The effect of egg ingestion on ovalbumin concentration in human milk.

by

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A thesis submitted for the Degree of Doctor of Philosophy

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Abstract

Maternal dietary avoidance has been recommended to treat food allergy in breastfed infants although the evidence base for this recommendation is limited. A survey was conducted to benchmark current dietetic practice and it revealed that the majority of Australian specialist paediatric allergy dietitians follow the current recommendations. The foods most commonly restricted are cow's milk, egg, peanut and tree nuts and complete rather than partial avoidance of the causal food protein from the maternal diet was commonly advised.

Maternal dietary restriction to treat breastfed infants with food allergy assumes that food proteins ingested by women are absorbed and excreted antigenically intact into breast milk. Egg is one of the most common food allergens and was chosen as the food challenge for my research because it can be restricted in the diet without nutritionally compromising lactating women. A randomised, blinded, crossover, intervention trial was undertaken in breastfeeding women at the same stage of lactation to determine whether the dietary dose of cooked egg influences ovalbumin content in human milk and whether cooked versus raw egg ingestion alters ovalbumin content in human milk. Breast milk samples were collected from 41 women at two hourly intervals for eight hours after maternal ingestion of no egg, one raw egg, half a cooked egg or one cooked egg and ovalbumin concentration measured by enzyme-linked immunosorbent assay (ELISA). The ELISA method was also developed as part of this thesis. The results demonstrated that at least two thirds of these women had breast milk ovalbumin detected after consuming one cooked egg. A direct, dose response between the amount of cooked egg ingested and human

milk ovalbumin concentration was found. The ovalbumin excretion in response to one raw egg did not differ from ingesting half a cooked egg.

A double-blinded, randomised controlled trial with the primary aim of determining the effect of maternal cooked egg ingestion on the ovalbumin concentration in breast milk fed to infants with egg sensitivity was then conducted. A secondary aim was to determine the effect of maternal egg ingestion on infant eczema severity. Each mother was randomly allocated to consume one muffin per day containing one egg (egg group, n=16) or egg-free muffins (control group, n=16) for 21 days, while mothers and infants followed an otherwise egg-free diet. Breast milk samples were collected on three days at two hourly intervals for six hours after muffin ingestion and ovalbumin concentration measured again by ELISA. Infant eczema assessments (SCORAD scores) were preformed at trial commencement and completion. More women in the egg group had consistent breast milk ovalbumin detected at higher peak concentrations and total excretion than the control group. Over the three week period SCORAD scores significantly reduced with time for both groups but the scores were independent of dietary treatment. This lack of difference in mean eczema symptom scores between the groups suggests that maternal dietary avoidance of egg may not be necessary for all breastfed infants with egg sensitivity.

This thesis provides new evidence concerning the effect of egg ingestion on ovalbumin concentration in human milk. This knowledge is crucial for health professionals who recommend maternal dietary restriction to treat food allergy in breastfed infants.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Debra Jane Palmer

Signed: ...

Date: .....16/02/06.....

Publications and presentations in support of thesis

Publications

Palmer DJ, Gold MS, Makrides M. Treatment and prevention of food allergies in breastfed infants: practice and evidence. Nutr Diet 2004;61:76-81.(See Appendix 10)

Palmer DJ, Gold MS, Makrides M. Effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomized, double-blind, cross-over trial. Clin Exp Allergy. 2005;35(2): 173-178. (See Appendix 11)

Published abstracts

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Conference presentations

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Palmer DJ, Gold MS, Makrides M. The effect of egg ingestion on ovalbumin concentration in human milk. *Nutrition Society of Australia Annual Scientific Meeting, December 2003*. (Oral presentation and abstract).

Palmer DJ, Gold MS, Makrides M. Maternal Dietary Restrictions for Treatment and Prevention of Food Allergy in Breastfed Infants. *14th Annual Scientific Meeting of the Australasian Society of Clinical Immunology and Allergy, October 2003.* (Poster and abstract).

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List of Abbreviations

AAP: American Academy of Pediatrics

ANOVA: analysis of variance

CI: confidence interval

CV: coefficient of variation

EAACI: European Academy of Allergology and Clinical Immunology

ELISA: enzyme-linked immunosorbent assay

EMBASE: Excerpta Medica database

ESPACI: European Society for Pediatric Allergology and Clinical Immunology

ESPGHAN: European Society for Pediatric Gastroenterology, Hepatology and Nutrition

IDQOL: Infants' Dermatitis Quality of Life Index

n: number of participants

NHMRC: National Health and Medical Research Council

NS: not significant

PBS: phosphate buffered saline

PEG: polyethylene glycol

RAST: radioallergosorbent test

SCORAD: standardised scoring system for atopic dermatitis

SD: standard deviation

SPT: skin prick test

USA: United States of America

WCH: Women's and Children's Hospital (Adelaide, Australia)

Chapter 1: Introduction and literature review

1.1 Introduction

Food allergy symptoms including atopic dermatitis/eczema, urticaria, colic, diarrhoea and vomiting have been observed in one out of every two hundred exclusively breastfed infants (1-3). Expert nutritional committees of the American Academy of Pediatrics, the European Society for Pediatric Allergology and Clinical Immunology and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (4, 5) have recommended maternal dietary restriction to treat breastfed infants with food allergy.

The majority of published evidence in favour of this recommendation comes from descriptions of case series (2, 6-9) where maternal dietary interventions were non-randomised and uncontrolled. There have only been three randomised trials of crossover design with double-blind food challenges and these trials have demonstrated conflicting results (10-12). Thus current evidence on the use of maternal dietary restriction to treat food allergy in breastfed infants is of limited strength.

The use of maternal dietary restriction during lactation to treat food allergy in breastfed infants led to the hypothesis that maternal dietary restriction during lactation may also prevent the development of food allergy in breastfed infants. For infants at risk of developing allergic disease due to a strong atopic family history, the

American Academy of Pediatrics, Committee on Nutrition (5) has recommended that women breastfeed for the first year of life and eliminate peanuts and tree nuts from their diets. It was also advised that eliminating eggs, cow's milk and fish should be considered. The evidence from clinical trials (13-20) investigating the use of maternal dietary restriction during lactation to prevent allergy in high-risk infants (due to an atopic family history) have shown varied results. Interestingly only the three studies (15, 19, 20), where the dietary interventions were not adequately randomised, have specifically investigated maternal dietary restriction during lactation alone. These three studies report no significant differences in rates of specific food sensitization measured by IgE levels or skin prick tests, but the presence of symptomatic food allergy was not documented by means of food challenges. Thus no trials to date have specifically investigated maternal dietary restriction during breastfeeding alone to prevent food allergy in breastfed infants and confirmed the presence of food allergy by means of food challenges.

Hence, lactating women maybe advised to exclude certain food proteins from their diets to prevent or treat food allergy in breastfed infants. The food proteins most commonly restricted are cow's milk, egg, fish and nuts. However the total dietary avoidance of one or more food proteins is difficult to achieve, can be nutritionally compromising, time consuming and socially restrictive for breastfeeding mothers. As a result many women question the need for total versus partial avoidance of a food protein.

The premise that maternal dietary restrictions during breastfeeding may benefit the infant assumes that ingested food proteins are absorbed and excreted antigenically

intact into breast milk. Previous studies have documented the presence of food proteins in human milk, including: ovalbumin (egg) (21-23), ovomucoid (egg) (21), bovine *B*-lactoglobulin (cow's milk) (2, 8, 21, 23-31), gliadin (wheat) (32, 33) and *Ara h* 1 and *Ara h* 2 (peanut) (34). Some of these studies (2, 21-23, 27, 31, 32, 34) have shown that even after the ingestion of the same challenge dose of a particular food, the frequency and concentration of the detected food protein in breast milk is highly variable between women.

However none of the previous studies investigating the presence of food proteins in human milk have examined whether there is a dose related effect from the ingestion of different quantities of a food protein on the concentration of that food protein appearing in breast milk samples from the same group of lactating women. Previous studies have also not done repeat breast milk food protein concentration measurements on different days on the same women after consumption of identical food challenges. Also lacking in previous studies is the investigation of what effect the ingestion of raw versus cooked foods has on the concentration of food proteins in human milk.

Thus for this thesis I undertook two randomised controlled trials. The primary aims of the first trial were to investigate whether the dietary dose of cooked egg consumed by lactating women influences ovalbumin content in human milk and whether cooked versus raw egg ingestion alters ovalbumin content in human milk. In the second trial the effect of maternal cooked egg consumption on the ovalbumin concentration in breast milk fed to infants with egg sensitivity was investigated. In order to achieve these aims a sandwich enzyme-linked immunosorbent assay

(ELISA) method to measure ovalbumin concentration in human milk was also developed as part of the work undertaken for this thesis.

Other outcomes as a result of my trials included a determination of whether maternal atopy status or usual dietary egg intake influences the ovalbumin content in human milk. The effect of maternal cooked egg ingestion on infant symptoms of atopic dermatitis/eczema was investigated. Total IgA levels in breast milk fed to infants with egg sensitivity and atopic dermatitis/eczema were measured.

I also conducted a survey to benchmark current Australian dietetic practice regarding the use of maternal dietary restrictions for treatment and prevention of food allergy in breastfed infants.

1.2 Literature review

Food allergies can occur in breastfed infants. Maternal dietary restriction has been recommended for the treatment and possible prevention of food allergies in breastfed infants. Studies investigating the use of maternal dietary restriction for these purposes are evaluated in this literature review. However the dietary avoidance of food proteins by lactating women is generally not without social and nutritional consequences which are discussed. A detailed examination of current evidence concerning the presence of food proteins in human milk is then undertaken. Finally the possible influence of IgA antibodies in human milk on the presence of allergy symptoms in breastfed infants is explored.

1.2.1 Food allergy

Food allergy is defined as an adverse immunological response to a food protein (35). Food allergic disorders can be divided into those which are mediated by IgE antibodies and those which are not (non-IgE mediated) (35, 36). The most common symptoms associated with food allergy in infants and children include urticaria, angio-oedema, atopic dermatitis/eczema, enterocolitis, enteropathy, colic, vomiting, diarrhoea and anaphylaxis (5, 36). Childhood food allergy has been shown to have a significant impact on general health perception, parental emotional distress and upon family activities (37).

There appears to be an increasing prevalence of atopic disease and food allergy seems to be part of this increase (36, 38). In a study conducted in the United States of America (39), an unselected population of 480 children were recruited as they attended a paediatrician's appointment at two weeks of age and prospectively followed to three years of age to assess the development of food allergies. A detailed history was taken if possible food allergy symptoms were reported and incriminated foods were then eliminated from the child's diet. If symptoms improved, then oral food challenges were used to confirm a food allergy diagnosis. In this group of American children, the food allergy incidence from birth to three years of age was found to be 8% and the most common food allergies were to cow's milk, soy, peanut and egg. Climate, food availability and cultural influences can influence local eating patterns. These along with genetic factors, may affect the incidence pattern of common food allergens in a particular country (36). In Australia (where the work for this thesis was undertaken) the most common food allergies have been calculated (from a combination of a population of at risk infants and infants randomly chosen from the community) to be egg at an incidence of 3.2%, cow's milk at 2.0% and peanut at 1.9% from birth to two years of age (40). The overall food allergy incidence in Australian children to my knowledge has not been reported.

The diagnosis of a food allergy usually involves a detailed history and physical examination, when potential food proteins are identified, a period of eliminating the food protein(s) from the diet is undertaken usually lasting one to six weeks (35, 36). If symptoms are alleviated then oral food challenges with that food protein(s) should be performed to confirm the food allergy diagnosis (35, 36). Skin prick tests (SPT) or radioallergosorbent tests (RAST) can be done to assist the identification of possible

food allergens causing IgE-mediated reactions. However both these tests detect sensitisation to the food protein and can occur without clinical reactivity. Thus these tests should only be used as a guide and food allergy diagnosis made with careful consideration of clinical history and confirmation by food challenge (36). The double-blind, placebo-controlled food challenge is the method least prone to observer bias (36) and is thus considered to be the gold standard for confirmation of food allergy diagnosis (38). Treatment for a diagnosed food allergy involves complete dietary avoidance of the causal food protein (4).

1.2.2 Food allergies in breastfed infants

Three European studies (1-3) conducted between 1979 and 2000, have followed cohorts of 1079, 1749 and 6209 newborns for the first year of their life and assessed these infants for cow's milk allergy. The cow's milk allergies in these infants were confirmed by oral cow's milk challenge and they reported a cow's milk allergy incidence of 1.9% to 2.2%.

These three studies (1-3) also determined the incidence of cow's milk allergy in exclusively breastfed infants (prior to the introduction of any infant formula or solids) to be from 0.4 – 0.8%. In two of these studies (1, 2) the diagnosis of cow's milk allergy in exclusively breastfed infants was made following the disappearance of symptoms after a maternal dietary elimination of cow's milk protein and recurrence of identical symptoms after maternal cow's milk challenge. The symptoms observed in the exclusively breastfed infants included atopic

dermatitis/eczema, urticaria, colic, diarrhoea, vomiting, recurrent wheezing and rhinitis (1, 2) and the age of onset of symptoms ranged from two to 12 weeks of age (2).

To the best of my knowledge I am not aware of any studies that have determined the general population incidence of cow's milk allergy in exclusively breastfed infants in Australia. I am also not aware of any general population studies conducted anywhere in the world determining the incidence of other common food allergies (for example egg and peanut) in exclusively breastfed infants where the diagnosis was confirmed by food challenge.

Expert nutritional committees of the American Academy of Pediatrics (AAP), the European Society for Pediatric Allergology and Clinical Immunology (ESPACI) and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (4, 5) have recommended the use of maternal dietary restriction for the treatment of breastfed infants with a food allergy.

1.2.3 Maternal dietary restriction to treat food allergy in breastfed infants

My aim in this section of the review is to assess studies that have reported the use of maternal dietary restriction to treat breastfed infants with food allergy symptoms. I decided to further limit my inclusion criteria to only those studies which involved both maternal dietary restriction and maternal dietary challenge. The Cochrane library, Medline, Excerpta Medica database (EMBASE) and Proquest electronic

databases were searched for published papers using the following terms: maternal diet restriction; food allergy treatment; maternal food challenge; food allergy and breastfed infants. The search was limited to human subjects and English language articles. Reference lists of identified papers were also checked for any other studies suitable for inclusion.

Ten relevant studies (as summarised in Table 1.1) and a Cochrane review were identified. The Australian National Health and Medical Research Council (NHMRC) levels of evidence criteria (see Appendix 1) (41) were used to assess the study design quality of these studies. Five of the ten studies (2, 6-9) were case series, two studies (42, 43) used a case-control study design and three studies (10-12) were randomised controlled trials.

Table 1.1: Studies on maternal dietary restriction and challenge to treat breastfed infants with food allergies.

Study	Study design & level of evidence [a]	Maternal dietary restriction (n^1)	Results of maternal dietary restriction	Maternal dietary challenge (n^2)	Results of maternal dietary challenge	Additional comments
Cavagni et al, 1988 (8)	Case series with open challenge **IV**[a]	cow's milk and egg (n^1=13)	11/13 infants had improved atopic dermatitis/eczema	cow's milk (n^2=9)	9/9 infants had atopic dermatitis/eczema exacerbation	No explanation of why only cow's milk and not egg challenges were done.
De Boissieu et al, 1997 (9)	Case series with open challenge **IV**[a]	cow's milk and egg or cow's milk and fish (n^1=6)	6/6 infants had symptom disappearance	cow's milk and egg or cow's milk and fish (n^2=4)	4/4 infants had return of symptoms (vomiting, diarrhoea, colic and/or atopic dermatitis/eczema)	2/6 mothers decided to use an extensively hydrolysed formula rather than to continue to breastfeed
Gerrard, 1979 (7)	Case series with open challenge **IV**[a]	cow's milk and/or egg, orange, apple, banana, strawberry, tomato, chocolate and coffee (n^1=18)	18/18 infants had symptom improvement	cow's milk and/or egg, orange, apple, banana, strawberry, tomato, chocolate and coffee (n^2=18)	18/18 infants had return of symptoms (diarrhoea, atopic dermatitis/eczema, vomiting, bronchitis, colic and/or rhinorrhea)	All 18 cases were chosen for inclusion when infants improved on maternal diet restriction and symptoms returned on challenge
Host et al, 1988 (2)	Case series with open challenge **IV**[a]	cow's milk (n^1=9)	9/9 infants became symptom-free	cow's milk (n^2=9)	9/9 infants had return of symptoms (atopic dermatitis/eczema, vomiting, diarrhoea and/or colic)	
Matsumura et al, 1975 (6)	Case series with open challenge **IV**[a]	egg (n^1=10)	10/10 infants had improved atopic dermatitis/eczema	egg (n^2=5)	5/5 infants had return of atopic dermatitis/eczema	No explanation of why only half of the mothers had an egg challenge.

NHMRC levels of evidence (41)

n^1 = number of women undertaking maternal dietary restriction
n^2 = number of women undertaking maternal dietary challenge

Table 1.1: Studies on maternal dietary restriction and challenge to treat breastfed infants with food allergies.

Study	Study design & level of evidence [(a)]	Maternal dietary restriction (n^1)	Results of maternal dietary restriction	Maternal dietary challenge (n^2)	Results of maternal dietary challenge	Additional comments
Jarvinen et al, 1999 (42)	Case-control study design with open challenge **III-2**[(a)]	cow's milk, oats, wheat, rye, barley, egg, fish, nuts, citrus fruits and tomato (n^1=27)	*No details given*	cow's milk (n^2=27)	16/17 infants with cow's milk allergy had return of symptoms (eczema, diarrhoea, abdominal pain, rhinorrhea). 10/10 healthy control infants had no symptoms	No explanation of why only cow's milk and not other food challenges were done.
Warner, 1980 (43)	Case-control study design with double-blind challenge **III-1**[(a)]	egg (n^1=2)	2/2 infants were symptom-free	cow's milk powder vs cow's milk + egg (=1 egg per day) powder (n^2=2)	Infant's eczema exacerbated with egg containing powder only. No reaction in healthy control infant.	
Cant et al, 1986 (12)	Randomised, crossover design with double-blind challenge **II**[(a)]	cow's milk, egg, chocolate, nuts, wheat, fish, beef, chicken, citrus fruits, colourings & preservatives (n^1=19)	*No period of maternal dietary restriction alone prior to challenges*	soy milk powder vs cow's milk and egg powder (=600ml milk and 1 egg per day) (n^2=19)	Individual infant eczema scores varied greatly, but mean scores not significantly different between the soy verus cow's milk and egg challenges.	No explanation of why only cow's milk and egg and not other food challenges were done.
Evans et al, 1981 (10)	Randomised crossover design with double-blind challenge **II**[(a)]	cow's milk (n^1=20)	*No period of maternal dietary restriction alone prior to challenges*	600ml soy milk vs 300ml soy + 300ml cow's milk (n^2=20)	No difference in infantile colic symptoms between challenge drinks.	
Jakobsson & Lindberg, 1983 (11)	Randomised, crossover design with double-blind challenge, followed by an open challenge **II**[(a)]	cow's milk (n^1=16)	16/16 infants had colic symptom disappearance	cow's milk vs potato starch capsules, followed by open challenge of cow's milk (n^2=16)	9/16 infants had infantile colic symptoms after cow's milk capsules and cow's milk drink, but not after placebo potato capsules.	

[(a)] NHMRC levels of evidence (41)

n^1 = number of women undertaking maternal dietary restriction
n^2 = number of women undertaking maternal dietary challenge

1.2.3.1 Case series

In the five studies (2, 6-9) which were case series, the dietary interventions were not randomised and uncontrolled. Although maternal dietary challenges were given they were open (non-blinded) food challenges and the amount of challenge food(s) given was not standardised. The sample sizes of these five case series were small with less than 20 participants per study and only in two of these case series (2, 7) did all of the women who commenced dietary restriction followed on with the dietary challenge.

Three of these studies (2, 7, 9) involved infants with a wide range of symptoms including diarrhoea, vomiting, atopic dermatitis/eczema, bronchitis, colic and/or rhinorrhea. In these studies there was no attempt to score these symptoms and therefore quantify the level of symptom improvement on maternal dietary restriction or deterioration on maternal dietary challenge. In the two studies (6, 8) which included only infants with symptoms of atopic dermatitis/eczema, Cavagni and co-workers (8) used a scoring system to assess and follow the progress each infant's atopic dermatitis/eczema, however the study by Matsumura and colleagues (6) only described the areas of the infants' bodies affected, but did not quantify the extent or severity of the atopic dermatitis/eczema.

Although the results in these five studies appear impressive, their case series study designs are associated with the lowest level of evidence (NHMRC level of evidence IV) (41) on this topic.

1.2.3.2 Case-control studies

The two studies (42, 43) using a case-control study design provide a moderate level of evidence (NHMRC level of evidence III) (41) on this topic.

In the study by Jarvinen and colleagues (42) investigating breastfed infants with and without cow's milk allergy. The maternal cow's milk challenge involved a standardised protocol of increasing doses of cow's milk protein over two days followed by free consumption of cow's milk. This enabled the determination of the volume of cow's milk ingested by the mother eliciting symptoms in her infant and it was found that the medium total volume eliciting symptoms in this group of infants was 700mL (range 100-2300mL) of cow's milk. Unfortunately in this study, as was the case in all the case series described above, the maternal cow's milk challenge was an open (non-blinded) food challenge and did not include a placebo maternal food challenge. Another criticism of this study was although both groups of infants had comparable age ranges, no clear indications were given as to how the 10 healthy control infants were selected for participation and what was the basis of matching these infants to the 17 infants with cow's milk allergy.

Warner (43) undertook a case-control study involving one infant with atopic dermatitis/eczema and one healthy control infant. In this study a two week maternal dietary challenge period involved breastfeeding mothers being given a powder supplement to mix with milk and take daily, while following an egg-free diet. For 1 week this powder contained milk powder and colouring, and the other week it

contained milk and egg powder (equivalent to one egg per day). The mothers and physician were blinded to which powder was given each week. In the week the mother of the infant with a history of atopic dermatitis/eczema consumed the egg containing powder, the infant had an exacerbation of his atopic dermatitis/eczema and also developed diarrhoea and vomiting. During the other week this infant had slightly dry skin but otherwise was well. The healthy control infant had no reaction to his mother's diet challenges. This well blinded, placebo-controlled study design was unfortunately limited to only one case and one control infant. As per the study by Jarvinen and colleagues (42), Warner also failed to describe how the healthy control infant was selected for participation and what was the basis of matching this control infant to the infant with a history of atopic dermatitis/eczema.

1.2.3.3 Randomised controlled trials

Only the three studies (10-12) which were randomised controlled trials, provide the highest level of evidence (NHMRC level of evidence II) (41) available on this topic from individual studies. All of these three trials (10-12) used a cross-over study design with double-blind, placebo-controlled food challenges.

The double-blind, placebo-controlled food challenge is the method least prone to observer bias (36) and is considered to be the gold standard for confirmation of food allergy diagnosis (38). The choice of an appropriate placebo challenge for such trials should be a low-allergenic food, such as potato starch which was used in the trial conducted by Jakobsson and Lindberg (11). Unfortunately the two other trials (10,

12) used potentially allergenic soy protein for placebo challenges, which may explain the lack of difference in symptoms observed between challenge foods.

The use of a cross-over study design can be criticised when used to assess the effect of an intervention on a clinical outcome which is known to naturally improve with time, for example infantile colic or atopic dermatitis/eczema. The two trials involving infants with colic symptoms were however of limited duration of only 3-12 days, thus reducing the chances of the infants spontaneously improving over the study period. The trial by Cant and co-workers (12) investigating infants with atopic dermatitis/eczema was of eight weeks duration, so although this was considerably longer it was still probably short enough to exclude the possibility of the majority of the infants having naturally improved with time.

Only the randomised controlled trial conducted by Jakobsson and Lindberg (11) claimed to show a benefit of maternal cow's milk dietary restriction for the treatment of infants with colic symptoms. However the participating mothers in this trial were pre-selected after an initial period of infant colic symptom improvement after maternal cow's milk avoidance and symptom return on open maternal cow's milk challenge. This may have biased the results towards a more positive response than if any group of infants with colic symptoms were studied.

The sample sizes of these three randomised controlled trials (10-12) were again small ($n \leq 20$) and none of these trials described the use of power calculations to determine the number of participating infants required to demonstrate a statistically significant response between the maternal challenge foods upon infant symptom outcomes.

The Cochrane review covering this topic is entitled "Maternal dietary antigen avoidance during pregnancy and/or lactation for preventing or treating atopic disease in the child" (44) and only included studies that were acceptably randomised. The primary outcome measure for this Cochrane review was the occurrence and severity of atopic disease in the child. One of the objectives of this review was to assess "the effects of prescribing an antigen avoidance diet to lactating mothers of infants with established atopic eczema on the severity of the eczema". Only the one trial by Cant and co-workers (12) (as previously discussed) was suitable for inclusion in this Cochrane review. The two other randomised trials (10, 11) had a primary focus on infantile colic rather than atopic eczema and were thus not included in this Cochrane review. The authors of this review concluded that the results of this trial (12) should be interpreted with caution because of its small sample size and that soy was used for the placebo challenge (as previously discussed). Larger future trials were recommended.

Thus despite recommendations by expert committees (AAP, ESPACI and ESPGHAN) (4, 5), current evidence on the use of maternal dietary restriction to treat food allergy in breastfed infants is of limited strength. In order to substantiate these recommendations, research evidence of improved quality is required from large, randomised, appropriately placebo controlled, double-blinded food exclusion and challenge trials to determine the extent of benefit offered by maternal food avoidance diets to breastfed infants for the treatment of food allergy.

1.2.4 Maternal dietary restriction to prevent food allergies in breastfed infants

Maternal dietary restriction during lactation to treat food allergy in breastfed infants has led to the hypothesis that maternal dietary restriction during lactation may also prevent the development of food allergy in high risk breastfed infants. For infants with a strong atopic family history, the American Academy of Pediatrics, Committee on Nutrition (5) has recommended that women breastfeed for the first year of life and eliminate peanuts and tree nuts from their diets. It was also advised that eliminating eggs, cow's milk and fish should be considered. However a recent critical review paper by a group of experts of the Section of Pediatrics, European Academy of Allergology and Clinical Immunology (EAACI) (45) determined that there was no conclusive evidence for the protective effect of maternal exclusion diets during lactation.

My aim in this section of the review is to assess the published evidence with regard to the use of maternal dietary restriction to prevent food allergies in breastfed infants. I searched for studies investigating the use of maternal dietary intervention during lactation to prevent allergy in high risk breastfed infants. The Cochrane library, Medline, EMBASE and Proquest electronic databases were searched for published papers using the following terms: maternal diet restriction; lactation (breastfeeding); food allergy prevention; allergy (atopic disease) prevention. The search was limited to human subjects and English language articles. Reference lists of identified papers were also checked for any other studies suitable for inclusion.

Eight relevant studies (as summarised in Table 1.2) and a Cochrane review were identified. The NHMRC levels of evidence criteria were again used to assess the study design quality of these studies (41). Although the intervention in all studies included dietary restriction in lactating women, the type and number of foods avoided and length of dietary restriction varied (Table 1.2). In two studies (19, 20) the participating women self-selected their dietary intervention group, one study (15) was a cluster trial with the maternal dietary intervention group allocated according to the town in which the women resided and five of the eight studies (13, 14, 16-18) were randomised controlled trials.

None of these eight studies were conducted double-blinded as the participating mothers were aware of their dietary group allocation. The infants in these studies underwent clinical examinations for atopic disorders of varied frequency up to either 12, 18 or 24 months of age as summarised in Table 1.2. However only in five studies (14, 16-18, 20) were these examinations conducted blinded to the maternal diet group allocation. However Zeiger and colleagues (16) did acknowledge that in their study, the examining physicians did occasionally become aware of the maternal diet group allocation after it was revealed to them by the participating mother or it had been noted in the infant's medical records by a paediatrician. Although not described it would be surprising if this did not also occur in some of the other "blinded" studies.

Table 1.2: Studies on maternal dietary restriction for allergy prevention in breastfed infants.

Study	Study design	Sample size	Maternal and infant interventions	Infant outcome measure	Main results
Herrmann et al, 1996 (19)	Participants self-selected their intervention group with non-blinded examination **III-2**[a]	150 enrolled, 12 withdrew, A=30, B=33 and C=41 completed (34 mothers breastfed <3 months)	A = cow's milk, egg-free diet for mothers for last trimester of pregnancy and during lactation (allowed goat and sheep milk) B = cow's milk, egg- free diet for mothers for lactation only (allowed goat and sheep milk) C = mothers consumed at least 1000ml cow's milk & 1 egg daily	Atopic dermatitis at 1, 3, 6 & 12 months. IgE specific to cow's milk & egg at 6 & 12 months.	NS for prevalence of atopic dermatitis at 6 & 12 months.
Kilburn et al, 1998 (20)	Participants self-selected intervention group with blinded examination **III-2**[a]	111 enrolled, 4 withdrew, D=13 and ND=94 completed	D= cow's milk, egg, fish, nut-free diet for mothers during lactation ND= no diet restrictions for mothers	Atopic disorders at 3, 6, 12 & 18 months. SPT at 6, 12 & 18 months to foods & inhalant allergens.	NS for incidence of atopic disorders at 3, 6, 12 & 18 months.
Hattevig et al, 1989 (15)	Cluster trial of two towns with non-blinded examination **III-1**[a]	115 enrolled, 6 withdrew, D=65 and ND=50 completed	D= cow's milk, fish, egg-free diet for mothers for first 3 months of lactation ND = no diet restrictions for mothers	Atopic disorders at birth, 3, 6, 9, 12 & 18 months. SPT at 9 months to egg, cow's milk and fish.	Atopic dermatitis at 3 months, D=3% < ND=22% and at 6 months, D=11% < ND=28%. NS for prevalence of atopic dermatitis at 9, 12 & 18 months
Arshad et al, 1992 (17)	Randomised trial with blinded examination **II** [a]	136 enrolled 16 withdrew, D=58 and ND=62 completed	D= cow's milk, fish, nut, egg-free diet for mothers for first 9 months of lactation [b] + solid restrictions for infants and anti-house dust-mite measures ND= no diet restrictions for mothers or infants	Atopic disorders at 3, 6 & 12 months. SPT to foods and environmental allergens.	Atopic disorders at 3 months, D=5% < ND=18%, at 6 months, D=12% < ND=32% and at 12 months, D=14% < ND=40%.
Chandra et al, 1986 (13)	Randomised trial with non-blinded examination **II** [a]	121 enrolled, 12 withdrew, D=55 and ND=54 completed	D= cow's milk, egg, beef, fish, peanut-free diet for mothers throughout pregnancy and lactation ND= no diet restrictions for mothers	Atopic eczema up to 12 months.	Atopic eczema up to 12 months, D=31% < ND= 44%.
Chandra et al, 1989 (14)	Randomised trial with blinded examination **II** [a]	112 enrolled, 15 withdrew, D=49 and ND=48 completed	D = cow's milk, egg, fish, peanuts, soybean-free diet for mothers for first 6 months of lactation ND = no diet restrictions for mothers	Atopic eczema up to 18 months.	Atopic eczema up to 18 months, D=22% < ND=44%.
Lovegrove et al, 1994 (18)	Randomised trial with blinded examination **II** [a]	44 enrolled, 6 withdrew, D=12 and ND=14 completed (12 non-atopic women)	D = cow's milk-free diet for mothers during lactation [b] ND= no diet restrictions for mothers	Atopic eczema at 6, 12 and 18 months.	Atopic eczema at 18 months, D=11% < ND=50%. NS for prevalence of atopic eczema at 6 & 12 months.
Zeiger et al, 1989 (16)	Randomised trial with blinded examination **II** [a]	379 enrolled 91 withdrew, D=103 and ND=185 completed	D = cow's milk, egg, peanut-free diet for mothers for last trimester of pregnancy and during lactation [b] + solid restrictions for infants ND= no diet restrictions for mothers or infants	Atopic disorders at 4, 12 & 24 months. SPT at 4, 12 and 24 months to food and inhalant allergens.	Atopic disorders at 12 months, D=16% < ND=27%. NS for prevalence of atopic disorders at 4 & 24 months

[a] NHMRC levels of evidence (41)
[b] infants had hydrolysed formula when breastfeeding ceased
NS = no significant differences
SPT = skin prick test

As described in Table 1.2, the lactating women in these studies were asked to avoid between one (cow's milk) (18) to five (cow's milk, egg, peanut, fish and beef/soybean) (13, 14) different food proteins in their diet. Thus a measure of their dietary compliance with such a restrictive diet should be an essential component of the study design. In five of the eight studies (13, 14, 16, 19, 20) diet compliance was monitored by food diaries kept by the participating women but compliance results from these records were not reported. In the three studies (15, 17, 18) which did report diet compliance results it was found that in one study 16/58 (28%) mothers reported occasional mistakes with dietary compliance to either the maternal or infant dietary restrictions (17), another study reported 12/65 (18%) women in the diet restricted group mistakenly ate foods containing small amounts of cow's milk protein (15) and in the third study most of the dietary non-compliance was described to have occurred while the women were in hospital in the immediate post-natal period, however the number of women in the diet restricted group who mistakenly ate foods containing cow's milk protein was not reported (18).

1.2.4.1 Self-selected dietary intervention studies

In two studies (19, 20) the quality of the results are diminished due to the lack of maternal diet group randomisation. In the study by Herrmann and co-workers (19) the participating women self selected to be in one of three diet intervention groups. Interestingly the sample sizes of the three groups turned out to be reasonably compatible as summarised in Table 1.2. However in the study by Kilburn et al (20)

the participating women self selected either a diet free from cow's milk, egg, fish and nuts during lactation or a diet without any restrictions and this resulted in an uneven sample size distribution with only 13 women completing the study in the restricted diet group compared to 94 women in the no diet group.

Herrmann and co-workers (19) found no difference in infant atopic dermatitis/eczema prevalence and no differences in infant sensitisation rates (measured by RAST) to cow's milk and egg between the three groups. Unfortunately goat and sheep milk proteins are highly cross-reactive with cow's milk protein (46, 47), thus another criticism of this study undertaken by Herrmann and co-workers (19) was that the women avoiding cow's milk were allowed goat or sheep milk and more than half of these women did consume these alternative mammalian milks regularly during the study.

Kilburn et al (20) also found no statistically different incidences of allergic disease symptoms between their two groups, however there was an unexpected trend towards increased atopic dermatitis/eczema incidence in the maternal diet group infants. Atopic dermatitis/eczema tended to be associated with food but not environmental allergen sensitivity in both groups and the total number of infants with food associated eczema decreased from six to 12 months. The authors of this paper suggest that exposure to low doses of food proteins via breast milk may promote infant tolerance rather than sensitisation to these food proteins.

1.2.4.2 Cluster trial

A study conducted by Hattevig and colleagues (15) was a cluster trial with the maternal dietary intervention group allocated according to the town in which the women resided. In this study infant atopic dermatitis/eczema was found to be significantly higher during the first six months in the maternal unrestricted diet group but not there after, and the incidences of other allergic disease symptoms were similar in both groups. Infant skin prick testing was done at nine months to cow's milk, egg and fish, however there were no differences in sensitisation rates between the two groups. As this study was non-randomised and non-blinded, the quality of this evidence is of limited strength.

1.2.4.3 Randomised controlled trials

In the five randomised controlled trials, the randomisation methods included using a random number table (13, 14) or a computer-generated list of random numbers (16, 17), with the randomisation method not described in one study (18).

In one study by Chandra and co-workers (13), the infants were followed for 12 months at which age 17/55 (31%) of the maternal diet intervention group infants had eczema compared to 24/54 (44%) of the control group infants. However as the maternal dietary restriction in this study was undertaken during both pregnancy and lactation one can not be sure whether any benefit of such intervention occurred by reducing in utero sensitisation or by reducing the food allergen content of breast milk

or a combined effect of both. Only 35/55 (64%) women in the maternal diet intervention group and 36/54 (67%) women in the control group breastfed their infants which diminishes the evidence from this study with regard to the effect of maternal dietary restriction during lactation on the development of atopic disease.

In a second trial by Chandra and co-workers (14), the maternal dietary interventions were only for the first six months of lactation. The infants whose mothers had undertaken dietary restrictions were found to have reduced severity and a lower incidence (11/49 (22%) diet compared to 21/48 (44%) control group) of eczema symptoms. These results support the use of maternal dietary restriction during lactation alone in reducing atopic eczema in infants up to 18 months of age.

Special note

In a recent article published in the *British Medical Journal* (48), the integrity of published work by Chandra has been questioned. In February 2005, the editor of *Nutrition* retracted a paper by Chandra that it had published in 2001 (49). The reasons for retracting this paper included doubts concerning the cognitive tests used in this particular study of elderly subjects and the statistical analyses reported. Another paper by Chandra published in the *Lancet* in 1992 (50), has also been questioned (51) again in relation to statistical problems and that "all individuals approached enrolled in the study". The two studies by Chandra and co-workers (13, 14) described in this section of my literature review have not to date been retracted. However it is worth mentioning that in an article in *The Scientist* (52) also discussing the questioning of Chandra's published work, it is stated that in 1994 Memorial University of Newfoundland at St John's had investigated Chandra "after a research

nurse complained about a study on the effects of maternal diet during lactation on allergy in infants. She said that the number of mothers studied was less than what had been reported." No further reports concerning doubts over the two studies by Chandra and co-workers (13, 14) described in this section of my literature review could be identified at the time of submitting this thesis.

In the study by Lovegrove et al (18) it was reported that the infant allergy incidence in the atopic maternal diet group of 1/12 was significantly lower compared with 7/14 in the atopic maternal unrestricted diet group when the infants were 18 months of age (no difference between these groups when the infants were six or 12 months old). However the analysis of this data was not based on "intention to treat" principles as this statistical difference was only found after the removal of three infants in the atopic maternal diet group who had been given cow's milk formula by mistake. Another finding from this study was that the non-atopic group had significantly lower atopic eczema than the atopic group when the infants were 12 and 18 months old, this was not at all surprising as family history of atopy is regarded to be the strongest predictor for allergy development in infants (53).

In two other randomised controlled trials (16, 17) in addition to maternal dietary restriction, the infants in the diet intervention group (but not the control group) received protein hydrolysate formula when breastfeeding needed supplementation or ceased and it was recommended that the introduction of allergenic solid foods be delayed for the diet intervention group infants only. In one of these two studies (17) the diet intervention group only also undertook anti-house dust-mite measures. This

study by Arshad and et al (17) found significantly less 8/58 (14%) of diet intervention group infants compared to 25/62 (40%) of control group infants had allergic disease by 12 months of age. Zeiger and colleagues (16) also found infant allergic disease in 16/99 (16%) of the diet restricted group infants to be significantly less when compared with 48/177 (27%) of control group infants at 12 months, but no such differences were found at four or 24 months. Unfortunately as infant dietary interventions (and environmental measures) varied between the diet compared with the control group, it is not possible to determine the relative importance of the maternal food avoidance during lactation alone on these results compared with the other interventions.

The Cochrane review covering this topic is entitled "Maternal dietary antigen avoidance during pregnancy and/or lactation for preventing or treating atopic disease in the child" (44) and only included studies that were acceptably randomised. One of the objectives of this review was to assess the effects of prescribing a maternal antigen avoidance diet during lactation on the occurrence and severity of atopic disease in the child. This Cochrane review included studies regardless of degree (number of foods eliminated from the diet) or duration of maternal dietary intervention, but excluded trials of multimodal interventions that included manipulation of the infant's diet or non-dietary aspects of the infant's environment. Thus although the studies by Arshad et al (17) and Zeiger et al (16) were randomised controlled trials they were not included in the Cochrane review, as both trials applied different infant dietary interventions between the diet intervention compared with the control group.

Thus only three (13, 14, 18) of the eight studies described above were included in this Cochrane review, which determined that the combined results of these three trials suggested a protective effect of maternal dietary restriction during breastfeeding on the incidence of atopic eczema during the child's first 12-18 months. However methodological shortcomings as previously described were identified in these three trials, including no details on the randomisation procedure used (18), analysis of data was not based on "intention to treat" principles (18), only one of these trials provided limited results on dietary compliance (18) and in one trial the physicians examining the children for atopic disease were not blinded to the maternal diet group allocation (13). It was therefore recommended that future studies address these methodological shortcomings and also aim to gain more information on women's experiences (as well as compliance) with such food antigen avoidance diets.

In order to determine whether maternal dietary restriction during lactation prevents the development of food allergy specifically, the outcome measures should include double-blind, placebo-controlled food challenges. Skin prick testing to food allergens (15-17, 20) or IgE levels specific to food allergens (19), as an indicator for food allergen sensitisation were performed in five of these studies, however food challenges to confirm the diagnosis of food allergy were only done in two studies (16, 17), with only one using double-blind, placebo-controlled food challenges (16). Both of these trials however also involved infant dietary intervention differences between the diet compared with the control group as discussed previously.

Interestingly only the three studies (15, 19, 20), where the dietary interventions were not adequately randomised, have specifically investigated maternal dietary restriction during lactation alone. These three studies report no significant differences in rates of specific food sensitization measured by IgE levels or skin prick tests, but the presence of symptomatic food allergy was not documented by means of food challenges. Thus no trials to date have specifically investigated maternal dietary restriction during breastfeeding alone to prevent the development of food allergy in breastfed infants and confirmed the presence of food allergy by means of food challenges.

1.2.5 Implications of maternal dietary restrictions during lactation

Both for primary prevention and secondary management of food allergy in breastfed infants, lactating women maybe advised to exclude certain food proteins from their diet. The food proteins most commonly restricted are cow's milk, egg, fish and nuts (Tables 1.1 and 1.2). The number and type of food proteins that are restricted in the mother's diet will influence the possibility that her diet may become nutritionally comprised. Maternal dietary restriction of cow's milk protein may significantly reduce calcium, energy and protein dietary intakes at a time when the maternal requirements for these nutrients are higher due to lactation. For example lactating women require an additional 400mg of calcium and 16g protein per day (54). Detailed and regular dietary advice is essential to ensure the aim of maternal nutritional adequacy is achieved (55).

Cow's milk protein was restricted in the diets of lactating women in six of the previously discussed studies on treatment of food allergy in breastfed infants (2, 7-9, 11, 42) and in all eight studies on maternal dietary restriction for allergy prevention in breastfed infants (13-20). The use of calcium supplementation was described in eleven of these studies (8, 11, 13-20, 42), but only two studies (18, 19) described the use of a milk substitute by the lactating women to assist energy and protein dietary intakes whilst avoiding cow's milk and diary products in their diets. The involvement of a dietitian in providing advice concerning the maternal dietary restrictions was specifically mentioned in six studies (9, 15-17, 19, 20), and six studies (9, 15, 17-20) described assessment of nutritional adequacy of the maternal diet.

However only three studies (14, 15, 18) reported results on maternal nutritional adequacy whilst undertaking dietary restrictions. Lovegrove et al (18) reported the mean nutrient intakes were within local reference ranges and not different between the diet restricted compared to the diet unrestricted groups of participating women. In a study by Chandra and co-workers (14), the nutritional state of lactating women who undertook diet restrictions for up to six months was reported to be comparable to women following an unrestricted diet using change in body weight, haemoglobin concentration and serum concentrations of albumin and pre-albumin as nutritional status markers, but actual data were not shown in this paper. However Hattevig and colleagues (15) found a significant difference between the return to pre-pregnant weights within three months after delivery in 43/65 (66%) of the diet restricted group of breastfeeding mothers compared to only 10/50 (20%) of the mothers in the unrestricted diet group, which suggests consumption of lower total energy intakes by the women in the diet restricted group.

The careful checking of food ingredient labels for the presence of certain food proteins is time consuming (55). Using the total dietary avoidance of egg as an example, this means checking labels from products including crumbed or battered foods, processed meat and fish products, pasta and noodles, cakes, biscuits, muffins, pancakes, puddings, ice cream, custard, mayonnaise, lollies and glazed baked goods (56). The home preparation of foods also requires knowledge of inclusion of substitute ingredients into recipes and can again be more time consuming than purchasing a product already made. Again detailed dietary advice from a dietitian who specialises in the area of food allergy will reduce the impact of maternal dietary restriction by increasing the mother's knowledge of label reading and suitable products to purchase and prepare (55).

Many social events often revolve around eating. The need to avoid cow's milk and egg for example will limit many restaurant menu options and even make morning tea with a group of fellow mothers awkward for a breastfeeding woman avoiding these foods. Thus some women find dietary restrictions to be socially restrictive (19, 55).

In a randomised controlled trial by Zeiger and colleagues (16) regarding the use of maternal dietary restriction during lactation to prevent allergy in high-risk infants there was a large loss to follow up. The women in the diet intervention group of this trial were asked to avoid cow's milk, egg and peanut in their diets commencing during the last trimester of pregnancy and continuing during lactation. 48/167 (29%) women in the diet intervention group withdrew from this allergy prevention study due to difficulties following the dietary restrictions. Another example of lactating

women finding the avoidance of food proteins difficult to achieve was in the paper by De Boissieu et al (9) which described a series of case studies on six breastfed infants with possible food allergy symptoms. Two of six (33%) mothers decided to use an extensively hydrolysed formula rather than to continue to breastfeed and undertake maternal dietary restriction for the treatment of their infants with possible food allergy symptoms.

Thus the total dietary avoidance of one or more food proteins can be difficult to achieve, is time consuming, socially restrictive and can be nutritionally compromising for breastfeeding mothers. As a result many women question the need for total versus partial avoidance of a food protein. Lactating women also commonly ask whether ingested food proteins pass into the breast milk of all women.

1.2.6 Evidence for presence of food proteins in human milk

The premise that maternal dietary restrictions during breastfeeding may benefit the infant assumes that ingested food proteins are absorbed and excreted antigenically intact into breast milk. The aim of this section of the literature review is to examine the current available evidence for the presence of food proteins in human milk.

I searched for published papers involving the detection of food proteins in breast (human) milk. The Cochrane library, Medline, EMBASE and Proquest electronic databases were searched using the following terms: food protein (+cow's milk, egg, fish, wheat, nut) and breast (human) milk. The search was limited to human subjects

and English language articles. Reference lists of identified papers were also checked for any other studies suitable for inclusion. Seventeen papers were identified as potentially relevant for inclusion.

Previous studies have documented the presence of food proteins in human milk, including: ovalbumin (egg) (21-23), ovomucoid (egg) (21), bovine *B*-lactoglobulin (cow's milk) (2, 8, 21, 23-31, 42), gliadin (wheat) (32, 33) and *Ara h* 1 and *Ara h* 2 (peanut) (34).

Some of these studies (24-26, 30, 33) have simply measured the concentrations of dietary food proteins in human milk while breastfeeding mothers followed their usual diet. One would expect that the mothers' dietary intake of these food proteins would influence the concentration of these proteins measured in breast milk. Most of these studies (24, 25, 33) did not report maternal dietary intake details. One study by Machtinger and Moss (30) recorded during the initial interview for each mother their usual intake of dairy products and reported that all but one mother ingested at least a pint of cow's milk or its equivalent in dairy products per day during lactation. However they did not describe any relationship between these dietary intakes of dairy products and the *B*-lactoglobulin concentrations in breast milk. In another study, Axelsson and colleagues (26) asked participating mothers to record their daily cow's milk intake as litres of cow's milk per day. However there was no mention of whether significant amounts of cow's milk protein were ingested from other dairy foods like yoghurt and cheese. Thus their conclusion that there was no correlation between cow's milk intake and the *B*-lactoglobulin concentrations in breast milk may

not appear to reflect the normal dietary situation of obtaining cow's milk protein from other dairy food sources.

In another study by Bertino and co-workers (28), breast milk samples from 14 women were analysed for their bovine *B*-lactoglobulin concentrations. Five women were asked to follow a high cow's milk diet (more than 500mL cow's milk per day and free intake of other cow's milk protein containing foods), four women were asked to consume a medium cow's milk diet (200mL cow's milk per day and complete avoidance of other cow's milk protein containing foods) and five women were asked to follow a strict cow's milk protein-free diet. This study reported that the detected *B*-lactoglobulin concentrations were not significantly different between the three groups of women. However the timing of the breast milk samples in relation to maternal cow's milk protein intake was not reported. There was also no description of any attempt to measure maternal dietary compliance in this study.

Table 1.3 summarises those studies which analysed human milk samples after maternal ingestion of a specific challenge dose of one or two food proteins. In most of these studies where more than one breast milk sample was taken over a period of at least six hours, the peak appearance of the food protein occurred from one to four hours after ingestion of the food (21, 22, 32, 34). In all of these studies (21, 22, 32, 34), the food proteins measured were avoided for 12 to 24 hours prior to ingestion of the food challenge. There was one other study (27), where the peak appearance of *B*-lactoglobulin occurred at 4-24 hours (median of 8 hours), interestingly cow's milk was not avoided by these mothers prior to the food challenge, but the women did avoid cow's milk for 24 hours post challenge.

Table 1.3: The detection of food proteins in human milk after food challenge ingestion.

Study	Participating women	Dietary avoidance pre-challenge	Food challenge dose	Timing of samples	Food protein concentration (ng/ml)	Detection frequency (% women)	Timing of peak appearance
Kilshaw and Cant, 1984 (21)	n=29 1 week to 12 months of lactation	cow's milk & egg for 24 hrs	½ pint cow's milk & one raw egg	Prior to and at 2, 4 & 6 hours after challenge	0.11 - 6.4 cow's milk 0.26 – 6.17 egg	52% cow's milk 59% egg	4 hours
Cant et al, 1985 (22)	n=19 < 6 months of lactation	egg for 24 hrs	one raw egg	Prior to and at 2, 4 & 6 hours after challenge	0.2 – 4.0	74%	2-4 hours
Troncone et al, 1987 (32)	n=53 (n=6) 1 week to 5 months of lactation	gluten for 12 hrs	20g gluten (as above)	2-4 hours (Prior to and at 2, 4 & 6 hours) after challenge	5 - 95	68%	1 sample only (at 2-4 hours)
Cavagni et al, 1988 (8)	n=13 Lactation stage not clearly described	overnight fast only	1 cooked egg & 100g cow's milk	3-5 hours after challenge	results only given as positive or negative	62%	1 sample only
Host et al, 1988 (2)	n=19 Lactation stage not clearly described	none	500ml cow's milk	4 hours after challenge	0.5 – 45	21%	1 sample only
Host et al, 1990 (27)	n=20 1-2 weeks (atopic) & 8-19 weeks (non-atopic) of lactation	none	500ml cow's milk	Prior to and at 4, 8, 12 & 24 hours after challenge	0.9 – 150	95%	4-24 hours (median 8 hr)
Sorva et al, 1994 (31)	n=53 2 to 12 months of lactation	cow's milk for 24 hrs	400ml cow's milk	Prior to and at 1 and 2 hours after challenge	0.00 - 8.6? (not clearly stated)	75%	only 1 mother studied >2 hours
Fukushima et al, 1997 (23)	n=24 4 to 10 months of lactation	none	200ml cow's milk	1-3 hrs, 4-8 hrs and 9-15hrs after challenge	up to 16.5	63%	not reported
Jarvinen et al, 1999 (42)	n=26 2 to 9 months of lactation	cow's milk for 1 week	100-400ml cow's milk	Prior to and at 1, 2, 3 & 4 hrs after challenge	0.03 – 11.54	52%	not reported
Vadas et al, 2001 (34)	n=23 Lactation stage not clearly described	legumes for 24 hrs	50g peanuts	Prior to and at 1, 2, 3, 4, 6, 8 & 12 hrs after challenge	120 - 430	48%	1- 2 hours

These studies have shown that even after ingestion of the same challenge dose of a particular food, the concentration of detected food protein in human milk is highly variable between women, 0.5-150 ng/ml of bovine *B*-lactoglobulin after consumption of 500ml of cow's milk (2, 27), 0.1-6.17 ng/ml of ovalbumin after consumption of one raw egg (21, 22), 5-95 ng/ml of gliadin after consumption of 20g gluten (32) and 120-430 ng/ml of peanut protein after consumption of 50g peanuts (34). The frequency of food protein detection in human milk has also varied from 48% to 95% of women in the studies where more than one breast milk sample was collected (see Table 1.3).

Apart from the natural biological variation that would be expected between individual women, some of the variability in results in previous studies may also be due to the influence of other factors including the stage of lactation, maternal atopy status, dietary intake prior to and after food challenge, other breast milk components or recruitment bias. The following sections of this chapter will explore these possible influencing factors.

1.2.6.1 Stage of lactation

In a study detecting gliadin in human milk (32), the concentration and frequency of detection of gliadin decreased with stage of lactation. This may relate to the decrease in total protein concentration in breast milk as lactation progresses. The protein concentration in breast milk is approximately 2.3g/100ml in colostrum during days 1-5 of lactation (57), approximately 1.3g/100ml at 2 weeks of lactation and this

concentration decreases to approximately 0.9g/100ml at 2-3 months of lactation (58). Table 1.3 demonstrates that in previous studies relating to food proteins in human milk, results have been included from women at a wide range of lactation stages, for example 1 week to 12 months (21) and 2 to 12 months (31). Thus the varying stage of lactation of the breastfeeding mothers may have accounted for some of the variability observed between the presence and concentration of food proteins in breast milk.

1.2.6.2 Maternal atopy status

In a recent position paper by the European Academy of Allergology and Clinical Immunology (EAACI) (59) the definition of atopy was proposed as follows: "Atopy is a personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis."

The investigation of whether maternal atopy influences the presence of food proteins in human milk has been attempted in many of the discussed studies (2, 8, 22, 23, 26, 27, 30, 31, 34). However only three of these studies have measured the participating mothers ability to produce IgE antibodies in response to allergens using either skin prick testing (SPT) (22, 31) or a radioallergosorbent test (RAST) (8). All of these three studies (8, 22, 31) also obtained a history of allergic disease in their breastfeeding mothers. Two of these studies (8, 31) were unable to find any statistically significant associations between the concentrations of *B*-lactoglobulin in breast milk samples after maternal cow's milk challenge and the mother's atopy

status. In the other study (22), although both a history of allergic disease was obtained as well as skin prick testing undertaken on their participating women, no results were described on the maternal atopy status nor whether there was a relationship of this to the ovalbumin concentration of the collected breast milk samples.

Despite family history of atopy being the strongest predictor for the development of childhood atopic disease (53), published studies so far have not demonstrated an association between maternal atopy status and food protein detection in human milk. Overall few atopic women have been studied. Thus larger studies are needed to investigate the direct relationship between maternal atopy status and the passage of a challenge dose of a particular food protein into breast milk. These studies should be designed to involve women whose atopy status is clearly defined as both the ability to produce IgE antibodies in response to allergens (measured by SPT or RAST) as well as a history of allergic disease.

1.2.6.3 Dietary intake prior to and after food challenge

In the study by Kilshaw and Cant (21) it was noted that trace quantities of egg and cow's milk proteins were detected in the initial breast milk samples prior to ingestion of the food challenges and was reported to be after a 24 hour avoidance of these food proteins. The extent of dietary advice provided to these participating women and their degree of compliance with this avoidance was not reported. Sorva and co-workers (31) also measured *B*-lactoglobulin in 23/47 (49%) of breast milk samples prior to the cow's milk challenge again after a 24 hour milk-free diet. These

breastfeeding mothers were staying in hospital at the time, but again there was no mention of compliance to the milk-free diet. If all the women in these two studies (21, 31) had in fact strictly adhered to a 24 hour avoidance of these food proteins then their detection in human milk would suggest that a 24 hour dietary avoidance may not have been long enough for food proteins to be cleared from the maternal circulation and thus they continued to be excreted in breast milk.

In those studies where a food challenge dose was given, only one study (27), described that the breastfeeding mothers followed a cow's milk free diet during the next 24 hours after consumption of their cow's milk challenge. Thus in the other studies, the limited reported details about the foods eaten on the day during the breast milk collection also makes their results difficult to interpret, as for example any ingestion of other foods containing even small amounts of cow's milk protein may have influenced the quantities of *B*-lactoglobulin measured in human milk.

In the study conducted by Fukushima and colleagues (23), the participating women were asked to record their daily intake of cow's milk from late pregnancy to the day of the breast milk sample collection. An interesting finding from this study was that *B*-lactoglobulin concentrations were low in the women whose cow's milk consumption during the entire lactating period was low, even though all women consumed >200ml per day for one week before breast milk sampling. Thus one could postulate that food protein concentrations in human milk may relate to average long term ingestion over a period of months rather than the immediate amount of food challenge ingestion prior to breast milk sampling.

1.2.6.4 Other breast milk components

One could propose that some of the variability between individual women observed in previous studies regarding food protein detection in human milk may also be due to the influence of other breast milk components. Two studies (23, 30) reported the presence and quantity of *B*-lactoglobulin was not correlated to IgA levels in the breast milk samples. As discussed earlier only one of these studies (23), analysed human milk after maternal ingestion of a specific challenge dose of cow's milk.

Bertino and co-workers (28) investigated bovine *B*-lactoglobulin concentrations in breast milk samples from women on diets containing different levels of cow's milk protein. After intensive laboratory analysis they concluded that the anti- *B*-lactoglobulin antibodies used in their ELISA method to quantify the presence of *B*-lactoglobulin in human milk were also cross-reacting with human *B*-casein, human α-lactalbumin and human lactoferrin proteins. Thus cross-reactivity to these human milk proteins may explain some of the variability in results observed in this and other previous studies investigating the presence of *B*-lactoglobulin in human milk.

1.2.6.5 Recruitment and participation bias

For those studies (2, 8, 21-23, 27, 31, 32, 34, 42) investigating the presence of food proteins in human milk after maternal ingestion of a specific challenge dose of food protein(s), there are limited details describing the recruitment process and participation rate of the recruited women. Fukushima and colleagues (23) report that 40 pregnant women were recruited and that the participation rate was 24/40, although

no details are given to the method of recruitment. The most recent study in this area conducted by Vadas and co-workers (34) did describe that volunteer women were recruited in response to notices posted at breastfeeding centres, but no details of the participation rate of recruited women were reported. Two other studies (27, 32) did not describe the recruitment process or how many women were approached to participate.

In the study by Kilshaw and Cant (21), 22 women had breast milk samples analysed for the presence of ovalbumin and 19 of these 22 for *B*-lactoglobulin. There was no indication why the breast milk of three of the women was not analysed for the presence of *B*-lactoglobulin. Again there was no description of the recruitment process or how many women were approached to participate. It was however noted that 5/22 women had infants with eczema symptoms present at the time of breast milk sampling. In the same published paper (21), the detection of ovomucoid in breast milk samples from 9 women was also reported. By matching the subject initials and weeks postpartum it appears that 2/9 of these women were also in the group of 22 women as described above. Interestingly 8/9 of these women had infants with eczema symptoms present at the time of breast milk sampling. Thus one could speculate that some of these women may have been recruited after eczema symptoms occurred in their infants and that the presence of allergy symptoms in breastfed infants would correlate with high levels of food proteins detected in their mother's breast milk. This may have biased the results, especially for the high frequency 7/9 (78%) of women with detectable ovomucoid in their breast milk.

Five studies (2, 8, 22, 31, 42) did specifically investigate mothers whose breastfed infants had suspected cow's milk allergy and/or eczema symptoms. The possible relationship between food protein detection in human milk and allergy symptoms in breastfed infants will be further explored in the next section of my literature review.

1.2.7 Food proteins in human milk fed to infants with allergy symptoms

Seven previous studies (2, 8, 22, 25, 26, 31, 42) have detected the presence of food proteins (ovalbumin or bovine B-lactoglobulin) in human milk fed to infants with allergy symptoms. Five of these studies (2, 8, 22, 31, 42) analysed breast milk samples after maternal ingestion of a specific challenge dose of food protein (egg or cow's milk) and two of these studies (25, 26) measured the concentration of *B*-lactoglobulin in human milk while women followed their usual diet.

Of the four studies (2, 22, 26, 42) which also included breast milk samples from mothers of infants without allergy symptoms, only the study by Axelsson and co-workers (26) found the presence of allergy symptoms (diarrhoea, vomiting, colic and eczema) in the infants to be significantly correlated with high levels of *B*-lactoglobulin detected in their mother's breast milk. Seven women had more than 50 ng/ml *B*-lactoglobulin in at least one breast milk sample, and 6/7 of these women had infants with allergy symptoms.

In the study by Cant et al (22) no difference was found for the ovalbumin frequency of detection or concentration in the breast milk between mothers of infants with

eczema symptoms and a positive skin prick test to egg compared to mothers of infants without eczema and negative skin prick test to egg. Jarvinen and co-workers (42) found comparable *B*-lactoglobulin concentrations between 9/16 mothers of infants with cow's milk allergy and 5/10 control mothers of healthy infants without allergy symptoms. In the other study by Host and co-workers (2) *B*-lactoglobulin was detected in the breast milk of 3/9 mothers of infants with cow's milk allergy and in 1/10 control mothers of healthy infants without allergy symptoms. Overall this was a low detection rate of 21% compared with other studies detecting *B*-lactoglobulin in human milk, which may be due to the collection of only one breast milk sample per women in this study.

It is important to note that these four studies (2, 22, 26, 42) do not appear to have adequately matched their control mothers for maternal characteristics like maternal age or stage of lactation.

Three studies (2, 8, 25) have reported breastfed infants to have improved allergy symptoms and reduced breast milk detectable *B*-lactoglobulin concentrations following the commencement of a maternal cow's milk-free diet. Unfortunately in all three of these studies (2, 8, 25), sample sizes were small ($n \leq 13$) and the maternal cow's milk dietary interventions were non-randomised, uncontrolled and non-blinded.

Thus randomised trials are required which are adequately powered to detect differences in human milk food protein concentrations from women whose breastfed infants have allergy symptoms compared to those from matched control women

whose infants do not have allergy symptoms. Large, blinded, randomised controlled trials are also needed to detect differences in human milk food protein concentrations following maternal dietary restriction and maternal dietary challenge.

1.2.8 IgA in human milk

Previous research (21-23, 27, 31, 34) has determined that food proteins can be detected in the breast milk of at least 48% of women. Thus the presence of food proteins in human milk appears to be a common phenomenon, so this is probably not the only factor leading to the possible sensitisation and continuation of symptoms in breastfed infants with food allergy. IgA antibodies in human milk may bind to food antigens to form immune complexes, decreasing the chances of these food antigens from crossing the intestinal surface in a breastfed infant. Approximately 90% of total IgA in human milk is present in the secretory form and the proportion of secretory to total measurable IgA remains constant throughout lactation (60).

Some factors which have been shown to affect IgA concentrations in human milk include parity (61), socioeconomic status (61) and stage of lactation (60). IgA levels in human milk have been demonstrated to decrease from 2.1 mg/mL at 2-3 days to 0.5 mg/mL by 12 weeks of lactation, and then slightly increase again to 0.6 mg/mL at 24 weeks, 0.9 mg/mL at 36 weeks and 1.0 mg/mL at 52 weeks (60). No significant measurable human milk IgA concentration differences have been shown for breast milk collected between left and right breasts on the same day, or in the course of a single breastfeeding or a 24 hour period (62).

Previous studies (30, 63, 64) have demonstrated that total IgA levels in human milk were significantly lower in mothers who had infants with cow's milk allergy symptoms compared with women of allergy-free infants. Infant symptoms of cow's milk allergy, including eczema, urticaria, colic, diarrhoea and respiratory symptoms, were particularly associated with maternal breast milk total IgA levels of <0.25 mg/mL (30, 63). The symptomatic infants in two of these studies (63, 64) had their cow's milk allergy verified by a cow's milk protein challenge, unfortunately the study design was weakened by this challenge being preformed non-blinded. One study (30) also demonstrated cow's milk specific IgA levels to be lower in the breast milk fed to infants with cow's milk allergy symptoms, but this finding was not corroborated in other studies (63, 64).

All three of these studies (30, 63, 64) were cohort studies and the infants with cow's milk allergy symptoms were not specifically matched to allergy-free infants. Only the study by Jarvinen et al (63) had a similar number of infants with a cow's milk allergy (n=48) compared to the number of allergy-free infants (n=39), whereas the Savilahti et al study (64) involved only 7/198 infants with cow's milk allergy. The earlier study by Machtinger and Moss (30) involved only 11/57 infants described to have high allergic symptom scores without a cow's milk challenge or SPT or RAST to verify the diagnosis of cow's milk allergy.

All three studies (30, 63, 64) used the radial immunodiffusion method to measure total IgA levels in breast milk samples which were collected at various stages of lactation from 3 days to 12 months. However all three studies (30, 63, 64) did

investigate the differences in the total IgA levels after they were matched for lactation duration and still found lower levels in the breast milk fed to infants with cow's milk allergy symptoms.

However another cohort study (65) found no difference between total S-IgA levels in breast milk from mothers of allergic (symptoms of atopic eczema, bronchial asthma, allergic urticaria and food allergy, n=44/120) as compared to non-allergic infants. This study measured total S-IgA levels by an ELISA method on breast milk samples taken at 2-4 days postpartum and at three months of lactation. Unfortunately again in this study (65) the infants with allergy symptoms were not specifically matched to allergy-free infants.

No relationship has been found between breast milk IgA levels and maternal atopy (30, 63, 66-68). Previous studies have also demonstrated specific IgA levels to food proteins (*B*-lactoglobulin and ovalbumin) are independent of consumption of these food proteins (23, 30, 67, 68) and are not correlated to the food protein concentrations in breast milk (23, 29, 30).

This evidence suggests further investigation of maternal total IgA levels in breast milk appears warranted and may point towards being an important influence on the prevention and treatment of food allergy in breastfed infants.

1.3 Summary of the literature review and rationale for the work presented in this thesis

Insufficient high quality evidence exists to determine the extent of benefit offered by maternal food protein avoidance diets to breastfed infants to treat and prevent food allergy. There is a significant knowledge gap in our understanding of the presence of food proteins in human milk. In particular none of the previous studies investigating the presence of food proteins in human milk have examined whether there is a dose related effect from the ingestion of different quantities of a food protein on the concentration of that food protein appearing in breast milk samples from the same group of lactating women. Previous studies have also not reported repeat breast milk food protein concentration measurements on different days on the same women after consumption of identical food challenges. Furthermore as cooking generally denatures protein, it is not known what effect the ingestion of raw versus cooked foods has on the concentration of food proteins in human milk. Providing answers to these questions is vital because the basis of maternal food protein avoidance diets relies on the implication that the presence of food proteins in human milk results in the sensitisation and continuation of symptoms in breastfed infants.

In this thesis I will focus on egg protein (ovalbumin). Egg protein was chosen in preference to other common food allergens for several reasons. As opposed to cow's milk protein, maternal dietary avoidance of egg is not generally nutritionally compromising to breastfeeding mothers. Of clinical relevance is that egg allergy is the most common food allergy in Australia affecting 3% of children under 2 years of

age (40). Also importantly from a practical study design perspective, egg can be disguised easier than some other food proteins in blinded food challenges containing different amounts and forms of egg.

The presence of egg protein (ovalbumin) in human milk has only been studied in three previous reports (21-23). Fukushima and colleagues (23) detected ovalbumin in breast milk samples from women following their usual dietary egg consumption. The other two studies (21, 22) detected ovalbumin in human milk after lactating women had ingested a challenge dose of one raw egg. However of practical dietary relevance is that egg is more commonly consumed in the Australian diet in the cooked form, thus evidence regarding the presence of ovalbumin in human milk after a maternal cooked egg challenge is required.

Egg white is known to contain six major proteins, these being ovalbumin, ovomucoid, ovotransferrin, ovomacroglobulin, ovomucin and lysozyme, with ovalbumin contributing the largest portion (greater than 50%). There may be differences in absorption and excretion into human milk between these egg proteins. Hoffman (69) discusses the difficulty in inducing antibody production in rabbits to ovomucoid, resulting in techniques to measure ovalbumin rather than ovomucoid in biological samples. Thus we, along with the other previous studies in this field (21-23), investigated the presence of dietary egg protein in breast milk focussing on ovalbumin.

Hence the studies presented in this thesis aim to assess the effect of egg ingestion on ovalbumin concentration in human milk fed to infants with and without allergy

symptoms. An investigation of immune mechanisms that may play a role in infant allergy responses to egg was not undertaken because it was necessary to first establish and characterise dietary exposure of ovalbumin to infants through human milk.

The specific primary aims of my thesis were:

- To benchmark current Australian dietetic practice regarding maternal dietary restrictions for treatment and prevention of food allergies in breastfed infants.

- To determine whether cooked versus raw egg ingestion alters ovalbumin content in human milk.

- To determine whether the dietary dose of cooked egg influences ovalbumin content in human milk.

- To determine the effect of eating cooked egg on the ovalbumin concentration in breast milk fed to infants with egg sensitivity.

CHAPTER 2: Materials and methods

This chapter describes the development of recipes for the dietary interventions used in the two randomised controlled trials (Chapters 4 and 5) and the development of a sandwich enzyme-linked immunosorbent assay (ELISA) method to measure the concentration of ovalbumin in human milk.

2.1 Dietary intervention recipe development

To enable blinding of both participants and researchers, the dietary interventions used in both randomised controlled trials (Chapters 4 and 5) needed to be indistinguishable by appearance and taste. The dietary interventions required for the first trial (Chapter 4) were four test breakfasts containing either one cooked egg or half a cooked egg or one raw egg or an egg-free breakfast. The dietary interventions required for the second trial (Chapter 5) were the consumption of one cooked egg per day or an egg-free diet. I choose muffins as the challenge food to disguise the cooked egg content and milkshakes to disguise the raw egg in the dietary interventions.

2.1.1 Muffins

Usually a standard muffin contains 1/6 of an egg, but muffins containing either ½ an egg or one whole egg were required. I commenced with a standard blueberry muffin recipe (70) containing two eggs per 12 muffins (1/6 of an egg per muffin). Initially a

recipe was developed for 12 muffins containing six eggs (½ an egg per muffin) before further modifications were made to achieve one egg per muffin. Lemon juice in the original recipe was replaced with vanilla essence to help disguise the taste of the increased egg content. The sugar content of the recipe was also increased to further assist in disguising the taste of increased egg content. The addition of extra eggs added more liquid, protein and fat to the mixture so the milk content was reduced as a counter balance. Plain flour was added to the recipe to reduce the rising effect of the additional egg content.

This recipe was then adjusted to include 12 eggs in 12 muffins (one cooked egg per muffin). The oil ingredient was removed, milk content further reduced and the plain flour amount increased to again balance the effect of additional eggs.

An egg-free muffin recipe was then developed using a commercial egg replacer product ("egg-like", Country Harvest, Bentleigh, Victoria, Australia) which contains potato and tapioca starches. I commenced with the ½ an egg per muffin recipe which I had developed as described above. More oil and milk were added to the ½ an egg per muffin recipe after the removal of the six eggs to counter balance the liquid, protein and fat contents.

However when these egg-free muffins were compared with the muffins containing ½ an egg per muffin and one egg per muffin the egg containing muffins could be distinguished by their egg taste. The egg containing muffins were also larger in size than the egg-free muffins.

I decided to use larger size muffins and increased the self-raising flour content of all three recipes which significantly reduced their differences in taste. However the one cooked egg per muffin recipe resulted in muffins which rose higher than the ½ an egg per muffin and egg-free muffins, enabling them still to be distinguished by appearance. Finally I added extra egg replacer to both the egg-free muffin recipe and the ½ an egg per muffin recipe which reduced their differences in appearance to the one egg per muffin recipe. In total 18 batches of muffins were cooked to achieve the final muffin recipe ingredients. Cooking times and temperatures were then optimised with the cooking of another 6 batches of muffins. The final muffin ingredients and recipes are shown in Table 2.1 and the macronutrient composition of the muffins are summarised in Table 2.2.

The muffins were trialled with 15 volunteers to assess visual appearance and taste detection of the egg content. When asked and given the options of determining on visual appearance alone whether the muffins contained one whole egg, ½ an egg or were egg-free, volunteers could not distinguish the muffins on egg content. 11/15 of the volunteers could detect egg by taste in the egg containing muffins, but only 5/15 correctly distinguished between those muffins containing ½ an egg compared with one whole egg.

Table 2.1: Final muffin ingredients and recipes used for dietary interventions.

egg-free muffin recipe ingredients	½ an egg per muffin recipe ingredients	one egg per muffin recipe ingredients
18 teaspoons of egg replacer beaten with 1cup water	9 teaspoons of egg replacer beaten with ½ cup water	
	6 eggs (beaten) of total weight of 330g	12 eggs (beaten) of total weight of 660g
1 cup milk	¾ cup milk	½ cup milk
1 cup oil	½ cup oil	
2 tablespoons vanilla essence	2 tablespoons vanilla essence	2 tablespoons vanilla essence
1 cup sugar	1 cup sugar	1 cup sugar
5 cups self-raising flour	4½ cups self-raising flour and	4 cups self-raising flour
	½ cup plain flour	1 cup plain flour
1cup drained tinned blueberries	1cup drained tinned blueberries	1cup drained tinned blueberries

Method for all muffin recipes:

Combine wet ingredients with sugar and mix well to evenly distribute egg/egg replacer content. Stir in flour. Gently fold in the blueberries. Makes 12 muffins.

Bake at 200 C in an electric oven for 20 minutes.

Table 2.2: Macronutrient composition of the muffins.

per muffin	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
egg-free	2115	9	20	74
½ (27.5g) egg	1912	12	13	71
one (55g) egg	1708	15	6	69

2.1.2 Milkshakes

Initially strawberry milkshakes (containing cow's milk, vanilla ice-cream and liquid strawberry flavouring) with and without one raw egg were taste tested by nine volunteers. The milkshakes were put in milkshake containers with a lid and straw ensuring their visual appearance was identical. 7/9 of these volunteers could determine which milk drink contained the raw egg because they were able to taste the egg or detect egg lumps in the milkshakes.

The milkshakes with and without raw egg were then re-tested by 16 volunteers after vanilla essence (to help disguise the egg taste), mashed banana (to help disguise the egg lumps) and liquid banana flavouring (to help disguise the egg taste)were added to the recipe instead of the liquid strawberry flavouring. When asked and given the options of determining on taste whether the milkshakes contained egg (yes or no), as would be expected from a random guess only 7/16 of the volunteers were then able to correctly identify which of the two banana milkshakes contained the raw egg. The final milkshake ingredients and recipes are shown in Table 2.3 and macronutrient composition of the milkshakes are summarised in Table 2.4.

Table 2.3: Final milkshake ingredients and recipes used for dietary interventions.

Egg-free milkshake ingredients	Raw egg milkshake ingredients
200ml low-fat (1%) milk	150ml low-fat (1%) milk
2 scoops egg-free vanilla ice cream	2 scoops egg-free vanilla ice cream
1 teaspoon vanilla essence	1 teaspoon vanilla essence
½ mashed banana	½ mashed banana
1 tablespoon of banana flavouring	1 tablespoon of banana flavouring
	55g well beaten raw egg

Method for both milkshake recipes:

Put well blended ingredients into a milkshake container with a lid and straw.

Each recipe serves 1 person.

Table 2.4: Macronutrient composition of the milkshakes.

per milkshake	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
egg-free	1214	11	7	46
one (55g) raw egg	1479	16	12	43

Table 2.5: Macronutrient composition of the test breakfasts served in the first trial (Chapter 4).

per breakfast	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
egg-free	3329	20	27	140
½ cooked egg	3126	23	20	117
1 cooked egg	2922	26	13	115
1 raw egg	3594	25	32	117

In summary, recipes were developed for muffins containing one cooked egg (55g), muffins containing half a cooked egg (27.5g) and egg-free muffins, as well as milkshakes containing one raw egg (55g) and egg-free milkshakes. Table 2.5 summarises the macronutrient composition of the four different test breakfasts each comprising a combination of a muffin and milkshake as served for breakfast to the women participating the first trial (Chapter 4). The picture in figure 2.1 illustrates one of these muffin and milkshake test breakfasts.

Figure 2.1: Muffin and milkshake as served for breakfast in the first trial.

2.2 Laboratory method development: The measurement of ovalbumin concentration in human milk by enzyme-linked immunosorbent assay (ELISA)

In the mid-1980s, a radioimmunoassay method was used to measure the concentration of ovalbumin in human milk (21, 22), the sensitivity of this assay was 0.1ng/mL and ovalbumin was detected at concentrations of 0.1-6.17ng/mL after lactating women consumed one raw egg. More recent studies (23, 27-29, 31, 33, 34) have measured food protein concentrations in human milk using enzyme-linked immunosorbent assay (ELISA) methods. ELISA methods can be as sensitive as radioimmunoassay methods and have the advantage of being safer and less costly (71). Table 2.6 summarises food protein in human milk detection methods and assay sensitivity levels from previous studies. In order to measure the concentration of ovalbumin in human milk for my studies, my aim was to find or develop an ELISA method to measure the concentration of ovalbumin in human milk with an assay sensitivity of 0.1ng/mL.

Table 2.6: The detection of food proteins in human milk.

Study	Food protein	Detection method	Assay sensitivity (ng/mL)
Stuart et al, 1984 (24)	β-lactoglobulin (cow's milk)	ELISA	not reported
Kilshaw and Cant, 1984 (21)	β-lactoglobulin (cow's milk) ovomucoid and ovalbumin (egg)	radioimmunoassay	0.1
Cant et al, 1985 (22)	ovalbumin (egg)	radioimmunoassay	0.1
Jakobsson et al, 1985 (25)	β-lactoglobulin (cow's milk)	radioimmunoassay	5
Axelsson et al, 1986 (26)	β-lactoglobulin (cow's milk)	radioimmunoassay	5
Machtinger and Moss, 1986 (30)	β-lactoglobulin (cow's milk)	ELISA	0.1
Troncone et al, 1987 (32)	gliadin (wheat)	ELISA	5
Cavagni et al, 1988 (8)	β-lactoglobulin (cow's milk)	radioimmunoassay	0.01
Host et al, 1988 (2)	β-lactoglobulin (cow's milk)	ELISA	0.3
Host et al, 1990 (27)	β-lactoglobulin (cow's milk)	ELISA	0.3
Sorva et al, 1994 (31)	β-lactoglobulin (cow's milk)	ELISA	0.002
Bertino et al, 1996 (28)	β-lactoglobulin (cow's milk)	ELISA	not clear
Lovegrove et al, 1996 (29)	β-lactoglobulin (cow's milk)	ELISA	0.08
Fukushima et al, 1997 (23)	β-lactoglobulin (cow's milk) and ovalbumin (egg)	ELISA	0.1
Chirdo et al, 1998 (33)	gliadin (wheat)	ELISA	1
Vadas et al, 2001 (34)	*Ara h* 1 and *Ara h* 2 (peanut)	ELISA	not reported

The availability of a commercial ELISA test kit for ovalbumin was investigated. The Veratox Egg Allergen test kit (Neogen Corporation, Lansing, MI, USA) was identified. This is a sandwich ELISA for the quantitative analysis of egg protein in food products, however the limit of quantitation of this assay was 2500ng/mL whereas I was aiming for an ELISA with a sensitivity minimum of 0.1ng/mL. This option was not assessed any further because of the large difference between the sensitivity of this commercial test kit and that required for my studies.

At our research institute a sandwich ELISA method to detect ovalbumin in rat serum had previously been developed. The method was as follows. Flat bottom, polystyrene, high binding 96 well assay plates (3590 Costar, Corning Incorporated, Corning, NY, USA) were coated with 100µL per well of mouse anti-chicken egg albumin monoclonal antibody (Sigma BioSciences, St Louis, MO, USA) diluted at 1 in 5000 in 0.05mol/L carbonate-bicarbonate buffer at pH 9.6 (Sigma-Aldrich, Saint Louis, Missouri, USA) and incubated overnight at 4°C. The wells were washed 3 times with phosphate buffered saline (PBS) and blocked with 300µL per well of 1% fetal calf serum in PBS, incubated for 30 minutes at 37°C then 30 minutes at room temperature. Each well was washed 3 times with PBS. A standard curve for this ELISA method used 1/6 normal rat serum (diluted in PBS) spiked with known quantities of ovalbumin (chicken egg albumin grade V, Sigma-Aldrich) with a concentration range of 62.5 - 16 000ng/mL. These standards, along with the samples to analyse, were added in triplicate at 100µL per well and incubated for 2 hours at room temperature. After washing each well 3 times with PBS + 0.05% Tween 20,

100μL per well of horseradish peroxidase conjugated rabbit anti-ovalbumin polyclonal antibody (Rockland), diluted at 1 in 10 000 with 0.1% fetal calf serum + PBS with 0.05% Tween 20, was added and incubated for 2 hours at room temperature. The wells were washed 5 times with PBS + 0.05% Tween 20 and 150μL per well of the substrate o-phenylenediamine dihydrochloride (SIGMA FAST OPD tablets, Sigma-Aldrich) solution was added and the colour reaction allowed to proceed in the dark for 30 minutes at room temperature. The reaction was stopped by the addition of 50μL per well of 0.5mol/L H_2SO_4 and read on an automatic microplate reader (Opsys MR, Dynex Technologies Inc, Chantilly, Virginia, USA) at optical density wavelength of 490nm. Unfortunately this ELISA method had an assay sensitivity minimum of 100ng/mL, which was 1000 times higher than my aim of 0.1ng/mL.

Factors that can influence ELISA limit of assay detection include the type of assay plates used, blocking agents, composition of dilution and incubation buffers, type (monoclonal versus polyclonal), specificity and concentrations of antibodies, the assay speed and incubation temperatures (72). Comparing the previous ELISA methods used to measure food protein concentrations in human milk, the method developed by Makinen-Kiljunen and Palosuo (73) and used in the study by Sorva and co-workers (31), measured bovine *B*-lactoglobulin concentration in human milk with the lowest assay limit of detection of 0.002ng/mL. This method was examined to identify possible modifications that may improve our assay's detection limit. There were several differences in the method by Makinen-Kiljunen and Palosuo (73), including the overnight plate incubation at 4°C of samples and β-lactoglobulin standards, the use of polyethylene glycol 6000 added to the incubation buffers to

improve the assay sensitivity, the overnight at 4°C incubation used for the secondary detection antibody and polyclonal antibodies used for both the primary coating antibody and secondary detection antibody.

2.2.1 Ovalbumin in human milk standards plate incubation conditions experiment

Background: On comparison of the ELISA method used to detect ovalbumin in rat serum at our research institute with those previously published ELISA methods used to measure food protein concentrations in human milk, the methods described by Makinen-Kiljunen and Palosuo (73), Lovegrove et al (29) and Bertino et al (28) all incubated the human milk samples and standards overnight at 4°C.

Aim: To determine whether a change from 2 hours at room temperature to an overnight at 4°C plate incubation of ovalbumin in human milk standards improved the assay sensitivity.

Method: Using the sandwich ELISA to detect ovalbumin in rat serum method as described above, I undertook an experiment where ovalbumin in human milk standards were incubated for 2 hours at room temperature on two plates and compared with the incubation overnight at 4°C on another two plates.

Commercial known ovalbumin concentration in human milk standards were not available. Thus ovalbumin in human milk standards to use for the ELISA standard curve were produced using a pooled breast milk sample collected from ten women after 48 hours on an egg-free diet. The breast milk samples were centrifuged (Sigma

Laboratory Centrifuge 4K15, Osterode am Harz, Germany) at 2000g for 20 minutes at 4°C on the day of collection and the aqueous supernatant frozen at -20°C. This aqueous supernatant was thawed and then spiked with known quantities of ovalbumin (chicken egg albumin grade V, Sigma-Aldrich) with a concentration range of 0-1000ng/mL.

Result: As illustrated in Figure 2.2 this change in plate incubation conditions resulted in an improvement of the assay sensitivity from 100 to 10ng/mL.

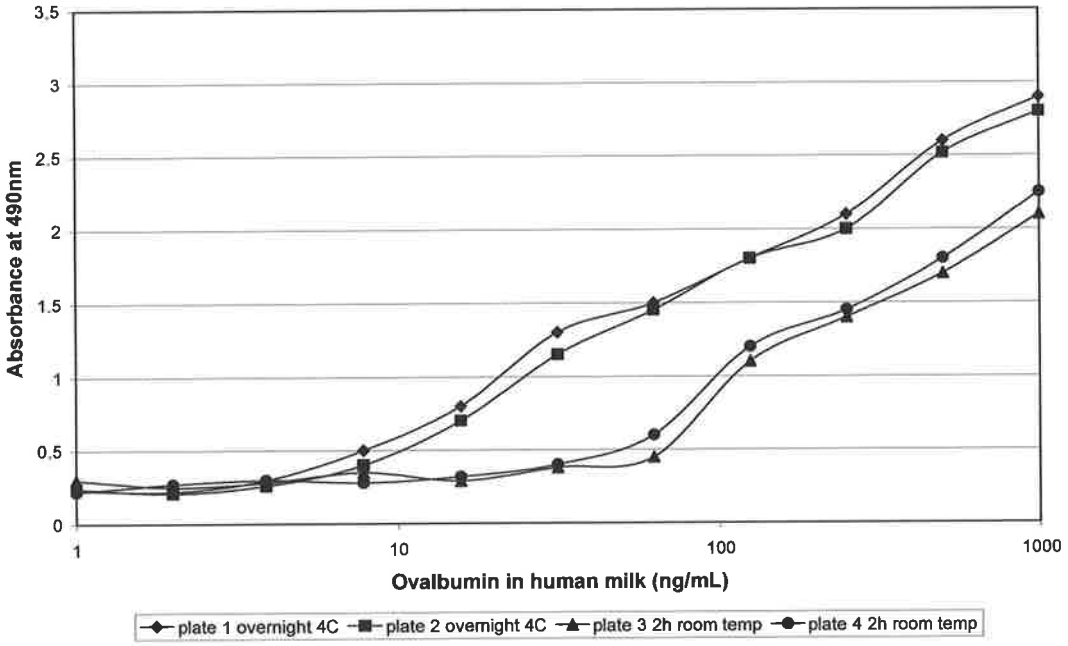


Figure 2.2: Comparison of plate incubation conditions for ovalbumin in human milk standards. On plates 1 and 2 the ovalbumin in human milk standards were incubated overnight at 4°C which resulted in an improved assay limit of detection to 10ng/mL, compared to 100ng/mL for plates 3 and 4 incubated for 2 hours at room temperature.

2.2.2 Use of polyethylene glycol 6000 added to ovalbumin standards experiment.

Background: Makinen-Kiljunen and Palosuo (73) described the addition of 3% polyethylene glycol 6000 to their human milk samples and standards to improve their assay sensitivity.

Aim: To determine whether the use of polyethylene glycol 6000 added to the ovalbumin in human milk standards improved the assay sensitivity.

Method: In this experiment the ovalbumin in human milk standards were prepared with the addition of 3% w/v polyethylene glycol (PEG) 6000 on two plates and without the addition of polyethylene glycol (PEG) 6000 on two plates. The original ELISA method to detect ovalbumin in rat serum was otherwise followed with the change of the overnight at 4°C plate incubation of the ovalbumin in human milk standards as this was found to be beneficial in the previous experiment.

Result: As illustrated in Figure 2.3 this addition of polyethylene glycol 6000 resulted in no further improvement of the assay sensitivity.

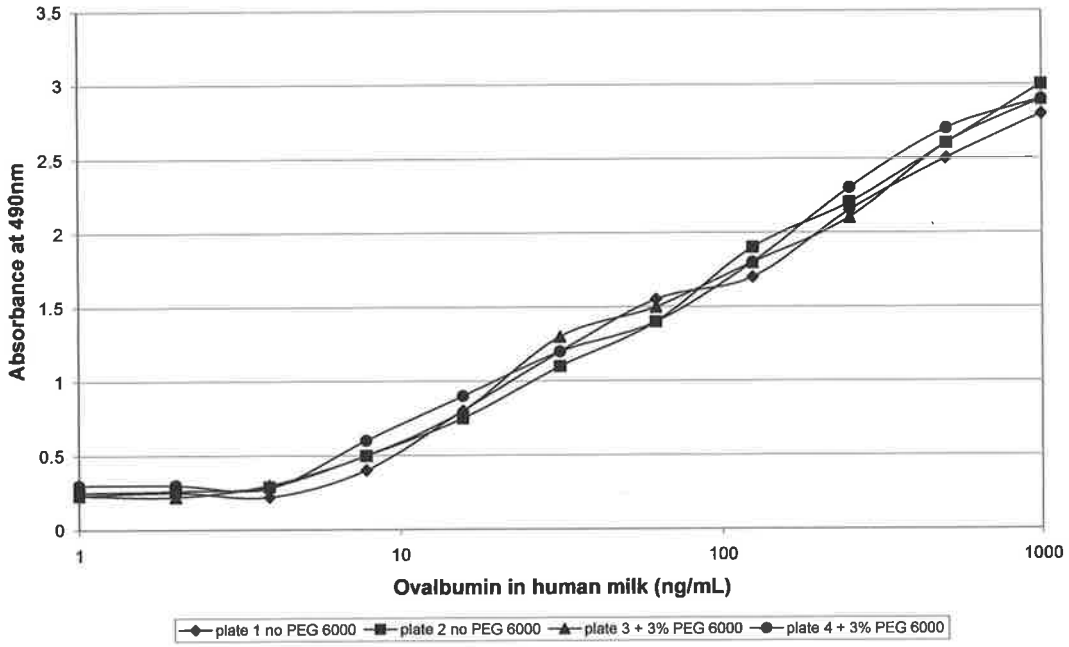


Figure 2.3: Comparison of ovalbumin in human milk standards prepared with and without the addition of 3% w/v polyethylene glycol (PEG) 6000. The addition of PEG 6000 to the ovalbumin in human milk standards did not further reduce the ELISA limit of detection for ovalbumin in human milk.

2.2.3 Addition of polyethylene glycol 6000 to the secondary antibody experiment.

Background: In the method published by Makinen-Kiljunen and Palosuo (73) polyethylene glycol 6000 was also added to the secondary antibody incubation step.

Aim: To determine whether the addition of polyethylene glycol 6000 to the secondary antibody may further improve the assay sensitivity.

Method: Polyethylene glycol 6000 was added at concentrations of 1% w/v, 3% w/v and 5% w/v and compared with no addition of polyethylene glycol 6000 to the secondary horseradish peroxidase conjugated rabbit anti-ovalbumin polyclonal antibody (Rockland, Gillertsville, PA, USA). For this experiment I also changed the serial dilution concentration range for the ovalbumin in human milk standards from 1-1000ng/mL to 0.4-100ng/mL, as the assay sensitivity was currently down to 10ng/mL and in preparation of developing a final ELISA method using an ovalbumin in human milk standards range of 0.1-25ng/mL.

Result: As illustrated in Figure 2.4 the addition of 1% w/v polyethylene glycol 6000 resulted in a further reduction in assay sensitivity to 3ng/mL. The higher concentrations of 3% w/v and 5% w/v polyethylene glycol 6000 caused background non-specific binding.

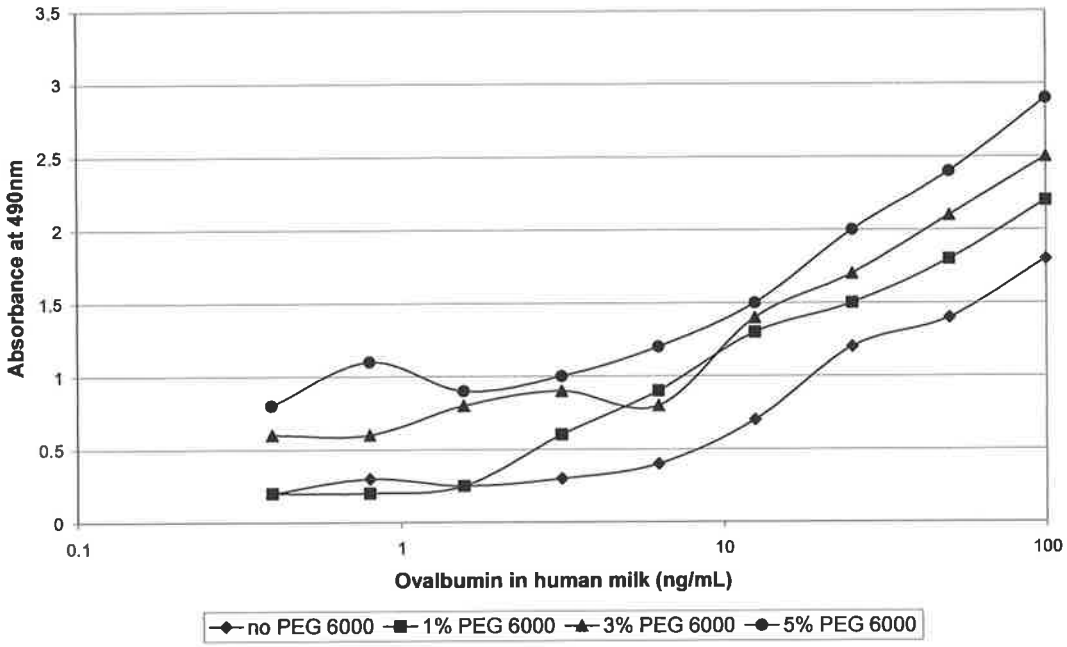


Figure 2.4: Comparison of the addition of polyethylene glycol 6000 to the secondary anti-ovalbumin polyclonal antibody. The addition of PEG 6000 at a concentration of 1% w/v resulted in a further improvement of the assay limit of detection to 3ng/mL.

2.2.4 Secondary detection antibody plate incubation conditions experiment.

Background: On comparison of the previously published ELISA methods used to measure food protein concentrations in human milk, the plate incubation conditions for the secondary detection antibody varied. Makinen-Kiljunen and Palosuo (73) used an overnight at 4°C incubation, Lovegrove et al (29) used a 37°C for 2 hours incubation and Bertino et al (28) a 37°C for 4 hours incubation.

Aim: To determine whether a change from 2 hours at room temperature to an overnight at 4°C or 3 hours at 37°C plate incubation of the secondary detection antibody improved the assay sensitivity.

Method: In this experiment the incubation conditions of 2 hours at room temperature, 3 hours at 37°C, 2 hours at room temperature followed by 2 hours at 4°C then 1hour at room temperature, 4hours at 4°C and overnight at 4°C for the secondary detection antibody were compared.

Result: Interestingly it was found that the combination of 2 hours at room temperature followed by 2 hours at 4°C then 1hour at room temperature resulted in a further reduction in the assay sensitivity for ovalbumin detection to 1.5ng/mL, as illustrated in Figure 2.5.

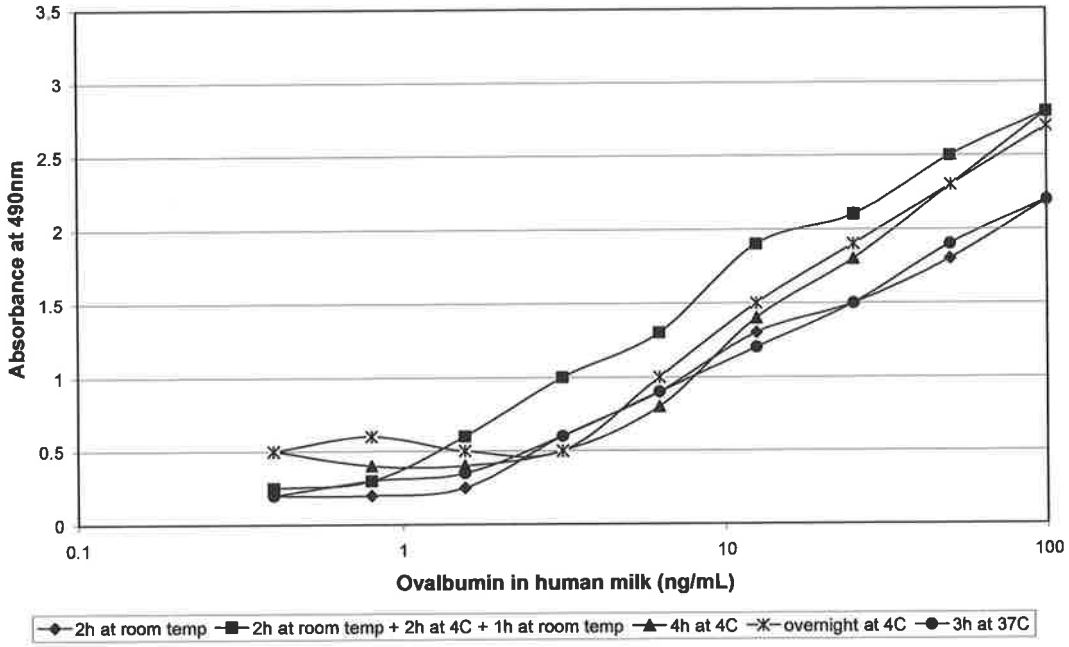


Figure 2.5: Comparison of the effects of different incubation times and temperatures for the secondary antibody. A combination of 2 hours at room temperature followed by 2 hours at 4°C then 1hour at room temperature resulted in a further improvement of the assay limit of detection to 1.5ng/mL.

2.2.5 Use of a polyclonal antibody for the primary coating antibody experiment.

Background: Polyclonal antibodies recognise a variety of epitopes on the antigen and are therefore more tolerant of minor changes in the antigen (eg slight denaturation) than are monoclonal antibodies (74). Previous ELISA methods (28, 29, 73) used to measure food protein concentrations in human milk have all used polyclonal antibodies for both the primary coating antibody and secondary detection antibody.

Aim: To determine whether a change from the use of a monoclonal to a polyclonal antibody for the primary coating antibody improved the assay sensitivity.

Method: In this experiment the use of the mouse anti-chicken egg albumin monoclonal antibody (Sigma BioSciences, St Louis, MO, USA) as the primary coating antibody and a rabbit anti-ovalbumin polyclonal antibody (Rockland, Gillertsville, PA, USA) as the primary coating antibody were compared. Checker-board titration was used to optimise the concentration of this primary coating antibody. The antibody dilutions compared were 1:500, 1:1000, 1:2000, 1:5000 and 1:10000. As per previously with the monoclonal antibody, the polyclonal antibody was also diluted in 0.05mol/L carbonate-bicarbonate buffer at pH 9.6.

For this experiment I further reduced the serial dilution concentration range for the ovalbumin in human milk standards from 0.4-100ng/mL to 0.1-50ng/mL, as the assay sensitivity was currently down to 1.5ng/mL.

Result: The use of the polyclonal antibody at a dilution of 1 in 2000 for the primary coating antibody was found to enable the ELISA sensitivity for ovalbumin detection in human milk to be reduced from 1.5 to 0.1ng/mL, as illustrated in Figure 2.6.

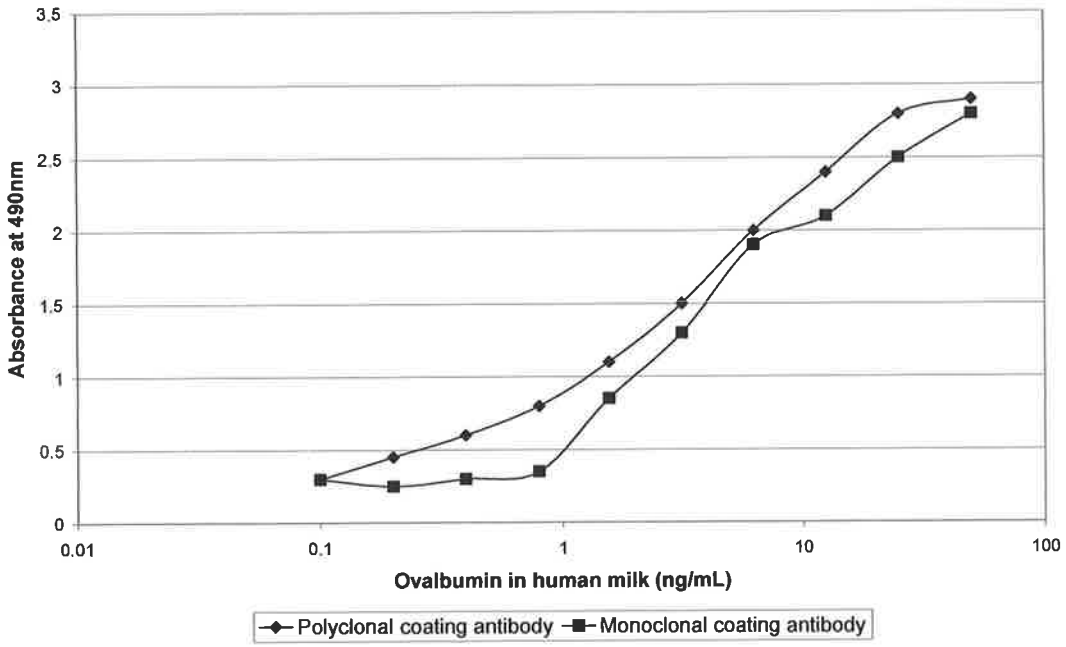


Figure 2.6: Comparison of mouse anti-chicken egg albumin monoclonal antibody versus rabbit anti-ovalbumin polyclonal antibody for the primary coating antibody. The use of the polyclonal antibody for the primary coating antibody resulted in a further enhanced ELISA limit of detection down to 0.1ng/mL.

2.2.6 Final ELISA method used and coefficient of variation results.

The final sandwich ELISA method used to measure the concentration of ovalbumin in human milk for the studies conducted in my thesis is as described below. Flat bottom, polystyrene, high binding 96 well assay plates (3590 Costar, Corning Incorporated, Corning, NY, USA) were coated with 100µL per well of rabbit anti-ovalbumin polyclonal antibody (Rockland, Gillertsville, PA, USA) diluted at 1 in 2000 in 0.05mol/L carbonate-bicarbonate buffer at pH 9.6 (Sigma-Aldrich, Saint Louis, Missouri, USA) and incubated overnight at 4°C. The wells were washed 3 times with phosphate buffered saline (PBS) and blocked with 300µL per well of 1% fetal calf serum in PBS, incubated for 30 minutes at 37°C then 30 minutes at room temperature. Each well was washed 3 times with PBS. A standard curve for this ELISA method used breast milk (from women after 48 hours on an egg-free diet) spiked with known quantities of ovalbumin (chicken egg albumin grade V, Sigma-Aldrich) with a concentration range of 0-50ng/mL. These breast milk standards, along with the breast milk samples collected from the participating women, were added in triplicate at 100µL per well and incubated overnight at 4°C. After washing each well 3 times with PBS + 0.05% Tween 20, 100µL per well of horseradish peroxidase conjugated rabbit anti-ovalbumin polyclonal antibody (Rockland), diluted at 1 in 10 000 with 0.1% fetal calf serum + 1% w/v polyethylene glycol 6000 + PBS with 0.05% Tween 20, was added and incubated for 2 hours at room temperature, 2 hours at 4°C, then 1 hour at room temperature. The wells were washed 5 times with PBS + 0.05% Tween 20 and 150µL per well of the substrate o-phenylenediamine

dihydrochloride (SIGMA FAST OPD tablets, Sigma-Aldrich) solution was added and the colour reaction allowed to proceed in the dark for 30 minutes at room temperature. The reaction was stopped by the addition of 50μL per well of 0.5mol/L H_2SO_4 and read on an automatic microplate reader (Opsys MR, Dynex Technologies Inc, Chantilly, Virginia, USA) at optical density wavelength of 490nm. This ELISA method had an assay sensitivity of 0.1ng/mL.

For my studies described in Chapters 4 and 5, all breast milk samples collected from the same woman were assayed together on the same plate. Samples from four women were assayed on the same ELISA run along with a standard curve plate. An Immunoassay Data Management program from Pharmacia (Uppsala, Sweden), known as Multicalc, was used to quantitate the ovalbumin concentration of each breast milk sample provided by the women.

Quality control samples were prepared and used to validate results within and between assays. Negative control wells contained breast milk aqueous supernatant (prepared as described previously) collected from women after 48 hours on an egg-free diet. 'High' quality control samples contained this same breast milk aqueous supernatant spiked with ovalbumin (chicken egg albumin grade V, Sigma-Aldrich) at a concentration of 12.5ng/mL and 'low' quality control samples were spiked with ovalbumin at a concentration of 0.8ng/mL. Blank wells contained PBS only. Each plate assayed contained the negative quality control sample, the 'high' quality control sample, the 'low' quality control sample and blank sample in triplicate wells. Variability within (intra-assay) and between (inter-assay) ELISA runs was assessed by the determination of the coefficient of variation (CV) for the quality control

samples. The intra-assay coefficient of variation (CV) was found to be 3-11% and the inter-assay CV was 9-15%.

Possible limitations of my ELISA method include:

- the use of a pooled breast milk sample from women after 48 hours on an egg-free diet to prepare the ovalbumin human milk standards assumes that all these women had complied with a strict egg-free diet.
- cross-reactivity of the anti-ovalbumin polyclonal antibodies with other human milk proteins. Human α-lactalbumin and human serum albumin were not checked for any cross-reactivity with the anti-ovalbumin polyclonal antibodies used in this ELISA method.
- the presence of maternal anti-ovalbumin antibodies (for example sIgA) which may bind to the human milk ovalbumin thus reducing its detection in the breast milk samples.

Chapter 3: Maternal dietary restrictions for treatment and prevention of food allergies in breastfed infants: current Australian dietetic practice.

3.1 Introduction

Expert nutritional committees of the American Academy of Pediatrics (AAP), the European Society for Pediatric Allergology and Clinical Immunology (ESPACI) and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (4, 5) have recommended the use of maternal dietary restriction for the treatment of breastfed infants with a food allergy.

To prevent food allergy in infants at high-risk due to an atopic family history, these expert nutritional committees have recommended exclusive breastfeeding or use of a formula with reduced allergenicity for at least 4-6 months and not introducing solid foods before 5-6 months of age (4, 5). The use of maternal dietary restriction during lactation to prevent food allergy is controversial. The American Academy of Pediatrics, Committee on Nutrition (5) has recommended that mothers of high risk infants should eliminate peanuts and tree nuts, and also consider eliminating eggs, cow's milk and fish from their diets while breastfeeding, while the ESPACI/ESPGHAN (4) joint committee have made no such recommendation.

The aim of this survey was to benchmark current Australian dietetic practice regarding maternal dietary restrictions for treatment and prevention of food allergies in breastfed infants.

3.2 Subjects and Methods

A survey was mailed to all major paediatric hospitals in Australia including Australian Capital Territory Community Care, Flinders Medical Centre, John Hunter Hospital, Mater Health Services Brisbane, Monash Medical Centre, Princess Margaret Hospital, Royal Children's Hospital Brisbane, Royal Children's Hospital Melbourne, Royal Hobart Hospital, Sydney Children's Hospital and The Children's Hospital at Westmead. In addition other known specialist paediatric allergy dietitians were invited to participate in this survey. A total of 15 specialist dietitians were identified.

This survey was constructed to determine the extent of maternal dietary restrictions advised by dietitians to breastfeeding mothers to treat or prevent food allergy in their infants. Where maternal dietary restriction was used, questions were asked regarding circumstances, the extent of foods avoided and whether partial or complete avoidance of these foods was recommended. Where dietary restriction was not used, the reasons for this practice were also ascertained. The survey was approved by the Research Ethics Committee of the Women's and Children's Hospital. See Appendix 2 and Appendix 3 for copies of the survey used.

3.3 Results

3.3.1 Treatment of food allergy

All 15 major paediatric hospitals and other known specialist paediatric allergy dietitians surveyed responded. The majority (13/15) of the dietitians surveyed would recommend the use of maternal dietary restriction for breastfed infants with food allergy symptoms (see Figure 3.1) and the foods most commonly restricted are cow's milk, egg, peanut and tree nuts. Nine out of thirteen dietitians advise complete rather than partial dietary avoidance of these food proteins. Treatment was individualised with the most common circumstances for advising maternal dietary interventions being if the food allergy symptoms commenced when the infant was exclusively breastfed and/or the breastfed infant had severe symptoms. Two of the dietitians surveyed never recommend the use of maternal dietary restrictions for treatment as they believe there is insufficient evidence that food proteins ingested by lactating women pass into breast milk and adversely affect the infant.

3.3.2 Prevention of food allergy

Nine out of fifteen dietitians surveyed reported that they do not advise maternal dietary restrictions for lactating women with a family history of allergy in order to prevent food allergy in the infant (see Figure 3.1). The most common reason given was the lack of scientific evidence that food proteins in breast milk may adversely affect the infant. Six dietitians reported that they would advise maternal dietary restrictions for food allergy prevention, especially if the infant has a sibling with

food allergies. Again the advice was individualised, however all of these six dietitians would recommend that peanuts be completely avoided.

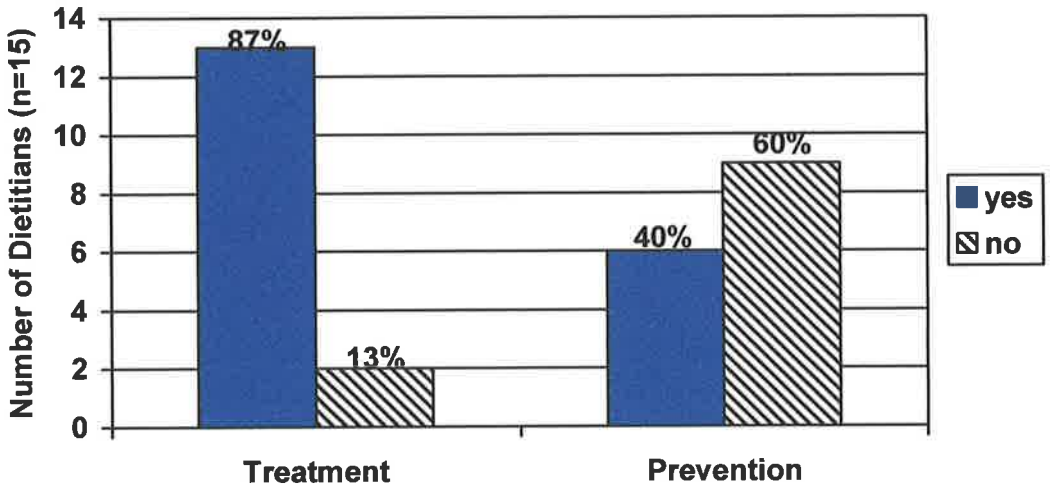


Figure 3.1: Use of maternal dietary restriction to treat or prevent food allergy in breastfed infants.

3.4 Discussion

In the absence of any Australian recommendations, the majority of Australian specialist paediatric allergy dietitians surveyed follow the recommendations from both European and American expert committees (AAP, ESPACI, ESPGHAN) (4, 5) concerning the use of maternal dietary restriction for the treatment of breastfed infants with a food allergy. There was also agreement that the foods most commonly restricted are cow's milk, egg, peanut and tree nuts. Most of the dietitians surveyed would advise complete rather than partial avoidance of the causal protein from the diet of the lactating mother to treat food allergy in breastfed infants.

However as detailed in Chapter 1 of this thesis, the majority of published evidence in favour of the use of maternal dietary restriction for the treatment of food allergy in breastfed infants comes from descriptions of case series where maternal dietary interventions were non-randomised and uncontrolled. There have only been three randomised trials of crossover design with double-blind food challenges and these trials have demonstrated conflicting results (10-12). Thus despite widespread recommendations and current dietetic practice of maternal food protein avoidance for treatment of breastfed infants with food allergy, the strength of published evidence in this area is limited.

The practice of advising maternal dietary restriction (especially peanuts) for food allergy prevention in breast fed infants was limited, but was in keeping with the recommendation from the American Academy of Pediatrics, Committee on Nutrition (5). Chapter 1 of this thesis details the evidence from previous trials investigating the

use of maternal dietary restriction during lactation for allergy prevention in high-risk infants (due to an atopic family history) which have shown varied results. In a recent review on this topic, Zeiger (75) concluded that maternal dietary restriction should be individualised and only advised after an evaluation of each family's atopic risk and circumstances.

The total dietary avoidance of one or more food proteins is difficult to achieve, can be nutritionally compromising, time consuming and socially restrictive for breastfeeding mothers. Thus improved quality evidence is required from randomised controlled trials to establish these recommendations and the current dietetic practice of maternal dietary restriction for food allergy treatment or prevention in breastfed infants.

Chapter 4: Effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomised, double-blind, cross-over trial

4.1 Introduction

Previous studies (2, 21-23, 27, 31, 32, 34) have shown that after ingesting the same challenge dose of a particular food, the frequency and concentration of detected food protein in breast milk is highly variable between women. Apart from the natural biological variation that would be expected between individual women, some of this variability may be due to other factors including the stage of lactation, maternal atopy status or usual dietary intake of these food proteins. Furthermore as cooking generally denatures protein, it is not known what effect the ingestion of raw versus cooked foods has on the concentration of food proteins in human milk.

As egg can be restricted in the diet without nutritionally compromising lactating women and is one of the most common food allergens, this study along with others (21, 22) investigated the presence of dietary egg protein in human milk focussing on ovalbumin. As a first step to further the understanding of food proteins excreted into human milk, a randomised, cross-over trial was conducted in breastfeeding women at the same stage of lactation to determine whether the dietary dose of cooked egg influences ovalbumin content in human milk and whether cooked versus raw egg ingestion alters ovalbumin content in human milk. A secondary aim was to determine

whether maternal atopy status and usual dietary egg intake influenced the ovalbumin content in human milk.

4.2 Subjects and Methods

4.2.1 Participants

Breastfeeding mothers of term (gestational age at birth >37 weeks) singleton, newborn infants were approached on the postnatal ward at Women's and Children's Hospital (WCH) prior to discharge. Women with known egg allergy, or with any other food allergies (eg wheat) which were contra-indicated by the clinic day menu were excluded. The trial was explained to the mother and verbal consent obtained to telephone her at eight weeks post-partum. At eight weeks if the women were continuing to exclusively breastfeed they were invited to participate in the trial and information sheets (see Appendix 4 for a copy of the information sheet used in this study) were then mailed to eligible and interested families.

4.2.2 Study design and treatments

Written informed consent (see Appendix 5 for a copy of the consent form used in this study) was obtained prior to an appointment at ten weeks post-partum according to the protocol approved by the Human Research Ethics Committee, WCH. At this appointment, mothers were instructed how to follow an egg-free diet, sociodemographic information (age, parity, smoking habits and education level) was collected and a diet history taken to estimate usual egg intake.

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

The test breakfasts were identical (blueberry muffin and banana milkshake) with the exception of the egg content. Prior to commencement of the study, I developed recipes (see Chapter 2) to prepare test breakfasts indistinguishable in taste and appearance to aid blinding. A standard muffin contains 1/6 of an egg, but for my trial I required each muffin to contain either ½ an egg or one whole egg. The test breakfasts were: egg-free muffin and egg-free milkshake, muffin containing half a cooked egg (27.5g) and egg-free milkshake, muffin containing one cooked egg (55g) and egg-free milkshake or egg-free muffin and milkshake containing one raw egg (55g). All milkshakes contained fresh banana and banana flavouring to mask the raw egg. All muffins were cooked at 200°C for 20 minutes.

4.2.3 Randomisation and blinding

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

4.2.4 Breast milk analyses

Ovalbumin concentration in human milk

The breast milk samples were centrifuged (Sigma Laboratory Centrifuge 4K15, Osterode am Harz, Germany) at 2000g for 20min at 4°C on the day of collection. The aqueous supernatant was frozen at -20°C until analysis using the sandwich enzyme-linked immunosorbent assay (ELISA) method as described in Chapter 2. The assay limit of detection for breast milk ovalbumin was 0.1ng/mL. The mean intra-assay CV for the quality control samples was 7.3% and the inter-assay CV was 12.1%.

Total protein content

To reduce the influence of variation in total volume of breast milk produced by each woman over the eight hours of breast milk sample collection, the total protein content of the aqueous supernatant of each breast milk sample was analysed using the Bio-Rad protein assay (Bio Rad Laboratories, Hercules, CA, USA) based on the Bradford dye-binding procedure (76). Bovine serum albumin was used as the protein standard. The ovalbumin concentration (ng/mL) of each breast milk sample was then expressed relative to the total protein concentration (mg/mL) of that sample to give the ovalbumin per protein content (ng ovalbumin/mg protein).

4.2.5 Maternal atopy status

Atopy status was assessed by an allergy questionnaire (77) (see Appendix 6) at ten weeks post-partum and by conducting skin prick testing to six allergens with a control and histamine challenge. An atopic person was defined as one whom had at least one atopic disorder (asthma, eczema or hay fever) identified from the allergy questionnaire and a positive skin prick test to at least one common environmental allergen (78). A positive skin prick test was defined as a mean of two perpendicular weal diameters of 3mm or greater in size than the mean weal of the negative control site at 15 minutes. I performed all the skin prick testing after being trained by an experienced allergy clinic nurse. The allergens tested were whole egg (AUST R32582) (1:20 w/v), standardised grass pollen perennial ryegrass (strength 10 000 BAU/mL), Alternaria Tenuis (AUST R32618) (1:10 w/v), standardised cat hair

(strength 10 000 BAU/mL), standardised mite (Dermatophagoides farinae) (strength 30 000 AU/mL) (AUST R32429), standardised mite (Dermatophagoides pteronyssinus) (strength 30 000 AU/mL) (AUST R32421), negative control containing 50% (v/v) glycerin as per all six allergens above and Histamine (10mg/mL) (by Stallergenes, Antony, France). All allergens (except the histamine) and negative control were supplied by Hollistier-Stier, Spokane, Washington, USA.

4.2.6 Sample size and statistical methods

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

The breast milk ovalbumin concentration (ng/mL) of each woman in response to each test breakfast was plotted against time. The resulting curve was used to determine the peak ovalbumin concentration (ng/mL) and total ovalbumin excretion (ng/mL/h) by calculating the area under the curve as described by Matthews (79).

This process was also repeated after the breast milk ovalbumin concentration had been corrected for the total protein content of each sample.

The effect of different test breakfasts on breast milk peak ovalbumin concentration, total ovalbumin excretion, peak ovalbumin per protein content and total ovalbumin excretion per protein content were assessed by Wilcoxon Signed Ranks Tests (as the data were not normally distributed). Pearson Chi-Square tests were used to determine the association between the frequency of ovalbumin detection after ingesting the different test breakfasts and maternal atopy status. Spearman's Rank Order Correlations were used to determine whether breast milk peak ovalbumin or total ovalbumin excretion were correlated with usual daily egg intake. All statistical computations were completed with SPSS for WINDOWS version 10.0 (SPSS Inc, Chicago). Statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Participation

A total of 585 women (see Figure 4.1) were screened for eligibility to participate in the trial between September 2001 and August 2002. 92 women did not meet the inclusion criteria (the majority had ceased breastfeeding by ten weeks post-partum), 16 women could not be re-contacted after the initial screening and 428 women declined to participate. The main reasons were because the mother was too busy with other children to accommodate the study (n=141), four visits to the hospital were too

many (n=63), too far to travel back to the hospital (n=58) and the eight hour clinic days were too long (n=51).

Forty nine women agreed to participate with 41 women successfully completing all aspects of the trial. Eight women withdrew from the study, six women were unable to attend the clinic days due to family illnesses and two women withdrew after having difficulty with the breast milk expression on their first clinic day.

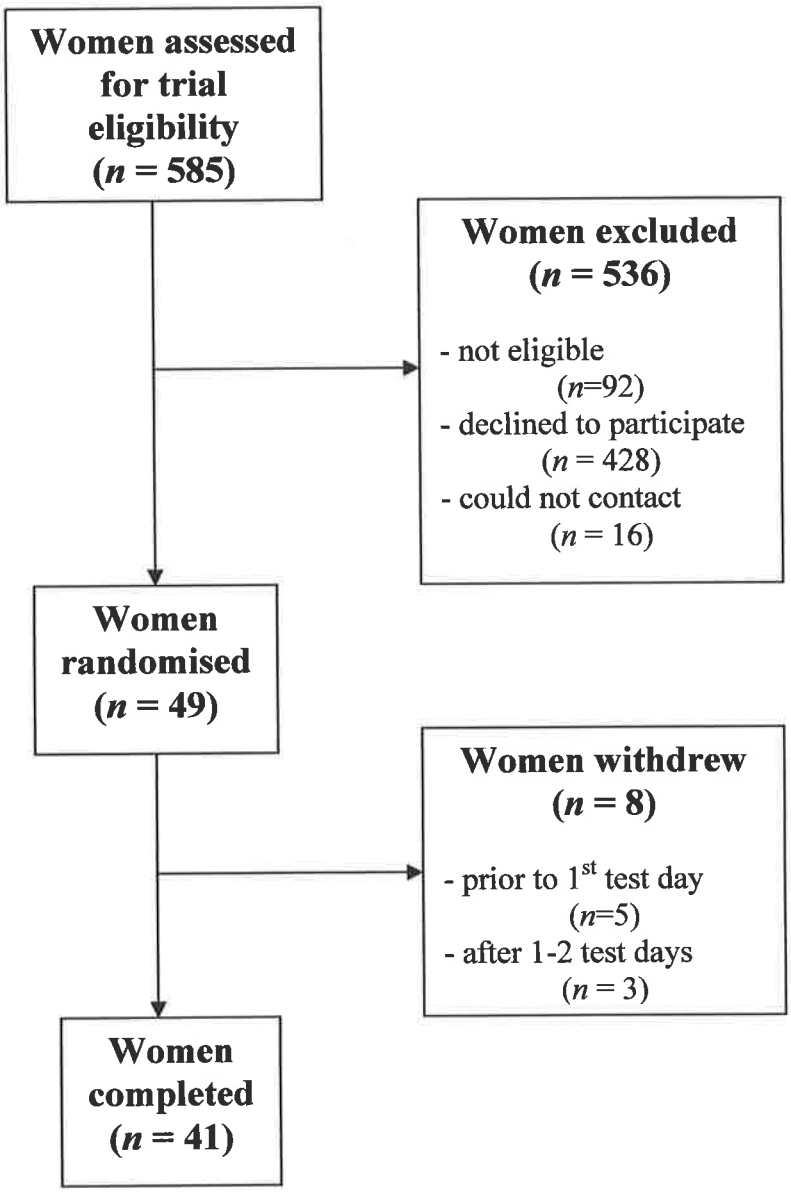


Figure 4.1: Trial participation

4.3.2 Maternal characteristics, dietary compliance and maternal atopy status

Mean age of the women who completed the trial was 30.6 years and 83% had completed secondary education (see Table 4.1). Most women (80%) were compliant with the egg-free diet prior to each test day. Only one woman consumed two scrambled eggs for breakfast 48 hours prior to one of the test days. In all other cases of non-compliance, small quantities of egg were consumed as an ingredient in mayonnaise, ice cream, cake, biscuits and confectionery.

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

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

Table 4.1: Characteristics of the women completing the trial

Variable	***n* = 41**
Age (y)	30.6 ± 5.7 [1]
Parity (no.)	1.5 ± 0.8 [1]
Completed secondary education	34 (83%) [2]
Smoking	2 (5%) [2]
Usual daily egg intake (eggs/day)	0.47 ± 0.36 [1]
Atopic women [3]	18 (44%) [2]

[1] Mean ± SD

[2] *n* (%)

[3] history of at least one allergic disease plus a positive skin prick test to at least one allergen

4.3.3 Ovalbumin in human milk

Ovalbumin was detected in the breast milk of 3/41 (7%) women after consuming the no egg breakfast. Ovalbumin detection frequency increased to 19/41 (46%) after ingesting the ½ cooked egg breakfast and further increased to 28/41 (68%) after consuming the one cooked egg breakfast (see Table 4.2). Ten women (24%) had no ovalbumin detected in their breast milk on all four clinic days.

Combined plots of all 41 women for ovalbumin concentration in human milk as a function of time post test breakfast are illustrated in Figure 4.2. A direct, dose response was demonstrated between the amount of cooked egg ingested and the peak ovalbumin concentration (no egg mean 0.05ng/mL [95%CI, 0.01-0.11], ½ cooked egg mean 2.24ng/mL [95%CI, 0.57-3.91], one cooked egg mean 3.16ng/mL [95%CI, 1.41-4.91], $P<0.05$) and between the amount of cooked egg ingested and the total ovalbumin excretion (no egg mean 0.18ng/mL/h [95%CI, 0.04-0.39], ½ cooked egg mean 4.93ng/mL/h [95%CI, 1.40-8.46], one cooked egg mean 9.14ng/mL/h [95%CI, 4.25-14.03], $P<0.05$) (Table 2). The peak concentration and total ovalbumin excretion in response to one raw egg did not differ from the ½ cooked egg breakfast. Correcting for total protein content of each breast milk sample did not alter the pattern of results in response to each of the test breakfasts (see Table 4.2).

Table 4.2: Ovalbumin detection in human milk after four different dietary interventions

Ovalbumin Detection	No egg (n = 41)	1 raw egg (n = 41)	½ cooked egg (n = 41)	1 cooked egg (n = 41)
Frequency [1]	3 (7%) [a]	22 (54%) [b]	19 (46%) [b]	28 (68%) [c]
Peak concentration [2]				
ng/mL	0.05 [0.00] (-0.01-0.11) [a]	1.08 [0.28] (0.43-1.74) [b]	2.24 [0.00] (0.57-3.91) [b]	3.16 [1.18] (1.41-4.91) [c]
ng/mg protein	0.01 [0.00] (0.00-0.02) [a]	0.14 [0.03] (0.06-0.22) [b]	0.29 [0.00] (0.08-0.51) [b]	0.40 [0.16] (0.18-0.61) [c]
Total excretion [2]				
ng/mL/h	0.18 [0.00] (-0.04-0.39) [a]	2.38 [0.41] (0.90-3.85) [b]	4.93 [0.00] (1.40-8.46) [b]	9.14 [3.46] (4.25-14.03) [c]
ng/mg protein/h	0.03 [0.00] (-0.01-0.06) [a]	0.30 [0.05] (0.12-0.49) [b]	0.64 [0.00] (0.19-1.10) [b]	1.16 [0.39] (0.54-1.78) [c]

[1] [n (%)], [a,b,c] values with different superscripts indicate significant differences (P<0.05) by Pearson Chi-Square Test

[2] mean [median] (95% CI), [a,b,c] values with different superscripts indicate significant differences (P<0.05) by Wilcoxon Signed Ranks Test

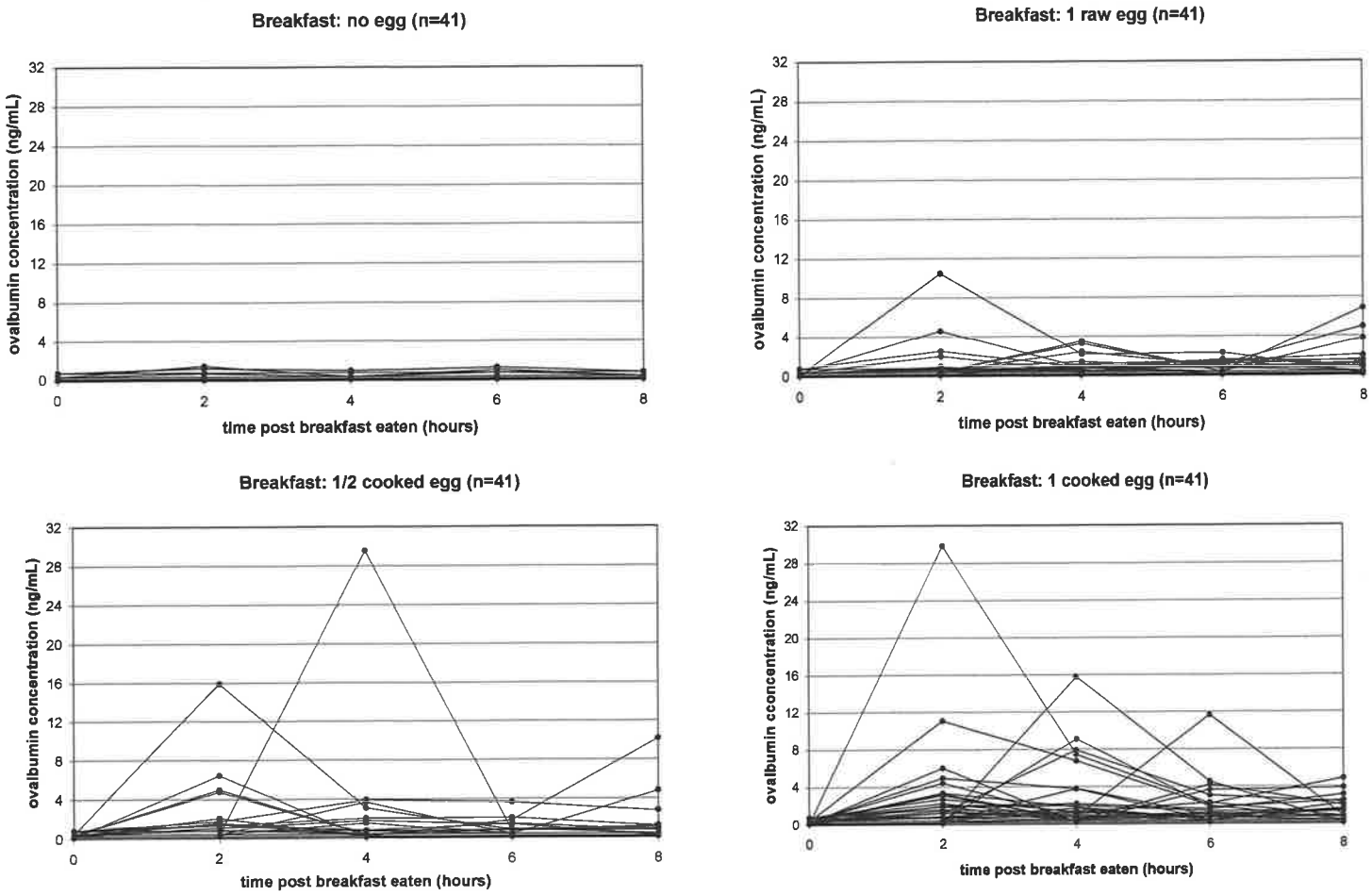


Figure 4.2: Ovalbumin excretion in human milk as a function of time post test breakfast.

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

4.4 Discussion

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

The consistency observed in response to increasing concentrations of cooked egg in the diet is particularly noteworthy. The increasing quantity of cooked egg in the challenge breakfasts resulted in increasing proportions of women with detectable

ovalbumin in their breast milk, an increasing peak ovalbumin concentration as well as increasing total ovalbumin excretion. These data clearly suggest that the quantity of cooked egg in the diet is directly related to the amount of egg protein that appears in breast milk. These observations were made in healthy women with no known metabolic or gastrointestinal illness and no known egg allergy, indicating that a proportion of egg protein finds its way into human milk despite the processes of digestion. Whether the ovalbumin detected remains in its native form could not be ascertained from this trial, but previous studies involving raw egg challenges have demonstrated that the ovalbumin detected in human milk matches native ovalbumin (21). Perhaps the more clinically relevant question is whether the ovalbumin detected in breast milk retains its allergenicity. Matsumura et al (6) described a series of case reports involving breastfed infants with eczema, where the infant's eczema resolved after elimination of eggs in the maternal diet and returned on maternal dietary egg challenge. However the maternal egg challenges in these cases varied in type of egg containing food (mayonnaise, hard boiled egg); there was no standard dose of egg eaten for the maternal challenge nor was breast milk ovalbumin measured.

Previous studies that measured ovalbumin concentration in human milk have only done so in response to a raw egg challenge (21, 22). Our results, using an ELISA detection method, are comparable with these previous studies that used a radioimmunoassay detection method (21, 22). 22/41 (54%) of women in this trial had ovalbumin detected in their milk after one raw egg compared with 13/22 (59%) and 14/19 (74%) in earlier reports (21, 22), and ovalbumin peak concentrations of those women with detectable ovalbumin after raw egg ingestion ranged from 0.17 to

10.48ng/mL in this trial compared with 0.20 to 6.17ng/mL (21, 22). Despite this similarity, I observed a consistently greater response of ovalbumin in human milk after one cooked egg compared with one raw egg. Anecdotal evidence suggests that raw egg ingestion may be more allergenic than cooked egg and this can be supported by case reports of egg-allergic children (80) who tolerated one cooked (hard boiled) egg on dietary challenge but later adversely reacted to ingestion of raw egg of unknown quantity. In my trial I focused on measuring the concentration of ovalbumin in human milk after the extremes of raw versus well cooked egg and this trial was not designed to assess human milk ovalbumin allergenicity. In a recent study Evenepoel et al (81) determined that human small intestine digestion and absorption of cooked egg was 91% compared with 51% for raw egg, suggesting that a greater proportion of egg protein from cooked eggs enters the mother's circulation, and this may explain the greater concentration of ovalbumin in human milk in response to cooked rather than raw egg. Another influence on the results in my trial may have been the higher total fat content of the one raw egg test breakfast (32g fat, as detailed in Table 2.5) slowing the rate of maternal absorption and decreasing the ovalbumin in human milk detection within the eight hour period compared to the one cooked egg test breakfast (13g fat).

The majority of previous studies which analysed breast milk samples after maternal ingestion of a specific challenge dose of food protein found the peak appearance in breast milk occurred between one and four hours after ingestion of the challenge (21, 22, 32, 34). Only one study (27) showed the peak appearance between four and 24 hours (median of eight hours). Thus in this study, I collected breast milk samples at two hourly intervals up to eight hours post-dietary challenge. Interestingly 24% of

the women had no ovalbumin detected in their breast milk on any of the four clinic days, suggesting that some women either have a delayed excretion or may not excrete ovalbumin in their breast milk, at least when challenged with one whole egg. Further studies with breast milk collections over a longer period of time (24-48 hours) are needed to determine whether dietary restrictions in up to one quarter of women may be unwarranted. However the practicalities of such a study would probably limit both participation and compliance.

A high proportion (44%) of the women who participated in this trial were atopic. I suspect that their history of allergic disease may have motivated involvement in this time consuming study for mothers of young infants. Despite this apparent selectivity towards atopic women, there was no relationship between ovalbumin detection in human milk and maternal atopy status. Family history of atopy is regarded to be the strongest predictor for allergy development in infants (53). Previous studies were also unable to find any evidence that maternal atopy status influences the passage of food proteins into human milk (23, 27, 34).

The total dietary avoidance of a food protein can be difficult to achieve, may be nutritionally compromising, time consuming and socially restrictive, and many mothers question the need for total versus partial avoidance of a food protein. This study has provided important information about the frequencies and concentrations of ovalbumin in human milk after the ingestion of raw versus cooked egg and after an increasing dose of cooked egg consumption. As none of the infants in this study had known egg allergy, further studies need to examine whether the results from this study are reproducible in a group of lactating women whose infants have an egg

allergy. To improve the quality of dietary advice to breastfeeding mothers, future studies also need to determine the threshold of ovalbumin excretion in human milk that leads to symptoms in egg allergic breastfed infants.

Chapter 5: Ovalbumin and total IgA levels in human milk fed to infants with egg sensitivity: a randomised, double-blinded, controlled trial.

5.1 Introduction

Expert nutritional committees (AAP, ESPACI, ESPGHAN) (4, 5) have recommended maternal dietary restriction to treat breastfed infants with a food allergy. However as detailed in Chapter 1, the majority of published evidence in favour of this recommendation comes from descriptions of case series (2, 6-9). There have only been three relevant randomised controlled trials (10-12) which have produced conflicting results. Thus the evidence on the use of maternal dietary restriction to treat food allergy in breastfed infants is of limited strength.

The premise that maternal dietary restrictions during breastfeeding may benefit the infant assumes that ingested food proteins are absorbed and excreted antigenically intact into breast milk. My first study (Chapter 4) determined that the median total ovalbumin excretion into breast milk of mothers with healthy infants was 3.5ng/ml/hr after the consumption of one well cooked egg. One could postulate that the presence of allergy symptoms in infants would correlate with high levels of food proteins detected in their mother's breast milk.

Four case-control studies (2, 22, 26, 42) have detected the presence of food proteins (ovalbumin or bovine *B*-lactoglobulin) in human milk fed to infants with and without allergy symptoms. Only Axelsson and co-workers (26) found the presence of allergy symptoms (diarrhoea, vomiting, colic and eczema) in the infants to be significantly correlated with high levels of *B*-lactoglobulin (>50 ng/ml) detected in their mother's breast milk. It is important to note that these four studies (2, 22, 26, 42) do not appear to have adequately matched their control mothers of infants without allergy symptoms for maternal characteristics like maternal age or stage of lactation.

Three case series (2, 8, 25) have reported improved allergy symptoms in breastfed infants and demonstrated reduced breast milk detectable *B*-lactoglobulin levels following the commencement of a maternal cow's milk-free diet. Unfortunately these studies (2, 8, 25) had small sample sizes ($n \leq 13$) and the maternal dietary interventions were non-randomised, uncontrolled and non-blinded.

Randomised trials are required which are adequately powered to detect differences in human milk food protein concentrations from women whose breastfed infants have allergy symptoms compared to those from matched control women whose infants do not have allergy symptoms. Large, blinded, randomised controlled trials are also needed to detect differences in human milk food protein concentrations fed to infants with food allergy symptoms following maternal dietary restriction and maternal dietary challenge.

My primary aim in this study was to determine the effect of eating cooked egg on the ovalbumin concentration in breast milk fed to infants with egg sensitivity. The

secondary aims were to determine the effect of maternal egg ingestion on infant eczema severity and breast milk total IgA levels.

5.2 Subjects and Methods

5.2.1 Eligibility

Mother-infant pairs were eligible for the trial if infants were singleton, breastfed, had egg sensitivity as indicated by a positive skin prick test to egg and symptoms of moderate to severe eczema (objective standardised scoring system for atopic dermatitis (SCORAD) score ≥15) (82). The infants did not need to be exclusively breastfed. As the test muffins given to the mothers in this study contained wheat and soy, women with Coeliac Disease or food allergies to wheat or soy were excluded, as were infants with soy or wheat sensitivity as indicated by a positive skin prick test to wheat or soy.

5.2.2 Screening of participants

Initial recruitment for this trial was undertaken by placing newspaper advertisements, interested women telephoned me or my office and trial information sheets (see Appendix 7 for a copy of the information sheet used in this study) were then mailed to potentially eligible and interested families. A receptionist at a specialist paediatric allergy clinic also asked potentially eligible families for permission to be contacted about the trial when they were telephoning this clinic in order to be placed on the

clinic's waiting list for an appointment. I telephoned those families providing permission to be contacted and again if they were interested in participating trial information sheets were then mailed out to them. Written informed consent (see Appendix 8 for a copy of the consent form used in this study) was obtained prior to participation in a screening process for the trial according to the protocol approved by the Human Research Ethics Committee, Women's and Children's Hospital.

Due to the strict inclusion criteria, recruitment involved an initial voluntary screening appointment for each infant to be skin prick tested and to score the clinical severity of the infant's eczema using a SCORAD assessment (82) (see Appendix 9 for a copy of the SCORAD assessment sheet used). The SCORAD (82) is a widely used and accepted method of quantifying the extent and severity of an infant's eczema. It relies on a clinical examination of the infant with the extent of eczema determined as one would estimate the extent of burn injury: the so-called rule of nines. An average representative eczema lesion is then scored for erythema, oedema, excoriation, and crusting and the general skin scored for thickening and dryness. Together these assessments form the objective SCORAD score. The parents are also asked to rate their child's sleep disturbance and itchiness on a visual analogue scale to form a subjective symptoms score. The total SCORAD score was determined by a mathematical formula that combines both objective SCORAD and parental subjective symptoms scores. In order to reduce inter-observer bias I performed all the SCORAD assessments during this initial screening process.

An experienced nurse preformed skin prick testing on the back of each infant using six food allergens with negative and positive (histamine) controls. A positive skin

test was defined as a mean weal diameter greater than 3mm compared to the weal of the negative control site at 15 minutes. The general approach as recommended by Burks (83) in a recent review paper was followed, which is to screen children with atopic dermatitis to egg, cow's milk, peanut, soy and wheat. The allergens tested in this study were egg white (1:20 w/v), whole cow's milk (1:20 w/v), peanut (1:10 w/v), cashew nut (1:10 w/v), soybean (1:10 w/v), and wheat (1:10 w/v), negative control containing 50% (v/v) glycerin as per all six allergens above and histamine (10mg/ml) (by Stallergenes, Antony, France). All allergens (except histamine) and negative control were supplied by Hollistier-Stier (Spokane, WA, USA).

Oral egg challenges were not performed to confirm egg allergy in these infants, only egg sensitivity from positive skin prick testing. This is because all the infants were under two years of age and in agreement with an American expert committee (5), the introduction of egg containing solids to the infant diet is not recommended until 2 years of age in infants at risk of allergy.

5.2.3 Study design and dietary interventions

This was a randomised, double-blind, controlled trial that assessed the effect of maternal ingestion of cooked egg on human milk ovalbumin concentrations and infant eczema severity over a three week period. Breast milk total IgA concentrations were also measured.

Demographic information was collected about the mothers (age, parity, education level, usual daily egg intake) and their infants (age, breastfeeding frequency, solids and any formula dietary intakes).

An initial home visit appointment was arranged (Day 0), at which dietary advice was provided on following an egg-free diet. Commencing the next day (Day 1), these dietary restrictions applied to both the mother and her infant and were for the duration of the trial. No infants or their mothers were on dietary restrictions prior to commencement in this trial.

To replicate the study design of my previous study (Chapter 4), the mothers followed an egg-free diet for two days (Days 1-2) prior to commencing the dietary intervention. From the third day (Day 3), each mother consumed one muffin per day for 21 days (Days 3-23). The muffins used in this trial were egg-free muffins (control group) or muffins containing one (55g) whole cooked egg (egg group). Thus all the infants had egg-free solids and the only egg ingested by the mothers was well controlled in the form of test muffins provided.

For some infants and their mothers, depending on their food skin prick testing results, additional dietary restrictions (cow's milk, peanut and cashew nut) were required. I provided individualised dietetic support and the nutritional adequacy of both the maternal and infant diets were monitored.

The only modification to the recipes for the muffins used in this trial compared with those developed (see Chapter 2) and successfully used in the previous trial (see

Chapter 4) was the substitution of soy milk instead of cow's milk. This was necessary because some of the infants in this trial were skin prick test positive to cow's milk.

The adherence to the egg-free diet was assessed by detailed dietary food intake records for both the mother and her infant for three days on three occasions (Days 1-3, 10-12, 21-23).

5.2.4 Randomisation and blinding

An independent consultant produced a computer generated randomisation schedule for this trial. A supervisor not involved in the day to day management of the trial and who had no contact with participants then used this randomisation schedule to allocate either egg-free muffins (control group) or muffins containing one (55g) whole egg (egg group) to an unique study number. At trial entry each woman was given a three week supply of frozen muffins that corresponded with her assigned study number.

The muffins were identical in appearance to enable the mothers and researchers to be blinded to the egg content of the allocated muffins. As I undertook the day to day management of the trial and also analysed the breast milk samples, I remained blinded to the muffin allocation of each participant until all laboratory analyses were completed.

5.2.5 Eczema assessments

SCORAD assessments were performed on each infant at the commencement (Day 0) and completion (Day 24) of the trial. I performed the majority of the SCORAD assessments, with a back-up research nurse only required on two occasions during the whole trial. On Days 0, 12 and 23, the mother completed an Infants' Dermatitis Quality of Life Index (IDQOL) (84) to assess the impact on quality of life caused by the infant's eczema. This questionnaire was designed to be completed by parents of children less than 4 years of age and includes questions on amount of itching and scratching, sleep disturbance and interference of eczema with daily living tasks over the preceding week (see Appendix 10 for a copy of the IDQOL questionnaire used) (84).

Details of topical steroid cream usage were recorded at Day 0. As this study was investigating the dietary treatment of atopic eczema/dermatitis syndrome (AEDS), no medical consultation was given as part of this trial. However a letter was sent to the infant's general practitioner about the study and included guidelines for the management of eczema should an acute flare up occur. At Day 24 each mother was asked whether her infant had required any additional eczema treatment over the preceding week.

5.2.6 Ovalbumin concentration in human milk

On Days 3, 12 and 23 the mother manually expressed 5mL of breast milk into sterile containers, prior to and at two, four and six hours after eating the test muffin for

breakfast. The mothers continued to breastfeed their infants as per their usual feeding routine. The breast milk samples were stored in the home freezer until I collected them during the second home visit on Day 24. The collected frozen breast milk samples were thawed, centrifuged (Sigma Laboratory Centrifuge 4K15, Osterode am Harz, Germany) at 2000g for 20min at 4°C, and the aqueous supernatant frozen at -20°C until analysis of the breast milk ovalbumin concentration using the sandwich enzyme-linked immunosorbent assay (ELISA) method as described in Chapter 2. The assay limit of detection for breast milk ovalbumin was 0.1ng/mL. The mean intra-assay CV for the quality control samples was 6.2% and the inter-assay CV was 10.6%.

As correcting for the total protein content of each breast milk sample did not alter the pattern of results for ovalbumin concentration in human milk in Chapter 4, the measurement of the total protein content of the aqueous supernatant of each breast milk sample was not undertaken in this trial.

5.2.7 Total IgA in human milk

Pooled samples collected from each woman on the first (Day 3, n=30) and last (Day 23, n=30) breast milk collection days were analysed for total IgA content using a human IgA ELISA quantification kit (Bethyl Laboratories Inc, Montgomery, TX, USA). The assay limit of detection for total IgA was 7.8ng/mL. These samples had previously been centrifuged as described above and the aqueous supernatant frozen at -20°C until analysis. Pooled breast milk samples were used for this analysis as no

significant measurable human milk IgA concentration differences have been shown for breast milk collected between left and right breasts on the same day, or in the course of a single breastfeeding or a 24 hour period (62).

5.2.8 Post-trial telephone contact

A Paediatric Allergist (who was not involved in the day to day management of the trial) telephoned each participating mother after the end of the three week study to notify them as to which muffins they had eaten and provide his recommendations regarding the need for any continued maternal dietary restriction. The unblinding of the mothers at this stage was considered to be ethically responsible to assist in the ongoing care for their infants with eczema and egg sensitivity.

One month after completing the trial, a research nurse telephoned each mother to ask about her current egg consumption pattern and to complete a fourth IDQOL questionnaire for her infant.

5.2.9 Sample size and statistical methods

In my previous study ovalbumin was detected in breast milk samples from 3/41 (7%) women who ate egg-free muffins and 28/41 (68%) women who ate muffins containing one cooked egg. Based on the assumption of 90% power, an alpha-value of 0.01 and using the previous study results, the sample size estimate for this trial was calculated to be 13 women per group to enable a detectable difference between

the two groups for the primary outcome of determining the effect of eating cooked egg on the ovalbumin concentration in breast milk fed to infants with egg sensitivity.

The breast milk ovalbumin concentration (ng/mL) of each woman on breast milk collection Days 3, 12 and 23 in response to test muffin consumption was plotted against time. The resulting curve was used to determine the peak ovalbumin concentration (ng/mL) and total ovalbumin excretion (ng/mL/h) by calculating the area under the curve as described by Matthews (79).

Independent Samples T-tests or Mann-Whitney U Tests (when the data were not normally distributed) were used to investigate any statistical differences between the egg and control groups. For categorical data, the Pearson Chi-Square Test examined any statistical differences between the maternal diet groups. The effect of consuming muffins containing egg (egg group) versus egg-free (control group) muffins on breast milk peak ovalbumin concentration and total ovalbumin excretion were assessed by Mann-Whitney U Tests (as the data were not normally distributed) and Pearson Chi-Square tests were used to determine the association between the frequency of ovalbumin detection.

As the total IgA levels in breast milk were not normally distributed, results between the maternal diet groups were compared using the Mann-Whitney U Test. Spearman's Rank Order Correlations were used to determine whether breast milk total IgA concentrations were correlated with breast milk ovalbumin concentrations and infant age.

A mixed between-within subjects analysis of variance (ANOVA) was performed to assess the combined effect of time and maternal diet group on infant objective and total SCORAD scores at Day 0 and Day 24, and IDQOL at Days 0, 12, 23 and post-trial. The relationship between observed eczema (objective SCORAD) and parents reporting of quality of life due to eczema symptoms (IDQOL) was investigated using Pearson product-moment correlation coefficient. Spearman's Rank Order Correlation was used to determine whether objective SCORAD results were correlated with breast milk total ovalbumin excretion. All statistical computations were completed with SPSS for WINDOWS version 11.0 (SPSS Inc, Chicago, USA). Statistical significance was set at $P < 0.05$.

5.3 Results

5.3.1 Participation

A total of 75 breastfed infants with eczema were screened for inclusion (60 answered newspaper advertisements and 15 from a paediatric allergist's waiting list) to participate in the trial between November 2003 and March 2004 (see Figure 5.1). Skin prick testing revealed 44 (59%) infants had sensitivity to at least one food protein, with 37 (49%) positive to egg, 22 (29%) positive to cow's milk and 20 (27%) positive to peanut. 43 infants did not meet the inclusion criteria, 38 had negative skin prick testing to egg and 5 had positive skin prick testing to wheat as well as egg. All infants with a positive skin prick test to egg also had an objective SCORAD score ≥15. Thus 32 mothers and their infants were eligible to participate and all agreed to do so, with 30 women successfully completing all aspects of the trial. Two women (in the egg group) withdrew because they ceased breastfeeding.

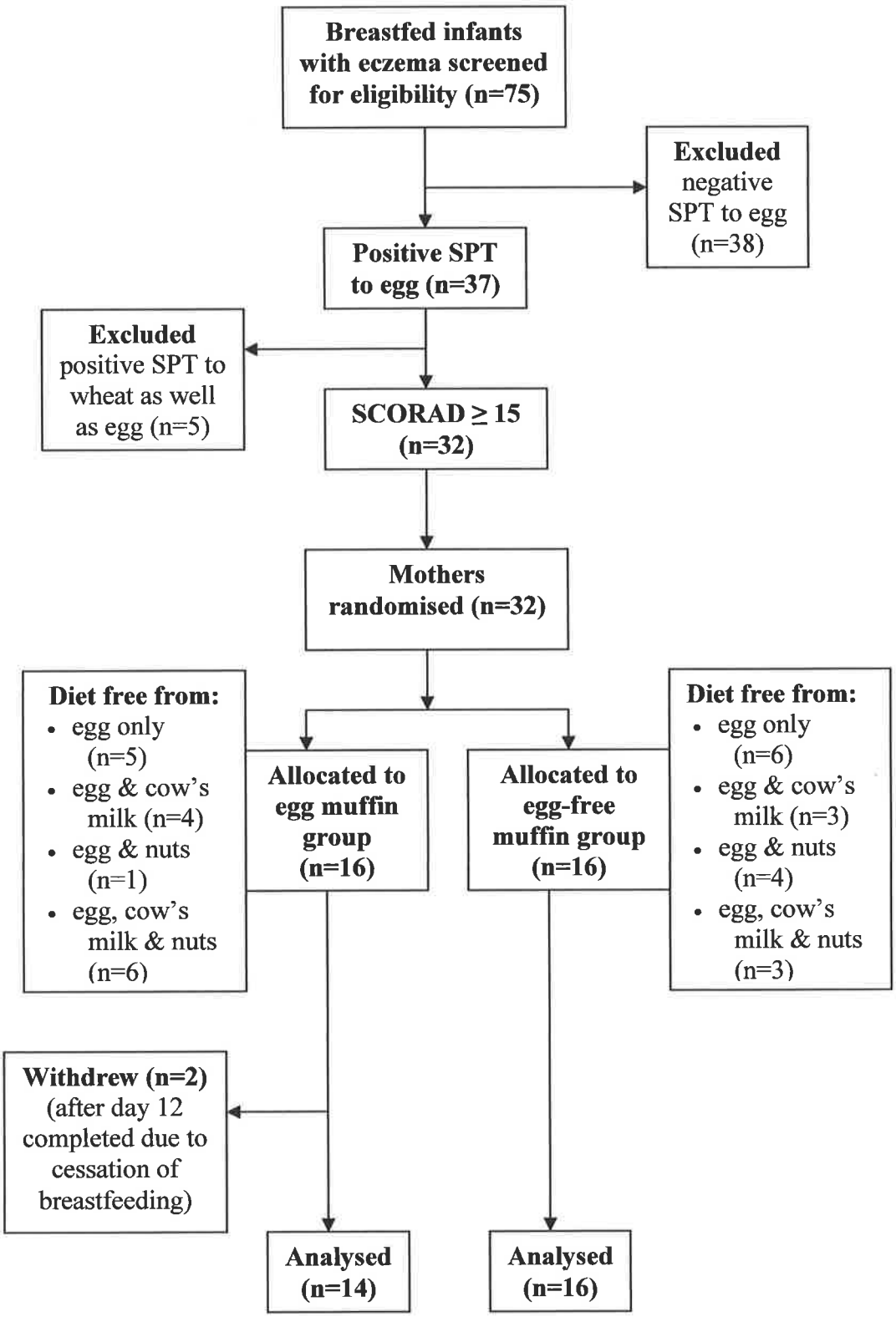


Figure 5.1: Trial flow diagram

5.3.2 Maternal and infant characteristics

The women in the egg group (n=16) were not different to the women in the control group (n=16) with regard to age, education level, parity, breastfeeding frequency and usual daily egg intake (see Table 5.1). The infants in the two groups were also not statistically different for age, sex, skin prick test results and potency of topical steroid cream used (see Table 5.1).

5.3.3 Dietary restrictions and compliance

As a result of positive skin prick testing, 16 mother-infant pairs avoided cow's milk protein, 13 avoided peanut and eight avoided cashew nut in addition to egg (see Figure 5.1). Food records kept for nine of the 23 days during the trial revealed that no woman ate obvious sources of egg, however five women (three in egg group) on one to three occasions each, ate egg hidden in foods such as biscuits, pasta, mayonnaise and puddings. To my knowledge no infant consumed any egg protein (obvious or hidden) during the study period.

Table 5.1: Maternal and infant characteristics

	egg group (*n*=16)	control group (*n*=16)	*P*=
Maternal variable			
Age (years)	32 ± 3 [1]	32 ± 4 [1]	0.71 [a]
Completed secondary education	13 (81%) [2]	14 (88%) [2]	1.00 [b]
Parity (no.)	2.0 (1.3-2.3) [3]	1.0 (1.2-1.7) [3]	0.27 [c]
Usual daily egg intake (eggs/day)	0.6 ± 0.2 [1]	0.6 ± 0.4 [1]	0.68 [a]
Breastfeeding frequency (feeds/day)	5.0 (4.4-7.1) [3]	5.0 (4.3-5.7) [3]	0.52 [c]
Infant variable			
Age (months)	7.1 ± 1.8 [1]	7.0 ± 2.5 [1]	0.94 [a]
Sex (male)	9 (56%) [2]	6 (38%) [2]	0.48 [b]
Egg SPT weal (mm)	6.0 (4.4-8.3) [3]	6.0 (4.7-7.6) [3]	0.91 [c]
Egg only +ve SPT	5 (31%) [2]	6 (38%) [2]	1.00 [b]
Egg + cow's milk only +ve SPT	4 (25%) [2]	3 (19%) [2]	1.00 [b]
Egg + nut only +ve SPT	1 (6%) [2]	4 (25%) [2]	0.33 [b]
Egg + cow's milk + nut +ve SPT	6 (38%) [2]	3 (19%) [2]	0.43 [b]
Mild potency topical steroid cream used	12 (75%) [2]	12 (75%) [2]	1.00 [b]
Moderate potency topical steroid cream used	4 (25%) [2]	4 (25%) [2]	1.00 [b]

[1] mean ± SD [2] *n* (%) [3] median (95% CI)
+ve: positive SPT: skin prick test
[a] Independent Samples T-test [b] Pearson Chi-Square Test
[c] Mann-Whitney U Test

5.3.4 Ovalbumin in human milk

More women in the egg group had ovalbumin detected in their breast milk within six hours of eating the test muffin than women in the control group (see Table 5.2). Four women (25%) in the egg group had no ovalbumin detected in their breast milk up to six hours after eating the egg muffin on all three breast milk collection days. Figure 5.2 illustrates the time course of ovalbumin excretion for each woman in the egg and control groups. The data are separately plotted for each maternal group on each breast milk collection day (3, 12 and 23). The women in the egg group demonstrated a greater response (Mann-Whitney U Test, $P<0.05$) for both the peak ovalbumin concentration and total ovalbumin excretion on days 3, 12 and 23 than the control group (see Table 5.2). The frequency of ovalbumin detection, peak ovalbumin concentration and total ovalbumin excretion results were consistent within each group at days 3, 12 and 23 (see Table 5.2).

Table 5.2: Ovalbumin detection in human milk

Ovalbumin detection	egg group	control group
Frequency [1] (total)	12/16 (75%)[a]	1/16 (6%)[b]
Day 3	10/16 (63%)[a]	1/16 (6%)[b]
Day 12	10/16 (63%)[a]	1/16 (6%)[b]
Day 23	8/14 (57%)[a]	1/16 (6%)[b]
Peak concentration [2](ng/mL)		
Day 3	1.67 [0.63] (0.01-3.32)[c]	0.20 [0.00] (-0.23-0.63)[d]
Day 12	1.93 [0.81] (0.04-3.81)[c]	0.18 [0.00] (-0.20-0.56)[d]
Day 23	1.36 [0.89] (0.38-2.35)[c]	0.22 [0.00] (-0.25-0.68)[d]
Total excretion [2] (ng/mL/h)		
Day 3	4.28 [1.11] (0.29-8.26)[c]	0.55 [0.00] (-0.62-1.72)[d]
Day 12	3.96 [1.72] (0.15-7.77)[c]	0.48 [0.00] (-0.54-1.50)[d]
Day 23	3.45 [2.19] (1.23-5.66)[c]	0.63 [0.00] (-0.71-1.98)[d]

[1] *n* (%), [a,b] values with different superscripts indicate significant differences ($P<0.05$) by Pearson Chi-Square Test.

[2] mean [median] (95% CI), [c,d] values with different superscripts indicate significant differences ($P<0.05$) by Mann-Whitney U Test.

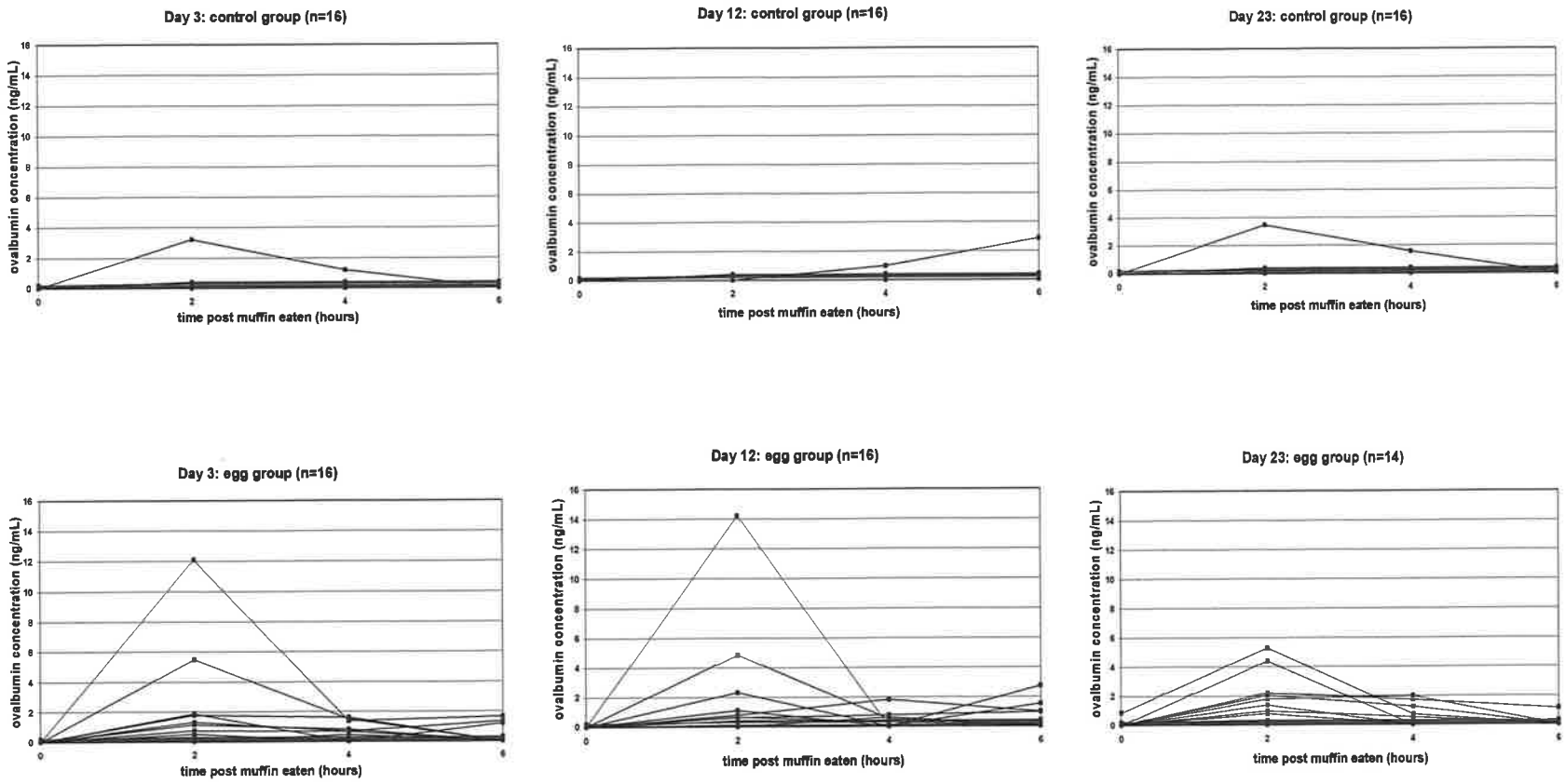


Figure 5.2: Ovalbumin excretion in human milk as a function of time post test muffin consumption.

5.3.5 Total IgA in human milk

No significant differences were found between breast milk total IgA levels measured from the women in the egg and control groups on Days 3 and 23 (see Table 5.3). There were no relationships (Spearman rank order correlations, $P>0.05$) between breast milk total IgA concentrations and ovalbumin peak or total excretion concentrations on Days 3 or 23. A strong positive correlation (Spearman's rho correlation coefficient, $r = 0.84$, $P<0.01$) was found between total IgA concentrations on Days 3 and 23 (see Figure 5.3). The outlier points on the graph illustrated in Figure 5.3 are attributed to six women who all had infants that were older than eight months of age. These women were not different to the other 24 participating women for characteristics including maternal age, parity, infant food protein sensitivity or breast milk ovalbumin concentrations.

Table 5.3: Total IgA detection in human milk

	egg group (*n*=14)	**control group (*n*=16)**
Total IgA [1] (mg/ml)		
Day 3	0.476 [0.310] (0.256-0.697) [a]	0.281 [0.236] (0.167-0.396) [a]
Day 23	0.592 [0.280] (0.191-0.994) [b]	0.374 [0.243] (0.138-0.611) [b]

[1] mean [median] (95% CI)
[a] no significant difference between egg and control groups on Day 3 (P=0.09) by Mann-Whitney U Test
[b] no significant difference between egg and control groups on Day 23 (P=0.27) by Mann-Whitney U Test

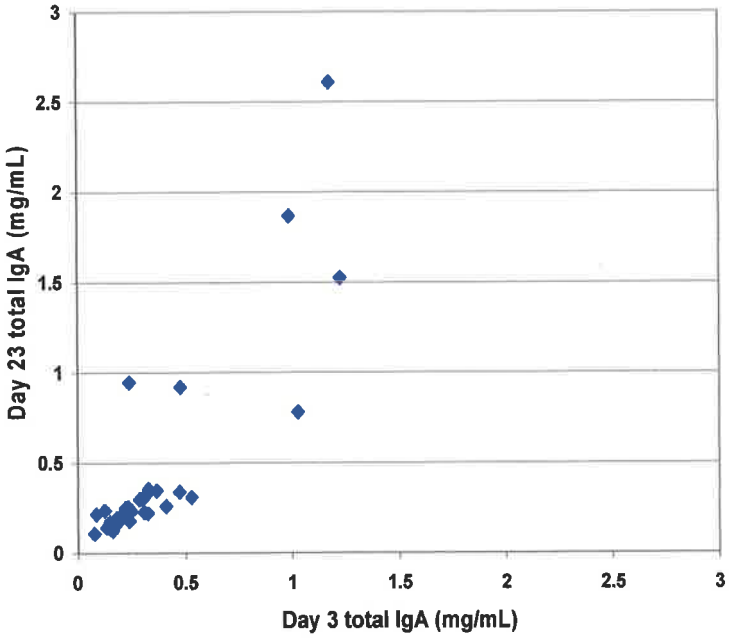


Figure 5.3: Comparison of total IgA concentrations on Days 3 and 23.

5.3.6 Eczema assessments

The mixed between-within subjects analysis of variance determined a significant reduction on mean infant objective SCORAD (Wilks' Lambda=0.23, $p<0.001$), total SCORAD (Wilks' Lambda=0.26, $p<0.001$) and IDQOL questionnaire (Wilks' Lambda=0.55, $p<0.001$) scores with time for both groups, but there was no statistical difference between the groups (see Table 5.4). Although not significantly different (P =0.14, Pearson Chi-Square Test) a greater proportion of infants in the egg group (43%) continued to have symptoms of moderate eczema with an objective SCORAD score $\geq$ 15 at Day 24 compared to those in the control group (13%) (see Table 5.4).

A positive correlation (Spearman's rho correlation coefficient, r = 0.44, n = 30, $P<0.05$) was found between breast milk total ovalbumin excretion and infant eczema severity (objective SCORAD score) at the completion of the trial.

There were strong correlations both at Day 0 (Pearson product-moment correlation coefficient, r=0.41, $P<0.05$) and at Day 24 (Pearson product-moment correlation coefficient, r=0.71, $P<0.05$) between the infants' observed eczema (objective SCORAD) and parental reporting of quality of life due to eczema symptoms (IDQOL), with increased observed eczema decreasing the infant's quality of life.

Table 5.4: Eczema assessment results for infants who completed the trial

Eczema assessment method and day	egg group (*n*=14)	control group (*n*=16)
Objective SCORAD		
day 0 [1]	21.9 ± 6.5 [a]	21.4 ± 4.8 [a]
day 24 [1]	11.1 ± 7.3 [b]	9.5 ± 5.5 [b]
day 0 score ≥ 15 [2]	14 (100%)	16 (100%)
day 24 score ≥ 15 [2]	6 (43%)	2 (13%)
Total SCORAD		
day 0 [1]	34.3 ± 9.9 [a]	32.0 ± 6.4 [a]
day 24 [1]	17.6 ± 12.1 [b]	14.3 ± 7.1 [b]
IDQOL		
day 0 [1]	10.9 ± 5.3 [a]	8.4 ± 3.5 [a]
day 12 [1]	7.6 ± 3.8 [b]	6.4 ± 4.2 [b]
day 23 [1]	5.9 ± 4.9 [b]	5.1 ± 3.4 [b]
post-trial [1]	5.1 ± 3.7 [b]	3.5 ± 2.9 [b]

[1] mean ± SD

[2] *n* (%), no significant difference, by Pearson Chi-Square Test

[a,b] values with different superscripts indicate significant differences ($P<0.001$) by mixed between-within subjects ANOVA

Objective SCORAD: dietary group, NS; dietary group x time, NS; time, $P<0.001$.
Total SCORAD: dietary group, NS; dietary group x time, NS; time, $P<0.001$.
IDQOL: dietary group, NS; dietary group x time, NS; time, $P<0.001$.

At Day 0 the groups were the same for their regular eczema treatment usage of topical steroid creams (see Table 5.1). The mothers of only five infants (three in egg group) reported an additional frequency of topical steroid cream usage on their infants at Day 24 compared to Day 0. All five of these infants had moderate eczema with an objective SCORAD score ≥ 15 at Day 24, suggesting that the observed eczema improvement in the majority of infants was not due to increased topical steroid cream usage.

No significant relationships were found between infant objective SCORAD results and breast milk total IgA concentrations on Day 3 (Pearson correlation coefficient, r = 0.33, P=0.07) and Day 23 (Pearson correlation coefficient, r =0.36, P=0.05).

5.3.7 Post-trial telephone questionnaire

All 30 women who completed the trial were successfully followed up by telephone one month after the end of the trial to determine their current egg consumption pattern and to complete a fourth IDQOL questionnaire. A further three women (two in egg group) had ceased breastfeeding. Fifteen women (six in egg group) who continued to breastfeed also continued to follow a strict maternal egg-free diet. Ten women (five in egg group) had re-introduced small amounts of egg containing foods into their diet up to four times per week and only two (one in egg group) of the

breastfeeding mothers had re-commenced eating eggs and all egg containing foods. All 30 infants continued on a strict egg-free diet.

A mixed between-within subjects analysis of variance determined that the post-trial IDQOL questionnaire scores were significantly reduced from those on Day 0 (Wilks' Lambda=0.38, $p<0.001$) for both groups, but there was no statistical difference between the groups (see Table 5.4). No difference could be determined between the Day 23 and post-trial IDQOL questionnaire scores for both groups. There was also no difference in the post-trial IDQOL questionnaire mean scores for breastfed infants whose mothers continued on a strict egg-free diet (3.9 ± 3.1, n=15, six in egg group) and those who had re-introduced maternal egg consumption (4.0 ± 3.4, n=12, six in egg group).

5.4 Discussion

My study is the first randomised, double-blinded, controlled trial to demonstrate a consistent difference between human milk ovalbumin concentrations fed to infants with egg sensitivity after daily maternal ingestion of one cooked egg compared with maternal dietary egg avoidance. My results, which consistently demonstrate comparable detection and excretion of ovalbumin across time, suggest that ovalbumin does not accumulate despite the women in the egg group consuming 55g of cooked egg per day for 21 days. These results provide crucial background knowledge relevant to the use of maternal dietary restriction to treat breastfed infants with food allergy.

The detection of ovalbumin in human milk appears to be of the same order of magnitude between breastfeeding mothers of infants with egg sensitivity compared to those infants without known egg sensitivity. In this trial the ovalbumin in human milk detection frequency of 12/16 (75%) women, within six hours of eating one cooked egg is in accordance with the only other previous study investigating human milk fed to infants with egg sensitivity (22) where ovalbumin was detected in the breast milk of 8/11 (72%) women, within six hours of consumption of one raw egg and with my previous study involving infants without known egg sensitivity (Chapter 4) where ovalbumin was detected in the breast milk of 28/41 (68%) women, after ingestion of the same muffins containing 55g of cooked egg.

This trial investigated only dietary rather than medical intervention to treat atopic eczema/dermatitis in breastfed infants. The overall eczema symptom improvement in both groups of infants may be largely explained by dietary avoidance of egg (plus cow's milk, peanut and/or cashew nut) in the infants' solid foods, thus adding to existing evidence for the benefit of dietary intervention in the treatment of atopic eczema/dermatitis (85, 86). However the lack of significant difference in mean eczema symptom scores at Day 24 between the groups indicates that despite the women in the egg group consuming one well cooked egg per day for 21 days, as a group the infants' eczema symptoms still improved. Thus the small quantities of breast milk ovalbumin detected may not have been sufficient to trigger the infants' eczema symptoms. This may suggest that maternal dietary avoidance of egg may not be necessary for breastfed infants with egg sensitivity. These results are consistent with the only other published randomised controlled trial investigating the effect of

maternal dietary restriction on infant atopic eczema in 17 mothers and their breastfed infants (12). In this previous trial (12), individual infants varied in their response to maternal avoidance of cow's milk and egg, however overall mean eczema scores were not significantly different. However both my trial and that of Cant et al (12) were small and the results should be verified in a larger double-blind, randomised, controlled trial adequately powered for primary clinical outcomes. If confirmed this finding would lead to changes in current clinical dietary practice regarding the use of maternal dietary restriction to treat breastfed infants with food allergy.

Interestingly examination of the proportion of infants who still had moderate eczema (objective SCORAD score $\geq$ 15) at Day 24 revealed three times the number in the maternal egg versus control group. One explanation for this result which did not reach statistical significance may relate to the positive correlation found between breast milk total ovalbumin content and infant eczema severity at the completion of the trial. This would suggest that those women who excrete higher concentrations of ovalbumin in their breast milk may be the ones who require maternal dietary egg restriction to benefit their infants. However as this correlation was weak ($r=0.44$, $P<0.05$) it should be further investigated in a larger trial adequately powered for primary clinical outcomes to be able to clearly determine whether some breastfed infants with egg sensitivity may require maternal dietary egg restriction to reduce their eczema severity. Another possible explanation for this result is the direct exposure of infants to egg protein via contaminated hands (4) as the mothers in the egg group were consuming and therefore handling egg containing muffins.

All the women and their infants who were eligible for this trial agreed to participate. I suspect that a search for a treatment for their infant's moderate to severe eczema would have motivated their involvement. The convenience of all the participation in my study (other than the initial screening appointment) occurring in their own home, the limited three week study duration and no blood collection were also commented on by the participating women as benefits of the study design.

Eighty percent of the infants screened for this study were recruited via newspaper advertisements, thus representing the greater population of infants with atopic eczema/dermatitis than only those referred to specialist dermatological or allergy clinics, which has been a criticism of previous studies in this field (85). IgE-mediated food allergy has been estimated to be associated with atopic eczema/dermatitis in 33-65% of infants depending on the population studied (87-90). Interestingly in this study we found IgE-sensitisation to at least one food protein in 59% of those infants screened. Again as in other studies (87-89), the most common foods to be sensitised to were egg, cow's milk and peanut.

I acknowledge that oral egg challenges were not performed with the infants in this study to confirm egg allergy. Food allergy diagnosis requires the determination of a causal relationship between the improvement of symptoms after commencing a strict avoidance diet for a suspected food protein followed by the return of these symptoms on food challenge (4). However the egg skin prick test mean weal size of both groups of infants was >6mm and it has been shown that a weal ≥5mm is predicative of a positive egg challenge for children under two years of age (91, 92).

As the presence of food proteins in human milk appears to be a common phenomenon in infants with and without food protein sensitivity, this is probably not the only factor leading to the possible sensitisation and continuation of symptoms in breastfed infants with food allergy. Previous studies have demonstrated specific IgA levels to food proteins (B-lactoglobulin and ovalbumin) are not correlated to the food protein concentrations in breast milk (23, 29, 30) and in my study no relationship was found between breast milk total IgA concentrations and ovalbumin peak or total excretion concentrations. However in my trial the infants ranged from 4 to 14 (mean=7) months of age and mean total IgA levels were lower than those previously measured in human milk fed to healthy infants, which are reported to decrease from 2.1mg/mL at 2-3 days to 0.5 mg/mL by 12 weeks of lactation, and then slightly increase to 0.6 mg/mL at 24 weeks, 0.9 mg/mL at 36 weeks and 1.0 mg/mL at 52 weeks (60). Median total IgA levels were $\leq$0.31 mg/mL, which is of similar magnitude to that found previously for breast milk fed to infants with cow's milk allergy, where infant symptom scores were particularly associated with total IgA levels of <0.25 mg/mL (30, 63). Thus consistent with other researchers (30, 63), my study appears to have found lower total IgA concentrations in human milk fed to infants with allergic symptoms. This finding supports the hypothesis that lower concentrations of IgA antibodies may result in less binding of food antigens to form immune complexes, thus increasing the chances of these food antigens crossing the intestinal surface in a breastfed infant. However it is important to caution comparison of the results between these studies (30, 60, 63) as each study has used a different detection assay for the measurement of human milk total IgA concentrations and unfortunately the human IgA ELISA quantification kit used in my trial has not been validated to measure IgA specifically in human milk samples.

5.5 Summary and conclusion

My trial has determined that the presence of ovalbumin in human milk fed to infants with known egg sensitivity, within six hours of maternal cooked egg ingestion is a common phenomenon. After maternal ingestion of the same egg challenge dose, the frequency of detection, peak concentration and total excretion of ovalbumin in breast milk were consistent when lactating women were measured on three separate occasions over a three week period. No relationship was found between breast milk ovalbumin content and total IgA levels, however human milk fed to infants with egg sensitivity and atopic eczema/dermatitis appears to have lower total IgA levels than those previously published for healthy infants.

The marked improvement in eczema symptoms observed in the majority of infants regardless of maternal dietary intervention would advocate the use of dietary avoidance for the infant's diet. Within the limits of this trial the findings would suggest that maternal dietary avoidance of egg may not be necessary for all breastfed infants with egg sensitivity. Until further adequately powered trials for clinical outcomes are conducted each clinical case should be treated on an individual basis. However these results suggest that maternal dietary avoidance should only be used for those breastfed infants whose moderate to severe eczema persists after an initial period of infant solids dietary restriction.

Chapter 6: General discussion

My survey to benchmark current dietetic practice revealed that the majority of Australian specialist paediatric allergy dietitians recommend the use of maternal dietary restriction to treat food allergy in breastfed infants and egg is one of the most commonly restricted foods.

My research adds evidence concerning the effect of maternal egg ingestion on ovalbumin concentration in human milk and is the first to specifically investigate maternal cooked egg consumption. The results from the two randomised controlled trials demonstrated that ovalbumin can be consistently detected in the breast milk of women after the consumption of one well cooked egg. Both studies involved muffins indistinguishable in taste and appearance to ensure adequate blinding. A strength of my research is that this form of cooked egg is more representative of that commonly consumed. The avoidance of cooked egg containing foods like cake, muffins and biscuits can make women feel socially restricted and the need to prepare or purchase such foods in an egg-free form can be expensive and time consuming for breastfeeding mothers.

In both trials one quarter of the women studied had no ovalbumin detected in their breast milk up to six to eight hours after egg consumption on any of the breast milk collection days. This suggests that some women either have a delayed excretion or may not excrete ovalbumin in their breast milk, at least when challenged with one whole egg. Even in the second study one quarter of the women in the egg group still had no ovalbumin detected in any of their breast milk samples after consuming one

cooked egg per day for three weeks. Future research may be able to examine the detection of multiple food proteins in human milk from the same group of women after maternal food challenge to determine whether some women who appear to be "non-excretors" of one food protein (for example egg) may also be "non-excretors" of other food proteins (for example cow's milk, peanut). Additionally it would be useful to determine whether those women who had higher than average concentrations of ovalbumin detected would have higher than average concentrations of other food proteins detectable in their breast milk too. The weak but positive correlation found between breast milk ovalbumin concentration and infant eczema severity at the completion of my second trial, may point towards the concept that those women who excrete higher concentrations of ovalbumin in their breast milk may be the ones who need maternal dietary avoidance to benefit their infants and the identification of any predictors to determine which particular women may have the predisposition to transport higher concentrations of food proteins into their breast milk may enable more targeted maternal dietary advice.

Many Australian specialist paediatric allergy dietitians surveyed would advise complete rather than partial avoidance of the casual food protein from the diet of the lactating mother to treat food allergy in breastfed infants. In my first trial the amount of ovalbumin in human milk was found to be dose dependent and in the second trial no accumulation effect on the presence of ovalbumin in human milk was found after daily egg intake. Thus one could argue that the intake of small amounts of egg found in foods like biscuits, pancakes, glazed baked foods, crumbed foods, pasta, ice cream and lollies would result in minimal if any breast milk ovalbumin content and thus

should not be restricted in the diet of breastfeeding mothers with egg allergic infants and therefore reduce the burden of such diets on lactating women.

Kilburn et al (20) proposed that exposure to low dose food allergens via breast milk may produce infant oral tolerance to these foods rather than sensitisation/symptom development. This concept has been explored in a recent rat study by Korotkova and colleges (93) who examined the influence of different maternal diet omega-6 (n-6) to omega-3 (n-3) long chain polyunsaturated fatty acid ratios on the induction of neonatal oral tolerance to ovalbumin in rats. This study (93) found lower hypersensitivity reactions and antibody responses in neonatal rats against ovalbumin indicating the induction of oral tolerance with maternal n-3 long chain polyunsaturated fatty acid and ovalbumin dietary exposure. Korotkova and colleges (93) concluded that the quality of maternal dietary fatty acid intake may effect the development of infant immunological tolerance to food proteins ingested by the mother.

In agreement with other researchers (30, 63), lower total IgA levels in human milk fed to infants with allergy symptoms were demonstrated in the second study of my thesis. Further investigation into the role of IgA, other immune factors and the fatty acid composition within human milk was beyond the focus of this thesis, but is relevant to the whole picture regarding the prevention and treatment of food allergy in breastfed infants and therefore should be included in future research projects.

My aim was to focus on the question of whether maternal dietary restrictions are necessary for lactating women whose breastfed infants have allergy symptoms rather

than an examination of immune mechanisms that may play a role in infant food allergy responses. An investigation of the measurement of ovalbumin in infant serum after ingestion of human milk following maternal egg challenge was initially considered. However it was decided that minimal infant intervention including not taking infant blood samples as part of my research would maximise maternal participation in both time consuming trials in order to achieve the primary aim of investigating the effect of maternal egg ingestion on ovalbumin concentration in human milk.

It is well known that breastfeeding provides health, social, economical and environmental benefits to infants, their mothers and society as a whole (94). Thus the current recommendation by the World Health Organisation to attempt to exclusively breastfeed all infants for the first six months of life should be encouraged. For those breastfed infants with food allergy symptoms there is no clear evidence that maternal dietary restriction will benefit the infant and thus should not be recommended to the point that the mother finds the dietary restriction too difficult to endure and ceases breastfeeding as a result.

It is also important to consider that human milk may not be the only source of exposure to food proteins even for exclusively breastfed infants. Other possible routes of exposure may include contaminated hands (4), inhaled food proteins (4), contact with inflamed skin (95) and the presence of food proteins in house dust samples (96, 97). In a study by Witteman and co-workers (97) ovomucoid (egg protein) was detected at quantities of 170-6300ng/g dust and *B*-lactoglobulin (cow's milk protein) was detected at quantities of 16-71ng/g dust in house dust samples.

These researchers suggest that the ovomucoid levels detected were probably high enough to cause sensitisation and/or symptoms in infants. Thus the possible environmental exposure of breastfed infants to food proteins may be a confounding factor for maternal dietary avoidance studies and would be worth considering in future studies designed to investigate the treatment and prevention of food allergy.

As outlined in this discussion further research is required in many related topics surrounding the presence of food proteins in human milk and the use of maternal dietary restriction for the treatment of food allergy in breastfed infants. The next logical step would be to conduct a larger double-blind, randomised, controlled trial with primary clinical outcomes, involving around 150 mother-infant pairs, to investigate whether breastfed infants with egg sensitivity benefit from maternal dietary egg restriction to reduce their eczema severity. Until such a trial is performed, it is difficult to make broad clinical practice guidelines and practitioners will continue to treat each clinical case on an individual basis. However my results suggest that maternal dietary avoidance should only be used for those breastfed infants whose moderate to severe eczema persists after an initial period of infant solids dietary restriction.

Appendices

Appendix 1: Australian National Health and Medical Research Council (NHMRC) levels of evidence criteria (41)

Designation of levels of evidence

I evidence obtained from a systematic review of all relevant randomised controlled trials.

II evidence obtained from at least one properly designed randomised controlled trial.

III-1 evidence obtained from well-designed pseudo-randomised controlled trials (alternate allocation or some other method).

III-2 evidence obtained from comparative studies with concurrent controls and allocation not randomised (cohort studies), case-control studies, or interrupted time series with a control group.

III-3 evidence obtained from comparative studies with historical control, two or more single-arm studies, or interrupted time series without a parallel control group.

IV evidence obtained from case series, either post-test or pre-test and post-test.

Appendix 2: Survey on current Australian Dietetic practice: Maternal dietary restrictions to treat food allergy in breastfed infants

1. If a breastfed infant is suspected of having a food allergy, do you ever advise the breastfeeding mother to undergo any maternal dietary restriction?
   - ☐ Yes *(please continue with questions 2, 3, 4 & 5)*
   - ☐ No *(please see directly below & question 5)*

   If no, is this because of the following reasons? *(Please tick all that apply)*
   - ☐ do not wish to nutritionally compromise the breastfeeding woman
   - ☐ restriction of food proteins in solids or supplemental formula is all that is needed to improve the infant's symptoms
   - ☐ not enough evidence exists that food proteins ingested by breastfeeding women pass into their breast milk to adversely affect the infant
   - ☐ food proteins in breast milk induce tolerance to these foods in infants
   - ☐ other *(please specify)*

     _____

2. If yes, in what circumstances would you advise maternal dietary restriction? *(Please tick all that apply)*
   - ☐ always
   - ☐ if the infant has severe symptoms
   - ☐ if the infant is still exclusively breastfed
   - ☐ if symptoms commenced while the infant was exclusively breastfed
   - ☐ if initial dietary restriction of solids and/or supplemental formula did not improve symptoms
   - ☐ other *(please specify)*

     _____

3. If yes, which foods do you find you usually advise to avoid? *(Please tick all that apply)*
   - ☐ cow's milk
   - ☐ egg
   - ☐ peanut
   - ☐ tree nuts
   - ☐ soy
   - ☐ fish
   - ☐ shellfish
   - ☐ sesame seeds
   - ☐ other seeds
   - ☐ wheat
   - ☐ other *(please specify)*

     _____

4. If yes, do you advise partial or complete avoidance of these food proteins (for example egg in biscuits allowed but not scrambled eggs)?
   - ☐ partial avoidance
   - ☐ complete avoidance

Any other comments? _____

Appendix 3: Survey on current Australian Dietetic practice: Maternal dietary restrictions to prevent food allergy in high-risk breastfed infants.

1. Do you ever advise maternal dietary restrictions in breastfeeding women with a family history of allergy in order to prevent food allergies in their infant?
   - ☐ Yes *(please continue with questions 2, 3, 4, & 5)*
   - ☐ No *(please see directly below & question 5)*

   If no, is this because of the following reasons? *(Please tick all that apply)*
   - ☐ do not wish to nutritionally comprise the breastfeeding woman
   - ☐ restriction of food proteins in solids or supplemental formula only ever
   - ☐ needed to prevent food allergy in infants
   - ☐ not enough evidence exists that food proteins ingested by breastfeeding
   - ☐ women pass into their breast milk to adversely affect the infant
   - ☐ food proteins in breast milk induce tolerance to these foods in infants
   - ☐ other *(please specify)*

     _____

2. If yes, in what circumstances would you advise maternal dietary restriction? *(Please tick all that apply)*
   - ☐ always
   - ☐ if both parents have a history of allergy
   - ☐ if the infant's has a sibling with food allergies
   - ☐ if a sibling had food allergy symptoms while being breastfed
   - ☐ if the mother specifically asks to do so
   - ☐ other *(please specify)*

   _____

3. If yes, which foods do you find you usually advise to avoid? *(Please tick all that apply)*
   - ☐ cow's milk
   - ☐ egg
   - ☐ peanut
   - ☐ tree nuts
   - ☐ soy
   - ☐ fish
   - ☐ shellfish
   - ☐ sesame seeds
   - ☐ other seeds
   - ☐ wheat
   - ☐ other *(please specify)*

   _____

4. If yes, do you advise partial or complete avoidance of these food proteins (for example egg in biscuits allowed but not scrambled eggs)?
   - ☐ partial avoidance
   - ☐ complete avoidance

5. Any other comments? _____

   _____

Appendix 4: Information sheet used in study 1 (Chapter 4)

Women's and Children's Hospital

Does the amount of egg eaten effect the amount of egg protein in breast milk?

Information Sheet

Why is this study being done?

Breastfeeding mothers who have babies with an egg allergy or at high risk of developing an egg allergy are usually advised to go on an egg free diet. An egg free diet can be difficult because egg is an ingredient in many foods we cook and buy. The aim of our study is to work out how much egg mothers can eat before egg components appear in their breast milk.

What does this study involve?

We plan to study how much and how quickly egg protein may appear in breast milk after egg is eaten.

To do this you will be given 4 breakfasts on different days containing different amounts of egg. The 4 breakfasts will either include an egg free muffin, an egg containing muffin, 1 whole boiled egg or 2 whole boiled eggs. You will not know the amount of egg in each breakfast until the study day. The order you receive the breakfasts will not be known to you nor will the order be your choice.

For the remainder of the 8 hours whilst participating in the study day, egg-free food and drinks will be supplied to you to prevent any further ingestion of egg.

You will be asked to provide us with breast milk samples at regular intervals after eating the breakfasts, which we will later test for the amount of egg protein in these samples.

What will happen during the study?

This study involves an initial appointment and then spending one day per week for 4 consecutive weeks at 10, 11, 12 and 13 weeks of lactation at the Women's and Children's Hospital.

An appointment with the research dietitian will be made during the week prior to the first study day. You will be provided with advice on how to completely avoid eating any form of egg for 48 hours (2 days) prior to each test day. The avoidance of egg only will not affect your nutrition. You will be asked to keep a detailed dietary food intake record for these 48 hours.

At this first appointment, along with the egg free diet education, additional information about you will be collected, including your age, how many other children you have, education level, any history of allergic disease (asthma, eczema, hay fever and reactions to food) and recent immunisation history.

On the subsequent 4 appointments, you will come in after an overnight fast and be supplied with a test day breakfast. The test day breakfasts will be identical in food choice, with the exception of the egg content.

You will be provided with food while at the hospital for each of the 4 test days and be accommodated in a room at the WCH with other mothers also participating in the study.

For assessment of the egg content of your breast milk, you will be asked to provide breast milk samples of 10 mL (2 teaspoons) prior to and at 2, 4, 6 and 8 hours after eating the test day breakfast, thus 5 samples per day at the hospital.

You will receive $15 per test day for reimbursement for car parking or travel costs to the hospital.

Your Rights

You are free to withdraw from the study at any time without any explanation of why you have chosen to do so and without prejudice to you and your treatment.

All information gathered will be treated with confidence and no information that could identify you will be released to any person not associated directly with the study. These results may eventually be published in medical journals or at professional meetings, but you will not be identified in any way.

Any questions ?

If at any time during the study you have any queries or questions, please ring Debbie Palmer on

This study has been reviewed by the Research and Ethics Sub-Committee (WCH). Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study of your rights as a participant, or should you wish to make a confidential complaint, you may contact the executive secretary of this committee, Ms Brenda Penny, WCH on

Appendix 5: Consent form used in study 1 (Chapter 4)

WOMEN'S & CHILDREN'S HOSPITAL RESEARCH ETHICS COMMITTEE

CONSENT FORM

I _____

hereby consent to my involvement in the research project entitled:

'Does the amount of egg eaten effect the amount of egg protein in breast milk?'

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it, and agree to taking part.

2. I understand that I may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that while information gained in the study may be published, I will not be identified and information will be confidential.

5. I understand that I can withdraw from the study at any stage and that this will not affect medical care or any other aspects of my relationship with this hospital.

6. I understand that there will be no payment to me for taking part in this study unless specified in the Information Sheet.

7. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

8. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

9 a) I consent to following the dietary instructions provided, eating the test meals, having skin prick testing and to supply breast milk samples for use in the above project.

b) I do / do not consent to the breast milk samples being used in any other research project, provided the project has the approval of the Women's & Children's Hospital Research Ethics Committee.

10. I understand that I am free to stop donating breast milk samples at any stage, without giving any reason, and that my action of donating/not donating a sample will not affect (i) my prospects in any position; (ii) any academic prospects; or (iii) any other conceivable situation.

Signed: .....

Full name of patient: .....

Dated: .....

I certify that I have explained the study to the patient and consider that she understands what is involved.

Signed: ..... Title: .....

Dated: .....

Appendix 6: Maternal allergy questionnaire used in study 1 (Chapter 4) (77)

1. **Have you ever had wheezing or whistling in the chest at any time in the past ?**

   Yes[ ] No [ ] If you answered "**No**" please skip to **Question 6**

2. **Have you had wheezing or whistling in the chest in the last 12 months ?**

   Yes[ ] No [ ] If you answered "**No**" please skip to **Question 6**

3. **How many attacks of wheezing have you had in the last 12 months ?**

   None [ ] 1 to 3 [ ] 4 to 12 [ ] More than 12 [ ]

4. **In the last 12 months, how often, on average, has your sleep been disturbed due to wheezing ?**

   Never [ ] Less than one night per week [ ] One or more nights per week [ ]

5. **In the last 12 months, has wheezing ever been severe enough to limit your speech to only one or two words at a time between breaths ?**

   Yes[ ] No [ ]

6. **Have you ever had asthma ?**

   Yes[ ] No [ ]

7. **In the last 12 months, has your chest sounded wheezy during or after exercise ?**

   Yes[ ] No [ ]

8. **In the last 12 months, have you had a dry cough at night, apart from a cough associated with a cold or a chest infection ?**

   Yes[ ] No [ ]

9. **Have you ever had a problem with sneezing, or a runny, or a blocked nose when you DID NOT have a cold or the flu ?**

   Yes[ ] No [ ] If you answered "**No**" please skip to **Question 14**

10. **In the past 12 months, have you had a problem with sneezing, or a runny, or a blocked nose when you DID NOT have a cold or the flu ?**

    Yes [ ] No [ ] If you answered "**No**" please skip to **Question 14**

11. In the past 12 months, has this nose problem been accompanied by itchy-watery eyes ?

Yes [ ] No [ ]

12. In which of the past 12 months did this nose problem occur ? (please tick any which apply)

Jan [ ] Feb [ ] March [ ] April [ ] May [ ] June [ ]

July [ ] August [ ] Sept [ ] Oct [ ] Nov [ ] Dec [ ]

13. In the past 12 months, how much did this nose problem interfere with your daily activities ?

Not at all [ ] A little [ ] A moderate amount [ ] A lot [ ]

14. Have you ever had hay fever ?

Yes[ ] No [ ]

15. Have you ever had an itchy rash which was coming and going for at least 6 months?

Yes [ ] No [ ] If you answered "**No**" please skip to **Question 20**

16. Have you had this itchy rash at any time in the last 12 months ?

Yes [ ] No [ ] If you answered "**No**" please skip to **Question 20**

17. Has this itchy rash at any time affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes ?

Yes[ ] No [ ]

18. Has this rash cleared completely at any time during the last 12 months ?

Yes[ ] No [ ]

19. In the last 12 months, how often, on average have you been kept awake at night by this itchy rash ?

Never [ ] Less than one night per week [ ] One or more nights per week [ ]

20. Have you ever had eczema ?

Yes[ ] No [ ]

Appendix 7: Information sheet used in study 2 (Chapter 5)

Women's and Children's Hospital

Do breastfeeding mothers of babies with egg allergy need to avoid eating egg?

Information sheet

Why is this study being done?

Babies with an egg allergy need to have egg-free solids. This means not eating obvious sources of egg, like boiled egg and scrambled egg, but also involves reading ingredient labels of other common foods which may contain egg, like crumbed or battered foods, pasta, cakes, biscuits, muffins, pancakes, puddings, pavlova, ice cream, custard, mayonnaise, lollies and glazed baked goods.

When a baby is breastfed as well as eating solids, the solids should be egg-free, but there is controversy as to whether the breastfeeding mother's diet also needs to be egg-free. As complete egg avoidance in the diet is often more difficult for adults, is time consuming and can be socially restrictive, many mothers question the need for total egg avoidance in their diet.

Previous research has shown that after consuming one egg not all women will have egg detectable in their breast milk and that there maybe immune factors in breast milk that reduce the effect of any egg present in breast milk from affecting the baby. Whilst ensuring all babies have egg-free solids, the question we would like to ask is whether the breastfeeding mother's diet also needs to be egg-free. This study will investigate whether the amount of egg in breast milk and the baby's eczema is related to breastfeeding mothers eating egg when their babies have egg sensitivity. The results of this study may prevent unnecessary dietary restriction in mothers whose breastfed infants have egg sensitivity and eczema symptoms.

What does this study involve?

You and your baby will follow an egg-free diet. Each mother will be provided with either egg-free muffins or egg containing muffins and will consume one muffin per day for 3 weeks. You will not know the egg content of the muffins that you are provided with nor will the type of muffin provided be your choice.

At the beginning and at the end of this 3 week study, an assessment of your baby's eczema will be made. This involves looking at the baby's skin, it should not cause any discomfort for your baby and only takes about 10 minutes to do. We ask that during the study, you also complete a questionnaire about the impact of your baby's eczema on their sleep, bath-time, dressing, play-time and mood. This questionnaire will be done at the beginning, half way through and at the end of the study.

On 3 days at home, you will be asked to provide us with small 5mL breast milk samples at regular intervals after eating the muffin for that day. We will later test for the amount of egg protein and antibodies in these breast milk samples. The breast milk samples should be stored in your home freezer until collected by the researcher.

What will happen during the study?

An initial appointment will be made for an egg-free diet education session. Depending on clinical history and other food skin prick testing results additional dietary restrictions (for example cow's milk) may also be required. Commencing the next day, both you and your baby will follow this diet for the duration of the study. An experienced food allergy Dietitian will provide this advice and ensure that both of your diets are nutritionally adequate. We ask that a record of all food and drinks eaten by you and your baby be kept for 3 days on 3 occasions.

At the first appointment, additional information will be collected about your age, how many other children you have, education level and about your baby's age, weight and length, breastfeeding frequency and solids eaten.

The researcher will telephone you during the study to check on how you are going with the egg-free diet, muffin eating, completion of food records and baby eczema questionnaires, and collection of the breast milk samples. At each of these telephone calls the researcher will ask about any other symptoms your baby may have had over the previous week (for example loose stools, vomiting, hives, cough), any visits to the general practitioner, any additional treatment required for eczema symptoms or any medication changes.

A final appointment at home will be made for the second assessment of your baby's eczema. At this appointment the researcher will collect the completed questionnaires, food records and breast milk samples.

Should you be concerned about your baby's health at any stage during this study visit your general practitioner. A letter will be sent to your general practitioner explaining this study and will have details of who to contact should they require further information.

Your Rights

You are free to withdraw from the study at any time without any explanation of why you have chosen to do so and without prejudice to you and your treatment. All information gathered will be treated with confidence and no information that could identify you will be released to any person not associated directly with the study. These results may eventually be published in medical journals or at professional meetings, but you will not be identified in any way.

Any questions?

If at any time during the study you have queries or questions, please ring **Debbie Palmer**, Research Dietitian on **8161 7512,** or 8161 7000 (Hospital switchboard) and ask for pager 4452.

This study has been approved by the Research Ethics Committee of the Women's and Children's Hospital. Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study, your rights as a participant, or should you wish to make a confidential complaint, you may contact the Secretary of this Committee, Ms Brenda Penny, Research Secretariat, Women's and Children's Hospital on 8161 6521.

Appendix 8: Consent form used in study 2 (Chapter 5)

WOMEN'S & CHILDREN'S HOSPITAL RESEARCH ETHICS COMMITTEE

CONSENT FORM

I _____

hereby consent to my and my child's involvement in the research project entitled:

Do breastfeeding mothers of babies with egg allergy need to avoid eating egg?

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it, and agree to myself and my child taking part.

2. I understand that I and my child may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that while information gained in the study may be published, I and my child will not be identified and information will be confidential.

5. I understand that I can withdraw myself and my child from the study at any stage and that this will not affect medical care or any other aspects of my own or my child's relationship with this hospital.

6. I understand that there will be no payment to me or my child for taking part in this study.

7. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

8. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

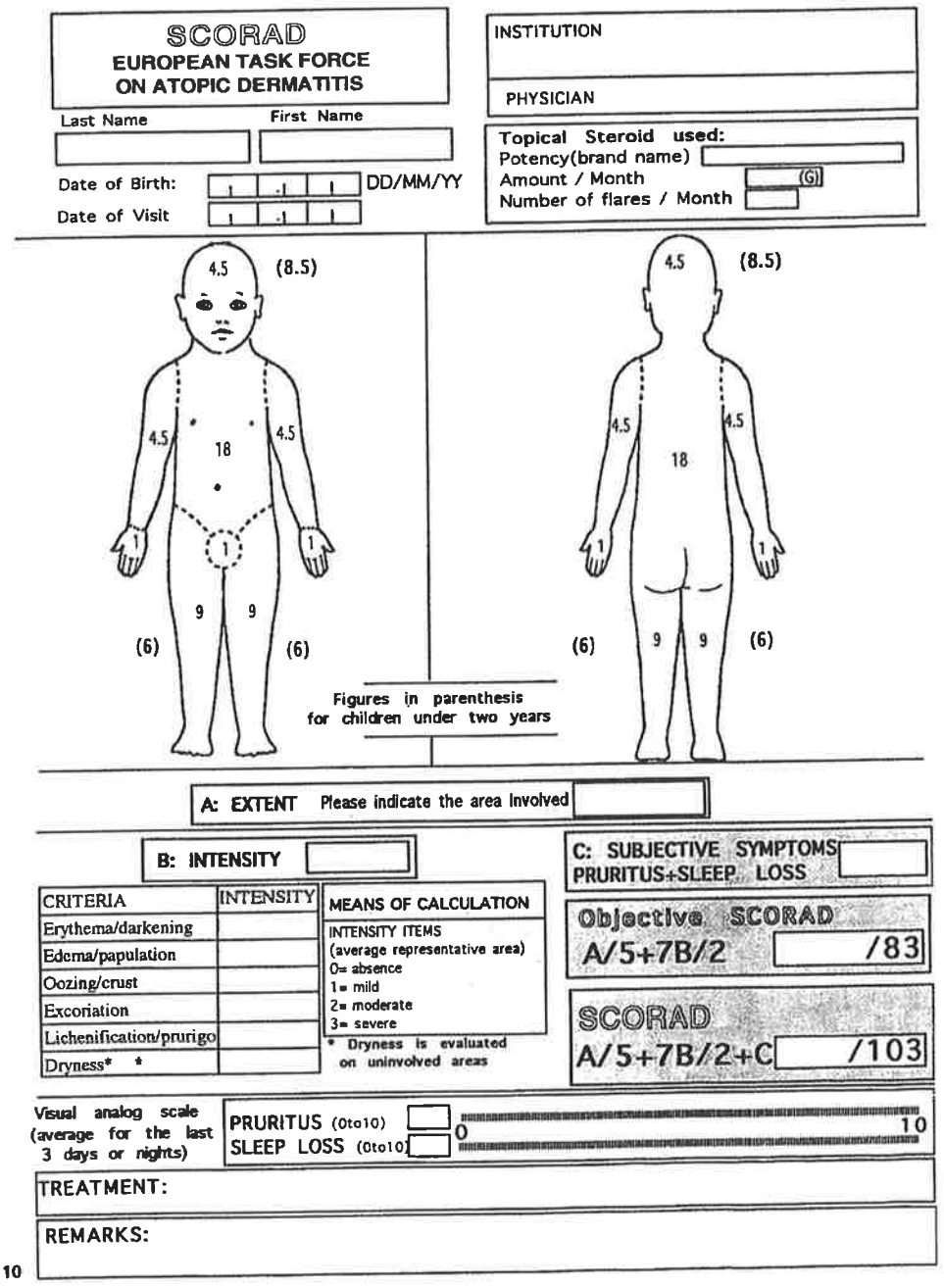
9. a) I consent to my baby's eczema being assessed, following the dietary instructions, completing the questionnaires about my baby's eczema, eating the muffins provided and to the supply of breast milk samples for use in the above project.

   b) I do / do not consent to the breast milk samples being used in any other research project, provided the project has the approval of the Women's & Children's Hospital Research Ethics Committee.

Signed: ..... Dated:.....
Full name of mother: .....
Full name of child: .....

I certify that I have explained the study to the mother and consider that she understands what is involved.
Name: ..... Signed: .....
Status in project: ..... Dated: .....

Appendix 9: SCORAD assessment sheet (82)



SCORAD
EUROPEAN TASK FORCE
ON ATOPIC DERMATITIS

INSTITUTION

PHYSICIAN

Last Name | First Name

Topical Steroid used:
Potency(brand name)
Amount / Month (G)
Number of flares / Month

Date of Birth: DD/MM/YY
Date of Visit

4.5 (8.5) 4.5 4.5 18 1 1 1 9 9 (6) (6)

4.5 (8.5) 4.5 4.5 18 1 1 1 9 9 (6) (6)

Figures in parenthesis
for children under two years

A: EXTENT Please indicate the area involved

B: INTENSITY

CRITERIA	INTENSITY
Erythema/darkening	
Edema/papulation	
Oozing/crust	
Excoriation	
Lichenification/prurigo	
Dryness* *	

MEANS OF CALCULATION
INTENSITY ITEMS
(average representative area)
0= absence
1= mild
2= moderate
3= severe
* Dryness is evaluated on uninvolved areas

C: SUBJECTIVE SYMPTOMS
PRURITUS+SLEEP LOSS

Objective SCORAD
A/5+7B/2 /83

SCORAD
A/5+7B/2+C /103

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

PRURITUS (0to10) 0 10
SLEEP LOSS (0to10)

TREATMENT:

REMARKS:

10

Appendix 10: Infants' Dermatitis Quality Of Life Index (IDQOL) (84)

Child's name: **Date:**

The aim of this chart is to record how your child's eczema (dermatitis) has been.
Each question concerns **THE LAST WEEK ONLY**. Please answer every question.

Eczema severity		
Over the last week, how severe do you think your child's eczema has been? i.e. how red, scaly, inflamed or widespread?	Extremely severe	☐
	Severe	☐
	Average	☐
	Fairly good	☐
	None	☐
Life Quality Index		
1. Over the last week, how much has your child been itching and scratching?	All the time	☐
	A lot	☐
	A little	☐
	None	☐
2. Over the last week, what has your child's mood been?	Always crying	☐
	Extremely difficult	☐
	Very fretful	☐
	Slightly fretful	☐
	Happy	☐
3. Over the last week, approximately how much time on average has it taken to get your child off to sleep each night?	More than 2 h	☐
	1-2 h	☐
	15 min to 1 h	☐
	0–15 min	☐
4. Over the last week, what was the total time that your child's sleep was disturbed on average each night?	5 h or more	☐
	3-4 h	☐
	1-2 h	☐
	Less than 1 h	☐
5. Over the last week, has your child's eczema interfered with playing or swimming?	Very much	☐
	A lot	☐
	A little	☐
	Not at all	☐
6. Over the last week, has your child's eczema interfered with your child taking part in or enjoying other family activities?	Very much	☐
	A lot	☐
	A little	☐
	Not at all	☐
7. Over the last week, have there been problems with your child at mealtimes because of the eczema?	Very much	☐
	A lot	☐
	A little	☐
	Not at all	☐
8. Over the last week, have there been problems with your child caused by the treatment?	Very much	☐
	A lot	☐
	A little	☐
	Not at all	☐
9. Over the last week, has your child's eczema meant that dressing and undressing the child has been uncomfortable?	Very much	☐
	A lot	☐
	A little	☐
	Not at all	☐
10. Over the last week, how much has your child having eczema been a problem at bath-time?	Very much	☐
	A lot	☐
	A little	☐
	Not at all	☐

Appendix 11:

Palmer DJ, Gold MS, Makrides M. Treatment and prevention of food allergies in breastfed infants: practice and evidence. Nutr Diet 2004;61:76-81.

Palmer D.J., Gold M.S. and Makrides M. (2004), Treatment and prevention of food allergies in breastfed infants: practice and evidence.
Nutrition and Dietetics, v. 61, pp. 76-81.

Appendix 12:

Palmer DJ, Gold MS, Makrides M. Effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomized, double-blind, cross-over trial. Clin Exp Allergy. 2005;35(2): 173-8.

D.J. Palmer, M.S. Gold and M. Makrides, (2005) Effect of cooked and raw egg consumption on ovalbumin content of human milk : a randomized, double-blind, cross-over trial. Clinical and Experimental Allergy, v. 35 (2) pp. 173-178.

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