Epidemiology of food hyper-sensitivity in school children

Validation with double-blind placebo-controlled food challenges and biomarkers

The Obstructive Lung Disease in Northern Sweden (OLIN) Studies, Thesis XV

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PROLOGUE

Bright eyed insight...

During our research travels in Northern Sweden, Åsa - my dear friend and fellow musketeer - and I - were still in denial regarding our uprising presbyopia. Due to our visual impairment we spent an absurd amount of time trying to decipher our study protocols, which, for some reason, were written in ridiculously small letters. During a food challenge series, one of our study participants sat watching us for a very long time and then he bursted out:

Ah! Now I understand what you mean by double-blind!

To all the amazing children who made this thesis possible...
# Table of Contents

Table of Contents  
Abstract  
Sammanfattning på svenska  
Abbreviations  
Original Papers  
Introduction  
Background  
Definition of food hypersensitivity (FHS)  
Prevalence of food hypersensitivity  
Prevalence of food allergy  
Prevalence of lactose intolerance  
Factors associated with food hypersensitivity  
Risk factors of food allergy  
Risk factors of lactose intolerance  
Immunological aspects of food allergy and tolerance development  
IgE-mediated food allergy  
Non-IgE-mediated and mixed-immune food allergies  
Cytokine patterns in tolerance development and IgE-mediated food allergy  
Allergic inflammation in the intestinal mucosa  
Symptoms of food allergy  
Symptoms of IgE-mediated food allergy  
Symptoms of non-IgE mediated and mixed-immune food allergies  
Establishing a food allergy diagnosis  
Clinical history  
IgE-tests  
Oral food challenges  
Open challenges  
Blinded challenges  
Complementary diagnostic tests and future prognostic biomarkers  
Endoscopy  
Inflammatory and regulatory makers in faeces  
Atopy patch tests  
Serological tests  
Basophil activation tests  
Cytokines  
Objectives  
Materials and methods
| 1. Method: Paper I                              | 27 |
| Method: The OLIN studies                       | 29 |
| Study population                               | 29 |
| Questionnaires                                 | 30 |
| Skin Prick Tests                               | 30 |
| Child interview                                | 31 |
| Serological tests                              | 31 |
| Statistical analyses                           | 32 |
| Definitions                                    | 33 |
| 2. Method: Paper II                            | 37 |
| 4. Method: Paper IV                            | 43 |
| 5. Method: Paper V                             | 45 |
| **Results**                                    | 49 |
| Validation of new DBPCFC recipes (Paper I)     | 49 |
| Incidence and remission of food hypersensitivity (Paper II) | 50 |
| Assessment of allergy to milk, egg, cod and wheat (Paper III) | 53 |
| Milk hypersensitivity phenotypes (Paper IV)    | 54 |
| Biomarkers in blood and stool samples (Paper V) | 59 |
| **Discussion**                                 | 63 |
| Discussion of methodology                      | 63 |
| Sensory testing                                | 63 |
| Epidemiologic study design                     | 63 |
| Reported data                                  | 65 |
| Analyses of associated risk factors            | 66 |
| IgE-tests                                      | 67 |
| Validation of reported data                    | 68 |
| The DBPCFC methodology                         | 69 |
| Cytokine mRNA expression in PBMC               | 69 |
| Analyses of fecal biomarkers                   | 70 |
| Discussion of main results                     | 71 |
| Epidemiology of food hypersensitivity          | 71 |
| Diagnosing food allergy                        | 73 |
| Ethical considerations                         | 75 |
| Personal point of closure                      | 77 |
| **Acknowledgements**                           | 79 |
| **Funding**                                    | 83 |
| **References**                                 | 84 |
| **Supplement**                                 | 101 |
| Questions from structured interviews           | 101 |
Abstract

Background
This thesis focuses on the incidence and remission of reported food hypersensitivity in schoolchildren followed from 8 to 12 years of age and the prevalence of hypersensitivity to milk, egg, cod and wheat among 12-year olds investigated by reported data, clinical investigation and double-blind placebo-controlled food challenges and biomarkers.

Methods
The studies are mainly based on a population based cohort recruited in 2006 from three municipalities in Northern Sweden. All children in first and second grade, aged 7-8 years, were invited to a parental questionnaire study and 2585 (96% of invited) participated. The children in two of the municipalities were also invited to a skin prick test with airborne allergens. At age 11-12 years, there was a follow-up of the cohort using the same methods, with the addition of a child interview and assessment of body mass index (BMI).

At the follow-up, children who reported milk hypersensitivity were invited to structured interviews and children reporting complete elimination of milk, egg, cod or wheat due to perceived hypersensitivity were invited to a clinical examination and blood sampling. According to test results, the children were categorized into different food hypersensitivity phenotypes according to preset criteria. Children categorized as current food allergy were then invited to further evaluation with a double-blind placebo-controlled food-challenge using newly developed recipes. Before their use, the recipes were successfully validated regarding detectable sensorial differences between the active and placebo substances in a separate cohort of healthy schoolchildren (n=275).

Before and after the challenge series blood samples were collected for analyses of cytokine mRNA expression in peripheral blood mononuclear cells including hallmark cytokines for the humoral allergy-promoting T helper (Th) 2 response, cellular cytotoxicity-promoting Th1 response, inflammatory-, and T regulatory responses. Fecal inflammatory biomarkers were also analyzed before and after the challenge series.
Results

Reported food hypersensitivity increased from 21% at age 7-8 years to 26% at 11-12 years. There was a high incidence (15%) as well as a high remission (33%) of reported food hypersensitivity. Risk factors associated with incidence and remission were different for milk hypersensitivity and hypersensitivity to foods other than milk. The agreement between reported symptoms to milk, egg, cod, wheat, soy and peanut and sensitization to the culprit food was poor. At 11 to 12-years of age the prevalence of reported allergy to milk, egg, cod or wheat was 4.8% while the allergy prevalence according to clinical evaluation was 1.4%. This figure was further halved when evaluated with double-blind placebo-controlled food challenges.

The majority of children with reported allergy to milk, egg, cod and wheat were categorized as other food hypersensitivity phenotypes, the most common being probable lactose intolerance (40%) and outgrown food allergy (19%). Even though reported milk hypersensitivity among the 11-12 year olds was 14.5%, only 3% were categorized as current milk allergy. Current and outgrown milk allergy was associated with other atopic disorders and a lower BMI (OR 0.8, 95% CI 0.80-0.98). Before the challenge series, the mRNA expression of the cytokines IL-13 and IL-10 were higher among children with a positive compared to a negative challenge outcome.

Conclusion

Reported food hypersensitivity was common among school children in Northern Sweden and increased from 7-8 years to 11-12 years of age, and both the incidence and remission of reported hypersensitivity was high. There was an 8-fold difference in the prevalence of allergy to milk, egg, cod or wheat when reported data was assessed by clinical examinations and double-blind placebo-controlled food challenges. Allergy to milk, egg, cod and wheat was an uncommon cause of complete avoidance of these foods due to perceived hypersensitivity. Some of the analyzed biomarkers might serve as prognostic markers for symptomatic, IgE-mediated food allergy but need further validation.
Sammanfattning på svenska

Bakgrund

Födoämnesöverkänslighet håller på att bli ett stort och kostsamt hälsoproblem i västvärlden. Prevalensen av rapporterad födoämnesöverkänslighet bland barn ökar, men det är fortfarande oklart om detta avspeglar en sann ökning i populationen. Det finns en stor spridning mellan uppmätta prevalenstal i olika studier och i de få studier där man följt upp rapporterade data med objektiva metoder ses en hög överrapportering.

Data saknas om reell prevalens av födoämnesöverkänslighet bland skolbarn i Sverige. Befintliga prevalensdata baseras på rapporterad födoämnesöverkänslighet och studier saknas där angiven födoämnesöverkänslighet i en barnpopulationskohort validerats med objektiva metoder. Även om dubbelblinda provokationer räknas som "gold standard" används i praktiken endast sjukhistoria, pricktest och analys av specifikt Immunoglobulin E (IgE) samt öppna provokationer för diagnostik. Metoderna har flera felkällor och mer tillförlitlig diagnostik behövs, särskilt vid sena och svårtolkade symptom. Korrekt diagnos är särskilt viktig när baslivsmedel har eliminierats eftersom kostrestriktionerna riskerar att leda till negativa konsekvenser för livskvalitet och intag av viktiga näringsämnen.

Syfte

Studierna i denna avhandling fokuserade på incidens och remission av födoämnesöverkänslighet bland skolbarn i Norrbotten, som följdes från 7-8 år till 11-12 års ålder, och på prevalens av överkänslighet mot mjölk, ägg, fisk och vete bland 12-åringar, undersökt med rapporterade data, klinisk undersökning samt dubbelblinda placebokontrollerade födoämnesprovokationer och biomarkörer.

Metod

Den huvudsakliga delen av det här projektet är baserat på en populationsbaserad kohort som rekryterades 2006 från 3 kommuner i norra Sverige. Föräldrar till alla barn i klass 1 och 2 (7-8 år) bjöds in till ett frågeformulär, som besvarades av 96% (n=2585) av de inbjudna. Barnen från två av kommunerna, Luleå och Kiruna, bjöds också in till ett pricktest med 10 vanliga luftburna allergen och 90% (n=1700) av de inbjudna deltog. År 2010, när barnen var 11-12 år, gjordes en studieuppföljning med samma metoder och med ytterligare tillägg av en intervju med barnet och bestämning av body mass index (BMI). Studiedeltagandet i enkäter och pricktest var lika högt vid uppföljningen som vid studiestart.
Vid studieuppföljningen bjöds barn med rapporterad mjölköverkänslighet in till en strukturerad intervju och barn som helt undvek mjölk, ägg, fisk eller vete på grund av upplevd överkänslighet, bjöds in till klinisk undersökning och provtagning. Baserat på testresultaten kategoriserades barnen i olika fenotyper av födoämnesöverkänslighet utifrån förutbestämda kriterier. Barn som bedömdes ha en aktuell födoämnesallergi bjöds därefter in till vidare utredning med dubbelblind placebokontrollerad födoämnes-provokation. De recept som användes vid de dubbelblinda provokationerna hade dessförinnan validerats avseende detekterbara smak- och konsistensskillnader mellan aktiv- och placebo substans i en separat kohort av friska skolbarn (n=275).

Före och efter den dubbelblinda provokationen samlades blodprover in för analys av cytokin mRNA-uttryck i mononukleära celler. Analyserna inkluderade cytokiner kännatecknande för humoralt allergidrivande T-hjälpar 2 (Th2) svar, cellulärt cytotoxiskt drivande Th1 svar samt inflammatoriskt- och T-reglerande svar. Vidare insamlades avföringsprover för analys av inflammatoriska biomarkörer före och efter genomgången provokationsserie.

**Resultat**

Prevalensen av föräldrarapporterad födoämnesöverkänslighet ökade från 21% vid 7-8 år till nästan 26% vid 11-12 års ålder. Incidensen av rapporterad födoämnesöverkänslighet var hög (15%), liksom remissionen (33%). Riskfaktorer associerade med incidens och remission var olika för mjölköverkänslighet och överkänslighet mot andra födoämnen. Vi såg också en bristande samstämmighet mellan föräldrarapporterad överkänslighet mot mjölk, ägg, fisk, vete, soja och jordnöt och IgE-sensibilisering mot det aktuella födoämnet.

Vid 11-12 års ålder var prevalensen av rapporterat allergi mot mjölk, ägg, fisk eller vete 4.8%, medan prevalensen baserad på klinisk undersökning och provtagning var 1.4%. Prevalenssiffran halverades ytterligare när kliniskt bedömd födoämnesallergi validerades med dubbelblinda placebokontrollerade födoämnesprovokationer. Majoriteten av barnen med rapporterad allergi mot mjölk, ägg, fisk eller vete klassificerades som andra fenotyper av födoämnesöverkänslighet, varav de vanligast förekommande var möjlig laktosintolerans (40%) och utläkt födoämnesallergi (19%).

Även om förekomsten av rapporterad mjölkköverkänslighet bland 11-12 åringarna var så hög som 14.5%, kategoriserades bara 3% av dessa som en aktuell mjölkallergi. Mjölkallergi, aktuell eller utläkt, var associerat med
andra atopirelaterade tillstånd och ett lägre BMI (OR 0.82, 95% CI 0.80-0.98) jämfört med barn som inte undvek mjölkprodukter.

Före den dubbelblinda provokationsserien var mRNA-uttrycket av den Th2-relaterade cytokinen IL-13 och den regulatoriska cytokinen IL-10 högre bland barn med provokationspåvisad födoämnesallergi jämfört med barn med en negativ födoämnesprovokation. Såväl före som efter provokationsserien kunde högre nivåer av inflammationsmarkörerna eosinofil-deriverat neurotoxin (EDN) och kalprotektin uppmätas i avföringsprover från barn med positivt provokationsutfall jämfört med barn med negativ födoämnesprovokation. Skillnaderna i uppmätta nivåer av biomarkörer i faeces uppnådde dock inte statistisk signifikans.

Slutsats

Rapporterad födoämnesöverkänslighet var vanligt förekommande bland skolbarn i Norrbotten och ökade från 7-8 år till 11-12 års ålder. Incidensen av rapporterad födoämnesöverkänslighet var hög, liksom remissionen. Prevalensen av rapporterad allergi mot mjölk, ägg, fisk eller vete var 8 gånger högre än den prevalens som kunde påvisas med dubbelblind placebokontrollerad födoämnesprovokation. Allergi mot mjölk, ägg, fisk och vete var en ovanlig orsak till att barn helt undvek dessa födoämnen på grund av upplevd överkänslighet. Några av de biomarkörer som analyserades innan provokationsserierna visade lovande resultat som möjliga, framtida prognostiska markörer för symptomatisk, IgE-medierad födoämnesallergi. Dessa resultat behöver dock valideras med ytterligare studier.
Abbreviations

BAT Basophil Activation Test
BMI Body Mass Index
CI Confidence Interval
DBPCFC Double-Blind Placebo-Controlled Food Challenge
EAACI European Academy of Allergology and Clinical Immunology
EDN Eosinophil-derived neurotoxin
ELISA Enzyme-Linked Immunosorbent Assay
EPX Eosinophilic cation protein X
FHS Food Hypersensitivity
FPIES Food Protein Induced Enterocolitis Syndrome
HBD2 Human Beta-Defensin 2
IgA Immunoglobulin A
IgE Immunoglobulin E
IgG Immunoglobulin G
IL- Interleukin
IFN-γ Interferon gamma
ISAAC International Study of Asthma and Allergy in Childhood
kU/L Kilo Units per Liter
MHC Major Histocompatibility Complex molecules
mRNA Messenger Ribonucleic Acid
OAS Oral Allergy Syndrome
OLIN Obstructive Lung Disease In Northern Sweden studies
OR Odds Ratio
PBMC Peripheral mononuclear blood cells
PUFA Polyunsaturated Fatty Acid
qRT-PCR Quantitative Real Time Polymerase Chain Reaction
SPT Skin Prick Test
Tc T-cytotoxic cell
Th T-helper cell
TGF-β Transforming Growth Factor beta
tTGA Tissue Transglutaminase A
Treg T-regulatory cell
USA United States of America
Original Papers


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Introduction

Personal point of departure

When I started working in pediatric Allergy around the millennium shift, one of the greatest challenges in our clinic was the increasing number of children reporting perceived food hypersensitivity. There was a lack of knowledge about different types of food hypersensitivity, their underlying mechanisms and the diagnostic tools available were insufficient. Much has happened since then. Emerging interest and research in the area of food hypersensitivity has broadened our understanding about the different presentations of food hypersensitivity and how to manage this in the clinical setting. Nevertheless, there is still a lack of knowledge about the prevalence of hypersensitivity to different foods at different ages as well as the prevalence of different phenotypes underlying reported food hypersensitivity in children and adolescents. There is also a shortage of easily available prognostic markers determining presence of tolerance.

Also, despite improved diagnostic methods, the number of children with perceived food hypersensitivity seems to be continuously increasing. There are data suggesting that this, at least partially, could be explained by a true increase and changing patterns of food allergy, though there is still a lack of available data on the prevalence, incidence and remission of food hypersensitivity in Sweden as well as globally (1). This awoke the idea in our pediatric allergy team to investigate the epidemiology of food hypersensitivity among school children in Northern Sweden and for this we joined the Obstructive Lung disease in Northern Sweden (OLIN) studies. The extensive experience of population based epidemiological research within the OLIN group made the project in this thesis possible.
Figure 1. Definition of food hypersensitivity (FHS).
(Modified from Johansson et al. Allergy 2001 (2) and printed with permission from Wiley, Copyright © Munksgaard 2001)
Background

Definition of food hypersensitivity (FHS)

There is inconsistency in the terminology of food hypersensitivity (FHS). In this work we have chosen to use the terminology stated in the World Allergy Organization (WAO) position paper published in 2001 (2), where FHS is defined as an umbrella term for adverse reactions to food including reactions of both immunological origin i.e. allergies and reactions driven by non-immunological mechanisms i.e. intolerances (Fig 1).

Allergies – can be either Immunoglobulin E (IgE) mediated or non-IgE mediated. While the immunological mechanisms in IgE-mediated allergies are well described (3) less is known about the causative mechanisms in non-IgE mediated allergies, though T-cells seem to play important roles (4-6).

Intolerances – can be either enzymatic, pharmacological or driven by other, still unknown mechanisms. The most common intolerances are caused by a shortage of or a decreased function of an enzyme necessary for the digestion of a component in food (4,7).

Prevalence of food hypersensitivity

Reported FHS has emerged as a costly health problem in Western countries (8) affecting 3-35% of the population (9-11). The prevalence in different studies is difficult to compare due to heterogeneity in methodology, study populations and definitions of FHS. Most studies focus on the prevalence of IgE-mediated food allergies.

Prevalence of food allergy

Food allergy is thought to affect nearly 8 % of young children and 5 % of adults in Western countries (12) and there are data suggesting that the prevalence is increasing (13). In a recent European study the pooled life-time prevalence and point prevalence of reported food allergy was 17.9% and 5.6% respectively (14). The prevalence of self-reported food allergy is usually higher compared to challenge-proven allergy (9,15). In another recent European meta-analysis, the prevalence of self-reported allergy to the 8 common foods: milk, egg, wheat, soy, fish, shellfish, peanuts and tree nuts was 6.0% while the prevalence of challenge-proven allergy to these foods was
0.6%. In this meta-analysis, allergies to most foods except peanut and soy were more common in Northern Europe (16).

Independent of geographic setting, 90% of IgE-mediated allergies in children are elicited by eight foods; cow’s milk, soy, hen’s egg, peanut, tree nuts, wheat, fish and shellfish (17). Milk, egg and peanut are the main triggers in infants and young children (16,18,19), while peanut, tree nuts, fish and shellfish are the main food allergy triggers in adults (16). The majority of cow’s milk and hen’s egg allergic children develops tolerance during infancy and early school age (20-22).

Allergies to peanuts and tree nuts are more likely to become permanent and less than 20% become tolerant (23,24). Allergy to fish and shellfish can emerge in early childhood, but more commonly at school age (5,16). In addition, raw fruits and vegetables are common triggers of the oral allergy syndrome (OAS) affecting more than 50% of individuals with birch pollen allergy, which is common in the Swedish population (25). Population based studies from the north of Sweden show a prevalence of IgE-sensitization to birch among young adults of 23% (26) and 13% in children aged 7-8 years (27).

While allergies to hen’s egg and cod are predominantly IgE-mediated, IgE and non-IgE mediated allergy to cow’s milk is equally common as is tolerance development in both of these conditions (15,28,29). In a recent European study, 69% of children diagnosed with cow’s milk allergy at one year of age had become tolerant by the age of 2 years (15). In this study, children IgE-sensitized to cow’s milk were less prone to develop tolerance compared to children with no detectable milk-specific IgE. Less is known about the prevalence and natural development of non-IgE-mediated allergy to other foods, like soy and cereals, but studies indicate that most of the affected children develop symptoms during the first years of life and the majority develop tolerance during childhood (4,6,22).

**Prevalence of lactose intolerance**

The most common variant of food intolerance is the adult-type lactose intolerance, which is caused by down regulation of the intestinal enzyme lactase resulting in a decreased capacity to digest lactose (30). The onset of lactose intolerance symptoms most commonly begins during adolescence and rarely before school age (7). The down regulation of lactase is caused by a specific genotype that is common in most parts of the world, except in Northern Europe, where the prevalence of lactase persistence is high (31). The prevalence of lactose intolerance has not been well studied, neither
globally nor in Sweden (7). One Swedish study showed a higher-than-expected prevalence of lactase down-regulation among Swedish school children, the prevalence increasing from 8% to 14% during a 10-year period (32). Others have shown that over-reporting of lactose intolerance is frequent even in populations in which lactase down-regulation is common (33), suggesting that symptoms of lactose intolerance often are caused by underlying mechanisms other than lactase down-regulation (34).

**In conclusion:**

Food hypersensitivity (FHS) is common in children as well as in adults, though the prevalence varies. This is likely due to the use of different study methods, age and origin of the study population and the definition of FHS. Most studies focus on IgE-mediated allergies, which affect around 8% of young children and 5% of adults in Western countries and the prevalence of food allergy seems to be increasing. The prevalence of reported food allergy exceeds the prevalence of allergy established by an oral challenge.

**Factors associated with food hypersensitivity**

A number of intrinsic and environmental factors have been associated with reported food hypersensitivity. These include female sex, number of siblings, allergic heredity, allergic sensitization and presence of atopy related conditions like asthma, rhinitis and eczema (35-37). The prevalence of reported food hypersensitivity also seems associated with age and native country of the study population (10,14,38). Since the risk factors associated with food hypersensitivity are related to the background mechanisms of the different types of food hypersensitivity, risk factors will be discussed separately for food allergy and lactose intolerance.
**Risk factors of food allergy**

Food allergy is a polygenic, complex disease in which the risk of developing symptoms seems to depend on a number of genetic and environmental factors (39). The strong relationship between allergic heredity and development of food allergy is a compelling evidence of the contribution of genetic factors in this disease (39,40). Nevertheless, the specific genetic loci, which modulates food allergy remains to be identified (41) as does the roles of gene-environment interaction, gene-gene interaction and epigenetics (39).

Most studies investigating risk factors of food allergy are focusing on IgE-mediated allergies and early life environmental exposures, given their possibility to modulate developing immune responses and tolerance (42) and being plausibly modifiable. Since the introduction and extension of the hygiene hypothesis (43), these possible early modulating factors include exposures affecting gut microbial composition and diversity: such as family size and place of upbringing (44,45), early infections (46,47), delivery mode (48), use of antibiotics, breast feeding and pre- and probiotics (49,50). In addition, many studies have investigated dietary components as vitamin D (51,52) and polyunsaturated fatty acids (PUFA) (53) as modifiers of allergy development.

Still, there are many questions in the search of the origin of food allergies. Recent data suggest that early introduction of allergenic foods in a child’s diet can steer the immune system towards tolerance development (54,55) while a route of IgE-sensitization through a disrupted skin barrier might do the opposite (56). Childhood eczema is a risk factor associated with development as well as persistence of food allergy (36,57,58). In addition, food allergies often co-exist with other atopy related conditions like asthma, rhinitis and allergic sensitization, which suggests that these conditions may have common risk factors (12,59,60).

Low-level IgE-sensitization and IgE-sensitization to one or a few food allergens may predict remission of a food allergy, while multi-sensitization and high levels of specific IgE is associated with development and persistence of food allergy symptoms (20,23,61). As discussed above, food allergy in children is strongly associated with allergies and atopic diseases in their parents (59). Except for genetic factors, other variables that could affect this association are exposure patterns including allergen avoidance in families at risk of having a food allergic child and report bias (39,63,64).
Some of the differences in the prevalence of food allergy between populations have been related to race. Studies from the USA have shown a higher risk of IgE-sensitization to common food allergens and higher levels of IgE-sensitization in children with self-reported black race and/or African ancestry compared to Hispanic or Caucasian children (65). Further, the prevalence of food allergy may be affected by sex (58,63). Even though food allergies and allergic sensitization are more common among boys than girls (59) there is a shift during adolescence making food allergies more common among women (37,66). The reason for this shift is still unknown, though contribution of hormonal factors has been suggested (67).

Similar to IgE-mediated allergies, non-IgE mediated food allergies are thought to emerge from a failed immunological tolerance development (68). However, the risk factors specifically related to non-IgE mediated food allergies are currently un-explored (64).

Risk factors of lactose intolerance

As in non-IgE mediated food allergies, there is a lack of studies investigating the risk factors of lactose intolerance (7). Since lactose intolerance is caused by a genotype coding for down-regulation of the intestinal enzyme lactase and different mutations of the lactase down-regulating gene are more or less prone to cause symptoms (69), the occurrence of this condition is strongly associated with heredity. Notably, large differences in the prevalence of lactose intolerance have been found between populations (30,70).

In most cases, symptoms of lactose malabsorption develop successively during adolescence making lactose intolerance uncommon in younger children (71). Even though the distribution of the lactase down-regulating genotypes is similar among men and women, symptoms of lactose intolerance are more frequently reported by women than men (70,72).
**Immunological aspects of food allergy and tolerance development**

Food allergy is a general term for adverse immunological reactions to food and includes a number of different disease phenotypes (4,5). A food allergy can be IgE-mediated, non-IgE mediated or a combination of the two. Even though the underlying immunological mechanisms are diverse, and in the case of non-IgE mediated food allergies largely unknown, they are all thought to emanate from failed or abrogated tolerance, which involve an aberrant regulatory T-lymphocyte response (73).

The development of tolerance normally occurs in the immune system of the gut (74). Even though most of our knowledge about tolerance emanates from animal studies, the reigning current paradigm is that the interaction between the gut microbiota, the immune cells of the gut and exposure of food allergens through an oral route are essential for a successful tolerance development also in humans (49).
T-lymphocytes have been given their designation letter because they mature in the thymus (75). There are three subpopulations of T-cells: T-helper cells (Th), T-cytotoxic cells (Tc) and T-regulatory cells (Treg). Following recognition of cell membrane bound antigens called major histocompatibility complex molecules (MHC), Th-cells differentiate into effector cells that enhance the activation of various other cells that participate in the immune response e.g. B-cells, Tc-cells and macrophages. Alternatively, some Th-cells differentiate into memory cells (76). The immune environment of cytokine and chemokine signaling steers the differentiation of Th-cells into different subsets (77). The first two subsets discovered were Th1 and Th2. A predominate Th1-activation is associated with autoimmunity while a dominant Th2-response relates to IgE-mediated allergy (78).

In recent years a number of additional Th-subsets have been discovered, including Th3 and Th17, contributing to the complexity of immune response patterns (73,79). Th17 cells are involved in autoimmunity and has also been suggested a role in neutrophilic asthma (80), whereas Th3 cells are part of T-regulatory cells. T-regulatory cells can suppress the immune response, either by cell to cell contact and/or by the production of down-regulatory cytokines (81,82). T-regulatory cells include CD4+CD25+ T-regulatory (Treg) cells, inducing suppressive effects via cell contact and type 1 regulatory (Tr1) cells and Th3 cells mediating their effects via cytokines e.g. interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) (83).

Treg cells are thymus-derived and express CD25 (the IL-2 receptor) and the transcription factor FoxP3. The suppressor function of Treg cells appear to depend in part on TGF-β, but less on IL-10 (84). Th3 cells are gut derived and characterized by production of TGF-β (+ IL-10), exerting their effects by mediating mucosal tolerance and antigen-specific IgA production (85). Tr1 cells are derived from the periphery and have the capacity to produce IL-10. However, their origin is unclear and as of yet it is not known whether they represent a distinctive developmental pathway or are derived from Th or Treg cells (86).

**IgE-mediated food allergy**

The allergic process starts with exposure of an allergen to the immune system, in food allergy usually via the intestinal mucosa (87,88). In the surface of the mucosa, dendritic cells can sample luminal allergens and present them to naïve T-cells in nearby lymph nodes (88). An environment of Th2-related cytokines and chemokines facilitates clonal expansion and differentiation of the naïve T-cells into Th2 subsets and memory cells.
Further, some of the encountered food allergens are transferred and presented to B-cells, triggering them to mature into plasma cells, that subsequently produce and secrete allergen specific IgE. This process is called allergic sensitization (87,89) (Figure 2).

Re-exposure to the food allergen leads to allergen binding to circulating and cell surface bound specific IgE-antibodies. Cross-linking of IgE on the surfaces of mast cells and basophils can, under the right circumstances, lead to release of inflammatory mediators e.g. histamine and tryptase, capable of triggering an allergic response. Partly due to cross-binding IgG4-antibodies and cross-reactivity between allergens, IgE-sensitization to food allergens is far more common than food allergy (90, 91).

**Figure 2.** Schematic illustration of allergic sensitization resulting in circulating and receptor-bound food antigen specific IgE-antibodies.
**Non-IgE-mediated and mixed-immune food allergies**

Less is known about the background mechanisms resulting in a non-IgE mediated food allergy (6). The non-IgE mediated food allergies are a heterogeneous group of diseases thought to be driven by cell-mediated immunity that can be separated into different phenotypes following improved methods of clinical investigation including increased use of endoscopy. These phenotypes include: food protein induced reflux, eosinophilic esophagitis, eosinophilic gastroenteritis and colitis and food protein induced proctitis (6,89). Non-IgE mediated food allergies often comprise symptoms from the gastrointestinal tract (92) and in some of these conditions signs of inflammation and disrupted cytokine responses have been detected in the local intestinal mucosa (93).

In recent years, it has been shown that in some food allergic diseases, IgE and non-IgE mechanisms can co-occur. In these conditions, T-cell mediated mechanisms are thought to cause a disrupted barrier in the skin or gastrointestinal mucosa and thereby facilitate allergic sensitization (94). Examples of these mixed immune food allergies are eosinophilic gastrointestinal diseases e.g. eosinophilic esophagitis and atopic dermatitis. Much is yet to be discovered about these complex diseases (64).

**Cytokine patterns in tolerance development and IgE-mediated food allergy**

Cytokines are mediators and communicators of the immune system, steering the immunological responses to antigens in predisposed directions (95,96). Food allergens are mainly presented to the immune system in the gut where interactions between the gut microbiota and T-regulatory cells induce a tolerogenic IL-10/TGF-β response that appears essential for tolerance development (49,97,98). IL-10 is a major regulatory cytokine of inflammatory responses (99) that inhibits the production of IgE and upregulates the secretion of IgG4 (100) and seems to play a critical role in tolerance development and outgrowth of food allergy (101-103). In addition, the cytokine TGF-β has been shown to enhance tolerance development by down-regulation of the immune system e.g. by inhibiting proliferation of T-cells and activation of macrophages (104). IL-10 and TGF-β gene polymorphisms are associated with persistent milk allergy in children (105).

Similarly to children with IgE-associated asthma and eczema, children with IgE-mediated food allergy have a cytokine response pattern with increased expression of Th2-related cytokines e.g. IL-4, IL-5 and IL-13 and reduced
production of the Th1-related cytokine interferon gamma (IFN-γ) (106). This dysregulated cytokine response is usually present already at a very young age (107, 108). Th2-associated cytokine patterns have even been detected in cord blood from children who eventually developed food allergy (109). Following stimulation with relevant food allergens, elevated levels of pro-inflammatory cytokines e.g. IL-6 have been detected in challenge-proven milk and egg allergic children compared to children who had developed tolerance to these foods, indicating an inflammatory response triggered by the food allergen in children with persistent allergy (110).

**Allergic inflammation in the intestinal mucosa**

The majority of children with food allergy have at least one symptom from the gastrointestinal tract, which comports with the common route of food allergen exposure (92). In gastrointestinal allergies signs of allergic inflammation can often be detected where the vulnerable intestinal mucosa has been exposed to the culprit food allergen. By examining mucosal biopsies, a skilled pathologist can distinguish eosinophilic inflammation caused by food allergy from other inflammatory diseases of the gut e.g. gastroesophageal reflux and celiac disease (6,92). The increased use of endoscopy in food allergy diagnosis opens up the possibility of measuring cytokines and other biomarkers at the site of the allergic inflammation (6,111). In addition, decomposing products emanating from an allergic inflammation in the intestinal mucosa may be detected in stool samples from food allergic children (112).

**In conclusion:**

*Food allergy is a general term for a heterogeneous group of disease phenotypes resulting from immunological reactions to food with failed or abrogated tolerance and aberrant regulatory T-cell response as common nominators. Cytokines are mediators and communicators of immunological responses. From very early life, dysregulated cytokine patterns can be detected in children eventually developing IgE-mediated food allergies.*
Symptoms of food allergy

Food allergy can cause a broad palette of symptoms and many food allergic children develop symptoms from more than one organ system (5). The most common locations for food allergy symptoms are the skin and the gastrointestinal tract including the oral mucosa (36). Symptoms of food allergy can occur within minutes of allergen exposure or require repeated exposures over time to be elicited. In addition, food allergy mostly causes benign symptoms but it can also be a life-threatening disease (3,113).

To further complicate the management and diagnostics of food allergies, the liability to develop symptoms may differ in the same individual due to co-factors like infections, drugs, simultaneous exposure to other allergens and physical activity (114,115). Even though symptoms caused by different types of food allergies may be confusingly similar, there are some reaction patterns and combination of symptoms that may help in the differential diagnostics (116).

Symptoms of IgE-mediated food allergy

The first symptoms of an IgE-mediated allergy classically occur within 2 hours of exposure and often within minutes (5). As a result, IgE-mediated allergies are also called immediate-type allergies. In some cases, the IgE-mediated food reaction may be biphasic with a symptom relapse within 1-6 hours and in rare cases within days of allergen exposure (91).

Symptoms associated with IgE-mediated food allergies are rapidly emerging itching of the mouth, vomiting, urticaria, angioedema and symptoms from the airways (5,94). IgE-mediated food allergies are also strongly associated with acute life-threatening anaphylaxis, which comprises airway and/or cardiovascular symptoms (117). However, even though food allergies are common triggers of anaphylactic reactions in childhood, these reactions can also be triggered by mechanisms other than IgE-sensitization (118).

Following IgE-sensitization to tree or grass pollens, birch pollen in particular, the pollen-specific IgE-antibodies may bind to other proteins similar to the original allergen occurring in numerous plants and vegetables (119). The majority of these pollen cross-reactive IgE-sensitizations do not cause clinical symptoms (120). Risk factors for symptom development are clinically relevant pollen IgE-sensitizations and IgE-sensitization to multiple pollens. This secondary type of IgE-mediated food allergy is called oral allergy syndrome (OAS) or pollen-associated food allergy syndrome (94).
The clinical symptoms of this condition are usually limited to itching and sometimes erythema of the oral mucosa shortly following allergen exposure. Except for some rare conditions, symptoms of OAS are usually local and benign. The oral symptoms may however be confused with oral symptoms as a first sign of a primary IgE-mediated food allergy (5).

**Symptoms of non-IgE mediated and mixed-immune food allergies**

The most common symptoms of a non-IgE mediated food allergy are skin and/or gastrointestinal symptoms occurring hours and sometimes days after allergen exposure (6,92). These symptoms are also common in mixed-immune food allergies, though symptom onset may be quicker due to the IgE component (94). The symptoms of non-IgE mediated and mixed immune food allergies e.g. eczema, diarrhea, malabsorption, feeding difficulties and failure to thrive are usually not as typically associated to food allergy as the symptoms of an IgE-mediated allergy (5,94). Together with the late onset symptoms, these non-specific symptom patterns often lead to a delayed diagnosis (6,64).

During recent years, we have learned that food allergies affecting the gut can be sorted into different phenotypes according to their location and/or distinct symptom patterns (6). Examples of such gastrointestinal food allergy phenotypes are: food-induced reflux, eosinophilic esophagitis, eosinophilic enterocolitis or food induced proctitis, the latter strictly limited to the most distal part of the colon resulting in blood-tinged stools in an otherwise unaffected infant (6, 94).

As in IgE-mediated food allergies, the non-IgE and mixed immune food allergies contain a broad variety of disease severity, ranging from mild eczema to a potentially life-threatening disease (92). The food protein induced enterocolitis syndrome (FPIES) is a non-IgE mediated food allergy resulting in dramatic symptoms including profuse repeated vomiting, pallor and apathy and in some cases hypovolemic shock. The symptoms of FPIES, that usually occur 1-4 hours following allergen exposure, are often misinterpreted as caused by other acute onset diseases like septicemia, meningitis, severe gastroenteritis or an acute gastrointestinal event (121).
Establishing a food allergy diagnosis

Diagnosing food allergy is a challenge, in every meaning of the word (64,116). There is a broad variety in symptoms, severity and background mechanisms of food allergy. This, together with insufficient diagnostic tools, means that investigation of a suspected food allergy requires knowledge of the different disease phenotypes, potential differential diagnoses as well as the possibilities and limitations of the tests available (122,123). To date, the most reliable method to diagnose or rule out a suspected food allergy is to perform an oral food challenge and the double-blind placebo-controlled food challenge is considered gold standard (5,116,123).

Clinical history

Even though it cannot be used as a solitary tool for food allergy diagnostics, a thorough clinical history can often narrow down the area of search to a few differential diagnoses and be helpful in the choice of proper diagnostic methods (5,116,123). A clinical history for food allergy diagnostics should include: possible food triggers, type and severity of symptoms, time to onset and duration of symptoms after allergen exposure, extrinsic co-factors, age of onset, allergic heredity, other atopy related diseases, growth deviation and eating habits of the child (5,116).
Recently, a standardized diet history tool has been developed and published in order to support the diagnosis of food allergy (124).

**IgE-tests**

The most common laboratory tests in allergy diagnostics are measurement of allergen specific IgE in patient sera and skin prick tests (SPT), measuring the reaction of allergen specific IgE clad mast cells in the subcutaneous tissue when exposed to allergen through a disrupted skin barrier (90,125) (Figure 3). Both of these tests demonstrate IgE-sensitization and not allergy, which also requires the presence of symptoms upon allergen exposure (5,126).

In population-based studies a relationship can be seen between the level of IgE-sensitization and the presence of allergic symptoms following allergen exposure (127). Due to the lack of reliable tests of individual tolerance and diverse allergenic properties in commercially available allergens, there are no valid cut-off levels of specific IgE that guarantee or exclude food allergy (128,129). Nor can the level of specific IgE be used for determining the disease severity (113). Since these tests measure IgE-sensitization, the results must always be interpreted in light of the clinical history (5) and the tests are consequently of no use in diagnosing a non-IgE mediated food allergy (6).

The first generation of IgE-assays was based on crude allergen extracts derived from natural sources, including both allergenic and non-allergenic compounds (90). Today, many allergenic molecules have been identified and by the use of recombinant DNA technology allowing cloning and large-scale production of recombinant allergens, component-resolved analyses of molecular allergens are now available for a number of food allergens (126,130). This new technique has improved the sensitivity and specificity of the IgE-assay, among other things it helps in differentiating cross-reactions from true IgE-sensitization (126). However, even though component resolved diagnosis allows a more individualized IgE-testing, a thorough clinical history is still essential for its interpretation (131).
Oral food challenges

In some cases of food allergy, such as severe immediate onset allergy to a single food trigger, the allergy diagnosis can be based on the clinical history in combination with a concordant IgE-test (132). In most cases of food allergy, however, the clinical history and the results of skin prick tests or serological analyses are not as clear-cut. In these cases further evaluation of the suspected food allergy is needed and the recommended method is the use of a diagnostic elimination diet followed by an oral challenge (5,116).

Figure 3. Schematic illustration of IgE-tests: 1. Measurement of free circulating food allergen specific IgE-antibodies in patient sera and 2. Skin prick test measuring the effects of mast cell degranulation in the subcutaneous tissue following allergen exposure through a disrupted skin barrier.
Most commonly when performing a diagnostic food elimination, a single suspected food allergen is eliminated from the child’s diet over a limited period of time. The duration of the food elimination is determined by the type and severity of symptoms, though an elimination period of 2-4 weeks is usually sufficient (116,132). Even if the child’s symptoms resolve during the elimination period, a food allergy diagnosis cannot be established until a food challenge has been performed, resulting in recurrence of symptoms (116,123,132).

In most cases of food allergy where the food-induced symptoms are benign, the diagnostic food challenge can be performed at home. However, if the parents are not properly instructed and motivated the diagnostic elimination/provocation test is often not completed and the child remains on an elimination diet (133). Therefore, the advice on food elimination should always be evaluated (132). Children with severe food allergic reactions should be followed by a pediatrician and food challenges, when appropriate, should be performed in a hospital setting (5,116).

**Open challenges**

Open challenges are most commonly used in clinical practice. Without blinding and the use of placebo the open challenge is easier to perform and less time consuming. It is, however, limited by the chance of bias, which may result in falsely positive outcomes (116,134). The risk of false positive results is highest if the patient is anxious about the challenge and if the patient’s previous symptoms have been late onset and/or subjective (132).

During a food challenge the food is introduced in gradually increasing doses, usually aiming at a final dose close to a portion size of the challenge food. In most cases a standardized challenge schedule is used, though the food amounts and dose intervals might need to be adjusted according to the severity and eliciting doses of previous reactions (5,123).

**Blinded challenges**

In a single-blinded challenge the serving of challenge food or placebo is blinded to the patient but not to the personnel responsible for the testing. This procedure might reduce the risk of bias on the part of the patient, but can still affect the observer in the interpretation of the challenge outcome (132,135).
Therefore, the double-blind placebo-controlled food challenge (DBPCFC) is considered gold standard in diagnosing food allergy and it is the preferred method for research protocols (5,116,135). Double-blind challenges reduce the patient and observer bias to a minimum and is particularly useful in diagnosing or ruling out adverse reactions to food presenting with delayed, chronic or subjective symptoms (132,136).

However, since the DBPCFC is time and labor consuming it is rarely used in daily clinical practice (116). Also, even though several elaborate guidelines have been published during recent years the procedure of DBPCFC has not yet been fully standardized and there is still a lack of validated DBPCFC recipes (114,136).

**In conclusion:**

Even though a thorough clinical history and the easily available IgE-tests might be helpful, they are usually not sufficient to diagnose or rule out a food allergy. An oral challenge is usually necessary to establish a food allergy diagnosis and the double-blind placebo-controlled food challenge (DBPCFC) is considered gold standard. Since DBPCFCs are costly and time consuming they are rarely used in daily clinical practice. Also, the procedure of DBPCFC has not yet been fully standardized and there is still a lack of validated DBPCFC-recipes.

**Complementary diagnostic tests and future prognostic biomarkers**

The discovery of new food allergy phenotypes brings about the need of new diagnostic methods (64). Some old diagnostic methods are being tested for new and more specific purposes and new diagnostic methods are being developed following deeper understanding of the background mechanisms of food allergy (126,138). Also, since DBPCFCs are time and labor consuming and puts the patient at risk of an allergic reaction, there is an ongoing search for easily available objective prognostic markers that would make double-blind challenges superfluous (131).
**Endoscopy**

Since many years endoscopy has been used to diagnose inflammatory diseases in the gastrointestinal tract. The possibility to take biopsies and visualize the site of inflammation has contributed to the understanding of different phenotypes of gastrointestinal allergies, their distribution and immunological mechanisms (92,111). For some types of food allergy i.e. eosinophilic esophagitis, endoscopy is essential for the diagnosis (137). In the future, endoscopy will probably be more commonly used for measurement of diagnostic markers on site (6,64,122,138).

However, diagnosis by endoscopic methods are invasive and especially for children, not easily available. Establishing a food allergy diagnosis through biopsies from the intestinal mucosa also requires access to experienced pathologists. So far, the fairly extensive resources necessary for endoscopic investigations has limited its use to the larger University hospitals (6,92).

**Inflammatory and regulatory makers in faeces**

Using endoscopic methods, signs of allergic inflammation can often be detected where the vulnerable intestinal mucosa has been exposed to the culprit food allergen (111). A less invasive upcoming diagnostic method is to determine decomposing products from an intestinal allergic inflammation by analyzing stool samples (112). Previous studies have shown that eosinophils are activated in food allergic intestinal inflammation (139) and that eosinophil-derived neurotoxin (EDN) also known as eosinophilic protein X (EPX), one of the major proteins released by eosinophils, might be used as a marker of food allergic reactions (139,140).

It has also been shown that measurement of the cytosolic, immunomodulatory protein calprotectin in faeces can be a helpful tool in the follow-up of response to treatment and determination of reoccurrence of cow’s milk allergy (141). In addition, the antibiotic peptide human beta-defensin 2 (HBD2) and secretory IgA have been investigated in stool samples as possible markers of food-induced inflammation and tolerance in the gut mucosa (142,143).

**Atopy patch tests**

Patch tests are commonly used to identify delayed-type immune reactions to allergens (138). In a patch test the allergen is placed in a small chamber, which is then applied to the skin of the patient for 48 hours. The test reaction is measured after 48 and 72 hours respectively.
In atopy patch testing, the same method is performed using food antigens, usually fresh foods (144). Atopy patch testing was first used in studies of patients with eczema, where it was found to be a useful tool in diagnosing food allergy (145). The test has also been shown to be helpful in identifying food triggers in children with gastrointestinal allergy i.e. FPIES and eosinophilic esophagitis (146,147). Nevertheless, the roles of atopy patch testing in food allergy diagnosis is conflicting and large-scale clinical studies are lacking. Also, the use of fresh foods as allergen extracts in patch testing has not been standardized and neither has the method of reading the test results, making it dependent on the skills of the investigator (148).

**Serological tests**

As previously discussed, the available tests measuring specific IgE to allergenic molecules in foods are rapidly increasing (126). Studies of patients allergic to nuts and peanuts have showed that IgE-sensitization to storage proteins more often correlate to the presence of allergy symptoms, while cross-sensitizations to pollen allergens are usually asymptomatic or causes local, benign symptoms (120). With a more widespread availability, the use of molecular allergen analyses to determine the relevance of food sensitization will probably increase, as will our understanding of the relationships between food allergen IgE-sensitization and symptoms (149).

The occurrence of food-specific IgG is thought to indicate previous exposure to the food and the presence of tolerance, though the role of specific IgG in the mechanism of tolerance development is not yet fully understood (88). During oral and sublingual desensitization therapy, clinical improvement has been associated with a decrease in specific IgE and an increase in allergen specific IgG4 (150). In addition, specific IgG4 has been shown to inhibit peanut-induced basophil and mast cell activation in children that are sensitized but clinically tolerant to major peanut allergens (151). However, the level of increased specific IgG4 does not correlate well with the degree of improvement or severity of the allergic disease which limits the use of specific IgG4 as a sole marker of tolerance development (5).

In addition to being detectable in stool samples, the immunomodulatory protein calprotectin can also be measured in sera. Calprotectin is a cursor of neutrophil inflammation and elevated serum calprotectin levels have been shown to be a useful marker of some rheumatic diseases (152). Recently it has also been suggested as a possible biomarker of neonatal sepsis (153). To our knowledge, no studies have so far been published investigating the levels of serum calprotectin in children with suspected food allergy.
**Basophil activation tests**

In cellular allergy testing, an allergic response is induced *in vitro* (126). The most common cellular allergy test is the basophil activation test (BAT), an upcoming tool for the diagnosis of IgE-mediated food allergy (154,155). During a BAT, allergen-induced changes in the expression of proteins on the surface of basophils are detected using flow cytometry (126). Studies have shown that BAT might be superior to IgE-tests and clinical history in distinguishing a symptomatic peanut allergy from tolerance, especially in ambiguous cases (156,157). The BAT has also been shown to complement more established allergy tests in improving the diagnostic sensitivity (158).

In addition, measurement of basophilic sensitivity as activation at graded dilutions of allergens (CD-sens) offers the possibility of monitoring treatment with anti-IgE and oral tolerance induction in children with severe food allergy (154,159). Up to now performing BATs requires a fairly rapid and costly processing of the collected samples and expertise in performing and interpreting the tests. Increased availability and standardization of methods are necessary for a more wide-spread use of BATs (155).

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**In conclusion:**

New diagnostic test and prognostic markers are emerging, hopefully facilitating future food allergy diagnostics. Analyses of IgE-sensitization to molecular allergens and cellular allergen tests appear promising in improving the diagnosis of symptomatic IgE-mediated allergies while an increased use of endoscopy brings about the possibility of sampling diagnostic markers on the site of inflammation. In addition, decomposing products emanating from local allergic inflammation in the intestinal mucosa may be detectable in stool samples from food allergic children.
**Cytokines**

Food allergic children have a cytokine response pattern with increased expression of Th2-related cytokines e.g. IL-4, IL-5 and IL-13 and reduced production of the Th1-related cytokine IFN-γ (106,110,160). These, and other cytokines including the regulatory cytokine IL-10, have been examined as possible prognostic markers using *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with relevant food allergens and more seldom *in vivo* with oral food challenges (103,161,162).

After stimulation of PBMCs with specific food allergens, the expression of the Th2 cytokine pattern is enhanced and the expressed levels of the regulatory cytokine IL-10 diminished in children with symptomatic IgE-mediated allergy compared to tolerant children (107,161). Due to its active role in tolerance development, IL-10 has been discussed as a possible biomarker for symptomatic IgE-mediated food allergy (101) and so has the Th2-related cytokine IL-13 (163,164).

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**In conclusion:**

Following stimulation with relevant food allergens, decreased expression of regulatory cytokines and increased levels of Th2-related and pro-inflammatory cytokines have been detected in blood samples from children with symptomatic food allergy compared to tolerant children. Some cytokines, including the regulatory cytokine IL-10 and the Th2-related cytokine IL-13 have been suggested as possible prognostic markers of a symptomatic IgE-mediated food allergy.
Objectives

The main objective of the project in this thesis was to examine the clinical epidemiology of food hypersensitivity in a population-based cohort of schoolchildren in Northern Sweden followed from age 7-8 to 11-12 years of age.

The specific aims were:

- To validate new recipes for double-blind placebo-controlled challenges with cow's milk, hen's egg, soy, cod and wheat (Paper I).

- To investigate the incidence and remission of parentally reported food hypersensitivity in a population based cohort followed from 8 to 12 years of age (Paper II).

- To assess the prevalence of allergy to cow's milk, hen's egg, cod and wheat among 11-12-year old Swedish children using reported data, clinical investigations and double-blind placebo-controlled food challenges (Paper III).

- To describe the food hypersensitivity phenotypes among Swedish 11-12-year old children reporting hypersensitivity to cow's milk and to analyze how the different phenotypes correlated to body mass index (BMI), living conditions, allergic sensitization, allergic heredity and physician diagnosed asthma, rhinitis and eczema (Paper IV).

- To investigate the spontaneous cytokine mRNA expression in peripheral mononuclear cells from children with possible food allergy and healthy controls before and after a double-blind placebo-controlled food challenge series. Also, to investigate inflammatory markers in stool samples from children with possible food allergy before and after the completed food challenge and in relation to the challenge outcome (Paper V).
Materials and methods

The first part of this study emanated from a clinical need of improving the methods for diagnosing or ruling out food allergy in our pediatric patients. At the time of the study, there were very few validated recipes for DBPCFC. To allow a wider spread of our developed recipes for double-blind challenges with the foods; cow’s milk, hen’s egg, soy, cod and wheat and to be able to use them in research protocols, we decided to perform a study with the aim of validating these recipes.

1. Method: Paper I

Study population

Whole school classes in the town of Umeå were invited to participate in our test panels. Apart from validating our DBPCFC recipes we also wanted to investigate whether the taste and texture of the challenge substances were acceptable for children of different ages. We therefore invited children from two different age groups; classes 2 and 3 (8-10 years of age) and class 8 (14-15 years of age). Children were excluded from the study if they had a hypersensitivity to the food that was tested.

We aimed to invite around 50 children in each age group (165). During the study, the recipes for milk and wheat had to be improved due to detectable differences between the active and placebo substances. New school classes were thus invited as test panels for the sensorial testing of the improved milk and wheat DBPCFC recipes. In all, 275 children (81% of invited) participated in the testing of at least one DBPCFC recipe.

Sensorial testing

Triangle tests were used for the sensorial testing (165,166). In a Triangle test the panelists are served three samples in coded covered mugs/canisters with a straw, whereof two samples are identical and one is odd (Figure 4). The task of the panelists is to pick out the odd sample. The Triangle test is a forced-choice procedure, meaning that the test panelist has to choose a sample whether or not a perceivable difference is detected (165).
**Statistical analysis**

To determine whether there was a statistically significant detectable difference between our active and placebo substances the numbers of correct responses were calculated and compared to the total number of participants. Further, *p*-values were calculated with a standard binomial distribution and a *p*-value <0.05 was considered statistically significant. One-sided testing was used, since the outcome of interest was whether the odd sample could be detected with significant frequency. The minimum numbers of correct responses to conclude a difference were calculated according to the formula: \[ x = (n/3) + 1.64\sqrt{(2n/9)} \] (165).

**Figure 4.** Arrangement of covered, coded cups with test substances and a worksheet for the performance of a Triangle test.
Method: The OLIN studies

The four remaining papers in this thesis are the results from a collaboration project between the Department of Clinical Sciences, Pediatrics, Umeå University and the Obstructive Lung disease In Northern Sweden (OLIN) studies. For 30 years, the OLIN-studies have pursued epidemiological, population based research with focus on obstructive lung disease and allergies in adults and children in Norrbotten, in the north of Sweden and the studies has so far resulted in more than 250 original scientific papers and 14 doctoral theses.

Study population

This project is based on the second pediatric OLIN cohort recruited in 2006. When recruited, the cohort consisted of all children in classes 1 and 2 (aged 7-8 years, medium age 8 years) in 3 municipalities in Northern Sweden; Luleå and Piteå located by the coast of the Baltic Sea and Kiruna located in the innermost parts of the Norrbotten County (Figure 5). The children invited to this cohort study composed almost 60% of all children in this age group in Norrbotten County in 2006. In 2010, there was a study follow-up, using the same methods. This time, all children in classes 5 and 6 (aged 11-12 years, medium age 12 years) in the same three municipalities were invited to participate in the study (27).
**Questionnaires**

**Procedure**

The parental questionnaire included the International Study of Asthma and Allergies in Childhood (ISAAC) core questions (167) with added questions about symptoms, physician diagnosis, medication and possible determinants of asthma, rhinitis and eczema. This questionnaire has been used in the OLIN cohort studies since 1996 (27,168-170). In 2006, queries about allergy/hypersensitivity to foods were added to the questionnaire (36). The parental questionnaires were distributed by school personnel.

**Participants**

When the cohort was recruited in 2006, the parents of 2585 children, 96% of invited, participated in the questionnaire. At the 2010 study follow-up, the parents of 2612 children, 96% of invited, answered the questionnaire, whereof whom 2378 (89% of the original cohort) also had participated at study start (Figure 6).

**Skin Prick Tests**

**Procedure**

The SPT followed the European Academy of Allergology and Clinical Immunology (EACCI) recommendations (171). The Solu-Prick panel included: birch, timothy, mugwort, cat, dog, horse, two house dust mite extracts (Dermatophagoides pteronyssius and farinae) and two mold extracts (Claudosporium and Alternaria) (ALK, Denmark).

The potency of the extracts was 10 histamine equivalent prick (HEP) except for the two molds, which were 1:20 w/v. Histamine 10 mg/ml and glycerol were used as the positive and negative controls. A positive test was defined as at least one wheal ≥3mm in diameter, recorded after 15 minutes.

**Participants**

In 2006, the 1895 children from the two municipalities Luleå and Kiruna were invited to the SPT and 1700 (90%) participated. At the 2010 study follow-up, 1657 children (86% of invited) participated in the SPT, whereof 1450 (77 % of the children invited in 2006) also had participated in the skin prick testing at study start in 2006 (Figure 6).
**Child interview**

**Procedure**

At the 2010 follow-up, all children in the two municipalities Luleå and Kiruna were also invited to a short interview, hence called Child interview, including questions about perceived allergy/hypersensitivity and avoidance of the basic foods; milk, egg, cod and wheat. The interviews were performed at the children’s schools along with the skin prick tests and assessment of BMI.

**Participants**

A total of 1824 11-12 year old children (98% of invited) participated in the Child interview (Figure 6).

**Serological tests**

**Procedure**

Serum samples were analyzed using an fx5 ImmunoCAP food screening test (ThermoFisher Diagnostics, Uppsala, Sweden) including cow’s milk, hen’s egg, cod, soy, wheat, and peanut. If the screening test was positive, specific IgE was analyzed separately for all of the included foods. An ImmunoCAP inhalant allergen screening was also conducted (ThermoFisher Diagnostics, Uppsala, Sweden) and included the following allergens: birch, timothy, mugwort, cat, dog, horse, dust mites (Dermatophagoides pteronyssius and farinae), and Cladosporium.

An IgE serum level > 0.35 kU/L was considered positive for all of the IgE tests. Further, tissue transglutaminase A (tTGA) was measured in serum as a marker of celiac disease using the EliA™ Celikey™ IgA test (ThermoFisher Diagnostics, and a tTGA serum level < 7 U/L was considered negative.

**Participants**

At the 2010 follow-up all children in Kiruna and 25% randomly selected children from Luleå were invited to donate blood samples for analyses of specific IgE-tests and tTGA. Of 997 invited, 695 children (71 %) participated in the blood sampling.
**Statistical analyses**

The statistical analyses were performed using the Statistical Package for Social Science Software 21 (SPSS Inc, Chicago, IL, USA). In Papers II-V, the Chi square test, and Fisher’s exact test when appropriate, or bivariate logistic regression was used for bivariate comparison of categorical variables. Multivariate regression models were used in Paper II and IV when adjusting for possible confounding factors. The multivariate regression models included the variables that were significant or borderline significant in the bivariate analyses. When comparing continuous variables between groups, the Student’s T-test or ANOVA was used if the variable was normally distributed and the Mann-Whitney test or the Kruskal-Wallis 1-way ANOVA if not. In all papers, a 95% confidence interval (CI) was used and a p-value < 0.05 was considered statistically significant.
Definitions

Paper II:
▫ Any FHS - was defined as questionnaire-reported symptoms to at least one of the specific foods: cow’s milk, hen’s egg, fish, wheat, soy, kiwi, orange, apple, raw carrots, banana, nuts, peanuts and almonds.

▫ Non-milk FHS - was defined as questionnaire-reported symptoms to one or more of the specific foods; hen’s egg, fish, wheat, soy, kiwi, orange, apple, raw carrots, banana, nuts, peanuts and almonds, cow’s milk excluded.

▫ Milk FHS - was defined as questionnaire-reported symptoms to cow’s milk only.

Paper III:
▫ Reported food allergy - was defined as reported allergy or hypersensitivity to and complete avoidance of one or more of the foods; cow’s milk, hen’s egg, fish, or wheat in the Child interview (children in Luleå and Kiruna) or in the parental questionnaire (children in Piteå). Children were not included in this definition if they had a questionnaire-reported physician diagnosed celiac disease.

Paper III-V:
▫ FHS phenotypes – were based on data collected from the structured interviews and analyses of food specific IgE and tTGA. The food hypersensitivity phenotypes were determined by the following preset criteria:

▫ IgE-mediated food/milk allergy phenotype – was defined as the fulfilled mandatory criteria:
  • A positive food specific IgE-test – provided exposure to the culprit food within the last two years
  • Symptoms triggered by less than 100 ml of milk or less than a portion size of egg, cod or wheat products

...and at least two fulfilled secondary criteria:
  • Onset before five years of age – provided that the food had been introduced in the child’s diet prior to that age
  • First symptom within 15 minutes of exposure
  • Symptoms from more than one organ system
  • Symptoms triggered by treasure amounts of the culprit food
• Symptoms triggered by skin exposure
• Symptoms triggered by airborne exposure
• Anaphylaxis / exercise induced anaphylaxis

\( ^\circ \)Non-IgE mediated food/milk allergy phenotype\(^\)– was defined as the fulfilled mandatory criteria:
• A negative food specific IgE-test
• Symptoms triggered by less than 100 ml of milk or less than a portion size of egg, cod or wheat products
• No celiac disease

...and at least two fulfilled secondary criteria:
• Onset before five years of age – provided that the food had been introduced in the child’s diet prior to that age
• First symptom more than 1 hour after exposure
• Symptoms from more than one organ system
• Symptoms triggered by treasure amounts of the culprit food

\( ^\circ \)Suspected food allergy – was defined as all but one fulfilled criteria for the IgE-mediated or non-IgE mediated allergy phenotypes.

\( ^\circ \)Outgrown food/milk allergy phenotype – was defined as a convincing history of an IgE-mediated or non-IgE mediated allergy, and continued avoidance of the culprit food despite tolerance of at least 100 ml of milk or a portion size of egg, cod or wheat products without symptoms.

\( ^\circ \)Probable lactose intolerance phenotype – was defined as fulfilled mandatory criteria:
• Symptom onset after five years of age
• Symptoms limited to flatulence, stomach-ache and/or diarrhea
• Symptoms triggered by 100 ml of milk or more
• Symptom-free on a lactose free/reduced diet
• No milk allergy
• No Celiac disease

\( ^\circ \)Suspected lactose intolerance – was defined as all but one fulfilled mandatory criteria for the probable lactose intolerance phenotype.

\( ^\circ \)Celiac disease phenotype - was defined as a reported physician diagnosis of Celiac disease and/or a positive tTGA-test.
Non-definable phenotype – was defined as avoidance of milk, egg, cod or wheat but did not fulfil the criteria for any of the listed phenotypes or declined donation of food specific IgE and/or tTGA, which were essential for the FHS phenotyping.

Discontinued elimination diet – was defined as reported food allergy at the Child interview (children in Luleå and Kiruna) or in the parental questionnaire (children in Piteå) and cancellation of the food avoidance prior to the structured interview.

Paper II-V:

Allergic heredity – was defined as questionnaire-reported parental asthma, rhinitis and/or eczema.

FHS heredity – was defined as questionnaire-reported parental food hypersensitivity

Allergic sensitization – was defined as at least one positive SPT or positive IgE screen test.
Figure 6. Overview of the study participation: total number of children (and percentage of invited) in the different parts of investigation of the pediatric OLIN-cohort that was recruited in 2006 and followed-up in 2010.
2. Method: Paper II

**Study population**

The main objective of Paper II was to investigate the incidence and remission of parentally reported food hypersensitivity between 7-8 and 11-12 years of age. For this longitudinal study we included the 2378 children (89% of invited) from the OLIN cohort who participated both at study start and at the 2010 follow-up (Figure 6).

**Questionnaires and Skin prick tests**

Questionnaire data regarding parentally reported hypersensitivity to foods at age 7-8 years and 11-12 years were collected. Variables from the parental questionnaire and SPT results analyzed as possible risk factors for incidence and remission of reported hypersensitivity to foods were collected from the 2006 investigation when the children were 7-8 years of age.

**Serological tests**

Among the serological analyses collected from the random population sample, results from the fx5 ImmunoCAP food screen test (ThermoFisher Diagnostics, Uppsala, Sweden) were available for 652 children (71% of invited) of those who had participated in the questionnaire both at study start and study follow-up. The presence of a positive IgE to any of the foods; milk, egg, cod, wheat, soy and peanut was analyzed in relation to parentally reported hypersensitivity to the corresponding food.
Figure 7: Study overview paper III

(Winberg et al. PlosOne 2015)
3. Method: Paper III

Study population

The main objective of Paper III was to assess the prevalence of allergy to cow’s milk, hen’s egg, cod and wheat at 11-12 years of age. In this cross-sectional study we included all of the 2612 children who participated in the 2010 questionnaire (Figure 6). A study overview is presented in Figure 7.

Questionnaires and Child interviews

The inclusion criteria for invitation to the clinical investigation was a reported allergy to the basic foods; milk, egg, cod or wheat, defined as:

- Reported allergy/hypersensitivity to milk, egg, cod or wheat
- Complete avoidance of the culprit food
- No physician diagnosed celiac disease

The children from the two municipalities Luleå and Kiruna were included according to their answer to the Child interview question:

“To what extent do you avoid the following foods; cow’s milk, hen’s egg, fish, or wheat, due to allergy/ hypersensitivity?” The possible answer alternatives for each food were; not at all, partially or completely.

Since the children in the municipality of Piteå were not invited to the Child interviews and SPTs, these children were included according to the answer to the following question in the parental questionnaire:

“To what extent does your child avoid the following foods; cow’s milk, hen’s egg, fish, or wheat, due to allergy/ hypersensitivity?” The possible answer alternatives for each food were; not at all, partially or completely.

Data on celiac disease was collected from the parental questionnaire query:

“Has your child been diagnosed by a physician as having celiac disease?”
**Clinical examinations**

The children with reported allergy were invited to a clinical examination, including:

- A structured interview (Supplemental file)
- A physical examination
- Blood samples for analysis of tTGA, food specific IgE, an IgE food screen test and an IgE inhalant screen test

The clinical examination included measurement of length (cm) and weight (kg) and a clinical investigation including inspection of ears, mouth and skin, auskultaion of heart and lungs and palpation of abdomen, thyroid and nodular glands. All clinical examinations were performed by the same pediatric allergist (AW).

**Food hypersensitivity phenotypes**

According to the results from the clinical examinations, children were sorted into the following food hypersensitivity phenotypes according to preset criteria (see Definitions):

- Current food allergy
  - IgE-mediated allergy
  - Non-IgE-mediated allergy
  - Suspected allergy
- Outgrown food allergy
- Lactose intolerance
- Celiac disease
- Non-definable cases/ Non-avoidance diet

**Double-blind placebo-controlled food challenges**

The DBPCFC was performed as a 3-session challenge series, the sessions set one week apart (Figure 9). At two of the challenge sessions the test substance contained the challenge food and at one session the test substance was free of the challenge food (placebo).

The serving order was drawn by lot and unknown to both the patients and the personal undertaking the challenges (AW and ÅS). The test substances were prepared immediately prior to the challenge session and the same dietician (LN) was responsible for the preparation during all the challenge series. The DBPCFC recipes used were those previously validated in Paper I.
Due to safety reasons, the DBPCFC series were performed in a hospital setting. The test substance was served in increasing doses 30 minutes apart and the child remained for observation for 2 hours after the completed challenge. The test session was aborted at the dose step where the child developed clear, objective symptoms or when all dose steps had been completed. If the symptoms were severe, the whole challenge series was terminated. Following each challenge session, the child performed a 3-day symptom diary.

The DBPCFC code was broken at a follow-up visit one week after the completed challenge series. The same pediatric allergist (AW) and study nurse (ÅS) were responsible at all challenge-sessions and follow-up visits. A DBPCFC series was considered positive if the child during or following the challenge sessions with active substance developed symptoms that were objective and/or repeatable.
Figure 8. Study overview paper IV
4. Method: Paper IV

Study population

The low prevalence of challenge-proven allergy to basic foods initiated further examination of the children reporting partial milk avoidance, as we had only included children reporting complete milk avoidance in Paper III. For this cross-sectional study we included all 1824 children (98% of invited) from the 2010 follow-up who had participated in the Child interview (Figure 6). The main aim of this study was to investigate the milk hypersensitivity phenotypes among children reporting milk avoidance due to perceived hypersensitivity and whether the phenotypes were different between children reporting complete or partial milk avoidance.

Child interviews, structured interviews and serological tests

The inclusion criterion was a report of milk avoidance, partially or completely, due to perceived milk hypersensitivity. The inclusion was based on the following question from the Child interview:

“To what extent do you avoid the following foods; cow’s milk, hen’s egg, fish, or wheat, due to allergy/ hypersensitivity?” The possible answer alternatives for each food were; not at all, partially or completely.

Children with physician-diagnosed celiac disease were not invited to the structured interview. Data on celiac disease was collected from the parental questionnaire query:

“Has your child been diagnosed by a physician as having celiac disease?”

Children participating in the child interview were also invited to assessment of BMI and 1808 children (97% of invited) participated in measurement of both length and weight.

Data regarding living conditions, allergic sensitization, allergic heredity and physician-diagnosed asthma, rhinitis and eczema were collected from the 2010 parental questionnaire.
The structured interviews were performed 6-12 months after the Child interview.

- Children reporting complete milk avoidance were invited to the Clinical examination described in Paper III, which included a structured face-to-face interview.
- Children reporting partial milk avoidance were invited to the same structured interview but over telephone.

Children, who at the time of the telephone interview still avoided milk, were invited to donate blood for analyses of tTGA, milk specific IgE, an IgE food screen test and an IgE inhalant screen test (ThermoFisher Diagnostics, Uppsala, Sweden). Results from analyses from the same tests were already available for the children reporting complete milk avoidance who participated in the Paper III study.

**Food hypersensitivity phenotypes**

According to the results from the structured interviews and blood sample analyses, the children were sorted into the following milk hypersensitivity phenotypes according to preset criteria (see Definitions):

- Current milk allergy
  - IgE-mediated allergy
  - Non-IgE-mediated allergy
- Outgrown milk allergy
- Probable lactose intolerance
- Celiac disease
- Non-definable cases
- Discontinued milk avoidance

The prevalence of the different milk hypersensitivity phenotypes was compared between children reporting complete and partial milk avoidance. In addition, the association between the different milk hypersensitivity phenotypes and BMI, living conditions, allergic sensitization, allergic heredity and physician diagnosed asthma, rhinitis and eczema was analyzed.
5. Method: Paper V

Since DBPCFC are time and labor consuming and put the child at risk of an allergic reaction, there is ongoing search of objective, prognostic markers that could predict the food challenge outcome. We examined the spontaneous cytokine mRNA expression of PMBCs, quantified for a panel of 15 cytokines discriminating between a humoral Th2-, cytotoxic Th1-, regulatory Th3/Tr1- and inflammatory responses in children with suspected food allergy and healthy controls before and one week after a completed food challenge-series. In addition, inflammatory and regulatory biomarkers in faeces were analyzed in children with suspected food allergy. The main objective was to investigate whether the included biomarkers had the potential of determining the challenge outcome.

Study population

We included all 18 children with suspected food allergy who participated in the DBPCFC series in Paper III. Complete analyzable blood samples were available from 17 of these children.

In addition, 7 children without any allergies randomly selected from the OLIN cohort participants living in Luleå, were invited to participate in a DBPCFC series with egg. All of the invited controls participated in the challenge series and complete blood samples were available from 6 of these children.

Serum sampling and serological tests

Blood samples for the analyses of expressed cytokine mRNA patterns were collected immediately before the first challenge session (baseline samples) and one week after the last session (post challenge samples) (Figure 9). Sera for analysis of food specific IgE and IgG4 (ThermoFisher Diagnostics, Uppsala, Sweden) were collected from all children at baseline and from children with suspected food allergy post challenge.

Cytokine expression in PBMCs

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-paque gradient centrifugation. Then, using quantitative real time polymerase chain reaction (qRT-PCR), a cytokine gene expression analysis was made.
The messenger ribonucleic acid (mRNA) expression of the 12 included cytokines were analyzed as markers of T-cell response; IFN-γ as a marker of Th1-response, IL-4, IL-5 and IL-13 as markers of Th2-response, TGF-β and IL-10 as markers of T-regulatory response, IL17-A as a marker of Th-17 response, IL-2 and IL-15 as markers of T-cell proliferation and TNF α, IL-1β, IL-6 and IL-8 as markers of pro-inflammatory response. The expression of the different cytokines at baseline and post challenge was then analyzed according to DBPCFC outcomes and in relation to the control group.

Figure 9. Study overview of paper 5 and analyzed cytokine mRNA expression in peripheral blood mononuclear cells including hallmark cytokines for the humoral allergy-promoting T helper (Th) 2 response, cellular cytotoxicity-promoting Th1 response, inflammatory-, and T regulatory responses.
Biomarkers in stool samples

The 18 children with suspected food allergy were also invited to donate stool samples at baseline and post challenge. From the complete stool samples available from 12 of these children, five fecal markers where quantified using enzyme-linked immunosorbent assay (ELISA) methods (Immundiagnostik, Bensheim, Germany): PhiCal®Calprotectin, EDN (Eosinophil-derived neurotoxin), sIgA (Secretory IgA), HBD-2 (human β-Defensin-2) and α1-Antitrypsin. The levels of the different fecal markers at baseline and post challenge were then analyzed according to DBPCFC outcomes.
Results

Validation of new DBPCFC recipes (Paper I)

In this study we aimed to validate new recipes for low-dose double-blind placebo-controlled food challenges in school children, by investigating if there were any sensory differences between the active materials containing cow’s milk, hen’s egg, soy, wheat or cod, and the placebo materials. The challenge materials contained the same amino acid based product Elemental 028 extra liquid orange/pineapple flavour (Nutricia, Liverpool, England), with or without added food allergens. Further, to match the sensorial properties of the challenge foods, supplementary ingredients had been added to the different test materials. The test panels consisted of 275 school children, aged 8-10 and 14-15 years respectively, from five Swedish schools. Each participant tested at least one recipe.

Standardized blinded triangle tests were performed to investigate if any sensory differences could be detected between the active and placebo materials. In our final recipes, no significant differences could be detected between the active and placebo materials for any challenge food ($p>0.05$). These results remained after stratification for age and gender. The taste of challenge materials was acceptable to the children in both age groups and no unfavourable side effects related to test materials were observed.

**Main results:**

New recipes for low-dose double-blind placebo-controlled food challenges for milk, egg, cod, wheat and soy were validated regarding detectable differences between the active and placebo substances. The taste and texture of the test substances were accepted by the children in the test panels, i.e. at 8-10 and 14-15 years of age.
Incidence and remission of food hypersensitivity (Paper II)

**Prevalence, incidence and remission**

In this longitudinal study, we investigated parentally reported FHS in a population based cohort of schoolchildren, followed from 8 to 12 years of age. The prevalence of reported hypersensitivity to any food increased from 21% at 8 years to 26% at 12 years of age (p < 0.001). During the same 4-year period, the prevalence of reported hypersensitivity to the basic foods: milk, egg, cod and wheat increased from 10% to 15% (p < 0.001) and the prevalence of reported milk hypersensitivity from 9% to 13% (p < 0.001) (Figure 10). The cumulative incidence of reported FHS was high (15%), as was remission (33%). This pattern was recognizable for hypersensitivity to many of the 13 common foods investigated, but it was particularly evident for hypersensitivity to cow’s milk.

![Figure 10](image.png)

**Figure 10.** Comparison of prevalence of food hypersensitivity, asthma, rhinitis and eczema respectively, at the age of 7-8 and 11-12 years of age.
Factors associated with incidence and remission

Female sex, allergic heredity, current rhinitis and allergic sensitization were associated with the incidence of any FHS. Allergic sensitization was negatively associated with remission. While the factors associated with the incidence and remission of non-milk FHS were similar to the factors associated with any FHS, the risk factor patterns for milk hypersensitivity were different. Female sex and allergic heredity were associated with the incidence of milk FHS and living in Kiruna was negatively associated with its remission, though no association was found to other atopy related conditions (Figure 11).

**Figure 11.** Association of listed variables at 8 years of age to the incidence of non-milk FHS and milk FHS presented as odds ratios (OR) and 95% confidence intervals (CI).
**Reported FHS and IgE-sensitization**

Among the 652 children (71% of invited) who participated in the serological tests, parentally reported hypersensitivity to the foods; milk, egg, cod, wheat, soy and peanut were analyzed in relation to the presence of a positive IgE-test to the culprit food. Generally, the agreement between reported food hypersensitivity and IgE-sensitization to the implicated food was poor. This was particularly true for the foods: milk, wheat and soy. However, even for peanut, only one third of the peanut sensitized children reported symptoms and only one third of the children with reported hypersensitivity to peanut had a positive IgE-test.

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**Main results:**

*There was a high incidence as well as a high remission of reported food hypersensitivity from age 8 to 12 years of age. Risk factors associated with incidence and remission were different for milk hypersensitivity and hypersensitivity to foods other than milk. The agreement between reported symptoms to milk, egg, cod, wheat, soy and peanut and IgE-sensitization to the culprit food was poor.*
Assessment of allergy to milk, egg cod and wheat (Paper III)

In this cross-sectional study we investigated the prevalence of allergy to the basic foods; milk, egg, cod and wheat among 11 to 12-year-old children in a population based cohort using:

1. Reported data
2. Clinical investigations (structured interviews, physical examinations and analyses of serum specific IgE and a celiac screening test)

The prevalence of reported hypersensitivity to cow’s milk, hen’s egg, cod, and/or wheat among the 11 to 12 year olds was 14.7% and the prevalence of reported allergy to these foods according to our definition was 4.8% (Figure 12).

The 125 children with reported allergy to milk, egg, cod and wheat were invited to the clinical examination and 94 (75%) participated. Based on the results, children were categorized into the following FHS phenotypes: current allergy (29%), outgrown allergy (19%), lactose intolerance (40%) and unclear (12%). Among children categorized as having a current allergy, positive food (63%) or inhalant (74%) allergen screen test were common, as were reported physician diagnoses of asthma (52%), rhinitis (44%), and eczema (67%). Assuming a similar distribution among the non-participants, the prevalence of allergy to milk, egg, cod and wheat according to the clinical examination was 1.4% (Figure 12).

The 27 children categorized as current food allergy were then considered for further evaluation with a DBPCFC series. Two of these children fulfilled preset exclusion criteria and were therefore not included. Of 25 invited, 18 children (72%) participated in a total of 20 challenge series with milk, egg or cod. Thus, 2 children participated in 2 challenge series with different basic foods.

Out of the 20 DBPCFCs, 9 were considered positive. All but one child with a positive challenge outcome were IgE-sensitized to the culprit food. Four of the nine children with positive challenge outcomes reacted with an anaphylaxis. Only one of these children had previously been prescribed an epinephrine auto-injector and none of these children were on daily treatment for their concomitant asthma. Assuming a similar distribution of
positive DBPCFC outcomes among the non-participants, the food allergy prevalence following DBPCFC was 0.6% (Figure 12).

**Figure 12:** Prevalence of reported food hypersensitivity and allergy to the basic foods; milk, egg, cod, and wheat according to reported data, clinical investigation and double-blind placebo-controlled food challenges.

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**Main results:**

The prevalence of reported allergy to cow’s milk, hen’s egg, cod, or wheat was 4.8%, whereas the prevalence according to preset diagnostic criteria was 1.4%. This figure was further halved when possible food allergy was evaluated with double-blind placebo-controlled food challenges. The majority of children with reported allergy to basic foods were categorized as other food hypersensitivity phenotypes, the most common being probable lactose intolerance and outgrown food allergy.
Milk hypersensitivity phenotypes (Paper IV)

In this cross-sectional study we investigated the milk hypersensitivity phenotypes among 11-12-year old children with self-reported milk avoidance due to perceived milk hypersensitivity. Of the whole study sample, n=1824, the prevalence of self-reported milk hypersensitivity was 14.5% (n=265). Of these, 193 children (73%) reported partial milk avoidance and 72 children (27%) reported complete avoidance of milk. Among the 265 children with reported milk hypersensitivity, 236 (89%) participated in a structured interview.

Only 3 % of the 11 to 12-year-olds with self-reported milk hypersensitivity were categorized as current milk allergy; of whom all had reported complete milk avoidance. The most common milk hypersensitivity phenotype was probable lactose intolerance (40%) followed by outgrown milk allergy (23%).

![Figure 13. Distribution of the different milk hypersensitivity phenotypes among children reporting complete and partial milk avoidance](image)

Figure 13. Distribution of the different milk hypersensitivity phenotypes among children reporting complete and partial milk avoidance
Children with outgrown milk allergy had a convincing history of milk allergy but tolerated at least 100 ml of milk without any symptoms at the time of the evaluation. Despite this, they had remained on an elimination diet. At the time of the structured interview, 30% of the children had discontinued their milk elimination diet. All children with discontinued elimination diet had previously reported partial milk avoidance. Except for the phenotypes current milk allergy and discontinued milk avoidance, the distribution of the different milk hypersensitivity phenotypes was similar between children reporting complete and partial milk avoidance (Figure 13).

**Figure 14.** Association of the listed variables to milk allergy, current or outgrown expressed as odds ratios (OR) with 95% confidence intervals (CI).
Children with current or outgrown milk allergy had a lower BMI (OR 0.82, 95% CI 0.80-0.98) compared to children with no milk avoidance. Also, among children with milk allergy the variables female sex, physician diagnosed eczema and heredity for food hypersensitivity were more common compared to children not avoiding milk (Figure 14). In contrast, children with probable lactose intolerance were less often sensitized to aeroallergens and food allergens compared to children with no reported milk hypersensitivity.

Only 9% of the children currently avoiding milk reported that they in previous health care contacts had ever been referred to a dietician for nutritional advice and only 2% reported that their milk hypersensitivity diagnosis had been established by an oral challenge.

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**Main results:**

Even though the prevalence of reported milk hypersensitivity among the 11-12 year olds was as high as 14.5%, only 3% of them were categorized as current milk allergy. The most common milk hypersensitivity phenotypes were probable lactose intolerance and outgrown milk allergy. Current and outgrown milk allergy was associated with other atopic disorders and lower BMI. Only 9% of the children currently avoiding milk reported that they had ever been referred to a dietician for nutritional advice and only 2% had a milk hypersensitivity diagnosis established by an oral challenge.
If only immunology was this simple...
Biomarkers in blood and stool samples (Paper V)

Here, we analyzed the spontaneous cytokine mRNA expression pattern of PMBCs before and one week after a completed DBPCFC series with milk, egg or cod in 12–13 year old children with suspected food allergy and in age-matched healthy controls. Blood samples were available from 17 children with suspected food allergy; 9 children with negative challenge outcomes and 8 with positive challenge outcomes. Of the children with positive challenge outcomes, 3 had reacted with anaphylaxis during challenge. In addition, 7 children from the OLIN cohort without any allergies participated in a DBPCFC series with egg and blood samples were available from 6 of these controls.

Further, inflammatory and regulatory biomarkers in faeces were analyzed in children with suspected food allergy before and one week after the DBPCFC series. Stool samples were available for 12 children, 6 with positive and 6 with negative challenge outcomes.

Figure 15. Median mRNA expression of the cytokines IL-13 and IL-10 at baseline and post challenge in children with positive and negative challenge outcomes and in controls.
At baseline, children with suspected food allergy expressed higher mRNA levels of the Th2-related cytokine IL-13 compared to controls. The baseline mRNA level of IL-13 was higher in children with a positive challenge outcome compared to children with a negative challenge outcome. The baseline levels of the regulatory cytokine IL-10 were similar in the controls and in children with a negative challenge outcome, though significantly higher among children with a positive challenge outcome, i.e. challenge-proven food allergy (Figure 15).

Post challenge, children with suspected food allergy expressed higher mRNA levels of the pro-inflammatory cytokines IL-1β and IL-6 compared to controls but there were no significant differences detected in the post challenge cytokine mRNA profiles between children with positive and negative food challenges.

Figure 16. Median values of fecal markers at baseline and post challenge among children with a positive (n=6) and negative (n=6) challenge outcome.
Three of the children in our study reacted with anaphylaxis during DBPCFC. Compared to healthy controls, children with anaphylaxis expressed 10-fold higher mRNA levels of the Th2-related cytokines IL-4 and IL-13 at baseline and 30-100 fold higher levels of the pro-inflammatory cytokines IL-1β and IL-6 post challenge. Children with anaphylaxis also expressed a 10-fold higher post challenge mRNA level of the regulatory cytokine IL-10 compared to controls. As opposed to this finding, the post challenge mRNA level of the regulatory cytokine TGF-β1 was decreased among children with anaphylaxis compared to healthy controls. Due to the low number of children with anaphylaxis, no statistical analyses were performed on this subgroup.

The levels of calprotectin and EDN in feces were higher among the children with a positive food challenge compared to children with a negative challenge outcome both at baseline and post challenge, though the differences did not reach statistical significance (Figure 16). There were no detectable differences between children with challenge-proven allergy and children with a negative challenge outcome for the fecal biomarkers: sIgA, β-Defensin-2 and α1-Antitrypsin.

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**Main results:**

At baseline, children with suspected food allergy had elevated expression levels of the Th2-associated cytokine IL-13 compared to controls. IL-13 mRNA and mRNA for the regulatory cytokine IL-10 were higher in challenge-proven food allergy compared to the cytokine levels seen in negative challenges. Post challenge, IL-1β and IL-6 mRNA were elevated in the allergic children compared to controls. Fecal calprotectin and EDN levels were higher in challenge proven food allergy compared to a negative challenge although not statistically significantly.
Discussion

Discussion of methodology

Sensory testing

There are two types of sensorial differentiation tests; Overall difference tests that are designated to show whether subjects can detect any difference between samples and Attribute difference tests where different samples are ranked according to one or more attributes i.e. sweetness (166). Since our aim was to investigate whether children in different age groups could detect any sensorial difference between the active and placebo materials of our recipes, we choose to use the Overall difference test called Triangle test. Compared to the alternative Overall difference test called Duo-trio-test, the Triangle test is statistically more efficient (165,166).

It has been suggested that for sensorial testing, professional adult panelists should be used (166,172). However, since a secondary aim of our validation study was to examine whether the taste and texture of our test samples were acceptable to children of different ages, we chose to include children aged 7-10 years and 14-15 years in our test panels. To increase the validity of the tests, we used large test panels. In a triangle test, a test panel of at least 25-30 people is required to obtain the requisite sensitivity of the test (165,172). We therefore aimed to invite around 50 children in each age group. Further, before starting the study tests the participating children were educated in the triangle test technique using two different kinds of fruit juices.

Epidemiologic study design

A major strength of our project including Papers II-V, is the longitudinal and population-based design and the high participation-rates, supporting the representativeness of the population i.e. the internal validity. The internal validity describes to what extent the study outcome defines the true situation in the study population, while external validity describes whether the results of the study are applicable to other populations or settings (173).

Since the investigated cohort represent almost 60% of the children in Norrbotten County, we have reason to believe that our findings are representative for schoolchildren in the North of Sweden.
Supported by the findings of others (16,32,35,38), we also think that our results might be applicable to children in other parts of Sweden and perhaps even to children with similar socio-economic conditions in other parts of the Western world, even though the very high percentage of Caucasians in our cohort could affect the prevalence of FHS (32,65).

The relatively large study cohort and the commonness of the investigated conditions, support the reliability of our studies. The pediatric cohort was originally recruited for investigating prevalence of asthma, rhinitis, eczema and allergic sensitization and for this the power of the study was excellent. The calculated power for investigating incidence and remission of these conditions was also satisfactory.

In line with this, the power of our questionnaire studies investigating prevalence, incidence and remission of reported food hypersensitivity was good. Even though the participation in the structured interviews and clinical examinations were somewhat lower, 89% and 75% respectively, the very high participation (96-97% of invited) in the parental questionnaires allowed us to perform non-responder analyses based on questionnaire data. In Paper III and IV these analyses did not show any significant differences for variables like: sex, living conditions, older siblings, parental smoking, allergic heredity, allergic sensitization and physician diagnosed asthma, rhinitis and eczema between participants and non-participants in the structured interviews and clinical examinations. Thus the study participants were representative of the entire cohort.

A possible limitation with the population-based design, was that despite a large cohort, the high participation rate and a high prevalence of reported hypersensitivity to the basic foods: milk, egg, cod and wheat in the entire cohort, the final prevalence of current food allergy following clinical examination was low. As a consequence, a small group of children were finally invited to the DBPCFC series, affecting the power in the analyses of the challenge outcomes in Paper III and more so in the statistical analyses of biomarkers in Paper V. The findings of the latter study therefore needs to be verified by larger studies, by either inviting a larger cohort or by inviting study participants from a hospital material (173).
In Paper II we investigated the cumulative incidence of reported food hypersensitivity i.e. the onset of new cases with time, and the remission i.e. the number of study participating recovering with time (173), between the ages 7-8 and 11-12 years. A longitudinal study design, where a cohort is followed over time, is preferable when examining incidence or remission of a condition. In Papers III and IV, we used cross-sectional studies to investigate the prevalence of allergy to basic foods and other food hypersensitivity phenotypes at 11-12 years of age. Prevalence is a measure of the percentage of individuals affected by a condition or exposure at a certain point of time. Combining longitudinal and cross-sectional data on FHS rendered a broader picture and a better understanding of the investigated condition and was a major strength of our study.

**Reported data**

A significant part of this work is based on reported data, making it susceptible to information bias (175). Bias stands for a systematic error that causes distortion of the study, which might affect the study outcome (173,174). Information bias is caused by measurement errors and can arise when some people are more or less inclined to report the presence of a disease or an exposure, defined as reporting bias, or if certain people are more or less prone to remember conditions and exposures of the past, defined as recall bias (175). Information bias may also occur if different questionnaires or study methods are used at different study occasions, when using study methods that are highly dependent on the investigator or when the same reported data are collected from different individuals, e.g. children and either of their parents.

In our study we used a comprehensive questionnaire, which was almost identical at study start and follow-up. The questionnaire has also been used in previous OLIN-studies since 1996 (27,168-170). Further, the clinical examinations in Paper III were all performed by the same pediatric allergist (AW). We did not, however, have access to the children's medical journals to verify reported data on food hypersensitivity diagnoses and previous diagnostic tests, which could have added further information and reduced the risk of report bias.

In Paper III, the inclusion criteria for the children in the two municipalities Luleå and Kiruna and the municipality of Piteå differed. The children in Luleå and Kiruna were included according to self-reported FHS at the Child interview, while children in Piteå were included according to parentally reported FHS.
Of the children in Luleå and Kiruna who participated in the parental questionnaire, 98% also participated in the Child interview, which allowed comparison of parentally reported FHS and FHS reported by the child. This comparison showed a good agreement between parental and children reports of allergy/hypersensitivity to milk (Table 1) as well as FHS to egg, cod and wheat. The agreement was highest for complete avoidance of these foods. This is in line with previous study findings from another OLIN cohort including children of the same ages as in the current cohort, where a very high agreement between parental and child reports of allergic diseases was found (176).

Table 1. Comparison between milk avoidance, complete or partially, reported by the child and parentally reported milk avoidance of the child.

<table>
<thead>
<tr>
<th>Milk avoidance reported by child</th>
<th>Non-avoidance</th>
<th>Milk avoidance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parentally reported milk avoidance</td>
<td>(n=1559)</td>
<td>(n=265)</td>
</tr>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Non-avoidance</td>
<td>1507 (97%)</td>
<td>63 (24%)</td>
</tr>
<tr>
<td>Milk avoidance</td>
<td>52 (3%)</td>
<td>202 (76%)</td>
</tr>
</tbody>
</table>

Analyses of associated risk factors

In the analyses of possible risk factors in Papers II-IV, we included available variables, which in previous studies have been associated with increased or decreased risk of food hypersensitivity or food allergy (39,56,57,65). Further, variables with significant or borderline significant differences in the bivariate analyses, were included in multiple logistic regression analyses to adjust for possible confounding factors (173,177).
Confounding factors are sometimes regarded as sources of bias (173,174). A confounding factor is an extraneous variable that is correlated with the investigated variable of interest as well as with the outcome. If an existing confounding factor is not regarded in the analyses, a false association between the investigated variable and the outcome might appear. Despite the use of multiple logistic regressions, it is often difficult to rule out all possible confounding factors, since many confounding factors are still unknown (173).

It has been suggested, though not established, that when analyzing many possible risk factors, the p-value should be diminished in order to reduce the risk of significant associations appearing by chance (173,177). However, the tested variables in our study were not included by chance but selected according to previous study findings (39,56,57,65), suggesting these variables as possibly associated to our study outcomes.

**IgE-tests**

The current project was part of a larger longitudinal population-based study, investigating prevalence and associated risk factors for atopy related conditions i.e. asthma, rhinitis, eczema and allergic sensitization. To determine the prevalence of allergic sensitization, the children in the two municipalities Kiruna and Luleå were invited to a SPT with 10 common airborne allergens. Further, all children in Kiruna and 25% randomly selected from Luleå were invited to donate blood for serological analyses and 695 children (71% of invited) participated. To facilitate participation, the SPTs were performed at the children’s schools.

For the benefit of our project, it would have been preferable to add common food allergens, especially milk, egg, cod and wheat to the SPT panel and this was also discussed prior to study start. However, even though SPT with food allergens are considered to be a safe procedure in recent management guidelines (178), anaphylactic reactions during SPT with food allergens have been described. During my 15 years practice as a pediatric allergist I have witnessed two cases of anaphylaxis caused by SPT with food allergens, one triggered by hen’s egg and the other by cod. Since severe reactions due to SPT with food allergens are rare and when early recognized, fairly easily treated, we would not have considered this a problem if the study SPT had been performed in a health care facility. However, in this study SPTs were made on 1700 children and performed at the children’s schools, some located very far from a Health Care Center. During these circumstances we could not guarantee the tests to be completely safe for the study participants.
In a previous pediatric OLIN cohort study the correlation between a positive SPT and a specific IgE > 0.35 KU/L to aeroallergens included in the SPT was validated and found to be excellent (179). Therefore, instead of including foods in our SPT panels, food-specific IgE-analyses were added to the serological tests in the random population sample (n=695).

With the exception of Paper V, specific IgE was only analyzed to the crude food allergens. Before study start, analyses of specific IgE to allergen molecules were also discussed for Papers II-IV. We did not, however, prioritize this since by that time, only a few IgE-tests for molecular allergens in foods were commercially available and the knowledge about their usefulness in food allergy diagnostics was limited (126). There is however a need for population based studies investigating the prevalence of IgE-sensitization to these newly discovered allergenic molecules and how they relate to the presence of allergic symptoms, for food allergens as well as inhalant allergens (180). There is also a need for clinical research investigating whether patterns and levels of IgE-sensitization to different molecular food allergens might be useful in predicting food challenge outcomes (126).

**Validation of reported data**

Another major strength of this project is the validation of reported data on allergy to milk, egg, cod and wheat, first with the more easily available clinical examinations and serological tests and then further with DBPCFCs (135,178). The FHS phenotyping following the clinical examination and serological tests, was useful for identifying the children with current food allergy who were invited for further evaluation with a DBPCFC series. The categorization also gave us a good overview of the distribution of FHS phenotypes underlying reported hypersensitivity to milk, egg, cod and wheat in the examined cohort.

Previous studies have reported that the prevalence of perceived food allergy exceeds that of challenge-proven allergy (9,15,16) and the use of DBPCFC renders the lowest prevalence (9,135). However, even though considered gold standard for diagnosing food allergy, DBPCFC are rarely used in daily clinical practice and until recently, few population based studies have validated reported food allergy with double-blind challenges (15,16,181).

In Paper III, we used a DBPCFC series to validate reported allergy to milk, egg, cod and wheat. Since our resources were limited, we chose to focus on these four basic foods due to the high reported prevalence of FHS to milk, egg, cod and wheat in this cohort (36), the possible negative nutritional
effects of elimination of these foods (182-184) and the lack of knowledge about the phenotypes underlying perceived FHS to milk, egg, cod, and wheat in schoolchildren (4,12). The omitted validation of the other FHS phenotypes could be considered a study limitation, as could the exclusion of allergies to other foods. However, validating all FHS phenotypes or including hypersensitivity to more foods was not possible in our project. Instead, this will be an eligible task for future population based studies on food hypersensitivity.

**The DBPCFC methodology**

When we planned this study, very few validated recipes for DBPCFC in children were available, whereof the wheat recipe required baking and no validated recipe at all was available for cod (185). Therefore we developed our own DBPCFC recipes, which were successfully validated regarding detectable sensorial differences between the active and placebo substances in a separate cohort of healthy schoolchildren (n=275) in Paper I.

The recipes all contained the same liquid amino acid based product with supplementary ingredients to disguise the taste and texture of the challenge food. The use of a liquid test substance made it easy to prepare and easy to dose. The limitation was the difficulty to hide a large enough amount of challenge food in a drinkable amount of test substance. Therefore, the doses of allergenic food given during the DBPFC sessions were low, ranging from 2.0-2.4 g of food protein. The challenge foods, except for cod, was used in raw form in order to keep their allergenicity (186) and to increase the sensitivity of the test, the DBPCFC was performed as a 3-session challenge series including two challenge occasions with active substance (114,187).

**Cytokine mRNA expression in PBMC**

In Paper V, we chose to measure cytokine mRNA expression profiles in PBMCs instead of measuring levels of biologically active cytokine proteins. The rationale for this decision was the high sensitivity and specificity of qRT-PCR as a method and access to a well-established qRT-PCR for measurement of cytokine mRNA expression. Importantly, a previous study by our group, showed an excellent correlation between the levels of cytokine mRNA measured by this method and detected levels of corresponding cytokines at the protein levels (188).

The strengths of the study in Paper V, were the broad palette of cytokines and fecal markers analyzed before and after in vivo stimulation with relevant food allergens and the well-defined study participants. A study weakness was
that the study groups were small and the children heterogeneous according to type of food allergy, symptom severity, challenge food and the serving order of test samples during the DBPCFC series. The limited number of participants was due to a low prevalence of allergy to basic foods, even though we initially invited all children with reported allergy to milk, egg, cod and wheat from a large population-based cohort with a high prevalence of reported FHS to these foods. The difference in the serving order of active and placebo substances was a consequence of serving orders being drawn by lot, which was necessary to achieve a double-blind food challenge series (135).

The transport time was an important issue in our study, since the food challenges were performed in hospitals 250-500 km from the laboratory that performed the cytokine analyses. Thus, for practical and logistic reasons, the post challenge samples in our study were collected one week after the completed challenge series. The optimal time for measuring post challenge biomarkers is a matter of discussion. The mRNA expression of different cytokines peaks at different time intervals, which are different depending on the detection method used. A Polish study investigating cytokine protein levels in sera, showed a significant increase in the levels of IL-4 and IL-5 following double-blind challenges with active substance but not with placebo in children with asthma and IgE-mediated food allergy. The maximum secretion for these cytokines were seen after 24 hours (162). In contrary, the levels of fecal inflammatory markers and specific IgE and IgG4 are likely to peak weeks after allergen exposure (87,90,141,142).

**Analyses of fecal biomarkers**

For the analyses of inflammatory and tolerogenic markers fecal markers we used commercially available ELISA methods (Immundiagnostik, Bensheim, Germany). The ELISA method is the conventional method used for detecting fecal calpotectin levels in inflammatory bowel disease, though other, more rapid tests are emerging on the market. A previous study showed that the sensitivity and specificity is somewhat different in different methods of measuring fecal calprotectin and that the same detection method therefore should be used when comparing fecal levels of calprotectin at different time intervals or between individuals (189).
Discussion of main results

Epidemiology of food hypersensitivity

The prevalence of parentally reported FHS in this population-based cohort in Northern Sweden was high, 21%, at 7-8 years of age and even higher, 26%, at 11-12 years of age. Although the majority of children with allergies to basic foods i.e. milk and egg develop tolerance before school age (5,15) and lactose intolerance is thought to be uncommon among Swedish schoolchildren (31), the prevalence of reported hypersensitivity to milk, egg, cod and wheat increased from 10% at 7-8 years of age to almost 15% at 11-12 years of age. The prevalence of reported FHS to basic foods found in this study was high compared to other studies on older schoolchildren and adolescents (9,11,16).

According to our definition, the prevalence of reported allergy to the basic foods: milk, egg, cod and wheat at 11-12 years of age was 4.8%. When validated with a clinical examination and analyses of food specific IgE and a celiac screen test, the prevalence of current allergy to these foods was 1.4%. This prevalence was further halved following a DBPCFC series. The prevalence of challenge-proven allergy to milk, egg, cod and wheat of 0.6% was consistent or somewhat lower compared to the few other studies using a similar study approach (9,16,181) and 25-fold lower than the prevalence of reported hypersensitivity to these foods in our study cohort.

In Papers III and IV we showed that current food allergy was an uncommon cause of reported hypersensitivity to milk, egg, cod and wheat. The most common FHS phenotypes were probable lactose intolerance followed by outgrown allergy. At the time of the evaluation, children with outgrown allergy could tolerate a portion size of the culprit food without any symptoms, but they had nevertheless remained on an elimination diet. This is consistent with previous studies, showing that many children with food allergy keep avoiding the culprit food even after they have become tolerant (133).

In Paper IV we also showed that milk allergy, current or outgrown, was associated with a lower BMI compared to children not avoiding milk. A possible reason for this finding is that children with milk allergy had eliminated dairy products since very early life and still were avoiding them, even though children with outgrown milk allergy probably did not have to. This finding is in line with other studies showing that a milk eliminated diet due to allergy may lead to impaired growth and lower intake of important nutrients like vitamin D (182-184, 190).
Of the children in Paper IV still avoiding milk, 43% reported that their milk hypersensitivity had been diagnosed by a physician and a previous physician diagnosis was more common among children who were categorized as having milk allergy. Only 2% of the children reported that their milk hypersensitivity had been established by an oral challenge and 9% of the children still avoiding milk, reported that they had ever been referred to a dietician for nutritional advice. Together, these data supports previous findings that food hypersensitivity diagnoses given early in life may have dietary and nutritional consequences in adolescence (189,191) and pinpoints the importance of following up and properly evaluating medical advice given regarding elimination of basic foods (5,192,193).

In Paper III, four out of nine children with a positive challenge outcome reacted with anaphylaxis. The food-specific IgE among these children were moderately elevated, ranging from 3.3-13.1 kU/L. This is consistent with the findings of others, showing that food allergy severity cannot be determined by the level of specific IgE (90,122,128). None of the children who reacted with anaphylaxis had reported previous anaphylactic reactions to the challenge food and only one of them had been prescribed an epinephrine auto-injector. None of these children were on daily medication for their concomitant asthma. These findings highlight the importance of recurrent evaluations of children with suspected food allergy (5,116).

By the time of the structured interviews in Paper IV, 30% of the children reporting milk avoidance due to perceived hypersensitivity at the Child interview, had discontinued their milk-eliminated diet. All of these children had previously reported partial milk avoidance. Stated reasons for discontinuing the milk elimination diet were that the children had tried to eliminate milk during a period, seeking alleviation for various symptoms, but had found that the elimination diet did not result in any improvement or that they were symptom-free independently of diet. This high prevalence of discontinued milk elimination was in line with our findings in Paper II, where we found a high incidence (15%) as well as a high remission (33%) of parentally reported FHS between 7-8 years to 11-12 years of age. This pattern was recognizable for hypersensitivity to many of the investigated foods, but was most obvious for reported hypersensitivity to milk.

Reported hypersensitivity to milk had different symptom patterns as well as different associated risk factors compared to non-milk FHS. In Paper II, incidence of non-milk FHS was associated with allergic heredity as well as current rhinitis and allergic sensitization, while asthma and allergic sensitization were negatively associated with the remission of non-milk FHS.
Similar relationships between atopy related conditions and prevalence of FHS have been found by others (35,37), although there is a lack of population-based studies investigating risk factors associated with incidence and remission of FHS in older schoolchildren and adolescents (15,19).

Neither incidence nor remission of milk FHS were associated with any other atopy-related conditions. A possible explanation for this finding could be that the current allergy phenotype was more common among children with non-milk FHS compared to children with milk FHS (20,194). In Paper IV, where children avoiding milk due to perceived hypersensitivity were sorted into different milk hypersensitivity phenotypes, only 3% of these children were categorized as current milk allergy and 23% as outgrown milk allergy. The most common phenotype was probable lactose intolerance. While milk allergy, current or outgrown, was associated with a number of atopy related variables, children in the probable lactose intolerance phenotype were less sensitized to aeroallergens and food allergens compared to children not avoiding milk.

Female sex was associated with incidence of any FHS, non-milk FHS and milk FHS. It was also associated with milk avoidance due to perceived hypersensitivity as well as having a milk allergy phenotype at 11-12 years of age. This is in line with the findings of others, showing that while IgE-sensitization to food allergens and food allergy is more common among younger boys than girls, food allergy is more common among adolescent girls and women compared to men (59,66). The reason for this shift is unknown, but thought to be related to hormonal factors (67). In addition, previous studies have shown that women more often report symptoms of lactose intolerance than men (70,72).

In Paper II, we also investigated the relationship between IgE-sensitization to milk, egg, soy, cod, wheat and peanut and parentally reported symptoms to the corresponding food in their child. Generally, there was a poor agreement between reported symptoms and a positive specific IgE to the culprit food and the agreement was weakest for milk, soy and wheat. This is consistent with other studies (90, 195) as well as with our findings in Paper III and IV showing that current IgE-mediated allergy was an uncommon course of reported hypersensitivity to basic foods in this cohort of 11-12 year olds.

Diagnosing food allergy

In this project, we successfully validated recipes for low-dose DBPCFC with cow’s milk, hen’s egg, soy, cod and wheat in children. These recipes were
then used in DBPCFC series for validating reported allergy to basic foods, but the validation of our recipes has also allowed a spread of these recipes in order to facilitate the use of DBPCFC in daily clinical practice. It has been shown in previous studies that the performance of an oral food challenge can reduce the fear of future adverse reactions to the culprit food but may also improve quality of life in families with children with suspected food allergy (196). Even though DBPCFCs are considered gold standard for diagnosing or ruling out food allergy, the methodology has not yet been fully standardized (135,136). The access to validated DBPCFC recipes may facilitate such a standardization, which will make results from studies using DBPCFCs easier to compare.

Since DBPCFCs are time and resource consuming, there is a search of objective prognostic markers that could predict challenge outcome and thereby make DBPCFCs superfluous. In Paper V, we showed that the baseline mRNA levels of the Th2-related cytokine IL-13 were higher in children with challenge-proven food allergy compared to children with a negative challenge outcome compared to healthy controls. It has been shown in other studies that high levels of IL-13 are expressed after stimulation of PBMCs with relevant food allergens in food allergic patients and that the IL-13 level decreases after tolerance development (163,164). In addition, Metcalfe et al recently showed that an elevated egg-specific Th2 cytokine response – of IL-13 and IL-5 in particular – at 4 months of age could predict egg allergy at 12 months of age (161), which could be in line with our findings.

Further, we showed that the expressed mRNA level of the regulatory cytokine IL-10 at baseline was higher among children with challenge-proven food allergy compared to children with a negative challenge outcome. Higher IL-10 levels have been shown following stimulation of PBMCs with cow’s milk protein in children with IgE-mediated cow’s milk allergy compared to children with non-IgE mediated milk allergy (163,197) and in children during tolerance development compared to children with symptomatic food allergy (164). Previous studies have also shown that after stimulation of PBMCs with specific food allergens the expression of the Th2 cytokine pattern is enhanced and the expressed levels of the regulatory cytokine IL-10 diminished in children with symptomatic IgE-mediated allergy compared to children who have developed tolerance (107,161). Due to its active role in tolerance development, IL-10 has been discussed as a possible biomarker for symptomatic IgE-mediated food allergy (101). However, since data are not univocal and study results vary with study population and investigated food (108,164,198) it has been suggested that analyzing IL-10 as part of a broader Th2-profile would be preferable (103,199,200).
In Paper V, we also investigated the fecal levels of EDN as a marker of eosinophilic inflammation and calprotectin as a marker of neutrophilic inflammation of the gastrointestinal tract, in relation to DBPCFC outcome. The baseline levels of EDN and the baseline and post challenge levels of fecal calprotectin were almost twice as high among children with a positive compared to a negative challenge outcome, although none of these differences reached statistical significance. This was probably due to a lack of power caused by the low number of participants together with the large natural variance in faecal calprotectin levels (141,189). Our findings are, however, in line with the results from other studies showing that fecal levels of EDN might be used as a marker of food allergic reactions (139,140) and that measurement of fecal calprotectin can be a helpful tool in the follow-up of response to treatment and determination of reoccurrence of cow’s milk allergy (141,142).

**Ethical considerations**

The benefit of our study participants was the possibility to be diagnosed or freed from a suspected allergy to basic foods by the use of gold standard methods (5,116). To secure the safety of our study participants during DBPCFC, the double-blind challenges were performed in a hospital setting with drugs and equipment for the treatment of allergic symptoms, including anaphylaxis, immediately available. Also, the same experienced allergist (AW) and allergy nurse (AS) were responsible at all challenge occasions. Before each challenge occasion, an intravenous line was set in case of need to administer intravenous drugs or rehydration fluids. This intravenous line was also used for blood sampling when appropriate, to avoid unnecessary venous punctures. Children with positive tTGA-tests and no previous diagnosis of celiac disease, were informed about the test result and referred to a pediatric clinic for further investigation.

The children participating in the validation of our DBPCFC recipes and the analyses of cytokine mRNA expressions, had no immediate personal benefits of participating in the study, neither were they exposed to any health-related risks caused by their participation, except for the discomfort of donating blood samples in Paper 5. These children have, however, contributed to research that will hopefully improve future diagnostics and care-taking of children with suspected food allergy and consequently decrease unnecessary food avoidance.
ADVANCED
BIO
STATISTICS
Personal point of closure

I have learned a lot during these years of doctoral studies, adding new dimensions to my clinical work. A better understanding of food hypersensitivity in schoolchildren, its prevalence, clinical expressions and underlying phenotypes has increased the interest and engagement in our allergy team to disentangle the cause of adverse reactions to foods in our pediatric patients and to improve our diagnostic methods. The most significant lessons I have learned during this project were that perceived food hypersensitivity to basic foods was even more common, and that the percentage of children with current allergy to these foods lower, than I first thought. Further, that medical advice on food avoidance early in life, may have nutritional consequences in adolescence.

Even though I find our attempts to diagnose or free children with suspected food hypersensitivity important, this study has made me realize that to achieve significant changes, we have to start much earlier on. Since most children with allergies to basic foods develop tolerance before school age and since the older the child the more difficult it is for them to accept new tastes and textures, we must become better at evaluating diagnostic elimination diets and at following-up children with suspected food hypersensitivity to avoid unnecessary food avoidances. It is of course not necessary for everyone to eat all kinds of foods, but since I believe it to be a good start of a healthy relationship to food, I want as many children as possible to have the opportunity to do so. Following-up and properly diagnosing children with suspected food hypersensitivity will also diminish the risk of children with severe food allergy being disbelieved and thereby liable to risks of unnecessary exposure to the culprit food.

Our developed recipes for double-blind challenges in children have been used frequently in our daily clinical practice and the validation of these recipes has allowed a spread to other clinics in the country. Further, we have gained knowledge about how schoolchildren experience food hypersensitivity and food avoidance, which has facilitated improvement of our patient follow-ups. The upcoming theses of my friend and colleague Åsa Strinnholm, will contribute additional knowledge on quality of life in adolescents with perceived food allergy. Also, continuous studies and new research from the OLIN group and the department of Pediatrics, Umeå University will contribute to future knowledge on epidemiology and risk factors of food hypersensitivity – and I will probably not be able to keep my fingers out of that jar...
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Supplement

Questions from structured interviews
considered in Paper III and/or Paper IV.

1. Was your child breastfed?
   □ No
   □ Yes
   If yes, for how long?
   □ < 3 months □ 3-6 months
   □ 6-12 months □ >12 months

2. Did your child suffer from any symptoms during breastfeeding?
   □ No
   □ Yes
   If yes, what kind of symptom/s?
   □ Colic □ Gastrointestinal
   □ Eczema □ Urticaria
   □ Respiratory □ other: ____________________

3. At what age was your child introduced to taste portions?
   □ 3-6 months □ 6-12 months
   □ >12 months

4. Did your child suffer from any symptoms during introduction of
   taste portions?
   □ No
   □ Yes
   If yes, what kind of symptom/s?
   □ Colic □ Gastrointestinal
   □ Eczema □ Urticaria
   □ Respiratory □ Other: ____________________

5. Has your child had a growth deviation?
   □ No
   □ Yes
   If yes, which type of growth deviation?
   □ Length □ Weight
   □ Head circumference
6. Parental allergy/atopy related conditions?
□ No
□ Yes, mother
If yes, what type of allergy/atopy related condition in mother?
□ Asthma
□ Rhinoconjunctivitis
□ Eczema
□ Gastrointestinal
□ Allergy to airborne allergen/s, which?
__________________________
□ Food hypersensitivity;
□ During childhood
□ In adulthood
To which food/foods? -
__________________________
□ Yes, father
If yes, what type of allergy/atopy related condition in father?
□ Asthma
□ Rhinoconjunctivitis
□ Eczema
□ Gastrointestinal
□ Allergy to airborne allergen/s, which?
__________________________
□ Food hypersensitivity;
□ During childhood
□ In adulthood
To which food/foods? -
__________________________

7. Other parental (mother or father) diseases/conditions?
a. Celiac disease
□ Yes □ No
b. Diabetes Mellitus type 1
□ Yes □ No
c. Inflammatory bowel disease
□ Yes □ No
d. Thyroid disease
□ Yes □ No
e. Gastritis/Esophagitis
□ Yes □ No
f. Irritable bowel disease
□ Yes □ No
8. Atopy related diseases/conditions in child (except for food hypersensitivity)
   a. Asthma  □ Yes, now  □ Yes, before  □ No, never
   b. Rhino-conjunctivitis  □ Yes, now  □ Yes, before  □ No, never
   c. Eczema  □ Yes, now  □ Yes, before  □ No, never
   d. Allergy  □ Yes, now  □ Yes, before  □ No, never

If yes, what kind of allergy?
□ Birch pollen
□ Grass pollen
□ Ragweed
□ Furred animals
□ Bee/Wasp
□ Drugs, type: __________________________

9. Other diseases/conditions in child?
   a. Celiac disease  □ Yes  □ No
   b. Diabetes Mellitus type 1  □ Yes  □ No
   c. Inflammatory bowel disease  □ Yes  □ No
   d. Thyroid disease  □ Yes  □ No
   e. Gastritis/Esophagitis  □ Yes  □ No
   f. Irritable bowel disease  □ Yes  □ No

10. Has the child ever had anaphylaxis caused by food?
□ No
□ Yes
If yes, by what food trigger/s?
__________________________________________
At what age/s?
__________________________________________
Questions on symptoms caused by the specific foods: milk, egg, cod and wheat.

If the child reported hypersensitivity to more than one of these foods, the questions were asked separately for the different foods.

11. Current symptoms caused by intake of milk/egg/cod/wheat:

- Airways:
  - Rhinitis
  - Asthma
  - Laryngospasm

- Heart/circulation (decreased blood pressure/ circulatory failure)

- Eyes (conjunctivitis/kerato-conjunctivitis)

- Oral:
  - Itching
  - Aphtae
  - Lip swelling
  - Itching/bloated feeling of the throat

- Skin:
  - Eczema
  - Urticaria
  - Angioedema

- Gastrointestinal:
  - Vomiting
  - Diarrhea
  - Constipation
  - Flatulence
  - Stomach ache
  - Affected growth

- Other/s:

12. Symptoms caused by intake of milk/egg/cod/wheat at symptom onset:

- Airways:
  - Rhinitis
  - Asthma
  - Laryngospasm

- Heart/circulation (decreased blood pressure/ circulatory failure)

- Eyes (conjunctivitis/kerato-conjunctivitis)

- Oral:
  - Itching
  - Aphtae
  - Lip swelling
  - Itching/bloated feeling of the throat

- Skin:
  - Eczema
  - Urticaria
  - Angioedema

- Gastrointestinal:
  - Vomiting
  - Diarrhea
  - Constipation
  - Flatulence
  - Stomach ache
  - Affected growth

- Other/s:
13. Has the child had symptoms upon
   a. Airborne exposure of the culprit food?
      □ Yes □ No
   b. Skin exposure of the culprit food?
      □ Yes □ No

14. Are co-factors necessary for the development of food hypersensitivity symptoms?
    □ No
    □ Yes
    If yes, what kind of co-factors?
    □ Exercise □ Infection
    □ Pollen season □ Coldness
    □ Drugs
    □ Other: ________________________________

15. Childs age at symptom onset?
    □ 0-2 years □ 2-5 years
    □ 5-8 years □ 9-12 years
    □ >12 years

16. Time to symptom onset after intake of the culprit food?
    □ 0-15 minutes □ 15-60 minutes
    □ 1-3 hours □ 4-24 hours
    □ 1-3 days

17. Symptom duration after intake of the culprit food?
    □ 0-15 minutes □ 15-60 minutes
    □ 1-3 hours □ 4-24 hours
    □ 1-3 days □ > 3 days

18. Current elimination of the culprit food from child’s diet?
    □ Not at all
    □ Partially □ Completely
19. If hypersensitivity to milk: does lactose-free products trigger symptoms?
   □ No
   □ Yes
   If yes, at what dose?
   ____________________________________________________________

20. If hypersensitivity to milk: What replacement products does the child eat/drink?
   □ Lactose free/reduced products
     □ < 1 dl/day  □ 1-3 dl/day  □ >1 dl/day
   □ Soy products
     □ < 1 dl/day  □ 1-3 dl/day  □ >1 dl/day
   □ Oat products
     □ < 1 dl/day  □ 1-3 dl/day  □ >1 dl/day
   □ Rice products
     □ < 1 dl/day  □ 1-3 dl/day  □ >1 dl/day
   □ Calcium supplement
   □ Other:
   ____________________________________________________________

21. If hypersensitivity to fish, what kind of fish causes symptoms?
   □ Cod fish  □ Salmon  □ Shell fish

22. Physician diagnosis of celiac disease?
   □ No
   □ Yes
   If yes, at what age?
   ____________________________________________________________

23. If hypersensitivity to wheat: does other grains cause symptoms?
   □ No
   □ Yes
   If yes, what kind of grains?
     □ Barley  □ Rye  □ Oats
24. At what age was the culprit food introduced in the child’s diet?

- □ < 3 months □ Through breastmilk □ Own diet
- □ 3-6 months □ Through breastmilk □ Own diet
- □ 6-12 months □ Through breastmilk □ Own diet
- □ > 12 months □ Through breastmilk □ Own diet

25. Amount and type of trigger food necessary to cause symptoms:

<table>
<thead>
<tr>
<th>Food</th>
<th>Baked food</th>
<th>Treasure amount</th>
<th>Table spoon</th>
<th>&lt; 1 dl</th>
<th>&gt; 1 dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>Baked milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treasure amount</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Table spoon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt; 1 dl</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 1 dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>Raw (pasteurized) milk</td>
<td>Treasure amount</td>
<td>Table spoon</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt; 1 dl</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>&gt; 1 dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>Baked egg</td>
<td>Treasure amount</td>
<td>Tablespoon of muffin</td>
<td>Whole muffin</td>
<td>Pancake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treasure amount</td>
<td>Table spoon</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Table spoon</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Portion size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raw egg</td>
<td>Treasure amount</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Tablespoon of sponge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cod</td>
<td>Cooked cod</td>
<td>Treasure amount</td>
<td>Table spoon</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Table spoon</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Raw cod (sushi, pickled herring)</td>
<td>Treasure amount</td>
<td>Table spoon</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Table spoon</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Wheat</td>
<td>Baked wheat</td>
<td>Treasure amount</td>
<td>Tea spoon of bread</td>
<td>Table spoon of bread</td>
<td>Portion size</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tea spoon of bread</td>
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<td>Table spoon of bread</td>
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<td></td>
<td></td>
<td>Portion size</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Raw wheat</td>
<td></td>
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</tr>
</tbody>
</table>
26. When was the child last exposed to the culprit food?
   □ < 3 months          □ 3-6 months
   □ 6-12 months         □ 1-2 years
   □ >2 years

27. How long ago did the child last have a reaction due to intake of the culprit food?
   □ < 3 months          □ 3-6 months
   □ 6-12 months         □ 1-2 years
   □ >2 years

28. Has the child been diagnosed with hypersensitivity to the culprit food through a previous health care contact?
   □ No
   □ Yes
   If yes, by whom?
   □ By a physician
   □ By a child health care nurse
   □ Other: ________________________

29. Has the child performed any investigations/diagnostic test before the food hypersensitivity diagnosis?
   □ No
   □ Yes
   If yes, what kind of investigation/test?
   □ Clinical history
     □ Positive □ Negative
   □ Specific IgE/Skin prick test
     □ Positive □ Negative
   □ Oral challenge
     □ Positive □ Negative
   □ Lactose challenge
     □ Positive □ Negative
   □ Gene test (lactase down-regulating gene)
     □ Positive □ Negative
   □ Other: ________________________

30. Has the child ever been referred to a dietician for nutritional advice due to the food hypersensitivity?
   □ No
   □ Yes