Can fish oil in pregnancy and lactation alter maternal and infant immunological responses and prevent allergy in the offspring?

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Linköping 2010
To my family
ABSTRACT

Background: A connection has been proposed between the increase of allergic disease and the altered composition of fatty acids in the diet in the westernised world. Less oily fish and more vegetable oil are consumed today compared to 50-100 years ago. Programming of the immune responses takes place very early in life and environmental factors, such as fish in the diet, have been suggested to protect from infant allergy.

Aim: The general aim of this thesis was to assess the effects of maternal dietary supplementation with ω-3 long chain polyunsaturated fatty acids (LCPUFA), *i.e.* fish oil, in pregnancy and lactation on the development of allergic symptoms and sensitisation in the infants as well as some immunological markers in mothers and infants.

Subjects: This thesis is based on the results from a prospective double-blind placebo-controlled multi-centre trial comprising 145 families.

Methods: Pregnant women, at risk of having an allergic infant, were recruited at the antenatal clinics in Linköping and Jönköping and randomised to daily supplementation with 1.6 g eicosapentaenoic acid (EPA, C20:5ω-3) and 1.1 g docosahexaenoic acid (DHA, C22:6ω-3) or placebo, starting in the 25<sup>th</sup> gestational week and continuing through 3.5 months of breastfeeding. Phospholipid fatty acids in maternal and infant serum/plasma were analysed before, during and after the intervention to assess compliance. Prostaglandin E2 (PGE2), leukotriene B4 (LTB4) and infant vaccine induced responses were analysed with ELISA technique. Maternal cytokines and infant chemokines were analysed with Luminex technique. Clinical outcomes were allergic disease and positive skin prick test/detectable circulating IgE antibodies to common allergens.

Results: Phospholipid proportions of ω-3 LCPUFA increased significantly in the ω-3 supplemented women and their infants. Lipopolysaccharide-induced PGE2 secretion from whole blood culture supernatants decreased in a majority of the ω-3-supplemented mothers (p<0.01). The decrease in PGE2 production was more pronounced among non-atopic than atopic mothers. No difference in the prevalence of allergic symptoms was found between the intervention groups. The cumulative incidence of IgE associated eczema and IgE mediated food allergy was though reduced in the ω-3 group during the first years (OR= 0.3 and 0.1 compared to placebo, p<0.05 for both) and up to two years (OR= 0.2 and 0.3 compared to placebo, p<0.05 for both). The cumulative incidence of any IgE associated disease during the first two years of life was 13% in the ω-3 supplemented group compared to 30% in the placebo
group (p=0.01, OR 0.3, p<0.05). This effect was most evident in infants of non-allergic mothers. Higher maternal and infant proportions of DHA and EPA were associated with lower prevalence of IgE associated disease (p=0.01-0.05), in a dose dependent manner. In addition, no allergic symptoms as compared to multiple allergic symptoms, in the infants, regardless of sensitisation, were related to higher maternal and infant ω-3 LCPUFA status (p<0.05).

High infant Th2-associated CC-chemokine ligand 17 (CCL17) levels were associated with infant allergic disease (p<0.05). In infants with non-allergic mothers the ω-3 supplementation was related to lower CCL17/ CXC-chemokine ligand 11 (CXCL11) (Th2/Th1) ratios (p<0.05). This was not seen in infants whose mothers had allergic disease. Furthermore in non-allergic, but not in allergic infants, ω-3 supplementation was linked with higher Th1-associated CXCL11 levels (p<0.05), as well as increased IgG titres to diphtheria (p=0.01) and tetanus (p=0.05) toxins.

**Conclusions:** A decreased cumulative incidence of IgE associated disease in the infants was found after maternal ω-3 LCPUFA supplementation in pregnancy and lactation. This result was supported by a reverse dose response relationship between maternal ω-3 LCPUFA status and infant IgE associated disease. Higher proportions of DHA and EPA in maternal and infant serum/plasma were also associated to less severe allergic disease. A tendency towards strengthened Th1 associated response after maternal ω-3 LCPUFA supplementation was indicated in the analysis of maternal and infant immunological markers. These effects, as well as the clinical outcomes, were more pronounced in non-allergic individuals, suggesting gene-by-environment interactions.
SAMMANFATTNING

Bakgrund: I takt med att sammansättningen av fettsyror i vår kost förändrats har också allergifrekvensen ökat i västvärlden. Vi äter mindre fisk och mer vegetabiliska oljor idag än för 50-100 år sedan. Immunförsvaret programmeras tidigt i livet och epidemiologiska studier talar för att ökat fiskintag under graviditeten och barnets första år kan förebygga allergi hos barnet.

Syfte: Avhandlingens syfte är att ta reda på om tillskott av fiskolja under graviditet och amning kan påverka några av de immunologiska markörer hos mor och barn som har betydelse för allergisjukdom, samt förekomsten av allergiska symptom och sensibilisering hos barnen upp till två års ålder.

Metod: Blivande mödrar i Jönköping och Linköping med allergisjukdom hos dem själva eller i närmaste familjen, rekryterades via mödravårdscentraler och slump fördelades till att äta 2.7 g fiskolja dagligen eller icke aktiva kapslar från graviditetsvecka 25 till och med 3.5 månaders amning.

Andelen fiskolja i blodets fosfolipider mättes upprepade gånger hos mödrar och barn. LumineX- och ELISA- tekniker användes för att analysera immunfaktorer som prostaglandiner, leukotriener och cytokiner i mödrarnas blod samt kemokiner i barnets blod och barnens immunstatus efter vaccination. Barnen undersöktes upprepade gånger och genomgick pricktest för vanliga allergiframkallande ämnen. Även cirkulerande IgE-antikroppar mot dessa ämnen mättes.

Resultat: Nivåerna av ω-3-fettsyror ökade i den ω-3-behandlade gruppen hos både mödrar och barn och var högre än i placebo gruppen upp till ett år efter barnets födelse. De stimulerade cellkulturerna från mammor som fått omega-3-fettsyror visade mindre produktion av prostaglandin E2 (PGE2), som visat sig ha betydelse för allergisk sensibilisering. Produktionen av cytokiner hos mödrarna samt kemokiner hos barnen skiljde sig inte mellan omega-3-gruppen och placebo gruppen.

Under sina första två levnadsår hade barnen till mammor som fått fiskoljetillskott mindre ofta positiv pricktest mot födoämnen, speciellt ägg. Däremot var det ingen skillnad mellan grupperna med avseende på förekomst av allergiska symptomer, så som eksem, astma, födoämnesallergi eller hösnuva. De barn som hade både allergiska symptom, oftast i form av eksem eller födoämnesreaktioner och sensibilisering (positiv pricktest/detekterbara IgE-antikroppar i blod), s.k. IgE associerad allergisk sjukdom var dock vanligare i placebo gruppen än i den ω-3-behandlade gruppen (30 % mot 13 %, p=0.01).
Det visade sig också att högre andel omega-3-fettsyror både i mammans och i barnets blod gav lägre sannolikhet för barnet att utveckla IgE-associerad allergisk sjukdom under sina första två år. Dessutom hade barn med mer än ett allergiskt symptom, dvs en allvarligare form av allergisk sjukdom, lägre nivåer av omega-3-fettsyror i blodet än barn utan allergiska symptom.

Effekten av fiskoljetillskottet var tydligast i den grupp där mammorna själva inte hade allergisjukdom, både gällande PGE2 hos mamman och IgE associerad allergisk sjukdom samt positiv allergitest hos barnet. Hos barn till icke allergiska mammor fanns dessutom lägre nivåer av kemokiner aktiva i allergisk inflammation i omega-3-gruppen jämfört med kontrollgruppen (p<0.05). Icke allergiska barn byggde också upp ett bättre skydd mot stelkramp och difteri efter vaccination om deras mammor fått ω-3-tillskott (p<0.05 respektive 0.01).

**Slutsats:** En skyddande effekt sågs av fiskoljetillskott mot IgE-associerad allergisk sjukdom hos barnen upp till två års ålder. Det verkar som höga nivåer av fiskfetter i barnets och moderns blod var associerat till en minskad omfattning av den allergiska sjukdomen hos barnen, möjlichen pga. mindre allergisk sensibilisering. Effekterna av fiskoljetillskottet, både på immunologiska markörer och på klinisk sjukdom, var tydligast hos barn till mödrar som själva inte hade allergisk sjukdom vilket kan tyda på ett samspelet mellan gener och kostfaktorer.
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ORIGINAL PUBLICATIONS

I. The effects of omega-3 fatty acid supplementation in pregnancy on maternal eicosanoid, cytokine and chemokine secretion.
Kristina Warstedt, Catrin Furuhjelm, Karel Duchén, Karin Fälth-Magnusson, Malin Fagerås Böttcher

II. Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy.
*Catrin Furuhjelm*, Kristina Warstedt, Johanna Larsson, Mats Fredriksson, Malin Fagerås Böttcher, Karin Fälth-Magnusson, Karel Duchén

III. Allergic disease in infants up to two years of age in relation to plasma omega-3 fatty acids and maternal fish oil supplementation in pregnancy and lactation.
*Catrin Furuhjelm*, Kristina Warstedt, Malin Fagerås, Karin Fälth-Magnusson, Johanna Larsson, Mats Fredriksson, Karel Duchén

IV. Th1 and Th2 chemokines, vaccine induced immunity and allergic disease in infants after maternal ω-3 fatty acid supplementation during pregnancy and lactation.
*Catrin Furuhjelm*, Maria C. Jenmalm, Karin Fälth-Magnusson, Karel Duchén
*Pediatr Res. Accepted.*
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>ARC</td>
<td>allergic rhinoconjunctivitis</td>
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<tr>
<td>CAPS</td>
<td>the Childhood Asthma Prevention Study</td>
</tr>
<tr>
<td>CBMC</td>
<td>cord blood mononuclear cells</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-chemokine ligand</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CV</td>
<td>coefficient of variance</td>
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<tr>
<td>CXCL</td>
<td>CXC-chemokine ligand</td>
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<td>day</td>
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<tr>
<td>DBPCFC</td>
<td>double-blind placebo controlled food challenge</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>Der P</td>
<td>Dermatophagoides pteronyssinus</td>
</tr>
<tr>
<td>DHA</td>
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<td>dns</td>
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<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>FABPpm</td>
<td>plasma membrane fatty acid binding protein</td>
</tr>
<tr>
<td>FADS</td>
<td>fatty acid desaturase encoding gene</td>
</tr>
<tr>
<td>FcεRI</td>
<td>high affinity IgE receptor</td>
</tr>
<tr>
<td>FLG</td>
<td>filaggrin gene</td>
</tr>
<tr>
<td>GST</td>
<td>glutation s-transferase</td>
</tr>
<tr>
<td>gw</td>
<td>gestational week</td>
</tr>
<tr>
<td>HDM</td>
<td>house dust mite</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ISAAC</td>
<td>the International Study of Asthma and Allergies in Childhood</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
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LCPUFA  long chain polyunsaturated fatty acids
LNA    alfa-linolenic acid
LOX    lipoxygenase
LPS    lipopolysaccharide
LT     leukotriene
MHC    major histocompatibility complex
NFκβ   nuclear factor kappa beta
NK     natural killer
PAF    platelet aggregating factor
PBMC   peripheral blood mononuclear cells
PCB    polychlorinated biphenyles
PGE    prostaglandin E
PHA    phytohemagglutinin
PKC    protein kinase C
PL     phospholipid
PLA    phospholipase A
PPAR   peroxisome proliferator-activating receptor
PRR    pathogen recognition receptors
PUFA   polyunsaturated fatty acids
RDI    recommended daily intake
RSV    human respiratory syncytial virus
SIgA   secretory IgA
SNP    single nucleotide polymorphism
SPT    skin prick test
TCR    T cell receptor
Th     T helper
TLR    toll like receptor
TNF    tumour necrosis factor
Treg   regulatory T-cell
VCAM   vascular cell adhesion molecule
INTRODUCTION

Until recently, a common allergy preventing advice for mothers of infants at risk of developing allergic disease was to avoid fish in the infant diet until the end of first year (1). Now the former risk factor “early fish intake” is becoming a potentially beneficial element in the prevention of allergic disease. Some of the ideas, theories and facts behind this change are discussed in this thesis.

The prevalence of allergic disease varies widely between different countries. This indicates that there are many factors, including genetic and environmental aspects, accounting for the occurrence of allergies (2). The reason for the increased prevalence of allergic disease seen in affluent countries during the last decades is not clear (2) but among other environmental factors, the quality of dietary fat might be of importance (3, 4). The International Study of Asthma and Allergies in Childhood (ISAAC) indicates that countries with a low frequency of allergic disease, such as Asian countries (including India and China but excluding Hong Kong and Japan), Eastern European countries (including Russia and Poland), and some Southern European countries (including Greece), share a relative low dietary intake of oils containing the omega (ω)-6 fatty acid linoleic acid, i.e. various vegetable oils. Instead their diets contain olive oil (ω-9) and/or fish oil (ω-3). Thus, the ω-6 (vegetable oil)/ω-3 (fish oil) ratio is low (4).

Furthermore, the prevalence of sensitisation to inhalant allergens in schoolchildren from Greenland, who supposedly consume considerable amounts of fish, is low compared to European children (5). Still, the prevalence of allergic disease has increased in the Arctic, just like in the Western countries (6). Efforts to treat already established asthma in children and adults with ω-3 fatty acids, thus lowering the ω-6/ω-3 ratio, have not been successful (7). On the other hand, attempts to influence early foetal immune responses with
maternal ω-3 fatty acid supplementation in pregnancy have been more fortunate (8, 9). In this thesis we have investigated the effects of ω-3 fatty acid supplementation in pregnancy and lactation on infant allergic sensitisation and disease in addition to maternal and infant immunological markers.
REVIEW OF THE LITERATURE

Introduction to allergic disease

Allergic disease is caused by a hypersensitivity reaction initiated by specific immunologic mechanisms. An allergen is an antigen causing allergic disease and allergy can be antibody- or cell-mediated. The reaction is IgE mediated (type I hypersensitivity) in most patients with allergic symptoms from the skin and mucosal membranes in the airways and gastrointestinal tract. In non-IgE mediated allergy, the inflammation can be brought about by antibodies of the IgG isotype (type II or III, involving antibodies directed against cell surface/matrix associated antigens or soluble antigens, respectively) or by allergen specific T-lymphocytes (type IV). However, in the more chronic stages of the IgE mediated allergy, the inflammation causing the symptoms is dominated by allergen specific T lymphocytes and eosinophils as well (10). Primarily, IgE mediated allergy in the first years of life will be discussed in this thesis.

The allergic march

The term “allergic march” refers to the natural history of sensitisation to allergens and allergic symptoms. It starts in infancy as IgE-related eczema, sometimes accompanied by allergic gastrointestinal disease, and then progresses to respiratory allergy. The initial IgE reactivity is directed to food allergens and reactivity to inhaled allergens develops later (11). The term “allergic march” has been questioned lately from a clinical perspective. It is quite clear that there are many different phenotypes in the temporal sequence of asthma and allergic diseases (12). For example, the majority of children with eczema and asthmatic symptoms at age 7 already wheezed in early childhood, indicating an early co-
manifestation of these allergic phenotypes rather than an allergic march (13). The IgE mediated allergic reaction varies between different tissues. For instance, asthma is dominated by the long lasting reactions mediated by leukotrienes and cytokines while histamine effects dominate in urticaria. Individual sensitivity in mast cells in different organs regulates where the symptoms appear (14).

**Allergic sensitisation - atopy**

Individuals who are sensitised (i.e. atopic (10)) synthesise IgE antibodies towards food or inhalant proteins. These IgE antibodies can be demonstrated through a skin prick test where an allergen is injected in the skin. Mast cells with IgE towards the allergen bound to high affinity IgE receptors (FεRI) on their surface subsequently degranulate and release histamine. The skin reaction, a wheal appearing where the allergen was injected, can be measured with a ruler. Circulating IgE antibodies to allergens can also be detected in serum (14). Eczema, food reactions, asthma and rhinoconjunctivitis are allergic diseases where IgE mediated mechanisms can be involved. The IgE associated disease is also entitled extrinsic (15, 16). There are children who have symptoms without measurable IgE sensitisation (i.e. intrinsic), as well as sensitised children who are asymptomatic (10). For example, sensitisation to foods in young children without food related symptoms seems to be a common phenomenon (17). About 40% of chronic allergic disorders was attributable to sensitisation (extrinsic) and 60% to organ based and other factors (intrinsic) in a study of four year old children in the United Kingdom (18).

Nevertheless, sensitisation can be a predictive marker of future allergic manifestations. In the Danish DARC birth cohort study, IgE sensitisation to at least one food allergen between 3 and 18 months was significantly associated with eczema and asthma at the age of 6 years. Around 50% of children suffering from eczema, rhinoconjunctivitis or asthma at 6 years were sensitised to food at 6 months (19). In agreement with this, children with IgE associated eczema at 2
years had a greater risk of developing asthma at 6 years than non-sensitised children in a recent study (20). Hence, recognition of IgE sensitisation can provide useful information on the risk of childhood asthma and allergic rhinoconjunctivitis. However, early IgE responses to allergens are often transient in infants who remain non allergic whereas they reach higher and more persistent levels in those who develop allergy (21). In a German report, children persistently sensitised to food had a 3.4 fold higher risk of developing allergic rhinitis and a 5.5 fold higher risk of developing asthma than infants who were only transiently food sensitised. Persistent food sensitisation in combination with a positive atopic family history was a strong predictor for the development of allergic rhinitis and asthma at five years of age (22).

In conclusion, although sensitisation to food allergens during the first years of life is not compulsory in allergic disease, it represents a predictive factor for future allergic manifestations, especially if the early sensitisation persists through the first two years.

**Food allergy**

Any food can cause a reaction, but there are few foods responsible for the large majority of the symptoms: milk, eggs, wheat, peanuts, nuts, fish and shellfish. Of these, cow’s milk and egg allergy is frequently presented in infants (23). The major food allergens involved in children’s allergies are heat, acid, and protease stable water-soluble glycoproteins 10 to 70 kD in size. Heating or preparing foods might reduce (egg) or enhance (roasted peanut) allergenicity by modifying conformational epitopes (24).

The term food allergy can be further split into IgE and non-IgE mediated reactions (Fig. 1). IgE mediated food allergy begins in the first 1-2 years of life
with the process of sensitisation. For an allergic reaction to occur, re-exposure is needed with binding of the allergen to allergen-specific mast cell- or basophil-bound IgE antibodies (23). The symptoms of IgE mediated food allergy include angioedema, urticaria, rashes, flushing, anaphylaxis, bronchospasm, rhinoconjunctivitis and immediate gastrointestinal symptoms. The non-IgE mediated immune reactions that can arise in the gastro-intestinal tract are not so well defined and more difficult to recognise. Eosinophil eosophagitis and gastroenteritis as well as eczema are considered mixed IgE and cell mediated while more delayed gastrointestinal symptoms, contact dermatitis, dermatitis herpetiformis and pulmonary hemosiderosis are considered cell mediated (25). Indeed, it has been well established that approximately 30% of children suffering from moderate to severe eczema present an associated food allergy that worsens their eczema. Double blind placebo controlled food challenge (DBPCFC) is considered the “gold standard” for diagnosing any kind of food allergy (26) but IgE testing and epicutaneous tests can be helpful to diagnose IgE mediated and non-IgE mediated food allergy respectively (23).

Figure 1. Classification of food hypersensitivity reactions. Modified from (10).
A diagnosis of food allergy is very often suspected in early childhood with at least 25% of parents reporting one or more adverse food reactions. IgE mediated food allergy can be confirmed in 5-10% of young children in a normal population and the peak prevalence is at approximately 1 year of age (27). Most patients with egg allergy and egg IgE level less than 50 kU/L are likely to develop egg tolerance by late childhood (28). In a population based study, 76% of children with IgE mediated milk allergy and 100% of those with non-IgE mediated milk allergy were tolerant by the age of three years (29). In addition, adverse reactions to fruits, vegetables and other cereal grains are typically very short-lived (27). Children who present with one food allergy, especially if it is IgE mediated, have a very high risk of developing additional food and inhalant allergies (reviewed in (27)). Interestingly, reported food hypersensitivity at young ages, even though transient, increased the risk for other allergic diseases at 8 years in one study (30).

**Sensitisation and tolerance in the gut**

In the gastrointestinal tract, a single-cell layer of columnar intestinal epithelial cells separates the internal sterile environment from the external world (31). An intricate “gastrointestinal mucosal barrier” has evolved consisting of physiologic and immunologic components to process food to a form that can be ingested but also prevent the penetration of harmful pathogens into the body. However, the components of this mucosal barrier are immature in infants (31) and this may contribute to the increased prevalence of both gastrointestinal tract infections and food allergies seen in the first years of life. Factors that influence the outcome of an immune response to oral antigens include antigen availability, the immune environment and actions by immune cells with concomitant cytokine secretion. However, how the mucosal immune system “decides” whether and when to induce tolerance or sensitisation when exposed to fed antigens remains largely unclear (32).
In conclusion, different forms of food hypersensitivity are common during the first two years of life, possibly due to the immaturity of the gut immune system. Although most of the children become tolerant after a few years, the risk of future allergic disorders is increased if the food allergy is IgE mediated.

**Eczema**

Eczema is the most common inflammatory skin disease in childhood with a course marked by exacerbations and remissions. In a Swedish cohort of high risk infants 35% developed eczema before 2 years of age and 28% had IgE associated eczema (33). It usually clears in about one third of the patients after 2 years, and in another third after 5 years of age (13, 34). Eczema often coincides with food allergy (35).

The definition of eczema is complicated due to variability of the distribution of symptoms and morphology, the inconsistency of the time course of disease and the lack of a diagnostic test. The diagnostic criteria, defined by Hanifin and Rajka (36), to identify children with eczema has been a matter of debate, especially with respect to the significance of some minor features in younger children. Seymour (37) and Oranje (35) modified the criteria for infants. After comparing five different sets of diagnostic criteria for children up to 18 months of age, Johnke et al. found the agreement acceptable but the importance of repeated examinations was underlined (38).

Major determinants of the prognosis of early eczema are severity of disease and early allergic sensitisation (13). The mechanism of sensitisation may differ between individuals. In some children with eczema, an intrinsic defect of the skin barrier facilitates sensitisation due to damaged skin, while sensitisation in the gastrointestinal tract precedes the development of eczema in other children.
Filaggrin is a key protein for the skin barrier. It consolidates the keratin filaments into dense bundles, thus being crucial for development of the cornified cell envelope, maintaining the barrier function of the uppermost layer of the skin (40). Two independent loss-of-function genetic variants (R510X and 2282del4) in the gene encoding filaggrin (FLG) are very strong predisposing factors for eczema (39). The FLG gene is located on the human chromosome 1q21, within the epidermal differentiation complex, composed of more than 30 genes, all involved in terminal differentiation of the epidermis (41). Weidinger et al (42) evaluated an association of FLG null alleles with eczema, allergic sensitisation and asthma in a German population of 476 families. Strong associations were found between the FLG variants and eczema in combination with allergic sensitisation. In agreement with this, eczema has been proposed to be a predictor for subsequent development of sensitisation in non-sensitised children (43, 44). Consequently, restoring skin barrier function in filaggrin deficient infants may help prevent the development of sensitisation and halt the development and progression of allergic disease (45). Filaggrin gene mutations also increased the risk of asthma in people with atopic eczema.

In summary, eczema is the most common symptom of allergic disease in early childhood, appearing with or without sensitisation. The skin barrier defect associated with eczema seems to facilitate sensitisation but sensitisation can also precede the onset of eczema. The combination of eczema and allergic sensitisation is a strong predictor of future allergic disease in the airways.

**Asthma and rhinoconjunctivitis**

Asthma is a chronic inflammatory disease in the lower respiratory tract where mast cells, eosinophils and T lymphocytes take part. This inflammation causes repeated attacks of wheeze and cough, especially at night. The attacks may be
induced by allergen or viral infections, primarily infections, in early childhood (46). About 10% of children in a high risk cohort in Sweden developed IgE mediated asthma (33), also called allergic asthma (10). Without concomitant sensitisation or asthma in the family, infants with non-allergic asthma do not have an increased risk of asthma at 7 years of age according to the German Multicentre Allergy study (47). Rhinoconjunctivitis mediated by allergens is uncommon in the first two years of life but the incidence increases with age and is about 20% in the teen-age years (48).

In summary, allergic asthma and rhinoconjunctivitis are not as common as eczema during the first two years of life. Wheeze is a frequent symptom in early childhood but it is commonly triggered by infections and does not always predispose for future asthma.

Introduction to the immune system

The immune system consists of innate and acquired immune defences. The actions of macrophages and neutrophils, the phagocytes of the innate immune system, do not depend on prior exposure to a particular antigen. They engulf and degrade the microbe by phagocytosis. Complement, natural killer (NK) cells, mast cells, basophiles and eosinophils also participate in the inflammatory processes destined to destroy microbes (14, 49). Innate immune cells express pathogen recognition receptors (PRRs), e.g. Toll like receptors (TLR), recognising microbial associated molecular patterns, for instance lipopolysaccharide from Gram- bacteria and lipoteichoic acid from Gram+ bacteria (50). TLR ligation induces secretion of cytokines including tumour necrosis factor (TNF), IL-1β, IL-6, IL-10 and IL-12 and up regulation of cell surface proteins (50).
The cells in the acquired immune system are T- and B-lymphocytes and they respond to specific antigens and provide an immunologic memory. There are two types of T lymphocytes: CD4+ (helper/regulatory) and CD8+ cells (cytotoxic) (14). B-lymphocytes that mature into plasma cells produce antibodies; soluble molecules that can neutralise microbes and toxins, activate complement, enhance phagocytosis and trigger cell degranulation.

Communication between the innate and the acquired immune systems is brought about by cell-to-cell contact involving adhesion molecules and by the production of chemical messengers (14). The immune responses vary between individuals due to age, sex, smoking habits, alcohol consumption, menstrual cycle, stress and infections, vaccination and early environment (49).

**Immune components involved in allergic inflammation**

The allergens are ingested, internalised and expressed as peptides bound to major histocompatibility (MHC) molecules at the surface of antigen presenting cells (APC). The APC present the antigen to T-lymphocytes which may promote the transformation of B-lymphocytes to plasma cells that secrete IgE (23). Once formed and released into the circulation, IgE binds to high affinity receptors on mast cells, ready for future interaction with allergen (Fig. 2) (23).

**T-cells and cytokines**

The APCs carrying out the antigen presentation to naïve CD4+ T cells are dendritic cells (DC), principal regulators of T-cell memory function (51). The antigen presentation takes place in the lymph nodes where the naïve T-cells circulate. DC’s process protein antigens and express peptide fragments of the antigen at the membrane with MHC class II molecules. To fully activate a naïve T-cell, a second signal is needed; B7 on the APC that has been up regulated binds to the CD28 on the T cell (Fig. 2) (52). T cell activation also requires
cytokines, a heterogeneous group of proteins that mediate intercellular signalling and induce proliferation and differentiation. Cytokines are secreted from different cells, such as activated antigen presenting cells, monocytes, macrophages, mast cells and lymphocytes (53). Conventional CD4+ Th cells control the adaptive immunity by activating, in an antigen-specific fashion, other effector cells such as CD8+ cytotoxic T cells, B cells and macrophages (54).

Thus far, four CD4+ Th cell lineages are documented based on their cytokine profiles and regulatory properties: namely Th2, Th1, Treg and Th17 cells (55). Allergens that are presented to the naïve T cell makes it mature into a IL-4, IL-5, IL-9, and IL-13 producing Th 2 lymphocyte (Fig. 2) (56). IL-4 and induces production of IgE from plasma cells (53). This cytokine also contributes to the differentiation of naïve Th lymphocytes toward a Th2 phenotype and prevent apoptosis of Th2 lymphocytes (57). Another important activity of IL-4 in allergic inflammation is its ability to induce expression of vascular cell adhesion molecule 1 (VCAM-1). This produces enhanced adhesiveness of endothelium for T cells, eosinophils, basophiles, and monocytes, but not neutrophils, as is characteristic of Th2-mediated allergic reactions (58). IL-13 shares much of IL-4’s biologic activities on mononuclear phagocytic cells, endothelial cells, epithelial cells, and B cells but it also induces mucus production and airway hyper responsiveness (53). IL-5 is the most important cytokine for recruiting and enhancing the survival of eosinophils and their progenitors and primes these cells for activation and chemo taxis. Mast cells are recruited by IL-9 (56).

Antigens like bacteria and viruses induce IL-12 production from the APC and the naïve T cell, which then stimulates CD4+ Th cells to a Th1 immune response. This is characterised by production of interferon gamma (INFγ) and TNF, with activation of macrophages and cytotoxic T- cells that eliminate intracellular microbes (14).
Figure 2: Schematic overview of the differentiation of Th cells with emphasis on allergic sensitisation. The allergen is presented to the naïve T cell by the antigen-presenting cell (APC) and the T-cell receptor (TCR) binds to the Major Histocompatibility Complex (MHC) class II and the peptide; CD4 stabilises this interaction and the local cytokine milieu is essential for the differentiation. It is dominated by IL-4 in this example of Th2 differentiation (solid arrows) that causes IgE production by plasma cells. Upon re-exposure of the allergen the mast cell is activated. Modified from (52, 53).
There is a regulatory element in immune responses to allergens in healthy individuals (14). It consists of regulatory T cells (Treg) cells that can suppress Th2 responses to allergen, airway eosinophilia, mucous hyper secretion, and airway hyper responsiveness, partly through the cytokines IL-10 and TGFβ. Suppression by these cells may be decreased in allergic individuals (59).

Th17 cells produce many cytokines including IL-17A, IL-17F, IL-22 and IL-21. In addition to their involvement in autoimmune diseases, Th17 cells also play critical roles during immune responses against fungi and extracellular bacteria, e.g. via neutrophil activation (60).

**Chemokines**

Chemokines, *i.e.* small chemotactic proteins attracting immune cells, are produced by macrophages, DCs, keratinocytes, bronchial epithelial cells and fibroblasts (61). They are characterised by the presence of 3 to 4 conserved cysteine residues and can be subdivided into 4 families based on the positioning of the N-terminal cysteine residues: CC, CXC, C and CX₃C (Fig. 3) (53).

*Figure 3: Chemokines of the CC and CXC families. Black line: peptide chain. Grey line: disulphide bond C: cystein residue, X: amino acid*
The differential display of chemokine receptors by Th1 and Th2 subsets of T lymphocytes after polarisation allows the cells to selectively respond to multiple chemokines (62). The CC chemokine family has been extensively studied in allergic diseases because of some of its members’ ability to recruit eosinophils, T-lymphocytes, and monocytes to regions of inflammation. The CXC chemokines, on the other hand, target neutrophils (53). For example, the CC-chemokine receptor (CCR) 3 and CCR4 are frequent on cells involved in the allergic inflammation (63).

The chemokines analysed in this thesis are CXCL10, CXCL11, CCL17 and CCL22 (Table 1). CCL17 and CCL22 bind to the CCR4 receptor on the Th2 cells (14). Up regulation of CCL17 and CCL22 has been associated with presence and severity of eczema in children and adults (64, 65). Both IL-4 and IL-13 promote CCL17 expression, amplifying Th2 responses (66). In addition, nasal epithelial cells express CCL17, and expression of this chemokine and its receptor, CCR4, was higher in patients with allergic rhinitis compared with non-allergic control subjects in one study (66). Furthermore, CCL17 and CCL22 levels clearly differentiate asthmatic children from non-atopic children with chronic cough (67). On the other hand, the chemokines CXCL10 and CXCL11 are induced by IFNγ from Th1 cells and NK cells and act chemotactically on Th1 cells by binding to their specific receptor CXCR3 (68). They are linked to Th1-like diseases like Crohn’s disease (68). As CXCL10 also may be produced in airway epithelial cells during the early phases after allergen exposure (69), possibly induced by contact with the allergen (53), its role in allergic disease is not completely clear. Nevertheless, a Th2 like deviation, *i.e.* increased plasma levels of CCL22 and CCL22/CXCL10 ratios, in cord blood (63) and during the first year (33) was associated to development of allergic disease in recent studies.
Table 1. The chemokines analysed in this thesis, their corresponding receptors and cells.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Cells</th>
<th>Induced by</th>
</tr>
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<tbody>
<tr>
<td>CCR4</td>
<td>CCL17</td>
<td>Th2 lymphocyte</td>
<td>IL-4, IL-13</td>
</tr>
<tr>
<td></td>
<td>CCL22</td>
<td>Dendritic cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Natural killer cell</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Mast cell</td>
<td></td>
</tr>
<tr>
<td>CXCR3</td>
<td>CXCL10</td>
<td>Th1 lymphocyte</td>
<td>IFNγ</td>
</tr>
<tr>
<td></td>
<td>CXCL11</td>
<td>Mast cell</td>
<td></td>
</tr>
</tbody>
</table>

B cells and immunoglobulins

The immunoglobulins are produced by plasma cells derived from B cells. IgE, IgA and IgG subtypes are linked to the allergic inflammation. IgE activates mast cells and it is produced by plasma cells that have encountered a specific allergen and have been stimulated by IL-4 and IL-13 from activated CD4+T helper cells (Fig. 2) (70). The primary physiologic function of IgE might be to protect against parasites as high levels of IgE are found in worm-infected subjects (14). IgE is very biologically active but has a short half-life (<1d) when not bound to mast cells and it is present in exceptionally low concentrations in the circulation. However, IgE responses over long time can be attributed to the long life of IgE producing B cells and stability of mast cells in the skin (70).

IgG responses to a range of purified house dust mite (HDM) allergens showed that both IgG1 and IgG4 antibodies are present in sensitised individuals (71). IgG1 activates complement and stimulates phagocytosis (70). IgG4 production has been associated to high exposure to cat (70) and food antigens (72). It is dependent of IL-4, a feature of Th2 responses (70), but is considered a physiological response of the immune system (72) that has been associated to tolerance (73, 74). The regulatory cytokine IL-10 can stimulate production of IgG4 from B-cells while IgE production is decreased. In fact, data from experimental animal studies have shown that high-dose allergen exposure
independent of the route of administration favours immune tolerance, while low-dose allergen exposure favours immune responsiveness. Observational epidemiological studies in humans suggest that exposure to pets decreases the risk of pet allergy, possibly involving this mechanism, reviewed in (75).

IgA exists in two forms: the monomeric form in blood and secretory IgA on mucosal surfaces. The secretory IgA is dimeric and very resistant to acid and enzymes of the gastrointestinal tract. It represents the primary line of protection against incoming pathogens (e.g. allergen), preventing attachment to the underlying epithelium (76). The very high concentration of secretory IgA in human colostrum and milk strongly suggests that it must play an important role in the immune protection of the newborn (76).

**Eosinophils**

Eosinophils are recruited to the allergic inflammation site and activated mainly by the cytokine IL-5 and the chemokine CCL11 (eotaxin) (77). They adhere to VCAM1 on endothelial cells, reach the tissues, degranulate and synthesise the lipid metabolites LTC4 and platelet-activating factor (PAF). Eosinophilia in the blood is present in severe allergic reactions (53). Eosinophils are important in the chronic allergic inflammation and in the airways they can contribute to a reconstruction of lung tissue, which may cause progressing hyper reactivity. Attempts to reduce eosinophilia in humans by anti-IL-5 antibody therapy has resulted in decreased eosinophilia and improved asthma control (77). This is, however, not in clinical use.

**Mast cells**

The mast cells are situated around the vessels in the airways, the intestinal mucosa, in connective tissue and the epidermis. They have receptors for the Fc part of IgE on the surface, *i.e.* FceR, as well as receptors for C3a from
complement activation and substance P from nerves. The mast cell is activated through binding of any of these receptors or by heat, cold or pressure (14). When the mast cell becomes activated, histamine is immediately released (Fig. 2) and makes plasma pass through the vessel walls and into the tissues. The endothelial cells produce nitric oxide that dilates capillaries and venules and the axon reflex, which involves substance P, also generates dilatation of the vessels. The swelling increases over several hours. Eosinophilic granulocytes and T cells are recruited from the bloodstream by chemokines and the cytokines IL-4, IL-5, IL-9 and IL-13 are secreted by the mast cell (70). Phospholipase A2 is mobilised when the mast cell is activated and it releases the arachidonic acid (AA) from the intracellular membrane phospholipids. Lipooxygenase (LOX) and cyclooxygenase (COX) convert AA into eicosanoids; lipid mediators that maintain allergic inflammation (78). These mediators are released to the tissues several days after the activation of the mast cell and they contribute to the late symptoms in the allergic reaction (14).

In summary, several cells and immune mediators are active in the allergic inflammation. The cytokine, chemokine and eicosanoid responses associated with allergic inflammation are considered Th2 deviated, as opposed to the Th1 deviated responses that take part in the defence against intracellular microbes. The T regulatory cells are thought to balance the Th2/Th1 responses.

Intrauterine sensitisation and foetal immune responses

Maternal immune responses during pregnancy

As foetal tissue is regarded as semi-allogeneic, expressing human leukocyte antigen (HLA) from the father, a successful pregnancy depends upon tolerance of a genetically incompatible foetus by the maternal immune system (79). The innate branch of the immune system is activated during pregnancy in healthy
women, possibly to compensate for the increased immunoregulation of the adaptive immune system (80). The presence of maternal Th2/Treg cytokines, such as IL-4, IL-10, and IL-13, during pregnancy is thought to suppress Th1-mediated immunity, usually associated with transplant rejections, and to promote acceptance of the foetus in the womb by the mother (81). In contrast, the Th1 cytokine, IFN-γ, is an abortificant, whose effect may be mediated through the promotion of cytotoxic lymphocyte and NK cell development (82, 83). Consequently, cytokine responses to phytohemagglutinin (PHA), allergen extracts and lipopolysaccaride (LPS) are influenced by pregnancy regardless if the mother is allergic or not. Further, there are findings that support the view of hormones, especially progesterone, as critical regulators of the Treg populations that influence the Th2/Th1 balance in pregnancy (84). Hence, lower IgE levels 2 years after pregnancy than during pregnancy have been detected in unselected women (85). However, the levels of total IgE in early pregnancy were higher in sensitised women with allergic symptoms than in healthy women in a recent study, indicating that the Th2 deviation is augmented by allergic disease (86). Combined information from old and new studies indicates that interaction between Th1 and Th2 type cytokines is important for reproductive success (87).

The maternal CD4+ T-cell is assumed to play a central role in the interface between maternal and foetal immune systems. T-cells release regulatory cytokines (IL-4, IL-13, IL-10) that may cross the placenta and interact with the foetus (82). Maternal cells themselves may also cross the placenta and cells within the placenta may be stimulated to release various cytokines. The transfer of maternal CD4+ Th2 skewed T cells across the placenta could potentially skew foetal immune development toward a Th2 bias (88). The exact nature and mechanism of this maternal influence and how it might be associated with the development of allergic sensitisation and asthma in the child is not clear (82).
The foetal immune system

Stem cells are present in the human yolk sac at 21 days of gestation and the first lymphocytes appear in the thymus at the end of the ninth week of gestation. From 14 weeks, the lymphocytes can be seen in a range of organs, including the lungs and gut, but the maturation of these cells occur in the third trimester of pregnancy. By 19–20 weeks, circulating B-cells have detectable surface immunoglobulin M (81). At delivery, the immune system is quite complete although some cells, like phagocytes and dendritic cells, are not yet adequate in number and function. The microbial flora colonising the gut facilitates the expansion of the lymphoid population (89). If the neonatal immune system is unable to down-regulate the pre-existing Th2 dominance effectively, then an allergic phenotype may develop (79).

Early T cell responses to allergens

IgE and allergens might be amniotically transferred and ingested by the foetus through the skin, respiratory tract, and gastrointestinal tract (90). The second route of allergen exposure is by direct transfer across the placenta, possibly mostly in complexed form with immunoglobulin G in the third semester of pregnancy (91). Jones et al found that infants of mothers who were exposed to birch pollen at 22 weeks of gestation or beyond have significantly higher cord blood mononuclear cell (CBMC)-proliferative responses to birch pollen at the time of birth than infants of mothers who had either not been exposed at all during their pregnancy or whose exposure had been prior to 22 weeks. These findings suggest that inhaled allergens might have crossed the placenta during a critical period during foetal development and primed the infant T cells (92).

Allergen specific CD45 RA+ CD3 T cells have been found in cord blood (93, 94) but recent evidence suggests that they might be immature and cross reactive. They are recent thymic emigrants that respond to antigens/allergens not
previously encountered and express altered antigen receptors that lack the fine specificity of conventional T cells. These cells rapidly apoptose after allergen triggered proliferation but have a greater chance of survival if IL-2 is present. There is no correlation between cytokine pattern from recent thymic emigrants in cord blood and future atopy (93, 95).

Generally the T cells from infants at high risk of developing atopy seem to secrete less Th1 and Th2 cytokines than T cells from low risk individuals, the reduction being more profound in Th1 cytokines (96), for example reduced IFNγ production was associated to the development of eczema in a recent study (97). This reduced capacity to generate Th1 polarising mediators, such as IL-12p70 from dendritic cells and macrophages (98) during infancy may compromise or delay the transition to a Th1-competent immune response to allergens (reviewed in (82)). Except for the influences of the maternal Th2 skewed environment another possible explanation for this is that the neonatal T lymphocytes have a reduced ability to activate mitogen-activated protein kinases, which are crucial for cytokine production. This is associated with reduced expression of several protein kinase C (PKC) isozymes. Variations in PKC isozyme expression in CD4+Th cells lead to corresponding differences in maturation characteristics, patterns of cytokine response, and disease susceptibility. Lower levels of PKCzeta have been associated to allergic disease (99).

**Early specific IgE**

The issue of intrauterine sensitisation is controversial. The foetal gut, with its Peyers patches built up of T cells and other immune cells, is the principle route by which sensitisation might occur through allergen in the amniotic fluid (100). Intrauterine sensitisation may have evolved to facilitate enhanced neonatal host response to maternal helminths. Indeed, infants born to helminth infected
mothers have Th2 biased immune responses to helminth antigen and IgE antibodies to these antigens (81). Contrarily, recent studies indicate that allergen specific IgE in cord blood does not reflect intrauterine sensitisation. For example, allergen specific IgE was found in 14% of cord blood samples from mothers with asthma and specific IgE in cord blood completely matched specific IgE in maternal blood with respect to allergen specificity and level of specific IgE. The IgE was no longer detectable at 6 months (n=411) and small placental bleedings during late pregnancy or delivery may be a plausible mechanism (101). These findings are supported by Bertino et al (102) but not by Nambu et al (103).

However, epidemiological studies show that prenatal exposure to stables or farm life is protective from inhalant allergies (104, 105). The nature of this response may be regulated by the maternal environment, particularly the mother's IgE/IgG ratio (106). For instance, protective specific IgG4 antibodies may cross the placenta after maternal allergen exposure (107). After birth, sensitisation to foods in infants without food related symptoms seem to be a common phenomenon (17). Children who remain non-allergic produce detectable but low levels of specific IgE up to 12 months of age before returning to baseline (95), whereas the IgE levels reach higher and more persistent levels in those who develop allergy (21).

**Total IgE in cord blood**

Some infants destined to have allergic disease have raised total IgE in cord blood. This has proved to be a specific but very insensitive marker of later disease (108, 109). In average, 23 % of cord blood samples in a group of women from Taiwan (n=334) had levels above 0.35 IU/ml. This was associated with maternal total serum IgE > 100 IU/l one month before delivery, maternal allergy to dog dander and atopy in maternal grand parents (110). Maternal history of
Asthma was shown to be the most important determinant for high cord blood total IgE in two different studies. There was no relationship with paternal asthma, demonstrating the impact of the maternal environment (111, 112). Maternal atopic history, elevated total IgE levels and allergic sensitisation were associated with infants with elevated CB IgE levels and infantile eczema (113). A predictive value has been shown for cord blood IgE for urticaria due to food allergy at 12 months (111), atopy at 18 months (114) and asthma at 11 years (109). Other studies have shown no associations between cord blood total IgE and future sensitisation and allergic symptoms in the child (108). The question remains if cord blood IgE levels merely reflect the maternal atopic status or if the immune responses in the child are influenced by the IgE antibodies in cord blood (81).

The mechanisms are not completely clear and some results are contradictory on the subject of intrauterine sensitisation and immune responses of the foetus. There is epidemiological evidence that pre- and perinatal events like low maternal vitamin E (115) and fish (116, 117) intake in pregnancy as well as smoking in pregnancy (118) and delivery by caesarean section (119, 120) all increase the risk of sensitisation and allergic disease in childhood. This indicates that future sensitisation and allergic disease is associated with some kind of pre- and perinatal immune programming.

In summary, all infants are born with a somewhat Th2 skewed immune response but infants who develop allergic disease seem to have less Th1 immune responses (IFNγ and IL-12) at birth and more sustained and elevated production of IgE antibodies (Th2) than healthy infants. This immune deviation might be influenced by early environmental factors through mechanisms that need further characterisation.
**Prevention of allergic disease**

As discussed earlier, the intrauterine environment seems to have an impact on the risk of developing allergic disease. Interestingly though, studies on adopted children who move to industrialised countries before 2 years of age indicate that they develop allergies to the same extent as children born in the country as opposed to children who move here later in life (14), which indicates environmental effects during the first years of life. The hypothesis of an environmentally based deviation of the immune response towards a Th2 and away from a Th1 response in allergic disease is questionable from an epidemiological point of view. The occurrence of Th1 diseases such as type 1 diabetes is positively rather than negatively associated with asthma (121) and both Th1 and Th2 diseases have increased under the same environmental influences (122). A lack of Treg cells might be the factor of importance as they may suppress Th2 and Th1 immune responses through IL-10 (123).

On the other hand allergic disease is a hereditary disorder. When both parents have the same expression of atopic disease, their child has a 70% risk of developing the same manifestations. If both parents are atopic there is a 30-50% risk for the child to develop atopic disease (124-126). Ten percent of children without atopic heredity and 30% with a single heredity develop disease. As many as around 30% of newborns have at least one parent/ or older sibling with previous or current atopic disease (124, 126).

Apart from the genetic predisposition for allergic disease, there are mechanisms for interaction between genes and environment, *i.e.* epigenetic mechanisms and gene-by-environment interaction that have an impact on the individual risk of developing allergies. The term epigenetics is defined as environmentally caused changes in gene expression, inherited in the absence of mutations in the DNA.
sequence as well as the event that initiated the change. DNA methylation (binding of a methylene group to a DNA CpG dinucleotide), and methylation, acetylation or phosphorylation of histones and chromatin are examples of epigenetic modifications. Factors that may have an impact on the epigenetic mechanisms are nutrition, toxic chemicals, radiation, exposure to air pollution and tobacco smoke (127). Gene-by-environment interaction, on the other hand, describes that different genotypes imply different susceptibility to environmental factors and their ability to protect from or increase the risk of disease (128, 129).

In conclusion: when working with prevention of allergic disease, knowledge of genetic and environmental influences, as well as their complex interaction, is essential.

Environmental factors

There are several environmental factors that have been considered regarding their impact on the development of allergic disease. The most studied ones are early allergen exposure, smoking and air pollution, psychological factors, microbial exposure, respiratory infections, use of antibiotics and maternal and infant dietary content including micronutrients. They are each discussed separately below and for some of them gene-by-environment interaction mechanisms have been reported.

Early allergen exposure

Inhalants

Early-life exposure to pets or lifestyle factors associated with exposure to pets seem to reduce the risk of developing atopy-related diseases in early childhood. However, these findings might also be explained by selection for keeping pets (130). There is a dose dependent relationship between early exposure to aeroallergens, e.g. HDM, and sensitisation and asthma in many but not all
studies (126). Exposure to HDM antigen together with dampness at home was a significant risk factor for the persistence of bronchial hyper reactivity and respiratory symptoms in children with asthma in one study (131). In another report, the level of prenatal exposure to *Dermatophagoides pteronyssinus* (Der p) 1 influenced the immune profile of cord blood T lymphocytes and the prevalence of eczema in early life (132). Yet, prevention studies addressing the environmental issue with drastic avoidance measures show disappointing results (133).

**Foods**

Repeated intervention studies have not shown a protective effect of an allergen-poor diet on the part of the mother during pregnancy and lactation (134-136). On the contrary, recent findings indicate that early introduction to food allergen, at 4 months rather than at 6 months, can be protective of allergic disease (discussed in (137)). Høst *et al* state that the most effective dietary regimen for the child at high risk of developing allergic disease is exclusive breastfeeding for at least 4-6 months or, in absence of breast milk, formulas with documented reduced allergenicity for at least the first 4 months, combined with avoidance of solid food and cow's milk for the first 4 months (138). This, however, is questioned in a recent update where the authors declare that hypoallergenic formula does not prevent the allergic symptoms, it merely delays them (139). Consequently, the Swedish Paediatric Society recommends extensively hydrolyzed formulas for the first 4 months in a very limited number of cases; only for infants in families with at least two family members with documented severe and long lasting allergic disease and where breast-feeding is not sufficient (144).

**Smoking and air pollution**

Smoking during pregnancy can increase recurrent wheezing during infancy (reviewed in (126)) but the possible effect on allergic sensitisation is
contradictory (118, 140). Thus, if passive smoking early in life has any impact on allergies, it is likely to increase the severity of disease in those who have additional factors promoting the development of allergic sensitisation (140). The Glutation S-transferase (GST) –enzymes (GSTM1, GSTP1 and GSTT1) protect cells from toxic substances and oxidative stress. In some genotypes, these proteins do not function properly and there is an increased risk of damage to the airway epithelium after exposure of air pollution and second hand smoke (141). There are indications that children with the GSTP1-genotype (Valin 105) have an increased risk of allergic disease in areas with high air pollution (142) and children with a GSTM1-deletion have a susceptibility to develop asthma from maternal smoking during pregnancy (143).

**Psychological factors**

Psychological stress can influence the balance between Th1 and Th2 cytokines which might strengthen the allergic inflammation especially in allergic individuals (144). A meta analysis revealed a robust relationship between psychosocial factors and atopic disorders. In addition to conventional physical and pharmacological interventions, psychological intervention might be beneficial for successful prevention and management of atopic disorders (144).

**Microbial components - the hygiene hypothesis**

Epidemiological studies suggest that microbial components in the environment early in life are mediators of allergy-preventing effects (145). For example, the International Study of Asthma and Allergies in Childhood (ISAAC), including more than 200 000 children, found that an increase in the tuberculosis notification rate was associated with an absolute decrease in wheeze. Mycobacterial notification rate served here as a surrogate marker for mycobacterial exposure (146). Increased levels of microbial exposures
recognized by innate immune cells may affect adaptive immune responses resulting in decreased risk of atopic sensitisation and asthma (104).

Poverty, multiple older siblings and growing up on a farm with animals reduce the risk of developing allergy with good evidence (14, 104), maybe due to increased microbial exposure. A number of gene-by-environment interactions have been observed with polymorphisms in genes of innate immunity receptors and exposure to farm surroundings. One example involves the CD14-gene, coding for a receptor that influence immune responses towards endotoxin that is frequent in farming environment. The protective effect on the development of asthma and allergies from drinking farm milk seems to be influenced by variations of this gene (147). Some studies (33, 148), but not all (149) find a protective effect from day-care on different allergic manifestations and there are indications of genotype specific responses (150).

It was suggested that changes in the microbial gut flora may mediate changes in the prevalence of atopy (151). Delivery by caesarean section has been found to be associated with a moderately increased risk of allergic rhinitis, asthma, hospitalisation for asthma, and perhaps food allergy (119), but not with eczema (120). These findings demonstrate that the mode of delivery may have significant effects on immunological functions in the infant, possibly via gut micro biota development. Trials using supplementation with different strains of probiotics early in life have been performed in order to enrich the gut flora and prevent allergic disease and the results have been slightly favourable (152, 153).

**Infections - Respiratory Syncytial Virus (RSV).**

Whether the RSV bronchiolitis is a risk factor for allergic sensitisation and asthma is a matter of debate (154, 155). RSV infection that is severe enough to require hospitalisation does not cause asthma but is an indicator of the genetic
predisposition to asthma, is the conclusion of a recently published twin study (156). A deficient cytokine response in relation to infection with RSV has been proposed as an indicator of host susceptibility to severe disease. Several polymorphisms in cytokine genes have been proposed. In particular, polymorphisms in the IL-4, IL-10, IL-13, CCR5, TGFβ, and TLR-4 genes have been related to RSV severity (157).

**Antibiotics**

Early life exposure to broad-spectrum antibiotics may have a causative role in sensitisation in combination with expression of wheeze (158). In the KOALA birth cohort in the Netherlands, early antibiotic use preceded the manifestation of wheeze but not eczema or allergic sensitisation during the first 2 years of life (159). The effect of antibiotics on respiratory disease may though be due to confounding by chest infections at an early age when asthma may be indistinguishable from infection (160).

**Protective factors in the diet of mother and child**

*Breast feeding*

In a recent meta analysis of 21 studies there was no strong evidence of a protective effect of exclusive breastfeeding for at least 3 months against eczema, even among children with a positive family history (161). In another meta analysis the conclusion was that breast-feeding fully for 3 months and partly for 3-4 months reduces the risk of eczema in children with atopic heredity during the first 4 years of life (162). This is supported by observational studies from a Swedish group where at least 4 months of breast feeding offered protection from eczema up to 4 years (163), as well as asthma and sensitisation up to 8 years of age (164). However, there are also opposing results (165, 166). A possible preventive effect of breastfeeding on the development of allergic disease might be due to either a protective effect of human milk or avoidance of high dose cow
milk protein, or a combination of these factors. There is also a risk of reverse caution in some studies: mothers in families with a high risk of allergy breastfeed longer (126). The diverse results from the studies could be caused by the varying composition of breast milk and individual susceptibility to the protective factors in the milk. In line with this, the protective effect of breastfeeding on eczema in children was found in non-allergic mothers but not in allergic mothers in the Dutch KOALA Birth Cohort Study (167).

**Maternal diet**

There might be components of the maternal diet in pregnancy that protect from development of allergies. For example adequate intake of anti-oxidants (168), apples (117) and fish (116, 117) have been associated with lower risk of allergic disease in the child. Vitamin E is an antioxidant which has been reported to possibly decrease the risk for asthma, eczema (168) and sensitisation (169) in childhood. Murine studies indicate that dietary folate intake in pregnancy can alter the allergic predisposition through epigenetic mechanisms (170). The possible preventive effects of fish are discussed further on in this thesis.

*In summary: so far no special diet in pregnancy or lactation is recommended in Sweden, in order to prevent allergic disease. Very high-risk children, who have two family members with severe allergic disease, may benefit from hypoallergenic formula before 4 months of age in absence of breastfeeding (primary prevention). Children with established allergic disease should avoid tobacco smoke and exposure to relevant allergens (secondary prevention) (171). For the general health of the children, the Swedish primary childcare advice all families to avoid tobacco smoke during pregnancy and lactation and to breastfeed exclusively for 4-6 months followed by the introduction of solid foods. Cows milk formula is the supplement of choice for most infants not fully breast-fed.*
Long chain polyunsaturated fatty acids

As early as in the 1940’s a relationship between atopic eczema and abnormal serum essential fatty acids was found (172). Fatty acids are long carbon chains with a methylene at one side and a carboxyl at the other side. The number of carbons varies and there are one or multiple double bonds between the carbons. Polyunsaturated fatty acids (PUFAs) contain multiple double bonds and the two families of essential PUFAs; ω-6 and ω-3 are named according to the position of the terminal double bond at the methylene end of the chain. Each fatty acid also has the number of carbons in the chain and the number of double bonds in its denomination.

\[ \text{eicosapentaenoic acid (EPA)} \]

**Figure 4:** C20: 5 ω-3 - 20 carbons, 5 double bonds, and the terminal double bond is situated at the 3rd carbon counted from the methylene end.

The essential fatty acids, which have to be provided from the diet, are linoleic acid (LA, 18:2ω-6) and α-linolenic acid (LNA, 18:3ω-3). By insertion of additional double bonds into, and elongation of, the acyl chain, long chain PUFA (LCPUFA) can be produced from these fatty acids (173). The metabolic steps take place in the liver cells, mainly in the endoplasmatic reticulum, except for the last step that requires β oxidation in the peroxisomes (174) (Fig.5).
PUFA comprise 15% of dietary fat in the European diets and 5-6% of the total energy intake (175, 176). LA comprises 85-90% of the PUFA intake, and LNA about 10% while AA, DHA and EPA contribute to less than a few percent of this according to data from the diets of women in Australia and Belgium (176, 177). All vegetable oils are rich in LA while rapeseed oil and linseed oil also contain significant amounts of LNA. Additionally, walnuts and green plants are important sources of LNA. Meat and eggs provide high quantities of AA while DHA and EPA are abundant in seafood and fatty fish like herring, mackerel, salmon and tuna.

\[
\begin{align*}
\text{ω-6 PUFA} & \quad \text{enzymes} & \quad \text{ω-3 PUFA} \\
18.2 \text{ω-6 LA} & \quad \Delta^6\text{-desaturase} & \quad 18.3 \text{ω-3 LNA} \\
18.3 \text{ω-6} & \quad \text{elongase} & \quad 18.4 \text{ω-3} \\
20:3 \text{ω-6} & \quad \Delta^5\text{-desaturase} & \quad 20:4 \text{ω-3} \\
20:4 \text{ω-6 AA} & \quad \text{elongase} & \quad 20:5 \text{ω-3 EPA} \\
22:4 \text{ω-6} & \quad \text{elongase} & \quad 22:5 \text{ω-3} \\
24:4 \text{ω-6} & \quad \Delta^6\text{-desaturase} & \quad 24:5 \text{ω-3} \\
24:5 \text{ω-6} & \quad \beta\text{-oxidation} & \quad 24:6 \text{ω-3} \\
22:5 \text{ω-6} & \quad \text{β-oxidation} & \quad 22:6 \text{ω-3 DHA} \\
\end{align*}
\]

**Figure 5.** Conversion of LA and LNA into long chain polyunsaturated fatty acids (LCPUFA). LA: linoleic acid, AA: arachidonic acid, LNA: α-linolenic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, ER: endoplasmatic reticulum
The ratio of ω-6 to ω-3 has increased in the northern European diet during the last half of the 20th century, mainly because of increased intake of vegetable oils and decreased intake of fish oil (178). The ratio used to be close to 1:1 (ω-6/ω-3) (178) but is currently between 5:1 and 20:1 (179). An additional explanation to this increase is that meat and dairy products from ruminant animals contain less ω-3 fatty acids (i.e. LNA) nowadays because of the practice of feeding them concentrated cereal grain-based feed rather than green grass (178).

On the other hand, the conversion of LNA into EPA and DHA in the human body is quite inefficient as less than 10% of LNA is converted to DHA in adults (180). In women of reproductive age and in neonates, particularly prematures, this process may be a little more proficient (181, 182). In adults DHA is most abundant in retina, sperm and the cerebral cortex while AA can be found mostly in red blood cells, spleen and liver (180). The elongation and desaturation of PUFA seems very important. In a patient, with reduced Δ-6-desaturase activity, there were skin abnormalities, growth failure, corneal ulcerations and feeding intolerance shortly after birth and this was corrected with LCPUFA supplementation (183).

In pregnancy, fatty acids are released from maternal triglycerides by lipoprotein lipase on the maternal surface of the placenta and bound to plasma membrane fatty acid binding protein (p- FABPpm) and cytoplasmatic transport proteins. In order to meet the foetal demand, the placental transfer is selective and results in higher levels of both DHA and AA in cord blood compared to maternal blood (184). Omega-3 LCPUFA are incorporated into phospholipids and sphingolipids in cell membranes of primarily the liver and the brain during the last trimester of pregnancy (180) and they are important for growth of the foetus and development of the CNS and the retina. LCPUFA status in the child is related to gestational age at birth, maternal diet and maternal LCPUFA status in
phospholipids (185). There is evidence that the maternal requirements of DHA in pregnancy and lactation are not always met by our western diet and the maternal stores are not easily replenished after pregnancy (186, 187). Consequently, women who are pregnant with their second or third child have less available \( \omega-3 \) fatty acids, especially DHA (186, 188).

In summary, the \( \omega-3 \) LCPUFs DHA and EPA are abundant in oily fish and especially DHA is important for foetal development. There is a selective transport of DHA across the placenta to the foetus.

**Fish oil intake early in life and infant allergic disease**

Observational studies indicate that fish consumption 2-3 times a week during pregnancy reduces the risk of atopic eczema and sensitisation in the children at one year (116) and atopic eczema at five years of age (117). Fish consumption during the first year of life may also reduce the risk of allergic rhinitis (189) and any allergic disease at 4 years of age (190). There are however a few studies that show negative effects or no effect at all from fish consumption on allergic disease in childhood (reviewed in (191). Low levels of EPA and DHA and a high ratio of AA/EPA in maternal milk were reported to relate to increased risk of allergic disease during the first 18 months of life (192).

These findings have encouraged the performance of supplementation studies. Due to the inefficient conversion of LNA into LCPUFA, the most proficient way of substituting EPA and DHA is by giving the pure substance, not a precursor (193). Trials with EPA and DHA supplementation in pregnancy have thus resulted in increased levels of these LCPUFAs in both maternal and infant plasma phospholipids (194, 195) as well as in cord blood erythrocytes (196).
Supplementation with EPA and DHA to adults led to a decreased ratio of AA/EPA acid in the membrane phospholipids of mononuclear cells (197).

**Clinical outcomes**

Dunstan et al reported beneficial effects on the severity of infant eczema (SCORAD≥25, p=0.045) and SPT to egg (p=0.055) at one year of age after supplementing 98 atopic mothers with 3.3 g EPA and DHA during the last half of pregnancy. The positive effect was noted even though the study was not designed to analyse clinical outcomes (9).

The Danish study of Olsen et al reported that fish oil supplementation in late pregnancy is associated with a marked reduction in allergic asthma in the offspring at age 16 years of age compared to placebo (olive oil), suggesting a long-term effect of any immunologic change that occurred in pregnancy and early life of those children. Unexpectedly, the group who did not take any supplementation had a risk of asthma similar to the ω-3 supplemented group. The study was originally designed to investigate pregnancy related effects of the supplementation (198).

In the Australian Childhood Asthma Prevention Study (CAPS), cooking oils and spreads with low ω-6 content starting in the last month of pregnancy and tuna fish oil supplementation (500 mg/d starting at weaning or at 6 m of age) decreased the prevalence of wheeze at 18 months of age. High plasma ω-3 PUFA levels were associated with less bronchodilator use, irrespective of the supplementation group but there was no effect on sensitisation (199, 200). Follow-up at 3 years showed that the atopic children in the fish oil group had a reduced prevalence of cough, but not wheeze (201). However, no effect of fish oil was seen on the other endpoints: eczema, serum IgE concentration or doctor diagnosis of asthma. At 5 years of age, there was no significant effect of fish oil.
on any of the clinical outcomes relating to lung function, allergy, or asthma (202). At 5 years of age the children stopped taking the supplementation but the children were examined at 8 eight years of age and no significant effects on atopic disorders were reported (203). The possible reasons for the lack of beneficial effects of long-chain ω-3 PUFAs after 3 years of age may be related to suboptimal adherence to and/or implementation of the intervention (50% and 56% compliance in the intervention and control group, respectively), as well as to the low dose of fish oil used. The intervention started in late pregnancy and this may have been too late to have an impact on the development of the foetal immune system.

Immunological outcomes

High intakes of ω-3 LCPUFA suppress a wide range of immune variables such as lymph proliferation (204-206), CD4+ cells antigen presentation (207, 208), adhesion molecule expression (209) as well as proinflammatory cytokine (206) and eicosanoid production (205, 210) in humans and rodents. A few human studies of these effects early in life were performed:

An observational study reported that increased levels of cord blood EPA and AA in cell membranes attenuated cord blood lymphocyte proliferation and decreased allergen stimulated IFNγ secretion. A lower AA/EPA ratio was also associated to reduced IFNγ secretion (211), indicating that the balance between ω-6 and ω-3 fatty acids is important.

In a ω-3 supplementation study of pregnant women, lower neonatal IL-13 (Th2 related cytokine) levels were noted in the ω-3 group (8) and there was a trend towards lower IL-5, IL-13, IL-10 and IFNγ responses to all allergens in the fish oil group (9). Further, fish oil supplementation modulated expression of infant T cell PKC zeta, one of the mitogen-activated protein kinases, which are crucial
for cytokine production, in the neonatal period. Allergic disease is associated with a decreased expression of PKC zeta (99).

The Danish study of maternal fish oil supplementation during lactation (212) investigated immune outcomes in the offspring. Infants of lactating mothers who received fish oil supplementation had a higher IFNγ production at 2.5 years of age compared to children whose mothers had received olive oil as a placebo, an observation that may reflect faster maturation of the immune system.

The “early-in-life” ω-3 LCPUFA supplementation trials reporting immunological outcomes in maternal and infant blood and clinical outcomes in the infants are summarised in Table 9.

**Breast milk**

Maternal ω-3 LCPUFA supplementation increases the content of these lipids in breast milk (213, 214) but a low dose did not alter milk cytokine content (214). After supplementation with 3.3 g ω-3 LCPUFA daily during pregnancy, the levels of S-IgA and soluble CD14 in breast milk were increased (213), which may have allergy preventing effects by influencing immunological responses in the intestine of the infant (215). Furthermore, addition of small amounts of AA and DHA (close to the content in human milk) to formula for preterm babies may influence the concentration, proportion, maturation and production of peripheral blood lymphocytes in the children (216).

*In summary: Fish in the diet during pregnancy and lactation has been associated to decreased risk of allergic disease in the early years. The fish oil supplementation studies performed so far indicate a slight protective effect but the studies have either not been designed to investigate the clinical outcomes or*
used very small doses of fish oil. Nevertheless, they point out that ω-3 LCPUFA in pregnancy and lactation have effects on neonatal immune responses.

**Immune modulating mechanisms.**

The immune modulating actions of long chain ω-3 PUFAs are executed at the gene level, in the cell membranes, and in the metabolism of lipid mediators (217) and it is not clear which mechanism is the most important. The previously mentioned effects on cytokines, cell functions and clinical symptoms may be executed through these different mechanisms.

**Gene level**

Omega-3 LCPUFAs have been reported to decrease the activity of inflammatory genes such as COX 2 and VCAM-1 through modification of nuclear factor κβ (NFκβ) activity, and thereby direct alteration of gene expression (218, 219). The ω-3 LCPUFA are also natural ligands of nuclear receptors such as peroxisome proliferators activated receptors (PPAR) α and γ that are transcription factors in inflammatory cells (218). These implications on gene expression may contribute to possible epigenetic changes by ω-3 LCPUFA.

**Membrane fluidity**

AA, and to a lesser extent LA, are major fatty acid components of lymphocyte membrane phospholipids. For example, CD4+ T cells, which play a central and critical role in immune regulation, have a high proportion of membrane AA (25%), but very little DHA (4%) (210). Recent data indicate that the organisation and trafficking of LCPUFAs play an important role in the stabilisation of cell membrane lipid rafts and the acylation of membrane proteins involved in cell regulation. Lipid rafts are small domains of the plasma
membrane that are platforms for cell activation and signalling between cells (220). Th1 cell activation, opposite to Th2 cell activation, is proposed to be dependent on lipid rafts. Increased ω-3 LCPUFA proportions in cell membranes, which can be obtained by a fish oil rich diet, alter membrane fluidity and modulate lipid raft composition and hereby may influence T cell responses (197, 217, 219), supposedly by down regulation of Th1 responses (221). Additionally, increased content of ω-3 LCPUFA in the APC cell membranes may reduce the capacity to present antigens by inhibiting the up-regulation of MHC class II receptors, cytokines and co stimulatory molecules in murine cells (207, 208). This has been difficult to prove in neonatal APCs probably due to their immaturity and reduced antigen presenting capacity (222).

**Lipid mediators**

When an inflammatory reaction takes place, AA in the cell membranes is released by the enzyme phospholipase A2. Further on, eicosanoids are synthesised through the enzymes COX, that induces production of prostaglandins and tromboxanes, and LOX that generates leukotrienes. COX is present in all types of cells but LOX only in inflammatory cells like monocytes, macrophages, mast cells and granulocytes (14). Lipid mediators like prostaglandins and leukotrienes are also formed from EPA in the cell membranes. However, the membrane fatty acid composition influences the production of lipid mediators in the cells (223).

**Omega-6 lipid mediators**

Eicosanoids formed from AA are the two-series of prostaglandins (e.g. PGD2 and PGE2), and the four-series of leukotrienes (e.g. LTB4). Lipoxines are also formed from AA through the 5-LOX pathway and they are active in the late phase of inflammation resolution (224).
Activated endothelial cells and macrophages release PGE2, that can inhibit T cell proliferation (225), regulate APC function, inhibit IL-1, IL-2, IL-6 and IFNγ and possibly enhance the production of IL-4 and IL-5 (226). PGE2 also stimulates the production of IgG1 and IgE antibodies in LPS and IL4 stimulated mature B cells (225). In line with this, it has been reported that PGE2 enhances antigen-specific IgE responses in murine lymphocytes (227). On the other hand PGE2 is suggested to be protective against airway inflammation (228). It is possible that PGE2 promotes sensitisation via its effect on T- and B-cells but is protective against the subsequent manifestations of inflammation (191).

**Figure 6.** AA, EPA and DHA are released from cell membranes and form substrates for cyclooxygenases (COX) and 5- lipooxygenase (5-LOX). PGE: prostaglandin E, TXA: Thromboxane, LT: Leukotriene. Solid lines: pro inflammatory mediators, striped line: less inflammatory mediators, dotted line: anti-inflammatory mediators. Modified from (14, 174).
PGD2 is released from mast cells and causes bronchoconstriction, dilatation of vessels and chemotaxis for inflammatory cells. It also suppresses activation of neutrophil granulocytes (14). LTB4, released from macrophages and neutrophils, and LTC4 from eosinophils and mast cells, increase vascular blood permeability, enhance local blood flow and stimulate mucus secretion and constriction of the airways (14). They are also chemotactic for leukocytes and promote cytokine production, such as TNFα, IL1, IL6, IL2, IFNγ (229).

**Omega-3 lipid mediators**
Eicosanoids, such as PGE3 and LTB5, are formed from EPA in the cell membranes. It has been assumed that these mediators have lower biological potency than those formed from AA (230). Yet, more recent results indicate that PGE3 and PGE2 may have equivalent inhibitory effects upon production of TNF and IL-1 and consequently, EPA and AA derived eicosanoids do not always have different potencies (223). However, there are recently revealed mediators formed from DHA and EPA through COX2 and 5-LOX that have anti-inflammatory properties, *i.e.* resolvins and protectines (Fig. 6) (231). They block production of inflammatory mediators and stop infiltration of polymorphonuclear cells and thereby help resolve inflammation (224). This indicates that DHA might have similar potency as EPA as an anti-inflammatory fatty acid (232).

*In summary, when the availability of DHA and EPA is increased, *i.e.* by a diet rich in fish oil this might influence gene expression, modify immune cell responses through altering the composition of the cell membranes and decrease the eicosanoid production from AA in favour of lipid mediators from EPA and DHA. These mechanisms together or separately might prevent the development of allergic sensitisation and hamper the allergic inflammation.*
Gene-by-environment interaction

The fatty acid desaturases genes (FADS) 1 and 2, code for the rate limiting PUFA desaturases Δ-5 desaturase and Δ-6 desaturase respectively (Fig. 5) (233, 234). Genetic variants of FADS1/FADS2 influence breast milk essential fatty acid composition and plasma phospholipid content during pregnancy (235). It has been shown that the biological effects of LCPUFAs differ between individuals or population subgroups and this may partly depend on different polymorphisms in the FADS genes (174, 236, 237). For example, Caspi et al studied the association between breastfeeding and IQ in children with different polymorphisms of the FADS1/FADS2 genes. Children carrying the C allele inrs174575 polymorphism had marked advantage in IQ due to breastfeeding (and supposedly its content of LCPUFAs) while children without this allele had no advantage from breastfeeding (237).

Similar mechanisms might contribute to the diverse data on the protective effect from breast milk on allergic disease. Allergic disease and sensitisation have been associated with a disturbed fatty acid metabolism in maternal blood (238) and low ω-3 LCPUFA in mature breast milk (192). It has also been linked to the same region in chromosome 11 as the FADS1/FADS2 genes (233). Moreover, a lower prevalence of allergic rhinitis and eczema was associated to several single nucleotide polymorphisms in the FADS genes. However, these associations were not significant after correction for multiple testing (234).

In summary: The biological effects of ω-3 LCPUFA might differ between subgroups of individuals due to gene-by-environment interaction. In future studies of the effects of ω-3 LCPUFA, gene analyses would be useful.
AIMS AND HYPOTHESIS

The general aim of this thesis was to assess the effect of maternal dietary supplementation with ω-3 LCPUFA, i.e. fish oil, in pregnancy and lactation on the development of allergic symptoms and sensitisation in the infants and to explore the associations between some maternal and infant immunological markers and the ω-3 LCPUFA supplementation.

Specific aim and hypothesis of each paper:

I. To evaluate the effect of ω-3 LCPUFA supplementation in pregnancy on maternal eicosanoid and cytokine production. We hypothesise that PGE2 and LTB4 as well as the proinflammatory cytokines IL-1, IL-6 and TNF will decrease during ω-3 LCPUFA supplementation.

II. To assess the effect of ω-3 LCPUFA supplementation during pregnancy and lactation on the risk of allergic sensitisation, food reactions and eczema during the first year of life. We hypothesise that the ω-3 LCPUFA supplementation will decrease the incidence of these clinical outcomes.

III. First, to investigate the effect of ω-3 LCPUFA supplementation during pregnancy and lactation on the risk of allergic sensitisation and disease during the first two years of life. Second, to explore the relationship between the proportions of DHA, EPA and the AA/EPA ratios in maternal and infant serum/plasma phospholipids and the frequency of IgE associated disease as well as the severity of eczema and number of allergic symptoms in the infants. We hypothesise that the ω-3 LCPUFA supplementation will decrease the incidence of the clinical outcomes. We also believe that high maternal and infant proportion of ω-3 LCPUFA will be associated with low prevalence of allergic
symptoms and sensitisation, lower SCORAD index in eczematous children and a reduced number of allergic symptoms.

IV. To relate circulating Th2- and Th1-associated chemokines and IgG antibody responses to tetanus and diphtheria vaccines to the ω-3 fatty acid supplementation and allergic disease in mother and child. We hypothesise that the infant Th2 chemokines will be suppressed by the ω-3 LCPUFA supplementation in favour of Th1 chemokines, especially in infants of non-allergic mothers. We also believe that the vaccine-induced responses will be strengthened by the ω-3 LCPUFA supplementation.
MATERIALS AND METHODS

Inclusion of participating families

All papers in this thesis are based on a prospective double blind placebo controlled randomised trial conducted at the Department of Paediatrics at the University hospital in Linköping and in the county hospital of Jönköping, all in South Eastern Sweden. 185 families were willing to participate when asked at the antenatal clinic between March 2003 and May 2005. The inclusion criteria were allergic disease in at least one family member (mother, father and/or sibling of the future child) and an intention to breastfeed the child for at least three months. The mothers had to be included before gestational week 25. A structured interview by one of three experienced allergy nurses covered the family history of eczema, asthma, allergic urticaria, gastrointestinal allergy or allergic rhinoconjunctivitis before inclusion of the family.

Women with allergy to soy or fish, those under treatment with anticoagulants and those who were unwilling to stop taking their own fish oil supplements were not included in the study. In all, 145 families were enrolled. Two mothers gave birth to twins but only the first born in each couple was included. The reasons for non-inclusion and for discontinuing the intervention are displayed in Fig. 7. Some of the families missed planned visits due to loss of interest or relocation but they were encouraged to a phone call or visit at 24 months. Finally, data on allergic symptoms in the infant during the first two years was obtained from 143/145 families.
Assessed for eligibility (n=185)

Excluded (n=40):
Not meeting inclusion criteria (n=31)*
Declined participation (n=7)
Other reasons (n=2)**

Randomized (n=145)

ω-3 group (n=70)
Discontinued intervention (n=16)
Moved (n=1)
Lost interest (n=1)

Placebo group (n=75)
Discontinued intervention (n=9)
Moved (n=1)
Lost interest (n=1)

Intervention not completed (n=25)***
lost to follow up (n = 1)

Assessment at 24 months (n=143)

6 months visit (n=65)
Moved (n=1)
Lost interest (n=1)

12 months visit (n=63)

24 months visit (n=63)

24 months telephone contact and allergy questionnaire (n=8)

Lost interest (n=2)

Missed 6 months visit (n=7)

6 months visit (n=15)
Lost interest (n=3)

24 months visit (n=19 )

Assessment at 24 months (n=143)
Figure 7: Inclusion and clinical follow up.
*: Treated with anticoagulants (n=2), had passed 25th gw (n=12), allergy to soy (n=2), wished to continue their own fish oil supplements (n=1), no allergies in the family (n=4), planned birth date after end of study (n=10).
**: moved (n=1), miscarried (n=1).
***: Reasons for discontinuing intervention: inability to swallow capsules (n=9), nausea (n=6), abdominal pain (n=3), ignorance (n=3) and miscellaneous (n=4). The content of the capsules did not have an impact on reasons for withdrawal.

Intervention

The content of the capsules

Block randomisation was performed by the producer of the supplements (Pharma Nord, Vejle, Denmark) and the material was double blinded until the end of the two-year follow-up of the children. The women in the ω-3 group (n=70) were supplemented with Bio Marin capsules (Pharma Nord, Vejle, Denmark), containing a minimum of 35% EPA and 25% DHA. The recommended daily dose of nine 500-mg capsules contained in average 1.6 g EPA, 1.1 g DHA and this corresponds to a meal of approximately 100 g salmon daily. It also contained 23 mg α-tocopherol as an antioxidant, a necessary ingredient, according to the standard procedure of the manufacturer, to assure the durability of the oil.

The mothers in the placebo group (n=75) received nine soy oil capsules a day, containing mainly the ω-6 PUFA LA (58%, 2.5 g/day), a small amount of the ω-3 PUFA LNA (6%, 0.28 g/day) and 36 mg α-tocopherol. The fatty acid content of the capsules is presented in detail in Table 2.
Table 2. The content of the capsules.
Analyses performed at professor Birgitta Strandviks laboratory in Gothenburg, resulting in slightly higher proportions of EPA and DHA than reported as a minimum content by the manufacturer. This is due to the variation in EPA and DHA content of the fish used in the production. The placebo capsules do not contain EPA or DHA and the analysis of the placebo content agreed well with the data from the manufacturer.

<table>
<thead>
<tr>
<th></th>
<th>ω-3 mol %</th>
<th>Placebo mol %</th>
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<tbody>
<tr>
<td>12:0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>14:1ω-5 *</td>
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</tr>
<tr>
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<td>0.1</td>
</tr>
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<tr>
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<td>0.3</td>
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<tr>
<td>22:6ω-3 (DHA)</td>
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</table>

The choice of soy oil as a placebo was based on the ability of the producer to manufacture the capsules. Estimated intakes of LA in women according to a nation wide study in Sweden in 1989 was 7.8 g daily (239), which means that the placebo capsule would add about one third of the daily intake to the mothers’ diets. The daily intake of LNA (1.2 g) was increased by about 25%. However, there are indications that the ω-6/ω-3 ratio has an impact on inflammatory responses (211, 240) and in that respect the placebo capsule, with
a ratio of \( \omega-6/\omega-3 \) (LA/LNA) of about 9 was close to the ratio LA/LNA of 7, registered in the diet of Swedish women (239). The ratio LA/LNA in the \( \omega-3 \) capsule was 1 and the total \( \omega-6/\omega-3 \) was <1.

The metabolism of LNA into DHA and EPA is inefficient so it can be presumed that only insignificant amounts of DHA and EPA were added through the LNA in the placebo capsule (181). On the other hand, the \( \omega-3 \) capsule increased the EPA and DHA intake 8-10 times (239) and the ratio of the active metabolites AA/EPA (\( \omega-6/\omega-3 \)) was more than ten times less than in the normal diet (239). This supports the idea that the possible changes in LCPUFA composition of blood and breast milk caused by the placebo capsule should be negligible, particularly compared to those of the \( \omega-3 \) LCPUFA oil.

The content of \( \alpha \)-tocopherol (vitamin E) in the capsules differed somewhat between the two groups. Vitamin E is an antioxidant with a recommended daily intake during pregnancy of 15-20 mg. It has been reported to possibly decrease the risk of developing asthma, eczema (168) and sensitisation (169) in childhood. However, the reduced risk of sensitisation has been seen at intakes of vitamin E up to 7 mg/day, but higher doses did not give significant additional effects (169). All mothers in this study received more than 25 mg/day. Furthermore, 30 mg vitamin E daily did not modify the immunomodulatory effects of fish oil in a study of healthy men (241). The content of polychlorinated biphenyles (PCB) and dioxins in the capsules was 20 times less than in a corresponding portion of oily fish according to the manufacturer (Pharma Nord).
Compliance

Dietary supplementation started at the 25th (range 23.0-27.1) week of gestation, which is around the time when the first immune responses to allergen have been detected (92). The women, who completed the intervention, took the capsules for an average time of 30.9 weeks, range: 15.1–42.1(66.1), SD ±5.6 weeks, (Table 4, Fig. 8).

The nurses called the mothers twice during pregnancy to remind them of the capsules and compliance was assessed by analysis of phospholipid fatty acids in maternal and infant serum/plasma. Each mother received enough capsules to last through the 3-4 first months of breastfeeding but there were some mothers who did not manage to take full dose and consequently the capsules lasted longer. These mothers still fulfilled the study. Nine capsules daily were taken once a day or, if there were swallowing difficulties 3 capsules 3 times a day were recommended. There was one extreme outlier, as one woman in the placebo group took a lower daily dose for 66 weeks (Fig. 8). Additionally, not all women breastfed fully during the whole study period (Table 6) and therefore the babies’ daily dose and length of treatment varied. This emphasises the importance of measuring LCPUFA proportions in phospholipids in order to monitor compliance and detect associations to allergic disease but it makes this study unable to determine the appropriate dose for maternal ω-3 supplementation in allergy prevention. When breastfeeding was not sufficient extensively hydrolysed formula was recommended for infants with signs of cow’s milk allergy and cow’s milk formula was suggested for everyone else.
Figure 8. Duration of supplementation. Grey: placebo Black: ω-3 supplementation. Dotted line: Minimum 15 weeks of supplementation was required for inclusion in the analysis of outcomes in the intervention groups (“as treated analysis”).

Blinding

After about 4 weeks of supplementation, 11/80 (14%) mothers reported belching. Ten of them were supplemented with ω-3 fatty acids (p= 0.001). Belching has been reported previously when similar doses of fish oil have been given in pregnancy (242, 243). Thus, the blinding to the subjects might have been unmasked. This could be a problem as diagnosis of clinical symptoms is dependent on the mother’s description and expectations. However, positive SPTs and detection of specific IgE antibodies in blood samples are objective measures. Further, all staff members working with the intervention and follow-up were blinded throughout the whole study.
Safety

Increased consumption of fish oil in pregnancy could theoretically increase bleeding tendency at delivery (244) and prolong gestation but also reduce the risk of reoccurrence of preterm delivery (245). Replacement of AA by EPA in the cell membranes induces vasodilatation and decreases platelet aggregation (244). However, supplementation with the commonly used dose of 3 g EPA+DHA per day, which is 10-20 times the average intake of most pregnant women in westernised countries, has not been associated with increased risk of maternal bleeding complications (reviewed in (246)). That risk is clinically significant only if the consumption of ω-3 fatty acids is 7-10 g per day (244). Further, the mean length of infant hospital stay or the relative risks of admission to neonatal care, congenital malformation, neonatal bleeding and non-bleeding disorders have not been affected by 3g EPA and DHA daily during pregnancy (reviewed in (245, 246)).

On the other hand, consumption beneath 150g ω-3 LCPUFA per day may increase the risk of preterm delivery and low birth weight (247) and therefore a minimum intake of 200-300 mg daily for pregnant women has been recommended (217, 246). The saturation dose of DHA was 1.2 g / day when a combination of DHA and EPA was provided to healthy adult volunteers (180, 248), which supports our choice of supplementing the mothers with 1.1 g DHA daily.
Study subjects

**Paper I.** Fifty-nine mothers were included in this paper. Inclusion was based on the availability of plasma samples for whole blood cultures. Twenty-eight of these mothers were supplemented with $\omega-3$ LCPUFA and 31 with placebo.

**Paper II.** 117 families, who completed the follow-up to one year after delivery, were included in this paper. Fifty-two of the mothers were supplemented with $\omega-3$ LCPUFA and 65 with placebo. Infants were excluded from sub-analyses if data were missing from any time point. This was the case for sensitisation and IgE associated eczema at one year, since blood samples were not obtained from all infants.

**Paper III.** 119 families (54 $\omega-3$ and 65 placebo) were included in this paper and the outcome for 24 of the 25 mothers who did not complete the intervention was also discussed. Infants were excluded from sub-analyses, if data were missing from any time point. This was the case for sensitisation and IgE associated disease up to two years, since blood samples were not obtained from all infants.

**Paper IV.** The chemokine analyses in this paper were confined to infants from Linköping, from whom blood was available from any time point during the first two years of life (n=80). Chemokines were analysed in plasma from the umbilical cord (n=72), 3 months (n=42), 12 months (n=61) and 24 months (n=61). For analysis of vaccine induced immunity, samples from Jönköping and Linköping were used and blood from 94 infants were available for analysis. Number of subjects in each intervention group is given in Table 1, paper IV.
**Clinical methodology**

**Table 3. The families were assessed according to the research protocol below.**

<table>
<thead>
<tr>
<th>MOTHER</th>
<th>gw25</th>
<th>partus</th>
<th>1m</th>
<th>3m</th>
<th>6m</th>
<th>12m</th>
<th>24m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heredity + clinical history</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Questions maternal diet</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood sample</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast milk samples*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>colostrum</td>
<td>X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHILD</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical investigation (nurse)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Physical investigation (paediatrician)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Allergy questionnaire</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Blood sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cord blood</td>
<td>X</td>
</tr>
<tr>
<td>Saliva sample*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Faeces sample*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

* Samples collected but not analysed in the papers included in this thesis.

The infants were examined by an experienced allergy nurse at 3, 6 and 12 months and in case of possibly allergic symptoms, a paediatrician, CF or JL, re-examined the child (Table 3). At 24 months of age all children were examined by a paediatrician (CF or JL). Serum (obtained from families in Jönköping) and plasma samples were immediately frozen and stored in −70 °C. An allergy questionnaire used in prior studies (192), covering allergic symptoms, diet and environmental factors, such as exposure to pets, tobacco and day-care were filled out by the parents regularly. Records from primary care units, private paediatricians and the paediatric clinics were scrutinized if the parents reported that a paediatrician had examined the child.

Twenty-four-hour maternal food diaries were registered 3 days in a row at gestational week 25 and at 6 months after delivery. A certified dietician assessed the maternal intake of energy and fat according to routine clinical praxis using
the software ‘Dietist XP’ (Kost- och näringsdata, Bromma, Sweden, www.kostdata.se). The amount of each provision was estimated from the registered weight, volume or number of portions (Table 4).

**Diagnostic criteria**

Symptoms of eczema, food reaction, asthma or rhinoconjunctivitis were considered as clinical symptoms of allergic disease. Concomitant sensitisation, *i.e.* at least one positive SPT and/or detectable circulating allergen specific IgE antibodies against tested allergens, defined IgE associated allergic disease.

The diagnostic criteria for *eczema* used in this thesis are modified from the criteria by Sampson (249) adapted by Seymour (37), for AEDS < 2 years.

*The major features were:*

- Evidence of chronic pruritic dermatitis
- Typical facial or extensor eczematous or lichenified or nummular dermatitis
- Eczema-free skin of nose-mouth area and /or diaper area

*The minor features were:*

- Xerosis/ichthyosis/hyperlinear palms
- Peri-auricular fissures
- Chronic scalp scaling
- Perifollicular accentuation

For diagnosis of eczema 3 criteria were needed and pruritus was compulsory. At least two of the criteria needed to be major. All children in the study had allergic disease in the family, hence that criteria, originating from the definitions by Samson (249), Oranje (35) and Seymour (37) was fulfilled for all children. If detectable IgE antibodies or a positive SPT were present in combination with eczema, it was defined as *IgE associated eczema*. Internal workshops were
arranged for the two examining paediatricians before and during the follow-up of the children in order to achieve uniform assessments of eczema.

A *food reaction* was defined as gastrointestinal symptoms, hives, aggravated eczema or wheezing following ingestion of a certain food with recovery after food elimination and reoccurrence of symptoms after re-ingestion of the particular food. If food specific positive SPT or serum IgE antibodies were present, the food reaction was considered as *IgE mediated food allergy* (*food allergy* in paper II).

*Asthma* was defined as doctor diagnosed wheezing at least three times during the first two years. *IgE associated asthma* was defined as asthma with the presence of any IgE antibodies or positive SPT.

*Rhinoconjunctivitis* was seasonal itching and running eyes and nose. It was considered as *IgE mediated rhinoconjunctivitis* if there was a corresponding positive SPT or detectable specific IgE antibodies.

*Allergic mothers* were mothers with current, or a history of, eczema, asthma, food reactions or rhinoconjunctivitis according to interviews at inclusion. *Atopic mothers* were mothers with any of the allergic symptoms and a positive Phadiatop®, *i.e.* detection of IgE in a panel of inhalant allergen. Mothers with positive Phadiatop® and no symptoms or vice versa were excluded from the sub-analyses of atopic/non-atopic women (paper I).
Laboratory methodology

The laboratory methods used in the papers included in this thesis are listed below. The methods are described in detail in each paper.

- **ELISA**
  Analyses of PGE2 and LTB4 from whole blood cultures (paper I).
  Analyses of tetanus IgG and diphtheria IgG (paper IV).

- **Luminex**
  Analyses of cytokines from whole blood cultures (paper I).
  Analyses of chemokines (paper IV).

- **Gas chromatography:**
  Analyses of phospholipids (PL) fatty acids in serum or plasma (paper I-IV).
  We have chosen to analyse LCPUFA in phospholipids of serum/plasma instead of investigating the immune cell membranes, which might be the preferable method when assessing immunological effects. Unfortunately, we did not obtain enough cells to accomplish this. Phospholipids are transporters of fatty acids that have not yet been incorporated into cellular membranes and they give the best reflection of the overall PUFA status (188). The relative concentrations of PUFA in plasma PL correlate to fatty acid content of erythrocytes in pregnancy (250, 251) and both are reasonable markers for tissue LCPUFA content (180). Analyses of PUFA in PL in serum and plasma provide analogous results.

- **Skin prick testing (paper II-IV).**

- **UniCap®, Immunocap100 Phadiatop®**
  Analyses of total IgE, circulating IgE against allergens (paper I-IV).
Statistics

Power calculations for planning adequate sample size were performed based on a previous study (252). In order to detect a 40% difference in the prevalence of clinical symptoms of allergic disease, with 80% power and a probability of 0.05, we needed at least 134 women to be included in this study. Preferably there should be 67 in each intervention group. With a presumed drop out frequency of 20% around 160 mothers would have been desirable in this study. We did not reach this goal due to the difficulties in recruiting mothers to this kind of intervention and a limited durability of the capsules that did not allow us to continue the recruitment of mothers for more than two years.

The categorical data of the two groups, outcome variables and background data, were analysed by chi-2 tests (\(\chi^2\)). Fisher’s exact test was used when the expected frequency for any cell was less than five. The means of the continual variables were analysed by Student’s t-test. For the variables that were not normally distributed, such as some of the fatty acids, the chemokines, PGE2, LTB4, the cytokines and the vaccine induced IgG, Mann Whitney U-test was used for unpaired analyses and Wilcoxon signed rank test for paired analyses (paper I). Spearman’s correlation was used for correlation of non-parametric variables (paper I and IV). Samples with a concentration below the limit of detection were assigned a value corresponding to half the cut-off value (paper I and IV). Multiple logistic regression was employed to identify effect modifiers among the possible confounders and calculate adjusted and unadjusted odds ratios (paper II and III). Number needed to treat (NNT) was calculated with the substitution method described by Daly (253) (paper I). \(\chi^2\) and \(\chi^2\) trend (Extended Mantel-Haenszel chi-square test for linear trend) were used to assess the differences in prevalence of IgE associated disease between the quartiles of
phospholipids fatty acid proportions in paper III. Friedman’s test was used for
analysis of repeated measures of CCL17 in paper IV.

A difference with a $p$-value < 0.05 was considered to be statistically significant,
except for the analysis of cytokines and chemokines in paper I, where
differences were considered significant at a $p$-value of <0.01 as a consequence
of multiple comparisons. Statistic analyses were performed using Stat View for
Windows Version 5.9 (SAS Institute Inc., Cary, North Carolina, USA) in paper I
and SPSS software 15.0 for Windows (SPSS Inc, Chicago, Illinois, USA) in
paper II-IV.

**Ethical considerations**

The effect of maternal LCPUFA supplementation on the development of allergic
disease early in life was not documented before 2003, when this study was
planned, but there was enough data to assure the safety of this study design (see
paragraph on *safety*). Pain connected with blood sampling was minimised with
topical anaesthesia.

Pharma Nord had no impact on the study design, data collection, data
interpretation or publication of the results. An informed consent was obtained
from both parents before inclusion. In fact, the close surveillance and early
diagnosis of infant allergic disease might even have been an advantage for the
participating families. The Regional Ethics Committee for Human Research at
Linköping University approved the study.
RESULTS AND DISCUSSION

Maternal diet and fatty acid status.

The average registered dietary intake of DHA, EPA and LA at inclusion was 0.2 g/day, 0.1 g/day and 8 g/day respectively (Table 4), which corresponds well with data from previous diet registrations in Sweden (239).

The proportions of ω-3 and ω-6 fatty acids in serum/plasma phospholipids of the mothers were similar in the two groups at the time of inclusion, with a few exceptions (Table 5). The placebo group had higher proportions of LA and the ω-3 group had higher proportions of AA before the intervention (p<0.05 for both). Dietary habits did not seem to explain these differences as both groups showed similar diet registrations (Table 4). In the analysis of clinical outcomes AA or LA levels at inclusion were adjusted for when they turned out to be significant effect modifiers (paper II and III).

Higher relative levels of EPA and DHA in maternal serum/plasma phospholipids and lower AA/EPA ratio were found in the ω-3 supplemented group compared to the placebo group one week after delivery (p for all <0.001). Still 8 months after ending the supplementation, i.e. 12 months after delivery AA/EPA ratios remained lower (p=0.008) in the ω-3 supplemented group compared to the placebo group indicating a long lasting effect of the ω-3 supplementation (Table 5). The proportions of both ω-6 PUFA, AA and LA, in the ω-3 supplemented group were lower compared to the placebo group (p<0.001) one week after delivery, an effect of the ω-3 supplementation seen in previous studies (180). At 12 months after delivery, however, ω-6 PUFA levels were similar in both groups.
DHA and EPA increased in the ω-3 supplemented group between week 25 of gestation and one week after delivery (p<0.001). Eight months after the end of the intervention the DHA proportions were the same as in pregnancy but the EPA levels were still higher than the mid pregnancy levels (p<0.001, Table 5).
Children are provided ω-3 fatty acids from maternal stores, her diet and her metabolism of LNA during gestation. LCPUFA are delivered through the placenta and, after delivery, through breastfeeding (254). We found a decrease in DHA proportions during pregnancy and lactation in our placebo group (p<0.01) but the EPA levels increased after delivery (p<0.001). This most likely reflects the utilization of DHA for breast milk as supported by the work of Otto et al, 2001 (255). They also found an increase in the other ω-3 PUFAs after parturition, represented by the increase in EPA in our placebo group, suggesting that there is a selective transport of DHA into breast milk without any increased conversion of EPA into DHA. In agreement with this, Jorgensen et al found a decrease of DHA in maternal red blood cell-phosphatidylcholine during the first 4 months of lactation (256). Relative DHA levels increased in plasma phospholipids during normal pregnancy until gestational week 18 and thereafter the proportions decreased until >6 months after delivery in a Dutch study (257).

Current studies indicate that pregnant women today have difficulties meeting the demands for ω-3 fatty acids from the foetus, i.e. women who are pregnant with their second or third child have less available ω-3 fatty acids (186, 256). In our study, there were weak but significant negative correlations between DHA levels and parity in the whole group at inclusion (rho= -0.21, p=0.01) and in the placebo group 12 months after delivery (rho= -0.27, p=0.03). No correlation was found one week after delivery in the placebo group. The maternal relative levels of DHA in mid pregnancy were generally a little higher than reported from the Netherlands (186) which could explain, like in the study by Dunstan (196), that the stores were not significantly compromised during pregnancy. Interestingly, the levels of LNA decreased during the study period in both our intervention groups.
The AA content in maternal serum/plasma phospholipids increased in the placebo group (p<0.001) until delivery and remained at the higher level at 12 months after delivery. The LA proportions in the placebo group also increased during the study period, but not until after delivery (p<0.001). Compared to the Dutch studies of pregnant women our placebo population tended to have slightly lower proportions of ω-6 PUFA and higher proportions of ω-3 PUFA in their serum/plasma phospholipids in mid pregnancy (186, 187).

There were no differences in serum/plasma phospholipid fatty acid levels of allergic and non-allergic mothers prior to supplementation. Other studies have shown inconsistent associations between ω-6 and ω-3 PUFA levels in serum phospholipids or cell membranes and allergic conditions (reviewed in (258)). There are also studies reporting no associations between levels of ω-6 or ω-3 fatty acids in serum phospholipids and allergic disease in adults (n=740, 427 men) (259) and in cord blood vs. allergic disease in the infants (n=1238) (260). However, mothers who are allergic tend to have an abnormal balance of fatty acids in their breast milk (254, 261). In our study the changes in serum/plasma phospholipid fatty acid proportions due to ω-3 LCPUFA supplementation were similar in the allergic and non-allergic mothers, data not shown (dns).
**Table 5. Proportions of ω-6- and ω-3-PUFA in serum/plasma phospholipids of the mothers before, during and after the intervention.**

<table>
<thead>
<tr>
<th>Fatty acid (mol%)</th>
<th>Gestational week 25</th>
<th>First week after delivery</th>
<th>12 months after delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ω-3 (n=52) mean(SD)</td>
<td>Placebo (n=64) mean(SD) t-test p</td>
<td>ω-3 (n=51) mean(SD) Placebo (n=61) mean(SD) t-test p</td>
</tr>
<tr>
<td>18:2ω-6 (LA)</td>
<td>18.9(2.5)</td>
<td>19.9(2.1)</td>
<td>15.2(2.3) **</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.1(1.6) **</td>
</tr>
<tr>
<td>20:4ω-6 (AA)</td>
<td>9.3(1.7)</td>
<td>8.6(1.5)</td>
<td>0.4(0.1) **</td>
</tr>
<tr>
<td>18:3ω-3 (LNA)</td>
<td>0.5(0.1)</td>
<td>0.5(0.1)</td>
<td>7.6(2.5) **</td>
</tr>
<tr>
<td>20:5ω-3 (EPA)</td>
<td>1.3(0.8)</td>
<td>1.2(0.6)</td>
<td>7.9(1.5) **</td>
</tr>
<tr>
<td>22:6ω-3 (DHA)</td>
<td>5.5(1.1)</td>
<td>5.4(1.2)</td>
<td>1.4(1.3) **</td>
</tr>
<tr>
<td>20:4ω-6/20:5ω-3 AA/EPA</td>
<td>9.1(4.2)</td>
<td>8.6(4.0)</td>
<td>4.8(1.9) **</td>
</tr>
</tbody>
</table>

*: p<0.01 compared to gestational week 25 (paired t-test),
**: p<0.001 compared to gestational week 25 (paired t-test)
ns : not significant.

**Pregnancies and deliveries**

The data on pregnancies and deliveries are presented in Table 6. The increase in gestational length reported in several large ω-3 supplementation trials (reviewed in (245)) was not seen in this study. This could be explained by two facts, the relatively small size of the study but also by the significant increase of pharmacological induction of deliveries in the ω-3 group (p=0.04, Table 6B). Even though the inductions due to prolonged gestation were as common in the two groups there were more inductions for various other reasons in the ω-3 group. This could conceal a significant difference in gestational length. The need of emergency caesarean sections was similar in the two groups, though planned sections were only performed in the placebo group. The motivations for planned caesarean sections were humanitarian reasons, *i.e.* anxiety (n=5),

- 79 -
osteoporosis (n=1) and narrow pelvis (n=1). These results seem peculiar in such a small study. However, in trials designed to investigate the psychological effects of ω-3 supplementation by assessment of test anxiety (262), and other psychological stress (263, 264), both biochemical and behavioural effects of PUFA supplementation have been reported. An association between low seafood consumption in pregnancy and maternal depression has been observed (265) and ω-3 LCPUFAs have been considered in treatment of perinatal depression (266).

Table 6 A and B. The pregnancies, the deliveries and the babies.

<table>
<thead>
<tr>
<th></th>
<th>ω-3</th>
<th>Placebo</th>
<th>p§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n value(SD)</td>
<td>n value(SD)</td>
<td></td>
</tr>
<tr>
<td>Maternal age at delivery (years)</td>
<td>53 31.1(4.0)</td>
<td>65 31.7(3.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Parity, including study child</td>
<td>54 1.4(0.6)</td>
<td>66 1.5(0.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight gw 8-10 (kg)</td>
<td>53 69.6(10.2)</td>
<td>65 68.1(14.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight gain until delivery (kg)</td>
<td>45 13.1(3.8)</td>
<td>60 13.8(4.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>53 167(5)</td>
<td>65 165(6)</td>
<td>ns</td>
</tr>
<tr>
<td>BMI gw 8-10 (kg/m²)</td>
<td>53 25(3.5)</td>
<td>65 24.8(3.6)</td>
<td>ns</td>
</tr>
<tr>
<td>BMI increase until delivery (kg/m²)</td>
<td>45 4.7(1.4)</td>
<td>60 5(1.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Gestational length (days)</td>
<td>54 282(9.8)</td>
<td>66 281(11.1)</td>
<td>ns</td>
</tr>
<tr>
<td>Bleeding at delivery (ml)</td>
<td>52 490(258)</td>
<td>64 492(279)</td>
<td>ns</td>
</tr>
<tr>
<td>APGAR 1 minute</td>
<td>54 8.5(1.1)</td>
<td>66 8.3(2.0)</td>
<td>ns</td>
</tr>
<tr>
<td>APGAR 5 minute</td>
<td>54 9.7(0.7)</td>
<td>66 9.6(1.0)</td>
<td>ns</td>
</tr>
<tr>
<td>APGAR 10 minute</td>
<td>54 10(0.2)</td>
<td>66 9.8(0.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Head circumference baby (cm)</td>
<td>53 35(1.1)</td>
<td>65 35.2(1.5)</td>
<td>ns</td>
</tr>
<tr>
<td>Birth weight baby (g)</td>
<td>54 3511(499)</td>
<td>66 3567(554)</td>
<td>ns</td>
</tr>
<tr>
<td>Birth length baby (cm)</td>
<td>54 50(2.0)</td>
<td>66 50.5(2.6)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight baby at 6 months (kg)</td>
<td>48 7.8(0.9)</td>
<td>57 7.8(1.0)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight baby at 12 months (kg)</td>
<td>49 9.9(1.1)</td>
<td>62 10(1.1)</td>
<td>ns</td>
</tr>
<tr>
<td>Weight baby at 24 months (kg)</td>
<td>51 13(1.4)</td>
<td>61 13(1.2)</td>
<td>ns</td>
</tr>
<tr>
<td>Duration of breastfeeding (months)</td>
<td>53 9.6(5.0)</td>
<td>66 9.3(3.6)</td>
<td>ns</td>
</tr>
</tbody>
</table>

§ = t-test, gw = gestational week, ns = not significant
Apart from the belching in the ω-3 group mentioned before, maternal nausea and abdominal pain occurred in both intervention groups (Fig. 7). Three to four percent of the infants had any kind of heart defect, i.e. vitium organicum cordis (VOC), which does not exceed the prevalence reported previously in a Swedish population (267). Only one VOC led to surgery during the first two years in our study.
**Allergic heredity and environmental factors**

There were no significant differences between the intervention groups in terms of allergies in the family and environmental factors of possible importance for allergic disease, except for caesarean sections (Table 7, data on parity, breastfeeding and caesarean sections are presented in Table 6). Nevertheless, variables that turned out to be effect modifiers were adjusted for in the odds ratio analysis of the clinical results (paper II and III).

All families used cow’s milk formula if the breast-feeding was insufficient and there were no signs of cow’s milk allergy in the children (Table 7). None of the families gave formula containing ω-3 fatty acids to their infant. Solid foods were introduced at 4.7 month of age (SD 0.7) in the placebo group and at 4.8 months (SD 0.9) in the ω-3 group (ns). Five infants were introduced to solid foods between 3 and 4 months of age (1 in the placebo group and 4 in the ω-3 group).

Fish was introduced in the diet of three infants in the placebo group and two infants in the ω-3 group between 4 and 6 months of age, *i.e.* 4 %, while 16-17 % of the families chose not to introduce fish to their child before the first birthday. This reflects the impact of the previous recommendations given to families with high-risk infants.
Table 7. Allergic history and environmental factors.

<table>
<thead>
<tr>
<th>Allergic history *</th>
<th>ω-3</th>
<th></th>
<th>Placebo</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mother</td>
<td>n</td>
<td>freq(%)</td>
<td>n</td>
<td>freq (%)</td>
<td>p#</td>
</tr>
<tr>
<td>father</td>
<td>54</td>
<td>40(74)</td>
<td>66</td>
<td>42(64)</td>
<td>ns</td>
</tr>
<tr>
<td>both parents</td>
<td>54</td>
<td>36(67)</td>
<td>66</td>
<td>44(67)</td>
<td>ns</td>
</tr>
<tr>
<td>sibling only</td>
<td>54</td>
<td>22(42)</td>
<td>66</td>
<td>21(32)</td>
<td>ns</td>
</tr>
<tr>
<td>Positive Phadiatop® mother</td>
<td>54</td>
<td>32(59)</td>
<td>66</td>
<td>35(53)</td>
<td>ns</td>
</tr>
<tr>
<td>Positive Phadiatop® and allergic history mother¶</td>
<td>44</td>
<td>31(71)</td>
<td>59</td>
<td>35(59)</td>
<td>ns</td>
</tr>
<tr>
<td>Eczema in the family</td>
<td>54</td>
<td>24(44)</td>
<td>66</td>
<td>25(38)</td>
<td>ns</td>
</tr>
<tr>
<td>Food reactions in the family</td>
<td>54</td>
<td>10(19)</td>
<td>66</td>
<td>14(21)</td>
<td>ns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure to furry pets (0-24m)§</td>
<td>54</td>
<td>35(65)</td>
<td>65</td>
</tr>
<tr>
<td>Exposure to smoking (0-24m) †</td>
<td>53</td>
<td>10(19)</td>
<td>62</td>
</tr>
<tr>
<td>Any AB treated infections (0-24m)</td>
<td>50</td>
<td>20(40)</td>
<td>58</td>
</tr>
<tr>
<td>More than 5 common colds (0-24m)</td>
<td>49</td>
<td>40(82)</td>
<td>57</td>
</tr>
<tr>
<td>Day-care</td>
<td>51</td>
<td>42(82)</td>
<td>59</td>
</tr>
<tr>
<td>Cow’s milk formula before 4 m</td>
<td>52</td>
<td>13(25)</td>
<td>65</td>
</tr>
<tr>
<td>No fish in infant’s diet during the first year</td>
<td>51</td>
<td>8(16)</td>
<td>64</td>
</tr>
</tbody>
</table>

*: Current or previous symptoms like eczema, asthma, allergic urticaria, gastrointestinal allergy or allergic rhinoconjunctivitis.

¶: atopic disease (paper I)

§: Exposure to furry pets at least once a week.

†: Tobacco smoke in the home or where the child spends the day and/or smoking family member.

AB: antibiotics
Maternal eicosanoid secretion

Eicosanoids, cytokines and chemokines were analysed in whole blood supernatant from the mothers at inclusion and at delivery with and without LPS. The results are presented in paper I. The mothers allocated to ω-3 fatty acids supplementation responded with higher LPS-induced PGE2 secretion from whole blood cell culture supernatants at inclusion compared with the mothers who were later treated with placebo (p = 0.01). To overcome the influence of this random difference already present at inclusion, we chose to analyse the change in secretion during supplementation, rather than the absolute levels. The LPS induced PGE2 secretion increased in the placebo treated group (p<0.001) between gw 25 and partus but remained unaltered in the ω–3 LCPUFA treated group. Furthermore, LPS-induced PGE2 secretion decreased during the supplementation period in a majority of the ω-3 LCPUFA-supplemented women but increased in the majority of the placebo-supplemented women (p=0.002, Fig. 1A, paper I).

The changes in LPS-induced PGE2 production during intervention correlated positively with changes in plasma phospholipid AA proportions and negatively with changes in EPA (Fig. 2 AB, paper I). A positive correlation was also observed between changes in LPS-induced PGE2 secretion and changes in AA/EPA ratios (Fig. 2C, paper I). The effects of PGE2 in allergic inflammation are complex, both facilitating and hampering inflammatory reactions (reviewed in refs. (225, 229)). However, PGE2 seems to facilitate sensitisation as it inhibits the production of Th1 cytokines, primes immature T cells to produce IL-4 and IL-5, and facilitates B-lymphocyte immunoglobulin isotype switching to IgE (225, 227, 268). Consequently a decreased PGE2 secretion may have a impeding effect on allergic sensitisation and influence the predisposition to IgE associated disease (191).
**Infant fatty acid status**

The infant serum/plasma EPA and DHA proportions were higher in the ω-3 supplemented group compared to the placebo group up to one year of age, about nine months after the end of supplementation (Table 3, paper III). At two years of age there were no differences between the groups. The DHA and AA concentrations were considerably higher in cord blood compared to maternal blood after delivery due to the preferential accretion of DHA and AA relative the precursors EPA and LA by the placenta (184, 269).

Until 12 months of age the LA and AA proportions in the ω-3 supplemented group were lower than in the placebo group, reflecting the response to the DHA and EPA supplementation seen in previous studies (180). The LA and AA proportions in the placebo group correspond with (188) or are lower (270) than in previous studies which makes a significant contribution from the placebo capsule less likely.

**Clinical outcomes**

Throughout the follow-up the number of children with eczema and food reactions were lower in the ω-3 group compared to the placebo group but these differences were not statistically significant (Table 1, paper III). Our sample size was calculated based on the cumulative incidence of allergic disease during the first 18 months of life (252). However, for instance the cumulative incidence of eczema in this study was 20% in the ω-3 and 31% in the placebo group, and thus less common than expected. In order to dismiss such a difference between the two groups as a type II error, with an 80% power at a 0.05 significance level, 246 mothers would have been needed in each group. Consequently our study
cannot rule out a protective effect of ω-3 fatty acids on allergic symptoms per se, due to the limited sample size.

On the other hand the cumulative incidence of positive skin prick tests up to two years of age was 19 % in the ω-3 group and 36 % in the placebo group (p=0.05), with the most obvious difference in SPT to food (15% vs 34%, p=0.02), especially egg (13% vs. 30%, p=0.04). This agrees with our hypothesis that EPA outstrips AA and the production of PGE2 is reduced, leading to less IgE antibodies (271). No significant differences in detectable specific IgE antibodies between the groups were found (Table 1, paper III), possibly due to the small number of samples.

IgE associated eczema (eczema and positive SPT/detectable specific IgE) during the first two years of life was less common in the in the ω-3 group compared to the placebo group (Fig. 9). It has been reported that sensitised infants with eczema are at increased risk for later development of allergic asthma and allergic rhinoconjunctivitis (ARC)(20, 272), which makes these results clinically important. Twenty-two children had any food reaction during the first two years and 17 of them were IgE mediated. The symptoms were hives: n = 14 (10 of them were IgE mediated), gastrointestinal symptoms: n = 11 (7 IgE mediated), aggravated eczema: n = 6 (3 IgE mediated), wheezing: n = 1, which was IgE mediated. Hence, some infants experienced more than one symptom. There were no significant differences between the ω-3 and placebo groups regarding the symptoms caused by the food reactions. Most of the food reactions and all of the IgE mediated ones were caused by milk or egg. One child developed hives after eating fish but no sensitisation to fish was found.
Figure 9 A. Cumulative incidence (0-24m) of eczema and IgE associated eczema in the placebo and ω-3 groups.
Grey diamond: any eczema in the placebo group,
Grey circle: any eczema in the ω-3 group,
Black square: IgE associated eczema in the ω-3 group.
Black triangle: IgE associated eczema in the ω-3 group.
Data from the three months visit is presented at six months.

B. Cumulative incidence of allergic symptoms and IgE associated disease 0-24 months. Grey bars: allergic symptoms, i.e. asthma, rhinoconjunctivitis, eczema and/or food reaction. Black bars: IgE associated disease: any allergic symptom and allergic sensitisation. Three children with symptoms and negative SPTs were excluded from the analysis of IgE associated disease because of missing IgE data.

Chi-2 test was used for calculation of p in A and B.

Up to one year of age the risk of developing food allergy (IgE mediated food reactions) was ten times higher in the placebo group compared to the ω-3 group (Table 2, paper II). The odds ratios for developing IgE associated eczema, IgE mediated food allergy or any IgE associated disease (e.g. atopic disease) (10) up to two years of age indicate a 3-5 fold increased risk in the placebo group compared to the ω-3 group (Table 2, paper III).
Intention-to-treat analysis

There were 25 women who did not complete the intervention but their infants were followed up according to Figure 7. Their infants did not differ significantly from the placebo group in terms of positive SPTs at 6 months or allergic symptoms or IgE associated disease during the first two years of life. However, 19/25 children of mothers who did not complete the intervention, were skin prick tested at 24 months and they had a slightly higher incidence of positive tests compared to the placebo group (Table 8). One explanation to that could be a higher interest in the skin-prick testing by families with allergic compared to healthy children. The families that did not attend the follow-up visits were interviewed by phone regarding allergic symptoms (Fig. 7) and only one of the mothers who did not complete the intervention was not reached (n=24).

An intention-to-treat analysis was performed regarding the main clinical outcomes and there was a trend toward lower cumulative incidence of IgE associated disease in the group allocated to ω-3 supplementation compared to the group receiving placebo capsules. However, most of the differences seen in the “as treated” analysis vanished when including the children whose mothers did not complete the intervention (Table 8). This indicates that compliance needs to be increased by making the supplementation more tolerable and easy to ingest in order to improve the results.
Table 8. The main clinical outcomes in “as treated” and “intention to treat” analysis.

<table>
<thead>
<tr>
<th></th>
<th>“As treated”</th>
<th></th>
<th>“Intention to treat”</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ω-3 n (%)</td>
<td>Placebo n (%)</td>
<td>Intervention not completed n (%)</td>
<td>Placebo n (%)</td>
</tr>
<tr>
<td>Any positive SPT at 6m†</td>
<td>6/52 (12)</td>
<td>16/65 (25)</td>
<td>3/15 (20)</td>
<td>8/62 (13)</td>
</tr>
<tr>
<td>Any positive SPT at 24 m‡</td>
<td>6/52 (12)</td>
<td>12/62 (19)</td>
<td>8/19 (42)*¶</td>
<td>12/64 (19)</td>
</tr>
<tr>
<td>Allergic symptoms up to 24 m</td>
<td>19/54 (35)</td>
<td>28/65 (43)*</td>
<td>15/24 (62)*</td>
<td>28/69 (41)</td>
</tr>
<tr>
<td>IgE associated disease up to 24 m</td>
<td>6/54 (11)</td>
<td>19/62 (31)**</td>
<td>7/18 (38)**</td>
<td>11/65 (17)</td>
</tr>
</tbody>
</table>

*: p< 0.05 vs. ω-3 group.
**: p= 0.01 vs. ω-3 group.
¶: p<0.05 vs. placebo group.
#: p=0.06 vs. ω-3 group.
†: egg, milk, wheat and/or cat.
‡: egg, milk, wheat, cat, birch, and/or timothy.

Infant chemokines and vaccine induced immune responses

High Th2-associated CCL17 levels and high CCL17/CCL11 (Th2/Th1) ratios were associated with allergic disease in the child (p<0.05 for both), which is in agreement with earlier research (64-67).

CCR4 mRNA levels, i.e. the receptor for Th2 associated chemokines CCL17 and CCL22, decreased in the fish oil intervention group compared with those seen in the placebo group in a recent supplementation study with 0.5 g DHA and 0.15 g EPA from the 22nd gestational week until delivery (273). In our study, including all children with available chemokine data, we found no significant differences in CCL17, CCL22, CXCL10 or CXCL11 in the ω-3 compared to the placebo group but we did not analyse the chemokine receptors.
Allergic children have been shown to be intrinsically hyporesponsive to vaccines, possibly due to Th2 skewed immune responses, even though this seems to be overcome by common vaccination regimens (274). In this study the vaccine induced responses did not differ significantly between allergic and non-allergic children (dns). Further, ω-3 fatty acids have been shown to enhance the Th1 responses through IL-2 and IFNγ production (219) and may therefore enhance vaccine antibody responses. However, anti-tetanus and anti-diphtheria IgG levels were similar in the placebo and the ω-3 groups (Table 2, paper IV).

**Previous ω-3 supplementation trials - design and outcomes**

Trials with ω-3 supplementation in pregnancy and/or lactation analysing clinical and/or immunological outcomes in the offspring are summarised in Table 9. There are though several methodological differences between those and our study. They were either not originally designed for investigating clinical outcomes in the children (9, 198, 212, 273), the dose of ω-3 LCPUFA was very low (200) or the supplementation was given merely in pregnancy (9, 198, 273) or only in infancy (200, 212). To the best of our knowledge, our study is the first originally designed to analyse clinical outcomes in the children after ω-3 supplementation during pregnancy and lactation. The intervention lasts for a 6 months period and the dose is appropriate (248) and safe (245).

Interestingly, a transient protective effect from black currant seed oil supplementation in pregnancy and childhood on eczema, but not sensitisation at 1 year was shown in an additional recent trial (275). The composition of black currant seed oil corresponds to the recommended optimal dietary intake of ω-3 and ω-6 PUFAs; in other words, the oil has a ratio of ω-3/ω-6 from 1/3 to 1/4. Besides, the black currant seed oil was well tolerated by the mothers (275).
Table 9. Omega-3 supplementation in pregnancy and/or lactation. Clinical and immunological outcomes.

<table>
<thead>
<tr>
<th>First authors</th>
<th>Country</th>
<th>Subjects</th>
<th>Intervention daily dose</th>
<th>Placebo</th>
<th>Exposure period</th>
<th>Outcome measured</th>
<th>Findings in ω-3 suppl. group</th>
<th>Year published</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dunstan Prescott</td>
<td>Australia</td>
<td>Atopic pregnant women</td>
<td>2.2 g EPA, 1.1 g DHA, 0.4 g other ω-3 PUFA n=52</td>
<td>Olive oil n=46</td>
<td>20th gw - delivery</td>
<td>Plasma cytokine concentrations in cord blood. Cord blood MC cytokine responses to allergens SPT, SCORAD 1 year</td>
<td>Cord blood plasma IL-13 ▼, MC response of IL-10 to cat allergen ▼, T cell PKC zeta ▲, SPT to egg at 1 year ▼ SCORAD ▼</td>
<td>2003-2007</td>
<td>(8, 9, 99)</td>
</tr>
<tr>
<td>Mihrshahi, Peat Marks</td>
<td>Australia</td>
<td>Infants with family history of asthma</td>
<td>Tuna fish oil 500mg (37% ω-3 LCPUFA) low ω-6 cooking oil n=149</td>
<td>Sunola oil n=149</td>
<td>6 months of age or at weaning - 5 years. Cooking oil from gw 36</td>
<td>S-IgE, SPT, eczema up to 5 years Allergy questionnaire Lymphocyte cytokine responses to allergen stimulation</td>
<td>Wheeze at 18 m ▼, Cough in atopic children at 3 years ▼</td>
<td>2003-2006</td>
<td>(199, 201)</td>
</tr>
<tr>
<td>Olen</td>
<td>Norway</td>
<td>Pregnant women</td>
<td>2.7 g ω-3 LCPUFA n=263</td>
<td>Olive oil n=136</td>
<td>30th gw - delivery</td>
<td>eczema, ARC, asthma and allergic asthma at 16 yrs.</td>
<td>asthma ▼, allergic asthma ▼ at 16 yrs</td>
<td>2008</td>
<td>(198)</td>
</tr>
<tr>
<td>Lauritzen</td>
<td>Denmark, Spain</td>
<td>Lactating women</td>
<td>1.5g ω-3 LCPUFA n=37</td>
<td>Olive oil n=28</td>
<td>First 4 m of lactation</td>
<td>In vitro LPS stimulated IFNγ, IL-10 production in children 2-12 yrs</td>
<td>IFNγ ▲, IL-10-no difference</td>
<td>2005</td>
<td>(212)</td>
</tr>
<tr>
<td>Krauss-Etschmann</td>
<td>Germany, Hungary</td>
<td>Pregnant women</td>
<td>0.5 g DHA, 0.15 g EPA n=49</td>
<td>Milk based suppl. n=50</td>
<td>22nd gw - delivery</td>
<td>Maternal and cord blood mRNA: CCR4, IL-13, IL-4, CRTH2, CXCR3, IFNγ, IL-1, TGFβ mRNA levels of CCR4 ▼, IL-4 ▼ and IL-13 ▼ in cord blood. IL-1 ▲ IFNγ ▲ in maternal blood</td>
<td></td>
<td>2007</td>
<td>(273)</td>
</tr>
</tbody>
</table>
**Phospholipid fatty acids in relation to allergic disease**

In order to assess compliance, fatty acid status in serum/plasma phospholipids were analysed in mothers and children. The dose and length of supplementation varied in spite of our instructions to the mothers (Fig. 8) but the analysis of fatty acid proportions in blood allowed us to relate maternal and infant ω-3 fatty acid status to the clinical outcomes regardless of intervention group. We found an inverse dose-response relationship between both maternal DHA status at delivery and infant DHA status at 12 months and the prevalence of infant IgE associated disease (Fig.10). This dose-response relationship supports causality between ω-3 LCPUFA status and allergic disease according to the criteria by Bradford-Hill (276). Still, it does not tell us the appropriate dose to prevent IgE associated disease or if the dose should be given in pregnancy and/or lactation.

A previous study by Mirshahi et al investigated plasma levels of ω-3 fatty acids in relation to allergic outcomes after ω-3 supplementation in early childhood. No dose dependent relationship could be found but levels of infant plasma ω-3 fatty acids in the higher quintiles at 18 months of age were associated with a reduced prevalence of some asthma symptoms (199). During the last four weeks of pregnancy and breastfeeding the mothers in the fish oil intervention group used cooking oils and spreads based on canola oil to reduce the ω-6 LCPUFA intake and the children were supplemented with 500 mg tuna fish oil daily, starting at weaning or at 6 months, at the latest, and continuing through childhood. The dose of ω-3 was low compared to our study and DHA and EPA were not analysed separately. Furthermore, Mirshahi et al analysed fatty acid levels in the infants at 18 months and not in maternal blood. These factors may explain the different results.
Figure 10. Mother-infant pairs divided into four groups according to quartiles of DHA, EPA proportions and AA/EPA ratios in maternal plasma phospholipids (PL) one week after delivery, in infant plasma PL at 12 months and in infant plasma PL at 24 months.

**Black bars:** Infants with IgE associated disease (allergic symptoms combined with positive SPT and/or circulating specific IgE).

**Striped bars:** Infants with allergic symptoms but no positive SPTs or detectable specific IgE.

**Dotted bars:** Infants with detectable IgE antibodies or positive SPT without allergic symptoms.

**White bars:** Infants without any allergic symptoms or sensitisation.

Thus, the outcomes of allergic disease and/or allergic sensitisation in all children in each quartile are represented.

#= Chi-2 for linear trend within black bars, p<0.01.
The ω-3 LCPUFA seem to have an impact on development of allergic sensitisation early in life but what is the immediate clinical consequence of this result? There were no significant differences between the intervention groups in terms of severity of allergic disease *i.e.* SCORAD (discussed in paper III) or number of allergic symptoms. However, the infants with more than one symptom of allergic disease had significantly lower serum/plasma proportions of DHA and EPA and higher AA/EPA ratios at several timepoints than the children with no symptoms (Table 4, paper III). In agreement with this, the risk reduction associated with regular fish consumption at age 1 in an observational study, appeared particularly pronounced for more severe allergic disease measured as multiple allergic disease, *i.e.* two or three diseases (aOR 0.56, 0.35–0.89) (190). We also found an association between sensitisation and number of allergic symptoms (p= 0.001, paper III). This indicates that IgE associated disease (atopic disease) presents itself with a more severe phenotype than allergic disease without detectable sensitisation. A previous study has found that children with severe eczema are sensitised to common allergens to a larger extent than children with milder forms of eczema, which agrees with our findings (277).

**Possible gene-by-environment interaction**

Children with allergies in the immediate family were recruited to this study in order to get the expected incidence of allergic disease according to the power calculation. Hence, both allergic and non-allergic mothers were included, even though the study was not designed to find out differences between these two groups of mothers. It was striking, however, how the differences between the intervention groups in terms of infant sensitisation and IgE associated disease were significant in the rather small group of non-allergic mothers while it was not at all as evident in the group of allergic mothers (Table 11).
Table 11. The main clinical outcomes from the study divided into groups regarding maternal allergic history.

<table>
<thead>
<tr>
<th>Cumulative incidence</th>
<th>No maternal history of allergy</th>
<th></th>
<th>Maternal history of allergy</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ω-3 n (%)</td>
<td>Placebo n (%)</td>
<td>p‡</td>
<td>ω-3 n (%)</td>
</tr>
<tr>
<td>Any pos SPT*</td>
<td>2/14(14)</td>
<td>11/22(50)</td>
<td>0.03</td>
<td>8/38(21)</td>
</tr>
<tr>
<td>Any detectable IgE*</td>
<td>4/13(31)</td>
<td>5/13(38)</td>
<td>1.0</td>
<td>7/31(23)</td>
</tr>
<tr>
<td>Allergic symptoms‡</td>
<td>3/14(21)</td>
<td>12/24(50)</td>
<td>0.10</td>
<td>16/40(39)</td>
</tr>
<tr>
<td>Food allergy</td>
<td>0/14(0)</td>
<td>8/24(33)</td>
<td>0.017</td>
<td>3/40(25)</td>
</tr>
<tr>
<td>IgE mediated eczema</td>
<td>0/14(0)</td>
<td>7/24(29)</td>
<td>0.033</td>
<td>5/40(12)</td>
</tr>
<tr>
<td>IgE associated disease</td>
<td>1/14(7)</td>
<td>9/23(39)</td>
<td>0.06</td>
<td>5/40(12)</td>
</tr>
</tbody>
</table>

*: towards egg, milk, wheat, birch, timothy, cat.
‡: eczema, food reactions, asthma or rhinoconjunctivitis.
‡‡: Chi-2 test (Fisher’s exact test if expected values were less than 5).

This is in agreement with the observational studies reporting that fish in the diet during pregnancy offers protection from allergic sensitisation in children of non-allergic mothers (278) or without allergic heredity (190). Further, children of non-allergic, but not of allergic mothers had a protective effect of breastfeeding (supposedly through its content of LCPUFA) on eczema at two years of age (167).

Our immunological findings follow the same pattern: the decrease in LPS-induced PGE2 secretion in the ω-3 LCPUFA-treated mothers was more pronounced among non-atopic mothers (Fig. 1B, paper I). Further, the correlation between changes in maternal LPS-induced PGE2 production during intervention and changes in serum/plasma phospholipid EPA, was stronger in non-atopic compared to atopic mothers (Fig. 2B, paper I). In children without, but not with, maternal history of allergy, the ω-3 supplementation was related to lower CCL17/CCL11 ratios (p<0.05, paper IV). When the cord blood samples in the study by Krauss-Etschmann et al was stratified for maternal allergic disease, the decrease of IL-13 mRNA levels in the fish oil group was more pronounced.
in cord blood samples from non-allergic mothers, which agrees with our results 
(273). In the study by Dunstan, where the mothers receiving ω-3 
supplementation (3.7 g/day) were all atopic, there was still a slight effect on 
sensitisation to egg the first year of life and corroborating immunological 
changes were noted (8, 9). The question is whether the allergic mothers need a 
larger dose of EPA and/or DHA in order to get a protective effect in the child, 
due to a FADS1/FADS2 genotype variant that has an impact on PUFA 
metabolism (279). However, in disagreement with this we did not see any 
differences in serum/plasma LCPUFA levels between allergic and non-allergic 
mothers in our study (dns).

Furthermore in the non-allergic, but not in the allergic children, ω-3 
supplementation was associated with higher Th1-associated CXCL11 levels 
(p<0.05), as well as increased IgG titres to diphtheria (p=0.01) and tetanus 
(p=0.05) toxins compared to placebo (paper IV). This could be representing a 
modification of the immune responses toward more Th1 dominance in the non-
allergic ω-3 supplemented children.

Even though these results should be interpreted carefully it may strengthen the 
idea that the allergic status of the individual has an impact on the balancing 
effect of ω-3 supplementation towards a less Th2 dominated immune response.
CONCLUSIONS

This thesis was based on a double-blind placebo controlled trial, supplementing women with ω-3 LCPUFA in pregnancy and lactation. Allergic disease in the offspring in addition to maternal and infant immunological markers were analysed.

Major observations:
I. Maternal lipopolysaccharide-induced PGE2 production was decreased in a majority of the ω-3 LCPUFA supplemented mothers in contrast to the placebo group who displayed an increase in the majority of cases (p=0.002). No effect on lipopolysaccharide induced cytokine secretion was observed.

II. Maternal ω-3 fatty acid supplementation decreased the risk of food allergy (i.e. IgE mediated food allergy) and IgE-associated eczema during the first year of life in infants with a family history of allergic disease (p=0.01 and <0.05 respectively). No effect was seen on the prevalence of allergic symptoms per se and the benefit for the families may be doubtful. However the decreased incidence of sensitisation in the eczematous children may indicate a lower risk of asthma later in childhood.

III. Maternal ω-3 fatty acid supplementation decreased the risk of any IgE associated disease (asthma, rhinoconjunctivitis, eczema and/or food allergy) during the two first years of life in infants with a family history of allergic disease (p=0.01). The effect on eczema and food allergy seen the first year remained during the second year. Maternal and infant serum/plasma phospholipid ω-3 LCPUFA proportions were related to infant IgE associated disease in an inversed dose response manner. Low ω-3 LCPUFA status in mother and infant was related to increased severity of allergic disease expressed
as number of allergic manifestations. This indicates a clinical benefit for the infants from the increased ω-3 LCPUFA proportions, which may be achieved with maternal ω-3 LCPUFA supplementation.

IV. The levels of the circulating Th2 chemokine CCL17 were higher in infants with eczema or any IgE associated disease but there were no significant differences in the Th1 or Th2 chemokines or vaccine induced responses between the ω-3 and the placebo group. Nevertheless, in the group of non-allergic mothers there were decreased infant levels of CCL17 after ω-3 supplementation and in the non-allergic children the immune responses against tetanus and diphtheria were strengthened in the ω-3 group.

Thus, the prospect of balancing the infant immune system towards a less Th2 dominated response, by maternal ω-3 fatty acid supplementation, seems to be influenced by allergic status. This is also supported by the findings in paper I-III where maternal allergic status seemed to influence the effect of the ω-3 LCPUFA supplementation on maternal eicosanoid production as well as clinical outcomes in the infants.
FUTURE PERSPECTIVES

Analysis of fatty acids in breast milk is the next step in this study and it is performed at present.

A follow-up of the children in this study at 8-9 years of age, focusing on respiratory disease and sensitisation towards inhalants, is currently being planned.

S-IgA and cytokines in faeces and saliva are interesting to analyse in the future and relate to clinical outcomes.

It would also be interesting to analyse maternal FADS1/FADS2 polymorphisms in relation to maternal allergy and clinical outcomes in the children.

Reports, mostly from animal studies, indicate that the anti-viral immune responses are depressed by ω-3 fatty acids (280). To investigate this further it would be appealing to assess vaccine-induced responses to morbilli, rubella and paramyxoviruses in this study group.
ACKNOWLEDGEMENT

I am grateful to everyone who has contributed to this thesis:

All the children, mothers and fathers who participated in this study. Thank you for your time and endurance.

My supervisor Karel Duchén for inviting me to participate in this project and for invaluable encouragement along the way in the clinical as well as in the scientific work.

My supervisor professor Karin Fälth-Magnusson for your endless support and patience. Thank you for meticulously reading my manuscripts and for all the scientific and clinical advice.

The midwives at the antenatal department “Kvinnohälsan” and at the maternal health centre “Storken” who recruited the families.

The research nurses Lena Lindell, Kicki Helander, Nina Timelin and Linnea Andersson for keeping track of our study-families, supporting and caring for them in an outstanding way.

Kristina Warstedt, my co-worker, for sharing the mysteries of laboratory work and leading the way through the PhD studies. Thank you for very important contributions to our publications and for being “on call” for the births in our study, day and night.

Malin Fagerås, co-author and supervisor, for lots of thoughtful ideas, and for your support at our conference trips to Barcelona and Gothenburg.

Johanna Andersson, co-author, for the clinical assessment in Jönköping and for always mailing me the data I need.

Maria Jenmalm, co-author, for enthusiasm and great knowledge.

Mats Fredriksson, co-author and statistician who never hesitates to answer questions, explain and explain again the secrets of statistics.

Professor Johnny Ludvigsson for support and wise comments on my work through the years.

Ammi Fornander for guidance in the laboratory and for performing some of the laboratory works in this study.
Lennart Nilsson and Thomas Abrahamsson for thoughtful comments on this work throughout the years.

Nina Nelson, my former boss, for making it possible for me to finish this work in a feasible way and for providing an outstanding PhD course on research in children.

Christina Johansson, my boss at the paediatric clinic, for patience and support during the final part of this work.

All friends at KEF for good discussions and scheduled coffee breaks.

All my friends, of all professions, working at the paediatric clinic, for believing in me, supporting me and making me enjoy clinical work. Special thanks to Farhad Vahedi and Emma Olsson and all co-workers at “BAVA”, for being patient with my absence.

My parents Eva and Jan for being role models and for actively sharing the delight of our children.

My mother-in-law Elisabeth for all the care you give our family.

Our children: Karin, Johan and Gustav for patiently sharing my computer with me these past years. You and baby David fill my life with joy!

My husband Jörgen for love, happiness and encouragement always.

This work was supported by grants from Medical Research Council of South-East Sweden (FORSS), Östergötland County Council, The Ekhaga Foundation, Swedish Asthma and Allergy association, The Research Council for Environmental, Agricultural Sciences and Spatial Planning (FORMAS), The Swedish Society of Medicine, The Swedish Medical Research Council (VR) Glaxo Smith Kline and Lions Club Gnesta. The fish oil capsules were supplied by Pharma Nord Denmark, at a reduced cost.
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