in food allergy, whereby plasma from peanut sensitised patients containing specific-IgG4, inhibited basophil and mast cell activation in vitro (221). This “blocking” effect of IgG4 was also seen in grass pollen immunotherapy, where IgG4 was shown to inhibit the formation of immune-complexes preventing complement activation (222).

Antigen specific T cell responses in response to OIT treatment are much less defined. It has been suggested that desensitisation from OIT is mediated via the suppression of Th2 cytokine responses (IL-4, IL-5, IL-13), and an increase in regulatory cytokines (IL-10 and IFN-γ), which is mediated by FOXP3+ Treg cells (223, 224). However, in peanut allergic children undergoing OIT, those children who maintained high IgE levels throughout treatment, Th2 reactivity to peanut persisted despite clinical desensitisation and an increase in specific IgG4 (225). An important consideration for this thesis is that these immune processes, which reverse the allergic immune response back towards a tolerant state, may be different to the natural course of tolerance induction, which occurs in healthy (non-allergic) individuals.
The ultimate goal in OIT studies and primary prevention studies is to establish sustained and lasting oral tolerance. Which, by definition, is sustained unresponsiveness to an allergen, even after long periods of allergen avoidance. As it stands the leap from food desensitisation to sustained oral tolerance is still a grey area in OIT or for allergy prevention, and continues to be an area of interest (162). The LEAP study is the only prevention study to investigate sustained desensitisation after a 12 month period of peanut avoidance. In a follow-up study Du Toit demonstrated that there was no significant increase in peanut allergy in the peanut consumption group after 12 months of peanut avoidance, whereby infants in this group maintained lower rates of Arah h2 IgE sensitisation and higher levels of peanut specific IgG4 than the avoidance group (226). Thus as the long-term efficacy for the treatment of food allergy remains largely unknown, and evidence of successful prevention strategies emerging, identification of prevention strategies which induce oral tolerance to food proteins in early life remains a priority.

1.15 Conclusions and future directions

With infant food sensitisation occurring early in infancy there is a need to understand the events that promote oral tolerance in the early postnatal period. The development of oral tolerance appears to be an allergen driven process, dependent on oral ingestion of relevant food proteins to induce regulated T cell mediated suppression of potential inflammatory responses to what may be otherwise perceived as foreign substances (227). However, potentially adverse conditions in the local environment (i.e relative deficits of immunomodulatory factors and increased exposure to pro-inflammatory agents) may be contributing to the ‘perfect storm’ that disrupts normal tolerance development mechanisms. To promote oral tolerance we require a better understanding of both how to optimise the conditions during allergen encounter, and how (and when) to optimise allergen exposure, which is the subject of this thesis.
In this regard, it is clear that allergen exposure is essential in both the primary induction of tolerance, and can also induce secondary tolerance in scenarios where sensitisation has already occurred. Numerous studies now demonstrate that controlled oral allergen exposure can achieve oral desensitisation in children with established food allergy. The immune mechanisms behind this are still not well defined, however oral tolerance has been consistently associated with increasing levels of specific-IgG4. In the prevention of food allergy, existing research has focused on the timing that allergenic foods are introduced into the infant diet (83, 84, 203). However, high rates of food specific sensitisation and clinical reactivity prior to the introduction of solid foods in these studies indicates immune dysregulation following allergen encounter earlier in life, however, allergen specific T cell responses around the introduction of allergenic foods into the diet, and with regular allergen exposure have yet to be fully explored.

Potential routes of allergen exposure in the early postnatal period are via impaired epithelial barrier and breast milk, where the route of exposure may have important consequences as to whether sensitisation or oral tolerance to food allergens is achieved. Very few studies have addressed infant allergen exposure during this period before solid foods introduction, and thus this is the focus of this thesis. With the notion that interventions starting with the introduction of solid foods are too late in a proportion of infants, alternate routes of allergen exposure earlier in the postnatal period will be explored. As animal studies suggest breast milk may play an integral role in facilitating oral tolerance induction in the early postnatal period by providing oral food allergen exposure in a tolerogenic milieu, there is a need to investigate these concepts in human neonates. Thus, this thesis will examine whether maternal dietary ingestion of foods during lactation can manipulate allergen exposure for breast fed infants, and whether this exposure influences the development of markers for sensitisation or tolerance in the infant.
Chapter 2  Aims and hypotheses
This thesis sought to investigate egg allergen exposure in the postnatal period to identify strategies for the primary prevention of egg allergy in infants at high risk of developing allergic disease. This PhD project was designed to assess the development of egg-specific immune responses both prior to and after the introduction of egg in solid foods, and to examine possible routes of egg exposure before the introduction of solid foods.

To address these aims, two study populations have been included in this thesis (Figure 2.1). The STAR trial clinical outcomes paper was previously published (Appendix A), and thus only aspects relating to the immune function analysis of this trial form part of the work undertaken in this thesis.

2.1 Aims

The primary aims of this thesis were to:

1. Investigate early immune dysregulation in infants prior to the introduction of egg in solids and whether these early patterns of immune responses can predict egg allergy later in infancy (STAR Study, Chapter 3).
2. Examine breast milk as a possible route of oral egg exposure prior to the introduction of solid foods, to create novel insight into the prevention of egg allergy through oral tolerance induction in the early postnatal period (QuEST Study, Chapters 4 and 5).

Specifically in relation to egg allergy prediction prior to solids introduction we aimed to:

1. Determine whether early egg-specific immune responses predict the presence of IgE mediated egg allergy later in infancy, in order to identify early life bio-markers involved in the development of egg allergy.
2. Examine egg-specific Th2 immune responses to a panel of four egg allergens, to examine which egg proteins are implicated in egg allergy.
3. Examine whether early introduction of egg at four months of age, versus a later introduction at 8 months, had any effect on egg specific Th2 immune responses at 12 months of age.

Specifically in relation to *egg allergy prevention* prior to introduction of solid foods we aimed to:

1. Determine the effect of maternal egg ingestion during early lactation on the egg protein (ovalbumin, OVA) concentration of human milk over the first six weeks of lactation, ultimately to examine egg allergen exposure in breastfed infants.
2. Investigate the impact of egg exposure via breast milk on infant egg-specific immune markers for sensitization and tolerance (IgE and IgG4 respectively).
3. Investigate the role of skin barrier function in the development of infant sensitisation and eczema in the early postnatal period. Additionally to examine the relationship between maternal and infant skin barrier function.

### 2.2 Hypothesis

We hypothesized in the context of allergy prediction:

1. Identification of egg proteins capable of eliciting inflammatory immune responses would be a useful tool to further characterize different phenotypes of egg allergy.
2. Early egg-specific immune programming will have lasting effects on the trajectory of egg allergy.

We hypothesized in the context of egg allergy prevention:

1. Maternal diet would influence the OVA concentration of breast milk, and therefore infant egg exposure in the early postnatal period could be manipulated through maternal dietary ingestion.
2. Infant egg exposure via breast milk would not result in increased levels of IgE, and may result in increased levels of the tolerance marker egg-specific IgG4.

3. Infant TEWL would relate to the presence of infant eczema and increased levels of IgE sensitization prior to the introduction of solids.

2.3 Study populations included in this thesis

The work presented in this thesis is comprised from the following two studies. How these studies fit together is illustrated in (Figure 2.1)

2.3.1 STAR (Solids Timing for Allergy Reduction) Study

A double-blind randomized controlled trial designed to assess the effect of early, regular egg exposure in solid foods in the prevention of egg allergy in infants with eczema. Infants with moderate to severe eczema, who had never ingested egg in solid foods were recruited at four months of age. Blood samples were collected at four and twelve months of age for egg-specific immune function analysis (both prior to and after the ingestion of egg in solid foods) alongside clinical allergy and eczema assessments. Blood samples from this study were used to examine whether early life egg-specific immune responses could predict egg allergy at one year of age, and whether early versus late egg introduction influences egg-specific immune responses (Chapter 3).

2.3.2 The QuEST Study (Questioning the role of Egg in lactation in the induction of Specific Tolerance)

A randomized controlled trial designed to assess the effect of maternal dietary egg ingestion on breast milk OVA levels over the first six weeks of lactation. Women were allocated to one of three dietary egg ingestion groups, and breast milk samples were taken at two, four and six weeks of lactation for the measurement of egg protein (OVA). Blood samples were taken at six
weeks alongside the breast milk sample and again at 16 weeks of age prior to the introduction of solid foods. This trial aimed to investigate whether egg exposure in breastfed infants can be modified through maternal egg ingestion (Chapter 4), and how maternal egg ingestion influences egg-specific infant sensitisation and tolerance markers (IgE and IgG4 respectively, Chapter 5).
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To address these aims, two study populations have been included in this thesis (Figure 2.1). The STAR trial clinical outcomes paper was previously published (Appendix A), and thus only aspects relating to the immune function analysis of this trial form part of the work undertaken in this thesis.

2.1 Aims

The primary aims of this thesis were to:

1. Investigate early immune dysregulation in infants prior to the introduction of egg in solids and whether these early patterns of immune responses can predict egg allergy later in infancy (STAR Study, Chapter 3).

2. Examine breast milk as a possible route of oral egg exposure prior to the introduction of solid foods, to create novel insight into the prevention of egg allergy through oral tolerance induction in the early postnatal period (QuEST Study, Chapters 4 and 5).

Specifically in relation to egg allergy prediction prior to solids introduction we aimed to:

1. Determine whether early egg-specific immune responses predict the presence of IgE mediated egg allergy later in infancy, in order to identify early life bio-markers involved in the development of egg allergy.

2. Examine egg-specific Th2 immune responses to a panel of four egg allergens, to examine which egg proteins are implicated in egg allergy.
3. Examine whether early introduction of egg at four months of age, versus a later introduction at 8 months, had any effect on egg specific Th2 immune responses at 12 months of age.

Specifically in relation to egg allergy prevention prior to introduction of solid foods we aimed to:

1. Determine the effect of maternal egg ingestion during early lactation on the egg protein (ovalbumin, OVA) concentration of human milk over the first six weeks of lactation, ultimately to examine egg allergen exposure in breastfed infants.
2. Investigate the impact of egg exposure via breast milk on infant egg-specific immune markers for sensitization and tolerance (IgE and IgG4 respectively).
3. Investigate the role of skin barrier function in the development of infant sensitisation and eczema in the early postnatal period. Additionally to examine the relationship between maternal and infant skin barrier function.

2.2 Hypothesis

We hypothesized in the context of allergy prediction:

1. Identification of egg proteins capable of eliciting inflammatory immune responses would be a useful tool to further characterize different phenotypes of egg allergy.
2. Early egg-specific immune programming will have lasting effects on the trajectory of egg allergy.

We hypothesized in the context of egg allergy prevention:

1. Maternal diet would influence the OVA concentration of breast milk, and therefore infant egg exposure in the early postnatal period could be manipulated through maternal dietary ingestion.
2. Infant egg exposure via breast milk would not result in increased levels of IgE, and may result in increased levels of the tolerance marker egg-specific IgG4.

3. Infant TEWL would relate to the presence of infant eczema and increased levels of IgE sensitization prior to the introduction of solids.

2.3 Study populations included in this thesis

The work presented in this thesis is comprised from the following two studies. How these studies fit together is illustrated in (Figure 2.1)

2.3.1 STAR (Solids Timing for Allergy Reduction) Study

A double-blind randomized controlled trial designed to assess the effect of early, regular egg exposure in solid foods in the prevention of egg allergy in infants with eczema. Infants with moderate to severe eczema, who had never ingested egg in solid foods were recruited at four months of age. Blood samples were collected at four and twelve months of age for egg-specific immune function analysis (both prior to and after the ingestion of egg in solid foods) alongside clinical allergy and eczema assessments. Blood samples from this study were used to examine whether early life egg-specific immune responses could predict egg allergy at one year of age, and whether early versus late egg introduction influences egg-specific immune responses (Chapter 3),

2.3.2 The QuEST Study (Questioning the role of Egg in lactation in the induction of Specific Tolerance)

A randomized controlled trial designed to assess the effect of maternal dietary egg ingestion on breast milk OVA levels over the first six weeks of lactation. Women were allocated to one of three dietary egg ingestion groups, and breast milk samples were taken at two, four and six weeks of lactation for the measurement of egg protein (OVA). Blood samples were taken at six
weeks alongside the breast milk sample and again at 16 weeks of age prior to the introduction of solid foods. This trial aimed to investigate whether egg exposure in breastfed infants can be modified through maternal egg ingestion (Chapter 4), and how maternal egg ingestion influences egg-specific infant sensitisation and tolerance markers (IgE and IgG4 respectively, Chapter 5).
Figure 2.1: Timeline and summary of the two randomised controlled trials included in this thesis
Chapter 3

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

Jessica R. Metcalfe, Nina D’Vaz, Maria Makrides, Michael S. Gold, Patrick Quinn, Christina E. West, Richard Loh, Susan L. Prescott, Debra J. Palmer
3.1 Introduction

Hen’s egg is one of the most common food allergens to induce Th2 allergic immune responses in young infants (57, 228, 229). IgE-mediated allergic reactions can occur early in infancy (83), often on first ingestion of egg in solid foods (82, 83, 230). 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 (83). 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 (231). 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 preceding 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 ovalbumumin as the most abundant protein in hen’s egg (49, 74, 75), 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 (65).
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.

3.2 Materials and methods

3.2.1 Subjects

The study population comprised a subset of infants who participated in a 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 informed parental consent was obtained from all participants. Full details of the RCT have been previously published (83). Briefly, infants with moderate to severe eczema determined using a standardized Scoring Atopic Dermatitis (SCORAD) (232) score of ≥ 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(83). The subset of RCT participants included in this study was determined by availability of sufficient blood volume collected for cell culture analysis.

3.2.2 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°C for a standardized controlled-rate of -1°C/minute cell freezing. Within 24 hours of freezing, PBMC’s were transferred to liquid nitrogen for long-term storage.

3.2.3 Mononuclear cell culture

PBMC cell culture was conducted using the methods detailed previously (49, 233). Briefly, cryopreserved mononuclear cells were thawed and transferred to RPMI culture media. Cells were counted, viability tested using trypan 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) OVA (100μg/ml), b) OM (1mg/ml), c) CON (200μg/ml) and d) LYS (500μg/ml), were 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% CO2 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.

3.2.4 Cytokine measurements

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 describ(49). 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.

3.2.5 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(83). 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(83). 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 sensitisation (positive skin prick test) to egg, or medical advice not to proceed with the challenge due to a previous serious allergic reaction to egg.
3.2.6 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.).

3.3 Results

3.3.1 Study Population

This study included 68 infants from the clinical trial who had blood samples available for cytokine analysis, as illustrated in Figure 3.1. The baseline characteristics of this subset, shown in Table 3.1 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.

3.3.2 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 3.2). There were also no differences between groups for IL-10, IFNγ or TNFα responses. (Data in Appendix D, Table 2).
Table 3.1: Baseline characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Present Study (n=68)</th>
<th>Original RCT (n=86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age at birth (years) *</td>
<td>32.7 (5.0)</td>
<td>32.5 (4.7)</td>
</tr>
<tr>
<td>Maternal Caucasian race ^</td>
<td>52 (76.5%)</td>
<td>68 (79.1%)</td>
</tr>
<tr>
<td>Caesarean-section birth ^</td>
<td>24 (35.3%)</td>
<td>28 (32.6%)</td>
</tr>
<tr>
<td>1st degree relative history of allergic disease ^</td>
<td>61 (89.7%)</td>
<td>79 (91.9%)</td>
</tr>
<tr>
<td>Infant male sex ^</td>
<td>46 (67.6%)</td>
<td>57 (66.3%)</td>
</tr>
<tr>
<td>Age of onset of eczema (months) *</td>
<td>1.8 (1.0)</td>
<td>1.8 (1.0)</td>
</tr>
<tr>
<td>Eczema severity (objective SCORAD score) 6</td>
<td>12.6 (4.6-25.8)</td>
<td>15.2 (7.2-26.53)</td>
</tr>
<tr>
<td>Use of prescription steroid cream ^</td>
<td>54 (79.4%)</td>
<td>68 (79.1%)</td>
</tr>
<tr>
<td>Ever breastfed ^</td>
<td>67 (98.5%)</td>
<td>85 (98.8%)</td>
</tr>
<tr>
<td>Breastfed at randomisation ^</td>
<td>54 (79.4%)</td>
<td>71 (82.6%)</td>
</tr>
<tr>
<td>Smoking in the household ^</td>
<td>8 (11.8%)</td>
<td>11 (12.8%)</td>
</tr>
</tbody>
</table>

Table 3.2: Baseline cytokine responses at 4 months of age. *IL-13 and IL-5 responses (pg.ml) per intervention group prior to the introduction of egg in solid foods*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Egg protein</th>
<th>Egg (n=22)</th>
<th>Control (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>OVA</td>
<td>215.9 (118.0-511.3)</td>
<td>202.4 (109.4-556.1)</td>
<td>0.819</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>226.1 (110.5-578.0)</td>
<td>220.3 (54.7-399.0)</td>
<td>0.299</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>152.3 (75.6-395.2)</td>
<td>73.1 (28.5-513.1)</td>
<td>0.585</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>198.6 (99.2-748.6)</td>
<td>395.8 (161.2-676.5)</td>
<td>0.528</td>
</tr>
<tr>
<td>IL-5</td>
<td>OVA</td>
<td>7.33 (1.4-51.0)</td>
<td>14.3 (1.0-40.6)</td>
<td>0.717</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>10.6 (1.3-43.6)</td>
<td>2.42 (1.0-25.5)</td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>2.42 (0.9-13.3)</td>
<td>2.42 (1.0-16.2)</td>
<td>0.644</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>15.4 (1.7-60.9)</td>
<td>22.0 (5.8-98.8)</td>
<td>0.377</td>
</tr>
</tbody>
</table>
Figure 3.1: CONSORT diagram for participants in this study

Potential participants initially screened (n=243)

Excluded (n=157)
- Not eligible (n=109)
- Did not consent (n=32)
- Lost to follow up (n=16)

Infants consented (n=86)

Infants Randomised (n=86)

Assigned to the egg (active) intervention (n=49)
Pre-randomisation blood sample available for cytokine analysis (n=22)

Discontinued intervention (n=15)
- Parent withdrew consent (n=7)

12 month of age assessment
Primary outcome (IgE sensitisation status + egg challenge) completed (n=35)
12 month of age blood sample available for cytokine analysis (n=33)

Assigned to the rice (placebo) intervention (n=37)
Pre-randomisation blood sample available for cytokine analysis (n=18)

Discontinued intervention (n=4)
- Parent withdrew consent (n=2)

12 month of age assessment
Primary outcome (IgE sensitisation status + egg challenge) completed (n=32)
12 month of age blood sample available for cytokine analysis (n=25)
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 3.2). 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 Appendix D, Table D.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 in Appendix table D.2. 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).

Figure 3.2: 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).
3.3.3 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 3.3). No differences between the groups were also found for IL-10, IFNγ or TNFα responses, data in Appendix D, Table D.3.

Table 3.3 Cytokine responses at 12 months of age. IL-13 and IL-5 responses (pg/ml) per intervention group.

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

3.3.4 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 3.3). There were no associations between IL-10, TNFα and IFNγ responses with IgE-mediated egg allergy, data in supplementary tables Appendix D.
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).

![Cytokine responses at 4 months of age](image1)

(a) Cytokine responses at 4 months of age

![Cytokine responses at 12 months of age](image2)

(b) Cytokine responses at 12 months of age

Figure 3.3: 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).

3.3.5 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 3.4). 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 3.4).

3.3.6 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 3.3). There were no significant differences for
any other egg allergen for cytokines IL-5 and IL-13 (Figure 3.3). Again there were no differences for IL-10, IFN\(\gamma\) or TNF\(\alpha\) for any of the egg allergens, data in Appendix D. At 12 months of age, LYS IL-13 and IL-5 predicted egg allergy status at that time point (\(\beta=0.4\); 95%CI 1.0-2.4; \(P=0.039\)) and (\(\beta=0.3\); 95%CI 1.0-1.8; \(P=0.05\)) respectively.

3.3.7 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(83). 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.

3.4 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 (88), and have been detected in breast milk (128), providing potential avenues of exposure. Transcutaneous exposure also may be a particularly important route of exposure in children with moderate to severe eczema (231). In addition to impaired skin barrier function, children with eczema also show evidence of increased gut mucosal permeability (28, 234), 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 recognised 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 (158). 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 (83). 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 (235). Whilst egg allergic children 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%) (236), up to 35% of egg allergic patients have been shown to produce LYS specific-IgE (237). 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 (57), 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.
Chapter 4

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

4.1 Introduction

Our previous studies presented in **Chapter 3** have shown that the processes leading to egg sensitisation are already strongly established in many high risk infants in the first months of life, before the introduction of solid foods (83, 238). Specifically, a significant proportion of 4 month old infants with eczema already had established egg sensitisation, and egg-specific Th2 cytokine responses prior to their ‘first’ introduction of egg, resulting in clinical reactivity (83). This implies that much earlier preventive interventions are ultimately needed to promote the development of tolerance to foods. 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.

Early oral exposure to allergens through the gut is critical for maintaining and reinforcing oral tolerance (147), and allergens secreted in breast milk provide an important source in the early postnatal period. In breastfed infants oral allergens are first encountered in the context of tolerogenic signals from maternal milk, including cytokines (187), and allergen-antibody complexes (239), thus variations in breast milk composition may influence the development of oral tolerance. Animal models are suggesting that allergens ingested in breast milk favour the development of oral tolerance in the offspring (110, 179). Although common food allergens have previously been detected in human breast milk; peanut (arah h1 and arah h2) (109, 110), cow’s milk (beta lactoglobulin), (113, 116, 117) and egg protein (ovalbumin) (88, 113, 116, 127-129), the role of breast milk derived food proteins in the development of oral tolerance for breast fed infants remains unknown.

The amount and type of maternal egg ingested has been previously shown to influence human milk egg protein (ovalbumin, OVA) concentrations within an eight hour period (128). There is
however limited understanding of the longer-term influences of dietary patterns including all forms of egg on allergen secretion in breast milk. Wide variability in OVA concentrations between women even after the consumption of the same amount and type of egg has been demonstrated (128), suggesting additional factors during digestion, absorption or excretion may influence the amount of food protein present in breast milk. While, increased permeability of the mammary epithelium has been correlated with an increased risk for atopic disease in infants with allergic mothers (130), no previous studies have examined whether mammary epithelium permeability affects the amount of food protein detected in human breast milk.

Before any new prevention strategies to potentially optimise food allergen delivery through breast milk can be considered, we firstly need a deeper understanding of factors that may influence the passage of food proteins into human milk. In this randomized controlled trial, I investigated the effect of a dietary intervention during the first six weeks of lactation, including standardised maternal egg ingestion (all forms of egg), and assess this in relation to OVA detection in human breast milk. I also examined whether permeability of the mammary epithelium is related to OVA breast milk concentrations.

4.2 Materials and Methods

4.2.1 Study Design

Pregnant women planning to breastfeed were initially screened for a history of medically diagnosed allergic disease (asthma, eczema, hay-fever or IgE mediated food allergy) at antenatal clinics and classes in Perth metropolitan area. 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 the University of Western Australia (RA/4/1/6115) and 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).

4.2.2 Randomisation and blinding

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. One of my supervisors not involved in the assessment of any outcome measurements prepared the randomisation 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.

4.2.3 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). Mothers allocated to the egg-free group were given dietary advice on avoiding egg and its derivatives, and an information sheet was provided (Appendix G).
4.2.4 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 (Appendix F). To accurately track egg consumption for a variety of foods, the diary card also contained a conversion table showing the amount of egg present in common egg containing foods. This was constructed by compiling recipes for egg containing foods (cake, quiche, meatballs, pancakes), whereby the average number of eggs ingested per standard serving of the food was calculated. Mother’s were told to use this as a guide if they were eating out or for pre-prepared meals, or to adjust it accordingly if they were making these foods at home. 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 a research nurse and myself 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 and recorded in the trial specific case report form (Appendix E).

4.2.5 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 both asked to consume the equivalent of one egg, between two and six hours prior to expressing their breast milk sample to optimise the detection of OVA (128), and to investigate the effects of long term patterns for dietary egg ingestion. Maternal blood samples were collected via venipuncture at six weeks of lactation, and serum was separated from 5 ml of whole blood. Both breast milk and serum samples were stored at -80 °C prior to analysis.
4.2.6 Detection of ovalbumin in serum and breast milk

I measured OVA concentration in serum and skim breast milk (fat was separated by centrifugation at 4000 g for 20 min at 4 °C) using 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 ELISA’s were analyzed using a MultiSKAN ELISA plate reader (ThermoFisher Scientific, NSW Australia), at a wavelength of 450 nm. SkanIt™ 2.5.1 Research Edition software (ThermoFisher Scientific, NSW Australia) was used to interpret the results. If any samples were above the detection limit of 4ng/ml, they were diluted and repeated to obtain fluorescence readings within range. Any samples that were below the lower limit of detection were assigned a value of one half the lowest limit of detection (0.05 ng/ml). All samples (breast milk from 2, 4, and 6 weeks of lactation and serum at 6 weeks) for each woman were run on the same plate. Additionally each plate contained a distribution of all three intervention groups (high egg, low egg and egg free). The samples were coded using unidentifiable numbers to ensure the researcher remained blinded throughout the detection assays.

4.2.7 Rationale for using OVA ELISA to measure egg protein in breast milk

I chose to measure OVA in breast milk as a measure of infant egg exposure as it is the most abundant allergen in egg making up 54% of total egg white protein (236). Numerous studies have documented its ability to induce inflammatory immune responses in infants (49, 74, 75), and it has been the chosen egg allergen for other trials investigating the detection of egg protein in breast milk (88, 128, 129). There are also now commercial kits available for the detection of hen’s egg ovalbumin, allowing these results to be more reproducible than using an in house method. OM is also now an allergen of interest due to its ability to withstand extensive heating, however as it only constitutes 11% of total egg white protein, and the levels of OVA detected in breast milk are very low (128, 129), there would have been an increased risk for OM allergen
levels to be below the detection sensitivity of our assay. As a result I decided to use OVA in breast milk as a measure of egg exposure in breastfed infants.

ELISA has been the consistently chosen method for the detection of food proteins in breast milk (Table 1.1). The ELISA method is highly specific down to trace amounts of protein, making it the optimal choice for the low levels of food allergen typically detected in breast milk (240). The commercial ELISA kits that I chose for OVA protein was specific to 0.1ng/ml, which is a similar sensitivity to ELISA’s previously designed in house(128, 129). The kits were checked for specificity using spiked breast milk, which had a mean recovery rate of 81.5% +/- 6%. This was considered as acceptable, as there is expected to be some blocking effects of the breast milk itself, and levels above expected would indicate non-specific binding was occurring. There are now other methods available for the detection of food allergens including mass spectrometry (241), however this methodology does not have the capacity to measure ovomucoid (due to protein stability), thus we would still only be able to measure OVA. This method has not yet been used in breast milk research, and hence it was not deemed beneficial to use this method over an ELISA at this time.

4.2.8 Breast permeability assay

The sodium: potassium ratio in breast milk is associated with the permeability of a lactating breast. The concentrations of sodium and potassium in the milk samples were measured by Dr. Ching Tat-Lai (under the supervision of A/Prof Donna Geddes) of Hartmann lactation group at the University of Western Australia and were determined using ion selective electrodes (C- 122 for sodium; C-123 for potassium, Horiba, Japan). Calibration of the electrodes was conducted according to the manufacturer recommendations. For each measurement, the whole milk samples were thawed at 37°C for 1 hour. Prior to the measurement, the milk sample was hand mixed for 15 seconds followed by 3 inversions. 300μl of the mixed milk was pipetted onto the sensor pad of the electrode. The reading was taken when results had stabilized for approximately 15 seconds. After each measurement, the milk sample was removed from the pad.
and returned to the storage tube. The pad was then rinsed with double deionised water and was
wiped with KimWipes prior to the next measurement. All samples were analysed at the same
time in duplicate. Same procedures were applied to both electrodes. The sodium: potassium
ratio was calculated in a 1:1 ratio.

4.2.9 Total protein measurement in breast milk

The protein content in the milk samples was also performed by the Hartmann Lactation group,
determined by a modified Bradford method using a commercial protein reagent (Bio-Rad
Laboratories, Richmond, CA, USA) (242). Protein standards were prepared from an aliquot of
human milk and the protein concentration determined by the Kjeldhal method, as described by
Atwood and Hartmann (243) and the protein assay as described by Mitoulas et al (244). The
recovery of a known amount of protein added to the milk samples was 98.4 ± 2.2 (n=5). The
detection limit of this assay was 0.021g/L and the inter-assay CV was 6.9% (n=10).

4.2.10 Total fat content of breast milk

The fat content of the milk samples was determined by a spectroscopic esterified fatty acid
(EFA) method (244, 245) by the Hartmann Lactation group. The detection limit of the assay
was 7.65g/l and the inter assay coefficient of variance (CV) was 10.2% (n=11).

4.2.11 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 previous RCTs of breast milk OVA
detection (128, 129) (128, 129). 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), 32 infants per group were required. 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 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. Comparisons between intervention groups at baseline were performed using analysis of variance (ANOVA), Kruskal-Wallis (non-parametric ANOVA) or Fisher’s Exact test as appropriate. Breast milk samples were assayed in triplicate at 2, 4 and 6 weeks and summarised as individual median OVA concentrations. Serum OVA concentrations at 6 weeks were similarly summarized. Median breast milk and serum OVA concentrations were log transformed prior to analysis using TOBIT regression, with a lower limit of log(0.1) ng/ml, an upper limit of log(4.0) ng/ml and either dietary group or average egg intake as the explanatory variable. Breast milk OVA to total protein ratio was calculated as a 1:1 ratio of OVA concentration to total protein concentration.

### 4.3 Results

Recruitment for this study commenced in August 2013 and the last 6 weeks of lactation appointment was completed in February 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 4.1). There were no significant differences in baseline characteristics between the three intervention groups (Table 4.1). 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. Overall 93.7% (105/112) women were still breastfeeding at six weeks of age. Three women randomized to the egg free group withdrew prior to commencing the intervention due to concerns about restricting their diet. One woman withdrew her consent prior to the six-week assessment due to being too busy, one woman could not attend the six-week appointment due to a family crisis, and four women were lost to follow-up during the intervention period (Figure 4.1).

4.3.1 Intervention and dietary compliance

Overall 109/117 (93%) participants completed the full six-week intervention and 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 36/36 (100%), where all of the women complied with their intervention and consumed an average of more than four eggs per week for six weeks. 40/42 (95%) women in the low egg group consumed an average of one to three eggs per week. In the egg free group compliance was lower with 23/31 (74%) of women eating an average of less than 0.25 eggs per week during the intervention period. Compliance in the egg free group improved over the six week intervention period, with the median (IQR) eggs eaten per week in week one was 0.00 (0.48) compared to week six 0.00 (0.00). In most cases accidental egg exposures in the egg free group were from small quantities of egg found in common foods such as cake, mayonnaise, biscuits, and noodles.
Table 4.1: Baseline characteristics per intervention group

<table>
<thead>
<tr>
<th></th>
<th>Egg Free (n=36)</th>
<th>Low Egg (n=44)</th>
<th>High Egg (n=40)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age at birth (y)*</td>
<td>33.1 (4.3)</td>
<td>33.1 (3.7)</td>
<td>33.0 (4.0)</td>
<td>.99</td>
</tr>
<tr>
<td>Maternal Caucasian race ^</td>
<td>29 (87.9%)</td>
<td>41 (93%)</td>
<td>37 (92%)</td>
<td>.27</td>
</tr>
<tr>
<td>Maternal completion of secondary school ^</td>
<td>32 (97%)</td>
<td>41 (93%)</td>
<td>37 (92%)</td>
<td>.79</td>
</tr>
<tr>
<td>Maternal smoking during pregnancy ^</td>
<td>1 (3%)</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>.74</td>
</tr>
<tr>
<td>Other smoking in the household ^</td>
<td>3 (9%)</td>
<td>4 (9%)</td>
<td>4 (10%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Dog at home ^</td>
<td>15 (45%)</td>
<td>19 (44.2%)</td>
<td>21 (52%)</td>
<td>.72</td>
</tr>
<tr>
<td>Cat at home ^</td>
<td>10 (30%)</td>
<td>11 (25.6%)</td>
<td>11 (27%)</td>
<td>.90</td>
</tr>
<tr>
<td>Antibiotics during pregnancy ^</td>
<td>8 (24%)</td>
<td>13 (43.3%)</td>
<td>9 (23%)</td>
<td>.83</td>
</tr>
<tr>
<td>Paternal allergy ^</td>
<td>16 (49%)</td>
<td>28 (64%)</td>
<td>22 (55%)</td>
<td>.43</td>
</tr>
<tr>
<td>Total weekly household egg intake*</td>
<td>6.8 (3.9)</td>
<td>7.3 (4.9)</td>
<td>8.6 (4.2)</td>
<td>.20</td>
</tr>
<tr>
<td>Maternal Egg Intake *</td>
<td>3.1 (1.9)</td>
<td>3.4 (2.1)</td>
<td>3.9 (2.1)</td>
<td>.20</td>
</tr>
<tr>
<td>Parity greater than one</td>
<td>7 (21%)</td>
<td>6 (14%)</td>
<td>11 (27%)</td>
<td>.30</td>
</tr>
<tr>
<td>Infant birth weight (g)*</td>
<td>3452 (425)</td>
<td>3469 (394)</td>
<td>3494 (318)</td>
<td>.90</td>
</tr>
<tr>
<td>Delivery method via C-section ^</td>
<td>17 (53%)</td>
<td>19 (45%)</td>
<td>16 (43%)</td>
<td>.57</td>
</tr>
<tr>
<td>Infant male sex ^</td>
<td>21 (64%)</td>
<td>17 (39%)</td>
<td>20 (50 %)</td>
<td>.10</td>
</tr>
<tr>
<td>Gestational age at birth (weeks)*</td>
<td>39.5 (1.2)</td>
<td>39.5 (1.2)</td>
<td>39.7 (0.8)</td>
<td>.62</td>
</tr>
</tbody>
</table>
Potential participants assessed (n = 2034)

Excluded (n=1914)
- Not eligible (n=1515)
- Did not consent (n=319)
- Unable to complete eligibility (n=80)

Randomised (n=120)

Egg Free Group
n=36
Did not commence the intervention (n=3)

- Withdrew consent (n=4)
- Non-compliant with study protocol (n=6)
- Did not complete intervention (n=1)
- Lost to follow-up (n=0)

- 6 weeks of lactation appointment (n=32)
- Breast milk for primary outcome (n=30)

Low Egg Group
n=44

- Withdrew consent (n=0)
- Non-compliant with study protocol (n=2)
- Did not complete intervention (n=0)
- Lost to follow-up (n=2)

- 6 weeks of lactation appointment (n=42)
- Breast milk for primary outcome (n=39)

High Egg Group
n=40

- Withdrew consent (n=0)
- Non-compliant with study protocol (n=0)
- Did not complete intervention (n=1)
- Lost to follow-up (n=2)

- 6 weeks of lactation appointment (n=37)
- Did not attend (n=1)
- Breast milk for primary outcome (n=33)

Figure 4.1: CONSORT diagram for QuEST trial participants
4.3.2 Breast milk ovalbumin content and dietary egg ingestion

At the end of the intervention (six weeks of lactation), the women allocated to the high egg diet had significantly higher levels of OVA in their breast milk than women following an egg free diet (P=0.036). The median levels for each group can be seen in Table 4.2. There were no detectable differences between the low egg and the egg free diet groups breast milk OVA levels at two, four or six weeks (P=0.43, P=0.47 and P=0.42 respectively). The OVA concentration for all three time points as per intervention are illustrated in Figure 4.2, and OVA concentration per individual are illustrated in Figure 4.3.

Table 4.2: OVA concentration (ng/ml) in breast milk between the three intervention groups

<table>
<thead>
<tr>
<th>Week of lactation</th>
<th>Egg free</th>
<th>Low egg</th>
<th>High egg</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>(high egg - egg free)</td>
</tr>
<tr>
<td></td>
<td>n=33</td>
<td>n=44</td>
<td>n=40</td>
<td></td>
</tr>
<tr>
<td>Breast milk 2 weeks</td>
<td>0.14 (0.05-0.28)</td>
<td>0.15 (0.05-0.68)</td>
<td>0.05 (0.05-0.54)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>n=28 BLQ= 43% (12/28)</td>
<td>n=40 BLQ= 45% (18/40)</td>
<td>n=35 BLQ=51% (18/35)</td>
<td></td>
</tr>
<tr>
<td>Breast milk 4 weeks</td>
<td>0.05 (0.05-0.25)</td>
<td>0.05 (0.05-0.48)</td>
<td>0.08 (0.05-0.65)</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>n=30 BLQ = 57% (17/30)</td>
<td>n=39 BLQ =54% (21/39)</td>
<td>n=36 BLQ=50% (18/36)</td>
<td></td>
</tr>
<tr>
<td>Breast milk 6 weeks</td>
<td>0.05 (0.05-0.20)</td>
<td>0.05 (0.05-0.41)</td>
<td>0.20 (0.05-0.96)</td>
<td>0.036</td>
</tr>
<tr>
<td></td>
<td>n=27 BLQ=63% (17/27)</td>
<td>n=39 BLQ=54% (21/39)</td>
<td>n=33 BLQ=42% (14/33)</td>
<td></td>
</tr>
<tr>
<td>Maternal Serum 6 weeks</td>
<td>0.72 (0.37-1.39)</td>
<td>0.54 (0.27-1.76)</td>
<td>0.63 (0.35-1.39)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>n=32 BLQ= 0% (0/32)</td>
<td>n=40 BLQ= 3% (1/40)</td>
<td>n=36 BLQ= 3% (1/36)</td>
<td></td>
</tr>
</tbody>
</table>

BLQ: Levels of OVA below the level of quantification for the assay (<0.1 ng/ml)
Figure 4.2: Breast milk OVA concentration (ng/ml) per intervention group at 2, 4, and 6 weeks of lactation.

Figure 4.3: Individual participant breast milk OVA concentration at two, four and six weeks of lactation.
When breast milk OVA concentrations were examined against actual maternal egg intake, the average number of eggs per week consumed during the intervention was significantly associated with log transformed OVA concentration in breast milk at six weeks (β=0.22, SE(β)=0.09, P=0.01). Ingesting an additional one egg per week resulted in a 25% increase in OVA concentration (95% CI 5%–48%) conditional on detectable OVA levels (Figure 4.4c). There were no associations between average egg ingestion at two or four weeks with OVA concentration in breast milk (P=0.83 and P=0.16 respectively) which is represented in Figure 4.4a and b.

There were 92 participants with three consecutive breast milk samples available at two, four and six weeks of lactation. A total of 31/92 (33.7%) women did not have any detectable OVA in their breast milk at any time point throughout the study. In the high egg group, 8/30 (26.7%) women did not have any detectable OVA in any of their breast milk samples despite eating a minimum of four eggs per week. In the low egg group 14/36 (38.9%) of women in did not have any detectable OVA in their breast milk. Despite following an egg free diet, only 9/26 (34.6%) of women in the egg free intervention group did not have any detectable OVA in their breast milk at any time point throughout the intervention.

4.3.3 Maternal serum OVA levels

Maternal serum OVA levels are reported in Table 4.2. No associations were detected between maternal serum OVA levels and either intervention diet group allocation or average actual egg ingestion. Serum OVA concentration did not correlate with breast milk OVA concentration at six weeks of lactation.
Figure 4.4: 95% predicted OVA in breast milk per increase in maternal egg ingestion at (a) 2 weeks lactation, (b) 4 weeks lactation and (c) 6 weeks lactation.
4.3.4 Breast Permeability

There were 95 women with measures of both mammary epithelium permeability and breast milk OVA concentration. No detectable associations were found between mammary epithelium permeability and breast milk OVA concentrations (unadjusted $P=0.14$, and adjusted for maternal dietary egg ingestion $P=0.18$). Mammary epithelium permeability did not differ between the three egg intervention groups at any time point.

4.3.5 Total protein in breast milk

There were no differences in total protein concentration between the three intervention groups at 2 weeks ($p=0.55$, $n=101$), 4 weeks ($P=0.27$, $n=100$) or 6 weeks ($P=0.42$, $n=95$) of lactation. OVA as a ratio of total protein in breast milk did not differ between the intervention groups at six weeks of lactation ($P=0.10$, $n=90$), and total protein in breast milk was not associated with OVA concentration at any time point. When breast milk OVA concentrations as a ratio of total protein were examined against actual maternal egg intake, the average number of eggs per week consumed during the intervention was significantly associated with the log transformed OVA to total protein ratio in breast milk at six weeks ($\beta=0.29$, $SE(\beta)=0.09$ $P=0.034$). In women with total protein measured at six weeks of lactation, 48% (33/68) of women who were ingesting egg (high egg and low egg groups), did not have detectable OVA in their breast milk. There was however no difference in total protein measured between women who had OVA levels above detection ($n=35$, mean 11.78 +/- 1.8) or below detection ($n=33$, mean 11.57 +/- 1.9) ($P=0.60$).
4.3.6 Total fat in breast milk

There were no differences in total fat content between the three intervention groups at 2 (P=0.94, n=103), 4 (P=0.88, n=102) or 6 (P=0.10, n=97) weeks of lactation. Actual maternal dietary egg intake over the six weeks also did not associate with breast milk total fat content at 6 weeks of lactation (P=0.75, n=91).

4.4 Discussion

This is the first randomized controlled trial to investigate the effect of an early postnatal intervention 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 build on the previously observed short-term dose relationship between maternal egg ingestion on the concentrations of OVA in breast milk (128).

The principal aim of this study was to explore the rationale that encouraging high intakes of egg in the maternal diet during lactation would result in increased oral egg allergen exposure for breastfed infants, potentially modulating tolerance to these foods. Here we have demonstrated that ingestion of one additional egg per week resulted in a 24% increase in breast milk OVA concentration, and additionally can conclude that all forms of egg (cake, quiche, meatballs, or whole egg) will contribute to the overall level of egg allergen present in human milk.

Consistent with previous studies (128, 129), there were considerable variations between women in OVA levels detected in the breast milk samples, with 26-38% of women in the egg ingestion intervention having undetectable breast milk levels of OVA at all time points. This may be due to delayed excretion of the allergen beyond six hours in some individuals, or perhaps a
proportion of women do not have the ability to excrete OVA in their breast milk due to differences in digestion, absorption, or excretion kinetics. Alternatively there were also low levels of OVA in the egg avoidance group despite reported compliance to eliminating egg from their diet, highlighting the difficulties of true egg avoidance, due to traces of egg in processed foods and in the environment (246). The concentration of OVA observed in this study were lower than previous studies measuring peak OVA concentration (128, 129), however this would be expected as peak concentration was not measured here, instead we measured OVA concentration from only one sample within a window of time. Interestingly, there were no associations found between breast milk OVA levels and measurement of mammary epithelium permeability. Further research is required to identify potential factors influencing these variations in food allergen passage from the diet into human milk, and the limitations of the results from this section are discussed further in Chapter 6.

Measurement of the total protein and total fat content of maternal breast milk demonstrated there were no differences in total fat or protein composition between the three intervention groups. When we included total protein in the analysis of OVA concentration in this study, actual egg ingestion over the six-week intervention period remained positively associated with OVA as a ratio of total protein. However, differences between the high egg and egg free intervention groups at six weeks were no longer significant, presenting a discord in the results. Contrasting our results, a previous study by Palmer et al, showed total protein concentration did not influence the results of maternal egg ingestion on OVA concentration (128). I also investigated if the presence or absence of OVA secreted in the breast milk of women who were ingesting egg, was associated with total protein at six weeks of lactation, however there were no differences noted between women who had OVA levels above detection versus women with levels below detection. Most other studies investigating breast milk allergen concentration have not measured total protein (with the exception of Palmer et al), and as OVA may be selectively absorbed in some but not all individuals, the relevance of total protein on breast milk allergen concentration could be questioned.
Strengths of this study include frequent breast milk collections in early lactation, with 77% of the participating women providing a breast milk sample at two, four and six weeks of lactation. Both of the egg inclusion groups had high rates of compliance for the whole six week intervention period (95-100%). The initially lower level of adherence and the higher rate of participant withdrawal from the egg-free group do highlight the practical difficulty of food allergen avoidance diets. However, the compliance in the egg-free dietary group did improve over time as the women became accustomed to which foods they needed to avoid, and thus a longer intervention may have seen further improved dietary compliance.

The discordance between serum and breast milk OVA concentration has also been demonstrated previously (88), and highlights the complexity of food protein absorption from the diet. The transient nature of OVA in breast milk after maternal ingestion has been demonstrated in multiple studies (110, 113, 120, 127, 128), and the number of samples in this study with OVA levels below the level of detection demonstrates the difficulty of measuring food proteins from a single breast milk sample. This is despite the sample being taken within a window of 2-6 hours post ingestion, which has previously been shown as the optimal window for OVA detection(128). Future studies should consider taking multiple samples to increase the overall detection rates of allergen in breast milk.

Conclusions
This is the first randomized controlled trial to show that increasing maternal dietary egg intake during early lactation, including all forms of egg, results in measurable increases in the concentration of OVA in human milk. This provides credence to the notion that maternal diet can be used to modify allergen exposure for breastfed infants in the postnatal period. This raises the question of whether encouraging high intakes of the major food allergens in the maternal diet during breastfeeding could modulate the development of early oral tolerance, and future studies need to determine the effect of oral allergen exposure through breast milk on tolerance induction markers in the infant.
Chapter 5

Maternal ingestion of hen’s egg protein during early lactation is associated with elevated levels of egg-specific IgG4 in breastfed infants

5.1 Introduction

Recent studies have shown a high incidence of early life food sensitisation in infants prior to oral exposure in solid foods (82-84). In Chapter 3, I demonstrated that early egg-specific T helper 2 cytokine responses at four months of age predicted egg allergy at 12 months (238). This suggests that allergen-specific immune dysregulation is already strongly established in the first months of life, and is evidence of much earlier allergen exposure. In order to reduce the increasing impact of food allergy in young children there is a need to identify routes of allergen exposure in the early postnatal period that promote the development of oral tolerance.

The initial route in which the immune system first encounters a food allergen may be an important determinant of whether oral tolerance or sensitisation is established. There is concern that allergen exposure via the skin, most notably in infants with a compromised skin barrier, may promote the development of sensitisation (93). Alternatively, oral exposure to food proteins results in an intricate allergen-immune interaction in the gut, which will lead to the induction of tolerance in a healthy intestinal milieu (147). As discussed in Chapter 4, breast milk provides an important source for neonatal exposure to oral allergens in the postnatal period. While animal models are suggesting a role for breast milk derived allergen exposure in the induction of oral tolerance (110, 179), the role of breast milk in the development of food allergy in humans is less clear, and is limited to epidemiological studies (reviewed in (247)).

The maternal elimination of allergens during lactation has not proven to be effective at reducing the risk for food allergies in the infant (144). Additionally, with the recent advances in both the treatment and prevention of allergy using controlled allergen exposure, there remains great interest in the role of breast milk as an optimal method to provide neonates with oral exposure to allergens in the first months of life.
In Chapter 4 we established that maternal dietary ingestion of egg influences the OVA concentration of breast milk, and thus infant egg exposure can be modified by maternal diet in early life. In this chapter we aim to determine whether allergen exposure through breast milk influences the induction of oral tolerance mechanisms in the infant. Using a randomised controlled trial, we examined the association between maternal dietary egg ingestion during the first six weeks of lactation and infant immune markers for oral tolerance (egg-specific IgG4) and sensitisation (egg-specific IgE) prior to the introduction of egg in solid foods. Additionally we also sought to investigate whether infant skin barrier function is compromised in those infants who developed food sensitisation in early life.

5.2 Methods

5.2.1 Study Design

This study included infants whose mothers were participating in a randomized controlled trial designed to investigate the effect of maternal dietary egg ingestion on the amount of detectable egg protein (OVA) in their breast milk (ACTRN12613000643774). This study was approved by the University of Western Australia (RA/4/1/6115) and local ethical institutional review boards (Human Research Ethics Committees) at Princess Margaret Hospital, St John Of God (SJOB) Hospitals: Murdoch, Mt. Lawley and Subiaco, and South Metropolitan Hospital: Kaleeya, in Western Australia. Full details on study design were discussed in Chapter 4. In brief, pregnant women planning to breastfeed were initially screened for a history of medically diagnosed allergic disease (asthma, eczema, hay-fever or IgE mediated food allergy). For the first six weeks of lactation each woman was randomly allocated to one of three dietary egg intervention groups: high egg diet (recommended consumption of 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). The participating mothers attended appointments with their infants at the end of the intervention at six weeks of lactation, and again when their infant was sixteen weeks of age.
5.2.2 Blood and breast milk sample collections

Infant blood samples (maximum of 5 ml) were collected via venipuncture from a vein in the cubital fossa area at six and sixteen weeks of lactation by trained nursing staff. Plasma was separated from whole blood by centrifugation within four hours of sample collection. Breast milk samples (10 ml) were collected from participating mothers in their homes at two and four weeks, and during the six week of lactation appointment. 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 optimise the detection of OVA (128). Both breast milk and plasma samples were stored at -80 °C prior to analysis. To avoid any study bias all blood and breast milk samples were de-identified and re-coded to ensure blinding during the analysis.

5.2.3 Antibody measurements

I measured whole egg–specific IgE, peanut-specific IgE and egg white–specific IgG4 antibody concentrations using the ImmunoCAP 250 system (Phadia AB, Uppsala, Sweden). Plasma samples were thawed, centrifuged, and non-aqueous matter (lipid) was removed prior to analysis. The lower limit of detection for specific-IgE was 0.1 kUA/L, and 0.07 mg of antibody/L for specific-IgG4. For analysis, values of less than the lower limit of detection were replaced by half the lower limit of detection.

5.2.4 Detection of ovalbumin in 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). Full details of methodology can be found in Chapter 4. In brief, 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. The ELISA’s were analyzed using a MultiSKAN ELISA plate reader (ThermoFisher Scientific, NSW Australia), at
a wavelength of 450 nm. SkanIt™ 2.5.1 Research Edition software (ThermoFisher Scientific, NSW Australia) was used to interpret the results. Any samples that were below the lower limit of detection were assigned a value of one half the lowest limit of detection (0.05 ng/ml). All samples for each woman were run on the same plate.

5.2.5 Trans epidermal water loss (TEWL) measurement

Trans epidermal water loss (TEWL) was measured using the AquaFlux 200 ((BIOX Systems Ltd, London, England) on both the mother and the infant at the six and sixteen week of age appointments. The participants were acclimatized to the room for at least 10 minutes prior to the TEWL measurement, and were advised to avoid any moisturizer on the forearms for twenty-four hours prior to the appointment. Measurements were taken on the forearm half way between the elbow and the wrist on skin unaffected by eczema. The TEWL measurement was taken three times, and an average reading was calculated based on the two closest measurements.

5.2.6 Infant eczema assessments

Infants were assessed for the presence of eczema at both six and sixteen weeks of age. This was defined as a history of dry, red, itchy skin. Infants with any possible symptoms of eczema, were assessed by trained study staff using the SCORAD assessment (232). SCORAD scores were assigned for each infant with signs of eczema on the day of the appointment. Unaffected infants did not have a SCORAD assessment.

5.2.7 Statistical Methods

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. Comparisons between intervention groups at baseline were performed using analysis of variance (ANOVA), Kruskal-Wallis (non-parametric ANOVA) or
Fisher’s Exact test as appropriate. Similarly, comparisons between intervention groups for eczema prevalence and SCORAD scores at six and sixteen weeks of lactation were analysed in the same manner. Breast milk samples were assayed in triplicate at 2, 4 and 6 weeks and summarised as individual median OVA concentrations. Median breast milk and serum OVA concentrations were log transformed prior to analysis using TOBIT regression, with a lower limit of log(0.1) ng/ml, an upper limit of log(4.0) ng/ml and either dietary group or average egg intake as the explanatory variable. Infant egg-specific immunoglobulin concentrations at six and sixteen weeks were summarized and analysed similarly using a lower limit of log(0.1) kUA/L for IgE and log(0.07) mg of antibody/L for IgG4. The correlation between log transformed maternal and infant TEWL was estimated using Pearson’s correlation coefficient. All analyses were performed in R software (version 3.2.1).

5.3 Results

5.3.1 Study population

This study was comprised of 120 mother and infant pairs: high egg (n= 40), low egg (n=44), and egg free (n=36) (Figure 5.1). Baseline characteristics were similar between all three intervention groups and were reported in Chapter 4 (Table 4.1). Blood samples for the measurement of egg-specific immunoglobulins were available from 82/111 (74%) infants who attended the six week appointment, and 61/109 (56%) infants who attended the sixteen week appointment (Figure 5.1). 86.1% (93/108) of participants were still breastfeeding at 16 weeks of age, and blood samples were only included for infants who were still being breastfed. Blood samples were not collected due to failure to obtain sufficient blood volume after a maximum of two venipuncture attempts, or parent refusal. Infants who did not have a blood sample taken successfully, still underwent the other clinical assessments at both six and sixteen weeks of age, including questionnaires, anthropometric assessment, the measurement of TEWL and an eczema assessment.
Figure 5.1 CONSORT diagram for participants in the QuEST Study
5.3.2 Maternal egg consumption and egg-specific IgG4

There was a positive association between maternal dietary ingestion of egg and the tolerance marker IgG4 in breastfed infants, where average maternal egg ingestion over the six week intervention period was significantly associated with infant egg-specific IgG4 (P=0.02) at six weeks. Ingesting one additional egg per week resulted in an average 22% increase in infant egg-specific IgG4 levels (95% CI 3%-45%) dependent on detectable IgG4 levels. At 16 weeks of lactation, the median egg-specific IgG4 levels in infants who were still breastfeeding were below the level of quantification and thus there was no relationship between maternal dietary egg ingestion and infant egg-specific IgG4.

Figure 5.2 Predicted level of IgG4 (mg of antibody/L) based on average maternal dietary egg ingestion at (a) 6 weeks and (b) 16 weeks
Infant levels of egg-specific IgG4 did not statistically differ between the three intervention
groups at six weeks of lactation (Table 5.1) and egg-specific IgG4 levels are illustrated in
Figure 5.3. At 16 weeks of lactation the median egg-specific IgG4 levels in infants who were
still breastfeeding were below the level of quantification in all three groups (Table 5.1). This is
despite women consuming a mean of 4.1 +/− 3.9 eggs per week at 16 weeks of age, which had
increased from average egg consumption at baseline (3.5 +/− 2.1) (Table 5.2). The egg free
group had the largest increase in egg consumption from baseline to sixteen weeks, with the
other two egg intervention groups also increasing their maternal egg ingestion slightly from
baseline (Table 5.2).

Table 5.1: Egg specific IgG4 levels (mg of antibody/L) per intervention group

<table>
<thead>
<tr>
<th>Week of lactation</th>
<th>Egg free Median (IQR)</th>
<th>Low egg Median (IQR)</th>
<th>High egg Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks</td>
<td>0.125 (0.035-0.915)</td>
<td>0.41 (0.08-1.20)</td>
<td>0.42 (0.08-1.1)</td>
</tr>
<tr>
<td></td>
<td>n=26</td>
<td>n=29</td>
<td>n=27</td>
</tr>
<tr>
<td></td>
<td>BLQ: 38% (10/26)</td>
<td>BLQ: 24% (7/29)</td>
<td>BLQ: 18% (5/27)</td>
</tr>
<tr>
<td>16 weeks (post intervention)</td>
<td>0.035 (0.035-0.39)</td>
<td>0.035 (0.035-0.14)</td>
<td>0.035 (0.035-0.15)</td>
</tr>
<tr>
<td></td>
<td>n=13</td>
<td>n=32</td>
<td>n=26</td>
</tr>
<tr>
<td></td>
<td>BLQ: 54% (7/13)</td>
<td>BLQ: 53% (17/32)</td>
<td>BLQ: 61% (16/26)</td>
</tr>
</tbody>
</table>

BLQ: Levels that were below the level of quantification for the assay (<0.07ng/ml)
5.3.3 Infant egg-specific and peanut-specific IgE levels

Infant sensitisation was assessed at six weeks of age (n=82), however none of the infants had detectable levels of egg or peanut-specific IgE at this time point. By 16 weeks of age 5/84 (6.0%) infants had detectable levels of peanut or egg-specific IgE. Egg sensitisation was the most frequent where 4/84 (4.8%) infants had detectable levels of egg-specific IgE. Of these, two infants (2.4%) had levels > 0.35 kUA/L, and the other two infants had low levels of detectable
egg specific IgE (0.1-0.35 kUA/L). Only one infant (1/84=0.8%) had a detectable peanut-specific IgE level with a concentration of 0.1 kUA/L at 16 weeks of age. Of these five sensitised infants, only one had been diagnosed with eczema by 4 months of age.

### 5.3.4 Infant eczema

Eczema symptoms were present in 5.4% (6/111) infants at six weeks of age and 13% (14/108) infants at sixteen weeks of age. At six weeks of age the frequency of eczema differed between the three intervention groups: egg free, 3.1%(1/32; low egg, 11.9% (5/42); and high egg 0% (0/37) (P=0.048). By sixteen weeks of age however there were no sustained detectable differences in eczema frequency between the three intervention groups: egg free, 6.9% (2/29); low egg 21% (9/42); and high egg 8.1% (3/37) (P=0.14). The median SCORAD score at six weeks of lactation was 5.55 (IQR 0.0-8.3, n=6) and at sixteen weeks was 7.2 (IQR 0.0-8.2, n=14). SCORAD scores for infants with a history of eczema did not differ between the three intervention groups at either six weeks or sixteen weeks (Table 5.3).

Table 5.3: SCORAD results per intervention group at 6 and 16 weeks of age

<table>
<thead>
<tr>
<th>Week of lactation</th>
<th>Egg free (SCORAD)</th>
<th>Low egg (SCORAD)</th>
<th>High egg (SCORAD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=33</td>
<td>n=44</td>
<td>n=40</td>
<td></td>
</tr>
<tr>
<td>6 weeks</td>
<td>7.4 (7.4-7.4)</td>
<td>3.7 (0.0-9.25)</td>
<td>n=0</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>n=1</td>
<td>n=5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 weeks</td>
<td>0.0 (0.0-0.0)</td>
<td>7.3 (7.2-10.9)</td>
<td>0.0 (0.0-8.2)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>n=2</td>
<td>n=9</td>
<td>n=3</td>
<td></td>
</tr>
</tbody>
</table>
5.3.5 *Trans epidermal water loss (TEWL)*

Positive associations were detected between maternal and infant TEWL at six weeks (P=0.002) and 16 weeks of age (P<0.0001) Figure 5.4. At 16 weeks of age there were 14/108 (13%) infants with symptoms of eczema. The infants with eczema symptoms had significantly higher TEWL readings than unaffected infants (P=0.003)(Figure 5.5). At six weeks of age there were only six infants with symptoms of eczema, and there were no detectable differences in TEWL readings at this age between infants with and without eczema. Infant TEWL readings (mean, +/- SD) at six weeks (13.4 +/- 4.13 g/m²h, n=100) did not differ from TEWL readings at sixteen weeks (14.5 +/- 6.1g/m²h, n=97).

Figure 5.4: Maternal and infant TEWL Ln(g/m2h) at (a) 6 weeks, n=100 and (b) 16 weeks, n=97.
Mothers with a history of eczema at any time over the lifespan (n=44) did not have higher TEWL readings at six or sixteen weeks lactation than mothers with no history of eczema (n=57) (P=0.65 and P=0.175 respectively). The mean TEWL reading for mothers at six weeks was 12.2 +/- 8.6 g/m²h (n=102), and at sixteen weeks was 13.1 +/- 7.3 g/m²h (n=99). There was a significant positive correlation between maternal TEWL measured at six weeks and TEWL at sixteen weeks (β=0.30, n=93, P=0.004), demonstrating minimal variability in TEWL over time.

5.4 Discussion

In this study, I have shown that increasing maternal dietary ingestion of hen’s egg during early lactation is positively associated with the production of egg-specific IgG4 in breastfed infants. 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. The finding that infant egg-specific IgG4 levels were positively associated with maternal egg ingestion during early lactation provides evidence that the infant immune system recognises egg in breast milk, however we cannot determine from this study whether this will have lasting effects on the development of
tolerance. Our results are consistent with recent findings from a cohort study by Jarvinen et al (184) 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 (184). IgG4 is a known marker for allergen exposure, and increased levels of IgG4 in relation to IgE has been associated with oral tolerance in allergy prevention studies using regular allergen exposure with solid foods in the first year of life (83, 84).

The waning of egg-specific IgG4 to between six and sixteen weeks to a mean level below the level of assay quantification observed in this study remains a point of interest. At 16 weeks, women were consuming an average of 4.1 eggs per week (which is approximately what mothers in the high egg group were consuming during the intervention), however there was no longer an association between maternal egg ingestion and infant egg-specific IgG4 levels. Although speculative, although speculative, it is possible that as the infant increases in body size, the dose of egg protein in the breast milk also needs to continue to increase in order to induce the production of IgG4. Additionally as the frequency of breastfeeding usually decreases with infant age, infants may be exposed to less OVA due to the transience of the protein through the breast milk. Whether there will be any lasting immune programming effects in those infants who had elevated levels of egg-specific IgG4 at six weeks of age remains unknown. We are now following up these infants at 12 months of age, where it will be interesting to investigate whether increased production of egg-specific IgG4 in early life, is associated with immunoglobulin levels or tolerance induction later in infancy.

The lack of any associations between OVA in breast milk and infant egg-specific IgG4, however positive association between infant egg-specific IgG4 levels and average maternal egg ingestion, suggests OVA measured from a single sample of breast milk is not a good indicator of overall egg exposure for the infant. Thus infant egg-specific IgG4 levels may be a more
indicative approach to quantify infant egg exposure through breast milk in the postnatal period. This is likely due to the fact that OVA is transient through the breast milk, and was not even quantifiable in some women due to unknown differences in absorption, digestion or excretion kinetics (Chapter 4). Serum-specific antibodies are much more stable, and do not lend to the same difficulties as measuring food proteins in breast milk. An alternative approach would be to measure the levels of infant egg-specific IgA, as was done by Jarvinen (184).

Ideally the maternal intervention period could have been extended to least 4-6 months duration to fully assess the effects of maternal food allergen intake during lactation on infant oral tolerance prior to the commencement of solid foods. For pragmatic reasons we chose to initially test this concept in the immediate postnatal period with a shorter intervention to reduce the participant burden. The initially lower level of adherence and the higher rate of participant withdrawal from the egg-free group does highlight the practical difficulty of food allergen avoidance diets. However, the adherence in the egg-free dietary group did improve over time, and compliance rates were high (95-100%) in both the low-egg and high-egg groups.

This study also has several novel aspects, including the measurement of infant immunoglobulin levels twice prior to the commencement of solid foods, which provides insight into early life sensitisation for a subset of infants at lower risk of sensitization than data currently available (82-84). The rate of infant egg sensitisation at 16 weeks of age (2.4%) is notably lower in this trial (in which only 13% of infants had eczema at 16 weeks) compared to another recent trial in which all infants had eczema (83) and very high rates of sensitisation; 36% had egg-specific IgE >0.35 kU/L at 4 months of age prior to first ingestion of egg in solid foods. This reflects the role of eczema as a major risk factor in food sensitisation, potentially the result of altered mucosal and cutaneous barrier function, and putative transcutaneous sensitisation (93, 248).
Another novel aspect of this trial was the measurement of skin barrier function in both mothers and infants throughout the study. The positive correlation detected between maternal and infant TEWL is interesting in light of recent evidence that skin barrier impairment at birth predicts food allergy at 2 years of age (249). With further investigation, maternal TEWL could be a potential strategy to identify high-risk infants for targeted intervention studies. While one of the planned outcomes of this study was to examine the effect of TEWL on the sensitisation status of the infant, low rates of infant sensitisation (2.4%) has made this impossible. Thus further investigation in larger studies, with an increased risk for food sensitisation, is required to examine the effect of skin barrier dysfunction on infant sensitisation prior to the introduction of solid foods.

Conclusions

This is the first randomized controlled trial to show that increasing maternal dietary egg intake during early lactation, and related higher breast milk egg protein (OVA) concentrations, are associated with increased infant egg-specific IgG4 levels at six weeks of age, especially when more than four eggs per week are eaten. This suggests that maternal diet during lactation can modify measures of infant immune tolerance to allergenic foods. This provides a platform for larger trials to assess the impact of similar interventions (extended throughout lactation) to assess clinical outcomes, such as challenge proven food allergy in later infancy and childhood.
Chapter 6

General Discussion and Future Directions
This thesis is constructed around the question of whether egg exposure in the early postnatal period can induce oral tolerance in infants, and thus act as a potential strategy for the primary prevention of egg allergy. To address this, I firstly examined the ‘four-to-twelve month postnatal window’ to determine how infant egg-specific immune responses from this age vary in relation to early or delayed introduction of egg in solid foods. Secondly, I focused on the earlier window of development, from ‘birth-to-four months’ to determine the effects of altering egg exposure through breast milk on markers of infant immune tolerance prior to starting egg in solids foods. This entailed the first randomised controlled trial designed to assess how altering maternal dietary egg ingestion in the early postnatal period influences egg allergen concentration in breast milk, and infant egg-specific IgG4 levels. Below I discuss the key findings in relation to my hypothesis and in the context of the existing literature. This is followed by study limitations, directions for future research and conclusions.

6.1 Summary of findings in relation to our hypothesis.

We hypothesized in the context of allergy prediction:

1. Identification of egg proteins capable of eliciting inflammatory immune responses would be a useful tool to further characterize different phenotypes of egg allergy.
   
   *We found:* In the STAR study multiple egg proteins were capable of eliciting Th2 cytokine responses that were associated with egg allergy, including OVA, OM and LYS.

2. Early egg-specific immune programming will have lasting effects on the trajectory of egg allergy.
   
   *We found:* The STAR study also demonstrated that elevated levels of IL-5 and IL-13 prior to the introduction of solid foods predicted egg allergy at 12 months of age, suggesting egg allergy is programmed early in life in some infants.
We hypothesized in the context of egg allergy prevention:

1. Maternal diet would influence the OVA concentration of breast milk, and therefore infant egg exposure in the early postnatal period could be manipulated through maternal dietary ingestion.

We found: In Chapter 4, the QuEST study showed that maternal ingestion of egg is associated with maternal breast milk OVA concentration in 2/3 of women. 1/3 of women however, did not produce any OVA in their breast milk at any time point, suggesting their infant would not be exposed to any OVA while breastfeeding.

2. Infant egg exposure via breast milk would not result in increased levels of IgE, and may result in increased levels of the tolerance marker egg-specific IgG4.

We found: In Chapter 5, maternal egg ingestion was associated with infant egg-specific serum IgG4. Rates of egg-specific IgE were very low however, and we were not able to draw any conclusions on the effect of breast milk OVA and egg sensitisation in infants.

3. Infant TEWL would relate to the presence of infant eczema and increased levels of IgE sensitization prior to the introduction of solids.

We found: In Chapter 5, infant TEWL was associated with infant eczema at 4 months of age, however we could not make any conclusions about the effects on egg sensitisation.

6.2 Summary of key findings in the context of existing literature

The primary aims of this thesis were to investigate the effects of early postnatal egg exposure on the development of egg specific immune responses by: 1) determining whether egg-specific Th2 driven immune responses prior to the introduction of solid foods predict later challenge-proven egg allergy at twelve months of age, 2) exploring whether maternal dietary ingestion of egg influences protein concentration in breast milk, as a possible strategy to manipulate egg exposure for breastfed infants in the promotion of oral tolerance and 3) investigating whether
breast milk derived allergen exposure leads to the development of markers for sensitisation or tolerance in infants.

Chapter 3: Elevated IL-5 and IL-13 responses predate the introduction of solid foods in infants with eczema (STAR study)

The key finding of this study was that Th2 responses (IL-5 and IL-13) to multiple egg proteins (OVA, OM, CON, LYS) were already clearly established in many infants with moderate to severe eczema prior to the introduction of egg in solid foods. Furthermore, this is the first study to demonstrate that these early T cell responses at four months of age to a range of egg proteins predicted challenge-proven egg allergy at twelve months of age. These findings indicate early sensitisation through other sources or routes of environmental egg exposure, prior to dietary ingestion, as discussed further below. Although speculative, the context, the timing and the route of exposure may be important factors determining the development and maintenance of egg-specific Th2 responses, which drive the subsequent allergic phenotype.

The study also provides novel insight into patterns of egg specific cytokine responses around the timing of egg introduction (at four months of age) and the subsequent development of clinical egg allergy at 12 months of age. While the actual age that egg was introduced did not have any association with egg-specific cytokine production at 12 months of age (IL-5, IL-13, IL-10, IFN-g, TNF-a), we noted a number of other interesting findings. In particular, early egg-specific responses at four months of age predicted IgE-mediated egg allergy at 12 months. Interestingly, cytokine results at twelve months (at the time of egg challenge) did not correlate with challenge outcomes, despite continued clinical reactivity. Longitudinal data analysis also revealed and that the Th2 responses in children with egg allergy at 12 months were more pronounced at 4 months before starting egg in solids and significantly waned with age.

As noted, all infants in this study had moderate to severe eczema with associated defective skin barrier function. This is the population with recognised increased risk of food allergy, as
explored further below. In infants with eczema, a plausible explanation for early life food sensitisation is through impaired skin and mucosal barrier function (discussed in Chapter 5). While we were able to analyse PBMC’s to identify egg-specific T cell responses indicative of egg allergy, extending this to examine longitudinal patterns of homing T cell populations (skin versus gut) (151, 250, 251) in relation to oral allergen exposure would be of great interest. Although hypothetical, based on animal models (94, 252), our findings suggest that early life allergen exposure (possibly through the skin) may disrupt the development of oral tolerance mechanisms that extend to 12 months of age. Thus a better understanding of whether oral exposure changes populations of tissue specific T cells, may shed light on the pathogenesis of food allergy in infants with eczema. Perinatal factors including frequent washing, use of soaps and detergents could be relevant to the increase in skin barrier dysfunction more generally (253, 254), and are important directions for future investigation.

Introducing rather than ‘avoiding’ allergenic foods has become a key focus of research studies aimed at reducing the risk of food allergy. Recent studies confirm the merits of this. The LEAP study demonstrated that the introduction of an allergenic food (peanut) during infancy to infants at high risk of peanut allergy (with eczema, egg allergy or both) significantly reduced the risk of peanut allergy at 5 years of age (84). This study showed a reduction of peanut allergy in both infants without sensitisation at study entry, as well as those with low-level sensitisation (SPT <4mm) at the time of recruitment (between 4-11 months). While this suggests ‘oral exposure’ is a viable approach for inducing tolerance in children who already have early sensitisation, it remains important to understand the events contributing to early sensitisation and how this may also be reduced.

My findings demonstrate elevated levels of egg-specific Th2 cytokines at four months of age suggesting that interventions prior to this period may also be important in reducing the burden of food allergy. Others have also observed high rates of egg-sensitisation in infants with eczema prior to the introduction of egg into the diet, resulting in allergic reactions on initial oral
exposure to whole egg (82, 83). This included severe reactions that may preclude ongoing oral exposure, unless immunotherapy eventually becomes a safe and viable option in this age group. The growing number of children developing early food sensitisation in the first months, culminating in reactions on the first taste, highlights the need for prevention strategies prior to this. This may include more general strategies to provide more tolerogenic conditions during initial allergen encounter (such as optimising early colonisation of the skin and the gut) or allergen-specific approaches, which may need to be more targeted according to genetic risk or early phenotype (such as eczema). As eczema is arguably the greatest risk factor for food allergy, strategies to improve the skin barrier function and prevent this condition are likely to be significant in reducing the burden of food allergy, although this was beyond the scope of this thesis. The phenomenon of early food sensitisation by four months of age however prompted the investigation of breast milk derived allergen exposure in Chapters 4 & 5 as a potential intervention strategy to induce oral tolerance to foods in the immediate postnatal period.

**Chapter 4 and 5: The QuEST Study**

The QuEST study is the first randomised controlled trial designed to investigate how maternal dietary egg ingestion over a six week period influences OVA concentration of human milk and measures of infant immune tolerance. Here, the key finding was that women ingesting at least four eggs per week, had higher levels of OVA in their breast milk after six weeks than women avoiding egg. Moreover, average ingestion of egg during the six-week period was correlated with breast milk OVA levels. These findings show that breast milk OVA concentration can be modified through patterns of maternal dietary ingestion of egg in the early postnatal period.

This is consistent with previous work of our team, by Palmer et al. who found a dose response effect for the amount of egg consumed and breast milk OVA concentration after a challenge dose of egg (128). They later reported higher breast milk OVA concentration in women consuming one egg per day for three weeks when compared to an egg free diet (129). In the present study we sought to investigate this further, and examine patterns of egg ingestion over a
duration of six weeks, including all forms of egg, which is more representative of dietary guidelines that could be implemented for the prevention of allergic disease. The current findings extend the existing knowledge and provide insight into the relationship between maternal dietary ingestion and breast milk allergen content.

Our results showed that increasing egg ingestion by one egg per week resulted in a 25% increase in breast milk OVA concentration, in women who had detectable levels of egg protein. Additionally we can conclude that to achieve an increase in breast milk OVA concentration, all forms of egg (including cake, meatballs, pasta and whole egg) can be included towards weekly totals. Thus it is possible for egg exposure in breastfed infants to be manipulated by the maternal diet during early lactation.

In accordance with past research this study confirmed wide inter-woman variability in the concentration of OVA in breast milk (128, 129). This includes some women who did not have detectable levels of OVA in their breast milk at any time point throughout the intervention, despite the regular ingestion of egg. This suggests that if recommendations were to encourage lactating mothers to include allergenic foods in their diet as a food allergy prevention strategy, it would not be beneficial for up to one third of mother infant pairs. Thus determining additional factors that may be involved, and in some cases inhibiting the passage of allergen into the breast milk would be beneficial.

Factors that influence the passage of allergen from the diet into human breast milk remain unknown, however it would seem plausible that complex biological differences in digestion, absorption or excretion kinetics are responsible for inter-woman variation. The present study is the first to investigate breast permeability as a possible influencing factor for OVA concentration in breast milk, however here I saw no associations between Na/K ratio of breast milk and OVA concentration of human milk. Despite this, Benn et al. have previously
demonstrated infants born to atopic mothers with increased Na/K ratio were at a significantly greater risk for a positive skin prick test, and/or atopy (130). Our results suggest that increased allergen load in the breast milk due to increased permeability is not the mechanism driving this association, however our sample collection may not have been optimised to examine this thoroughly (discussed in section 6.3) and thus further research is needed in this area.

Of particular significance, these studies also show for the first time that this had an effect on markers of infant immune tolerance development to egg protein. I demonstrated that increasing maternal egg ingestion from birth to six weeks was effective in increasing infant egg-specific IgG4 levels at six weeks of age. Each additional egg ingested per week resulted in a 22% increase in infant egg-specific IgG4 levels. Although significant differences in IgG4 levels were not noted between the three intervention groups, we have demonstrated that maternal inclusion of egg during lactation results in increased levels of IgG4 irrespective of how much egg was consumed in pregnancy. Similar effects on IgG4 levels has also been demonstrated in a cohort study by Jarvinen et al (184), who demonstrated cow’s milk elimination from the maternal diet in the first three months of life was associated with lower infant milk-specific IgG4. In contrast to the QuEST study where the primary focus was on allergen exposure for breastfed infants in the induction of oral tolerance, these investigators did not examine cow’s milk allergen but instead cow’s milk specific-IgA. Maternal dietary ingestion of cow’s milk products resulted in increased specific-IgA levels in the breast milk, which was positively associated with infant IgG4. The clinical implication for this was that low cow’s milk specific infant IgG4 was associated with increased risk for challenge confirmed cow’s milk allergy around 6 months of age. Thus whilst it remains unclear whether it is the allergen exposure through breast milk as measured in the QuEST study, or specific immunoglobulin complexes that are responsible for increasing infant specific IgG4 levels, maternal dietary ingestion of these foods is associated with infant food-specific immunoglobulin production in both instances.
These results are in accordance with studies investigating the immunomodulatory effects of regular allergen exposure. Allergy prevention studies have demonstrated infants ingesting an allergen regularly from the introduction of solid foods resulted in significantly increased levels of specific-IgG4 (83, 84). Oral immunotherapy studies also show increased levels of egg-specific IgG4 are associated with oral desensitisation in children with existing egg allergy (158, 255). Furthermore low levels of cow’s milk-specific IgG4 has been associated with confirmed clinical cow’s milk allergy around six months of age (184). Whilst the clinical implications of the elevated egg-specific IgG4 levels in the QuEST study are unknown, the association between maternal diet during lactation and the marker associated with tolerance, provides promise for the role of breast milk in oral tolerance induction.

In this study we did not have a sufficient number of infants with positive levels of food-specific IgE to investigate whether oral egg exposure via breast milk influences the rate of infant egg-specific sensitisation. Questions around the role of food proteins in breast milk, especially for high risk mothers has been a longstanding matter of contention. Here we had a population of high-risk mothers, two thirds of who were ingesting egg regularly in their diet and only (2.8%) of the infants were sensitised. Although speculative, this could suggest breast milk allergen exposure did not induce food-specific sensitisation in this instance.

The contrasting sensitisation rates in the two studies presented in this thesis confirms the significance of eczema as a risk factor for food allergy - that infants with eczema have more than a 15-fold increase in the rate of sensitisation prior to the introduction of solid foods. In the STAR study (83), 36% of infants with moderate to severe eczema were sensitised to egg at four months of age prior to eating egg in solid foods, compared to only 2.4% in the QuEST study (where only 13% of the infants had eczema). This is potentially the result of altered mucosal and cutaneous barrier function in infants with eczema (93, 248). Recent evidence of skin barrier impairment at birth predicting food allergy at 2 years of age (249), enhances the growing body of evidence that high rates of food sensitisation in infants with eczema are due to transcutaneous
sensitisation (91, 93, 248). Further investigation is required to examine the pathogenesis of putative transcutaneous food sensitisation in these infants. While maternal and immediate family history of allergy are important risk factors for allergy, increasing rates of eczema, often in the absence of family heredity, presents another arguably more significant risk factor for early food-sensitisation, and this population should be targeted in future prevention studies. This includes strategies to improve the skin barrier, optimise allergen delivery (form, route, timing and dose) and promote local and systemic conditions that favour tolerance during allergen exposure. Breast milk derived allergens are likely to play a key role in promoting oral tolerance during this period, and may be of particular importance in infants with eczema. The many immunomodulatory properties of breast milk components are likely to be of importance in the development of normal oral tolerance, including the induction of Treg and associated mucosal responses in offspring (181, 239). Finally, the role of maternal health, maternal nutrition and the maternal microbiome are also factors that are likely to influence the quality and composition of breast milk, as well as other aspects of infant development and immune maturation in ways that will determine the likelihood of tolerance to food allergens (256).

6.3 The limitations of these studies

While this thesis provides novel and important findings in relation to egg allergen exposure in the early postnatal period, there are a number of factors that need to be considered in the interpretation of these findings.

The STAR Study: Egg-allergen limulus testing

The potential for lipopolysaccharide LPS contamination is a factor in all studies using antigenic in vitro culture systems. To minimise this, prior to use, the allergens were passed over an endotoxin column to remove LPS and endotoxin constituents prior to use in PBMC culturing. Based on this we can be confident the allergens were free of contamination, although limulus testing for the egg-allergens would have confirmed the absence of LPS constituents.
Additionally, our results demonstrated egg-specific differences in Th2 responses that related with egg allergy status, strongly suggesting that the responses demonstrated in this thesis were allergen-specific, and not non-specific responses due to LPS contamination.

The QuEST Study dietary intervention

The dietary intervention in the QuEST study was designed to resemble dietary guidelines that could be implemented for the prevention of food allergy. Studies involving modifications to the diet can be difficult, especially when the complete avoidance of a common food is required. As thus only three quarters of mothers in the egg free group were able to eat less than \(\frac{1}{4}\) of an egg per week for six weeks. The compliance in the egg free group did improve over time, presumably as participants became more aware of which foods contain egg, and as they discovered alternate options for foods they usually eat. As women in the low egg group could eat a minimum of one egg per week, and many women in the egg free group were ingesting small amounts of egg, these two groups did not differ drastically in their weekly egg ingestion. This may contribute to why we saw no differences in breast milk OVA concentration between the low-egg and egg-free groups; yet saw an association when we looked at the actual amount of egg ingested during the intervention period irrespective of groups. Thus future studies would find it beneficial to introduce a greater difference between groups for a dietary intervention of this nature.

Blinding in the QuEST Study

Due to the nature of this intervention women were not blinded to their allocated dietary group, as they were required to make modifications to their usual egg intake at home. The lack of blinding led to higher initial rates of withdrawals in the egg free group, three of which withdrew immediately after randomisation and thus did not start the intervention. However despite higher rates of withdrawals in the egg-free group initially, overall withdrawal rates for this study were low, and thus would have had minimal impact on the presented results.
I acknowledge the risk of bias when conducting an open label study, however multiple precautions were undertaken to minimise participant bias. Participant dietary recall bias was minimised by providing a diary card to record any egg exposure on a daily basis, in combination with telephone calls to assess compliance, and to maintain participant accountability. The rates of compliance in the egg-free group demonstrate overall honesty with dietary reporting, however we acknowledge that relying on participant reporting has limitations. Unfortunately this is difficult to avoid in dietary studies that are not conducted in controlled environments. Research staff were not blinded to intervention group during phone calls or clinic assessments because compliance with the intervention was assessed at each time point. As the primary and secondary outcomes were objective lab based outcomes (breast milk OVA and serum immunoglobulin levels), and all laboratory work was conducted in a blinded manner, I am confident that my results were not compromised by the open label design of this study.

Breast milk samples
A high proportion of breast milk samples assayed for OVA were below the limit of detection, demonstrating that the sampling procedure was not optimal. Due to the transient nature of OVA in breast milk (128), my results demonstrate that taking a single sample within a window of two to six hours after ingestion does not capture breast milk OVA in all women. This is likely attributed to complex biological differences, which influence the passage of food protein from the maternal diet into breast milk. The foods ingested with or before their egg consumption could have influenced the passage of egg protein into breast milk by delaying excretion (110), which was also not captured in this study. Overall our primary aim in this study was to report differences in OVA concentration between intervention groups. As such these considerations do not affect the data presented in this thesis. However the consequence was a reduced number of breast milk samples with detectable levels of OVA, and this may have contributed to the lack of detectable differences between intervention groups at two and four weeks of lactation.
To adequately examine whether breast permeability influences OVA concentration, peak concentration of OVA in breast milk (as measured previously by Palmer et al. (128)) would more accurately assess this association. The flexibility that was created around breast milk and egg ingestion collection in this study was designed to optimise compliance for participating mothers. However the variation in the type of egg ingested and timing of the sample makes it difficult to rule out breast permeability as a factor that may influence the passage of OVA into breast milk. Other larger studies involving controlled egg challenges, varied states of fasting, and multiple samples are needed to investigate which biological factors could influence the transfer of allergen from the diet to breast milk, including breast permeability.

_Sensitisation and eczema rates_

Overall, both sensitisation and eczema rates were lower than anticipated in the QuEST study. This restricted our ability to draw any conclusions about the role of maternal dietary ingestion of egg during early lactation on egg-specific sensitisation in breastfed infants. The addition of a trial inclusion requirement to be an atopic mother, measured by objective measures of sensitisation (SPT or IgE), would have increased the allergy risk factor for the infants in this study, and potentially increased the number of atopic infants by four months of age.

### 6.4 Conclusions and future directions

This thesis contributes novel insights into egg allergen exposure for oral tolerance induction during the postnatal period, particularly prior to the introduction of solid foods. I have identified that infants with eczema require food allergy prevention strategies implemented prior to 4 months of age. I have also shown that maternal dietary ingestion of egg while breastfeeding may provide an important early source of egg allergen exposure in breastfed infants for the promotion of oral tolerance.
6.4.1 *STAR Study*

The STAR study highlighted the risk for early life food-specific immune dysregulation in infants with eczema. Here we showed that egg-specific immune responses prior to the introduction of solid foods predicted egg allergy at 12 months of age, and that age of egg in solid foods introduction had no effect on egg-specific cytokine production. The STAR study raises some important questions that should be addressed in future studies.

Firstly, while we were able to use PBMC’s to examine T cell function through induced cytokine production, there is scope to extend this to examine tissue specific T cell populations (skin homing versus gut homing) that are activated and programmed via allergen exposure at their corresponding sites (257). The longitudinal analysis of these populations in response to regular oral allergen exposure would provide a more comprehensive look at the plasticity of T cell function in two predominant sites of allergen exposure in infants with eczema.

Secondly, to further examine allergen exposure in the early postnatal period, and the role that impaired skin and mucosal barriers may play, future studies may consider measuring environmental allergen exposure (dust) in combination with TEWL to assess the risk for transcutaneous sensitisation. Furthermore, interventions to promote skin integrity in the perinatal period, or even before this, are an important avenue of future research. As a major risk factor for food allergy, any strategies that can prevent development of eczema will substantially reduce the risk of food allergy and possibly other aspects of the ‘atopic march’. Factors which contribute to gut inflammation and permeability may be of equal importance. Further studies are also needed to assess oral allergen exposure in the context of gut permeability to shed light on how this influences the development of oral tolerance. Again, the role of early colonisation (the developing gut microbiome) and breast milk composition are likely to be important determinants of gut permeability, the propensity for mucosal inflammation, and the success of local immune regulation.
6.4.2 The QuEST Study

The most significant conclusion of the QuEST RCT was that infant egg-specific IgG4 levels could be influenced by a maternal dietary egg intervention, which increased OVA concentration of human breast milk over a six week period. From these findings however, we cannot determine whether this has any influence on tolerance induction in the infant, and larger studies powered for clinical outcomes are required. This does demonstrate that infant egg allergen exposure during lactation can be manipulated via maternal diet, and is sufficient to increase infant production of specific IgG4 as an indicator of robust systemic immune responses following allergen exposure. At this time the clinical implications are unknown, and follow-up of these infants for the presence of IgE mediated egg allergy at 12 months of age is underway. However the sample size for this study is limited and not designed to assess clinical outcomes. This necessitates, larger, well designed randomised controlled trials to fully assess the role of food proteins in breast milk on the development of clinical measures of oral tolerance. It was also beyond the scope of this thesis to perform detailed examination of the underlying cellular responses to allergens (including flow cytometric studies and cytokine profile studies in cell culture), which will be the subject of future studies. Previously epigenetic studies by our group and others have revealed novel signatures in children with food allergy, including differential gene expressions in the mucosal network pathways (suggesting a roll of IgA pathways) (35, 42). Applying similar methodologies to samples from the QuEST population might also provide valuable insights in future mechanistic studies.

With data available from this thesis and the existing literature, future research into breast milk as a vector to provide allergen exposure for breastfed infants should consider the following:

i) The target population – as infants with a compromised skin barrier are at the greatest risk for early life sensitisation, maternal interventions during lactation may be of greater importance in this population. Identification of infants with a compromised skin barrier in the neonatal period prior to the onset of clinical symptoms (eczema) poses difficulty,
however with new evidence that TEWL at birth predicts food allergy later in life, there may be the potential to use TEWL as a screening tool to identify high risk infants at birth (249).

ii) *Duration of the intervention* – it is logical to address maternal diet over a longer period, including in pregnancy. As yet, the role of allergen exposure and the antenatal period (as a preventive measure) is not clear. Most previous studies examined maternal avoidance with no effect (144). For potential interventions directly targeting lactation, it remains unclear whether these should be throughout breastfeeding or at a minimum until solid foods are introduced. Earlier measures of oral tolerance and sensitisation may be important to assess the effectiveness of these interventions in future studies.

iii) *The nature of the dietary intervention* – Blinded studies are ideal for the highest level of study design quality but do not replicate real life scenarios. Futures studies need to balance the need for open label interventions that can be translated more readily to future dietary recommendations against the need for blinded interventions to eliminate study bias. The ideal scenario of a standardised study product, taken fasting (in the morning) to eliminate confounders from foods eaten with the allergen, may not be realistic. There is danger of ‘over-medicalising’ the normal process of eating.

iv) *Breast milk* measurement of allergens and immune factors - due to the transient nature and the difficulties associated with measuring food protein in breast milk, focusing on infant immune markers may be a more reliable measure of infant allergen exposure (such as serum-specific IgG4). Whilst this thesis focused on *allergen exposure* through breast milk to induce oral tolerance via the gut immune network, there remains significant questions about the role of various immune factors and allergen-immune complexes also found in human milk (258). The examination of various immunological
constituents of human milk, and their role in the development of oral tolerance remains an important question.

This study has explored the timing and mode of allergen exposure in relation to infant food-specific immune responses in two substantial periods in early life. With a particular focus on the period prior to the introduction of solids, the novel findings herein provides evidence that egg-specific immune dysregulation established prior to the introduction of solid foods may have lasting effects on the development of egg allergy in infants with eczema. However breast milk may be a possible route for oral egg exposure in the postnatal period, which induces systemic protective immune responses, evident through the production of specific-IgG4. Thus strategies to improve skin barrier function in early life, and promote maternal dietary ingestion of allergenic foods during lactation, may be future considerations for the induction of oral tolerance in breastfed infants. This research lays the foundation for future studies exploring the use allergen exposure in the prevention of food allergy, and makes important contributions in the pursuit to optimise tolerance induction in the early postnatal period.
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Appendix A

Published article

Early regular egg exposure in infants with eczema: A randomised controlled trial

Debra J Palmer, Jessica Metcalfe, Maria Makrides, Michael Gold, Patrick Quinn, Christina West, Richard Loh and Susan Prescott
Early regular egg exposure in infants with eczema: A randomized controlled trial

Debra J. Palmer, PhD,a,b Jessica Metcalfe, BSc,a Maria Makrides, PhD,b,c Michael S. Gold, MD,d,e,f Patrick Quinn, MBBS,d Christina E. West, MD, PhD,g,h Richard Loh, MD, and Susan L. Prescott, MD, PhD,i,j

Background: Observational studies suggest that early regular ingestion of allergenic foods might reduce the risk of food allergy.

Objective: We sought to determine whether early regular oral egg exposure will reduce subsequent IgE-mediated egg allergy in infants with moderate-to-severe eczema.

Methods: In a double-blind, randomized controlled trial infants were allocated to 1 teaspoon of pasteurized raw whole egg powder (n = 49) or rice powder (n = 49) daily from 4 to 8 months of age. Cooked egg was introduced to both groups after an observed feed at 8 months. The primary outcome was IgE-mediated egg allergy at 12 months, as defined based on the results of an observed pasteurized raw egg challenge and skin prick tests.

Results: A high proportion (31% [15/49]) of infants randomized to receive egg had an allergic reaction to the egg powder and did not continue powder ingestion. At 4 months of age, before any known egg ingestion, 36% (24/67) of infants already had egg-specific IgE levels of greater than 0.35 kilounits of antibody (kU/L). At 12 months, a lower (but not significant) proportion of infants in the egg group (33%) were given a diagnosis of IgE-mediated egg allergy compared with the control group (51%); relative risk, 0.65; 95% CI, 0.38-1.11; P = .11).

Egg allergy is the most common food allergy, now affecting 8.9% of children at 1 year of age in Australia.1 With increasing rates of food allergy,2 there is ongoing confusion and controversy over the role of allergenic foods in the development of food allergy. Until recently, it has been common practice to avoid egg and other allergenic foods for the primary prevention of food allergy.3 Although guidelines have been revised to indicate that there is insufficient evidence to support this,4,5 it is recognized that the level of evidence in this area is generally weak and largely based on observational studies with methodological limitations and that randomized controlled trials are needed to address this more conclusively.

Animal studies have shown that the development of oral tolerance is driven by regular allergen exposure and that avoidance strategies might increase the risk of adverse immune responses to allergens.6 The potential role of regular food allergen exposure to induce tolerance in human subjects is also illustrated by studies of specific oral tolerance induction in children with food allergy.6,7 Animal studies have also shown that early exposure to repeated doses of food proteins (allergens) can induce oral tolerance during a critical early window of development.8 Although the timing of this potential window is not clear in human subjects, delayed introduction of specific foods (egg, cow’s milk, fish, and oats) beyond 6 to 9 months of age has been associated with increased risk of allergic disease in nonintervention cohort studies.11,12 The Australian HealthNuts study13 found that delaying introduction of egg until 10 to 12 months of age (adjusted odds ratio, 1.6; 95% CI, 1.0-2.6) or after 12 months of age (adjusted odds ratio, 3.4; 95% CI, 1.8-6.5) was associated with significantly higher risk of egg allergy compared with earlier introduction at 4 to 6 months of age. Thus early oral exposure to egg might be an important strategy to prevent or reduce the risk of egg allergy.

Here we report the first randomized controlled trial to investigate whether early introduction of egg reduces the risk of egg allergy in infants with a history of eczema. Infantile eczema is an important risk factor for food allergies,19 and we targeted this population based on their greater burden of disease and because they are most likely to benefit from the prevention of food allergy.
METHODS

Study design

Singleton term infants with symptoms of moderate-to-severe eczema (determined by using a standardized SCORAD score of ≥15) were recruited at 4 months of age from 2 Australian centers (Adelaide and Perth). Infants who had commenced solids before 4 months of age or who had any previous known direct ingestion of egg were excluded. Written informed consent was obtained before trial participation. Approval was granted by the local institutional review boards (Human Research Ethics Committees) of each center and the Women’s and Children’s Health Network, Adelaide and Princess Margaret Hospital, Perth. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12609000415202).

The study was conducted by using a double-blind, randomized, controlled trial design. Baseline characteristics, including maternal age at birth, maternal race, cesarean delivery, smoking in the household, family (first-degree relative) history of allergic disease, infant sex, infant dietary information on breastfeeding and/formula feeding, and infant history of treatments used for eczema, were recorded at randomization at 4 months of age. A blood sample was collected before the first exposure to the study powder. Baseline egg-specific IgE and IgG levels were analyzed at the completion of the trial and did not influence eligibility.

Randomization and blinding

Each participating infant was assigned a unique study number and randomly allocated to one of two intervention groups. A computer-generated randomization schedule was produced by an independent consultant. The schedule was stratified by infant sex and feeding mode (breast-fed or formula fed if receiving ≥200 mL of infant formula per day) at 4 months of age.

Independent research assistants coded the identically packaged dietary intervention powders, and these research assistants were not involved in the dietary group allocation or assessment process, thus keeping the outcome assessments blinded.

Dietary intervention

The trial compared the effects of 2 food powders (egg and rice) in infants’ diets given daily from randomization at 4 months of age until 8 months of age. For both groups, the study powder was administered orally by mixing the powder with infant rice cereal. The intervention group was allocated to 1 teaspoon (≈ 0.9 g of egg protein, which is equivalent to one sixth of an egg) per day of pasteurized raw whole egg powder manufactured by Farm Pride Foods (Keysborough, Australia). The control group received 1 teaspoon (≈ 0.23 g of rice protein) per day of rice flour powder (ingredients: white rice only) manufactured by Ward McKenzie Pty Ltd (Altona, Australia). Rice was chosen as the placebo (control group) because rice cereal is commonly the first food introduced and IgE-mediated allergic reactions to rice are uncommon. A medical assessment, including an observed ingestion of the allocated study powder dose (where appropriate), was conducted to confirm any possible allergic reactions to the study powder before a decision was made to cease the powder use. Any infant whose powder use was ceased was still included in all follow-up assessments. Infants in both groups were advised to follow an egg-free diet (with avoidance of egg protein in any food, including foods cooked with egg as an ingredient) from 4 to 8 months of age by an experienced pediatric dietician and to introduce other solid foods based on family diet preferences and the infant’s individual feeding skill development.

Statistical analysis

A sample size estimate was calculated based on the assumption that the expected prevalence of IgE-mediated egg allergy at 12 months of age in a population of infants with eczema would be 40%,12 and therefore to detect an absolute reduction of 20% (relative reduction of 50%), from 40% to 20% (with 85% power, α = .05), we would have required 103 infants per group. Allowing for 10% loss to follow-up, the aim was to recruit a total of 226 infants into the trial. However, the study recruitment was paused in September 2011 at the request of the Human Research Ethics Committee at Princess Margaret Hospital, Perth, Australia, to examine the rate of allergic reactions to the study powder and cases of anaphylaxis. An independent, unblinded data safety monitoring committee review was undertaken, and the recommendation from this Committee was that the trial should continue. The decision was made by the ethics committee to reopen the trial for recruitment in May 2012; however, by this time, insufficient funds remained to recommence recruitment, and the chief investigators decided the trial should be terminated early without reaching the sample size originally estimated.

Analyses were performed according to the intention-to-treat principle. The proportion of infants with diagnosed IgE-mediated egg allergy at 12 months of age was compared between groups. Secondary comparisons between groups included the proportion of children with cooked egg allergy, eczema severity...
RESULTS
Enrollment for the trial began on July 15, 2009, and ended on September 7, 2011. Eighty-six infants were randomized into the trial, with 49 randomized to the egg group and 37 randomized to the rice group. There were no significant differences in baseline characteristics between the 2 groups (Table I). Data collection was completed on May 25, 2012. Ninety percent (77/86) of infants attended their final appointment, with 77 (90%) of 86 infants having SPTs and 67 (78%) of 86 infants undertaking an egg challenge. Nine (2 in the rice group) parents withdrew their infant’s consent to participate during the study for the following reasons: became too busy to attend hospital appointments (n = 4, 1 in the rice group), did not like the study powder (n = 2, 1 in the rice group), infant had repeated illnesses (n = 1), family moved overseas (n = 1), and parents did not want the raw egg challenge (n = 1).

Intervention, compliance, and safety
A high proportion (21% [18/86]) of infants randomized had an allergic reaction to their allocated study powder. The proportion of reactors was higher (31% [15/49]) in those allocated to receive egg. Most of these (10/15) had a reaction on first exposure to the egg powder, including 1 case of anaphylaxis. Three infants in the rice group had allergic reactions (all had generalized skin erythema and vomiting) to the rice powder, and these infants tended their final appointment, with 77 (90%) of 86 infants undertaking an egg challenge. Nineteen (10 in the rice group) parents withdrew their infant’s consent to participate during the study for the following reasons: became too busy to attend hospital appointments (n = 4, 1 in the rice group), did not like the study powder (n = 2, 1 in the rice group), infant had repeated illnesses (n = 1), family moved overseas (n = 1), and parents did not want the raw egg challenge (n = 1).

TA B LE I. Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Egg group (n = 49)</th>
<th>Control group (n = 37)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age at birth (y)^</td>
<td>32.8 (5.5)</td>
<td>32.1 (3.4)</td>
<td>.48</td>
</tr>
<tr>
<td>Maternal white race†</td>
<td>36 (73%)</td>
<td>32 (86%)</td>
<td>.14</td>
</tr>
<tr>
<td>Cesarean section birth†</td>
<td>17 (35%)</td>
<td>11 (30%)</td>
<td>.63</td>
</tr>
<tr>
<td>Maternal history of allergic disease†</td>
<td>37 (76%)</td>
<td>25 (68%)</td>
<td>.42</td>
</tr>
<tr>
<td>First-degree relative history of allergic disease†</td>
<td>44 (90%)</td>
<td>35 (95%)</td>
<td>.69</td>
</tr>
<tr>
<td>Infant male sex‡</td>
<td>31 (63%)</td>
<td>26 (70%)</td>
<td>.50</td>
</tr>
<tr>
<td>Age of onset of eczema (mo)^</td>
<td>1.8 (1.1)</td>
<td>1.8 (0.9)</td>
<td>.75</td>
</tr>
<tr>
<td>Eczema severity (objective SCORAD score)^‡</td>
<td>33.8 (29.2-37.5)</td>
<td>32.7 (25.0-39.5)</td>
<td>.46</td>
</tr>
<tr>
<td>Use of prescription steroid cream^</td>
<td>40 (82%)</td>
<td>28 (76%)</td>
<td>.50</td>
</tr>
<tr>
<td>Ever breast-fed‡</td>
<td>48 (98%)</td>
<td>37 (100%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Breast-fed at randomization‡</td>
<td>40 (82%)</td>
<td>31 (84%)</td>
<td>.96</td>
</tr>
<tr>
<td>Smoking in the household^</td>
<td>8 (16%)</td>
<td>5 (8%)</td>
<td>.34</td>
</tr>
</tbody>
</table>

Values are presented as follows: *means (SDs), †numbers (percentages), or  \( \bar{x} \) (medians (IQRs)).

For the primary outcome, a lower proportion of infants in the egg group (33% [14/42]) were given a diagnosis of IgE-mediated egg allergy at 12 months of age compared with the control group (51% [18/35]); however, the difference did not reach statistical significance (relative risk [RR], 0.65; 95% CI, 0.38-1.11; \( P = .11; \) Fig 1). Overall, 22 (33%) of 67 of the infants who underwent the pasteurized raw egg challenge had an allergic reaction. Ten infants did not have a pasteurized raw egg challenge because of an independent medical decision not to proceed based on a previous documented allergic reaction to egg and associated evidence of sensitization (positive SPT response) to egg. Secondary outcome analyses found a lower proportion of infants in the egg group (45% [19/42]) were sensitized to egg (positive SPT response) at 12 months of age compared with the control group (63% [22/35]); however, the difference did not reach statistical significance (RR, 0.72; 95% CI, 0.47-1.09; \( P = .12; \) Fig 1). There were no differences in the severity and extent of eczema (objective SCORAD score) at 8 months of age (median in the egg group of 7.6, with an interquartile range [IQR] of 3.6-14.5 \( [n = 42] \) and median in the control group of 7.8, with an IQR of 3.6-14.1 \( [n = 35] \); \( P = .80; \) or at 12 months of age (median in the egg group of 7.2, with an IQR of 0.0-12.2 \( [n = 42] \) and median in the control group of 8.2, with an IQR of 0.0-14.4 \( [n = 35] \); \( P = .35 \). There was also no difference in the proportion of infants using prescription steroid cream between 4 and 12 months of age (90% vs 97% in the egg and control groups, respectively; \( P = .37; \) or in the number of visits to a doctor for eczema (1 visit on average in each group, \( P = .75 \). At 8 months of age, the rate of allergic reactions to cooked egg was 16% (12/75): 6 (15%) of 40 in the egg group and 6 (17%) of 35 in the control group (RR, 0.88; 95% CI, 0.31-2.47; \( P = .80; \)
TABLE II. Clinical outcomes of infants (n = 18) who had an allergic reaction to the study powder

<table>
<thead>
<tr>
<th>Allocated study powder</th>
<th>Doses of study powder before powder use ceased</th>
<th>Cooked egg exposure</th>
<th>Pasteurized raw egg challenge</th>
<th>IgE-mediated egg allergy at 12 mo of age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg</td>
<td>6 Allergic reaction</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>3 Tolerated</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 Tolerated</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 No exposure</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 No exposure</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>5 Allergic reaction</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>3 Allergic reaction</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 No exposure</td>
<td>Withdrawn</td>
<td>Unknown (withdrawn)</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 Tolerated</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>43 Tolerated</td>
<td>Tolerated</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 Tolerated</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 Allergic reaction</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 Tolerated</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 No exposure</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>1 No exposure</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>7 Allergic reaction</td>
<td>No challenge (anaphylaxis to cooked egg exposure)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>3 Tolerated</td>
<td>Allergic reaction</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>3 Allergic reaction</td>
<td>No challenge</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

Eleven infants did not have cooked egg exposure: 4 because of independent medical advice after an allergic reaction to the study powder, 1 because of repeated illnesses, and 6 because they were withdrawn. Twenty-one (95%) of 22 infants (6 in the egg group and 15 in the control group) who reacted to the pasteurized raw egg challenge were able to tolerate cooked egg previously.

**FIG 1. IgE-mediated egg allergy and positive SPT response (+ SPT) to egg at 12 months of age. A, Proportion of infants. B, RR between the egg and control groups.**

IGE and IgG antibody measurements

There were no differences in baseline egg-specific IGE levels between the groups or at any other time point (Table III). At 4 months of age, before any known ingestion of egg, 36% (24/67) of the infants already had egg-specific IGE levels of greater than 0.35 kU/L. Within the egg group at 4 months of age, the egg-specific IGE concentrations were significantly greater (P = .001) for those who had an allergic reaction to the egg powder (median, 0.78 kU/L; IQR, 0.55-2.07 kU/L; n = 11) compared with those who tolerated the powder (median, 0.05 kU/L; IQR, 0.05-0.39 kU/L; n = 24).

Early ingestion of egg (egg group) was associated with significantly (P < .001) and persistently higher egg-specific IgG levels (Fig 2 and Table III). The median IgE/IgG ratio at 12 months of age in the egg group (0.39; IQR, 0.05-4.15) was significantly lower (P = .001) than in the control group (5.14; IQR, 1.43-25.28). In infants with IgE-mediated egg allergy, the median IgE/IgG ratio at 12 months of age (median, 0.35; IQR, 0.05-1.43; Fig 3). The egg-specific IgE concentrations at 12 months of age in infants with IgE-mediated egg allergy (median, 2.37; IQR, 1.23-9.72) were also significantly higher (P < .001) than for infants who tolerated the raw egg challenge (median, 0.13; IQR, 0.05-0.76; Fig 4).

**DISCUSSION**

This is the first reported randomized controlled trial to investigate the hypothesis that early regular oral exposure to an allergenic food can induce oral tolerance and reduce the risk of subsequent food allergy. We specifically targeted children with moderate-to-severe eczema in this study because of their particularly high risk of food allergy. Recognizing that neither the rate of sensitization nor the rate of clinical reaction has previously been described in this population at this very young age, we adopted a “community scenario” approach in this study and elected not to pretest or exclude children on the basis of an egg-specific IgE levels at randomization. As a result, we observed a high proportion (36%) of infants already sensitized to egg before randomization at 4 months of age, and 31% who were allocated to receive egg powder had a clinical reaction, including 1 case of anaphylaxis. This clearly indicates that a high proportion of young infants with moderate-to-severe eczema are already
sensitized to egg before commencing solid foods (in all cases there was no previous history of known direct ingestion of egg) through other routes, potentially in utero across the placenta, through the defective skin barrier, or through breast milk, much earlier than 4 months of age and emphasizes the need for caution when first introducing allergenic foods to this high-risk group. Importantly, it is also increasingly clear that the processes leading to food sensitization are already strongly established by 4 months of age, indicating that much earlier preventive interventions will ultimately be needed. Differences in neonatal immune function of children with subsequent food allergy suggest that these events are initiated in utero and consolidated during the very early postnatal period. With such a dramatic increase in food allergy, there is a pressing need to define events around much earlier allergen encounter.

This study was terminated early for logistic reasons (see the Methods section), and we acknowledge that this is a major limitation because of the resulting insufficient power to show statistically significant definitive results. Even so, the trend for lower incidence of egg allergy in the egg group (33%) compared with the control group (51%) reduces previous concerns that early introduction of this allergenic food would be associated with increased egg allergy risk. In fact, the data point to the contrary and deserve further study. There are now at least 3 other randomized controlled trials (Trial Registry details: ACTRN 12610000388011, ACTRN 12611000535976, and JPRN-UMIN000008673) investigating early regular egg exposure to reduce the risk of egg allergy development. However, each of these trials is targeting infants at lower risk of egg allergy than those in the present study. Our present findings in this very high-risk population will therefore contribute a valuable dimension to the composite picture that will emerge as the results of each of these trials come to light.

TABLE III. Egg-specific IgE and IgG₄ antibody concentrations

<table>
<thead>
<tr>
<th></th>
<th>Egg group</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg-specific IgE level (kU/A/L) at 4 mo of age</td>
<td>0.23 (0.05-0.78), n = 35</td>
<td>0.05 (0.05-0.31), n = 31</td>
<td>.40</td>
</tr>
<tr>
<td>Egg-specific IgE level (kU/A/L) at 8 mo of age</td>
<td>0.34 (0.05-0.86), n = 36</td>
<td>0.52 (0.05-3.92), n = 23</td>
<td>.22</td>
</tr>
<tr>
<td>Egg-specific IgE level (kU/A/L) at 12 mo of age</td>
<td>0.54 (0.05-2.55), n = 40</td>
<td>0.40 (0.05-2.32), n = 29</td>
<td>.88</td>
</tr>
<tr>
<td>Egg-specific IgE level (mg/A/L) at 4 mo of age</td>
<td>0.04 (0.04-0.04), n = 35</td>
<td>0.04 (0.04-0.07), n = 30</td>
<td>.23</td>
</tr>
<tr>
<td>Egg-specific IgE level (mg/A/L) at 8 mo of age</td>
<td>1.00 (0.06-3.00), n = 36</td>
<td>0.04 (0.04-0.04), n = 23</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Egg-specific IgE level (mg/A/L) at 12 mo of age</td>
<td>1.76 (0.16-9.00), n = 40</td>
<td>0.04 (0.04-0.74), n = 29</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Values are presented as medians (IQRs).

mg/A/L, Milligrams of antibody per liter.

FIG 2. Egg-specific IgG₄ (in milligrams of antibody per liter) concentrations at 4, 8, and 12 months of age. NS. Not significant.

FIG 3. IgE/IgG₄ ratios at 12 months of age in infants with IgE-mediated egg allergy compared with those seen in infants who tolerated the egg challenge. For infants with IgE-mediated egg allergy, the median IgE/IgG₄ ratio in the egg group was 2.42 (IQR, 1.58-7.50), and that in the control group was 2.32 (IQR, 1.91-11.40). For infants who tolerated the egg challenge, the median IgE/IgG₄ ratio in the egg group was 0.13 (IQR, 0.05-0.84), and that in the control group was 0.05 (IQR, 0.05-0.60).

FIG 4. Egg-specific IgE concentrations at 12 months of age in infants with IgE-mediated egg allergy compared with those seen in infants who tolerated the egg challenge. For infants with IgE-mediated egg allergy, the median IgE concentration in the egg group was 2.42 (IQR, 1.58-7.50), and that in the control group was 2.32 (IQR, 1.91-11.40). For infants who tolerated the egg challenge, the median IgE concentration in the egg group was 0.13 (IQR, 0.05-0.84), and that in the control group was 0.05 (IQR, 0.05-0.60).
We chose a particularly allergenic form of egg for the intervention group study powder, namely pasteurized raw egg, which has equivalent allergenic properties to those of raw egg. The rationale was to induce tolerance to the range of epitopes encountered in the most allergenic forms of egg by using a powder form that could be easily mixed with the infant’s solid foods. However, this form of egg is also more likely to induce reactions in infants who are already sensitized. It is possible that early intervention with cooked or baked egg might achieve tolerance with less risk of reactivity, although the observational Australian HealthNuts study suggested that first exposure to more allergenic (unbaked) egg was more likely to reduce the egg allergy risk. More intervention studies are needed to determine how best to deliver the allergen, although ideally, this should be in natural foods.

In conclusion, induction of immune tolerance pathways and reduction in the egg allergy rate can be achieved by early regular oral exposure to egg from 4 months of age in infants with moderate-to-severe eczema. The earlier introduction of egg in oral exposure to egg from 4 months of age in infants with moderate-to-severe eczema already and clinical reactivity by 4 months of age. This points to much earlier events in the initiation of food sensitization and reactivity in these high-risk infants are first exposed to egg because many have sensitization already and clinical reactivity by 4 months of age. The points to much earlier events in the initiation of food sensitization, wellness before the introduction of complementary feeding.

We thank the families who participated and the following research staff and students who supported the data collection: Vicki Barrett, Daniella Calderisi, Patricia-Cathelbert, Carol Garfield, Heather Garrefa, Joanne Gooden, Henning Johannsen, Michaela Lucas, Suzi McCarthy, Alison McQueen, Sharon Nicholls, Diane Videky, Rachel West, and Brianna White. We also thank the trial’s Serious Adverse Event committee (Philip Ryan, Nick Manton, and Robert Heddle) and Data Safety Monitoring Committee (Philip Ryan, Robert Heddle) and Data Safety Monitoring Committee (Philip Ryan, Robert Heddle, and Jo Zhou).

**REFERENCES**


Appendix B
Published article

Randomised controlled trials investigating the role of allergen exposure in food allergy: Where are we now?

Jessica Metcalfe, Susan L Prescott and Debra Palmer
Randomized controlled trials investigating the role of allergen exposure in food allergy: where are we now?

Jessica Metcalfe, Susan L. Prescott, and Debra J. Palmer

Purpose of review
The dramatic increase in food allergy stresses the need for more definitive treatment strategies that induce lasting oral tolerance in tandem with more effective approaches to primary prevention. Allergen-induced oral tolerance is now of prime interest in both of these settings as a potentially more effective approach to traditional avoidance strategies. Here, we review the recent randomized controlled trials (RCTs) examining the controlled allergen exposure in both treatment and prevention of food allergy.

Recent findings
Collectively, RCTs of oral immunotherapy (OIT) for the treatment of food allergy increase the amount of food allergen that can be tolerated. Allergic side-effects are common and this remains a major obstacle to general use in clinical practice. There are also at least eight RCTs currently in progress investigating early allergen exposure for the primary prevention of food allergy.

Summary
OIT is showing promise as a possible treatment for food allergy; however, more large, longitudinal studies are needed to optimize both safety and efficacy and to assess the long-term effects, before this can be considered in clinical practice. The results of the primary prevention studies will be of great importance in determining the role of earlier introduction of allergenic foods in reducing the burden of food allergy.

Keywords
allergen exposure, food allergy, oral immunotherapy, oral tolerance, randomized controlled trial

INTRODUCTION
The incidence of food allergy has increased dramatically over the last 10–20 years, with the greatest burden of this new epidemic affecting young children particularly in their first years of life. In developed regions such as Australia, recent studies have found more than 20% of 1-year-olds are now sensitized to foods and that more than 10% have clinical challenge-proven, IgE-mediated food allergy [1]. Food anaphylaxis in preschool children has also substantially increased, with a five-fold rise in over just 10 years [2]. Similar trends are now emerging in the developing regions [3]. Along with increasing persistence of disease [4], this is contributing to a growing burden of food allergy in many communities.

Traditionally, the only treatment available for a food allergy has been a strict avoidance diet of the food allergen. These diets are difficult to maintain, and accidental exposure is still likely, with many individuals on avoidance diets still experiencing severe reactions [5]. The ever-present fear of potentially life-threatening reactions places the families under enormous stress, and has been shown to substantially decrease the quality of life when compared with the general population, and to an even greater extent than seen in other chronic conditions [6].

There has been recent debate around whether allergen ‘controlled exposure’ rather than ‘complete avoidance’ may be the ultimate solution for both treatment and prevention of food allergy. The merits of various allergen-based delivery

School of Paediatrics and Child Health Research, University of Western Australia, Perth, Western Australia, Australia

Correspondence to Debra J. Palmer, PhD, School of Paediatrics and Child Health Research, University of Western Australia, PO Box D184, Princess Margaret Hospital, Perth, WA 6001, Australia. Tel: +61 8 9340 8171; fax: +61 8 9389 2097; e-mail: debbie.palmer@uwa.edu.au

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DOI:10.1097/ACI.0b013e3283609671
Table 1. Recent randomized controlled trials on food allergen exposure for the treatment of food allergy

<table>
<thead>
<tr>
<th>Study</th>
<th>Inclusion criteria</th>
<th>Oral immunotherapy (OIT) protocol</th>
<th>Food challenges and efficacy measures</th>
<th>Withdrawals</th>
<th>Main results</th>
<th>Safety</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burks et al. 2012 [20] USA Hen’s egg Double-blind placebo controlled (n = 59)</td>
<td>Egg allergic children 5–18 Years old without a history of anaphylaxis Defined as: 1. Six years and older specific IgE greater than 5 kU/L 2. Between 5 and 6 years old specific IgE greater than 12 kU/L No DBPCFC at baseline</td>
<td>Intervention group (n = 40): 3. Maintenance phase: 2 g of egg white powder daily Treatment duration: 22 months Control group (n = 19): Placebo for 10 months only</td>
<td>Oral food challenge (OFC) at 1. 10 months of OIT: 5 g of egg white powder (both groups) 2. 22 months of OIT: 10 g of egg white powder (intervention group only) 3. 24 months after 4–6 weeks egg-free period: 10 g egg white powder and one whole-cooked egg SPT at baseline and 22 months egg-specific IgE and IgG4 measured at all time points</td>
<td>Total: 7 Five in active group because of allergic reactions and Two in placebo group: one because of reaction and one because of transport issues</td>
<td>Challenge outcomes: 10 months: 22 of 40 (55%) of intervention group passed OFC and 0 of 15 in the control group 22 months: 30 of 40 (75%) passed OFC 24 months: 11 of 27 (40%) passed OFC (after egg-free period) Immune outcomes: IgG4: Median IgG4 of tolerant patients at 10, 22 and 24 months was significantly higher than children who reacted SPT: Decreased SPT wheal size from baseline at 22 months was associated with sustained desensitization at 24 months (P &lt; 0.01)</td>
<td>No serious adverse events Adverse events were highest in the first 10 months and mostly associated with OIT dosing</td>
</tr>
<tr>
<td>Varshney et al. 2011 [21] USA Peanut Double-blind placebo controlled (n = 28)</td>
<td>Peanut allergic children 1–16 years of age Defined as: Reaction within 6 min of peanut ingestion and a peanut-specific IgE &gt;15 kU/L SPT to peanut &gt;3 mm No DBPCFC at baseline</td>
<td>Intervention group OIT (n = 19): 1. Peanut four Control group (n = 9): Placebo powder Maintenance phase: 4000 mg protein for 44 weeks Treatment duration: 48 weeks</td>
<td>DBPCFC at 48 weeks. Cumulative intake of 5000 mg peanut protein Peanut-specific total IgE, IgG and IgG4 measured at 2, 6, 9 and 12 months (OFC) Th2 cytokines IL-5 and IL-13 measured from PBMC’s cultured with crude peanut extract</td>
<td>Total: 3 All in peanut OIT group early in study because of allergic side-effects</td>
<td>Challenge outcomes: All peanut OIT reaching OFC (16 of 19) consumed entire dose 3.5 g/500 mg protein compared with control group 4. Peanut-specific total IgE, IgG and IgG4 measured at 2, 6, 9 and 12 months (OFC) Th2 cytokines IL-5 and IL-13 measured from PBMC’s cultured with crude peanut extract</td>
<td>Doses of epinephrine: 2 Both in the active OIT group during the initial daily escalation phase No epinephrine required during the home doses in the peanut OIT group</td>
</tr>
<tr>
<td>Martorell et al. 2011 [22] Spain Cow’s milk (CM) Open, controlled (n = 60)</td>
<td>CM allergic children 24–36 months old Defined as: SPT &gt;3 mm and specific IgE level &gt;0.35 kU/L for milk and CM proteins Clinically reactive during DBPCFC at baseline</td>
<td>Intervention group (n = 30): Maintenance phase: 200 ml CM daily plus inclusion of dairy in the diet Control group (n = 30): CM-free diet Treatment duration: 12 months</td>
<td>DBPCFC after 12 months with 200 ml pure cow’s milk only infants in OIT group not tolerating full dose at home and all of control group challenged CM-specific IgE, IgG and IgG4 measured at baseline and</td>
<td>Total: 2 1 because of moving house 1 because of poor tolerance of desensitization protocol</td>
<td>Challenge outcomes: 27 of 30 (90%) patients in active group made it to full dose of 200 ml CM daily by 12 months 3 of 23 (13%) in control group passed OFC at 12 months Immune outcomes: Significant decrease in Peanut IgG4 levels: increased significantly (P &lt; 0.001) Peanut IgG4 levels: increased significantly (P &lt; 0.001) Peanut IgG4 levels: increased significantly (P &lt; 0.001) Peanut IgG4 levels: increased significantly (P &lt; 0.001)</td>
<td>Doses of epinephrine: 2 80% of children in OIT group had clinically significant reactions</td>
</tr>
<tr>
<td>Study</td>
<td>Inclusion criteria</td>
<td>Oral immunotherapy (OIT) protocol</td>
<td>Food challenges and efficacy measures</td>
<td>Withdrawals</td>
<td>Main results</td>
<td>Safety</td>
</tr>
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</tr>
<tr>
<td>Pajno et al. 2010 [23] Italy Cow’s milk Single-blind, placebo controlled (n = 30)</td>
<td>Milk allergic children S—10 years old Defined as: IgE levels not specified, but had to be present SPT &gt;5 mm for CM DBPCFC positive at baseline</td>
<td>Intervention group: Cow’s milk (n = 15) Control group: Soy milk (n = 15) OIT ¼ weekly dosing dose doubled every week in clinic for 18 weeks. Maintenance phase: 200 ml daily Treatment duration: 18 weeks</td>
<td>DBPCFC after 18 weeks of OIT Cumulative dose of 200 ml of pure CM CM-specific IgE and IgG4 levels measured at baseline, 13 weeks and at OFC after 18 weeks</td>
<td>Total: 3 2 withdrawals in the active group 1 withdrawal in the placebo group</td>
<td>Challenge outcomes: 10 of 15 (77%) achieved total tolerance of 200 ml No change in threshold for the placebo group Immune outcomes: No significant change in IgE levels in either groups from baseline to the end of OIT. Three children with serious adverse events had elevated IgE levels. Significant increase in IgG4 levels from 4.5 (2.4) to 23.8 (5.3) mg/ml in the active group (P &lt; 0.003), but not in the control group</td>
<td>Doses of epinephrine: 2 of 15 had severe adverse events requiring termination of OIT</td>
</tr>
<tr>
<td>Skripak et al. 2008 [24] USA Cow’s milk Double-blind, placebo controlled (n = 20)</td>
<td>CM allergic children 6–17 Years old and no history of anaphylaxis Defined as: SPT &gt; histamine Milk-specific IgE &gt;0.35 kU/l Positive DBPCFC to 2.5 g or less of milk protein</td>
<td>Intervention group (n = 13): Nonfat powdered milk Control group (n = 7): Placebo powder Maintenance phase: 500 mg milk protein for 13 weeks Treatment duration: 23 weeks</td>
<td>OFC at 23 weeks of OIT to cumulative 8 g CM protein. CM-specific α-lactalbumin, β-lactoglobulin and casein S1Gp, IgE and IgG4 measured at baseline, when maintenance dose reached (week 10) and after 13 weeks of the maintenance dose (week 23)</td>
<td>Total: 1 1 because of persistent eczema during dose escalation phase</td>
<td>Challenge outcomes: Median threshold quantity Significant increase in the median threshold dose of CM in the OIT group from baseline: 40–5100 mg (P &lt; 0.002) by the end of treatment No change in the control group: 40 mg. Immune outcomes: Increase in IgG4 levels from baseline in the OIT group (P = 0.002). No change in IgE levels in either groups</td>
<td>Doses of epinephrine: 4 (all in OIT group: 2 during initial (n hospital build-up doses and 2 at home)</td>
</tr>
<tr>
<td>Longo et al. 2008 [25] Italy Cow’s milk Open, controlled (n = 60)</td>
<td>Severely CM allergic children Age 5–17 years with IgE levels &gt; 85 kU/l and 1 severe allergic reaction DBPCFC positive reactions to small amounts of milk selected for desensitization (n = 60)</td>
<td>Intervention group (n = 30): Control group (n = 30): CM and all other forms of dairy foods Control group (n = 30): CM-free diet for 1 year followed by DBPCFC Treatment duration: 12 months</td>
<td>OIT group open feeding of 150 ml of cow’s milk at 1 year Elimination group underwent second DBPCFC at 1 year IgE levels and SPT to whole milk measured at enrolment 6 months and 1 year in all individuals</td>
<td>No data available on withdrawals</td>
<td>OIT intervention group: 11 of 30 (38%) of OIT group could ingest 150 ml of CM with no adverse reaction 16 of 30 (54%) had achieved partial tolerance ranging from 5 to 150 ml 3 of 30 (10%) could not continue because of allergic reactions: failed treatment Placebo group: 0 of 30 achieved an increase in oral tolerance Difference between to the two groups significantly different (P &lt; 0.001) CM-specific 5IgE levels decreased significantly in 15 of 30 within the OIT group</td>
<td>Doses of epinephrine: 5 All doses in OIT group, with 4 during in hospital dosing and 1 given during home dosing (in ED) 1 more child attended ED for a reaction whilst on home dosing</td>
</tr>
<tr>
<td>Study Authors</td>
<td>Year</td>
<td>Country</td>
<td>Allergens</td>
<td>Study Design</td>
<td>Inclusion Criteria</td>
<td>Intervention Details</td>
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</tr>
<tr>
<td>Staden et al. 2007 [26]</td>
<td>Germany</td>
<td>Hen’s egg and cow’s milk</td>
<td>Open, controlled</td>
<td>Hen’s egg allergic or CM allergic (n = 47) (median age 2.5 years) Defined as: Confirmed positive DBPCFC at baseline No specific IgE or SPT inclusion criteria</td>
<td>Intervention group: Egg OIT (n = 14) pasteurized cow’s milk (n = 11) Maintenance phase: 3300 mg CM protein or 1600 mg egg protein Control group: Strict avoidance diet (n = 20, 10 in cow’s milk and 10 in hen’s egg) Treatment duration: 11–59 months</td>
<td>DBPCFC 18–24 months after enrolment for both groups depending on when induction phase and maintenance were achieved OIT intervention group went on strict elimination diet 2 months before final food challenge</td>
</tr>
<tr>
<td>Morisset et al. 2007 [27]</td>
<td>France</td>
<td>Hen’s egg (HE) and cow’s milk</td>
<td>Open, controlled (n = 60 CM and n = 90 HE)</td>
<td>CM allergic children aged 13 months to 6.5 years Hen’s egg allergic children aged 1–8 years Allergy defined as positive SPT, the presence of specific IgE antibodies and a positive OFC at baseline</td>
<td>CM intervention group (n = 27): Maintenance phase: 250 ml/day CM, and all dairy products, then unlimited Egg intervention group (n = 49): Maintenance phase: hard-boiled egg daily and then unlimited egg products CM and egg control group (n = 30 and 35): strict elimination diet for 6 months Treatment duration: 6 months</td>
<td>SBPCFC performed after 6 months of treatment with 200 ml CM SPT and specific IgE measured at baseline and 6 months</td>
</tr>
</tbody>
</table>

DBPCFC, double-blinded, placebo-controlled food challenge; ED, Emergency Department; SPT, skin prick test.
Food allergy

concentrations. Some investigators have measured clinical efficacy as an increase in the median threshold amount of allergen tolerated in a DBPCFC at both baseline and at the end of treatment [21,24]. Alternatively some determined efficacy by a pass or fail determination, based on the ability to ingest a total fixed amount of allergen after the intervention period [20,22,23]. Reporting an ‘increase in threshold’ gives a more comprehensive picture than that of a simple ‘pass or fail’ determination, as even children who ‘fail’ a challenge may have shown a marked increase in threshold from their initial baseline challenge. Results have been also reported as ‘full tolerance’, which has been defined as an ability to ingest the entire challenge amount, or ‘partial tolerance’ if the child was able to ingest more than at baseline, but experienced allergic symptoms before completing the challenge [25,26].

Regardless of the varying protocols and allergens, all of these RCTs demonstrated an increase in threshold from baseline to the end of treatment in participants receiving OIT (Table 1). In the study by Skripak et al. [24], the median threshold dose of cow’s milk tolerated significantly increased in the active group from 40 to 5140 mg of protein after OIT, compared with no change in the placebo group. Additionally, the seven children in the placebo group went on to receive the active OIT treatment and achieved an increase in the median dose threshold from 40 to 1840 mg. These findings are also consistent with Varshey et al.’s [21] study with peanut, in which there was an increase in the median threshold in the OIT group from 280 to 5000 mg of protein (approximately 20 peanuts), compared with no increase in the placebo group. Despite these promising observations, a recent meta-analysis deemed there to be insufficient evidence to show any greater clinical benefit of OIT for patients compared with the use of a standard elimination diet [28]. In this meta-analysis however, there were only three RCTs available [24–26] that fit the inclusion criteria and these studies were greatly heterogeneous, making it difficult to provide any statistical significance.

The ultimate goal in the treatment of food allergy is to induce complete and ‘permanent’ immune tolerance to the food allergen, where the individual remains tolerant even after long allergen-free periods. Although OIT appears to be effective whilst the allergen is being consumed regularly, the long-term effects are still uncertain and likely to vary between individuals. Two of the aforementioned studies [20,26] investigated this by including an allergen-free period prior to the final OFC, addressing the question of efficacy with regard to long-term immune tolerance. The study by Staden et al. [26] included a 4–6-week food allergen (cow’s milk and egg) free period before the final OFC and demonstrated a lower efficacy rate than other studies, with only 9 of 25 (36%) children in the OIT group able to fully pass the OFC. Interestingly, there was no difference between intervention and control groups, as the control group also had 35% of children exhibiting total tolerance (fully passing the OFC). Burks et al. [20] also demonstrated a decrease in tolerance after an allergen-free period. After 22 months of egg OIT, 75% of children passed an OFC; however, after a further 2 months on an egg-free diet, this number dropped to 28%. This is consistent with the notion that OIT induces ‘transient tolerance’, whereby the tolerance state is maintained through regular oral ingestion of the allergen, but is not sustained with periods of allergen avoidance [29]. This however has not been studied extensively and more research is needed to assess the long-term efficacy of OIT.

The best age to commence OIT and the food allergens best targeted also require further investigation. Cow’s milk and egg allergy are more prevalent in early childhood, yet also more transient as resolution is common with age [4,30]. This has led to questions about the timing and efficacy of OIT with cow’s milk and egg, as it can be difficult to differentiate between the natural resolution and the active induction of tolerance by OIT. However, RCTs in which the intervention and control groups are age-matched should address this issue.

IMMUNOLOGICAL EVIDENCE FOR TOLERANCE INDUCTION FOLLOWING ORAL IMMUNOTHERAPY

Oral tolerance is described as an active immunological process to facilitate nonresponse to any antigen administered orally, and allergy reflects a breakdown of this process [31]. Allergen-specific immunoglobulins are well recognized markers reflecting the underlying cellular processes involved in the development of an allergic or tolerant phenotype. Most OIT studies have examined the changes in specific IgE and IgG4 in response to the treatment. As with many studies of subcutaneous immunotherapy, allergen-specific IgG4 has been a useful marker of the development of immune tolerance, without necessarily any sustained decline in IgE levels [32]. Whereas some studies observed that specific IgE levels decreased significantly after cow’s milk and egg OIT [22,25,26], some of these saw the same pattern in the control group children who developed natural tolerance on an elimination diet [26]. Other studies reported no change in the IgE
levels in the OIT group from baseline or when compared to the control group [23,24]. Although specific IgE results are conflicting, changes in allergen-specific IgG4 are more consistent across the trials, with most showing significantly increased levels after OIT. Skripak et al. [24] saw an increase in the median cow’s milk specific IgG4 of 767% in the treatment group compared with no change in the placebo group. Burks et al. [20] established that IgG4 levels were significantly higher in participants who were tolerant to egg than those children who reacted at all challenges ($P < 0.02$). Little is known about the mechanistic role of IgG4 in tolerance induction, but it is thought to interfere with IgE antigen binding [29]. More recently, the allergen-specific IgE-to-IgG4 ratio has been investigated in egg allergy as the best predictor for clinical reactivity and has been shown to be more effective than either IgE or IgG4 alone [33]; however, this has not been reported as an outcome measure in any of the above-mentioned OIT trials.

THE SAFETY OF ORAL IMMUNOTHERAPY

OIT has shown promise in inducing tolerance in food allergic children, both clinically and immunologically; however, the safety of such treatment is still of concern and a key reason that this has not been translated into routine clinical practice. Most children receiving OIT have reportedly experienced allergic symptoms at some point during the treatment. The majority of these reactions occurred during the dose-escalation phase in hospital [20]. Most were mild-to-moderate symptoms and resolved spontaneously or with a dose of antihistamine. Minor cutaneous or abdominal reactions make up the bulk of the overall symptoms observed with OIT, but there have also been cases of serious adverse events including respiratory and systemic reactions requiring epinephrine. Nearly all of the serious adverse events occurred during the hospital visits and were treated by the medical staff; however, there were four cases that occurred at home in two of the cow’s milk studies [24,25]. Three of these four doses were related to the OIT treatment; however, one case was in the control group because of accidental exposure [24].

As the inclusion criteria between these trials were heterogeneous, the overall risk associated with the allergen exposure varies with the population characteristics of the children enrolled. Although most studies excluded children with a history of a severe reaction or anaphylaxis, Longo et al. [25] enrolled severely cow’s milk allergic children, of whom 78% had specific IgE levels above 100 kU/l, and also a history of at least one severe reaction to cow’s milk. As a result, this particular group of children experienced more adverse events than observed in other studies. Five doses of epinephrine were administered for serious reactions to OIT, which accounted for one-third of the total doses of epinephrine given across all the RCT OIT studies included in this review.

Adjunctive administration of recombinant monoclonal anti-IgE therapy may be a promising strategy to address some of the safety concerns associated with OIT [34-37]. This may be particularly useful during the early stages of OIT when reactions are most common and severe. The use of anti-IgE is expensive, but has been shown to be effective in reducing the number of severe allergic reactions during ragweed immunotherapy [35] and has also been used to reduce allergic symptoms observed during oral peanut challenges [36]. Research is ongoing to further explore this possibility.

Children with food allergies are at risk of an allergic reaction even on an elimination diet because of accidental exposure, and with the rise of processing in the food industry, it can be difficult to avoid the hidden allergens [37]. The increased risk of OIT when compared with a standard elimination diet is still uncertain, and more RCTs are needed on a larger scale for each particular allergen to deem whether this treatment is effective in a cost vs. benefit analysis. OIT dose escalations seem to induce the majority of allergic reactions and they are typically performed in a controlled environment, under the direct supervision of medical staff. This generally ensures the child is tolerant prior to home dosing, and even in the initial stages of these trials children are consuming more food allergens than they have previously, which could reduce the risk of a reaction because of accidental exposure.

Of concern for clinical practice is the somewhat unpredictable nature of immune tolerance, in which the immune responses and the threshold for reactivity can be affected by other factors such as infection and exercise [26]. It is therefore vital for all participants to have the necessary medication and auto-injectors at home, in case a previously tolerated dose causes a reaction when the immune system is compromised.

ALLERGEN EXPOSURE IN THE PRIMARY PREVENTION OF FOOD ALLERGY

There are currently a number of RCTs investigating the role of allergen exposure in the primary prevention of food allergies. These current trials (summarized in Table 2) are testing the hypothesis that early regular exposure to food allergens in infancy is more likely to induce oral tolerance, and reduce the risk of
Table 2. Current randomized controlled trials on food allergen exposure in infancy to prevent food allergy

<table>
<thead>
<tr>
<th>Study</th>
<th>Study type (sample size)</th>
<th>Trial registration details</th>
<th>Population characteristics</th>
<th>Intervention</th>
<th>Comparison</th>
<th>Primary outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEP (Australia) Early regular egg exposure during infancy to prevent egg allergy</td>
<td>Blinded, RCT (n = 1512)</td>
<td>ACTRN 12610000388011 Date of trial registration 13/05/2010</td>
<td>Intermediate risk – maternal history of allergy and positive maternal SPT</td>
<td>Daily consumption of egg powder from 4 to 6 months until 12 months of age. Egg introduced from 10 months</td>
<td>Daily consumption of placebo, rice powder from 4 to 6 months until 12 months of age. Egg introduced from 10 months</td>
<td>IgE-mediated egg allergy at 12 months of age based on positive SPT and egg challenge</td>
</tr>
<tr>
<td>BEAT (Australia) The effect of early introduction of egg in the diet of high risk for atopic infants and subsequent egg allergy</td>
<td>Blinded, RCT (n = 600)</td>
<td>ACTRN 1261100035976 Date of trial registration 24/05/2011</td>
<td>Intermediate risk – first-degree relative with atopy No infant IgE sensitization to egg at 4 months of age</td>
<td>Daily consumption of egg powder from 4 to 6 months until 12 months of age, with egg-free diet until 8 months, then introduction of unrestricted diet from 8 months</td>
<td>Daily consumption of placebo, rice powder from 4 to 6 months until 12 months of age, with egg-free diet until 8 months, then introduction of unrestricted diet from 8 months</td>
<td>IgE sensitization to egg measured by SPT at 8 and 12 months of age</td>
</tr>
<tr>
<td>EAT (UK) Early introduction of allergenic foods to induce tolerance in infants</td>
<td>RCT (n = 2500)</td>
<td>ISRCTN 14254740 Date of trial registration 31/03/2009</td>
<td>Normal risk – general population</td>
<td>Exclusive breastfeeding until 3 months of age then sequential introduction of cow’s milk, egg, wheat, sesame, fish and peanut from 3 months of age</td>
<td>Exclusive breastfeeding until around 6 months of age and no early introduction of allergenic foods before 6 months</td>
<td>Prevalence of IgE-mediated food allergy between 1 and 3 years of age, as defined by food challenge</td>
</tr>
<tr>
<td>LEAP (UK) Promoting tolerance to peanut in high-risk children</td>
<td>RCT (n = 640)</td>
<td>NCT00229784 Date of trial registration 23/05/2006</td>
<td>High risk – infant with eczema and egg allergy</td>
<td>Peanut consumption in the form of an age appropriate peanut snack commencing from 4 to 10 months of age</td>
<td>Peanut avoidance</td>
<td>Prevalence of clinically defined peanut allergy at 5 years of age</td>
</tr>
<tr>
<td>HEAP (Germany) Hen’s Egg Allergy Prevention</td>
<td>RCT (n = 800)</td>
<td>Personal communication</td>
<td>Normal risk – general population</td>
<td>Egg powder 3 times a week from 4 to 6 months of age</td>
<td>Placebo powder 3 times a week from 4 to 6 months</td>
<td>IgE sensitization to egg at 12 months of age</td>
</tr>
<tr>
<td>PEADA (Germany) Preventing Peanut Allergy in Atopic Dermatitis</td>
<td>Nonrandomized self-allocated controlled (n = 400)</td>
<td>Personal communication</td>
<td>High risk – eczema without peanut sensitization</td>
<td>Peanut snack 3 times a week from 5 to 30 months</td>
<td>Peanut avoidance</td>
<td>IgE-mediated peanut allergy after 1 year of consumption or avoidance</td>
</tr>
<tr>
<td>(Japan) Prevention of egg allergy in infants with atopic dermatitis</td>
<td>Blinded, RCT (n = 200)</td>
<td>JPRN-UJMII000000673 Date of trial registration 10/08/2012</td>
<td>High risk – infants with eczema</td>
<td>Daily consumption from 6 months of age of egg powder</td>
<td>Daily consumption from 6 months of age of placebo pumpkin powder</td>
<td>Negative egg food challenge at 1 year old</td>
</tr>
</tbody>
</table>

RCT, randomized controlled trial; SPT, skin prick test.
food allergy development, than avoidance or delayed exposure.

The majority of the current RCTs are investigating the timing of introduction to one specific food allergen, either hen’s egg or peanut. However, the inclusion criteria differ among these trials (see Table 2), in which the risk of food allergy development is varied; high-risk infants with eczema are being investigated in Starting Time for Allergy Reduction (STAR), Learning Early About Peanut (LEAP) and Preventing Peanut Allergy in Atopic Dermatitis compared to intermediate-risk (family history of allergic disease) infants in Starting Time for Egg Protein (STEP) and Beating Egg Allergy Trial (BEAT). Additionally, some of the trials (e.g. BEAT and LEAP) are excluding infants with specific food allergen sensitization prior to randomization compared with other trials that are not (STAR and STEP). The Enquiring About Tolerance study in the United Kingdom is unique in investigating the intervention of exclusive breastfeeding until 3 months of age, then sequential introduction of cow’s milk, egg, fish, wheat, sesame and peanut from 3 months of age compared with exclusive breastfeeding until around 6 months of age.

One of these RCTs, our own study investigating the role of early egg exposure in food allergy prevention (the STAR trial) is now complete and the results have been submitted for publication. One particularly interesting finding from this trial was that a significant number of high-risk infants with eczema already had established food sensitization and clinical reactivity (including anaphylaxis) prior to the introduction of solid foods at 4–5 months of age. This indicates that the processes leading to food allergen sensitization are already strongly established by this age in some infants, and that even earlier preventative interventions will ultimately be required. This underscores the pressing need to better define the events around much earlier to the food allergen encounter and it is essential to have a better understanding of the antecedent events that lead to the establishment of food allergy so early in infancy.

In the primary prevention of food allergy, exposure to ‘food allergens’ is only one component of a much bigger story, in which optimizing conditions during early critical periods of immune programming is vital to promote oral tolerance induction. The dose, timing and delivery method of the food allergen require investigation in tandem with other strategies that promote tolerogenic conditions such as optimizing early gut colonization, nutritional patterns and reducing exposure to pro-inflammatory factors.

**CONCLUSION**

Prevention strategies are of prime importance as rates of food allergies in children continue to increase. The results of current RCTs investigating the early regular exposure to food allergens in infancy for the primary prevention of food allergy are eagerly awaited. For the treatment of established food allergy, more studies are needed in relation to each of the specific food allergens in order to adequately assess both the efficacy and safety of food allergen exposure. Adjunctive administration of anti-IgE therapy with OIT may be a way forward to address the safety concerns associated with ‘intentional’ allergen exposure. Recent studies are adding to a body of growing evidence that exposure may be a better alternative to avoidance; however, at this point, there is not enough evidence available for a shift in the current, routine clinical practice.

**Acknowledgements**

None.

**Conflicts of interest**

The authors declare that no financial or other conflict of interest exists in relation to the content of the article. The authors are supported by the project grants from the Australian National Health and Medical Research Council, Australia.

**REFERENCES AND RECOMMENDED READING**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

Food allergy

21. Most recently, Burks et al. published a randomized placebo-controlled trial investigating OIT in egg allergic children. This study involved a staged OIT protocol in which children were challenged at three time points. They demonstrated that egg OIT is most effective (75% of patients) after 22 months of desensitization and when there has not been an ‘allergy-free’ period. With no serious adverse events, this study is an advocate for both safety and efficacy of OIT in the treatment of egg allergic patients.
23. This is the first randomized, placebo-controlled trial investigating peanut OIT as a treatment for peanut allergic children. In this study after 44 weeks of desensitization, 16 of 19 patients were able to consume the equivalent of 20 peanuts, compared with the control group, who could only tolerate 1 peanut. Unfortunately, because of a significant difference in the OFG outcomes between the groups, this study was terminated prematurely and they were not able reach their target of 60 peanuts. However, this level of desensitization is an advance in the treatment of peanut allergy and has the potential to decrease the potential severe reactions due to accidental exposure.
25. Martorel et al. enrolled 60 cow’s milk allergic infants between the age of 24 and 36 months for this OIT study. Although many other cow’s milk OIT studies include infants as young as 1 year of age, this study is the first with infants in a tight age range. This trial found a desensitization rate of 90% after 1 year of OIT.
Appendix C
Published Article

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

Jessica R. Metcalfe, Nina D’Vaz, Maria Makrides, Michael S. Gold, Patrick Quinn, Christina E. West, Richard Loh, Susan L. Prescott, Debra J. Palmer
Elevated IL-5 and IL-13 responses to egg proteins predate the introduction of egg in solid foods in infants with eczema

J. R. Metcalfe1, N. D’Vaz1,2, M. Makrides3,4,5, M. S. Gold6,7, P. Quinn6, C. E. West1,2, R. Loh8, S. L. Prescott1,2,8 and D. J. Palmer1,3

1School of Paediatrics and Child Health, The University of Western Australia, Perth, WA, Australia, 2Women’s & Children’s Health Research Institute, Adelaide, SA, Australia, 3School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia, 4Telethon KIDS Institute, The University of Western Australia, Perth, WA, Australia, 5Department of Paediatrics and Child Health, Adelaide, SA, Australia, 6School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia, 7Children, Youth and Women’s Health Service, Adelaide, SA, Australia, 8Department of Clinical Sciences, Pediatrics, Umeå University, Umeå, Sweden, and 9Department of Immunology, Princess Margaret Hospital, Perth, WA, Australia

Correspondence:
Dr Debra J. Palmer, School of Paediatrics and Child Health, University of Western Australia, PO Box D184, Princess Margaret Hospital, Perth, WA 6001, Australia.
E-mail: debbie.palmer@uwa.edu.au

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Summary
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 randomization to receive ‘early egg’ or a placebo as part of a randomized 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 egg’ 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.

Keywords allergy prevention, cytokines, eczema, egg allergy, egg protein, infancy

Submitted 24 November 2014; revised 30 June 2015; accepted 2 July 2015

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 sensitization 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 sensitization 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 recognized need to further characterize the proceeding immunological events leading to egg sensitization, as this may help define pathways to sensitization, 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-sensitized patients have focused on responses to ovalbumin (OVA) as the most abundant protein in hen’s egg [8–10], this study provided the opportunity to examine the 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 4 months of age predicted subsequent IgE-mediated egg allergy. In addition, 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 ≥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 h. 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™, Axis-Shield, Oslo, Norway) method. Peripheral blood mononuclear cells (PBMCs) were isolated, washed using Roswell Park Memorial Institute (RPMI) media (Gibco Life Technology, Grand Island, NY, USA) and stored in RPMI (49%), heat-inactivated foetal calf serum (43.5%) and dimethylsulphate (7.5%). Cells were stored in 1 mL aliquots at a concentration of no more than 15 × 10⁶ cells/mL, transferred to a CoolCell® and immediately stored at −80°C for a standardized controlled rate of −1°C/min cell freezing. Within 24 h of freezing, PBMCs were transferred to liquid nitrogen for long-term storage.

Mononuclear cell culture

PBMC cell culture was conducted using the methods as per detailed previously [8, 13]. 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, Sydney, NSW, Australia) at a concentration of 2 × 10⁶ cells/mL. Hen’s egg allergens: (a) ovalbumin (OVA 100 µg/mL), (b) ovomucoid (OV M 1 mg/mL), (c) conalbumin (CON 200 µg/mL) and (d) lysozyme (LYS 500 µg/mL), all purchased from Sigma-Aldrich Co. These concentrations were identified as optimal for in vitro T-cell stimulation in preliminary titration experiments. As OVA is routinely used to stimulate PBMCs at a concentration of 100 µg/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, while maintaining minimal responses in unaffected infants. A mitogen phytohaemagglutinin (PHA) was used as a positive control, to ensure that PBMCs were responding suitably to stimulation. Nonstimulated negative controls were also
included for each infant. Lymphocytes were cultured for 48 h in 5% CO₂ 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, MN, 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 10 000 and 30 000 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 three concurrent noncontact urticaria persisting for at least 5 min and/or generalized skin erythema, and/or vomiting, and/or anaphylaxis within 2 h 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. Nonparametric data were analysed between groups using Mann–Whitney U-tests. Chi-squared tests were used for comparisons of categorical data between groups. Where possible nonparametric data were log-transformed to achieve a normal distribution for the remaining statistics. Binary logistic regression was used to calculate the prediction of allergic outcomes. Paired t-tests were used to quantify changes over time. All statistics were performed using SPSS v20 (IBM, Chicago, USA), and figures were generated using Prism v 6 (GraphPad Software Inc., San Diego, CA, USA).

**Results**

**Study population**

This study included 68 infants from the clinical trial who had blood samples available for cytokine analysis, as illustrated in Fig. 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 were 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 nonreactors**

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 Table S1 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 were available for five 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 (Fig. 2). There was also a significantly higher production of IFNγ to lysozyme in the egg powder reactors (P = 0.011) than in the nonreactors (data in Table S2). 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 Table S2 available in the online repository. PHA stimulation was used to assess viability in all samples, and the level of cytokines produced did

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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 Table S3 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...
There were no associations between IL-10, with IgE-mediated egg allergy 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 (Fig. 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 (Fig. 4). While 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 (Fig. 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 (Fig. 3). There were no significant differences for any other egg allergen for cytokines IL-5 and IL-13 (Fig. 3). Again there were no differences for IL-10, IFNγ or TNFα for any of the egg allergens, data as summarized in Table S4 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 and 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 were not sufficient to accurately assess in relation to cytokine production.

Table 3. Cytokine responses at 12 months of age. IL-13 and IL-5 responses [pg/mL] per group

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13*</td>
<td>OVA</td>
<td>112.8 (34.9–208.8)</td>
<td>126.6 (44.5–204.7)</td>
<td>0.580</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>98.6 (123.6–342.8)</td>
<td>90.0 (49.8–207.3)</td>
<td>0.733</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>19.4 (0.9–39.4)</td>
<td>20.7 (1.0–60.7)</td>
<td>0.860</td>
</tr>
<tr>
<td>IL-5*</td>
<td>OVA</td>
<td>3.1 (1.0–10.6)</td>
<td>1.0 (1.0–5.24)</td>
<td>0.311</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>1.0 (1.0–3.4)</td>
<td>1.0 (1.0–1.2)</td>
<td>0.494</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>2.2 (1.0–24.5)</td>
<td>2.7 (1.0–15.0)</td>
<td>0.985</td>
</tr>
</tbody>
</table>

0VA, ovalbumin; OM, ovomucoid; CON, conalbumin; LYS, lysozyme.
*Median (interquartile range).
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 and 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 sensitization 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 sensitization, during lactation or even in utero. Egg proteins are known to cross the placenta [14] and have been detected in breast milk [15], 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 [16, 17], 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

![Fig. 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). Categorized 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.](image-url)
intervention group prior to the introduction of egg in solid foods.

**Table S2.** Cytokine Responses at 4 months of age to egg powder. IL-10, IFN\(\gamma\) and TNF\(\alpha\) responses (pg/mL) per egg powder reaction [egg group only].

**Table S3.** Cytokine responses at 12 months of age: IL-10, IFN\(\gamma\), TNF\(\alpha\) responses (pg/mL) per intervention group.

**Table S4.** Cytokine responses at 12 months of age: IL-10, IFN\(\gamma\), TNF\(\alpha\) responses (pg/mL) per IgE mediated egg allergy.
inducing Th2 cytokine responses associated with the presence of IgE-mediated egg allergy. In addition, 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.

Acknowledgements

We would like to sincerely thank the families and acknowledge the nurses and research assistants involved with this study. This trial was supported by grants from the Women’s and Children’s Hospital Foundation and the Ilhan Food Allergy Foundation.

Conflict of interest

The authors declare no conflict of interest.

References


Supporting Information

Additional Supporting Information may be found in this online version of the article:

Table S1. Baseline Cytokine Responses at 4 months of age. IL-10, IFNγ, TNFα responses (pg/mL) per

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Appendix D

Chapter 3 Supplementary Tables
Table D.1: **Baseline Cytokine Responses at 4 months of age.** IL-10, IFNγ, TNFα responses (pg/ml) per intervention group prior to the introduction of egg in solid foods.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Egg protein</th>
<th>Egg (n=22)</th>
<th>Control (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>272.1 (171.0-961.9)</td>
<td>237.9 (157.1-804.9)</td>
<td>0.798</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>181.3 (107.4-458.9)</td>
<td>171.2 (111.7-266.5)</td>
<td>0.677</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>372.2 (95.0-816.2)</td>
<td>349.8 (162.0-628.1)</td>
<td>0.878</td>
<td></td>
</tr>
<tr>
<td>LYS</td>
<td>694.2 (158.5-1069.1)</td>
<td>593.8 (335.3-1493.7)</td>
<td>0.726</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>231.7 (64.5-1018.9)</td>
<td>94.2 (1.0-295.0)</td>
<td>0.079</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>40.8 (1.0-376.1)</td>
<td>10.7 (1.0-91.3)</td>
<td>0.657</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>36.7 (1.0-36.7)</td>
<td>1.0 (1.0-141.1)</td>
<td>0.475</td>
<td></td>
</tr>
<tr>
<td>LYS</td>
<td>84.2 (2.5-342.8)</td>
<td>77.0 (1.0-114.3)</td>
<td>0.685</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>191.8 (57.8-389.8)</td>
<td>153.1 (93.1-420.4)</td>
<td>0.925</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>104.1 (14.2-249.4)</td>
<td>92.1 (26.2-178.4)</td>
<td>0.989</td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>173.4 (83.1-416.8)</td>
<td>197.2 (110.2-487.7)</td>
<td>0.769</td>
<td></td>
</tr>
</tbody>
</table>
Table D.2: Cytokine Responses at 4 months of age to egg powder. IL-10, IFNγ and TNFα responses (pg/ml) per egg powder reaction (egg group only).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Egg protein</th>
<th>Powder Reaction (n=5)</th>
<th>No Powder Reaction (n=17)</th>
<th>P value</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>OVA</td>
<td>423.8 (168.3-1731.9)</td>
<td>256.6 (169.7-1050.3)</td>
<td>0.493</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>453.8 (106.2-563.2)</td>
<td>179.6 (96.4-349.2)</td>
<td>0.543</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>490.6 (146.0-1469.4)</td>
<td>344.1 (94.8-782.1)</td>
<td>0.704</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>726.2 (241.5-1460.7)</td>
<td>662.3 (149.7-1200.6)</td>
<td>1.000</td>
</tr>
<tr>
<td>IFNγ</td>
<td>OVA</td>
<td>591.8 (215.2-1865.2)</td>
<td>139.3 (29.0-769.0)</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>180.9 (66.9-1411.7)</td>
<td>14.0 (1.0-310.2)</td>
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<td></td>
<td>CON</td>
<td>40.9 (1.0-601.7)</td>
<td>32.4 (1.0-81.6)</td>
<td>0.704</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>337.5 (171.4-717.6)</td>
<td>56.0 (1.0-117.2)</td>
<td>0.011</td>
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<tr>
<td>TNFα</td>
<td>OVA</td>
<td>188.0 (91.4-440.4)</td>
<td>195.5 (51.8-405.7)</td>
<td>0.940</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>110.8 (76.2-251.7)</td>
<td>56.7 (11.5-297.3)</td>
<td>0.595</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>167.4 (80.8-418.4)</td>
<td>200.3 (73.1-420.8)</td>
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<td></td>
<td>LYS</td>
<td>117.8 (60.1-293.1)</td>
<td>136.8 (84.6-646.3)</td>
<td>0.940</td>
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</table>
Table D.3: Cytokine responses at 12 months of age: IL-10, IFNγ, TNFα responses (pg/ml) per intervention group.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Egg protein</th>
<th>Egg (n=33)</th>
<th>Control (n=25)</th>
<th>P value</th>
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<tr>
<td></td>
<td>Median (IQR)</td>
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<td>IL-10</td>
<td></td>
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<tr>
<td>OVA</td>
<td>200.7 (53.8-962.7)</td>
<td>308.7 (120.1-562.1)</td>
<td>0.919</td>
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<tr>
<td>OM</td>
<td>54.4 (12.6-138.4)</td>
<td>38.6 (14.1-84.5)</td>
<td>0.734</td>
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</tr>
<tr>
<td>CON</td>
<td>274.4 (39.2-727.9)</td>
<td>346.7 (180.4-711.7)</td>
<td>0.276</td>
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</tr>
<tr>
<td>LYS</td>
<td>116.2 (25.2-660.3)</td>
<td>251.0 (102.4-507.3)</td>
<td>0.176</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
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<tr>
<td>OVA</td>
<td>84.4 (1.0-53.9)</td>
<td>15.4 (1.0-282.0)</td>
<td>0.411</td>
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<tr>
<td>OM</td>
<td>10.5 (1.0-53.9)</td>
<td>2.5 (1.0-42.8)</td>
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<td>CON</td>
<td>2.4 (1.0-68.5)</td>
<td>6.4 (1.0-64.8)</td>
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<td>LYS</td>
<td>5.7 (1.0-102.3)</td>
<td>1.0 (1.0-42.4)</td>
<td>0.377</td>
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<td>TNFα</td>
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<tr>
<td>OVA</td>
<td>86.5 (32.8-645.8)</td>
<td>170.6 (66.9-403.2)</td>
<td>0.525</td>
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<tr>
<td>OM</td>
<td>17.9 (3.2-87.4)</td>
<td>4.1 (1.0-59.2)</td>
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<td>CON</td>
<td>77.4 (10.4-415.1)</td>
<td>121.1 (39.8-370.3)</td>
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<tr>
<td>LYS</td>
<td>31.3 (2.3-120.5)</td>
<td>36.1 (10.5-233.7)</td>
<td>0.181</td>
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</table>
Table D.4: Cytokine responses at 12 months of age: IL-10, IFNγ, TNFα responses (pg/ml) per IgE mediated egg allergy

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Egg protein</th>
<th>IgE mediated egg allergy (n=25)</th>
<th>No egg allergy (n=33)</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>OVA</td>
<td>316.4 (117.7-588.9)</td>
<td>200.73 (81.7-807.2)</td>
<td>0.969</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>51.9 (17.8-103.4)</td>
<td>38.6 (12.7-125.6)</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>387.7 (128.2-544.5)</td>
<td>275.5 (91.0-1014.7)</td>
<td>0.928</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>211.3 (58.2-569.2)</td>
<td>189.0 (52.8-689.5)</td>
<td>0.758</td>
</tr>
<tr>
<td>IFNγ</td>
<td>OVA</td>
<td>47.44 (1.0-472.7)</td>
<td>34.5 (1.0-182.9)</td>
<td>0.531</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>1.0 (1.0-65.45)</td>
<td>10.6 (1.0-42.8)</td>
<td>0.907</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>1.0 (1.0-58.4)</td>
<td>2.4 (1.0-84.8)</td>
<td>0.403</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>11.6 (1.0-110.1)</td>
<td>1.0 (1.0-59.23)</td>
<td>0.475</td>
</tr>
<tr>
<td>TNFα</td>
<td>OVA</td>
<td>170.65 (29.3-490.6)</td>
<td>142.2 (48.2-471.5)</td>
<td>0.956</td>
</tr>
<tr>
<td></td>
<td>OM</td>
<td>14.7 (1.0-92.2)</td>
<td>11.5 (1.0-42.8)</td>
<td>0.857</td>
</tr>
<tr>
<td></td>
<td>CON</td>
<td>139.8 (25.6 – 369.6)</td>
<td>77.4 (20.3-547.0)</td>
<td>0.807</td>
</tr>
<tr>
<td></td>
<td>LYS</td>
<td>31.1 (6.7-118.6)</td>
<td>36.15 (3.7-163.2)</td>
<td>0.772</td>
</tr>
</tbody>
</table>
Appendix E

QuEST Trial Case Report Form
The QuEST Study

‘Questioning the role of Egg in lactation for the induction of Specific Tolerance’

THE EFFECTS OF MATERNAL DIETARY EGG INTAKE ON BREAST MILK EGG LEVELS DURING EARLY LACTATION: A RANDOMISED CONTROLLED TRIAL

CASE REPORT FORM

Childhood Allergy and Immunology Research (CAIR)
School of Paediatrics and Child Health
University of Western Australia
100 Roberts Road,
Subiaco, Western Australia 6008
Phone: 08 9340 8834
Email: CAIR-SPACH@uwa.edu.au

This Case Report Form contains a total of 38 pages

Investigator Statement

I confirm that the data recorded in this Treatment Phase of the Case Report Form accurately and completely represent the results of the examinations, tests and evaluations performed on the dates specified

_________________________
Investigator Signature

_________________________
Investigator Name

__ __ / __ __ / __ __ __ __
dd             mm             yyyy
Randomisation date: __ __ / __ __ / __ __ __ __  
Mother's Name: ____________________  ______________________  
                       First                   Surname  
Estimated Delivery Date: __ __ / __ __ / __ __ __ __  

Randomisation Information

Mother’s estimated usual egg intake  
☐ < 1 egg per week   ☐ 1-3 eggs per week   ☐ > 3 eggs per week  

Allocated study number: QST - ____ ____ ____  
Allocated dietary intervention: ☐ High Egg   ☐ Low Egg    ☐ Egg Free  

Section completed by (Sign):   Date:             Checked by: 
SECTION A: ANTENATAL APPOINTMENT

Date of Appointment: __ __ / __ __ / __ __

**A1 PERSONAL DETAILS**

<table>
<thead>
<tr>
<th></th>
<th>MOTHER</th>
<th>FATHER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FIRST NAME</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LAST NAME</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DOB</strong></td>
<td>__ __ / __ __ / __ __ __ __ dd  mm  yyyy</td>
<td>__ __ / __ __ / __ __ __ __ dd  mm  yyyy</td>
</tr>
<tr>
<td><strong>ADDRESS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>State ______ Post Code: __ __ __</td>
<td>State ______ Post Code: __ __ __</td>
</tr>
<tr>
<td><strong>TELEPHONE NUMBERS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Home: _______________________________</td>
<td>Home: _______________________________</td>
</tr>
<tr>
<td></td>
<td>Mobile: _________________________________</td>
<td>Mobile: _________________________________</td>
</tr>
<tr>
<td><strong>EMAIL ADDRESS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DESCRIPTION OF USUAL OCCUPATION</strong></td>
<td>_______________________________</td>
<td>_______________________________</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CONTACT #1</td>
<td>CONTACT #2</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td><strong>LAST NAME</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FIRST NAME</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RELATIONSHIP TO INFANT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ADDRESS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>______________________________</td>
<td>______________________________</td>
</tr>
<tr>
<td></td>
<td>______________________________</td>
<td>______________________________</td>
</tr>
<tr>
<td></td>
<td>State ______ Post Code: __ __ __</td>
<td>State ______ Post Code: __ __ __</td>
</tr>
<tr>
<td><strong>EMAIL ADDRESS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TELEPHONE NUMBERS</strong></td>
<td>Home: ________________________</td>
<td>Home: ________________________</td>
</tr>
<tr>
<td></td>
<td>Mobile: ______________________</td>
<td>Mobile: ______________________</td>
</tr>
</tbody>
</table>
**A2.2 Mothers Physician details**

<table>
<thead>
<tr>
<th>DOCTOR’S NAME</th>
<th>First Name</th>
<th>Last Name</th>
</tr>
</thead>
</table>

AREA OF CLINICAL PRACTICE

- ☐ GP
- ☐ Obstetrician
- ☐ Other: __________________________

<table>
<thead>
<tr>
<th>CLINIC NAME and ADDRESS</th>
<th>__________________________</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State _____________ Post Code: __ __ __ __</td>
</tr>
</tbody>
</table>

TELEPHONE NUMBER

---

**A3 FAMILY ALLERGY HISTORY**

<table>
<thead>
<tr>
<th></th>
<th>Hay Fever</th>
<th>Eczema/Dermatitis</th>
<th>Asthma</th>
<th>Food Allergies confirmed by skin prick test or RAST</th>
<th>Other/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
</tr>
<tr>
<td></td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
</tr>
<tr>
<td>Father</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
</tr>
<tr>
<td></td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
</tr>
<tr>
<td>Your Children</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
<td>☐ Yes</td>
</tr>
<tr>
<td></td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
<td>☐ No</td>
</tr>
</tbody>
</table>

---

**A4 PRESENT OBSTETRIC HISTORY**

**A4.1** How many pregnancies of gestation >20 weeks have you had in total, including this one? _____

**A4.2** Have you had any complications during this pregnancy? ☐ Yes ☐ No (go to A 4.3)

- ☐ Bleeding
- ☐ Gestational Diabetes
- ☐ Hypertension/ Pre-eclampsia
- ☐ Other complications, please specify; __________________________________________________________

---

**A4.3** What is your estimated delivery date? __ __ / __ __ / __ __ __ __

- Gestation at screening ___ weeks ___ days
- Hospital __________________________
- Obstetrician __________________________
A 4.4 Have you been on any prescribed medication during this pregnancy?  
☐ Yes  ☐ No (go to A 4.5)

<table>
<thead>
<tr>
<th></th>
<th>During pregnancy?</th>
<th>Last trimester (≥ 27 weeks)</th>
<th>Details (eg. name, duration etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotics</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Asthma preventers</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Asthma relievers</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Antidepressants</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Other prescription medication</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
</tbody>
</table>

A 4.5 Have you had any over the counter medication during pregnancy?  
☐ Yes  ☐ No (go to A 4.6)

<table>
<thead>
<tr>
<th></th>
<th>During pregnancy?</th>
<th>Last trimester (≥ 27 weeks)</th>
<th>Details (eg. name, duration etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hay fever medication</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Asthma medication</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Pain relief</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Heartburn or Reflux</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
<tr>
<td>Other over counter medication</td>
<td>☐ Yes ☐ No</td>
<td>☐ Yes ☐ No</td>
<td></td>
</tr>
</tbody>
</table>

A 4.6 From 27 weeks gestation have you taken any dietary supplements or health food preparations?  
(THIS INCLUDES MULTIVITAMINS, FISH OIL, PROBIOTICS, INDIVIDUAL VITAMINS OR MINERALS AND HERBAL SUPPLEMENTS)  
☐ Yes  ☐ No (go to A 4.7)

<table>
<thead>
<tr>
<th>Supplement Code (see codes below)</th>
<th>Manufacturer and Product Name</th>
<th>No. of tablets/wk</th>
<th>Duration of tablets (wks) (27wks – today)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Supplement Coding

- MV – Multivitamin
- FO – Fish Oil
- P – Probiotics
- V – Vitamin only
- M – Mineral only
- H – Herbal supplement
- O – Other
- U – Unknown
A 4.7 Have you had any vaccinations during this pregnancy?  □ Yes  □ No (go to A 4.8)

Name of vaccine:  □ FluVax  □ other, specify ___________________

A 4.8 Have you had any infections/conditions during this pregnancy?  □ Yes  □ No (go to A 5)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
</tr>
<tr>
<td>□ respiratory  □ ENT  □ mastitis</td>
<td>□ No □ Yes</td>
</tr>
<tr>
<td>□ urinary     □ GI    □ birth related</td>
<td></td>
</tr>
<tr>
<td>□ skin        □ other</td>
<td></td>
</tr>
<tr>
<td>Occasion 2</td>
<td></td>
</tr>
<tr>
<td>□ respiratory  □ ENT  □ mastitis</td>
<td>□ No □ Yes</td>
</tr>
<tr>
<td>□ urinary     □ GI    □ birth related</td>
<td></td>
</tr>
<tr>
<td>□ skin        □ other</td>
<td></td>
</tr>
<tr>
<td>Occasion 3</td>
<td></td>
</tr>
<tr>
<td>□ respiratory  □ ENT  □ mastitis</td>
<td>□ No □ Yes</td>
</tr>
<tr>
<td>□ urinary     □ GI    □ birth related</td>
<td></td>
</tr>
<tr>
<td>□ skin        □ other</td>
<td></td>
</tr>
<tr>
<td>Occasion 4</td>
<td></td>
</tr>
<tr>
<td>□ respiratory  □ ENT  □ mastitis</td>
<td>□ No □ Yes</td>
</tr>
<tr>
<td>□ urinary     □ GI    □ birth related</td>
<td></td>
</tr>
<tr>
<td>□ skin        □ other</td>
<td></td>
</tr>
</tbody>
</table>

A 5  PREVIOUS MEDICAL HISTORY

A 5.1 Have you had any medical problems prior to this pregnancy?  □ Yes  □ No (go to A 6)

____________________________________________________________________________________________

____________________________________________________________________________________________

A 6  LIFESTYLE AND ENVIRONMENT

A 6.1 Have you ever smoked cigarettes?  □ Yes  □ No (go to A 6.2)

6.1.1 How long have you/did you smoke for?  ___ years

6.1.2 Average amount cigarettes smoked per day?

Prior to pregnancy:  ___ ___ cigarettes

During pregnancy:  ___ ___ cigarettes
A 6.2 Does any other household member smoke?  
- Yes  
- No

A 6.3 How would you describe your exposure to passive smoking during pregnancy outside the home?  
- Heavy  
- Moderate  
- Light  
- Not Exposed

A 6.4 Did you drink alcohol before becoming pregnant?  
- Yes  
- No (go to A 6.5)

6.4.1 What type of drink did you usually have?  
- Beer  
- Fortified Wines  
- Wine/Champagne  
- Spirits/Liqueurs

6.4.2 How many standard drinks per week?  
- <1  
- 1-3  
- 3-5  
- 6-8  
- >8

A 6.5 How many standard drinks have you had per week during your pregnancy?  
- None  
- <1  
- 1-3  
- 3-5  
- 5-7  
- >8

A 6.6 How many adults (>18 years of age) live in your home?  

A 6.7 How many children in each age group live in your home?  

0 – 3 yrs  
4 – 13 yrs  
14 – 18 yrs

A 6.8 Do you have any pets?  
- Yes  
- No (go to A 6.9)

<table>
<thead>
<tr>
<th>Type</th>
<th>Yes/No</th>
<th>How long (years)</th>
<th>Lives?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>Yes/No</td>
<td></td>
<td>inside</td>
</tr>
<tr>
<td>Dog</td>
<td>Yes/No</td>
<td></td>
<td>inside</td>
</tr>
<tr>
<td>Rabbit / Guinea Pig</td>
<td>Yes/No</td>
<td></td>
<td>inside</td>
</tr>
<tr>
<td>Bird</td>
<td>Yes/No</td>
<td></td>
<td>inside</td>
</tr>
</tbody>
</table>

A 6.9 Is there a free-standing gas heater without a chimney in your home?  
- Yes  
- No
# Household Egg Intake

Please record the frequency **per week** that each household member consumes the following egg containing foods. Please include all household members who live in the household at least 3 days per week.

<table>
<thead>
<tr>
<th>Food</th>
<th>Mother</th>
<th>Mother's egg equivalence (use table)</th>
<th>Father Or N/A</th>
<th>Another household member yrs</th>
<th>Another household member yrs</th>
<th>Another household member yrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 egg (scrambled, boiled, fried, poached or omelette)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 slice quiche</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 slice of frittata</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 egg in a hamburger/steak sandwich/in a drink</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 slice French Toast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 serve (1 cup) of egg containing fried rice or pasta dish (e.g. carbonara)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 muffin or slice of cake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 serve pavlova/meringue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 pancake (= 2-3 pikelets)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**For Randomisation**

Maternal egg intake equivalence: __ __ __ __ eggs per week
### A8 SUN EXPOSURE HABITS

#### A 8.1 General Questions

<table>
<thead>
<tr>
<th>8.1.1</th>
<th>In the past 2 months, when outdoors did you use sunscreen?</th>
<th>□ Always</th>
<th>□ Sometimes</th>
<th>□ Never (Go to A 8.1.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1.2</td>
<td>What factor sunscreen (if known)?</td>
<td>□ &lt; Factor 30</td>
<td>□ Factor 30</td>
<td>□ &gt; Factor 30</td>
</tr>
<tr>
<td>8.1.3</td>
<td>Do you usually wear a hat?</td>
<td>□ Yes</td>
<td>□ No</td>
<td></td>
</tr>
</tbody>
</table>

#### A 8.2. Sun Exposure Last Weekend

(Please place a cross in appropriate boxes)

<table>
<thead>
<tr>
<th>8.2.1</th>
<th>When did you spend time in the sun last <strong>Saturday</strong>? (Please estimate for how long)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Before 11.00AM</td>
</tr>
<tr>
<td></td>
<td>□ 11.00AM to 3.00PM</td>
</tr>
<tr>
<td></td>
<td>□ After 3.00PM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>8.2.2</th>
<th>When did you spend time in the sun last <strong>Sunday</strong>? (Please estimate for how long)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Before 11.00AM</td>
</tr>
<tr>
<td></td>
<td>□ 11.00AM to 3.00PM</td>
</tr>
<tr>
<td></td>
<td>□ After 3.00PM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>8.2.3</th>
<th>Where did you spend most of your time over last weekend?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Indoors</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>8.2.4</th>
<th>Amount of skin exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Face and hands</td>
</tr>
</tbody>
</table>

#### A 8.3. Sun Exposure Last week

<table>
<thead>
<tr>
<th>8.3.1</th>
<th>When did you spend time in the sun on a <strong>regular weekday</strong>? (Please estimate for how long)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Before 11.00AM</td>
</tr>
<tr>
<td></td>
<td>□ 11.00AM to 3.00PM</td>
</tr>
<tr>
<td></td>
<td>□ After 3.00PM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>8.3.2</th>
<th>Where did you spend most of your time last week?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Indoors</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>8.3.3</th>
<th>Amount of skin exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>□ Face and hands</td>
</tr>
</tbody>
</table>
A9 Blood Samples

A 9.1 Blood Sample Collected?

- Yes
- No, specify reasons (check one only):
  - Unsuccessful
  - Refused
  - Other specify: __________________________

A 9.2 Who collected/attempted the blood sample?

A 9.3 Cord blood kit given?

- Yes, number: __ __
- No, reason ________________________________

A 10 Study information provided

- Obstetrician letter
- Egg dietary advice
- Diary Card
- Breast milk collection pots and instructions
- Consent forms signed and copies given to participant

Pre-pregnancy weight: __ __ __ . __ kg
Pregnancy weight at visit: __ __ __ . __ kg
Height: __ __ __ . __ cm
<table>
<thead>
<tr>
<th>QuEST Study</th>
<th>Dietary Group</th>
<th>Study No: Q S T - ___ ___</th>
</tr>
</thead>
</table>

QuEST Study Case Report Form
Version 1 28.06.13
12

194
SECTION B: 2 WEEK TELEPHONE CALL

Date of telephone call  __ / __ / ___ ___

B 1 Infant information

<table>
<thead>
<tr>
<th>INFANT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>First name</td>
<td></td>
</tr>
<tr>
<td>Last name</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>☐ Male ☐ Female</td>
</tr>
<tr>
<td>Infant DOB</td>
<td>dd mm yyyy</td>
</tr>
</tbody>
</table>

B 2 Maternal dietary egg intake compliance

B 2.1 Which egg containing foods have you eaten between 0-2 weeks of lactation?

<table>
<thead>
<tr>
<th>Food</th>
<th>Eaten</th>
<th>Amount</th>
<th>Egg Equivalence (after phone call)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw egg (1)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Boiled egg (1)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fried egg (1)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Scrambled egg (1)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Poached egg (1)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Omelette (3 eggs)</td>
<td>☑ No ☑ Yes</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Quiche (1 slice)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Frittata (1 slice)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>French toast (1 slice)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing fried rice (1 cup)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing fresh pasta (e.g. noodles, Lasagne etc.) 1 cup</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing sauce (e.g. carbonara, hollandaise etc.) 1 serve</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing patties/balls</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing cake or muffin (1 piece/muffin)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing biscuit</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pavlova/Meringue (1 serve)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg containing pancake/pikelets (1)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egg custard (tinned or homemade) (1 serve)</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Crumbed chicken</td>
<td>☑ No ☑ Yes</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Total eggs eaten over 2 weeks (use egg conversion sheet)

After the phone call

B 2.2 Maternal average weekly egg intake: ___ ____ . ____ eggs (total eggs consumed / 2)
B 2.3 Have you been able to follow your recommended dietary egg intake?

- Yes (Go to B3)
- No, please specify reason
  - practical issues (did not count properly, forgot etc)
  - does not want to follow intervention
  - health professional advice
  - perceived adverse event, please specify ______________________
    ___________________________________________________________
  - Other, please specify: ________________________________
    ________________________________________________________

B 3 Have you (mother) had an infection since birth?

- No
- Yes, how many times __ __ (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>ENT</td>
</tr>
<tr>
<td>urinary</td>
<td>GI</td>
</tr>
<tr>
<td>skin</td>
<td>birth related</td>
</tr>
<tr>
<td>other</td>
<td></td>
</tr>
<tr>
<td>Occasion 2</td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>ENT</td>
</tr>
<tr>
<td>urinary</td>
<td>GI</td>
</tr>
<tr>
<td>skin</td>
<td>birth related</td>
</tr>
<tr>
<td>other</td>
<td></td>
</tr>
</tbody>
</table>

B 4 Breastfeeding

B 4.1 Has your baby ever been fed any breast milk?

- Yes
- No (go to B 6)

B 4.2 Is your baby currently breastfed?

- Yes; number of feeds in the last 24 hours __ __
- No; how old was your baby when you stopped breastfeeding? __ __ (days)

B 4.3 Is there any aspect of breastfeeding that you would like more support/advice?

- No (go to B 5)
- Yes, please specify ________________________________
B 5 Breast milk sample

B 5.1 Has a breast milk sample been collected?

☐ Yes, please complete below

- Volume: ___ ___ ml
- Date of collection: ___ ___ / ___ ___ / ___ ___
- Time of collection: ___ : ___
- Amount and type of egg eaten prior: __________________________
- Time of egg consumption: ___ : ___

☐ No, please specify:

☐ planning to collect later or tomorrow
☐ does not want to give a sample
☐ having difficulties expressing
☐ ceased breastfeeding

B 5.2 Is follow-up required regarding breastfeeding/expressing difficulties?

☐ No
☐ Yes; please provide details: __________________________

*Complete after telephone call*

Egg equivalence consumed prior to breast milk sample: ___ ___ . ___ eggs
Time from egg consumption to breast milk sample collection: ___ ___ hrs

B 6 Formula Feeding

B 6.1 Has your baby been fed any infant formula since birth?

☐ No ☐ Yes

B 6.2 What was your baby’s age when they were first given any infant formula?

___ ___ (weeks) ___ ___ (days)

B 6.3 Which infant formula has your baby been fed most since birth?

____________________________________

Full name of formula

B 6.4 Is your baby currently fed infant formula?

☐ Yes, number of feeds per 24 hours ___ ___ volume ___ ___ ml/day

☐ No, how old was your baby when you stopped feeding infant formula? ___ ___ (days)
**After Telephone call**

**B 7  What advice has been given regarding breastfeeding/expressing difficulties?**

- None, there are no breastfeeding difficulties
- None, following up with birth hospital lactation services
- Provided the Australian Breastfeeding Association contact details
- Lactation consultant has been notified, date: __ __ / __ __ / __ __ __ __

Section completed by (Sign):  
Date:  
Checked by:
SECTION C: 4 WEEK TELEPHONE CALL

Date of telephone call ___ / ___ / ___

C 1 Maternal dietary egg intake compliance

C 1.1 Which egg containing foods have you eaten between 2-4 weeks of lactation? (Please complete the table below)

<table>
<thead>
<tr>
<th>Food</th>
<th>Eaten</th>
<th>Amount</th>
<th>Egg Equivalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw egg (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boiled egg (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fried egg (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scrambled egg (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poached egg (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omelette (3 eggs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quiche (1 slice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frittata (1 slice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>French toast (1 slice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing fried rice (1 cup)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing fresh pasta (e.g. noodles, Lasagne etc.) 1 cup</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing sauce (e.g. carbonara, hollandaise etc.) 1 serve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing patties/balls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing cake or muffin (1 piece/muffin)</td>
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<td></td>
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</tr>
<tr>
<td>Egg containing biscuit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pavlova/Meringue (1 serve)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing pancake/pikelets (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg custard (tinned or homemade) (1 serve)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crumbed chicken</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total eggs eaten over 2 weeks (use egg conversion sheet)

Total eggs eaten over two weeks (use egg conversion sheet)

After the phone call

C 1.2 Maternal average weekly egg intake: ___ ___ _ ___ eggs (total eggs consumed / 2)
C 1.3 Have you been able to follow your recommended dietary egg intake?
  □ Yes (go to C2)
  □ No, please specify reason
    □ practical issues (forgot etc)
    □ does not want to follow intervention
    □ health professional advice
    □ perceived adverse event, please specify ________________________

  □ Other, please specify: __________________________

C 2 Have you (mother) had any infections between 2 - 4 weeks of lactation?
  □ No  □ Yes, how many times ____ (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>□ No □ Yes</td>
</tr>
<tr>
<td>ENT</td>
<td></td>
</tr>
<tr>
<td>mastitis</td>
<td></td>
</tr>
<tr>
<td>urinary</td>
<td></td>
</tr>
<tr>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>birth related</td>
<td></td>
</tr>
<tr>
<td>skin</td>
<td></td>
</tr>
<tr>
<td>other</td>
<td></td>
</tr>
</tbody>
</table>

| Occasion 2                                             |             |
| respiratory                                           | □ No □ Yes  |
| ENT                                                    |             |
| mastitis                                               |             |
| urinary                                                |             |
| GI                                                     |             |
| birth related                                          |             |
| skin                                                   |             |
| other                                                  |             |

C 3 Breastfeeding

C 3.1 Has your baby ever been fed any breast milk?
  □ Yes  □ No (go to C 5)

C 3.2 Is your baby currently breastfed?
  □ Yes; number of feeds in the last 24 hours ____
  □ No; how old was your baby when you stopped breastfeeding? ____ (days)

C 3.3 Is there any aspect of breastfeeding that you would like more support/advice?
  □ No (go to C 4)
  □ Yes, please specify __________________________

C 3.4 Is follow-up required regarding breastfeeding/expressing difficulties?
  □ No
  □ Yes; please provide details: __________________________
### C 4 Breast milk sample

**C 4.1** Has a breast milk sample been collected?
- [ ] Yes, please complete below
  - Volume: ___ ___ ml
  - Date of collection: ___ / ___ / ___ ___
  - Time of collection: ___ : ___
  - Amount and type of egg eaten prior: ___________________________________________
  - Time of egg consumption: ___ : ___
- [ ] No, please specify:
  - planning to collect later or tomorrow
  - does not want to give a sample
  - having difficulties expressing
  - ceased breastfeeding

**Complete after telephone call**

- Egg equivalence consumed prior to breast milk sample: ___ ___ . ___ eggs
- Time from egg consumption to breast milk sample collection: ___ ___ hrs

### C 5 Formula Feeding

**C 5.1** Has your baby been fed any infant formula between 2 and 4 weeks?
- [ ] Yes  [ ] No (Phone call finished)

**C 5.2** What was your baby’s age when they were first given any infant formula?
- ___ (weeks) ___ (days)  or  [ ] Previously recorded

**C 5.3** Which infant formula has your baby been fed most since birth?
- ______________________________________
  - Full name of formula

**C 5.4** Is your baby currently fed infant formula?
- [ ] Yes, number of feeds per 24 hours ___ ___ volume ___ ___ ml/day
- [ ] No, how old was your baby when you stopped feeding infant formula? ___ ___ (days)

**After Telephone call**

**C 6** What advice has been given regarding breastfeeding/expressing difficulties?
- [ ] None, there are no breastfeeding difficulties
- [ ] None, she is following up with birth hospital lactation services
- [ ] Provided the Australian Breastfeeding Association contact details
- [ ] Lactation consultant has been notified, date: ___ / ___ / ___ ___ ___
<table>
<thead>
<tr>
<th>Section completed by (Sign):</th>
<th>Date:</th>
<th>Checked by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SECTION D: 6 WEEK APPOINTMENT

D 1 Date of appointment __ ___ / __ ___ / __ ___ (dd/mm/yyyy)

D 2 Maternal dietary egg intake compliance

D 2.1 Which egg containing foods have you eaten between 4-6 weeks of lactation? (Please complete the table below)

<table>
<thead>
<tr>
<th>Food</th>
<th>Eaten</th>
<th>Amount</th>
<th>Egg Equivalence (after appointment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw egg (1)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boiled egg (1)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fried egg (1)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scrambled egg (1)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poached egg (1)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omelette (3 eggs)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quiche (1 slice)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frittata (1 slice)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>French toast (1 slice)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing fried rice (1 cup)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing fresh pasta (e.g. noodles, Lasagne etc.) 1 cup</td>
<td>No</td>
<td>No</td>
<td>Yes →</td>
</tr>
<tr>
<td>Egg containing sauce(e.g. carbonara, hollandaise etc.) 1 serve</td>
<td>No</td>
<td>No</td>
<td>Yes →</td>
</tr>
<tr>
<td>Egg containing patties/balls</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing cake or muffin (1 piece/muffin)</td>
<td>No</td>
<td>No</td>
<td>Yes →</td>
</tr>
<tr>
<td>Egg containing biscuit</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pavlova/Meringue (1 serve)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg containing pancake/pikelets (1)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg custard (tinned or homemade) (1 serve)</td>
<td>No</td>
<td>No</td>
<td>Yes →</td>
</tr>
<tr>
<td>Crumbed chicken</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total eggs eaten over 2 weeks (use egg conversion sheet)

After the appointment

D 2.2 Maternal average weekly egg intake: ____ ____ . ____ eggs (total eggs consumed / 2)
D 2.3 Have you been able to follow your recommended dietary egg intake?
  ❑ Yes (Go to D3)
  ❑ No, please specify reason
    ❑ practical issues
    ❑ does not want to follow intervention
    ❑ health professional advice
    ❑ perceived adverse event, please specify: ______________________
    ______________________
  ❑ Other, please specify: ______________________

D 3 Maternal General Health

D 3.1 Have you (mother) had any infections between 4 - 6 weeks of lactation?
  ❑ No ❑ Yes (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection</th>
<th>Antibiotics</th>
<th>Over the counter medication (specify)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ❑ Yes</td>
<td>No ❑ Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occasion 1</td>
<td>No ❑ Yes</td>
<td>No ❑ Yes</td>
</tr>
<tr>
<td>❑ respiratory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ ENT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ mastitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ urinary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ GI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occasion 2</td>
<td>No ❑ Yes</td>
<td>No ❑ Yes</td>
</tr>
<tr>
<td>❑ respiratory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ ENT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ mastitis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ urinary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ GI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ skin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>❑ other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D 3.2 Have you (mother) had any vaccinations between birth and 6 weeks of age?
  ❑ No
  ❑ Yes: ❑ FluVax ❑ Other, please specify: ______________________

D 4 Infant Birth Details

D 4.1 Gestational age at birth: ___ weeks ___ days

D 4.2 Birth Anthropometry

D 4.2.1 Weight: ___ ___ ___ grams
D 4.2.2 Recumbent Length: ___ . ___ cm
D 4.2.3 Head circumference: ___ . ___ cm

D 4.3 Mode of delivery
  ❑ Natural vaginal
  ❑ Assisted vaginal (vacuum, forceps)
  ❑ Caesarean section
D 4.4 Baby’s Physician Details

<table>
<thead>
<tr>
<th>Doctor’s Name</th>
<th>First Name</th>
<th>Last Name</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Area of clinical practice
- [ ] GP
- [ ] Paediatrician
- [ ] Other: _______________________

Clinic name:

Clinic address:

State _____________ Post Code: __ __ __ __

Telephone number:

D 5 Infant Feeding History

D 5.1 Breastfeeding

D 5.1.1 Has your baby been fed any breast milk between 4 - 6 weeks of age?
- [ ] Yes
- [ ] No (go to D 5.2)

D 5.1.2 Is your baby currently breastfed?
- [ ] Yes, number of feeds in last 24 hours __ __
- [ ] No, how old was your baby when you stopped breastfeeding?
  __ __ months & __ __ weeks

D 5.2 Formula Feeding

D 5.2.1 Has your baby been fed any infant formula between 4 – 6 weeks of age?
- [ ] No (Go to D 5.3)
- [ ] Yes

D 5.2.2 What was your baby’s age when they were first given any infant formula?
  __ __ (weeks) __ __ (days) Or [ ] Previously recorded

D 5.2.3 Which infant formula has your baby been fed most since birth?
  ___________________________________________________________

Full name of formula

D 5.2.4 Is your baby currently fed infant formula?
- [ ] Yes, number of feeds in the last 24 hours __ __ volume __ __ __ ml/day
- [ ] No, how old was your baby when you stopped feeding infant formula?
  __ __ (weeks) __ __ (days)
D 5.3 Is your baby currently consuming any other fluids?

- Yes, please complete table below
- No (go to D6)

<table>
<thead>
<tr>
<th>Fluid</th>
<th>≤ 1 times/week</th>
<th>2-3 times/week</th>
<th>4-5 times/week</th>
<th>≥ 6 times/week</th>
<th>Amount (ml/serve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow’s milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit juice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D 6 Infant General Health

D 6.1 Has your baby seen a doctor because he/she was unwell between birth and 6 weeks of age? (Do not include vaccinations or well baby checkups)

- No (Go to D 6.3)
- Yes, specify number of times: __ __

D 6.2 Have any of these symptoms been reasons for the consultations (cross all that apply)

- Noisy breathing (wheeze or stridor)
- Floppy unresponsive baby
- Vomiting
- Swelling of face or body
- Loose watery stools
- Hives
- Blood stained stools
- Generalised skin rash
- Poor sleeping
- None of these symptoms
- Irritability

D 6.3 Has your baby taken any antibiotics between birth and 6 weeks of age?

- No
- Yes, how many courses? __ __ (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Course 1</td>
</tr>
<tr>
<td>respiratory</td>
</tr>
<tr>
<td>Course 2</td>
</tr>
<tr>
<td>respiratory</td>
</tr>
</tbody>
</table>
D 6.4 Has your baby been taken any over the counter medication between birth and 6 weeks of age?

☐ No  ☐ Yes, indicate reason and provide details

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Medication &amp; Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
</tr>
<tr>
<td>☐ Fever</td>
<td>☐ Stuffy nose</td>
</tr>
<tr>
<td>☐ Constipation</td>
<td>☐ Cough/ cold</td>
</tr>
<tr>
<td>☐ Skin</td>
<td>☐ Other</td>
</tr>
<tr>
<td>Occasion 2</td>
<td></td>
</tr>
<tr>
<td>☐ Fever</td>
<td>☐ Stuffy nose</td>
</tr>
<tr>
<td>☐ Constipation</td>
<td>☐ Cough/ cold</td>
</tr>
<tr>
<td>☐ Skin</td>
<td>☐ Other</td>
</tr>
</tbody>
</table>

D 6.5 Has your baby been admitted to hospital or attended a hospital emergency department between birth and 6 weeks of age?

☐ No (Go to D 7)  ☐ Yes, how many times? ___ (Please complete the table)

For each admission to hospital or attendance at a hospital emergency department document the date, name of the hospital, length of stay and reason in the table below.

<table>
<thead>
<tr>
<th>Admission date</th>
<th>Hospital Name</th>
<th>Length of stay days (1 day min)</th>
<th>Primary diagnosis or reason for hospitalisation</th>
<th>Secondary diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>__ / __ / ____</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>__ / __ / ____</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D 7 Sun Exposure

D 7.1 What times of day do you normally take your baby outdoors? (estimate times)

☐ Before 11am  Time in direct sunlight:    ____ minutes

Time in shade:    ____ minutes

☐ 11am to 3 pm   Time in direct sunlight:    ____ minutes

Time in shade:    ____ minutes

☐ After 3pm      Time in direct sunlight:    ____ minutes

Time in shade:    ____ minutes
D 7.2 When outdoors, does your baby wear sun screen?
- Always
- Sometimes
- Never (go to D 7.4)

D 7.3 What factor sunscreen?
- < Factor 30
- Factor 30
- > Factor 30
- Unknown

D 7.4 Amount of skin exposed
- Face and hands
- Face, hands and arms
- Face, hands, arms and legs

D 8 Eczema / atopic dermatitis history

D 8.1 Has your baby shown signs of dry, red, itchy, scaly skin (eczema) between birth and 6 weeks of age?
- No (Go to D 9)
- Yes, complete D 8.5 SCORAD

D 8.2 At what age did the symptoms first occur? ___ months & ___ weeks

D 8.3 Has a medical doctor diagnosed your baby as having eczema/atopic dermatitis?
- No
- Yes

D 8.4 Is your baby currently on any treatments for eczema/atopic dermatitis?
- No
- Yes, please indicate all treatments currently used:
  - Dietary changes, please specify ________________________________
  - Oral medications, please specify ______________________________
  - Prescription steroid cream, please specify _______________________
  - Over the counter steroid cream, please specify ____________________
  - Moisturizer, please specify _________________________________
  - Other, please specify_________________________________________
D 8.5 SCORAD Assessment

SCORAD INDEX
EUROPEAN TASK FORCE ON ATOPIC DERMATITIS

A: EXTENT Please indicate the area involved
B: INTENSITY
C: SUBJECTIVE SYMPTOMS PRURITUS + SLEEP LOSS

Objective SCORAD
A/I5+7B/I2 /83
SCORAD A/I5+7B/I2+C /103

CRITERIA INTENSITY
Erythema
Oedema/Polypopulation
Oozing/Incrust
Excoriation
Lichenification
Dryness*

Visual analog scale (average for the last 3 days or nights)

* Dryness is evaluated on uninvolved areas

MEANS OF CALCULATION
INTENSITY ITEMS
(average representative area)
0 = absence
1 = mild
2 = moderate
3 = severe

PRURITUS (0 to 10) 
SLEEP LOSS (0 to 10) 

Derived from SCORAD INDEX, Dermatology 1993; 150:23-31
D 9 Infant Assessment

D 9.1 Current Anthropometry
D 9.1.1 Weight ___ ___ ___ grams
D 9.1.2 Length ___ . ___ cm
D 9.1.3 Head circumference ___ . ___ cm

D 9.2 Infant TEWL Assessment:
D 9.2.1 Is the measurement site free from moisturiser for at least 24 hours?
☑ Yes ☐ No
D 9.2.2 Temperature: ___ . ___ (°C) Humidity: ___ . ___ (%)
D 9.2.3 TEWL 1: ___ ___ . ___ ___ g/m2h
    TEWL 2: ___ ___ . ___ ___ g/m2h
    TEWL 3: ___ ___ . ___ ___ g/m2h

D 9.3 Biological samples
D 9.3.1 Infant blood collected?
☑ Yes, time of collection: ___: ___ (use 24 hr clock) Volume: ___ . ___ ml
☐ Yes, time of collection: ___: ___ (use 24 hr clock) Volume: ___ . ___ ml
☐ No, specify reasons (check one only):
☐ Unsuccessful ☐ Refused ☐ Other specify: ____________________________
D 9.3.2 Who collected/attempted the blood sample: ____________________________

D 10 Maternal Assessment

D 10.1 Maternal TEWL assessment
D 10.1.1 Is the measurement site free from moisturiser for at least 24 hours?
☐ Yes ☐ No
D 10.1.2 Temperature: ___ . ___ (°C) Humidity: ___ . ___ (%)
D 10.1.3 TEWL 1: ___ ___ . ___ ___ g/m2h
    TEWL 2: ___ ___ . ___ ___ g/m2h
    TEWL 3: ___ ___ . ___ ___ g/m2h

D 10.2 Maternal Biological samples
D 10.2.1 Maternal blood collected?
☑ Yes, time of collection: ___: ___ (use 24 hr clock) Volume: ___ . ___ ml
☐ Yes, time of collection: ___: ___ (use 24 hr clock) Volume: ___ . ___ ml
☐ No, specify reasons (check one only):
☐ Unsuccessful ☐ Refused ☐ Other specify: ____________________________
D 10.2.2 Who collected/attempted the blood sample: ____________________________
D 10.2 Biological samples

D 10.2.1 Maternal blood sample collected?

☐ Yes, time of collection: ___ : ___ (use 24 hr clock) Volume: ___ . ___ ml

☐ No, specify reasons (check one only)
  ☐ unsuccessful    ☐ Refused    ☐ Other specify: ______________________

D 10.2.2 Who collected/attempted the blood sample: ______________________

D 10.3 Breast milk sample collected?

☐ Yes; volume: ___ . ___ ml (please complete below)

  10.3.1 Date of collection: ___ / ___ / ___ ___ ___  Time: ___ : ___

  10.3.2 Type of egg containing food consumed: _________________________

  10.3.3 Amount of egg containing food consumed: _________________________

  10.3.4 Time of egg consumption: ___ : ___

☐ No, please specify:

  ☐ does not want to give a sample
  ☐ having difficulties expressing
  ☐ Ceased breast-feeding

D 10.4 Home breast milk received?

☐ Day 14 breast milk samples
☐ Day 28 breast milk samples
☐ Samples yet to be received
☐ Samples not collected by participant on: ☐ Day 14  ☐ Day 28

Complete after visit  (Egg Free diet not applicable)

Egg equivalence consumed prior to breast milk sample: ___ . ___ eggs

Time from egg consumption to breast milk sample collection: ___ . ___ hrs
<table>
<thead>
<tr>
<th>QuEST Study</th>
<th>Dietary Group</th>
<th>Study No:</th>
<th>Q</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
</table>

**SECTION E: 16 WEEK APPOINTMENT**

**E 1** Date of appointment  __ __ / __ / __ __ __ __ (dd/mm/yyyy)

**E 2** Maternal dietary egg intake compliance

**E 2.1** Which egg containing foods have you eaten over the last week? *(Please complete the table below)*

<table>
<thead>
<tr>
<th>Food</th>
<th>Eaten</th>
<th>Amount</th>
<th>Egg Equivalence (after appointment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw egg (1)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Boiled egg (1)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Fried egg (1)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Scrambled egg (1)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Poached egg (1)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Omelette (3 eggs)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Quiche (1 slice)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Frittata (1 slice)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>French toast (1 slice)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing fried rice (1 cup)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing fresh pasta (e.g. noodles, Lasagne etc.) 1 cup</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing sauce(e.g. carbonara, hollandaise etc.) 1 serve</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing patties/balls</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing cake or muffin (1 piece/muffin)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing biscuit</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Pavlova/Meringue (1 serve)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg containing pancake/pikelets (1)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Egg custard (tinned or homemade) (1 serve)</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
<tr>
<td>Crumbed chicken</td>
<td>☐ No</td>
<td>☐ Yes  →</td>
<td></td>
</tr>
</tbody>
</table>

Total eggs eaten over the last week *(use egg conversion sheet)*

---

**After the appointment**

**E 2.2** Maternal average weekly egg intake:  ____ . ____ . ____ eggs *(total eggs consumed)*
E 3  Maternal General Health

E 3.1 Have you (mother) had any infections in the last 2 weeks?

- No
- Yes (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Antibiotics</th>
<th>Over the counter medication (specify)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>ENT</td>
<td>mastitis</td>
</tr>
<tr>
<td>urinary</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>skin</td>
<td>other</td>
<td></td>
</tr>
<tr>
<td>Occasion 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>ENT</td>
<td>mastitis</td>
</tr>
<tr>
<td>urinary</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>skin</td>
<td>other</td>
<td></td>
</tr>
</tbody>
</table>

E 3.2 Have you had any other infections since your last appointment (between 6 -14 weeks)?

- No
- Yes (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Antibiotics</th>
<th>Over the counter medication (specify)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>ENT</td>
<td>mastitis</td>
</tr>
<tr>
<td>urinary</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>skin</td>
<td>other</td>
<td></td>
</tr>
<tr>
<td>Occasion 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>respiratory</td>
<td>ENT</td>
<td>mastitis</td>
</tr>
<tr>
<td>urinary</td>
<td>GI</td>
<td></td>
</tr>
<tr>
<td>skin</td>
<td>other</td>
<td></td>
</tr>
</tbody>
</table>

E 3.3 Have you (mother) had any vaccinations between 6 -16 weeks of lactation?

- No
- Yes: FluVax
- Other, please specify: __________________________

E 4  Infant Feeding History

E 4.1 Breastfeeding

E 4.1.1 Has your baby been fed any breast milk between 6 and 16 weeks of age?

- Yes
- No (go to D 4.2)

E 4.1.2 Is your baby currently breastfed?

- Yes, number of feeds in last 24 hours __ __
- No, how old was your baby when you stopped breastfeeding?
  __ __ months & __ __ weeks
E 4.2 Formula Feeding

E 4.2.1 Has your baby been fed any infant formula between 6 – 16 weeks?
   - No (go to E 4.3)  - Yes

E 4.2.2 What was your baby’s age when they were first given any infant formula?
   __ __ (weeks) __ __ (days) or  - Previously recorded

E 4.2.3 Which infant formula has your baby been fed most since 6 weeks?
   ____________________________________________________________
   Full name of formula

E 4.2.4 Is your baby currently fed infant formula?
   - Yes, number of feeds in the last 24 hours __ __ volume __ __ __ __ ml/day
   - No, how old was your baby when you stopped feeding infant formula?
     __ __ (weeks) __ __ (days)

E 4.3 Is your baby currently consuming any other fluids?
   - Yes, please complete table below  - No (go to E 4.4)

<table>
<thead>
<tr>
<th></th>
<th>≤ 1 times/week</th>
<th>2-3 times/week</th>
<th>4-5 times/week</th>
<th>≥ 6 times/week</th>
<th>Amount (ml/serve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow’s milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit juice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E 4.4 Introduction of Solid Food

E 4.4.1 Have you introduced solid foods to your baby?
   - No (Go to E5)  - Yes

E 4.4.2 How old was your baby when solid foods were first introduced?
   __ __ months & __ __ weeks
E4.4.3 For the following foods, please record the age when the foods were introduced for the first time and any reactions the infant may have experienced to the food?

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>Study No:</th>
<th>QuEST Study Case Report Form</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Have any of these foods been introduced (directly ingested)?</th>
<th>How old was your child when first introduced?</th>
<th>Did your child have a reaction to this food?</th>
<th>Symptoms Insert number/s according to index below</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg</td>
<td>❑ No (go to Wheat)</td>
<td>❑ No (go to cow's milk)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>❑ Yes →</td>
<td>❑ Yes between 2-6 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>❑ Yes after 6 hours</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>❑ No (go to peanut)</td>
<td>❑ No (go to fish)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>❑ Yes →</td>
<td>❑ Yes within 2 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>❑ Yes between 2-6 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>❑ Yes after 6 hours</td>
<td></td>
</tr>
<tr>
<td>Peanut</td>
<td>❑ No (go to cashew nut)</td>
<td>❑ No (go to cashew nut)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>❑ Yes →</td>
<td>❑ Yes within 2 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>❑ Yes between 2-6 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>❑ Yes after 6 hours</td>
<td></td>
</tr>
</tbody>
</table>

### Symptoms Index

1. Red skin rash on face or body  
2. Hives  
3. Swelling of face or body  
4. Wheeze or stridor  
5. Cough  
6. Vomiting  
7. Loose watery stools  
8. Blood stained stools  
9. Floppy unresponsive baby

### E 5 Infant General Health

E 5.1 Has your baby seen a doctor because he/she was unwell between 6 - 16 weeks of age? (Do not include vaccinations or well baby checkups)

❑ No (go to E 5.3)  ❑ Yes, specify number of times: __ __

E 5.2 Have any of these symptoms been reasons for the consultations (cross all apply)

❑ Noisy breathing (wheeze or stridor)  ❑ Floppy unresponsive baby  
❑ Vomiting  ❑ Swelling of face or body  
❑ Loose watery stools  ❑ Hives  
❑ Blood stained stools  ❑ Generalised skin rash  
❑ Poor sleeping  ❑ None of these symptoms  
❑ Irritability
E 5.3 Has your baby taken any antibiotics 6-16 weeks of age?

- [ ] No
- [ ] Yes, how many courses? __ __ (please complete the table below)

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Course 1</th>
<th>Course 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>respiratory</td>
<td>ENT</td>
</tr>
<tr>
<td></td>
<td>respiratory</td>
<td>ENT</td>
</tr>
</tbody>
</table>

E 5.4 Has your baby been taken any over-the-counter medication between 6-16 weeks of age?

- [ ] No
- [ ] Yes, indicate reason and provide details

<table>
<thead>
<tr>
<th>Reason / Type of Infection (cross all that apply below)</th>
<th>Medication &amp; Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occasion 1</td>
<td></td>
</tr>
<tr>
<td>- [ ] Fever</td>
<td></td>
</tr>
<tr>
<td>- [ ] Constipation</td>
<td></td>
</tr>
<tr>
<td>- [ ] Skin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Occasion 2</td>
<td></td>
</tr>
<tr>
<td>- [ ] Fever</td>
<td></td>
</tr>
<tr>
<td>- [ ] Constipation</td>
<td></td>
</tr>
<tr>
<td>- [ ] Skin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E 5.5 Has your baby been admitted to hospital or attended a hospital emergency department between 6-16 weeks of age?

- [ ] No (go to E 6)
- [ ] Yes, how many times? __ __ (Please complete table)

For each admission to hospital or attendance at a hospital emergency department document the date, name of the hospital, length of stay and reason in the table below.

<table>
<thead>
<tr>
<th>Admission date</th>
<th>Hospital Name</th>
<th>Length of stay: days (any part of day = 1)</th>
<th>Primary diagnosis or reason for hospitalisation</th>
<th>Secondary diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>__ / __ / ___</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>__ / __ / ___</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
E 6 Sun Exposure

E6.1 What times of day do you normally take your baby outdoors? (estimate times)

- Before 11am
  - Time in direct sunlight: _______ minutes
  - Time in shade: _______ minutes
- 11am to 3 pm
  - Time in direct sunlight: _______ minutes
  - Time in shade: _______ minutes
- After 3pm
  - Time in direct sunlight: _______ minutes
  - Time in shade: _______ minutes

E 6.2 When outdoors, does your baby wear sun screen?

- Always
- Sometimes
- Never *(go to E 6.4)*

E 6.3 What factor sunscreen?

- < Factor 30
- Factor 30
- > Factor 30
- Unknown

E 6.4 Amount of skin exposed

- Face and hands
- Face, hands and arms
- Face, hands, arms and legs

E 7 Eczema / atopic dermatitis history

E 7.1 Has your baby shown signs of dry, red, itchy, scaly skin (eczema) between 6 and 16 weeks of age?

- No *(go to E 8)*
- Yes, complete SCORAD

E 7.2 At what age did the symptoms first occur? ___ months & ___ weeks

E 7.3 Has a medical doctor diagnosed your baby as having eczema/atopic dermatitis?

- No
- Yes

E 7.4 Is your baby currently on any treatments for eczema/atopic dermatitis?

- No

- Yes, please indicate all treatments currently used:
  - Dietary changes, *please specify* ________________________________
  - Oral medications, *please specify* ________________________________
  - Prescription steroid cream, *please specify* _________________________
  - Over the counter steroid cream, *please specify* _____________________
  - Moisturiser, *please specify* ________________________________
  - Other, *please specify* ________________________________________
E 7.5 Eczema SCORAD (complete at the same time as anthropometrics)

**SCORAD INDEX**
*European Task Force on Atopic Dermatitis*

**A: EXTENT** Please indicate the area involved

**B: INTENSITY**

**C: SUBJECTIVE SYMPTOMS**
- Pruritus
- Sleep Loss

**Objective SCORAD**

A/I+7B/2  83

**SCORAD**

A/I+7B/2+C  103

**CRITERIA**
- Erythema
- Oedema/Papulation
- Oozing/ooze
- Excoriation
- Lichenification
- Dryness

**INTENSITY**

* Dryness is evaluated on uninvolved areas

**Visual analog scale**
(average for the last 3 days or nights)

<table>
<thead>
<tr>
<th>PRURITUS (0 to 10)</th>
<th>SLEEP LOSS (0 to 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Derived from SCORAD INDEX, *Dermatology* 1993; 180:23-39
E 8 Infant Assessment

E 8.1 Current Anthropometry
E 8.1.1 Weight __ __ __ __ grams
E 8.1.2 Length __ __ . __ cm
E 8.1.3 Head circumference __ __ . __ cm

E 8.2 Infant TEWL Assessment:
D 10.1.1 Is the measurement site free from moisturiser for at least 24 hours?
☑ Yes ☐ No
D 10.1.2 Temperature: __ __ . __ (°C) Humidity: __ __ . __ (%)
D 10.1.3 TEWL 1: __ __ . __ __ g/m²h
   TEWL 2: __ __ . __ __ g/m²h
   TEWL 3: __ __ . __ __ g/m²h

E 8.3 Infant Biological sample
E 8.3.1 Infant blood collected?
☑ Yes, time of collection: __ __ : __ __ (use 24 hr clock) Volume: __ __ ml
☐ No, specify reasons (check one only):
☐ Unsuccessful ☐ Refused ☐ Other specify: __________________________

E 8.3.2 Who collected/attempted the blood sample: __________________________

E 9 Maternal Assessment and Biological Samples

E 9.1 Maternal TEWL assessment:
D 10.1.1 Is the measurement site free from moisturiser for at least 24 hours?
☑ Yes ☐ No
D 10.1.2 Temperature: __ __ . __ (°C) Humidity: __ __ . __ (%)
D 10.1.3 TEWL 1: __ __ . __ __ g/m²h
   TEWL 2: __ __ . __ __ g/m²h
   TEWL 3: __ __ . __ __ g/m²h
E 9.3 Breast milk sample collected?

☐ Yes, volume: __ __ . __ ml (please complete below)

9.3.1 Date of collection: __ __ / __ __ / __ __ __ __ Time: __ __: __ __

☐ No, please specify:

☐ does not want to give a sample

☐ having difficulties expressing

☐ ceased breast-feeding

Section completed by (Sign): Date: Checked by:
Appendix F

QuEST Study Diary Card
You have been allocated to:

**Infant DOB**  ___ / ___ / ___

Please start diary card on day that infant was born, **which was a _____________________** (day of week)

Fill in all of line 1 completely before moving onto line 2. Record your egg intake for the first 6 weeks of lactation.

<table>
<thead>
<tr>
<th>Week of lactation</th>
<th>Monday # eggs</th>
<th>Tuesday # eggs</th>
<th>Wednesday # eggs</th>
<th>Thursday # eggs</th>
<th>Friday # eggs</th>
<th>Saturday # eggs</th>
<th>Sunday # eggs</th>
<th>Total # eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Volume of any infant formula**

<table>
<thead>
<tr>
<th>Week of lactation</th>
<th>Monday (mls)</th>
<th>Tuesday (mls)</th>
<th>Wednesday (mls)</th>
<th>Thursday (mls)</th>
<th>Friday (mls)</th>
<th>Saturday (mls)</th>
<th>Sunday (mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>3</td>
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<td>6</td>
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</tr>
</tbody>
</table>

**Amount of egg in common egg containing foods:**

<table>
<thead>
<tr>
<th></th>
<th>1 egg</th>
<th>1 serve pavlova/meringue</th>
<th>1/2 egg</th>
<th>1 pattie or large meat ball</th>
<th>1/6 egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg containing sauce carbonara/hollandaise</td>
<td>3/4 egg</td>
<td>Egg containing fresh pasta or lasagne</td>
<td>1/4 egg</td>
<td>1 slice cake or 1 muffin</td>
<td>1/6 egg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1 egg</th>
<th>1 crumbed chicken or fish fillet</th>
<th>1/4 egg</th>
<th>1 serve pudding/custard</th>
<th>1/6 egg</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1/2 egg</th>
<th>1 serve quiche</th>
<th>1/4 egg</th>
<th>1 serve pudding/custard</th>
<th>1/6 egg</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1/2 egg</th>
<th>1 slice French Toast</th>
<th>1/4 egg</th>
<th>1 pancake</th>
<th>1/12 egg</th>
</tr>
</thead>
</table>

**If you have any questions about the completion of this Diary Card please contact Jessica on 9340 8834.**
**BREAST MILK SAMPLES**

- You are asked to collect a **10ml** breast milk sample on **day 14** and **day 28** of lactation.
- Please read the collection instructions inside your breast milk collection kit.

**IMPORTANT** store in your freezer immediately after collection to preserve the sample.

---

### DAY 14 OF LACTATION: BREAST MILK SAMPLE

- Date of collection: ____ / ____ / ______
- Time: ____ : ____ (24hr clock)
- Volume: ____ . ____ ml
- Time of egg consumption: ____ : ____ (24hr clock)
- Type of egg containing food consumed: ________________________________________
- Amount of egg containing food consumed: _______________________________________

---

### DAY 28 OF LACTATION: BREAST MILK SAMPLE

- Date of collection: ____ / ____ / ______
- Time: ____ : ____ (24hr clock)
- Volume: ____ . ____ ml
- Time of egg consumption: ____ : ____ (24hr clock)
- Type of egg containing food consumed: ________________________________________
- Amount of egg containing food consumed: _______________________________________

**IMPORTANT** store in your freezer immediately after collection to preserve the sample.

---

If you have any questions about the completion of this Diary Card please contact Jessica on 9340 8834.
Appendix G

Egg Free Diet Sheet
QuEST TRIAL EGG-FREE DIET INFORMATION

You will need to follow an egg-free diet for the first 6 weeks of lactation.

This includes avoiding all types of eggs (eg hen, duck and goose).

Eggs may be used as an ingredient in many food products, you should always check the ingredient list carefully. In Australia, the presence of egg in a food must be declared on the label. If you are not sure, check with the manufacturer or avoid the food.

Avoid foods containing the following ingredients:

- albumin
- apovitellin
- avidin
- dried egg
- egg
- egg protein
- egg powder
- egg solids
- egg white
- egg yolk
- flavoproteins
- globulin
- livetin
- lysozyme
- ovalbumin
- ovoglycoprotein
- ovomucin
- ovomucoid
- ovomuxoid
- sili ci albuminate
- simplesse
- vitellin
- whole egg

Foods which usually contain egg:

- Eggs – boiled, fried, frozen, poached, powdered, raw, scrambled
- egg nog
- egg noodles
- egg pasta
- frittata
- omelettes
- quiche
- pavlova
- meringues
- soufflés

Foods which may contain egg (check the food ingredient label carefully):

- biscuits, cakes, muffins, cake mixes, buns, doughnuts, pastries, macaroons, quick breads
- pancakes, puddings, mousses, royal icing (eg on traditional wedding and christmas cakes)
- custard, ice cream, sherbets, some ice blocks, frozen desserts
- chicken, fish, meat or vegetarian patties, rissoles, meat balls, processed meats, meat loaf
- crumbed or battered foods, fritters, hamburgers
- fresh pasta, lasagne, some canned tuna, soups, salad dressings
- egg mayonnaise, tartar sauce, coleslaw dressing, béarnaise sauce, hollandaise sauce
- glazed fruits, fruit bars, health food bars, protein bars
- egg nog, malted drinks, some milk drink mixes/shakes
- some lollies, confectionary and marzipan

Eggs maybe used to glaze baked goods, eg buns, pastries, pretzels and bread rolls

You can avoid egg and still have a healthy diet.

QuEST Trial

May 2013
Appendix H

ASCIA Infant Feeding Guidelines 2016
Infant feeding and allergy prevention

Key recommendations

- When your infant is ready, at around 6 months, but not before 4 months, start to introduce a variety of solid foods, starting with iron rich foods, while continuing breastfeeding.
- All infants should be given allergenic solid foods including peanut butter, cooked egg and dairy and wheat products in the first year of life. This includes infants at high risk of allergy.
- Hydrolysed (partially and extensively) infant formula are not recommended for prevention of allergic disease.

Introduction

ASCIA has developed these guidelines to outline practices that may help reduce the risk of infants developing allergies, particularly early onset allergic diseases such as eczema and food allergy.

These guidelines are based on current published evidence, including information published after 2010. The revised recommendations listed above are based on a consensus agreement by participants in the Infant Feeding Summit hosted by the Centre for Food & Allergy Research (CFAR) in May 2016.

The reasons for the continued rise in allergic diseases, such as food allergy, eczema, asthma and allergic rhinitis (hay fever) are complex and not well understood. Although infants with a family history of allergic disease are at higher risk of allergies, infants with no family history can also develop allergies. Therefore, these guidelines are relevant for all families, including those in which siblings or parents already have food allergies or other allergic conditions.

If your infant already has an allergic disease (such as severe eczema or food allergy), you should discuss what specific measures might be useful with your doctor.

Maternal diet during pregnancy and breastfeeding

- ASCIA recommends a healthy balanced diet, rich in fibre, vegetables and fruit. This provides many health benefits to the mother and infant during pregnancy and breastfeeding.
- Exclusion of any particular foods (including foods considered to be highly allergenic) from the maternal diet during pregnancy or breastfeeding is not recommended, as this has not been shown to prevent allergies.
- Up to 3 serves of oily fish per week may be beneficial, as there is some evidence that omega-3 fatty acids (found in oily fish) during pregnancy and breastfeeding may help prevent eczema in early life.
- Whilst there is moderate evidence that probiotics during pregnancy and breastfeeding may help prevent eczema in early life, recommendations about probiotic supplements cannot currently be made because the optimal species and dose of probiotics that might have an effect is unclear. More research is required in this area before clear and specific recommendations can be made.

Breastfeeding and infant formula

- Breastfeeding is recommended for at least 6 months and for as long as mother and infant wish to continue. There is no consistent evidence that breastfeeding is effective for the prevention of allergic disease. However, breastfeeding is recommended for the many benefits it provides to mother and infant.
• Breastfeeding during the period that solid foods are first introduced to infants from around 6 months may help reduce the risk of the infant developing allergies, although evidence for this is low.

• If breastfeeding is not possible, a standard cow’s milk based formula can be given. There is no evidence that soy or goat’s milk formula reduce the risk of allergic disease when used in preference to standard cow’s milk based formula.

• Based on a recently published review of studies, there is no consistent convincing evidence to support a protective role for partially hydrolysed formulas (usually labelled “HA” or Hypoallergenic) or extensively hydrolysed formulas for the prevention of eczema, food allergy, asthma or allergic rhinitis in infants or children.

• Regular cow’s, goat’s milk (or other mammal derived milks), soy milk, nut and cereal beverages are not recommended for infants as the main source of milk before 12 months of age.

Introduce solid foods from around 6 months, but not before 4 months, when your infant is developmentally ready whilst continuing to breastfeed

• Foods should not be introduced before 4 months.

• Infants differ in the age that they are developmentally ready for solid foods.

• Signs that your infant may be developmentally ready to start solids include: being able to sit relatively unaided, loss of the tongue-thrust reflex that pushes food back out, and trying to reach out and grab food.

• ASCIA recommends the introduction of solid foods around 6 months, but not before 4 months, and preferably whilst breastfeeding. There is some evidence this is protective against the development of allergic disease.

• When your infant is ready, introduce foods according to what the family usually eats, regardless of whether the food is considered to be a common food allergen. There is some evidence that the introduction of common allergenic foods (including cooked eggs as raw egg is not recommended, peanuts, nuts, wheat, fish) should not be delayed. However further evidence is required to clarify optimal timing for each food.

• You may choose to introduce one new food at a time so that if a reaction occurs, the problem food can be more easily identified. If a food is tolerated, continue to give this as a part of a varied diet.

• If possible, continue to breastfeed whilst you introduce foods to your infant. There is some limited evidence that this may reduce the risk of allergies developing, and there are many other health benefits of continued breast feeding.

• Cow’s milk or soy milk (or their products, such as cheese and yoghurt) can be used in cooking or with other foods if dairy products/soy are tolerated.

• There is good evidence that for infants with severe eczema and/or egg allergy, that regular peanut intake before 12 months of age can reduce the risk of developing peanut allergy. If your child already has an egg allergy or other food allergies or severe eczema, you should discuss how to do this with your doctor.

• There is moderate evidence that introducing cooked egg (raw egg is not recommended) into an infant’s diet before 8 months of age, where there is a family history of allergy, can reduce the risk of developing egg allergy.

• When introducing foods that other family members are allergic to, it is important to follow risk minimisation strategies to prevent cross contamination of allergens, for those who are allergic to the foods.

• It is important to understand that the facial skin in babies is very sensitive and that many foods (including citrus, tomatoes, berries, other fruit and vegemite) can irritate the skin and cause redness on contact – this is not food allergy. Smearing food on the skin will not help to identify possible food allergies.

• Some infants will develop food allergies. If there is any allergic reaction to any food, that food should be stopped and you should seek advice from a doctor with experience in food allergy.

Other measures

• Do not smoke during pregnancy, or in the presence of the infant, or in enclosed spaces where the infant sleeps or plays.