MASTER THESIS

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Maternal omega-3 and omega-6 fatty acid status in pregnancy and asthma in children at 3 years

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Preface

My time at Oslo Met has come to an end. The years at Oslo Met have been educational and challenging and I am grateful for the experience. I find the subject of this master's thesis interesting and very important as asthma is a global health problem, and I find the connection between fatty acids and chronic disease fascinating. That is the main reasons I chose to write this thesis.

The process of completing this master's thesis has been comprehensive and challenging, but most of all a very interesting journey. I have learned a lot and would never be without this experience, I have also gained knowledge and experience I will take with me further in life.

First, I would like to thank my supervisor Christine Louise Parr. Thank you for all the help and support throughout this process. Thank you for all your patience, guidance and feedback. I could not have done this without you. And I would like to thank the Norwegian Institute of Public Health for giving me the opportunity to use data from the Mother, Father and Child cohort study.

Finally, I would like to thank my family and fellow students for all the support, for always believing in me and for all the encouragements along the way.

Sande, May 2019

Ida Sofie Andreassen

Abstract

Background: Asthma is chronic disease characterized by airway inflammation and is common in children. The risk factors are largely unknown. Fatty acids are precursors for metabolites with anti-inflammatory and pro-inflammatory properties. According to Developmental Origins of Health and Disease hypothesis, exposures in fetal life and the intrauterine environment may have a role in later disease development. Earlier research on maternal fatty acid status during pregnancy and later asthma development is limited and shows inconsistent results.

Objective: Purpose of this study was to see if there is an association between maternal status of selected omega-3 and omega-6 fatty acids in pregnancy and childhood asthma at 36 months, and if results differ for fatty acids in relative and absolute concentrations.

Methods: The master thesis is based on data from the Norwegian Mother, Father and Child Cohort study conducted by The Norwegian Institute of Public Health. It is a prospective cohort study with questionnaire data and biological material. Whole blood phospholipid fatty acids (absolute and relative) and plasma biomarker concentrations were measured in blood samples taken in approximately pregnancy week 18. Child's asthma was reported by mother in a questionnaire at 36 months. Risk of asthma in relation to maternal fatty acid status is presented as adjusted odds ratios from multivariable logistic regression models.

Results: The adjusted results indicated a statistically significant reduced risk of total long chain omega-3 when presented in relative concentrations on childhood asthma. It was a significant reduced risk of specific long chain polyunsaturated docosapentaenoic acid and docosahexaenoic acid in relative concentrations. Specific long chain polyunsaturated omega-6 arachidonic acid indicated significant increased risk for asthma when presented in absolute concentration. Ratio arachidonic acid and eicosapentaenoic acid in absolute concentration show significant increased risk for asthma.

Conclusion: Maternal fatty acid status in pregnancy was associated with the development of asthma in childhood. There was a protective effect of higher total omega-3 and docosahexaenoic acid status and a possible adverse effect of higher arachidonic acid status, but result differ by scale of measurement.

Sammendrag

Bakgrunn: Astma er en kronisk sykdom karakterisert av inflammasjon i luftveiene og er vanlig hos barn. Risikofaktorer er stort sett ukjente. Fettsyrer er forløpere for metabolitter med anti-inflammatoriske og pro-inflammatoriske egenskaper. I henhold til hypotesen «Developmental Origins of Health and Disease» kan eskponeringer i fosterlivet og intrauerine miljøet ha en rolle i senere sykdomsutvikling. Tidligere forskning på mors fettsyrestatus under graviditet og senere astmautvikling er begrenset og usikre funn.

Hensikt: Hensikten med denne studien var å se om det er en sammenheng mellom mors fettsyrestatus for utvalgte omega-3 og omega-6 fettsyrer i svangerskapet og astma ved 36 måneder, og om resultatene er forskjellige for fettsyrer i relative og absolutte konsentrasjoner.

Metode: Masteroppgaven er basert på data fra Den norske mor, far og barn kohortstudien utført av Folkehelseinstituttet. Det er en prospektiv kohortstudie med spørreskjemaopplysninger og biologisk materiale. Fosfolipidfettsyrer i fullblod (relativ og absolutt) og biomarkør fra plasmakosentrasjon ble målt i blodprøver som ble tatt rundt graviditetsuke 18. Barnets astma ble rapportert av mor i spørreskjema ved 36 måneder. Risiko for astma i forhold til mors fettsyrestatus presenteres som justerte odds ratio fra multivariabel logistisk regresjonsanalyse.

Resultater: De justerte resultatene viste en statistisk signifikant redusert risiko for total langkjedet omega-3 når de ble presentert i relative (%) konsentrasjoner for astma ved 36 måneder. Det var også en statistisk signifikant redusert risiko for de lankjedede flerumettet fettsyrene dokosapentaensyre og dokosaheksaensyre i relative (%) konsentrasjoner. Langkjedede flerumettede fettsyren omega-6 arakidonsyre viste en statistisk signifikant økt risiko for astma i absolutt (nmol/L) konsentrasjon. Ratio av arakidonsyre og eikosapentaensyre i absolutt konsentrasjon viste en signifikant økt risiko for astma.

Konklusjon: Mors fettsyrestatus i graviditeten viste en sammenheng med utviklingen av astma i barnet ved 36 måneder. Det var en beskyttende effekt av høyere total omega-3 og dokosaheksaensyre status og en mulig negativ effekt av høyere arakidonsyrestatus, men resultatene ble forskjellig etter hvilket mål som ble benyttet (relativ/absolutt).

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List of abbreviations

AA	Arachidonic acid
BMI	Body Mass Index
CI	Confidence interval
DHA	Docosahexaenoic acid
DOHaD	Developmental Origins of Health and Disease
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
HDL	High Density Lipoprotein
MoBa	The Mother, Father and Child Cohort Study
NIPH	Norwegian Institute of Public Health
NNR	The Nordic Nutrition Recommendations
OR	Odds Ratio
PL	Phospholipid
PUFA	Polyunsaturated Fatty Acid
VLDL	Very Low Density Lipoprotein

1.0 Introduction

The burden of asthma has increased globally and is one of the most common chronic diseases in childhood (Asher & Pearce, 2014). The Global Burden of Disease study has estimated that about 339 million people suffered from asthma worldwide in 2016 (Global Asthma Network, 2018) and asthma is seen as one of the most important non communicable diseases (NCD). There is various occurrence of asthma around the world. The disease is more prevalent in high-income countries and in early ages, but the causes are not yet known (Asher & Pearce, 2014). The prevalence of asthma and wheezing in preschool children and infants is high and still increasing in developing countries (Global Asthma Network, 2018). In a nationwide study from 2005-2007 the increase of asthma was highest in children at 12 years and younger, where prevalence was highest in 2-year-old children (Karlstad, Nafstad, Tverdal, Skurtveit, & Furu, 2010). Asthma is a heterogenous disease which is most often characterized by chronic inflammation in the airways (Global Asthma Network, 2018). Genetic predisposition is also an important risk factor for asthma. Asthma can develop at any age, but symptoms most commonly develop in childhood (Asher & Pearce, 2014). Asthma is partly hereditary, but genes are not the only explanation as asthma is a complex disease (Henderson & Warner, 2012). Asthma commonly originates in early life and children with asthma has often started with wheezing in the first years of life, but there has been more focus on maternal diet during pregnancy and the effect on the fetus.

All risk factors for asthma have not yet been determined, but exposure to tobacco smoke in childhood and also pre-natally during pregnancy is a known risk factor (Global Asthma Network, 2018). The Global Asthma report states the importance of identifying environmental risk factors that can lead to interventions to reduce asthma worldwide. Asthma contributes to reduced quality of life and it is important to understand the mechanisms behind the disease and do further research to find the causes and risk factors. Over time several hypotheses on asthma risk have been raised, as the hygiene hypothesis related to microbial exposure, exposure to other environmental factors, and the developmental origins hypothesis where exposures in fetal life could influence and cause disease later in life (Sharma et al., 2014). Fetal factors as birth weight and preterm birth may be associated with respiratory outcomes as asthma weight (Duijts, 2012; Henderson & Warner, 2012) Maternal diet may be a factor that can affect fetal outcome but further research is needed (Guilleminault et al., 2017; Henderson & Warner, 2012). Western diet is characterized by a lot of processed and fried foods, red meat and a low intake of fruit, vegetables and seafood (Guilleminault et al.,

2017). This then leads to a higher intake of fatty acids from the n-6 (omega-6) family. Fatty fish as salmon, mackerel and sardines are a great source of long-chain fatty acids from the n-3 (omega-3) family. These n-3 fatty acids may have anti-inflammatory capabilities. Because the western diet includes plenty of processed and fried foods, the diet is rich on long-chain n-6 fatty acids and this diet is considered to be pro-inflammatory and can possibly increase airway inflammation (Guilleminault et al., 2017). The composition of maternal dietary fatty acids is very important during pregnancy because of the transfer across placenta (Innis, 2011). Because of changes in intake of dietary fat such as increased intake of vegetable oils rich in n-6 fatty acids, it is possible that the imbalance between n-6 and n-3 fatty acids could be a risk factor for the development of asthma. Mothers fatty acid status in pregnancy may affect the child's development of asthma. Asthma is still a complex disease with unknown causes, and findings in the field on fatty acid status connected to childhood asthma is limited with inconsistent results and further research is needed.

1.2 Aims

Epidemiology is about finding the cause of disease, where nutritional epidemiology focus on dietary causes (Willett, 2013, p. 2). This study is an epidemiological study of maternal fatty acid status in pregnancy and the development of asthma in childhood carried out within a national pregnancy cohort called The Norwegian Mother, Father and Child Cohort study (MoBa) with questionnaire data and biological material from over 100 000 pregnancies. The purpose of this study was to investigate the association between maternal whole blood fatty acid status in pregnancy and childhood asthma by 36 months, using data on a subsample.

The specific aims were to investigate

1) the association between the following maternal fatty acid exposures and childhood asthma at age 36 months
    - The individual long chain n-3 fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)
    - Total long chain n-3 fatty acids defined as the sum of EPA, DPA and DHA
    - The n-6 fatty acid arachidonic acid (AA)
    - The ratio of arachidonic acid (AA) to eicosapentaenoic acid (EPA) as measure of the n-6 to n-3 ratio
2) If associations differ for absolute and relative values of the fatty acid concentrations
3) If associations are mediated through birth weight and gestational length

2.0 Theoretical background

In this chapter relevant theory is presented.

2.1 Epidemiology

Epidemiology is the study of health in a population and attempts to find the cause of disease and risk factors by looking at associations between exposure and outcome (Thelle, 2015, p. 24). Nutritional epidemiology focus on nutritional factors and health related to food, where the food or nutrients are the exposure (Rothman, Greenland, & Lash, 2008). There are different types of research design in epidemiology and the two research designs cohort and nested case-control study are relevant for this master thesis. Prospective cohort studies follow a group of healthy people over time to observe their health and the factors they are exposed to (Thelle, 2015, pp. 112-114). It makes it possible to study exposures and detect potential health risks in the population. Birth cohorts follow pregnant women through pregnancy and birth and may also follow offspring further as in the prospective birth cohort study MoBa (Wilcox, 2010). Prospective pregnancy cohorts as MoBa gather information about pregnant women and their offspring and assess many possible exposures, mediators, effect modifying variables and outcomes (Magnus et al., 2016). A nested-case-control study is a case-control study conducted within a cohort study, and the participants are followed from before the outcome occurs as in a cohort study (Rothman et al., 2008). The participants with the studied disease are defined as cases and a selection of the rest of the participants of the cohort as controls.

Prospective cohort studies are avoiding some possible methodological bias by collecting the exposure information before disease occurs (Willett, 2013, pp. 6-7). Retrospective recall of exposure after disease can be a source of methodological bias in many case-control studies. But as this study is based on a nested case-control design the data on mothers and children were collected before disease.

Bias are systematic errors in estimates of studies (Rothman et al., 2008). The opposite of bias is validity and in cohort studies loss of subjects to follow-up may affect the validity of the study. Validity is divided in intern validity and extern validity or generalizability. The intern validity is classified as selection bias, information bias and confounding. External validity or also called generalizability is about the validity of the conclusion for the people in the general population. Selection bias are errors that comes from how the subjects are selected and how it can affect the ones that are participating in the study (Rothman et al., 2008). This type of bias can lead to differences between the participants and population that would have been eligible for participation. Information bias is about how the information is collected in a

study (Rothman et al., 2008). Errors in measurement of risk factors or disease can lead to bias in the measurement of the effect of exposure on disease. Measurement error, also called misclassification can be divided in differential misclassification and non-differential misclassification. Retrospective cohort studies may be influenced by recall bias as the participants report their history of exposure, but by initiating prospective cohort studies the information or measure on disease is collected at the same time as the study is conducted (Rothman et al., 2008).

A direct acyclic graph (DAG) shown in **Figure 1**, can be used as a tool in epidemiology to help visualize exposure and outcome with confounding and mediating factors (Rothman et al., 2008). A covariate can be defined as a confounder or a mediating factor. Confounders are associated with exposure and outcome and can thereby inflict the results (effect of exposure on outcome) if not adjusted for. This can be a misclassification by residual confounding, as it is common in epidemiologic studies that assess nutrients and disease. The confounder is not in the causal pathway to the disease and therefore important to include in analysis. Mediating factors are factors or variables that are in the causal path between exposure and outcome (Rothman et al., 2008). It is a factor that can come with time or after the exposure. The mediator can affect the outcome in the end but occurs along the way. If the interest is in the total effect of exposure it is not necessary to adjust for mediating factors, but when the aim is to look at the direct effect between exposure and outcome there is a need for adjustment (Rothman et al., 2008).

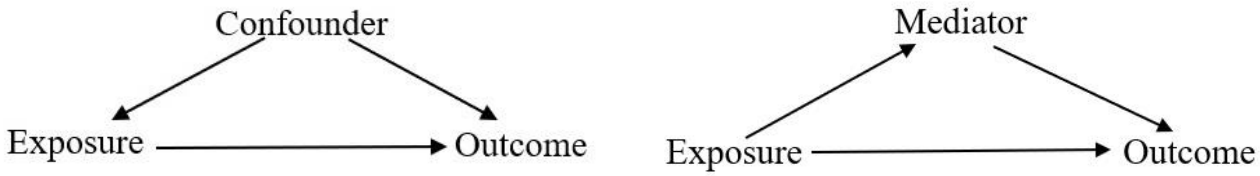

Figure 1. DAG of confounding and mediating factors

2.1.1 Biomarkers of fatty acid status

It is challenging to study dietary causes as the human diet is complex and because of many exposures and compositions that affect each other. (Willett, 2013, p. 71). Also, self-reported dietary intake may be inaccurate. Biomarkers can be used alone or provide important information in addition to dietary assessment methods, such as FFQs, diet records and 24-hour recall (Willett, 2013, p. 151). Biomarkers are objective measures used to measure dietary intake and nutrient status of individuals. The fatty acids that can't be synthesized endogenously in the body are the ones that are the best to measure dietary intake by

biomarkers (Willett, 2013, p. 182). This is the essential n-3 fatty acids and essential n-6 fatty acids, trans fatty acids and some saturated fatty acids. Whole blood, plasma and adipose tissue is some of the biological samples that are used to measure fatty acid concentrations. Biomarkers of fatty acids will give more accurate measurement on the actual amount of fatty acids as the metabolism and endogenous synthesis varies from one person to another (Willett, 2013, pp. 182, 191).

2.1.2 Composition of whole blood

The current study uses whole blood to assess fatty acid status during pregnancy. Whole blood consists of plasma, platelets, white blood cells and red blood cells as seen in **Figure 2** (Sand & Toverud, 2018, p. 364). The blood plasma is important for transport of many substances as plasma proteins, glucose, lipids, hormones, cells, and wastes. Plasma protein are transport molecules for substances that are not water soluble, as fatty acids or lipids. Lipoproteins are packages of lipid molecules that are wrapped in by proteins so that they are water soluble. Triglycerides are lipids from the diet and the fatty acid storage in the body. From food, triglycerides are transported from the intestine in form of chylomicrons. Chylomicrons are lipoproteins with triacylglycerols, phospholipids and proteins (Frayn, 2010). Phospholipids are found in red blood cells (erythrocytes), white blood cells (leucocytes) and platelets (Calder, 2018). Both measurements of fatty acids in plasma and whole blood has been shown to be correlating with reported intake of fatty acids (Willett, 2013, p. 186).

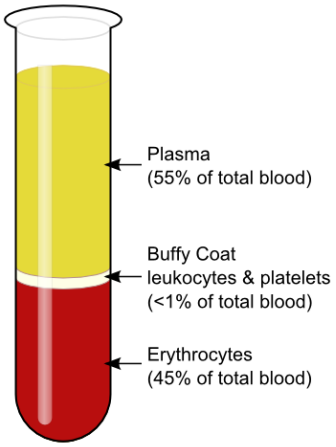

Figure 2. "Blood Composition", 2008 (By KnuteKnudsen). Retrieved from (https://commons.wikimedia.org/wiki/File:Blood-centrifugation-scheme.png). CC BY 3.0

2.2 Fatty acids

Fatty acids are essential for adequate nutrition, a great source for energy and important for human and fetal development (Wiktorowska-Owczarek, Berezinska, & Nowak, 2015). Regulation through eicosanoids and structural functions as phospholipids are also essential for good health and many studies are assessing the relation on dietary fatty acids and development of chronic diseases (Willett, 2013, p. 182). Current study assess fatty acid exposure and therefore relevant theory are presented to understand fatty acid composition and background theory.

2.2.1 Structure and classification

Fatty acids are carboxylic acid consisting of one methyl end, a hydrocarbon chain and a carboxyl terminus (Tvrzicka, Kremmyda, Stankova, & Zak, 2011). Fatty acids are divided in groups based on their structure and effect. They are classified as saturated fatty acids that contain no double bonds, mono-unsaturated fatty acids with one double bond and polyunsaturated fatty acids with multiple double bonds (Frayn, 2010). The structures of the fatty acids can be expressed with first the carbon number, number of double bonds and then the position of first double bond from methyl terminus (Tvrzicka et al., 2011). For the polyunsaturated fatty acids linoleic acid and α-linolenic acid, the structures are 18:2n-6 and 18:3n-3.

Fatty acids are amphipathic, that means they have a hydrophilic head and a hydrophobic tail (Wiktorowska-Owczarek et al., 2015). The largest part of long chain fatty acids are the hydrophobic part which are water insoluble and transported with a protein in the circulation (Ferrier, 2017, p. 181). Fatty acid esters as triacylglycerol, cholesteryl esters and phospholipids are more than 90% of the fatty acids found in plasma in the lipoproteins. Lipoproteins are particles consisting of chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Ferrier, 2017, p. 227). These are different particles with a variety of density and content but are built up by a core of triacylglycerol and cholesteryl esters with a hydrophilic layer of apolipoproteins, phospholipids and non-esterified cholesterol.

2.2.2 Essential fatty acids (re precursors of n-3 and n-6 fatty acids)

Saturated fatty acids can be synthesized endogenously in the body, but polyunsaturated fatty acids as the n-3 α-linolenic acid (18:3n-3) and n-6 linoleic acid (18:2n-6) are called essential

fatty acids and do not have the ability to synthesize endogenously (Frayn, 2010). The essential fatty acids must be provided by the diet. The two essential fatty acids linoleic acid and α-linolenic acid has different structures. Linoleic acid has 18 carbon atoms and two double bonds where the first one is located on the sixth carbon atom from the methyl end and are therefore termed n-6 (Tvrzicka et al., 2011). α-linolenic acid has also 18 carbon atoms but three double bonds where the first is on the third carbon atom and termed n-3 fatty acid. By further elongation and desaturation these essential fatty acids work as pre-cursors of the long chain polyunsaturated fatty acids (Tvrzicka et al., 2011).

2.2.3 Polyunsaturated fatty acids

Polyunsaturated fatty acids have multiple double bonds and are primarily the essential fatty acids n-3 and n-6 (Ferrier, 2017, p. 182). N-6 fatty acids have their first double bond located on the sixth carbon atom counting from the methyl end of the hydrocarbon chain while n-3 fatty acids have the first double bond located at the third carbon atom. The very long chain polyunsaturated fatty acids eicosapentaenoic acid (TEPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) are products of α-linolenic acid after conversion (Calder, 2012). In the diet α-linolenic acid (n-3) can be collected from seeds, plants and some nuts. Also soybeans, linseeds and blackcurrant seeds contain α-linolenic acid in small amounts (Calder, 2012; Tvrzicka et al., 2011). N-3 are most commonly found in oily fish as sardines, mackerel, trout, salmon, fresh tuna and herring, including fish oils (Tvrzicka et al., 2011). Marine n-3 are a greater source and can provide up to 2 gram of EPA and DHA per portion.

Linoleic acid (n-6) are found in high concentrations in soybean oil, sunflower seed oil, safflower oil, grape seed oil, poppy seed oil, borage seed oil and lower amounts can be collected from corn oil, walnut oil and sesame oil (Tvrzicka et al., 2011). During the last 50 years there has been a higher consumption of n-6 fatty acid linoleic acid from vegetable oils in western nations and make up to 80-90% of the polyunsaturated fatty acids, and the intake of n-3 fatty acids has declined compared with the 1900s (Innis, 2011).

2.2.4 Fat intake recommendations in pregnancy

Several studies are looking at intake and supplementation of fatty acids in pregnancy and 1 are included in discussion from previous studies. An overview of intake and supplementation is therefore presented in this chapter.

The Nordic Nutrition Recommendations (NNR) recommends pregnant women to include at least 5 E% from the essential fatty acid linoleic acid (n-6) and α-linolenic acid (n-3), including 1 E% from n-3 fatty acids where 200 mg/d comes from DHA (Nordic Council of Ministers, 2014, p. 236). According to NNR fish oil is cleansed of high doses of vitamin A and pollutants and is a source to the recommended intake of EPA and DHA, and vitamin D (Nordic Council of Ministers, 2014, p. 109). In pregnancy there is a higher recommended dose than for the general population as the PUFAs are important for fetal development (Guesnet, Marmonier, Boyer, & Delplanque, 2018). The FAOs recommendation for pregnant and lactating women are a minimum intake of 0,3 g/day of EPA and DHA where minimum 0,2 g/day is from DHA for the optimal fetal and adult health (Food and Agriculture Organization, 2010, p. 17). Both WHO/FAO and NNR share almost the same recommendations for fat intake.

Fish oil is considered as food in Norway and not as supplementation, but it is recommended because of the provision for the n-3 fatty acids EPA and DHA and vitamin D (Nordic Council of Ministers, 2014). The Norwegian Health Directorate recommends pregnant women to take 5 ml of fish oil or the same amount in fish oil capsules per day (Helsedirektoratet, 2017). In a sub-study from MoBa during 2002 and 2005 fish oil or cod liver oil are the most used or reported supplement (Haugen, Brantsaeter, Alexander, & Meltzer, 2008). Daily intake from diet and supplements among 40 108 women in MoBa reported median total n-3 fatty acids to 2.35 g/day, median EPA 0.21 g/day and median DHA 0.32 g/day.

2.2.5 Biosynthesis of long chain n-3 and n-6 fatty acids

Both α-linolenic acid and linoleic acid can convert to long chain polyunsaturated fatty acids (PUFA) by desaturation and elongation (Calder, 2012). When long chain fatty acids are desaturated, it means adding double bonds (Ferrier, 2017, p. 187). Elongation means adding two carbon units and by desaturation and elongation different PUFAs can be synthesized. α-linolenic acid converts first to stearidonic acid by Δ6-desaturase who further can convert to eicosatetraenoic acid (Calder, 2012). Δ5-desaturase can convert eicosatetraenoic acid to the long chain PUFAs EPA, DPA and DHA. The pathway from EPA to DHA is limited because of β-oxidation of DHA. β-oxidation is a process where the two-carbon units are removed (Ferrier, 2017, p. 190). Retro conversion can also have an impact on levels of DHA (Calder, 2012). DHA can be retro converted by β-oxidation to DPA and EPA (Arterburn, Hall, &

Oken, 2006). In the n-6 family arachidonic acid (AA) is the product of linoleic acid (Tvrzicka et al., 2011).

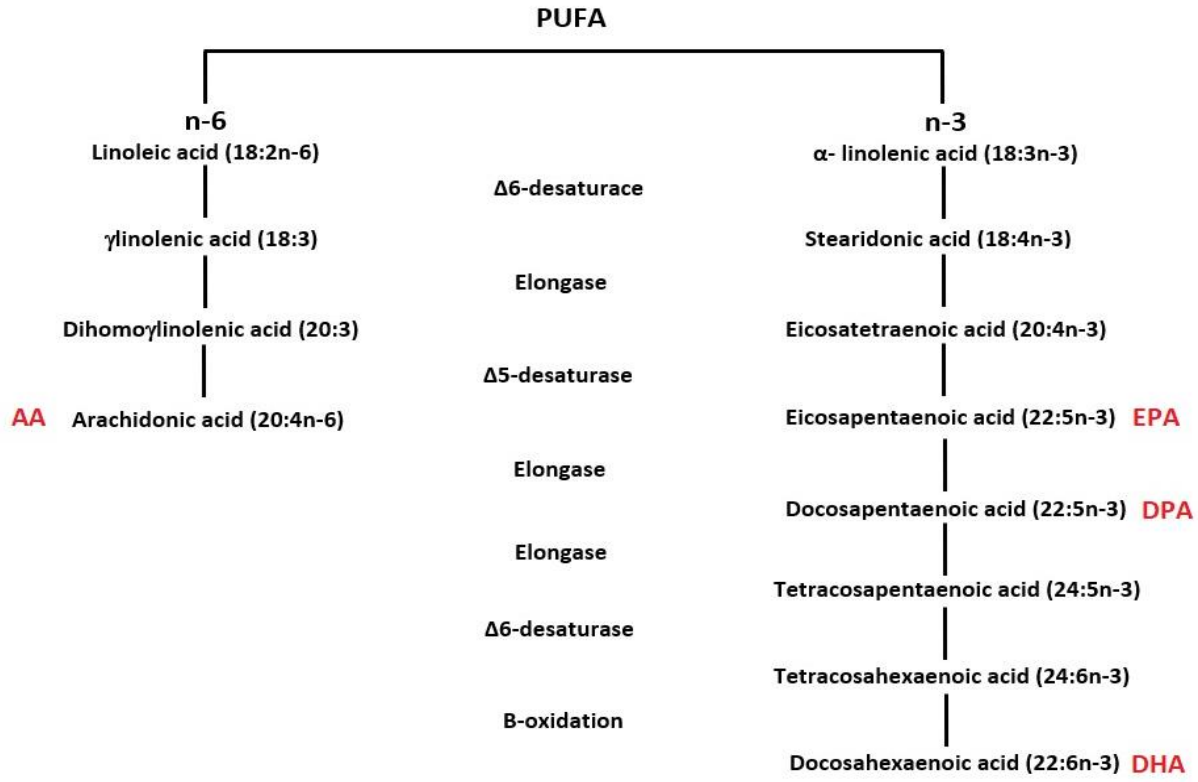

Figure 3. PUFA synthesis of linoleic acid and α-linolenic acid with enzyme path. Inspired by (Bishop et al., 2015) and (Burdge & Calder, 2005).

There is found differences between gender where desaturase activity has shown to be higher in women than in men (Lohner, Fekete, Marosvölgyi, & Decsi, 2013). The ability to synthesize long chain n-3 PUFAs α-linolenic acid to DHA are greater in women. Δ-6 desaturase has higher activity in women and give higher values of AA and DHA in plasma phospholipids. In erythrocyte lipids women had higher DHA than men. This may be due to the sex hormone estrogen. Fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS 2) have effects on the metabolism on PUFAs and the long chain PUFAs (Glaser, Lattka, Rzehak, Steer, & Koletzko, 2011). The Δ-5 and Δ-6 desaturase are products of the genes FADS1 and FADS2 and are important for the synthesis of long chain PUFAs. Variation in FADS1 and FADS2 can change the function of desaturase, and in maternal red blood cell phospholipids DHA is affected by FADS (Glaser et al., 2011). This can influence the provision of DHA to the child in pregnancy. Conversion of essential fatty acids to PUFAs in placenta is almost non existing because the placenta lack Δ-6 and Δ-5 desaturase (Kremmyda, Tvrzicka, Stankova, & Zak, 2011). It is therefore crucial for the fetus to get supplies of fatty acids through placenta, and this is dependent on maternal intake and availability of these fatty

acids. In the Westernized diet n-6 is dominating and therefore unfortunate because of the competition between the two PUFA families, n-6 and n-3. For the child's development and growth there is important to balance dietary n-6 and n-3 (Dutta-Roy, 2000; Guilleminault et al., 2017).

2.2.6 Metabolism and functions of n-3 and n-6 in pregnancy

After fatty acids are consumed some of the fatty acids are stored through metabolic pathways while other fatty acids are used right away (Hodson, Skeaff, & Fielding, 2008). After the consumption fatty acids, they become chylomicrons by the enterocytes and continues to circulate. The liver can take up remaining chylomicrons and the fatty acids from diet can be formed in to VLDL consisting of triacylglycerol, cholesteryl esters and phospholipids. Further fatty acids are distributed within- and between lipids in tissues and blood to provide for the whole body (Hodson et al., 2008). Long chain fatty acids known as PUFAs are transported in plasma bound to plasma protein albumin because they are insoluble in water. Triacylglycerols are transported in plasma as packages of lipoprotein (Frayn, 2010).

Fatty acids in pregnancy are very important for maternal and fetal health, and the supply of fatty acids through placenta are important for fetal development (Kremmyda et al., 2011). α-linolenic acid may increase during gestation as DHA synthesis have shown to be higher in women, possible because of the need of DHA in fetal development (Burdge & Calder, 2005). In placenta phospholipids account for 88% of the lipids with highest level of PUFAs, with highest concentration of AA followed by DHA and EPA (Kremmyda et al., 2011). Third trimester in pregnancy fatty acids stored in maternal adipose tissue are released to free fatty acids and diffused in to the bloodstream where the fatty acids can be transferred by the placenta to fetus (Kremmyda et al., 2011). Triacylglycerol will during the last time of pregnancy increase in plasma where LDL and HDL increases. Cholesterol ester transfer protein that control the exchange between VLDL, LDL and HDL increase its activity and may be the reason why LDL and HDL increases. This can make maternal fatty acids more available for the fetus. DHA and AA are very important for visual and cognitive development and also growth of the fetus, and may also affect gestation length and birth weight (Kremmyda et al., 2011).

At delivery maternal fatty acid status in cord blood plasma are different in lipid fractions. Umbilical cord plasma holds more AA and DHA which can be because of selective transfer of certain fatty acids from placenta to the fetus and in pregnancy maternal plasma

holds lower levels of AA and DHA and can be the explanation of the selective transfer to the fetus (Kremmyda et al., 2011).

2.2.7 Inflammation

Both n-6 and n-3 are synthesized by the same enzymes of desaturation and elongation and therefore there may be competition between these two PUFAs (Burdge & Calder, 2005). Because of the competition for the enzyme, the conversion of n-3 α-linolenic acid to EPA is thought to compete with the n-6 fatty acid AA (Calder, 2012). Because there is found more linoleic than α-linolenic acid in cells, conversion of n-6 PUFA may be better than n-3 PUFA. But if there is more EPA and DPA in the phospholipid cell membrane the amount of AA will decrease (Calder, 2018). Inflammation are an important and normal immune reaction in the body that works as a healing and protecting mechanism (Duvall, Bruggemann, & Levy, 2017). When the mechanism fails to turn off the acute inflammation it can become chronic and cause disease. This is the pathophysiology to many lung diseases, as asthma. The PUFAs AA, EPA and DHA are also known as substrates for eicosanoids that regulates inflammation (Calder, 2018).

AA converts to an inflammatory mediator but the products of EPA are different than AA by developing products that are anti-inflammatory (Wiktorowska-Owczarek et al., 2015). Resolvins are mediators that solve and reduce inflammation and are made by EPA and DHA (Calder, 2013). The anti-inflammatory resolvins the E-series are made by EPA and D-series are made by DHA. Animal studies have shown that the resolvins turned out to reduce inflammatory diseases as asthma (Calder, 2013). This may explain why n-3 have the anti-inflammatory capabilities.

PUFA has many functions and some of them is already mentioned (Calder, Kremmyda, Vlachava, Noakes, & Miles, 2010). These fatty acids also make sure there is a good environment for membrane protein function and regulate cell signaling. This can influence the function of immune cells that can affect development of atopy. The eicosanoids are derived from 20 carbon PUFAs, and connects PUFAs and immunological processes that are related to atopy. The immune cells have a high level of n-6 AA and therefore the largest foundation for making eicosanoids. There is also other PUFAs as n-3 but in lower amounts. The eicosanoids prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) originates from n-6 and n-3 PUFAs with 20 carbons (Ferrier, 2017, p. 213). The n-6 fatty acid AA contains 20 carbon atoms with four double bonds are the precursor for PG. AA is

incorporated in membrane phospholipid at carbon 2 that is released in response to different signals. 3 PG are derived from EPA the n-3 fatty acid that has 3 double bonds (Ferrier, 2017, p. 213). AA and EPA are precursors for several eicosanoids but AA and EPA produce eicosanoids with opposing effects (Kremmyda et al., 2011). PG has several tasks, but one of them is to stimulate inflammation. PG produced by AA is made in response to stress or injuries where PG from EPA is set to inhibit the response from AA and protect from inflammatory diseases. That is why fish oil is seen to have anti-inflammatory properties.

2.2.8 Phospholipids

In this current study phospholipids were isolated from frozen whole blood for the analysis of fatty acids. Fatty acids are an essential group of lipids and there are different types of classes divided in triacylglycerol, phospholipids, cholesteryl esters and non-esterified fatty acids (Tvrzicka et al., 2011). Lipids are hydrophobic esters molecules that floats in the blood as lipoproteins, that are composed by the cholesterol esters, triacylglycerols and phospholipids. That means they are water-insoluble and are often transported in packages of triacylglycerol in adipocytes or transported in the blood bound to proteins (Ferrier, 2017, p. 173). In cell membranes phospholipids are the most frequent lipids (Ferrier, 2017, p. 201). They have a hydrophilic head that the phosphate group and alcohol is attached to. The hydrophobic tail stores the fatty acids or fatty acids derived hydrocarbons. In the hydrophobic tail the nonpolar membranes as glycolipids, proteins and cholesterol are stored. The largest lipoprotein particles are the chylomicrons. In chylomicrons the triacylglycerols enters after a meal and will later be transported to tissues through enzyme lipoprotein lipase (Frayn, 2010). Phospholipids are important for all cell membranes and essential for fluidity and function (Tvrzicka et al., 2011).

Phospholipids are one of the lipid classes in lipoproteins (Hodson et al., 2008). Most of the synthesis of phospholipid takes place in the liver where other proteins and lipids form lipoproteins that are transported to the blood. HDL has stored most phospholipids of the lipoproteins. N-3 and n-6 are integrated in cell membranes and from phospholipid membranes they are released and form the base of eicosanoid synthesis (Wiktorowska-Owczarek et al., 2015). EPA and DHA are divided differently in phospholipids, as DHA are dominant in the phospholipids in/of brain and eyes. When there is more EPA and DHA in the phospholipid cell membrane the amount of n-6 fatty acids as AA will decrease (Calder 2017). This will also

decrease the production of eicosanoids from AA that are known to be inflammatory. It is therefore crucial that EPA and DHA are incorporated in phospholipids (Calder 2017).

2.2.9 Measurement of PUFAs and relative vs. absolute values

Biomarkers for fatty acids are most often presented in percentages of the sum of fatty acids. Relative values of fatty acids must be summed up to 100%. There have been debated to use absolute values of fatty acids instead because the values of each individual fatty acid are not independent as relative values (Song et al., 2016). Many factors can influence the biomarker concentrations as intake of other fatty acids, diet and lifestyle. Also, individual differences as metabolic and genetic factors are important factors that can affect the biomarker concentrations.

EPA and DHA are shown to be very low in adipose tissue, plasma free fatty acids and triglycerides but a much higher concentration in plasma total fatty acids and plasma, platelet and erythrocyte phospholipid fractions (Willett, 2013, p. 184). Studies on fatty acid composition in tissues or plasma indicate a variance in concentrations and how well they can reflect dietary intake. Fatty acids from diet have shown to incorporate to plasma triglycerides hours after a meal (Willett, 2013, p. 184). The use of plasma total fatty acids can be influenced by recent food consumption and complicate the findings of the results as triglycerides are included in total plasma (Willett, 2013, p. 186). When analyzing phospholipid fraction there is not that crucial to collect fasting samples as when triglyceride fractions are analyzed (Willett, 2013, pp. 190-191). EPA and DHA are shared differently between the phospholipid components (Calder, 2018). EPA incorporates more rapid than DHA but it has a lot to say which lipid pool it incorporates to, as white blood cells have shown to incorporate EPA and DHA faster than red blood cells. Biomarker measurement in adipose tissue are considered a gold standard to reflect intake but for the long-term diet (Baylin et al., 2005; Hodson et al., 2008). Phospholipids measured in whole blood are most likely to reflect the short-term intake where it reflects days or months.

2.3 Asthma

Asthma was defined by Global Initiative for Asthma (2020) report as a heterogeneous disease characterized by chronic inflammation of the airways. The symptoms of the condition can vary between wheeze, shortness of breath, chest tightness, cough and airflow obstruction.

In 2016 The Global Burden of Disease estimated that 339,4 million people were affected by asthma and one of the most important chronic respiratory diseases (Global Asthma Network, 2018). It is challenging to study asthma because of different definitions and diagnostic criteria (Global Initiative for Asthma, 2020). As a heterogeneous disease, asthma have various symptoms and varies from one person to another (Papi, Brightling, Pedersen, & Reddel, 2018). This also make it more difficult to fully diagnose. Asthma is a common chronic disease in early childhood and is seen earlier in boys than girls (Global Initiative for Asthma, 2019). Even though there are more boys than girls that has asthma in childhood there is a higher prevalence of asthma in women than men which can indicate a turn in puberty (Papi et al., 2018).

Chronic asthma is observed to develop in preschool age and many adults with chronic asthma started to have symptoms at 3 years (Bisgaard & Bønnelykke, 2010). In the review by Bisgaard and Bønnelykke they found that 80 to 90% of the asthma cases had developed asthma before 5 years of age. The symptoms often begin in early childhood but children younger than 5 years is difficult to fully diagnose (Global Initiative for Asthma, 2020). Wheeze can be an indication of asthma but at this young age group wheezing occurs among many children that does not necessarily get diagnosed with asthma when they get older. Diagnose on young children is based on their symptoms as repeated episodes of wheeze, cough, breathlessness by activity, and family history of asthma.

The International Study of Asthma and Allergies in Childhood (ISAAC) is a study of global prevalence and severity of asthma consisting of three phases, where the first phase conducted in 1993-1995 and the third phase was conducted in 2000-2003 (Lai et al., 2009). The study included children at the age 6-7 and 13-14 years globally. The prevalence in the group of 6 to 7 years old children included 61 countries with 388 811 participants and found that 4.9% of the children had symptoms of severe asthma with a wide range from around the world. The range were from 3.2% in Asia-Pacific and Northern- and Eastern Europe to 9.5% in Oceania. Highest prevalence was found in Latin America. The nationwide prescription study from 2005-2007 by Karlstad et al. (2010), indicated that 5.5% of the population in Norway in 2007 between the age 2-29 years had asthma based on prescription for asthma medications. 2 to 5-year-old males had highest increase in prevalence of asthma. In 2007 the prevalence was highest among 2-year-olds with prevalence on 13% for males and 9% of females. It indicated that the prevalence of asthma decreased almost by half by 10 years of age and continued to decrease to aged 20. Prevalence of asthma varies globally and children from 6 to 7 years old have high variation reported (Papi et al., 2018). In Indonesia 2.8% and

in Costa Rica 37.6%. Asthma prevalence may be decreasing or stabilizing in developed countries but increasing in developing countries possibly because of the trend of western diet. Still the prevalence in developed countries are high. It is an important factor that some developing countries may have a problem with underdiagnosis due to lack of resources (Papi et al., 2018).

There is no gold standard definition or diagnose of asthma and because of many different definitions it causes a variety in estimates of asthma prevalence and diagnose in studies (Sá-Sousa et al., 2014). Definition of asthma should be identified by combination of symptoms, clinical diagnose and tests, but in epidemiological studies it is most often not possible. This makes it difficult to diagnose asthma by questionnaires where misclassification can occur (Sá-Sousa et al., 2014).

Asthma in childhood are often connected with allergy, but not all children have asthma included with allergies (Henderson & Warner, 2012). There are found different phenotypes of asthma which means different clustered characteristics based on pathophysiology and demography (Global Initiative for Asthma, 2019). The most common phenotypes are allergic-asthma, non-allergic asthma, asthma with persistent airflow limitation and asthma with obesity. Allergic asthma is the most common in phenotype that originate in childhood. Asthma with persistent airflow limitation are remodeling of the airways and exposure to cigarette smoke are one of the risk factors for this phenotype. Children with this phenotype can be at risk for reduced growth in lung function and a higher risk for declined lung function later in life (Global Initiative for Asthma, 2019). Allergic asthma is caused by inflammation mediated by immunoglobulin E (IgE) mechanisms (Douwes & Pearce, 2014, pp. 2270-2271). The bronchial muscles and edema tighten in the airways and this will lead to symptoms as tightness in the chest, wheeze, coughing and short breath when exposed to allergens. Non-allergic asthma is still difficult to completely understand because of many complex mechanisms.

There are some known factors that can trigger asthma symptoms and exacerbation (Global Initiative for Asthma, 2019). Triggers as viral respiratory infections, allergen exposure, air pollution, smoking and tobacco exposure. For children under 5 years of age activity, laughing, crying, exposure to tobacco smoke and air pollution are characteristics that can trigger asthma exacerbation.

2.3.1 Risk factors for asthma

Asthma is as mentioned a heterogenous disease which is influenced by genes and environment (Global Initiative for Asthma, 2020). This can alter already in early life and even in utero. Factors from the environment as nutrition, allergens, pollution, microbes and physiological factors are important risk factors for development of asthma. For many years asthma were considered as an allergenic disease but today it is known that less than half of the cases are allergy related where the complex mechanisms involved is not yet well understood (Asher & Pearce, 2014; Global Asthma Network, 2018). There has been different hypothesis through the years. The hygiene hypothesis or also called microbial exposure hypothesis is about change in environmental- and lifestyle factors that have caused children to grow up in an environment with lack of natural burden to microbes that leads to under stimulation of the immune system, also called westernization (Ferrante & La Grutta, 2018; Henderson & Warner, 2012). Environmental factors as tobacco smoke is well known to increase the risk of asthma and especially in utero (Global Asthma Network, 2018). The interaction between environmental- and genetic factors are complex (Asher & Pearce, 2014).

There are debated on how to reduce asthma or prevent asthma prevalence in childhood, and how nutrition in intrauterine life may affect this development (Stratakis et al., 2017). Oily fish rich in the n-3 fatty acids are thought to decrease the risk because of the anti-inflammatory properties. Already in 1997 Black and Sharpe had a hypothesis about the connection between dietary fat and asthma because of an increasing prevalence of asthma among children in developed countries at that time (Black & Sharpe, 1997). At the same time there was an increase in consumption of vegetable oils and decrease in intake of oily fish. Other factors as lifestyle has changed over many years and many factors can be involved in these trends.

2.4 Developmental Origins of Health and Disease/ Early life hypothesis

According to the Developmental origins of health and disease (DOHaD) hypothesis, some chronic disease may originate during early life and development (Aris, Fleisch, & Oken, 2018). The original study by Barker on fetal growth and coronary disease in adult life researched factors that affected health later in life that originated in utero(Barker, 1995). This was where DOHaD hypothesis originated and Barker have done further research on this field. Later the hypothesis of poor prenatal nutrition in early life to various non-communicable

diseases (NCDs) later in life were developed by Barker (Suzuki, 2018). Over time the hypothesis has also included environmental factors that also can be connected to NCDs.

The hypothesis about fetal origin can be applied to development of asthma in childhood, where maternal exposure in fetal life can influence disease later in life (Sharma et al., 2014). Environmental factors can affect fetal development and programming as the development of fetus's lungs. Smoke exposure in utero are one factor that can cause reduced lung function at birth, and this can lead to a greater risk of disease later in life (Sharma et al., 2014). Asthma is a complex disease and may be developed by both environmental- and genetical interactions (Henderson & Warner, 2012). Early symptoms as wheezing can be a risk factor for later development of asthma.

Environmental factors and exposures that the mother are exposed to during pregnancy can be at risk for the fetus later in life (Kremmyda et al., 2011). During pregnancy the fetus is in a phase that is critical for development of organs. Exposures during this time can change health outcomes. Fatty acids are essential for maternal and fetal health during pregnancy (Kremmyda et al., 2011). In the third trimester fatty acids are transported to the fetus by placenta. There may be multiple ways PUFAs can be transported from mother to fetus. In placenta, receptors can retrieve lipoproteins and long chain PUFAs can as well be transported through uptake of triacylglycerols. PUFAs are important for cognitive and visual development of the fetus and during the last trimester in pregnancy the fetus have high levels of DHA in the membranes of cells in the brain and retina, nervous- and visual system (Kremmyda et al., 2011). It may also be important for the fetus development of immune functions. The research on DOHaD hypothesis is important to improve public health and insights on risk factors through the life course (Suzuki, 2018).

2.5 Previous studies

Previous research on this field are mostly on fatty acid supplementation and few studies are based on maternal fatty acid status. Today there are conflicting results and more research are needed. There are few studies that look at maternal fatty acid status. In this current study 6 articles from Cohort studies on maternal fatty acid status as exposure, where 1 of the included are an RCT, will be presented.

The study by Rosa et al. (2020) used data from the American cohort Conditions Affecting Neurocognitive Development and Learning in Early Childhood study (CANDLE) assessed the relationship between second-trimester n-3 and n-6 PUFAs and the n-6/n-3 PUFA

ratio and wheeze/asthma at 4 to 6 years. Maternal exposure was collected from plasma phospholipid specimen pool with unknown fasting sample at 16-27 weeks in pregnancy. Information on outcome was obtained by questionnaire at 4- to 6-year postnatal visit. The study found higher maternal plasma n-6 PUFAs to be associated with higher risk for asthma. They did not find a significant association between n-6/n-3 PUFA ratio and respiratory outcomes.

From the Avon Longitudinal Study of Parents and Children (ALSPAC) in England, Newson et al. (2004) published a study on umbilical cord and maternal blood red cell fatty acids and early childhood wheezing and eczema. Maternal blood sample were collected after pregnancy-week 20 and red blood cells membrane phospholipids were separated from the sample. Information on wheezing were collected by questioning mother when the child were 30 to 42 months. The study found it unlikely that exposure to n-6 and n-3 fatty acids in fetal life were determinants of early childhood wheezing and atopic disease.

A study of the KOALA Birth Cohort Study in Netherland by Notenboom et al. investigated the prenatal fatty acid exposures association with atopy in childhood. Blood samples of the mother were taken in week 34 to 36 to assess fatty acid status in plasma phospholipids. Wheeze were measured until child were 6-7 years old by questionnaire filled out by parents. The study found wheezing as the most frequently reported symptom in first year of life but declined with age. On childhood atopic outcomes the study found no association between maternal fatty acid status and development of asthma at age 6-7 years (Notenboom, Mommers, Jansen, Penders, & Thijs, 2011).

The Generation R Study in Netherland examines the association of maternal fatty acid levels during pregnancy with airway resistance and inflammation, asthma and eczema, in school-age children at 6 years. Maternal blood samples were collected in second trimester and analyzed in plasma glycerophospholipids. Childhood asthma were reported by parents in questionnaire at 6 years. The study observed that higher levels of maternal total PUFA and total n-6 PUFA levels during pregnancy were associated with decreased risk of asthma, but the underlying mechanisms need to be further explored (Rucci et al., 2016).

The Asian birth cohort study Growing Up in Singapore Towards healthy Outcomes (GUSTO) investigated the relationship between maternal PUFA status and potential offspring allergic diseases up to the age of 18 months. Maternal blood samples were collected in pregnancy week 26-28 where plasma phosphatidylcholine was separated. Information on wheezing was collected until 18 months of age by questionnaire in interview and by doctor

diagnose. The study did not find any significant protective effects of higher n-3 PUFA or lower n-6 PUFA on offspring allergic diseases in early childhood (Yu et al., 2015).

The Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) cohort study are a double-blind, randomized, controlled trial on supplementation by Bisgaard et al. (2016) in Denmark. The study assessed the effect on risk of persistent wheeze and asthma in offspring by n-3 long chain PUFA supplementation during the third trimester of pregnancy. In pregnancy week 24 the participating women were randomly assigned to receive 2.4 g/day of n-3 long chain PUFA with 55% EPA and 37% DHA in form of triacylglycerol, the placebo containing olive oil with 72% n-9 oleic acid and 12% n-6 linoleic acid. Maternal whole blood levels of EPA and DHA were collected at the randomization and 1 week after birth. Supplementation continued to 1 week after delivery. Information on child asthma were collected by clinical visits until 5 years of age. The asthma diagnose was termed persistent wheeze until the child reached 3 years and termed asthma thereafter. The study concluded that supplementation of n-3 long chain PUFA in third trimester reduced the absolute risk of persistent wheeze or asthma in offspring (Bisgaard et al., 2016).

3.0 Subjects and methods

The current study uses existing data from a previous nested case-control sub-study of the Norwegian Mother, Father and Child Cohort Study (MoBa). MoBa is a national population-based birth cohort study administered by the Norwegian Institute of Public Health. Recently MoBa changed name from The Norwegian Mother and Child Cohort study to The Norwegian Mother, Father and Child Cohort study to highlight the influence of the father and that data from father was also collected from the beginning. This master thesis is an epidemiological study with a quantitative approach analyzed as a nested case-control study. This method was chosen because of the epidemiological study design.

3.1 The Norwegian Mother, Father and Child Cohort Study

MoBa is a prospective birth cohort with questionnaire data and biological material from more than 100 000 pregnancies in Norway, collected from 1999 to 2008 (Rønningen et al., 2006). The recruitment ended in 2008, but the follow-up is still ongoing through questionnaires and population registry linkages. In addition to biological material, questionnaires in pregnancy

and offspring childhood are answered. MoBa is linked to the Medical Birth Registry of Norway (Magnus et al., 2016).

The aim of MoBa is to discover causes of complex diseases by studying exposures as genes and lifestyle among children and parents (Magnus et al., 2016). The exposure and outcome data are important to gain better insight of the children's health. MoBa see it as important to conduct more studies to prevent non-infectious diseases as they are a global burden today (Magnus et al., 2016).

3.2 Study sample

When the Norwegian Institute of Public Health started the MoBa study all women who was to give birth at one of the 50 included hospitals in Norway could join, but the participants had to be able to read Norwegian because the questionnaires and information were only available in Norwegian (Magnus et al., 2016). Invitations were sent by mail to pregnant women before the first routine ultrasound examination. Invitations were sent to 277 702 pregnant women with a participation rate of 41%. Recruitment took place at 50 out of 52 hospitals in Norway (Magnus et al., 2016). Biological material was collected from mother in approximately pregnancy week 18 during the first ultrasound appointment, and when giving birth at the hospital (Rønningen et al., 2006).

The current study is based on a previous case-control study of maternal folate status in pregnancy and child asthma at 36 months nested within the MoBa cohort, seen in flow chart **Figure 4** (Håberg et al., 2011). Children eligible for the case-control study were born in 2002-2004 and had complete follow-up information to age 36 months. A sub-sample of the case-control study had data on newborn DNA methylation (Joubert et al., 2012) and maternal fatty acid status was measured in the same sub-sample. The present study includes 986 mother-child pairs of singleton births with available maternal whole blood. The participants are subsample from a previous nested case-control study of maternal folate levels in pregnancy and asthma in children at age three years (Håberg et al., 2011), and a meta-analysis on maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns (Joubert et al., 2012).

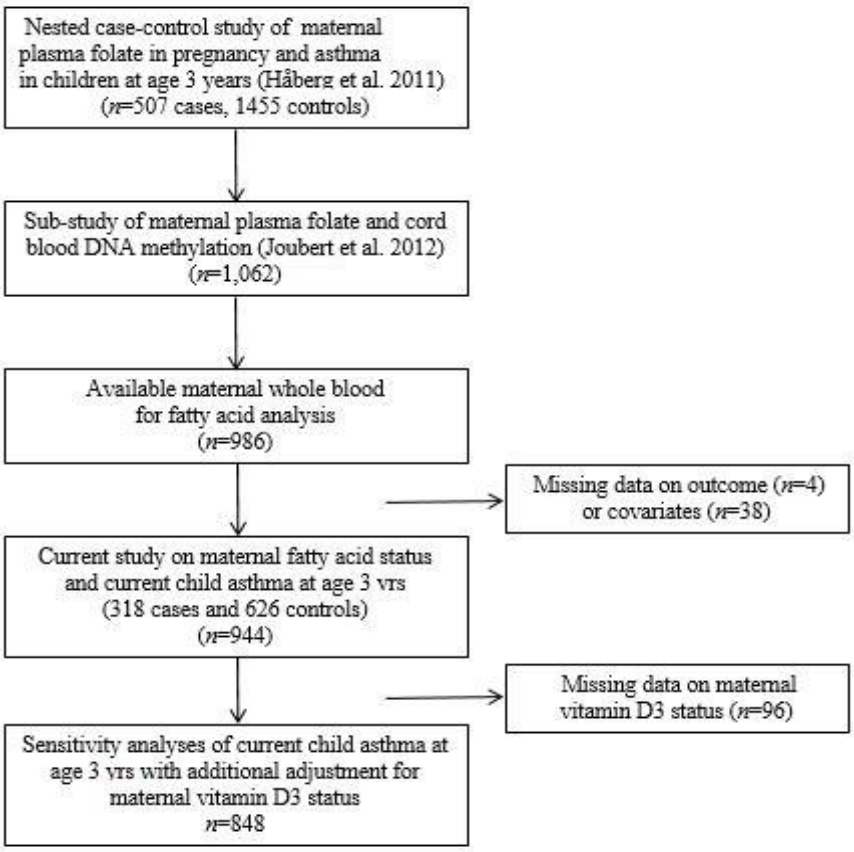

Figure 4. Flow chart to illustrate study sample selection.

Whole blood from mothers had to be available in the MoBa biobank in order to measure fatty acids, therefore less than 1062 mothers were available from Joubert (n=986). Available follow-up questionnaire on the child at 36 months were also a requirement to be included in this study. Missing data on outcome and covariates results in data on 944 participants.

3.3 MoBa questionnaire data

The first questionnaire was received in pregnancy week 13-17 where the questions asked about health history before and health during pregnancy, see appendix 1 (Magnus et al., 2006). In week 22 a food frequency questionnaire (FFQ) was sent with questions about diet. A new questionnaire was sent out in pregnancy week 30 with questions about health status during pregnancy and lifestyle. Six months after birth the questionnaire asked questions regarding the child's health and nutrition. New questionnaires at 18 and 36 months after birth asked about the child's development and health. The MoBa cohort is still ongoing as new questionnaires are continuing to be sent out as the children are aging (Magnus et al., 2016).

One of the questionnaires used in the study is administered when the child was 36 months (Appendix 3).

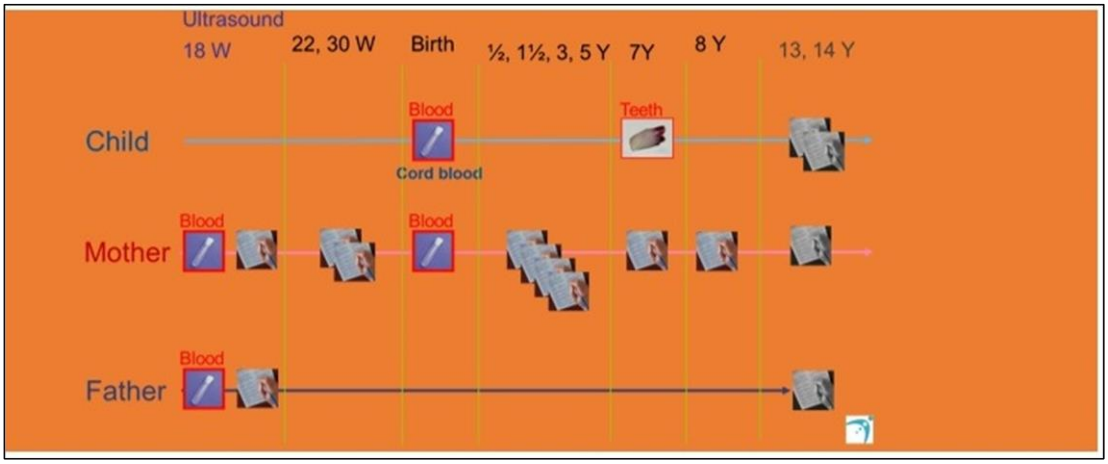

Figure 5. Collection of data (blood, cord blood, teeth, questionnaire) in the MoBa study from enrollment of study until the child have turned 14 years. With permission. Source: Norwegian Institute of Public Health

3.4 Current child asthma at age 36 months (outcome)

Child asthma at age 36 months was based on the questionnaire administered at 36 months after birth. Asthma were registered by maternal questionnaire report when the child was 36 months. The question was: has your child suffered any long-term illness or health problems since the age of 18 months? One of the health problem alternatives was asthma and the choice of answers was “no”, “yes, has now” and “yes, had previously”. An additional question: If so, has the child been referred to a specialist? with the answers “yes” and “no”. The next question was: Has your child taken any medication during the last 12 months? If the answer were “yes” a new question asked after name on medication and over how long time it has been used. Current asthma at 36 months was defined by mothers reply “yes, has now” to child’s current asthma and reported use of asthma medication the last 12 months.

3.5 Maternal fatty acid status in pregnancy (exposure)

Non-fasting blood samples were collected one time during pregnancy, in approximately median gestation week 18 (Rønningen et al., 2006). The samples were drawn in two EDTA-

tubes where one of the tubes is spun to separate plasma before shipping to the MoBa biobank, leaving one tube with whole blood and one with plasma. Most samples were received at the biobank the day after blood samples were taken. Whole blood and plasma are stored at -80 ºC at the Biobank (Rønningen et al., 2006). The phospholipid fraction of the whole blood samples was extracted at VITAS laboratory. The concentration of a panel of 18 fatty acids was measured at VITAS laboratory by gas chromatography - flame ionization detection method (GC-FID). See appendix 4. The fatty acids were measured in both relative (%) and absolute (nmol/L) concentrations.

3.6 Covariates

To identify and get an overview of confounders we used a direct acyclic graph, **figure 6** (look at confounder and mediating factors). In this current study the exposure is maternal fatty acid status that were measured in median gestation week 18. The outcome is childhood asthma at 36 moths and adjusted for confounding factors from maternal characteristics.

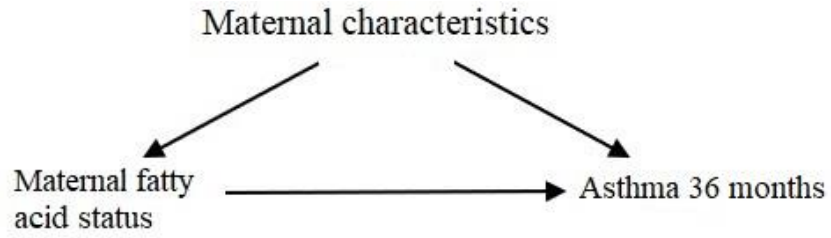

Figure 6. DAG of exposure, outcome and confounding in current study

Covariates included in this study can be potential confounding factors with asthma and were chosen before the analyses based on previous study (Håberg et al., 2011). The covariates included maternal age that is based on records in Medical birth registry. Education level reported by mother in first questionnaire were included as a measure of socioeconomic status which may be an important factor. Maternal pre-pregnancy body mass index (BMI) was calculated from height and weight reported in the first questionnaire by mother, and as it is found to be an important risk factor for asthma, we included it as a covariate. Parity was based on Medical birth registry where the mother's previous births are registered. Smoking during pregnancy were reported in first questionnaire by mother, where it is possible to get prospective over time assuming the mother report the actual smoking habit. Smoking are well

established factor for asthma and therefore important to include as covariate. Cotinine levels measured from plasma is a short measure of tobacco smoke exposure from smoking or passive smoking. Maternal allergies were reported in first questionnaire on: hay fever, animal hair allergy, atopic dermatitis and other. Mothers allergies may be hereditary and can pass on to the child. Asthma reported by mother in first questionnaire was included to control for genetic predisposition to asthma as it may be hereditary. Addition in sensitivity analysis, vitamin D and folate measured in plasma based on results in previous studies. Vitamin D and folate are both thought to reduce asthma (Håberg et al., 2011).

3.6.1 Laboratory measurements

Plasma vitamin D, plasma folate and plasma cotinine were available from previous studies in MoBa. Maternal plasma 25-hydroxyvitamin D3, folate and cotinine were measured at Bevital AS laboratories in Bergen, Norway (www.bevital.no). The method used to analyze 25 hydroxyvitamin D3 were by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Maternal plasma folate and plasma cotinine were measured around 18 gestational weeks by microbiological assay, *Lactobacillus casei* (O'Broin & Kelleher, 1992), and LC-MS/MS (Midttun, Hustad, & Ueland, 2009), respectively, at Bevital AS laboratories in Bergen, Norway (www.bevital.no). Cotinine reflects smoking exposure from the last three to five days and has a half-life of 15 to 40 hours, but is shorter during pregnancy (Dempsey, Jacob, & Benowitz, 2002) and maternal cotinine levels during pregnancy was categorized as previously in MoBa (Joubert et al., 2012) undetectable ≤0 nmol/L, low >0-56.8 nmol/L, moderate >56.8-388 nmol/L, high >388 nmol/L. Levels on 56.8 nmol/L was indicated as active maternal smoking.

3.6.2 Covariates from Medical birth registry

Maternal age at delivery was retrieved from the Medical birth registry as continuous variable. Parity retrieved from Medical birth registry are categorized after number of pregnancies as first-time delivery, 1, 2, 3 or more.

3.6.3 Covariates from questionnaire

Education level was reported in maternal questionnaire where the variable is categorized in <high school, high school, some college, ≥4 years of college/university. Maternal pre-pregnancy body mass index (BMI) calculated from height and weight reported in maternal questionnaire and categorized as <18,5 low, 18,5-24,9 normal, 25-29,9 high, ≥30 obese. Smoking during pregnancy reported in maternal questionnaire categorized as no, stopped and sometimes/daily. Maternal allergies reported in questionnaire categorized as hay fever, animal hair allergy, atopic dermatitis and other. Maternal asthma reported in questionnaire and categorized as yes, no.

3.7 Statistical analyses

The statistical analyses were conducted in STATA version 16 SE with remote access to the data which were stored at the Norwegian Institute of Public Health. The data material was available in a remote access at The Norwegian Institute of Public Health.

The analyses included 944 of 986 singleton births after exclusion of 42 mother-pairs due to missing data on maternal covariates and child asthma (**Figure 4**). Descriptive analyses were performed to assess if fatty acid variables were normally distributed and to get an overview of differences between cases and controls. Categorical variables for maternal characteristics distributed (age, parity, education, BMI, smoking, cotinine level, asthma status, cod liver oil use and folate supplement use) are presented as % in cases and controls. Continuous variables for maternal characteristics (plasma vitamin D and folate) are given as median (25^{th}, 75^{th} percentile). Chi square test were used to test the associations between maternal characteristics and asthma at 36 months for categorical characteristics, and Wilcoxon rank-sum (Mann-Whitney) test for maternal continuous variables for plasma biomarkers. Categorical variables for characteristics of the children (sex, birth weight and premature birth) are presented in % and chi square test were used to test the association with asthma. The data were analyzed as a case control study, therefore all covariates are presented by case-control status. The missing values were not included in the analyses as seen in flow chart **figure 4** (missing, outcome, covariates, Vitamin.D3).

Some variables had to be re-coded for the analyses and some new variables were created to be able to conduct the analyses needed to the current study, from MoBa data. Exposure values are the fatty acids EPA, DPA, DHA, AA in relative (%) and absolute (nmol/L) values, and AA:EPA ratio. The individual n-3 fatty acids EPA, DPA and DHA were

added together to get the variable sum n-3 for relative and absolute values. Variable for AA:EPA ratio was created by dividing relative AA on EPA. All fatty acid exposure variables EPA, DPA, DHA, AA and AA:EPA ratio were divided into quartiles where the cut offs were based on the asthma control group. Then the cases were categorized according to the same cut offs.

The differences in maternal fatty acid concentration by child asthma were analyzed with Wilcoxon rank-sum (Mann Whitney) test to calculate the association between the fatty acids and asthma. They were divided in cases and controls, and in absolute and relative concentrations (**Table 3**).

The data had an underlying nested case-control design and multivariable logistic regression models were used to calculate adjusted odds ratios (ORs) with 95% confidence interval (CI) for the relative risk for asthma by mother's fatty acid status. Three different models were used: A crude/unadjusted OR model where each fatty acid was individually analyzed by asthma at 36 months. The first model adjusted OR (adj1) was for each fatty acid where odds ratio is adjusted for potential confounders. The second model adjusted OR (adj2) is additionally adjusted for (maternal education level, maternal age, parity, maternal BMI, maternal smoking in pregnancy week 17, maternal allergy, maternal asthma and plasma folate) covariates from questionnaire, birth registry and plasma folate.

Additional analysis of sensitivity analyses was done to see what happens when vitamin D status were adjusted for in the regression analyses. Adjusted for vitamin D in a smaller sample with available data. The sensitivity analysis was performed by multivariable logistic regression analyses with vitamin D n=848 for relative and absolute concentrations, with one crude/unadjusted model. The first adjustment was for all individual fatty acids, the second adjustment included confounding variables, and the third additionally adjusted for vitamin D and folate.

The Spearman correlation coefficient (**Table 4 and Table 7)** was used to measure the correlation between first the individual fatty acids, and second between fatty acids and the potential mediating variables birth weight and gestational week. The variable for smoking reported in questionnaire by mother in approximately week 18 were used in the regression test, as the distribution of the variable were the same as the variable for measured cotinine. Two-way scatter plot (**Figure 7**) was performed to examine the correlation between relative and absolute values of the fatty acids. A p-value <0.05 were considered statistically significant for all tests.

3.8 Ethical considerations

To be a participant in the study MoBa mothers had to give written informed consent (Magnus et al., 2006). This states that MoBa can use biological material in the study and access information in health registries in relation to the content of the study. The study is voluntary, and participants can quit the study at any time and if requested all data and information can be deleted (Magnus et al., 2006). The data is anonymous where all pregnancies get an identification number (Magnus et al., 2006).

The MoBa study has been approved by the Norwegian Data Protection Authority (ref. 01/4325) and the Regional Committee for Medical Research Ethics (ref. S-97045, S-95). The current MoBa study was approved by the Regional Committee for Medical Research Ethics of South/East Norway.

3.8.1 Funding

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4.0 Results

4.1 Study population

Table 1 shows background characteristics of the 944 participating mothers presented by the case-control status of the children at age 36 months. Maternal age at delivery indicated that case mothers was somewhat younger than the control mothers, but no significant difference in age overall (p=0.08). The control mothers showed highest percent at 30-34 years old (40.9%) and the case mothers 25-29 years old (39.0%) at delivery. Regarding maternal education, a high proportion of both control mothers (42.7%) and case mothers (49.1%) had some college, but not statistically significant in education overall (p=0.29). Maternal pre-pregnancy BMI shows that over the half of the women had a normal weight in both controls (67.1%) and cases (63.2%). With a BMI <18.5 there was almost no difference between case mothers and control mothers (4.1%) vs. (2.9%) and overall, not statistically significant (p=0.59).

Most of the mothers reported no smoking in pregnancy with 71.1% in controls and 73.3% in cases, which was quite consistent with measured cotinine levels below detection in 70.3% of controls and 70.1% in cases. Reported smoking sometimes/daily was higher in control group (12.9%) than in case group (8.5%). Cotinine levels also showed a significant p-value at 0.02. Cod liver oil supplement use was very similar in cases and controls with 41.2% and 40.9% respectively. Supplement of folate were highest in the case group with 79.9% and 72.2% in the control group and had a significant p-value on 0.02. Maternal current asthma shows a significant association with child asthma (<0.001) where mothers with child diagnosed with asthma was 11% and mothers with current asthma with non-asthmatic child was 3%. Plasma measure of folate status was similar in cases (median 9.5) and in controls (median 9.4), p=0.24. There was statistically higher median in plasma vitamin D in control group (75.2) than in case group (68.8) with p-value 0.01.

Table 1. Maternal background characteristics by child asthma status at age 36 months (n=944)

Characteristic	n	Controls n=626 %	Cases n=318 %	p-value[1]
Maternal age at delivery, yrs.				0.08
<25	100	10.1	11.6	
25-29	329	32.7	39.0	
30-34	377	40.9	38.1	
≥35	138	16.3	11.3	
Parity				0.06
First time delivery	384	40.6	40.9	
1	384	38.5	45.0	
2	123	14.7	9.7	
3 or more	53	6.2	4.4	
Education				0.29
<High school	67	7.5	6.3	
High school	304	33.7	29.2	
Some college	423	42.7	49.1	
≥4 years of college/univ	150	16.1	15.4	
Maternal BMI (pre-pregnancy), kg/m^2				0.59
<18.5	31	2.9	4.1	
18.5-24.9	621	67.1	63.2	
25-29.9	197	20.3	22.0	
≥30	95	9.7	10.7	

Characteristic	n	Controls n=626 %	Cases n=318 %	p-value[1]
Reported smoking in pregnancy				0.11
No	678	71.1	73.3	
Stopped	158	16.0	18.2	
Sometimes/daily	108	12.9	8.5	
Cotinine level in pregnancy, nmol/L				0.02
Below detection	663	70.3	70.1	
>0-56.8	168	15.8	21.7	
>56.8-388	58	7.2	4.1	
>388	55	6.7	4.1	
Current asthma, reported				<0.001
Yes	54	3.0	11.0	
No	890	97.0	89.0	
Cod liver oil, reported bef. blood sample				0.92
Yes	388	41.2	40.9	
No	556	58.8	59.1	
Folate, reported bef. blood sample				0.02
Yes	709	72.7	79.9	
No	235	27.3	20.1	
		Median (p25, p75)	Median (p25, p75)	p-value[2]
Plasma folate, nmol/L	944	9.4 (6.1, 16.2)	9.5 (6.9, 17.2)	0.24
Plasma vit. D, nmol/L	848	75.2 (56.1, 89.4)	68.8(51.3, 86.5)	0.01

[1] p-value calculated by chi-square test for categorical variables.

[2] p-value for Mann-Whitney two sample test

Table 2 shows the background characteristics of the 944 children at birth divided in cases and controls. There were significantly higher male cases (65.4%) than female cases (34.6%). There was a higher proportion of low birthweight children in cases (3.8%) compared with controls (1.3%) with an overall p-value of 0.04. More premature births in case group with 5% and 3% in control group.

Table 2. Characteristics of children by asthma status at age 36 months (n=944)

Characteristics	n	Controls n=626 %	Cases n=318 %	p-value[1]
Sex				<0.001
Female	432	51.4	34.6	
Male	512	48.6	65.4	
Birth weight, gram				0.04
Low (≤2500)	20	1.3	3.8	
Normal (2500-4500)	876	93.6	91.2	
High (≥4500)	48	5.1	5.0	
Premature birth, gest. wks.				0.13
Yes (≤36)	35	3.0	5.0	
No (≥37)	905	97.0	95.0	
Missing	4	<0.01	<0.01	

[1] p-value calculated chi-square by asthma

4.2 Maternal fatty acid concentrations by child's asthma at 36 months

In **table 3**, when measured on a relative scale, the concentration of all the n-3 fatty acids (sum n-3, EPA, DPA, DHA) was higher in the control group than the case group, but the difference was only borderline statistically significant for EPA (p=0.06). Sum n-3 had a significant higher concentration in controls (p=0.001), DPA a significant p-value on 0.003 and DHA significant p-value on 0.001. The n-6 fatty acid AA and the ratio AA:EPA were higher in the case group where median AA was 7.69 in control and 7.76 in case but not significant (p=0.34). AA:EPA do not have a significant p-value (p=0.5) where median was 11.76 in control group and 12.74 in case group.

For the absolute fatty acid concentrations, all statistically significant differences in n-3 in relative concentrations were lost. There was a higher median concentrations of n-6 fatty acid AA in the case group. AA shows median 71.22 for control and 75.32 for case with p-value 0.01. The n-3 fatty acids were higher in the control group for sum n-3, DPA and DHA but EPA had a higher median in the case group but not statistically significant. Total phospholipids were significantly higher in case group (p=0.003). DHA and AA was the specific fatty acids with highest concentrations in both relative and absolute. P values were calculated by Wilcoxon rank-sum test.

Table 3. Maternal whole blood relative and absolute fatty acid concentrations in pregnancy by child asthma status at age 36 months (n=944)

	Control n=626 (median, Q1-Q3)	Case n=318 (median, Q1-Q3)	p-value[1]
Relative (%)			
Sum n-3	6.77 (5.67-8.11)	6.38 (5.48-7.62)	0.001
EPA	0.65 (0.45-1.04)	0.61 (0.42-0.88)	0.06
DPA	0.97 (0.85-1.12)	0.93 (0.81-1.08)	0.003
DHA	5.10 (4.33-5.98)	4.85 (4.08-5.66)	0.001
AA	7.69 (6.94-8.50)	7.76 (7.02-8.58)	0.34
AA:EPA ratio	11.76 (7.12-18.13)	12.74 (8.14-19.02)	0.05
Absolute (nmol/L)			
Sum n-3	64.77 (51.86-79.48)	61.96 (51.65-80.08)	0.41
EPA	6.20 (4.17-9.98)	6.23 (4.07-8.97)	0.36
DPA	9.28 (7.70-10.90)	9.22 (7.57-10.76)	0.65
DHA	47.82 (38.98-58.93)	46.82 (38.08-58.63)	0.44
AA	71.22 (61.98-83.96)	75.32 (64.51-87.10)	0.01
Tot phospholipids	947.49 (856.29-1033.85)	965.11 (885.79-1080.82)	0.003

[1] Wilcoxon rank-sum test

4.3 Correlations between fatty acids

Figure 7 of the scatter plot indicated a positive linear relationship. But with higher concentration the plots are little more spread and low concentrations was more linear. Correlation coefficients (Spearman) for presented scatter plot shows sum n-3 relative and absolute with correlation 0.87. EPA relative and absolute show correlation 0.97. DPA relative and absolute show correlation 0.81. DHA relative and absolute show correlation 0.86.

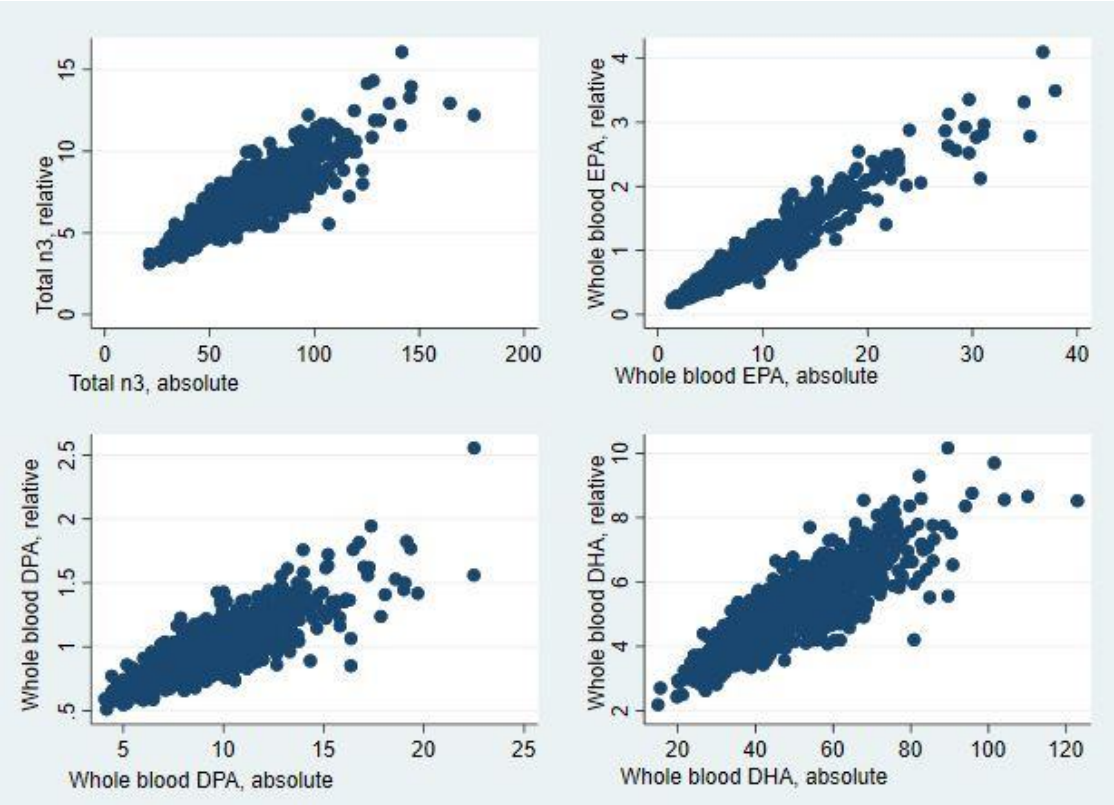

Figure 7. Scatter plot of absolute versus relative fatty acid concentrations (n=944). Correlation: total n-3 0.87, EPA 0.97, DPA 0.81, DHA 0.86.

Table 4 show the correlation between the long chain PUFAs included in this study. The correlation shows various correlation coefficient for the relative and absolute concentrations. Relative EPA shows a positive correlation to DHA and DPA, and DPA a positive correlation to DHA. Relative EPA show a negative correlation to AA (-0.23). DPA and DHA show a weak negative correlation to AA, and DPA and DHA also show a negative correlation to AA:EPA. For the absolute values, EPA shows a positive correlation to DPA and DHA. DPA also shows a positive correlation to DHA. EPA shows close to zero correlation to AA, but DPA and DHA show a weak positive correlation to AA. DPA and DHA show a negative correlation to AA:EPA.

Table 4. Spearman correlation matrix for relative and for absolute concentrations of maternal fatty acid status (n=944)

	Relative (%)					Absolute (nmol/L)			
	EPA	DPA	DHA	AA		EPA	DPA	DHA	AA
EPA	1.00				EPA	1.00			
DPA	0.68	1.00			DPA	0.72	1.00		
DHA	0.76	0.67	1.00		DHA	0.76	0.81	1.00	
AA	-0.23	-0.11	-0.07	1.00	AA	0.10	0.39	0.43	1.00
AA:EPA		-0.64	-0.71		AA:EPA		-0.54	-0.57	

4.4 Logistic regression analysis for risk of asthma

P-values are not shown in **table 5** and **table 6**. **Table 5** shows the results from the multivariable logistic regression analysis of childhood asthma risk in relation to maternal fatty acid status in relative concentrations before and after adjustment for confounding factors. Sum n-3 show 42% reduced risk for asthma in the highest quartile (Q4) in crude/unadjusted (OR 0.58, 95% CI 0.39-0.86, p=0.006) compared with the reference quartile. Also when adjusted for all fatty acids (adj.1) the model show 42% reduced risk for asthma in highest quartile (OR 0.58, 95% CI 0.39-0.87, p=0.008), and when adjusted for potential confounding factors (adj.2) the model show 40% reduced risk for asthma (OR 0.60, 95% CI 0.40-0.91, p=0.017) in the second highest quartile (Q3). In highest quartile (Q4) there was 53% reduced risk for asthma (OR 0.47, 95% CI 0.30-0.74, p=0.001) when all confounding factors were controlled for. For EPA adjustment for other fatty acids had a large impact, there was 80% increased risk for asthma (OR 1.80, 95% CI 1.11-2.92, p=0.018) in the third quartile (adj1), but the effect was weaker after adjustment for other maternal factors (adj2) and there was no significantly increased risk in the upper quartile for any of the models.

DPA show 35% reduced risk for asthma (0.65, 95% CI 0.45-0.95, p=0.027) when unadjusted in the second highest quartile, and 41% reduced risk for asthma (OR 0.59, 95% CI 0.40-0.87, p=0.008) in highest quartile. When adjusted with other fatty acids and potential confounding factors (adj.2) the second highest quartile show 41% reduced risk for asthma (OR 0.59, 95% CI 0.37-0.93, p=0.024).

For DHA in crude/unadjusted the model shows 35% reduced risk for asthma (OR 0.65, 95% CI 0.45-0.96, p=0.029) in the second highest quartile, the highest quartile shows

41% reduced risk for asthma (OR 0.59, 95% CI 0.40-0.87, p=0.008). When adjusted for all fatty acids (adj.1) the model shows 42% reduced risk for asthma (OR 0.58, 95% CI 0.35-0.94, p=0.028) in second highest quartile, and in highest quartile 46% reduced risk for asthma (OR 0.54, 95% CI 0.31-0.95, p=0.033). When adjusted for fatty acids and potential confounding factors (adj.2) the model showed 45% reduced risk for asthma (OR 0.55, 95% CI 0.33-0.91, p=0.021) and highest quartile show 50% reduced risk for asthma (OR 0.50, 95% CI 0.28-0.90, p=0.020). AA:EPA ratio show an increased risk for asthma (OR 1.52, 95% CI 1.02-2.25, p=0.04) when not adjusted. AA show no significant reduced or increased risk.

Table 5. Odds ratio for child asthma at age 36 months in relation to categories[1] of maternal whole blood phospholipid fatty acid status as relative concentration (%), n=944

Sum-n3 (%)	Crude OR	(95% CI)	OR adj.1[2]	(95% CI)	OR adj.2[3]	(95% CI)
<5.67	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
5.67-6.77	0.93	(0.65-1.34)	0.92	(0.64-1.33)	0.88	(0.60-1.29)
6.78-8.11	0.72	(0.50-1.06)	0.72	(0.49-1.05)	**0.60**	**(0.40-0.91)**
≥8.12	**0.58**	**(0.39-0.86)**	**0.58**	**(0.39-0.87)**	**0.47**	**(0.30-0.74)**
EPA (%)						
<0.45	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
0.45-0.65	0.91	(0.63-1.33)	1.18	(0.78-1.79)	1.14	(0.74-1.76)
0.66-1.03	1.05	(0.72-1.51)	**1.80**	**(1.11-2.92)**	1.64	(0.99-2.72)
≥1.04	0.69	(0.46-1.02)	1.48	(0.81-2.69)	1.41	(0.75-2.65)
DPA (%)						
<0.85	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
0.85-0.97	0.80	(0.56-1.15)	0.81	(0.55-1.20)	0.79	(0.53-1.19)
0.98-1.12	**0.65**	**(0.45-0.95)**	0.67	(0.43-1.04)	**0.59**	**(0.37-0.93)**
≥1.13	**0.59**	**(0.40-0.87)**	0.67	(0.40-1.12)	0.61	(0.36-1.05)
DHA (%)						
<4.33	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
4.33-5.10	0.92	(0.64-1.31)	0.81	(0.54-1.22)	0.80	(0.52-1.23)
5.11-5.98	**0.65**	**(0.45-0.96)**	**0.58**	**(0.35-0.94)**	**0.55**	**(0.33-0.91)**
≥5.99	**0.59**	**(0.40-0.87)**	**0.54**	**(0.31-0.95)**	**0.50**	**(0.28-0.90)**
AA (%)						
<6.94	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
6.94-7.69	1.06	(0.72-1.56)	1.07	(0.72-1.58)	1.12	(0.74-1.68)
7.70-8.50	1.00	(0.68-1.48)	1.01	(0.68-1.51)	1.05	(0.69-1.58)
≥8.51	1.19	(0.82-1.74)	1.23	(0.83-1.84)	1.23	(0.81-1.88)
AA:EPA (%)						
<7.12	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
7.12-11.76	1.43	(0.96-2.13)	1.20	(0.78-1.87)	1.24	(0.78-1.96)
11.77-18.13	1.42	(0.95-2.12)	0.99	(0.60-1.63)	1.09	(0.65-1.84)
≥18.14	**1.52**	**(1.02-2.25)**	0.90	(0.52-1.57)	0.99	(0.56-1.78)

[1] The cut-off values corresponds to the quartile cut-points among controls.

[2] All single fatty acids (EPA, DPA, DHA, AA) mutually adjusted for; AA:EPA ratio adjusted for the remaining fatty acids DPA, DHA; and sum n-3 adjusted for AA (all relative concentrations).

[3]Adjusted as adj. 1 with additional adjustment for maternal education level, maternal age, parity, maternal BMI, maternal smoking in median pregnancy week 18, maternal allergy, maternal asthma, plasma folate.

Table 6 shows the absolute fatty acid concentrations. Sum n-3 show 40% reduced risk for asthma (OR 0.60, 95% CI 0.39-0.91, p=0.016) in the second highest quartile (Q3) when adjusted for all fatty acids (adj.1). When adjusted for all fatty acids and potential confounding factors (adj 2.) the model shows 52% reduced risk for asthma (OR 0.48, 95% CI 0.31-0.76, p=0.002) in second highest quartile. No other n-3 fatty acid showed reduced risk.

When adjusted for all fatty acids (adj.1) AA show 65% increased risk for asthma (OR 1.65, 95% CI 1.09-2.48, p=0.018) in second highest quartile. In the highest quartile AA show 71% increased risk for asthma (OR 1.71, 95% CI 1.09-2.69, p=0.021). When adjusted for all fatty acids and confounding (adj.2) AA show 64% increased risk for asthma (OR 1.64, 95% CI 1.07-2.52, p=0.022) in second highest quartile. In the highest quartile AA show 87% increased risk for asthma (OR 1.87, 95% CI 1.16-3.03, p=0.010) when adjusted for all fatty acids and confounding. For AA:EPA ratio the OR show 52% increased risk for asthma (OR 1.52, 95% CI 1.02-2.25, p=0.04) in crude. Also, borderline statistically significant 50% increased risk for asthma (OR 1.50, 95% CI 0.97-2.32, p=0.06) when adjusted for all fatty acids in second highest quartile. In highest quartile AA:EPA ratio show borderline statistically significant 60% increased risk for asthma (OR 1.60, 95% CI 1.00-2.57, p=0.051).

When adjusted for all fatty acids and confounding factors AA:EPA ratio show 55% increased risk for asthma (OR 1.55, 95% CI 1.01-2.37, p=0.044) in second quartile, and 70% increased risk for asthma (OR 1.70, 95% CI 1.07-2.70, p=0.024) in second highest quartile. In the highest quartile AA:EPA ratio show 83% increased risk for asthma (OR 1.83, 95% CI 1.10-3.02, p=0.019).

Table 6. Odds ratio for child asthma at age 36 months in relation to categories[1] of maternal whole blood phospholipid status as absolute concentration (nmol/L), n=944

Sum-n3 (nmol/L)	Crude OR	(95% CI)	OR adj.1[2]	(95% CI)	OR adj.2[3]	(95% CI)
<51.86	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
51.86-64.77	1.05	(0.73-1.52)	0.94	(0.64-1.37)	0.80	(0.53-1.20)
64.78-79.48	**0.71**	**(0.48-1.06)**	**0.60**	**(0.39-0.91)**	**0.48**	**(0.31-0.76)**
≥79.49	0.96	(0.66-1.40)	0.79	(0.53-1.19)	0.65	(0.41-1.02)
EPA (nmol/L)						
<4.17	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
4.17-6.20	0.93	(0.64-1.37)	1.00	(0.64-1.55)	0.94	(0.59-1.50)
6.21-9.98	1.20	(0.83-1.74)	1.49	(0.91-2.42)	1.35	(0.81-2.25)
≥9.99	0.78	(0.52-1.16)	1.08	(0.60-1.97)	0.96	(0.51-1.08)
DPA (nmol/L)						
<7.70	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
7.70-9.28	0.83	(0.57-1.22)	0.77	(0.50-1.19)	0.76	(0.49-1.19)
9.29-10.90	0.95	(0.66-1.38)	0.87	(0.53-1.41)	0.78	(0.47-1.31)
≥10.91	0.83	(0.57-1.22)	0.76	(0.42-1.38)	0.70	(0.38-1.32)
DHA (nmol/L)						
<38.98	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
38.98-47.82	1.08	(0.74-1.57)	0.98	(0.63-1.53)	0.89	(0.56-1.41)
47.83-58.93	0.84	(0.57-1.24)	0.68	(0.39-1.20)	0.67	(0.37-1.20)
≥58.94	0.97	(0.66-1.42)	0.76	(0.40-1.47)	0.76	(0.39-1.51)
AA (nmol/L)						
<61.98	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
61.98-71.22	0.87	(0.58-1.32)	0.96	(0.63-1.47)	0.94	(0.60-1.46)
71.23-83.96	1.45	(0.99-2.13)	**1.65**	(1.09-2.48)	**1.64**	(1.07-2.52)
≥83.97	1.40	(0.96-2.06)	**1.71**	(1.09-2.69)	**1.87**	(1.16-3.03)
AA:EPA (nmol/L)						
<7.12	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
7.12-11.74	1.43	(0.96-2.13)	1.47	(0.98-2.21)	**1.55**	**(1.01-2.37)**
11.75-18.12	1.42	(0.95-2.12)	**1.50**	**(0.97-2.32)**	**1.70**	**(1.07-2.70)**
≥18.13	**1.52**	**(1.02-2.25)**	**1.60**	**(1.00-2.57)**	**1.83**	**(1.10-3.02)**

[1] The cut-off values corresponds to the quartile cut-points among controls.

[2] All single fatty acids (EPA, DPA, DHA, AA) mutually adjusted for; AA:EPA ratio adjusted for the remaining fatty acids DPA, DHA; and sum n-3 adjusted for AA (all absolute concentrations).

[3] Adjusted as adj. 1 with additional adjustment for maternal education level, maternal age, parity, maternal BMI, maternal smoking in median pregnancy week 18, maternal allergy, maternal asthma, plasma folate.

The results from the regression analysis indicate that for the relative sum n-3 there was a higher reduced risk for asthma when the fatty acid status was increasing from Q3 to Q4 is

increasing from medium high to high. Relative DHA show that increasing status gives reduced risk for asthma. The sum n-3 absolute values show reduced risk for asthma with a medium high status. Absolute AA and AA:EPA ratio may indicate that medium high to high status increase the risk for asthma at 36 months.

4.5 Additional analyses

The sensitivity analysis of the main results from the regression analyses (**Table 6** and **Table7**) included additional adjustment for maternal plasma vitamin D in the sample with available data (**Figure 1**, Flow chart, n=848). The results showed that after adjusting for vitamin D there was still a reduced risk for asthma for the relative values of sum n-3, DPA and DHA. For absolute values there was still a higher risk for asthma by AA. In AA:EPA the increased risk in Q3 are lost.

The correlation between maternal absolute fatty acid concentrations and potential mediating variables for childhood asthma birth weight and gestation week show that the mediating factors are close to zero. The variables for premature birth (gestation week) and low birth weight (LBW) were excluded from further analysis due to no association in spearman correlation, and no further mediation analysis was performed. Mediators was therefore not adjusted for in regression analysis.

Table 7. Spearman correlation matrix for relative concentrations of maternal fatty acid status and potential mediating variables for child asthma (n=944)

Fatty acids	Birth weight	Gestation week
EPA	-0.002	0.061
DPA	0.014	0.056
DHA	-0.019	0.053
AA	-0.019	0.031
AA:EPA	-0.050	0.003

5.0 Discussion

The purpose of this study was to investigate the association between maternal fatty acid status in pregnancy and childhood asthma at 36 months. In this chapter the main results of this study will be discussed in relation to previous studies and methodological considerations.

5.1 Main findings of present study

Results from this study shows that there may be an association between maternal fatty acid status and asthma in the offspring, but the interpretation is affected by the scale of measurement for fatty acids.

The main findings was a significant protective effect of the total n-3 PUFAs and DHA on asthma (relative scale) and significantly increased risk of high AA status which supports the results in some studies (Bisgaard et al., 2016; Rosa et al., 2020; Yu et al., 2015) but also in conflict with other studies (Newson et al., 2004; Notenboom et al., 2011; Rucci et al., 2016). For the total long chain PUFA n-3 (sum n-3) fatty acid there was a significant reduced risk for asthma. The relative sum n-3 fatty acids indicated a reduced risk for the two highest quartiles after adjustment for fatty acids and potential confounding. The absolute sum n-3 fatty acids indicated a significant reduced risk for asthma in the third quartile when adjusted for fatty acids and potential confounding. Relative specific long chain n-3 DPA indicated significant reduced risk for asthma in the two highest quartiles in crude/unadjusted and significant reduced risk for asthma in second highest quartile when adjusted for fatty acids and confounding. For relative DHA the two highest quartiles indicated reduced risk in crude and both adjusted. EPA showed no significant association in relative or absolute values, but increased risk for asthma was indicated in second highest quartile when adjusted for other fatty acids. For the absolute specific long chain n-6 AA there was significant increased risk for asthma when adjusted, in the two highest quartiles. Absolute DHA and DPA had no significant reduced risk for asthma. Relative ratio AA:EPA indicated a risk for asthma when not adjusted. For absolute ratio AA:EPA there was significant increased risk for asthma in crude and both adjusted, in the higher quartiles.

There are some similar results for absolute and relative values on fatty acids in sum n-3 in third quartile where both are statistically significant and show higher concentrations in control group. The fatty acid concentrations in **table 3** show significant higher concentrations for most of the n-3 fatty acids and ratio in relative values, while in absolute values it is the n-6

fatty acid that has significant higher concentration in case group. Fatty acid concentration for relative values seems to be a higher concentration of maternal sum n-3, EPA, DPA, DHA in controls and higher concentration of AA and AA:EPA ratio in cases. For absolute values sum n-3 is also higher for controls together with DPA and DHA. EPA are higher in cases together with AA and AA:EPA ratio. Total phospholipids are significantly higher in case group. There was a high correlation between absolute and relative sum n-3, EPA, DPA and DHA fatty acid concentrations in scatter plot but not quite similar. In relative values EPA, DPA and DHA show a negative correlation with AA but in absolute values there was a weak positive correlation. DPA and DHA had a negative correlation to AA:EPA in both relative and absolute.

5.2 Interpretation of findings

The results showed highest concentration of DHA between the individual fatty acids. The results for sum n-3 was very much alike to the DHA concentration, but an effort was made to separate the effects of EPA, DPA and DHA. This could present problems due to high correlation between the individual fatty acids. By looking at the individual fatty acids there is possible to see the distribution of concentrations of the n-3 fatty acids, and how they correlate to each other. There is shown that the individual fatty acids vary (Song et al., 2016) and results from the current study can not be turned over to intake . To look at the total n-3 fatty acids can be important to see the total effect of the n-3 fatty acids.

5.2.1 The individual long chain n-3 fatty acids EPA, DPA and DHA

The current study found that there is variation between the individual long chain n-3 fatty acids which also have been shown in study by (Kremmyda et al., 2011). For EPA in relative concentration there was no statistically significant indication for a reduced risk for asthma, but to our surprise an increased risk for asthma when adjusted for the other fatty acids DHA, DPA and AA in the multivariable regression. It may therefore have been affected by DPA, DHA or AA as there is no effect in crude or further adjustments. It is thought that the synthesis from EPA to DHA through DPA is limited and as the fatty acids are products of the shorter chain α-linolenic acid (n-3) (Calder, 2012), therefore the correlation between the n-3 fatty acids included in this study was not unexpected. It is difficult to compare fatty acid profile from this study to other studies because there are many factors that can affect the

results as diet and supplementation, and endogenous synthesis. These factors can be influenced by genes and possible pregnancy. Few studies have used whole blood to measure fatty acid status in pregnancy and therefore it is difficult to compare with previous studies as the different fractions varies.

Relative DHA had highest concentration between the n-3 fatty acids in both relative and absolute, and indicated highest reduced risk for asthma between the individual n-3 fatty acids. DHA indicated a reduced risk up to 50% when in Q4. There was also a gradient with reduced risk in Q3 and Q2, relative to Q1. Of the n-3 fatty acids DHA seemed to be the one that contributed the most to the total n-3 as it had the highest concentration in **Table 3**. Study by Lohner et al. (2013) found that the synthesis of α-linolenic acid to DHA were higher in women than in men because of a higher activity of Δ-6 desaturase. This can be a possible explanation to why there is much higher concentration of DHA in current study. During pregnancy α-linolenic acid may increase because of the need of DHA to the fetus (Burdge & Calder, 2005). There are many factors that can influence the fatty acid status and there is also found that polymorphisms in the fatty acid desaturase genes (FADS) can influence the metabolism (Glaser et al., 2011). Higher values of AA and DHA during pregnancy may possibly be seen in current study. However, a study by Kremmyda et al. (2011) reported that during pregnancy there are lower concentrations of AA and DHA during pregnancy due to selective transfer to the fetus. There may be that maternal fatty acid concentration decreases during late pregnancy. In this current study measured fatty acid status was in early second trimester and maternal fatty acid status can change during pregnancy. This can possibly be connected to the high status of DHA in the current study as the first two trimesters, the mother collects nutrients for the last trimester of pregnancy where the fatty acids are released to the placenta for fetal growth and development (Kremmyda et al., 2011).

5.2.2 Total long chain n-3 fatty acids defined as the sum of EPA, DPA and DHA

The total long chain n-3 fatty acids defined as sum n-3, indicated a high statistically reduced risk for asthma with 53% reduced risk when in Q4 relative to the reference (Q1). As mentioned, most of the sum n-3 comes from DHA as shown in **Table 3** in concentrations of the fatty acids in the current study. There was a correlation between the individual fatty acids that may affect the results. As many of the mothers in MoBa used cod liver oil it (**Table 1**) can also contribute to higher intake of DHA. However, biomarkers are objective measures (Willett, 2013, p. 151) and it may rather be genes than intake that affects the fatty acid status.

FADS affect DHA by change in the function of desaturase and have an effect on metabolism of PUFAs (Glaser et al., 2011). The ability to synthesize α-linolenic acid to DHA are greater in women (Lohner et al., 2013) and can protect from inflammatory diseases by eicosanoids from the n-3 fatty acid DHA that stimulates inflammation and act as anti-inflammatory (Kremmyda et al., 2011).

5.2.3 The n-6 fatty acid AA

The n-6 fatty acid AA had a statistically significant higher concentration in the case group than the control group and indicated an increased risk on 87% for asthma from reference (Q1) to Q4. The concentration of AA was also higher than the total n-3 fatty acids. This could be a possible explanation why EPA have low concentration, as there may be a competition between EPA and AA for the same enzymes (Calder, 2012). The correlation between AA and EPA indicated a negative correlation in relative concentrations, but a correlation close to zero in absolute concentrations. The Westernized diet that is characterized by dominating n-6 fatty acids with pro-inflammatory capabilities can be a cause to the high concentration of AA (Guilleminault et al., 2017). However, the fatty acid concentrations are an objective measure of the fatty acids (Willett, 2013, p. 151) and can also be more influenced by endogenous synthesis as it shows that Δ-6 desaturase is higher in women (Lohner et al., 2013) and therefore also higher values of AA. This can increase the eicosanoid inflammatory mediator from AA if there is a higher concentration of AA than EPA or DHA that can be related to atopy and inflammatory diseases (Calder, 2018; Kremmyda et al., 2011)

5.2.4 The ratio of AA to EPA as measure of the n-6 to n-3 ratio

The AA:EPA ratio in relative concentrations had a statistically significant increased risk for asthma when there was no adjustment in the regression analysis, but with further adjustments in the regression analysis there was not statistically significant. In the absolute concentrations the AA:EPA ratio had a statistically significant higher risk for asthma in Q3 and Q4 when compared to reference Q1. AA:EPA had a negative correlation to DPA and DHA in both relative and absolute. Concentration of AA:EPA was statistically higher in the case group but only by 1 percentage point. As mentioned the Westernized diet with dominating n-6 fatty acids can be unfortunate because of the competition between the two PUFA families n-3 and

n-6 (Innis, 2011). AA are also thought to have larger foundation to make eicosanoids and affect the development of atopy (Calder et al., 2010).

5.2.5 Absolute and relative values of the fatty acid concentrations

The relative concentrations had more difference between cases and controls than the absolute concentrations. The relative n-3 fatty acids were statistically significant in sum n-3, DPA, DHA and borderline statistically significant for EPA. For AA there was no statistically significant difference between cases and controls, but for AA:EPA borderline statistically significant. However, the absolute concentrations had no statistically significant n-3 fatty acids, but AA had statistically significant difference between cases and controls. Whether to present fatty acids as relative or absolute concentrations have been debated and Song et al. (2016) found that if the values were presented in relative or absolute it may not affect the results of the study. However, in the current study the interpretations would be different if only presented in relative or absolute. Even though the scatter plot (**Figure 7**) indicated a linear positive correlation between the sum n-3, EPA, DPA and DHA fatty acids there were more difference in regression analysis and fatty acid concentrations by asthma. Whether absolute or relative are the best to measure fatty acids concentrations are uncertain. As Song et al. (2016) found relative and absolute values were correlated there is still limitations with both relative and absolute values.

5.2.6 Mediated through birth weight and gestational length

PUFAs as DHA and AA can affect gestation length and birth weight of the fetus during pregnancy (Kremmyda et al., 2011). However, in this current study birth weight and gestation length showed no correlation with the fatty acids included in the current study (**Table 7**). The effect of fatty acids did not mediate through birth weight and gestation length, these are potential mediating factors that can affect the result of analysis but as it indicated no correlation these was not included in further analysis.

5.3 Compared with previous studies

In chapter of presented previous studies (chapter 2.5), 3 cohort studies on fatty acid status found no statistically significant association between maternal n-3 fatty acids and childhood asthma at 18 months, 30-32 months and 6-7 years (Newson et al., 2004; Notenboom et al.,

2011; Yu et al., 2015). In addition 1 cohort study found total omega-6 fatty acid to be associated with reduced risk for asthma at 6 years (Rucci et al., 2016). On the contrary the latest conducted cohort study CANDLE found higher maternal n-6 to be associated with higher risk for asthma at 4-6 years (Rosa et al., 2020), and 1 RCT on supplementation (Bisgaard et al., 2016) found reduced risk of persistent wheeze and asthma at 3-5 years. Of the studies included no other study look at phospholipid fraction in whole blood.

In this current study we found that higher maternal n-3 PUFAs can reduce the risk for asthma at 36 months and that the n-6 PUFA AA increased the risk for asthma. Partly in line with our findings the study from CANDLE by Rosa et al. (2020) found that higher maternal plasma n-6 PUFAs was associated with higher risk for asthma. Unlike this current study they included 6 n-6 PUFAs and 4 n-3 PUFAs. Maternal blood samples were collected in second trimester, similar to this current study where the maternal blood samples were also collected in the second trimester that will reflect the mid phase of pregnancy. Similar to this current study they found DHA to be the most dominant n-3 fatty acid of the total n-3. They also had a gradient from Q1 to Q4, but not statistically significant. They did not find a significant association between n-6/n-3 PUFA ratio where this current study found a higher risk for asthma. Unlike the current study, they studied the ratio between total n-6 and total n-3 that included several n-6 fatty acids where we studied the ratio between the long chain PUFAs AA and EPA which can lead to different results. Concentrations in the CANDLE study was presented in relative values, and unlike this current study they found higher risk for asthma where this current study found absolute n-6 fatty acids to be statistically significant. Unlike the current study, they also adjusted for effect modification by maternal asthma, atopic disease, child sex and maternal race with PUFA status and found that the effect of n-6 PUFAs were modified by maternal asthma.

The ALSPAC Cohort study by Newson et al. (2004) did not find an association between maternal fatty acid status and early childhood wheezing, unlike the current study. The study of ALSPAC measured maternal red blood cells membrane phospholipids after pregnancy week 20. Current study measured maternal phospholipids fractions in whole blood at median pregnancy week 18 and due to different measurement, it is difficult to compare level of effect. In present study the fatty acids are presented in both relative (%) and absolute (nmol/L) values where the ALSPAC study measured only in relative values (%). Unlike current study they measured fatty acid status to reflect late pregnancy where this current study reflects mid pregnancy. There may be a factor that fatty acid status change during pregnancy, and that this current thesis, including CANDLE study found an association earlier in

pregnancy. 4202 children were included with available maternal blood sample in the table of descriptive, but 2764 children were included with maternal red blood cell fatty acids exposure after controlled for potential confounders. Unlike this current study the OR for wheeze by fatty acid exposures in the ALSPAC study indicated no effect and OR close to 1 for all fatty acids.

The KOALA Birth Cohort Study by Notenboom et al. (2011) did not find an association between maternal fatty acid status and development of asthma at age 6-7 years, in line with the ALSPAC study. The KOALA study measured plasma phospholipids which is difficult to compare to current study due to measurement in different fractions, as whole blood phospholipids was used to measure exposure in the current study measures. Similarly to current study, they examined whether n-6 AA may increase the risk on atopic disease as wheeze and asthma. As the KOALA study measured asthma in 6-7-year-old children the study is not completely comparable to the current study on children at 3 years. They have presented fatty acids in relative values (%) as well as this current study On the contrary to our collection of maternal blood samples in median week 18 reflecting mid pregnancy, the KOALA study collected maternal blood samples in week 34-36 of pregnancy which reflect fatty acid status in late pregnancy. As the authors in KOALA study point out the measurement of fatty acids in the end of pregnancy are uncertain whether reflect fatty acid status during the whole pregnancy. Similar to the previous study by Newson et al. (2004) they found no significant reduced risk for wheeze or asthma at 6-7 years.

The Generation R Study by Rucci et al. (2016) found higher maternal total PUFA and total n-6 PUFA levels to be associated with reduced risk for childhood asthma which is the opposite result than The CANDLE study. Total PUFA was the sum of the included n-6 and n-3 fatty acid, linoleic acid and its products that included total 7 n-6 fatty acids, and α-linolenic acids and its products that included total 5 n-3 fatty acids. Unlike this current study they included more PUFAs in the analysis. The Generation R. Study collected maternal blood samples in approximately week 20 of pregnancy and reflects mid pregnancy that was similar to present study. However, analysis of maternal exposure was in plasma glycerophospholipids which make it difficult to compare the levels with this current study as it was measured in whole blood phospholipids. Fatty acids were expressed as concentrations but used as relative (wt%) in analysis and included more PUFAs in analysis than this current study. The Generation R. Study included the confounding factors as maternal gestational age, child's sex, age at follow-up, folic acid levels, total daily calorie intake in first trimester, psychological distress, child's ethnicity, pet keeping, child's gestational age at birth, birthweight,

breastfeeding, lower respiratory infection, inhalant allergies and body mass index at age 6 years. These are confounding factors and mediators that are not included in the present study. However, in the present study factors on the child (birth weight and premature birth) which may mediate the association between maternal fatty acid status and child asthma were not included because the correlation with maternal fatty acid status was found to be close to zero.

In line with the previous studies (Newson et al., 2004; Notenboom et al., 2011; Rucci et al., 2016) the Asian birth cohort GUSTO by Yu et al. (2015) did not find significant protective effects of higher maternal n-3 PUFA or lower n-6 PUFA on offspring allergic diseases in early childhood. The measurement of maternal fatty acid in plasma phosphatidylcholine was obtained in week 26-28 which was over 9 weeks later than the current study's blood samples were taken. That can be a factor as mentioned earlier that the fatty acid status can change during pregnancy, and as they mentioned in their discussion. Due to the blood sample in GUSTO was measured in a different fraction than in this current study there is difficult to compare. The cohort focused on the percentage of total n-3 PUFA, total n-6 PUFA and n-6:n-3 PUFA ratio. Also, the specific n-3 and n-6 PUFAs from α-linolenic acid, EPA, DPA, DHA, EPA+DHA, and from linoleic acid and AA. One of the outcomes in the GUSTO study was wheeze where information was obtained up to 18 months. The children in the GUSTO study are younger than the children in the current study and can be a factor to why they did not find an association. However, the GUSTO study also found lower OR for wheezing in infants with increasing quartiles of maternal fatty acids as this current thesis, but they had not statistically significance. They included more confounding for the child than the current thesis as sex, ethnicity, birth weight, gestational age, length of breast-feeding, child care attendance and pets. However, birth weight and gestational age was not correlated to fatty acids in the current study.

Few of the included studies assessed outcome asthma at 36 months, and studies with older children was included to get a wider perspective. Symptoms on asthma have been seen to develop at the age of 3 years and also develop asthma later in childhood (Bisgaard & Bønnelykke, 2010). In the younger children wheeze are often an early indicator for asthma and therefore studies include both wheeze and asthma in study. The definitions vary and diagnose criteria can make it difficult to compare studies with different estimates. However, most of the studies included in this current study adapted questionnaires based on the International Study of Asthma and Allergies (ISAAC). However, study by Sá-Sousa et al. (2014) found that even by same questionnaire by ISAAC there was variation in definitions of asthma. Dietary patterns vary between different countries and there have been measured high

blood levels of EPA and DHA in healthy adults in the Scandinavian countries Norway, Denmark, and Greenland (Stark, Van Elswyk, Higgins, Weatherford, & Salem, 2016), and compared with other Scandinavian countries, Sweden and Finland had moderate blood levels of EPA and DHA. The included previous studies are from different countries and there may be variations in status. There was few studies on whole blood and in a review by Hodson et al. (2008) measurement of fatty acid composition in whole blood found most linoleic acid 18:2 n-6 (30 mol%) and less AA 20:4 n-6 and DHA 22:6 n-3 (2 mol% and 9 mol%).

The study on supplementation from The COPSAC cohort study by Bisgaard et al. (2016) found that supplementation of long chain n-3 PUFAs in third trimester reduced the absolute risk for persistent wheeze and asthma in offspring. Maternal whole blood levels of EPA and DHA was collected 1 week after birth. The study used a high dose of long chain n-3 PUFAs (2.4 g/d). A study on supplementation is not comparable to the measurements in current study, however, the RCT study may contribute to a wider perspective on the association between maternal fatty acid and asthma in offspring. The dose of long chain n-3 PUFAs on 2.4 g/d was much higher than the recommended intake of EPA and DHA by the NNR are 200 mg/d of DHA and by FAO 0,3 g/d EPA + DHA and minimum 0,2 g/d DHA (Food and Agriculture Organization, 2010; Nordic Council of Ministers, 2014). The Norwegian Health Directorate recommends 5 ml/d of cod liver oil that give approximately 0.4g EPA and 0.6g DHA (Helsedirektoratet, 2017). In current study there was found higher reduced risk for asthma in the highest concentrations than the reference quartile.

5.4 Methodological considerations

The main strength to this current study was the prospective data collection by the large cohort study of MoBa with over 100 000 pregnancies, one of the world's largest cohort studies (Magnus et al., 2016). Even though this study only has a sub-sample of the data from MoBa, the cohort study has collected information in a large time-span and therefore much valuable information was collected. Because of this it is possible to explore the relation between independent factors of each participant, environmental factors, and the potential confounding factors that can be controlled for, and in studies assessing exposure and outcome it is important to adjust for factors that can affect the results of the study (Rothman et al., 2008)

The limitations of the current study will be further discussed in the following chapters. In epidemiology the study of dietary causes is complex. The fatty acids were analyzed separately and a variable for sum n-3 to explore the correlation and the difference in concentration

between the individual fatty acids. It is difficult to separate the fatty acids as there is complex mechanisms that can influence. That is why the correlation matrix was performed to see the correlation between the individual fatty acids in relative and absolute values. There have been shown that EPA and DHA may be the protective n-3 PUFAs and AA of the n-6 PUFAs the opposing fatty acid that can cause inflammation and therefore fatty acids was analyzed individually to the n-6 AA (Kremmyda et al., 2011; Wiktorowska-Owczarek et al., 2015).

In this current study multivariable regression analysis was performed to include potential confounding variables and estimate OR. There are multiple methodological considerations in epidemiological studies that are important to discuss. Discussion of intern validity by selection bias, information bias and confounding, and further extern validity are presented in the following chapters.

5.4.1 Selection bias

Selection bias are systematic differences between the groups in study or between the participants in the study and the general population (Rothman et al., 2008). This could cause that the participants are not representative for the population. In the main study of MoBa the participating rate for all invited pregnancies was approximately 41% (Magnus et al., 2016). Loss to follow-up or failure to return follow-up questionnaires can be a source of selection bias. In MoBa there have been a fall in the returned questionnaires. When participants did not return FFQ or questionnaire at 6 months, 18 months and 36 months they were not included in analysis in the current study.

Many of the MoBa mothers were higher educated and from the characteristics over half of the study population had education of “some college” or more. This can explain the high use of cod liver oil in cases as about 40% of the mothers used supplementation in current study. This was only cod liver oil and in the MoBa study there could be higher use of supplement by other fish oils and casules. There is known that people with higher education are more health aware (Haugen et al., 2008). However in a study by Nilsen et al. (2009) there was found that self-selection in MoBa were probably not a validity problem. Minority groups in Norway may probably be under-represented in the MoBa study, due to the criteria to join the study was to understand Norwegian. The questionnaires were only available for the participants in Norwegian.

5.4.2 Information bias

As in all case-control and cohort studies there is a possibility for bias. In case-control studies the disadvantage with retrospective design can affect the validity by recall bias, but due to this current study is based on data from the prospective cohort study MoBa it is not an issue for this study. The data are collected prospectively and before the disease occurred (Willett, 2013, pp. 8-9). Cohort studies avoid many of the methodological biases but losses to follow-up may be a problem as it can affect the validity of the study (Rothman et al., 2008). Prospective cohorts with a long-time span may experience loss to follow up as missing on questionnaires and participants leaving the study.

The gold standard for measuring fatty acids are in adipose tissue. However, there is variance in concentration and the PUFAs EPA and DPA have shown to be low in adipose tissue but higher in plasma, platelet and erythrocyte phospholipids. Whole blood phospholipids can therefore be considered to be preferred to measure the PUFAs EPA and DPA.. Phospholipids measured in whole blood are most likely to reflect the short-term intake where it reflects days or months and would reflect second trimester of pregnancy (Baylin et al., 2005; Hodson et al., 2008).

As seen in characteristics of study population the MoBa mothers seem to be in general health conscious when looking at reported education, BMI, intake of cod liver oil and reported smoking in pregnancy. This can lead to mothers over-reporting asthma in self reported questionnaires, but seen in characteristics of mothers there is no association between maternal education, maternal pre-pregnancy BMI, use of cod liver oil or reported smoking with childhood asthma. As mentioned earlier study by Nilsen et al. (2009) found that self-selection in MoBa were probably not a validity problem.

In exposure, outcome and covariates misclassification can occur (Rothman et al., 2008). Misclassification of exposure in cohort studies are most likely non-differential due to the collection of exposure variable is collected from all participants, as non-differential misclassification is equal misclassification between the subjects of the study or cases and controls (Rothman et al., 2008). This bias can cause dichotomous variables of exposure or outcome to show an association towards null (no relation). In this current study, collection of exposure variables was obtained by biomarkers and analyzed at VITAS laboratory (vitas.no). The blood samples were collected at one point during pregnancy, at median gestation week 18 that reflect mid pregnancy. Phospholipid membrane was extracted from a single non-fasting whole blood sample and therefore not guaranteed the sample was influenced by latest meal. However, phospholipids are not as much influenced by diet as triglycerides (Willett, 2013, pp.

190-191). The blood sample was only collected at approximately pregnancy week 18 that would reflect second trimester. Whether the single phospholipid sample was a good measure for maternal fatty acid status during pregnancy for fetal asthma development is yet uncertain.

Misclassification of outcome in the current study are the measurement of asthma and lack of a gold standard measurement for diagnose or classify as wheeze and asthma a challenge as well as in all epidemiological studies. Due to no gold standard measurement there is variance in estimation and results of childhood asthma. However, the definition on asthma in this study was based on mother reporting in questionnaire on symptoms but also the child's use of medication the last 12 months that will strengthen the definition. The study by Furu et al. (2011) found that the child's use of anti-asthmatics reported by mother for 7 year old children in MoBa were highly valid.

In current study measurement (did not separate asthma and wheeze) was only on asthma and not wheeze. In younger than school aged children there is most often reported wheeze, and asthma after 3 years. It is also difficult to fully diagnose children younger than 6 years as many children have symptoms on wheeze in young age but does not develop asthma later with age (Global Initiative for Asthma, 2020). However, there are many children that develop asthma before the age of 5 and symptoms develops at early age (Bisgaard & Bønnelykke, 2010).

5.4.3 Confounding

In this current observational study potential confounding factors was identified from previous studies (Håberg et al., 2011). Confounding factors are important to include in analysis as unmeasured confounders can lead to bias (Rothman et al., 2008). However, we can not exclude the possibility of confounding. In large cohort studies as the MoBa study there are available data on many factors that can be adjusted for as possible confounding factors. However, not all confounders may have been identified. Also, if included confounders are imperfectly measured. The adjustment for the confounding factor may be incomplete, leading to residual confounding (Rothman et al., 2008).

5.4.4 Extern validity

Selection bias may reduce the extern validity of the study (Rothman et al., 2008). Findings from this study can possibly not be generalized to the general population because the current study is based on a subsample from a cohort where additional asthma cases were included

(Håberg et al., 2011; Joubert et al., 2012). However, when examining risks and associations to disease there may be better to have a study population that are more homogenous and that are better for control of confounding (Nilsen et al., 2009). The importance of representative samples can be seen more important when examining prevalence in a population than disease risk factor. There could still be found an association between maternal fatty acid status and asthma even though if the whole cohort were included. Whether the associations in the current study are generalizable to the population are therefore uncertain.

6.0 Conclusion and further research

Main findings of this master thesis were a significant protective effect of high maternal total n-3 PUFA and DHA status measured on a relative scale on risk of child asthma. While a high status of the n-6 fatty acid AA measured on an absolute scale, indicated a significant adverse effect on asthma. The results differ by the scale of measurement and the interpretation of the study is affected by the scale of measurement.

The findings in this master thesis supports the hypothesis of DOHaD, that exposures in early life during pregnancy can increase the risk for chronic disease as asthma later in life as maternal exposure can be a risk factor. Further research is needed to identify more risk factors for asthma, and hopefully this master thesis can contribute to inspire for further research.

For further research there would be interesting to explore whether there is a long-term effect, does the effect last for older children/adolescents or is it transient. In school age children and adolescents asthma with allergy becomes more prevalent and it would also be interesting to explore whether there are differences in the effect of maternal fatty acid status for asthma with and without atopy/allergy.

Further to explore whether there are differences in genetic variants in fatty acid metabolism genes between cases and controls (mother and children). A recent study reported that the association between maternal fatty acid status and child asthma was modified by maternal asthma and child sex ((Rosa et al., 2020)). Potential interaction effects between fatty acids and between fatty acids and other factors should be explored, but was beyond the scope of this thesis.

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Appendix 1

folkehelseinstituttet

Samtykke fra mor

Jeg har lest informasjonsbrosjyren om Den norske mor og barn undersøkelsen og er kjent med at opplysningene jeg gir vil bli behandlet strengt fortrolig. Jeg er informert om at undersøkelsen er vurdert av Regional komité for medisinsk forskningsetikk og godkjent av Datatilsynet.

Deltakelse i Den norske mor og barn undersøkelsen innebærer følgende:

- at jeg fyller ut spørreskjemaer, under og etter svangerskapet, om min egen og barnets helse og levekår.
- at jeg gir en blodprøve og en urinprøve i svangerskapet og en blodprøve etter fødselen, og at det tas en blodprøve fra barnets navlesnor etter fødselen.
- at blodprøvene/urinprøven fra meg, og blodprøven fra mitt barn lagres i en biobank på Nasjonalt folkehelseinstitutt. Prøvene blir avidentifisert og lagres med et prøvenummer. Blod-/urinprøver skal kun benyttes til forskning i forbindelse med årsaker til sykdom, herunder samspill mellom arvelige faktorer og miljøpåvirkninger. Dette vil bli gjort i laboratorier i Norge og andre land, etter at den aktuelle bruken av blodprøven er vurdert av Regional etisk komité og godkjent av Datatilsynet.
- at resultatet fra ultralydundersøkelsen i svangerskapet blir stilt til rådighet for prosjektet.
- at blodprøven som blir tatt av barnet til undersøkelse på Føllings sykdom kan stilles til disposisjon for prosjektet.
- at det ikke meldes noen resultater tilbake til meg om min eller mitt barns helse, heller ikke resultater fra blodprøvene.
- at opplysninger om meg og barnet kan hentes fra andre kilder, slik som Medisinsk fødselsregister og sykehusregistre, etter Datatilsynets godkjennelse.
- at jeg kan bli spurt om å bli med i undersøkelser som innebærer innsamling av nye opplysninger (herunder prøver). Slike delprosjekter vil separat bli vurdert av Datatilsynet og Regional etisk komité. Deltakelse er frivillig, og er ikke nødvendig for videre deltakelse i hovedprosjektet.
- at det ikke er satt tidsbegrensning for hvor lenge opplysningene og blodprøvene kan lagres. Prosjektet er langvarig og kan inkludere årsaker til sykdom som oppstår i voksen alder. Mitt barn vil bli informert om prosjektet ved 15-års alder, og vil bli spurt om samtykke til fortsatt deltakelse når han eller hun er 18 år.
- at ingen opplysninger eller prøver stilles til rådighet for forskere uten at navn og fødselsnummer er fjernet.
- at jeg på hvilket som helst tidspunkt kan trekke meg fra videre deltakelse ved å skrive eller ringe til Den norske mor og barn undersøkelsen. I tillegg kan jeg be om at innsamlede opplysninger og blodprøver blir slettet/destruert, uten å oppgi noen grunn.

Jeg har lest informasjonen ovenfor og samtykker i å delta i Den norske mor og barn undersøkelsen.

Navn: _____

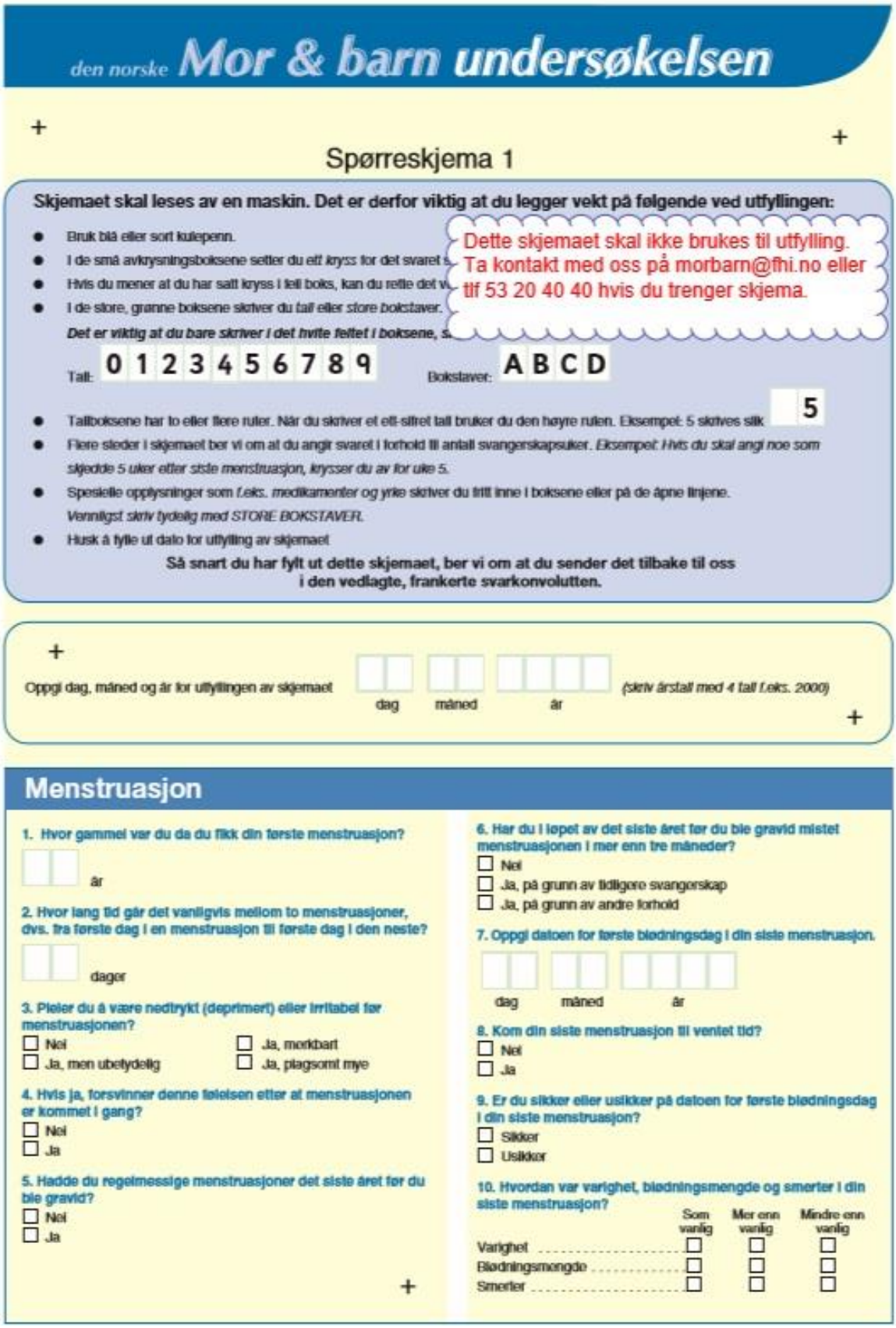
Fødselsnr. (11 sifre): _____

Dato: _____ Underskrift: _____

Min adresse på invitasjonsbrevet er feil, den korrekte adressen er:

_____

Appendix 2



den norske **Mor & barn undersøkelsen**

Spørreskjema 1

Skjemaet skal leses av en maskin. Det er derfor viktig at du legger vekt på følgende ved utfyllingen:

- Bruk blå eller sort kulepenn.
- I de små avkrysningsboksene setter du *ett kryss* for det svaret s…
- Hvis du mener at du har satt kryss i feil boks, kan du rette det v…
- I de store, grønne boksene skriver du *tall* eller *store bokstaver*.

Det er viktig at du bare skriver i det hvite feltet i boksene, s…

Tall: 0 1 2 3 4 5 6 7 8 9 Bokstaver: A B C D

Dette skjemaet skal ikke brukes til utfylling. Ta kontakt med oss på morbarn@fhi.no eller tlf 53 20 40 40 hvis du trenger skjema.

- Tallboksene har to eller flere ruter. Når du skriver et ett-sifret tall bruker du den høyre ruten. Eksempel: 5 skrives slik [ ][5]
- Flere steder i skjemaet ber vi om at du angir svaret i forhold til antall svangerskapsuker. *Eksempel: Hvis du skal angi noe som skjedde 5 uker etter siste menstruasjon, krysser du av for uke 5.*
- Spesielle opplysninger som f.eks. *medikamenter og yrke* skriver du trlll inne i boksene eller på de åpne linjene. *Vennligst skriv tydelig med STORE BOKSTAVER.*
- Husk å fylle ut dato for utfylling av skjemaet

Så snart du har fylt ut dette skjemaet, ber vi om at du sender det tilbake til oss i den vedlagte, frankerte svarkonvolutten.

Oppgi dag, måned og år for utfyllingen av skjemaet [ ][ ] dag [ ][ ] måned [ ][ ][ ][ ] år *(skriv årstall med 4 tall f.eks. 2000)*

Menstruasjon

1. Hvor gammel var du da du fikk din første menstruasjon?

[ ][ ] år

2. Hvor lang tid går det vanligvis mellom to menstruasjoner, dvs. fra første dag i en menstruasjon til første dag i den neste?

[ ][ ] dager

3. Pleier du å være nedtrykt (deprimert) eller irritabel før menstruasjonen?

☐ Nei ☐ Ja, merkbart
☐ Ja, men ubetydelig ☐ Ja, plagsomt mye

4. Hvis ja, forsvinner denne følelsen etter at menstruasjonen er kommet i gang?

☐ Nei
☐ Ja

5. Hadde du regelmessige menstruasjoner det siste året før du ble gravid?

☐ Nei
☐ Ja

6. Har du i løpet av det siste året før du ble gravid mistet menstruasjonen i mer enn tre måneder?

☐ Nei
☐ Ja, på grunn av tidligere svangerskap
☐ Ja, på grunn av andre forhold

7. Oppgi datoen for første blødningsdag i din siste menstruasjon.

[ ][ ] dag [ ][ ] måned [ ][ ][ ][ ] år

8. Kom din siste menstruasjon til ventet tid?

☐ Nei
☐ Ja

9. Er du sikker eller usikker på datoen for første blødningsdag i din siste menstruasjon?

☐ Sikker
☐ Usikker

10. Hvordan var varighet, blødningsmengde og smerter i din siste menstruasjon?

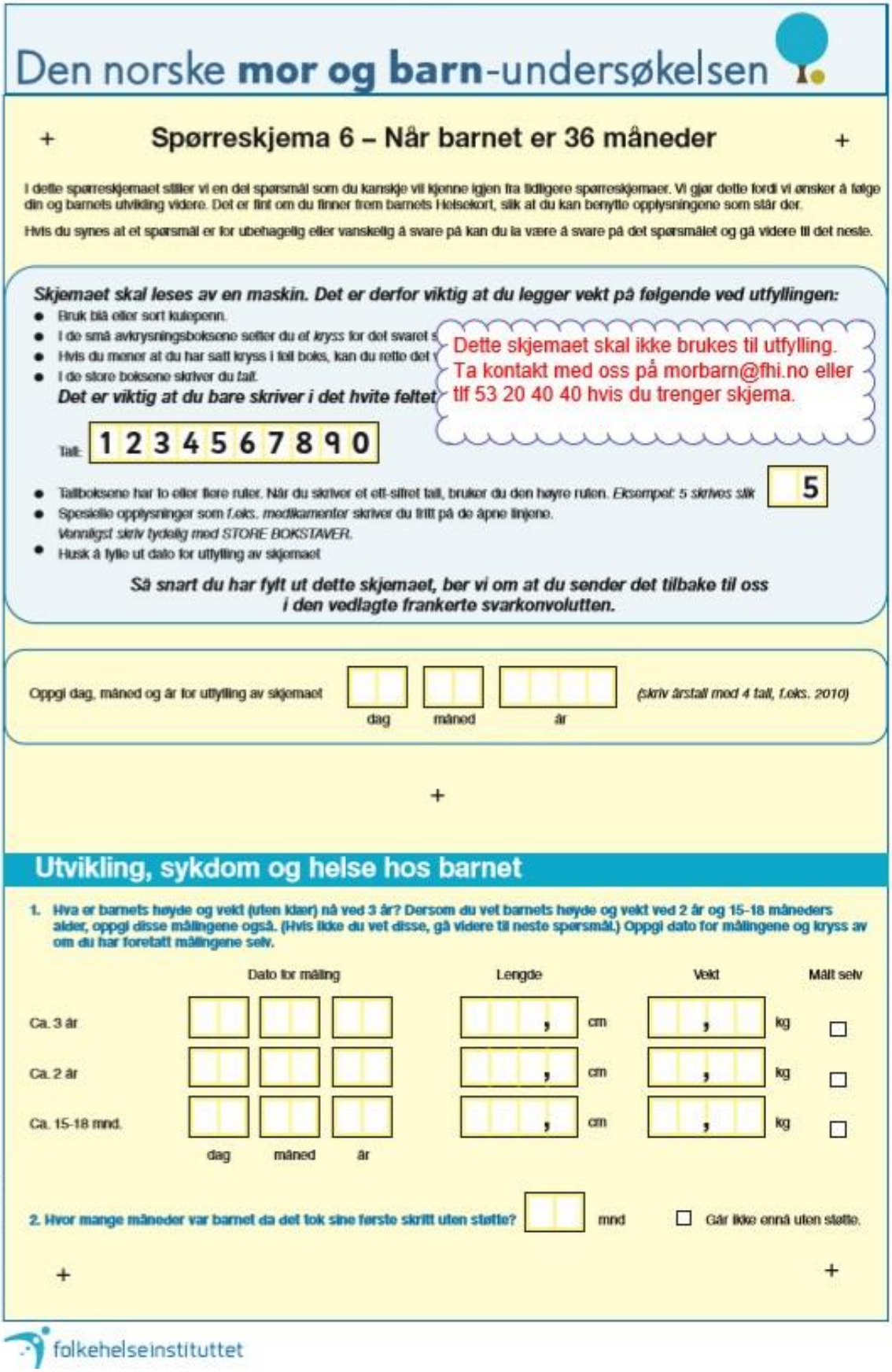
	Som vanlig	Mer enn vanlig	Mindre enn vanlig
Varighet	☐	☐	☐
Blødningsmengde	☐	☐	☐
Smerter	☐	☐	☐

Tidligere og nåværende sykdommer og helseplager

39. Kryss av hvis du har eller har hatt noen av følgende sykdommer eller helseplager. Hvis du har brukt tabletter, miksturer, stikkpiller, inhalasjoner, salver osv. i forbindelse med sykdommen eller helseplager, oppgi navnet på medisinen(e) og når du brukte disse.

Sykdommer / helseplager			Bruk av medisiner					
Sykdom / helseplage +	Før svanger-skapet	I svanger-skapet	Navn på medisiner	Siste 6 mnd. før svanger-skapet	I svangerskapsuke 0-4	5-8	9-12	13+	Antall dager brukt
Astma / Allergi / Hud									
1 Astma	☐	☐		☐	☐	☐	☐	☐	
2 Høysnue, pollenallergi	☐	☐		☐	☐	☐	☐	☐	
3 Dyrehårsallergi	☐	☐		☐	☐	☐	☐	☐	
4 Annen allergi	☐	☐		☐	☐	☐	☐	☐	
5 Atopisk eksem *(ofte kalt barneeksem)*	☐	☐		☐	☐	☐	☐	☐	
6 Elveblest *(urticaria)*	☐	☐		☐	☐	☐	☐	☐	
7 Psoriasis	☐	☐		☐	☐	☐	☐	☐	
8 Annen eksem	☐	☐		☐	☐	☐	☐	☐	
9 Munnsår *(herpes)*	☐	☐		☐	☐	☐	☐	☐	
10 Akne/kviser *(alvorlig)*	☐	☐		☐	☐	☐	☐	☐	
Diabetes / Sukkersyke				+					
11 Diabetes behandlet med insulin	☐	☐		☐	☐	☐	☐	☐	
12 Diabetes ikke behandlet med insulin	☐	☐		☐	☐	☐	☐	☐	
Hjerte / Blod / Stoffskifte / Blodkar									
13 Medfødt hjertefeil	☐	☐		☐	☐	☐	☐	☐	
14 Annen hjerte-/karsykdom	☐	☐		☐	☐	☐	☐	☐	
15 Forhøyet kolesterol	☐	☐		☐	☐	☐	☐	☐	
16 For høyt blodtrykk	☐	☐		☐	☐	☐	☐	☐	
17 For høyt eller for lavt stoffskifte	☐	☐		☐	☐	☐	☐	☐	
18 Anemi/lav blodprosent	☐	☐		☐	☐	☐	☐	☐	
19 B-12-/folat/folsyremangel	☐	☐		☐	☐	☐	☐	☐	
Mage / Tarm +									
20 Hepatitt/leverbetennelse	☐	☐		☐	☐	☐	☐	☐	
21 Gallestein	☐	☐		☐	☐	☐	☐	☐	
22 Magesår	☐	☐		☐	☐	☐	☐	☐	
23 Crohns sykdom / Ulcerøs colitt	☐	☐		☐	☐	☐	☐	☐	
24 Coeliaki	☐	☐		☐	☐	☐	☐	☐	
25 Annen mage-/tarmplager	☐	☐		☐	☐	☐	☐	☐	
Muskel / Skjelett / Bindevev +				+					
26 Leddgikt *(reumatoid artritt)*, Bekhterevs sykdom	☐	☐		☐	☐	☐	☐	☐	

Appendix 3



Den norske **mor og barn**-undersøkelsen

Spørreskjema 6 – Når barnet er 36 måneder

I dette spørreskjemaet stiller vi en del spørsmål som du kanskje vil kjenne igjen fra tidligere spørreskjemaer. Vi gjør dette fordi vi ønsker å følge din og barnets utvikling videre. Det er fint om du finner frem barnets Helsekort, slik at du kan benytte opplysningene som står der.

Hvis du synes at et spørsmål er for ubehagelig eller vanskelig å svare på kan du la være å svare på det spørsmålet og gå videre til det neste.

Skjemaet skal leses av en maskin. Det er derfor viktig at du legger vekt på følgende ved utfyllingen:

- Bruk blå eller sort kulepenn.
- I de små avkrysningsboksene setter du *et kryss* for det svaret s
- Hvis du mener at du har satt kryss i feil boks, kan du rette det
- I de store boksene skriver du *tall*.

Det er viktig at du bare skriver i det hvite feltet

Tall: 1 2 3 4 5 6 7 8 9 0

Dette skjemaet skal ikke brukes til utfylling.
Ta kontakt med oss på morbarn@fhi.no eller
tlf 53 20 40 40 hvis du trenger skjema.

- Tallboksene har to eller flere ruter. Når du skriver et ett-sifret tall, bruker du den høyre ruten. *Eksempel: 5 skrives slik* 5
- Spesielle opplysninger som *f.eks. medikamenter* skriver du fritt på de åpne linjene.
  Vennligst skriv tydelig med STORE BOKSTAVER.
- Husk å fylle ut dato for utfylling av skjemaet

Så snart du har fylt ut dette skjemaet, ber vi om at du sender det tilbake til oss i den vedlagte frankerte svarkonvolutten.

Oppgi dag, måned og år for utfylling av skjemaet — dag / måned / år *(skriv årstall med 4 tall, f.eks. 2010)*

Utvikling, sykdom og helse hos barnet

1. Hva er barnets høyde og vekt (uten klær) nå ved 3 år? Dersom du vet barnets høyde og vekt ved 2 år og 15-18 måneders alder, oppgi disse målingene også. (Hvis ikke du vet disse, gå videre til neste spørsmål.) Oppgi dato for målingene og kryss av om du har foretatt målingene selv.

	Dato for måling (dag / måned / år)	Lengde	Vekt	Målt selv
Ca. 3 år		, cm	, kg	☐
Ca. 2 år		, cm	, kg	☐
Ca. 15-18 mnd.		, cm	, kg	☐

2. Hvor mange måneder var barnet da det tok sine første skritt uten støtte? mnd ☐ Går ikke ennå uten støtte.

folkehelseinstituttet

Nå følger spørsmål om sykdom og helseproblemer hos barnet. Først spør vi om mer langvarige plager, og deretter om sykdommer og plager av mer forbigående type.

3. Har barnet hatt langvarig sykdom eller helseproblemer siden barnet var 18 måneder?

Helseproblem +	Nei	Ja, har nå	Ja, hadde tidligere	Hvis ja, er barnet henvist til spesialist: Nei	Ja
1. Nedsatt hørsel	☐	☐	☐	☐	☐
2. Nedsatt syn	☐	☐	☐	☐	☐
3. Forsinket motorisk utvikling (f.eks. sitter/går sent)	☐	☐	☐	☐	☐
4. Cerebral parese	☐	☐	☐	☐	☐
5. Leddproblemer	☐	☐	☐	☐	☐
6. Diabetes	☐	☐	☐	☐	☐
7. For liten vekstakning	☐	☐	☐	☐	☐
8. For stor vekstakning	☐	☐	☐	☐	☐
9. Hjertefeil	☐	☐	☐	☐	☐
10. Testiklene ikke kommet ned i pungen	☐	☐	☐	☐	☐
11. Astma	☐	☐	☐	☐	☐
12. Allergi i øyne eller nese, f.eks høysnue	☐	☐	☐	☐	☐
13. Atopisk eksem (barneeksem)	☐	☐	☐	☐	☐
14. Annen eksem	☐	☐	☐	☐	☐
15. Ofte diare	☐	☐	☐	☐	☐
16. Ofte magesmerter	☐	☐	☐	☐	☐
17. Matallergi/intoleranse	☐	☐	☐	☐	☐
18. Andre mage-/tarm problemer	☐	☐	☐	☐	☐
19. Forsinket eller avvikende språkutvikling	☐	☐	☐	☐	☐
20. Søvnproblemer	☐	☐	☐	☐	☐
21. Kontaktvanskar	☐	☐	☐	☐	☐
22. Hyperaktivitet	☐	☐	☐	☐	☐
23. Autistiske trekk	☐	☐	☐	☐	☐
24. Andre atferdsproblemer	☐	☐	☐	☐	☐
25. Annen langvarig sykdom/ tilstand	☐	☐	☐	☐	☐

Hvilken da? _____

+

4. Hvis barnet har vært hos spesialist eller på sykehus, hva viste undersøkelsen?

☐ Alt var i orden
☐ Fortsatt tvil/ utredes videre
☐ Har ikke vært til undersøkelse ennå

☐ Fikk diagnose I: _____

_____

☐ Fikk diagnose II: _____

_____

☐ Fikk diagnose III: _____

_____

5. Hvis barnet har en alvorlig eller langvarig sykdom, beskriv eventuelt nærmere:

_____

_____

6. Har barnet ditt vært utsatt for eller vært involvert i en alvorlig hendelse?

☐ Nei ☐ Ja +

7. Hvis ja, beskriv:

_____

_____

_____

_____

_____

_____

_____

8. Synes du det har påvirket barnets væremåte eller utvikling?

☐ Nei ☐ Ja +

Appendix 4

Procedure at VITAS AS laboratories for quantitative determination of fatty acids in the phospholipid fraction of whole blood

Blood stored at -20°C were thawed overnight at 4°C and vortexed for 10 sec immediately prior to pipetting. 100µl blood and internal standard (1,2 diheptadecaonyl-sn-glycero-3-phosphatidylcholin) were extracted with dichloromethane/methanol. After shaking and centrifugation the supernatant was transferred to new glasses and washed in 0.9% NaCl solution. Lower phase was transferred to solid phase extraction (SPE) columns. Neutral lipids were washed out with dichloromethane/isopropanol and methyl tert-butyl ether (MTBE)/formic acid. Finally, phospholipids were eluted with methanol. After evaporation to dryness on thermoblock at 70C, phospholipids were transmethylated with sodium methoxide and fatty acid methyl esters were extracted to hexane prior to gas chromatography analysis.

Analysis was performed on a 7890A gas chromatograph with a split/split less injector, a 7683B automatic liquid sampler, and flame ionization detection (Agilent Technologies, Palo Alto, CA). Separation was performed on a SP 2380 (30 m × 0.22 mm i.d. × 0.25 µm film thickness) column (Supelco, Inc., Bellefonte, PA).

Feasibility of using whole blood

Fasting whole blood has been described as a suitable biomarker of long-term essential fatty acid intake, comparable to that of fasting plasma, but with the advantage of requiring less sample processing (Baylin et al., 2005). After phospholipid extraction, the ranking of the predominant fatty acids in our study is compatible with phospholipid fatty acid profiles described for plasma, platelets, and erythrocytes (Hodson et al., 2008), when considering that whole blood reflects relative contributions by each of these blood pools. The proportion of LC omega-3 fatty acids was modified by dietary intake of marine foods and omega-3 supplement use and correlated negatively with markers of inflammation. Taken together, this confirms the feasibility of using frozen whole blood to measure fatty acid status in population based studies.