1 Introduction

1.1 Motivation

Nutrition plays a key role during the first years of life where we undergo an incredible development and accelerated growth. Getting sufficient nutrients is of utmost importance to ensure optimal development and growth according to our genetic potential.

In Denmark, an increased intake of n-3 long chain polyunsaturated fatty acids (LCPUFA) is recommended during the first years of life to support brain docosahexaenoic acid (DHA) accretion, which matches the old Nordic tradition of supplementing infants with cod liver oil. However, the optimal intake is not known and only a few studies have examined to what extent DHA status is affected by endogenous synthesis from α-linolenic acid (ALA) relative to the influence of dietary intake and other potential determinants in infancy and childhood.

The endogenous synthesis of LCPUFA is influenced by genetic variation in the fatty acid desaturase (FADS) gene cluster. I was inspired to include *FADS* genotype by Schaeffer *et al* (2) who published the first study showing that *FADS* genotype is associated with fatty acid composition of serum phospholipids in adults. Another study of great inspiration was the study by Caspi *et al* (1) which used *FADS* genotype to support that n-3 LCPUFA are relevant in the mechanism behind the effects of breastfeeding on IQ. These findings led me to think that *FADS* genotype should be included to get the full picture of what determines DHA status since endogenous synthesis is high in infants. Furthermore, I included functional genetic variation in genes encoding proteins involved in alleged mechanisms (e.g. *PPARG2*, *COX2*, and *NFKB1*) as effect modifiers to support functional effects of n-3 LCPUFA.

An efficient metabolism is necessary to ensure optimal growth and development. This includes a well-functioning glucose and insulin regulation with insulin having important anabolic effects during growth. Results from rodent studies supports that n-3 LCPUFA positively affects insulin resistance and prevents obesity but presently the results in humans are inconsistent.

Lipid metabolism is also of importance during growth and development and one previous study has shown that n-3 LCPUFA can affect plasma triacylglycerol levels in infants in the same way as seen in adults (3). In adults, n-3 LCPUFA have been shown to have positive effects on a number of risk markers of disease i.e. lowering plasma triacylglycerol and blood pressure. The long term consequences of having a lower TAG and blood pressure (BP) in infancy and early childhood are not known. However the mere fact that these metabolic markers can actually be affected in young children is interesting and needs to be considered.

Another aspect of great importance in relation to ensuring optimal growth and development is maturation of the immune system. Theoretically, n-3 LCPUFA have immune suppressive and anti-inflammatory effects, but results from randomized controlled trials (RCT) are inconsistent and more studies in infants and young children are warranted.

In my PhD project, I investigated potential determinants of infant and young child DHA status including genetic variation in *FADS*. Furthermore, I explored whether functional effects of n-3 LCPUFA on metabolic markers and immune maturation in young children can be supported by polymorphisms in genes involved in the mechanisms.

1.2 Determinants of DHA status in early life

Total body DHA status is dynamic and depends on intake and endogenous synthesis of DHA as well as the endogenous use of DHA. In the body, DHA is incorporated into various tissues with high concentrations in the brain and retina (4). In tissues DHA can be used for production of docosanoids and may also be used as an energy source. Growth affects DHA status by requiring more DHA for growing tissues. If intake and endogenous supply of DHA do not keep up with the increasing demand during periods of growth, then total body DHA status will decrease.

Infancy and early childhood are periods of rapid growth and DHA demands are high especially for the growing brain (5). During the first 6 months of life exclusive breastfeeding is recommended (6) and considered sufficient in supplying DHA to the infant. Breastfeeding continues during the complementary feeding period and according to data from 2005 14% are still breastfed at one year of age in Denmark (7). A high intake of n-3 PUFA is continuously recommended during the complementary feeding period, but the optimal intake of DHA in infancy and early childhood is not known. In the Nordic countries it is recommended that n-3 PUFA should comprise at least 1 E% for children between 6 and 11 months and 0.5 E% from 12 to 23 months (6). Presently, there are no national data on whether or not infants and young children consume n-3 fatty acids (FA) according to the recommendations. However based on findings from previous studies, Danish children do not fulfill the recommendations (3;8;9).

In Norway, it is recommended that infants are supplemented with cod liver oil from 4 weeks of age. During the first 6 months of life the amount of cod liver oil should increase gradually from 2.5 to 5 mL per day and from 6 months, the daily supplement should be 5 mL (10). The Norwegian recommendation is primarily based on tradition and on the rationale that formula fed infants benefit from the high contents of DHA in cod liver oil and breastfed infants benefit from the vitamin D content (10). Cod liver oil contains 13.5 g DHA per 100 g (11) and if the Norwegian recommendations are followed, infants will receive ~1 E% from DHA from cod liver oil during the first year of life. However no studies have investigated whether the provided DHA confers beneficial effects on development and health of infants.

In the following sections, I will introduce various determinants of DHA status and give a brief overview of the literature. **Figure 1** gives an overview of the possible determinants of DHA status. The novelty of my PhD project is the inclusion of *FADS* genotype as a possible determinant of DHA status and in section 1.2.6 Genetic variation in *FADS*, I will present an overview of the studies that have investigated associations between *FADS* genotype and LCPUFA status with emphasis on DHA status.

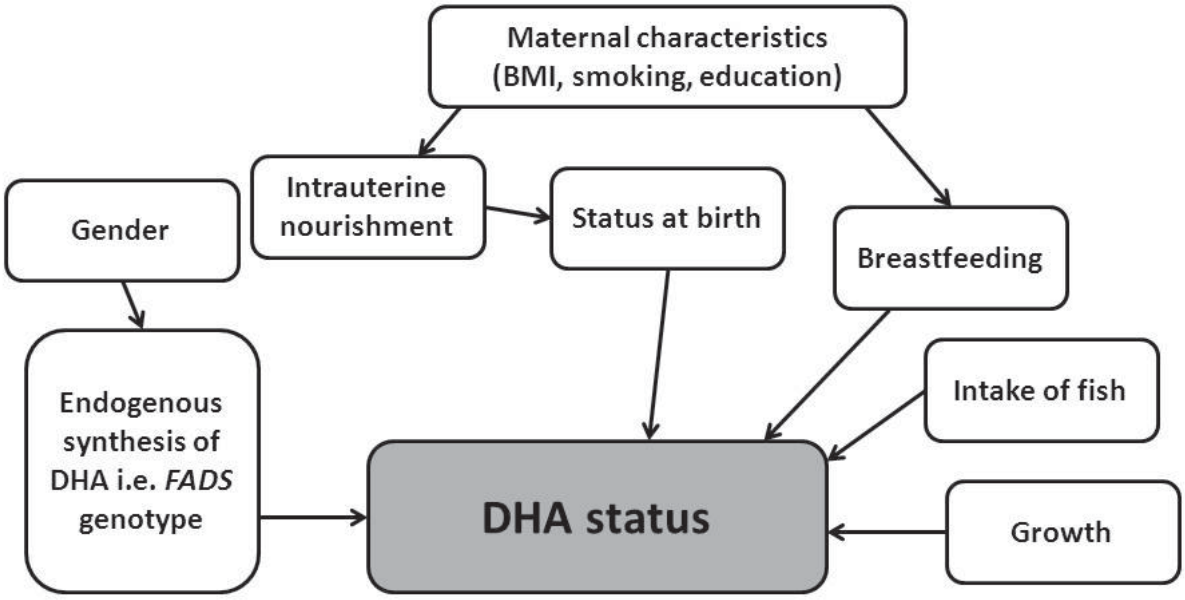

Figure 1 Possible determinants of docosahexaenoic acid (DHA) status of infants and young children

1.2.1 Intrauterine nourishment and status at birth

During pregnancy, the fetus relies on maternal supply of DHA since the fetal capacity for endogenous synthesis of DHA is low (12). Maternal DHA is transferred across the placenta to the fetal blood circulation via specific fatty acid transport proteins and membrane binding proteins and LCPUFA are preferred over PUFA at placental membrane binding sites (12;13). A concentration gradient between maternal and fetal circulation drives the transfer of DHA and a gradual increase in the gradient occurs during pregnancy which ensures an increasing supply of DHA as the fetus grows (12). In line with this, longer gestational age has been shown to result in increased DHA status measured in umbilical cord plasma of newborns (14). As the fetus is supplied with sufficient DHA at increasing amounts during pregnancy, maternal DHA status declines over the course of pregnancy (15) and women who have been pregnant more than once have been shown to have a lower DHA status compared to women who were pregnant for the first time (16). Birth order therefore also affects fetal DHA status with infants of a higher birth order having a lower DHA status at birth (16). Birth weight may be used as a proxy measure of DHA status at birth since higher birth weight is presumed to reflect an increased growth and thereby increased accretion of DHA in utero.

1.2.2 Maternal characteristics

Maternal DHA status is highly influenced by dietary intake (14) and studies where women were supplemented with DHA during pregnancy have shown that their offspring have an increased status at birth (17;18).

Maternal BMI has been shown to be inversely associated with breast milk DHA concentration among Danish mothers (19). This was confirmed in a recent study by Storck *et al* (20) where breast milk from Swedish mothers with a high BMI > 30 kg/m^2 had a lower DHA concentration compared to breast milk from normal weight mothers. Furthermore DHA levels in plasma from their 3-day-old infants were also lower among mothers with a high BMI. However the study also included a group of mothers with a high BMI > 30 kg/m^2 who were intervened to follow healthy dietary advice and increase their physical activity and these mothers did not differ from the normal weight mothers with regards to breast milk and infant DHA levels. BMI per se may therefore not affect DHA levels but rather a high BMI correlates with an unhealthy lifestyle which is low in DHA.

Maternal smoking has been shown to negatively affect DHA status in 4-day-old infants (21;22). Furthermore, a lower increment in breast milk DHA from the first day of lactation and until 3 and 6 months of lactation was found among smoking mothers compared to non-smoking mothers (23). This negative association between maternal smoking and breast milk DHA levels may be explained by reduced DHA synthesis in the mammary gland when exposed to products of cigarette smoke as shown in an *in vitro* study (24).

Maternal educational level and age is associated with duration of breastfeeding. In a systematic review it was concluded that there is strong evidence for early introduction of complementary foods among young mothers and mothers with a low educational level (25). Furthermore it is well-known that a higher level of education is associated with a healthier lifestyle which includes increased consumption of fish and fish products (26;27).

1.2.3 Breastfeeding

The content of DHA in breast milk varies between mothers (28;29) and depends on maternal intake of DHA (19;29). The content of DHA in breast milk varies from a low content of ≤ 0.15 wt% in e.g. USA, Netherlands, and Pakistan to a very high content of >1.0 wt% in Japan and in the Canadian Arctic (30) with the two latter being countries with high consumption of marine foods. In Danish mothers with a fish intake below the 50th percentile, the DHA concentration in breast milk has been measured to 0.30 and 0.41 FA% at two and four months of lactation, respectively and among mothers with a fish intake above the 75th percentile, the DHA in breast milk was measured to 0.6 and 0.74 FA% at the same time points (31). Therefore, the average DHA content among Danish women in general is probably around 0.5 FA%. Although, the DHA concentration in breast milk changes over the course of lactation, Mitoulas *et al* (28) found no significant difference in the amount of DHA consumed by infants during the first 12 months of lactation probably due to varying intake of breast milk over the course of lactation. However, the study sample was relatively small consisting of only 5 mothers. An estimation of the average daily intake of DHA in breast fed infants based on a DHA content in breast milk of 0.5 FA% is 148 mg DHA for an infant consuming 800 mL/d of breast milk, with a fat content of 3.9 g/100 mL (32) and ~95% fatty acids in fat. The optimal intake of DHA during infancy is unknown. Breast milk is considered sufficient in supplying DHA to the infant, but with the large variation in content of DHA in breast milk worldwide, the DHA status of breastfed infants is expected to vary considerably.

The content of DHA in breast milk has also been shown to increase in women who were supplemented with DHA during pregnancy and or lactation (33-36). Furthermore, an increase in DHA concentration in breast milk has been shown to result in an increased DHA status among breastfed infants (37). Gibson *et al* (38) showed a dose-response relation between maternal breast milk DHA concentration in the range 0.21-1.13 FA% (achieved by supplementing the mothers with 5 different doses of DHA during lactation) and infant DHA status measured in red blood cells (RBC) and plasma. However there was no significant difference in infant DHA status at the two highest breast milk DHA concentrations, which indicates that the infant DHA status is saturated at a breast milk DHA concentration of ~0.9 FA% (38). An alternative approach to the fish oil supplementation studies was used in the "Salmon in Pregnancy" study, where 123 women were randomly allocated to two portions of salmon per week or their habitual diet, which was low in oily fish, from week 20 of pregnancy and until delivery (39). This study also found a higher DHA status in the salmon group among the mothers measured in plasma in week 34 and 38 and in umbilical cord plasma at delivery (39).

Several studies have investigated fatty acid status of breastfed infants and infants fed formula. From these studies it is evident that breastfed infants have a higher LCPUFA status compared to infants who have been fed formula only containing the LCPUFA precursors, ALA and LA (5;40-43). The endogenous synthesis of DHA from ALA is thereby not sufficient to bring formula fed infants up to a DHA status comparable to that of breastfed infants. Auestad *et al* (40) showed that infants who are fed formula with DHA and arachidonic acid (AA) have around the same level of DHA and AA in their RBC as breastfed infants. However, it is worth noting that the participating infants were from USA and as mentioned above the content of DHA in breast milk in USA is in the low end worldwide. Based on these findings and with breast milk being considered the golden standard, the majority of infant formulas are now produced to contain DHA and AA to ensure that formula fed infants are sufficiently supplied. However the optimal content of DHA in formula is unknown and to the best of my knowledge the content of DHA in formulas are based on an estimation of the average content in breast milk worldwide such as estimated by Brenna *et al* (30). Therefore in countries with a high DHA content in breast milk, infants fed a formula with DHA may still have a lower status compared to breastfed infants and conversely in countries with a low DHA content in breast milk, infants fed a formula with DHA may have a higher status compared to breastfed infants. In Denmark, the average content of DHA in formula is 8 mg/100 mL. Infants fed formula will therefore on average consume 68 mg DHA per day assuming a formula intake of 800 mL which is only half of what Danish breastfed infants consume.

1.2.4 Fish intake

In Denmark, the complimentary feeding period usually starts sometime between 4 and 6 months of age and lasts until around 9 months of age where the infant's diet is very similar to the family diet with only seasoning and texture prepared appropriate to the infant's developmental stage (6). During the complementary feeding period, DHA supplied via breast milk and formula consumption is still presumed to be an important determinant of DHA status. However, as infants are gradually introduced to a varied diet with solid foods and eventually consume more or less the family diet, the

intake of DHA from other food sources than breast milk and formula is presumed to play an important part in determining DHA status. Currently fish intake is recommended to begin from 6 months of age and should be consumed regularly according to Danish recommendations (6).

In adults, fish and fish products are the major dietary sources of DHA and therefore presumed also to be the major dietary DHA source in young children. However, the amount of DHA in fish varies depending on species, feed and geographical location (44). The content of DHA is high in fatty fish such as mackerel, salmon, and herring with around 1.8 g DHA per 100 g and lower in lean fish such as plaice, tuna, and cod with around 0.2 g DHA per 100 g (11).

In Denmark, there is currently no national data available on the fish intake among young children. However based on previous studies, the median fish intake among 9 months old Danish children is ~5 g/d (3;9). Since Danish children predominantly consume fatty fish the daily intake of DHA from 5 g fish is roughly estimated to be ~100 mg.

1.2.5 Gender

Gender may also theoretically influence DHA status in early life. No studies have investigated gender differences among infants and young children, but women have been shown to have higher endogenous synthesis of DHA from ALA (45) and a higher RBC DHA status has also been shown in teenage girls compared to boys (46). Steer *et al* (47) examined gender differences among 7-year-old children but found equivocal results as girls had higher plasma concentrations of ALA but this did not result in higher levels of plasma eicosapentaenoic acid (EPA) and DHA. Furthermore boys, who were expected to have lower levels of LCPUFA, turned out to have higher level of the DHA precursor, n-3 docosapentaenoic acid (DPA) (47).

The proposed explanations for these gender differences are that estrogen increases Δ^6-desaturase (D6D) activity and that women, possibly due to lower muscle mass, use less ALA as a substrate for β-oxidation which makes more ALA available for conversion to DHA (45). The production of estrogen does not increase in girls before puberty and thus should not differ between girls and boys during infancy. However, boys have an intrauterine testosterone production, which continues during infancy (48) and results from animal studies and studies of trans-sexual humans suggest that testosterone may reduce the endogenous synthesis of DHA possibly by reducing the activity of Δ^5-desaturase (D5D) and D6D (49).

1.2.6 Genetic variation in *FADS* – an overview of the literature

Conversion of ALA to DHA

DHA is synthesized endogenously from ALA through several desaturation and elongation steps. The n-3 FA, ALA and the n-6 FA, LA are metabolized by using the same desaturases and elongases which causes competition between the n-3 and n-6 PUFA families, especially at the rate-limiting D6D step in the initial conversion of ALA and LA to 18:4n-3 and 18:3n-6, respectively (**Figure 2**) (45). The affinity of D6D for ALA is greater than for LA. However, the concentration of LA is typically higher compared to ALA, which ultimately causes a greater conversion of n-6 PUFA (45).

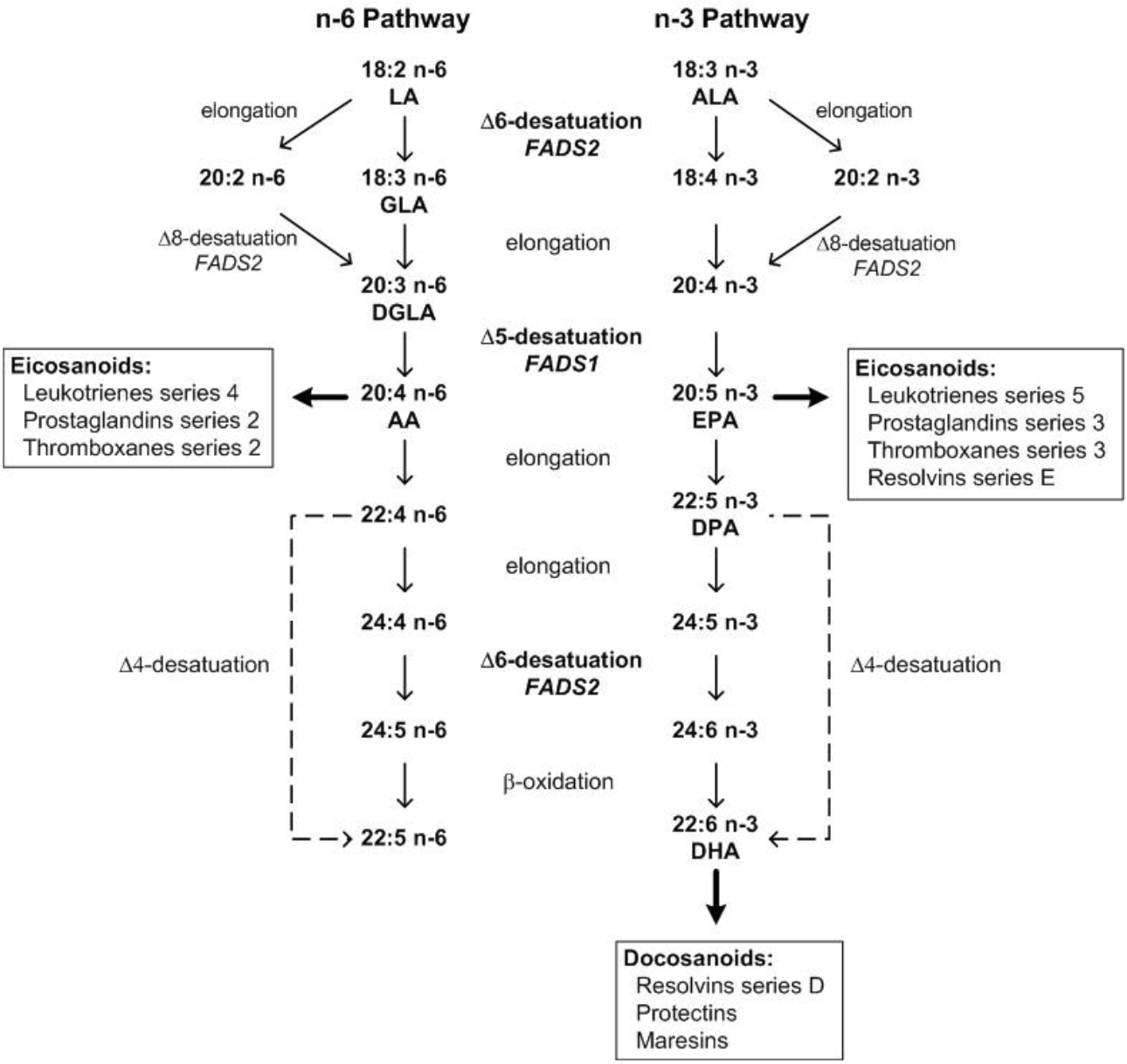

Figure 2 Pathway of n-6 and n-3 long-chain polyunsaturated fatty acid synthesis. Broken lines are routes presently not known to exist in humans. AA: arachidonic acid; ALA: α-linolenic acid, DGLA: dihomo-γ-linoleic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FADS: fatty acid desaturases; GLA: γ-linoleic acid; LA: linoleic acid (Redrawn from references (50-52)).

Competition also occurs between PUFA and LCPUFA. This has been demonstrated by Blank *et al.* (53) who by altering the ratio between ALA and LA in the diet of piglets, showed that the proportions of DHA in tissues was lower at both high and low proportions of ALA. This may be explained by the competition for D6D, which is used when ALA is converted to 18:4n-3 and later in the pathway when 24:5n-3 is converted to 24:6n-3, which following oxidation results in formation of

DHA. The liver is the primary site for conversion of PUFA to LCPUFA and all reactions occur in the endoplasmatic reticulum, however before the last step, 24:6n-3 is translocated to the peroxisomes where DHA is formed by β-oxidation (45;54).

Humans of all ages are capable of DHA biosynthesis (55). However, the activity of the conversion is limited in both women and men (56). Pawlosky *et al* (57) investigated the conversion capacity using stable isotopes in 8 adults (4 men and 4 women) and found that merely 0.2% of plasma ALA was available for synthesis of EPA. On the other hand dietary EPA may be a rather efficient source as 37% of EPA was accessible for production of DHA (57). These findings are supported by animal studies and supplementation studies in humans, which have consistently shown that ALA intake causes a dose-dependent increase in EPA and n-3 DPA whereas there is almost no effect on DHA in plasma and RBC (45;54;55). However Brenna *et al* (30) point out that the duration of ALA intake should be considered since the plasma content of DHA is greater than the content of EPA and consequently it may take a longer time to detect a small contribution of endogenously synthesized DHA.

Studies using administration of ALA labeled with stable isotopes have established that both term and preterm infants can convert ALA to DHA (58;59). This was confirmed in a recent study by Lin *et al* (60) with 11 one-week-old infants, who were given $^{2}H_{5}$-ALA and ^{13}C-U-EPA. Furthermore, it was also shown that endogenously formed EPA compared to externally supplied EPA was more efficiently converted to DHA. But provision of preformed EPA was ~3.5 times more effectively converted to DHA compared to ALA (60). Furthermore, the conversion of ALA to DHA appears to be greater in infants (in the order of 1%) compared to adults (55) and greater in women compared to men (45). According to findings by Pawlosky *et al* (61), women may use a nearly 3-fold greater proportion of n-3 DPA for DHA synthesis compared to men.

The genes *FADS1* and *FADS2* encoding D5D and D6D, respectively are located on chromosome 11 (11q12-q13.1) in a head-to-head orientation along with *FADS3* (62;63). To date numerous single nucleotide polymorphisms (SNPs) in the *FADS* gene cluster have been identified and a selection can be viewed in **Figure 3**. A person's capability of forming LCPUFA has been shown to depend on *FADS* genotype and in the following sections, I will give an overview of the studies investigating associations between SNPs in the *FADS* genes and PUFA composition in RBC, plasma, serum, umbilical cord plasma and breast milk.

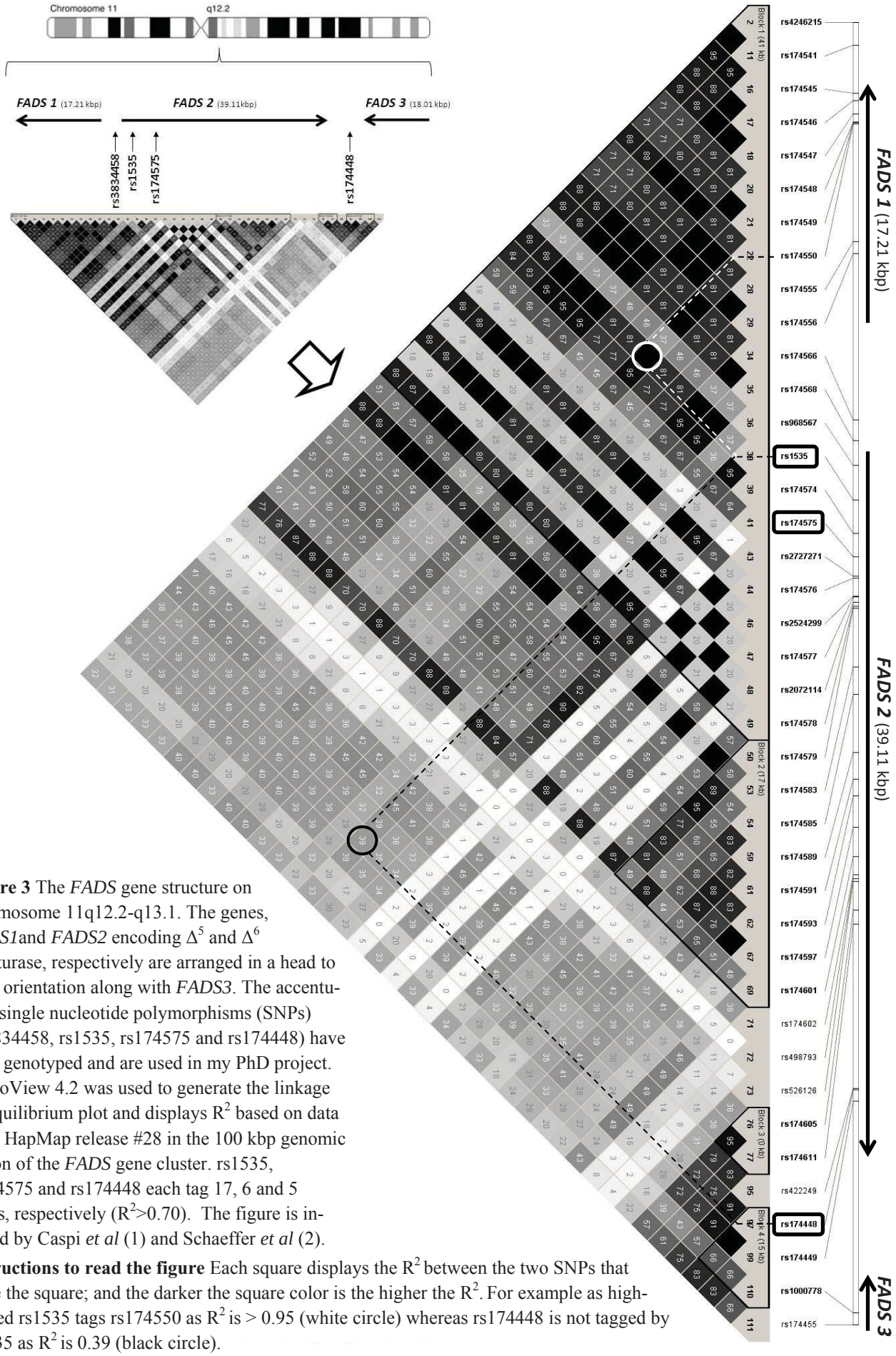

Figure 3 The *FADS* gene structure on chromosome 11q12.2-q13.1. The genes, *FADS1*and *FADS2* encoding Δ^5 and Δ^6 desaturase, respectively are arranged in a head to head orientation along with *FADS3*. The accentuated single nucleotide polymorphisms (SNPs) (rs3834458, rs1535, rs174575 and rs174448) have been genotyped and are used in my PhD project. HaploView 4.2 was used to generate the linkage disequilibrium plot and displays R^2 based on data from HapMap release #28 in the 100 kbp genomic region of the *FADS* gene cluster. rs1535, rs174575 and rs174448 each tag 17, 6 and 5 SNPs, respectively (R^2>0.70). The figure is inspired by Caspi *et al* (1) and Schaeffer *et al* (2).

Instructions to read the figure Each square displays the R^2 between the two SNPs that share the square; and the darker the square color is the higher the R^2. For example as highlighted rs1535 tags rs174550 as R^2 is > 0.95 (white circle) whereas rs174448 is not tagged by rs1535 as R^2 is 0.39 (black circle).

FADS polymorphisms and RBC FA status

Several studies have investigated associations between FA composition in RBC and *FADS* genotype and the overall conclusion is that carriers of the minor alleles of the various *FADS* polymorphisms tend to have a lower RBC content of LCPUFA and increased concentrations of precursor PUFA (**Table 1**). Therefore it seems as though minor allele carriers have lower conversion rates of PUFA which may be due to decreased expression or lower activity of desaturases.

Generally, the studies showed that associations were stronger for n-6 FA than n-3 FA and only the two largest studies (>4000 participants) found a distinct effect of *FADS* genotype on DHA status (47;64). These two studies were based on the same study group of pregnant women with Koletzko *et al* (64) investigating 17 SNPs (including rs3834458 and rs174448) and Steer *et al* (47) two additional SNPs (rs1535 and rs174575). Among 1076 adults, Tanaka *et al* (65) also found lower DHA status among minor allele carriers of rs174537 (located near *FADS1* and tagged by rs1535) and this association tended to be statistically significant (p=0.068).

Dietary intake was only included as a potential confounder in the study by Koletzko *et al* (64) but did not change the results. The information on dietary intake was based on food frequency questionnaires (FFQ) and 81 dietary variables were included in the analyses. Dietary intake of foods with DHA – mainly fish and fish products – affects the RBC DHA status if fish consumption is stable and preferably frequent since mainly the RBC formed on the same day where fish was consumed will be affected (section 2.2 RBC content of DHA as a measure of DHA status). A FFQ provides a very rough estimate of food intake and probably is a good measure of food items consumed infrequently such as fish. But on the other hand healthy foods such as fish tend to be overestimated when recalled and reported in a FFQ. All in all, these studies consistently show that minor allele carriers have higher proportions of precursor PUFA and lower content of LCPUFA in their RBC with stronger associations for n-6 FA.

Table 1. Studies investigating associations between *FADS* polymorphisms and fatty acid status in red blood cells

Reference	Participants and cohort	Diet adjustment	SNPs		Association findings
Steer *et al* 2012 (47)	4342 pregnant women *ALSPAC*	No	**rs1535** **rs174575**		Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA, DHA)
Koletzko *et al* 2011 (64)	4457 pregnant women *ALSPAC*	Yes, FFQ	rs174548 rs174556 rs174561 **rs3834458** rs986567 rs174570 rs174574 rs2727271 rs174576	rs174578 rs174579 rs174602 rs498793 rs526126 **rs174448** rs174449 rs174455	Minor allele carriers of most SNPs (incl. rs3834458 and rs174448): ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA, DHA)
Tanaka *et al* 2009 (65)	1076 adults *GOLDN Study*	No	rs174537		Minor allele carriers: ↑Precursor PUFA (n-6: LA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA, DHA*) *tendency p=0.068
Xie & Innis 2008 (66)	69 women	No	rs174553 s99780* *100% identical frequency distribution	**rs174575** rs174583*	Minor allele carriers: ↑Precursor PUFA (n-6: LA) ↓LCPUFA (n-6: AA)
Martinelli *et al* 2008 (67)	876 adults *Verona Heart Study*	No	rs174545 rs174556 rs174561 **rs3834458** rs174570 rs2524299 rs174583	rs174589 rs498793 rs174611 rs17831757 rs174627 rs1000778	Minor allele carriers of most SNPs (incl. rs3834458): ↑Precursor PUFA (n-6: LA; n-3: ALA) ↓LCPUFA (n-6: AA) ↓Ratio: EPA/ALA

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Rzehak *et al* 2008 (68)	535 adults *Bavarian Nutrition Survey II*	No	rs174556 rs174561 **rs3834458**		Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA) ↓LCPUFA (n-6: AA) Results for n-3 PUFA were in the same direction, but not significant
Malerba *et al* 2008 (69)	658 adults *Verona Heart Study*	No	rs174545 rs174556 rs174561 **rs3834458** rs174570 rs2524299 rs174583	rs174589 rs498793 rs174611 rs17831757 rs174627 rs1000778	Minor allele carriers of most SNPs (incl. rs3834458): ↑Precursor PUFA (n-6: LA (only 3 SNPs)) ↓LCPUFA (n-6: AA)

SNPs highlighted in bold corresponds to the four SNPs I have genotyped as part of my PhD project. Abbreviations: AA: arachidonic acid; ALA: α-linolenic acid, DGLA: dihomo-γ-linoleic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FADS: fatty acid desaturases; FFQ: food frequency questionnaire; LA: linoleic acid; LCPUFA: long chain polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid; SNPs: single nucleotide polymorphisms

FADS polymorphisms and plasma or serum FA status

Associations between *FADS* genotype and FA status measured in plasma or serum were examined in a number of studies. In line with the findings from associations with RBC FA status, the general conclusion from these studies was that carriers of the minor alleles tend to have lower plasma or serum concentrations of LCPUFA and increased concentrations of precursor PUFA (**Table 2**). Furthermore, the associations were stronger for n-6 FA than n-3 FA with the exception of findings from the ALSPAC study, where Steer *et al* (47) found that both child and maternal *FADS* genotype were associated with lower concentrations of EPA and DHA in child plasma at 7 years of age. However maternal genotype was no longer associated with child plasma FA after adjustment for child genotype. Significant associations between *FADS* genotype and plasma DHA were also found among 879 two-year-old children (52) and among 309 women (51). In a genome wide association (GWA) study with 1075 adults, Tanaka *et al* (65) found that rs174537 resulted in the most significant associations with AA.

None of the studies on *FADS* genotype and FA status measured in plasma or serum adjusted for dietary intake of fish with the exception of Moltó-Puigmartí *et al* (51) who found that increased intakes of fish and fish oil were associated with increased levels of plasma DHA regardless of genotype. A high intake of fish and fish oil thereby seems to compensate for being a minor allele carrier (i.e. having a lower capacity for endogenous synthesis of DHA). This supports the notion that adult DHA status is primarily influenced by intake of preformed DHA due to the generally low capacity for endogenous synthesis of DHA in adults (55;56).

Table 2. Studies investigating associations between *FADS* polymorphisms and fatty acid status in plasma or serum.

Reference	Participants and cohort	Diet adjustment	SNPs	Association findings
Steer *et al* 2012 (47)	5240 seven-year-old children *ALSPAC*	No	**rs1535*** **rs174575***	*Associations between child genotype and child plasma* Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA, DHA) *Associations between maternal genotype and child plasma** Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA, DHA) *Not significant when adjusted for child genotype
			*Both maternal and child genotypes were analyzed	
Rzehak *et al* 2010 (52)	879 two-year-old children *KOALA Birth Cohort Study* and *LISA Study*	No	rs174545 rs174546 rs174556 rs174561 **rs3834458**	Minor allele carriers of most SNPs (incl. rs3834458): ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA(only 2 SNPs)) ↓LCPUFA (n-6: AA; n-3: DHA)
Bokor *et al* 2010 (70)	1144 teenagers *HELENA Cross-sectional study*	No	rs174546 rs968567 rs174570 rs174572 rs2072114 rs174589 rs174602 rs498793 rs526126 rs174611 rs174616	Minor allele carriers of most SNPs: ↑Precursor PUFA (n-6: LA, DGLA) ↓LCPUFA (n-6: AA; n-3: EPA(only 3 SNPs))
Moltó-Puigmartí *et al* 2010 (51)	309 women *KOALA Birth Cohort Study*	Yes, FFQ	rs174561 **rs3834458** **rs174575**	Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA) ↓LCPUFA (n-6: AA; n-3: DHA) Plasma DHA increased with increasing fish and fish oil intake irrespective of genotype
Tanaka *et al* 2009 (65)	1075 adults *InCHIANTI Study*	No	GWA Study rs174537 resulted in the most significant associations with AA	Minor allele carriers of rs174537: ↑Precursor PUFA (n-6: LA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA)

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Rzehak *et al* 2008 (68)	163 adults *Bavarian Nutrition Survey II*	No	rs174556 rs174561 **rs3834458**		*Similar results as in RBC (table 1)*
Malerba *et al* 2008 (69)	658 adults *Verona Heart Study*	No	rs174545 rs174556 rs174561 **rs3834458** rs174570 rs2524299 rs174583	rs174589 rs498793 rs174611 rs17831757 rs174627 rs1000778	Minor allele carriers of most SNPs (incl. rs3834458): ↑Precursor PUFA (n-3: ALA (only 3 SNPs)) ↓LCPUFA (n-6: AA)
Schaeffer *et al* 2006 (2)	727 adults *European Community Respiratory Health Survey I*	No	rs174544 rs174545 rs174546 rs174553 rs174556 rs174561 rs174568 **rs3834458** rs968567	rs99780 rs174570 rs2072114 rs174583 rs174589 rs174602 rs526126 rs174620 rs482548	Minor allele carriers of most SNPs (incl. rs3834458): ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: EPA)

SNPs highlighted in bold corresponds to the four SNPs I have genotyped as part of my PhD project. Abbreviations: AA: arachidonic acid; ALA: α-linolenic acid, DGLA: dihomo-γ-linoleic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FADS: fatty acid desaturases; FFQ: food frequency questionnaire; LA: linoleic acid; LCPUFA: long chain polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid; SNPs: single nucleotide polymorphisms

FADS polymorphisms and umbilical cord plasma FA status

The influence of maternal and child *FADS* genotype on FA status in umbilical cord plasma has also been investigated. Lattka *et al* (71) investigated 17 SNPs (including rs3834458 and rs174448) and Steer *et al* (47) two additional SNPs (rs1535 and rs174575) in the same study population. The general conclusion from these studies is also that carriers of the minor alleles are associated with lower concentrations of LCPUFA and increased concentrations of precursor PUFA in umbilical cord plasma (**Table 3**). Interestingly, maternal *FADS* genotype was primarily associated with precursor n-6 PUFA in umbilical cord plasma when adjusted for child genotype; whereas child *FADS* genotype was mainly associated with n-6 LCPUFA in umbilical cord plasma when adjusted for maternal genotype. The associations with precursor n-3 PUFA and n-3 LCPUFA were generally weaker when maternal genotype was adjusted for child genotype and vice versa (47;71). This indicates that for n-6 fatty acids, the newborn child relies on the mother for supply of precursor n-6 PUFA while supply of n-6 LCPUFA comes from endogenous synthesis of the newborn child. Significant associations were also found between *FADS* polymorphisms and DHA in umbilical cord plasma as the level of DHA was associated with both maternal and child *FADS* genotype (47;71).

FADS polymorphisms and breast milk FA status

A few studies have investigated associations between maternal *FADS* genotype and FA status in breast milk (**Table 4**). The first study by Xie and Innis (66) found that women who were minor allele carriers had a lower proportion of AA, EPA and DHA in their breast milk at 1 month postpartum. This is in line with the findings for RBC and plasma. In addition to investigating associations between *FADS* genotype and breast milk FA concentrations, Moltó-Puigmartí *et al* (51) also examined gene-diet interactions. In contrast to the findings in plasma the EPA and DHA concentrations in breast milk increased as expected with fish and fish oil intake but only among major allele carriers who have a normal endogenous capacity for production of n-3 LCPUFA. In other words, a higher fish and fish oil intake did not compensate for the lower DHA content in breast milk among those who had a genetically determined lowered capacity for n-3 LCPUFA production (minor allele carriers). Therefore, women who are minor allele carriers may not benefit from increased n-3 LCPUFA intake as much as women who are major allele carriers (51). Interestingly Lattka *et al* (72) measured FA concentration at two time points during lactation in order to examine whether *FADS* genotype influences the increases and decreases in FA concentration which occur over the course of lactation. Using a rather conservative statistical approach with a significance level of 0.001, only AA was lower among homozygous minor allele carriers at both 1.5 and 6 months postpartum.

Altogether, these three studies come to the same conclusion namely that minor allele carriers have a lower content of LCPUFA in their breast milk and may not benefit from a higher n-3 LCPUFA intake, but more studies are needed to confirm this preferably with multiple measurements of breast milk FA content and inclusion of dietary intake of PUFA and LCPUFA.

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Table 3. Studies investigating associations between maternal and infant *FADS* polymorphisms and fatty acid status in umbilical cord plasma

Reference	Participants and cohort	Diet adjustment	SNPs		Association findings
Lattka *et al* 2013 (71)	3750 **children** 2035 **mother-child pairs** *ALSPAC*	Yes, FFQ	rs174548 rs174556 rs174561 **rs3834458** rs968567 rs174570 rs174574 rs2727271 rs174546	rs174578 rs174579 rs174602 rs498793 rs526126 **rs174448** rs174449 rs174455	*Associations between child genotype and cord plasma* Minor allele carriers of most SNPs (incl. rs174448 and rs3834458): ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: DHA) *Associations between maternal genotype and cord plasma* Minor allele carriers of most SNPs (incl. rs174448 and rs3834458): ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: DHA)
Steer *et al* 2012 (47)	3343 umbilical cord blood samples 4342 women 5240 seven-year-old children *ALSPAC*	No	**rs1535** **rs174575**		*Associations between child genotype and cord plasma* Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA; n-3: ALA) ↓LCPUFA (n-6: AA; n-3: DHA) *Associations between maternal genotype and cord plasma* Minor allele carriers: ↑Precursor PUFA (n-6: LA, DGLA) ↓LCPUFA (n-6: AA; n-3: DHA)

SNPs highlighted in bold corresponds to the four SNPs I have genotyped as part of my PhD project. Abbreviations: AA: arachidonic acid; ALA: α-linolenic acid, DGLA: dihomo-γ-linoleic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FADS: fatty acid desaturases; FFQ: food frequency questionnaire; LA: linoleic acid; LCPUFA: long chain polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid; SNPs: single nucleotide polymorphisms

Table 4. Studies investigating associations between maternal *FADS* polymorphisms and fatty acid status in breast milk

Reference	Participants and cohort	Measuring time point postpartum	Diet adjustment	SNPs		Association findings
Lattka *et al* 2011 (72)	772 women *Ulm Birth Cohort study*	1.5 months (n=772) 6 months (n=463)	No	rs174547 rs174556 rs174602 rs498793	rs526126 rs174626 rs1000778 rs174455	Minor allele carriers : ↓LCPUFA (n-6: AA) ↓Ratio: DGLA/AA No association between *FADS* genotype and time course of FA concentrations during lactation.
Moltó-Puigmartí *et al* 2010 (51)	309 women *KOALA Birth Cohort Study*	1 month	Yes, FFQ	rs174561 **rs3834458** **rs174575**		Minor allele carriers: ↑Precursor PUFA (n-6: LA(rs3834458 only), DGLA) ↓LCPUFA (n-6: AA; n-3: EPA, DHA) EPA and DHA content in breast milk increased with increasing fish and fish oil intake in major allele carriers
Xie & Innis 2008 (66)	54 women	1 month	No	rs174553 rs99780*	rs174575 rs174583*	Minor allele carriers of most SNPs (incl. rs174575): ↓LCPUFA (n-6: AA; n-3: EPA, DHA)
				*100% identical frequency distribution		

SNPs highlighted in bold corresponds to the four SNPs I have genotyped as part of my PhD project. Abbreviations: AA: arachidonic acid; ALA: α-linolenic acid, DGLA: dihomo-γ-linoleic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FADS: fatty acid desaturases; FFQ: food frequency questionnaire; LA: linoleic acid; LCPUFA: long chain polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid; SNPs: single nucleotide polymorphisms

1.2.7 Summary and relevance of our study

In summary, many factors influence infant DHA status. An optimal supply from the mother to the fetus is important during pregnancy and after birth, breastfeeding or intake of formula with DHA is a major determinant of infant DHA status. As the infant is gradually introduced to solid foods, fish intake becomes an important determinant. Furthermore gender and various maternal characteristics such as fish intake, smoking, and BMI have also been shown to influence infant DHA status. No study has investigated all these determinants in the same study population and thereby been able to differentiate the individual degree of influence that these determinants have on infant DHA status.

Numerous studies have investigated associations between *FADS* polymorphisms and RBC, plasma, serum, umbilical cord plasma or human milk FA composition. Overall, the conclusion is that carriers of the minor alleles of *FADS* genotype tend to have a lower content of LCPUFA and increased concentrations of precursor PUFA. None of the studies have investigated associations between *FADS* polymorphisms and RBC DHA status in infancy. Therefore, it is still unknown whether genetic variations in *FADS* have the same influence during infancy as consistently shown in adults. Furthermore only a few studies have adjusted for dietary intake of PUFA and LCPUFA. In infancy the diet is fairly simple consisting of breast milk and formula during the first 6 months of life. Although the diet becomes more and more complex as infants are gradually introduced to the family diet from 6 to 12 months of age, their dietary intake is more easily controlled and parents often know rather precisely what their infant has consumed during a day.

Therefore we found it relevant to investigate potential determinants of infant and young child DHA status, including genetic variation in *FADS*, and our findings are presented in **Paper 1**.

1.3 n-3 LCPUFA and metabolic markers

1.3.1 n-3 LCPUFA, glucose, insulin, growth and body composition

A normal functioning metabolism is a prerequisite for optimal growth and development. This includes a well-functioning glucose homeostasis controlled by insulin and glucagon. Glucose is the primary energy source for cells in the body and thereby important for maintaining cell functions. Insulin has growth promoting effects by controlling tissue accretion of carbohydrate and lipid and as a key regulator of carbohydrate and lipid metabolism. Thus insulin plays a significant role during periods of growth. Furthermore insulin can function as a growth factor both directly by binding to the insulin-like growth factor-1 (IGF-1) receptor and indirectly by stimulating hepatic release of IGF-1 (73;74).

Effects of n-3 LCPUFA on growth has been investigated in numerous studies and in two Cochrane reviews, it was concluded that LCPUFA supplementation of formula fed to both term and preterm infant has no clear beneficial or harmful effects on growth measured as weight, length and head circumference throughout the first three years of life (75;76). In another Cochrane review, Delgado-Noguera *et al* (77) concluded that LCPUFA supplementation to breastfeeding mothers also had no clear effects on growth. However, breastfed infants of LCPUFA supplemented mothers were shorter and had a larger head circumference beyond two years of life. However, the effect size with regards to length was relatively small with a mean difference of merely -0.75 cm (95% CI: -1.38 to -0.12). In all three Cochrane reviews results from several different studies were pooled and only group differences were investigated without considering dose and duration of LCPUFA supplementation which varied between the included studies. Furthermore, in many of the studies a mix of DHA and AA supplementations were used and results from these studies were pooled with the studies investigating DHA supplementation alone which made it difficult to distinguish between specific effects of DHA and AA.

Besides being related to growth, insulin also has important metabolic effects. Intake of n-3 LCPUFA has consistently been shown to beneficially affect insulin sensitivity in rodents (78). Furthermore, mice fed n-3 PUFA have been shown to gain less weight and adipose tissue compared to mice fed n-6 PUFA (79). This led Ailhaud and coworkers to hypothesize that the increasing intake of n-6 PUFA that has occurred in Western Countries over the past decades may be an important factor in the development of obesity and the metabolic syndrome (80). No effect of n-3 LCPUFA on insulin sensitivity and markers of glucose metabolism has been observed in human RCTs with fish oil among adults (81;82). Few randomized controlled trials have investigated effects of LCPUFA on adiposity in infants (9;83). Andersen *et al* (9) found no difference in weight, length or BMI z-scores among 133 infants who had been randomized to n-3 LCPUFA or n-6 PUFA from 9 to 18 months of age. However, the n-3 LCPUFA supplemented infants had a lower triceps/subscapular skinfold ratio compared to the n-6 PUFA supplemented infants. In a study where women were randomized to n-3 LCPUFA or no supplementation during pregnancy and until 4 months of lactation, Hauner *et al* (83) found no difference in fat mass estimated by skinfold thickness among 165 infants during their first year of life.

Summary and potential mechanisms

All in all it remains to be clarified whether n-3 LCPUFA affects glucose, and insulin in childhood. Some of the effects of n-3 LCPUFA on insulin sensitivity may be explained through the actions of peroxisome proliferator-activated receptors (PPARs). There are three different types of PPARs: PPAR-α, PPAR-β/δ and PPAR-γ (84). They are nuclear receptors and function as ligand-inducible transcription factors controlling expression of numerous genes involved in maintenance of metabolic homeostasis, lipid metabolism, adipogenesis and inflammation (84). PPAR-γ is found in two isoforms PPAR-γ1 and PPAR-γ2 with the latter primarily expressed in adipose tissue where it regulates adipogenesis, lipid metabolism and insulin sensitivity (84;85). Both eicosanoids and free n-3 LCPUFA can act as natural ligands for PPARs (86). Activation of PPAR-γ has been shown to enhance the ability of adipose tissue to store lipids through various target genes and this reduces accumulation of fat in muscle and liver tissues and thereby improves insulin sensitivity (85;87). Furthermore activated PPAR-γ stimulates the production of adiponectin which increases oxidation of FA and improves insulin sensitivity in muscle and liver (87). Other mechanisms by which PPAR-γ activation can affect insulin sensitivity involves leptin, tumor necrosis factor (TNF)-α and resistin (87).

1.3.2 n-3 LCPUFA and lipid profile

There is convincing evidence for a dose-dependent TAG-lowering effect of n-3 LCPUFA both in adults with hyperlipidemia (88) and among healthy adults (89). On the other hand it was concluded in a Cochrane review that n-3 LCPUFA appears to have no effect on total or HDL cholesterol but raises LDL cholesterol (90).

To my knowledge only one previous randomized intervention study has investigated whether fish oil supplementation affects lipid profile in infants (3). In the study by Damsgaard *et al* (3), 83 infants were randomized to receive a daily supplement from 9 to 12 months of age of either 5 mL fish oil (+FO) or no supplement (–FO) in a 2×2 factorial design that also included randomization to two different milk types. Plasma TAG decreased during the intervention in the +FO group but there was no significant difference between the +FO group and –FO group. However TAG was negatively associated with RBC EPA at 12 months of age, indicating that intake of EPA had a TAG-lowering effect. The lack of a significant difference between the groups may be ascribed to lack of power, since there were only ~20 infants in each group in the adjusted analysis. Plasma total and LDL cholesterol were higher in the +FO group than in the –FO group at 12 months (3). However, since fat intake was unbalanced in the two groups the effects on total and LDL cholesterol could be unrelated to fish oil intake as such, and may be caused by the higher fat intake in the +FO group.

In a longitudinal observation study by Thorsdottir *et al* (91), higher consumption of PUFA between 9 and 12 months of age was associated with higher total cholesterol at 12 months among 103 Icelandic infants. Additionally cod liver oil consumption was positively associated with total and LDL cholesterol among girls at 12 months of age (91). The effects on LDL cholesterol in infants based on these two studies are in accordance with findings in adults (90). Based on data from two prospective cohort studies with 2006 children, Standl *et al* (92) found that a higher n-3 PUFA intake

was associated with higher total, LDL and HDL cholesterol and lower TAG levels at 10 years of age.

FADS polymorphisms and lipid profile

During the past few years several studies have explored whether lipid profile is associated with *FADS* genotypes (**Table 5**). In a GWA study, *FADS* polymorphisms were found to be associated with HDL and TAG. The major allele of rs174547 (normal capability of endogenous LCPUFA synthesis) was associated with higher HDL and lower TAG (93). By including *FADS* polymorphism in the analysis of possible associations between n-3 PUFA intake and lipid profile it is possible to link any observed significant associations with the capability of endogenous synthesis of n-3 LCPUFA according to *FADS* genotypes. Overall the conclusion seems to be that those who have a low capability of synthesizing n-3 (and n-6) LCPUFA i.e. minor allele carriers (both homozygous (mm) and heterozygous (Mm)) have lower total, LDL and HDL cholesterol and higher TAG compared to those who have a normal capability of synthesizing n-3 LCPUFA (homozygous major allele carriers (MM). These findings are in accordance with the TAG lowering effects of n-3 LCPUFA.

Two studies have been done among 2-year-old children (94) and 10-year-old children (92) and based on these studies, minor allele carriers seem to have lower total, LDL and HDL cholesterol and possibly also higher TAG compared to major allele carriers, as was seen among adults. Furthermore, Standl *et al* (92) investigated potential interaction between *FADS* genotypes and n-3 PUFA intake but were unable to find such an interaction. However interactions between genotype and n-3 PUFA or n-3 LCPUFA intake have been observed in the following studies.

Dumont *et al* (95) only observed significant associations between *FADS* genotype and total cholesterol and non-HDL cholesterol among adolescents who had a high intake of ALA (>1.4 g/d). Therefore those who had a normal capability of n-3 LCPUFA synthesis and the highest amount of available precursor PUFA (and thereby the theoretically highest potential for endogenous synthesis of n-3 LCPUFA) had a higher total cholesterol and non-HDL cholesterol compared to those with a lower amount of available precursor PUFA (ALA intake ≤ 1.4 g/d). The findings are in accordance with those of Lu *et al* (96) who observed more pronounced associations between genetic variation in *FADS* and total cholesterol at high intakes of n-3 PUFA (≥0.5 E%.) and significant association with non-HDL were only observed at high intakes of n-3 PUFA (≥0.5E%). In line with this, Hellstrand *et al* (97) also found significant interaction between *FADS* genotype and n-3 LCPUFA intake as LDL was lower among those who had the lowest intake of n-3 LCPUFA (≤0.14 E%) and were minor allele carriers. This indicates that lower n-3 LCPUFA (both as intake and endogenously synthesized) is associated with lower LDL. Combined these three studies suggest that increased n-3 LCPUFA (both as intake and endogenously synthesized) is associated with increased cholesterol. To some extent this is in accordance with the finding that n-3 LCPUFA appears to raise LDL as concluded in the previously mentioned Cochrane review (90).

Table 5 Overview of studies investigating associations between *FADS* genotype and lipid profile

Reference	Participants and cohort	SNPs	Association findings	Diet	Interaction with dietary PUFA intake
Children and adolescents					
Moltó-Puigmatí *et al* 2013 (94)	521 Two-year-old children *KOALA Birth Cohort*	rs174545† rs174546† rs174556 rs174561 **rs3834458†**	**mm *versus* MM:** ↓Total cholesterol ↓HDL cholesterol (only †SNPs) **Mm *versus* MM:** ↓Total cholesterol	No information about diet.	Not investigated
Standl *et al* 2012 (92)	2006 Ten-year-old children *GINIplus and LISAplus*	rs174545‡ rs174546‡ rs174556†‡ rs174561†‡ **rs3834458** **rs174575**	**mm *versus* MM:** ↓Total cholesterol (only †SNPs) ↓LDL cholesterol (only †SNPs) **Mm *versus* MM:** ↑TAG ↓HDL cholesterol (only ‡SNPs)	FFQ	No interaction with n-3 PUFA intake.
Dumont *et al* 2011 (95)	573 adolescents *HELENA study*	rs174546	**mm+Mm *versus* MM** ↓Total cholesterol§ ↓non-HDL cholesterol§ §Only at high intakes of ALA >1.4g/d	Two 24 hour recalls	Yes, significant associations were only observed at high intakes of ALA >1.4g/d.
Adults					
Hellstrand *et al* 2012 (97)	4635 Adults	rs174547	**mm *versus* MM** ↓LDL cholesterol **mm *versus* MM:** ↑TAG	168 item dietary questionnaire, 7 day menu book and 1h diet history inter-view	Yes, among mm: lower LDL was observed only within the lowest tertile of n-3 LCPUFA intake (≤ 0.14 E%). Among mm and mM: lower HDL was observed with-in the lowest and mid-tertile of ALA/LA intake.
Lu *et al* 2010 (96)	3575 Adults *Doetinchem Co-hort Study*	rs174546† rs482548 rs174570	**mm *versus* MM** ↓Total cholesterol (only †SNPs) ↓non-HDL cholesterol†§ §Only at high intakes of n-3 PUFA ≥0.5E%	FFQ	Yes, the association with total cholesterol was more pronounced at high intakes of n-3 PUFA ≥0.5E%. Significant association with non-HDL were only observed at high intakes of n-3 PUFA ≥0.5E%
Tanaka *et al* 2009 (65)	2142 Adults *InCHIANTI & GOLDN Study*	rs174537	**mm *versus* MM:** ↓Total cholesterol ↓LDL cholesterol		Not investigated

SNPs highlighted in bold corresponds to the four SNPs I have genotyped as part of my PhD project. Abbreviations: ALA: α-linolenic acid; FADS: fatty acid desaturases; FFQ: food frequency questionnaire; LA: linoleic acid; LCPUFA: long chain polyunsaturated fatty acid; MM: homozygous major allele carriers; Mm: heterozygotes; mm: homozygous minor allele carriers, PUFA: polyunsaturated fatty acid; SNPs: single nucleotide polymorphisms

Only one intervention study by Cormier *et al* (98) has investigated whether supplementation with n-3 LCPUFA could influence associations between *FADS* genotype of 17 SNPs and plasma TAG. In the study 208 adults were supplemented with 3 g/d of n-3 LCPUFA (1.9 g EPA and 1.1 g DHA) for 6 weeks but no effect modification by *FADS* genotype on plasma TAG was observed. However *FADS* genotype (rs174546) and the n-3 LCPUFA supplementation independently resulted in the same effect size on TAG.

Summary and potential mechanisms

All in all, it seems as though n-3 LCPUFA affects lipid profile in infants and children in the same way as observed in adults, but more randomized controlled intervention studies are needed to support findings by Damsgaard *et al* (3). Whether the effect is modified by *FADS* polymorphisms or by polymorphisms in genes encoding proteins (e.g. PPARs) involved in the mechanisms behind the effect remains to be clarified. The mechanisms by which n-3 LCPUFA may reduce plasma TAG is at the gene transcriptional level (99) and includes 4 nuclear receptors that regulates TAG synthesis in liver cells: the liver X receptor, hepatocyte nuclear factor-4α, farnesol X receptor, and PPARs (99). As previously mentioned both n-3 LCPUFA and eicosanoids can activate PPARs, which then modulates gene transcription by binding in a heterodimer with the retinoic X receptor to a specific DNA sequence, the peroxisome proliferator responsive element (100). Activation of PPAR-γ promotes lipid uptake and storage in adipose tissue (85). Upregulation of fatty acid oxidation via PPARα and down-regulation of fatty acid biosynthesis, through inhibition of sterol regulatory element-binding protein (SREBP)-1c in the liver and skeletal muscle may explain the TAG lowering effects of n-3 LCPUFA (99).

1.3.3 n-3 LCPUFA, blood pressure and heart rate

In the most recent meta-analysis by Campbell *et al* (101) comprising 17 studies (15 parallel RCTs and two crossover trials), it was found that fish oil supplementation for a minimum of 8 weeks significantly reduced systolic blood pressure (SBP) and diastolic blood pressure (DBP) among hypertensive adults and among normotensive adults although the latter not significantly. This is in accordance with two earlier meta-analyses (102;103). Campbell *et al* (101) also performed a meta-regression analysis which resulted in a non-significant dose-response association where SBP decreased by 0.16 and 0.01 mmHg per additional 1 g of fish oil among hypertensive and normotensive adults, respectively. The authors conclude that no clear dose-response association was observed between fish oil supplementation and BP and suggest that the BP lowering effects of fish oil may be found at relatively low doses of fish oil.

To my knowledge, the randomized intervention study by Damsgaard *et al* (3) is the only study that has investigated whether fish oil supplementation affects BP in infants. In addition one other study investigating fish oil supplementation among teenagers with BP as a primary outcome has been published (104). Furthermore, several follow-up studies of effects of fish oil supplementation in early life (pregnancy, lactation, infancy and up to 5 years of age) on BP in later childhood and young adulthood has been conducted (8;105-108). An overview of the studies is presented in **Table 6**.

Table 6 Overview of intervention studies investigating the impact of fish oil supplementation on blood pressure

Reference	Participants	n	Study design	Intervention	Dose of n-3 LCPUFA	Control	Duration	Findings	Dose-response	Effect
Studies with blood pressure as primary outcome										
Damsgaard *et al* 2006 (3)	9 months old infants	83	Randomized intervention	5 mL/d of FO from 9 to 12 months of age	~1g EPA and ~0.7g DHA per day	No FO	3 months	↓SBP (by 6.3 mmHg) in the FO group	No association with RBC-EPA	Yes
Pedersen *et al* 2010 (104)	13-15 year-old boys	78	Randomized controlled double blinded intervention	Bread with FO or vegetable oil	~0.2g EPA and ~0.8g DHA per day	Vegetable oil (6:1:1 mix of palm shortening, soy oil and rapeseed oil)	16 weeks	↓SBP (by 3.8 mmHg) and ↓DBP (by 2.6 mmHg) in the FO group	Yes association with RBC-EPA	Yes
Follow-up studies of FO intervention early in life with later investigation of blood pressure										
Larnkjær *et al* 2006 (8)	2.5 year-old children[†]	98	Randomized controlled double blinded intervention	Maternal FO supplementation among women with a low fish intake (<0.4 g n-3 LCPUFA/d)	1.5 g n-3 LCPUFA per day	Maternal olive oil supplementation and a reference group of mothers with a high fish intake (>0.8 g n-3 LCPUFA/d)	The first 4 months after delivery	No difference among the groups	No association with RBC-LCPUFA measured at 4 months	No
Forsyth *et al* 2003 (105)	6 year-old children	235	Randomized controlled intervention	147 newborn infants randomized to formula with or without LCPUFA	Formula with 0.15-0.25 g DHA per 100 g of fat	Formula without n-3 LCPUFA and a reference group who were breastfed	The first 4 months of life	Formula with *vs.* without LCPUFA ↓DBP (by 3.6 mmHg) ↓MAP (by 3 mmHg) Breastfed *vs.* formula without LCPUFA ↓DBP (by 3.4 mmHg) Breastfed *vs.* formula with LCPUFA No difference	NA	Yes

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Asserhøj *et al* 2009 (106)	7 year-old children[†]	98	Randomized controlled double blinded intervention	Maternal FO supplementation among women with a low fish intake (<0.4 g n-3 LCPUFA/d)	1.5 g n-3 LCPUFA per day	Maternal olive oil supplementation and a reference group of mothers with a high fish intake (>0.8 g n-3 LCPUFA/d)	The first 4 months after delivery	Boys; FO *vs.* olive oil ↑DBP (by 6.4 mmHg) ↑MAP (by 6.6 mmHg) Girls; FO vs. olive oil No difference	No association with RBC LCPUFA measured at 4 months	Yes
Ayer *et al* 2009 (107)	8 year-old children	616	Randomized controlled intervention	Active intervention to increase n-3 and decrease n-6 PUFA (n-3/n-6-ratio of 1:5) or control (n-3/n-6-ratio of 1:15-20)	Tuna oil capsules providing ~0.9 g n-3 FA per day and oils and spreads low in n-6 PUFA	Sunola oil capsules (mostly MUFA) and oils and spreads containing 40% n-6 PUFA	The first 5 years of life	No difference among the groups	No association with plasma LCPUFA measured at 18 months, 3, 5 and 8 y	No
Rytter *et al* 2012 (108)	19 year-old offspring	180	Randomized controlled intervention	FO or olive oil during the last trimester of pregnancy	2.7 g n-3 LCPUFA per day	Olive oil	Last trimester of pregnancy	No difference among the groups	NA	No

[†]Same study population. Abbreviations: DBP: diastolic blood pressure; FO: fish oil; LCPUFA: long chain polyunsaturated fatty acid; MAP: mean arterial pressure; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acid; SBP: systolic blood pressure.

The two intervention studies investigating acute effects after 3-4 months of fish oil supplementation in infants and teenagers both show a lower SBP and DBP (teenagers only) in the fish oil group compared to the control group (3;104). Follow-up studies of children and young adults who had been intervened early in life or whose mothers had been supplemented during pregnancy or lactation provide inconsistent results. Forsyth *et al* (105) found lower DBP and mean arterial pressure (MAP) among 6 year-olds, who had received formula supplemented with LCPUFA during the first four months of life compared to children who had received formula without LCPUFA. A follow-up of 7 year-olds showed a higher DBP and MAP among boys of mothers who had been supplemented with fish oil compared to olive oil during the first 4 months of lactation (106). No difference in BP was observed among the same children at 2.5 years of age (8). Likewise no difference in BP was seen among 19 year-olds whose mothers had been supplemented with either fish oil or olive oil during the third trimester of pregnancy (108). No effects on BP was observed at 8 years of age among children who had been assigned to an increased intake of n-3 FA (and decreased n-6 FA) or a control diet from introduction of complementary foods and until 5 years of age (107).

The findings from intervention studies are in contrast to results from observational studies where n-3 LCPUFA status is positively associated with BP. In the recent OPUS School Meal Pilot Study with 73 8-11 year-old school children, an increased n-3 LCPUFA status was associated with increased MAP, but only among the boys. A high RBC DHA status was also associated with increased SBP among 109 Danish teenagers (46). In a recent Dutch study, they were unable to find associations between DHA status and BP among 973 12 year-olds (109). However, the RBC DHA content in the Dutch 12 year-olds was rather low compared to the Danish teenagers (1.4±0.8 *versus* 5.0±1.2 (boys) and 5.8±1.0 (girls), respectively).

In a meta-analysis by Mozaffarian *et al* (110) comprising 30 trials (22 parallel RCT and eight cross-over trials), fish oil supplementation has been shown to reduce heart rate (HR) particularly among adults with a higher baseline HR and longer duration of fish oil supplementation. In infants, formula with DHA and AA have been shown to reduced HR (111;112) and in the previously mentioned study where infants were supplemented with fish oil from 9 to 12 months of age, the mean RR interval was 6% longer (equivalent to a lower HR) among FO-supplemented boys (113).

Summary and potential mechanisms

All together it seems as though n-3 LCPUFA may have acute BP lowering effects in infants and teenagers, but more randomized controlled intervention studies are needed to support these findings. A possible mechanism by which n-3 LCPUFA may affect BP is through conversion by cyclooxygenase-2 (COX-2) to eicosanoids with vasodilatory effects. The EPA-derived eicosanoids, such as the vasocontrictory thromboxane A, are generally less potent than the corresponding eicosanoids formed from AA (114). Those who have increased COX-2 activity may therefore produce more of the less potent eicosanoids when the intake of n-3 LCPUFA is increased since more EPA becomes available relative to AA. It is also possible that the increase in EPA from fish oil affects the competition for D6D so that less AA is synthesized from LA since D6D primarily will be used to covert EPA to DHA. This will cause a lower synthesis of the eicosanoids produced from AA including thromboxane A_2 which is a more potent vasoconstrictor compared to EPA-derived vasoconstrictors

(114). Another potential mechanism behind effects of n-3 LCPUFA on BP includes increased nitric oxide production which may result from changes in the membrane fatty acid composition that has been shown to modify raft association and function of endothelial nitric oxide synthase (115). Both of these mechanisms result in reduced systemic vascular resistance which causes lower blood pressure. Effects on BP may also lead to change in HR and furthermore effects of n-3 LCPUFA on HR may be related to incorporation of DHA into myocardial cells membranes where it modulates ion channels (116;117) and thereby may affect heart rate (110). Furthermore n-3 LCPUFA may affect both BP and HR through effects in the autonomic nervous system possibly via altered production of neurotransmitters (118).

1.3.4 Summary and relevance of our study

The evidence for effects of n-3 LCPUFA on metabolic markers in young children is limited due to lack of randomized controlled intervention studies and whether n-3 LCPUFA affects glucose and insulin in childhood remains to be clarified. Based on the few available studies, n-3 LCPUFA may have an effect on lipid profile and BP in infants and children as is seen among adults but the only study by Damsgaard *et al* (3), that has investigated this did not provide a control oil and it is therefore not possible to determine whether the observed effect where due to the PUFA content of fish oil or in fact the n-3 LCPUFA content of FO.

In addition to confirming these results it would be interesting to explore whether *FADS* genotype modifies the effects in a randomized controlled trial and whether polymorphisms of genes encoding proteins involved in the mechanisms behind the effect (such as *PPARG2* and *COX2*) can support the findings. Therefore we attempted to explore whether polymorphisms in *PPARG2* and *COX2* could modify the effects of n-3 LCPUFA on metabolic markers in an intervention study. In our study infants were supplemented with n-3 LCPUFA or n-6 PUFA from 9 to 18 months of age and the findings are presented in **Paper 2**.

1.4 n-3 LCPUFA and immune modulation

The immune system is very complex and describing it thoroughly in detail is beyond the scope of my thesis. I will briefly introduce the immune maturation which occurs in early childhood and review the studies that have investigated effects of n-3 LCPUFA on immune function in infants and children. The novelty of my PhD project is the inclusion of genotypes of genes involved in the mechanisms behind the effects of n-3 LCPUFA on immune modulation and I will therefore describe the alleged mechanisms and argue for the choice of genes used in our study.

1.4.1 Immune maturation

The immune system is developed and begins maturation already in utero. Infants are born with an immature immune system which continues to mature especially during early infancy (119). At birth, infants are capable of immune responses with components of both the innate and adaptive immune systems. However their immune responses are characterized by a reduced ability to produce cyto-

kines compared to adults (120) and their T-lymphocyte functions are poorly developed and dominated by Th2 cells (119;121).

The two types of Th-polarization are characterized by different cytokine production. Th1 cells produce high levels of interleukin (IL)-2 and interferon (IFN)-γ and both of these promote a cell-mediated immune response which characterizes the adaptive immune system (121) and IFN-γ furthermore inhibit induction of Th2 cells. Th2 cells produce IL-4, IL-5, IL-10, IL-13 and these promote a humoral response (121) and furthermore, IL-4 inhibit induction of Th1 cells. Secretion of IL-4 by Th2 cells induces production of immunoglobulin (Ig)-E (122) which play an important role in type 1 hypersensitivity reactions seen in e.g. atopic dermatitis and asthma (123). During early childhood the immune system matures as the capacity to produce IL-12 increases with age (124). IL-12 is required during the initial phases of Th1 polarization and for maintenance of IFN-γ production (125). If the immune system fails to down-regulate the Th2 dominance, e.g. by expansion of the Th1 population, then the risk of developing allergic diseases is increased (119).

1.4.2 Immuno-modulatory effects of n-3 LCPUFA

Breast milk contains several immunological components such as macrophages, lymphocytes, and cytokines, which may influence development and maturation of immune cells in breastfed infants (126). Furthermore Field *et al* (127) have shown that preterm infants fed breast milk or preterm infant formula with LCPUFA experience a faster immune maturation compared to preterm infants fed formula without LCPUFA during the first 6 weeks of life. This was seen as similar higher levels of IL-10 and lymphocyte populations among breastfed preterm infants and preterm infants fed formula with LCPUFA at 6 weeks of age than in preterm infants fed formula without LCPUFA (127). Similar findings were also observed by Field *et al* (128) among term infants during the first 6 weeks of life as infants who were fed formula with LCPUFA had a TNF-α production and lymphocyte distribution comparable to that of breastfed infants but different from infants fed formula without LCPUFA. This indicates that LCPUFA may have immuno-modulatory effects in early infancy.

To the best of my knowledge only one study has specifically aimed at investigating whether fish oil supplementation affects immune response and immune maturation by measuring *ex vivo* cytokine production in infancy (129). In a randomized intervention study infants were allocated to fish oil supplements or no supplements from 9 to 12 months of age and 64 infants (of the 83 completers) had plasma C-reactive protein and IgE measured as well as *ex vivo* cytokine production of several cytokines. The fish oil supplemented infants had a higher production of IFN-γ at 12 months compared to the non-supplemented infants. This indicates that n-3 LCPUFA may cause a faster immune maturation (129). However the study is limited by the small sample size, no use of control oil and the infants were only supplemented for 3 months, which only allows for measurements of short-term acute effects.

n-3 LCPUFA, allergy and illness

Many studies have investigated potential effects of n-3 LCPUFA on development of allergic diseases in infants and children. In a recent systematic review including 20 observational studies and 5

intervention studies, Kremmyda *et al* (130) concluded that results are inconclusive and more evidence is needed to clarify the implications of n-3 LCPUFA on childhood allergy and to establish whether the observed effects persist.

In addition to the studies examining effects of n-3 LCPUFA on allergy, several studies have investigated effects of n-3 LCPUFA on prevalence of respiratory illness and respiratory-related hospitalization rates among infants and children. US toddlers (mean age ~26 months) who had been supplemented with 130 mg DHA per day for 60 days had a lower number of respiratory events compared to infants who were not supplemented with DHA (131). However, in a large randomized controlled trial with 657 preterm infants who were allocated to either high DHA (1%) or standard DHA (0.3%) from the first week of life and until term equivalent, there was no difference between the two groups in the number of hospitalizations reported by parents during the first 18 months of life calculated from term equivalent (132). Lower relative risk of admission to neonatal intensive care was found among 726 children whose mothers had been supplemented with fish oil (800 mg DHA) or vegetable oil daily from the 19th week of gestation and until birth (133). However, as pointed out by the authors, this may have been driven by the lower prevalence of very preterm births in the FO-group.

The effects of n-3 LCPUFA on illness among school children have also been investigated. In a study with 180 Thai school children, it was found that those who consumed milk fortified with fish oil (1g DHA and 200 mg EPA) for 5 days a week for 6 months experienced fewer days and shorter duration of illness compared to school children consuming placebo milk (134). The lower prevalence of illness among the fish oil supplemented school children may be due to an improved immune function but this was not clearly verified by the cytokine findings as plasma IL-6 tended to decrease in the fish oil group while plasma IL-10 was not affected by either fish oil or placebo (134). In a study with 598 Indian school children, fewer episodes of respiratory illness were also observed among those who had received foods with 100 mg DHA plus 900 mg ALA for 1 year compared to children receiving food with 140 mg ALA (135). In a study with 133 Indonesian school children it was found that those who were given a fish oil supplement daily (650 mg DHA and 100 mg EPA) for 3 months had fewer days of inability to attend school compared with controls receiving soy oil (136). The reason for absence from school was not noted during the study but may be due to increased prevalence of illness in the control group. These studies indicate that n-3 LCPUFA may decrease the prevalence of illness – possibly respiratory illness in particular. But more studies are needed for confirmation of these findings and to find the optimal dose of n-3 LCPUFA since the dose varied from 0.1 to 1.2 g.

Potential mechanisms

The mechanisms by which n-3 LCPUFA may affect immune response and immune maturation remain to be fully clarified. Studies have shown that increased intake of n-3 LCPUFA resulted in an increased content of EPA and DHA in blood immune cells. The increased content of EPA and DHA in immune cells was mostly at the expense of AA and correlated with the amounts consumed (137). The altered LCPUFA composition of membrane phospholipids of immune cells results in increased

production of n-3 LCPUFA derived bioactive lipid mediators such as prostaglandins (PGs), leukotrienes (LTs), lipoxins, resolvins, protectins and maresins (Figure 2) which influence the immune response (137;138). COX-2 is induced in activated macrophages and other cells at inflammation sites and plays an important role in initiating inflammation through production of eicosanoids derived from AA. Additionally COX-2 is essential in resolution of inflammation by a shift towards the production of n-3 LCPUFA-derived anti-inflammatory mediators. As previously mentioned, EPA is a substrate for COX-2 as wells as lipoxygenase in the formation of PGs and LTs of the 3- and 5-series which are less potent compared to the 2- and 4-series derived from AA and therefore dampens inflammation (139). EPA and DHA are also precursors for E- and D-series resolvins, respectively via acetylated COX-2 and DHA is a precursor for the newly discovered protectins and maresins (138). All these anti-inflammatory mediators play an important role in the regulation of inflammatory responses and are produced to resolve inflammation, which is necessary to limit tissue damage and prevent chronic inflammatory conditions (138). Some of their actions include reducing chemotaxis and transendothelial migration in addition to decreasing activation of neutrophil granulocytes and down-regulating cell adhesion molecules on immune cells. Furthermore they inhibit formation and actions of pro-inflammatory mediators (138).

Regulatory T cells (Treg) are highly involved in suppression of immune responses including functions of Th1, Th17 and Th2 cells. Treg cells may be induced from naïve T cells through the action of transforming growth factor (TGF)-β. However in the presence of IL-6 and TGF-β, Th17 cells are induced, which produces the pro-inflammatory cytokine IL-17 as well as IL-6 and TNF-α (140;141). Thus, IL-6 may overrule Treg differentiation induced by TGF-β (141). Therefore IL-6 seems to play an important role in balancing the differentiation of Treg and Th17 (141). Differentiation of Tregs has been shown to be induced by n-3 LCPUFA via PPAR-γ activation (142). PPAR-γ is expressed in immune cells where is has been shown to down-regulate the expression of inflammatory cytokines such as IL-6 and TNF-α (143).

Furthermore it has been hypothesized that the anti-inflammatory effects of n-3 LCPUFA could be due to reduced nuclear factor (NF) κB activity. The transcription factor, NFκB induces expression of several immune components involved in inflammation including a number of cytokines (e.g. TNF-α) and COX-2 (144). NFκB is activated by phosphorylation and subsequently dissociation of its inhibitory subunit, IκB, allowing for translocation of NFκB to the nucleus (145). n-3 LCPUFA seem to be able to decrease phosphorylation of IκB resulting in decreased NFκB activity (146). There may also be interaction between NFκB and PPAR-γ as activated PPAR-γ may prevent NFκB from translocation to the nucleus and thereby n-3 LCPUFA could prevent upregulation of NFκB target genes by activating PPAR-γ (144).

1.4.3 Summary and relevance of our study

n-3 LCPUFA seems to play a role in immune maturation, but this is only based on a few studies in early and late infancy. More studies are needed especially to confirm the findings in late infancy by Damsgaard *et al* (129). The evidence for effects of n-3 LCPUFA on childhood allergy and illness

also remain inconclusive and more studies are therefore needed to fully clarify the implications of the observed effects of n-3 LCPUFA.

Furthermore I propose that inclusion of genotypes of genes involved in the mechanisms behind the effects of n-3 LCPUFA (such as *PPARG2, COX2* and *NFKB1*) may support the findings and provide evidence of possible mechanisms of action by identifying the involved pathways and genes.

Therefore we attempted to explore whether polymorphisms in *PPARG2, COX2* and *NFKB1* could modify the effects of n-3 LCPUFA on immune maturation and function in an intervention study. In our study infants were supplemented with n-3 LCPUFA or n-6 PUFA from 9 to 18 months of age and our findings are presented in **Paper 3**.