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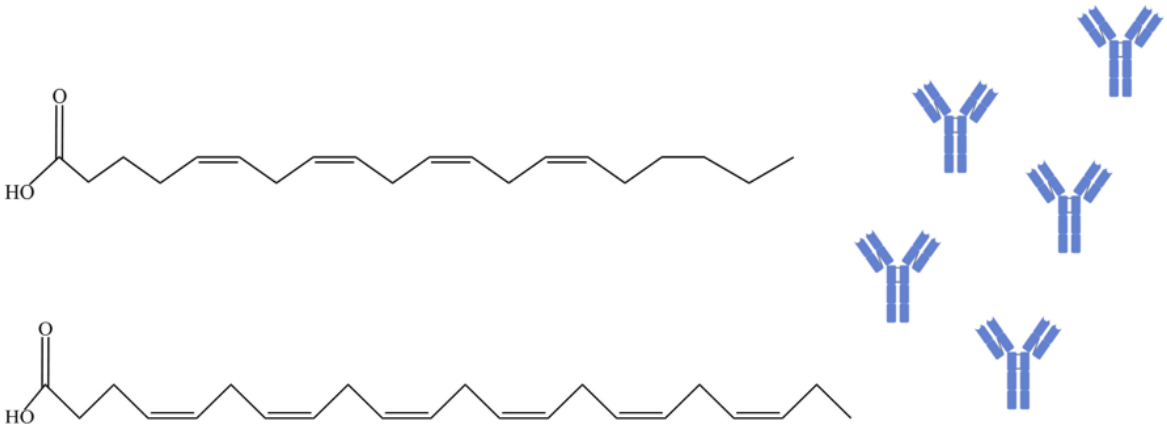

Long chain polyunsaturated fatty acids and allergy development

Analysis of phospholipids in maternal and cord serum and their relationship with each other, diet and development of allergy

Master's thesis in Biotechnology

ELIN JOHANSSON

Department of Biology and Biotechnology
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2019

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ELIN JOHANSSON

Supervisor: Malin Barman, Department of Biology and Biotechnology
Examiner: Ann-Sofie Sandberg, Department of Biology and Biotechnology

Department of Biology and Biotechnology
Division of Food and Nutrition Science
Chalmers University of Technology
SE-412 96 Gothenburg
Telephone +46 31 772 1000

Typeset in LaTeX
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Abstract

Allergy is an increasing problem today in the Western world. Even though the mechanism causing the allergic response is understood is it not known why some people develop allergy and some does not. The aim of the thesis was to investigate relation between long chain polyunsaturated fatty acids (LCPUFA) in the phospholipid fraction of maternal and cord serum and development of allergy. Phospholipids were extracted from maternal and cord serum using solid phase extraction columns. Composition of phospholipids was then analyzed using GC-MS. Wilcoxon signed rank test, Spearman's correlation, Mann-Whitney U test and independent t-test was used to analyze the data. Relation between levels of fatty acids in mother and children were compared to each other as well to allergy at one year of age. Influence of diet on levels in mother and children as well as allergy was also examined.

Levels of LCPUFA was found to correlate between mothers and children. Osbond acid and total n-6 PUFA was found in higher levels in children that developed eczema, whereas lower levels of docosahexaenoic acid (DHA) and total n-3 LCPUFA was found in mothers to children developing any allergy. Linoleic acid (LA) and n-6:n-3 PUFA ratio was also found to be higher in mothers to children developing any allergy. Daily intake of arachaidonic acid (AA) and DHA during the pregnancy was found to be lower in mothers to children who developed food allergy and asthma than in mothers to children who did not develop any allergy. No correlation between the diet and levels of fatty acid in mother and children were found. To conclude, a high proportion of n-6 PUFA in cord serum phospholipids and a low proportion of n-3 PUFA in maternal serum phospholipids was observed in relation to allergy and may influence the development of allergy.

Keywords: LCPUFA, PUFA, allergy, cord serum, GC-MS, phospholipid

Acknowledgements

First of all I would like to thank my supervisor Malin Barman for supporting me in this project, for always answering all of my many questions and for keeping me motivated. This project would not have been the same without you. Secondly I want to thank everyone at Food and Nutrition Science department who welcomed me with open arms.

Thanks to my amazing family for supporting me during my studies and for always encouraging me to take on new adventures, even if it means moving three hours away from you. Lastly - thanks to all my lovely friends in 42 who kept me motivated and happy through my five years of studies: you truly are the meaning of life.

Elin Johansson, Gothenburg, June 2019

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List of Abbreviations

AA	Arachaidonic acid
ALA	α-linolenic acid
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FBP	Fatty acid binding protein
GC-MS	Gas chromatography-mass spectrometry
HDL	High-density lipoprotein
IgE	Immunoglobin E
IgG	Immunoglobin G
LA	Linoleic acid
LCPUFA	Long chain polyunsaterated fatty acid
LDL	Low-density lipoprotein
NEFA	Non-esterified fatty acid
PUFA	Polyunsaterated fatty acid
QC	Quality control
SPE	Solid phase extraction
RWBC	Red and white blood cells
RSD	Relative standard deviation

1
Introduction

Long chain polyunsaturated fatty acids (LCPUFA) are essential during fetal development. They have various functions during the pregnancy but one of the most important function is to help develop brain, eyes and nervous system. The fatty acids are also essential components for the phospholipids in lipid membranes which maintain cell integrity.[1] Another important function LCPUFA are involved in is the immune system since they have the ability to regulate it by modulating inflammatory and immune responses.[2] Allergy is an increasing problem today in the Western world but it is still unknown why some develop allergy and some does not. Even though the immune system is poorly developed at birth, it is important that the infant is immunocompetent, meaning that it has the ability and components to form an immune response once exposed to an antigen. Because of this LCPUFA are considered as important components in the innate immune system, since they allow for an immune response.[3] Previous studies have found that low levels of LCPUFA in breast milk as well as in infants were associated with risk of allergy[4, 5], as another study found that high levels may also be associated with risk of allergy, as it is hypothesized to hinder full maturation of the immune system.[6] It is therefore of interest to examine levels of LCPUFA in the infant in relation to allergy to further understand their role in allergy development.

Serum can be used to examine levels of LCPUFA since it contains chylomicrons, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). These are proteins which are responsible for transporting fatty acids such as triglycerides and phospholipids to the cells. The lipoproteins are also believed to be an important component of the innate immune system. Levels of LCPUFA in the phospholipid fraction of the serum are therefore interesting to study.[7]

In addition to relation between allergy and LCPUFA in serum is the transportation mechanism of the fatty acids from mother to fetus across the placenta also of interest to examine. The exact transportation mechanism of LCPUFA is still unknown but many studies has found that the levels of LCPUFA are higher in the blood serum of the new-born infant than in the serum of the mother. This indicates that the placenta has the ability to active and selectively transport LCPUFA from mother to fetus and regulate its own need of fatty acids. It is suggested that the transportation is mediated by several plasma membrane-located binding/transport proteins but more research is required.[1] By investigating the relation between LCPUFA in mother and infant serum more information may be provided about the transport over the placenta.

The project was a part of Nutritional impact on Immunological maturation during Childhood in relation to the Environment (NICE) study and the samples used where from mothers and children born at Sunderby hospital in Norrbotten. NICE is an on-

going birth cohort with purpose to study the effect diet, microbes and environmental toxins may have during pregnancy on maturation of infant's immune system as well as development of immune mediated and neurological diseases. Families were recruited during mid pregnancy and biological samples (e.g blood, breast milk, placenta, urine) are to be collected at 10 time points from pregnancy until children reaches 4 years of age. Further information about lifestyle, socioeconomic status and diet was collected using questionnaires. When children reached 1 year of age skin prick test was used to test for sensitization to common allergens and allergy was also diagnosed by a specialist in paediatric allergology. Diagnose was based on certain clinical examination and history. Allergies diagnosed was food allergy, animal allergy, pollen allergy, atopic dermatitis (eczema) and asthma. Diagnose for food, animal and pollen allergy required an allergic reaction on at least two consecutive occasion when exposed to an allergen where allergic symptoms disappeared once the allergen were eliminated. Eczema and asthma were diagnosed based on certain criteria which needs to be fulfilled.[8]

Food allergy affects approximately 5 % of children in Western countries where egg, milk, peanut and soy are common allergens.[9] Animal allergy, especially allergy to furry animals (animal dander), are spreading in the Western world and are considered to be a risk factor for developing asthma and rhinitis (hay fever).[10] Pollen from grass, tree and weed cause allergic response in form of rhinitis and is also believed to trigger respiratory allergies.[11] Between 10-20 % of children has eczema and it is suggested that eczema may give rise to other allergic diseases such as food allergy, rhinitis and asthma.[12] Asthma is a chronic inflammation in the upper airway. The cause for asthma is not known but environmental exposures as well as sensitization to inhaled allergens are identified as risk factors for development of asthma.[13]

1.1 Aim of thesis

The thesis was divided into two parts: 1) method development and 2) analysis of phospholipid fraction in maternal and cord serum. The aim of the method development part was to develop a solid phase extraction (SPE) method for extraction of phospholipids In order to do this three different aminopropyl (NH_2) SPE columns were tested, each with different particle and pore size.

The aim of the second part of thesis was to examine the relation between LCPUFA in maternal serum and cord serum at the time of childbirth. Furthermore the relation between amount and type of LCPUFA in cord serum and eventual allergies at one year was also examined. To examine this the method developed in the first part of the thesis was used: phospholipids were extracted using SPE columns and analyzed by gas chromatography-mass spectrometry (GC-MS). The data produced was then aimed to be statistically analyzed after processing.

1.2 Demarcations

The first part of the project was limited to only examine and compare the SPE columns which were already available at the lab. Only aminopropyl columns of the brand Strata (500 mg/3mL), Supelco (500 mg/3mL) and Telos (500 mg/6mL) was therefore tested.

The second part of the project was limited to only analyze maternal and cord serum which was collected at childbirth, so samples from other time points were not examined. As the sample consist of maternal and cord serum it was only the phospholipids in chylomicrons, LDL and HDL that was analyzed. This meant that phospholipids in the red and white blood cells (RWBC) was not analyzed.

2
Theoretical background

2.1 Long chain polyunsaturated fatty acids

LCPUFA are defined as fatty acids containing 20 or more carbon atoms with at least two double bonds.[14] They are categorized into two different groups depending on the position of the first double bond counting from the methyl end group of the fatty acid: ω3 (n-3) and ω6 (n-6). The n-3 group consist of eicosapentaenoic acid (EPA, 20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Arachaidonic acid (AA, 20:4 n-6) belongs to the n-6 group. The LCPUFA can be provided by the diet or be elongated from the polyunsaturated fatty acids α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6).[15] For structure of the fatty acids, see figure 2.1.

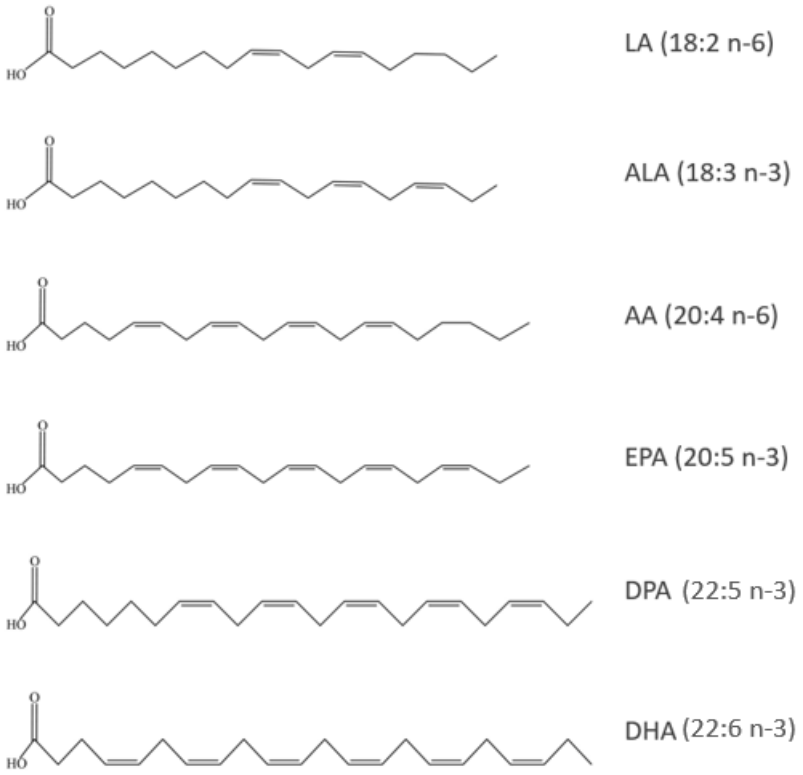

Figure 2.1: Structure of n-3 and n-6 PUFA.

ALA and LA are two polyunsaturated fatty acids (PUFA) which are classed as essential

since the human body lacks the ability to produce them and therefore they need to be provided from the diet.[15] Vegetable oils such as flaxseed, soybean, canola, sunflower oil as well as fatty fish (e.g. salmon) are good sources of the essential fatty acids.[16] As previously mentioned, the LCPUFA can be elongated from these two essential fatty acids by desaturase and elongase enzymes in the human body.[17] The conversion rate is however very low as one study showed that only 0.05 % of the ALA is converted into DHA by the human body.[18] Dietary recommendations of n-3 LCPUFA during pregnancy is 250 mg of EPA and DHA plus an additional 100-200 mg preformed DHA per day according to the European Food Safety Authority (EFSA).[19] Since the conversion into LCPUFA is low, it can be hard to satisfy the fetal demand by only consuming ALA and LA during pregnancy. Therefore it may be necessary for the mother to include LCPUFA in her diet during pregnancy.

LCPUFA are essential during the fetal development as they are involved in the development of brain, eyes and nervous system. They are especially important during the brain growth spurt that occurs during the last trimester.[20] DHA and AA seems to be particularly important during pregnancy. DHA is involved in the brain growth and starts to rapidly accumulate in the brain during the last trimester. This allow the brain to grow and develop normal cognitive and visual function.[2] AA act as a precursor to eicosanoids, such as leukotrines and prostaglandins, which are signaling molecules that are involved in several functions in the human body. One important functions is to modulate immune and inflammatory responses. AA is however not the only LCPUFA which has a role in the immune system as DHA also has been reported to be able to modulate inflammation and immune responses.[21]

2.2 Transportation of LCPUFA across the placenta

Fatty acids are transported from mother to child across the placenta. The exact transportation mechanism of LCPUFA is however still unknown, but the placenta seems to be able to selectively transport LCPUFA from the mother to the fetus. When comparing levels of PUFA in the phospholipid fraction of maternal and cord serum: LA, ALA, EPA and DPA have been found in higher levels in maternal plasma while AA and DHA have been found in higher levels in the cord plasma.[22, 23] It has previously been shown that the placenta has the ability to prioritize transportation of LCPUFA over other fatty acids, as well as selectively prioritize the transport of DHA.[24]

The placenta is made of genetic material from both the fetus and the mother and consist of three main components: decidua, intervillous space and chorionic villi, see figure 2.2. Blood from the mother is transported to the placenta via the decidua and fills the intervillious space where it surrounds the chorionic villi.[25] The maternal blood in the intervillous space in the placenta is separated from the fetal blood in the chorionic villi by the placental membrane. The placenta membrane consist of four layers during the beginning of the pregnancy: syncytiotrophoblast (phospholipid bilayer), cytotrophoblast, connective tissue of villus and endothelium which are lining the fetal vessel wall. During week 20 of the pregnancy the cytotrophoblast layer disappears.[2]

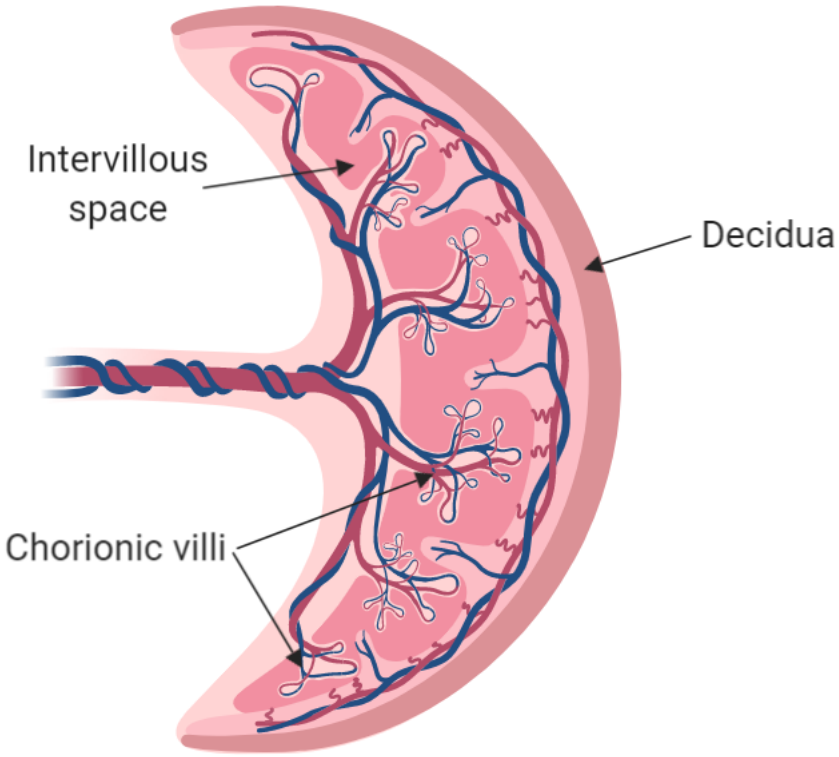

Figure 2.2: Different components of the placenta.

The fatty acids that are transported across the placenta are mainly provided from triglyceride rich lipoproteins such as LDL, HDL and chylomicrons. The lipoproteins are able to bind to the placental microvillus membrane where the triglycerides are hydrolyzed into non-esterified fatty acids (NEFA) by lipoprotein lipase. This mechanism will allow some of the fatty acids to cross the syncytiotrophoblast layer through passive diffusion.[26] The hydrolysis is believed to play an important role in the transportation of LCPUFA but it is not clear how. Further, there is a NEFA concentration gradient between mother and fetus which increases steadily during pregnancy. This gradient work as a driving force for transportation of fatty acids.[2]

Fatty acid binding proteins (FABPs) are also thought to be of importance for transportation of LCPUFA. There are three main membrane associated FABPs in the placenta: FABPpm, FAT/CD36 and FATP. The mechanism of how the FABPs are facilitating the transportation of fatty acids are still unknown but suggestions of their functions have been made. FABpm is a membrane fatty-acid binding protein that is believed to act as an extracellular fatty acid acceptor and facilitate diffusion through the lipid bilayer in the syncytiotrophoblast. It is proposed that the protein helps by locally increasing the fatty acid gradient and decreasing the diffusion distance, allowing LCPUFA to be actively transported against a concentration gradient. FATP and FAT/CD36 are integral proteins and it is believed that they function as fatty acid transporters or translocases.[26]

2.3 Allergy

2.3.1 Mechanism of allergic disease

Allergy can be defined as an abnormal immune response against a non-infectious substance (allergen). The immune response is caused by allergic sensitization. Once the body has been exposed to an antigen, an antigen-presenting cell (e.g. dendritic cell) envelops it and introduces it to a T cell. In a non-allergic person the T cell differentiates into a Th1 cell which activates a pathway leading to production of Immunoglobin G (IgG) antibodies.[27] IgG recognizes the antigen, neutralizes and destroys it without causing any allergic reaction.[28]

In an allergic person the T cell differentiates to a Th2 cell instead, which activates a different pathway leading to production of Immunoglobin E (IgE) antibodies. Once produced, IgE binds to high-affinity receptors on mast cells present in tissue where it can remain for months. This is the allergic sensitization. Next time the sensitized person is exposed to an antigen it binds to an epitope on the IgE antibody which causes an allergic reaction by making the mast cells to release pro-inflammatory components such as histamine, prostaglandins, tryptase, and leukotrienes.[29] This results in allergic disorders such as ezcema, hay fever, food allergies and allergic asthma. Even though the mechanism for development of allergic diseases is fairly known is it still unknown why some go through the sensitization process while other develop a resistance to the antigen.[27]

2.3.2 Influence by PUFA on allergy

Many factors are believed to mediate allergic responses or inhibition of them and lipid mediators are thought to be one of those factors. These mediators are often derived from n-3 PUFA and n-6 PUFA where n-3 PUFA are associated with anti-inflammatory effects and n-6 PUFA are associated with pro-inflammatory effects. Because of this is the ratio of n-6:n-3 PUFA thought to be important to influence the immunological activity.[30] The ratio in the Western diet is today around 15:1, which is considered to promote pathogenesis of many diseases e.g. cardiovascular and inflammatory diseases.[31] This imbalance in the ratio is also suggested to be a cause of development of allergic diseases.

The n-6 LCPUFA AA is as previously mentioned precursor to eicosanoids which are involved in modulation of intensity and duration of inflammation. One eicosanoid is prostaglandin which has the ability to make dendritic cells mature into a Th2 promoting phenotype.[32, 33] LCPUFA are incorporated into the phospholipid membrane of cells, e.g. effector cells (cells which responds to stimulus and performs a specific function). The composition of the phospholipid membrane can be altered as EPA and DHA is incorporated into the membrane at the cost of AA. This may lead to a decrease in production of inflammatory eicasanoids.[30]

A number of studies has been conducted to investigate which effect n-3 and n-6 PUFA have on allergic disease. Different effects has been connected to different types of allergy (e.g. food allergy, eczema, asthma etc.) As the first year in life is important for developing a good immune system nutrition and PUFA provided by maternal milk (

as well as other factors such as environment, genetics etc) may also be an important factor for development of allergy. Since the study focus is on relation between allergy and LCPUFA in maternal and cord serum some findings of previous studies on the subject are summarized here.[30]

One study concluded that increasing concentration of n-3 PUFA in cord serum was related to a reduced risk of eczema whereas another study found n-3 PUFA and n-6 PUFA concentrations was higher at birth in subjects that later developed eczema when comparing to subjects who did not develop any allergy.[34, 6] When it comes to relation between LCPUFA and asthma it has been suggested by a study that n-3 LCPUFA may decrease risk of asthma while AA may increase the risk.[35] Intake of fish, which is rich in n-3 PUFA such as EPA and DHA, during pregnancy has also been connected to a decreased risk of allergic disease in the infant. One study showed that consumption of fish 2-3 times a week decrease the risk of eczema and another study concluded that consumption of fish reduced risk of food allergy.[36, 37] Whether it is the LCPUFA in the fish or the combination of different components in the fish that give rise to these protective functions is still unclear.

2.4 Solid phase extraction

Solid phase extraction is a useful sample preparation technique with a broad application base. The technique is often used to prepare liquid samples and can be used to extract analyte, concentrate analyte and to clean sample.[38] The SPE column consist of three parts: barrel, frits and sorbent (SPE packing), see figure 2.3. The barrel is made of polypropylene or glass. Barrels made of polypropylene can contaminate sample with stearic acid and palmitic acid equivalents (0.07-0.14 mg/mL).[39] Samples which are sensitive to this kind of contamination should therefore be treated in SPE column with glass barrel. The frits are made of polyethylene, Teflon or stainless steel. The SPE packing vary with the type of sample but for 90 % of the SPE columns silica gel based packing is used.[40]

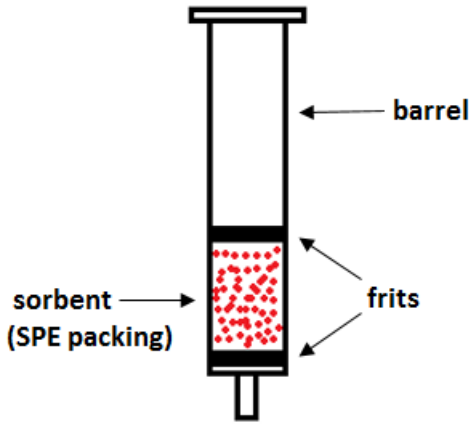

Figure 2.3: SPE column with its different parts.

Silica gel is a partially hydrate form of silica (SiO_2) and the reason for using silica gel based packing in the majority of the SPE columns is because of its distinctive properties.

Silica gel is highly porous and amorphous and it has a rigid backbone which does not swell or shrink in a broad range of solvent. The water in the silica gel has the ability to form silanol groups (SiOH) where a hydroxyl group is attached to the silica. Because of this a variety of functional groups can be added to the silica backbone and therefore increase selectivity.[40] For extraction of phospholipids aminopropyl (NH_2) columns are used.[41] The structure of the aminopropyl sorbent can be seen in figure 2.4.

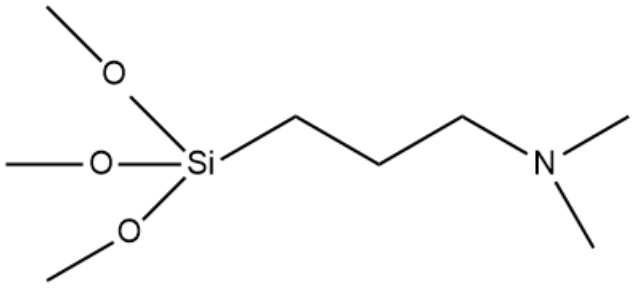

Figure 2.4: Structure of aminopropyl silica sorbent.

The sorbent consist of small particles of silica gel. The particles can be either irregular or spherical, where irregular is most common for smaller analytes and spherical most common for bigger analytes. On the surface of the particles there are pores which goes through the particles. It is within the pores that the interaction between analyte and sorbent occurs. Therefore it is important that the pore is big enough to let the analyte pass through. The size of the particles affect the flow characteristics of the columns. Bigger particles give better flow characteristics but if the concentration of the analyte is low, bigger particles may decrease the recovery. It is however unclear if this depends on the size of the particle itself or if it depends on the increased flow rate which comes along with bigger particles.[40]

There are four different types of SPE phases suitable for different types of analytes. The phases are reversed phase, normal phase, ion exchange and adsorption. For extraction of phospholipids normal phase SPE is used. During normal phase extraction the polar group of the analyte will interact with the polar group on sorbent surface. The analyte will thereafter be bound to the sorbent surface until something disrupts the interaction. This can for example happen when the sorbent is washed with an even more polar sorbent or by a change of pH.[42] The aminopropyl group attached to the sorbent is polar which allows it to interact with the polar head of the phospholipid.[43]

2.5 Statistical tools

2.5.1 Relative Standard deviation

Relative standard deviation (RSD) or coefficient of variation (CV) asses the variability within the sample group relative to the mean. In other words can RSD be described as

the standard deviation, σ, as percentage of the mean, μ see equation 2.1. RSD can be used to express precision and repeatability of a method. [44]

$$RSD = \frac{\sigma}{\mu} * 100 \tag{2.1}$$

2.5.2 Kolmogorov-Smirnov test

Kolmogorov-Smirnov test is a nonparametric test which compares the data to a normal distribution. This test will tell if the data is normal distributed and if a parametric or a non-parametric test should be used for further analysis of the data. [44]

2.5.3 Comparison of two related groups

Wilcoxon signed-rank test is the nonparametric version of a paired t-test and is used to compare two related groups. The test calculate differences between the two variables in each pair and assign them ranks. The ranks are then summed up and the p-value for each pair is calculated. This will tell if there is any significant difference between the two variables in the pair. To be able to find correlation between the two variables in each pair, Spearman's rank correlation can be used.[45]

2.5.4 Comparison of two unrelated groups

When comparing two unrelated groups against each other an independent t-test or a Mann-Whitney U test can be used, depending on if the data is under normal distribution or not. If the data is normal distributed an independent t-test can be used to test if there is a significant difference between the means in two unrelated groups. If the data does not display a normal distribution a Mann-Whitney U test can be used. The Mann-Whitney U test will rank each sample in the groups, sum the ranks in each group and thereafter calculate p-value to examine if there is any significant difference between the sum of ranks in the two groups.[45]

3
Methods

This section aims to describe the methods and materials which were used in the thesis. The work flow can be summarized as following: samples was collected at birth, serum samples were ordered in a random order and thereafter lipids were extracted from the serum. From the total lipids, SPE technique was used to extract the phospholipid fraction. This was followed by methylation of the fatty acids which then were analyzed by GC-MS. The data obtained was then processed and evaluated using Wilcoxon signed-rank test, Spearman's correlation, Mann-Whitney U test and independent t-test.

3.1 Sampling

3.1.1 Sampling of serum

Sampling of blood was done at Sunderby Hospital in Norrbotten. Blood samples were collected from mothers by a study nurse in a 6mL EDTA tube at childbirth and blood from child was collected from the umbilical cord in 6mL EDTA tubes. The plasma was separated from red and white blood cells via centrifugation and stored at 4 °C until aliquoting. Samples were then stored at -20 °C for maximum of 12 weeks until they were transferred to long-term storage at -80 °C.[8]

3.1.2 Assessment of dietary intake in mothers

Information about diet was collected using a food frequency questionnaire MealQ. Mothers received the questionnaire during gestational week 34 and answered 102-174 questions about their diet during the previous month. The nutritional composition of the diet was calculated from the questionnaires using database of nutrient content from the Swedish National Food Agency.[8]

3.1.3 Allergy diagnosis and sensitization test

At 12 months children went through skin prick testing to test for senzitisation against common allergens. Sensitization for milk, egg, timothy, birch, cat and dog was tested and histamine dihydrochloride was used as a positive control. A specialist in paediatric allergology also diagnosed children with allergy based on certain criteria. A diagnose for food, pet and pollen allergy required a medical history of allergic reactions in presence of the allergen, where the the symptoms was improved or disappeared when the allergen was removed. Children were also diagnosed with asthma or eczema at 12 months if they met the criteria for each disease.[8]

3.2 Sample order

To avoid any confounding error the experimental order was randomized. This was done by randomizing the order using R code, which can be seen in Appendix A.

3.3 Sample preparation

The samples used in this project was a part of the NICE study. The samples were thawed for 20 minutes in ice water and thereafter was 200 µL serum and 50 µL of internal standard heptadecanoic acid 17:0 added to a glass tube. For the method development part the serum used was quality control (QC) samples. QC samples contained the same conce«ntration of fatty acids making it possible to evaluate the method. For the second part of the project maternal and cord serum was used together with three QC samples. Total of 207 samples was analyzed where 103 was from mothers and 104 was from children. In the method development part of the project two different extraction methods for lipids from the serum was tested: reversed SPE and liquid-liquid extraction. For extraction of lipids from maternal and cord serum liquid-liquid extraction was used.

3.3.1 Total lipid extraction

3.3.1.1 Reversed SPE

Fatty acids were extracted from the serum using reversed phase SPE columns (Isolute, C18 Bonded Silica (EC), 100 mg/3 mL). The SPE columns were first conditioned with 500 µl acetonitrile followed by 500 µl MQ water. Samples were loaded to the columns and washed with 5 % methanol in distilled water. After that, samples were eluted with 500 µl acetonitrile and evaporated at 40 °C under nitrogen gas. After evaporation samples were dissolved in 200 µl chloroform.

3.3.1.2 Liquid-liquid extraction

Fatty acids were extracted by first adding 4 mL of chloroform:methanol 1:1(v/v) to the tubes containing serum and the tubes were vortexed for one minute. After vortexing, 2 mL of 0.5 % NaCl-solution was added and the samples were vortexed for an additional minute. The samples were then centrifuged at 3000xG for 6 minutes and after centrifugation was 1 mL of the chloroform phase collected and evaporated at 40 °C under nitrogen gas. After evaporation samples were dissolved in 200 µl chloroform.

3.3.2 Phospholipid extraction

After lipid extraction the phospholipids were extracted using SPE columns. For the method development part of the project phospholipids were extracted using aminopropyl SPE columns of the brand Strata (NH_2, 500 mg/3mL), Supelco (NH_2, 500 mg/3mL) and Telos (NH_2, 500 mg/6mL). Each column had a different particle and pore size, which can be seen in table 3.1.

Table 3.1: Particle and pore size of aminopropyl SPE columns of different brands.

	Strata	**Supelco**	**Telos**
Particle size (µm)	55	45	50
Pore size (Å)	70	60	70

For extraction of phospholipids from mothers and their children, SPE columns of brand Supelco (NH_2, 500 mg/3mL) were used. First columns were conditioned with 2x2 mL hexane. Samples were added to columns and 2x2 mL chloroform:isopropanol 2:1(v/v) was added followed by 2x2 mL of 2 % acetic acid in diethyl ether. Finally samples were eluted with 2x2 mL of methanol evaporated at 40 °C under nitrogen gas. [46]

3.3.3 Methylation of fatty acids

After phospholipid extraction, samples were methylated and thereby converted into fatty acid methyl esters (FAMEs) to enable detection of the lipids by GC-MS. This was done by adding 2 mL of toluene and 2 mL of 10 % acetyl chloride in methanol to the evaporated sample followed by one minute of vortexing. Samples were then methylated at 70 °C for 2 hours and vortexed every 30 minute. After methylation 1 mL of MQ water and 2 mL of petroleum ether was added to the samples. They were vortexed for one minute followed by centrifugation at 2500xG for 5 minutes. After centrifugation, 3 mL of the organic phase was collected and once again evaporated at 40 °C under nitrogen gas. Samples were dissolved in 100 µL iso-octane and added to the GC-MS for analysis.

3.4 GC-MS analysis

3.4.1 Conditions

The samples were analyzed by GC-MS together with the external standard GLC-463 which contained a mixture of FAMEs. The gas chromatograph separated the FAMEs on a VF-WAXms column (30 m × 0.25 mm × 0.25 µm) and the mass spectrometer quantified them after ionization. Electrical and chemical ion source was used. Helium was used as a carrier gas and the FAMEs were initially separated at a temperature of 100 °C. The temperature was increased until it reached 205 °C and thereafter the temperature increased with an increase rate of 1 °C/min until it reached 230 °C where the temperature was held for 5 minutes. Sample injection volume was 1 µL and the split ratio was 1:10, meaning that 0.1 µL actually was injected onto the column.

3.4.2 Assessment of variation from GC-MS

To examine if the GC-MS itself caused any variation in the data ten samples with the same concentration was analyzed. This was done by preparing 20 QC samples according to the method described above. When all 20 samples had been dissolved in 100 µl iso-octane each, they were pooled together and vortexed. Thereafter the pooled samples were divided into ten new samples and added to GC vials. This ensured that the ten new samples had the same concentration of each fatty acid. After the analyze, data obtained was processed and concentrations as well as relative proportions of each

fatty acid were calculated. The RSD of each fatty acid was then calculated for both concentrations and relative proportions.

3.5 Raw data processing

The fatty acids were identified by comparing retention times of the external standard with the retention times of the samples. The mass spectrum of each peak was also compared with mass spectrum's registered in a library which was available in the lab. The following fatty acids were identified and quantified in the phospholipid fraction of the serum: 14:0, 16:0, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6 (LA), 18:3 n-3 (ALA), 20:0, 20:3 n-6 (DGLA), 20:4 n-6 (AA), 20:5 n-3 (EPA), 22:0, 22:4 n-6, 22:5 n-6, 22:5 n-3 (DPA) and 22:6 n-3 (DHA). The fatty acids were quantified using the internal standard (mg/mL) and the relative amount of each fatty acid was also calculated (% of total phospholipids).

3.6 Statistical analysis

3.6.1 Relative standard deviation for Quality Control samples

RSD was calculated for each fatty acid in the QC samples according to equation 2.1 using Excel. RSD for each fatty acid was then compared between the different SPE columns and a RSD below 10 % was considered acceptable. RSD was also calculated for the three QC samples in each batch of maternal and cord serum to ensure good precision and repeatability of the method.

3.6.2 Analysis of relation between phospholipids in maternal and cord serum

Statistical analysis was done using the statistical program IBM SPSS. First a Kolmogorov-Smirnov test was used on the data to test if it displayed a normal distribution. Wilcoxon signed-rank test was used to test if there was any significance difference between relative proportions in mother and child. Spearman's rank correlation was also used to investigate if levels of phospholipids in maternal and cord serum correlated.

3.6.3 Analysis of phospholipids in relation to allergy

Mann-Whitney U test was used to examine if there was any relation between allergy and each individual fatty acid in both maternal and cord serum, as well as intake of fish in relation to allergy. When examining relation between allergy and total amount of n-3/n-6 PUFA, total n-3/n-6 LCPUFA as well as n-6:n-3 PUFA ratio, an independent t-test was used since this data was normal distributed. Relative proportions of phospholipids were compared between non-allergic children (control group) and children with any allergy. The allergy group could further be divided into five different types of allergy: eczema, food allergy, pet allergy, pollen allergy and asthma.
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4
Results

This section aims to provide an overview of the results obtained. The result consist of three major parts: establishment of the SPE column which gave lowest variation in data, relation between phospholipids in maternal and cord serum and relation between LCPUFA and allergy. No results will be presented from reversed phase SPE since no LCPUFA could be identified when looking at the chromatograms.

4.1 Method development

4.1.1 Comparison between different SPE columns

RSD values for the three different SPE columns Strata, Supelco and Telos are presented in table 4.1. RSD was calculated for both concentration as well as proportion. The results were similar and only RSD for proportions are shown below since it corrects for any eventual pipetting errors. SPE columns of the brand Supelco (particle size 45 µm and pore size 60 Å) resulted in lowest RSD values for each fatty acids, with values $<$ 6%. Strata and Telos who both had larger particle and pore size than Supelco gave a greater variation between samples. RSD was higher in all columns for arachidic acid (20:0), EPA, behenic acid (22:0) as well as DPA. In Strata and Telos there was also a high variation for ALA. Worth mentioning is that the peaks in the chromatogram were smaller for the fatty acids mentioned above.

Table 4.1: RSD values for relative phospholipid proportions in QC serum for SPE columns Strata, Supelco and Telos.

Fatty acid	**RSD$_{\text{Strata}}$ %**	**RSD$_{\text{Supelco}}$ %**	**RSD$_{\text{Telos}}$ %**
14:0	9.78	2.70	12.0
16:0	2.78	1.77	6.44
18:0	2.67	3.60	9.40
18:1 n-9	8.42	1.92	4.94
18:1 n-7	5.40	2.69	9.98
18:2 n-6 (LA)	3.66	3.54	7.01
18:3 n-3 (ALA)	25.6	3.31	35.3
20:0	13.7	5.93	22.8
20:3 n-6 (DGLA)	8.45	3.88	11.8
20:4 n-6 (AA)	9.48	4.32	15.8
20:5 n-3 (EPA)	17.9	4.61	9.66
22:0	17.3	5.48	6.31
22:5 n-3 (DPA)	28.9	5.68	24.6
22:6 n-3 (DHA)	18.1	4.23	16.1

4.1.2 Assessment of variation from GC-MS

To verify that the GC-MS itself did not cause the data variation 20 QC samples were pooled and divided into ten samples. They were then analyzed by the GC-MS and both concentration and relative proportions of phospholipids were calculated. RSD was then calculated for both concentration as well as proportion. The results were similar and only RSD for proportions are presented in table 4.2. As seen in the figure RSD was below 2.5 % for the majority of the fatty acids. ALA and arachidic acid and EPA had an RSD below 4.2 % and behenic acid (22:0) and DPA had an RSD around 6 %.

Table 4.2: RSD values for proportions of phospholipids for pooled QC samples (n=10).

Fatty acid	**RSD (%)**
14:0	1.85
16:0	0.88
18:0	0.94
18:1 n-9	0.87
18:1 n-7	0.96
18:2 n-6 (LA)	1.07
18:3 n-3 (ALA)	4.19
20:0	4.07
20:3 n-6 (DGLA)	2.17
20:4 n-6 (AA)	1.50
20:5 n-3 (EPA)	3.46
22:0	6.00
22:5 n-3 (DPA)	6.30
22:6 n-3 (DHA)	2.23

4.2 Phospholipids in maternal and cord serum

4.2.1 Relation between phospholipids in maternal and cord serum

Concentration and relative proportion was calculated for each fatty acid in the maternal and cord serum samples as well as the QC samples. RSD was also calculated for the three QC samples to investigate precision and repeatability of the method. The intrabatch RSD range for all fatty acids was from 1.21%-8.01% where highest RSD was for fatty acids found in low concentration in maternal and cord serum. One of the fatty acids with lowest concentration was ALA which had an intrabatch range of ∼5-8 %. Another low concentration fatty acid was DPA which had an RSD intrabatch range of ∼5-7 % whereas the intrabatch range for palmitic acid which was found in higher concentrations in maternal and cord serum was ∼0.3-3 %.

Kolmogorov-Smirnov test was performed on the data for each fatty acid to test if it displayed a normal distribution or not. The results showed that the majority of the data had a non-normal distribution and therefore non-parametric statistical tests were used. Wilcoxon signed-rank test was performed to investigate if there was any difference between the relative proportions of each fatty acid in mothers and children (n=103 pairs), see table 4.3. Spearman's rank correlation (r) was used to investigate if there were any significant correlation between proportions of fatty acids in the mother and the child. There was a significant difference for proportions of all fatty acids between mothers and children ($p \leq 0.001$) except for palmitic acid (16:0).

Correlation of fatty acids between mother and children varied depending on the fatty acids, but correlation obtained was found significant for all fatty acids ($p \leq 0.01$) expect for myristic acid (14:0) and oleic acid (18:1 n-9).

Table 4.3: Relative proportions of phospholipids in maternal and cord serum at birth in mothers and children and correlation between phospholipids in mother and children.

Fatty acid	**Mother** %	**Child** %	p^1	r	p^2
14:0	0.42 (0.34-0.51)	0.32 (0.24-0.37)	$<10^{-9}$	0.127	0.204
16:0	30 (29-31)	29 (28-35)	0.923	0.449	<0.001
18:0	12 (11-13)	18 (17-20)	$<10^{-24}$	0.313	0.001
18:1 n-9	9.6 (8.9-10)	6.5 (5.9-7.2)	$<10^{-26}$	0.045	0.654
18:1 n-7	0.86 (0.76-0.92)	1.4 (1.3-1.5)	$<10^{-23}$	0.349	<0.001
18:2 n-6 (LA)	22 (21-25)	7.9 (6.8-8.7)	$<10^{-28}$	0.404	<0.001
18:3 n-3 (ALA)	0.10 (0.08-0.13)	0.014 (0.012-0.018)	$<10^{-30}$	0.264	0.008
20:0	0.31 (0.27-0.36)	0.51 (0.44-0.60)	$<10^{-22}$	0.433	<0.001
20:3 n-6 (DGLA)	4.4 (3.9-5.4)	6.3 (5.5-7.0)	$<10^{-18}$	0.373	<0.001
20:4 n-6 (AA)	10 (8.5-11)	19 (15-20)	$<10^{-25}$	0.473	<0.001
20:5 n-3 (EPA)	0.75 (0.58-1.1)	0.44 (0.35-0.59)	$<10^{-21}$	0.503	<0.001
22:0	0.76 (0.65-0.87)	0.94 (0.82-1.2)	$<10^{-9}$	0.337	0.001
22:4 n-6	0.30 (0.24-0.35)	0.62 (0.53-0.73)	$<10^{-22}$	0.268	0.006
22:5 n-6	0.31 (0.23-0.44)	0.46 (0.34-0.55)	$<10^{-8}$	0.330	0.001
22:5 n-3 (DPA)	0.70 (0.56-0.90)	0.46 (0.32-0.70)	$<10^{-10}$	0.494	<0.001
22:6 n-3 (DHA)	6.2 (5.0-7.2)	6.7 (5.3-8.6)	0.001	0.359	<0.001

Data are presented as medians (range: 25th percentile and 75th percentile). [1]p-value for Wilcoxon signed-rank test. [2]p-value for Spearman's rank correlation.

Figure 4.1 shows levels of n-6 PUFA in mothers and children. Relative proportions of LA was higher in mothers and DGLA, AA, adrenic acid (22:4 n-6) and osbond acid (22:5 n-6) was found to be higher in children, indicating an active transport across the placenta. Spearman's rank correlation showed that the n-6 PUFA in mother and child significantly correlated with each other more or less. Correlation between mother and child for LA, DGLA and AA was r=0.404, r=0.373 and r=0.473 respectively. A somewhat weaker correlation between fatty acids was found for adrenic and osbond acid with r=0.268 and r=0.330, respectively.

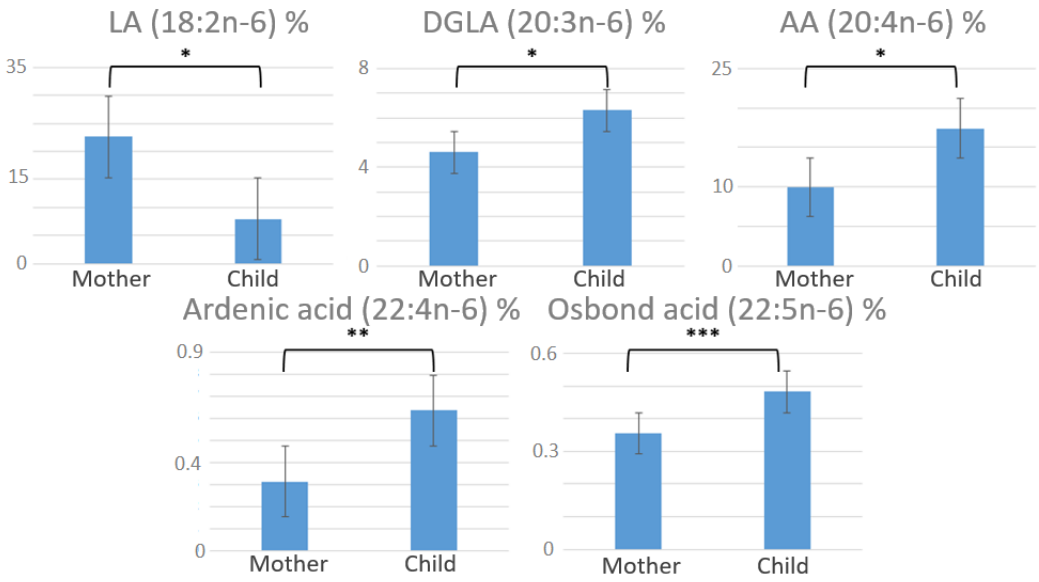

Figure 4.1: Mean proportions of n-6 PUFA in mother and child. *p<0.001, **p=0.006, ***p=0.001

Figure 4.2 shows levels of n-3 PUFA in mothers and children. Proportions of ALA, EPA and DPA was higher in mothers than in childre while DHA was higher in children than in mothers. This may indicate an active transport of DHA across the placenta. n-3 PUFA in mother and child correlated significantly with each other more or less. ALA showed a correlation of r=0.264 and DHA a correlation of r=0.359. EPA and DPA showed a strong correlation between mother and child with r=0.503 and r=0.494 respectively.

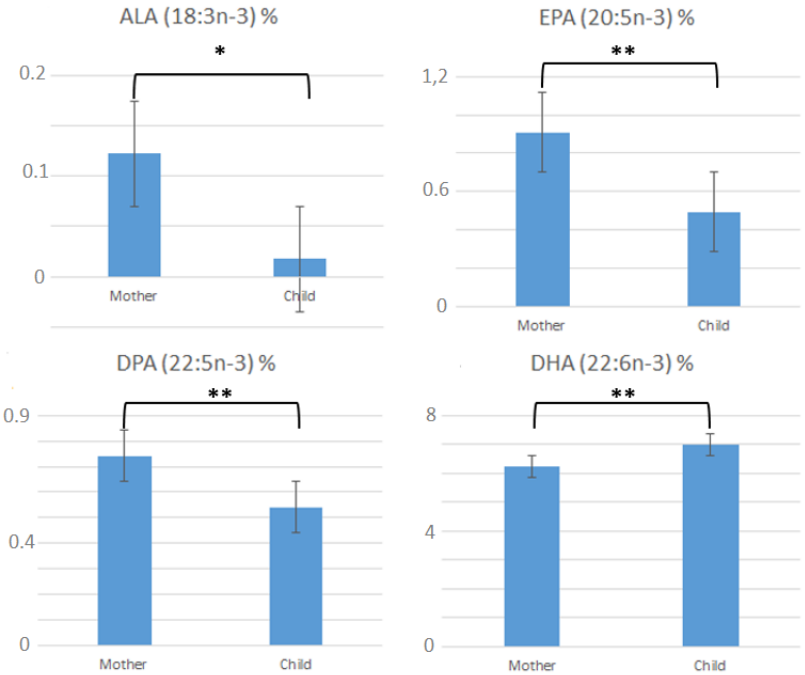

Figure 4.2: Mean proportions of n-3 PUFA in mother and child. *p=0.008. **p<0.001

4.3 Relation between LCPUFA and allergy

4.3.1 Relation between fatty acids and allergy in children

Since the data displayed a non-normal distribution Mann-Whitney U test was performed to compare non-allergic (control) group (*n=64*) to the allergic group (*n=20*). Differences of total n-3/n-6 PUFA, LCPUFA as well as the ratio of n-6:n-3 PUFA in cord serum was also compared between non-allergic children and allergic children using an independent t-test. The results are presented in table 4.4 and graphs for significant findings are presented in figure 4.3. No significant differences of relative proportions of fatty acids in cord serum was found between the control groups and the allergic group. Levels of total n-3/n-6 PUFA, LCPUFA as well as the ratio of n-6:n-3 PUFA in cord serum was also compared between the two groups but no significant differences were found.

Table 4.4: Relative proportions of phospholipids in cord serum at birth in non-allergic children and allergic children

Fatty acid	**Non-allergic**	**Allergic**	***p***
14:0	0.31 (0.24-0.36)	0.32 (0.22-0.38)	0.807
16:0	29 (28-34)	29 (27-33)	0.475
18:0	18 (17-19)	18 (17-19)	0.938
18:1 n-9	6.5 (5.7-7.2)	6.5 (6.0-7.2)	0.782
18:1 n-7	1.4 (1.2-1.5)	1.4 (1.3-1.6)	0.613
18:2 n-6 (LA)	7.4 (6.6-8.6)	7.9 (7.4-8.9)	0.172
18:3 n-3 (ALA)	0.014 (0.012-0.018)	0.015 (0.012-0.017)	0.913
20:0	0.50 (0.44-0.60)	0.53 (0.46-0.61)	0.367
20:3 n-6 (DGLA)	6.2 (5.3-6.9)	6.4 (5.8-6.9)	0.367
20:4 n-6 (AA)	19 (15-20)	20 (16-21)	0.443
20:5 n-3 (EPA)	0.47 (0.36-0.61)	0.39 (0.32-0.47)	0.098
22:0	0.94 (0.83-1.2)	0.97 (0.84-1.3)	0.658
22:4 n-6	0.64 (0.53-0.73)	0.62 (0.60-0.66)	0.815
22:5 n-6	0.46 (0.35-0.54)	0.47 (0.38-0.62)	0.456[1]
22:5 n-3 (DPA)	0.52 (0.32-0.75)	0.44 (0.32-0.82)	0.831
22:6 n-3 (DHA)	7.1 (5.3-8.7)	6.8 (5.5-8.7)	0.880
Total n-3 PUFA	8.0 (6.1-9.9)	7.5 (6.2-10)	0.676
Total n-6 PUFA	33 (30-36)	35 (33-36)	0.141
Total n-3 LCPUFA	8.0 (6.1-9.9)	7.5 (6.1-10)	0.681
Total n-6 LCPUFA	26 (22-28)	27 (24-28)	0.102
n-6:n-3 PUFA ratio	4.2 (3.4-5.6)	4.6 (3.4-5.8)	0.510

Data are presented as medians (range: 25th percentile and 75th percentile). Mann-Whitney U test was used to compare each individual fatty acid between groups. Independent t-test was used to compare n-3/n-6 PUFA, LCPUFA and n-6:n-3 ratio between groups. [1]Osbond acid (22:5 n-6) was significant (p=0.031) when comparing children with eczema to non-allergic children .

When comparing children with eczema (n=*6*) to the control group the n-6 LCPUFA osbond acid (22:5 n-6) was found in significantly higher proportions in children with eczema (0.49 % vs. 0.46 %, p=0.031), which is illustrated in figure 4.3. A significant difference was also found between total n-6 PUFA in allergic and non-allergic group. Proportions of total n-6 PUFA was higher in children with eczema than in the control group (36 % vs. 33 %, $p < 0.001$).

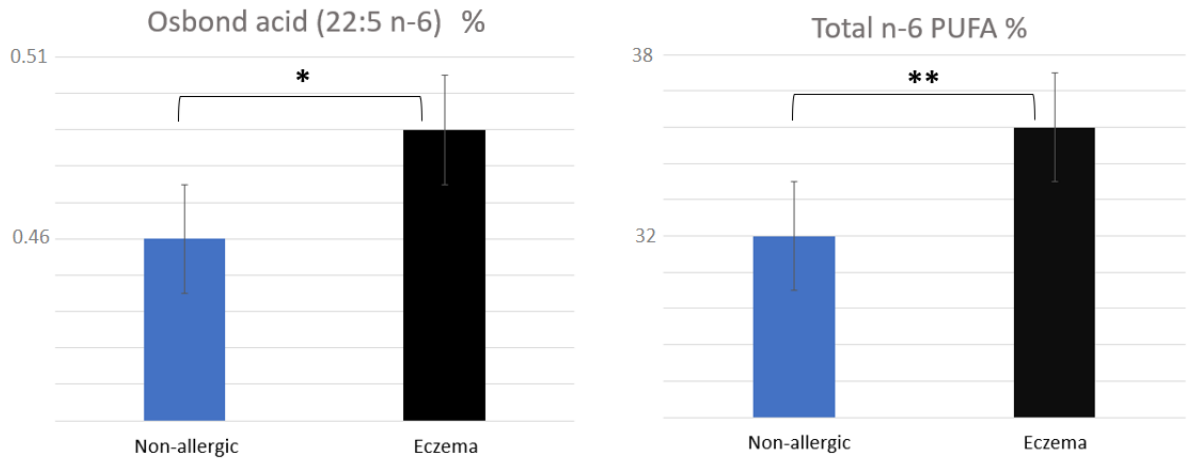

Figure 4.3: Medians for relative proportions of osbond acid (22:5 n-6) and total n-6 PUFA in non-allergic children and children with eczema. *p<0.05. **p<0.001.

4.3.2 Relation between fatty acids in mothers and allergy in children

Differences of relative proportions of phospholipids in maternal serum was compared between non-allergic (control) children and allergic children using a Mann-Whitney U test. Differences of total n-3/n-6 PUFA, LCPUFA as well as the ratio of n-6:n-3 PUFA in maternal serum was also compared between non-allergic (control) children and allergic children using an independent t-test (table 4.5).

Table 4.5: Relative proportions of phospholipids in maternal serum at birth in mothers to non-allergic children and mothers to allergic children.

Fatty acid	**Non-allergic**	**Allergic**	***p***
14:0	0.45 (0.34-0.50)	0.42 (0.34-0.49)	0.501
16:0	30 (29-31)	30 (28-31)	0.233
18:0	12 (11-13)	12 (11-12)	0.126
18:1 n-9	9.5 (8.8-10)	9.5 (9.0-10)	0.902
18:1 n-7	0.86 (0.76-0.90)	0.88 (0.79-0.92)	0.186
18:2 n-6 (LA)	22 (21-24)	24 (22-26)	0.036
18:3 n-3 (ALA)	0.10 (0.08-0.13)	0.11 (0.07-0.15)	0.609
20:0	0.31 (0.27-0.37)	0.30 (0.29-0.33)	0.648
20:3 n-6 (DGLA)	4.4 (4.0-5.5)	4.4 (3.4-5.1)	0.550
20:4 n-6 (AA)	11 (8.7-11)	10 (9.0-11)	0.727
20:5 n-3 (EPA)	0.81 (0.62-1.1)	0.73 (0.58-1.1)	0.357
22:0	0.76 (0.67-0.89)	0.76 (0.65-0.87)	0.703
22:4 n-6	0.31 (0.25-0.36)	0.29 (0.20-0.34)	0.158
22:5 n-6	0.30 (0.23-0.43)	0.29 (0.21-0.45)	0.876
22:5 n-3 (DPA)	0.75 (0.58-0.92)	0.70 (0.57-0.86)	0.416
22:6 n-3 (DHA)	6.6 (5.0-7.6)	5.8 (4.4-6.6)	0.041[3]
Total n-3 PUFA	8.1 (6.7-9.8)	7.4 (5.7-8.3)	0.070
Total n-6 PUFA	38 (36-39)	39 (37-40)	0.338
Total n-3 LCPUFA	8.0 (6.6-9.7)	7.3 (5.3-8.2)	0.051[4]
Total n-6 LCPUFA	15 (14-17)	16 (13-17)	0.384
n-6:n-3 PUFA ratio	4.7 (4.0-5.7)	5.2 (4.5-6.2)	0.029[5,6]

Data are presented as medians (range: 25th percentile and 75th percentile). Mann-Whitney U test was used to compare each individual fatty acid between groups. Independent t-test was used to compare n-3/n-6 PUFA, LCPUFA and n-6:n-3 ratio between groups. [3]DHA showed significance (p=0.026) when comparing mothers to children with food allergy and mothers to control group. [4]Total n-3 LCPUFA showed significance (p=0.032) when comparing mothers to children with food allergy and control group. [5]Ratio showed significance (p=0.012) when comparing mothers to children with eczema to control group. [6]Ratio showed significance (p=0.027) when comparing mothers to children with food allergy to control group.

Significant results are illustrated in figure 4.4. Proportions of LA was significantly higher in mothers to allergic children compared to the control group (24 % vs. 22 %, p=0.036). For DHA, proportions were significantly higher in the control group than in mothers to allergic children (6.6 % vs. 5.8 %, p=0.041). When comparing control group with mothers to children with food allergy (n=*11*), DHA had higher significant proportions in the control group (6.6 % vs. 5.4 %, p=0.026). The n-3:n-6 ratio was

significantly higher in mothers to allergic children when comparing to the control group (5.2 % vs. 4.7 %, p=0.029). The ratio was also significantly higher in mothers to children with eczema (5.7 % vs. 4.7 %, p=0.012) and in mothers to children with food allergy (5.6 % vs. 4.7 %, p=0.027). Total n-3 LCPUFA was not significant (although p was very close to 0.05) when comparing control group and the allergic group, but it showed that levels were significantly lower in mothers to children with food allergy when comparing it to the control group (6.5 % vs. 8.0 %, p=0.032).

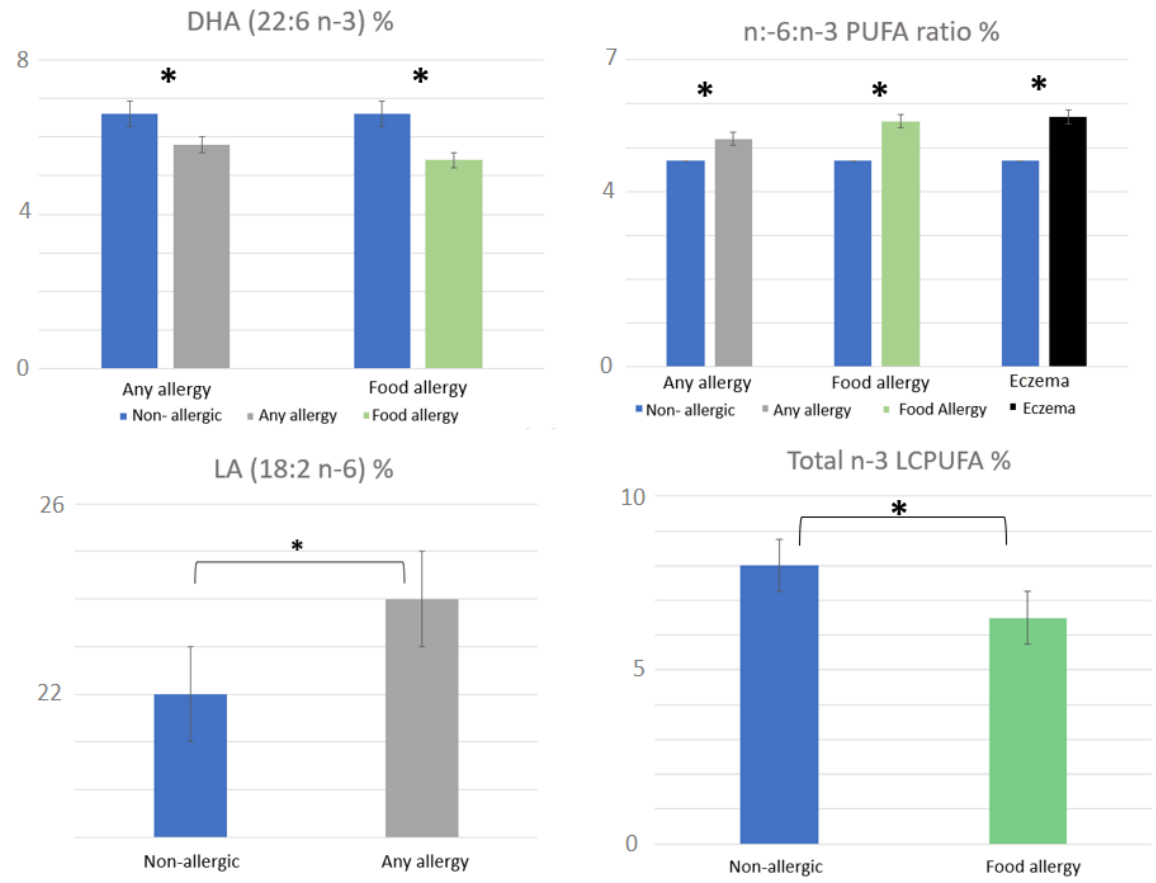

Figure 4.4: Median for relative proportions of DHA, n-6:n-3 PUFA ratio and total n-3 LCPUFA in non-allergic children, children with any allergy, children with food allergy and children with eczema. *p<0.05.

4.3.3 Relation between consumption of LCPUFA and allergy

Since fish is believed to have protective effects against allergy [36, 37, 47], weekly consumption of fish during pregnancy was compared between the control group and the allergic group. No significant differences were however obtained. Thereafter daily consumption of LCPUFA was compared between the two test groups and some significant results were obtained (table 4.6, figure 4.5).

Table 4.6: Relation between daily intake of LCPUFA and allergy

	Food allergy			**Asthma**		
	Non-allergic	Allergic	p	Non-allergic	Allergic	p
AA (mg/day)	89 (67-114)	60 (47-93)	0.041	89 (67-114)	50 (24-67)	0.003
DHA (mg/day)	177 (109-72)	119 (81-144)	0.048	-	-	ns

ns=not significant. Mann-Whitney U test was used.

Levels of consumed AA (mg/day) was found to be significant lower in food allergic children compared to non-allergic (60 mg/day vs. 89 mg/day, p=0.041). The daily intake of AA was also found to be lower in children with asthma when comparing to non-allergic children (50 mg/day vs. 89 mg/day, p=0.003). In addition to that, daily intake of DHA was found to be significantly higher in non-allergic children when comparing to children with food allergy (177 mg/day vs. 119 g/day). Daily intake of EPA and DPA was not found to be significantly different in the two groups.

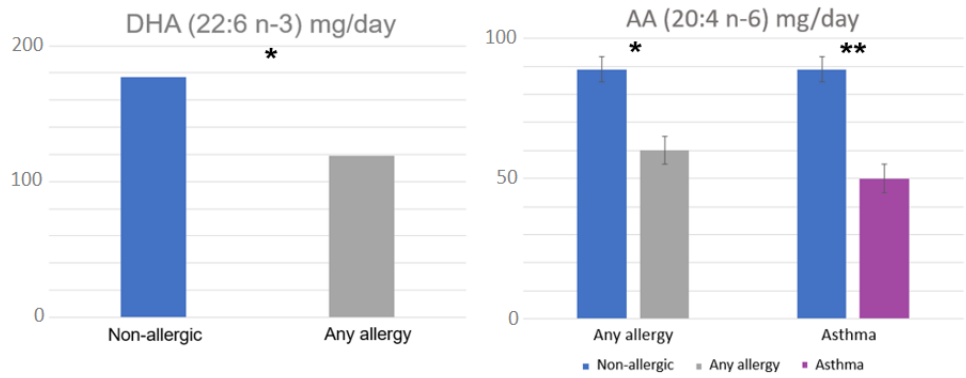

Figure 4.5: Median for daily intake (mg) of DHA and AA for mothers giving birth to non-allergic children and mothers giving birth to children with any allergy and children with asthma. *p<0.05. **p<0.01.

To further investigate the influence of the diet on levels of each individual PUFA, Spearman's rank correlation test was performed. Daily intake of PUFA as well as weekly intake of fish was tested against relative proportions as well as concentration (mg/ml) in both mother and children. No significant correlation was found between diet and levels in the serum of mother and children.

5
Discussion

In this section significant results will be discussed and compared to litterature. Future prospects for this study are also proposed.

5.1 Variation of RSD between fatty acids

When looking at RSD for each fatty acid a trend was observed where RSD tended to be higher for fatty acids which had a smaller peak in the chromatogram. This was observed for pooled samples, which should result in nearly no variation, as well as in the different SPE columns. The variation may depend on different factors. First of all is the human factor: since the peaks are all integrated manually there will always be a small variation and as the peaks get smaller the variation coming from the integration itself will get bigger.

Another factor affecting the variation might be the limit of detection. Since the peaks were very small and sometimes hard to differ from the baseline the concentration of that fatty acid might have been to low to be detected properly. This can however be corrected by increasing injection volume, changing the split ratio as well as increasing amount of sample used from the beginning. As suspected does pore and particle size of the SPE column also affect the variation between samples. The reason why this might be are discussed in the section below.

5.2 SPE

As shown in the results SPE column Supelco, with particle size 45 µm and pore size 60 Å, resulted in the lowest RSD for each fatty acid. This was also the column with smallest particle and pore size. The reason why this resulted in lowerd RSD can be discussed, but it is conclusive with previous studies which have used SPE columns with particle size $\leq$ 40 µm when extracting phospholipids in serum.[46, 48] According to theory, bigger particle size gives better flow characteristics of the SPE column which is the opposite of what has been observed in the study. However it was mentioned that bigger particles may decrease recovery if analyte concentration is low.[40] This might be an explanation why smaller particles works better when extracting phospholipids as concentration of phospholipids in the serum is around 0.20-0.25 mg/mL in an adult.[49]

The pore size 60 Å has also been used before when extracting phospholipids. One theory why this might work better may be that smaller pore size may allow better

interaction between sorbent and analytes. Regardless of the reason behind, a SPE column with particle size 45 µm and pore size 60 Å proved to be the best option when extracting phospholipids from serum in this study.

5.3 Relation between LCPUFA in mother and child

The findings in this study supports previous findings where LA, ALA, EPA and DPA was higher in maternal plasma and DHA and AA was found to be higher in cord plasma.[22, 23] Even though EPA and DPA are LCPUFA they are found in lower concentrations in the infant. It is not clear why this is the case. One reason may be that EPA and DPA are not prioritized by the placenta. Another reason might be that the majority of EPA and DPA are converted into DHA before it is transported across the placenta since DHA is very important for the development of a normal cognitive and visual function.[1] It may also be that the child convert EPA and DPA to DHA once it has crossed the placenta.

5.4 Relation between diet, levels of LCPUFA in serum and allergy

As previously mentioned is it probably more than one factor affecting the development of allergy. In this thesis, relation between diet and allergy, proportions of PUFA in children and allergy as well as proportions of mother and allergy has been investigated. The correlation of phospholipid levels between mother and children was also examined. One logical idea is that the diet affect levels of PUFA in mother which affects levels of PUFA in the child which affects the development of allergy. This line of thought is presented in figure 5.1.

Figure 5.1: Proposed linear relation between different factors and their relation to allergy.

Based on the results obtained in this study the impact of each factor will be discussed, starting with the dietary impact. Daily intake of AA and DHA during pregnancy was proved to be related to allergy at one year of age. AA and DHA was found to be consumed in lower levels by mothers which gave gave birth to children who developed food allergy, this was also true for AA in children with asthma. Fish intake as well as other PUFA was not significant in relation to allergy.

This indicates that intake of DHA and AA during pregnancy may influence the development of allergy. Since DHA comes from fish is it reasonable to say that fish intake is also related to the development of allergy even though no significant difference was found for the weekly fish intake between different groups. There is a possibility that it is the fish itself which provides protection against allergy development and that DHA is a biomarker for fish intake. It is however still a theory which need further validation before any conclusion like that can be made.

No correlation was found between diet and levels of PUFA in mothers and children. What is important to take into consideration here is that the levels was analyzed in serum and RWBC. Fatty acids in RWBC have a much slower turnover than fatty acids in serum and reflects diet more ($\sim$3 months vs. $\sim$ 1 week). Therefore does the findings not conclude that diet has no influence on fatty acid levels in mothers but it tells us that diet does not correlate with fatty acids in serum and may therefore not influence LCPUFA in the serum. Other reason for not finding an association between dietary intake and levels in serum might be that the food intake is assessed with an food frequency questionnaire and that this is not an optimal method for assessing dietary intake in detail. The findings may also be due to the low sample size. Although no correlation was found between diet and levels of LCPUFA in maternal and cord serum was correlation found between levels in mother and child. The correlation for most of the fatty acids where $r \geq 0.3$ which mean that levels of fatty acids in the infant is influenced by the mother. Further, a connection between proportions of the n-6 LCPUFA osbond acid as well as total n-6 PUFA and children with eczema was found. Proportions were found to be higher in allergic children compared to non-allergic children for both cases. This is consistent with previous studies as well as the theory stating that n-6 PUFA are pro-inflammatory.[6, 50]

Proportions of fatty acids in mothers and their relation to allergy were also compared. Proportions of LA and the ratio of n-6:n-3 PUFA was found to be higher in children with any type of allergy. The ratio was also higher in children with food allergy and eczema. Proportions of DHA and total n-3 LCPUFA was found to be lower in children with food allergy. Levels of DHA was also found lower for children with any type of allergy. These findings further support the theory stating that n-6 PUFA are pro-inflammatory and n-3 PUFA anti-inflammatory. It also shows that PUFA and LCPUFA might have some kind of influence on the development of allergy.

These finding indicates that the relationship between diet, mother, child and allergy might not be as linear as first thought. Diet seems to be connected to allergy but it was not found to be correlated with fatty acid levels in mother and children. Further, levels of PUFA in children as well as mothers was found to be related with allergy and might also influence development of this. In addition, a strong connection between levels in mothers and children was found for most of the fatty acids. This alters the initial thought and the pattern of influence of different factors on allergy is proposed to look more like what is presented in figure 5.2 based on the results in this thesis. However based on the gathered knowledge from previous studies it is suggested to be an association also between diet and maternal blood levels even though this was not shown in this material.

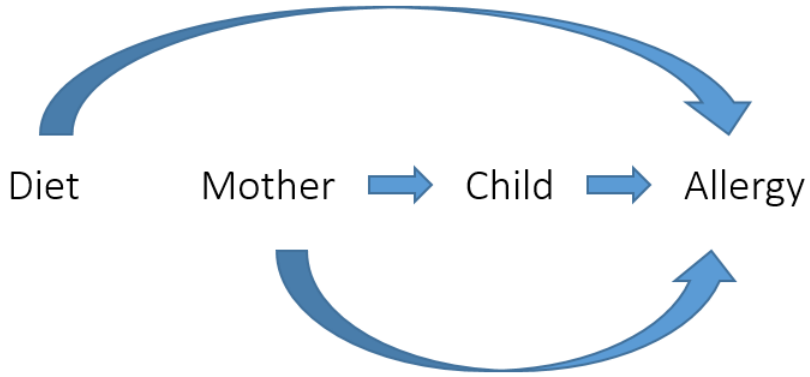

Figure 5.2: Proposed relation between different factors and allergy

5.5 Future prospects

To be able to further examine the relation between allergy and LCPUFA in maternal and cord serum the sample size should be increased. This may also increase the statistical significance of the findings already made. More research is also needed to fully understand the mechanism of transportation of LCPUFA across the placenta. One interesting subject to examine in the future are levels of triglycerides in maternal and cord serum in relation to levels of phospholipids since triglycerides are highly involved in transportation across the placenta. To further understand relation between LCPUFA and allergy it would be interesting to compare levels found in cord blood with children developing allergy later in life. It would also be interested to take more variables in to account, such as the child's living environment, mother's diet during breast feeding as well as the diet of the child during childhood, to further understand who develops allergy and why that is the case.

6
Conclusion

For the method development part of the project, it was concluded that the SPE column with particle size 45 µm and pore size 60 Å gave the lowest variation in data. This column was therefore used when extracting phospholipids from maternal and cord serum. When it comes to relative proportions of phospholipids in the mother and children was LA, ALA, EPA and DPA found in higher proportions in mother. DGLA, AA, adrenic acid, osbond acid was found in higher proportions in the child. Correlation was also found between levels of PUFA in mothers and children.

A couple of factors was found to be connected to allergy. Osbond acid (22:5 n-6) and total n-6 PUFA was found in higher levels in children who later developed eczema whereas lower levels of DHA and total n-3 LCPUFA was found in mothers to children developing allergy. LA and n-6:n-3 PUFA ratio was also found to be higher in mothers to children developing allergy. Daily intake of AA and DHA in the diet was found to be lower in mothers with children who developed food and/or asthma than in mothers with children who did not develop any allergy. However, no correlation between the diet and levels of fatty acid in mother and children were found.

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A

Appendix

The R code that was used to randomize the experimental order list is presented down below.

```
#Sample order to Elin

ElinIDs=read.csv2("\\\\file00.chalmers.se\\home\\ollehar\\.win
\\MyDocuments\\Doktorandstuffs\\motherchildgw28\\Sakertill␣Elin
\\StudieIDntillElin.csv")
Elinmatrix=matrix(nrow=(nrow(ElinIDs)*2), ncol=2)
Elinmatrix[,1]=rep(ElinIDs[,1],times=2)
Elinmatrix[,2]=c(rep('c', times=nrow(ElinIDs)),
rep('m', times=nrow(ElinIDs)))
randomizedorder=sample(ElinIDs[,1], replace=F)

randomizedElinmatrix=Elinmatrix[sample(nrow(Elinmatrix),
size=nrow(Elinmatrix),replace=F),]
randomizedElinmatrix2=randomizedElinmatrix
[order(match(randomizedElinmatrix[,1],'
as.numeric(randomizedorder))),]␣write.table(randomizedElinmatrix2,
"Elin␣Sample␣order2.csv",␣sep=";",␣row.names=F,␣quote=F)
}
```