

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Long chain polyunsaturated fatty acids in serum phospholipids

Relation to genetic polymorphisms, diet and allergy development in children

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Front cover: Foetus, DNA, IgE antibodies, droplet of blood and droplet of oil. *Illustrated by Karin Jonsson.*

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) are essential for human cell and tissue development. In foetus, PUFAs are supplied via placental transfer from maternal circulation. After birth, PUFAs are supplied via the diet. Long chain PUFAs (LCPUFAs) may also be synthesized from precursor fatty acids present in the diet. LCPUFAs have modulatory effects on the immune system. As maturation of the immune system in the neonatal period appears to be crucial for protection against allergy development, a major aim of the study was to study the impact of fatty acid composition in infant blood at birth on allergy development. Secondly, we sought to elucidate the sources of infant LCPUFAs with focus on polymorphisms in genes responsible for production of LCPUFAs in the body from shorter dietary fatty acids. Third, we studied whether LCPUFA and vitamin D metabolism differed in allergic and non-allergic adolescents.

High proportions of either n-6 or n-3 LCPUFAs, among cord serum phospholipids were positively associated with the risk of developing either respiratory allergy, or atopic eczema, diagnosed at 13 years of age. We hypothesized that LCPUFAs counteract activation of the infant's immune system in response to microbial stimuli in early life, thereby hampering the proper immune maturation necessary for healthy immune development.

Regarding determinants of cord serum LCPUFA composition, we found that single nucleotide polymorphisms in the *FADS* gene cluster affected the proportion of the main n-6 LCPUFA, arachidonic acid, in cord serum as well as in adolescent serum. *FADS* gene polymorphisms that were associated with decreased proportions of arachidonic acid were also associated with a low prevalence of atopic eczema. Increased proportions of the n-3 LCPUFAs DPA and DHA in cord serum phospholipids were instead related to increased length of pregnancy.

Adolescents with established allergy did not differ from non-allergic controls regarding proportions of LCPUFAs in serum phospholipids. Nor did they differ in vitamin D status. Proportions of n-3 LCPUFA in serum reflected dietary intake of fish in non-allergic adolescents, but not in adolescents with atopic eczema. The results may suggest that subjects with atopic eczema have a different LCPUFA metabolism, maybe because of enhanced usage of LCPUFAs during the allergic inflammation.

In conclusion, the results suggest that LCPUFA metabolism may affect the risk of allergy development and may also be altered as a result of the allergic state. The lack of relation between allergy and vitamin D status in adolescents does not exclude that neonatal vitamin D status may affect allergy development.

LIST OF PUBLICATIONS

This doctoral thesis is based on the work contained in five papers:

- I. Malin Barman, Sara Johansson, Bill Hesselmar, Agnes Wold, Ann-Sofie Sandberg and Anna Sandin. **High levels of both n-3 and n-6 long-chain polyunsaturated fatty acids in cord serum phospholipids predict allergy development.** *PLoS ONE*, 2013. **8(7)**: p. e67920.
- II. Malin Barman, Staffan Nilsson, Åsa Torinsson Naluai, Anna Sandin, Agnes Wold, and Ann-Sofie Sandberg. **Single nucleotide polymorphisms in fatty acid desaturases is associated with cord blood long chain PUFA proportions and development of allergy.** *Submitted*
- III. Malin Barman, Bill Hesselmar, Agnes Wold, Ann-Sofie Sandberg and Anna Sandin. **Proportion of DHA among cord serum phospholipids increases with gestational age.** *Manuscript*
- IV. Malin Barman, Karin Jonsson, Anna Sandin, Agnes Wold, and Ann-Sofie Sandberg. **Serum fatty acid profile does not reflect seafood intake in adolescents with atopic eczema.** *Acta Paed*, 2014. **103(9)**: p. 968-76.
- V. Malin Barman, Karin Jonsson, Bill Hesselmar, Anna Sandin, Ann-Sofie Sandberg and, Agnes Wold. **No association between allergy and current 25-hydroxy vitamin D in serum or vitamin D intake.** *Acta Paed*, 2015. **104(4)**: p. 405-13.

CONTRIBUTION REPORT

Paper I: The author, Malin Barman (MB), was involved in the design of the study, performed fatty acid analyses, performed statistical calculations, was involved in the interpretation of the data and was responsible for writing the manuscript.

Paper II: MB was involved in the design of the study, performed fatty acid analyses, was involved in the statistical calculations and interpretation of the data and was responsible for writing the manuscript.

Paper III: MB was involved in the design of the study, performed fatty acid analyses, performed the statistical calculations, was involved in the interpretation of the data and was responsible for writing the manuscript.

Paper IV: MB was involved in the design of the study, performed the fatty acid analyses, performed the statistical calculations, was involved in the interpretation of the data and was responsible for writing the manuscript.

Paper V: MB was involved in the design of the study, performed the 25-hydroxy vitamin D analyses, performed the statistical calculations, was involved in the interpretation of the data and was responsible for writing the manuscript.

ABBREVIATIONS

1,25(OH) ₂ D	1,25-dihydroxy vitamin D
25(OH)D	25-hydroxy vitamin D
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
<i>ELOVL</i>	elongation of very long chain fatty acids
EPA	eicosapentaenoic acid
<i>FADS</i>	fatty acid desaturase
GWAS	genome wide association studies
IFN- γ	interferon- γ
IgE	immunoglobulin E
IL	interleukin
LCPUFA	long chain polyunsaturated fatty acids (≥ 20 carbon atoms)
LT	leukotriene
PG	prostaglandin
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids (≥ 18 carbon atoms)
SNP	single nucleotide polymorphism
SPT	skin prick test
Th	T helper
TNF	tumour necrosis factor

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INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are essential for cell and tissue development in humans. PUFAs are supplied to the foetus via placental transfer from the maternal to the foetal circulation [1]. After birth, PUFAs are supplied via the diet, including breast milk or formula in infants and from fatty foods later in life. Long chain PUFAs, i.e. PUFAs consisting of more than 20 carbon atoms may either be supplied directly via the diet, or be synthesised from shorter precursor fatty acids present in the diet [2].

PUFAs are known modulators of immune function. Long chain PUFAs are incorporated into phospholipid membranes and are strong inhibitors of activation of T cells [3-5], particularly those of the Th1 subset [6]. Long chain PUFAs also inhibit secretion of interferon- γ by T cells stimulated by mitogen [6, 7]. Arachidonic acid, a long chain n-6 PUFA, is the precursor of prostaglandins, thromboxanes and leukotrienes that are inflammatory mediators [8]. Prostaglandins stimulate maturation of dendritic cells into a Th2 promoting phenotype [9]; Th2 cells are central in atopic (IgE-mediated) allergy.

The prevalence of IgE mediated allergies has increased markedly during the past decades and today allergy is the most common chronic disease among children in Western affluent societies like Sweden [10]. The risk of developing allergy is suggestedly caused by a paucity of stimulation by microbes in the neonatal period [11] and an immaturity of the immune system [12]. Changed dietary habits have been linked to the rise in prevalence of allergies during the 20th century, foremost a change in fatty acid consumption [13]. Margarine intake has been positively associated with increased risk for allergy [14].

Fish has been shown to be protective against allergy, both when introduced early to the infant's diet [15-18] and when consumed by children and adults [19]. Fish is rich in LCPUFAs. Fish also contains considerable amount of proteins, vitamin D, selenium and vitamin B12. Vitamin D has been shown to have immunomodulatory effects and the aim of the last paper was to study if low levels of vitamin D in serum or in diet were involved in allergy development.

This thesis investigates the relation between exposure to LCPUFAs and allergy in cases and controls selected from a birth cohort that was recruited in 1996-7. The cohort included all children born by vaginal delivery during one year in the county of Jämtland. Subjects with isolated respiratory allergy or isolated atopic eczema, diagnosed at 13 years of age, were selected from the cohort and examined regarding diet, serum fatty acid proportions, serum 25-hydroxy vitamin D levels and genetic variants of enzymes carrying out elongation and desaturation of PUFAs. Cord blood samples from the same individuals were retrospectively analysed for LCPUFA composition. The project is a collaboration between Food and Nutrition Science, Department of Biology and Biological Engineering, at Chalmers University of Technology, Clinical Bacteriology, Department of Infectious Medicine, at the University of Gothenburg and Paediatrics, Department of Clinical Sciences, at Umeå University.

OBJECTIVES

The overall aim of this thesis was to study the association between long chain polyunsaturated fatty acid (PUFA), vitamin D and allergy development. We investigated if the fatty acid status of the newborn was associated with the risk of subsequent allergy development and further, if fatty acid metabolism differed in allergic and non-allergic subjects. We also study if the proportion of different LCPUFAs in cord serum was determined by genetic factors and by the transport of LCPUFAs to the foetus in late pregnancy. Specific questions that were addressed in the thesis are (Figure 1):

- Does the fatty acid milieu in the neonate affect the risk of subsequent allergy development? (Paper I)
- Which factors determine the LCPUFA composition in cord serum? (Papers I, II and III)
- Does the fatty acid metabolism differ between allergic and non-allergic adolescents? (Papers II and IV)
- Are genetic polymorphisms in genes responsible for desaturation and elongation associated with allergy? (Paper II)
- Is there an association between vitamin D and allergy in adolescents? (Paper V)

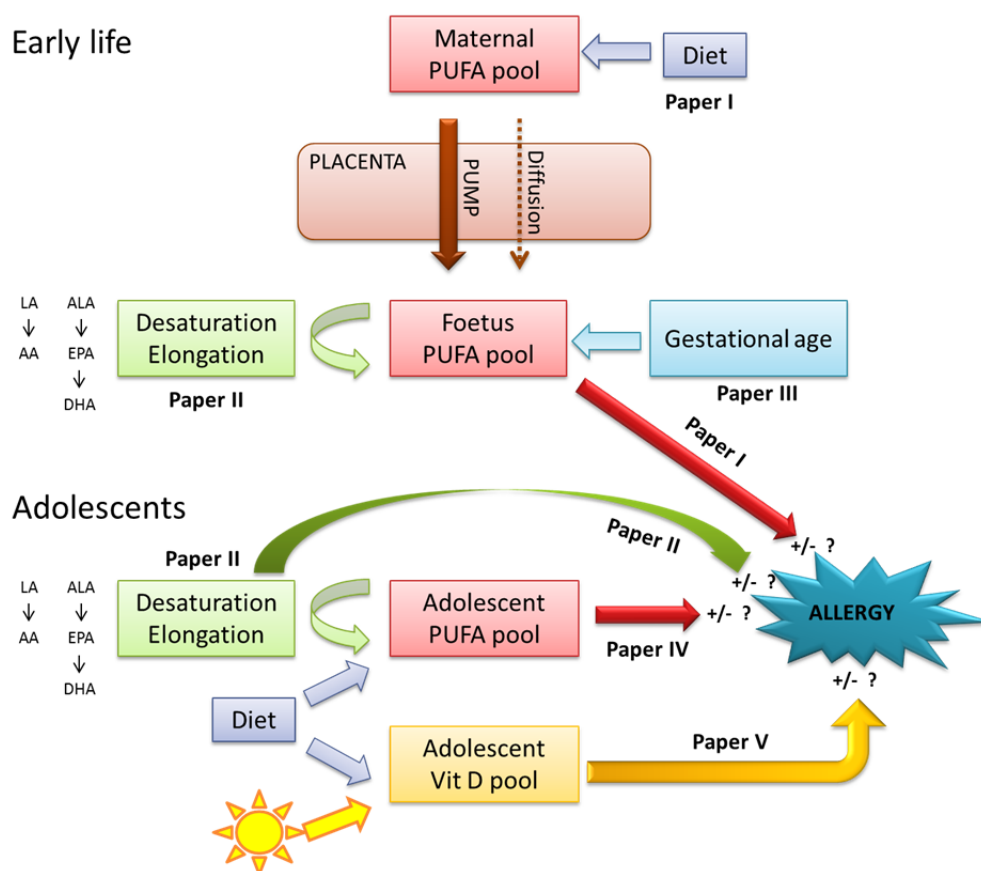


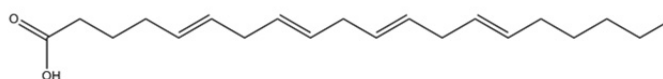
Figure 1: Overview of the five papers included in the thesis

LITERATURE OVERVIEW

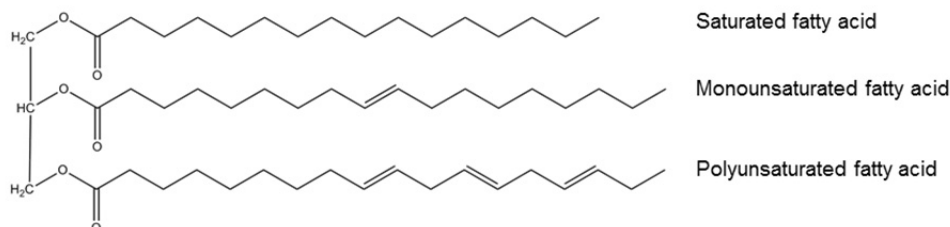
Fatty acid metabolism

Fatty acids consist of an unbranched hydrocarbon chain containing a terminal carboxylic acid (Figure 2a) [20]. Fatty acids are classified according to the number of double bonds that the hydrocarbon chain contains: saturated fatty acids are straight molecules with no double bonds, unsaturated fatty acids contain one (monounsaturated) or several (polyunsaturated, PUFA) double bonds and are flexible around the double bond positions (Figure 2b) [2]. Omega-3 (n-3) PUFAs have their first double bond positioned 3 carbon atoms from the methyl end, while n-6 PUFAs have their first double bond positioned 6 carbon atoms from the methyl end (Figure 3).

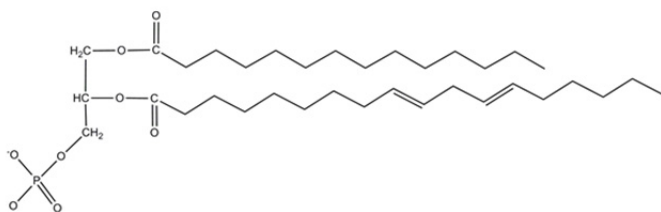
Fatty acids are incorporated into triacylglycerols, which constitute the largest proportion of dietary lipids (Figure 2b). The fatty acids incorporated into triacylglycerols are of many different varieties. Most of them are unsaturated with an even number of carbon atoms, ranging from 4 in milk fat to 30 in some fish oils [21].



a) Free fatty acid (arachidonic acid)



b) Triacylglycerol (1-palmitic-2-oleic-3-linolenic-triacylglycerol)



c) Phospholipid (L-α-1-myristoyl-2-linoleoyl-phosphatidic acid)

Figure 2: Structure of a free fatty acid (a), a triacylglycerol (b) and a phospholipid (c)

Endogenous production of LCPUFAs

All mammals can synthesise fatty acids *de novo* from acetyl-CoA, the end product being stearic acid (18:0) [21]. However, cell membranes require that a proportion of the fatty acids are unsaturated to maintain fluidity and function. A mechanism for the introduction of double bonds therefore exists, called desaturation. Δ -9-desaturase introduces a double bond between carbon atom 9 and 10 and converts stearic acid (18:0) to oleic acid (18:1 n-9). Both plants and animals have this enzyme.

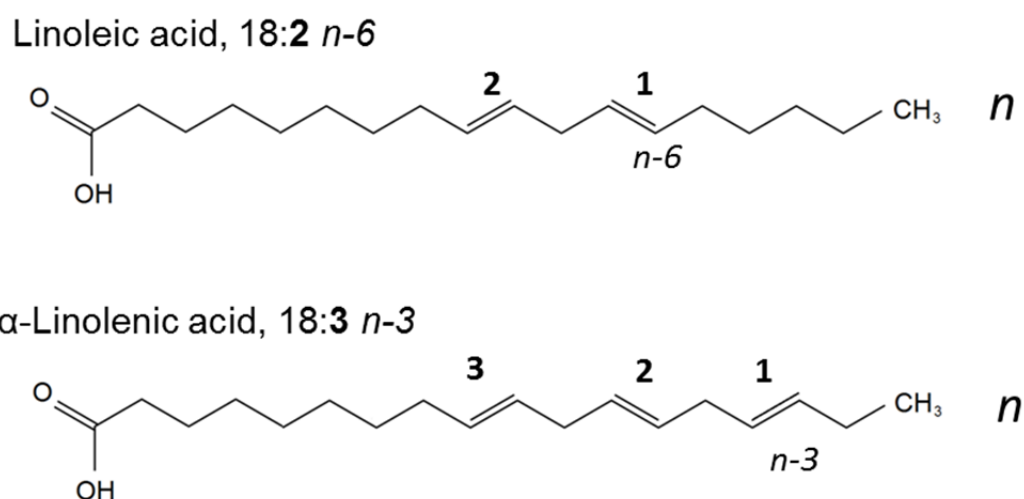


Figure 3: The essential fatty acids linoleic acid and α -linolenic acid

Linoleic acid (18:2 n-6) has two double bonds (marked with numbers in bold), where the first double bond is positioned after the sixth carbon (*n*-6), counted from the methyl end. α -linolenic acid (18:3 n-3) has three double bonds (marked with numbers in bold), where the first double bond is positioned after the third carbon (*n*-3), counted from the methyl end of the fatty acid.

Plants, but not animals, also have the enzymes Δ -12-desaturase, which converts oleic acid to linoleic acid (18:2 n-6), as well as Δ -15-desaturase, which converts linoleic acid to α -linolenic acid (18:3 n-3). Since animals can synthesise neither linoleic acid nor α -linolenic acid, both these fatty acids need to be present in the diet, and are therefore termed essential fatty acids [22] (Figure 3). Linoleic acid is found, for example, in oils from corn, rapeseed and sunflower, while α -linolenic acid is found primarily in rapeseed, flaxseed and soybean oils.

The long chain PUFAs of the n-6 family are formed from the n-6 fatty acid linoleic acid, while the n-3 family of PUFAs derive from α -linolenic acid (Figure 3). This takes place in the membranes of the smooth endoplasmic reticulum and is carried out by two different types of enzymes, the desaturases and the elongases (Figure 4 and 5). Both pathways involve the same enzymes, and, hence, there is competition between the two pathways. Thus, conversion of α -linolenic acid into its longer n-3 derivatives is reduced if n-6 linoleic acid is present in large amounts. Conversely, high proportions of n-3 α -linolenic acid hamper the conversion of n-6 linoleic acid to arachidonic acid and other long chain n-6 PUFAs. In male humans, approximately 5% of α -linolenic acid is

estimated to be converted to EPA, but only 0.5 % to DHA [23]. However, the capacity to convert α -linolenic acid to EPA and DHA differs; for example, the conversion seems to be higher in women during pregnancy [24, 25].

The elongation of fatty acids occurs through sequential addition of two carbon atoms and is catalysed by enzymes called elongases that are encoded by genes belonging to the *ELOVL* (elongation-of-very-long-chain-fatty-acids) gene family [26]. Seven enzymes, termed *ELOVL1-7*, have been identified [27-32]. *ELOVL1*, *ELOVL3*, *ELOVL6*, and *ELOVL7* are though involved in the elongation of saturated and monounsaturated fatty acids, while *ELOVL2*, *ELOVL4*, and *ELOVL5* elongate PUFAs [32]. Using *ELOVL2*-knock out mice, it has recently been shown that the major *in vivo* substrates of *ELOVL2* are 22:5 n-3 and 22:4 n-6 [33].

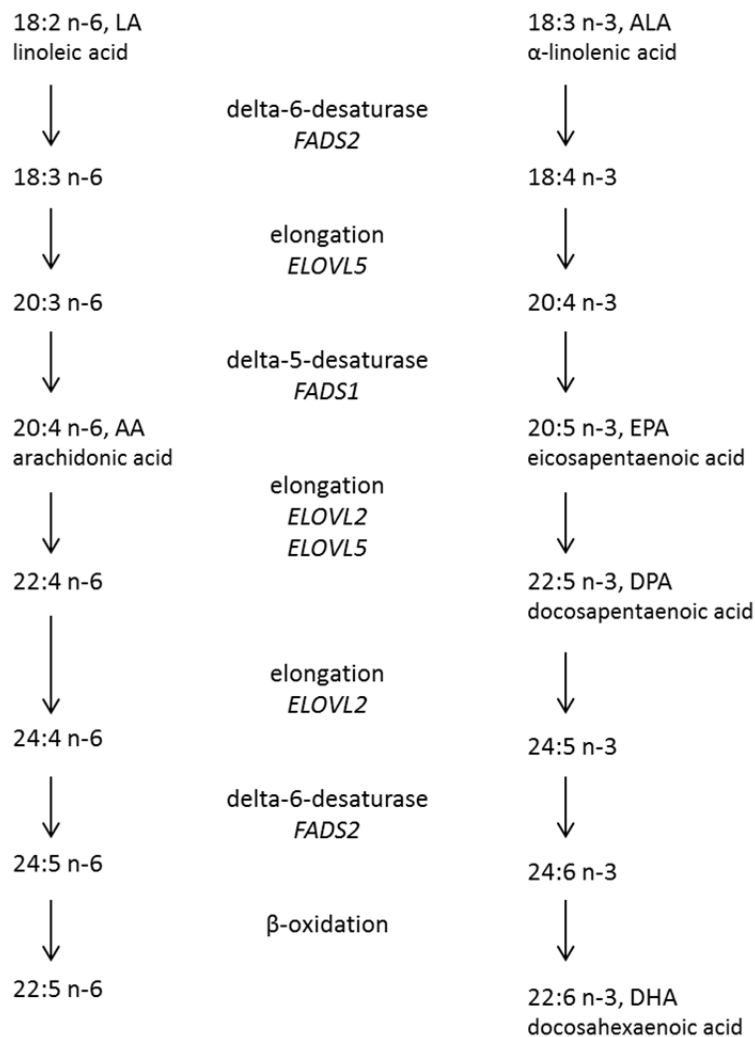


Figure 4: The metabolic pathways of polyunsaturated fatty acids in mammals [32]

The metabolic pathways from linoleic acid (18:2 n-6) and α -linolenic acid (18:3 n-3) to the longer PUFAs involve two different types of enzymes, desaturases and elongases. Since both pathways involve the same enzymes, there is competition between the two pathways, and the conversion of α -linolenic acid into its longer derivatives is influenced by the level of linoleic acid.

After a two-carbon atom unit has been added, a double bond is introduced to the carbon chain. This process is termed desaturation and is catalysed by enzymes termed desaturases. The two major desaturase species, Δ -5- and Δ -6-desaturase, are encoded by the *FADS1* and *FADS2* genes, respectively [34-36]. The enzymes are named after where they insert the double bond; Δ 6 desaturase inserts a double bond after the sixth carbon, counted from the carboxylic end of the fatty acid (i.e. opposite to the omega system, which is counted from the methyl end) (Figure 5). The desaturases are the rate limiting enzymes in the elongation pathway. The *FADS* genes are arranged in a head-to-head orientation and build a gene cluster on chromosome 11 (11q12-13.1) together with a third desaturase gene, *FADS3* [37]. The function of *FADS3* is still unidentified.

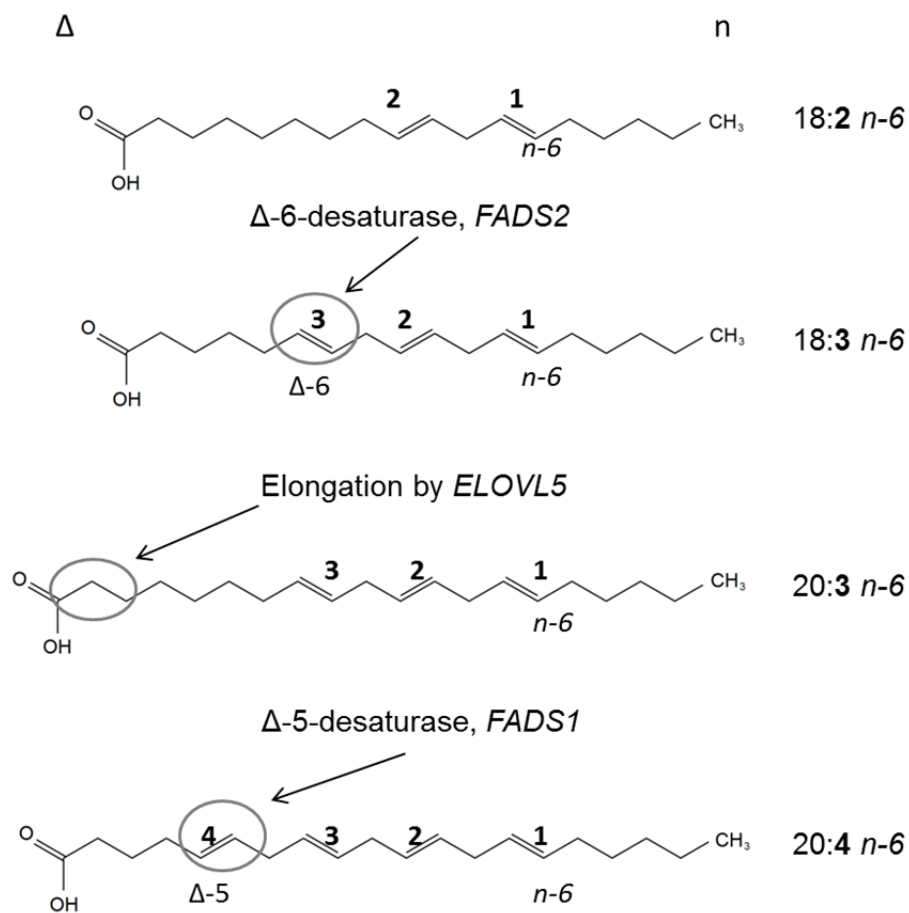


Figure 5: Desaturation and elongation in the n-6 pathway

Δ -6-desaturase inserts a double bond after the sixth carbon atom, counted from the carboxylic end of the fatty acid chain and converts linoleic acid (18:2 n-6) to γ -linolenic acid (18:3 n-6), which further is elongated by an addition of two carbon atoms to the carboxylic end to dihomoy-linolenic acid (20:3 n-6). Next, Δ -5-desaturase inserts a double bond after the fifth carbon, and arachidonic acid (20:4 n-6) is produced.

Polymorphism in *FADS* and *ELOVL* genes

Several studies in the past ten years have discovered that single nucleotide polymorphisms¹ (SNPs) in the *FADS* gene cluster influence PUFA and LCPUFA levels in human tissue [38-53]. Individuals with the minor *FADS2* allele² had lower Δ -6 desaturase activity and decreased proportions of the products arachidonic and EPA, while the substrates for the reaction, i.e. linoleic and α -linolenic acid, accumulate in the body (Figure 6). Whether polymorphisms in the *ELOVL* genes encoding the elongases, affect PUFA proportions has been less studied and overall results are inconclusive [54-57].

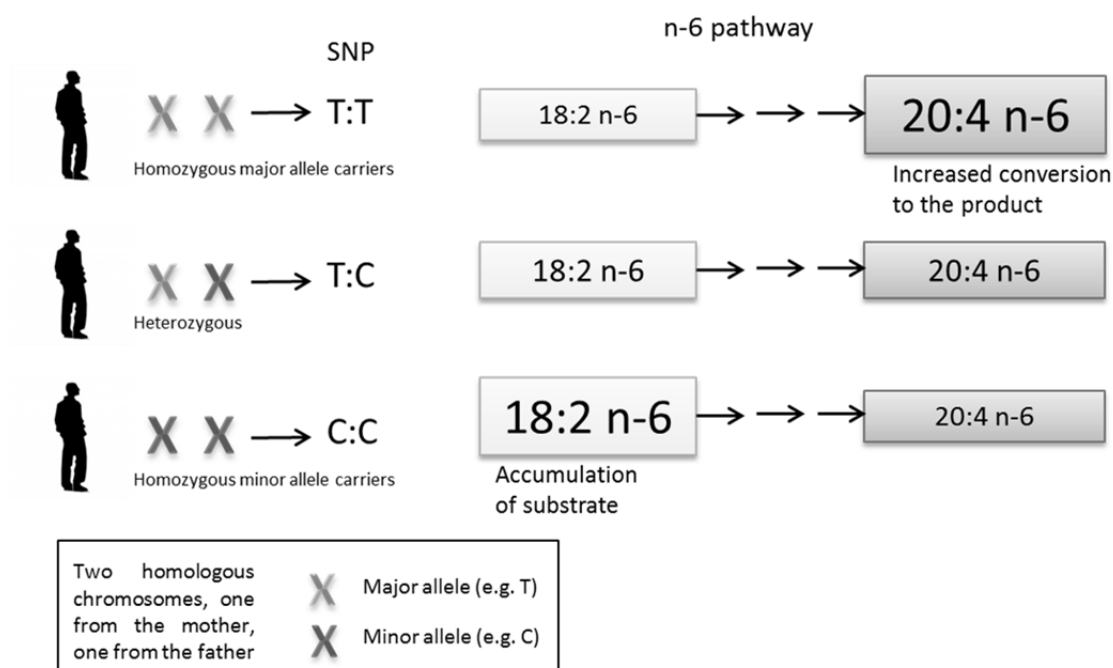


Figure 6: Single nucleotide polymorphisms in the *FADS* gene cluster influence the production of LCPUFAs

Several association studies have shown that carriers of the minor alleles have a decreased synthesis of the product LCPUFAs and an accumulation of the substrate.

FADS polymorphism and allergic disease

As PUFAs exert immunoregulatory actions, particularly long chain PUFAs, it is reasonable to believe that differences among individuals in the capacity to convert medium-chain PUFAs into longer PUFA species, may affect development of immunoregulatory diseases, such as allergy.

¹ Single nucleotide polymorphism – a genomic variation occurring commonly within a population (> 1 %), in which a single nucleotide differs between individuals. This can lead to a change in amino acid sequence of an enzyme and affect activity.

² Allele – An allele is a variant of the DNA sequence, i.e. one of two copies of a gene (one from the mother and one from the father). The alleles could be the same (homozygous) or different (heterozygous).

Studies exploring the association between allergic disease and polymorphism in the *FADS* gene cluster are summarized in Table 1. The first study by Schaeffer et al. [44] found the minor allele³ carriers to have reduced incidence of self-reported allergic rhinitis and atopic eczema in adulthood [44]. To the contrary, Rzehak et al. [43] found minor allele carriers of several SNPs to have a higher prevalence of eczema in the LISA-study, while no associations were found in the KOALA-study. The association between allergy and polymorphism in the subjects in the LISA-study has also been studied at six and ten years of age together with subjects from another German birth cohort, the GINI-study. No association was found between the same five SNPs as in the Rzehak study regarding cumulative prevalence of eczema, asthma, bronchitis or rhinitis at six years [58] or asthma at ten years of age [59]; all diseases were reported by the parents (Table 1). Hence, the overall result of the studies exploring the association between *FADS* polymorphism and allergic disease are inconclusive.

Table 1: Studies exploring the association between *FADS* polymorphism and allergic disease

Reference	Study (n)	Age (years)	Clinical criteria	<i>FADS</i> SNPs	Findings (minor allele carriers)
Schaeffer et al. 2006 [44]	ECRHS1 (German): N = 727 Rhinitis: n = 76 Eczema: n = 49	20-74	Self-reported allergy + specific IgE	18 in <i>FADS</i> cluster	↓ rhinitis, atopic eczema. No association with IgE levels.
Rzehak et al. 2009 [43]	LISA-study (German): N = 333 Eczema: 14 % KOALA-study (Dutch): N = 542 Eczema: 31 %	2	Cumulative parentally-reported eczema IgE-levels	<i>FADS1/FADS2</i> : rs174545, rs174546, rs174556, rs174561, rs3834458	LISA study: ↑ eczema No associations in KOALA-study No association with IgE levels.
Singmann et al. 2010 [58]	LISA and GINI-studies (German): N = 2718 Asthma: n = 110 Bronchitis: n = 636 Eczema: n = 776 Rhinitis: n = 235	6	Cumulative parentally reported doctor's diagnosis of eczema, asthma, bronchitis or rhinitis	<i>FADS1/FADS2</i> : rs174545, rs174546, rs174556, rs174561, rs3834458	No association with allergy
Standl et al. 2012 [59]	LISA and GINI-studies (German): N = 2245 Asthma: 11 %	10	Cumulative parentally reported doctor's diagnosis of asthma	<i>FADS1/FADS2</i> : rs174545, rs174546, rs174556, rs174561, rs3834458	No association with asthma

Abbreviations: SNP = single nucleotide polymorphism, *FADS* = fatty acid desaturase

³Minor allele – the version of the allele that is less common in a population

Nutrient supply to the foetus

During pregnancy nutrients and oxygen are transported from the maternal circulation to the foetus via the placenta, while waste products and carbon dioxide are transported back from the foetus to the mother (Figure 7).

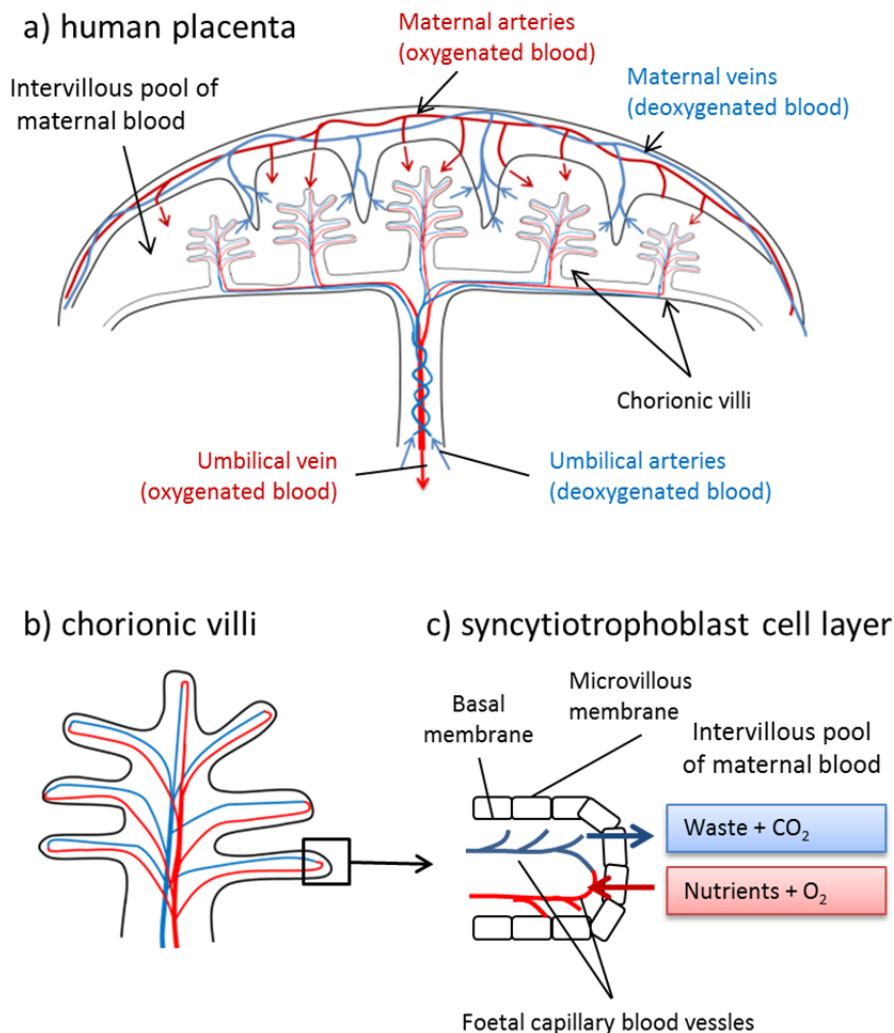


Figure 7: Structure of the human placenta (a), chorionic villi (b) and syncytiotrophoblast cell layer (c)

The placenta provides the foetus with all the requirements necessary for growth and development, such as nutrients and oxygen. Maternal blood flows in to the intervillous space that surrounds the chorionic villi and forms a pool of maternal blood that is in direct contact with the microvillous membrane of the syncytiotrophoblast cell layer that covers the chorionic villi. Foetal blood is not in direct contact with the syncytiotrophoblast cell layer but is contained in foetal capillary veins (red) and arteries (blue) that are situated close to the basal membrane of the syncytiotrophoblast cell layer. Hence, a single layer of cells separates the maternal and the foetal blood and nutrient and gas exchange can take place over the syncytiotrophoblast cell layer. An example of transportation of nutrients (in this case fatty acids) over the syncytiotrophoblast cell layer is shown in Figure 8.

Maternal blood enters the placenta through spiral arteries and fills the intervillous space with oxygenated and nutrient rich maternal blood (Figure 7). Foetal blood flows from the foetus into two arteries in the umbilical cord and enters the capillary network in the chorionic villi [60]. Maternal and foetal blood is separated by a single cell layer, the syncytiotrophoblast cell layer, covering the chorionic villi. A microvillous membrane is facing maternal blood in the intervillous space and a basal membrane is situated on the foetal side of the syncytiotrophoblast cell layer [61]. The maternal blood in the intervillous space is in direct contact with the microvillous membrane of the syncytiotrophoblast cell layer while foetal blood is contained in foetal capillary vessels (Figure 7).

Nutrient and gas exchange takes place across the syncytiotrophoblast cell layer. Oxygen and nutrients that crosses the syncytiotrophoblast cell layer are absorbed by the foetal capillary veins, which are situated close to the cell layer, and transported through the umbilical vein in the umbilical cord to the foetus. Waste products and carbon dioxide that crosses syncytiotrophoblast cell layer are distributed in to the intervillous pool of maternal blood and absorbed by maternal veins and transported out of the placenta to the maternal circulation (Figure 7).

Transport of fatty acids to the foetus

The two most important fatty acids for the foetus, arachidonic acid and DHA, are found in higher concentrations in the foetal than in the maternal circulation during the third trimester of pregnancy [62]. One can speculate if the higher concentration of arachidonic acid and DHA in the foetal circulation is due to placental or foetal production of LCPUFAs from precursor fatty acids. It has been suggested that the foetus itself is capable of synthesizing long chain fatty acids from linoleic acid and α -linolenic acid, supplied from the mother [63]. The foetal production is however generally regarded to be insufficient [63-65], and in addition, the placenta has been suggested to contain undetectable [66] or very low levels [67] of the enzymes Δ -5 and Δ -6 desaturases that are necessary for desaturation. Hence, it is assumed that the foetal requirements of LCPUFAs are met mainly by placental transfer of fatty acids from the maternal circulation [68] and it has been suggested that the placenta is capable of selective transport of PUFAs to the foetus. Haggarty et al [69]. found the order of preference to be DHA>arachidonic acid > α -linolenic acid>linoleic acid when human placentas were perfused with fatty acids in the ratios found in maternal circulating triglycerides. The selectivity might depend either on the transport proteins [66], or the tendency of placental lipases to release various PUFAs from triacylglycerols [70].

Which form the PUFA exists in, bound to triglycerides or circulating as free fatty acids bound to albumin, is important for the supply of PUFA/LCPUFA to the foetus. The absolute rate of placental transfer of DHA was 13 times higher for free fatty acids than for DHA bound to triglycerides while the transfer of arachidonic acid was eight times higher for free fatty acids than for triglycerides [69].

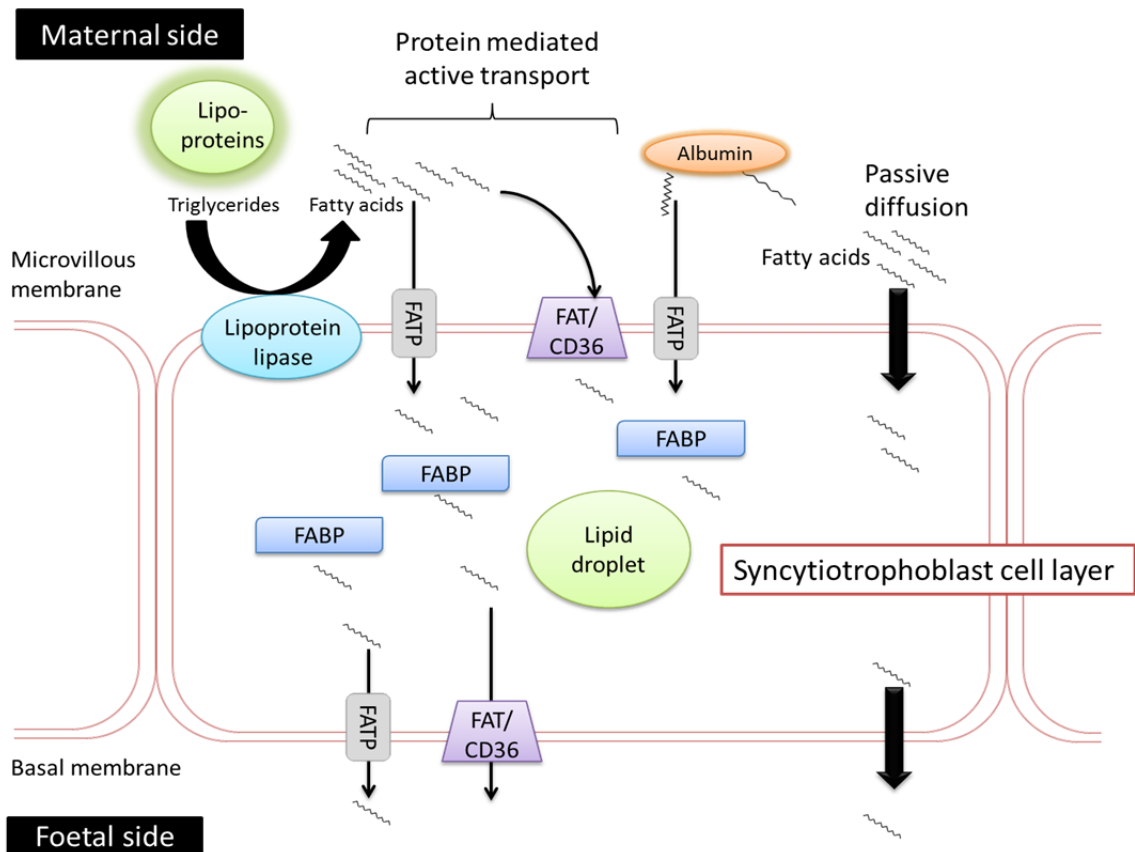


Figure 8: Fatty acid transport over the syncytiotrophoblast cell layer [66]

Fatty acids are transported from the maternal pools of blood in the intervillous space, across the syncytiotrophoblast cell layer, to the foetal capillaries (see Figure 7). The fatty acids can enter the syncytiotrophoblast cell layer either by protein mediated active transport or by passive diffusion. The microvillous membrane of the syncytiotrophoblast cells are in direct contact with the maternal blood, while the foetal blood is contained in foetal capillaries close to the basal membrane of the syncytiotrophoblast cell layer. Abbreviations: FATP = fatty acid transport proteins, FBP = fatty acid binding proteins, FAT/CD36 = fatty acid translocase.

Fatty acids are transported to the foetus in free, non-esterified form. They can derive either from free fatty acids in the maternal circulation, or they can be cleaved off from triacylglycerols by lipases at the maternal/foetal interface (Figure 8). In the maternal circulation lipids are transported either as free fatty acids bound to albumin or as triglycerides incorporated into lipoproteins or chylomicrons. Lipoprotein lipases and other triglyceride hydrolases are situated on the microvillous membrane on syncytiotrophoblast cells of the placenta. They release fatty acids from the lipoproteins and the albumin in the maternal blood circulation. The fatty acids can then be transported across the microvillous membrane either via passive diffusion [71] or carrier-mediated transport [72]. The most important carrier proteins are fatty acid transport proteins (FATP), fatty acid binding proteins (FABP) and fatty acid translocase (FAT/CD36) [66]. In the syncytiotrophoblast cell layer the free fatty acids are transported to different sites for esterification, oxidation, or direct transfer to the foetus. Lipids can be stored in lipid droplets in the cells. The fatty acids are then believed to cross the basal membrane by either simple diffusion or associated to FATPs or FAT/CD36.

Cord serum fatty acid composition

The umbilical cord connects the developing foetus with the placenta. The umbilical cord contains foetal blood vessels, i.e. one vein that supplies the foetus with oxygenated nutrient-rich blood from the placenta and two arteries that transport deoxygenated and nutrient-depleted blood back to the placenta (Figure 7a). Since the blood in both the arteries and the vein in the umbilical cord are part of the foetal circulation, blood drawn from the umbilical cord at birth will provide a picture of the nutritional status of the foetus.

The cord blood plasma phospholipid concentration is stable during pregnancy, but the relative amount of fatty acids change; linoleic acid and DHA increase, while arachidonic acid decreases as pregnancy progresses [73]. The fatty acid status of the newborn is also related to birth order [74, 75], the proportion of arachidonic acid and DHA being higher during the first pregnancy than during subsequent ones [75-77]. Furthermore, in single pregnancies, the concentration of arachidonic acid and DHA is higher in the umbilical artery vessel wall phospholipids, compared to twin or triplet pregnancies [75].

Maternal and foetal proportions of fatty acids correlate strongly for EPA and DHA, but more weakly for arachidonic acid [78]. EPA and DHA supplementation during pregnancy increase's the proportions of these fatty acids in maternal plasma and cord plasma phospholipids [79, 80] as well as cord blood erythrocytes [81] in some studies. However, others found no association between supplementation and proportions in cord blood [76].

Effects of PUFAs on the immune system

PUFAs affect immune functions. Several mechanisms have been identified [82].

1. PUFAs are incorporated into cellular membranes and affect cell membrane fluidity and thereby receptor signalling [83].
2. PUFAs are ligands for ligand-activated transcription factor peroxisome proliferator-activated receptors (PPARs) [84, 85].
3. PUFAs are precursors of lipid mediators affecting inflammation and immune functions, such as prostaglandins, thromboxanes and leukotrienes [86].
4. PUFAs bind to membrane-bound fatty acid receptors, such as the G protein-coupled receptor 120 (GPR120) on macrophages [87, 88].

1. Effect of PUFAs on membrane structure and function

All membranes are formed by phospholipid bilayer, in which the hydrophobic tails points inward and their hydrophilic heads outward (Figure 9). If the supply of n-3 fatty acid is abundant, n-3 fatty acids will constitute a larger part of the fatty acids in the membrane phospholipids than when n-3 fatty acids are limited. An increasing proportion of n-3 PUFAs in membrane phospholipids increases membrane fluidity. This in turn affects the function of membrane-bound receptors, secondarily affecting intracellular signal transduction [83].

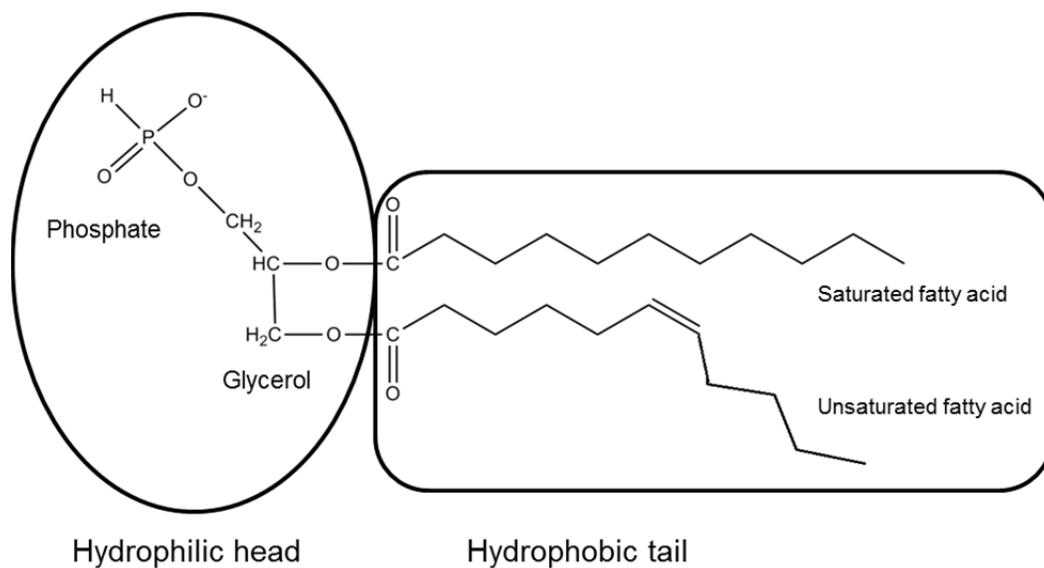


Figure 9: Phospholipid

A phospholipid consisting of a hydrophilic head with a negatively charged phosphate group and a glycerol molecule and a hydrophobic tail with two fatty acids, often one saturated and one unsaturated fatty acid.

Many receptors that induce signal transduction are concentrated in so called lipid rafts, which are platforms for cell activation and signalling between cells. Lipid rafts are specialized membrane domains that contain high concentrations of cholesterol, sphingomyelin and gangliosides. They are also enriched in phospholipids that contain saturated fatty acids and thus form semisolid “islands” floating in the fluid lipid bilayer. The T cell receptor and its associated signalling complex are located in lipid rafts, and an alteration in the fatty acid composition in the lipid rafts affects T cell responses [89].

2. PUFAs as ligands for peroxisome proliferator-activated receptors (PPARs)

The ligand-activated transcription factors PPARs function as regulators of lipid and lipoprotein metabolism and glucose homeostasis and influence cellular proliferation, differentiation and apoptosis [90]. PPAR- α stimulates β -oxidative degradation of fatty acids, and PPAR- γ triggers adipocyte differentiation and promotes lipid storage. More recently, PPAR- γ has also been recognized to exert regulatory effects on immune responses [90]. PPAR- γ is expressed in low amounts in monocytes, the expression is induced when monocytes differentiate towards dendritic cells or macrophages [91]. Also, activation of PPAR- γ can lead to differentiation of monocytes to macrophages. PPAR- γ activation enhances the capacity of dendritic cells to phagocytose apoptotic neutrophils. Moreover, PPAR- γ can induce apoptosis in a variety of cell types, including macrophages [92] and T cells [93].

PPAR- γ is activated by a variety of lipophilic ligands, including long chain PUFAs, such as arachidonic acid, DHA and EPA. Also, arachidonic acid derived metabolites, such as 15-hydroxyeicosatetraenoic acid (15-HETE) and 15-deoxy- Δ -12,14, prostaglandin J₂ (PGJ₂), have been found to be important, naturally occurring ligands (Figure 10).

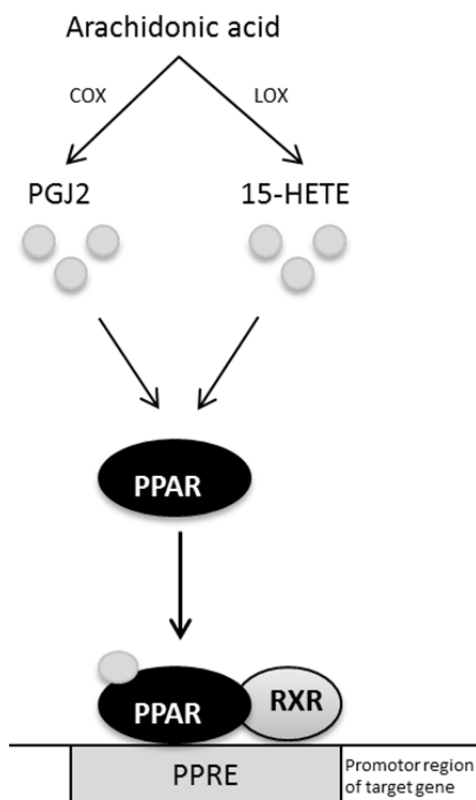


Figure 10: Activation of PPARs with arachidonic acid derived metabolites

Upon activation, PPARs forms a heterodimer with 9-cis retinoic acid receptor (RXR) and binds to peroxisome proliferator response elements (PPRE) located in the promoter of target genes, thus regulating their transcription.

3. PUFAs as substrates for production of prostaglandins and leukotrienes

Arachidonic acid is released from membrane phospholipids when cells are activated by inflammatory stimuli. Arachidonic acid is converted into prostaglandins and thromboxanes via the cyclooxygenase, COX, pathway, or into leukotrienes via the action of lipoxygenase, LOX (Figure 11). Collectively these mediators are called eicosanoids, which are signalling molecules made by oxidation of 20-carbon fatty acids. COX is found in all cells in the body but LOX is present only in inflammatory cells such as macrophages, granulocytes and mast cells. Hence, prostaglandins can be produced by a variety of cells in the body, while leukotrienes are only produced by inflammatory cells.

Different inflammatory cells produce different mixtures of eicosanoid metabolites, as they possess different enzymes that convert the intermediary product PGH₂ from COX into different types of prostaglandins and thromboxanes. Macrophages are the largest producer of

prostaglandins, producing mostly prostaglandin E₂ (PGE₂) but also producing prostaglandin I₂ (PGI₂). The main function of PGE₂ is to induce fever and vasodilation, and enhance pain. Prostaglandin E₂ also decrease inflammation by a number of mechanism: inhibition of lymphocyte activation and IFN-γ production, and suppression of TNF-α and IL-1 production from activated macrophages [94]. PGE₂ also acts on antigen presenting dendritic cells promoting Th2 differentiation of naïve T cells which interact with the dendritic cell: Th2 cells promote class switching to IgE in B cells [95]. Macrophages produce leukotriene B₄ (LTB₄) as well, that attract neutrophils and monocytes. LTB₄ also activates neutrophils and increases vascular permeability and production of TNF-α and IL-1β.

Eosinophils and mast cells produce the cysteinyl leukotrienes LTC₄, LTD₄ and LTE₄ and the prostaglandin PGD₂ which are all important mediators in the hypersensitivity reaction. PGD₂ is chemotactic for eosinophils and Th2 cells that are involved in the allergic reaction, while LTC₄, LTD₄ and LTE₄ promote endothelial cell permeability and airway smooth muscle constriction during anaphylactic reactions and asthma.

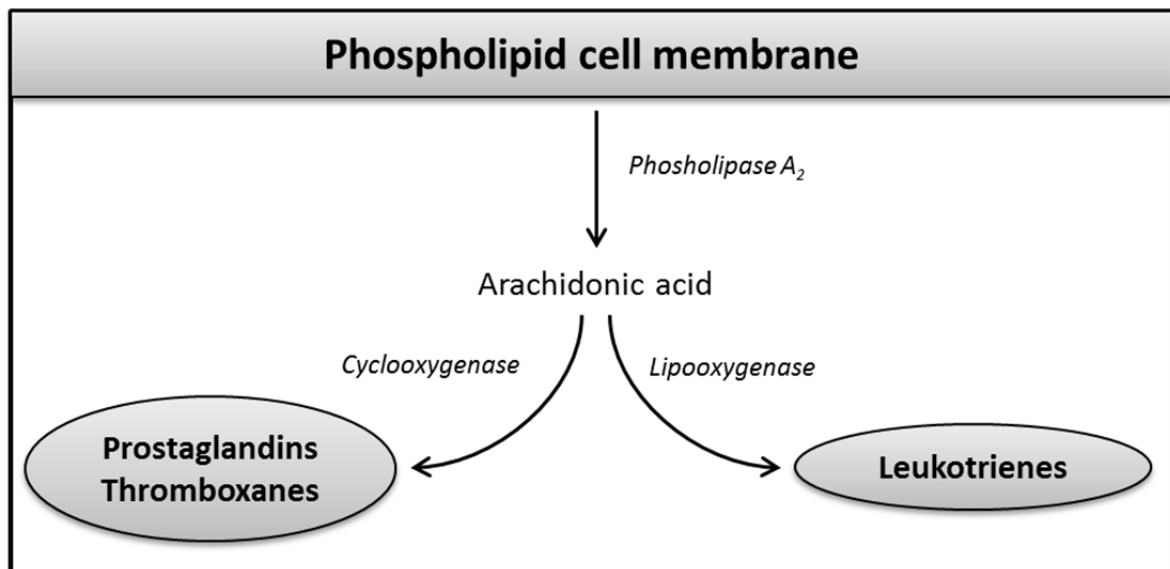


Figure 11: Conversion of arachidonic acid to its main metabolites

Upon an inflammatory signal phospholipase A₂ is activated and cleaves of arachidonic acid from the phospholipid cell membrane. Leukocytes isolated from rats fed a diet rich in arachidonic acid produced more PGE₂ when stimulated with concanavalin A, than leukocytes from rats fed regular diet. Conversely, rats fed a diet rich in EPA and DHA produced less PGE₂ [96]. Increased oral intake of EPA decreased the ex vivo production of PGE₂ by human mono nuclear cells in a dose-dependent fashion [97] and fish oil supplementation of humans decreased the production of LTB₄ [98].

Other C20 PUFAs than arachidonic acid, such as EPA, have also been suggested to be metabolized to some extent by COX and 5-LOX, leading to formation of EPA derived 3-series prostaglandins and 5-series leukotrienes. These are considered to be biologically less potent than corresponding arachidonic acid derived substances [99]. However, the full range of biological activities of the EPA derived eicosanoids has not been investigated [100]. In the addition to the eicosanoids, EPA and DHA have been suggested to be converted to mediators termed E-series resolvins and D-series resolvins and protectins that has been suggested to have resolving effects on inflammation [101, 102].

4. Effects of PUFA on membrane-bound fatty acid receptors

GPR120 is a G-protein coupled receptor that has been shown to have anti-inflammatory properties after binding n-3 PUFAs [87]. GPR120 is expressed in macrophages [88], as well as in the gastrointestinal tract and adipose tissue [103]. Binding of n-3 LCPUFA to GPR120 has been suggested to suppress inflammatory signalling via NF- κ B [104]. Both n-3 and n-6 PUFAs are natural ligand for GPR120 and a recent study found that both EPA, DHA and arachidonic acid caused the same signalling events, but with different kinetics and efficiency through GPR120 in Caco-2 cells [105]. This study also found that both n-3 and n-6 PUFAs inhibit NF- κ B activation in intestinal epithelial cells [105].

Effect of PUFA on T lymphocyte function

The proliferation of T cells in response to mitogens is strongly impeded by n-3 and n-6 PUFAs, as shown in both rodent [106-109] and human [4, 5, 109-118] *in vitro* systems, i.e. experiments where lymphocytes have been isolated from blood or lymphoid organs and stimulated with broad lymphocyte activators (mitogens) or specific antigen in the presence or absence of different PUFAs. Decreased lymphocyte proliferation has also been found in *ex vivo* studies. In this case, different PUFAs have been supplemented into the diet of laboratory animals [109, 119-126] or humans [4, 127, 128], e.g. n-6 linoleic acid, n-3 α -linolenic acid or complex natural mixtures (fish oil). Lymphocytes taken from these individuals are less prone to proliferate when stimulated, than lymphocytes from individuals not fed PUFAs.

Relatively few studies have investigated the influence of PUFAs on the spectrum of T cell derived cytokines produced. IL-2 is required for T cell proliferation and IL-2 production decreases with exposure to PUFAs both *in vitro* [108, 114, 116] and in *ex vivo* studies [4, 128-132].

Helper T cells are divided into different subsets where the Th1 subset produces interferon- γ and increases the bactericidal capacity of macrophages interferon- γ production by T-lymphocytes is reduced in the presence of n-3 PUFAs [6, 7, 131]. On the other hand, the Th2 cytokine IL-4 has been found to be upregulated [133] or unaffected [6] in mice fed a diet rich in fish oil.

Allergy

Allergy is defined as immune mediated hypersensitivity. An allergic individual mounts an immune response against “harmless” substances in the environment, called allergens. This process is termed sensitisation. In an allergic individual, subsequent exposure to the same allergen triggers an inflammatory reaction, leading to symptoms in the skin, gut and/or airways. Not everyone who is sensitised to an allergen will produce symptoms upon natural exposure to the allergen; i.e. one may be sensitised without being allergic.

Allergies can be IgE mediated or T cell mediated. It has been proposed that other immune effector mechanisms may also be involved in allergy, e.g. IgG antibodies [94].

IgE mediated (atopic) allergy

Immunoglobulin E (IgE) causes atopic allergy. Sensitisation involves B cells being transformed into antibody-producing plasma cells that produce IgE antibodies against common respiratory or dietary allergens (Figure 12, left panel). The antibodies become attached to tissue resident mast cells via their Fc ϵ receptors. Mast cells contain histamine stored in granules and are capable of producing a range of other potent inflammatory mediators when activated. The mast cells are situated around blood vessels in the gastrointestinal tract and the airways.

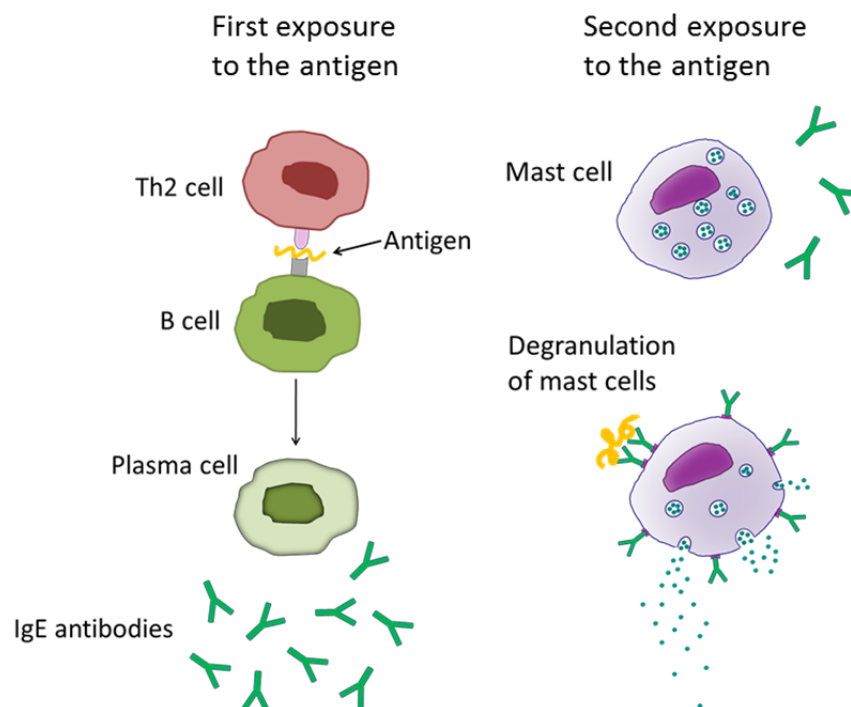


Figure 12: IgE mediated allergy

The first exposure to an antigen (left panel) gives rise to IgE antibody producing plasma cells (sensitisation). Exposure to the same antigen in sensitised individuals gives rise to cross-linkage of IgE antibodies and degranulation of the mast cell (right panel).

Upon exposure to an allergen in sensitised individuals, the IgE antibodies on the mast cells capture the allergen. If two IgE antibodies are cross-linked by an allergen molecule, the mast cells will release their granules and histamine will induce dilatation and leakage in local blood vessels (Figure 12, right panel). Activation of the mast cell also activates intracellular phospholipase A₂, which cuts off arachidonic acid from membrane phospholipids. Through the action of mast cell specific enzymes, arachidonic acid is converted to prostaglandins D₂ and leukotriene C₄ (LTC₄) (which are spontaneously converted to LTD₄ and LTE₄). The mast cell leukotrienes induce vascular leakage, tissue swelling and constriction of bronchial smooth muscle. Later, cytokines and chemokines are produced from mast cells, macrophages and T cells in the allergic focus, which induce infiltration by eosinophilic granulocytes and T cells of the Th2 type [94].

Eosinophilic granulocytes, which are signature cells of the allergic reaction, are avid producers of a wide range of lipid metabolites. They possess COX, LOX-5 and LOX-15 enzymes and produce, in addition to prostaglandins and leukotrienes, a range of other metabolites, such as 5-HETE and 5-HPETE [94].

Atopic march

The allergic disease may change the way it manifests during the life-course of an individual, a phenomenon termed “the allergic march” or “the atopic march”. The earliest manifestations are often atopic eczema. Atopic eczema is defined as a chronic, relapsing, inflammatory skin condition associated with epidermal barrier dysfunction [134], also called atopic dermatitis. IgE sensitisation to food allergens may be seen early in life (generally cow’s milk and egg proteins), which may progress into food allergy. Later, the individual displays sensitisation to airway allergens (cat, birch and grass being most common in Swedish individuals), asthma and allergic rhinitis. Many longitudinal studies have described this atopic march [135-137], but the biological background remains elusive. In accordance with the atopic march concept, an individual who displays atopic eczema in early age and also is sensitised to e.g. egg proteins is much more likely to develop hay fever and/or asthma to birch pollen in adolescence than an individual who displays no atopic manifestations during the first year of life.

Effect of fatty acid milieu on development and manifestation of allergic disease

It is known that the milieu during the first year(s) of life is of key importance to the risk of developing allergy [138], even if the allergic disease may manifest in school age. Hence, for the relation between PUFA and allergy the fatty acid milieu during the neonatal importance might be of highest interest. In individuals with manifest allergy, the fatty acid milieu may, secondarily, affect the allergic manifestations, given the role of PUFAs as substrates for inflammatory mediator production.

PUFAs may affect the risk of developing sensitisation and allergies in several ways. Prostaglandin E₂, produced from arachidonic acid may promote sensitisation to allergens, through actions on dendritic cells. Dendritic cells cultured in the presence of PGE₂ favour T cell differentiation along the Th2 pathway; Th2 cells produce IL-4, IL-5 and IL-13 and promote B cell class switching to IgE production [95]. In the allergic effector phase prostaglandins may instead dampen the inflammatory response, as suggested by the increased Th2 cytokine production and the increased airway reactivity in COX-1 and COX-2 inhibited mice [139]. Most studies have not made a clear distinction between these two different effects of PUFAs.

Dietary intake and allergic disease

Margarine

The incidence of allergies has increased in prevalence in parallel with a higher intake of n-6 PUFAs in the diet of the general population, which has led to the suggestion that there is a causal effect of n-6 PUFAs on allergic diseases [13, 140]. Margarine intake has been positively associated with allergy in both infants [141-146], adolescents and adults [147-149]. A systematic review on the association between fat intake and allergy suggested that margarine intake is a risk factor for allergy [14].

Fish

Consumption of fish has repeatedly been shown to be protective against allergy in children, both when consumed by pregnant and lactating mothers [142, 150-154] and by the children themselves [15, 155-158]. Also, early introduction of fish into the infant's diet protects against development of eczema, as shown in several studies [15-18].

Standl et al. [19] summarized epidemiologic studies published between 2005-2012, regarding the relation between intake of fatty acids and fat rich food items and allergy and concluded that intake of fish might protect against allergy. The preventive effect of fish has generally been attributed to its content of n-3 LCPUFAs, especially DHA and EPA, which are not found in any food items other than marine foods and whose production in the body is supposed to be limited. However, studies which investigated the relationship between intake of specific n-3 PUFAs α -linolenic acid, EPA and DHA and allergic diseases did not confirm these findings [19]. Fish

contains many compounds other than fatty acids that could be responsible for an effect on the immune system.

Cord blood fatty acid composition in relation to subsequent allergic disease

The fatty acid composition of cord blood reflects the fatty acid milieu in the foetus in late pregnancy. This milieu might affect early development of the immune system and, hence, affect the milieu in which the infant immune system encounters the first immunostimulatory events when the newborn infant is exposed to microbes directly after birth.

The fatty acid composition in several lipid fractions and cell types from cord blood has been investigated in relation to both allergies in the child, Table 2, as well as a family history of allergy, Table 3.

Data regarding cord blood fatty acid composition in relation to subsequent allergy development are conflicting (Table 2). Half of the studies recorded no differences in the proportion of individual fatty acids between infants who later developed allergy or stayed healthy [159-162]. The other four studies found subsequent allergic children to have higher proportions of linoleic acid [163], and lower proportions of 20:3 n-6 [163, 164], lower proportions of arachidonic acid [163, 164], lower DHA [163, 165], lower total n-3 LCPUFA [165] and lower total LCPUFA [165].

Notably, many of the studies had a relatively short follow-up time, assessing allergy at age 12 to 36 months (Table 2). It is possible to diagnose atopic eczema at early ages since it is at its maximum in prevalence at 12-24 months of age, but it not possible to properly diagnose respiratory allergies at such a low age. Only one study included allergy diagnoses later in childhood. Standl et al. [162] diagnosed the 243 included children both at 6 years of age and at 10 years of age. At 10 years of age, 17 (7 %) had eczema, 12 had asthma (5 %) and 32 had hay fever (13 %). This study found no differences in fatty acid proportions in cord serum phospholipids in relation to any allergy at any age. Standl et al. and most of the other studies included a small number of allergic subjects (Table 2). The largest of the studies, 301 subjects with eczema at 18-30 months of age compared to 937 non-allergic subjects, found no difference in the proportions of fatty acids, but a higher arachidonic acid/EPA ratio in infants developing eczema [160].

Table 3 summarizes studies examining differences in cord blood fatty acid composition between children born to allergic or non-allergic mothers or fathers [166-170]. The results are inconsistent (Table 3). Yu et al. performed two studies on infants with a family history of allergy (n= 33 and n= 25) and found that they had higher proportions of several n-6 and n-3 LCPUFAs compared to infants with no family history of allergy [168, 169]. The three other studies instead found lower proportions of LCPUFAs, foremost arachidonic acid, in infants with a family history of allergy [166, 167, 170]. In the largest of the studies Beck et al. [170] selected 50 subjects with allergy in the family and 50 subjects with no allergy in the family. The fatty acid composition was measured in different lipid fractions as well as in red blood cell phospholipids. The results varied

between the different lipid classes, but a pattern was found with lower proportions of arachidonic acid, α -linolenic acid and DHA, and higher proportions of EPA in infants with allergy in the family [170].

Serum or plasma fatty acid composition in individuals with manifest allergic disease

Some studies have also investigated PUFA pattern in serum from children or adults who are allergic, compared to subjects of similar age who have no allergy [142, 159, 162, 171-174], these studies are summarized in Table 4. As can be seen in Table 4, allergic subjects tend to have lower proportions of PUFA and LCPUFA in their blood, compared to non-allergic subjects and this is particularly evident for n-3 fatty acids. Most studies are small, although Dunder and colleagues studied 318 allergic and 318 non-allergic children [142]. This study had other merits, including that allergy was diagnosed by a physician and that allergy was diagnosed at older age compared to many of the other studies, which is important as the certainty of the allergy diagnosis improves with the age of the subjects. The results showed lower proportions of EPA and DHA in children with atopic eczema, compared to age and sex matched controls. Children with atopic rhinitis or asthma did not differ from matched controls regarding fatty acid pattern in blood [142].

Intervention studies – n-3 fatty acids/fish oil supplementation

Based on the favourable effect of fish diet on reducing subsequent allergy development, a number of trials have been carried out aiming to prevent allergy development in children by supplementing the diet of pregnant or lactating women, or the newborn infants with n-3 fatty acids.

Maternal fish oil supplementation during pregnancy and lactation

Studies in which pregnant or lactating women have been given fish oil are summarized in Table 5 [175-181]. Out of seven studies, three found reduction of eczema at one year of age [175, 179, 180]. In two out these three studies, allergy was also evaluated at two years of age [178] or three years of age [181], at these later time-points there was no longer any beneficial effect of PUFA feeding on eczema prevalence. No effect was seen on asthma in infancy in any of the studies evaluating allergy in young infants [175, 176, 178-181]. A single study assessed allergy in adolescents in relation to supplementation of pregnant women with LCPUFA; Olsen et al. [177] gave pregnant women either fish oil, olive oil, or no oil capsules, from gestational week 30 until parturition. The number of subjects with asthma, or any type of allergy, were assessed at 16 years of age. The olive oil group had the highest incidence, while both the fish oil and the no oil groups had lower incidence of allergy than the olive oil group. However the number of allergic subjects in this study were small, only seventeen of the children included in this study (n=533) were allergic at 16 years of age. Eight subjects had asthma in the olive oil group, compared to two in the fish oil group and none in the no-oil group.

Table 2: Fatty acid differences in cord blood from subsequent allergic and non-allergic children

Reference	Cases (n)	Controls (n)	Atopic criteria	Age for allergy diagnose	Sample	Cord serum fatty acid differences in subsequent allergic children
Strannegård et al. 1987, [163]	9 with atopic dermatitis	107	-	-	Serum PC	Higher 18:2 n-6, but lower 20:4 n-6, 22:6 n-3 and 20:3 n-6 in subsequent allergic children
Galli et al. 1994, [164]	10 with atopic dermatitis 3 with asthma	44	Hanifin for eczema	2-12 mo		Lower 20:3 n-6, 20:4 n-6 and 20:4 n-6/18:2 n-6 ratio in subsequent allergic children
Duchén et al. 2000, [159]	19 with atopic eczema, asthma or food allergy	40	Doctors diagnosed SPT	18 mo	Serum PL	No fatty acid differences
Newson et al. 2004, [160]	301 with eczema	937	Parental reported eczema	30-42 mo	RBC PL	Higher 20:4 n-6/20:5 n-3 ratio in subsequent allergic children No other fatty acid differences
Byberg et al. 2008, [161]	35 with atopic dermatitis	35	History of atopic dermatitis and positive SPT	3 y	Whole plasma	No fatty acid differences (Only a tendency for lower 20:5 n-3 in subsequent allergic children, p = 0.056)
Montes et al. 2013, [165]	41 with recurrent eczema	170	Parental reported recurrent eczema	6 and 14 mo	Plasma total lipid	Lower proportions of 22:6 n-3, tot n-3 LCPUFA, and tot LCPUFA in subsequent allergic children
Standl et al. 2014, [162]	6 y: 39 eczema, 8 asthma, 8 hay fever, 66 sens 10 y: 17 eczema, 12 asthma, 32 hay fever, 104 sens		Questionnaires SPT	2 y (only eczema), 6 y and 10 y	Serum PL	No fatty acid differences

Abbreviations: SPT = skin prick test, PL = phospholipid, PC = phosphatidylcholine, RBC = red blood cells, mo = months, y = years.

Table 3: Fatty acid differences in cord blood from children with a family history of allergy compared to no family history of allergy

Reference	Cases/controls (n)	Atopic criteria	Sample	Cord serum fatty acid differences in children with a family history of allergy
Galli et al. 1989, [166]	34/-	History of allergy, IgE ICB	CB lymphocytes	Lower proportions of 20:4 n-6 and 20:3 n-6 in children with a family history of allergy No association between 20:4 n-6 and IgE proportions
Ioppi et al. 1994, [167]	32/30	High risk for allergy	Mononuclear leukocytes	Lower proportions of 20:4 n-6 in children with a family history of allergy
Yu et al. 1996, [168]	33/35	Allergic mothers	Serum PL	Higher 20:4 n-6, 20:5 n-3, 22:6 n-3 and 20:3 n-6 in children with allergic mothers
Yu et al. 1998, [169]	25/22	Allergic mothers	Serum PL	Higher 20:4 n-6, 20:5 n-3, 22:6 n-3 and 22:4 n-6 in children with allergic mothers
Beck et al. 2000, [170]	50/50	Allergy in the family	Plasma and RBC PL Triglycerides Sterol esters	Lower proportions of 22:4 n-6 and lower proportions of 20:4 n-6, 22:4 n-6, tot n-6 in plasma PL in children with a family history of allergy

Abbreviations: CB=cord blood, PL= phospholipid, RBC=red blood cells,

Table 4: Fatty acid differences in serum from atopic and non-atopic subjects

Reference	Cases (n)	Controls (n)	Age	Atopic criteria	Sample	Fatty acid differences in children with allergy (atopics)
Duchen et al. 2000, [182]	16 with asthma, eczema or food allergy	35	FA analysis 3 mo Allergy at 18 mo	Doctors diagnosed SPT	Serum PL	Atopics had higher proportions of 22:4 n-6 and 22:5 n-6 and higher 20:4 n-6/20:5 n-3
Kankaanpaa et al. 2001, [171]	20 eczema or food allergy together with positive SPT	20	FA analysis 3 mo Allergy at 3 mo?	Questionnaires SPT	Serum PL, TG and CE	Atopics had lower 22:6 n-3 in CE, lower 18:3 n-6 in PL and higher 18:2 n-6 in TG.
Dunder et al. 2001, [142]	126 atopic eczema 145 atopic rhinitis 47 asthma	126 145 47	FA analysis and allergy at 3-18 y	Doctors diagnosed	Serum CE	Children with atopic eczema had lower 20:5 n-3 and 22:6 n-3 No fatty acid differences for children with atopic rhinitis or asthma
Focke et al 2005, [172]	22 atopic dermatitis 8 with atopic rhinitis and/or asthma	6	FA analysis and allergy at 1-16 y	Questionnaires SPT IgE	Plasma	Atopics had lower 18:3 n-6
Laitinen et al. 2006, [174]	6 atopic eczema (positive SPT) 6 eczema (negative SPT)	19	FA analysis median age 5.6 mo Allergy median age 2.5 mo	Questionnaires SPT	Serum PL	Atopics had lower 18:3 n-6, but higher 20:5 n-3
Johansson et al. 2011, [173]	14 eczema and respiratory allergy 7 only respiratory allergy	20	FA and allergy at median age 32	Questionnaires SPT IgE	Serum PL	Women with atopic eczema and respiratory allergy had lower proportions of 20:4 n-6 and 20:5 n-3. No fatty acid differences in women with only respiratory allergy
Standl et al. 2014, [162]	<u>2.y.</u> : eczema <u>6.y.</u> : 39 eczema, 8 asthma, 8 hay fever, 66 sens <u>10.y.</u> : 17 eczema, 12 asthma, 32 hay fever, 104 sensitised		FA analysis at age 0, 2, 6 and 10 y Allergy at 2 (only eczema), 6 and 10 y	Questionnaires SPT	Serum PL	<u>2.y.</u> : Children with eczema had lower tot n-3 LCPUFA and higher n-6/n-3 ratio <u>6.y.</u> : Children with sensitisation or asthma had lower tot n-3 LCPUFA <u>10.y.</u> : No fatty acid differences

Abbreviations: SPT= skin prick test, PL= phospholipid, CE=cholesteryl esters, TG=triglycerides, mo=months, y=year.

Table 5: Maternal fish oil supplementation during pregnancy or lactation and allergy in the infant of those mothers

Study	Subjects	Study design	Intervention	Exposure period	Clinical outcome	Findings
Perth, Australia Dunstan et al. 2003 [175]	83 atopic pregnant mother FO n=40 Control n=43	Double blinded RCT	FO: 3.7 g/day n-3 PUFA (56 % DHA, 28 % EPA) Control: 4 g olive oil/day	From week 20 of pregnancy until delivery	Asthma, wheeze, food allergy, atopic dermatitis SPT at 12 mo of age	Maternal FO: less severe dermatitis but no effect on clinical allergy
Copenhagen, Denmark Lauritzen et al. 2005 [176]	65 lactating mothers with low fish intake: FO n=37 Control n=28 26 lactating mothers with high fish intake	Double blinded parallel group RCT	1. FO: 4.5 g/ day (30 % LCPUFA) 2. Control: olive oil 3. Women with high fish intake – no supplementation	First 4 months of lactation	Parental reported allergy at 2.5 years of age	No differences in allergy between the groups.
Copenhagen, Denmark Olsen et al. 2008 [177]	533 pregnant women FO n=266 Olive oil n=136 No oil n=131	Double blinded RCT	1. FO: 4 g/day (32 % EPA, 23 % DHA) 2. Olive oil capsules (4 g/day) 3. No oil capsules	From week 30 of pregnancy until delivery	Allergic asthma, atopic dermatitis or allergic rhinitis at 16 years of age (from the National patient registry in Denmark)	Maternal FO: lower risk of asthma, allergic rhinitis and atopic dermatitis compared to olive oil group. No differences in allergy between FO and no oil group.
Linköping, Sweden Furuhjelm et al, 2009 and 2011 [178, 179]	145 pregnant women with family history of allergy FO n=70 Control n=75	Double blinded RCT	FO: 1.6 g EPA + 1.1 g DHA/day Control: soybean oil capsules	From week 25 of pregnancy until end of lactation (3- 4 months of breastfeeding	Clinical examinations at 12 month of age SPT IgE	12 mo: maternal FO lower prevalence of food allergy, IgE associated eczema, and of any positive SPT and SPT to egg 24 mo: No effect of supplementation on allergy or sensitisation
Adelaide, South Australia Palmer et al. 2012 and 2013 [180, 181]	706 pregnant women with child allergy follow up FO n=368 Control n=338		FO: 100 mg EPA + 800 mg DHA per day Control: Three capsules (500 mg rapeseed, 500 mg sunflower and 500 mg palm oil)	From week 21 of pregnancy	12 mo: Clinical diagnosis of IgE associated eczema or food allergy 3 y: Asthma, eczema, allergic rhinitis and food allergy with and without sensitisation	12 mo: FO associated with lower risk for eczema with sensitisation and sensitisation to egg 3 y: No effects of supplementation on allergy or sensitisation No effect of supplementation on cumulative allergy

Abbreviations: FO=fish oil, RCT=randomized controlled study, DHA=docosahexaenoic acid, EPA=eicosapentaenoic acid, SPT=skin prick test.

Vitamin D

Humans produce vitamin D in the skin from cholesterol under the influence of UV-irradiation from sunlight, or derive it from their diet [183]. It has been estimated that 90-95% of human vitamin D requirements can be produced in the skin [184]. Naturally, the concentration of synthesis will be affected by any factors altering the amount of UVB radiation entering the skin, e.g. time of season, time of day, latitude, cloudiness, ozone, air pollution, as well as pigmentation, age, time spent outdoors, clothing and use of sun protecting creams [184]. Dietary sources of vitamin D include fish and egg yolk. Margarines and low fat milk are fortified with vitamin D and constitute an important dietary source of vitamin D. In Sweden, where sunlight is scarce during the autumn and winter months, it is recommended that children up to 2 years of age receive vitamin D supplements (10 µg/day).

Vitamin D is metabolised in the liver to 25-hydroxy vitamin D (25(OH)D) which is transported in the blood bound to vitamin D binding protein to the mitochondria of the renal cortex where it is converted to the active metabolite 1,25-dihydroxy vitamin D (1,25(OH)₂D).

The main function of vitamin D is in the regulation of calcium absorption and homeostasis, and it is also involved in the phosphorous homeostasis. Together with the hormones calcitronin and parathyroid hormone (PTH), 1,25(OH)₂D maintains constant concentrations of serum calcium and phosphorous. The regulation of calcium is achieved by increasing the intestinal absorption of calcium, increasing the mineralization of bone and reduce the excretion of calcium [185].

Foetal concentrations of 25(OH)D at birth correlated significantly with maternal concentrations, suggesting that 25(OH)D diffuse easily across the placental barrier and that foetal concentrations are dependent on the concentrations in the maternal circulation [186, 187].

Vitamin D and allergy

The biological effects of vitamin D are mediated through the vitamin D receptor. The finding of the vitamin D receptor in peripheral blood mononuclear cells and leukocytes [188, 189] suggests that vitamin D might play an immunoregulatory role (Figure 13).

Vitamin D exposure in early life and allergy risk

A number of epidemiological studies have linked a higher vitamin D intake in pregnant women with lower risk of allergic disease in their children [190-193]. However, since dietary sources generally contribute to the human vitamin D status with less than 10% [184], measuring maternal 25(OH)D concentrations during pregnancy better reflects vitamin D exposure to the developing foetus *in utero*. Only a few observational studies have evaluated this association and the results are inconsistent: two studies found high maternal 25(OH)D concentrations in serum to be associated with higher prevalence of allergy in the offspring [194, 195], while three studies found no associations [196-198]. Cord blood 25(OH)D concentrations have been studied in relation to allergy development in a number of studies [194, 199-204]. Most of the studies failed

to find an association between cord blood 25(OH)D concentrations and allergy development [201, 202, 204]. However, an inverse association was found between cord blood 25(OH)D concentrations and wheeze in two studies [199, 200] and eczema in two studies [199, 203]. One of the studies found a positive association with food allergy [194], while none of the studies found cord blood 25(OH)D concentrations to be associated with asthma or allergic rhinitis [194, 199-204].

Two hypotheses about influence of vitamin D on allergy development

Two contradictory hypotheses have emerged regarding the effect of vitamin D on immune function and allergy development. The first hypothesis proposes that an increased vitamin D intake is responsible for the worldwide rise in incidence of allergies [205] and the second hypothesis suggests that vitamin D deficiency might have contributed to the increase in allergy [206].

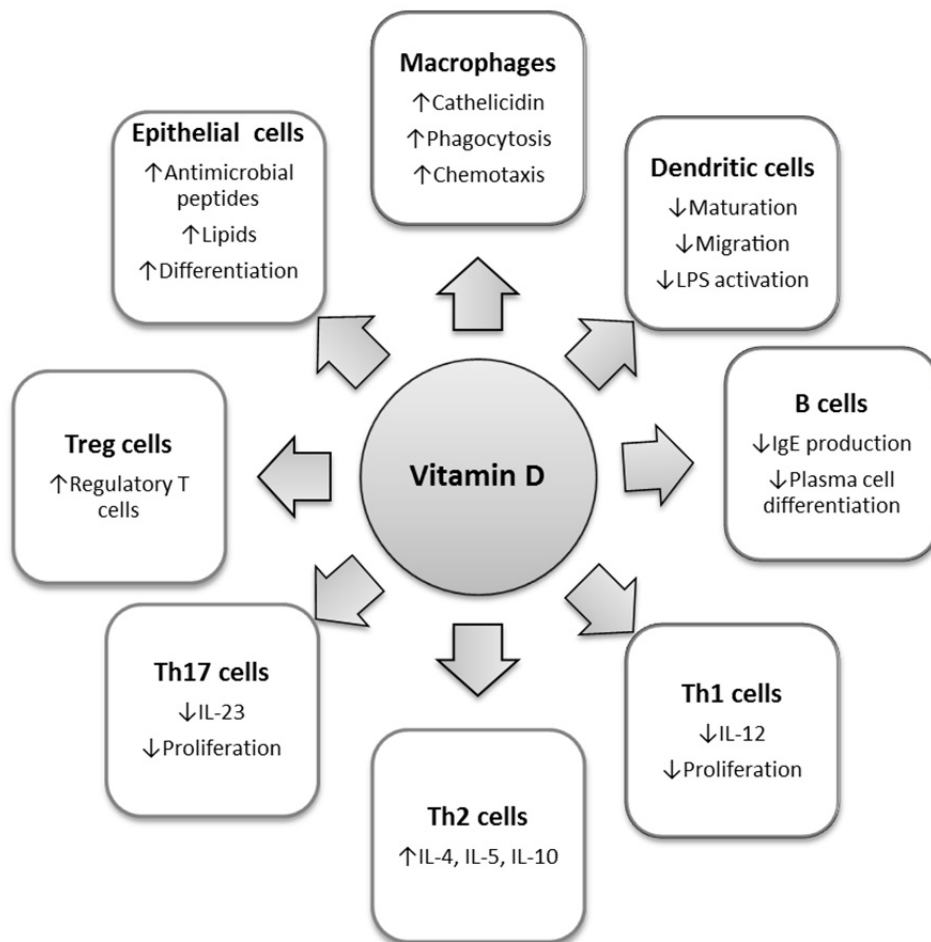


Figure 13: The impact of vitamin D on immune system

Vitamin D has modulatory effects on the immune system [207, 208]. Some of them are summarized in the figure. Abbreviations: LPS = lipopolysaccharide, Th = T helper, IL = interleukin, Treg = T regulatory.

Theory 1: Vitamin D is involved in the rise in allergies

The first theory is based on the coincidence of a rise in allergies and an increased supplementation of food products, such as dried milk, with vitamin D for the prevention of rickets [205]. Accordingly, intake of vitamin D [209] or supplementation with vitamin D [210] during the first year of life was associated with an increased risk of allergy at 6 or 31 years of age respectively. Also, epidemiological studies have found higher serum concentrations of 25(OH)D in individuals with atopic rhinitis [211, 212], and eczema [213] (Table 6).

In vitro studies that support the theory that vitamin D increases the risk of allergy have shown that high 1,25(OH)₂D concentrations reduce the secretion of typical Th1-type cytokines, such as IL-12 and IFN- γ [214] from T cells, while the secretion of cytokines of the Th2-type, such as IL-4 and IL-13 is increased [215], (Figure 13).

Table 6: Studies assessing dietary intake of vitamin D and allergy.

Reference	Conclusion
<i>Vitamin D intake is associated with decreased incidence of allergy</i>	
Sidbury <i>et al.</i> [216]	Supplementation with vitamin D improved the severity of atopic dermatitis more than placebo.
Amestejani <i>et al.</i> [217]	Supplementation with vitamin D improved the severity of atopic dermatitis, no such improvement was found in the placebo group.
Javanbakht <i>et al.</i> [218]	Supplementation with vitamin D improved the severity of atopic dermatitis, but no difference from placebo was found.
<i>Vitamin D intake is associated with increased incidence of allergy</i>	
Hyppönen <i>et al.</i> [210]	Infants receiving supplementation with vitamin D during the first year of life had a higher risk for allergy at 31 years of age
Bäck <i>et al.</i> [209]	Higher intake of dietary vitamin D during the first year of life was correlated with an increased risk of eczema at age 6 years
<i>No associations between vitamin D intake and allergy</i>	
Sidbury <i>et al.</i> [216]	No significant effect on severity of atopic dermatitis in subjects treated with vitamin D compared to placebo
Bäck <i>et al.</i> [209]	Intake of dietary vitamin D during the first year of life was not correlated with asthma or allergic rhinitis at age 6 years

Theory 2: Low concentrations of vitamin D might contribute to the increase in allergy

The alternative theory – that insufficient concentrations of vitamin D predisposes to allergy [206] has lent support from findings of lower 25(OH)D concentrations in serum in individuals with asthma [219-221], and atopic dermatitis [222-224] (Table 7). However, none of these studies are prospective and could perhaps be explained by increased consumption of vitamin D in allergic individuals.

Recently, randomized placebo-controlled trials have evaluated the effect of supplementation with vitamin D on the severity of atopic dermatitis and all found a reduced severity of the disease in the supplemented group compared to placebo [217, 225] (Table 6). The proposed mechanisms behind the beneficial effect of vitamin D on the severity of eczema involve reduction in epidermal permeability by vitamin D [226]. Keratinocytes express the enzyme 25(OH)D 1 α -hydroxylase that converts 25(OH)D to 1,25(OH)₂D, which has been shown to enhance keratinocyte differentiation [227].

Vitamin D has also been suggested to protect against airway remodelling that is a consequence of long-standing asthma and which contributes to symptoms such as hyper reactivity [228]. 1,25(OH)₂D decreased platelet-derived growth factor-induced airway smooth muscle cell growth in cells from both normal and asthmatic subjects *in vitro* [229]. However a recent RCT failed to show an effect on airway hyper reactivity when supplementing asthmatic subjects with vitamin D [230].

Table 7: Studies assessing vitamin D status and allergy

Reference	Conclusion
<i>Low serum 25(OH)D was associated with allergy</i>	
Bener et al. [219]	Lower serum concentrations of 25(OH)D in individuals with asthma
Searing et al. [220]	Lower serum concentrations of 25(OH)D in individuals with asthma
Baek et al. [222]	Atopic dermatitis was more severe in infants with vitamin D deficiency.
Peroni et al. [223]	Lower serum concentrations of 25(OH)D was associated with more severe atopic dermatitis
Oren et al. [224]	Atopic dermatitis was more severe in obese patients with vitamin D deficiency.
<i>High serum 25(OH)D was associated with allergy</i>	
Jung et al [134]	Higher serum concentrations of 25(OH)D in individuals with atopic rhinitis
Wjst et al [135]	Higher serum concentrations of 25(OH)D in individuals with atopic rhinitis
Heimbeck et al. [213]	Higher serum concentrations of 25(OH)D in eczematous individuals compared with those without eczema
<i>No association between serum 25(OH)D and allergy</i>	
Oren et al. [224]	No association between vitamin D status and either asthma or rhinitis in obese patients
Samochocki et al. [225]	No association between serum concentrations of 25(OH)D and atopic dermatitis
Chiu et al. [231]	No association between serum concentrations of 25(OH)D and atopic dermatitis
Gergen et al. [232]	No association between serum concentrations of 25(OH)D and asthma

METHODS AND MATERIALS

Study design

The cases (subjects with respiratory allergy or atopic eczema) and controls studied in this thesis were selected from a Swedish population-based birth cohort (BAS) enrolled during one year (February 1996 - January 1997). The children were all vaginally delivered at the Östersund Hospital in Jämtland in Northern Sweden. The families were enrolled either at the first visit to the antenatal clinic or at the birth of the child. In total, 1231 infants were included; three babies died during the first year of life, resulting in 1,228 children.

A questionnaire covering socioeconomic data and life style factors was filled out by the pregnant mothers at week 18 of gestation (Figure 14). The children were then followed regularly regarding sensitisation to common food and inhalant allergens (skin prick tests at 1, 4 and 13 years of age) and allergic symptoms (questionnaires at 1, 4, 7 and 13 years of age).

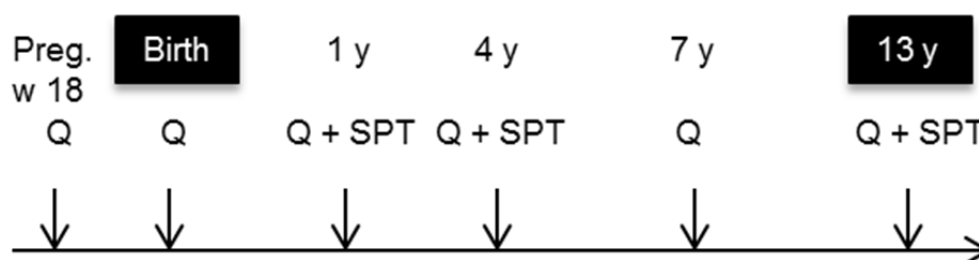


Figure 14: Overview of the questionnaires and skin prick test performed in the cohort
Abbreviations: Preg = pregnancy, Q = questionnaire, SPT = skin prick test.

Questionnaires

Questionnaires were distributed at gestational week 18. It included questions about home environment including food habits, smoking and allergic symptoms in the family. 857 families responded (Table 8).

Another questionnaire was distributed around the time of the birth of the child. 905 families returned the questionnaire. It included questions about housing and socioeconomics, parental smoking, pet keeping and allergic symptoms in family members.

At 5 months of age, a questionnaire concerning allergic symptoms, infectious diseases, antibiotic courses and amount of breastfeeding was filled in with the help of the child welfare centers (872 families responded). At 1 year of age, the same questions were asked in the form of a structured interview with the study nurse: questions concerned breastfeeding, infectious diseases, antibiotic courses, changes in home environment and allergic symptoms during the first year of life. 1043 families were interviewed.

At 4 years, the questionnaire was mailed to the parents of all children still living in the region. The parents of 801 children responded to the questions concerning home environment, including pet ownership, and symptoms of allergic diseases in the child. The parents of 736 children replied to questionnaires on symptoms of allergies at both 1 and 4 years of age. At 7 and 13 years of age, a new questionnaire concerning home environment and symptoms of allergic diseases was distributed to the parents of all children in the region with the aid of school teachers. The questionnaires were answered by 724 subjects at the age of 7 and by 834 subjects at 13 years of age (Table 8).

Table 8: Number of subjects that took part in the data collection at different time points

	W 18	Birth	5 mo	1 y	4 y	7 y	13 y
Questionnaire, <i>n</i>	857	905	872	1,043	877	724	834
Skin prick test, <i>n</i>	-	-	-	1,040	879	-	795
Blood sample, <i>n</i>	-	819	-	-	-	-	272

Abbreviations: W 18: pregnancy week 18, mo: months, y: years.

Skin prick tests

Parents were offered skin prick tests of their children at 1 and 4 years of age. At 13 years, both parents and adolescents were asked. The tests were performed on the volar side of the lower arm. At 1 and 4 years of age, standardized allergen extracts of egg, milk, birch, timothy grass and cat (Solu-Prick SQ, ALK-Abeló, Denmark) were applied with lancets from ALK. At 13 years of age, the response to ten allergens were tested, i.e. the above five, plus fish, wheat, soy, dog and horse, were used (ALK, Hørsholm, Denmark). The potency of the allergens was ten histamine equivalent prick units (HEP). The test was considered positive for a particular allergen if the mean wheal diameter was at least 3 mm in diameter after 15 minutes. Histamine dihydrochloride, 10mg/mL, was used as a positive control. Subjects were recommended to avoid antihistamines 72 hours before the test. The same study nurse carried out all the tests, and the reproducibility of the skin prick test technique was checked repeatedly according to recommendations in the ISAAC phase II manual [233]. The number of subjects that took part in the skin prick tests is shown in Table 8.

Allergy diagnoses

Allergy diagnoses were based on responses to questionnaires and skin prick tests and included: respiratory allergy and atopic eczema.

Respiratory allergy

Respiratory allergy, i.e. asthma and allergic rhinitis, was defined as fulfilling ≥ 1 of the following criteria: wheeze in the past year, doctor diagnosed asthma, asthma inhalation treatment, or a positive answer to the question "Have you had any signs of pollen allergy or allergy to furred pets during the last 12 months?" Further, at least one positive reaction in the skin prick test was required and the individual should have no other atopic diagnosis. In all, 130 adolescents fulfilled these criteria.

Atopic eczema

Atopic eczema was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution, fulfilling three of the main criteria suggested by Hanifin and Rajka [234]. Sensitisation was not required, but the individual should display no other allergic disease manifestation. In all, 79 adolescents fulfilled these criteria.

Non-allergic and non-sensitised controls

These were defined as having neither allergic symptoms nor any positive reaction in the skin prick test at 13 years of age ($n = 331$).

Subjects belonging to none of the groups

Subjects that did not belong to any of the three above mentioned groups included those who had a positive skin prick test in any of the measures at 1, 4 or 13 years, but no symptoms of allergy, who had more than one allergic manifestation at 13 years of age, or who had been diagnosed with an allergic manifestation previously (e.g. eczema), but who did not have symptoms at 13 years of age.

Selection of subjects for blood sampling at 13 years of age

The subjects that were invited to take part in the blood sampling at 13 years of age were selected on the basis of their allergy diagnosis at 13 years of age. The aim was to include as many subjects as possible from the two allergic groups, atopic eczema or respiratory allergy, as well as non-allergic controls, to take part in the blood sampling. In total, 272 subjects gave a blood sample at 13 or 14 years of age (Figure 15). The blood was collected at three time points: November 2009 ($n = 88$), April 2010 ($n = 52$) and January 2011 ($n = 132$) (Figure 15).

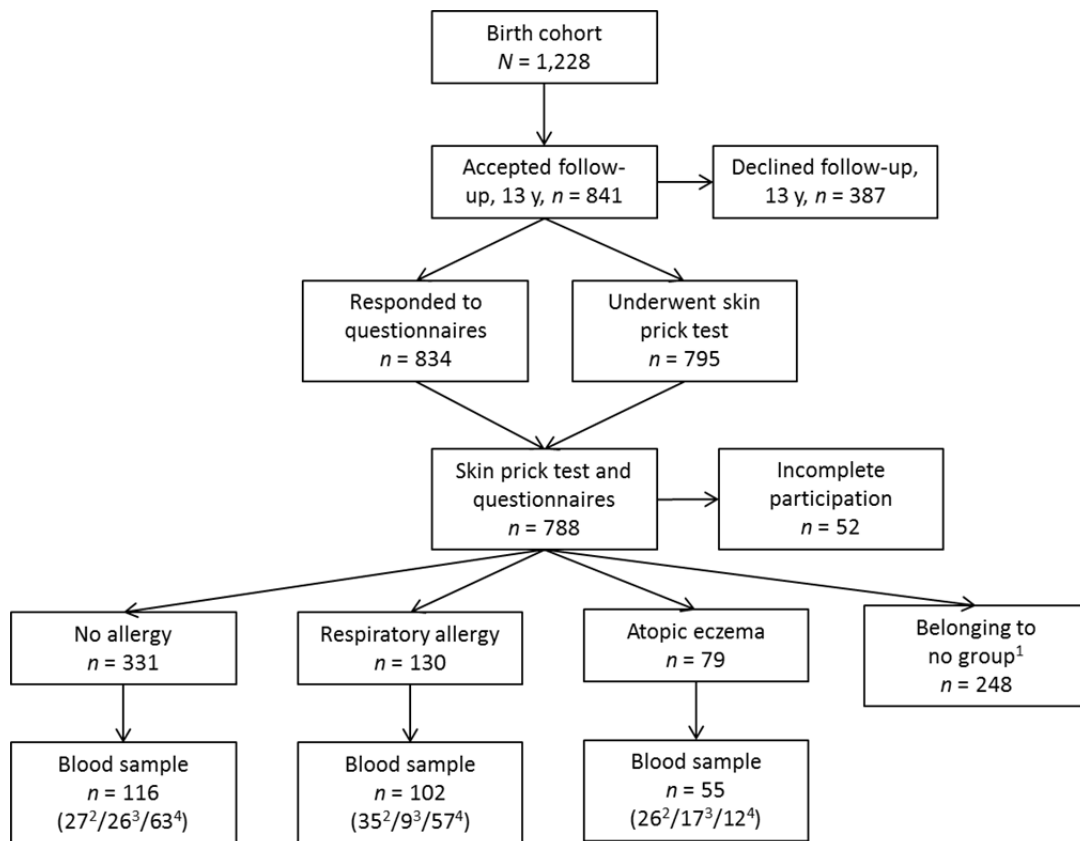


Figure 15: Flow chart of the cohort in the follow-up at 13 years of age

¹Subjects who were not included in any of the three groups were either sensitised but did not show any allergic symptoms or had more than one allergic symptom. ²Number of subjects that gave blood in November 2009. ³Number of subjects that gave blood in April 2010. ⁴Number of subjects that gave blood in January 2011.

Selection of cases and controls for the different papers

The studies were performed with a case-control design. Subjects from the two allergic groups (respiratory allergy or atopic eczema) were selected and compared to non-allergic controls.

Figure 16 shows an overview of the cohort and the number of subjects in the different papers. First, subjects were selected for fatty acid analysis in serum from the adolescents drawn at 13 years of age. The aim was to include around 50 subjects in each group (Paper IV). At birth, cord serum was stored for around 70 % of the subjects in the cohort. The subjects in Paper IV (n = 148) that had stored cord serum were included in Paper I (n = 129), and later also in Papers II (only subjects with genetic analysis, n = 120) and Paper III (only subjects born at term, n = 124).

During blood sampling at 13 years of age, the subjects were asked for participation in genetic analysis. 261 subjects gave blood for genetic analysis. From these, 211 subjects were selected for analysis of genetic polymorphisms (Paper II). This selection was done based on availability of cord serum.

For the analysis of 25(OH)D (Paper V), the season of blood sampling was considered in selecting subjects with or without allergy. We aimed to achieve similar numbers of subjects with blood samples in the autumn, winter and spring in the three groups: atopic eczema, respiratory allergy and no allergy.

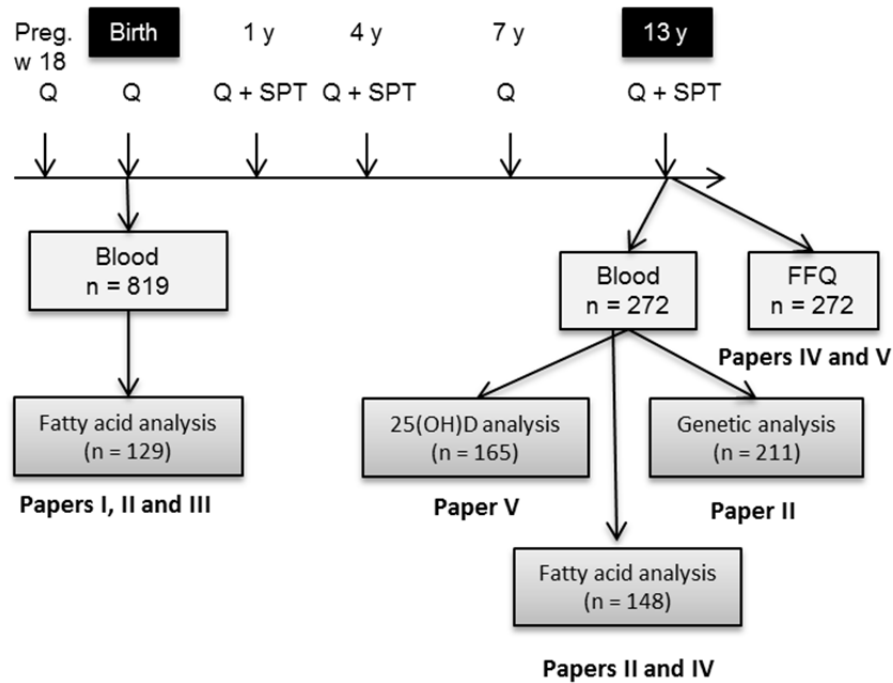


Figure 16: Overview of the cohort and the study designs

Abbreviations: Preg = pregnancy, Q = questionnaire, SPT = skin prick test, FFQ = food frequency questionnaire, 25(OH)D = 25-hydroxy vitamin D.

Paper I

The aim of Paper I was to investigate whether fatty acid composition of cord serum phospholipids affects allergy development up to age 13 years. For this study, 129 subjects were chosen for fatty acid analysis in the stored cord serum based on their allergy diagnosis at 13 years of age. The cases who had either respiratory allergy ($n = 44$) or atopic eczema ($n = 37$) and controls who were non-allergic and non-sensitised ($n = 48$). The allergic subjects were allowed to have only one type of allergy. The non-allergic controls also had not had any allergy diagnosis or positive skin prick test when examined at an earlier age (1, 4 and 7 years of age).

Paper II

The aim of Paper II was to investigate whether proportions of LCPUFAs in cord serum or adolescent serum phospholipids were determined by allele variants in genes that are involved in the elongation (*ELOVL*) and desaturation (*FADS*) of PUFAs. A second aim was to investigate whether these genetic variations were associated with allergy development.

Genotyping was performed in 211 subjects. Four SNPs were analysed: two SNPs in the *FADS* gene cluster and two SNPs in the *ELOVL2* gene. Eighty two of the subjects had respiratory allergy, 41 subjects had atopic eczema and 88 subjects were non-allergic. Proportions of LCPUFAs in cord serum phospholipids were available for 118 of the subjects and in adolescent serum phospholipids for 120 of the subjects.

Paper III

The aim of Paper III was to examine the relationship between cord serum LCPUFA pattern and gestational age, weight, length, and head circumference at birth in subjects that were born at term (n = 1052). Proportions of LCPUFAs in cord serum phospholipids were available for 124 subjects. Data on gestational age at delivery, as well as weight, length and head circumference at birth were derived from the Swedish Medical Birth Register kept by the Swedish National Board of Health and Welfare.

Paper IV

The aim of Paper IV was to investigate whether proportions of LCPUFAs in serum phospholipids at 13 years of age were related to allergic diagnosis and to seafood intake at 13 years of age. Further, the LCPUFA proportions in sera collected at 13 years of age were compared with those in cord blood from the same individuals.

Cases and controls from whom we had serum at 13 years of age were selected for fatty acid analysis. The subjects had either respiratory allergy (n = 53) or atopic eczema (n = 40), or were non-allergic and non-sensitised controls (n = 55) of the same age. Food frequency questionnaires were used to assess the intake of food items expected to affect fatty acids in serum and to calculate the intake of specific fatty acids.

Paper V

The aim of Paper V was to investigate whether serum levels of 25(OH)D at age 13 were related to 1) allergy diagnosis, 2) intake of foods containing vitamin D or total vitamin D intake, calculated from a food frequency questionnaire, or 3) season of blood sampling.

We selected cases who had respiratory allergy (n = 55) or atopic eczema (n = 55) and controls who were non-allergic and non-sensitised (n = 55) and measured 25(OH)D concentrations in serum using liquid chromatography coupled to mass spectrometry (LC-MS).

Dietary assessments

Maternal consumption of fish during pregnancy

The questionnaire distributed to the families at gestational week 18 contained questions about fish consumption habits. The first question was: "Do you eat fish?" The second question was: "What type of fish do you eat and how often?" According to these two questions, the mothers were manually divided into three groups based on how often they had written that they ate fish: < 2 times/month, 2 times/month, or > 2 times/month. The maternal fish consumption was correlated to fatty acid phospholipid proportions in cord and maternal serum at birth in Paper I. Not all mothers specified what type of fish (lean or fatty) they consumed; hence the type of fish consumed was not included in the analysis.

Food frequency questionnaires at 13 years of age

On the same occasion as the blood sampling, the adolescents were asked to fill out a food frequency questionnaire regarding intake frequency of 52 food items expected to affect the fatty acid pattern in serum, including dairy products, seafood, meat, eggs and bakeries. Options for intake frequencies included "never", "once a month", "once a week" and "daily". The questionnaire was simple in design with few options for frequencies to make it easier for the respondents to fill out, but each question was followed by a blank field where the respondents could provide more information if they wanted. For all questionnaires completed with less than eight missing values, the intake frequencies of the different food items were converted to grams using the weight table from the Swedish National Food Agency, dated 2001. The fatty acid and vitamin D intakes were then calculated by a nutritionist using the Diet32 software (Aivo AB, Stockholm, Sweden, 2008-04-11).

Blood sampling and analyses

Blood sampling

The first blood sampling took place at birth: cord serum was collected from 819 vaginally delivered children born at term. Maternal serum was also collected at the time of delivery. At 13 years of age, a subgroup of 300 adolescents was invited to take part in a new blood sampling. 272 adolescents gave blood on three different occasions: November 2009 ($n = 88$), April 2010 ($n = 52$) and January 2011 ($n = 132$).

Fatty acid composition of serum phospholipids

In human serum, fatty acid composition was analysed in the phospholipid fraction since it gives a more relevant estimation of long-term fatty acid intake than analysing all lipid fractions (triglycerides and free fatty acids as well). Serum phospholipid composition was determined extracting fat with chloroform and methanol according to the method of Lee *et al.* [235]. The

phospholipid fraction in serum was separated with solid phase extraction on aminopropyl solid phase extraction columns according to a method by Kaluzny *et al.* [236]. Methylation was then done with a direct-transesterification technique [237]. After methylation, the fatty acids were separated by gas chromatography on two different columns. The samples were first separated on an HP Ultra 1 (50 m × 0.32 mm × 0.52 μm d_f) silicon column (J&W Scientific, California) suitable for separation of the 20 - 22 carbon atom-long fatty acids. Where sufficient materials remained, we also performed separation of 16 - 18 carbon atom-long fatty acids on a DB-WAX (30 m × 0.25 mm × 0.25 μm d_f) column (J&W Scientific, California). Fatty acids that were found above the limit of quantification and could clearly be separated were selected for statistical analyses. The proportion of each fatty acid was expressed as area percentage of the total fatty acids, i.e. all fatty acids 16 - 22 carbon atoms long.

Fatty acid proportions in serum phospholipids were analysed in 148 of the subjects in the cohort at 13 years of age. The subjects were selected on the basis of their allergy diagnosis at 13 years of age and had either respiratory allergy (n = 53) or atopic eczema (n = 40), or were non-allergic and non-sensitised controls (n = 55). Proportions of fatty acids in cord serum phospholipids were measured retrospectively in stored serum samples from those of the 148 subjects (from Paper IV) that had cord serum available, i.e. 129 of the subjects (Papers I, II and III).

25-hydroxy vitamin D in serum

There are two forms of vitamin D in the blood: 25(OH)D and 1,25(OH)₂D. The most widely used indicator of vitamin D status is the measurement of 25(OH)D in either serum or plasma [238-241]. There are many commercially available methods for measuring 25(OH)D in serum to determine vitamin D status: high-pressure liquid chromatography (HPLC) and mass-spectrometry (MS), radioimmunoassays (RIA), enzyme immunoassays (EIA), competitive protein binding assay (DPBA), automated chemiluminescence protein-binding assay (CLPBA) and chemiluminescence immunoassays (CLIA) [242]. A high variability has been demonstrated between the different assays and between laboratory disagreements in the determination of 25(OH)D in serum [242-244]. Measurements with HPLC have been shown to have a higher accuracy than CLIA methods [242]. Vitamin D exists in two major forms. Vitamin D₃ (cholecalciferol) is produced in the skin from cholesterol after sun exposure, and vitamin D₂ (ergocalciferol) is produced in plants from ergosterol or can be extracted from yeast [245]. Both D₃ and D₂ are used to fortify milk and other food products in the United States. In Europe, vitamin D₃ is most commonly used in supplementation and in multivitamins [245]; hence serum samples from European populations seldom consist of measurable amounts of 25(OH)D₂. HPLC measurements can discriminate between 25(OH)D₂ and 25(OH)D₃, while RIA and CLIA methods measure the sum of the two forms of 25(OH)D. More recently, HPLC–Tandem Mass Spectrometry has been suggested to provide a rapid, accurate, sensitive and cost-effective alternative to other methods for detection of 25(OH)D₂ and 25(OH)D₃ at nanomolar concentrations [246, 247].

In Paper V we used an LC-MS method for measuring 25(OH)D in serum from 165 adolescents. The extraction part of the method is based on a method by Turpinen *et al.* [248] with some modifications. The main modification made was the use of an internal standard; 10 ng of 26, 26,

26, 27, 27, 27-D₆-25-OH Vitamin D₃ (Cerilliant, Round Rock, TX, USA) was added to all serum samples. In the mass spectrophotometer, selected-ion monitoring mode was used with ion 384 for 25(OH)D₃ and ion 389 for D₆-25(OH)D₃. 25(OH)D₃ and D₆-25(OH)D₃ were identified based on the retention time and mass of pure standard 25(OH)D₃ and D₆-25(OH)D₃.

We did not analyse 25(OH)D₂ in our samples. Only the 25(OH)D₃ form was determined since, overall, the subjects' levels of 25(OH)D₂ were too low in the samples to be measurable.

Determination of genetic variants of genes involved in fatty acid elongation and desaturation

Venous blood was collected in EDTA tubes for genetic analyses of adolescents at 13 years of age. Of the 281 subjects that took part in the blood sampling at 13 years of age, genetic analysis for polymorphisms was conducted in 211 subjects. The samples were sent to Kbiosciences (LGC genomics, Hoddeston, UK) for DNA extraction and genotyping.

DNA extraction

Genomic DNA was extracted from the blood samples using the standard protocol at Kbiosciences (LGC Genomics, Hoddeston, UK).

Genotyping

SNP genotyping in the present study was guided by previous GWAS studies that showed a significant association between fatty acids in serum phospholipids and SNPs in the *FADS* gene cluster or the *ELOVL* gene family [54, 249]. A total of six SNPs had been identified in the *FADS* gene cluster (rs102275, rs174547, rs174550, rs1535, rs174574, rs14448), and a total of four SNPs had been identified in the *ELOVL* gene family (rs17606561, rs3798713, rs3734398, rs2236212) [54, 249]. Of SNPs that were in complete linkage disequilibrium (LD, $r^2=1$) with one other, only one was analysed. Two SNPs in the *FADS* gene cluster (rs102275 and rs174448) and two SNPs in the *ELOVL2* gene (rs2236212 and rs17606561) that were not in LD with each other were selected for genotyping.

Statistical analysis

All univariate statistical analyses were made using SPSS Statistics version 19 (IBM Corporation, New York, USA); two-tailed $p \leq 0.05$ was considered significant. Student's T-test was used to compare means between any two groups when the variables were normally distributed, and the Mann-Whitney *U* test was used when the variables were not normally distributed. For correlation tests, Pearson's correlation test was used when both variables were normally distributed while Spearman's rank correlation was used if one or both of the variables tested were not normally distributed. Chi square test was used to analyse differences in background variables between the selected individuals and the groups from which they were selected.

Normality of variables was tested with the Kolmogorov–Smirnov test, a p-value > 0.05 indicates that the variable is normally distributed.

In Paper I, Kaplan–Meier curves were calculated to follow allergy development over time in subjects grouped according to cord LCPUFA proportions. Cox regression was used to control for confounders.

In Paper II, Hardy-Weinberg equilibrium was investigated with a chi-square goodness of fit. SNPs were analysed coded according to minor allele count and analysed as a numeric variable. The association between SNPs and fatty acids was analysed using linear regression and the association between SNPs and allergy was analysed with logistic regression.

In Paper IV, we used the multivariate method partial least squares-discriminant analysis (PLS-DA) (17) to find a model able to separate the three clinical classes: non-allergic, atopic eczema and respiratory allergy, based on the proportion of long chain PUFAs. The variables that contribute to the separation of the classes (allergic groups) can be identified using a PLS-DA model. The method permits an evaluation of differences in variable values without the risk of mass significance and need for normal distribution of data or strict independence among variables, as in conventional statistics. PLS-DA was carried out using Simca 13.0 (Umetrics, Umeå, Sweden).

Multiple logistic regression analysis was used in Paper V to analyse the association between allergy diagnosis and serum 25(OH)D as well as calculated dietary intake of vitamin D, taking into consideration potential confounders.

RESULTS

Fatty acids and allergy development

An important aim in the thesis was to study the association between LCPUFA composition in serum and the development of allergy. The association between cord serum fatty acid proportions at birth and allergy at 13 years of age was studied in Paper I and the association between adolescent serum fatty acid proportions at 13 years of age on allergy was studied in Paper IV.

Subjects who had atopic eczema or respiratory allergy at age 13 were found to have higher proportions of several n-3 and n-6 LCPUFAs among the fatty acids composing cord serum phospholipids, and a lower proportion of the monounsaturated fatty acid 18:1 n-9 (oleic acid), as compared to individuals who were non-allergic at age 13, and neither had any allergic manifestations nor had been sensitised up to that age (Paper I).

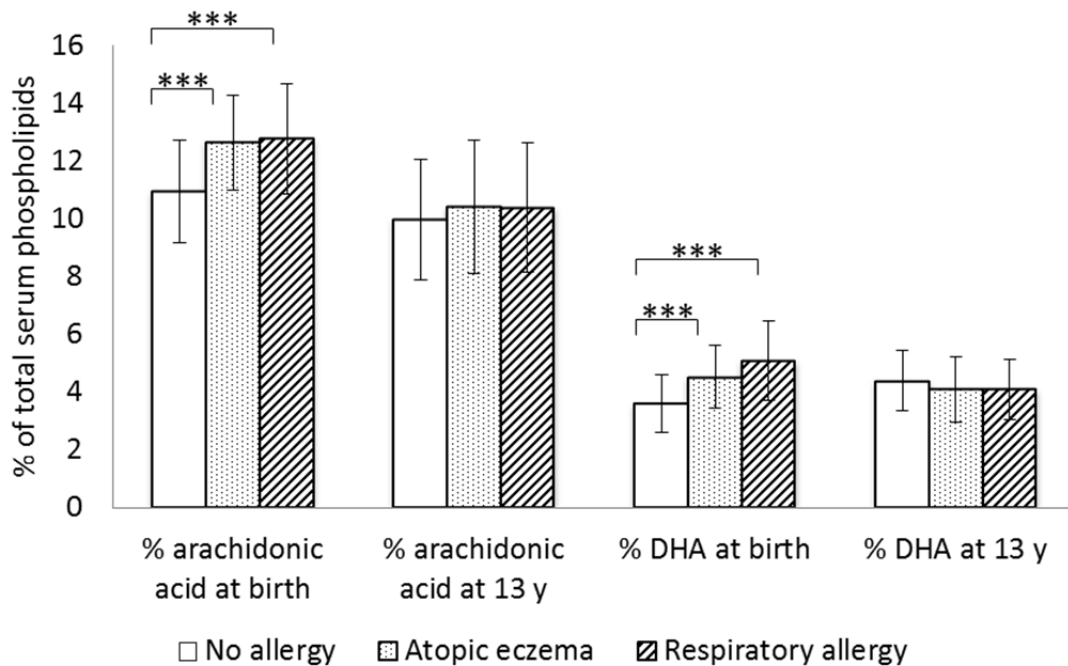


Figure 17: Proportions of arachidonic acid and DHA at birth and at 13 years of age

Proportions of fatty acids were expressed as area percentage of total phospholipids

*** $p < 0.001$

At 13 years of age, neither of the individual fatty acids differed in proportion among serum phospholipids in allergic compared with non-allergic adolescents (Paper IV). Figure 17 shows the proportions of the main n-6 (arachidonic acid) and the main n-3 LCPUFA (DHA) at birth and at 13 years of age.

The proportions of arachidonic acid were significantly higher in cord serum at birth than in adolescent serum at 13 years of age: 12 % vs 10 % (P < 0.001). The proportion of DHA tended to decrease from birth to 13 years of age: 4.4 % vs 4.2 % (P = 0.081). Instead, the proportion of linoleic acid (18:2 n-6) increased from birth to 13 years of age: 8.2 % vs 22 % (P < 0.001).

Fish intake and allergy development

At 13 years of age, the adolescents filled out a food frequency questionnaire regarding current food consumption. The questionnaire contained questions on frequency of intake of food items expected to affect the fatty acid pattern in serum, such as fish, meat and dairy products.

No differences were found between the allergic and non-allergic subjects in intake of fish, meat, butter, margarine or fish oil capsules (Table 9).

Table 9: Intake of fat rich food items¹ in relation to allergy

(g/day)	No allergy n = 73 ²	Atopic eczema n = 56 ²	Respiratory allergy n = 74 ²	p ³ ecz vs ctrl	p ³ resp vs ctrl
Fatty fish	8.0 (4.9-22)	10 (4.9-20)	7.1 (4.9-20)	ns	ns
Lean fish	8.1 (4.9-20)	20 (4.9-20)	8.1 (4.9-20)	ns	ns
Total fish ⁴	25 (12-36)	28 (18-39)	25 (12-39)	ns	ns
Total meat ⁵	92 (72-103)	90 (66-105)	87 (71-105)	ns	ns
Butter	5.2 (5.0-6.4)	5.7 (1.1-10)	5.7 (5.0-10)	ns	ns
Margarine	5.2 (0.7-8.6)	5.0 (0.7-9.3)	5.0 (0.2-9.3)	ns	ns
Fish oil capsules	0 (0-0)	0 (0-0)	0 (0-0)	ns	ns

¹Shown as median (Inter quartile range)

²Number of subjects that filled out a complete food frequency questionnaire in each group at 13 years of age

³p-value obtained by non-parametric Mann-Whitney U-test

⁴Including fatty and lean fish, as well as shellfish

⁵Including prepared meat, red meat and chicken

Abbreviations: resp=respiratory allergy, ctrl=non-allergic controls, ecz=atopic eczema, ns=no significant group difference

Determinants of LCPUFA composition in cord serum

Long chain fatty acids in the foetus are derived from maternal circulation (being actively transported or passively diffused through the placenta) and/or from synthesis by the foetus from essential PUFAs that are elongated and desaturated by enzymes termed elongases and desaturases. With the aim of examining what determines the composition of LCPUFAs in the circulation of the newborn infant, we analysed the association between cord serum fatty acid proportions and polymorphism in the *FADS* gene cluster and in the *ELOVL2* gene (Paper II), gestational age (Paper III) and maternal fish intake during pregnancy (Paper I).

Long chain PUFAs in cord serum and genetic variation

It is unknown how well the foetus can produce LCPUFAs from precursors, i.e. linolenic acid for the n-6 series of LCPUFAs and α -linolenic acid for the n-3 series of LCPUFA. Production is carried out by elongases encoded by *ELOVL* genes and desaturases encoded by *FADS* genes. In Paper II we analysed two single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster and two SNPs in the *ELOVL2* gene.

All subjects have two copies of each gene, one from the mother and one from the father, situated on two homologous chromosomes. An allele is one of two or more versions of a gene. Single nucleotide polymorphisms are the most common type of genetic variation. Each SNP represents a difference in a single nucleotide in the genome. Most SNPs have two different variants, i.e. two possible alleles. Within a population the allele that is less common is denoted the minor allele and the allele that is most common is denoted the major allele. For the SNP rs102275 (situated intergenic downstream of *FADS1*, see Figure 18), T is the major allele and C is the minor allele. A subject can inherit the polymorphism either from one of the parents (heterozygote) or from both parents (homozygote) (described in more details in Figure 6 in the background section).

Association between cord serum PUFAs and polymorphism in the *FADS* gene cluster

Two SNPs were analysed in the *FADS* gene cluster, rs102275 and rs174448 (Figure 18). The SNP rs102275 is situated intergenic downstream of *FADS1*, which encodes Δ 5-desaturase, the enzyme that converts 20:3 n-6 to 20:4 n-6 and 20:4 n-3 to 20:5 n-3, by insertion of an additional double bond to the carbon chain (Figure 4). The SNP rs174448 is situated intergenic between *FADS2* and *FADS3* (Figure 18). *FADS2* encodes Δ 6-desaturase (Figure 4) while the function of *FADS3* is unknown.

When the *FADS* SNPs were added together in the linear regression models with fatty acids only, rs102275 was significant. This indicates that the association with rs174448 is simply a consequence of linkage disequilibrium ($R^2 = 0.5$). Hence only the data from SNP rs102275 will be presented here.

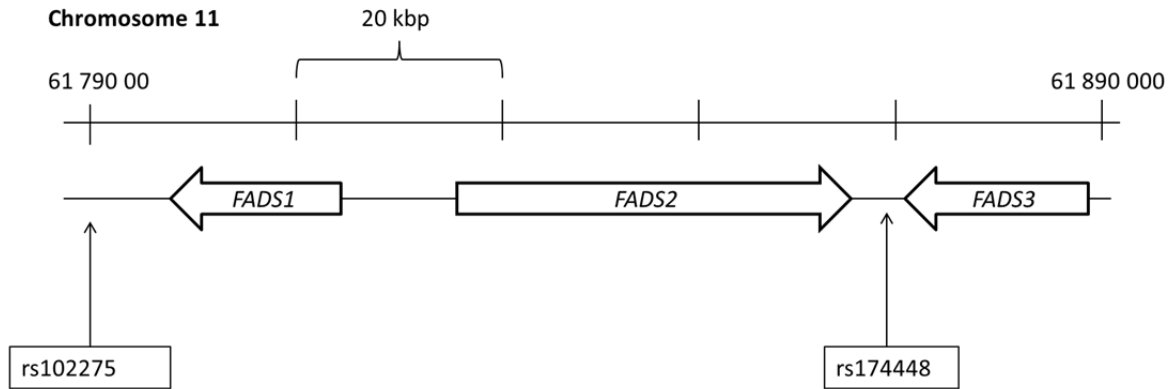


Figure 18: Location of the two single nucleotide polymorphisms analysed in the *FADS* gene cluster

Rs102275 is situated intergenic downstream of *FADS1* and SNP rs174448 is situated intergenic between *FADS2* and *FADS3*

As shown in Figure 19, carriers of two copies of the minor allele (C:C) had elevated proportions of the substrates dihomo- γ -linolenic acid (20:3 n-6) and decreased proportions of the product arachidonic acid (20:4 n-6), described schematically in Figure 20. Although the same enzymes are used to convert n-3 PUFAs into longer species, we found no correlation between n-3 LCPUFAs and polymorphism in the *FADS* gene cluster.

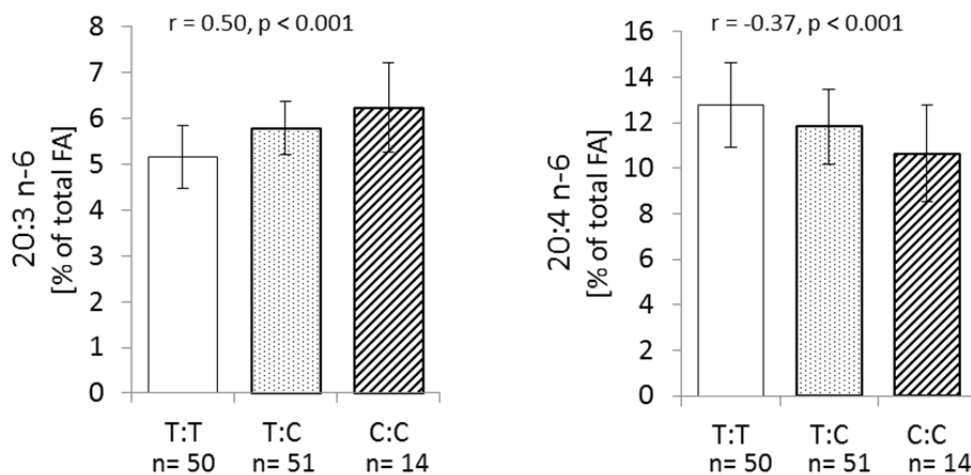


Figure 19: Association between cord serum proportions of 20:3 n-6 and 20:4 n-6 and single nucleotide polymorphism (rs102275) in the *FADS* gene cluster

For rs102275, T is the major allele and C is the minor allele (less common allele in this population). Single nucleotide polymorphisms were coded according to minor allele count and analysed as a numeric variable, i.e. T:T = 0, T:C = 1 and C:C = 2. A positive correlation equals that minor allele carriers (C) have higher proportions of the fatty acid than major allele carriers (T).

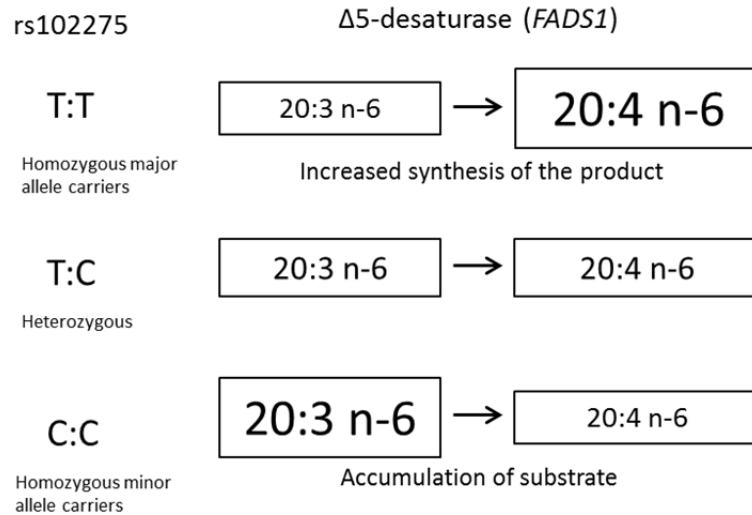


Figure 20: Association between rs102275 polymorphism and $\Delta 5$ -desaturase activity

For rs102275, T is the major allele and C is the minor allele. Minor allele carriers (C) had a lower conversion of the substrate 20:3 n-6 to the product 20:4 n-6.

The correlations between arachidonic acid (20:4 n-6) and dihomo- γ -linolenic acid (20:3 n-6) in cord serum and polymorphism in rs102275 were seen in both male and female subjects (Table 10).

Table 10: The effect of gender on the association between the *FADS* gene cluster polymorphism (rs102275) and cord serum proportions of dihomo- γ -linolenic acid and arachidonic acid

	Dihomo- γ -linolenic acid 20:3 n-6		Arachidonic acid 20:4 n-6	
	r	p	r	p
rs102275 C>T				
All subjects (n = 115)	0.50	< 0.001	-0.37	< 0.001
Females (n = 63)	0.48	< 0.001	-0.26	0.037
Males (n = 52)	0.53	< 0.001	-0.47	0.001

Correlations were calculated using the Pearson's correlation test

Number of subjects with different genetic combinations of alleles: T:T n= 50, T:C n = 51 and C:C n = 14

SNPs were coded according to minor allele count and analysed as a numeric variable: T:T=0, T:C=1, C:C= 2

A positive correlation equals that minor allele carriers have higher proportions of the fatty acid than major allele carriers

Association between cord serum PUFAs and polymorphism in the *ELOVL2* gene

Two single nucleotide polymorphisms were also analysed in the *ELOVL2* gene, rs2236212 and rs17606561 (Figure 21).

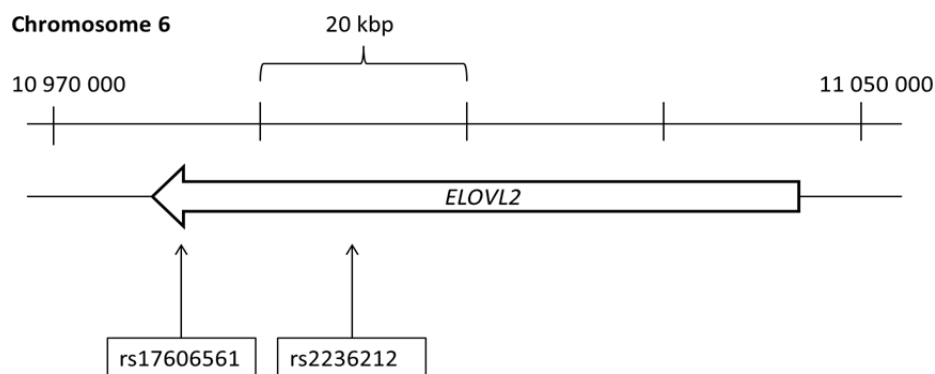


Figure 21: Location of the two single nucleotide polymorphisms analysed in the *ELOVL2* gene

Two single nucleotide polymorphisms in the *ELOVL2* gene were studied, rs2236212 and rs17606561.

ELOVL2 has been suggested to mainly be involved in the elongation of 22:4 n-6 to 24:4 n-6 and 22:5 n-3 to 24:5 n-3 [33] (see Figure 4 on page 5). These two products are then further converted by desaturation and β -oxidation to the end products of the n-6 and n-3 pathways; 22:5 n-6 and 22:6 n-3 (DHA).

Table 11: Associations between *ELOVL2* polymorphism (rs2236212) and proportions of LCPUFAs in cord serum phospholipids

	rs2236212 G>C	
	r	P
22:4 n-6	0.09	0.35
22:5 n-6	-0.12	0.20
22:5 n-6/22:4 n-6	-0.19	0.039
22:5 n-3 (DPA)	0.12	0.20
22:6 n-3 (DHA)	0.02	0.81
22:6 n-3/22:5 n-3	-0.19	0.038

Correlations were calculated using the Pearson's correlation test. G = major allele, C = minor allele. Number of subjects with different genetic combinations of alleles: G:G n= 41, T:C n = 48 and C:C n = 28. SNPs were coded according to minor allele count and analysed as a numeric variable: G:G=0, G:C=1, CC=2. A positive correlation equals that minor allele carriers have higher proportions of the fatty acid than major allele carriers.

The two SNPs were not associated with 22:4 n-6, 22:5 n-6, 22:5 n-3 or 22:6 n-3 alone. However, rs2236212 was significantly negatively associated with the ratio of the product and the substrate, 22:5 n-6/22:4 n-6 and 22:6 n-3/22:5 n-3 (Table 11). This suggests that carriers of two minor alleles had decreased proportions of the end products in the pathway (22:5 n-6 and 22:6 n-3) and increased proportions of the substrates (22:4 n-6 and 22:5 n-3), suggesting less conversion from the substrate to the product in carriers of two minor alleles.

Long chain PUFAs in cord serum and gestational age

Gestational age was a positive predictor for the proportion of DPA ($r = 0.36$, $p < 0.001$) and DHA ($r = 0.32$, $p < 0.001$) in cord serum. In contrast, gestational age did not correlated with the proportion of arachidonic acid, the major n-6 LCPUFA, in cord serum.

When the analysis was made for male foetuses and female foetuses separately gestational age was significantly correlated to the proportion of DPA ($r = 0.48$, $p < 0.001$) and DHA in cord blood ($r = 0.50$, $p < 0.001$) in male infants, but not in female infants (DPA: $r = 0.24$, $p = 0.054$, DHA: $r = 0.16$, $p = 0.21$) in cord serum (Figure 22).

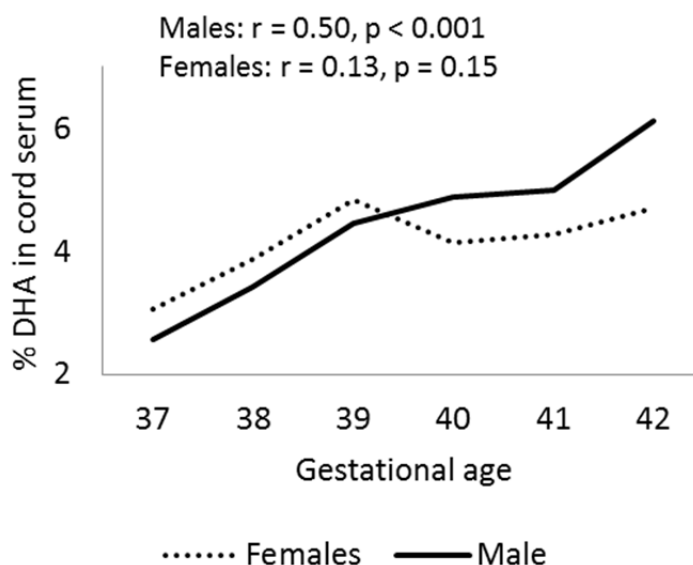


Figure 22: Proportions of DHA in cord serum in related to gestational age

Line chart showing how proportions of DHA (% of total fatty acids) in cord serum phospholipids was related to gestational age in female (dotted line, $n = 68$) and male (filled line, $n = 56$) subjects.

Long chain PUFAs in cord serum and maternal fish intake

Fish is a major dietary source of n-3 LCPUFAs, and fish intake in the pregnant woman may affect the amount of n-3 PUFAs in the maternal serum that is available for transport to the foetus. At gestational week 18, the mothers were asked to specify how often they consumed fish. The question was primarily posed to investigate whether pregnant mothers avoided fish as it may be a major source of allergens. The questionnaire responses were converted to intake frequencies in times per month and correlated to the proportions of total n-3 LCPUFA in cord serum phospholipids at birth, assuming that the fish intake did not differ between pregnancy week 18 and delivery. The results showed no association between frequency of maternal fish intake in pregnancy and total n-3 LCPUFA in cord serum at birth (Paper I).

Metabolism of long chain PUFA in adolescents

In order to study the fatty acid metabolism in adolescents, we investigated how the fatty acid pattern in serum of adolescents was associated with polymorphisms in the *FADS* gene cluster or in the *ELOVL2* gene (Paper II) and to dietary intake of fatty acids (Paper IV).

Endogenous production of long chain PUFAs in adolescent

As previously done for the subjects when they were newborn, the PUFA pattern of adolescent sera was related to polymorphisms of *ELOVL* and *FADS* genes. We found similar correlations as for the newborn infants, although the correlations were generally weaker for LCPUFAs in adolescent's serum, and only the proportion of arachidonic acid was significantly correlated with the *FADS* gene cluster polymorphisms.

When separating male and female subjects, the association between adolescent serum proportions of arachidonic acid and the *FADS* gene cluster polymorphism (rs102275) was stronger for female than for male subjects (Table 12). For the different allergic manifestations, the correlation between adolescent serum proportions of arachidonic acid and *FADS* gene cluster polymorphism (rs102275) was seen in both allergic and healthy persons, although it did not reach significance in the group with respiratory allergy (Table 12).

Table 12: Effect of gender and allergy on association between *FADS* polymorphism and dihomo- γ -linolenic acid and arachidonic acid proportions in adolescents

	Dihomo- γ -linolenic acid (20:3 n-6)		Arachidonic acid (20:4 n-6)	
	Pearson's correlation		Pearson's correlation	
rs102275 C>T	r	p	r	p
All subjects (n = 117)	0.12	0.21	-0.36	< 0.001
Females (n = 65)	-0.06	0.66	-0.50	<0.001
Males (n = 52)	0.22	0.12	-0.19	0.17
Non-allergic (n = 42)	0.17	0.28	-0.39	0.01
Atopic eczema (n= 35)	0.14	0.41	-0.49	0.003
Respiratory allergy (n= 41)	0.034	0.84	-0.23	0.15

SNPs were coded according to minor allele count and analysed as a numeric variable: T:T=0, T:C=1, C:C=2. A positive correlation equals that minor allele carriers have higher proportions of the fatty acid than major allele carriers. Number of subjects with different genetic combinations of alleles: T:T n= 50, T:C n = 51 and C:C n = 16.

Long chain PUFAs in adolescent serum and fat intake

Diet is a main source of LCPUFA. We have previously noted that women with a combination of eczema and respiratory allergy have lower proportions of LCPUFA in breast milk and serum compared to healthy women, despite higher fish intake [173]. This could indicate that more LCPUFA is used in the body during the allergic reaction [250]. In Paper IV we studied how well dietary intake of fatty acid-rich food items and intake of LCPUFAs correlated with serum proportions of corresponding LCPUFAs and whether the correlation differed between different clinical groups, i.e. those with respiratory allergy, atopic eczema or no allergy.

In non-allergic adolescents, serum proportions of DHA was significantly correlated to intake of seafood ($r = 0.46$, $p < 0.001$). In adolescents with respiratory allergy, there was a weak, but still significant, correlation between serum proportions of DHA and intake of seafood ($r = 0.29$, $p = 0.036$). In adolescents with atopic eczema, however, serum proportions of DHA was not significantly correlated to intake of seafood ($r = 0.16$, $p = 0.319$). The same results were found for fatty fish intake and n-3 LCPUFAs in serum (Paper IV).

Dietary assessment software was used to calculate intake of specific fatty acids (in grams per day) from the responses to the food frequency questionnaire that was distributed at the time of blood sampling at 13 years of age (see Methods). The n-3 LCPUFAs EPA and DHA are only found in marine foods, while arachidonic acid is found in meat, fish, eggs and a range of other foods. Table 13 shows the correlation between proportions among serum phospholipids of these fatty acids and intake of the same LCPUFA (in gram per day).

Table 13: Influence of allergy on the correlations between serum proportions of LCPUFAs and the intake of the same LCPUFAs

	AA		EPA		DHA	
	rho	p	rho	p	rho	p
No allergy (n = 55)	0.14	0.31	0.34	0.011	0.36	0.008
Atopic eczema (n = 40)	-0.07	0.67	-0.04	0.81	0.13	0.42
Respiratory allergy (n = 49)	0.02	0.88	0.20	0.17	0.39	0.006

Correlations were calculated with Spearman's correlation test since intake patterns were not normally distributed. Abbreviations: AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid.

The proportion of DHA in serum phospholipids correlated significantly with the intake of DHA in non-allergic subjects and subjects with respiratory allergy but not in subjects with atopic eczema, and EPA in serum correlated significantly with the intake of EPA in non-allergic individuals (Table 13). The proportion of arachidonic acid among serum phospholipids did not correlate significantly with the intake of arachidonic acid in any of the clinical groups.

Genetic variation in *FADS* and *ELOVL* genes and development of allergy

The association between prevalence of allergy and polymorphism in *FADS* and *ELOVL* genes was determined in Paper II. Figure 23 shows the association between allergy and *FADS* polymorphisms. Having the minor allele of either rs102275 or rs174448 was associated with a lower prevalence of atopic eczema. With logistic regression it could be calculated that carriers of these alleles had approximately half the risk of developing this particular atopic phenotype (OR = 0.48, $p = 0.018$ for rs102278 and OR = 0.52, $p = 0.035$ for rs174448).

No association between polymorphism in the *FADS* gene cluster and respiratory allergy was found. Also, no association between the two *ELOVL2* SNPs and risk for atopic eczema or respiratory allergy was found (Paper II).

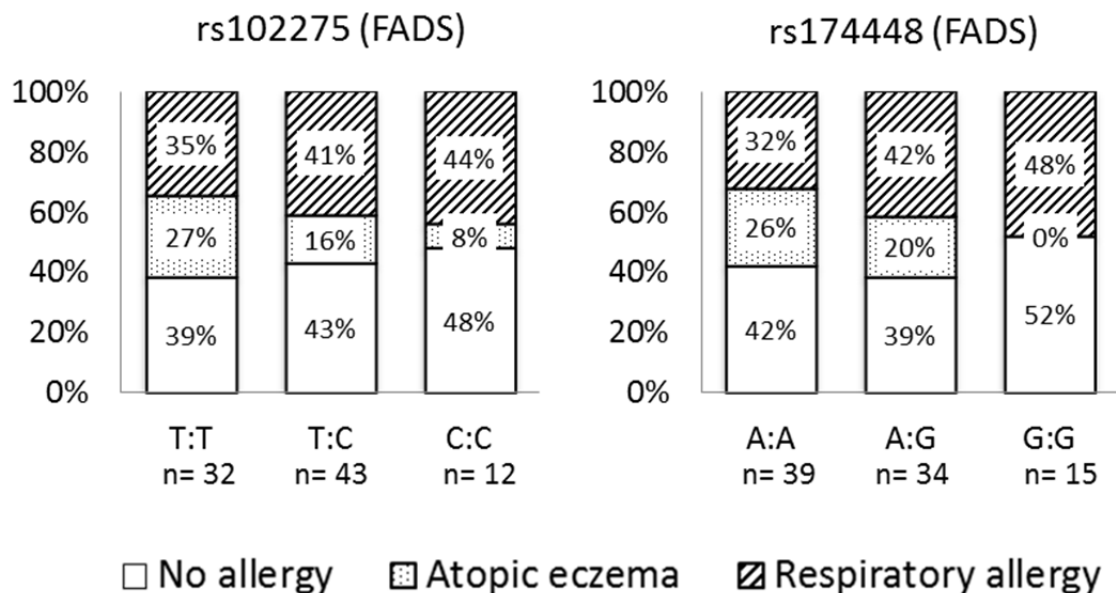


Figure 23: Number of subjects with different allergic manifestations at 13 years of age (%) in relation to rs102275 and rs174448 (*FADS*) polymorphism

For rs102275 C is the minor allele and T is the major allele, while for rs174448 G is the minor allele and A is the major allele. For rs102275, C:C denotes a subject that has two copies of the minor allele (least common allele in this population), T:C denotes a subject with one copy of the minor allele and one copy of the major allele, while T:T is a subject that carries two major alleles (the version of the gene that is most common in the population).

Vitamin D and allergy development

In paper V we investigate whether levels of 25-hydroxy vitamin D (25(OH)D) in serum, calculated intake of vitamin D and vitamin D containing food items differed between allergic and non-allergic adolescents.

Vitamin D is converted into 25(OH)D, which is the circulating form of the vitamin. Serum 25(OH)D levels did not differ between individuals with atopic eczema and non-allergic controls, nor between individuals with respiratory allergy and controls (Figure 24).

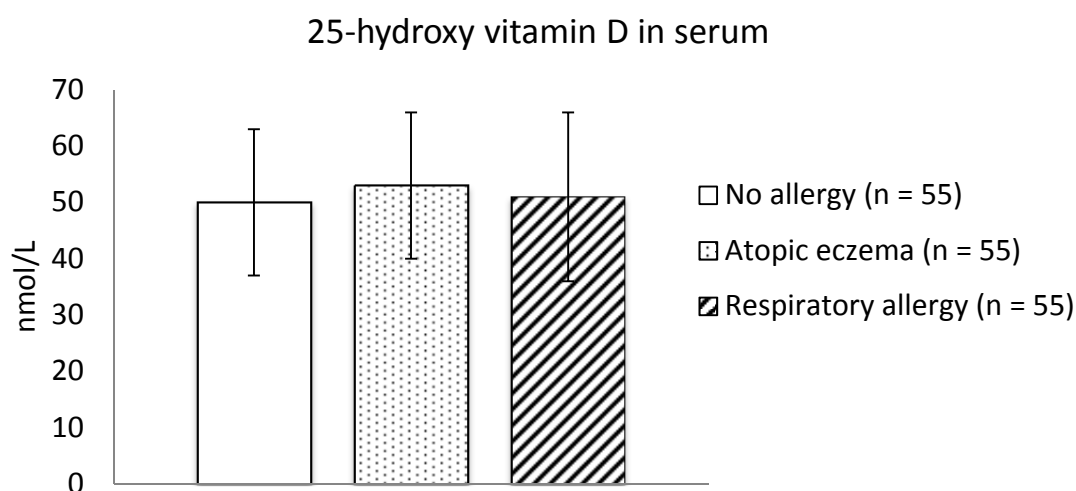


Figure 24: Concentration of 25(OH)D in serum from allergic and non-allergic subjects

No significant differences were found between any of the groups using student's t-test.

The blood samples were taken on three different occasions, i.e. in November, January and April. These time points were chosen to avoid blood sampling during or directly after the summer. Vitamin D has a half-life of approximately two months (26). Serum levels of 25(OH)D were significantly higher in samples collected in autumn (mean 54 nmol/L) compared to samples collected in winter/spring (mean 49 nmol/L, $p = 0.009$).

A food frequency questionnaire was filled out by the adolescents in conjunction with the blood sampling. It focused on fat-containing items that were considered important sources of vitamin D. No significant differences were found between the three groups, except that adolescents with respiratory allergy consumed less egg than did non-allergic controls, 1.8 g/day vs 3.8 g/day, $p = 0.002$.

Serum levels of 25(OH)D correlated significantly with intake of vitamin D fortified low fat milk ($Rho = 0.27$, $p = 0.001$) but not with vitamin D fortified margarine ($Rho = -0.02$), vitamin D rich fatty fish ($Rho = -0.02$) or with the calculated total daily intake of vitamin D ($Rho = 0.11$).

The current recommended intake of vitamin D for children and adults (<75 yr) in the Nordic countries is 10 µg/day. The average intake of vitamin D in Sweden is 5.7 µg/day in the 18 to 30 years age group (25). From the food frequency questionnaire we calculated an estimated daily intake of vitamin D. The mean intake of vitamin D was 3.8 µg/day in all subjects and did not differ significantly between either of the two allergic groups and the non-allergic controls (Figure 25).

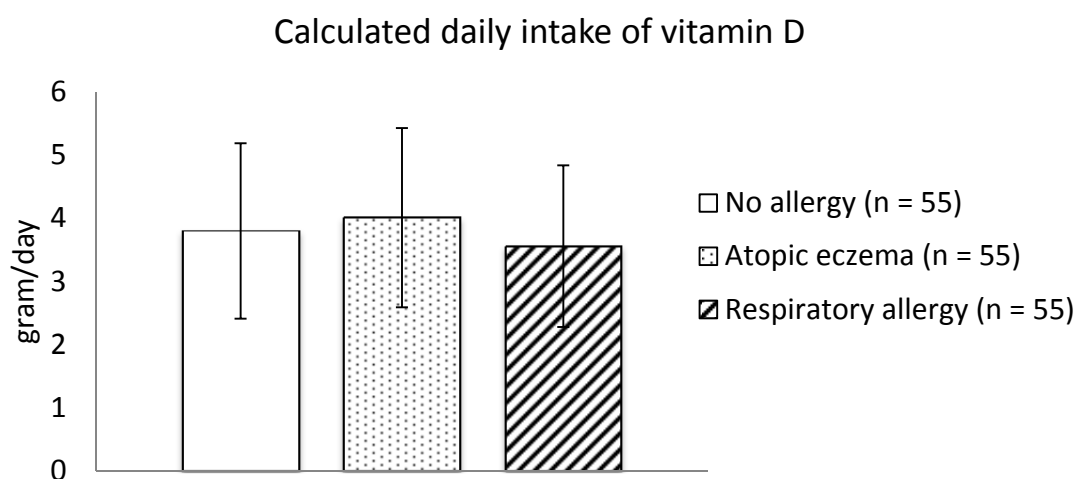


Figure 25: Calculated daily intake of vitamin D in allergic and non-allergic subjects
No significant differences were found between any of the groups using student's t-test.

DISCUSSION

Does the fatty acid milieu in the neonate affect the risk for subsequent allergy development?

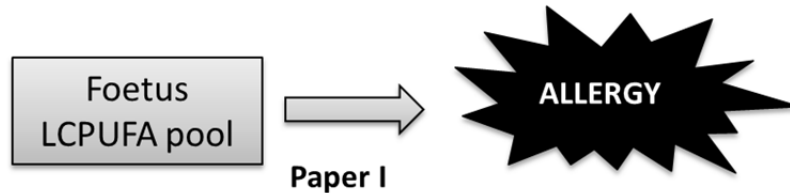


Figure 26: The impact of the fatty acid milieu in cord serum on allergy at 13 years of age was studied in Paper I

It is suggested that the infantile period is crucial for immune modulation and that the milieu during this period plays a key role for the risk of allergy development [138]. Soon after birth, the infant is exposed to microbes and foreign antigens in the food and air. The fatty acid composition affects the fate of naïve T cells that encounter their antigen for the first time. PUFAs reduce T cell activation and thereby dampen the immune system [4-6]. It is therefore relevant to ask whether the fatty acid composition of cord serum, which represents the milieu in which the infant immune system encounters the first immunostimulatory events, affects the development of allergy. The impact of the fatty acid milieu in cord serum on allergy at 13 years of age was studied in Paper I (Figure 26).

Both n-3 and n-6 PUFAs and their metabolites are immunosuppressive in that they reduce T cell activation and signalling [3-5, 116, 117, 251] as well as production of interferon- γ by T cells [6, 7]. Notably, LCPUFAs foremost target Th1 cells [133, 252-254] which are activated by microbial exposure [255], while Th2 cells, which are central in IgE-mediated allergy, are not suppressed by either n-3 or n-6 PUFAs to the same extent [6]. In fact, mice fed a fish oil diet had a somewhat increased Th2 reactivity [250, 256], which may be explained by the dampening effect of PUFAs on Th1 cells but not on Th2 cells (Figure 27).

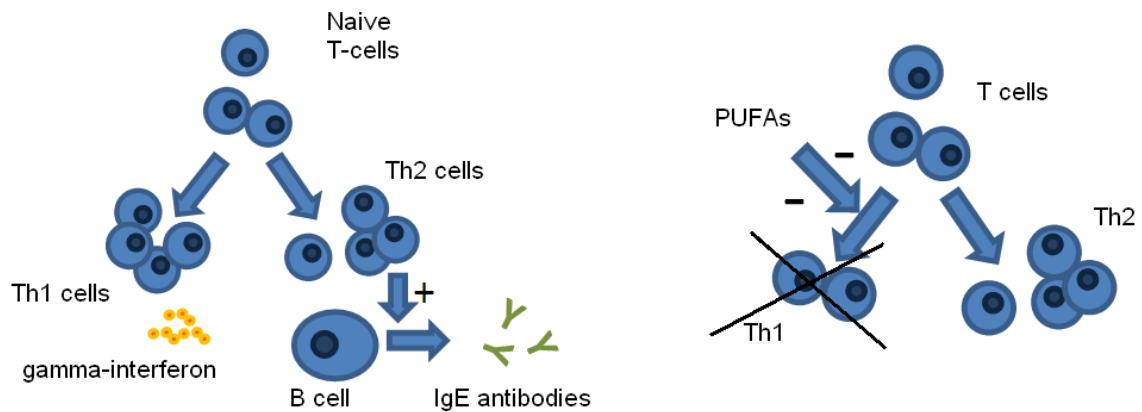


Figure 27: PUFAs reduce T cell activation and interferon- γ production

We suggest that exposure to PUFA dampens Th1-mediated inflammation but not Th2-mediated allergies.

In Paper I we found a positive association between high proportions of both n-3 and n-6 LCPUFAs in the phospholipid fraction of cord serum and development of allergic disease, i.e. respiratory allergy or atopic eczema, during the first 13 years of life. Our interpretation of the results is that high proportions of n-3 and n-6 LCPUFAs in cord serum counteract T cell activation in response to microbial exposure and thereby delay the maturation of the infant's immune system that is needed to develop tolerance to innocuous environmental antigens (Figure 28).

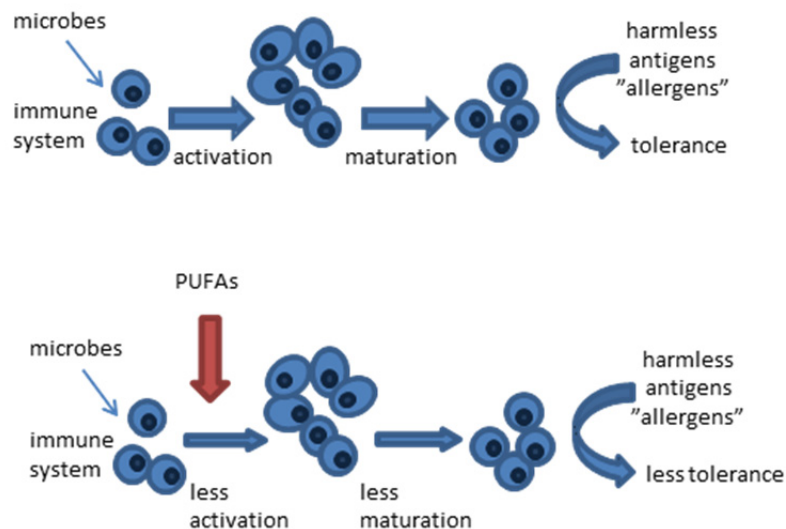


Figure 28: Our hypothesis that PUFA dampens the maturation of the immune system

The immune system needs to be activated early in life to be able to mature and develop tolerance to common antigens in the air and food. Early exposure to PUFAs leads to decreased activation and maturation of the immune system, which is needed to develop tolerance to antigens.

Few other studies have examined cord serum PUFA composition in relation to subsequent allergy development [159-165]. Some found no differences in single fatty acid proportions [159-162], while others found that subsequent allergic subjects had lower proportions of n-6 [163,

164] and/or n-3 LCPUFAs [163, 165] in their cord serum at birth. Most of these studies included rather few allergic subjects. The largest of the studies, in which 301 subjects with eczema were compared to 937 non-allergic subjects, found no difference in fatty acid proportions but a higher AA/EPA ratio in infants who developed eczema [160]. A difference between our study and previous ones was that we selected allergic and healthy individuals based on diagnosis at 13 years of age, supported by previous data from 1, 4 and 8 years of age. At that age, the allergic phenotype is fairly stable, while a diagnosis at early age is less certain. This is particularly true of respiratory allergy, which usually appears after 4 years of age, in young children a “wheezing” phenotype is often caused by viral respiratory infections, rather than allergy. Atopic eczema is possible to diagnose in early infancy since it is often most prevalent around one and two years of age. One previous study examined fatty acid pattern in cord blood in relation to allergy diagnosed after 4 years of age; Standl et al. [162] diagnosed the children at both 6 and 10 years of age. This study found no differences in fatty acid proportions in cord serum phospholipids in relation to parentally reported doctor diagnosed allergy, including asthma, hay fever/allergic rhinitis or eczema, neither at 6 years of age nor 10 years of age.

Even though our results are interesting, our study is small and no other studies have shown the same results as our study have. Hence, further studies are needed before any conclusions can be drawn about the influence of LCPUFAs in cord serum on the development of allergy.

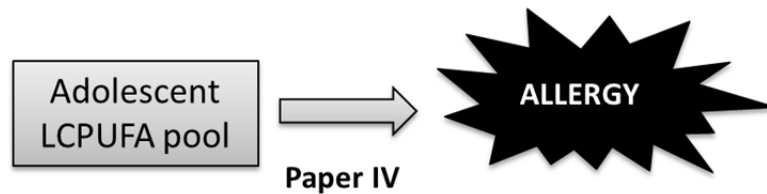


Figure 29: The relation between serum fatty acid composition and allergy at 13 years of age was studied in Paper IV

In Paper IV, we investigated whether the differences between allergic and non-allergic individuals regarding serum LCPUFA pattern in cord blood could be detected at 13 years of age (Figure 29) in the same subjects. No differences were found between allergic and non-allergic adolescents regarding fatty acid composition in serum at 13 years of age. Also, no differences were found between allergic and non-allergic subjects regarding intake of fat rich food items or fatty acids.

The results, which showed that the differences found at birth were completely absent at 13 years of age, suggest that nutrients available *in utero* are more important for future allergic disease than the actual nutrient state when the allergy has developed.

What determines the long chain PUFA composition in cord serum?

As discussed above, high proportions of LCPUFAs in cord serum at birth seems to be associated with allergy at 13 years of age. It is therefore relevant to study what factors that determine the fatty acid composition in cord serum focusing on 1) genetic polymorphism in the desaturase and elongase genes, 2) length of gestation and 3) maternal intake of fish during pregnancy (Figure 30).

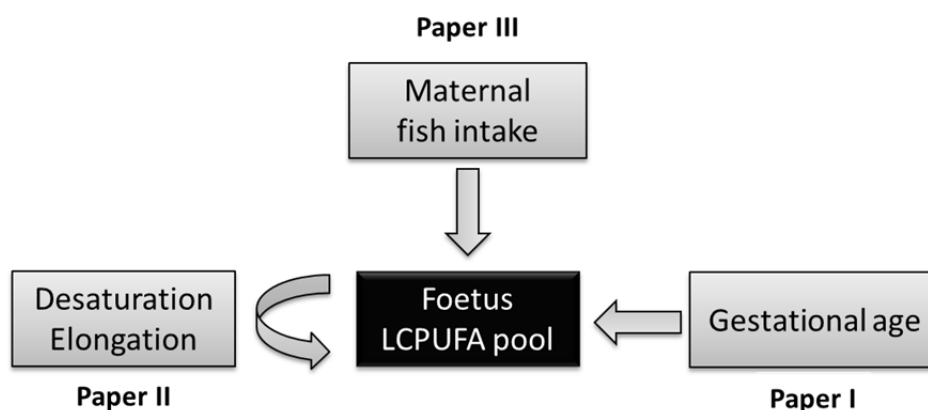


Figure 30: Overview of the factors that were studied in the thesis in relation to the LCPUFA composition in cord serum

We found a correlation between *FADS* polymorphism and the proportion of different n-6 LCPUFAs in cord serum. Carriers of the minor alleles of the single nucleotide polymorphisms in the *FADS* gene cluster displayed evidence of impaired conversion of the substrate, dihomo- γ -linolenic acid (20:3 n-6), to the products, arachidonic acid (20:4 n-6) and adrenic acid (22:4 n-6). This suggests that subjects with the minor C allele produce fatty acid desaturase enzymes that are less efficient at producing the products. No association was found for the fatty acids in cord serum in the competing pathway, the n-3 series of fatty acids in relation to *FADS* gene polymorphisms. Similar results were reported in the ALPSAC study, where both maternal and infant polymorphism in 17 single nucleotide polymorphisms in the *FADS* gene cluster were strongly correlated to n-6 PUFAs in cord plasma, but only a few SNPs correlated to n-3 cord plasma PUFAs [46, 53]. Hence, the lack of correlation between the n-3 LCPUFAs and polymorphism in the *FADS* gene cluster may be due to the n-3 LCPUFA production in foetuses being less sensitive to changes in the desaturase enzymes.

Adult females are more apt than adult men to produce long chain polyunsaturated fatty acids from the essential precursors, α -linolenic acid (for n-3 PUFAs) and linoleic acid (for n-6 PUFAs) [257]. It has been suggested that oestrogen is responsible for enhancing the metabolism of LCPUFAs in females [25]. In Paper II, female adolescents displayed stronger correlation between polymorphism in the *FADS* gene cluster and n-6 LCPUFAs, compared to male subjects of the same age. We speculated that this female trait would already affect fatty acid metabolism in

foetal life, i.e. that female foetuses would gain a greater proportion of their LCPUFAs from endogenous production than would male subjects. However, no differences were found between female and male subjects in the association between polymorphism in the *FADS* gene cluster and proportions of LCPUFAs in cord serum. The reason for this can only be speculated upon but it may be related to higher oestrogen levels in female adolescents than in female foetuses.

The association between genetic polymorphism in the *FADS* gene cluster and cord serum proportions of n-6 LCPUFAs suggest that the foetus itself may be able to produce LCPUFAs from precursor fatty acids derived from the maternal diet. We can, however, not rule out the possibility that foetal long chain PUFAs are chiefly determined by transport from the mother across the placenta and that the relation between foetal *FADS* and cord serum fatty acid pattern is caused by the fact that foetal and maternal polymorphisms are genetically linked, the foetus deriving half of its genes from the mother. In the ALPSAC study the association between maternal and foetal *FADS* polymorphisms and maternal and foetal LCPUFA plasma proportions has been studied [46, 49]. Both maternal and foetal polymorphisms in the *FADS* gene cluster were associated with, foremost n-6, cord plasma PUFA proportions [46]. Also, maternal polymorphisms in the *FADS* gene cluster were associated with both n-3 and n-6 maternal plasma PUFA proportions [49].

The pathway of endogenous production of long chain n-6 and n-3 PUFAs from shorter precursors involves another enzyme family, elongases, which elongate the fatty acid chain by adding a two-carbon atom unit. *ELOVL2* elongates 22:4 n-6 to 24:4 n-6 which is further converted by desaturation and β -oxidation to the end product 22:5 n-6. The same enzyme carries out elongation of 22:5 n-3 (DPA) to 24:5 n-3 which also is further converted to the end product of the n-3 pathway; 22:6 n-3 (DHA). In accordance, we found the ratio product/substrate (22:5 n-6/22:4 n-6 and DHA/DPA) to correlate negatively to a minor *ELOVL2* allele. This suggests that carriers of the minor allele in the *ELOVL2* gene have less capacity to convert the substrate to the product. This is in line with the findings in two genome-wide association studies [54, 55], where *ELOVL2* polymorphisms were associated with an increased proportion of plasma EPA (a precursor) and decreased proportions of DHA (the product) in plasma. Our results were, however, considerably weaker than those found in the genome-wide association studies.

Important LCPUFAs are actively and selectively transported across the placenta to meet the demand of the foetus. The demands for lipids peaks during the third trimester when the foetus starts to deposit fat in the adipose tissue and brain growth is maximal [258]. Several studies have found a correlation between high proportions of n-3 LCPUFA and increased gestational age [75, 152, 259-262]. In paper III we found that high cord serum proportions of DPA and DHA increased with increased gestational age in subjects born at term i.e. between 37 and 42 weeks' of pregnancy. This suggests that the maternal placental transport becomes more efficient with time, or that it continues at a similar rate, but that the foetal demands decline towards the end of the pregnancy. One aim of Paper III was to study if the association between gestational age and LCPUFAs differed in male and female foetuses. We found that the association between DHA in cord serum phospholipids and gestational age was somewhat stronger in male than female

subjects. The results are interesting but the differences are small and the association need to be further studied in larger population-based studies to evaluate the differences between the sexes.

The pregnant mothers were asked at one time point during pregnancy (week 18) how often they consumed fish. As the original aim of the question was to investigate whether pregnant women avoided certain food items deemed to be particularly allergenic, we did not have a complete record of the entire maternal diet. Also, the data on consumption of fish oil capsules was missing. Despite these drawbacks, we found that the reported maternal intake of fish during pregnancy correlated weakly to the proportion of long chain n-3 PUFAs in maternal serum phospholipids at delivery. In contrast, no correlation was found between maternal fish intake and the proportions of n-3 LCPUFAs in cord serum (Paper I). This might indicate that maternal intake of fish has little influence on cord serum n-3 LCPUFAs at birth, because of the active transport of n-3 LCPUFAs which occurs against a concentration gradient. However, the negative findings can also be due to an inability to capture the true maternal intake of n-3 LCPUFAs. Other studies have found EPA and DHA supplementation during pregnancy to be associated with increased proportions of these fatty acids in maternal plasma and cord plasma phospholipids [79, 80] and cord blood erythrocytes [81]. Nonetheless, there are also studies that have found no association between supplementation and proportions in cord blood [76].

A recent study [152] that aimed to identify the dietary and non-dietary determinants of DHA proportions in cord serum at delivery found the total number of DHA-rich intervention capsules consumed during pregnancy ($R^2=0.21$, $P=0.0001$) and gestational age at delivery ($R^2=0.16$, $P<0.0001$) to be the strongest predictors of cord blood DHA proportions. Other predictors, including parity⁴, maternal BMI, delivery mode, gestational diabetes, sex of the infant and induced delivery, together accounted <2 % of the variation in DHA concentration in cord blood [152]. Thus, the identified factors explain <50% of the cord serum DHA proportions, suggesting that other factors are of importance. These may include maternal and foetal endogenous synthesis of LCPUFAs, as well as differences in placental transport efficiency. In Paper II, we found genetic variation in the *FADS* gene cluster to be a relatively important predictor of cord serum proportions of n-6 PUFAs, explaining 14 % of the variation in the proportion of arachidonic acid in cord serum in our population.

⁴ Parity –number of pregnancies that the women have had.

Does the fatty acid metabolism differ between allergic and non-allergic adolescents?

The content of n-3 LCPUFAs in serum is often used as a biomarker for fish intake since it is generally considered that dietary intake of n-3 LCPUFAs is reflected in serum [263]. However, serum proportions of LCPUFAs are determined by additional factors, including endogenous production and the usage of fatty acids in the body for incorporation in cell membranes or as precursors for lipid mediators. Studies have found allergic subjects to have lower proportions of n-6 [172, 173] and n-3 [142, 173] PUFAs in serum than non-allergic subjects. Low serum PUFA proportions in allergic subjects may either indicate a protective effect of n-3 fatty acids, or that n-3 fatty acids are reduced as a result of the allergic state, through hampered endogenous production and/or an enhanced usage of PUFAs during the allergic inflammation.

Research from our laboratory has previously shown that women with both respiratory allergy and atopic eczema had lower proportions of n-3 LCPUFAs both in serum and in breast milk, as compared to non-allergic women, or women with isolated respiratory allergy, despite a greater intake of fish in the first group [173]. Similar results were found by Dunder et al. [142], who reported that children with atopic dermatitis had lower proportions of EPA and DHA than non-allergic controls, despite similar fish intake. Moreover, LCPUFAs were observed to be consumed in the body during allergic inflammation in a mouse model of respiratory allergy [250]. We have therefore previously suggested an enhanced usage of long chain n-3 fatty acids during allergic inflammation [250].

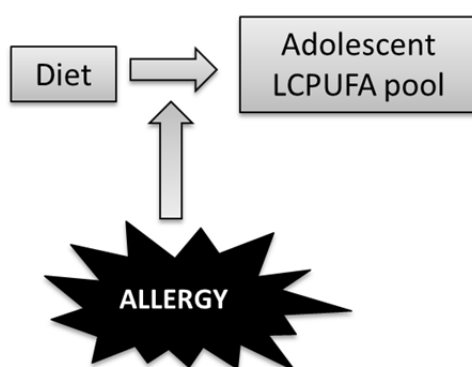


Figure 31: We studied whether allergic disease influenced the correlation between n-3 LCPUFAs in serum and dietary intake.

In Paper IV, we studied whether allergic disease influenced the correlation between n-3 LCPUFAs in serum and dietary intake (Figure 31). We found a significant correlation between intake of seafood and the proportion of n-3 LCPUFA in serum phospholipids in non-allergic adolescents and to some extent in adolescents with respiratory allergy, but not in subjects with atopic eczema at age 13. The latter group comprised subjects who had atopic eczema, but no other allergic manifestations. Atopic eczema is very common in early life, but often progresses into

other allergies, e.g. asthma and/or rhinitis, at school-age or adolescence [135-137]; a phenomenon termed the “atopic march”. Hence, it is unusual to have atopic eczema as the sole allergic symptoms beyond the first years of life. In the entire cohort from which we derived our study subjects, 79 individuals out of 788 (10 %) had this phenotype (atopic eczema with no other allergic manifestations). The pathogenesis of atopic eczema is complex and differs from asthma and hay fever, which are regarded as typical Th2-driven inflammatory conditions. In atopic eczema, skin-infiltrating T cells typically produce Th2 cytokines in the early lesion but Th1 cytokines at a later stage [264]. Moreover, CD8-positive T cells are numerous in the atopic eczematous lesions.

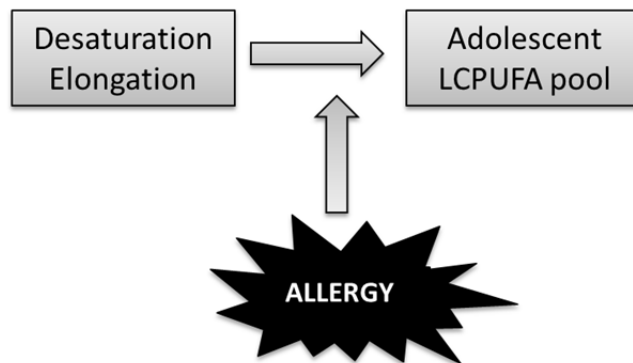


Figure 32: We also studied whether subjects with different allergic diseases had different associations between polymorphism in the desaturase gene cluster and PUFAs in serum at 13 years of age.

We also studied if allergic adolescents differed from non-allergic adolescents with respect to the association between *FADS* gene polymorphisms and LCPUFA proportions in serum (Figure 32). We found similar association between serum proportions of PUFAs and *FADS* gene cluster polymorphism in all three clinical groups. Hence, the lower correlation between n-3 LCPUFAs in serum and intake of fish in adolescents with atopic eczema does not seem to be due to a difference in endogenous production of LCPUFAs in the body. Instead the lower correlation between n-3 LCPUFAs in serum and intake of fish in adolescents with atopic eczema might be related to an enhanced usage of LCPUFAs during the allergic reaction in these subjects, in line with previous results from our laboratory [173, 250].

Are genetic polymorphisms in genes responsible for desaturation and elongation associated with allergy?

Variations in the *FADS* gene cluster have a strong influence on serum proportions of LCPUFAs [265]. Hence, genetic variation in the *FADS* gene cluster may be indirectly associated with inflammatory processes via their influence on endogenous LCPUFA production.

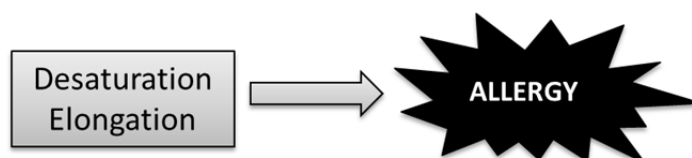


Figure 33: The influence of polymorphism in the *FADS* gene cluster or *ELOVL2* gene on the risk of allergy was studied in Paper II.

The influence of polymorphism in the *FADS* gene cluster or *ELOVL2* gene on the risk of allergy was studied in Paper II (Figure 33). We found that the minor allele of rs102275 (C) and of rs174448 (G) were protective against developing atopic eczema. Carriers of the minor alleles also had higher proportions of dihomo- γ -linoleic acid and lower proportions of arachidonic acid in cord serum, suggesting impaired conversion to longer-chain n-6 fatty acids in these individuals. This is in accordance with the findings in Paper I that high proportions of LCPUFAs, such as arachidonic acid, in cord serum are associated with development of atopic eczema.

Other studies that have evaluated the association between SNPs in the *FADS* gene cluster and allergic diseases found inconsistent results [44, 48, 58, 59]. Schaeffer et al found that adult minor allele carriers of several SNPs had a lower prevalence of self-reported allergic rhinitis and atopic eczema [44]. Rzehak et al found that minor allele carriers of several SNPs in the *FADS* gene cluster had a higher prevalence of parental reported eczema at 2 years of age in the LISA-study, while no associations were found in the KOALA-study [48]. Two studies found no association between 5 SNPs in *FADS1/FADS2* and asthma, bronchitis, eczema or hay fever at 6 years of age [58] or at 10 years of age [59] in subjects from the two German LISA and GINI birth cohorts. Hence, further studies are needed to confirm the protective effects of the minor allele in *FADS* genes polymorphism found on atopic eczema in this study.

Is there an association between vitamin D and allergy in adolescents?

Many studies have found consumption of fish to be protective against allergy both when consumed by pregnant mothers [142, 150-154] and by the children themselves [15, 155-158]. Fish is rich in LCPUFAs but fish also contains considerable amount of proteins, vitamin D, selenium and vitamin B12. Vitamin D has been shown to have immunomodulatory effects and the aim of the last paper was to study if low levels of vitamin D in serum or in diet were involved in allergy development (Figure 34).

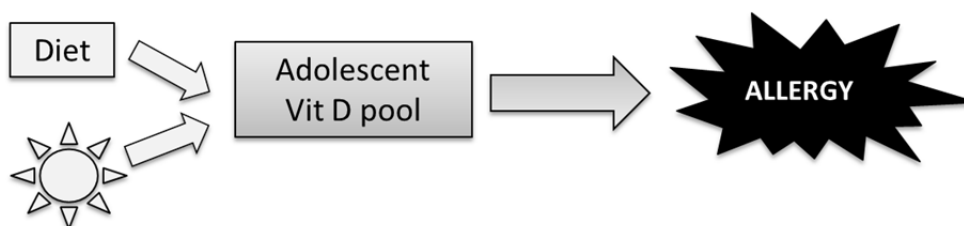


Figure 34: In Paper V, we studied the association between vitamin D and allergy in the adolescents.

The results showed that adolescents with respiratory allergy or atopic eczema at 13 years of age differed neither in serum levels of 25(OH)D nor in dietary intake of vitamin D from non-allergic and non-sensitised controls of the same age.

As shown in Tables 6 and 7, both observational studies and randomized controlled studies of the association between allergy and vitamin D show inconsistent results. There are studies that show both positive [125, 209-213] and negative [216-220, 222-224] correlations between vitamin D and allergy, as well as studies that found no effect [224, 225, 231, 232]. Our study supports the negative findings. However, based on our fatty acid results, it may be less relevant to measure vitamin D levels at an age when the allergic disease is already manifest, as the immune events that predispose to allergy take place very early in life. The association between the vitamin D status in early life and allergy development will be investigated in an ongoing study.

CONCLUSIONS

- ✓ Subjects with respiratory allergy or atopic eczema at age 13 years had higher proportions of several LCPUFAs of both the n-3 and n-6 series in cord serum compared to non-sensitised, non-allergic controls. The findings are interesting, however, our study is small and the results need to be confirmed in larger cohorts before any firm conclusions can be drawn.
- ✓ High LCPUFA proportions did not characterize adolescents with manifest allergy. This suggests a potential difference between exposures that occur in early infancy, when the immune system develops and the individual becomes primed to be sensitised to allergens, and exposure to the same factor later in life. PUFAs may exert different functions during these two phases of the allergic disease.
- ✓ Proportions of long chain n-3 PUFAs in cord serum were associated with duration of pregnancy; the longer the duration of pregnancy, the higher proportions of accumulating n-3 LCPUFAs. The accumulation of n-3 LCPUFAs during the late phases of pregnancy may be due to the active transport of these PUFAs across the placenta. Proportions of long chain n-6 PUFAs in cord serum were instead associated with polymorphisms in the *FADS* gene cluster. Subjects carrying the minor alleles displayed signs of less efficient $\Delta 5$ -desaturase, leading to accumulation of the precursor n-6 fatty acid (dihomo- γ -linolenic acid, 20:3 n-6) and decreased proportions of the products of the reaction (arachidonic acid, 20:4 n-6, and adrenic acid, 22:4 n6). In summary, the proportions of n-3 and n-6 LCPUFAs in cord serum may be regulated by different factors.
- ✓ The association between genetic polymorphisms and adolescent serum PUFA pattern was similar to that observed for cord serum PUFA pattern, but less pronounced. The reason for this can only be speculated upon, but may be due to a large influence of the diet on LCPUFA composition in adolescents.
- ✓ The minor alleles in the *FADS* gene cluster was less prevalent in individuals who had atopic eczema (but no other allergic manifestations) at age 13 years, compared to controls. The same polymorphism was associated with decreased production of arachidonic acid. This might suggest that reduced capacity to produce long chain n-6 PUFAs is associated with less prevalence of this particular allergic phenotype.

- ✓ No differences were found in intake of lean or fatty fish between allergic adolescents and non-allergic adolescents. The intake of lean or fatty fish was not correlated to n-3 LCPUFAs proportions in serum in adolescents with atopic eczema. Hence, long chain n-3 PUFAs may not be a good biomarker for fish intake in eczematous subjects. Since no effect of allergy diagnosis was found on the association between genetic polymorphism and n-3 LCPUFA proportions in serum, we suggest that usage of fatty acids in the body during the allergic reaction may explain the findings of absent correlation between n-3 LCPUFAs in serum and fish intake in subjects with atopic eczema.

- ✓ No evidence was found that insufficient 25(OH)D levels in serum were more prevalent among adolescents with respiratory allergies or atopic eczema compared to non-allergic control subjects. The lack of relation between allergy and vitamin D status in adolescents does not exclude that neonatal vitamin D status may affect allergy development.

FUTURE PERSPECTIVES

A number of new questions and hypotheses are raised by the findings in this thesis.

Most importantly, our findings suggesting increased risk of allergy development in individuals exposed to high proportions of LCPUFAs in the neonatal period need to be confirmed, as the subject is of importance to public health. Allergy is the number one chronic disease among the young and current practice is to recommend pregnant women to consume less saturated fats and more unsaturated fatty acids. To be able to confirm the findings in this cohort, the Bas cohort, we have initiated a new birth cohort, the NICE-study (Nutritional impact on Immunological maturation in Childhood in relation to the Environment). This cohort will follow 2000 pregnant mothers, their partners and children, from pregnancy week 18 until the children are 48 month old. The purpose of the NICE-study is to produce evidence based knowledge about the significance of pregnancy and early life exposure to environmental factors such as diet and microbiota for the development of (i) pregnancy complications and child outcomes such as preterm delivery and weight, (ii) allergic symptoms and other immune regulating diseases in children such as celiac diseases and type-I diabetes mellitus. We will be able to study immunological maturation over the first years of life in relation to PUFA pattern at birth. As exposure to microbes is thought to be the major environmental factor affecting allergy development, the protocol also involves measures of microbial exposure.

Given the fact that the neonatal (and maybe foetal) PUFA milieu may exert an influence on subsequent immune developing during the neonatal period, continuing during childhood into adolescence, it is important to elucidate the association between the diet of the pregnant woman, genetic variants in enzymes determining the PUFA synthesis in mother and foetus, and the capacity of the pregnant woman to transport LCPUFAs to the foetus. In the NICE cohort, we will study genetic polymorphisms and expression of placental proteins regulating fatty acid transport in samples of placental tissue, enabling us to dissect the factors that are most important in determining the PUFA pattern during foetal life and at birth.

Numerous observational studies show that fish diet in infancy is protective against development of allergy, particularly atopic eczema. Our observations suggest that it may not be the n-3 LCPUFAs that exert the protective effect of fish. We will try to identify other candidate compounds in fish that may be responsible for the allergy preventing effect [19]. Metabolomics studies will be performed to identify compounds in adolescent serum that correlate with intake of fish. Further, we plan to analyse zinc, iron and other minerals in relation to allergic state of the adolescents, as these minerals have shown to be associated with immune functions.

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