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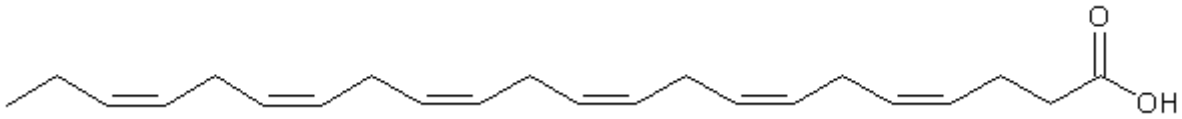
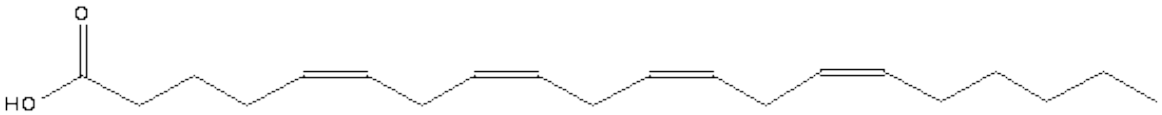


Docosahexaenoic acid (DHA) (22:6 n-3)



Arachidonic acid (AA) (20:4 n-6)

Long Chain Polyunsaturated Fatty Acids in Serum Phospholipids at Birth and the Development of Allergies in the Infants

Bachelor of Chemical Engineering Thesis

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Summary

Long Chain Polyunsaturated Fatty Acids in Serum Phospholipids at Birth and the Development of Allergies in the Infants

The main aim of this thesis was to investigate if the composition of the long chain polyunsaturated fatty acids AA and DHA in umbilical cord serum phospholipids or in maternal serum phospholipids could predict development of eczema, food allergy and asthma in the child at one, four and one and/or four years of age. Another aim was to analyze the correlation between fatty acid levels in maternal serum and cord serum in the mother-infant pairs.

Phospholipids of the long chain polyunsaturated fatty acids AA and DHA were extracted from stored maternal serum and cord serum respectively. The serum phospholipids were analyzed using GC-MS. Results was calculated using student's independent t-test. A questionnaire filled out by participating mothers, gave data of their children's allergies at one and four years. This data was correlated to the levels of the fatty acids AA and DHA in cord serum phospholipids in order to investigate possible connection.

Serum was collected in the year of 1996. Maternal serum samples showed a poor resolution probably due to sample degradation caused by the age of the samples. Cord serum samples, however, showed good resolution, indicating possible presence of antioxidants. Results from cord serum samples were therefore the main focus of this thesis.

No correlation between fatty acid levels in cord and maternal serum phospholipids was found, possibly due to the poor resolution of the maternal serum samples. Overall there were no consistently differences found in proportion or amount of AA and DHA in cord serum phospholipids between allergic and non-allergic children at one, four or one and/or four years. However, a pattern of lower levels of both amount and proportion of the fatty acids AA and DHA was found in cord serum phospholipids of allergic children at one and four years. This pattern was found for; eczema, food allergy and asthma.

This study showed no consistently differences in proportions or amounts of AA or DHA in cord serum phospholipids between allergic and non-allergic children. Also, the non-existing correlation between fatty acid composition in cord and maternal serum phospholipids implies that there is no association between the child's allergy development and the mother's fatty acid composition in serum phospholipids at birth and allergy at one or four years . Hence, the conclusion in this study is that allergy in children at 1 and 4 years of age is not dependent on the fatty acid milieu in serum at birth.

Keywords: fatty acids, serum, eczema, food allergy, asthma

Sammanfattning

Långkedjiga Fleromättade Fettsyror i Serum Fosfolipider vid Födsel och Utvecklingen av Allergier hos Barnet

Examensarbetets huvudsyfte var att undersöka om sammansättningen av de lång kedjiga fleromättade fettsyrorna AA och DHA i navelsträngsserum fosfolipider eller mammaserum fosfolipider skulle kunna förutspå allergiutveckling av eksem, födoämnesallergi och astma hos barn vid ett år, fyra år samt ett och/eller fyra år. Ett annat syfte var att analysera korrelationen mellan fettsyranivåer i serum från mödrar respektive navelsträng.

Fosfolipider av de lång kedjiga fleromättade fettsyrorna AA och DHA extraherades ur serumprover från mödrar respektive tillhörande barns navelsträngs serum. Serum fosfolipiderna analyserades med GC-MS. Resultat beräknades statistiskt med hjälp av oberoende t-test. Från en enkätundersökning ifylld av de deltagande mammorna erhölls uppgift om barnens allergier vid ett och fyra års ålder. Dessa data kopplades till halterna av fettsyrorna AA och DHA i navelsträngsserum för undersökning av eventuellt samband.

Serum till detta examensarbetet samlades in år 1996. Mödraproverna gav dålig upplösning i kromatogrammet vilket tyder på nedbrytning, troligen orsakad av provernas ålder. Navelsträngsproverna visade bra upplösning vilket indikerar möjlighet till förekomst av skyddande antioxidanter. Av denna anledning var navelsträngsproverna i fokus i fortsatt undersökning och diskussion.

Ingen korrelation hittades mellan fettsyranivåerna i navelsträngsserum fosfolipider och mammanserum fosfolipider vilket troligen var orsakat av den dåliga upplösningen hos mammaserum proverna. Överlag hittades inga genomgående skillnader mellan proportion eller halt av AA och DHA i navelsträngsserumfosfolipider mellan allergiska och icke-allergiska barn vid ett år, fyra år samt ett och/eller fyra år. Dock fanns ett övergripande mönster av längre nivåer av både proportion och halt av fettsyrorna AA och DHA i navelsträngsserum fosfolipider hos allergiska barn vid ett och fyra år. Detta gällde de undersökta allergierna: eksem, födoämnesallergi samt astma.

Denna studien påvisade inga genomgående skillnader i proportion eller halt av AA och DHA i navelsträngsserum fosfolipider mellan allergiska och icke allergiska barn. Då ingen koppling hittades mellan fettsyra nivåerna i navelsträngsserum och mammaserum fosfolipider var det heller inte möjligt att utröna om det fanns något samband mellan barntes allergiutveckling och fettsyranivåerna i mammaserum fosfolipider vid födseln och allergi vid ett och eller fyra års ålder. Därmed är slutsatsen av denna studien att allergi hos barn vid ett och fyra år inte beror på fettsyra miljön i serum vid födsel.

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Abbreviations

AA: aracadenoic acid

DHA**:** docosahexaenoic acid

SAFA: saturated fatty acids

MUFA: monounsaturated fatty acid

PUFA: polyunsaturated fatty acid

LA: linoleic acid

ALA: α-linolenic acid

APC: antigen presenting cell

Ig: immunoglobulins

T_H: T helper cells

SPT: skin prick test

SPE: solid phase extraction

GC-MS: gas ghromatograph (analyzer) and mass spectrometer (detector)

1. Introduction

1.1 Background

In the year of 1937 Hansen established a relationship between atopic eczema and abnormal levels of essential fatty acids in serum (1, 2). Today, allergies are an increasing problem and it is essential to discover allergy development as early as possible. This in order to avoid unnecessary elimination in the diet and also to ease the allergic reactions and symptoms. Early discovery of food allergy can make it possible to prevent development of future diseases (3). Children having one or both parents with allergies have higher risk of developing allergies (1, 4). Different types of allergens are found in different geographical regions and it is well known that the western world have more allergies than countries living in poverty (5, 6).

A variety of different substances are required in order for a fetus to grow properly. Two of these are the long chain omega-3 fatty acid docosahexaenoic acid (DHA) and the long chain omega-6 fatty acid arachidonic acid (AA). DHA is important for the development of the brain and eyes while AA is needed for cell division. These important fatty acids are actively transported with assistance of fatty acid transport proteins, from the mother's blood circulation to the fetus blood circulation.

It has been found that nutritional influences on the fetus during pregnancy plays an essential role for the development of future diseases. Investigations of the umbilical cord blood, which contains 100% fetal blood, make it possible to obtain exact information regarding different substances present in the fetus blood circulation system at birth.

The intake of fatty acids is a continuous process throughout life and begins early starting with the fetus. It gains the fatty acids through the umbilical cord while the infant gains the fatty acids either from the breast milk when nursed or by formula when nursing is not an option. Children and adults gain their fatty acids from the diet.

Studies have investigated fatty acid compositions of cord and maternal serum and the correlation to allergy development. Studies made by Beck *et al* (2000) and Strannegard *et al* (1987) found lower levels of AA in the cord serum of infants with

inherited higher risks of developing atopic diseases, while another study by Yu *et al* (1996) found higher levels of AA and DHA (31-33).

1.2 Objective

The main aim of this thesis was to investigate composition of the long chain polyunsaturated fatty acids AA and DHA in umbilical cord serum phospholipids and maternal serum phospholipids as a possible predictor of the allergy development of eczema, food allergy and asthma at one, four and one and/or four years of age. Another aim was to analyze the correlation between fatty acid levels in maternal serum and cord serum in the mother-infant pairs.

2 Theory

2.1 Fatty acids

Fatty acids consist of unbranched hydrocarbon chains with a carboxylic group attached at one end (7). This gives a biomolecule with both polar (carboxylic group) and nonpolar (hydrocarbon chain) properties (8).

Long chain polyunsaturated fatty acids are an essential incorporated component of cell membranes, affecting the fluidity and function of the membrane (9). Lack of polyunsaturated fatty acids leads to dry skin due to the impaired water barrier. Several studies have found a reduction of inflammatory skin diseases by dietary supplementation of fatty acids (2).

2.1.1 Saturated, unsaturated and polyunsaturated fatty acids

There are different families of fatty acids. Fatty acids with only carbon-to-carbon bonds and no double bonds are referred to as saturated fatty acids (SAFA). Fatty acids with one double bond are called monounsaturated fatty acids (MUFA) and when several double bonds are present, the fatty acid is called polyunsaturated fatty acid (PUFA) (7, 10). The most common hydrocarbon chain contains 16 to 18 carbons (8). An abbreviation system simplifies the collection of information about different fatty acids. The number of carbons is stated first, and then the number of double bond, for example, 18:0 (steric acid: 18 carbons and no double bonds) (8). The naturally occurring orientation of unsaturated fatty acids is usually cis and not trans (7, 8, 10).

2.1.2 Free fatty acids and triacylglycerols

Free fatty acids, the simplest form of lipids, act both as energy source for the formation of cell membranes and as signaling molecules (7). Free fatty acids are very rare in cells and in tissue since they are often bound in building blocks in triacylglycerols - consisting of fatty acids linked together with hydroxyl groups by esterification. The reason why fatty acids are appropriate for both energy storage and energy production is due to the fact that the hydrocarbon chains are in a highly reduced state (8).

2.1.3 Phospholipids

A phospholipid is build up by two fatty acid chains and one phosphate group attached combining the fatty acids (7). The fatty acids linked by ester bonds to the glycerol molecule, are found in the same variety as in triacylglycerols. The first fatty acid chain is often saturated while the second chain often is unsaturated. The special structure with a polar head and a nonpolar tail makes phospholipids especially appropriate in membrane structures (8).

2.1.4 Linoleic acid and α-linolenic acid

Linoleic acid 18:2 (LA) and α-linolenic acid 18:3 (ALA) are two very important fatty acids since they are both essential, meaning we cannot produce them ourselves; thus need to be added into our diet (7, 10).

LA is an omega-6 fatty acid while ALA is an omega-3 fatty acid and both can only be found in plants. LA is present in most vegetable oils such as corn oil, rapeseed oil and sunflower oils while ALA is found in the highest amount in flaxseed oil, perilla oil, canola oil and soybean oil (1, 7, 10).

LA and ALA are precursors of the production of long chain PUFAs undergoing enzymatic desaturation and chain elongation in the metabolic pathway (11). LA forms arachidonic acid (AA 20:4) while ALA forms docosahexaenoic acid (DHA 22:6) (1, 2, 4). The metabolic pathway is seen in figure 4 shown below.

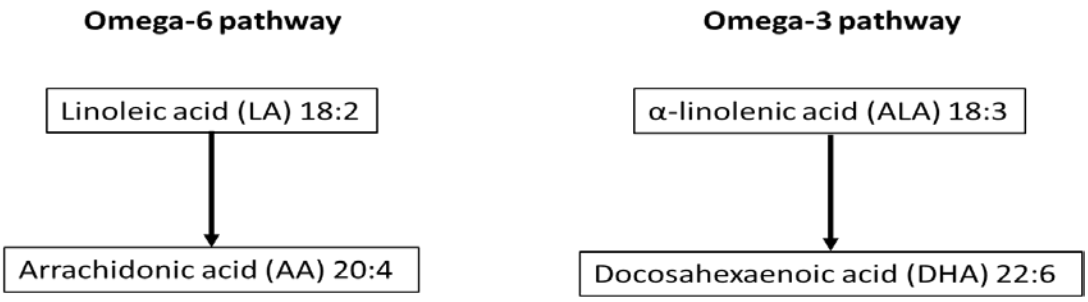


Figure 1. Metabolic pathway for the essential fatty acids Linoleic acid (LA) and α-linolenic acid (ALA) by the enzymes delta6- and delta5-denaturases forming arachidonic acid (AA), docosahexaenoic acid (DHA).Inspiration from(1) (17)

2.1.5 Long chain polyunsaturated fatty acids DHA and AA

Docosahexaenoic acid (DHA 22:6) is an omega-3 fatty acid while arachidonic acid (AA 20:4) is an omega-6 fatty acid (1, 7, 8, 10).

Long chain PUFAs, especially the PUFAs DHA and AA are important for the fetus development since they are used as building blocks for different purposes such as cells, organs, the brain and also the retina. DHA and AA are transported actively from the mother's blood circulation via the placenta to the fetus. Transportation takes place either by active or passive transport (7) which is explained more in section *1.2.6 Fatty acid transportation from mother to fetus.*

AA is involved in inflammatory and immunological processes (2, 7). AA is commonly found in meat and eggs while DHA is found in seafood and fatty fish such as herring, mackerel, salmon and tuna (1). Maternal intake of fish or supplements of fish oil during pregnancy has shown to reduce eczema and asthma in their infants. Early introduction of fish in children's diet has been shown to reduce risks for future allergic diseases (12-14).

2.1.6 Fatty acid transportation from mother to fetus

Maternal and fetal blood is separated by the placental membrane. Due to the limited fetal capacity to synthesize long chain PUFAs, the fetus nutritional supplementation is dependent on the transport of long chain PUFAs from the maternal blood circulation through the placenta to the fetus blood circulation.

Placenta lacks the desaturation enzymes necessary in the metabolic pathway, hence the conversation of essential fatty acids LA and ALA into long chain PUFAs is limited and PUFAs must therefore be supplied from the maternal blood circulation (16). Fatty acids can be transported either in the form of free fatty acids or be cleaved off from triacylglycerols by help of the lipoprotein lipase found on the surface of the placenta. Depending on lipases ability to release PUFAs and selectively transport them, an order of preference have been found to be DHA>AA>ALA>LA (7, 16-18).

As seen in figure 5, the transport of fatty acids can be either active or passive. Maternal levels of fatty acids can depend either on the dietary intake of food rich in fatty acids or on the mothers capacity to elongate shorter PUFAs to longer PUFAs.

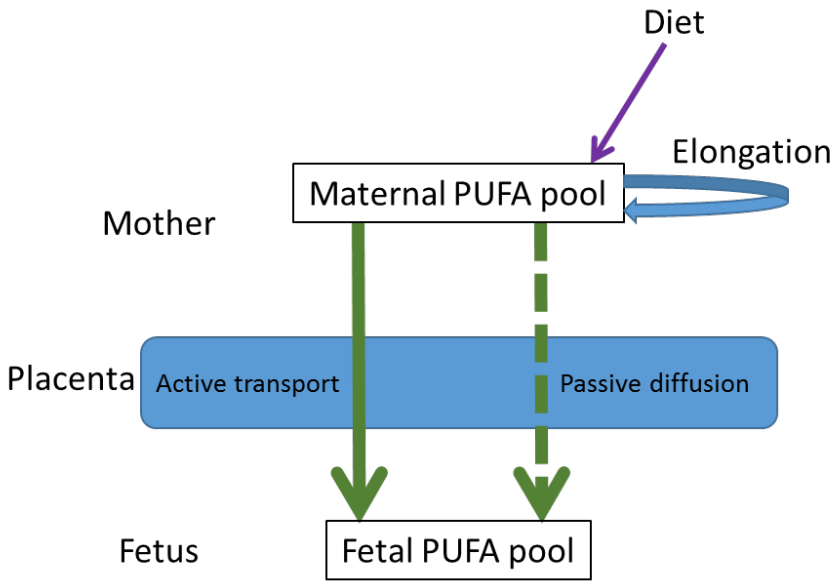


Figure 2. Transport of fatty acids from mother to fetus

2.2 The definition of hypersensitivity and allergy

The immune system is very complex thus only brief explanations will be made in this thesis. Different terms are addressed and reactions are explained with figures.

2.2.1 Sensitivity and hypersensitivity

Reaction towards a specific substance under special circumstances is defined as sensitivity while hypersensitivity is defined as initiation by exposure to a substance normally harmless causing recurring symptoms when exposed to that specific substance (19). During the first two years of life, the gut immune system is non-fully

developed. Since the food passes through the gut system hypersensitivity towards food is therefore common at this age (1).

2.2.2 Allergy

The term allergy is defined as symptoms occurring due to strong reactions of the immune system towards a harmless substance, such as food allergens, pollen or furry animals (20). These allergens can be introduced into the body either by oral or inhalant intake, or through the skin (1, 21). Allergy is a form of hypersensitivity which causes the body to produce antibodies towards intruding allergens (1, 6, 7, 19).

2.2.3 Allergen and different types of antibodies

An antibody is a molecule produced by B lymphocyte cells as a response to an antigen (allergen), specifically binding to that specific antigen. An allergen is a foreign substance or protein (i.e food allergen) which is recognized by the immune system when introduced into the body (21). Allergens are different types of proteins (4, 24), thus an allergic reaction can only occur if the allergen is a protein.

Antibodies have the formation of a Y, and are known as immunoglobulins (Ig). Five different types exist, IgA, IgE, IgD, IgG, and IgM all with special purposes and properties in the immune system (6, 19). IgG antibodies are the most abundant antibodyclass found in humam serum. It stands for about 80% while IgE antibodies only exist in trace levels (<0.1%). IgG antibodies protect the body by opsonization which means that IgG antibodies attaches to intruders in order to facilitate phagocytes. It is the only antibody which can cross over the placenta, hence it is important for the development of the fetal immune system (20).

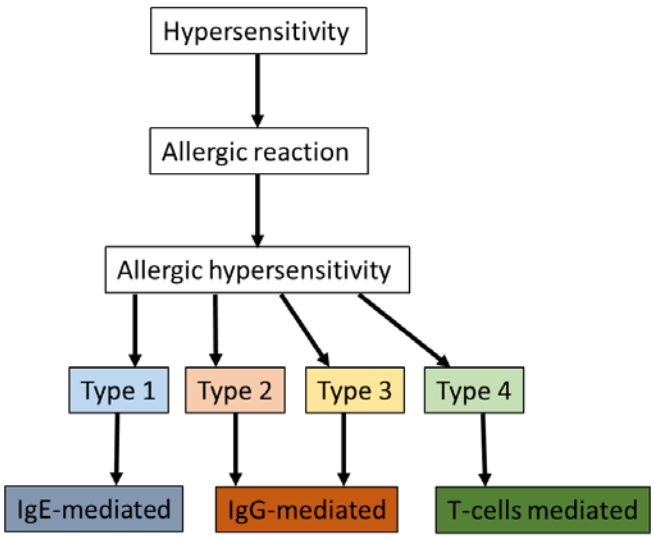


Figure 3. Hypersensitivity leading to allergic reaction and the four allergic hypersensitivity sub groups. Inspiration (23)

2.2.4 Allergy and the four types of hypersensitivity

As mentioned earlier, allergy is a form of hypersensitivity and can be divided into four types of reactions which can be either antibody mediated or cell mediated, see figure 3 (1, 7, 21, 23). The most

common form of allergy is the IgE mediated allergy also referred to as, type 1 hypersensitivity (23, 24).
It can also be referred to as immediate allergic reaction (7). Harmless allergens start a reaction in the immune system, which generates IgE-antibodies as a defense, releasing histamine and other substances which cause allergic symptoms. This mechanism is explained more in the sections 2*.2.5 Sensitization* and *2.2.7 Allergy and IgE-antibodies*.

Another form of allergy caused by IgG-antibodies, is referred to as type 2 and type 3 hypersensitivity (23). Less common is the cell mediated form of allergy which is the fourth type of hypersensitivity reaction. It is T-cells mediated caused by allergen specific T-lymphocyte cells (1, 23). This allergy usually gives allergic contact eczema (21) and can be referred to as delayed allergic reaction (7).

2.2.5 Atopy

An inherited formation of IgE-antibodies is referred to as atopy (21, 24, 25) Atopic allergies are caused by type 1 hypersensitivity (25) and individuals who are atopic have bigger risks of developing allergies to several allergens (1, 24). It is also known that infants to atopic parents have higher risks of developing atopy themselves (25).

2.2.6 Sensitization

The starting point for development of an allergy is defined as sensitization but it is important to note that one can be sensitized without being allergic (24). The first time an allergen is introduced into the body, it binds to an antigen presenting cell (APC) (7) which are strategically placed in the skin and mucosal membranes (26). The APC sends out signals to the T helper cells (T_H), which are constantly circulating, searching for invaders. The T_H cells interact with the B lymphocyte cells thus transforming these into antibody-producing plasma cells, producing IgE antibodies in large quantities (1, 8, 20). These IgE-antibodies attach to the surface of mast cells (1, 26). The individual have now been sensitized and the next time exposure to the specific allergen occurs, an allergic reaction will take place. See figure 4 for a schematic explanation of the sensitization phase.

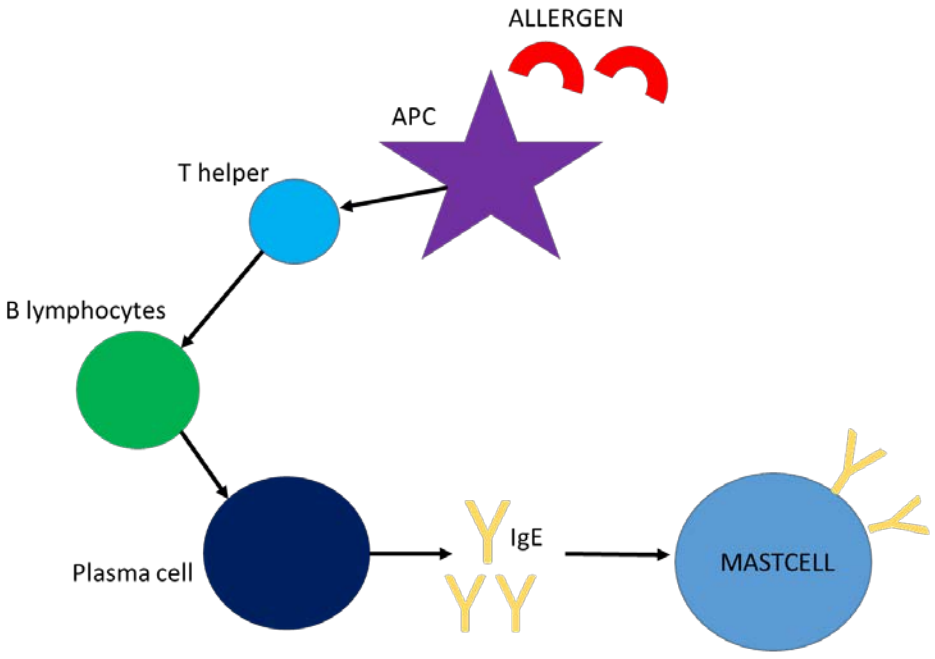


Figure 4. Sensitization phase including different cells and molecules such as APC, T helper, B lymphocytes, Plasma cells, IgE antibodies and Mast cells ready to defend when an invader comes in the body. Inspiration from (4)

B lymphocyte cells are developed in the bone marrow, hence the name B lymphocyte (8). T_H is an important cell in the cellular immune system since it sends out signals of an increased production of B lymphocyte cells and also natural killer cells (20). Unlike macrophages which circulate in the blood stream, (searching for invaders and killing them by phagocytosis) T_H can leave the blood stream and enter the lymphatic system (thymus gland, spleen and lymph nodes) where they keep constant watch for invaders. (8)

2.2.7 Allergy and IgE-antibodies

In the sensitization phase, IgE-antibodies are produced against allergens and attached to surface of mast cells. These mast cells are commonly found below the skin surface and the mucosal surface in nose, eyes, lungs, stomach and the intestine hence the allergic reactions show symptoms in these specific areas.

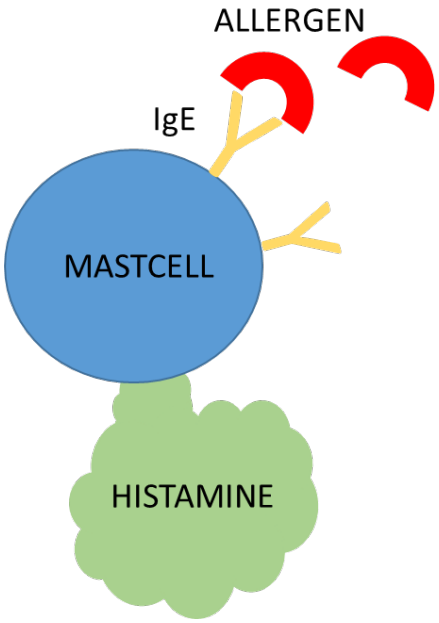


Figure 5. The allergic reaction that liberates histamine causing allergic reactions

The next time the specific allergen is introduced into the body, the defense reaction of the immune system is much faster since the sensitization phase have led to the production of IgE-antibodies bound onto the surface of mast cells enabling the IgE-antibodies to quickly capture the allergens and release histamine and other chemical substances initiating an inflammatory reaction, see figure 5 (26). This inflammatory reaction gives symptoms characteristic for allergic reactions (1, 4, 24, 25). Histamine and other chemical substances are released in order to extinguish or facilitate the removal of allergens (15).

2.2.8 Allergy diagnosis and skin prick test (SPT)

When diagnosing allergies it is important to start with a family history checkup, because it is common to inhere allergies especially if both parents are allergic (4, 21, 25). The next step is to perform a skin prick test which is a simple examination method used to see if an individual is allergic, by injection of different allergens into the top layer of the skin. If a small red dot, bigger than 3 mm forms on the skin were the allergen was injected within 20 minutes, the individual is sensitized towards that specific allergen (1, 28) IgE-antibodies can also be measured in serum.

2.3 Food allergy

Approximately 6-8% of children less than three years of age have food allergy but only a few percent of adults are allergic (21, 27). The most abundant form of food allergy is found to be infants having IgE-mediated allergy towards cow milk protein, which commonly progresses to allergy towards eggs, peanuts, nuts, wheat, fruits, vegetables, fish and shellfish (1, 21, 27). Although most children develop tolerance towards the food allergen causing allergies when they are a few years old, a child with IgE-mediated food allergy is more likely to develop future disorders (1).

The difference between being intolerant and having food allergy is that an intolerant individual is sensitive towards an allergen and the symptoms are often mild while an individual with food allergies can in worst case have deadly allergic reactions (1).

2.3.1 Symptoms and treatment

For an allergic reaction to occur, it is not necessary to consume large amounts of the food causing allergic reaction, since even very small amounts of a specific food product can cause an allergic reaction (4). In order to prevent allergic reactions and avoid unnecessary risky situations it is important to know what an individual is allergic against and how severe the allergy is.

Food allergy causes inflammatory symptoms (21) which usually appears as redness (erythema), nettle-rash, swelling of the lips and eyes, vomiting, diarrhea, abdominal cramps, clogged nose and wheezing breathing (21, 28). Reactions occurring in the gastrointestinal area are not easy to define and can be difficult to recognize (1). In some severe cases the allergic individual can react by anaphylaxis (1, 21, 28).

The only way to avoid an allergic reaction towards food allergens, is to exclude the food causing allergic reaction, consequently a changed diet is required (4). This can be very difficult to accomplice if an individual have many different food allergies. Another way to ease the everyday life is to continuously write a food journal. This can make it easier to see patterns of certain types of food which makes the individual feel better. One can also seek professional help, by consulting a dietitian. This is often made since they have much experience within the field hence giving very useful advices.

2.3.2 Progression of eczema to food allergy and asthma

For an infant with atopic eczema, it is likely that the allergy will progress into food allergy in the early years of life (1, 21). If clogged nose and wheezing breathing was symptoms during the first years of life, it is possible that the allergy will progress into allergic diseases in the airways, such as asthma. When the child is around six years old there are big chances that the allergy will develop into asthma and rhinoconjunctivitis. It is also common that the child develops tolerance towards the food allergen and out grows the allergy at around six years of age but the risk for future allergic disorders increase if the early allergy was IgE-mediated (1).

When the allergy starts with eczema, progresses into food allergy and later asthma, it is referred to as the allergic march (1)(21).

2.4 Atopic eczema

5-15% of the adult population in the western world is affected by atopic eczema which is a chronic skin disease (2). More than one of five children has eczema (29). Both immunological and inflammatory reactions are involved. The disease usually starts in the first two months of life and the first sign is dry skin which later progresses into eczema. Intake of fatty acids especially GLA have shown to improve the eczema (2). One cannot fully outgrow eczema since the water barrier of the skin is damaged but when reaching adult ages, the symptoms are often mild (29).

A child with one parent having eczema has a risk of about 20-25% of developing eczema. If both parents have eczema, the risk is as high as 75% that the child will develop eczema (21, 25).

2.4.1 Symptoms and treatment

Symptoms are recurrent weeping rash, blisters and very dry and itching skin (5). The size and placement of the eczema can vary from person to person but it is usually found on hands, elbows, knees, and other places on the body with very dry skin. Treatment is done by frequent use of moisturizing creams and lotions. The eczema is often worse during the winter due to the dry air, and much better in the summer since the sun has a positive effect on healing eczema (5) hence it is especially important to keep the skin as smooth and moisturized as possible during the winter. A stronger cream such as cortisone is usually very effective but can in the long run harm the skin hence should only be used when necessary. If the eczema is not treated correctly, the skin will harden, cracks will form and the skin can also start to form flakes. To ease the eczema the individual should not expose the skin to water too often, since it dries out the natural moist. It is important to wear lose fitting clothes made of cotton which breath much better then clothes made of synthetic materials (21).

2.5 Asthma

During the first four years of life, as many as 25 % of children have symptoms indicating asthma. If an individual develops asthma late in life, it probably depends on environmental factors and is not inherited (29). The disease is becoming more and

more common and is frequently found in the western world. This is due to increased urbanization, decreased infections in young ages and more pollution. Athletics with asthma often have abundant problems since exercise can trigger an asthma attack. Other triggers are pollen, particular food, breathing cold air or cigarette smoke. An individual with asthma should choose appropriate exercise such as swimming or bicycle and not running which stresses the body (20, 21).

2.5.1 Symptoms and treatment

An inflammation forms in the small braches of the bronchus in the airways giving respiratory distress, wheezing breathing and cough (20, 21, 29). Asthma is often described as breathing through a straw. Symptoms are often worse during evening and early morning and can vary much between individuals. Some only have mild symptoms while others have more severe symptoms. To ease the asthma it is important to avoid dust, cold environments, stress, pollutions, cooking fumes, moist and mold (5). Treatment of asthma is done by inhalant medication. There are two types of medication; one dilating bronchus hence making it easier to breath, while the other type consists of cortisol which dampen inflammations. Sometimes cortisol is prescribed to children to prevent future inflammations (21).

3. Method

3.1 Serum samples and data collection

This thesis is based on a birth cohort including all children born vaginally during one year (1996-1997) in the county of Jämtland at Östersunds hospital. Blood was collected from approximately 80 % of the mothers (venous blood) and from their infants (umbilical cord blood) at the time of delivery. A total of 76 blood samples collected from the mothers, will be analyzed in this thesis.

A questionnaire answered by the participating mothers gave us data of their children's allergies at both one and four years of age. Sensitization was reported with skin prick tests. Not all the participating mother's answered the survey at both child ages one and four years, hence we do not have complete data for all 76 children.

3.2 Definitions of the allergy diagnoses

Eczema diagnosis: Defined as a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution.

Food allergy diagnosis: Milk and/or egg allergy; defined as a positive answer to the question: "Have your child had a reaction against milk/egg the last 12 month?" and a positive skin prick test against the same allergen, i.e. milk/egg.

Asthma diagnosis: Fulfilling 1 or more of the following criteria; wheeze in the past year, 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?" together with at least one positive skin prick test to any allergen.

The tables in the result section, contain allergic and non-allergic children for each of the following allergies; eczema, food allergy and asthma. Note that an allergic child can have more than one allergy, and a child can be non-allergic towards i.e eczema but have asthma or food allergy.

3.3 Instrumental setup

Instrumental setups described in this thesis are solid phase extraction (SPE) and GC-MS since these play a key role for the separation and detection of the phospholipids in serum.

3.3.1 Solid Phase Extraction (SPE) column

SPE is a sample preparation method used for extracting phospholipids in serum. This extraction method is useful since it concentrates the phospholipids in serum by removing free fatty acids and triglycerides. Since SPE removes matrix effects, the analysis in GC-MS is simplified and gives more accurate results without interference (30).

A TELOS SPE column was used in this thesis with the surface area of 505 m^2/g, pore diameter of 70 Å and an average particle diameter of 50 µm.

3.3.2 GC-MS

Phospholipids are separated using a Gas Chromatograph (GC) and detected by a Mass Spectrometer (MS). Inserted phospholipids in GC are heated, vaporized and separated by help from helium as carrier gas (30). MS is used as a detector since it is very sensitive hence giving information about chemical structure very useful in this thesis.

In order to perform the separation using GC, the analytes need to be volatile. In this thesis, the analytes are phospholipids which are made volatile by methylation using methanol-acetyl chloride. The solution methanol-acetyl chloride is used since acetyl chloride is highly reactive and rapidly forms esters and HCl in the presence of an alcohol, and therefore methanol is used. The reaction needs to take place in a non-aqueous solution otherwise the reaction will be shifted backwards and methylation will not be achieved (30).

In this thesis the GC setup was: helium as carrier gas, inlet temperature of 100 degrees with an increasing temperature of 4 degrees/min for 42 min per sample. The final temperature was 250 degrees.

In this thesis the MS setup was: an extractor ion source, a heated gold quadrupole and a triple-axis detector. This type of MS is highly sensitive and gives a high signal-to-noise ratio and an increased confidence of trace analysis.

3.4 Material

For each sample the following is needed: 2 test tubes with tops, 2 regular test tubes without tops, 1 SPE column of type TELOS, 1 small vial with top, and many pipettes.

Chemicals needed: Internal standard 17:0, chloroform:methanol (1:1), 0,5% NaCl, chloroform, $CHCl_3$:isopropanol (2:1), 2% acetic acid in diethyleter, methanol, toluene, methanol-acetyl chloride, milliQ-water, petroleumether and isooctane. Equipment used: centrifugation machine, evaporator, GC-MS.

3.5 Evaluation of method

The experimental part of this thesis started with a test run of ten replicas of a control serum sample collected in 2009, to evaluate the method. The relative standard deviation was calculated and the result was within approved range, thus this method showed good precision and was applied for the real serum samples. See appendix for exact values.

3.6 Laboratory practical

3.6.1 Laboratory practical of serum samples

Samples were frozen hence needed to be thawed prior laboratory practical. 25 µl of internal standrad (17:0) with the concentration of 1 mg fatty acid/ml was added to 200 µl of serum. 4 ml chloroform : methanol (1:1) and 2 ml 0,5% NaCl was also added. The serum was vortexed and centrifuged at 3000xg for 6 min. A two phase system formed and as much as possible of the lower chloroform phase was collected. A second extraction of the long chain fatty acids present in the chloroform phase was done by addition of 2 ml chloroform. The samples were centrifuged again at 3000xg for 6 min and added to the previous test tube**.** Samples were thereafter evaporated at 40°C using nitric gas for 30-40 min.

The samples were extracted using a SPE which had been washed twice with 2 ml of hexane, too avoid contamination. The samples were dissolved using 200 µl of chloroform and poured into the SPE colon, the test tubes were washed with 100 µl of chloroform in order to recover small amounts left. The column was washed with 4 ml of $CHCl_3$: isopropanol (2:1) and 2% acetic acid in diethyl ether respectively which were put to waste. The column was washed using 4 ml methanol which eluted the phospholipids.

The methanol phase, including phospholipids, was evaporated in 40°C with nitric gas for 20 min. In order for methylation of the phospholipids, 2 ml toluene and 2 ml of methanol-acetyl chloride (10% acetyl chloride) was added to the samples which were vortexed and put to rest overnight.

Addition of 1 ml milliQ-water and 2 ml petroleumether was done prior to vortex and gentle centrifugation 2500xg for 5 min which formed a two phase system. The organic phase was taken out and evaporated under 40°C with nitric gas for 30 min. The samples were dissolved in 100µl isooctane prior to insertion into vials and later analyzed in the GC-MS.

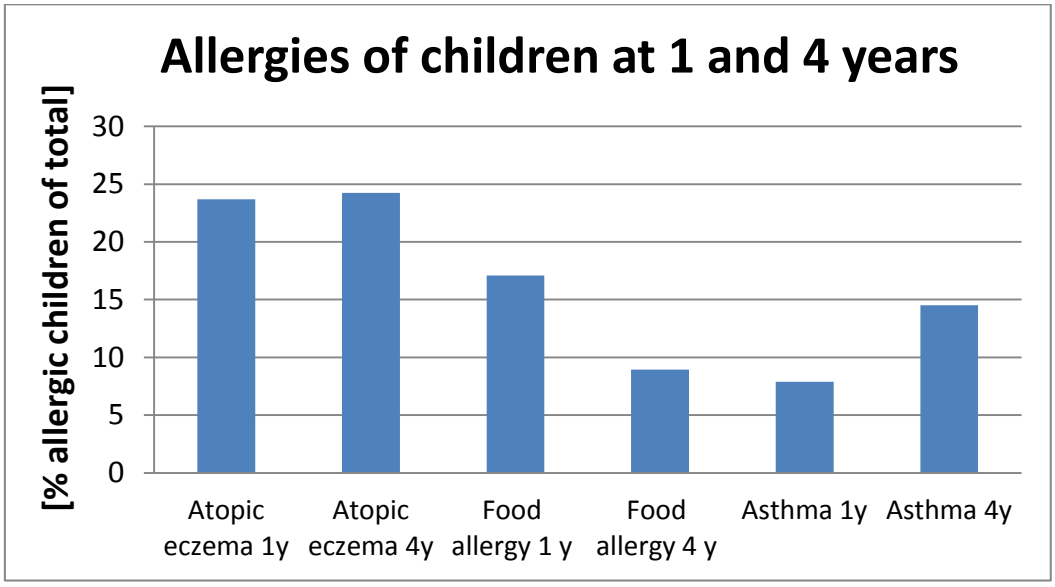
4. Results

The chromatograms obtained from the GC-MS of maternal serum samples, showed a poor resolution with many small extra peaks which should not have been seen. This indicates degradation and formation of byproducts non-relevant for this thesis. Cord serum samples, from respective children were better preserved, indicating a possible presence of protecting antioxidants in cord serum hence these results are more reliable and chosen as focus of the result and discussion section.

Due to the low number of allergic children at one and four years of age, in foremost the food allergy and asthma groups, also cumulative allergy were calculated. I.e. children having allergy at either one and/or four years of age.

4.1 Allergies in the children

Figure 6, displays the different allergies in children at one and four years of age based on the data collected from the questionnaire. It can be seen that a child with atopic eczema at one year is also likely to have atopic eczema at four years of age. Food allergy decreases from one year to four years, indicating developed tolerance. Asthma at one year is rare while asthma at four years is more common, this is explained by two factors, the diagnosing definition has not often been fulfilled during the first year of life and the allergic march which states that asthma comes later in childhood.

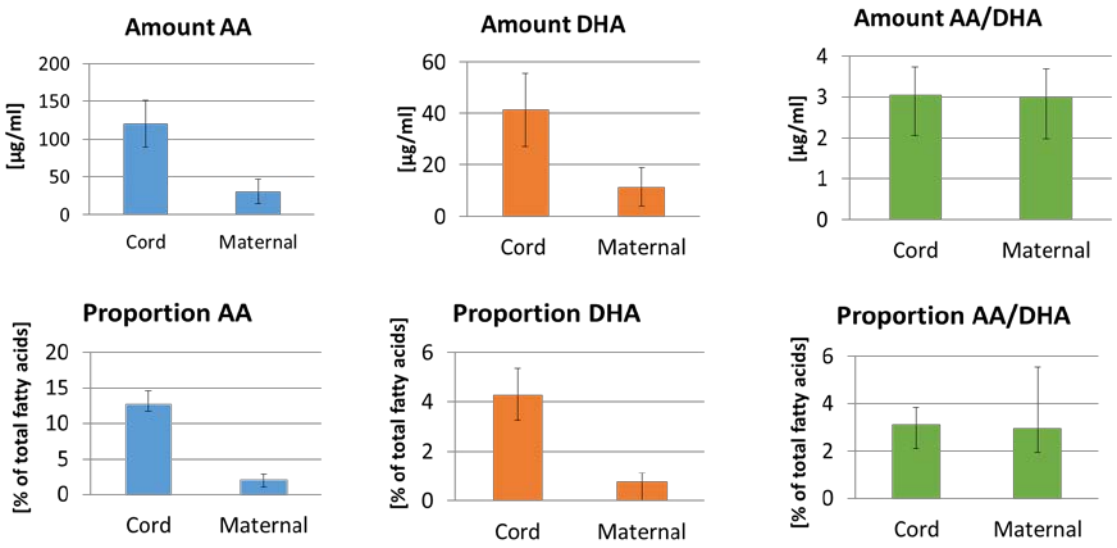


Figur 6. Proportions of allergic children.

4.2 Amount, proportions and ratio of AA and DHA in cord and maternal serum

Average values in amount (µg/ml) of AA and DHA, proportion of AA and DHA (% of total phospholipids) and the ratio of AA/DHA in both cord and maternal serum were calculated, as seen in figure 7. Errors bars as standard deviation was also calculated.

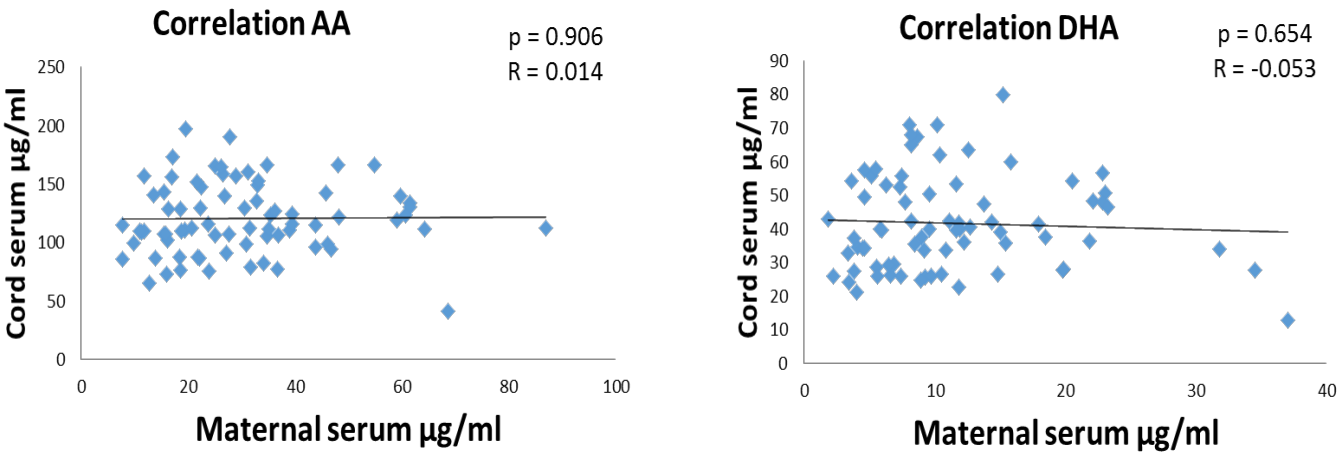
The amount and proportion of AA and DHA was much higher in cord compared to maternal serum phospholipids. Also, both the amount and proportion of AA was more than twice as high as DHA in both cord and maternal serum phospholipids. The ratio AA/DHA of both amount and proportion was on the other hand very similar for both cord and maternal serum phospholipids.



Figur 7. Average values in amount (µg/ml) of AA and DHA, proportion of AA and DHA (% of total phospholipids) and the ratio AA/DHA in both cord and maternal serum

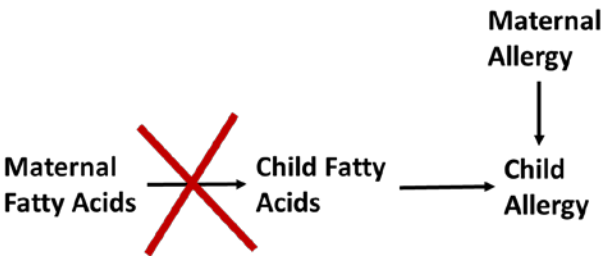
4.3 Correlations between AA and DHA in cord and maternal serum

In figure 8, the correlation between cord and maternal serum phospholipids is shown both for AA and DHA. No correlation was seen between either AA nor DHA in cord and maternal serum phospholipids.



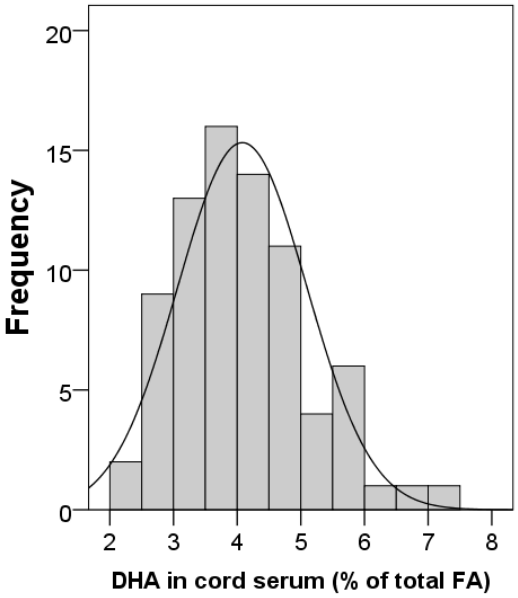
Figur 8. Correlations of AA and DHA between cord and maternal serum

Figure 9 illustrates the non-existing correlation between the maternal and cord fatty acid composition. Hence, the correlation between maternal levels of AA and DHA and allergy development in the children is probably also low. The amounts or proportions of AA or DHA in maternal serum samples are therefore not compared to allergy development in the children. The focus is instead on the levels of these two fatty acids in cord serum in relation to allergy development.



Figur 8. Non-existing correlation between maternal fatty acid and child allergy

4.4 Fatty acids in allergic vs non-allergic children



In order to investigate differences between allergic and non-allergic individuals, student's independent t-test was used. This could be used since the distribution was normally distributed as seen in figure 9.

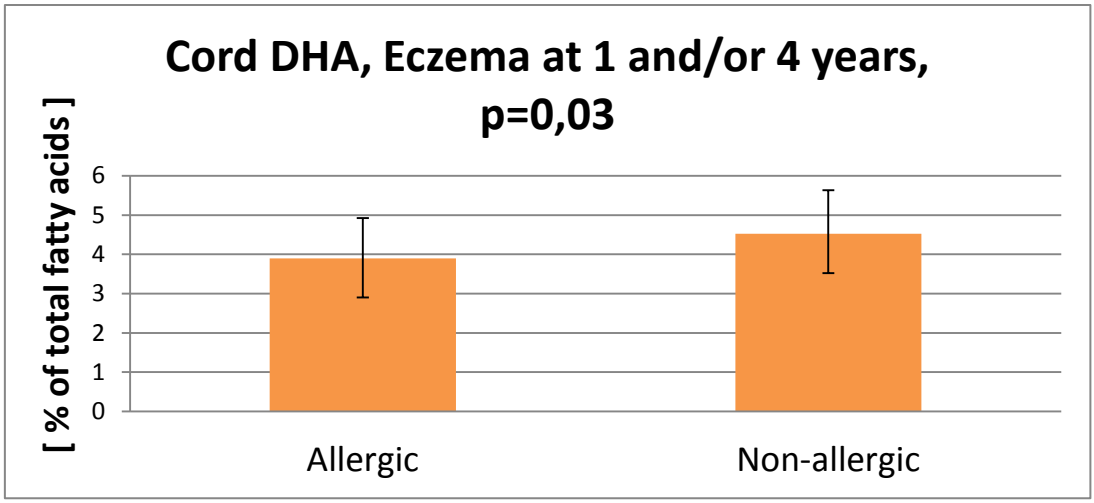
Figur 9. Gaussian distribution of DHA as proportion of total fatty acid (FA) composition in cord serum.

For the following tables significant values ($p \leq 0.05$), are marked in bold while trend values ($0.05 < p \geq 0.10$), are marked in italic.

4.4.1 Eczema

Of the one year old children 18 had eczema while 16 children had eczema at four years. In the cumulative group, 24 children had eczema.

There was only one significant value ($p=0.03$) found between children with eczema and non-eczematous children which is displayed in figure 10; children with eczema at four years of age had lower proportions of DHA in their cord serum phospholipids.



Figur 100. Proportion of cord DHA of eczema at 1 and/or 4 years.

A non-significant and contradicting trend (p=0.08) was found for the allergic four year old children with higher levels of the AA amount in their cord serum phospholipids.

A non-significant pattern was seen for the allergic children at the ages; one, four and one and/or four years with lower levels of the proportion of AA in cord serum phospholipids. For the allergic one year old children, the amount of AA was lower while the levels of AA was higher for the four year old and one and/or four year old children.

Allergic children at one year, four year and one and/or four years also showed a non-significant pattern of lower levels of both the proportion and amount of DHA in cord serum phospholipids. An exception was seen for allergic four year old children with higher levels of the amount of DHA in cord serum phospholipids.

The ratio of AA/DHA in allergic children at one and one and/or four years showed lower levels of the proportion in cord serum phospholipids while the proportion was higher for allergic children at four years, however, this was not found to be significant.

Allergic children at one year, four years and one and/or four years showed higher levels in cord serum phospholipids for the amount of the ratio AA/DHA, however, this was not found to be significant.

Table 1. Amount and proportions of AA and DHA and ratio AA and DHA in cord serum phospholipids in relation to eczema at one year, four years and one and/or four year old children.

Eczema	1 year			4 year			1 and/or 4 year		
	Allergic n=18	Non-allergic n=57	p-value	Allergic n=16	Non-allergic n=50	p-value	Allergic n=24	Non-allergic n=46	p-value
AA %	12.22 ± 1.56	12.90 ± 2.00	0.19	12.19 ± 1.72	13.02 ± 2.06	0.13	12.39 ± 1.64	12.97± 2.12	0.24
AA µg/ml	118.91 ± 30.72	120.60 ± 30.77	0.84	132. 65 ± 28.16	117.98 ± 29.25	*0.08*	124.94 ± 32.43	116.60 ± 29.50	0.28
DHA %	4.08 ± 1.01	4.33 ± 1.12	0.41	3.94 ± 1.14	4.45 ± 1.10	0.11	3.90 ± 1.02	4.52 ± 1.11	**0.03**
DHA µg/ml	39.46 ± 12.25	41.98 ± 14.5	0.51	42.92 ± 11.60	41.55 ± 14.11	0.72	40.29 ± 12.50	41.38 ± 14.44	0.76
AA/DHA %	3.09 ± 0.74	3.14 ± 0.72	0.79	3.12 ± 0.70	3.10 ± 0.73	0.90	3.10 ± 0.70	3.15 ± 0.74	0.78
AA/DHA µg/ml	3.16 ± 0.73	3.02 ± 0.68	0.46	3.07 ± 0.77	2.99 ± 0.65	0.67	3.04 ± 0.82	3.04 ± 0.61	1.00

4.4.2 Food allergy

At one year of age, 13 children had food allergy, while only 6 were allergic at four years, this because the developed tolerance. In the cumulative group, 13 had food allergy, see table 3.

As seen in table 2, no significant values were found for food allergy, however three non-significant trends were found. One non-significant trend was seen for the four year old children for the proportion of AA (p=0.07), another non-significant trend was seen for the one year old children for the ratio of AA/DHA of amount (p=0.07) and the last non-significant trend was seen for one and/or four year old children for the ratio of AA/DHA proportions (p=0.09). All non-significant trends indicate lower levels of AA in cord serum phospholipids in allergic compared to non-allergic children.

Allergic children showed a non-significant pattern of lower levels of AA in cord serum phospholipids which can be seen for both the proportion and amount of AA at one, four and one and/or four year old children.

A different and conflicting non-significant pattern was found for the allergic and non-allergic children at one, four or one and/or four years with regard to the proportion of DHA. Allergic children had higher levels of DHA in their cord serum phospholipids compared to non-allergic children. For the four year old children and the one and/or four year old children, the amount values of DHA were lower in allergic compared to non-allergic but the allergic one year old children showed higher values of DHA in their cord serum phospholipids.

A non-significant pattern was seen for allergic children at one, four and one and/or four years of age, indicating lower levels of both the proportion and amount of the ratio AA/DHA in their cord serum phospholipids.

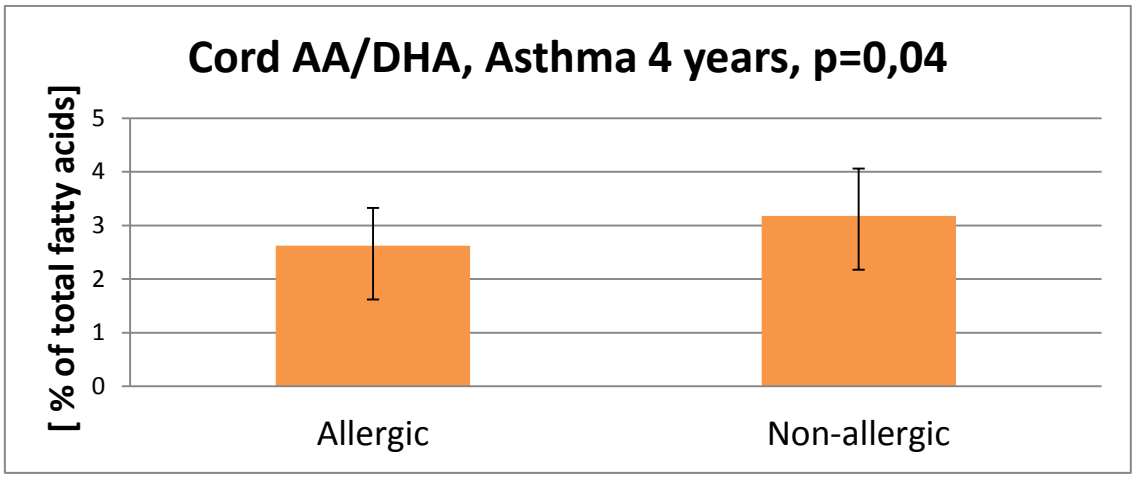
Table 2. Amount and proportions of AA and DHA and ratio AA and DHA in cord serum phospholipids in relation to food allergy at one year, four years and one and/or four year old children.

Food allergy	1 year			4 year			1 and/or 4 year		
	Allergic n=13	Non-allergic n=63	p-value	Allergic n=6	Non-allergic n=62	p-value	Allergic n=13	Non-allergic n=51	p-value
AA %	12.36 ± 1.96	12.78 ± 1.70	0.40	11.26 ± 3.27	12.80 ± 1.82	*0.07*	12.36 ± 1.96	12.78 ± 1.70	0.44
AA µg/ml	116.87 ± 29.26	120.88 ± 31.01	0.67	113.43 ± 30.46	120.91 ± 28.64	0.55	113.74 ± 30.49	122.09 ± 29.51	0.37
DHA %	4,32 ± 1.39	4.26 ± 1.04	0.84	4.37 ± 1.67	4.21 ± 1.00	0.72	4.67 ± 1.29	4.23 ± 1.06	0.20
DHA µg/ml	44.44 ± 15.60	40.75 ± 13.72	0.39	37.63 ± 12.80	42.32 ± 13.86	0.42	38.21 ± 10.01	42.44 ± 14.32	0.32
AA/DHA %	3.02 ± 0.82	3.14 ± 0.70	0.56	2.75 ± 0.95	3.17 ± 0.70	0.18	2.78 ± 0.66	3.17 ± 0.73	*0.09*
AA/DHA µg/ml	2.74 ± 0.49	3.11 ± 0.71	*0.07*	2.92 ± 0.55	3.12 ± 0.72	0.54	3.00 ± 0.44	3.04 ± 0.72	0.83

4.4.3 Asthma

Of the one year old children 6 had asthma while 9 had developed asthma at four years. In the cumulative group, 13 children had asthma.

As seen in figure 11, one significant value (p=0.04) was found for asthma. This was the four year old children with lower levels of the ratio AA/DHA in the allergic children in cord serum phospholipids with regard to proportion.



Figur 111. Significant value of p=0.036 for the proportions of AA/DHA ratio cord serum for asthma at 4 years.

Two non-significant trends were found for asthma. The first non-significant trend were (p=0.06) for the four year old children with higher levels of the proportion of DHA in allergic compared to non-allergic children in cord serum phospholipids. The other non-significant trend (p=0.09) showed lower levels in allergic compared to non-allergic children at one and/or four years for the ratio of AA/DHA proportions.

The allergic children at one, four and one and/or four years of age showed a non-significant pattern of lower levels of the proportion of AA in cord serum phospholipids. The amount of AA showed higher levels for the allergic four year old children and the one and/or four year old children, however the levels were lower for the one year old children.

One year old allergic children showed lower values of DHA with regard to the proportion while, allergic four year old children and one and/or four year old children

showed higher values. Allergic children at the ages; one, four and one and/or four years showed lower levels of the amount of DHA in their cord serum phospholipids compared to non-allergic children. However, these values were not significant.

Considering differences in ratios of AA/DHA in allergic children at the ages; one, four and one and/or four years, both ratios of AA/DHA, amounts and proportions, in cord serum, showed a non-significant pattern with lower levels of AA/DHA in the allergic children.

Table 3. Amount and proportions of AA and DHA and ratio AA and DHA in cord serum in relation to asthma at one year, four years and one and/or four year old children.

Asthma	1 year			4 year			1 and/or 4 year		
	Allergic n=6	Non-allergic n=70	p-value	Allergic n=9	Non-allergic n=54	p-value	Allergic n=13	Non-allergic n=51	p-value
AA %	12.11 ± 1.38	12.80 ± 1.96	0.41	12.30 ± 2.21	12.78 ± 1.68	0.44	12.36 ± 1.96	12.78 ± 1.70	0.44
AA µg/ml	108.13 ± 30.49	121.23 ± 30.50	0.32	124.00 ± 30.00	120.40 ± 29.76	0.74	123.75 ± 30.49	122.10 ± 29.51	0.37
DHA %	4.05 ± 0.51	4.29 ± 1.13	0.61	4.97 ± 1.14	4.22 ± 1.04	*0.06*	4.67 ± 1.29	4.23 ± 1.06	0.20
DHA µg/ml	35.74 ± 10.40	41.87 ± 14.24	0.31	41.39 ± 10.12	41.83 ± 14.20	0.93	38. 21 ± 10.10	42.44 ± 14.32	0.32
AA/DHA %	3.01 ± 0.38	3.14 ± 0.74	0.69	2.62 ± 0.71	3.17 ± 0.88	**0.04**	2.79 ± 0.66	3.17 ± 0.73	*0.09*
AA/DHA µg/ml	3.04 ± 0.28	3.06 ± 0.71	0.95	3.03 ± 0.54	3.04 ± 0.70	0.97	3.00 ± 0.44	3.04 ± 0.72	0.84

5. Discussion

The main aim of this thesis was to investigate if there was an association between fatty acid composition of AA and DHA in cord and maternal serum phospholipids and the development of allergy. Another aim was to investigate correlation between fatty acids in cord and maternal serum phospholipids.

No correlation was seen between the fatty acid composition in cord and maternal serum phospholipids hence the focus of the result and discussion is not the association between maternal fatty acid composition and development of allergies in the children, instead the focus in this thesis has been the cord serum fatty acid composition and the children's allergies at one and four years.

Both the amount and proportion of AA and DHA are found in higher levels in cord serum phospholipids compared to maternal serum phospholipids, implying that the transport of AA and DHA takes place by help from active pumps transporting the fatty acids through the placenta, against the concentration gradient. Beck *et al* (2000) have a theory stating that this active transport of fatty acids across the placenta against the concentration gradient and the placenta synthesis of both AA and DHA explain the higher amounts in cord serum phospholipids compared to maternal serum phospholipids (31).

Despite the order of preference, stating that DHA is transferred prior to AA, the levels of AA in cord serum phospholipids is more than twice as high compared to DHA, this is probably due to the fact that AA is more common both in our diet and in our bodies.

The ratio of AA/DHA show almost equal values of amount and proportion in cord and maternal serum phospholipids, indicating that both the amount and proportion of AA and DHA in cord serum phospholipids respective maternal serum phospholipids have the same relation of total fatty acid composition.

The non-existing correlation between maternal fatty acid composition and the child's fatty acid composition in serum phospholipids found in this thesis, implies that there is no link between maternal fatty acid composition and the child's fatty acid composition in serum phospholipids at birth. This result may be caused by the degradation of the maternal serum samples. Hence the child's allergy development is probably not

dependent upon the mother's fatty acid composition. The non-existing correlation implies an active and selective transportation of fatty acids from the maternal blood circulation to the fetus blood circulation across the placenta.

Even if the child's allergy was not shown to be dependent on the maternal fatty acid composition in this thesis, there might be another connection between the mother's allergy and her child's allergy development but this have not been investigated in this thesis. The child's fatty acid levels may depend on inner factors such as the active transportation of fatty acids from maternal blood circulation to the fetus during pregnancy rather than the fatty acid composition in the maternal circulation. It may also depend on other factors correlated to a not fully developed immune system of infants. The allergy development may also depend on other outer factors such as the well known factors included in the hygiene hypothesis.

The results from this thesis show only two significant differences between the proportion of the total fatty acid composition in allergic and non-allergic children. One with lower values of the proportion of the ratio AA/DHA in allergic compared to non-allergic four year old children with asthma. The other significant value was found for the proportion of DHA in the cumulative allergy at one and/or four year old children with eczema of lower values in allergic compared to non-allergic children.

However a non-significant pattern was seen for lower levels of the amount and proportion of both AA and DHA in cord serum of allergic compared to non-allergic children at one year, four year and one and/or four year old children. This pattern was found for eczema, food allergy and asthma. Other studies performed by Beck *et al* (2000) and Strannegardet al (1987) have also found lower levels of AA in the cord serum of infants with inherited higher risks of developing atopic diseases (31, 33). However another study, performed by Yu *et al* (1996), found higher levels of AA and DHA in cord serum phospholipids of allergic individuals (21). There have been resent research in this field made by Barman et al (2013) which found higher levels of AA and DHA to be a predictive factor for the development of allergies with the theory that reduced T cells activation might dampen inflammation leading to hinder of normal tolerance development.

The investigation of fatty acid composition in maternal and cord serum phospholipids and the correlation to allergy development needs more research since there are not many studies within this field and the existing data gives contradicting results.

From the results of this thesis, the child's fatty acid compositions have not been found to associate significantly to its allergy development. There are, instead, other well-known outer factors correlated to allergy development, for example those included in the hygiene hypothesis; poverty, large families, pets and farm animals, which have shown to reduce risk of allergy development. These factors were, however, not analyzed in this thesis.

6. Conclusion

No correlation was seen between levels of the two long chain polyunsaturated fatty acids, AA and DHA, in cord and maternal serum phospholipids.

Out of 108 calculated t-test values for the allergies; eczema, food allergy and asthma, for the ages one, four and one and/or four years, only two significant values was found. However an overall pattern was seen of lower levels of both amount and proportion of the fatty acids AA and DHA as well as the ratio AA/DHA in cord serum phospholipids in allergic compared to non-allergic children at one year, four year and one and/or four years. This pattern was found for eczema, food allergy and asthma.

In this study, the fatty acid compositions of AA and DHA in cord serum phospholipids have not been found to be predictive factors of the development of the investigated allergies; eczema, food allergy and asthma.

7. Future research

It would be interesting to expand this study to a larger group of participants and investigate other cohorts since this will give a more reliable result. To add a control group of healthy participants to compare results with would be useful.

In order to obtain better resolution of the maternal serum samples fresh new serum samples should be collected and analyzed immediately. This is recommend since the analyzed test serum samples, collected in 2009 (see appendix for standard deviation of the test samples), gave good resolution hence fresh serum samples are more reliable.

The participants need to have proper diagnosis by doctors and not only diagnosis by answering a questionnaire and SPT.

This study could also be expanded by investigation of several fatty acids, for example the essential fatty acids LA and ALA.

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9. Appendix

Test samples	**Date: 20130409**	
Sample	**20:4n6,AA**	**22:6 n3,DHA**
1.D	0,1248	0,0748
10.D	0,1157	0,0672
2.D	0,1262	0,0751
3.D	0,1334	0,0801
4.D	0,1372	0,0819
5.D	0,1219	0,0716
6.D	0,1231	0,0723
7.D	0,1214	0,0716
8.D	0,1211	0,0706
9.D	0,1235	0,0733
SD	0,006242516	0,004396274
Mean	0,12483	0,07385
RSD (SD/mean)	0,050008135	0,059529772
RSD i proc	5,0%	6,0%