RESEARCH

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Dietary aspects of cow's milk allergy in young children

ACADEMIC DISSERTATION

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Hyvä on hiihtäjän hiihdellä,
kun hanki on hohtava alla,
kun taivas kirkasna kaareutuu--
mut hauskempi hiihtää, kun ruskavi puu,
tuul' ulvovi, polku on ummessa
ja tuisku on taivahalla.

Hyvä on hiihtäjän hiihdellä,
kun ystävä häll' on myötä,
kun latu on aukaistu edessään--
mut parempi hiihdellä yksinään,
tiens' itse aukaista itselleen
ja yksin uhmata yötä.

Hyvä on hiihtäjän hiihdellä,
kun tietty on matkan määrä,
kun liesi viittovi lämpöinen,--
mut sorjempi, uljaampi hiihtää sen,
joka outoja onnen vaiheita käy
eikä tiedä, miss' oikea, väärä.

Ja hyvä on hiihtäjän hiihdellä,
kun riemu on rinnassansa,
kun toivo säihkyvi soihtuna yöss'--
mut käypä se laatuun hiihtää myös
hiki otsalla, suurissa suruissa
ja kuolema kupeellansa.

- Eino Leino -

Contents

Abstract

Cow's milk allergy (CMA) affects about 2-6% of infants and young children. Environmental factors during early life are suggested to play a role in the development of allergic diseases. One of these factors is likely to be maternal diet during pregnancy and lactation. The association between maternal diet and development of CMA in offspring is not well known, but diet could contain factors that facilitate development of tolerance. After an established food allergy, another issue is gaining tolerance towards an antigen that causes symptoms. The strictness of the elimination depends on the individual level of tolerance.

This study aimed at validating a questionnaire used to inquire about food allergies in children, at researching associations between maternal diet during pregnancy and lactation and subsequent development of cow's milk allergy in the offspring, and at evaluating the degree of adherence to a therapeutic elimination diet of children with CMA and factors associated with the adherence and age of recovery. These research questions were addressed in a prospective birth cohort born between 1997 and 2004 at the Tampere and Oulu University Hospitals. Altogether 6753 children of the Diabetes Prediction and Prevention (DIPP) Nutrition cohort were investigated.

Questionnaires regarding allergic diseases are often used in studies without validation. High-quality valid tools are therefore needed. Two validation studies were conducted here: one by comparing parentally reported food allergies with information gathered from patient records of 1122 children, and the other one by comparing parentally reported CMA with information in the reimbursement records of special infant formulae in the registers of the Social Insurance Institution for 6753 children. Both of these studies showed that the questionnaire works well and is a valid tool for measuring food allergies in children. In the first validation study, Cohen's kappa values were within 0.71-0.88 for CMA, 0.74-0.82 for cereal allergy, and 0.66-0.86 for any reported food allergy. In the second validation study, the kappa value was 0.79, sensitivity 0.958, and specificity 0.965 for reported and diagnosed CMA.

To investigate the associations between maternal diet during pregnancy and lactation and CMA in offspring, 6288 children were studied. Maternal diet during pregnancy (8^{th} month) and lactation (3^{rd} month) was assessed by a validated, 181-item semi-quantitative food frequency questionnaire (FFQ), and as an endpoint register-based information on diagnosed CMA was obtained from the Social Insurance Institution and complemented with parental reports of CMA in their children. The associations between maternal food consumption and CMA in offspring were analyzed by logistic regression comparing the highest and lowest quarters with two middle quarters of consumption and adjusted for several potential confounding factors. High maternal intake of milk products (OR 0.56, 95% CI 0.37-

0.86 p = 0.002) was associated with a lower risk of CMA in offspring. When stratified according to maternal allergic rhinitis or asthma, a protective association of high use of milk products with CMA was seen in children of allergy-free mothers (OR 0.30, 95% CI 0.13 – 0.69, p < 0.001), but not in children of allergic mothers. Moreover, low maternal consumption of fish during pregnancy was associated with a higher risk of CMA in children of mothers with allergic rhinitis or asthma (OR 1.47, 95% CI 0.96 - 2.27 for the lowest quarter, p = 0.043). In children of nonallergic mothers, this association was not seen. Maternal diet during lactation was not associated with CMA in offspring, apart from an inverse association between citrus and kiwi fruit consumption and CMA. These results imply that maternal diet during pregnancy may contain factors protective against CMA in offspring, more so than maternal diet during lactation. These results need to be confirmed in other studies before giving recommendations to the public.

To evaluate the degree of adherence to a therapeutic elimination diet in children with diagnosed CMA, food records of 267 children were studied. Subsequent food records were examined to assess the age at reintroduction of milk products to the child's diet. Nine of ten families adhered to the elimination diet of the child with extreme accuracy. Older and monosensitized children had more often small amounts of cow's milk protein in their diet (p < 0.001 for both). Adherence to the diet was not related to any other sociodemographic factor studied or to the age at reintroduction of milk products to the diet. Low intakes of vitamin D, calcium, and riboflavin are of concern in children following a cow's milk-free diet.

In summary, we found that the questionnaires used in the DIPP study are valid in investigating CMA in young children; that there are associations between maternal diet during pregnancy and lactation and the development of CMA in offspring; and that the therapeutic elimination diet in children with diagnosed CMA is rigorously adhered to.

Keywords: adherence, child, preschool, cow's milk allergy, elimination diet, etiology, food hypersensitivity, infant, lactation, pregnancy, validation studies, questionnaires

List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Jetta Tuokkola, Minna Kaila, Pirjo Pietinen, Olli Simell, Mikael Knip, Suvi M Virtanen. Agreement between parental reports and patient records in food allergies among infants and young children in Finland. J Eval Clin Pract 2008;14:984–9.

II. Jetta Tuokkola, Päivi Luukkainen, Minna Kaila, Heli Tapanainen, Timo Klaukka, Riitta Veijola, Olli Simell, Mikael Knip, Suvi M Virtanen. Validation of a questionnaire on cow's milk allergy: parental reports and physician's diagnosis. Acta Paediatrica 2010;99:1273-5.

III. Jetta Tuokkola, Päivi Luukkainen, Heli Tapanainen, Minna Kaila, Michael G Kenward, Lauri Virta, Riitta Veijola, Olli Simell, Jorma Ilonen, Mikael Knip, Suvi M Virtanen. Milk intake during pregnancy is inversely associated with milk allergy in the offspring. Submitted.

IV. Jetta Tuokkola, Minna Kaila, Carina Kronberg-Kippilä, Harri Sinkko, Timo Klaukka, Pirjo Pietinen, Riitta Veijola, Olli Simell, Mikael Knip, Suvi M Virtanen. Cow's milk allergy in children: adherence to a therapeutic elimination diet and re-introduction of milk into the diet. Eur J Clin Nutr 2010;64:1080-5.

In addition, some unpublished results are presented.

Abbreviations

CEA	Cereal allergy, i.e. allergy to wheat, barley, or rye
CI	Confidence interval
CMA	Cow's milk allergy
CMP	Cow's milk protein
DHA	Docosahexaenoic acid
DIPP	Type 1 Diabetes Prediction and Prevention study
EPA	Eicosapentaenoic acid
FFQ	Food frequency questionnaire
MUFA	Mono-unsaturated fatty acid
OR	Odds ratio
PUFA	Polyunsaturated fatty acid
SAFA	Saturated fatty acid
SOTI	Specific oral tolerance induction

1 Introduction

Cow's milk allergy (CMA) affects 2-6% of children under the age of 3-4 years in Finland (1-3). There are no publications of the time trends in incidence of CMA. However, according to the statistics of the Social Insurance Institution, the diagnosis of CMA, like all allergic diseases, has risen steeply over the past three decades (Social Insurance Institution, Statistical Branch, personal communication). Since a change in genetic susceptibility does not occur as fast as the rise in allergic diseases, environmental factors must play a role in this increase. The original hygiene hypothesis suggesting that allergy is linked to diminished infections in early childhood has been re-formulated as being a microbial deprivation disorder (4, 5). Excessive exposure to tobacco smoke, pollutants, and allergens has been identified to predispose to allergic diseases (6). Other factors suggested to be associated with development of allergic diseases are fetal growth (7), number of siblings (8), Cesarean section (9), maternal allergic status (10), use of probiotics (11), and daycare attendance (12). Another hypothesis is that alterations in the diet associated with the westernisation of societies are to blame for the increase in allergic diseases (6). These alterations include a diminished ratio of n-3 to n-6 polyunsaturated fatty acids (PUFAs) in the diet (studied in Finnish adults) (13).

There is also evidence of an early life period in which an infant is particularly susceptible to sensitization (14). The higher risk of allergic disease in offspring of mothers with allergic diseases than in offspring of allergic fathers suggests that pregnancy may be a critical period in determining subsequent disease development (10). Maternal diet during pregnancy and lactation is, therefore, an important research area in uncovering associations between early nutrition and subsequent development of allergic diseases. It is especially important in studying food allergy, which is often the first manifestation of allergic disease and may begin within hours of birth. This early onset suggests that the environmental factors leading to the development of allergic disease take place in utero. In fact, the fetus is dependent on the nutrients provided by the mother, transferred through the placenta. Moreover, the placenta modulates the fatty acid supply to the fetus (15, 16). Antigens are also transferred to the fetus, and reactions towards them can occur already in utero (17, 18).

Immunomodulation of the newborn gut continues postnatally. It is affected by, for instance, microbiota acquired from the mother at delivery, early infant feeding, and the composition of breast milk (reviewed in (19)). Breast milk contains antigens derived from the maternal diet, as well as several immunomodulatory compounds (reviewed in (20)). The link between breastfeeding and allergy has been debated since the 1940s, when Grulee and Sanford published their findings on the inverse association between breastfeeding and atopic eczema.

The gold standard study design in etiologic research is a double-blind randomized trial. However, in trials, only one or two exposures can be evaluated at a time. In the current situation, where we do not yet know which components of the diet can affect outcome, a randomized trial is not an option. Given these circumstances, a prospective birth cohort is the best study design to address the associations between maternal diet during pregnancy and lactation and the development of allergic diseases in offspring. The Type 1 Diabetes Prediction and Prevention (DIPP) cohort offers a unique opportunity to prospectively study the association between early nutrition and development of allergic diseases, including CMA. Food allergies are often queried from parents in questionnaires, but the correspondence between a parentally perceived allergy and a true food allergy is rarely good (21). Adequate research cannot be conducted without valid tools, which is why outcome variables must also be validated.

A recent review on food allergy and quality of life states that food allergy may have a profound psychosocial impact on children, adolescents, and their families (22). Prevention of food allergy is under continuous investigation. However, solid evidence of the effectiveness of any of the preventive measures is lacking (23). Maternal preventive dietary restrictions during pregnancy or delaying the introduction of complementary feeding of infants do not decrease the risk of food allergy (24). Unnecessary restrictions should be avoided because elimination diets always pose a risk for malnutrition during prevention and treatment of CMA (25, 26).

Winds of change have affected the dietary treatment of food allergy. How strict should the elimination diet be? For the past few decades, very strict elimination diets have been advised, but this may lead to inadequate nutrient intake and also to more severe reactions towards a food (27, 28). The Finnish Allergy Program is, in part, attempting to decrease the use of unnecessary elimination diets (29). However, neither strict nor more permissive elimination diets have been studied for their long-term effects, and the guidelines are based more on common practice than on evidence-based medicine. Specific oral immunotherapy towards food antigens may in the future be a useful tool in treating patients with prolonged food allergy (30). However, this treatment requires a significant amount of time from skilled allergologists. Could the same effect be achieved by simply not adhering to an elimination diet so meticulously? Further, could primary prevention of food allergy and promotion of tolerance be achieved by dietary intervention during pregnancy and lactation?

2 Review of the literature

2.1 Cow's milk allergy (CMA)

2.1.1 Prevalence and symptoms

The cumulative incidence of diagnosed CMA is between 2% and 6% in Finnish children aged less than 3-4 years (1-3). These figures are comparable twith those reported in other Western societies, ranging from 2% to 5% (31-34). Parentally perceived CMA is more common, ranging from 4.5% to 35% in infants and young children (2, 35-41).

Symptoms of CMA often occur within the first days after the introduction of cow's milk protein (CMP). Many children with CMA develop symptoms in at least two organ systems, with 50-60% having gastrointestinal, 50-60% skin, and 20-30% respiratory tract complaints (42) (Table 1).

Table 1. The most common symptoms of food allergy (adapted from (42)**,** (43)**,** (44)**, and** (45)**).**

Gastrointestinal	Cutaneous	Respiratory
frequent regurgitation	atopic dermatitis	runny nose
vomiting, nausea	angio-edema	otitis media
abdominal pain	urticaria	chronic cough
diarrhea	maculopapular rashes	wheezing
constipation	flushing	dyspnea
blood in stool	eczema	
iron deficiency anemia		
persistent distress or colic[1]		
oral allergy syndrome		

[1] Colic is not a specific symptom of CMA, and six infants need to be treated with CMP elimination to cure one child from colic in a selected population referred to a specialist (reviewed in (38, 46)).

Severe symptoms include failure to thrive due to chronic diarrhea or vomiting, iron deficiency anemia due to blood loss, hypoalbuminemia, enteropathy or severe colitis in the gastrointestinal tract as well as acute laryngoedema or bronchial obstruction with difficulty of breathing in the respiratory tract, or anaphylaxis. Food allergy is fairly common in children with moderate to severe atopic eczema (47). Also children with gastroesophageal reflux have CMA more often than others (48). Bloody stools may be a symptom of CMA, although they usually dissolve by themselves (49). In older children, chronic stomach pain and treatment-resistant constipation may be a sign of CMA (50). Allergic eosinophilic esophagitis and

gastroenteritis as well as food protein-induced enterocolitis, proctocolitis, and enteropathies are less common, non-IgE-mediated clinical manifestations of CMA (51, 52). For some reason, they are very rarely seen in Finland (46).

2.1.2 Diagnostics

The basis of diagnosis rests on a comprehensive history and a physician-supervised elimination-challenge test. During elimination, all sources of CMP are removed from the diet for 2-4 weeks, while keeping a symptom diary. In breastfed infants, the mother is advised to follow a CMP-free diet during the elimination test. Otherwise, the elimination diet is conducted by using a special infant formula: soy formula or an extensively hydrolyzed formula for infants after 6 months of age, extensively hydrolyzed formula for infants below 6 months of age, or if an extensively hydrolyzed formula does not improve the symptoms, an amino-acid formula. Children over the age of 12 months, whose growth is not compromised and who have no other major dietary restrictions, do not necessarily need a special infant formula. After the elimination period, a challenge test, supervised by a physician, is conducted. The golden standard for the diagnosis is considered to be a double-blind, placebo-controlled challenge (53), although it is rarely used in clinical practice. When a child has a delayed reaction, or when symptoms are difficult to interpret, a double-blind, placebo-controlled challenge is warranted. A double-blind, placebo-controlled challenge is not flawless; it is estimated to yield 3% false-negative results and 13% false-positive results (54). However, especially if symptoms are objective, an open challenge is sufficient. Children with a history of severe reaction are always challenged in a hospital. Internationally, all rechallenges are recommended to be performed in a setting with resuscitation facilities, as previously mild reactions may turn into severe ones after an elimination period (55). In Finland, this is not current practice.

After the elimination period, the challenge is started when the child is symptom-free or presenting with as few symptoms as possible, and does not have an infection (38, 46). Antihistamine use is discontinued 5 days before the challenge, but asthma medication is used without dosage changes. Atopic eczema is treated before the challenge, and topical treatment is listed in the symptom diary. The challenge starting dose can be determined by the history (lower than the amount that has previously caused a reaction) or it can be started with a drop on the lip, followed by a dose of 1-2 ml of cow's milk (44). Portion is increased every 15-30 min until symptoms appear or a typical for the age of the child is reached. Optimal dosage increase and time interval are yet to be determined – they may vary from doubling to a 10-fold increase and from 15 to 60 min (56). The challenge always begins supervised, and if the child shows no symptoms after the last dose on the first day, he/she can be discharged 1-2 h after the last dose. Cow's milk products are then consumed as usual, preferably at least 250 ml per day (44). A symptom diary is kept to track changes in symptoms, also recording the amount of the formula that the

child has used. If symptoms do not appear within a week, the challenge is considered negative, and the child can continue consuming CMP. Occasionally, the symptoms may appear after a period longer than one week (50), but in that case it is difficult to control for all the confounding factors. No other changes in the diet or in the topical treatment of atopic dermatitis should be made during the challenge.

Skin prick tests and determining specific IgE against cow's milk or individual CMPs are often used in the diagnostic work-up. Sensitivity and specificity of both of these tests vary according to population and children's age (57-59). Specific IgE levels can be measured by several commercial methods, but their units are not comparable. The IgE level that predicts CMA with 90% certainty varies from study to study, e.g. 88.8 kU/l in a German study (60), 23 kU/l in an American study (61), and 22 kU/l in a Swedish study (62). In skin prick tests, weal sizes of 6 mm at the age of 2 years and 8 mm at the age of 3 years (63) predict a positive oral challenge. Only IgE-mediated reactions can be detected with these tests, and an oral challenge is the only way to diagnose a delayed reaction. Atopy patch test, an unstandardized method attempting to detect late reactions, is currently not recommended for use in clinical practice (64). Specific IgA and IgG tests are not diagnostic, but rather show that the child has eaten the tested food (65). Due to lack of valid laboratory measurements and the long oral challenge period, diagnosing non-IgE-mediated food allergy is vulnerable to confounding factors and not as reliable as that of IgE-mediated reactions, stressing the importance of careful instructions on how to conduct the diagnostic procedure.

2.2 Perceived or diagnosed food allergy – validating questionnaires

If diagnosing food allergy is challenging, so is gathering reliable information on food allergies for research purposes. When conducting large, prospective cohort studies, one must rely on questionnaires for obtaining information on a number of issues. Querying food allergy in children is particularly cumbersome because parental beliefs about allergic reactions in their children greatly exceed actual food allergies. Accordingly, questionnaires overestimate the occurrence of food allergy (21).

Questionnaires must be developed to suit each study population. They are often used without description of contents or any method of validation. Between studies, the reported prevalence and incidence of food allergy may differ because of methodological differences, but also because of true differences between populations (66) and age groups (1, 2) in the occurrence of food allergies. Parentally reported food allergy varies from 4.5% to 35% in infants and young children (2, 35-41). In studies from developed countries, parentally perceived food allergy has been confirmed in 1/15 to 1/3 of the children (31, 34, 37, 40, 67, 68). However, food allergy may also go unnoticed by families even in the case of CMA (34, 50).

Formulating questions on special diets of the children is challenging. Since duration of food allergy or suspicion thereof can be short, current food allergies should be queried at short intervals to identify all of them (38); past food allergies would not be detected. On the other hand, retrospectively administered questionnaires introduce a possible recall bias in remembering food-related reactions. A report from a Norwegian cohort study describes a method for ascertaining parentally perceived reactions to CMP (34). Food-related reactions were queried in questionnaires at 6, 12, and 24 months of age, and a diagnostic work-up was conducted when the child was 2.5 years old. Probable point prevalences were calculated to take into account those lost to follow-up. A positive predictive value of parentally perceived food allergies ranged from 0.08 to 0.46, depending on duration of food allergy. Their study listed selection bias as the biggest limitation of their method; children who had never experienced a reaction to a food, i.e. the control group, had a harder time adhering to the diagnostic elimination period and were thus lost to follow-up.

The most reliable way to validate a questionnaire on food allergy is to have a physician confirm the diagnosis with a double-blind, placebo-controlled food challenge, accompanied by the necessary laboratory measurements. This, however, raises concerns about safety and ethics; implementing an elimination period could result in loss of tolerance towards an antigen in susceptible children. On the other hand, performing oral challenges on a child with severe reactions for the sole purpose of research is also not appropriate. When data on food allergies are collected prospectively and analyzed afterwards, a double-blind, placebo-controlled challenge is not possible because many of the children would have outgrown their food allergies by the time of validation, as seen in the study by Eggesbo et al. (34). Other ways to attempt validation are to compare parental information with medical history data and with registry-based data. The latter method has not thus far been used to evaluate the validity of parental responses to food allergy. A recent study from Norway reported a similar approach in a questionnaire regarding asthma, rhinoconjunctivitis, and eczema and dermatitis in children aged 2-6 years, where parental responses to patient records were compared (69). This study reported kappa values varying from 0.33 to 0.88 for parental responses to questions compared with information from medical records.

A review on questionnaire design states that ‘questionnaire bias is a result of unanticipated communication barriers between the investigator and respondents that yield inaccurate results’ (70). The review describes several potential sources of bias in questionnaire design as well as in administration of the questionnaire (Table 2). These notions emphasize the need to validate each questionnaire before putting it to use.

To summarize, questionnaire design and validation are important steps in a large cohort study and should not be overlooked.

Table 2. Potential sources of bias in questionnaires (adapted from (70)**).**

Source	Factors introducing bias in responses
Question design	
Problems with wording	• ambiguous or complex questions • double-barrelled question – how to interpret? • uncommon or vague words
Missing or inadequate data for the intended purpose	• belief vs. behavior; hypothetical questions • degraded data • insensitive measure (too few categories to differentiate respondents)
Faulty scale	• forced choice (insufficient categories) • missing or overlapping interval
Leading questions	• different wording gives different answers
Intrusiveness	• self-report response (selective suppression of information) • sensitive question (may affect all subsequent responses)
Inconsistency	• case definition (not precise enough) • change of scale or wording (comparison over time compromised) • diagnostic vague (different diagnosis/wording at different times)
Questionnaire design	
Formatting problem	• horizontal vs. vertical response format • juxtaposed scale (giving multiple responses to one item) • left or right alignment of the response choices
Lengthy questionnaire	• uniform, inaccurate answers • response fatigue, especially in open questions
Flawed questionnaire structure	• potential errors in skipping questions
Administration of questionnaire	
Respondent's subconscious reaction	• central tendency in answers • positive skewing
Respondent's conscious reaction	• faking "bad" (trying to appear sick to qualify for support) • faking "good" (giving answers that are considered 'desirable') • unacceptable disease or exposure more easily unreported
Respondent's learning	• guessing the hypotheses, giving expected answers
Respondent's inaccurate recall	• primacy and recency; in mailed surveys, tendency to choose the first alternative and in phone surveys the last alternative • proxy respondent; limits the type of matters that can be queried • underlying cause may affect recall of prior exposures • telescope; recalling that an event in the distant past has happened more recently
Cultural differences	• may affect interpretation of questions and answers

2.3 Hereditary and environmental risk factors for food allergy

2.3.1 Genetic factors

Asthma and allergic diseases are highly heritable. Family history of allergy is thought to be the strongest predictor of allergic diseases (71), and maternal heritage may be more strongly associated with allergic diseases in children than paternal heritage (10). There is a genetic predisposition also in food allergy (reviewed in (72) and (73)). Twin studies on peanut allergy and food sensitization show higher correlations for monozygotic than dizygotic twins, indicating a strong genetic component (74, 75). Polymorphism in several genes has been associated with food allergy, but genome-wide association studies are to date lacking (72).

Epigenetics is thought to play a role in the development of asthma and allergic diseases (Figure 1). Epigenetics is defined as accumulating heritable changes in gene expression potential (76). Thus, the field of epigenetics offers a mechanistic explanation for linking early exposures to later disease development. Immune development is under epigenetic regulation, and nutritional factors may in part be responsible for epigenetic programming (77).

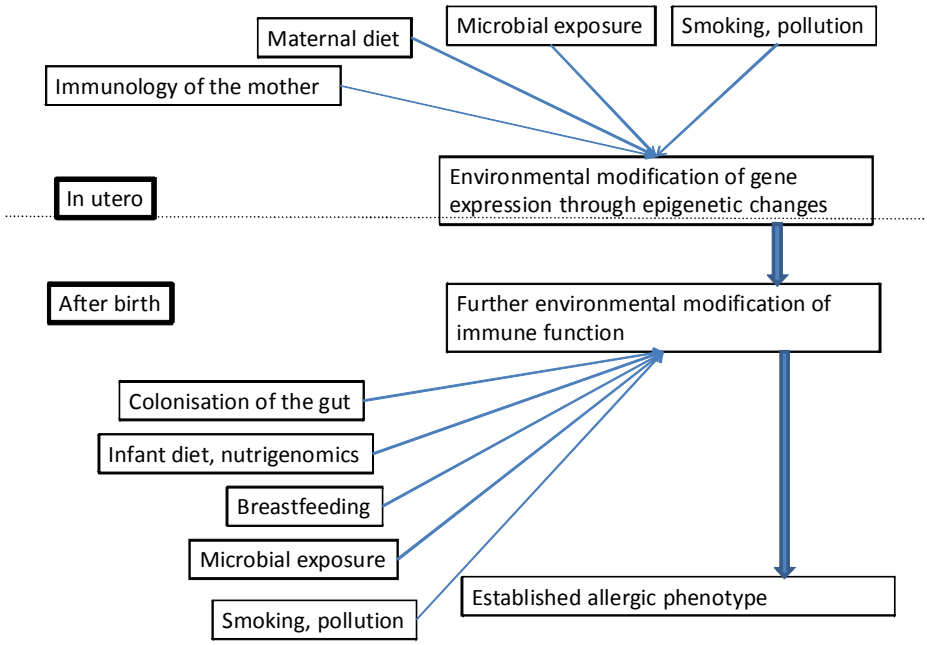

Figure 1. Interplay between hereditary and environmental factors in determining allergic phenotype (modified from (77)**).**

2.3.2 Hygiene hypothesis and other environmental factors

The hygiene hypothesis, first proposed by Strachan in 1989, states that:

> "Over the past century declining family size, improvements in household amenities, and higher standards of personal cleanliness have reduced the opportunity for cross infection in young families. This may have resulted in more widespread clinical expression of atopic disease, emerging earlier in wealthier people, as seems to have occurred for hay fever. "(78)

In addition to increased microbial contact with increasing number of children, pregnancies reduce maternal stores of nutrients, and frequent pregnancies provide a different nutritive environment for each fetus.

In line with the observations of Strachan et al. (79, 80), early daycare attendance is associated with a lower risk of asthma and allergic diseases, especially in children from small families. Moreover, living in a farm environment, consumption of farm milk (81-84), and pet keeping (84, 85) may decrease the risk of asthma and allergic diseases. A farm environment has abundant microbes, and especially endotoxins have been associated with a lower risk of asthma and allergic diseases (86). Furthermore, an anthroposophic lifestyle, with fewer vaccinations and less use of antibiotics and a diet that includes fermented products, has been linked to a lower risk of atopy (87). Another suggested risk factor for asthma and allergic diseases is Cesarean section (9), which deprives the newborn from contact with maternal vaginal flora. Exposure to tobacco smoke (6) and increased fetal growth (7) have been associated with a higher risk of asthma and allergic diseases.

Since 1989, the focus has shifted from the potential protective effect of infections and family size to microbial stimulation, after noting that lack of early microbial contacts may lead to later aberrant immune responses to antigens. Romagnani suggested two plausible explanations: first, innate immunity lacks stimulation that would lead to immune deviation from Th2 to Th1, and second, reduced stimulation of the immune system leads to reduced activation of regulatory T-cells (88). The latter now seems to be the focus of intensive research.

Probiotics offer the much needed stimuli to the immune system in order to decrease the risk of allergic diseases. Probiotics are defined as "live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host" (89). The effects of probiotic bacteria are strain-specific. Prebiotics, in turn, are "nondigestible food ingredients which are beneficial to the host because they stimulate the growth or activity of beneficial bacteria in the gut" (90). Probiotics have several potential modes of action; they increase antigen breakage by lowering pH in the gut, reduce intestinal permeability (91), and modulate immune responses (92, 93).

Probiotic use perinatally may decrease the risk of allergic diseases and sensitization in children, especially in those whose mother has some form of allergic disease (11, 94). A recent meta-analysis provides convincing evidence for a

preventive effect of probiotics on atopic dermatitis, but evidence for a beneficial effect in treating atopic dermatitis is less clear (95), although some data supports a beneficial effect in treating infants with CMA (96). The Cochrane reviews state that insufficient evidence exists to recommend the use of probiotics or prebiotics to prevent allergic diseases and food hypersensitivity (97, 98). However, the Finnish guidelines recommend periodic use of probiotics for diagnosed food allergy on a curative basis (46).

2.4 Dietary exposures and food allergy

2.4.1 Interactions between mother and fetus

A Th2 biased environment is essential for a successful pregnancy. Universal skewing towards a Th2 balance of allergen-specific responses in neonates has been suggested (99); a newborn is "atopic" to begin with, and environmental stimuli later switches on the shift towards development of tolerance – or allergic disease.

Most of the interaction between mother and the fetus occurs through the placenta. The main function of the placenta is the exchange of metabolic and gaseous products between maternal and fetal bloodstreams. Some substances, such as fat-soluble vitamins and carbohydrates, pass through the placental barrier freely, whereas others, such as amino acids, water-soluble vitamins, and some minerals, are actively transported. Also certain long-chain PUFAs, especially docosahexaenoic acid (DHA), are selectively transported and found in greater concentrations on the fetal side of the placenta than on the maternal side (15, 16). Ex vivo studies with placenta have shown that the fetus may also be exposed to dietary antigens in utero due to placental transfer (100, 101) and that food antigens consumed by the mother can be found in the amniotic fluid (reviewed in (18)). The antigens then come into contact with the fetal gastrointestinal tract due to fetal swallowing (102). Other routes of antigen contact are through the skin and respiratory tract. The fetus can have reactivity to allergens, including food allergens, as early as 22 weeks of gestation (reviewed in (18)). Antigen-specific T-cells are also present in cord blood, with the highest frequencies of response to cow's milk and hen's egg antigens (103, 104).

Of immunoglobulins, subclass IgG4 is associated with development of tolerance, and IgE with an allergic reaction (Table 3). IgG is actively transported through the placenta (101). The transfer of tolerance from mother to fetus in the form of IgG is allergen-specific. On the other hand, IgA passes the fetal membranes and is also present in the amniotic fluid. IgE is produced by the fetus from as early as 11 weeks of pregnancy, but in addition, maternal IgE may be transported across fetal membranes (reviewed in (17)). IgE exposure during fetal life may have an effect on determining allergic outcome later in life; high IgE/IgG ratio of the mother has been hypothesized to potentially increase the risk.

Table 3. Properties of certain immunoglobulin (Ig) subclasses (modified from (105)**).**

Immunoglobulin	Functions	Crosses placenta	use in allergy diagnostics
IgA	Mucosal response	no	no
IgE	Allergy	no	yes
IgG	Secondary response Neutralise toxins and virus	yes	IgG_4 subclass as a sign of development of tolerance

In mice, development of tolerance has been shown to be transferred from the mother to offspring when the mother is tolerized before conception (106). Tolerization can be obtained via the placenta and breast milk. Preconception immunization of the mother with ovalbumin inhibits IgE response of neonates and weaned immunized offspring (107). Thus, maternal atopic status plays a role in determining immunologic reactions in the offspring.

Considering the above, it is likely that maternal diet during pregnancy has an impact on development of tolerance prenatally and that the effect lasts until the postnatal period.

2.4.2 Fatty acids and immunology

The effects of fatty acids on immunology are mainly due to PUFAs. N-3 and n-6 PUFAs consist of two parent compounds, which are essential in human nutrition and must be obtained from the diet: linoleic acid, a fatty acid of the n-6 family with 18 carbon atoms and two double bonds, and α -linolenic acid, a fatty acid of the n-3 family with 18 carbon atoms and three double bonds (Figure 2). Linoleic acid can be elongated to arachidonic acid and α-linolenic acid to either eicosapentaenoic acid (EPA) or DHA, but there is competition between the n-6 and n-3 fatty acids for the elongase and desaturase enzymes that metabolize them. Along the pathway, desaturases are the major regulated steps. They are affected by dietary, hormonal, pharmacological, and toxicological factors (reviewed in (108)). Conversion of linoleic acid to arachidonic acid is more efficient than conversion of α-linolenic acid to EPA due to the higher intake of n-6 fatty acids than of n-3 fatty acids. Moreover, the affinity of the enzymes is stronger towards n-3 PUFAs than n-6 PUFAs. In addition to forming long-chain PUFAs from the precursor fatty acids, all of them can be derived from the diet; the main source of EPA and DHA is fish, and that of arachidonic acid, meat and meat products. As in Western countries in general, the intake of linoleic acid in Finland by far exceeds that of α-linolenic acid and other n-3 series of fatty acids, although the ratio of n-6 to n-3 PUFAs is lower in Finland due to the consumption of rapeseed oil than in countries using mainly olive oil. (109). Also notable is that bioavailability of fatty acids from foods seems to be better than from supplements (108).

Arachidonic acid and EPA are the precursors of eicosanoids, which are hormone-like substances with a number of physiological functions. The function of the eicosanoid is determined by the fatty acid from which it is derived. Arachidonic acid–derived series 2 prostaglandins guide immunologic responses towards allergic reactions, and most of the products in the arachidonic acid cascade lead to proinflammatory activities (reviewed in (110)). The generation of eicosanoids from EPA is less efficient than in the arachidonic acid cascade, and these metabolites have lower bioactivity than those derived from arachidonic acid. In addition, higher levels of n-3 PUFAs also dampen T-cell responses. Suggested pathways include membrane fluidity, intracellular signaling and gene transcription, reduced capacity of antigen-presenting cells to present antigen to T-cells, and cytokine production (111). The ratio of n-6 to n-3 fatty acids in tissues has been suggested to play an important role in mediating inflammatory responses; dietary intake of n-3 and n-6 fatty acids affects the availability of these fatty acids for eicosanoid synthesis (112). Nevertheless, the absolute needs for both series of fatty acids must be met. The optimal ratio of n-6 to n-3 fatty acids in the diet is yet to be determined, but it seems that, compared with present average intakes, more n-3 and less n-6 series fatty acids would be beneficial. Furthermore, fatty acid metabolism is suggested to be distorted in atopic individuals (113, 114), although this hypothesis is under debate (115, 116).

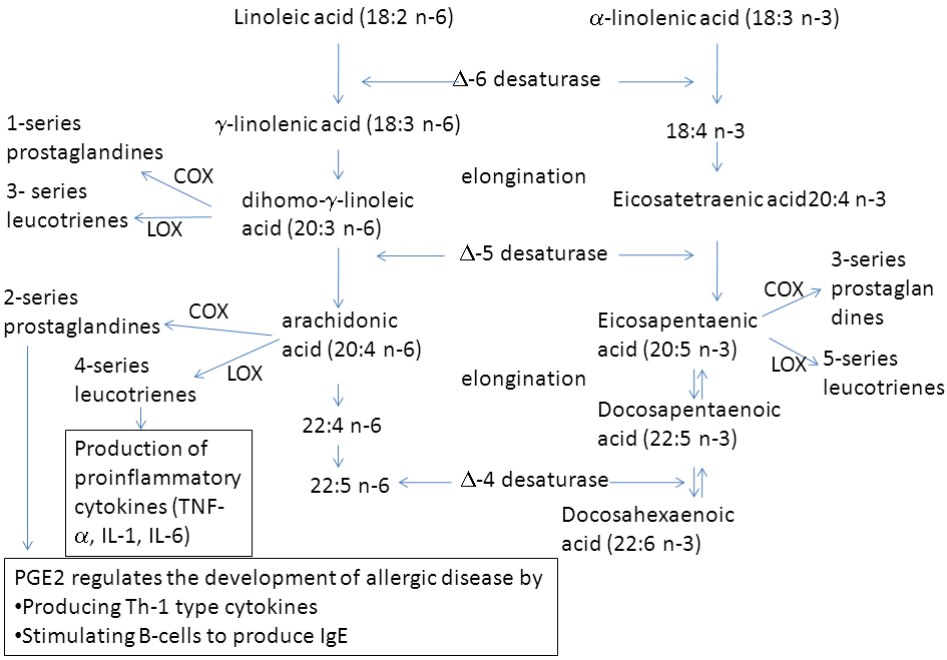

Figure 2. Metabolism of n-3 and n-6 polyunsaturated fatty acids (adapted from (110)**). COX, cyclooxygenase; LOX, lipoxygenase.**

In addition to long-chain PUFAs, other fatty acids may exert biological activities (reviewed in (108)). Mono-unsaturated fatty acids have been studied, and in general, they seem to be rather inert in terms of immunological interactions, but the evidence is limited. Trans-fatty acids and saturated fatty acids may have pro-inflammatory effects, but again, evidence is only just emerging (108).

2.4.3 Development of tolerance

What is tolerance?

Tolerance is defined as recognizing self from non-self and non-harmful foreign substances from harmful ones (Figure 3). Oral tolerance is 'a systemic immune unresponsiveness to a specific antigen which was previously orally administered'. In allergic individuals, the immune system may mistakenly consider food antigens as harmful and initiate a defense reaction towards them. The epithelial barrier in the gut of allergic individuals has defects, and abnormal passage of food antigens through Peyer's patches and tight junctions enables contact between the antigen and the immune system (117).

Tolerance can only be induced by contact with an antigen, as noted by studies of high-risk families where maternal food avoidance has not prevented food allergy in the child (118, 119). As mentioned earlier, tolerance may develop already in utero (106, 107). There, the fetus encounters food antigens in an environment which normally would lead to suppression of immune reactions against food antigens. Immunoprogramming and immunoregulation of the fetal immune system is controlled by maternal genetic and environmental exposure (120).

Window of opportunity

A 'window of opportunity' appears to exist for the induction of oral tolerance early in life. Intestinal flora is in active dialogue with the mucosal immune system – in fact, intestinal flora is required for oral tolerance induction (reviewed in (120)). The neonatal period is critical for priming of allergic diseases because the epithelial barrier and immunoregulatory network are underdeveloped (reviewed in (19)). The development of immune homeostasis depends on timing and dosage of dietary antigens. If an immature gut is exposed to antigens too early, or conversely too late, the child is at greater risk of developing food allergy as well as other allergic diseases. Therefore, the age of introduction of solids could influence the development of allergy or tolerance (121-126). The optimal age of introduction of solids remains unknown. This is also a difficult topic to study because randomized trials involving breastfeeding are not ethical and reverse causation among those presenting symptoms early will confound the associations between exposure and outcome. At present, the best academic guess is that mixed feeding, i.e. introducing solids while still providing the protection of breast milk, would promote optimal tolerance to food antigens. This is supported by the notion that children who receive breast milk with low levels of secretory IgA antibodies to CMP are at greater risk of

developing CMA than those receiving breast milk with high levels, although breast milk contains several other immunomodulatory factors as well (reviewed in (19)). Before maturation of the infant gut, secretory IgA in breast milk protects the child by dampening early immune activation. An increase in intestinal IgA is seen after one year of age in children in developed countries. Uptake of secretory IgA from breast milk is limited via the gut mucosa in newborns, but maturation of the mucosal barrier is nevertheless affected. In addition, small amounts of food antigens in maternal milk may enhance tolerance induction.

The association between maternal diet during pregnancy or lactation and development of food allergy has been studied very little. Breast milk composition, especially that of fatty acids, varies a great deal depending on maternal diet. According to meta-analyses, breastfeeding itself may protect the child from asthma and allergic diseases (127-129).

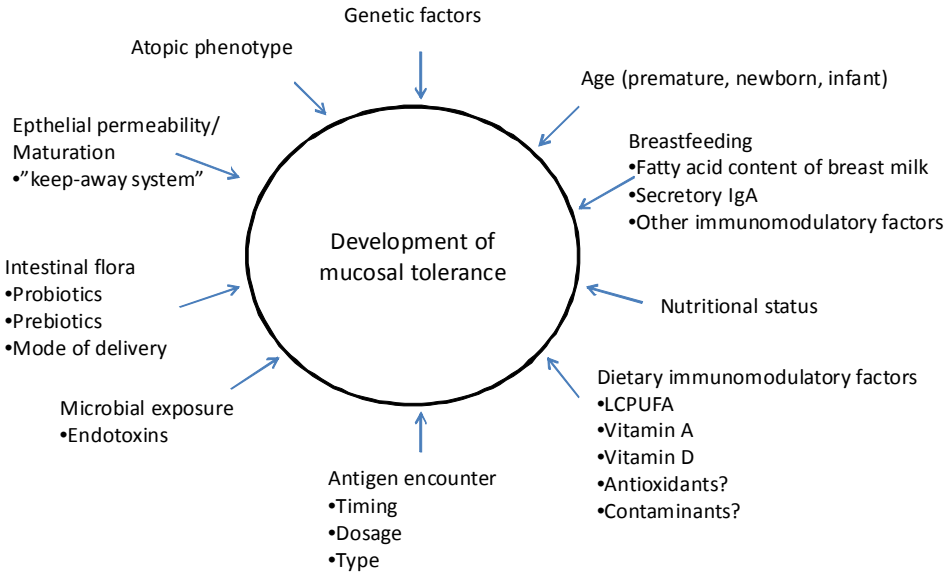

Figure 3. Factors suggested to affect the development of mucosal tolerance (modified from (19)**).**

Gaining tolerance after food allergy

An entirely different issue is the achievement of tolerance after having had allergic reactions towards a food. As discussed earlier, the prognosis of CMA is good; 96% of children with delayed reactions and 63% of children with immediate reactions become tolerant by the age of four years (130). Oral immunotherapy has proven to be a useful treatment in children with immediate reactions with a prolonged hypersensitivity, but whether true tolerance can be achieved is still questionable; exposure needs to be constant in order to maintain the tolerance (131, 132). A recent

study prospectively assessed whether using different special infant formulas influences the duration of CMA by feeding infants different types of special infant formulas (133). Infants with CMA were randomly allocated to receive either an extensively hydrolyzed formula, a soy formula, or a rice hydrolysate formula. Infants who received an extensively hydrolyzed formula recovered from CMA at an older age than children on a soy formula or a rice hydrolysate, and the authors speculated that the small amounts of CMP antigens in the formula could have delayed gaining tolerance. Unfortunately, they did not assess adherence to the CMP-free diet of the children apart from the formula used. Thus far, observational reports on adherence to a therapeutic elimination diet suggest neither a beneficial nor a harmful effect of minor and infrequent exposure to an allergen on the development of tolerance (134, 135).

2.4.4 Maternal diet during pregnancy

Maternal diet during pregnancy is proposed to have an impact on development of food allergy as well as asthma and allergic diseases (136), among other environmental factors. Prospective cohort studies on the associations between maternal diet during pregnancy and asthma or allergic diseases have yielded conflicting results thus far, most likely due to differences in study populations, definitions of endpoints, and methods of investigating the mothers' diets (Table 4). Small randomized studies have shown that allergic diseases are not prevented by food allergen avoidance during pregnancy (118, 119, 137). In one study, maternal dietary avoidance of egg had no effect on the amount of ovalbumin found in cord blood or breast milk, indicating that dietary exclusion is difficult and may not lead to removal of the antigen from the maternal system after all (138). On the contrary, certain dietary factors may protect the fetus from developing allergic diseases. Intake of fish during pregnancy may protect the child against the development of allergic diseases (139-145), although not all prospective cohort studies have detected this association (146-148).

A decline in the consumption of vegetables and fruit has been hypothesized to play a role in the increase of allergic diseases (6). However, this decline does not seem to be evident in Finland (109, 149). According to prospective cohort studies, intake of fruits and vegetables may give some protection against allergic diseases (140, 150), but contradictory findings have been reported as well (146, 148, 151-154). Isolated findings regarding specific fruits or vegetables further obscure the picture (Table 4).

The investigated association between maternal use of milk products and asthma and allergic disease in the offspring has also yielded conflicting results. In one Japanese prospective cohort study, maternal use of total milk products, full-fat (but not low-fat) milk, and cheese was inversely associated with infantile wheeze (155). In that study, no associations between maternal dairy consumption and eczema in the child were found. Another report from the same cohort showed no association

between maternal dairy product use and suspected eczema in infants aged 3-4 months (147). A cohort study from Germany revealed no associations between maternal use of milk, yoghurt, cheese, or cream and infant's eczema or allergic sensitization against any allergen, food allergens, or inhalant allergens (143). However, this study did report an increased sensitization against cow's milk with high maternal consumption of cream during pregnancy. A previous report from the DIPP cohort showed no associations between maternal use of milk, cheese, or fermented milk products during pregnancy and atopic sensitization in offspring aged five years (153). Only one cohort study from Japan evaluated the association between maternal diet during pregnancy and food allergy of children. They found no associations, including those with maternal consumption of milk (154).

In the past, elimination of such items as cow's milk, egg, and fish from the diet during pregnancy or lactation was hypothesized to decrease the risk of allergic diseases in offspring. Although two small trials suggested prevention of allergic diseases in early childhood with prophylactic elimination diet during pregnancy or lactation (156, 157), another two showed no risk or any protective effect (118, 119). A Cochrane systematic review with pooled analysis of randomized, controlled trials states that maternal antigen avoidance during pregnancy does not prevent allergic diseases in offspring (158).

A recent meta-analysis combining the results from four randomized, placebo-controlled trials using n-3 fatty acids (three studies using fish oil and one study using canola and tuna-based oil as an intervention and olive oil as the control) during pregnancy and lactation did not support a beneficial effect of the supplementation on atopic eczema, asthma, allergic rhinitis, or food allergy (159). However, the single included trial that was carried out only during pregnancy reported that maternal fish oil supplementation reduced the risk of having a diagnosis of allergic asthma, atopic dermatitis, or allergic rhinitis by the age of 16 years, compared with using a supplement containing olive oil (160). Furthermore, after the publication of the meta-analysis, Furuhjelm et al. (161) examined the effects of fish oil capsules containing 1.6 g of EPA and 1.1 g of DHA administered to mothers from the 25th week of pregnancy and during lactation in a randomized trial. They found a reduced risk in skin prick test positivity, atopic eczema, and food allergy with the use of fish oil, accompanied by a number needed to treat of 7.4 for food allergy. A downside to these trials is the question of what fish oil should be compared with: there is truly no placebo because most fatty acids affect immunological responses one way or another. For example, Furuhjelm et al. (161) used linoleic acid of the n-6 series, which is not immunologically inert. Nevertheless, fish oil supplementation as a source for n-3 fatty acids may be beneficial, especially during pregnancy, in mothers who are unable to consume fish due to fish allergy.

Table 4. Maternal diet during pregnancy and the risk of asthma, eczema, and sensitization in offspring according to prospective cohort studies (allergic outcomes according to how they were presented in the original publications; outcomes may overlap).

Dietary factor	Allergic outcome					
	Asthma	Wheeze	Eczema	IgE sensitization[1]		Reference
				Food	Inhalant	
Dietary patterns						
High Mediterranean Diet score[2]		−	−			(150)
Dietary patterns [3]	0	0	0			(152)
Fruits	− / 0	0	0	0	+	(140, 141, 153, 154, 162)
Apples	−	− / 0	0	0	0	(141, 143, 162)
Citrus fruit, kiwi	0	0	− / 0	0	+	(141, 143, 153, 162)
Exotic fruit, bananas, strawberries			0	0	0	(143)
Berries				0	0	(153)
Fruit juice	0	0	0	0	0	(141, 143)
Vegetables / roots	− / 0 / +	0	0	0	0	(140, 146, 153, 154, 162)
Potatoes	0		0	0	0	(153, 154)
Pulses and nuts				0	0	(153)
Green leafy vegetables	0		0			(141)
Green and yellow vegetables		0	−			(162)
Carrots, spinach, cabbage, tomatoes, salad, vegetable juice			0	0	0	(143)
Celery			0	+	0	(143)
Sweet pepper			0	0	+	(143)
Milk and milk products	0	−	− / 0	0	0	(143, 146, 147, 153-155)
Yoghurt		0	0	0	0	(143, 155)
Fermented milk products				0	0	(153)
Cheese		−	0	0	0	(143, 153, 155)
Cream			0	0 / + [4]	0	(143)

Dietary factor	Allergic outcome					
	Asthma	Wheeze	Eczema	IgE sensitization		Reference
				Food	Inhalant	
Fats	+			0	0	(153, 154)
Vegetable oils			+	0	0	(143, 153)
Deep-frying vegetable fat			0	0	+	(143)
Fat spreads	+					(140)
Fat from dairy products or butter use vs. margarine use	0		0			(141)
Butter			0	0	0	(143, 144, 153)
Margarine			+	0	0	(143, 144, 153)
Others						
Seeds			0	0	0	(143)
Nuts	0		0	0	0	(143, 146)
Nut products	+	+		0	0	(146)
Cereals				0	0	(153)
Whole-grain products	0		0			(141)
Fish intake in total	0	−	− / 0	0	0	(139, 141, 143-148, 153, 154)
Oily fish	− / 0				− [5]	(140, 141)
Eggs	0		0	0	0	(143, 146, 147, 153, 154)
Meat	0	0	0 / +			(145, 147, 154)
Chocolate and sweets				0	0	(153)

[1] as defined in original publications; either a positive response in skin prick test or specific IgE
[2] high consumption of vegetables, legumes, fruits, nuts, cereal, fish, dairy products, and olive oil and low consumption of sweets and fast foods
[3] Health-conscious, traditional, processed, vegetarian, confectionery; defined by principal components analysis
[4] specific IgE against cow's milk
[5] defined by parental response to a question about hay fever
+ a direct association
- an indirect association
0 no association

2.4.5 Maternal nutrient intake during pregnancy

What are the components of food items that mediate the effects on risk of asthma and allergic diseases? The strongest hypothesis concerns the effects of long-chain

PUFAs, EPA and DHA, derived from fish (163, 164). The association between dietary intake of SAFAs and allergic outcomes is contradictory (145, 165, 166). Among epidemiologic studies, only two reports from one birth cohort study have been published thus far that have evaluated the associations between maternal intake of n-6 and n-3 PUFAs and allergic diseases in offspring (145, 147). They showed either no associations or a direct association between intakes of n-6 PUFAs and linoleic acid with eczema in the child, and no associations with wheeze. Apart from the aforementioned associations, these studies report no associations between maternal intakes of total fat, SAFA, mono-unsaturated fatty acids (MUFA), n-3 PUFAs, α-linolenic acid, EPA, DHA, n-6 PUFA, linoleic acid, arachidonic acid, n-3/n-6 PUFA ratio, and eczema and wheezing of the child. A previous report from the DIPP cohort also showed no associations between maternal intake of total fat, SAFA, PUFA, or n-6 or n-3 PUFAs with IgE sensitization in offspring at the age of five years (153).

Another possible immunomodulatory nutrient in fish is vitamin D. However, the epidemiological evidence for vitamin D and the development of asthma or allergic diseases is inconsistent. Inverse (167, 168) or no (155) associations have been reported between maternal intake of vitamin D and wheeze in the child. Moreover, inverse relationships between vitamin D intake and asthma (169) and sensitization against food allergens (153) have previously been reported for our cohort. By contrast, no associations for eczema (155, 169), allergic rhinitis (169), or sensitization against inhalant allergens (153) have been found. Furthermore, maternal serum 25 (OH)-vitamin D concentration above 75 nmol/l in late pregnancy has been described to be associated with a higher risk of eczema and asthma in offspring in one prospective study (170). Adding to the confusion, the effect of vitamin D from food seems to be greater than that from supplements (153, 169). None of these studies have measured sunlight exposure as a source of vitamin D.

Although folate metabolism is important in DNA methylation and therefore has been suggested to play a role in epigenetics, no clear associations between maternal intake of folate or any other B-vitamin during pregnancy and allergic outcomes in offspring have been found (171-173), apart from one cohort study that suggested a higher risk of wheeze with folic acid supplementation (173). In that study, maternal folic acid supplementation was also associated with increased lower respiratory tract infections, indicating that the association detected was possibly not with allergic asthma.

Other potential immunomodulatory components in fruits and vegetables are antioxidant nutrients. Thus, another hypothesis concerns the effects of antioxidant intake on development of allergic disease (6). Vitamin A, retinoids, and carotenoids modulate immune development (reviewed e.g. in (174)). Epidemiological studies show conflicting results on the associations between maternal antioxidant nutrient intakes and development of asthma and allergic diseases. Higher maternal selenium serum levels during pregnancy (175) or in cord blood (176) may also protect

children from wheeze. High maternal vitamin E intake during pregnancy has been associated with a lower risk of asthma, wheeze, and eczema in one study (177), while another study found no association with eczema, except for an inverse association in children of atopic mothers (151). Interestingly, maternal intake of vitamin C has been identified as a risk factor for wheeze and eczema in one cohort in the UK (151). The same study reported no associations between wheeze or eczema and maternal intakes of β-carotene, selenium, magnesium, manganese, copper, and zinc during pregnancy, or between eczema and maternal intakes of vitamins E and C. A further report for the same cohort showed no associations between maternal intakes of vitamin C, β-carotene, magnesium, copper, manganese, and iron and wheezing, asthma, eczema, or hay fever, but they did find an inverse association between maternal intake of vitamin E and wheezing and asthma, and intake of zinc and asthma and eczema (178). Another cohort study conducted in the USA reported no associations between maternal intakes of vitamin C, vitamin E, α-carotene, β-carotene, β-cryptoxantin, folate, lutein, zeaxantin, lycopene, copper, or zinc and child's eczema or wheezing, except for an inverse association between zinc and vitamin E intake during pregnancy and wheezing (177).

To summarize, dietary n-3 fatty acids, derived from fish, and vitamin D intake during pregnancy may protect against the development of asthma and allergic diseases, but evidence for an association between antioxidant nutrient intakes and allergic diseases and asthma is less convincing.

2.4.6 Breastfeeding and maternal diet during breastfeeding

Breastfeeding has been proposed to decrease the risk of asthma and allergic diseases during childhood, but the evidence is contradictory. Meta-analyses have been conducted on the associations between breastfeeding and atopic dermatitis (128), bronchial asthma (127), and allergic rhinitis (129) in childhood. These analyses show an inverse association between exclusive breastfeeding during the first three months of life and atopic dermatitis in childhood in children with a family history of atopy; asthma in childhood, with a stronger protective effect in a subgroup of children with a positive family history of allergic disease; and allergic rhinitis in childhood with and without a family history of atopy. However, some studies have even suggested a higher risk of atopy in breastfed children, especially with prolonged (≥ 9 months) exclusive breastfeeding (124, 179).

One possible explanation for the inconsistent evidence on the association between breastfeeding and allergic diseases is that breast milk composition varies according to maternal diet during lactation. Accordingly, maternal diet during lactation is suggested to be associated with the risk of atopy. One prospective study with a small number of mother-child pairs suggests a diet rich in SAFA as well as low vitamin C intake from food to be risk factors for atopic dermatitis and sensitization in the infant (165, 180, 181). On the other hand, maternal intake of

milk protein during lactation was not associated with development of CMA or sensitization against CMP (182). Another small, observational study found no association between maternal diet during lactation and atopy in their offspring (116). In one trial conducted among high-risk families, maternal elimination of milk, egg, and fish during lactation reduced the risk of atopic eczema in children aged four years (OR 3.08, 95% CI 1.33-7.21 for the non-diet group), but several other allergic outcomes did not differ between the diet and non-diet groups (183). However, this study has been criticized because they used site-allocation by two different hospitals instead of a randomized design. Another trial introduced an elimination diet during pregnancy as well as during lactation (184). They restricted maternal consumption of eggs from the 20th week of pregnancy and during lactation. This intervention had no effect on incidence of atopic eczema, skin prick tests, or total allergic outcomes, compared with the control group. Other trials with dietary modifications during pregnancy or lactation have in addition used restrictions in infant feeding, and thus, conclusions cannot be drawn regarding maternal diet during pregnancy or lactation.

Some evidence suggests that lower EPA and DHA concentrations in mature breast milk predispose to allergic diseases (reviewed in (115)). Fatty acid profile in breast milk is dependent on both maternal dietary intake of fatty acids and those derived from maternal adipose tissue (185, 186). One Finnish study showed direct associations between high SAFA content of breast milk and atopic dermatitis, and between high maternal SAFA intake during lactation and atopic sensitization in offspring (165, 181).

2.5 Treatment of CMA

Once an allergic phenotype is established, the treatment of CMA has traditionally been total elimination of all CMP from the diet. In rare cases, an extended elimination has been associated with mild symptoms turning into severe ones (27). Therefore, the present line of treatment is to eliminate CMP from the diet to the extent that symptoms disappear. Threshold levels for provoking allergic reactions have been reported to vary from 0.6 to 180 mg of CMP, and the amount that triggers a reaction varies markedly from individual to individual (187). Thus, the strictness of the elimination needs to be considered individually (188). If the child's symptoms are mild or appear after large doses of milk, then milk products should be used up to the amounts that do not cause symptoms in order to maintain tolerance. However, children with severe symptoms and young infants are still advised to follow a strict elimination diet, as are patients during a diagnostic work-up.

To date, neither strict nor less rigorous elimination has been investigated for long-term effects, and the guidelines are based more on common practice than on evidence-based medicine. A recent study attempted to examine the long-term effects by randomly allocating infants with CMA to receive either an extensively hydrolyzed formula, a soy formula, or a rice hydrolysate formula (133). They found

that the children allocated to receiving an extensively hydrolyzed formula recovered from CMA at an older age than those receiving a rice hydrolysate or soy formula, and speculated that exposure to CMP antigen residue in the extensively hydrolyzed formula would delay the development of tolerance towards CMP.

Treatment of food allergy aims at controlling the symptoms, ensuring normal growth and development of the child, and at developing normal age-specific eating habits (46). Guaranteeing a sufficient intake of energy and all nutrients in the child's diet is essential. The child's growth chart, disappearance of symptoms, and the development of normal age-specific eating habits are monitored.

The prognosis of CMA is good, and the need for the elimination diet is evaluated at fixed intervals. Re-challenges are conducted every 6-12 months up to the age of four years, and thereafter, every 1-2 years. Children with non-IgE-mediated disease develop tolerance earlier than those with high specific IgE values (130). Sometimes the elimination is continued despite a negative oral challenge, posing a risk for malnutrition (189), stressing the importance of follow-up.

Without a balanced diet, a child is at risk of developing nutritional deficiencies and sustaining impaired growth and development (28). Besides providing diversity, the diet should provide enough calcium and vitamin D (190, 191). When cow's milk or infant formulas containing CMP are eliminated, they must be replaced with food items with a similar nutritional value (44, 46, 192, 193). Special hypoallergenic and soy infant formulae with nutritional values similar to cow's milk-based infant formulae are used at least to the age of one year. The extensively hydrolyzed formulae contain trace amounts of CMP. A small proportion of infants react to these traces and need an amino acid formula to control their symptoms. Soy formulae are not used in the diet of infants under the age of six months because they contain phytates, aluminum, and phytoestrogens (194). If the diet of the child is not balanced enough, a special infant formula should be used to the age of 1.5-2 years. The diet can be balanced without a special infant formula after the age of 1 year if only CMP is eliminated from the diet and the child's growth is sufficient (46). Other mammalian milks are not recommended due to cross-reactivity, even though some are tolerated better than others (195-197).

Oral immunotherapy, the so-called specific oral tolerance induction (SOTI), has thus far only been used in research, but it is likely to be a useful treatment for children with CMA in the future. In Finland, it is currently used at certain University Hospitals. In the protocol, an increasing dosage of the allergen is administered to the child, reaching a maintenance dose over several months (30, 198-200). With oral immunotherapy, even children with anaphylaxis may begin to tolerate CMP, at least to some extent (201). A downside is that maintenance of tolerance may require regular intake of CMP (131, 132). Daily consumption of cow's milk is more easily achieved than that of peanut, which is internationally another major target of SOTI (202). A rush protocol for oral immunotherapy has also been proposed (203, 204), attempting to achieve tolerance in days rather than months or years.

2.6 Diet in children with CMA

A very restricted diet may prevent normal growth and development of normal age-specific dietary habits. In Western societies, case reports from the last decade describe people consuming an unbalanced diet, due to true or perceived CMA, leading to kwashiorkor (25, 205). Parental belief of their child having multiple food allergies and following a very restricted diet has also led to failure-to-thrive (26, 206). Case reports document rickets resulting from a milk-free diet, when not adequately supplemented with vitamin D (205, 207, 208). In addition, a very restricted diet may also lead to pellagra (209). In children with CMA, bone mineralization may be decreased, which may lead to increased fracturing of bones (190, 210-212) and persisting height reduction and osteopenia (191), if the diet is not adequately supplemented with calcium and vitamin D.

In well-conducted elimination diets where the offending food is eliminated from the diet and replaced with a compensatory tolerated food, normal growth and development are secured. In practice, not all children following a milk-free diet receive all the necessary nutrients from their diet (213-215). The growth of these children may also be compromised even when intakes of nutrients are within reference values (215, 216). A possible delay in growth before the milk allergy diagnosis can be compensated by catch-up growth with proper treatment (217). In one study of 169 children, those who avoided two separate food items were shorter than those with only one avoided food item (218). In another study with 100 children, multisensitization did not, however, affect growth (214). The nutritional needs of milk-allergic children are not always adequately met; in different studies comparing diets or nutritional status of healthy children and children with CMA, reduced intakes or status outside reference values have shown deficiencies for energy, protein, calcium, iron, phosphorus, riboflavin, niacin, zinc, magnesium, and vitamins C, D, and E (213-216). Different soy and hydrolyzed infant formulae used by children with CMA have not been shown to cause differences in growth (217, 219), whereas the use of a rice-hydrolysate formula may result in decreased growth (220).

Even though elimination diet is the primary treatment for food allergy, it may also cause harm. A prolonged strict elimination diet may lead to worsening of allergic reaction and even acute reactions in children who have previously tolerated the offending food without acute symptoms (27, 55). Food allergy also may have significant effects on quality of life and activities of families with food-allergic children (221).

According to an EU directive (2003/89/EC), if a pre-packed food contains CMP, this must be mentioned on the food label (222). CMP may be present in such food items as vegetable margarines, meat products, bakery products, and several convenience foods. Butter is not always perceived as a CMP-containing product either. In the USA, only 7% and 22% of parents of milk- and soy-allergic children,

respectively, could identify the offending ingredient in all food labels handed to them, due to unclear food labeling (223).

Adherence to a therapeutic elimination diet has thus far only been assessed in three child series (134, 135, 224). In all of them, parents were asked about the completeness of the elimination by questionnaires. A Dutch study with 38 food-allergic children found that in one-third of the children's diets the elimination was incomplete and that in one-third the completeness of the elimination remained inconclusive (134). Reasons for the presence of an allergenic food in the diet were incorrect label reading, ambiguous labeling, undeclared ingredients, or accidental intake by the child. Half of the children who had consumed the offending food unintentionally reacted in a double-blind, placebo-controlled food challenge.

In a Norwegian study, one-third of 86 children with verified milk or egg allergy lacked the needed dietary restriction, as reported by parents (224). In this study, maternal age, education, and smoking were not associated with the completeness of the elimination. Furthermore, in a study conducted in Australia, in one-third of the diets of egg-allergic children, egg was not avoided all of the time (135). This was regardless of the advice given on how strict the elimination should be. That study also investigated the prognosis of an oral challenge and found that the strictness of the diet did not affect the outcome of an oral challenge. The phenotype of the disease determines whether a child can tolerate a little, perhaps baked or otherwise processed, CMP (225). Fermented milk products and long-ripened cheeses are other examples of processed milk products.

3 Aims of the study

The aims of this thesis were as follows:

- To study the validity of a parentally completed questionnaire on food allergies (I and II),
- To study the associations between maternal food consumption during pregnancy and lactation and CMA in the offspring (III),
- To characterize among children with diagnosed CMA (IV)
  - the adherence to a therapeutic elimination diet,
  - the diet composition of a CMP-free diet,
  - the reintroduction of milk products into the child's diet,
  - factors associated with adherence and age of reintroduction of milk products into the diet.

4 Subjects and methods

4.1 Study design

This study is a part of the Finnish Type 1 DIPP Nutrition Study, a multidisciplinary prospective population-based cohort study (226). After parental informed consent, all newborn infants from the catchment areas of three university hospitals in Finland (Turku, Oulu, and Tampere) were screened for HLA-conferred susceptibility to type 1 diabetes from cord blood samples. Infants who carried HLA genotypes (HLA-DQB1*02/0302 heterozygous and DQB1*0302/x-positive, where x ≠*02, *0301, *0602) conferring high and moderate risk, respectively, for type 1 diabetes (15% of those screened) were invited to participate. Exclusion criteria were children with severe congenital abnormalities or diseases or children whose parents were of non-Caucasian origin or did not understand Finnish, Swedish, or English. The local Ethics Committees approved the study protocol.

The DIPP Nutrition study is being performed in the framework of the Oulu and Tampere sections of the DIPP Study population (n = 7787). The nutrition study started in Tampere in 1997 and in Oulu in 1996. The children were followed for diet, growth, viral infections, diabetes–associated autoantibodies, and special diets at the ages of 3 and 6 months and 1 year, and annually thereafter.

4.2 Study subjects

Study I included all children in the DIPP Nutrition study born between October 1997 and June 2001 in the Tampere area (n = 2125)(Figure 4). In Studies II, III, and IV, special infant formula reimbursements from the Social Insurance Institution were checked in the DIPP Nutrition Study for all children for whom a personal identification code was disclosed to the study (n = 6753). Study II consists of the whole cohort with a known personal identification code (n = 6753). In Study III, the subjects comprised 6288 children for whom a personal identification code was known and who were born between August 1997 and September 2004, which is when information on maternal diet during pregnancy was collected. The lactation study started in August 1998. Dietary data were available from the mothers of 4921 children from 4861 pregnancies. Dietary information during lactation was available for 2940 children from 2915 mothers. Study IV comprised children who had received a reimbursement for special infant formulae, and who were born between October 1997 and May 2004 (n = 398, 6.2% of 6412 children).

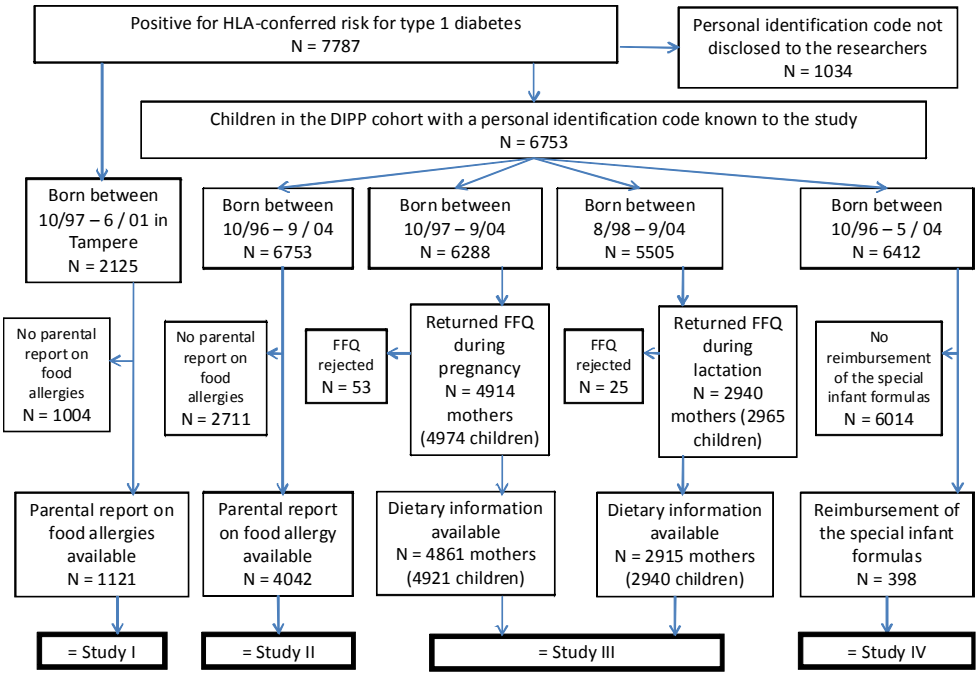

Figure 4. Study design.

4.3 Assessing CMA and validity of questionnaires (I and II)

Food allergies and other special diets were queried at routine study visits when children were aged 6 months, 1 year, and 2 years old with open questions and at 3 years with a structured questionnaire (Appendix I). The questionnaire was pre-tested among a small number of families to ensure that it is comprehensible before putting it to use. Open questions queried if the child was following a special diet at the time of filling in the questionnaire. Examples of the special diets were given: for instance, "milk-free diet" on some and "milk-free or other special diets due to food allergy, gluten-free diet, vegetarian diet without milk but including fish, vegan diet" in other forms. Structured questions included "Has the child ever had CMA, allergy to wheat, barley, rye and/or oats (cereal allergy, CEA), other food allergies?"; "Have commonly allergenic foods been avoided in the child's diet?"; and "Have vegetarian diets and other special diets been used?" (Appendix I). There were also questions for each of the diets about whether the diet was initiated by parents or a healthcare professional and about the age of the child when the diet started and ended. The format of the questionnaire was slightly altered during the course of the study. All information from the questionnaires was double-entered into a database (i.e. recorded twice to ensure the validity of the information). In addition, 10% of all

questionnaires were checked again to ensure valid pre-coding and recording of the answers.

In Study I, patient records of the children attending the DIPP clinic at the Tampere University Hospital and who had an elimination diet reported in the study questionnaire up to the age of three years were reviewed (Figure 4). Information from the patient records was then compared with information reported by the parents about the elimination diets. Children with any special diet reported were included in order to evaluate possible misreporting of food allergies as other special diets. In addition, the hospital database was searched to identify children in the study with an unreported diagnosis of food allergy by using the International Classification of Diseases (ICD-10) codes L27.2 (dermatitis due to ingested food) and K52.2 (allergic and dietetic gastroenteritis and colitis). The patient records of all participants assigned with either of the codes were also reviewed.

In Study II, information on the reimbursement of the special infant formulas up to the child's age of two years was obtained by record linkage from the Social Insurance Institution (figure 4). Parentally reported CMA up to the child's age of two years was validated against physician-diagnosed CMA.

4.4 Dietary methods

4.4.1 Food frequency questionnaire (III)

Maternal diet during pregnancy (8th month) and lactation (3rd month) was assessed by a validated 181-item semi-quantitative food frequency questionnaire (FFQ) (227) (Study III). The FFQ regarding diet during pregnancy was sent to the mothers by mail, and returned at a visit to the study clinic when the child was three months old, at which point it was checked by a trained study nurse. The FFQ regarding diet during lactation was given to the mothers when the child was aged three months and returned similarly at a visit to the study clinic when the child was six months old, again being checked by a trained study nurse. The date of filling in the questionnaire was queried and checked that it reflected the assigned time interval. In practice, there was slight variation in the times of filling in the questionnaire. The FFQ assessed the use of foods or food groups and the consumption frequency (not at all, number of times per day, week, or month) as common serving sizes, such as a glass of juice, two boiled potatoes, 2 dl of mashed potatoes, one slice of bread, 1 dl of muesli, or a piece of fruit. The questionnaire has been specifically targeted to reflect Finnish food consumption habits and had been validated for the present study design. Individual types of fat used in cooking, baking, and salad dressings were taken into account.

The food consumption data were double-entered into a database. If there were 10 or more missing frequencies, the FFQ was rejected (n = 53, 1.1% of FFQs during pregnancy and n = 25, 0.9% of FFQs during lactation). Other missing frequencies

were input as zero. Daily food consumption was calculated with the use of the in-house software of the National Institute for Health and Welfare (228). Two updated versions of the database and recipes were used: the first version for study years 1997-2002, and the second version for the years 2003-2004. Recipe compositions were changed from the first version to the second to reflect changes in food consumption habits and changes in the food market. The changes in recipes were mainly based on food consumption information of women aged 25–44 years from the national dietary surveys FINDIET 1997(229) and FINDIET 2002(149). Recording of the FFQ and the accuracy of the nutrient database of the National Public Health Institute were checked in dietary analysis at food use and nutrient intake levels.

4.4.2 Food records and adherence to a cow's milk protein-free diet (IV)

Three-day food records of the children in the cohort were collected at the ages of 3 and 6 months and at 1, 2, and 3 years (Study IV). Parents were given written and oral instructions on how to fill in the food records and were asked to record everything the child ate and drank during the recording period. Food consumption at daycare was also recorded on separate food record forms, inquiring also about the origin of the food. The three consecutive food record dates were assigned to the family in advance, containing two weekdays and one weekend day. Examples were given on how to report food consumption with household or weighed measures. Vitamin and mineral supplement usage was also recorded in the food records. Information on duration of overall and exclusive breastfeeding and long-term use of different types of infant formulae was gathered by questionnaires simultaneously.

Trained research nurses checked the food records with the families. In checking the food records, food picture booklets were used to help in estimating portion sizes (230, 231). Trained nutrition researchers entered all food consumption data into a food diary database. An in-house software and the Finnish national food composition databank Fineli were used for the data entry (228). Food consumption and nutrient intakes, as well as the amount of CMP, were calculated as average daily intake. The amount of CMP in sausages, fat spreads, and commercial biscuits could not be taken into account when calculating the exact amount of CMP in the food records. However, even the smallest amounts of CMP were taken into account when classifying adherence by going through the paper copies of the food records. The recording of food records was checked at food use, ingredient use, and nutrient intake levels, as well as for sources of CMP, to ensure valid recording and the validity of the databank used.

After gathering information on the month and year of the diagnosis of CMA from the Social Insurance Institution, the next food record within six months was assessed to determine the degree of individual adherence to the cow's milk

elimination diet (Study IV). Food records were classified as containing milk products, containing hidden or small amounts of CMP, containing no CMP, and containing no obvious sources of CMP, but including recipes for which some uncertainty exists about whether or not they contain CMP. Uncertain food items comprise for example, bakery products from small local bakeries and meat product manufacturers for which the detailed composition could not be traced. Also, in some cases, a commercial product name was not identified in the food record. To further study the reintroduction of milk products into the diet, subsequent food records of the same children either up to a food record containing milk products, up to the last food record, or up to three years of age were further analyzed (Study IV). As additional information, mothers were queried whether they followed any special diets during the breastfeeding period when the infant was approximately three months old. A total of 185 breastfeeding mothers reported avoidance of cow's milk products.

4.5 Sociodemographic and other characteristics of the study population

Sociodemographic and other background characteristics were queried in order to control for them in the statistical analyses as confounding factors. Families reported mothers and father's occupation, basic and vocational education, and age at birth of the child in structured questionnaires at recruitment. Information on place of residence at recruitment, pregnancy and delivery complications, gestational age, birth weight and height, earlier deliveries, and maternal smoking during pregnancy was received from the Finnish Medical Birth Registry. Parental allergic rhinitis and asthma were queried when the child was aged five years with a modified ISAAC questionnaire, including questions on mother's and father's asthma, allergic rhinitis, and atopic eczema (232). The questionnaire also contained questions about pet-keeping and farm animal contacts during the child's first year of life.

4.6 Statistical analyses

In Study I, the SPSS 12.0.1 software package (SPSS, Chicago, IL, USA) was used for data handling and statistical analyses. Reported special diets were classified into allergic and non-allergic categories. Avoidance of "commonly allergenic foods" was not considered to be due to diagnosed allergy. Agreement between reported and diagnosed food allergies was measured by means of cross-tabulation with Cohen's Kappa. Statistical tests were calculated for worst-case and best-case scenarios, i.e. 1) none of the children with insufficient information from the patient records would be diagnosed with food allergy and 2) all of the children with insufficient information from the patient records would be diagnosed with food allergy.

In Study II, cross-tabulation with Cohen's Kappa was also used to measure agreement between reported and diagnosed CMA. Sensitivity, specificity, positive

predictive value, and negative predictive value were also calculated. Analyses were performed with the SAS statistical program (SAS Institute Inc., Cary, NC, USA, version 8.2).

In Study III, associations between background factors and CMA were tested with logistic regression. The associations between maternal diet and CMA in the offspring were evaluated using a generalized estimating equation model for logistic regression. The particular model was used to account for correlations between siblings. Food consumption was divided into quarters, and highest and lowest quarters were compared with the combined middle quarters. Adjustments for possible confounding factors were done in two phases: first, adjustments were made for study area, gender, and birth weight of the child, maternal age and education, maternal smoking during pregnancy, type of delivery, number of older siblings, and length of breastfeeding, and second, we added maternal allergic rhinitis or asthma, urbanity of living environment, duration of gestation, season of birth, and visits to a stable and pet keeping during the first year of life. In the final model, maternal diet during lactation was adjusted for maternal diet during pregnancy. Confounding was handled in this sequential manner because information on parental allergic background and pet keeping was not queried until the child was five years old and not all families provided information at this point. Two-sided tests were used. Analyses were conducted with an SAS statistical package (SAS Institute Inc., Cary, NC, USA, version 9.1). Multiplicity issues were taken into account in cautious interpretation of the results.

In Study IV, statistical analyses were done using the SPSS software package 15.0 (SPSS Inc, Chicago, IL, USA, 2006) and R 2.7.0 (R Development Core Team, 2008). Multinomial logistic regression was applied to evaluate the associations of adherence to the therapeutic elimination diet with background factors. A Cox proportional hazards model with an accommodation to interval censored data was used to identify the factors associated with age of recovery.

In all analyses, p-values lower than 0.05 were considered statistically significant.

4.7 Ethical considerations

The purpose of the DIPP study was disclosed to the families prior to their making a decision to participate. Families were also informed that they were free to withdraw from the study at any point without explanation should they choose to do so. The food allergy section of the DIPP nutrition study involved no interventions, tests, or extra contacts with the participants; information was based on questionnaires, and validation studies required no additional participation. Thus, investigating food allergies in the DIPP nutrition study did not add any burden to participants. The DIPP Nutrition study protocol was approved by the Ethics Committees of the Northern Ostrobothnia and Pirkanmaa Hospital Districts. All parents gave their fully informed written consent.

5 Results

5.1 Cumulative incidence of CMA and special diets (I and II)

In the first validation study, 1122 questionnaires (52.8% of the 2125 initially invited) inquiring about special diets of children were returned by the parents at a three-year visit to the study clinic. Of the children, 52.8% were boys and 47.2% girls. Any food allergy was reported by the parents of 168 children (15.0%) and any special diet by 241 (21.5%) during the first three years of life. CMA was reported by parents of 103 (9.2%). Of the CMA elimination diets, 83.5% had been initiated by a heath professional, according to parents. CMA was diagnosed at the hospital for 6.3% of the study population. In 2.9% of the children, the diagnosis was possibly made by private practitioners or in primary healthcare, both of which are current practice in Finland.

In the second validation study consisting of the whole cohort for whom a personal identification code was given to the researchers (n = 6753 children), CMA was seen in 6.2%. Parents of 4042 children (59.9% of 6753) responded to the question about special diets for their children. In these questionnaires, CMA was reported for 10.5% of the 4042 children, and 7.6% of these 4042 had received a reimbursement for special infant formula by the age of two years.

5.2 Validity of the questionnaires (I and II)

The first validation study (Study I) concentrated on the structured questionnaire administered when the child was three years old (Appendix I). In this study, 263 children fulfilled the criteria of either a parentally reported special diet in a questionnaire administered at the child's age of three years (n = 241) or a diet not reported, but having a diagnosis code (ICD-10 K52.5 or L27.2) in the hospital database (n = 22). The patient records of these children were reviewed. Of the 263 children, 118 had visited the hospital because of food allergies. In addition, 50 children had visited the hospital for reasons other than food allergy, and for 28 of them patient records showed reasonable proof of food allergy diagnosed elsewhere. Searching the hospital database for food allergy-related diagnosis codes revealed an additional 22 children with a diagnosis code indicating a probable food allergy. Ten of these did have a food allergy and seven had CMA diagnosed at the hospital: the rest had visited the hospital for a suspicion of food allergy. Food allergies were unreported by 0.9% of this cohort. However, six of the seven unreported CMAs had been reported on questionnaires administered at an earlier age.

By the age of three years, 119 children (10.6% out of 1122) had been diagnosed with food allergy according to the patient records. Parents had reported food allergy in 168 children, 101 (60.1%) of whom had received the diagnosis at the hospital.

Other special diets (lactose intolerance, celiac disease, avoidance of "commonly allergenic foods", or special diet not specified) besides food allergy were reported by the parents of 73 children. Eight of these children had been diagnosed with food allergy at the hospital, but parents had not reported this as food allergy.

Forty-six (27.5%) of the 168 children for whom food allergy was reported had not visited the hospital at all. Possibly they had been diagnosed in primary healthcare or by private practitioners. Another possibility is that their food allergy had not been diagnosed by a physician. For these 46 children, we lacked sufficient data to conclude whether the child had been diagnosed with food allergy or not. Cohen's Kappa between reported and diagnosed allergies, calculated as worst-case and best-case scenarios with regard to insufficient data, was within a range of 0.71-0.88 for CMA, 0.74-0.82 for CEA, and 0.66-0.86 for any reported food allergy ($p < 0.001$ for all). The diagnosis of CMA was confirmed in hospital records for 62% and the diagnosis of CEA for 69% of the children whose parents had reported it (Table 5).

Table 5. Cumulative incidence of cow's milk allergy (CMA) up to the age of 2 years in the DIPP Nutrition cohort as reported by parents. Also noted is whether the diagnosis was found in patient records or in registers of the Social Insurance Institution.

Study I, n = 1122	CMA reported by parents (n)	CMA not reported by parents (n)	Total
Diagnosis of CMA in patient records	64	7	71
Diagnosis of CMA not found	32	1019	1051
Total	96	1026	1122

Study II, n = 4042	CMA reported by parents (n)	CMA not reported by parents (n)	Total
Special reimbursement	294	13	307
No special reimbursement	130	3605	3735
Total	424	3618	4042

The basis of the diagnosis was also reviewed, although this was not the main aim of the study. Of the 71 children diagnosed in the hospital with CMA, 41 (57.7%) had had a positive oral challenge test to confirm the diagnosis. The diagnosis of the remaining children had been based on a combination of suggestive history and skin prick tests, analyses of serum-specific IgE, or patch tests.

In the second validation study (Study II), 6.2% (419 of 6753 children) had received a reimbursement for special infant formulas (Figure 5.). Among these children, parents of 59.9% (4042 of 6753 children) had answered the question about food allergy in the questionnaires. Parents reported CMA for 424 children (10.5%),

and 307 children (7.6%) had received a reimbursement for the costs of special infant formulas (Table 5).

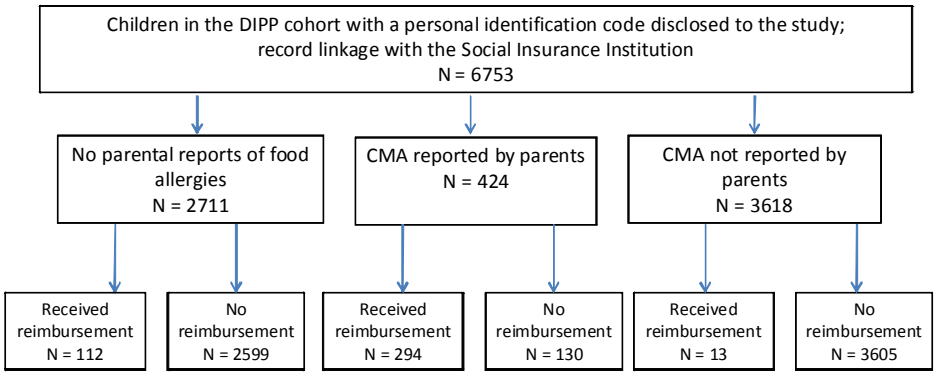

Figure 5. Parentally reported cow's milk allergy (CMA) and whether a reimbursement of special infant formulas was received from the Social Insurance Institution.

Parentally reported information on infant formulas used and length of breastfeeding was checked for those 130 children (3.2%) whose parents reported CMA, but the child did not have a record of the reimbursement in the registers of the Social Insurance Institution. For 83 of the 130 children, a plausible explanation was identified. Fifty-two children had been breastfed for one year or more. Forty-six children had become allergic to milk after the age of one year. In these cases, special formula is not always needed during milk elimination. Finally, the parents of eight children had reported an elimination diet lasting less than three months, and these children most likely had been on a diagnostic elimination diet. At the same time, CMA went unreported in 13 children (0.3%). Five children had been on a special infant formula according to parental reports, but for some reason parents did not note that the child had CMA.

Kappa test for parental report against reimbursement for special infant formulas was 0.79, sensitivity 0.958, specificity 0.965, positive predictive value 0.693, and negative predictive value 0.996. The exclusion of children for whom a short elimination diet (less than three months) was reported did not essentially change the results. Validity of the questionnaire varied slightly according to background factors. The questionnaire seems to work better for older and more highly educated mothers (Table 6). Our validation does not detect those children breastfed for more than one year who do not apply for reimbursement of the special infant formula, because they are not included in the registers of the Social Insurance Institution. Kappa value seems lower for this group than for those breastfed for less than 1 year.

Table 6. Kappa values, sensitivity, and specificity of the questionnaire with parental reports compared with physician-diagnosed cow's milk allergy (CMA) as reported by parents according to selected background factors. *SII = Social Insurance Institution

		Distribution n (%)	Children with parentally reported CMA, n (%)	Children with CMA according to SII* registry, n (%)	Kappa	Sensitivity	Specificity
Length of overall breastfeeding, months	<6	1473 (36)	157 (11)	131 (9)	0.86	0.962	0.977
	6-11.9	1692 (42)	177 (10)	133 (8)	0.82	0.977	0.970
	≥ 12	805 (20)	73 (9)	34 (4)	0.51	0.853	0.943
Age of mother at delivery, years	<25	655 (16)	53 (8)	38 (6)	0.68	0.842	0.966
	25-29.9	1434 (35)	155 (11)	112 (8)	0.79	0.964	0.964
	30-34.9	1221 (30)	137 (11)	101 (8)	0.81	0.980	0.966
	≥35	732 (18)	79 (11)	56 (8)	0.80	0.982	0.964
Maternal vocational education	No professional education	194 (5)	17 (9)	13 (7)	0.64	0.769	0.961
	Vocational school or course	1026 (25)	80 (8)	59 (6)	0.82	0.983	0.977
	Upper secondary vocational education	1806 (45)	204 (11)	148 (8)	0.78	0.953	0.962
	Academic education	929 (23)	117 (13)	82 (9)	0.78	0.976	0.956
All		4042	424	307	0.79	0.958	0.965

5.3 Associations between maternal diet during pregnancy and CMA in offspring (III)

High maternal consumption of cow's milk and milk products was associated with a lower risk of CMA (OR 0.56, 95% CI 0.37-0.86; p=0.002; Table 3 in Study III). The associations were not restricted to a specific fat content of milk products or to fermented vs. non-fermented products. In addition, low consumption of fish in general was associated with a higher risk of CMA, but the association did not reach significance after adjustment for maternal and paternal allergic rhinitis or asthma. However, maternal consumption of lean fish was inversely associated with CMA in offspring (Table 7).

The associations between maternal diet and CMA in the child were also analyzed separately in mothers with and without allergic rhinitis or asthma. In these analyses, the inverse association of high maternal milk consumption and CMA was seen in the offspring of non-allergic mothers (OR 0.30, 95% CI 0.13 – 0.69, $p < 0.001$) (Figure 1 in Study III). This association was not seen in the children of allergic mothers (OR 0.76, 95% CI 0.45 – 1.29, $p = 0.330$).

Maternal consumption of fish in total was associated with risk of CMA in the offspring of allergic mothers (OR 1.47, 95% CI 0.96 - 2.27 for the lowest quarter, p-value for term including both highest and lowest quarters = 0.043), but the association was not evident in the offspring of non-allergic mothers (OR 1.15, 95% CI 0.69 - 1.92 for the lowest quarter, $p = 0.826$) (Figure 6). In addition, the use of vegetable oil -based margarines was associated with a lower risk of CMA in children of non-allergic mothers (OR = 0.64, 95% CI 0.42-0.97, $p = 0.043$), in comparing users with non-users.

Dietary intakes of food items and food groups did not differ between allergic and non-allergic mothers, except for slight differences in the consumption of apple (allergic mothers consumed 42.8 g and non-allergic mothers 44.3 g per day, $p = 0.009$) and vegetables and roots (allergic mothers consumed 234 g and non-allergic mothers 221 g per day, $p = 0.016$).

Adjusting maternal food consumption during pregnancy for maternal food consumption during lactation did not remove the association between maternal milk consumption during pregnancy and CMA in offspring (OR 0.30, CI 0.13-0.68, $p = 0.014$). Likewise, the association remained after adjusting for age at introduction of milk products to the infant's diet (OR 0.59, CI 0.38-0.92; $p = 0.011$).

Table 7. Risk (odds ratio (OR) and 95% CI) of cow's milk allergy in the offspring associated with maternal consumption of fat-yielding foods during pregnancy. P-value is for the term including both highest and lowest quarters.

		Maternal diet during pregnancy					
		Unadjusted		Adjusted [1]		Adjusted [2]	
		OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
		n = 448 / 4921		n = 409 / 4014		n = 222 / 2189	
Fish and fish products in total	1st	1.15 (0.92-1.45)		1.27 (0.99-1.61)		1.30 (0.93-1.80)	
	2nd & 3rd	1	0.1242	1	**0.0458**	1	0.1035
	4th	0.87 (0.67-1.11)		0.86 (0.66-1.12)		0.85 (0.59-1.22)	
Fatty fish	1st	1.01 (0.81-1·26)		1.07 (0.84-1.36)		1.29 (0.94-1.76)	
	2nd & 3rd	1	0.4232	1	0.3590	1	0.3034
	4th	0.86 (0.67-1.11)		0.86 (0.66-1.13)		1.13 (0.79-1.63)	
Lean fish	1st	**1.29 (1.04-1.59)**		**1.29 (1.03-1.60)**		1.29 (0.95-1.74)	
	2nd & 3rd	1	**0.0007**	1	**0.0013**	1	**0.0215**
	4th	0.78 (0.60-1.02)		**0.73 (0.55-0.97)**		0.75 (0.51-1.11)	
Butter and butter spreads	1st	0.94 (0.74-1.19)		0.88 (0.68-1.15)		1.09 (0.78-1.53)	
	2nd & 3rd	1	0.4811	1	0.2703	1	0.5466
	4th	0.86 (0.67-1.10)		0.82 (0.63-1.06)		0.86 (0.59-1.25)	
Vegetable fat spreads	0	1	0.2265	1	0.2558	1	0.3758
	1	0.88 (0.73-1.08)		0.88 (0.72-1.09)		0.88 (0.66-1.17)	
Vegetable oils	1st	1.00 (0.79-1.27)		1.04 (0.80-1.35)		1.10 (0.78-1.56)	
	2nd & 3rd	1	0.9979	1	0.8315	1	0.4113
	4th	1.00 (0.79-1.26)		0.95 (0.73-1.22)		0.84 (0.60-1.19)	

[1]Adjusted for energy intake, study centre, gender, birth weight of the child, maternal age and education, maternal smoking during pregnancy, type of delivery, number of older siblings, and length of breastfeeding.

[2]Adjusted for maternal allergic rhinitis or asthma, duration of gestation, season of birth, urbanity of living environment, visits to a

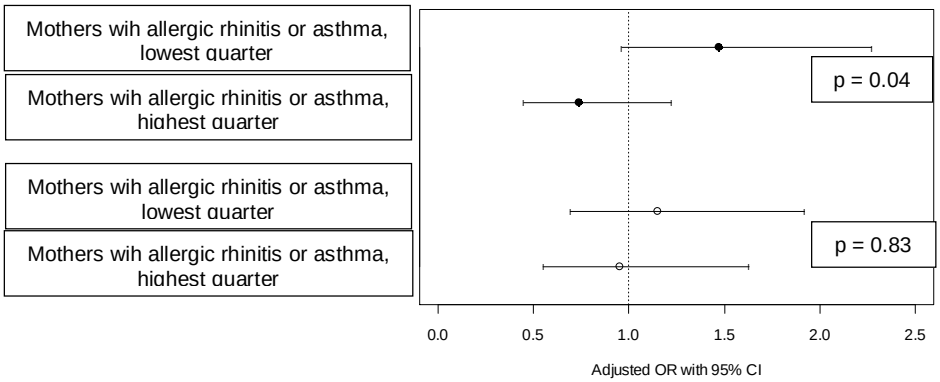

Figure 6. Maternal fish consumption during pregnancy and risk of CMA, stratified according to maternal allergic rhinitis or asthma. P-value is for the term including both highest and lowest quarters.

5.4 Associations between maternal diet during lactation and CMA in offspring (III)

We detected no association between maternal cow's milk intake during lactation and CMA in offspring (Table 3 in Study III). The most pronounced association was seen in maternal consumption of citrus fruit and kiwi, where use compared with non-use was associated with a lower risk of CMA (OR 0.54; 95% CI 0.34-0.86; p = 0.015 in adjusted model). By contrast, high maternal use of fruits and berries in general was associated with a higher risk of CMA, although this association was significant only in the model where diet during lactation was adjusted for diet during pregnancy in addition to other confounding factors.

5.5 Associations between sociodemographic factors and CMA in offspring (III)

Parental asthma and allergic rhinitis were associated with a higher risk of CMA in children (Table 1 in Study III). Maternal smoking was associated with a lower risk of CMA, and so was having pets inside the house during the first year of life. In addition, CMA was more common among boys than girls, and in children of highly educated and older mothers compared with those with a lower level of education and a younger age at delivery. Furthermore, high birth weight was associated with a greater risk of CMA. Season of birth, having older siblings, duration of gestation, type of delivery, study center, urbanity of the place of residence, visiting a stable during the first year of life, and duration of overall breastfeeding were not associated with risk of CMA.

5.6 Sensitivity analysis (III)

The reliability and suitability of our data for the analysis were checked in several ways. In defining the endpoint, we compared register-based and parentally reported CMA separately and combined, and the results were essentially the same (Study III; data not shown). A comparison between groups conferring high and moderate risk for type 1 diabetes revealed no differences in cumulative incidence of CMA ($p = 0.85$). The cumulative incidence of CMA was 8.3% (80/966) in the group with a high risk (HLA genotype HLA-DQB1*02/0302 heterozygous) and 9.3% (368/3955) in the group with a moderate risk (DQB1*0302/x-positive, where x ≠*02, *0301, *0602). Maternal use of milk products showed slightly different associations between the groups in stratified analyses; in the high-risk group in the lowest quarter OR was 1.25 (95% CI 0.51-3.1) and in the highest quarter 1.32 (95% CI 0.50-3.53, $p = 0.796$), whereas in the moderate-risk group in the lowest quarter OR was 1.26 (95% CI 0.87-1.83) and in the highest quarter 0.48 (95% CI 0.30-0.79, $p < 0.001$).

Because the number of children was reduced when adding more variables to the models while adjusting for potential confounders, we checked whether the differences in results were due to selective drop-out or due to the variables for which we adjusted. The final analysis of the pregnancy dietary data included 2189 mother-child pairs of the initial 6288 who were invited. This was done by comparing the different adjustments within the group in which we did the final analysis, which included all of confounding variables. This comparison suggests that the differences in results between adjustments are not due to selection but to the variables used in the adjustment (data not shown).

To further investigate the U-shaped association between maternal cereal consumption during pregnancy and CMA in offspring, the analysis was repeated omitting those with allergy to wheat, barley, or rye. The omission did not essentially change the association between maternal cereal intake and CMA in offspring.

Comparison between those who did or did not return the questionnaires regarding maternal diet during pregnancy and lactation and in the results of the questionnaire administered at the child's age of five years revealed that firstborns, highly educated persons, non-smokers, individuals born in Tampere area, and individuals living in an urban environment are overrepresented in the analysis (data not shown). The trends were similar with regard to the response to all the three questionnaires. In addition, drop-out rate was higher among children who did not have a CMA diagnosis, compared with children who were diagnosed with CMA according to the records of the Social Insurance Institution (data not shown).

5.7 Diet of children with CMA (IV)

Food records within six months from a diagnosis of CMA were available for 67% of the children within the time limit. Of the 267 children whose food records were studied for adherence to the therapeutic elimination diet, 226 had followed the diet

extremely accurately (Figure 7). In addition, among the breastfeeding mothers of infants with diagnosed CMA, 16% (29/185) had avoided cow's milk while breastfeeding.

Eight children had used actual milk products. These milk products comprised cheese, ice cream, infant formulas, and milk-containing infant porridges and gruels. They had a mean intake of 350 (range 76 – 1136) g of milk products per day during the three study days. The amount of CMP ranged from 2.0 to 36.5 g, with a mean intake of 8.5 g and median intake of 4.0 g. Small amounts of CMP were seen in the diet of 14 children, possibly as advised. These food items were industrial baby foods, bakery and meat products, and fat spreads containing CMP as an ingredient, cookies with milk chocolate, or a few cheese puffs. In these 14 children, the mean intake of milk products was 6.8 (range 0.0 – 42.7) g per day during the three days. The amount of CMP from these foods ranged from 0.0 to 9.1 g, with a mean intake of 1.0 g and a median intake of 0.02 g per day. In addition, in 19 children no obvious sources of CMP were found, but the food records included some recipes or foodstuffs for which there is uncertainty about whether or not they contained CMP. Examples of these uncertain food items are meat and bakery products for which a recipe was not known, or meat or bakery products or ready-to-use baby foods for which the commercial name was not specified. For three children, the parents had reported an ongoing oral challenge test with milk. They were classified as adherent and their CMP intake was not included in the figures mentioned above. Four of the eight children who had used milk products had also used special infant formulas.

Intake of energy and nutrients was studied in children who were not breastfed. Of the total energy intake of non-breastfed children, 96% at the age of three months, 48% at the age of six months, 34% at the age of one year, and 18% at the age of two years was derived from special infant formulas.

Intakes of energy-yielding nutrients, calcium, riboflavin, and vitamin D (including intake from both diet and supplements) are presented in Table 8. Mean intakes of calcium were below recommendations, but due to supplementation, mean intakes of vitamin D were sufficient. In addition, the intakes of riboflavin and calcium were especially low in 89 food records, which contained neither milk products nor special infant formulae (data not shown).

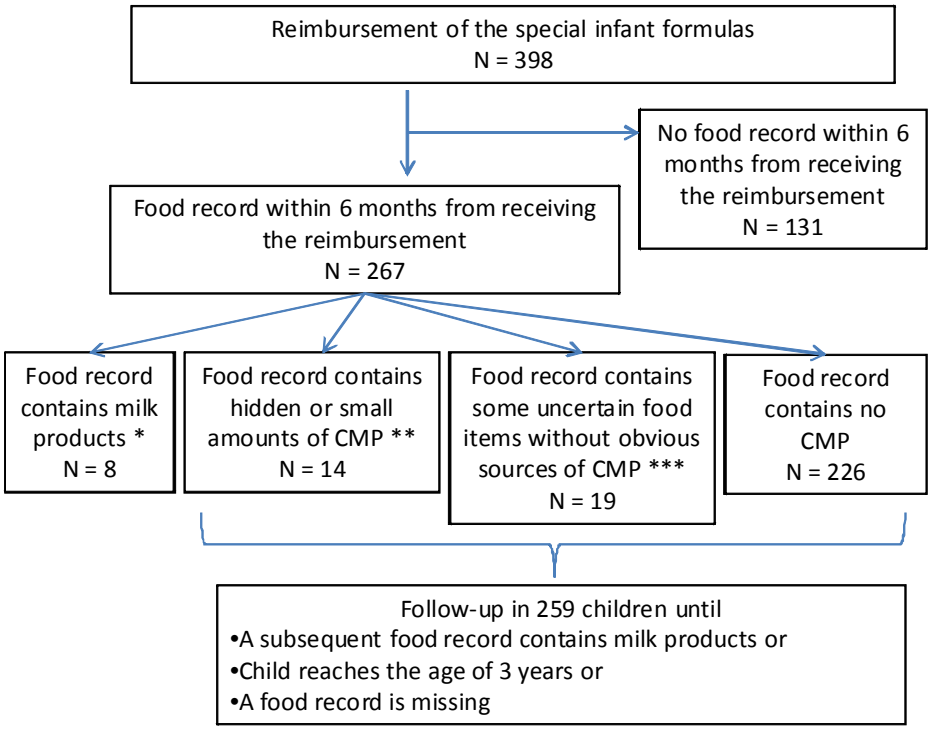

Figure 7. Adherence to a cow's milk protein (CMP) elimination diet within six months after the diagnosis of cow's milk allergy (those who had reported an ongoing oral challenge test with cow's milk are not included).

*Milk products used were cheese, ice cream, infant formulas, and milk-containing infant porridges and gruels.
**CMP in food items such as industrial baby foods, bakery and meat products, fat spreads containing CMP as an ingredient, cookies with milk chocolate, or a few cheese puffs.
***Meat and bakery products for which a recipe was unknown or meat or bakery products or ready-to-use baby foods for which the commercial name was not specified.

Table 8. Adherence to the cow's milk elimination diet study: mean (SD) intakes of energy and certain nutrients (intake from foods and supplements together) of non-breastfed children by age within six months of receiving a reimbursement for the special infant formulas, studied by 3-day food records.

	3 months n = 22	6 months n = 67	1 year n = 89	2 years n = 4
Energy intake, kJ	2430 (277)	2800 (554)	3910 (760)	4370 (325)
Protein intake, % of energy	10.2 (0.8)	10.2 (1.6)	12.2 (2.7)	12.1 (2.9)
Carbohydrate intake, % of energy	40.7 (5.3)	50.8 (7.4)	55.0 (6.7)	56.1 (5.5)
Fat intake, % of energy	49.0 (5.1)	38.7 (7.0)	32.4 (7.2)	31.2 (7.7)
Calcium intake, mg	467 (60)	430 (130)	473 (191)	780 (248)
Recommended intake of calcium, mg		540	600	600
Vitamin D intake, µg	14.7 (4.2)	14.1 (4.0)	13.3 (4.2)	12.3 (10.7)
Recommended intake of vitamin D, µg		10	10	7,5
Riboflavin intake, mg	0.84 (0.26)	0.74 (0.25)	0.81 (0.38)	1.14 (0.55)
Recommended intake of riboflavin, mg		0.5	0.6	0.7

Parental age and education, number of siblings, gender, duration of breastfeeding, urbanity of place of residence, and study center were not associated with adherence to the therapeutic elimination diet. Older age of the child increased the risk of having small amounts of CMP in the diet. Also those who did not have food allergies other than CMA more often had small amounts of CMP in their diet than multisensitized children ($p < 0.001$ for both; adjusted for maternal age and education, number of siblings, sex, duration of breastfeeding, and study center).

To study the reintroduction of milk products into the child's diet, subsequent food records were reviewed for children who had initially adhered to a CMP-free diet. Of the 259 children who had followed the CMP-free diet, subsequent food records were available for 215. The food records of these children were then observed further. The presence of CMP in the elimination diet within six months of receiving the reimbursement did not predict the age at reintroduction of CMP to the diet. In addition, place of residence, sex, and parental age or education level were not associated with the age of re-introducing cow's milk products. The children of smoking mothers were reintroduced to milk products at a younger age than the children of non-smoking mothers, when adjusted for sex and age of the child, adherence to the elimination diet, maternal education and age, duration of breastfeeding, and study center ($p < 0.05$).

The main milk products used after the period of milk elimination, documented by 3-day food records, were milk (87% of children), cheese (67% of children), and yoghurt (45% of children). However, amounts of milk products used were small, and only 17% of children consumed three glasses of milk, 23% used at least two slices of cheese, and 15% used at least 1 dl of yoghurt daily.

6 Discussion

6.1 Strengths and weaknesses of the study

6.1.1 Study design and endpoints

A prospective birth cohort study is the strongest possible study design for research questions for which randomized trials are not feasible. It allows multiple exposures and outcomes to be assessed simultaneously. When very little is known about a particular subject, one must first search for possible risk or protective factors before moving on to trials in which only one or two factors can be studied simultaneously. In the case of food allergy in children, the association between maternal diet during pregnancy and lactation has been studied very little. A cohort must be large enough to detect associations between exposures and outcomes and to find potential interactions. A major strength of the present study is the large cohort in which the study questions were addressed. Altogether 6288 children born between 1997 and 2004 were recruited for the long-term follow-up.

The prospective nature of the study diminishes the possibility of recall bias. To investigate maternal diet during pregnancy, FFQs were administered retrospectively after the child was born (Study III). This was necessary because the mothers were recruited after delivery, based on the results of a test from a cord blood sample. In this particular setting, it is possible that the newly gained knowledge about an infant's increased risk for type 1 diabetes would lead to recall bias of diet during pregnancy. However, the validity of the FFQ has proven to be good in the same setting as that used in the DIPP nutrition study (227). The questionnaire reflects the 8^{th} month of pregnancy, which was chosen because it is the last month before maternity leave and most likely represents the dietary habits of the mother throughout pregnancy. Apart from the FFQ for pregnant women and a structured questionnaire asking about special diets in children at three years of age, all information from parents was gathered prospectively. Researchers working with recording and checking of any of the information were unaware of the disease status of the children, minimizing the risk of bias in interpretation.

Loss to follow-up is a common problem in cohort studies. The response rate in our study was moderate. In the first validation study (Study I), 53% of those initially invited to participate did so at the age of three years. When studying the elimination diet of children with CMA, food records within six months of the diagnosis of CMA were available for 67% within the time limit (Study IV). A downside was that those children who had received the diagnosis right after the age of one year could not be included because the next food record was requested at the age of two years.

However, this did not cause a selection bias with regard to any of the background factors.

When investigating the associations between maternal diet and future CMA in offspring, mothers of nearly 5000 children chose to participate in the DIPP Nutrition study and to return the FFQ concerning diet during pregnancy (Study III). Unfortunately, the information gathered at five years of age was available for only one-third of the cohort, affecting the number of endpoints in some of the analyses. Thus, selective drop-out may potentially affect our results. The background factors in which there were differences in loss to follow-up (e.g. smoking and education level of the mother) leaves us with a study population that is more likely to express health-seeking behaviour than those who were no longer taking part in the study, but also with a study population that may be more susceptible to developing allergic diseases. Accordingly, drop-out rate in the cohort was higher in children without a CMA diagnosis than in those with a diagnosis, which may distort the figures of cumulative incidence of food allergy in the cohort. In fact, the cumulative incidence of CMA may be somewhat higher than found in two other Finnish cohort studies (2, 3) as well as in studies from other Western countries (32-34).

The number of endpoints (n = 529 in the whole cohort, n = 448 for diet during pregnancy and n = 168 for diet during lactation) was sufficiently large to evaluate the associations between maternal diet during pregnancy and lactation and CMA in offspring, as well as several associations with sociodemographic background factors. Even after adjusting for the information from the five-year questionnaire, the number of endpoints was sufficient (n = 222 for diet during pregnancy and n = 92 for diet during lactation). The number of analyses conducted between different dietary aspects and CMA is quite large. Therefore, cautious interpretation of the results is necessary. Only those results that are systematic and appear at each step of the adjustments are considered relevant, despite statistical significance.

Information must be gathered about numerous potential confounding factors in order to examine specific questions. In large cohort studies, researchers are not always aware at the beginning of the study of all the interesting issues that may emerge during a long follow-up. Because of this, the gathered information on exposures is not always optimal for all outcomes, and sometimes information may lack adequate detail. An example is lack of knowledge about consumption of foodstuffs containing probiotics because they were not on the market when the FFQ was designed. In addition, the questions regarding CMA were added to the study protocol approximately two years after the beginning of the study, so that the structured questionnaire investigating food allergies was distributed to all those who still took part in the study at the age of three years.

Another limitation of our study is having to rely on register-based information on CMA and not knowing the symptoms or the immunological route through which the symptoms are presented. IgE- and non-IgE-mediated diseases may have different etiological factors, a fact which could not be accounted for in our study. CMA was

chosen as the outcome in the etiological study because it is the most rigorously diagnosed; other food allergies were therefore not included. Because CMA was not diagnosed by the research staff, some uncertainty about the basis for the diagnosis is unavoidable (233). However, in a large birth cohort study, this is the only feasible way to define the endpoint. Register-derived information on CMA was complemented with information from parental reports because those children who are breastfed beyond the age of one year and those who receive the diagnosis of CMA after the age of one year usually do not need a special infant formula if no other staple foods are eliminated from the diet and the child is growing according to expectations. Therefore these children are not in the reimbursement registers of the Finnish Social Insurance Institution. We compared the endpoints (register-based and parentally reported) separately and in different combinations, and the results were essentially the same.

6.1.2 Subjects and generalizability of the results

The subjets were recruited among families who gave birth at two University hospitals, Oulu and Tampere. Apart from isolated exceptions, all mothers in Finland deliver their babies in hospitals. Thus the coverage of the study population within each study area is good. Dietary habits differ in different regions of Finland (109), as do other lifestyle factors. Having two separate study areas in different parts of the country, however, increases the representativeness of the study population. This could affect the generalizability of our results.

The most cumbersome limitation of this study is that the cohort was primarily gathered to evaluate the risk factors for type 1 diabetes and not to investigate CMA. The primary aim of the DIPP Nutrition study is to evaluate dietary predictors of β-cell autoimmunity. Secondary aims of the DIPP Nutrition study include evaluation of dietary predictors of allergic diseases and asthma.

All of the subjects carry an increased HLA-conferred risk for type 1 diabetes. No studies report the coexistence of CMA and type 1 diabetes in a general population. Some studies have found an inverse association between type 1 diabetes and some forms of allergic diseases (234, 235), while others suggest a positive association between allergy and diabetes (236). An increased intestinal permeability has been suggested in those with the HLA-DQB1*02 allele (237), representing approximately one-quarter of our cohort (226). Increased intestinal permeability has also been reported in persons with pre-type 1 diabetes and clinical type 1 diabetes, as well as in their relatives, but these studies do not describe the genetic background of their study population (238-240). Despite all of the confirmatory analysis, the generalizibility of our results to the general population may be compromised.

Furthermore, knowledge of the higher risk for type 1 diabetes may alter the families' behavior. They may be more eager to seek medical assistance, and thus, increase the likelihood of receiving a diagnosis of CMA and following medical

advice, e.g. a therapeutic elimination diet of children with CMA (Study IV). Knowledge of the increased risk for type 1 diabetes may also alter dietary habits. The likelihood of altering behavior due to gaining knowledge of the increased risk for type 1 diabetes is greatest just after receiving the information. It could affect maternal dietary habits during lactation, length of breastfeeding, and infant feeding patterns. All of the aforementioned factors could alter the risk of CMA.

6.1.3 Food frequency questionnaire and food record in studying food consumption and dietary intake of nutrients

A self-administered FFQ is the most useful tool for evaluating the diet of a large subject group. However, like all of the methods for studying a diet, the FFQ does not come without flaws. The FFQ has a tendency to overestimate food consumption and nutrient intake (241). However, when the same questionnaire is administered to the whole study population, it can be used to rank individuals, i.e. to identify individuals with high and low intakes compared with the others, and to examine the associations of different food consumption habits with disease outcomes. Report bias usually leads to underreporting of unhealthy food items and overreporting of what are thought to be healthy food items. In addition, obese individuals tend to underreport more than their lean counterparts. Therefore, absolute food consumption should be used with caution; energy-adjusted intakes are preferred instead. Furthermore, an FFQ is not optimal for investigating food items that are rarely consumed, or in exploring the diet overall; however, it is well suited for study of specific food groups such as milk products.

The FFQ used in Study III has been specifically designed and validated to investigate the diet of pregnant Finnish women (227). The validation study showed the FFQ to be a valid tool for measuring the diet of pregnant women in the precise population and setting that were used. The food consumption measured by the FFQ correlates well with that measured by two 5-day food records. In the validation of the FFQ, the correlation between the FFQ and food records for the use of low-fat milk products was 0.86, the use of fish 0.44, and the use of berries 0.68; the correlations for oils and low-fat spreads were the lowest (Pearson correlation coefficients 0.22 and 0.25, respectively). The FFQ was also shown to be reproducible with repeated measurements. Cross-classification in fifths revealed decent correlations for all food items and food groups (227).

If the FFQ has the disadvantage of possible recall bias, the food record overcomes this because it is filled in prospectively. However, other types of biases are incurred with the use of food records (241). Parents may alter their child's dietary habits while keeping the food record to provide an embellished picture of food consumption habits or to make it easier to fill in the food record. However, when examining adherence to a therapeutic elimination diet in children with CMA, food records were collected without the parents knowing that adherence to a CMP-

free diet was being investigated, so embellishment in this regard is unlikely (Study IV). Another disadvantage of using food records is considerable intraindividual variation in food consumption, and thus, nutrient intake (242). Weekdays especially differ from weekends, which is why the subjects in our cohort were advised to keep the food record for two weekdays and one day on the weekend. Three-day food records are accurate enough to gather information on a group level regarding energy nutrients when the record-keeping days are assigned in advance and the days are consecutive. For nutrients derived from only a few food items, such as vitamin D, the necessary period of record keeping is longer. Filling in a food record does not require a good memory, but some knowledge of cooking methods and recipes is needed.

We used food records collected blind to the purpose of the study when investigating adherence to the therapeutic elimination diet in children with CMA (Study IV). Because the collection of dietary data was not primarily aimed at evaluating adherence to the elimination diet, there is no bias with regard to receiving more socially desirable answers. However, some of the food records contain vague elements. These include imprecise recording of food items consumed (e.g. lack of knowledge of a brand name), but also not having sufficiently detailed information on products from, for instance, small local bakeries and meat product manufacturers dating years back. These inaccuracies were present in a small portion of the food records even after the food records were checked by a trained study nurse upon their return. Another limitation is the short period of the food record, three days at each study point, and lack of knowledge about the children's diets during the rest of the year. To conclude, both the FFQ and food records as methods for collecting information on diets have their advantages and disadvantages, and choosing the most suitable method for each study question is essential.

6.2 Associations between maternal diet and CMA in offspring

The most prominent finding was the lower risk of CMA in the offspring of mothers with high use of cow's milk products during pregnancy. This issue has not been addressed in a large, epidemiological study to date. In the 1980s, two small randomized trials showed no difference in associations between maternal intake of cow's milk products during pregnancy and development of allergic disease in high-risk populations (118, 119). Reports from a Japanese cohort have suggested an inverse association between maternal milk consumption during pregnancy and wheezing, but not eczema, in children (147, 155), and another Japanese study found an inverse association with atopic dermatitis in children (154). In these Japanese studies, the consumption of milk products was much lower and in no way comparable with the consumption seen in our study. In addition, a Dutch cohort study found no association between maternal milk consumption during pregnancy and wheezing or asthma in offspring (146). By contrast, the association between

maternal milk product consumption and sensitization to cow's milk in offspring, measured by specific IgE, was analyzed in a German cohort, revealing a direct association for the maternal use of cream during pregnancy (143). We found that high overall use of milk products was protective of CMA (III), and no particular fat content of milk stood out. The results of the German study are also not directly comparable with our results, because sensitization is not equivalent to clinical food allergy.

The components of milk contributing to the protective effects of milk products warrant further study. It is, of course, possible that the protective effects are not due to milk itself, but rather to some other lifestyle factor associated with milk consumption, which was not controlled for in our study. However, socioeconomic factors are more associated with preferences regarding the fat content of milk products, and less with overall use of milk. In any case, our study suggests an inverse association between abundant maternal milk consumption during pregnancy and subsequent development of CMA in offspring, taking into account several potential confounding factors. This association was only seen in children of non-allergic mothers. A possible explanation for this phenomenon is that tolerogenic mechanisms may not function in allergic individuals as they do in non-allergic individuals and tolerance towards antigens consumed by the mother is not transferred to the fetus. The mechanisms that facilitate development of tolerance should be studied further.

The strongest association arising from the literature, linking maternal diet during pregnancy with the development of asthma and allergic diseases, concerns n-6 and n-3 fatty acid intakes (108, 243). High intakes of the long-chain PUFAs EPA and DHA have especially been linked to a lower risk of allergic diseases (243). Accordingly, epidemiological studies have found a lower risk of asthma and allergic diseases with increasing consumption of oily fish and fish in general (139-145), although other studies report no associations (146-148, 153). No previous studies have reported associations between lean fish and allergic outcomes. In line with the majority of the previously reported findings, we observed a trend towards an inverse association between maternal consumption of fish during pregnancy and CMA in offspring, albeit not quite as significant as the finding regarding milk consumption (III). The association was stronger for lean fish than fish intake in general, suggesting that other components in fish besides fatty acids could exert the immunologic effects. The trend was particularly evident in children of allergic mothers; in this subgroup, maternal intake of fish was inversely associated with subsequent CMA in offspring, whereas in non-allergic mothers the association was not significant. This may be due to deficient conversion of α-linoleic acid to EPA and DHA in allergic individuals (113, 114) and is supported by, for instance, a report by Wijga et al. (244), who found an inverse association between breast milk n-3 PUFAs and allergic diseases in children of allergic mothers, but no associations in children of non-allergic mothers. Previously, a German study suggested a direct

association between maternal margarine consumption during pregnancy and atopic eczema in the offspring (245). However, there are differences in margarine composition between countries. By contrast, Hoppu et al. reported that a maternal diet rich in SAFA during lactation was associated with atopic sensitization in offspring and that SAFA content of breast milk was directly associated with development of atopic dermatitis in infants (165, 181). While we have not analyzed the quality of fat in the diet yet, we did not detect any associations between maternal intake of butter, butter spreads, vegetable oils, or vegetable fat spreads and the risk of CMA in offspring. Thus, our results (Study III) do not support the notion of either an inverse association between the use of butter or a direct association between the use of margarines and the risk of allergic diseases in offspring.

Some concern has arisen from the notion that fish contain environmental contaminants, such as methyl mercury, dioxins, and polychlorinated biphenyls, which are harmful to the developing fetus. The European Food Safety Authority has evaluated the risks of fish consumption and concluded that women of childbearing age consuming two meals of fish per week, which is the recommended level during pregnancy and lactation, should generally not exceed the provisional tolerable weekly intake of environmental pollutants (246). Plant sources of n-3 fatty acids are safe with regard to contaminants, but evidence on their association with allergic diseases is scarce.

The majority of studies assessing maternal fruit and vegetable consumption indicate an inverse association between consumption and allergic diseases in offspring (247). Why the use of vegetables and fruits and berries was directly associated with the development of CMA in our cohort is unknown, but could be explained by other lifestyle factors related to fruit and vegetable consumption. Previously, antioxidant intake has been linked to such social characteristics as maternal age and education in our cohort (248), and therefore, the intake of vegetables, fruits and berries may well be a surrogate for some other lifestyle factor. Another explanation is tendered by Murr *et al*. (249), who argue that increased antioxidant intake could suppress cytokines, leading to Th1 differentiation and thus promoting Th2 reactivity. The association between antioxidant nutrient intake during pregnancy and subsequent development of food allergy should be further addressed. In this study, the consumption of citrus fruits during pregnancy was not associated with CMA, unlike in a previous report from our cohort, which suggested a direct association between maternal intake of fruit in total and citrus fruit and allergic sensitization in the offspring (153). On the other hand, maternal use, compared to non-use, of citrus fruit and kiwi during lactation was inversely associated with the development of CMA in offspring (III). The likely explanation for this association is a prophylactic elimination diet in high-risk families and infants with early symptoms of food allergies. One previous study reported that maternal consumption of apples was inversely associated with allergic disorders at the age of five years (141), but this relationship has not been confirmed in other studies,

including ours. A further report from the same cohort showed an inverse association of fruit consumption with wheezing (146).

We found an association forming a U-shaped curve between maternal use of cereal products and CMA in offspring, the association being significant for diet during pregnancy in the unadjusted model but not after final adjustments. These associations were the same when children with allergy to wheat, barley, or rye were omitted from the analysis. We have no explanation for this association, but given the amount of analysis that was performed and the fact that the final adjustment removed the association, this finding could be due to chance. The only earlier study investigating cereal intake and allergic diseases found no association between maternal consumption of whole-grain products during pregnancy and asthma or eczema in offspring (141).

Maternal diet during lactation was less strongly associated with CMA in offspring. The association between maternal milk intake and CMA in the child was actually U-shaped and not statistically significant – thus, the effect of high maternal consumption was not associated with a lower risk of CMA during lactation, like it was during pregnancy. Fish intake during lactation was also not associated with CMA in offspring. As mentioned earlier, fatty acid profile of breast milk is affected by maternal fatty acid intake during lactation as well as by fatty acids derived from maternal adipose tissue (185, 186). Therefore diet during pregnancy would also be of importance. In addition, it is not possible to distinguish between maternal diet during pregnancy and lactation in our study, even after adjusting one for the other, if they are highly correlated. The smaller sample size in studying diet during lactation could account for these differences in results in Study III.

Dietary patterns are rather stable and maternal food consumption habits continue from pregnancy to lactation. Our results therefore leave open the question of whether diet during lactation or diet during pregnancy is more important in determining future risk of CMA in offspring. Furthermore, maternal food consumption habits are transferred to the child. A recent study by Katz et al. (250) suggests that the age of introduction of milk to the infant diet may influence the development of tolerance or allergy against cow's milk. In our study, adjusting maternal milk consumption during pregnancy with the age of introduction of milk products to the infant's diet did not abolish the association between maternal milk consumption and CMA in offspring (III), indicating that diet during pregnancy has an independent association with future development of CMA.

6.3 Validity of the questionnaire

Information on food allergies in children, provided by their parents in the questionnaire, correlated well with the data collected from the patient records and with the registry data from the Social Insurance Institution (studies I and II). The validity of the questionnaire was assessed retrospectively without conducting a pilot

study on how the questionnaire functions in reality. However, it was pre-tested among a small number of families before putting it to use to ensure that is comprehensible. Open questions in questionnaires administered to parents when their children are aged 6 months and 1 and 2 years provide varied information, which may leave room for interpretation. The structured questionnaire at the child's age of three years overcomes this problem because the questions are more precise and easier in terms of data handling. The arrangement of the structured questionnaire was changed once during the course of the study. As discussed in the review by Choi et al (70), this introduces a potential bias in gathering information on CMA, among other special diets. The different versions of the questionnaire were not investigated separately.

Since we addressed the validity of the questionnaire afterwards, most children with CMA would have become tolerant by the time of the validation. Therefore, confirming these allergies by an oral challenge was not possible within the present study. Instead, parental reports were compared with hospital patient records and register-based information. Practices for diagnosis of food allergy are known to vary to some extent (251), but this was the best available method to gather information on diagnosis of CMA in these children. Another pitfall in going through patient records was that information was not available from private practitioners or from primary health care. About one-quarter of the children whose parents reported that the child had had a food allergy had not visited the hospital for allergy-related reasons. They could have been diagnosed in primary healthcare or at private clinics, both of which are common practice following the guidelines given by the Pediatric Society (46). It is also possible that the child did not actually have a diagnosed food allergy. Therefore best-case / worst-case scenarios were calculated, and the true correlation between parentally perceived food allergy and actual diagnosis of food allergy lies somewhere in between.

Diagnosing food allergy is a challenging task, and patient records are not always flawless in depicting what has been discussed between a physician and the parents (Figure 8). Double-blind, placebo-controlled challenges, which are the most rigorous possible method for diagnosis (44), are often not used in clinical practice. In practice, an open challenge is applied in the process of diagnosing food allergy, and the diagnosis is based on medical history, skin prick tests, serum-specific IgE, and a positive response to a diagnostic elimination diet and oral challenge (46).

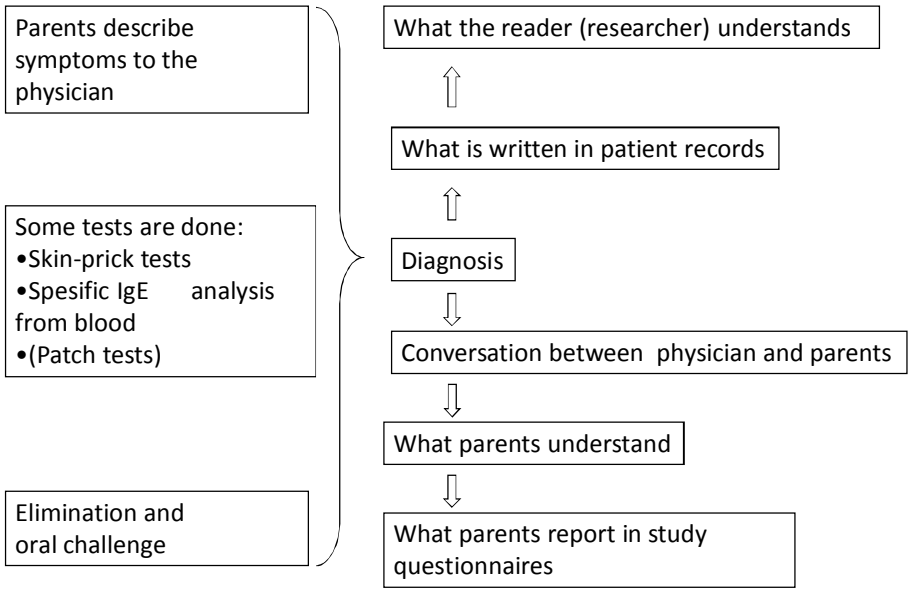

Figure 8. Diagnosis, what parents report on study questionnaires, and what is understood from patient records are not always the same thing. In addition, terminology, such as cow's milk allergy vs. lactose intolerance, and wheat allergy vs. celiac disease, is often unclear.

Some parents reported that their child's diet was started by the family, although a diagnosis was found in patient records. These diets were most likely initiated by the family and later confirmed by a physician. Due to these inaccuracies, the information on of whose initiative the diet was started was not included when using the parental reports of food allergy as an outcome in the etiological studies. The questionnaire was also found to underestimate the occurrence of egg and fish allergy because these were not specifically queried, and several parents had reported them as "commonly allergenic foods", a response that was misleading in this sense. These were therefore also not used as an endpoint in etiological studies. Moreover, CEA, egg, and fish allergies have not been as rigorously studied and their diagnosis includes even more uncertainties than that of CMA.

Unreported food allergy in the cohort was rare, only one in a hundred according to patient records. Six of seven unreported CMAs had, however, been reported in open-ended questions for children aged 3 and 6 months, and 1 and 2 years. Compared with registers of the Social Insurance Institution, CMA was not reported by the parents of three in a thousand children. Also, over-reporting is not as large a problem as previous studies suggest (21, 31, 34, 40); only three in a hundred families over-reported CMA in their child.

We also looked at sociodemographic groups in which our questionnaire works and does not work well. Suitability was lower for young mothers as well as for mothers with no professional education. In addition, children who were breastfed for more than 12 months were less likely to be included in the register of the Social Insurance Institution because breastfed infants do not usually need a special infant formula. For these children, the questionnaire would seem a more reliable source of information on food allergies than the aforementioned register. This is why we have used the combined outcome in etiological studies.

6.4 Diet of children with CMA

Our study is unique in determining adherence to a therapeutic elimination diet in children with CMA in an objective setting. This is the first study to prove what has been presumed in clinical practice; the therapeutic elimination diet in children with diagnosed CMA is adhered to very well. Adherence was extremely good in 85% of the families for whom food records included no CMP. We did not identify any sociodemographic groups that would have trouble in adhering to the diet. Those who had consumed small amounts of CMP were also more often monosensitized, which may reflect having a milder form of food allergy. In the "small amount" group, median intake of CMP was 20 mg, which may be less than is needed to trigger symptoms (187). On the other hand, children who are allergic only to CMP do not often receive nutritional counseling, which might also explain the association between monosensitization and consuming small amounts of CMP hidden in foodstuffs. Ingestion of these foods may be considered to be accidents or ignorance of treatment, the latter of which could be counteracted with dietary counseling. The necessity of the strictness of the diet in our study subjects is unknown because we do not have knowledge about the presenting symptoms in the children or possible inaccuracies in the clinical diagnosis. Those with mild symptoms may well tolerate small amounts of CMP. Unfortunately, information on visits to a nutritionist was not available in our study.

The CMP-free diet was discontinued at a rather early age; by three years of age about half of the children had begun to use milk products, and only one-quarter of the children were still avoiding CMP. This follows the recovery rate presented by Vanto et al. (130), who also included both IgE- and non-IgE-mediated CMA in their study. On the other hand, IgE-mediated CMA resolves slower (252). In our study, one-fifth of the subjects had left the study before they begun to use milk products so we do not know the age of reintroduction of milk products to their diet.

Eight children had consumed milk products within six months of receiving a reimbursement for special infant formulas. Two of these eight children with milk products in their diet had used a special infant formula for only two weeks, according to the information provided by their parents. These children can be speculated to have received the reimbursement because of a diagnostic elimination

diet. In addition, four children had consumed both a special infant formula and some milk products. Reimbursement for the special infant formulas is costly, and if a child can tolerate a cow's milk-based infant formula, reimbursement is not warranted.

Compared with previous studies addressing adherence to a therapeutic elimination diet in children with food hypersensitivities, in which approximately one- to two-thirds of the populations were fully adherent, our subjects show a much higher adherence (134, 135, 224). This discrepancy may arise from methodological differences, but may also be due to differences in the age groups and food allergies studied. Our results are based on three-day food records, and some milk products may have been consumed beyond this period. A dietary history interview would have increased the coverage and precision of the dietary information. However, food records collected blind to the purpose of the study are presumed to give a fairly reliable answer.

Compared with reported adherence to asthma treatment by others, adherence to the elimination diet here was much more thorough; internationally approximately 55% of asthma patients follow the guidelines for treatment (253, 254), and adherence to asthma treatment is also a concern in Finland (255). Therefore, the society itself does not explain the exceptionally good adherence seen in our study (IV). Being aware of the higher genetic risk for type 1 diabetes may, however, increase adherence to a dietary treatment.

Contrary to our original hypothesis, initial adherence to the elimination diet was not associated with the age of discontinuing the CMP-free diet. This notion is supported by other studies addressing adherence to an elimination diet; Vlieg-Boerstra et al. (134) reported that unintentional consumption of an allergenic food did not predict the outcome of a double-blind, placebo-controlled food challenge of the allergenic food. Likewise, adherence to an egg elimination diet did not predict outcome of an egg challenge (135). We did not check whether the milk used was baked, heated, or otherwise processed – a factor that has later been proposed to play a role in tolerating and maintaining tolerance to CMP (202). The precise amount of allergenic protein needed to induce tolerance remains to be elucidated.

The only background factor associated with an earlier reintroduction of milk products was maternal smoking during pregnancy. Discontinuing smoking is strongly encouraged in Finnish maternity clinics, attendance which is mandatory for all pregnant women in Finland (256). We assume that this association reflects motivation for following the prescribed diet rather than a quicker recovery from CMA. Factors that we did not include in our analyses were, for example, use of antibiotics or probiotics, infant feeding patterns, other food allergies, allergic diseases of the mother and father, and other nutritional factors from children's food records. All of these could potentially affect gaining tolerance to CMP.

Children following a CMP-free diet are at risk of inadequate intakes of protein, energy, calcium, vitamin D, and riboflavin, unless their diet is supplemented with the aforementioned nutrients. Eating patterns are rather stable throughout life, and

likes and dislikes are developed in early infancy. Most likely due to this, children who had recovered from CMA consumed less milk products than children on average (257). The inclusion of milk products in the child's diet does not correct these inadequacies if the quantity is not sufficiently large.

The question of whether ingesting small amounts of CMP in a home setting would induce tolerance and act as an oral immunotherapy should be addressed in a setting with more frequent follow-up (188). In our study, the lack of an association could be due to the fairly long intervals of evaluating the inclusion of CMP in the diet.

7 Summary and conclusions

Valid tools are essential in any research. The validation studies in this thesis show that the questionnaire used in the DIPP Nutrition study to investigate food allergies in children works well. Parents of Finnish infants and young children report their children's food allergies concordantly with patient records and with register-based information on CMA.

Our findings strengthen the hypothesis that maternal diet during pregnancy has an impact on future disease development in offspring. Maternal diet can be modified with dietary counseling (258), but risk factors and protective factors must first be identified. This identification should be sufficiently reliable to avoid unnecessary fluctuations in the advice given to the general public, and the advice must be clinically relevant.

CMA in offspring is associated with maternal diet during pregnancy. Our study suggests development of tolerance in offspring towards the foods that the mother consumes during pregnancy; abundant maternal consumption of milk and milk products was associated with a lower risk of CMA in the child, especially in children of non-allergic mothers. In addition, children, especially those of allergic mothers, may benefit from maternal fish consumption in the form of a lower CMA risk.

As for the clinical implications of these results, health authorities should exercise caution in interpreting these results to the public; the results need to be confirmed in other prospective birth cohort studies, or better yet, in randomized, controlled trials. Future studies will reveal whether the associations found here are due to chance, reflecting other lifestyle factors that are actually responsible for these effects, or whether they are causative. The current recommendation not to use any elimination diets for the primary prevention of food allergy seems justified.

How strict an elimination diet of a food-allergic child should be is a much debated issue. According to current recommendations, the level of avoidance depends on the severity of symptoms and individual tolerance. In our study, the therapeutic elimination diet of children with CMA was adhered to rigorously. Increasing age and monosensitization were associated with less stringent adherence, but apart from these, no sociodemographic risk groups were identified. Adherence to the diet was not associated with the age of reintroduction of milk products to the diet. However, our study was purely observational, with limited information available.

8 Implications for future research

In future studies investigating etiologic factors of food allergy, outcomes should optimally be divided into IgE-mediated and non-IgE –mediated food allergies, as these different types of hypersensitivities may have different etiological factors. Analysis of maternal nutrient intake during pregnancy could clarify which components of milk products and fish are beneficial in lowering the risk of food allergy. A more holistic approach in investigating associations between maternal diet and CMA in offspring should also be administered, instead of looking at different dietary exposures separately. Searching for interactions among dietary components and nutrients and dietary patterns would facilitate identification of different combinations of potential risk and protective factors.

Whether a less strict diet hastens the recovery from food allergy should be addressed in a setting with information on symptoms and sensitization, a confirmed diagnosis, and more frequent follow-ups. The idea of a home-based program for desensitization is attractive and may be feasible in children who do not have severe reactions. It would demand less effort and resources from health services than the present oral immunotherapy programs. Being able to consume even small amounts of cow's milk products without developing allergic symptoms could improve the quality of life in families who live with food allergies and encounter the daily challenges that accompany a restricted diet.

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