FOOD ALLERGY IN CHILDREN

Symptoms, diagnosis, relation to mode
of delivery and to atopic dermatitis

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Desember 2008
13 GENERAL DISCUSSION ........................................................................................................... 33

13.1 COW’S MILK ALLERGY IN CHILDREN .............................................................................. 33
13.2 CONJUNCTIVAL TESTING FOR FOOD ALLERGY ............................................................... 34
13.3 FOOD ALLERGY AND THE MODE OF DELIVERY ............................................................... 35
13.4 ATOPIC DERMATITIS IN PREMATURE AND TERM CHILDREN ........................................ 36
13.5 FUTURE CHALLENGES ........................................................................................................ 37

14 CONCLUSIONS ....................................................................................................................... 38
1 Acknowledgements

I want to thank Merete Eggesbo, who had the idea to and the initiation of this project, in which I am grateful for the opportunity to participate.

My gratefulness to my two excellent supervisors, Ragnhild Halvorsen and Morten Jacobsen. They have taught me the fundamental of research methodology. They have been as patient as necessary, as encouraging as I needed, and responded as quickly as I wanted. Without their hard work this project would not have been realized.

I want to thank all my colleagues at the Department of Pediatrics, especially Jan Henrik Lund and Helge Stene-Johansen, who supported me and agreed in my decision of entering this project, despite the increased amount of work for my colleagues.

I also want to thank all the staff in the Maternity Department and Children’s Department of Oestfold Hospital Trust for their participation in the project. A special thank to nurse Mette Valen Andreassen in the Outpatient Clinic, and secretaries Bente Aasheim and Ragna Sandnesaunet.

I am grateful for the practical support from the staff in the Department of Research and Development, as well as the staff in the Medical Library who was very helpful with literature service.

I want to thank all the families for their participation in this project. Especially I want to mention the mother who many years ago visited the Outpatient Clinic with her baby son, in whom she suspected cow's milk allergy. A drop of cream had splashed in his eye while cream was whipped, immediately the baby's eye got red, swollen and itching. During the following examination cow's milk allergy was proven. Then we asked the question, is it possible that conjunctival testing of food allergens may be an additional method for diagnosing food allergy? Thus, we started further studies.
I also want to thank MSc Petter Mowinckel, Ulleval University Hospital, for the help with the statistics.

I am grateful to Julie Rudihagen, Lovisenberg Hospital, and Suzanne Crowley, Voksentoppen, RH, for the help with the English language of the manuscripts.

This study has received financial support from Helse Oest and Oestfold Hospital Trust. In addition I very much appreciate grants from NAAF (The Norwegian Asthma- and Allergy Association), the support has been invaluable.

My family has participated in some of the practical work, for which they need a special thank, but even more they have been my best support, my best encouragement, and my love – thanks a lot my dear Sigurd, and my dearest daughters Line, Maria and Hanna – you are my pride and joy.

And last, but not least, I want to thank my dog, Maja, for forcing me out every day, no matter how bad the weather was – to keep me mentally and physically healthy.
2 Abbreviations

AD – atopic dermatitis
Th1 cell – T helper cell 1
Th2 cell – T helper cell 2
Treg cell – regulatory T cell
SPT – skin prick test
DBPC – double blind placebo controlled
PCA – principal component analyses
kU/l – kilo units / litre
OR – odds ratio
SD – standard deviation
ROC – Receiver Operator Characteristic
3 SUMMARY

Aims
Allergic diseases have increased markedly the last 100 years. The challenges concerning research and clinical approach are numerous. The aims of these papers were to study adverse reactions to cow's milk in the first year of life, to evaluate whether conjunctival testing for food allergy could facilitate the diagnosis, to study whether delivery by caesarean section increases the frequency of food allergy, as well as to develop more efficient methods for studying gut flora in infants. Additionally, we wanted to study atopic dermatitis (AD) and food allergy in term and preterm children.

Patients and Methods
609 children were included during the first four days of life, and followed closely for two years concerning development of food allergy and AD. 512 children participated at two years of age. Seventy-three more children were included as controls to be able to evaluate the conjunctival test. The children with any symptom had an examination which included SPT, specific IgE, conjunctival testing and food challenges. Stool samples were collected at day four and ten, as well as at one, four, twelve and twenty-four months.

Results
We found adverse reactions to milk in twenty-seven children, which equals 4.9%. We found that the symptoms started early, in all but one, before three months of age, and were mainly gastrointestinal (twenty children) and respiratory (six children), less often skin symptoms (one child). The symptoms and age of onset were the same in term and preterm children. Nine of the children were premature and eighteen were born at term. Half of the children, fourteen, were tolerant to milk at one year of age. In our cohort twenty-six children had non-IgE-mediated adverse reactions, just one child had an IgE-mediated cow's milk allergy.

Forty children with fifty possible diagnoses of food allergy had elevated IgE / positive SPT to a food allergen and negative conjunctival test to the same food allergen. Food challenges revealed no serious allergic reactions. Forty-six children with fifty possible diagnoses of food allergy had elevated IgE / positive SPT and a strongly positive conjunctival test, score
II, to the food in question. According to the diagnostic criteria they were all demonstrated
to have food allergy.
Twenty-one children with twenty-four possible diagnoses of food allergy, elevated IgE /
positive SPT and conjunctivalt test score I, revealed eight diagnoses of food allergy, in
sixteen cases there were no food allergy.
We found that thirteen out of 171 children born by caesarean section (7.6 %) and twenty-
two out of 341 children born by vaginal delivery (6.5 %) were diagnosed with food allergy.
The difference is not significant. We additionally studied children with elevated IgE,
allergic or sensitized, finding no over representation in the children born by caesarean
sections compared to vaginally delivered children, 3.5 % and 10.3 % respectively.
A new and promising method for examining the gut flora was introduced, which is DNA
alignment independent, using 16S rRNA gene clone libraries. The new method is more
efficient with respect to computer operation time, and thereby more applicable.
We found ninety-five (18.6 %) children with AD during the first two years of life, thirty-
two (19.9 %) were premature and sixty-three (17.9 %) were term children. The prevalence
of food allergy in the children with AD was 15.8 %, 15.6 % in premature and 15.9 % in
term children. IgE-mediated allergy was found in 8.4 % of the children. The children with
IgE-mediated food allergy had the most serious AD, with a higher SCORAD index. The
mode of delivery did not affect the prevalence of AD.

Conclusions
Adverse reactions to milk are common in the first year of life, occurring in 4.9 % of the
children. Symptoms start early in life, most often during the first weeks. Gastrointestinal
and respiratory symptoms were the most common onset symptoms. Frequency, age of
onset and symptoms were the same in premature as in term children. IgE-mediated allergy
seemed to be much less common than non-IgE-mediated adverse reactions. Half of the
children were tolerant at one year of age.
We have demonstrated that with a negative conjunctival test to a food allergen, despite a
positive SPT and an elevated IgE to the same food allergen, an oral challenge can safely be
performed without risking serious allergic reactions.
We have demonstrated that in children with a powerful conjunctival reaction, score II, to a
food allergen, food allergy could be proven.
We have found no increased frequency of food allergy in the first two years of life in children delivered by caesarean section compared to those delivered vaginally. A promising new method for analysing the gut flora, using 16S rRNA clone libraries is demonstrated. The prevalence of AD in the first two years of life was 18.6 %, the same prevalence in term and preterm children. The prevalence of food allergy was 15.8 %, IgE-mediated allergy in 8.4 %. There were no difference between preterm and term children according to food allergy. The children with IgE-mediated food allergy had the most serious AD, with a higher SCORAD index. Mode of delivery did not affect prevalence of AD.

4 Norwegian Summary (Resyme)

Mål
De siste 100 år har det vært en betydelig økning i forekomst av allergi, uten at man har kunnet finne sikker årsak til dette. Utfordringene i denne sammenheng er mange. Målet med dette arbeidet har vært å studere kemikreaksjoner i første leveår, evaluere bruk av conjunctival testing som ledd i diagnostisering av matvareallergi hos barn, samt studere om det er økt forekomst av matallergi i de to første leveår hos sectioforløste barn sammenliknet med vaginalt forløste. I tillegg ønsket vi å utvikle enklere metoder for å kunne studere forskjeller i tarmflora hos barn. Avslutningsvis ville vi se på forekomst av AD og matallergi i de to første leveår hos premature og terminbarn.

Pasienter og metode
609 barn ble inkludert i studien i løpet av de første fire levedager. De ble fulgt tett i de to første leveår med tanke på utvikling av matallergi og AD. 512 barn deltok ved to års alder. Ytterligere syttitre barn ble inkludert som kontroller for å kunne evaluere bruk av conjunctival testing som ledd i diagnose matallergi. Barna ble utredet med klinisk undersøkelse, priktest, spesifikk IgE, conjunctival test og matvareprovokasjoner. I tillegg ble det tatt avføringsprøver av barna ved fire og ti dager, samt ved en, fire, tolv og tjuefire måneder.
Resultater
Vi fant tjuesju barn med kemelkreaksjoner i første leveår, 4,9 %. Debut av symptomer forekom før tre måneders alder hos alle bortsett fra en. Debutsymptomer var i hovedsak gastrointestinal, hos tjue, og respiratoriske, hos seks. Kun en hadde symptom fra hud. Det var ikke forskjeller i symptomer eller debutalder hos premature kontra terminbarn. Ni av barna var premature og atten var terminbarn. Halvparten av barna, fjorten, var tolerante ved et års alder. I vår kohort var det kun et barn med IgE-medierte kemelkallergi. Førti barn med femti mulige diagnoser matallergi hadde positiv prikktest / forhøyet IgE, men negativ conjunctival test mot det aktuelle matallergen. Matvareprovokasjoner avslørte ingen alvorlige allergiske reaksjoner. Førtiseks barn med femti mulige diagnoser matallergi hadde positiv prikktest / forhøyet IgE og kraftig positiv conjunctival test, score II, mot det aktuelle matallergen. I henhold til de diagnostiske kriterier kunne alle påvises å ha sikker matallergi. Hos tjuefire barn med positiv conjunctival test, score I, forhøyet IgE / positiv prikk test, kunne det påvises matallergi hos åtte, mens seksten ikke hadde matallergi. Tretten av 171 sectioforløste barn (7,6 %), og tjueto av 341 vaginalt forløste (6,5 %), ble diagnostisert med matallergi. Forskjellen var ikke signifikant. Heller ikke da vi studerte barn med forhøyet IgE, allergiske eller sensibiliserte, fant vi øket forekomst hos sectioforløste barn i forhold til vaginalt forløste, 3,5 % kontra 1,3 %. En ny og lovende metode for undersøkelse av tarmflora er introdusert. 16S rRNA klone bibliotek benyttes. Den nye metoden er effektiv og arbeidsbesparende i forhold til de mer omfattende DNA analyser. I vår kohort fant vi nittifem barn (18,6 %) med atopisk dermatitt i de første to leveår, tjue (19,9 %) var premature og seksttre (17,9 %) var terminbarn. 15,8 % av barna hadde matallergi, 15,6 % av de premature og 15,9 % av terminbarna. 8,4 % var IgE-mediert. De barna som hadde IgE-mediert matallergi hadde mest alvorlig eksem, med høyere SCORAD index. Fødselsmåte affiserte ikke forekomst av AD.

Konklusjon
Kemelkreaksjoner er vanlig i første leveår. I vår kohort forekom debut av symptomer hyppigst i de første veveuker. Vanligste debutsymptomer var gastrointestinal og respiratoriske. Det var samme forekomst av symptomer og debutalder hos premature som
hos terminbarn. Halvparten av barna var tolerante ved et års alder. I vår kohort var ikke-IgE-mediert kumelkallergi mye hyppigere forekommende enn IgE-mediert allergi.
Med en negativ conjunctivaltest mot et matvareallergen, selv med positiv prikktest og forhøyet IgE mot samme allergen, kan åpen matprovokasjon trygt gjøres, uten risiko for alvorlige allergiske reaksjoner.
Ved en kraftig positiv conjunctivaltest, score II, mot et matallergen kan man forvente en sikker matallergi.
Vi har ikke kunnet påvise økt forekomst av matallergi i de to første leveår hos sectioforløste barn sammenliknet med vaginalt forløste.
Forekomst av AD i de første to leveår var 18,6 %, med påvist matallergi hos 15,8 %. Det var samme forekomst av AD og matallergi hos terminbarn og premature. Barn med IgE-mediert matallergi hadde mest alvorlig eksem. Forløsningsmetode affiserte ikke forekomst av AD.
5 LIST OF PAPERS

Adverse reactions to milk in infants. Kvenshagen B, Halvorsen R, Jacobsen M


Is there an increased frequency of food allergy in children delivered by caesarean section? Kvenshagen B, Halvorsen R, Jacobsen M
Accepted in Acta Paediatrica on 16th of September 08.


Atopic dermatitis in premature and term children. Kvenshagen B, Jacobsen M, Halvorsen R
Arch Dis Child. 2008 Oct 1. [Epub ahead of print]
6  INTRODUCTION

6.1 Epidemiology

Allergic sensitization and allergic diseases have increased markedly over the last 100 years in industrialised countries, from being rare to become among the most common diseases. Although asthma was well known as early as antiquity, it was a rare disease at that time. Allergy to pollen was described as an unusual phenomenon at the beginning of the 20th century. The incidence of cow’s milk allergy in Sweden in the first decades of the 20th century was 0.1-0.3/1000 according to a study from Vendel, 1948.

For the time being about 20 % of Norwegian children < 10 years of age have or have had a diagnosis of asthma (1), 12-15 % of European children have atopic dermatitis (AD) (2,3,4), nearly 20 % allergic rhinitis (5,6), and about 6 – 8 % (7,8) of children < 3 years of age have food allergy, defined as an abnormal or exaggerated immunological response to food proteins, that may be IgE or non-IgE mediated (9).

6.2 The hygiene hypothesis, immunological and microbial mechanisms

Much scientific work has been performed to unravel the reasons for the increase of allergic diseases. One leading theory the last decades has been that the microbial composition / diversity in the intestine is of great importance for the development of allergic diseases, as well as for the development of oral tolerance, and that environmental factors concerning birth and neonatal period, such as mode of delivery, use of antibiotics and possibly probiotics, are of importance for the composition of the gut flora. Genetic factors may as well play a part (10,11,12,13,14,15).

The current view of cellular and molecular mechanisms responsible for these phenomena includes the balance between T1 helper cells (Th1), Th2 and regulatory T cells (Treg). The previous understanding was that the altered microbial composition suppressed the Th1 responses, and up regulated the Th2 responses. More recently reduced activity of the T regulatory cells has been shown to be of importance. Both mechanisms may play a part. The processes seems to start already in utero, meaning that prenatal as well as early postnatal developmental stages may represent windows for allergy-preventing environmental influences (15,16).
These theories are supported by studies on germ-free animals in which there is reduced tolerance to food. A down regulated Th1 response and a maintained Th2 response have been found. When a successful colonisation of the intestine has occurred, the immune system is up regulated, and tolerance is achieved (11).

The foetal intestine is sterile. Following delivery, numerous allergens influence the intestine of the newborn. The maternal intestinal flora is the major source of bacteria for the neonatal gut in vaginally delivered children, though environmental influences also play a part. The bacterial flora is usually heterogeneous during the first few days of life. After the first week of life, a stable bacterial flora is usually established. In full-term and breast fed infants a flora rich in Bifidobacterium spp. is found. Other anaerobes, such as Clostridium spp. and Bacteroides spp., are more rarely encountered, and enterobacteria and enterococci are relatively few. Formula-fed babies are often colonized by other anaerobes in addition to bifidobacteria (17).

Environmental factors may be more important than breastfeeding in gut colonization after delivery. In children delivered by caesarean section, the establishment of a stable flora is delayed, and dominated by Clostridium spp and Enterobacteriaceae, with lower counts of bifidobacteria, bacteroides spp and E.coli. The differences have been detectable for weeks after delivery (18,19,20). In extremely low-birth weight infants, hospitalization in neonatal intensive care units, with following prolonged antibiotic therapy, parenteral nutrition, delayed oral feeding and intubation seem to affect the composition of the intestinal microbiota. The gut is colonized by a small number of bacterial species. Lactobacillus and Bifidobacteria spp. are seldom. The species found are Enterococcus faecalis, E. coli, Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus epidermidis and Staphylococcus haemolyticus. Hygienic conditions and antimicrobial procedures strongly influence the intestinal colonization pattern (17).

### 6.3 Allergic diseases and mode of delivery

Much scientific work has been performed in order to decide whether or not caesarean sections predispose to allergic diseases, but the issue is still debated, as previous studies have inconsistent findings:

One study has stated that children delivered by caesareans section have an enhanced risk of food allergy (21). The incidence was even more increased when the mother was allergic
The children delivered by caesarean section do not get the same contact with their mothers gut flora as the children born vaginally. According to the hygiene hypothesis above, this might explain some of the increase in allergic deceases, as the incidence of delivery by caesarean section has increased 10 fold the last 70 to 80 years (22,23).

On the other hand a relatively new paper from Adlerberth et al concludes that there seems to be no relation between lack of any particular cultivable intestinal commensal bacteria and development of food allergy or atopic dermatitis (20).

Renz-Polster and co-workers found an increased risk of allergic rhino-conjunctivitis in children delivered by caesarean section, but no increased risk of food allergy (24).

Recently a meta-analysis, bases on twenty-six studies, was published, in which the question asked was if caesarean section was associated with any increase in atopy or allergic diseases. A correlation was found between mode of delivery and allergic rhinitis, asthma and possibly food allergy, but not for inhalant atopy and atopic dermatitis (25).

6.4 Examination of microbial flora

Whatever the results so far, the composition of the intestinal microbial flora seems to be of great importance concerning the development of the immune system of the infant. Much scientific work has been performed in order to evaluate the connection, as a detailed knowledge might explain some of the increase in immune dependent diseases in general, and allergic diseases specially. However, a major problem has been an exact examination of the gut flora, the different methods being complex, time consuming, expensive and partly inaccurate.

Earlier, the only way of examining the gut flora was by culturing (20). This was, however, an inaccurate method, as anaerobe bacteria might die during the process, or there might be inappropriate conditions for growth.

During the last decades the use of DNA analyses has enabled a detailed examination of the gut flora, but the problem with the DNA sequence alignments and phylogenetic reconstruction is the large data sets that cannot easily be analysed. Multiple sequence alignment is too complicated for practical purpose (26,27,28).

The last years a promising new method for evaluating the microbial flora in human beings has been presented, comparing microbial communities that are DNA sequence alignment
independent, using 16S rRNA gene clone libraries. The new method is more efficient with respect to computer operation time, and thereby more applicable (29,30,31).

6.5 Food allergy in children

6.5.1 Definitions
The World Allergy Organisation in 2005 defined food allergy as an abnormal or exaggerated immunological response to food proteins that may be IgE or non-IgE mediated (9). Earlier there has been a debate concerning the terminology of food allergy, as for the time being, only IgE-mediated food allergy can be supported by laboratory findings in daily clinical practice.

Intolerance has been used to describe an abnormal physiological response to an agent which is non-immune-mediated. When a mechanism other than an immune one can be proven, the term non-allergic hypersensitivity is now recommended (32).

However, in the present papers the term “adverse reactions to cow's milk” has been used synonymously with non-IgE-mediated allergy. The reason for this was arguments against the use of the term food allergy, when IgE-mediated allergy could not be proven.

6.5.2 Consequences of food allergy
The frequency of food allergy in children below the age of three is for the time being about 6 – 8 % (33,34,35), which means at least ten thousand affected Norwegian children.

The obviously most feared consequence of food allergy is anaphylaxis, which may be life threatening (36). Consequently, the diagnosis of food allergy, correct or incorrect, in a child, may lead to constant fear of dramatic adverse reactions, especially for parents and other caretakers. The child may not be allowed to take part in normal daily life activities, as the diagnosis requires caretakers watching the child all day. An extra burden for the caretakers may be the fear that they are incompetent to handle an emergency situation, despite instructions, with the following constant fear of fatal events (37). Even in cases when anaphylaxis is excluded, food allergy may be considered a serious disease, as symptoms may be numerous and uncomfortable for the child.
Additionally, the diagnosis of food allergy may have further practical and social, as well as economic consequences. The elimination diet may be a complicated one, especially when the food in question is cow’s milk, as cow's milk proteins are present in numerous types of food. Special education in nutrition will be needed, at least for the parents. The elimination diet may be a boring one, or at least not as well tasting as a normal diet. In social connections a child with food allergy may be an outsider, because of the special needs. Furthermore, an elimination diet usually has the disadvantage of being more expensive than a normal diet, affecting the society as well as the individual families.

6.5.3 Adverse reactions to food

The diagnosis of food allergy may be difficult because the symptoms vary a lot, and because there is no laboratory test to prove or exclude an allergy with a 100 % certainty. Allergy to food may, as pointed out above, be IgE-mediated or non-IgE-mediated (32,38,39). IgE-mediated allergy is characterized by elevated specific IgE and positive SPT to the food allergen in question. If there is a history of serious adverse reactions to a food allergen shortly after intake of the food, and elevated specific IgE and / or positive SPT can be demonstrated, food allergy is probable. Much more difficult are the delayed allergic reactions, in which adverse reactions may appear hours or even days after the intake (32,40). In these cases elevated specific IgE / positive SPT to a food allergen may represent true allergy or a sensitization without clinical importance (41). Adverse reactions to food with no elevated IgE / negative SPT, may be a non-IgE-mediated food allergy or an non-allergic hypersensitivity (9,32). Non-IgE-mediated allergy are supposed to be cell-mediated reactions, but there is no clinical test to prove such allergic reactions (9,32).

Non-allergic hypersensitivity is not a well defined condition, meaning any adverse reaction to food that is not allergy. Among these conditions, however, the histamine reaction is well known and clearly defined. Lactose intolerance is another well known non-allergic reaction to food (9,32,42).

6.5.4 Symptoms

The symptoms of food allergy may be cutaneous, from a discrete rash to fulminate urticaria (9,24,33), gastrointestinal; with abdominal pain, vomiting, diarrhoea or
constipation (31,32) or respiratory; wheezing, coughing and breathing problems (9,32,43). In the worst cases food allergy may elicit anaphylaxis (32,36,44).

In a child with atopic dermatitis food allergy is regularly suspected, usually allergy to cow's milk, as the onset of atopic dermatitis most often is during the first months of life, when the child is fed with milk only (45). The reported frequency of food allergy in children with AD, however, varies from 4 up to 85.7 % in different studies, most often estimated about 30 %, representing a risk over diagnosing milk allergy (46,47,48,49).

Many children experience discrete rashes that might be indifferent or viral rashes, but often is thought to be caused by food allergy, with following groundless elimination diets (44). This might as well be the case when a child experiences urticaria. Idiopathic or viral ethiology is common, but food allergy is often suspected (50,51).

There are many reasons for abdominal symptoms. Abdominal pain may be caused by harmless colic, inflammatory or infectious diseases, disturbances of motility, adverse reactions to food, or rare diseases as congenital malformations or others (51,52). Although the possible diagnoses are numerous, the child with abdominal pain will often be suspected to have food allergy, especially if there is an elevated IgE to a food allergen. The result may be an elimination diet, often groundless or at least difficult to interpret. This might as well be the case when a child experience vomiting, diarrhoea or constipation, which may be caused by viral infections, disturbances of motility, gastroesophageal reflux and coeliac disease in addition to food allergy and more rare conditions (52,53,54). Especially in these circumstances the diagnosis of food allergy may be challenging, as the symptoms may be delayed for hours or days (32).

Even more challenging are the respiratory symptoms. A child with coughing, wheezing or apnoe is regularly suspected to have a viral infection or an early onset of asthma. Even for an experienced doctor a diagnosis of food allergy may seem unlikely under these circumstances.

The affected child may have a single symptom, or more often, a combination of symptoms (55).

Overestimation of food allergy in children is common. Up to 28 % of children have been reported by their parents to have suspected food allergy (32). To have an exact diagnosis of
food allergy an oral challenge is necessary, but in our experience, often not performed because of fear of serious adverse reactions.

School medicine, as well as alternative medicine, are consulted with possible diagnoses of food allergy, resulting in numerous types of examinations, as various blood tests, hair analysis and even more speculative types of tests.

In general, a doctor diagnosis of food allergy is based on a history of possible adverse reactions to a food allergen and positive SPT or elevated IgE, without oral challenge.

The majority of children with food allergy in the first years of life outgrow their allergy (56). The diagnosis of tolerance may be as challenging as the diagnosis of allergy. IgE may be elevated and SPT positive long after tolerance is achieved (41). Tolerance has to be confirmed by oral challenge, but the earlier described fear of adverse reactions often leads to delay of challenge, and thus the diagnosis of tolerance.

6.6 Atopic dermatitis in the first two years of life

Atopic dermatitis and food allergy often occur in the same patients (57). AD is common is common in children in the first years of life, now accounting for 12-15 %, with increasing prevalence during the last decades (4,58,59). Most children, however, outgrow their dermatitis, by twelve years of age two thirds are reported well (60).

6.6.1 Immunological mechanisms

A multifactorial background for AD has been suggested, with genetic as well as environmental factors influencing disease development (61). Genes expressed in the skin may play an important role in AD pathogenesis, in addition to genes influencing atopic diathesis. No "AD gene" has been identified, but candidate gene studies have identified nineteen genes that were shown to be associated with AD. These genes differs from genes associated with asthma, suggesting different genetic background (62). AD is a chronic inflammatory skin disease due to activation of T-cell lymphocytes in the skin. The physiopathology of AD implies dendritic cells, specific T-cell lymphocytes, type 1 and 2 cytokines and inflammatory chemokines (63).

Defects in skin barrier function, with increased water loss and allergen permeability are
distinctive of the disease (59,63,64), which clinically is characterized by itching and dryness of the skin (58,59). Many specific exogenous factors have been associated with the disease. These factors include, dietary allergens, house dust mites, irritants, air pollution, etc. (62).

6.6.2 Atopic dermatitis and the association with food allergy

Because of the association with exogenous factors, AD is often described as an "allergic" disease, however, an allergic causation is most often difficult to document (65). Despite no factual evidence of allergy in the majority of cases, it seems inevitable for parents, as well as health workers, to suspect food allergy to cause the dermatitis (45). Studies, however, are diverging. Sinagra reported 4 % allergy to cow's milk in infants with AD, whereas Ito found the prevalence of food allergy to be 85.7 % in children referred with AD to a specialist clinic (66,67). Most authors, however, indicate the prevalence to be around 30-40 % (49,57,58,68).

There are few studies on AD and prematurity, and with different results. One concludes that there is less AD dermatitis in premature children (69), another that male premature children are over represented (70), and a third one that there might be a marginal lowered risk in the premature (71).

The unsolved problems concerning atopic diseases and allergies in children in general, and food allergies in particular, are numerous - which are the symptoms of food allergy? - in what way could we more easily make a correct diagnosis? - what is the reason for the increase?

What is the actual prevalence of AD in preterm and term children, and what is the frequency of food allergy among these children?

This thesis deals with these topics.
7 AIMS OF STUDY

Main aims

• To study milk allergy in infancy, incidence, presentation, and course of disease up till two years of age.

• To study a possible increased frequency of food allergy in children delivered by caesarean section compared to those delivered vaginally.

• To study the prevalence of AD in preterm and term children, as well as the frequency of food allergy in the same children.

Secondary aims

• To evaluate different diagnostic methods in comparison with conjunctival testing.

• To evaluate new genetic methods to bacterial classification.
8 PATIENTS AND METHODS

8.1 Patients
According to the protocol, approved by the Ethical Committee, 200 premature children and 400 term children were planned enrolled in this study.

Inclusion took place during the first four days of life. When a premature child was born, the mother, and father, if present, were seen in the Maternity Ward during the first four days of the infant's life, and asked to participate. Verbal and written information were given. If the parents agreed immediately, a written consent was collected during the first visit. If the parents wanted some time to think it over, a new visit was arranged the day after.

When a child was born prematurely, the birth protocol was used to find the next two children born at term. The parents of both these children were asked to participate. If one of these children was excluded, or if the parents didn't want to participate, the next child born at term was selected.

8.2 Methods

8.2.1 Questionnaires and interviews
The first questionnaire was filled in at the time of inclusion. In order to follow these children closely with regard to symptoms consistent with possible food allergy and AD, phone interviews with one of the parents were conducted at one, four and twelve months of age. In addition a questionnaire was filled in at six, twelve, eighteen and twenty-four months of age. The parents were also given the cell phone number to the chief investigator to call if the child was not entirely healthy, regardless of the suspected reason.

8.2.2 Faecal samples
One of the main questions in the study protocol was if there was a different gut flora in children with food allergy compared to those without allergy. Thus, faecal samples were collected from the children at day four and ten, as well as at one, four, twelve and twenty-four months. The samples were immediately frozen at -20°C. The samples were transported
in an ice box to the microbial community test laboratory. Upon arrival the samples were dissolved in 5 ml of 50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA. The samples were stored at -40ºC, and processed further within one month.

The mothers were asked for a stool sample at day four after delivery. These samples were handled the same way as those of the children.

A lot of work has been done in order to work out the most exact method for examination of the flora. The new method using 16S rRNA gene clone libraries was taken in use.

8.2.3 Examinations

The children reported by their parents to have possible symptoms of food allergy, or symptoms from the skin, were invited to a clinical examination at the Oestfold Hospital Trust, usually within the following four to five days. No one failed to appear.

In addition to a general clinical examination, in which there was special attention to skin-lung- and abdominal conditions, SPT and specific IgE to different food allergens were performed.

8.2.4 Skin prick test (SPT)

SPT was performed on both lower arms with a 1mm tip lancet, (ALK-Abello, Copenhagen, Denmark) with whole cow's milk, raw and boiled egg, (prick to prick test), as well as allergen extracts from milk, egg, cod, hazel nuts, peanuts, wheat and soy (ALK-Abello, Copenhagen, Denmark). Histamine chloride10mg/ml (ALK-Abello, Copenhagen, Denmark) was used as a positive control, and NaCl 0.9 % as a negative control. The SPT was read after 13 minutes. The contours of the wheals were encircled by a pen, the two diameters perpendicular to each other were measured in mm, added and divided by two. The result was presented in mm. SPT equal to or greater than the positive control was considered positive.
8.2.5 Total and specific IgE

Total and specific IgE: Blood was drawn to determine specific IgE against food and inhalant allergens (cow's milk, egg white, soy, peanuts, hazelnuts, fish, wheat, birch, timothy, mugwort, house dust mite, mold, dog, cat) (Alastat, DPC, California, USA). The analyzes were performed at the Clinical Chemistry Laboratory of Oesfold Hospital Trust (OHT). Some of the children were examined at a specialist clinic in Oslo. Blood was drawn and analyzed at the Department of Clinical Chemistry, Ullevaal University Hospital, Oslo. The method used was CAP (FEIA, Phadia, Uppsala, Sweden).

8.2.6 Conjunctival tests

Solutions
Whole milk was used for cow’s milk, and boiled and raw eggs when this was the food in question. For other food allergens commercially available extracts were used (ALK, Copenhagen, Denmark). The test solutions, including cow's milk and raw eggs, were diluted with NaCl 0.9 %, initially 1:40, then 1:20, 1:10, 1:5, and finally undiluted.

Testing
The conjunctival test was performed by placing one drop of the test solution of the suspected allergen in the lower conjunctival pouch. The eye was observed for 15 minutes for any sign of reaction, rubor, itching, swelling or blisters. Signs of rhinitis were noted as well. If no reaction appeared, the concentration was increased every 15 minutes, using one eye only. For eggs the test was performed by rubbing a cotton stick in a boiled egg, and thereafter applied to the lower conjunctival pouch. If there was no reaction, the procedure was performed with raw egg. The child was observed until the eye normalized, or at least until the reaction was markedly reduced, usually within 30-60 minutes. To minimize a powerful reaction, the eye was washed with NaCl 0.9 % and the child given a dose of antihistamine. The conjunctival test was performed twice, on different days, to verify the result. Two physicians independently scored the reactions.
Scoring

0  No conjunctival reaction
I  Rubor / itching, undiluted test solution
II Rubor, itching and oedema, may include blistering and rhinitis symptoms, occurring within 5 minutes.

All the children had conjunctival tests to food allergens. For children in whom a specific food allergy was suspected, this allergen was chosen. For children with no suspected food allergy, various food allergens, milk, eggs, peanuts, hazelnuts and fish, were tested.

8.2.7 Food challenges

Elimination / challenge test
The infant and / or the mother had a diet totally free from the suspected food allergen for 14 days. Detailed dietary instruction was given. The challenge was performed by introducing the suspected food allergen to the infant or to the breastfeeding mother. A symptom score diary card was filled out daily by one of the parents during elimination and challenge. The challenge was considered positive when symptoms reappeared with reintroduction of the allergen, but had to be reproducible, and thus the elimination / challenge test was repeated at least once.

Open food challenge
Performed at the outpatient or specialist clinic: The clinic kitchen prepared the food. 1.0 mg of the food in question, or 0.1 ml of cow’s milk was given as the initial dose. The amount
was increased 3, 10, 30, 100, 300, 1000 fold every 20 minutes until an adverse reaction appeared or the last dose was reached. The children were observed for two hours after last challenge dose. A symptom score diary card was filled out during the following days.

**Double blind placebo controlled challenge (DBPC)**

Performed at the outpatient or specialist clinic: The clinic kitchen prepared food with and without the suspected allergen. The investigator, unaware of the food content, was responsible for feeding the child. Beginning with a minimal dose, the amount was increased and the child observed in the same way as for the open food challenge. The same symptom score diary card was filled out the following days. The alternative challenge was performed the following week. (72)

The author (BK) performed all the clinical examinations, as well as the SPT. The staff at the Clin Chem Lab was responsible for drawing and analysing the blood tests.

**8.3 The diagnosis of food allergy**

A diagnosis of food allergy was based on the following criteria:

1. A history of an immediate reaction to a food, and positive SPT and elevated IgE to the same food allergen,
   or
2. A history of a suspected food allergy, no immediate reaction to a food allergen, a negative or a positive SPT, and elevated or not elevated IgE to the food allergen in question
   - children < one year of age: positive elimination / challenge test, performed twice
   - children > one year of age: positive DBPC challenge

In order to evaluate the conjunctival test, all the children regardless of age were meant to have a DBPC challenge to verify the diagnosis of food allergy. However, we found it unethical to carry out a challenge in children with a resent history of very serious allergic reactions, as anaphylaxis, airway obstruction and angio-oedema. Infants entirely breastfed were as well unsuitable for DBPC, and an exception was made.

A negative open food challenge was accepted as proof of no food allergy.
9 METHODOLOGICAL CONSIDERATIONS

The present study was a prospective cohort study. Inclusion took place from November 2002 until May 2005.

According to the protocol 200 premature and 400 term children were planned included in the study. However, a few premature babies turned out to be very ill during their first days of life, and were therefore excluded. The term matches had already been included. Thus, we ended up with somewhat more than 1:2 ratio, 193 premature and 416 term children.

One main aim of this study was to investigate the prevalence of food allergy in children delivered by caesarean section compared to those delivered vaginally. To ensure a proper number of children born by caesarean sections a protocol was chosen in which one premature child was included, followed by the next two children born at term, as preterm children more often are delivered by caesarean section. It might be argued that choosing premature children would make the interpretation of the results difficult, as premature children have been reported to have less allergy (73). However, this possible bias has been adjusted for, by studying the prevalence of food allergy individually in the different groups, premature children born by caesarean section, term children born by caesarean section, premature children born vaginally and term children born vaginally, finding that prematurity did not affect the prevalence of allergy in our cohort.

The golden standard of diagnosis of food allergy is DBPC challenge (74). In the present study children below the age of one were diagnosed with food allergy by open elimination / challenge tests, as DBPC challenge is extremely difficult in breastfed infants.

Additionally, in children with a history of very serious allergic reactions, as anaphylaxis, airway obstructions, angio-oedema and generalized urticaria, we found it unethical to perform DBPC challenge. Most of these children had an open challenge to verify their allergy.

One of the questions asked in the protocol was if there were differences in gut flora in allergic children compared to non-allergic children. The method for examinations of the gut flora is presented in these papers, but due to delay in development of this new method the examination of the gut flora of the whole population is not finished yet.

As there were few children in our cohort with IgE-mediated food allergy, the Ethical Committee approved the inclusion of additional children as controls, in order to evaluate
the conjunctival test. Seventy-three additional children were included. These children had symptoms consistent with food allergy, just as the original children. They were referred to a specialist clinic, located in Oslo, with symptoms consistent with possible food allergy. The same examinations were carried out, but in these children blood was drawn and analyzed at the Department of Clinical Chemistry, Ullevaal University Hospital, Oslo. The method used was CAP (FEIA, Phadia, Uppsala, Sweden). A disadvantage was the use of two laboratories, having different systems, CAP FEIA and DPC ALAstat. The values of the laboratories are, however, regularly compared, as part of laboratory quality testing, and the values are consistently within the same range. This would have no consequences for the outcome of the study, as all patients with elevated IgE were identified as such, irrespective of the laboratory method applied. SPT was performed in all the children as well.

For children in whom a specific food allergy was suspected this food allergen was chosen for conjunctival testing. In children with no suspected food allergy different allergens were tested, in order to make sure there would be no conjunctival reaction to the test procedure whatever the allergen was.
10 **STATISTICAL CONSIDERATIONS**

The mean or median when appropriate, was used for continuous variables as index of location and standard deviation (SD) as index of dispersion. Counts with percentage were used for categorical variables. One-way analysis of variance was used to assess group differences for continuous variables with the Student-Newman-Keuls procedure for pairwise comparisons. Pearson’s Chi square test was used to assess group differences for categorical variables.

A Receiver Operator Characteristic (ROC) analysis was performed in order to assess the suitability of the IgE and conjunctival score index as diagnostic tools for diagnosing food allergy. The ROC analysis was performed using Number Cruncher Statistical System (NCSS, Kaysville, Utah, USA) version 2007.

The analysis was performed using the Statistical Package for Social Sciences version 15 (SPSS Inc. Chicago, Illinois).

All p-values equal to or below 0.05 were considered significant.
**11 Ethical Considerations**

The study protocol was approved by the Region Ethical Committee.

After inclusion some of the premature children turned out to be very ill. One child died, the others were excluded after ethical considerations. However, most of them might have been part of the follow up.

Only children with symptoms were examined, including SPT and blood test, in order not to perform unnecessary painful procedures in children. Consequently, some children sensitized to one or more allergens have probably been lost in follow up.

The golden standard of diagnosing food allergy is DBPC challenge. Some investigators argue that DBPC challenge should be performed even in infants, who may be entirely breastfed. We found this extremely difficult and even unethical, as to ensure a proper diet both mother and child would have to be hospitalized, for at least several days. Thus, we diagnosed cow's milk allergy in infants after elimination / challenge, performed twice. In older children we aimed at DBPC challenge, but some children had a recent history of serious allergic reactions, as anaphylaxis, airway obstruction and angio-oedema. In these children we found oral challenges unethical.

It might be argued that performing a conjunctival test, that may elicit troublesome oedema and itching, would be unethical. However, if this test may prove that some of the oral challenges would be superfluous, the ethical aspect would be indisputable, as a positive oral challenge might be much more uncomfortable for the child, as well as possibly frightening for the parents. Additionally, the value of a negative open food challenge following a negative conjunctival test is important as this immediately labels the child as healthy, and eliminates any parental concerns.
12 RESULTS – REVIEW OF PAPERS

Paper I
Adverse reactions to milk in infants.
Kvenshagen B, Halvorsen R, Jacobsen M
193 premature children and 416 term children were included and followed concerning symptoms consistent with allergy to cow’s milk in the first year of life. Twenty-seven children were diagnosed with adverse reactions to cow’s milk. All but one had début of symptoms before three months of age. The symptoms were mainly gastrointestinal and respiratory, less often there were skin symptoms. Premature and term children had the same symptoms and the same age of onset. Just one child had IgE-mediated allergy to cow’s milk. Half of the children were tolerant to cow’s milk at one year of age. We conclude that cow's milk allergy is common in the first year of life, that onset of symptoms most often occur during the first weeks of life, that non-IgE-mediated allergy seems to be much more common than IgE-mediated, and that symptoms and age of onset is the same in premature and term children.

Paper II
Conjunctival testing facilitates the diagnosis of food allergy in children.
Kvenshagen B, Jacobsen M, Halvorsen R
Submitted.
The aim of this study was to evaluate whether a powerful conjunctival test would indicate a true food allergy, whereas a negative conjunctival test would predict that an oral challenge could safely be performed. The conjunctival tests were scored: 0) no conjunctival reaction, I) rubor and itching, undiluted solution, II) rubor, itching, oedema or blistering, possibly sneezing. Forty-six children with fifty possible diagnosis of food allergy and powerful conjunctival reactions (score II) to the food allergens were all shown to have true allergy to the food in question. Forty children with fifty possible diagnosis of food allergy, with elevated specific IgE and positive SPT to a food allergen, but negative
conjunctival test, demonstrated no adverse reactions when challenged with the food in question. Twenty-one children with twenty-four possible diagnoses of food allergy had a score I reaction, eight were diagnosed with food allergy and fifteen without. This study demonstrates that with a negative conjunctival test, even with elevated IgE and positive SPT, oral challenge can safely be performed without risking serious allergic reactions. A score II positive conjunctival test indicates true food allergy.

**Paper III**

**Is there an increased frequency of food allergy in children delivered by caesarean section compared to those delivered vaginally?**

Kvenshagen B, Halvorsen R, Jacobsen M

Accepted in Acta Paediatrica on 16th of September 08.

At two years follow up, 512 children participated, and were studied with regard to an increased prevalence of food allergy in the children delivered by caesarean section compared to those delivered vaginally. Thirty-five children were diagnosed with food allergy, twenty-seven of these had non-IgE-mediated allergy to cow’s milk. Food allergy was diagnosed in 7.6 % of the children delivered by caesarean section and in 6.5 % of children delivered vaginally. Consequently, we did not find any significant increased frequency of food allergy in children delivered by caesarean section. As the numbers of non-IgE-mediated allergies were much higher than IgE-mediated, we looked at the relation between any elevated IgE and mode of delivery. We found that the frequency of elevated IgE was even less in those delivered by caesarean section compared to those delivered vaginally, 3.5 % and 10.3 %, respectively. We conclude that the present investigation does not confirm that children born by caesarean section have a higher prevalence of food allergy during the first two years of age.

**Paper IV**

**Alignment-independent comparisons of human gastrointestinal tract microbial communities in a multidimensional 16S rRNA gene evolutionary space.**

Rudi K, Zimonja M, Kvenshagen B, Rugtveit J, Midtvedt T, Eggessbø M

We present here a new method for examining the human gastrointestinal tract microbiota, which is independent of DNA sequence alignment. DNA is purified from all the bacteria in the stool sample. The next step is to transform DNA sequence data into DNA n-mer frequencies. The n-mer frequency data are obtained by sliding a window of size n (e.i.n nucleotider) and are identified by the base combination present for each step. The number of contributions for each of the 4^n combinations is then counted, and used as input for Principal Component Analyses (PCA). This new method is efficient, and thereby applicable to large-scale screening for microbial communities.

**Paper V**

**Atopic dermatitis in premature and term children**

Kvenshagen B, Jacobsen M, Halvorsen R

Arch Dis Child. 2008 Oct 1. [Epub ahead of print]

512 children, 171 premature and 341 term children, were followed with regard to AD and adverse reactions to food. Ninety-five (18.6 %) children were diagnosed with AD, thirty-two (19.9 %) were premature and sixty-three (17.9 %) were term children. The overall prevalence of food allergy was 15.8 %, 15.6 % in premature and 15.9 % in term children. IgE-mediated allergy was found in 8.4 % of the children. We conclude that the prevalence of AD is the same in preterm and term children, 18.6 %, and that food allergy occurs in a minority of cases, with no statistical difference between preterm and term children. The children with IgE-mediated food allergy had the most serious AD, with a higher SCORAD index. The mode of delivery did not affect the prevalence of AD.
13 General Discussion

13.1 Cow's milk allergy in children

Our studies demonstrate that adverse reactions to cow’s milk start early in life, most often before three months of age. This is in accordance with other investigators (55,75). In our cohort of children there were none with onset of adverse reactions to cow’s milk after the age of six months.

We also found that the majority of children (66 %) had gastrointestinal symptoms, including pain behaviour, while 37 % had respiratory symptoms, and 37 % had atopic dermatitis. Ten children (37 %) had more than one of the listed symptoms. This is in accordance with other investigators (35,76), but interestingly we found that most of the children with atopic dermatitis and cow’s milk allergy had onset of their eczema several months after the onset of allergy. Consequently they were on an elimination diet, indicating that the dermatitis was independent of the food allergy.

Most of the children, all but one in our cohort, had non IgE-mediated adverse reactions to cow's milk. This is surprising, as other investigators have found a much higher frequency of IgE-mediated reactions, 14 to 73 % respectively (77,78). There is no obvious reason for this discrepancy, but the apparent over representation of non-IgE-mediated adverse reactions might partly be explained by the very close follow up, from the first days of life. As some children have an early development of tolerance, especially those with non-IgE-mediated allergy (79), some of the diagnoses might have been lost if the interviews and questionnaires had been postponed until later in the first year, or in the second year of life.

Half of the children were tolerant to cow’s milk at one year of age. This is in accordance with other investigators (35,80).

Frequency and symptoms of adverse reactions to cow’s milk seemed to be the same in premature as in term children. The age of onset seemed to be comparable in term and preterm children as well. To our knowledge there is no study investigating this topic in particular, but some investigators have found less allergy in general among premature children (71,73,81).

More term than premature children were tolerant to cow’s milk at one year. The numbers are too small for statistical analysis, but theoretical consideration could support the
findings, as the premature children may have a delay in the maturation of their immune system.

13.2 Conjunctival testing for food allergy

The diagnosis of food allergy is difficult. Because there is no test to prove an allergy, the diagnosis is often made on the background of a history of possible adverse reactions to a food allergen, and positive SPT and / or elevated IgE. A positive SPT or an elevated IgE, however, only indicate sensitization, and are not diagnostic of clinical allergy. In order to diagnose food allergy with some degree of certainty oral challenge, open or double blind, should generally be carried out. Challenge is, however, often not performed because of fear of serious adverse reactions. The result may be a false diagnosis of food allergy, with practical, social and economic consequences, as well as groundless fear of serious allergic reactions for the parents and other caretakers.

Any clinical sign that would facilitate the diagnosing of food allergy would be very helpful. In the literature there are a few reports concerning conjunctival testing in diagnosing allergy to pollen (82,83), but to our knowledge there is no report on conjunctival testing for food allergy.

In our clinical experience with several hundred children we find the conjunctival testing very useful in facilitating the diagnoses of food allergy. The conjunctival test is simple and efficient to perform in daily clinical practice, not very time consuming, not expensive, well tolerated and very illustrative.

We have shown that with a negative conjunctival test to a food allergen, even if SPT is positive and IgE to the same allergen is elevated, an oral challenge can be carried out without fear of serious adverse reactions. One child in our study had a markedly elevated IgE to hazelnut, 93 kU/L, and positive SPT (4 mm), but a negative conjunctival test to hazelnut. The oral challenge was completely negative. Another child with IgE to peanut > 100 kU/l and a minimal reaction to the conjunctival test, demonstrated to be tolerant to peanuts when challenged.

On the other hand the exact diagnosis of a true food allergy is just as important. In some cases of food allergy the symptoms are relatively week, as for instance worsening of eczema. In such cases oral challenge is unproblematic, for the child as well as for the
parents and the doctor. In most cases of food allergy, however, one may expect serious symptoms; anaphylaxis or respiratory symptoms, or other symptoms very uncomfortable for the child, as general urticaria or sickness / vomiting. We have shown that the children in this cohort with an explicit conjunctival reaction, score II, all were demonstrated to have food allergy. However, as the number of patients in our study is relatively small, and this is the first study on this topic, it is too early to conclude that a powerful conjunctival reaction always prove a food allergy.

Though, in the cases of possible allergy to milk and eggs, when one expects development of tolerance, it seems reasonable to anticipate the diagnosis and postpone an oral challenge until the conjunctival reaction has become weaker. If oral challenge is performed in a child with a powerful conjunctival reaction, it should be carried out very carefully.

The conjunctival testing is only suitable for IgE-mediated allergy, but is most important in these cases, where the most serious reactions may be expected. In our cohort twenty-seven children with non-IgE-mediated allergy to cow's milk, all had a negative conjunctival test. None of these children had serious adverse reactions.

Overall, in our experience, the conjunctival testing is well tolerated by the patients, parents and staff.

13.3 Food allergy and the mode of delivery

We found no increased incidence of food allergy, neither IgE-mediated nor non-IgE-mediated, in children delivered by caesarean section compared to children delivered vaginally. This is contrary to the results from other investigators, where a three times increased risk in children delivered by caesarean section has been demonstrated, with an even more elevated risk if the mother was allergic (20). However, in this retrospective study, with 2803 children included, the diagnosis of food allergy was based on parentally perceived reactions to eggs, nuts and fish, objectively confirmed reactions to eggs only, at 2 ½ years of age. In our study the diagnosis of food allergy was based on objective signs. Furthermore, in our cohort the age group was different, and with an over representation of non-IgE-mediated allergy to cow's milk, which might explain some of the difference. However, when we studied the children with elevated IgE to any allergen, no matter if the child was allergic or just sensitized, we found no over representation in children delivered by caesarean section.
In our cohort no over representation of food allergy among children born by caesarean section, who had a mother with an elevated IgE to any allergen, could be shown. We have studied children with a sensitized mother, well aware that this is not synonymous with allergy. The study by Eggesbø et al (21), in which there was an even higher prevalence of food allergy in children born by caesarean section and with an allergic mother, was based on self-reported allergy in the mothers, and consequently, the results are not comparable.

On the other hand, our results are in accordance with Renz-Polster (24), who found no over representation of food allergy in children delivered by caesarean section. The study from Renz-Polster et al was, however, a retrospective study, and the age of the children was 3-10 years. Consequently the findings are not directly comparable neither to the Eggesbø or our study.

A prospective study from Adlerberth (20), included children at birth, followed for eighteen months. They found no correlation between differences in gut colonisation and sensitization to food allergens at eighteen months, and no increased frequency of sensitization to food allergens in children delivered by caesarean section. However, this study was not designed for evaluation of food allergy and delivery.

A meta-analyses recently published stated a possible correlation between delivery by caesarean section and food allergy, clearly demonstrating the uncertainty of this question (25).

13.4 Atopic dermatitis in premature and term children

In our cohort of 512 children, the prevalence of AD was 18.6 %, in accordance with other investigators (2,3,4). We found the same prevalence in premature and term children. Other investigators have different results, one that term children have a double risk of AD (71), another that male premature children are over represented (70), and a third one that there is a marginal lower risk in the premature children (69).

We found the highest frequency of AD in the first year of life, (overall 10.9 %), with reduction in prevalence during the following year, being 7.6 % and 6.5 % for premature and term children, respectively, at two years of age, a reduction in prevalence with age in accordance with earlier studies (60).

We found the overall prevalence of food allergy in children with AD to be 15.8 %, 15.6 %
and 15.9 % in preterm and term children, respectively, whereas the frequency of IgE-mediated allergy was 8.4 %. This relatively low prevalence differs from most other investigators, reporting about 30-40 % food allergy in children with AD (47,48,49,57,58). However, these investigators studied children who were referred to a specialist clinic, and thus, probably had a more serious dermatitis than the children in our cohort, and also probably an increased frequency of IgE-mediated food allergy as well (5,47). That children with IgE-mediated food allergy have a more serious dermatitis than those without, would be in accordance with our findings.

We found the same frequency of adverse reactions to food in preterm and term children with AD. To our knowledge there is no other study on food allergy in preterm children with AD. However, this would be in accordance with our previous findings that the prevalence of adverse reactions to cow's milk is the same in premature and term children.

We did not find any over representation of AD in children delivered by caesarean section compared to vaginally delivered children, which is in accordance with other investigators (20,24).

13.5 Future challenges
The diagnosis of food allergy is difficult. Conjunctival testing may facilitate the diagnosis. A major question is whether a score II always is a proof of allergy. If this may be ascertained in future studies, the diagnostic procedure will become safer and easier. The immunological mechanisms occurring in the conjunctival mucosa is essential to study in order to understand the described reaction in an organ usually not affected in food allergy.

The role of the intestinal flora for the development of allergy is not fully understood and to a certain extent relying on experiments in animals. Detailed examination of the microbes in the intestine, and increased understanding of the biological effect of each strain in the human intestine may add valuable information to the topic. However, the main question is why there has been enormous increase in allergic diseases, and the composition of the gut flora is probably not the whole explanation. Thus the main future challenge will be the mechanisms of increase.
14 Conclusions

Adverse reactions to cow's milk are common in the first years of life. The symptoms usually start very early, often during the first weeks of life, in term as well as in preterm babies. We have found gastrointestinal and respiratory symptoms being the most common, less often there are cutaneous symptoms. About half of the children are tolerant to cow's milk at one year of age. We found that non-IgE-mediated reactions are much more common than IgE-mediated in infants and very young children.

We have demonstrated that in diagnosing food allergy the conjunctival test may be helpful. If there is a negative conjunctival test to a food allergen, even with positive SPT or elevated IgE to the same food allergen, an oral challenge can be carried out without risking serious allergic reactions. Additionally, we have demonstrated that in children with a powerful conjunctival reaction (score II) to a food allergen, food allergy could be demonstrated in all cases.

We have studied a possible relationship between delivery by caesarean section and frequency of food allergy in the first two years of life, compared to children delivered vaginally. We did not find a higher prevalence of food allergy in children delivered by caesarean section.

In order to more efficiently examine the gut flora a new technique has been introduced, which is DNA sequence alignment-independent, instead using 16S rRNA clone libraries. The new method is more efficient with respect to computer operation time, and thereby more applicable, and promising for future research.

We have found that the prevalence of AD in the first two years of life is 18.6 %, regardless of gestational age. The frequency of food allergy in children with AD was 15.8 %, with no statistical difference between preterm and term children. Children with IgE-mediated food allergy had a more serious AD, with a higher SCORAD index. We found no over representation of AD in children delivered by caesarean section compared to those delivered vaginally.
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