Polyunsaturated fatty acids, maternal and infant immune responses and allergic disease in infancy

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To my astonishment
ABSTRACT

Background: The incidence of allergic diseases in industrialized countries has increased, and a relation between allergy and dietary fatty acids has been proposed. Modulation of the maternal immune function during pregnancy may have an impact on future clinical outcome in the child.

Aim: The aim of this thesis was to add knowledge on the relationship between long chain polyunsaturated fatty acids, sensitization and allergic disease and possible immunological events regulating this.

Subjects: The thesis is based on results obtained from two cohorts. The first, including 300 cord blood samples collected from 1985-2005. The second, a double-blind placebo controlled multi-centre study comprising 145 families with allergic disease.

Methods: Phospholipid fatty acids and total IgE antibodies were analyzed in cord blood samples with gas chromatography and Uni-CAP™, respectively. The families participating in the double-blind placebo controlled multi-centre study were recruited at antenatal units in Linköping and Jönköping and the mothers were supplemented with 2.6 g ω-3 long-chain polyunsaturated fatty acids (LCPUFA) or placebo daily from gestational week 25 until 3 months of breast feeding. Phospholipid fatty acids in maternal serum were analysed before and during the intervention to assess compliance. Prostaglandin E₂, leukotrienes B₄ and cytokines were analyzed with ELISA technique in supernatants from maternal LPS-stimulated whole blood cultures. Clinical outcome was allergic disease with positive skin prick test and/or specific circulating IgE to food allergens at one year of age. Cytokines, chemokines, SIgA antibodies and prostaglandin E₂ were analyzed in breast milk with Luminex and ELISA techniques.

Results: The proportions of cord serum linoleic acid (LA, C18:2 ω-6) and α-linolenic acid (LNA, C18:3 ω-3) decreased significantly from 1985 to 2005. However, the LA/LNA ratio did increase, revealing a relatively larger decrease in LNA than in LA. The proportions of both arachidonic acid (AA; C20:4 ω-6) and docosahexaenoic acid (DHA, C22:6 ω-3) as well as other ω-6 and ω-3 fatty acids increased significantly during the same time period. No correlations were found between ω-6 and ω-3 fatty acids and total IgE antibodies. Proportions of ω-3 LCPUFA increased in the ω-3 supplemented group of mothers. Lipopolysaccharide-induced prostaglandin E₂ secretion in whole blood culture decreased in a majority of ω-3 PUFA supplemented mothers (18 of 28, p < 0.002). The decreased prostaglandin E₂ production was more pronounced among non-atopic than atopic mothers. Lipopolysaccharide induced cytokine and chemokine secretion was not affected. The period prevalence of food allergy was lower in the ω-3 group (1/52, 2%) compared to the placebo group (10/65, 15%, p < 0.05) as well as the incidence of IgE-associated eczema (ω-3 group: 4/52, 8%; placebo group: 15/63, 24%, p < 0.05) at one of year. There were no differences in breast milk cytokine, SIgA and PGE₂ levels between the two intervention groups. However, the levels of several cytokines tended to be higher in colostrum from non-atopic ω-3 supplemented mothers as compared to non-atopic placebo supplemented mothers. Higher levels of TGFβ2 and SIgA in 3 months milk were associated with allergic disease at one year of age both with and without detectable IgE.

Conclusions: Cord blood LA proportions decreased and LA/LNA ratio increased over the 20 year period between 1985 and 2005 this was not related to total IgE. ω-3 fatty acid supplementation of pregnant and lactating mothers resulted in a lower period prevalence of IgE-associated eczema and food allergy in the children at one year of age. This was most pronounced in children of non-allergic mothers. The underlying mechanism requires further clarification.
SAMMANFATTNING

Bakgrund: Allergiska sjukdomar har blivit allt vanligare i den industrialiserade delen av världen och det har föreslagits att det finns ett samband mellan den ökade förekomsten av allergi och kostens innehåll av fettsyror. Påverkan på mammas immunsvar under graviditeten kan ha betydelse för om barnet senare i livet utvecklar allergisk sjukdom.

Syfte: Den här avhandlingen har syftet att öka kunskapen om sambandet mellan långa fleromättade fettsyror och allergi, samt om bakomliggande faktorer i immunsvarret som reglerar detta samband.


Metoder: Förekomst av fleromättade fettsyror och IgE mättes i de 300 navelsträngsproven, som fanns insamlade och nedfrysta. Familjerna i den andra studien engagerades via mödravårdscentraler i Linköping och Jönköping. De blivande mödrarna fördelades slumpmässigt till två grupper, där den ena gruppen fick antingen aktiv substans innehållande 2.6 g omega-3 fettsyror/dag eller icke-aktiv kapslar. Förrådet tillamnades med ovannämnda kapslar, och tillmätningen genomfördes ofta med ELISA teknik av vätska som uppsamlats från stimulerade cellkulturer. Allergisk sjuklighet hos barnet bedömdes utifrån om barnet hade allergisk sjukdom, positiv hudpricktest och/eller IgE mot mat vid ett års ålder. Immunologiska faktorer i form av cytokiner, kemokiner, SIgA antikroppar och prostaglandiner i bröstmjölk mättes med Luminex och ELISA tekniker.


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REFERENCES
ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Decreased proportions of linoleic acid (LA) in cord blood samples collected between 1985 and 2005.
   **Warstedt K, Duchén K**
   Manuscript

II. The effects of omega-3 fatty acid supplementation in pregnancy on maternal eicosanoid, cytokine, and chemokine secretion.
   **Warstedt K, Furuhjelm C, Duchen K, Fälth-Magnusson K, Fagerås M.**

III. Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy
   Furuhjelm C, **Warstedt K**, Larsson J, Fredriksson M, Böttcher MF, Fälth-Magnusson K, Duchén K.

IV. Omega-3 long chain polyunsaturated fatty acid supplementation in pregnancy and lactation and immune components in breast milk
   Submitted
ABBREVIATIONS

AA  arachidonic acid
AEDS atopic eczema/dermatitis syndrome
AD atopic dermatitis
ANOVA analysis of variance
APC antigen presenting cell
CAPS the Childhood Asthma Prevention Study
CBMC cord blood mononuclear cells
CD cluster of differentiation
CCL2 CC-chemokine ligand 2 (MCP-1, monocyte chemotactic protein-1)
CCL3 CC-chemokine ligand 3 (MIP-1α, macrophage inflammatory protein-1 alpha)
COX cyclooxygenase
CS cord serum
CV coefficient of variance
d day
DBPCFC double-blind placebo controlled food challenge
DC dendritic cell
DHA docosahexaenoic acid
DHGLA di-homo gamma-linolenic acid
DPA docosapentaenoic acid
EPA eicosapentaenoic acid
ER endoplasmatic reticulum
FAO Food and Agricultural Organization
FABPpm plasma membrane fatty acid binding protein
FAT fatty acid translocase
FATP fatty acid transport protein
FceRI high affinity IgE receptor
GALT gut associated lymphoid tissue
GINA The Global Initiative for Asthma
GLA gamma-linolenic acid
GM-CSF granulocyte-macrophage colony-stimulating factor
GPI glycosylphosphatidylinositol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</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>
</tr>
<tr>
<td>LCPUFA</td>
<td>long-chain polyunsaturated fatty acids</td>
</tr>
<tr>
<td>LNA</td>
<td>alfa-linolenic acid</td>
</tr>
<tr>
<td>LOX</td>
<td>lipoxygenase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PGE</td>
<td>prostaglandin E</td>
</tr>
<tr>
<td>pIgR</td>
<td>polymeric-Ig-receptor</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipids</td>
</tr>
<tr>
<td>PLA</td>
<td>phospholipase A</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activating receptor</td>
</tr>
<tr>
<td>PRP</td>
<td>pathogen recognition receptors</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RSV</td>
<td>human respiratory syncytial virus</td>
</tr>
<tr>
<td>SIgA</td>
<td>secretory IgA</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol-regulatory-element binding protein</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>T-reg</td>
<td>regulatory T-cell</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
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</table>
INTRODUCTION

The prevalence of allergic diseases has increased dramatically in the affluent world during recent decades \(^1\,^2\). The reasons for this increase are not completely identified but the impact of life style factors seems to be important. A variety of environmental factors such as pollution, cigarette smoke, allergen exposure, microbial pressure and diet are proposed explanations. To explain the geographical differences in allergic prevalence we need to think “outside the box” and look for factors that might have been lost in our westernized lifestyle. Factors that used to protect us from allergic diseases, which still are present in traditional societies. One such factor could be the quality of fat, including the omega (ω)-6/ω-3 fatty acid ratio. Earlier studies aiming to treat children and adults with allergic diseases with ω-3 fatty acids have been disappointing possibly because the intervention in established disease comes too late \(^3\). Intervention during the perinatal period might be a way to influence future health and disease.

This thesis is based on work where I and my colleagues have investigated the relationship between long-chain polyunsaturated fatty acid (LCPUFA) and allergic disease. We have investigated changes in the phospholipid LCPUFA profile in cord serum samples collected during a period when the prevalence of allergic changed dramatically. We have also investigated clinical and immunological effects of ω-3 LCPUFA intervention during pregnancy and lactation.
REVIEW OF THE LITERATURE

Fatty acids

Biochemistry

Fatty acids are essential for human life. They are substrates for energy generation by β-oxidation and may be stored in adipose tissue when energy intake exceeds spending. In addition, fatty acids act as components in cell membrane phospholipids, precursors for the formations of bioactive lipid regulators of gene expression.

Lipids consist mainly of hydrogen and carbon atoms and are characterized by their insolubility in water. The most common component of dietary fat is triacylglycerol formed by linking together glycerol (Figure 1a) with three fatty acids (FA) (Figure 1b) and this is also the most common fat component in the body. Fatty acids consist of a sequence of carbon atoms with a carboxyl component at one end and a methyl group at the other. The carboxyl end is reactive and forms ester links with alcohols such as glycerol.

Figure 1. a) Glycerol, b) Triacylglycerol formed by glycerol and three fatty acids.
Most fatty acids have an even number of carbon atoms because the human body synthesizes the chain by fusing 2-carbon fragments. The carbon chain lengths vary from 4 (e.g. in milk) to 30 (e.g. in some fish oils) but are usually between 14 and 24 carbon atoms long. A fatty acid is classified as saturated if all the carbon atoms are linked together by single covalent bonds and unsaturated if the carbon chain has one or more double bonds. Monounsaturated fatty acids have one double bond and polyunsaturated fatty acids (PUFA) have two or more double bonds (Figure 2). Long chain polyunsaturated fatty acids have a carbon chain with 20 or more carbon atoms. The configuration of the double bond is usually cis, although trans- isomers do occur in some animal fats, for instance in cow’s milk. The configuration of the acyl chain, the length and the degree of unsaturation of a fatty acid determines the physiological properties of a fat or oil. Triacylglycerols made up of mainly saturated fatty acids have high melting points and are solid in room temperature. They are what we in general call fats. Oils consist of triacylglycerols with a high proportion of monounsaturated and polyunsaturated fatty acids. They have lower melting points and are therefore more fluid.

![Fatty acid structures](image)

**Figure 2.** Fatty acid structures. *The fatty acids are called saturated if they have no double bonds, monounsaturated if they have one double bond and polyunsaturated if they have two or more double bonds.*
Nomenclature

Fatty acids can be designated in several ways. The systemic name numbers the carbons from the carboxylic end and is derived from the name of its parent hydrocarbon, substituting \(-oic\) for the final \(-e\). Alternatively, the short hand way states the number of carbon atoms, number of double bonds and the position of the double bond adjacent to the terminal methyl group (ω- or n- carbon) (Figure 2). In addition to these nomenclatures, fatty acids are often referred to by their trivial name (Table 1, Figure 2). For example, the saturated fatty acid with 18 carbon atoms is designated octadecanoic acid as systematic name, C18:0 as short hand and Stearic as trivial name (Table 1). The short hand notation is often used in PUFA classification, for example C18:2 \(\omega\)-6, meaning that the acyl chain consists of 18 carbon atoms with two double bonds; the first one positioned at carbon atom 6 counting from the methyl end (Figure 2).

<table>
<thead>
<tr>
<th>Systemic name</th>
<th>Trivial name and abbreviation</th>
<th>Shorthand notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acids (SFA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decanoic</td>
<td>Capric</td>
<td>C10:0</td>
</tr>
<tr>
<td>Dodecanoic</td>
<td>Lauric</td>
<td>C12:0</td>
</tr>
<tr>
<td>Tetradecanoic</td>
<td>Myristic</td>
<td>C14:0</td>
</tr>
<tr>
<td>Hexadecanoic</td>
<td>Palmitic</td>
<td>C16:0</td>
</tr>
<tr>
<td>Octadecanoic</td>
<td>Stearic</td>
<td>C18:0</td>
</tr>
<tr>
<td>Monounsaturated fatty acid (MUFA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-9-hexadecenoic</td>
<td>Palmitoleic</td>
<td>C16:1 (\omega)-7</td>
</tr>
<tr>
<td>cis-9-octadecenoic</td>
<td>Oleic</td>
<td>C18:1 (\omega)-9</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids from the (\omega)-6 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-9, cis-12-octadecadienoic</td>
<td>Linoleic (LA)</td>
<td>C18:2 (\omega)-6</td>
</tr>
<tr>
<td>all cis -6,9,12-octadecatrienoic</td>
<td>γ-linolenic (GLA)</td>
<td>C18:3 (\omega)-6</td>
</tr>
<tr>
<td>all cis -8,11,14-eicosatrienoic</td>
<td>Di-homo-γ-linolenic (DHGLA)</td>
<td>C20:3 (\omega)-6</td>
</tr>
<tr>
<td>all cis -5,8,11,14-eicosatetraenoic</td>
<td>Arachidonic</td>
<td>C20:4 (\omega)-6</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids from the (\omega)-3 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>all cis -9,12,15-octadecatrienoic</td>
<td>α-Linolenic (LNA)</td>
<td>C18:3 (\omega)-3</td>
</tr>
<tr>
<td>all cis -5,8,11,14,17-eicosapentaenoic</td>
<td>Eicosapentaenoic (EPA)</td>
<td>C20:5 (\omega)-3</td>
</tr>
<tr>
<td>all cis -7,10,13,16,19-docosapentaenoic</td>
<td>Docosapentaenoic (DPA)</td>
<td>C22:5 (\omega)-3</td>
</tr>
<tr>
<td>all cis -4,7,10,13,16,19-docosahexaenoic</td>
<td>Docoahexaenoic (DHA)</td>
<td>C22:6 (\omega)-3</td>
</tr>
</tbody>
</table>
Essential fatty acids and their longer derivatives

Linoleic acid (LA, C18:2 ω-6) and α-linolenic acid (LNA, 18:3 ω-3) belong to the two principle families of PUFA, the ω-3 and ω-6 families. They are regarded as essential fatty acids, as they cannot be synthesized de novo in humans, since mammalian cells lack the delta-12 and delta-15 desaturase enzymes facilitating the insertion of double bonds at the ω-3 or ω-6 position carbon. Hence, these fatty acids must be retrieved from the diet. Linoleic acid is found in large quantities in many vegetable oils such as corn, sunflower and soy bean oils and also in products derived from these oils, e.g. margarine. α-linolenic acid is abundant in e.g. walnuts, green leafy vegetables, rapeseed oil and flaxseed. Even though they cannot be synthesized by the human body, LA and LNA can be converted to a wide range of more unsaturated fatty acids with longer chain. The first desaturation inserts double bonds at the 6th carbon catalyzed by Δ6-desaturase, followed by additional elongation and desaturation. Consequently, LA is converted to arachidonic acid (AA, C20:4 ω-6) and Osbond acid (C22:5 ω-6) via γ-linolenic acid (GLA, C18:3 ω-6) and dihomo- γ-linolenic acid (DHGLA, C20:3 ω-6). In the same way, LNA is converted to eicosapentaenoic acid (EPA, C20:5ω-3), docosapentanoic acid (DPA, C22:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3) (Figure 3.). The formation of LCPUFA can take place in multiple organs e.g. the liver, the brain, the retina and the intestine. All reactions occur in the endoplasmatic reticulum with the exception of the final reaction taking place in the peroxisome, resulting in the formation of DHA and C22:5 ω-6, respectively. The formation of C22:5 ω-6 and DHA was earlier thought to be catalyzed by a Δ4 –desaturase, but is in fact a retroconversion: elongation and Δ6-desaturation followed by translocation to the peroxisome and β-oxidation shortening the acyl chain to a 22 carbon LCPUFA (Figure 3).

If there is an insufficient supply of PUFA to meet the physiological requirements, the body starts to synthesize certain other fatty acids with a similar molecular structure but without the same functions. These fatty acids are normally not present (or present in low proportions) and can therefore be used as PUFA status markers. A general deficiency in PUFA is indicated by a higher level of Mead acid (C20:3 ω-9) and a deficiency in DHA results in increased production of Osbond acid (C22:5 ω-6).
The conversion of LNA to longer chain ω-3 PUFA in humans seems to be limited. Studies with male volunteers given increased amounts of stable isotop-tracer-marked LNA show low conversion to EPA and DPA and constrained conversion to DHA. However, studies on women of reproductive age showed that the conversion of LNA to longer metabolites was 2.5 to >200-fold greater than in comparable studies on men. This discrepancy is thought to be due to the action of estrogen and could be important to meet the increasing demand of the fetus and the newborn for DHA during pregnancy and lactation. However, efficient tissue accretion of ω-3 LCPUFA depends to a significant degree on the delivery of EPA and DHA directly from dietary sources.
Plasma membranes and lipid rafts

The plasma membrane is composed principally of sphingolipids, phospholipids and cholesterol. Phospholipids consist of a glycerol backbone with two fatty acids at the sn1 and sn2 positions, and at the sn3 position there is a phosphate group attached to an alcohol group. A saturated fatty acid is often attached at the sn1 position and an unsaturated fatty acid at the sn2 position (Figure 4).

Figure 4. Schematic picture of a phospholipid. It consists of 2 fatty acids and one negatively charged phosphate group bound to carbon atoms in glycerol.

The unsaturation of the fatty acid influences the fluidity of the cell membrane. Phospholipids with an unsaturated fatty acid tend to be more loosely packed, forming into a liquid-disordered phase, allowing rapid lateral movements within the bilayer. Sphingolipids, on the other hand, have two long saturated acyl-chains, allowing them to be tightly packed in the bilayer, forming a gel-phase in which there is very little lateral movement or diffusion. Lipid rafts are small domains in the outer leaflet of the plasma membrane rich in cholesterol, sphingolipid and glycosylphosphatidylinositol (GPI) linked proteins (Figure 5). They are platforms for cell activation and facilitate the connection between signaling molecules and the cross-talk and contacts between different cell types. The T-cell receptor (TCR) within a lipid raft clusters when it gets in contact with an antigen presenting cell, and forms a contact zone
where the intracellular signaling is facilitated. The role of membrane rafts in Th1 and Th2 cells seems to be different with TCR activation in Th1-cells being dependent on rafts, whereas that in Th2-cells is not. Since the plasma membrane is rich in fatty acids it may also be responsive to diet-induced changes having the potential to modulate cellular function on many levels.

**Figure 5.** Plasma membrane bilayer with a lipid raft with GPI linked protein and Src-linked protein facilitating the intracellular signal.

**Eicosanoids and other bioactive lipids**

One of the most important functions of cell membrane phospholipids are as precursors of eicosanoids (e.g. prostaglandins, leukotrienes and thromboxanes). Eicosanoids are mediators derived from 20-carbon LCPUFAs (greek *eicosi* = twenty) and can be considered as biologically active lipids. They are not stored, but are synthesized *de novo* in response to inflammatory stimuli. The synthesis of eicosanoids is maintained through the enzymes cyclooxygenase (COX) and lipoxygenase (LOX), resulting in the production of prostaglandins, tromboxanes and leukotrienes respectively (Figure 6).
Figure 6. Eicosanoids from arachidonic acid (AA) are generated through the action of a variety of enzymes. The production of PGE₂ and LTB₄ is measured in this study.

The eicosanoids are produced in a cell specific manner depending on accessibility of the different enzymes in different cell types and the nature, timing and duration of the stimuli and also of the phospholipid fatty acid content in the cell membrane. Membrane AA raise the 2-series of prostaglandins (e.g. PGE₂), tromboxanes (e.g. TXA₂) and the 4-series of leukotrienes (e.g. LTB₄) (Figure 6). Both PGE₂ and LTB₄ are involved in adjusting the intensity and duration of inflammation and immune responses. PGE₂ possesses several pro-inflammatory properties, such as induction of fever, enhanced vascular permeability, vasodilatation and pain. In addition, PGE₂ has the ability to suppress lymphocyte proliferation and natural killer (NK) cell activity and to inhibit the production of tumor necrosis factor (TNF), interleukin (IL)-1, IL-2 and IL-6. LTB₄, on the other hand has the ability to enhance the production of these cytokines and also to increase vascular permeability and blood flow. LTB₄ is also a powerful chemotactic agent and can promote NK-cell activity and inhibit lymphocyte proliferation. PGE₂ also has the ability to down-regulate LTB₄.
production through inhibition of 5-LOX activity. Taken together, PGE₂ and LTB₄ have several opposing effects.

**Immunological mechanisms**

The central task for the immune system is to distinguish non-pathogens from pathogens and to eliminate these pathogens *e.g.* bacteria, viruses, fungi, parasites and tumors with minimal pathological outcome for the host. Immune responses can be divided into innate immunity mediated by monocytes, macrophages, dendritic cells and natural killer cells (NK-cells), and adaptive immunity mediated by lymphocytes.

Innate immunity offers a first line of defence and responds rapidly with phagocytosis of the intruding pathogen followed by secretion of cytokines and chemokines *e.g.* tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, IL-10 and IL-12 further activating and regulating the immune system (Table 2). Pathogen recognition receptors (PRPs) *e.g.* Toll like receptors (TLR) are evolutionary conserved receptors crucial for the innate immunity. They recognize microbial associated molecular patterns (MAMPs) *e.g.* lipopolysaccharide from Gram-negative bacteria *e.g.* *E. coli.*

The adaptive immune system is slower and responds to specific antigens and provides an immunologic memory. This system is maintained by T-cells and B-cells via production of cytokines (Table 2) and antibodies. Naïve T-helper cells differentiate upon stimulation and so far four different CD4 cell lineages have been isolated in vivo in humans, T-helper (Th)1, Th2, Th17 and T-regulatory (reg) cells. The local cytokine milieu is crucial for the differentiation of naïve T-helper cells. The differentiation of Th1 is promoted by IL-12/IFN-γ, Th2 is promoted by IL-4 + IL-2 (and TSLP), T-reg by TGFβ + IL-2 and finally Th17 differentiation depends on the presence of TGFβ + IL-1 and also IL-6, IL-21 and IL-23 (Figure 7). The Th1-like pathway typically producing IFN-γ, is important for the host defence against intracellular pathogens such as viruses. The Th2 lineage secreting IL-4 and IL-13 induce IgE-production and also IL-5, inducing eosinophil growth and differentiation for the protection against parasites. Regulatory T-cells play a critical role in maintaining self-tolerance and suppression of immune responses and Th17 cells mediate responses against extra cellular bacteria and fungi and participate in the induction of auto immune diseases.
Table 2. Summary of cytokines and chemokines referred to in this thesis.

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Produced mainly by…</th>
<th>Target cell, function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>Fibroblast, macrophages</td>
<td>Growth and differentiation of DC</td>
</tr>
<tr>
<td>IL-1</td>
<td>Monocytes;macrophages; dendritic cells</td>
<td>Co-stimulation of Th-cells, maturation and proliferation of B-cells, activation of NK-cells</td>
</tr>
<tr>
<td>IL-6</td>
<td>Many cells most of all macrophages</td>
<td>Stimulate antibody secretion, induce fever and acute phase response, induce production of cortisol, counteract the production of IL-1 and TNF</td>
</tr>
<tr>
<td>IL-12</td>
<td>Dendritic cells; macrophages</td>
<td>Stimulate the production of IFN-γ from T-cells and NK-cells, increased cytotoxic activity of CD 8+ T-cells and NK-cells.</td>
</tr>
<tr>
<td>TNF</td>
<td>Macrophages; activated T-cells; epithelial cells, mast cells</td>
<td>Induce fever, fatigue, drowsiness. Induce acute phase response, induce COX2 which induce the production of PGs and induce adhesion molecules.</td>
</tr>
<tr>
<td>CCL2 (MCP)</td>
<td>Macrophages</td>
<td>Chemotactic for monocytes and T-cells</td>
</tr>
<tr>
<td>CCL3 (MIP-1α)</td>
<td>Macrophages</td>
<td>Chemotactic for activated T-cells, involved in allergic inflammation</td>
</tr>
<tr>
<td>CXCL8 (IL-8)</td>
<td>Macrophages; endothelial cells; epithelial cells</td>
<td>Chemotactic and activating for neutrophil granulocytes</td>
</tr>
<tr>
<td>TGFβ</td>
<td>T-cells; monocytes</td>
<td>Anti-inflammatory, required for T-reg differentiation</td>
</tr>
<tr>
<td>IL-10</td>
<td>Monocytes, macrophages, DC and some T- and B-cells</td>
<td>Stimulate antibody secretion, counteract the activation of T-cells and the production of IFN-γ. Downregulate MHC II on APC. Considered anti-inflammatory.</td>
</tr>
<tr>
<td>IL-2</td>
<td>Th1 cells</td>
<td>Activation, growth and proliferation of T-cell, B-cells and NK-cells and required for Treg differentiation</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cells, mast cells</td>
<td>Th2 cell differentiation, proliferation and activation of of B-cells</td>
</tr>
<tr>
<td>IL-5</td>
<td>Th2 cells, mast cells</td>
<td>Growth and differentiation of eosinophils</td>
</tr>
<tr>
<td>IL-13</td>
<td>Th2 cells</td>
<td>Stimulate mast cells. Involved in the allergic inflammation with increased mucus production and airway hyperreactivity.</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Th1 cells, NK cells</td>
<td>Activation of macrophages, increased expression of MHC, Ig class switch, suppresses Th2</td>
</tr>
<tr>
<td>TSLP</td>
<td>Epithelial cells, fibroblasts, and different types of stromal or stromal-like cells</td>
<td>Maturation of DC, Promote Th2 immunity</td>
</tr>
</tbody>
</table>

Modified from 19,22,23
Figure 7. Schematic overview of the immunological synapse and the differentiation of Th-cells. The antigen presenting cell (APC) presents a degraded antigen/allergen as peptides bound to MHC kl II. This complex is recognized by the T-cell receptor (TCR) and delivers the first signal for T-cell activation. The T-cell receives a second signal through the interaction between B7 and CD28. Low-dose allergen exposure is suggested to favor Th2 associated immune responsiveness with high IgE production while high-dose exposure leads to development of clinical tolerance. Modified from 19-20.
The allergic reaction

The allergic reaction starts with the initial sensitization upon which an allergen enters the body. It is taken up by antigen presenting cells e.g. dendritic cells (DC) which migrate to a local lymph node. The processed allergen is presented as peptides together with major histocompatibility complex (MHC) II to the T-cell receptor on a naïve CD4 T-cell. The local cytokine environment, the dose of allergen and the route of presentation are essential for the maturation of naïve CD4 T-cells. Low-dose allergen exposure is suggested to favor Th2 associated immune responsiveness with high IgE production while high-dose exposure leads to development of clinical tolerance. IL-4 is the major mediator of B-cell switch, from IgM antibody production to IgE antibody production. The Th2-cells themselves, besides secreting IL-4 also, produce IL-5, IL-9 and IL-13, hence maintaining an environment beneficial for further Th2 cell differentiation. In addition, PGE\textsubscript{2} has the potential to inhibit the production of Th1 like cytokines and prime naïve T-cells to produce IL-4 and IL-5. Prostaglandin E\textsubscript{2} also promotes immunoglobulin class switching toward the production of IgE.

The IgE antibodies bind to the high affinity IgE receptor (FcεRI) on mast cells present under the skin and in the mucosal-associated lymphoid tissue of the airways and the gut, but also on basophils in blood. On re-encounter, the allergen cross-links IgE antibodies on the mast cell triggering the cell to release its content of immune mediators. These mediators can be pre-formed, crowded within secretory granules e.g. histamine, or newly synthesized lipid mediators e.g. prostaglandins and leukotrienes or cytokines and chemokines.

The release of these preformed or rapidly produced mediators generates a so called immediate hypersensitivity reaction starting within seconds from allergen exposure causing an instant increase in vascular permeability and smooth muscle contraction. This is often followed by a late phase reaction occurring hours or days later as an effect of leukotrienes and additional cytokines and chemokines secretion and influx of neutrophils, eosinophils, lymphocytes and mast cells to the site of inflammation which prolongs immune activity and tissue damage.

Immune responses in children

The immune system at birth is immature and infants are highly susceptible to infections. The neonate has poor cell-mediated immunity, poor inflammatory responses, impaired defence mechanisms against intracellular pathogens and inability to secrete certain immunoglobulin
isotypes. Newborns have a higher proportion of naïve T-cells and a lower proportion of memory T-cells, not reaching adult levels until teenage years. The proliferation and cytokine production of T-cells are reduced in neonates and naïve T-cells in neonates are more easily Th2 skewed than cells from adults.

During the first year of life a suppression of the Th2-like responses has been observed in non-allergic but not in allergic children. This may indicate that the postnatal immune maturation is delayed in children who develop allergy compared to those who do not.

Allergic diseases occur early in life and maternal atopy has been reported to represent a higher risk for development of asthma and eczema in children than paternal atopy. The question whether sensitization occurs prenatally or postnatally has been intensely debated during recent years. Circulating T-cells can be identified as early as in the 15 gestational week and specific immune responses to allergens have been demonstrated in gestational week 22.

**General aspects of allergic disease**

**Classification of hypersensitivity reactions**

There are four types of hypersensitivity reactions. Type I hypersensitivity reactions induce mast cells activation mediated by immunoglobulin (Ig) E antibodies against antigens, i.e. allergens. Other type of hypersensitivity immune reactions, e.g. Type II and III reactions involve IgG antibodies to cell/matrix associated antigens or soluble antigens, respectively causing immune complexes followed by tissue damage. The type IV hypersensitivity reactions are T-cell mediated.

**Atopy**

Atopy is a clinical definition of an IgE-antibody high-responder and the term atopy must not be used until the presence of in vivo IgE antibodies has been recognized by a positive skin prick test or by the presence of circulating IgE antibodies. Food allergy, atopic eczema, rhinitis, conjunctivitis and asthma are manifestation of IgE mediated reactions and are commonly occurring in infancy and childhood. The prevalence of allergic symptoms among children worldwide has been investigated by the International Study of Asthma and Allergy in Childhood (ISAAC) epidemiological research program. Among 6-7 years old Swedish
Eczema, food allergy and asthma

Eczema during childhood is very common and affects 20-30 % of the general population and 40-50 % of infants with atopic heredity. Eczema can be divided into atopic eczema when allergic sensitization can be demonstrated and non-atopic eczema in the absence of proven sensitization.

The pathogenesis of eczema is complex and multifactorial. A dual-allergen-exposure hypothesis has been proposed suggesting that low dose exposure to food allergens through dust, hands etc occurs and that the allergen is taken up by skin Langerhan’s cells leading to Th2 responses and IgE production. In contrast, early, high oral doses of food allergens induce tolerance through Th1 and regulatory immune responses in the gut associated lymphoid tissue (GALT).

An undamaged epidermal is crucial for the skin to function as a barrier against chemical and physical interference. Filaggrin, a filament-associated protein that binds to keratin fibers in epithelial cells is a vital part of this function and a strong genetic association of filaggrin gene (FLG) loss-of-function mutations and eczema is now clear.

The term food allergy is used to describe an adverse immune reaction to food stuffs and approximately 5 % of infants and children in affluent countries are affected. The infant is particularly prone to sensitization with food allergens since the gut barrier and immune system is not fully developed. The sIgA system preventing micro-organisms and allergens from adhering to the mucosa and activating the clearance of allergens is immature until 4 years of age. In addition, animal studies show hampered mucin production during the first weeks of life and increased permeability of the gut mucosa shortly after birth.

Food allergy can be expressed as abdominal pain, diarrhea, eczema, urticaria, rhinoconjunctivitis and asthma. The offending food items in infancy are usually cow’s milk, hen’s egg and wheat.

Children with food allergies or sensitization to food allergens, especially hen’s egg possess a greater risk of developing respiratory allergic manifestations later in life.
Food allergy can be indicated in multiple ways, e.g. accurate clinical history, positive skin-prick test (SPT) to food, circulating IgE to food. The gold standard for the diagnosis of food allergy is double-blind placebo controlled food challenge (DBPCFC). However, a large SPT wheal size and a high concentration of circulating specific IgE have been shown to correlate with a positive DBPCFC. A wheal diameter at or above 7 mm for hen’s egg and 8 mm for cow’s milk were always associated with a positive DBPCFC and a specific IgE value of ≥ 0.35 kUA/l to egg white predicted a positive clinical reaction in 94% of the cases. On the other hand, small wheal sizes and a low IgE value do not prove absence of food allergy.

No curative treatment for food allergies is currently available. The traditional advice given is to strictly avoid the offending foods temporarily, unfortunately influencing the quality of life in a negative way for both the child and its family. Almost 80% of children with food allergy will outgrow their disease before the age of five, i.e. develop tolerance.

Asthma is a disease of chronic airway inflammation. It is characterized by infiltration of eosinophils, increased mucus secretion and airway hyper-responsiveness. The inflammation may cause frequent episodes of wheezing (high-pitched whistling sounds when breathing out), cough, breathlessness and chest tightness. Not all children with wheeze have asthma since wheeze and cough is common in children having a regular cold. Recurrent wheezing is common many years after an early infection with Respiratory Syncytial Virus (RSV). The RSV infection seems to be a risk factor for future wheeze but the association to future sensitization is debated.

The so called atopic march is the natural history of atopic manifestations starting with atopic eczema and food allergy in infancy being replaced by asthma and rhinitis in pre-school age.
The mother/baby dyad

The mother as an immune deviating environment

The mother contributes to her child’s immune system not only genetically but also as an environmental factor. This is facilitated during fetal life via the placenta and later via breast milk.

Profound immunological changes occur in the mother during pregnancy. In 1993, Wegmann et al suggested that successful pregnancy in mice demands a shift away from cell-mediated immunity potentially harmful for the fetus towards a humoral immunity. It is now recognized that pregnancy involves a polarization towards Th2 like immunity and Treg cell responses. Differences in cytokine profile during pregnancy depending on the allergic status of the mother have also been demonstrated with significantly higher IL-13 Th2 responses to allergens in allergic women (i.e. clinical history and positive sensitization) compared to non-allergic women. The allergic mothers sustained their high Th2 responses to allergens both during and after pregnancy but the non-allergic mothers gradually down regulated their already low response. This enhanced Th2 response in allergic mothers might be an environmental factor contributing to the increased risk for allergic diseases in infants born to allergic mothers.

Omega-3 fatty acids in pregnancy and lactation

Pregnancy is associated with a generalized lipidemia and maternal plasma phospholipids are doubled during the course of pregnancy. The reason for this accretion is the increasing demands for energy supplies from the growing fetus and the formation of the placenta. Human gestational length and parturition is regulated by complex interactions of hormones, cytokines and eicosanoids. The exact mechanisms are however still unknown. The LCPUFAs play important roles during pregnancy and lactation as precursors of prostaglandins and as structural components of membranes.

The concentrations of AA are elevated in the amniotic fluid during labour and furthermore are the levels of PGE₂, PGF₂α, LTC₄ and LTB₄ elevated in the maternal circulation preceding the
onset of spontaneous labour. Increasing levels of prostaglandin (PG) metabolites in the peripheral circulation during labor indicate that PG synthesis increases during parturition at term. Administration of vaginal PGE\(_2\) has been a successful way to induce labour since the 1960s.

Observational studies from the Faroe Islands and Canada indicate that a high maternal intake of ω-3 fatty acids due to a high seafood intake during pregnancy is associated with a prolonged gestation and an increased birth weight as compared to infants born in Denmark and southern Québec respectively. Inspired by this, Olsen et al performed a randomized, controlled fish-oil supplementation study to investigate the effects on pregnancy duration, birth weight, and birth length after a daily addition of 1.3 g EPA and 0.92 g DHA from gestational week 30 or olive-oil or no supplementation as a control. Gestational length was prolonged by 4 days on average with no harmful effect on growth of the baby or course of labor in the fish-oil group as compared to the control. The mechanisms behind this could be that the ω-3 fatty acids inhibit the production of PGE\(_2\) and PGF\(_{2\alpha}\) thereby delaying labour and cervical ripening. A substantial number of studies have been performed within this context, both observational and randomized trials. Two independent meta-analyses have recently been published indicating a prolonged gestational length by 1.6-2.6 days after ω-3 LCPUFA supplementation during pregnancy. A similar effect was observed in a meta-analysis on high-risk pregnancies while no other effects were detected.

Normal pregnancy is characterized by increasing levels of total fatty acid phospholipids of both ω-6 and ω-3 fatty acids families in maternal plasma. The relative amounts of LA are similar during the course of pregnancy but the AA and DHA proportions are declining as pregnancy proceeds. The proportions of AA start to increase immediately after delivery in contrast to DHA proportions which still 6 months post-partum are lower than in gestational week 10. The maternal DHA stores become more depleted after each pregnancy.

LCPUFAs are crucial for the fetus with respect to the development of the central nervous system, body growth and the eicosanoids synthesis. Especially, AA and DHA are very important for the development of the retina and brain. All fatty acids accumulated by the fetus must be derived from the mother by placental transfer. The relative proportions of AA and DHA are higher, while LA is lower in phospholipids in fetal than maternal plasma. LCPUFA can diffuse passively from the maternal side through the placenta to the fetal side. However, new data indicate that LCPUFA uptake is tightly regulated by several plasma membrane-located transport- or binding proteins.

Fatty acid translocase (FAT/CD36),
plasma membrane fatty acid binding protein (FABPpm), a family of fatty acid transport protein (FATP 1-6) and intracellular FATP have been identified in several tissues including placenta. The biochemical mechanisms responsible for the selective transport and concentration of certain LCPUFA in fetal tissues are not fully understood. The National Food Administration in Sweden (Livsmedelsverket) recommends pregnant Swedish women to consume fish 2-3 times a week including for example all farmed fish, mackerel, salmon and trout. They are advised to avoid Baltic herring and tuna fish but also salmon and salmon trout from the Baltic, Lake Vänern and Vättern due to its high content of pollutants such as dioxin and mercury.

**Human milk**

Human milk comprises both nutrients and energy for growth and development but also an immune system. It contains numerous immune components aiming to facilitate active and passive immunity during the vulnerable early period of life when the neonatal mucosal immune system is inexperienced. Specific protecting is provided by e.g. viable lymphocytes and antibodies. Non-specific protective factors such as lactoferrin inhibit growth of certain pathogens by competing with bacteria for ferric ion. Lysozyme also act against bacteria by cleaving peptidoglycans in the bacterial wall and oligosaccharides function as receptor analogues inhibiting the binding of bacteria or their toxins. The immune composition of breast milk differs from one woman to another and can be altered by e.g. maternal allergy, inflammation, infection, supplementation of probiotics or ω-3 LCPUFA.

The enteromammary link provides the infant with specific secretory IgA (SIgA) reflecting the antigenic environment of the infant and mother. Antigen in the gut is taken up by M-cells and passed by antigen presenting cells to Peyer’s patches where presentation to T-cells provides signals for B-cell activation and dimeric IgA production. IgA production is induced at the basolateral side of the mammary cells and the antibody binds to a polymeric-Ig-receptor (pIgR) on the epithelial cell. The IgA-pIgR complex is transported to the apical surface, where the pIgR subsequently is cleaved, leaving a secretory component attached to the IgA dimer. The secretory component protects the antibody from degradation in the gut. SIgA in human milk subsequently enters the gut and protect the infant from invading pathogens by binding to the mucus layer coating the epithelial surface and prevent adherence of pathogens and their toxins. SIgA has little capacity to activate the classical pathway of
complement and these anti-inflammatory properties can be beneficial for the growing infant as exaggerated inflammation may cause reduced nutrient intake, gut damage and illness.\textsuperscript{85}

Breast feeding is a natural source of fatty acids during early infancy. We have previously reported lower levels of LA, LNA, total \( \omega-6 \) and \( \omega-3 \) LCPUFA in breast milk from atopic mothers than from non-atopic mothers. Particularly, low levels of LNA and EPA, expressed as higher LA/LNA and AA/EPA ratios, were found in milk from atopic compared to non-atopic mothers.\textsuperscript{86-87} Low levels of \( \omega-3 \) LNA and EPA and a high AA/EPA ratio in breast milk seemed to be associated with development of allergic disease in the children at 18 months of age.\textsuperscript{87}

A variety of immune modulatory agents have been identified in breast milk including cytokines, chemokines and eicosanoids.\textsuperscript{76-77,88} They originate primarily from the mammary gland. The extent to which these compounds survive through the gastrointestinal tract is mostly unknown, but previous work suggests that TGF-\( \beta \) survives passage\textsuperscript{89}, it even demands a lower pH for activation.\textsuperscript{90} This cytokine also seems to have important postnatal immune regulatory effects as TGF\( \beta \)-null newborn mice were able to survive and develop normally only if TGF\( \beta \) was present in maternal milk.\textsuperscript{89}

It is difficult to assess the role of individual cytokines and chemokines in breast milk in the development of the infant immune system as the levels vary significantly between different mothers. High concentrations of TGF-\( \beta \) in colostrum has been associated with post-weaning onset of atopic disease, whereas low TGF-\( \beta \) concentrations was associated with pre-weaning onset.\textsuperscript{91} Böttcher et al showed that infants who were sensitized (positive SPT and/or circulating allergen-specific IgE) at 6, 12 and 24 months of age had received colostrum with higher levels of TGF-\( \beta 2 \) than infants who were not sensitized.\textsuperscript{91} Moreover, a low colostral level of IgA was associated with a higher risk for allergic symptoms and atopy at 4 years of age.\textsuperscript{92} These results challenge the common idea of TGF\( \beta \) as an anti-inflammatory mediator that suppresses IgE responses.\textsuperscript{93}

Breastfeeding is the preferred way to nourish an infant but the allergy preventing role of breastfeeding is a constant issue for debate. A meta analysis, indicated a decreased risk of breastfeeding on the development of allergic rhinitis after three months of exclusive breast
feeding. Kull et al showed that exclusive breast feeding for four months or more reduced the risk for eczema and asthma at the age of 4 and this effect remained for asthma at the age of 8. On the other hand, another large Swedish study including more than 8300 children showed no protective effect of breast feeding on atopic dermatitis during the first year of life.

Interestingly, both breastfeeding and extended breastfeeding have even identified as risk-factors for asthma and eczema at least in children with atopic heredity. This could however be an effect of deliberate prolonged breast-feeding of high-risk infants in order to postpone the introduction of solid food. On the other hand, breastfeeding seems to decrease infection associated wheezing episodes often seen in young children.

**Atopy and fatty acids**

“The Black and Sharpe hypothesis”

Our fat consumption has gradually changed over time. We started out as gatherer and hunters and moved on to settle down as farmers. Our food has been more and more produced by the industry during the last century.

![Figure 8. Pattern of dietary intake of fatty acids over time.](image-url)
The human genome is supposed to have evolved during a period when our diet consisted of almost equal parts of ω-6 and ω-3 fatty acids. This ratio has changed dramatically and in some parts of the world does ω-6 fatty acid consumption exceed ω-3 fatty acid consumption by 10-20 times. The reason for this is a reduced intake of ω-3 fatty acids due to decreased fish consumption but also an increased intake of vegetable oils rich in ω-6 fatty acids. Further, the modern industrialized animal husbandry results in meat with high ω-6 fatty acid content, which also applies to the egg and fish industry.

In parallel with the introduction of a westernized lifestyle including altered fatty acid consumption there has been an increase in the prevalence of allergic diseases. The change in consumed fatty acids has been proposed as one explanation for the increase in allergic diseases.

The International Study of Asthma and Allergies in Childhood (ISAAC) has reported a 20-60 fold variation between different countries worldwide regarding the prevalence of asthma, rhinoconjunctivitis and eczema. The symptoms were most common in Australia, North- and South America, Western and Northern Europe and less common in China, Russia, India and Estonia, Latvia and Lithuania. A comparison between high and low allergy prevalence countries with respect to dietary intake of vegetable oil, mainly consisting of ω-6 fatty acids, reveals that low prevalence countries also are low vegetable oil consumers and vice versa for high allergy prevalence countries.

A modern diet comprising high amounts of ω-6 PUFA and low levels of ω-3 PUFA yields AA as the most abundant LCPUFA within cell membranes. The phospholipid composition of human peripheral blood mononuclear cells (PBMC) derived from healthy volunteers comprise of >10% LA, 12-25 % AA, only traces of LNA, >1 % EPA and 2-4 % DHA. Consequently, AA is the major substrate for eicosanoids synthesis in most humans. Cells from the innate immune system e.g. monocytes and macrophages are major producers of eicosanoids.
A proposed link between AA and inflammatory immune responses is the production of eicosanoids from 20 carbon PUFAs. As a consequence of high LA dietary intake, AA is the most abundant fatty acid in cell membrane phospholipids. AA acts as precursor for the production of PGE$_2$ and LTB$_4$, among other eicosanoids. PGE$_2$ restrains the production of Th1-like cytokines e.g. IFN-$\gamma$ and promotes the production of Th-2 cytokines, IL-4 and IL-5. The Th2-like immunity is thereby promoted as IL-4 stimulates B-cells to produce IgE. In addition, PGE$_2$ facilitates B-cell isotype switching to IgE. LTB$_4$ and other leukotrienes from the 4-series can also promote allergic disease.
Proposed mechanism of ω-3 LCPUFA in atopy

The above mentioned hypothesis also includes a low intake of ω-3 PUFA. A dietary supplement of ω-3 LCPUFA may lower the AA/EPA ratio and as ω-3 and ω-6 LCPUFA compete for the same enzyme system, an increased access to ω-3 LCPUFA may counteract the formation of 2-series prostaglandins and 4-series leukotrienes. This has also been shown in several studies \(^{106-108}\). Besides a reduced formation of AA derived eicosanoids may instead PGE3 and LTB4 eicosanoids originating from EPA be produced, suggested to be far less inflammatory \(^{109}\).

Prostaglandins E\(_2\), besides well known pro-inflammatory effects such as induction of fever and pain, also have an impact on the Th1/Th2 balance. As earlier mentioned it decreases the production of Th1-like IFN-γ and IL-2 \(^{110-112}\) and enhances the production of Th2-like IL-4 \(^{113}\) and IL-5 \(^{110-112}\). In addition, PGE\(_2\) also promotes B-cell switch to IgE synthesis \(^{25}\).

These effects on atopy is outlined in Figure 9 and is the base from which the “Black and Sharpe” hypothesis has been outlined linking increased intake of ω-6 LA with allergic disease through AA and eicosanoids.

Other mechanisms may also be affected by elevated access to ω-3 LCPUFA.

Animal studies with fish oil-rich diets revealed that ω-3 LCPUFA are indeed incorporated into raft lipids thereby reducing the content of sphingomyelin involved in the stability of lipid rafts \(^{114}\). In mice, ω-3 LCPUFA promote activation-induced cell death in Th1, but not in Th2, cells \(^{115}\). Th1 activation is suggested to be dependent on lipid rafts, whereas Th2 cells activation is not. Consequently, it has been suggested that modulation of lipid rafts composition with ω-3 LCPUFA could have a role in the down regulation of Th1 responses and therefore in the anti-inflammatory properties of ω-3 LCPUFA \(^{114}\).

ω-3 LCPUFA can also influence gene expression involving multiple and complex processes \(^7\). ω-3 LCPUFA may regulate multiple genes through the action of e.g. peroxisome proliferator-activated receptors (PPAR) and sterol- regulator- element binding protein (SREBP) involved in a wide range of effects on metabolism, cellular proliferation and immune responses and in the regulation of fatty acid and cholesterol metabolism \(^7,116\).
A novel group of mediators called resolvins and protectins formed from EPA and DHA appear to have anti-inflammatory and inflammation resolving properties. Two recent studies on mice showed that resolvin E1 decreased airway eosinophil and lymphocyte recruitment after methacholine provocation. In addition, the production of allergy promoting IL-13, circulating IgE antibodies and hyper responsiveness were also reduced and resolution of inflammatory airway responses were promoted by suppression of IL-6 and IL-23 in the lung.

Previous studies on ω-3 LCPUFA and allergic disease

A variety of studies have been performed to evaluate the effect of ω-3 LCPUFA on atopic disease outcome. There are studies aiming to treat already established allergic diseases with ω-3 LCPUFA. A Cochrane review assessing the effect of ω-3 fatty acids supplementation or a diet rich in ω-3 fatty acids on asthma in adults and children has been performed. The outcome variables were FEV1, peak flow rate, asthma symptoms, asthma medication use and bronchial hyper-reactivity but no consistent effect was shown. On the other hand, no adverse effects were reported either.

Several epidemiological studies exploring the consequences of maternal fish intake during pregnancy on atopic or allergic outcomes in the children showed protective effects. The effect of dietary fish intake during infancy and childhood is however more inconclusive. Some studies showed a protective effect with a risk reduction of 50 to 60 % and others showed no effect. A few even showed an increased risk. Interestingly, introduction to fish earlier than at one year of age reduced the risk for allergic manifestations compared to later introduction.

Reports on the effect of intervention studies with ω-3 LCPUFA during pregnancy and/or lactation on the effect of immune components and/or related allergic outcome in the children have been published previously from four different groups. Some characteristics of the studies are presented in Table 3. The studies share the feature that the ω-3 PUFA status increased with fish oil supplementation both in mothers and children.
The study by Dunstan et al was designed to detect a 15% difference in cytokine levels between control and intervention groups and clinical outcome was reported as a secondary outcome measure. They have published an impressive amount of results on the effect of ω-3 LCPUFA supplementation during pregnancy on early immune development. Lower levels of IL-5, IL-10, IL-13 and IFN-γ in cord blood mononuclear cell were reported as response to allergen and mitogen stimulation in the fish oil group. However, this was only statistically significant for IL-10 in response to cat allergen (p=0.046). They also showed, even though the study was not designed for it, that infants in the fish oil group were 3 times less likely to have a positive SPT to egg at one year of age. They did not observe any effect in the frequency of atopic dermatitis at one year of age in the fish oil group but the severity of the disease was significantly less severe in that group (odds ratio, 0.09; 95% CI, 0.01-0.94; p=0.045). In addition, they showed a higher percentage of cord blood CD34+ hemopoietic progenitor cells (p<0.002), more IL-5 responsive eosinophil/basophil colony forming units (p<0.03) and lower cord plasma IL-13 (p<0.05) in the ω-3 LCPUFA supplemented group as compared to the placebo group. They also reported significantly reduced neutrophil LTB₄ production in neonates belonging to the fish oil group (p=0.031) and also a trend for higher production of LTB₅ in the same group.

The study by Krauss-Etschmann et al was originally designed to compare the effects of combined supplementation of pregnant women with ω-3 LCPUFA and folate on maternal and fetal plasma ω-3 LCPUFA proportion. They also studied the pregnancy outcome concerning

<table>
<thead>
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<th>Supplementation</th>
<th>Exposure time</th>
<th>Clinical follow up</th>
<th>Author Year Reference</th>
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<td>3.7 g/d ω-3 PUFA (27.7% EPA, 56% DHA)</td>
<td>gw 20 → delivery</td>
<td>1 year of age</td>
<td>Dunstan et al 2003 82, 109, 140-145</td>
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<td>atopic and non-atopic pregnant women</td>
<td>0.5 g/d DHA, 0.15 g/d EPA</td>
<td>gw 22 → delivery</td>
<td>no clinical follow up</td>
<td>Krauss-Etschmann et al 2008 146, 149</td>
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<td>non-atopic lactating mothers</td>
<td>4.5 g/d olive oil or high fish intake</td>
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<td>no clinical follow up</td>
<td>Lauritzen et al 2005 146, 150</td>
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<td>atopic and non-atopic pregnant women</td>
<td>2.7 g/d EPA+DHA</td>
<td>gw 30 → delivery</td>
<td>16 years</td>
<td>Olsen et al 2008 64, 147</td>
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</table>

<table>
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<th>Subjects Exposures</th>
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<td>non-atopic lactating mothers</td>
<td>4.5 g/d olive oil or high fish intake</td>
<td>first 4 months of lactation</td>
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<td>atopic and non-atopic pregnant women</td>
<td>2.7 g/d EPA+DHA</td>
<td>gw 30 → delivery</td>
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</table>
the separate effects of ω-3 LCPUFA and folate. Th2 related IL-4, IL-13 and CCR4 cord blood mRNA levels were significantly lower after fish oil supplementation (all p<0.001). Interestingly, when they stratified for maternal allergic disease they noted that the decrease of cord blood IL-13 mRNA levels in the fish oil group was more pronounced if the mother was non-allergic. No clinical data were collected.

The Danish study by Olsen et al was a three armed study originally designed to evaluate the effect of fish oil supplementation from gestational week 22 on the pregnancy duration, birth weight and birth length compared to placebo or no supplementation at all. Sixteen years later they performed a registry-based follow-up regarding offspring asthma and found a reduced hazard rate of allergic asthma by 87% in the fish oil group compared with the olive oil placebo group.

The study by Lauritzen et al was designed to examine whether fish oil supplementation to lactating mothers from one week after delivery and for 4 months could influence ω-3 LCPUFA levels in infant erythrocytes and visual acuity during the first year. Another report from this study aimed to examine the effect of ω-3 LCPUFA supplementation in lactation on later immune function. This report showed a four-fold higher LPS-induced production of IFN-γ in children in the fish oil group (p=0.034) than in the placebo group at 2 ½ years whereas the IL-10 production was similar. The percentage of atopic children and the levels of plasma IgE were the same in the groups but the study was on the other hand not powered to evaluate atopic sensitization. The increased production of IFN-γ in the fish oil group was interpreted as a faster maturation of the immune system. An increased ω-3 LCPUFA intake early in life may polarize the immune response later in life toward Th1-like immunity.

Finally, five groups have examined the effects of intervention with ω-3 LCPUFA during infancy and childhood. Four of these also assessed the clinical outcome of allergic diseases. These studies all showed that fish oil supplementation resulted in a higher ω-3 PUFA status in the infants, children and teenagers.

A large study performed in Australia, the Childhood Asthma Prevention Study (CAPS), had four arms and included a total of 616 children supplemented with tuna oil capsules, canola margarine and canola oil as ω-3 fatty acid intervention or sunola oil (monounsaturated high-oleic sunflower oil) as placebo with or without house dust mite reduction from 6 months of age till 5 years of age. Clinical assessment for eczema and parental questionnaires
regarding wheeze, cough, asthma history and eczema and also measurement of total serum 
IgE and SPT were performed at 18 months and at 3 and 5 years of age. A decreased 
prevalence of wheeze and lower serum IgE was reported at 18 months of age in the fish oil 
group (p=0.02) and higher plasma ω-3 PUFA levels were associated with less bronchodilator 
use regardless of supplementation group (p<0.001) 153-154. At three years of age, the fish oil 
supplementation was associated with a reduction in prevalence of cough (p=0.003) but no 
effects on the other endpoint parameters were observed 152. Follow up at five years revealed 
no association between ω-3 or ω-6 fatty acids and wheeze, eczema, or atopy 151.

A Danish study investigated the effect of fish oil mixed in milk or formula on healthy children 
from 9th to 12th months of age. Fish oil supplementation increased IFN-γ production from 
whole blood cultures (p=0.05) and showed a tendency to decrease the production of IL-10 
(p=0.08) 158. The increased IFN-γ production was in agreement with a previous study 146.

Higher levels of TNF, IL-1β, IL-6, IL-1ra and IL-10 was associated with 3 months fish oil 
supplementation in a study on healthy children aged 8-12 years 159. None of these two studies 
on healthy children reported clinical outcome.

Two additional studies supplied asthmatic older children and teenagers with fish oil for 6 or 10 
months 155-156. Nagakura et al 156 supplementing for 10 months showed significantly reduced 
asthma severity score and improved lung function from 6 months of fish oil supplementation 
while Hodge et al 155 showed no effect of fish oil supplementation possibly due to an 
intervention period of only 6 months.
AIMS OF THE THESIS

The overall aim of this thesis was to add knowledge on the relationship between LCPUFA, sensitization and allergic disease and possible immunological events regulating this.

The specific aims of the papers were:

Paper I: To explore if the proportions of cord serum phospholipid fatty acids have changed during a 20 year period from 1985 to 2005 and if possible changes are correlated with changes in total IgE levels.

Paper II: To evaluate the effect of ω-3 LCPUFA supplementation in pregnancy on maternal eicosanoid and cytokine production.

Paper III: To evaluate the effect of ω-3 LCPUFA supplementation during pregnancy and lactation on the risk of allergic sensitization and disease during the first year of life in children.

Paper IV: To evaluate the effect of ω-3 LCPUFA supplementation on immune components of breast milk in relation to sensitization and IgE associated disease during infancy.
HYPOTHESIS

- Paper I: We hypothesized that the proportions of LA and the LA/LNA ratio in CS collected between 1985 and 2005 had increased over time.

- Paper II: We hypothesized that the production of PGE$_2$ and LTB$_4$ would be lower after 15 weeks of $\omega$-3 LCPUFA supplementation as compared to before start of the intervention. In addition, we hypothesized that the production of pro-inflammatory IL-1, IL-6 and TNF would decrease during $\omega$-3 LCPUFA intervention.

- Paper III: We hypothesized that the incidence of sensitization and IgE associated disease would be reduced in the $\omega$-3 LCPUFA supplemented group as compared to the placebo group.

- Paper IV: We hypothesized that the levels of PGE$_2$ and pro-inflammatory IL-1, IL-6 and TNF would be lower in breast milk from $\omega$-3 LCPUFA supplemented mothers.
MATERIAL AND METHODS

The results of the papers included in this thesis are based on two cohorts. The first cohort is the study population in paper I and the second cohort in Papers II-IV.

Design and study subjects in Paper I

This paper presents a descriptive, observational pilot study investigating cord serum phospholipid fatty acid proportions in samples collected from children born between 1985 and 2005 at the delivery ward at the University Hospital in Linköping. The midwives were instructed to collect cord blood after every uncomplicated delivery with a full term healthy baby and the sample collection comprises approximately 39000 samples. In order to detect a change in the proportions of LA of 1 % over a twenty year period 60 samples were needed. Selection of cord serum samples from the first ten children (five boys and five girls) born during uneven months (i.e. January, March, May, July, September and November) every fifth year (i.e. 1985, 1990, 1995, 2000 and 2005) yielded a total of 300 samples for analyses. Phospholipid fatty acids were extracted and analyzed in the samples as well as total IgE.

Design Paper II-IV

These papers describe a double-blind, randomized, placebo controlled intervention study. The women were recruited through the antenatal ward or by advertisement in the local newspapers in Linköping and in Jönköping between March 2003 and June 2005.
Figure 10. Flow of participants through the study. Reasons for exclusion before randomization were: not meeting inclusion criteria n=28, declines participation n=15, other reasons n=7. Twenty five women discontinued the study before 15 weeks of supplementation 16 (23%) in the ω-3 group and 9 (12%) in the placebo group (n.s.). Reasons for discontinuation before delivery were inability to swallow capsules (n=9), nausea (n=6), abdominal pain (n=3), obstipation (n=1), urticaria (n=1), urinary tract infection (n=1), premature rupture of membranes (n=1), preeclampsia and missed experimental supplements (n=3) and were similar in the two groups.

The allergic status of the family was assessed by structured interviews performed by an experienced allergy research nurse. Present and former symptoms associated to allergic disease i.e. bronchial asthma diagnosed by a doctor, atopic eczema, allergic food reaction, itching and running nose at exposure to pollen, furry pets or other known allergens were investigated. At least one member of each family had to have ongoing or previous allergic symptoms. Exclusion criteria due to safety and methodological reasons were: maternal fish or
soybean oil allergy, ongoing treatment with anticoagulants and already ongoing supplementation with fish oil capsules.

The intervention started at gestational week 25 and the women were allocated to daily treatment with either 9 capsules á 500 mg containing 1.6 g EPA and 1.1 g DHA, i.e. a total of 2.7 g ω-3 LCPUFA (n=70) and 23 mg α-tocopherol as an antioxidant, or placebo treatment, i.e. 9 soybean oil capsules á 500 mg, in total 2.8 g soybean oil containing 2.5 g LA and 0.28 g α-linolenic (LNA) and 36 mg α-tocopherol (n=75).

The capsules were produced by Pharma Nord, Vejle, Denmark and the randomization was stratified at their facility. The intervention and placebo capsules had the same exterior and the fatty acid composition of the capsules was verified by gas chromatography at professor Strandvik’s lipid laboratory in Gothenburg (Table 4) since this was done before this method was established at our laboratory.

**Table 4. Fatty acid content of the capsules (mol %)**

<table>
<thead>
<tr>
<th></th>
<th>ω-3</th>
<th>placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>1.1</td>
<td>11.7</td>
</tr>
<tr>
<td>16:1ω-7</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>0.9</td>
<td>3.0</td>
</tr>
<tr>
<td>18:1ω-9</td>
<td>2.8</td>
<td>19.7</td>
</tr>
<tr>
<td>18:2ω-6</td>
<td>0.3</td>
<td>58.5</td>
</tr>
<tr>
<td>18:3ω-6</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>20:0</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>18:3ω-3</td>
<td>0.3</td>
<td>6.2</td>
</tr>
<tr>
<td>20:2ω-6</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>20:3ω-9</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>20:3ω-6</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>22:0</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>20:4ω-6</td>
<td>2.6</td>
<td>0.0</td>
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<td>24:0</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>24:1ω-9</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>22:6ω-3</td>
<td>35.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The fish oil was extracted from wild fat fishes such as sardines, menhaden and anchovies. The exact procedure for the extraction was confidential, but the process involved several steps of extraction of the entire fish followed by enrichment and purification. A daily dose of 9
capsules adds approximately the same amount of ω-3 fatty acids as one portion of 100 g salmon daily. The current recommendation for the whole population including pregnant women from National Food Administration in Sweden is 2-3 fish meals a week\textsuperscript{74}. The average daily intake of ω-3 fatty acids in Sweden is approximately 0.3 g/day and the fish oil supplementation in this study gave almost 10 times this amount daily.

Soy bean oil was used as placebo. It comprises mainly of LA and oleic acid. The LA/LNA ratio in the ω-3 and the placebo capsule was 1 and 9.4 respectively. A supplementation with 9 placebo capsules corresponds to 1/3 of the daily average intake of LA in Sweden.

The choice to start the intervention at the 22\textsuperscript{nd} gestational week was based on earlier studies showing no adverse effects from supplementation with the same amount of ω-3 LCPUFA starting in gestational week 25 and also on data demonstrating the first fetal T-cell response to allergen at approximately 22 weeks of gestation\textsuperscript{33,160}.

The research schedule is presented in Table 5.

\textbf{Table 5.} Sampling and examination schedule for mother and child. A serum sample from the father was also collected once during the study period (CB, cord blood; SPT, skin prick test).
Study subjects paper II-IV

**Paper II**

This paper was based on 120 (ω-3: n=54 and placebo: n=66) women completing the study from the start of intervention at gestational week (gw) 25 and at least until one week post partum. Blood samples were drawn before the start of intervention and approximately one week post partum. Phadiatop (n=120; ω-3: n=54 and placebo: n=66) was analyzed at baseline and phospholipid fatty acids were analyzed at both sampling occasions; in gw 25 (n=118; ω-3: n=53 and placebo: n=65) and one week post partum (n=112, ω-3: n=51 and placebo: n=61); to assess compliance. Characteristics for the study population are presented in Paper II in Table 1.

LPS stimulated whole blood cultures for eicosanoids and cytokine analysis were established, but due to logistic reasons only in Linköping and cultures from both occasions were available from 59 (ω-3: n=28 and placebo: n=31) mothers.

**Paper III**

This paper includes clinical data on 117 children whose mothers completed the intervention for a minimum of 15 weeks. Detailed information about the mothers and their babies at inclusion and delivery is presented in Paper II in Table 1. Two women gave birth to twins but only the first born was included in the analysis. The children were followed to one year of age.

**Paper IV**

This paper includes data on breast milk samples collected from mothers completing the intervention study for a minimum of 15 weeks. Samples were collected as colostrum (n=107; ω-3: n=47 and placebo: n=60) and mature milk at 1 month (n=96; ω-3: n=42 and placebo: n=54) and 3 months (n=91; ω-3: n=40 and placebo: n=51). Cytokines and antibodies were related to allergic symptoms *i.e.* eczema and/or food reaction and IgE associated allergic symptoms in the infant at one year of age.
Clinical methodology

The clinical examinations were similar in Paper III and IV. The examinations of the infants were performed by an experienced allergy research nurse at 3, 6 and 12 months. A pediatrician was consulted if allergic symptoms were present and the pediatrician also performed the clinical assessment of all children at 12 months of age.

An internal work shop for pediatricians and research nurses involved in the study was arranged to ensure consistency of the diagnosis in both study centers.

Food reaction was defined as gastrointestinal symptoms, hives, aggravated eczema or wheeze after food intake. Recovery from symptoms after elimination of the particular food from the diet and reoccurrence after ingestion of the food was required for the classification.

The diagnostic criteria used in this thesis are from the criteria by Sampson adapted by Seymour, for atopic eczema/dermatitis syndrome (AEDS) < 2 years (Table 6). Three criteria were needed for diagnosis of eczema and pruritus (itching) was mandatory. At least two of the criteria needed to be major. All children in the study had allergic disease in the family, hence that criterium, which is part of the definitions by Samson and Seymour was fulfilled for all children.

<table>
<thead>
<tr>
<th>Major features</th>
<th>Minor features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evidence of chronic pruritic dermatitis</td>
<td>Xerosis/ichthyosis/hyperlinear palms (dry skin/thick scaly skin/</td>
</tr>
<tr>
<td>Typical facial or extensor eczematous or lichenified or nummular dermatitis</td>
<td>Peri-auricular fissures (Cracks under the ears)</td>
</tr>
<tr>
<td>Eczema-free skin of nose-mouth area and/or diaper area</td>
<td>Chronic scalp scaling</td>
</tr>
</tbody>
</table>

Table 6. Diagnostic criteria for infants and toddlers used in Paper III and IV.

Ref. 161-162
Allergic symptoms were defined as one or both of food reaction and eczema. The allergic symptoms were classified as IgE associated allergic disease if the child was sensitized i.e. had a positive skin prick test or positive test for circulating specific IgE.

Skin prick tests (SPT) were performed as single tests, at 6 and 12 months on the volar aspects of the forearms with skimmed cow’s milk, egg white and wheat flour mixed with water. Histamine hydrochloride (10 mg/ml) served as a positive control and albumin diluent as negative control, both from ALK-ABELLÓ, Hørsholm, Denmark (Soluprick®). A wheal diameter ≥ 3mm was considered as positive.

**Questionnaires**

At inclusion, the family answered a questionnaire about family members, family history of allergic disease, if the mother planned to breast feed her child, maternal soy bean allergy, ongoing fish oil supplementation and the present gestational length. The mothers were asked to complete a 3-days food diary at inclusion and when the baby was 6 months old. The data was processed by a dietician using the software “Dietist XP” (Kost- och näringsdata, Bromma, Sweden) to assess the maternal intake of energy and fat according to routine clinical praxis. The amount of each provision was estimated from the registered weight, volume or number of portions.

When the children were 3, 6 and 12 months the parents were asked to complete questionnaires regarding their child on breast feeding, introduction to food stuffs, allergic symptoms, living conditions, pets etc.
Laboratory methodology

Whole blood cultures (Paper II)

Whole blood cultures were established as described in Paper I. Median time between blood sampling and establishment of whole blood culture was 1.6 h (SD: ± 1.8h) at gw 25 weeks and 1.3 h (SD: ± 1.6 h) at the first week after partus.

Analysis of phospholipids (Paper I-IV)

Phospholipid fatty acids in serum or plasma were extracted and analyzed as described in Paper I, II and IV. The analysis in Paper II and III were performed before that in paper I. C21:0 (30 mg/50 ml hexane) was used as an internal standard (IS) for the relative determination of phospholipids in Paper II and III, and C17:0 (570 mg/L) in Paper I. The establishment of a new internal standard had to be done since C21:0 was not available in a new reference standard from Nu-Chek Prep Inc, MN, USA. The choice of C17:0 as a suitable alternative was based on a report by Krauss-Etschmann et al.148.

The principle for gas chromatography is based on a distribution between a mobile phase (carrier gas) and a stationary phase (in the column). The sample is vaporized in the injector and transported in the carrier gas through the column. The velocity through the column for the different compounds in the sample is determined by their distribution between the two phases. The distribution depends on their affinity to the stationary phase and the temperature. Differences in distribution ratio for different compounds results in a separation and the compounds leave the column one by one and are registered by a detector resulting in a chromatogram. The chromatogram gives both qualitative and quantitative information of the sample composition. The area of each individual peak is proportional to the amount of the compound. The retention time (the time it takes for a specific amount to reach the detector) is used for identification of the compound.

A reference standard comprise of pre-defined concentrations of the fatty acids to be analyzed. Thus, the peak area and a response factor, consisting of the ratio between the amount and the area of every fatty acid, were reported for every fatty acid within the reference standard. An internal response factor was calculated for every included fatty acid by dividing the response factor for a single fatty acid from the reference standard with the response factor for the IS. When calculating the concentrations of an individual sample the amount of the IS was
multiplied with the area for the specific fatty acid and with the internal response factor for the specific fatty acid and the product was divided with the area of the IS. The relative concentration was calculated as the ratio between the amount of the single fatty acid and the total amount of fatty acids analyzed multiplied by 100. A serum sample from a blood donor was extracted and analyzed together with the samples in every run as a quality control. The inter-assay CV% was 3.5 %, 4 % and 7 % for LA, LNA and EPA, respectively, in Paper II and III. In Paper I the inter-assay CV% was 1.2 % for LA and 1.7 % AA and 2.7 % for EPA.

**Analysis of PGE$_2$, LTB4 and cytokines from whole blood cultures (Paper II)**

Eicosanoids in cell supernatants were analyzed with commercial ELISA kits from R&D Systems (Abingdon, UK) according to the manufacturer’s instructions. We evaluated the addition of indometacin 10 μg/ml (a COX1 and COX2 inhibitor, counteracting the formation of new eicosanoids) to the cell supernatant as recommended by the manufacturer but no effect was observed. Since indometacin is toxic we therefore excluded it from the procedure.

Detection limits were 11.9 pg/ml for LTB$_4$ and 39.0 pg/ml for PGE$_2$. An LPS-stimulated whole blood culture supernatant from a blood donor was analyzed in every run as a quality control. The intra assay coefficient of variance was 7 % for PGE$_2$ and 30 % for LTB$_4$. Inter assay CV were 3 % and 5 % respectively. It was not possible to draw any conclusion from the LTB$_4$ results possibly due to its high CV %. The large CV% for the LTB$_4$ analysis was a great disappointment since the CV% was much lower, < 5 % when the test was initially investigated.

Cytokine secretion in cell supernatants from the 24 hour incubation was analysed on the XMap Luminex® 100 System using Bio-Plex Cytokine Assay (Bio-Rad, Stockholm, Sweden) including reagents for the detection of IL-1β, IL-5, IL-6, CXCL-8, IL-10, IL-12p70, IFN-γ, TNF, CCL-2 (MCP-1) and CCL3 (MIP-1α), according to the manufacturer’s instructions. Detection limit was 1pg/ml for IL-1β, IL-6, CXCL-8, IL-10, IL-12p70 and CCL3 (MIP-1α), 2 pg/ml for TNF, 0.8 pg/ml for IL-5 and 10 pg/ml for IFN-γ and CCL-2 (MCP-1). CV% for the Luminex quantified samples varied between 1 and 20 %.
Analysis of PGE$_2$ and cytokines in human milk (Paper III)

The breast milk analyses were performed at the laboratory in Linköping but also at Danone Research - Centre for Specialised Nutrition, Wageningen, The Netherlands. An extensive cytokine panel including, IL-1ß, IL-2, IL-4, IL-5, IL-6, CXCL-8, IL-10, IL-12p40/p70, IL-13, GM-CSF, TNF, IFN-γ was analyzed with Luminex® xMAP® technology at Danone Research centre. This panel was supplemented in Linköping with some additional parameters i.e. PGE$_2$, SIgA, TSLP and TGFβ2 which were not available at the Danone laboratory.

Two different multiplex kits from Bio Rad and Biosource with Luminex® xMAP® technology were carefully evaluated for breast milk analyses. Comparisons between the two kits were performed for whole milk vs. aqueous phase. In addition, treatment of milk in order to improve recovery, e.g. sonification, acidification, determination of recovery of cytokines added to milk samples, control for matrix effect of milk in the assay and determination of dilution of milk samples were also evaluated. The best result was achieved with undiluted, sonificated whole milk samples analyzed with the kit from Biosource. The samples were measured in triplicates.

Analysis of SIgA, TGFβ2, PGE$_2$ and TSLP were performed on milk whey in Linköping. The thawed milk samples were centrifuged twice in order to remove milk fat and cellular debris. The first centrifugation was performed at 680 x g for 10 minutes at 4°C and the interface below the fat layer was then collected. A second centrifugation was performed at 10000 x g for 30 minutes at 4°C. The interface between the fat layer at the top and the cells in the bottom was collected and re-frozen. A mature breast milk sample from a donor was used for evaluation and as internal control sample for parameters analyzed in Linköping.

Prostaglandin E$_2$ was analyzed with Amersham Prostaglandin E2 Biotrak Enzyme immunoassay (EIA) system (cat no RPN222) and TSLP Quantikine® Human Immunoassay kit (cat no DTSLLP0). A purification kit was evaluated as recommended by Laitinen et al. but as 90 % of both PGE$_2$ and TSLP were lost in the column we excluded this step. To evaluate the influence of breast milk components on the measured parameter, known amounts of PGE$_2$ and TSLP from the standard included in the kit were added to milk samples and then analyzed. Recovery for PGE$_2$ varied between 81-102 % and for TSLP between 94-98%. Mean
intra assay coefficient of variation for PGE\(_2\) was 5 % and mean inter assay coefficient of variation was 14 % for the control sample.

The levels of breast milk SIgA were analyzed with an in-house ELISA established in an earlier project\(^{76-77}\). In Paper IV a new standard (human IgA, I-1010, Sigma) and new coating and detection antibodies (monoclonal anti-human secretory component clone Ga-I I6635 and anti-human IgA peroxidase conjugate, A0295, Sigma) were evaluated as the previous reagents were not available any longer. Mean intra assay coefficient of variation was 3 % and mean inter assay coefficient of variation was 14 % for the control sample.

TGF\(_\beta\)2 was analyzed using a Quantikine® Human TGF\(_\beta\)2 Immunoassay kit from R&D (cat no DB250). In order to activate latent TGF\(_\beta\)2, the samples were acidified according to\(^{90}\) before analysis. The samples were analyzed in duplicate and the intra assay CV was 3 % and inter assay CV was 5 % for the control sample.

**Total and specific IgE (Paper I-IV)**

Total IgE and circulating IgE against specific allergens were analyzed according to ImmunoCAP™ technology provided by Phadia AB (Uppsala, Sweden). The methods and allergens are described in more detail in the respective papers.

**Statistical methods**

The power calculation in paper I was based on data from a previous interventional study\(^{164}\) where we reported the mean proportion of LA to be 6.3 +/- 1.0 mol% in CS phospholipids. To reveal a difference of 1% unit on LA concentrations over a 20 year period with a p-value < 0.05 and 80% power 60 samples were needed. However, 300 samples were analyzed in order to overcome possible quality problems.

The power calculations in paper II-IV were based on information from a previous study including families with a history of previous or present allergic disease. They found that the cumulative incidence of allergic symptoms within the first 18 months of life was 60%\(^{165}\). In order to be able to detect a 40 % difference in the development of allergic symptoms between the intervention group and the placebo group, with a power of 85% and a probability level of 5 %, we had to include 61 women in each group. Experiences from earlier studies at our
research facility show that approximately 10% chose to leave the study before it is completed. In order to compensate for this we invited 185 pregnant women to the study. Samples with cytokine and chemokines levels below the detection limit were given half the cut-off value to enable statistical analyses. As the data of cytokine, chemokines, eicosanoids, SIgA and IgE were not normally distributed, non-parametrical statistical tests, corrected for ties, were employed. Unpaired data were analyzed with Mann Whitney U-test and paired data with Wilcoxon signed rank test. Correlations were calculated with Spearman rank correlation test. The $\chi^2$ test was used for nominal variables and Fischer’s exact test when the expected frequency for any cell was less than 5. Friedman’s test was used in analyses of multiple longitudinal measures obtained from the same object and Kruskal Wallis test was used in subjects of different groups. Student’s t-test was used for normally distributed data to compare the means of the continual variables. ANOVA was used in analyses of multiple longitudinal measures of phospholipid fatty acids in subject in different groups. Binary logistic regression was used to calculate the odds ratios for developing allergic sensitization and disease in the $\omega$-3 supplemented groups compared to the placebo group. A difference together with a $p$-value < 0.05 was considered to be statistically significant, except for the analysis of cytokines and chemokines in Paper II where differences were considered significant at a $p$ value < 0.01 as a consequence of multiple comparisons. Statistic analyses in Paper I, II and IV were performed using Stat View for Windows Version 5.9 (SAS Institute Inc. NC, USA). Analyses in Paper III and Figure 1 in Paper I were made with SPSS 15.0 or 18.0 (SPSS Inc, Chicago, IL, USA).

**Ethical considerations & Safety**

Both studies were approved by the Regional Ethics Board at Hälsouniversitetet, Linköping University, Linköping, Sweden. Since the samples in Paper I were coded and analyzed with knowledge about sex and date of birth only no informed consent from the parents or the now grown up children was needed according to the Regional Ethics Board.

All families participating in the intervention study reported in Paper II-IV gave written informed consent. Pharma Nord had no impact in the study design, data collection, data analysis, data interpretation or publication of the results presented in Paper II-IV.
Clinical studies on pregnant women and their infants may be hazardous to implement and are legitimate only if similar results cannot be obtained on adults, as this study on the development of allergy and immunity during childhood.

The parents were thoroughly informed on study aims, the background and practical details regarding sample collection and clinic examinations. They were also carefully instructed about the confidentiality protection and that their participation was voluntary and they could withdraw their participation anytime without giving any reason. Informed consent can of course not be given by the infant itself and it may seem unethical to include such subjects. On the other hand, excluding these groups from research can also be unethical and discriminating.

Participation in a research project is associated with both disadvantages and advantages. Both the mother and the child are subject to blood sampling at several occasions. This constitutes a minimal risk unless too large amounts are drawn at too close intervals. Sensitization from skin prick testing, when an allergen is inoculated in the skin, has not been shown even in high-risk individuals. To minimize pain at blood sampling a topical anesthetic cream, EMLA, was used which diminishes the discomfort for the child. The sampling is only performed by experienced personnel. The skin prick test can also be painful but is part of normal clinical practice for children with allergic disease.

The family may benefit from an earlier allergy diagnosis including professional advice and care through the close contacts with experienced research nurses and pediatricians.

Regarding the pregnancy safety, there have been several studies on ω-3 LCPUFA supplementation performed over the years. Slightly longer gestational length has been reported in some but not all studies. Taken together, ω-3 LCPUFA supplementation during pregnancy did not cause any foetal growth impairment or serious adverse outcome during delivery that could be related to the supplementation. Adverse events reported particularly by mothers supplied with ω-3 LCPUFA were belching and unpleasant taste related to the intake of the capsule. Thus, ω-3 LCPUFA supplementation from mid-pregnancy does not seem to endanger pregnancy outcome or foetal development.

Sea food and fish oil may contain toxic contaminants, including polychlorinated biphenyls (PCB) and methyl mercury. The benefit of fish intake seems to exceed the potential risks at
least among adults. Women of childbearing age also benefit from a modest fish intake as long
as they avoid selected species from lakes. The fish oil capsules provided in this study are
manufactured by Pharma Nord in Denmark and the procedure involves removal of pollutants.
According to the supplier, an independent quality control with respect to dioxin and PCB
levels in fish oil preparations was recently performed on the capsules included in this study.
The content of pollutant was below the limits and they claim that you have to eat 826 capsules
a week before the limit for PCB and dioxin is reached. The investigation also revealed that
100 grams of salmon contain 20 times more dioxin than 9 fish oil capsules.

These kinds of studies also raise other ethical questions. What would happen if a large
proportion of the population in the affluent world would demand a daily dietary supply of ω-3
LCPUFA capsules? Would it be possible to meet such demands? And would it be possible
without compromising nutritional needs for people in the developing world?
RESULTS AND DISCUSSION

Paper I

The set of cord blood serum samples collected during two decades between 1985 and 2005 offers a unique possibility to study compositional changes over time. We aimed to reveal alterations in the content of ω-6 and ω-3 fatty acids in response to the intake of fatty acids i.e. to a putatively increased consumption of vegetable oils containing especially LA and a decreased intake of ω-3 fatty acids coincide with the increased prevalence of allergic diseases. The phospholipid fatty acid composition of umbilical blood should be indicative of fatty acid exposure of the fetus during fetal life; it is influenced by multiple factors, such as maternal diet and metabolism, but also by active placental transport of fatty acids. A higher maternal intake of LA yields higher concentrations of cord blood LA.

The proportions of LA and LNA decreased between 1985 and 2005. However, the LA/LNA ratio increased during the same period revealing that LNA decreased relatively more than LA. Arachidonic acid remained unchanged between 1985 and 1995, increased significantly from 1995 to 2000 and leveled off thereafter. Also the DHA proportions were similar between 1985 and 1995 but increased steadily thereafter.

We could not find any correlation between CS PL fatty acids and CS total IgE.

Cord blood total IgE was chosen as a predictor for allergic disease. It has been suggested as a predictor for IgE-mediated disease, but the value has been questioned. An alternative method could have been to analyze chemokines in the cord blood samples, as they can readily be measured in serum. A recent study showed that development of allergic disease during the first two years of life was associated with a more Th2- like immunity, i.e. with increased levels of Th2-associated CCL22 and higher ratios of CCL22/CXCL10 and CCL22/CXCL11 in cord serum.

The long storage-time could have influenced the condition of the phospholipid fatty acids in the serum samples, but to the best of our assessment there were no alarming alterations. Thus, the quantity of the total phospholipid fatty acids in mg/L, was not significantly changed between 1985 and 2005. More over qualitatively the chromatograms, of samples with different storage time were almost identical with the same number of peaks and the same appearance.
The impact of storage time was previously evaluated as a condition of concern in a study on serum phospholipid fatty acids and breast cancer\textsuperscript{177}, where some degradation of fatty acids was observed over time especially for \(\omega-3\) fatty acids. However, \(\omega-3\) fatty acids levels correlated with fish intake, indicating that the fatty acid measurements reflected the diet rather well even though some \(\omega-3\) fatty acids may have been lost during the storage. Their study was therefore performed as planned\textsuperscript{177}.

The effect of long-time storage in \(-20^\circ\text{C}\) on IgE antibodies has also been evaluated in a couple of studies suggesting that the antibodies are stable up to 30 years in the freezer\textsuperscript{178-179}.

Contrary to the first part of our hypothesis, this study disclosed a significant decrease in both LA and LNA proportions in CS phospholipids in samples collected from 1985 to 2005. However, the second part of the hypothesis was corroborated, LNA decreased relatively more than LA resulting in an increased LA/LNA ratio over this period of time. In addition, the proportion of AA increased together with EPA and DHA. The relative decrease in LA in this study is surprising, as the common belief is that the intake of LA has increased markedly during recent decades\textsuperscript{180}, this ought to be reflected in CS PUFA phospholipid PUFA profiles as shown be others\textsuperscript{172}.

The Food and Agricultural Organization Statistical Database (FAOSTAT) provides statistics from over 200 countries on agriculture, nutrition, fisheries, forestry, food aid, land use and population. They present food balance sheets that represent a comprehensive picture of the pattern of a country's food supply during a specified reference period. Data from the FAO food balance sheet has been related to ISAAC data on the prevalence of asthma, rhinitis and eczema by Black \textit{et al}\textsuperscript{103}. They suggested that differences in the consumption of LA could to some extent explain the variation in the prevalence of allergic diseases. The main source of LA is from vegetable oils, such as soybean oil, sunflower oil and safflower oil. Fat supply from vegetable oils has decreased in Sweden between 1985 and 2005 and this may explain the unexpected results in this study. We are well aware that the data from FAO is crude and only represent an estimation for the whole population and not for pregnant women specifically. As diet recommendations for pregnant women do not include vegetable oil one might suspect that the intake is an average also for pregnant women. Nevertheless, the decrease in fat supply from vegetable oils corresponds to some degree with the decreasing LA levels in CS phospholipids reported in this study.
**Paper II-IV**

*Effects of the supplementation on phospholipid fatty acids*

Phospholipids are the richest source of PUFA in the human body and changes in the LCPUFA profile are most evident in the PL fraction. In addition, phospholipids in general have a slower turnover than free fatty acids, triglycerides and cholesterol, consequently giving the best reflection of a long term PUFA status.

Phospholipid fatty acids were analyzed in maternal serum/plasma at inclusion, before the intervention started and one week post partum after 15 weeks of supplementation.

Incorporation of ω-3 LCPUFA reaches peak levels after approximately four weeks of supplementation according to Yaqoob *et al* [105] and significant changes were also observed in this study (Figure 12).

In the ω-3 supplemented group did the proportions of EPA and DHA increase at the expense of both LA and AA but also LNA. Importantly, the proportions of LA were not altered in the placebo group despite the additional LA provided in the capsules. The proportions of AA was however increased but we consider this as a physiological phenomenon as this has been observed previously in women soon after delivery [181]. Both EPA and DHA proportions and the AA/EPA ratio remained unchanged in the placebo group during the intervention.
Figure 12. Changes in serum/plasma phospholipid fatty acids after 15 weeks of supplementation. (LA, linoleic acid; LNA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid).

Optimization of whole blood cultures

The optimal LPS dose and incubation times for PGE\(_2\) and LTB\(_4\) measurements were chosen based on pilot experiments. The tested LPS doses and culture times were 10, 100 and 1000 ng/ml at 1, 4, 18, 24 and 48 hours. The cytokine levels were not indicative in this experiment. One hundred ng/ml LPS (Escherichia coli 026:B, Sigma Chemicals Co) was chosen for stimulation, for 1 h for the measurement of LTB\(_4\) and for 24 h for the measurement of PGE\(_2\) according to Figure 13 and 14.
Figure 13. LTB₄ kinetics in supernatants from whole blood cultures stimulated with 10, 100 and 1000 ng/ml LPS and incubated for 1, 4, 18, 24 and 48 hours. The spontaneous response was subtracted from cultures including LPS.

Figure 14. PGE₂ kinetics in supernatants from whole blood cultures stimulated with 10, 100 and 1000 ng/ml LPS and incubated for 1, 4, 18, 24 and 48 hours. The spontaneous response was subtracted from cultures including LPS.

This is, to the best of our knowledge, the first study exploring eicosanoid production in whole blood cultures after ω-3 LCPUFA supplementation to pregnant women. The advantage of whole blood cultures includes the fact that all blood components are retained and all cell types and soluble components at the same ratios as in vivo. Blood cells are also relatively unaffected since they have not been subjected to aggravation due to cell isolation and washing steps. Yaqoob et al have shown that cytokine measurements from whole blood cultures are comparable with the commonly occurring peripheral blood mononuclear cell cultures but also that there are substantial inter-individual variations although the production of any given cytokine by a particular person is stable ¹⁸².
von Aulock et al. measured eicosanoids from whole blood cultures in response to LPS with the same culture time and the same amount of LPS, as used in Paper II, although extracted from Salmonella abortus equi in contrast to ours extracted from Escherichia coli. They reported four times higher levels of PGE\(_2\). This could possibly be due to a higher availability to cell culture media providing greater access to nutrients or the proposed altered possibilities by different bacterial LPS to stimulate blood cells.

One of the disadvantages with whole blood cultures is the lack of information about the number of cells included in the culture. We were especially interested in the number of CD14+ monocytes as LPS acts through the CD14 receptor, or if there were any more precise differences between the numbers of monocytes in the cultures from the two intervention groups. To control this we used a Flow Cytometry Absolute Count Standard™ from Bangs Laboratories, Inc, Fishers, IN, USA. This standard is a precise counted population of microspheres for estimating counts of cells via flow cytometry. The microspheres are ~7-9\(\mu\)m in diameter and were internally labeled with multiple fluorophores for excitation with common lasers and discrimination from the cell population. By evaluating the ratio of microspheres to CD 14+ cells gated as monocytes, the volumetric number of cells may be determined.

There was no difference between the two intervention groups in the absolute count of monocytes in whole blood cultures established either in gw 25 or one week post partum (Table 7).

<table>
<thead>
<tr>
<th></th>
<th>(\omega-3) group</th>
<th>placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ((10^6/ml))</td>
<td>SD</td>
</tr>
<tr>
<td>gestational week 25</td>
<td>20</td>
<td>0.24</td>
</tr>
<tr>
<td>one week after partus</td>
<td>28</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The LPS-induced secretion of PGE\(_2\) or LTB\(_4\) did not correlate with the amount of CD14+ monocytes in the culture. This might possibly imply that the secretion of eicosanoids was not determined by the number of cells but by something else, possibly the amount of AA as phospholipids in the cell membrane.
Eicosanoid secretion in whole blood cultures

Eicosanoids, cytokines and chemokines were analyzed in whole blood supernatant with or without LPS. The results from the mothers are presented in Paper II. Since there was an initial difference in LPS-induced PGE\(_2\) secretion between the two intervention groups already at inclusion (Figure 15) we chose to analyze the changes in PGE\(_2\) secretion between the sampling occasions at inclusion and 1 week post partum rather than of absolute levels.

![Graph showing LPS-induced PGE\(_2\) secretion](image)

**Figure 15.** There was an initial difference in LPS-induced PGE\(_2\) secretion before the start of intervention.

The LPS-induced PGE\(_2\) 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 (Figure 16). However, LPS-induced PGE\(_2\) secretion decreased in a majority of ω-3 supplemented mothers but increased in the placebo-supplemented mothers.
The LPS-induced PGE$_2$ secretion increased in the placebo treated group ($p<0.001$) between gw 25 and partus but remained unaltered in the ω-3 treated group. LPS-induced PGE$_2$ secretion decreased in a majority of ω-3 supplemented mothers but increased in a majority of placebo-supplemented mothers ($\chi^2, p=0.002$).

Similarly to the mothers, whole blood cultures were established in blood samples from the children at birth and at 3 and 12 months of age. Analyses of PGE$_2$ in these cultures revealed that the levels of spontaneous PGE$_2$ were highest in cord blood and decreased thereafter (Figure 17), this was however not seen for LPS-induced PGE$_2$ secretion (data not shown).

Samples from children whose mothers were induced to labour with prostaglandin were excluded from this analysis. The higher levels of PGE$_2$ observed in cord samples might be a reflection of labour since the levels of several eicosanoids including PGE$_2$ are elevated in circulation during labour$^{186}$. No differences were observed with respect to the intervention.

The significantly higher spontaneous PGE$_2$ secretion in the ω-3 LCPUFA group compared to the placebo group (Figure 17) was unexpected and difficult to explain. No differences between the delivery time and the time point for the establishment of whole blood cultures could be found (ω-3: 9 hours; placebo: 11 hours, n.s).
Clinical outcome and sensitization

The period prevalence of eczema was similar in the ω-3 group and the placebo group during the first year (21% vs. 29%). Neither was the period prevalence of food reactions affected by the intervention (Table 7). The period prevalence of IgE associated eczema tended to be significantly lower in the ω-3 treated group compared to the placebo group at the age of 6 months (8% vs. 20%, p<0.06) and reached a significant difference at the age of 12 months (8% vs. 24%, p<0.02). Food allergy was also significantly less frequent in the ω-3 group compared to the placebo group up to the age of 12 months (2% vs. 15%, p<0.01).

The period prevalence of any positive SPT was significantly lower (15% vs. 32%, p=0.04) and fewer children had detectable circulating IgE (16% vs. 29%), although not statistically significant, in the ω-3 group during the first year (Table 8). The prevalence of positive SPT to egg tended to be significantly lower in the ω-3 group as compared to the placebo group at both 6 and 12 months. However, the period prevalence of positive SPT to egg was significantly lower in the ω-3 group up to 12 months of age (Table 8). No difference for sensitization to milk was observed between the groups.

We also found that infants of mothers without allergic symptoms to a lesser degree had any positive SPT, 2/14 (14%) in the ω-3 supplemented group and 11/23 (48%) in the placebo group.
group (p<0.05). The results were similar regarding food allergy (0% vs. 25%, p<0.05) and IgE associated eczema (0% vs. 29%, p<0.05). Corresponding differences were not observed among children of mothers with allergic symptoms (Paper III). This observation might be caused by polymorphisms in genes regulating the fatty acids metabolism and will be discussed later.

Table 8. The prevalence and period prevalence of skin prick test ≥ 3 mm, circulating IgE ≥ 0.35 kU/L against egg and milk and allergic diseases in children completing the study until 12 months of age.

<table>
<thead>
<tr>
<th></th>
<th>ω-3</th>
<th>placebo</th>
<th>p-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eczema 0-12 m</td>
<td>11/52 (21%)</td>
<td>19/65 (29%)</td>
<td>ns</td>
</tr>
<tr>
<td>Food reaction 0-12 m</td>
<td>4/52 (8%)</td>
<td>10/65 (15%)</td>
<td>ns</td>
</tr>
<tr>
<td>IgE associated eczema 0-6 m</td>
<td>4/52 (8%)</td>
<td>13/65 (20%)</td>
<td>0.06</td>
</tr>
<tr>
<td>IgE associated eczema 0-12 m</td>
<td>4/52 (8%)</td>
<td>15/63 (24%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Food allergy 0-12m #</td>
<td>1/52 (2%)</td>
<td>10/65 (15%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Any SPT 0-12 m</td>
<td>8/52 (15%)</td>
<td>20/63 (32%)</td>
<td>0.04</td>
</tr>
<tr>
<td>IgE 12m</td>
<td>7/45 (16%)</td>
<td>14/48 (29%)</td>
<td>ns</td>
</tr>
<tr>
<td>Pos SPT egg 6m</td>
<td>4/52 (8%)</td>
<td>13/65 (20%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Pos SPT egg 12m</td>
<td>6/52 (12%)</td>
<td>16/63 (25%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Pos SPT egg 0-12m</td>
<td>6/52 (12%)</td>
<td>18/63 (29%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Circulating IgE egg 12m</td>
<td>4/46 (9%)</td>
<td>10/48 (21%)</td>
<td>ns</td>
</tr>
<tr>
<td>Pos SPT milk 6m</td>
<td>3/54 (6%)</td>
<td>5/65 (8%)</td>
<td>ns</td>
</tr>
<tr>
<td>Pos SPT milk 12m</td>
<td>2/54 (4%)</td>
<td>5/65 (8%)</td>
<td>ns</td>
</tr>
<tr>
<td>Pos SPT milk 0-12m</td>
<td>4/54 (7%)</td>
<td>7/65 (11%)</td>
<td>ns</td>
</tr>
<tr>
<td>Circulating IgE milk 12m</td>
<td>5/48 (10%)</td>
<td>8/48 (17%)</td>
<td>ns</td>
</tr>
</tbody>
</table>

* χ²
$ Eczema with positive SPT and/or detectable circulating IgE towards egg, milk or wheat
# Food reaction with positive SPT for the offending food stuff and/or positive circulating IgE

Previous intervention studies investigating the effect of ω-3 LCPUFA supplementation during pregnancy and/or lactation on the atopic outcomes in infancy and adolescence and are
described earlier in this thesis. To the best of our knowledge, our study is the only one originally designed to evaluate the effect on allergic manifestations and also the only one supplementing both during pregnancy and lactation. Our clinical results are in agreement with those in the previous studies but differences in design among these studies make comparisons difficult. The studies differ with respect to dose of ω-3 LCPUFA (0.65-3.7 g/d), intervention during only pregnancy, only lactation or both and follow up time (1-16 years). They also differ with respect to the choice of placebo (olive oil or soybean oil) (Table 2).

In the study by Dunstan et al, children in the fish oil group were three times less likely to be sensitized to egg (OR, 0.34; 95% CI, 0.11-1.02; p=0.055) and 10 times less likely to have a SCORAD index >25 (OR, 0.09; 95% CI, 0.01-0.94; p=0.045). In addition, children in the fish oil group were overall less likely to have food allergy, asthma, chronic cough, recurrent wheeze, angioedema or anaphylaxis or a positive SPT to egg, peanuts, cow’s milk, house dust mite or cat. None of these differences were however statistically significant.

The study by Olsen et al was initiated in 1990 and diagnoses were extracted from a registry 16 years later. In the fish oil group were 8/263 children in the fish oil group and 11/136 children in the olive oil group diagnosed with asthma (hazard rate ratio, 0.37; 95% CI: 0.15-0.92; p=0.03). In addition, were 2/263 children in the fish oil group and 8/136 in the olive oil group diagnosed with allergic asthma (hazard rate ratio, 0.13; 95% CI, 0.03-0.6; p=0.01).

There was no difference in SCORAD index between infants in the two intervention groups in our study. A SCORAD index was calculated at every visit but was consistently very low, mean value in the fish oil group was 13.3 and in the placebo group 17.8 (ns). A severe eczema has a SCORAD index > 25. We speculate that the low figures in our study is due to the fact that the infants belong to families already familiar with the phenotype of eczema and therefore starting the treatment at an early stage. Pruritus is a substantial part of the SCORAD judgment but is difficult to evaluate in an infant.

The less frequent prevalence of sensitization among ω-3 LCPUFA supplemented infants in our study suggests some kind of impact on IgE synthesis which also is in agreement with the theory behind the intervention. As a consequence of the supplementation do the proportions of EPA and DHA increase in cell membranes, counteracting the proportions of AA and
thereby decreasing the synthesis of PGE$_2$. This may lead to a less Th2 like immunity and thereby hamper the development of IgE.

\textit{Adverse events}

The most common adverse event in the $\omega$-3 LCPUFA supplemented group were belching and this has previously been reported when similar doses of fish oil was given in pregnancy\textsuperscript{64,160}. The increase in gestational length reported in several large $\omega$-3 LC-PUFA supplementation trials\textsuperscript{66} was not seen in this study (Paper III). Caesarean sectios were more common in the placebo group compared to the $\omega$-3 group (21\% vs. 7\%; $p=0.035$). Acute sectio was equally common in both groups but planned caesarean sectio was significantly more common in the placebo group compared to the $\omega$-3 group (11\% vs. 0\%; $p=0.02$). The reasons for planned sectio were: humanitarian reasons $n=5$, osteoporosis $n=1$ and tight pelvis $n=1$. One mother had two reasons for planned sectio. One could only speculate on the reasons for the discrepancy between the two intervention groups. The concept of "humanitarian reasons" may include depressive and anxiety symptoms occurring during pregnancy. Evidence suggests that low levels of $\omega$-3 fatty acids are correlated with depressive symptoms during pregnancy and after delivery. Omega-3 fatty acids may produce antidepressant effects due to their role in serotonin functioning\textsuperscript{188}.
Immune components in breast milk

The highest concentrations of immune parameters were observed in colostrum with declining concentrations in one and three months samples (Paper IV, Table 3). Supplementation with ω-3 LCPUFA did not alter the levels of investigated immune parameters (Paper IV, Table 3) in colostrum, 1 month and 3 months breast milk, except for PGE$_2$ which was higher in 3 months milk from ω-3 supplemented mothers compared to placebo mothers.

When we analyzed non-atopic and atopic mothers separately we found that among non-atopic mothers ω-3 supplementation was or tended to be associated with higher colostral levels of most cytokines and chemokines compared to placebo supplementation (Paper IV, Figure 1 A, B). No such pattern was observed in atopic mothers (Figure 1 C, D), or in mature milk samples collected at 1 and 3 months of lactation regardless of intervention or atopic status (data not shown).

There were no associations between maternal AA, EPA, DHA or AA/EPA serum/plasma phospholipid proportions one week post-partum and colostrum immune factors with respect to the maternal ω-3-supplementation. However, once again, when stratifying for maternal atopic disease we saw differences between atopic and non-atopic mothers (Paper IV).

We speculate that the differences in cytokine levels observed in the group of non-atopic mothers but not among atopic mothers and differences in association with fatty acids could be genetically determined. This assertion is based on previous studies showing that SNPs (single nucleotide polymorphisms) in the FADS1/FADS2 (fatty acid desaturase) cluster, coding for the rate-limiting enzymes delta-5-desaturase and delta-6 desaturase, which catalyze the desaturation of LA (18:2ω-6) and LNA (18:3ω-6), is associated with differences in ω-6 and ω-3 fatty acids in plasma and erythrocyte membrane lipids during pregnancy and later in breast milk during lactation.$^{189}$

In this study we correlate colostral immune components and serum/plasma fatty acid phospholipids. High correlations between maternal serum and mature breast milk composition has been reported for AA ($r=0.56$, $p=0.001$) as well as for both EPA ($r=0.94$, $p<0.001$) and DHA ($r=0.89$, $p<0.001$)$^{163}$ suggesting that the relationship between breast milk immune components and serum/plasma phospholipid fatty acids is as adequate as with breast milk fatty acids.

High levels of SIgA and in 3 months mature milk samples were associated with both allergic symptoms ($p=0.014$ and 0.003) and IgE associated disease ($p=0.005$ and $p=0.006$ respectively). None of the other analyzed breast milk components were associated with infant
allergic symptoms or IgE associated disease (data not shown). Both TGFß2 and SIgA have previously been reported to be related to allergic disease in children. Higher TGFß2 levels have been reported in colostrum if children developed atopic dermatitis (AD) after weaning compared to those developing allergic symptoms before weaning. We did not have this distinction in our study which may explain our result regarding colostrum. Böttcher et al have reported significantly higher levels of TGFß2 in colostrum from mothers of children developing allergic sensitization up to 24 months of age. This finding was though not significant when assessing the children at 12 months of age and similar trends, i.e. a tendency for higher TGFß2 in mature milk related to sensitization on the children, were reported for mature milk. The higher slgA levels in mature milk related to development of allergic symptoms and IgE associated allergic disease most probably reflects the TGFß2 levels as slgA production is regulated by TGFß.

To the best of our knowledge, this is first study exploring the presence of thymic stromal lymphopoietin (TSLP) in breast milk. It was detected in both colostrum and mature milk but the levels decreased substantially over time. Thymic stromal lymphopoietin is highly expressed in skin lesions of patients with atopic dermatitis but undetectable in normal skin. No association between colostral TSLP and the presence of atopic eczema up to one year of age was however found. Thymic stromal lymphopoietin is primarily produced by epithelial cell and able to activate human myeloid dendritic cells to induce and enhance inflammatory Th2 response.

**LCPUFA metabolism and atopic disease**

The observed decrease in LPS-induced PGE2 secretion in the ω-3 LCPUFA supplemented group was more pronounced among non-atopic (4/5, 80%) than among atopic (11/16, 69%) mothers (Figure 18) (Paper II). In addition, the colostral cytokine levels were higher among non-atopic than atopic ω-3 LCPUFA supplemented mothers (Paper IV). Furthermore, we observed that infants of mothers without allergic symptoms to a lesser degree had any positive SPT, 2/14 (14%) in the ω-3 supplemented group and 11/23 (48%) in the placebo group (p<0.05). The results were similar regarding food allergy (0% vs 25%, p<0.05) and IgE associated eczema (0% vs 29%, p<0.05). Corresponding differences were not observed among children to mothers with allergic symptoms.
Several others studies have revealed different effects of dietary fish intake or fish oil supplementation with regard to allergic diseases. Stronger protective effect for sensitization to food but not for sensitization to inhalants after increased oily-fish intake for children born to non-allergic mothers compared to those born to allergic mothers have been reported. In addition, Kull et al showed that the dose-dependent reduction in risk of atopic disease with increased fish consumption frequency by the child was significant only in children without parental allergy. The multi-centre study by Krauss-Etschmann et al on fish oil supplementation to pregnant women showed a strong association between fish oil supplementation and decreased cord blood mRNA levels of Th2 related CCR4, IL-13 and IL-4 compared to placebo. Furthermore, the decrease of IL-13 mRNA levels in cord blood was more pronounced in cord blood samples from non-allergic mothers. The first report on abnormal metabolism of essential fatty acids and the relation to atopic disease came already in 1937. A similar study in 1984 suggested a relation to Δ6 desaturase. We have described an altered metabolism of Δ-6 and Δ-3 fatty acids in several reports from our group both in newborn babies that later develop allergic disease, in already allergic children and in allergic mothers at the time of delivery and during early lactation. We have also reported low levels of both Δ-6 and Δ-3 PUFA in transitional milk from atopic mothers, a relation between...
low levels of ω-3 fatty PUFA and a high AA/EPA ratio in mature milk and serum phospholipids in infants and the development of allergic disease in the children.  

These differences between atopic and non-atopic individuals regarding fatty acid proportions and eicosanoids secretion could possibly be explained by polymorphisms in the FADS1 and FADS2 genes. Besides dietary supply of LCPUFA (AA from meat and egg and EPA and DHA from fatty fishes) can LCPUFA be derived through the metabolism from essential fatty acids as outlined in Figure 3 side 23. The rate-limiting enzymes are the Δ5- and Δ6 desaturases. The two Δ5- and Δ6 desaturase genes FADS 1 and FADS 2 are located at chromosome 11q12-11q13, a chromosome which has been associated with allergic diseases. The importance of these two enzymes were shown in a recent study on fads2+/− knockout mice showing adverse effects on membrane integrity and fluidity causing infertility and disturbed eicosanoid synthesis. All these functions were furthermore restored after treatment with LCPUFA such as AA, EPA and DHA. In a study by Schaeffer et al. on the association of FADS polymorphisms with serum phospholipid fatty acids they reported a lower prevalence of allergic rhinitis and atopic eczema in carriers of the rare alleles of several single nucleotide polymorphisms (SNPs). Furthermore, a major influence on breast milk fatty acid composition by FADS genotype has also been shown.
Did the placebo capsules induce allergic disease?

The choice of soybean oil can be questioned as it contains the ω-6 fatty acid, LA. One might fear that the placebo would actually increase the risk for allergic disease. The additional intake of LA in the placebo group via the capsules was 2.5 g/d, corresponding to approximately 1/3 of the dietary daily intake reported in the dietary diaries completed by the mothers. The supplemented dose of ω-3 fatty acids is ten times higher than the daily average dietary intake. Maternal serum phospholipid fatty acids levels measured after 15 weeks of intervention were not altered with respect to LA proportions. A study on the allergy preventing effect of supplementation of Lactobacilli Reuteri during pregnancy and lactation has recently been performed at our department. The selection of mothers participating in our study and the L.reuteri study was performed similarly including pregnant women in families with allergic diseases meaning that the expected child has an increased risk to develop allergic diseases compared to children without allergic heredity. The placebo in the L.reuteri consisted of an oil mix of ¾ refined coconut oil and ¼ refined peanut oil (the refined oil did not contain peanut proteins (detection level < 0.005%)). The period prevalence for sensitization and IgE associated eczema were similar in the placebo groups in the two cohorts (Table 9). This data imply that we have not induced disease by using soybean oil as placebo.

Table 9. Period prevalence of sensitization and IgE associated eczema

<table>
<thead>
<tr>
<th></th>
<th>Placebo group</th>
<th>L. reuteri study</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any eczema</td>
<td>31</td>
<td>34</td>
<td>ns</td>
</tr>
<tr>
<td>Any rhinoconjunctivitis</td>
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<td>ns</td>
</tr>
<tr>
<td>Any positive SPT</td>
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<td>29</td>
<td>ns</td>
</tr>
<tr>
<td>IgE egg</td>
<td>23</td>
<td>27</td>
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<td>IgE food</td>
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<tr>
<td>IgE associated eczema</td>
<td>24</td>
<td>28</td>
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</tr>
</tbody>
</table>

*χ2 test
# Fisher’s exact test
CONCLUSIONS

This thesis was based on two different cohorts. The first one was a descriptive observational study describing changes in phospholipid fatty acids in cord blood samples collected in Linköping between 1985 and 2005. The second was a double-blind placebo controlled study aiming to investigate the effect of ω-3 long-chain polyunsaturated fatty acid supplementation to women during pregnancy and lactation with respect to allergy preventing effects in the offspring and mechanisms behind this.

Observations:

- The proportions of LA decreased while the LA/LNA ratio increased in cord serum samples collected in the years 1985-2005, the latter is in agreement with our hypothesis. The decreased LA proportion is, however, surprising but in agreement with data from the Food and Agricultural Organization at the World Health Organization for Sweden specifically, but not for other Western countries with a high prevalence of allergic diseases.

- Studying the effects of ω-3 LCPUFA supplementation in the mothers in blood, lipopolysaccharide-induced PGE$_2$ production was decreased in a majority (66%) of the ω-3 LCPUFA supplemented mothers in contrast to the placebo group who displayed an increase in the majority of cases (77%) (p=0.002). The decrease among ω-3 LCPUFA supplemented mothers tended to be more pronounced among the non-atopic mothers.

- No effect on lipopolysaccharide-induced cytokine secretion was observed.

- In the ω-3 LCPUFA intervention study, children of mothers supplemented with ω-3 LCPUFA during pregnancy and lactation had a lower cumulative risk at one year of age for IgE-associated eczema and food allergy; this was most evident in those of non-allergic mothers.

- In the breast milk samples, the cytokine expression patterns were complex, but in the group of non-atopic ω-3 LCPUFA supplemented mothers, the levels of the different cytokines were generally higher.
FUTURE PERSPECTIVES

Analysis of chemokines in the cord blood cohorts between 1985 and 2005 are planned in order to reveal changes in the Th1/Th2 balance and if this is associated with changes in the phospholipid fatty acid profile.

Children of ω-3 fatty acid supplemented mothers had a lower period prevalence of IgE associated eczema and food allergy at one year of age. Possibly, they have a reduced risk to develop respiratory allergic disease in higher ages. The mechanisms underlying this effect require further clarification.

Several immunological analyses still remain to be done to further elucidate the effects of ω-3 LCPUFA supplementation during pregnancy, on both the mother and her offspring. Cord blood chemokines are currently being analysed. Next in line are probably analyses of fatty acids in breast milk. It would be interesting to investigate the effect of ω-3 supplementation on T-cell reactivity in relation to the clinical outcome. If possible it would also be very interesting to investigate FADS1/FADS2 polymorphisms in the study group.
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