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EFECTOS DE LA SUPLEMENTACIÓN CON ACEITE DE PESCADO Y/O 5-METILTETRAHIDROFOLATO DURANTE EL EMBARAZO SOBRE EL PERFIL MATERNO-FETAL DE ÁCIDOS GRASOS Y EL DESARROLLO NEUROLÓGICO DE LOS NIÑOS: Estudio NUHEAL Follow-up

Effects of fish oil and/or 5-methyltetrahydrofolate supplementation during pregnancy on materno-fetal fatty acid profile and neurological development of the children: : The NUHEAL Follow-up Study

TESIS DOCTORAL

M. VICTORIA ESCOLANO MARGARIT

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M. Victoria Escolano Margarit

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Mª Victoria Escolano Margarit

Esta Tesis Doctoral ha sido dirigida por:

Prof. Dra. Dña. Cristina Campoy Folgoso
Departamento de Pediatría
Universidad de Granada. (España)

Prof. Dr. D. Berthold V. Koletzko
Department of Paediatrics.
Nutrition and Metabolism Division
Ludwig Maximilians Universität of München (Germany)

Prof. Dr. D. Támas Decsi
Department of Paediatrics
University of Pécs (Hungary)

Dña. Cristina Campoy Folgoso, Profesora del Departamento de Pediatría de la Universidad de Granada.

D. B.V. Koletzko, Profesor del Departamento de Pediatría de la Universidad LMU de Munich.

D. T. Decsi, Profesor del Departamento de Pediatría de la Universidad de Pécs.

CERTIFICAN: Que los trabajos de investigación que se exponen en la Memoria de Tesis Doctoral: **"EFECTOS DE LA SUPLEMENTACIÓN CON ACEITE DE PESCADO Y/O 5-METILTETRAHIDROFOLATO DURANTE EL EMBARAZO SOBRE EL PERFIL MATERNO-FETAL DE ÁCIDOS GRASOS Y EL DESARROLLO NEUROLÓGICO DE SUS HIJOS",** han sido realizados en el Departamento de Pediatría de la Universidad de Granada, correspondiendo fielmente a los resultados obtenidos. La presente Memoria ha sido revisada por los abajo firmantes, encontrándola conforme para ser defendida y aspirar al grado de Doctor Europeus en Medicina.

Y para que conste, en cumplimiento de las disposiciones vigentes, extendemos el presente en el mes de Mayo de 2005.

Prof. Dra. Dña. C. Campoy Folgoso

Prof. Dr. D. B.V. Koletzko **Prof. Dr. T. Decsi**

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Prof. Cristina Campoy Folgoso

Responsable de la Universidad de Granada de los proyectos NUHEAL y EARNEST

Departamento de Pediatría

Facultad de Medicina

Universidad de Granada

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BACKGROUND

This study is part of a larger multicenter study undertaken to investigate the effects of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) supplementation during pregnancy on pregnancy and pregnancy outcome. The study reported herein is part of the NUHEAL and the NUHEAL follow up European studies which have been carried out with partial financial support from the Commission of the European Communities, specific RTD Programme "Quality of Life and Management of Living Resources", within the 5th Framework Programme (Contract No. QLK1-CT-1999-00888 NUHEAL [*Nutraceuticals for a healthier life*]) and within the 6th Framework programme (Priority 5.4.3.1. Food quality and safety. Contract No. 007036-EARNEST Project [***EAR****ly* ***N****utrition programming-long term follow up of* ***E****fficacy and* ***S****afety* ***T****rials and integrated epidemiological, genetic, animal, consumer and economic research*]). This manuscript does not necessarily reflect the views of the Commission and in no way anticipates the future policy in this area. Some data from the NUHEAL cohort have already been analysed and published.

Franke et al. (1) analysed the dietary intake of docosahexaenoic acid (DHA) and folate in pregnant women in the three participating countries and concluded that dietary DHA and folate intake of pregnant women differs significantly across the three European samples. Only 7% of the participants reached the recommended folate intake during pregnancy, whereas nearly 90% reached the DHA recommended intake of 200 mg per day.

Preliminary data on erythrocyte membrane fatty acid composition after fish oil (FO) and/or folate supplementation from the 20th week gestation to delivery showed a significant enhancement of erythrocyte DHA in maternal, placental and venous cord blood lipids (2). The effects of FO and/or 5-Metyltetrhydrofolate (5-MTHF) supplementation during pregnancy on plasma lipids have also been reported. Krauss-Etschmann et al. (3) conclude that FO supplementation improves fetal DHA and eicosapentaenoic acid (EPA) levels in plasma phospholipids (PLs) and attenuates depletion of maternal stores.

A study in the Spanish cohort showed higher DHA increments in the FO compared to the FO+5-MTHF group in plasma PLs, on the contrary the increment was higher in the FO+5-MTHF in the trygliceride lipid fraction. Thus 5-MTHF may contribute to a preferential DHA incorporation into triglycerides than into PLs. However, supplementation with folate alone did not prove to enhance maternal DHA proportions (4).

Regarding infants neurological development, results on the Spanish cohort did not show differences between groups. Supplementation did not modify the latencies, amplitudes or visual acuity determined by cortical visual evoked potentials at 2 months of age. Furthermore, no differences between supplementation groups were found in performance in the Bayley Scale of Infant Development (BSID) but children with higher DHA levels in cord blood PLs showed better scores in the test.

1 INTRODUCTION

1.1 Metabolic programming

The term "metabolic programming" refers to the "fetal origin of adult diseases". It denotes the concept that "stimulus or insult operating at a critical or sensitive period of development could result in a long-standing or life-long effect on the structure or function of the organism"; this hypothesis associates the risk of adult diseases with a suboptimal in utero nutritional environment (5). It postulates that environmental factors, particularly nutrition, act in early life and program a number of organ structures and functions during embryonic and fetal life which are related with the risk for adverse health outcomes in adult life (6-8). Nutritional programming has been demonstrated convincingly in animals. The animal data show that brief periods of early dietary manipulation have lifelong effects on neurodevelopment and other health outcomes like metabolism, blood lipids, atherosclerosis, obesity, diabetes, blood pressure, increased in plasma insulin and cholesterol, which would be of considerable public health significance if they applied to humans. Human epidemiological associations have been found between malnutrition and reduced cognitive performance; furthermore, retrospective observations showed a relationship between adult disease and size in early life (9-13). However, these retrospective data cannot prove nutritional cause. Alan Lucas and co-workers provided some of the first experimental data on the sensitivity of humans to nutritional programming. They reported that a brief period of early dietary management had a major impact on later neurodevelopment and IQ, pattern of allergic reactions and atopy, waist-hip ratio, linear growth, and bone mineralisation (14). Long term follow up of these experimental studies show that

nutrition in early life has a major impact on health into early adulthood, notably on cardiovascular disease risk, bone health and cognitive function (5). These new findings have major biological, social and medical implications and should increasingly underpin health practices. These findings also justified investment in the study of the fundamental biological mechanisms involved. Possible biological mechanisms for storing throughout life the "memory" of early nutritional experience and its expression in adulthood include adaptive changes in gene expression, preferential clonal selection of adapted cells in programmed tissues and programmed differential proliferation of tissue cell types (5;15).

With the exception of special groups, pregnant women in Europe can easily meet their increased energy and protein requirements. However, requirements of a number of micronutrients are far higher than the increases of energy and protein needs and maternal dietary intake may not meet requirements. There has been in the last years growing interest in investigating the role of these micronutrients in promoting fetal growth and development. There are periods during perinatal development in which specific nutrients are required for optimal development, and there is growing evidence that optimal dietary intake of these nutrients, which includes iodine, DHA, choline and folate, among others, is important.

Epigenetics, that is the interindividual variation in DNA methylation patterns and chromatin remodelling, provide a potential explanation for how environmental factors can modify the risk for development of many common diseases. Epigenetic marking on genes can determine whether or not genes are expressed. That permits the fetus and the infant to adapt gene expression to the environment in which it is growing. If this adjustment goes awry, the risk of chronic disease is increased. Recent progress in the understanding of nutritional influences on epigenetics suggests that nutrients that are part of methyl-group metabolism can significantly influence epigenetics. During critical periods in development, dietary methyl-group intake

(choline, methionine, and folate) can alter DNA and histone methylation, which results in lifelong changes in gene expression (16-19).

Concern should also be given to the common genetic variations existing among individuals which may influence nutrient requirements. Though humans share the same genes, there are many individual variations (single nucleotide polymorphism; SNPs) in the codon sequences for these genes. Most humans have at least 50,000 SNPs across their genes. Over ten million SNPs exist that occur in more than 1% of the population and some common SNPs occur in >50% of the population. Some of these SNPs result either in alteration of regulation of gene expression or in changes in the gene product so that protein structure and function are altered, thereby altering metabolism and cell function. Based on this genetic variation, individuals may require different amounts of nutrients to achieve comparable biological effects (20;21). Genes regulating the metabolism of both, folate and LC-PUFA, have been reported to be polymorphic. Recent studies have shown that genetic variants of fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS2) influence blood lipid, erythrocyte membrane and breast milk n-6 and n-3 fatty acids in pregnancy and lactation (22-24). Genes regulating the metabolism of folate are also polymorphic.

Further human studies should be conducted for a better understanding of how diet may influence pregnancy and pregnancy outcome and whether the diet is particularly low in some nutrients. We also need to understand how genetic variations among individuals influence nutrient requirements during these periods and whether supplementation of the diet in expecting women with particular nutrients may or not improve fetal development.

1.2 Long Chain Polyunsaturated Fatty Acids (LC-PUFA)

1.2.1 Metabolism

There are two families of essential fatty acids (EFA), the n-3 and the n-6 families. Mammals including humans are unable to synthesize fatty acids with double bounds in n-3 or the n-6 carbon because neither ω-3 nor Δ12 desaturase are present in mammals. Thus, the parent EFA of the n-6 family, linoleic acid (LA; 18:2n-6), and that of the n-3 family, α-linolenic acid (ALA; 18:3n-3), must be obtained from the diet, which is the reason why these fatty acids are known to be essential fatty acids. These precursor polyunsaturated fatty acids (PUFAs) can be desaturated and elongated in the human body to longer chain, more unsaturated fatty acids, the LC-PUFAs. LA is this way converted to arachidonic acid (AA; 20:4n-6) and ALA is converted to eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). This metabolic interconversion of fatty acids occurs primarily in the liver in the endoplasmic reticulum and involves a series of elongation enzymes that sequentially add 2 carbon units to the fatty acid and desaturation enzymes that insert double bounds into the molecules. Three desaturases, Δ9, Δ6 and Δ5, are present in humans. Δ9 catalyzes the synthesis of monounsaturated fatty acids. Oleic acid, a main product of Δ9 desaturase, is the major fatty acid in mammalian adipose triglycerides, and is also used for phospholipid and cholesteryl ester synthesis. Δ6 and Δ5 desaturases are required for the synthesis of polyunsaturated fatty acids (PUFAs), which are mainly esterified into phospholipids and contribute to maintaining membrane fluidity. These enzymes are known to be the key enzymes of this pathway and are expressed in a majority of human tissues, with the highest levels in liver but also with major amounts in brain, heart and lung. The synthesis of DHA (22:6n-3) shares a pathway with AA (20:4n-6) up to the EPA (20:5n-3) step. EPA (20:5n-3) is afterwards elongated to tetracosapentaenoic acid (24:5n-3) and then desaturated to tetracosahexaenoic (24:6n-

3). Recent studies suggest that this final desaturation in the synthesis of DHA (22:6n-3) may be catalyzed by the same Δ6 desaturase that catalyzes the first step. Tetracosahexaenoic (24:6n-3) is then retroconverted to DHA (22:6n-3), it has been suggested this last step of the DHA (22:6 n-3) syntheses occurs by means of *β*-oxidation in peroxisomes. A direct transformation of DPA (22:5n-3) to DHA (22:6n-3) is not possible in humans, probably due to the absence of Δ4 desaturase. The final step in the synthesis of DHA (22:6n-3) is therefore more complex than that for AA (20:4n-6) as it requires a translocation to the peroxisomes for a β oxidation reaction (25;26) (Fig. 1).

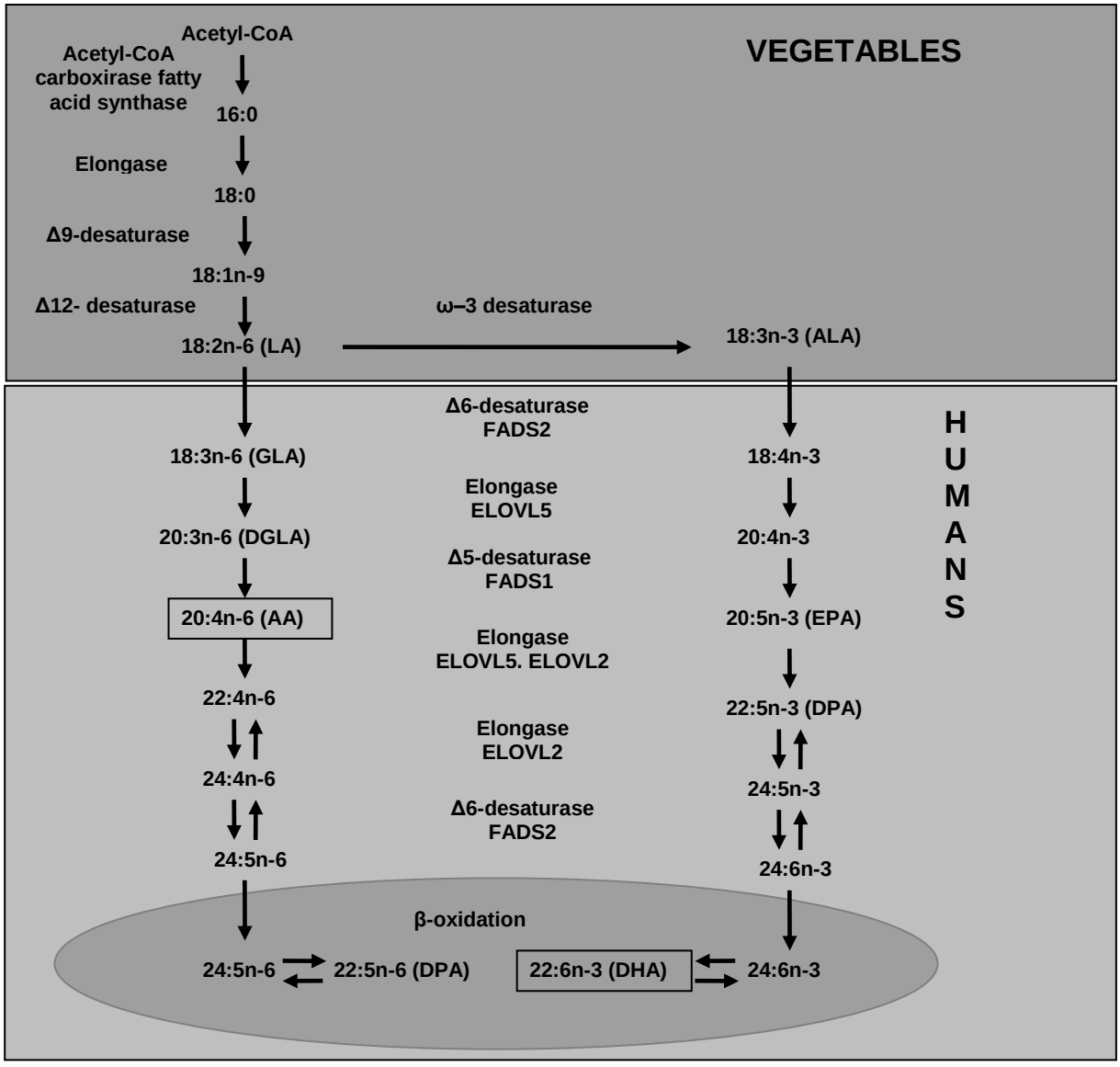

Fig1. Metabolic conversion of the essential fatty acids to long chain polyunsaturated fatty acids (Modified from Koletzko et al. J Perinat Med 2008).

The activity of desaturases is sensitive to nutritional, metabolic and hormonal regulation. Enzymes of the endogenous chain elongation and desaturation system show a higher affinity to n-3 PUFA as compared with n-6 PUFA; however, due to a much higher proportion of n-6 PUFA in the diet, more AA than EPA is formed (23). Because the same enzymes are involved in the n-3 and n-6 series interconversion, there is a competitive inhibition of desaturases that control the conversion of EFA into LC-PUFA. Background diet can, thereby, influence the conversion of these fatty acids. Thus, an excess of certain fatty acids may inhibit the synthesis of certain LC-PUFA that could be essential for fetal growth. The limiting step in the conversion of ALA to DHA is the conversion of DPA to DHA. An excessive consume of DHA and EPA rich oils have shown to inhibit the Δ5 and Δ6-desaturase activity, which is responsible for a lower plasma AA. Besides, an excessive dietary intake of LA inhibits the Δ6-desaturase through substrate competition, as a result formation of DHA decreases by 40%. Paradoxically, diets rich in ALA appear to increase the rate of ALA oxidation, limiting its accumulation in plasma and reducing its conversion rate to EPA and DHA. In addition, DHA itself also serves as a substrate for metabolic retroconversion to EPA and DPA through a β-oxidation process. Retroconversion rate has been calculated to be of 1,4% in subjects receiving normal dietary DHA intakes and up to 12% in subjects receiving high chronic DHA consumption (25-27).

Moreover, LC-PUFA synthesis is under strong feedback regulation. LC-PUFAs in serum PLs in healthy adults are maintained in a narrow range despite differences in intake of precursor PUFAs. Δ3 and Δ6 desaturases are fully induced only in EFA deficient conditions, and are suppressed when adequate precursor PUFAs are supplied from the diet, indicating that the capacity of endogenous synthetic pathway is sufficient to meet the requirement of LC-PUFAs in healthy adults. However, dietary LC-PUFA supplementation may become necessary for certain populations. Supplying LC-PUFAs from diets means bypassing the regulation of the endogenous synthetic pathway, and poses potential problems. First, dietary LC-PUFAs markedly

change the n-6/n-3 LC-PUFA ratio in PLs because n-6 and n-3 LC-PUFAs compete for the esterification to PLs. Second, one group of dietary LC-PUFAs would shut down the synthetic pathway shared by both n-3 and n-6 fatty acids, exacerbating the imbalance of n-6/n-3 LC-PUFAs. This indicates that a balanced supply of dietary n-3 and n-6 fatty acids is important to meet the requirement of both n-3 and n-6 LC-PUFAs (26).

Desaturases in mammals are regulated by induction of the enzyme at the transcriptional level. Desaturases share a common mechanism of a feedback regulation to maintain products in membrane phospholipids. Combinations of multiple transcription factors achieve this sophisticated differential regulation. Two transcription factors, sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator activated receptor-α (PPARα), play a key role in the regulation of desaturases by PUFAs. In liver, SREBP-1c activates entire genes of fatty acid synthesis and metabolism, including all three desaturases and, at the same time, PUFAs suppress the target gene transcription by reducing the active form of SREBP-1c. PPARα is a transcription factor of the nuclear receptor family. Binding of a ligand causes a conformational change to PPARα, which then forms a heterodimer with retinoid X receptor and activates transcription of target genes by binding peroxisome proliferator response element (PPRE) located in promoter regions of the targets. Nonesterified LC-PUFAs are considered endogenous ligands of PPARα that induces genes for fatty acid oxidation. However, PPARα also directly activates the Δ6-desaturase gene and plays a crucial role in the feedback regulation of LC-PUFA synthesis. Available data suggest that these two mutually antagonistic transcription factors (SREBP-1c and PPARα) act as sensors of LC-PUFA status and together mediate feedback regulation of LC-PUFA synthesis (26).

Although the metabolic pathways to synthesize LC-PUFA from EFA exist in the human body, the metabolic conversion rate of ALA to DHA is low (25) and as a

result, the contribution of endogenous DHA synthesis to overall DHA content in human body is limited. Studies of the in vivo conversion of ALA to EPA, DPA and DHA showed that more than 15-35% of dietary ALA is oxidized for energy, and that only a small proportion, estimated to be <1%, is converted to DHA. The fractional conversion of ALA to EPA varies between 0.3% and 8% and the conversion of ALA to DHA is <4% in men. This conversion seems to be more efficient in women with a reduction in the rate of ALA oxidation (22% compared with 33% in men). Approximately 21% of ALA is converted to EPA and up to 9% is converted to DHA in women. Moreover, there is considerable variability in the conversion rates between individuals, even when they are consuming similar diets. Common genetic variations in the genes encoding fatty acid desaturases (FADS1 and FADS2) are thought to be responsible for these inter-individual variations in the ability to metabolise ALA and LA to DHA and AA and influence blood lipid fatty acid concentrations (25;27).

FADS1 and FADS2 are localized as a cluster, with FADS1 and FADS2 oriented head to head, exon 1 of the genes separated by an 11-kb region on chromosome 11 (11q12-q13.1), and both FADS1 and FADS2 containing 12 exons and 11 introns (22;26). There are 18 SNPs reported in public databases in the FADS1 FADS2 cluster being polymorphic (24) (Fig. 2). Recent studies have shown that single nucleotide polymorphisms (SNP) in the FADS1 FADS2 cluster are associated with differences in (n-6) and (n-3) fatty acids in plasma and erythrocyte membrane lipids in gestation. Schaeffer et al. (24) observed that the minor alleles of the SNPs rs174544, rs174553, rs174556, rs174561, rs3834458, rs99780 and rs174583 were mostly associated with a decrease in the level of desaturase products while accumulating the precursors in plasma PLs. When compared with the homozygotes for the major alleles, the changes in the desaturases substrates and products of the homozygotes for the minor alleles were twice as high as the changes in the heterozygotes. This could be an indication for a decline in the transcriptional levels or in the conversion rates of the desaturases

in those subjects with minor alleles of the SNPs. However, other fatty acids in serum phospholipids belonging to other fatty acid pathways like oleic acid and DHA, which is mainly derived from dietary intake, did not show any significant associations with the genetic variants. Rzehak et al. (23) observed a similar association between the minor alleles of the SNPs rs174544, rs174553, rs174556, rs174561 and rs3834458 and fatty acid levels in erythrocyte membrane PLs. They found a higher proportion of the substrates and a lower proportion of the products of desaturases in the n-6 series in plasma phospholipids and erythrocyte membrane. They also noted similar but less pronounced effects on n-3 PUFA that did not reach statistical significance. Xie et al. (22) conducted the first study on the effects of SNP in the FADS1 FADS2 cluster on PUFA during pregnancy and provided data showing higher LA and ALA and lower levels of their products in plasma and erythrocyte PE PLs, in minor allele homozygotes of rs174553, rs99780, and rs174583. They also provided evidence that beast milk fatty acids were influenced by genotype, with significantly lower 14:0, ARA, and EPA but higher 20:2n-6 in the minor allele homozygotes of rs174553, rs99780, and rs174583 and lower ARA, EPA, 22:5n-3, and DHA in the minor allele homozygotes of rs174575. Although experimental data are missing, these data indicate a lower activity of desaturases in carriers of the minor allele haplotype compared to those carrying the major allele haplotype.

Although PUFA levels in human blood have been significantly related to PUFA dietary intake, the contribution of the liver desaturase-elongase enzyme system in seems likely on the basis of the effects of the genetic variation in FADS1 and FADS2 genes on the composition of PUFA observed. The extent to which maternal SNP in FADS1 and FADS2 interact with the maternal dietary fatty acid composition need to be considered in addressing those fatty acid requirements that best support human growth and development.

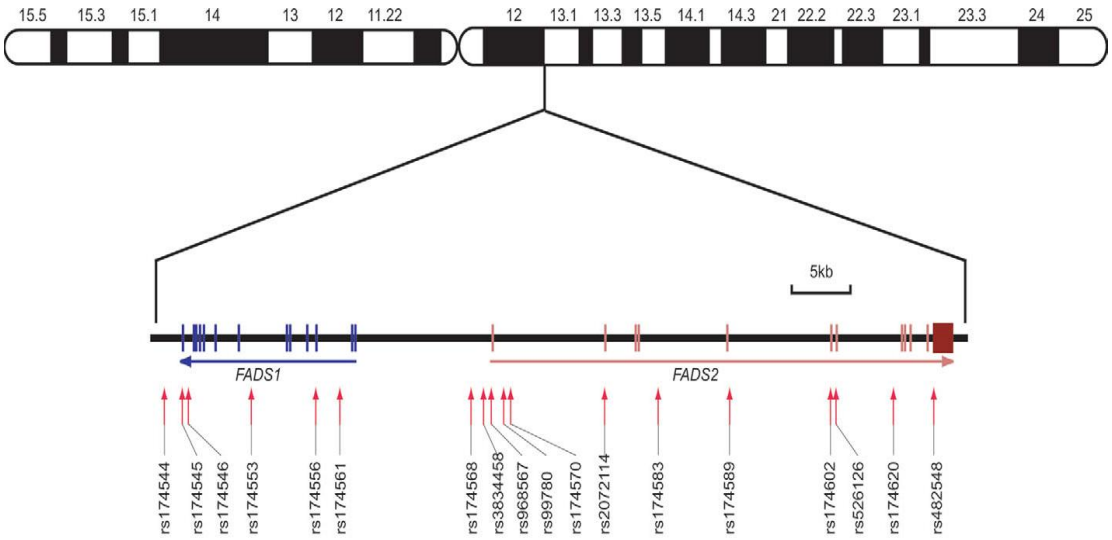

Fig 2. Structure of the FADS1 FADS2 gene cluster (Schaeffer et al. Human Molecular Genetics 2004)

Animal studies showed that the precursors 18:2n-6 and 18:3n-3 are readily stored in adipose triglycerides (TGs), which work as a reservoir of EFAs. In humans, fatty acid composition in adipose tissue reflects dietary fatty acids. In contrast to MUFAs and precursor PUFAs, LC-PUFAs are poor substrates for TG synthesis, and mainly incorporated into PLs, contributing to maintenance of membrane fluidity. However, maintaining membrane fluidity is not the main function of LC-PUFAs in mammals. LC-PUFAs are required for many other functions, such as eicosanoid signalling, pinocytosis, ion channel modulation, and regulation of gene expression (26).

As LC-PUFA can be synthesized in human tissues from their precursors they are not considered strictly essential. However, LC-PUFA perform essential metabolic and structural functions and it is uncertain whether there is sufficient enzymatic capacity to satisfy the high demands during fetal and early postnatal development. As LC-PUFAs in the fetus proceed mainly form the mother, they must be incorporated in the maternal diet especially during pregnancy when LC-PUFA demands are enhanced.

1.2.2 Biological functions

LC-PUFAs have important structural and metabolic functions in the human body. The two major LC-PUFA synthesised by the Δ5 and Δ6 desaturases pathway are AA and DHA. They are both essential constituents of structural lipids in membranes and play an important role in maintaining their permeability and fluidity. Fatty acids in cell membranes are incorporated primarily in PLs, sphingolipids and plasmalogens. The most abundant n-3 fatty acid in membranes is DHA, it is present in all organs but it is particularly abundant in neural tissues such us the brain and retina where, in addition to its structural function, it also plays an important functional role. The role of DHA in the central nervous system (CNS) will be further discussed in another chapter of the present review. AA is also relative abundant and is widely distributed in all tissues, although its distribution differs from that of the DHA. Other fatty acids like EPA or ALA are also present in membrane lipidic structures but only in small quantities. The relative proportion of fatty acids in cell membranes varies depending on fatty acid availability and influences their biophysical properties (fluidity, permeability, thickness) which may also affect the activity of transmembrane proteins and thereby the overall cell function. In addition, differences in the membrane's microenvironment might furthermore affect binding characteristics of embedded proteins or other interacting molecules. LC-PUFA may modulate this way membrane receptors activity, transport of substances, enzymes and other membrane-bound biochemical activities (25;28;29).

While LC-PUFA may be required for cold tolerance in plants and fish, the primary role of LC-PUFA in mammals is cell signalling. AA is the main precursor for eicosanoids, prostaglandins, tromboxanes and leukotriens. In many tissues and cell types AA is sterified to the sn-2 position of membrane PLs and this is how AA is stored in the cells. AA is upon stimulation released from PLs by phospholipase A2 and then enzimatically transformed to eicosanoids and other antiinflamatory

molecules. Eicosanoids work as autocrine/paracrine hormones and mediate a variety of localized reactions, such as inflammation, haemostasis, and protection of digestive tract epithelium (26). Other metabolically important LC-PUFA are EPA and the dihomogammalinolenic acid (DGLA; 18:3n-6) which can also be used as prostanoids or leukotrienes precursors (25;30;31). These molecules derived from LC-PUFA play a key role in modulating inflammation, immune response and the allergic phenomenon as well as platelet aggregation, thrombosis and vascular reactivity (28).

There are several mechanisms by which n-3 LC-PUFA influence immunological responses. The first of them is mediated by the competition between n-6 and n-3 LC-PUFA as substrateds for cyclooxygenase and lipoxygenase enzymes for the production of eicosanoids. The increment of n-3 LC-PUFA in membrane PLs leads to decrease of AA derived eicosanoids and leukotrienes (PGE_2 and LTB4) and an increase of the less biologically active 3-series prostaglandins (PGE_3) and 5-series leukotriens (LTB5). In addition to proinflammatory effects, PGE2 regulates cellular immune responses through distinct receptors in different cell populations. PGE_2 inhibit T-cell proliferation and exerts effect on the Th1/Th2 balance. It decreases the production of the Th1-type cytokines interferon (IFNγ) and interleukin-2 (IL-2), enhances the production of Th2-type cytokines IL-4 and IL-5. It also promotes IgE synthesis by B cells regulate antigen presenting cell functions. As membrane constituent modulates membrane structure and function. N-3 LC-PUFA alters lipid rafts in T-cell receptors which inhibits the T-cell response to the antigen-presenting cell. In addition, n-3 LC-PUFAs also regulate the immune and inflammatory response by their direct action on gene expression through their union to nuclear receptors and modification of the transcription factors activity. The n-3 LC-PUFA decrease the activity of the nuclear factor-kB (NF-kB) which plays a role in inducing a range of inflammatory genes including COX-2, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, tumor necrosis factor-a (TNFa), IL-1b, inducible nitric oxide synthase (iNOS), acute phase protein, in

response to inflammatory stimuli. Furthermore, n-3 LC-PUFAs are natural ligands of nuclear receptors such as peroxisome proliferator activated receptors PPAR-α and $-\gamma$ which are present in a variety of cell types including inflammatory cells (32-34).

In addition to the PPAR, fatty acids have also been reported to be ligands for other nuclear receptors such as retinoid X receptor, HNF4 and SREBP which are powerful transcription factors for several genes and can therefore, influence gene regulation which coordinates the expression and repression of enzymes involved in metabolism, thermoregulation, energy partitioning, growth and differentiation and inflammatory responses (26;28).

1.2.3 Biomarkers for the assessment of LC-PUFA status in humans

Given the importance of LC-PUFA in fetal development, crescent interest has been given in the last years to maternal fatty acid status and the best way to determine it. Fatty acids can be measured in various blood fractions and tissues (35;36). Adipose tissue has been demonstrated to correlate well with long-term fat intake; however the availability of adipose tissue limits its use in studies (36-39). Instead blood is more readily available and widely used for this purpose. It is generally accepted that LC-PUFA in plasma PLs reflect tissue LC-PUFA status, so that it has been generally assessed by analyzing plasma PLs. Recent discussion has centred on whether erythrocyte membrane PLs can be used to asses DHA status. However, a preferred blood constituent, plasma or erythrocyte, is difficult to establish given the available data (36).

Several studies have been conducted in order to establish the most confident compartment to asses the LC-PUFA status of individuals. Some observational studies examined the relation between dietary fatty acid intake reported by food frequency questionnaires and their concentration in plasma or erythrocyte. In general they all

observed good correlations between intake and plasma or erythrocyte fatty acid levels (38;40-51). Whether correlations were stronger for the association between intake and plasma fatty acid levels or for the association with erythrocyte fatty acid levels is difficult to conclude due to the methodological differences between studies. Qi Sun et al. compared dietary fatty acid intake with fatty acid levels in plasma and erythrocyte in the same population and reported stronger correlations for the association between dietary intake and fatty acid levels in erythrocytes than for the association with plasma fatty acid levels (37). A meta-analysis of interventional studies concluded that both, plasma and erythrocyte phospholipids, appear to bee good markers for DHA status in humans, but special consideration should be given to particular population subgroups (52).

Fatty acid status of humans depends only partially on intake; non dietary factors should also be taken into account. Genetic and metabolic factors, as well as lifestyle determinants may affect fatty acids concentrations in human tissues. Pregnancy may modify plasma and erythrocyte fatty acids concentrations as a result of the metabolic changes occurring during its course and may alter the association between dietary intake and plasma and erythrocyte fatty acid levels. In general, the few studies conducted in pregnant women also found a good correlation between intake and maternal plasma and/or erythrocyte n-3 PUFA content at delivery (42;53;54). However, while some studies conducted in early pregnancy showed a positive association between frequency of self-reported fish consumption and EPA and DHA relative content in erythrocyte (55), other studies showed no association between dietary intake and maternal plasma and erythrocyte fatty acid content in early pregnancy (56). In addition, interventional studies showed that mothers consuming n-3 PUFA supplements have higher n-3 PUFA levels in both, plasma and erythrocytes, during pregnancy and at delivery (57-61). In contrast, some authors report a different evolution of erythrocyte and plasma fatty acids during the course of pregnancy, and consequently conclude that they are not both equally suitable to

determine fatty acid status in pregnancy (62). On the opposite side, some other published data are consistent with a similar fatty acid evolution in both plasma and erythrocyte during pregnancy. Furthermore, strong significant correlations between maternal plasma and erythrocyte fatty acid levels and fatty acid changes throughout gestation have been reported. Plasma and erythrocyte fatty acid levels in cord blood have also been correlated (54;63;64).

There are several options available to asses the fatty acid status of an individual; however the type of study is a major consideration when determining which body compartment reflects a better measure of fatty acids. It has been suggested that fatty acid composition of cell membranes is less influenced by short term changes in fatty acids intake, given that fatty acids in erythrocyte phospholipids have a slower turnover than plasma lipoproteins (54;65;66). Thus, erythrocyte PLs may be a more reliable measure in the evaluation of long term fatty acids intake. As dietary intake of LC-PUFAs does not typically vary over time, both, plasma and erythrocyte PLs, could be indicative of maternal DHA status in observational studies. In contrast, plasma PLs are more sensitive to short-term changes in the intake of LC-PUFAs and may be more suitable to monitor the fatty acid status of individuals in intervention studies.

1.2.4 LC-PUFA and the fetus

Over the past decades increasingly concern has been given to the need of an adequate supply of LC-PUFA to the developing fetus and the infant in early life. The developing fetus requires fatty acids as a source of energy as well as for the conformation of membranes in which they play an important role in maintaining their physical and chemical properties and as precursors of important molecules such us prostaglandins, prostacyclins, leukotrienes and tromboxanes. While all fatty

acids can act as energy source, the structural and metabolic functions mainly depend on the LC-PUFA supply.

The prenatal brain accretion of DHA has been estimated to be approximately 15-22 mg/week during the last trimester of pregnancy and the postnatal accretion only 3.6 mg/week (29). Some other authors have suggested an accretion of 76 mg/week during the first year postnatal life while Lauritzen et al. report that the DHA content of the brain increases approximately 35 mg/week from the beginning of the third trimester till the end of the first year (29). However, DHA accumulation also takes place in other tissues during the last trimester of pregnancy, thus n-3 fatty acid requirements have been estimated to be 50 mg/kg/day during this period.

The most significant effects of LC-PUFA in early life are related to neural and retinal development. Studies on maternal LC-PUFA supplementation during pregnancy as well as those conducted on dietary LC-PUFA supplementation in term and preterm infants during early life have associated supplementation to beneficial effects of these fatty acids on mental development and visual function. Recently, two reviews of the Cochrane Collaboration concluded that there was no evidence for a clear long term benefit of LC-PUFA supplemented formula for term or preterm infants on the basis of the current information (67;68). There are reasons to believe that maternal dietary supplementation may show positive effects on neurological and visual development of their offspring, as fetal LC-PUFA status depends on maternal LC-PUFA supply. The effects of LC-PUFA supplementation are probably related to changes in the membrane properties and membrane microenvironment which may modify specific cell functions. Another possible explanation for the effects observed after LC-PUFA supplementation is the influence of these fatty acids on regulation of gene expression which has been associated with cell differentiation (28). Most of the studies conducted provided data on early infancy and preschool age, but long term beneficial effects of LC-PUFA supplementation have not been

proved yet. Results of the follow-up of children included in LC-PUFA supplementation clinical trials will help to elucidate this issue.

Fatty acids have also been reported to induce the gene expression of specific proteins involved in the differentiation of fibroblast to adipocytes and therefore to modulate adipogenesis. Thus, LC-PUFA supplementation may have potential long term effects on weight by means of regulating the expression of genes responsible for adipocyte development (28).

Fetal and neonatal periods are key periods for immunological adaptation, and many of the allergic and inflammatory diseases of adults are thought to originate during these crucial periods. Several events occur during the first months of life that allow the immune system to become both competent and functional. The development of immune tolerance is the result of active immune mechanisms, and both development and maintenance of tolerance are lifelong processes that start very early in life, even prenatally. Regulation of tolerance and active immune responses is critical to health, and failure to regulate these responses can lead to recurrent infections, inflammatory diseases, and allergic reactions (33). There are indications that n-3 PUFA may regulate immune response in early life, particularly during pregnancy. Some studies have observed that higher levels of n-3 LC-PUFA are associated with reduction in neonatal oxidative stress, reduced production of inflammatory leukotriennes (LTB4) and altered T cell function (69). Epidemiological studies have positively associated high n-6 PUFA/n-3 PUFA ratios with eczema and symptoms of asthma in the first years of life (32;33;69). Although there have been no effects of postnatal fish oil supplementation on allergy prevention, there is evidence to suggest that intervention with n-3 LC-PUFA in pregnancy may prevent the development of allergic and possibly other immune-mediated diseases in the offspring (33;69). DHA supplementation during pregnancy has been related to lower IL-13 concentrations and higher percentages of CD34+ cells (haematopoietic

progenitors altered in children with atopy) in cord blood. Moreover, although no difference was observed for the incidence of food allergy, asthma, chronic cough and angioedema, children born from mothers receiving DHA supplements during pregnancy have been reported to be less likely to have a positive skin prick test at 1 year of age and have less severe symptoms of atopic dermatitis (70). There are ongoing pregnancy intervention studies to address the influence of LC-PUFA on the complex process of the perinatal immune programming and its long term consequences.

Although it is well known that the main LC-PUFA source for the fetus is the mother, there is evidence for some degree of LC-PUFA synthesis also in fetal tissues. Δ5 and Δ6 desaturase activity have been demonstrated in fetal liver in the last trimester of pregnancy. Moreover, in vivo studies with stable isotopes in premature infants have demonstrated that LC-PUFA synthesis from their precursors occurs as early as 26 weeks gestation (71). Placenta has also been thought to be able to produce LC-PUFA from their parent precursors; however there is no consistent evidence to support this theory. Some authors have measured low Δ5 desaturase levels as well as Δ5 desaturase mRNA while others did not detect any desaturase activity in placental tissue. Moreover, placenta perfusion studies were unable to detect LC-PUFA synthesis from their precursors (31). In conclusion, there is no consistent evidence to support the LC-PUFA synthesis in the placenta but the fetus seems to have certain capacity to produce LC-PUFA from their precursors. However, the degree to which the fetus is capable of fatty acid desaturation and elongation is not clear and may not adequately meet requirements. Thereby, the fetus is considered to be dependent on its mother to adequately meet LC-PUFA requirements.

Brain accumulation of DHA starts in utero, but it continues after birth especially during the first year of life. Both, preterm and term infants are capable of synthesizing LC-PUFA from their parent fatty acids, but the synthesis rates are

insufficient to maintain stable LC-PUFA levels in the newborn. DHA levels rapidly decrease by approximately 50% in plasma PLs and erythrocytes within 4 months after birth without an exogenous source of DHA, but are maintained by human milk or DHA supplemented formula (25;72). Thus, human milk provides LC-PUFA to the infant, mean AA in human milk varies between 0.35 and 0.7% weight % of total fatty acids, and DHA varies in a more wide range 0.17 % - 1.0% and depends on maternal DHA intake (72). Nevertheless, a high in utero accretion of AA and DHA in fetal adipose tissue has been reported in the third trimester gestation. The stored DHA is mobilised from adipose tissue in the first 2 months of postnatal life if the diet is devoid of preformed DHA, thus the LC-PUFA stores appear to support brain and retinal development in the first months of postnatal life (31).

1.3 Folate and pregnancy

Folic acid supplementation during pregnancy is one of the most common nutritional interventions in developing countries. Maternal folate deficiency during pregnancy has been traditionally associated to neural tube defects. This congenital malformation of the brain and spinal cord results from failure of normal developmental processes in the fetus occurring in the first trimester gestation (21-28 days postconception). Thus, folate availability is very important during the first few weeks of pregnancy (21). Little attention has been given to folate nutritional deficiency later in the course of pregnancy; however, significant negative effects of folate deficiency in later gestation have been described in rodent models. Folic acid or choline deficiency late in gestation, when the hippocampus develops, diminishes neurogenesis and increases neural cell death in fetal brain. As a result, pups born from supplemented rats perform better in memory test. However, there are not equivalent studies in humans (20).

Dietary folates, in the form of tetrahydrofolates (THF), are essential cofactors for several biochemical reactions that transfer methyl groups. 10-formylTHF (formed from formate and THF by the enzyme C1-THF synthase, the product of the MTHFD1 gene) is required for the biosynthesis of purines. 5,10-methylTHF, derived from serine and THF, is required for thymidylate biosynthesis. 5,10-methyleneTHF can also be reduced to 5-MTHF (formed by methyleneTHF reductase, the product of the MTHFR gene) and this is needed for the biosynthesis of methionine, eventually influencing biosynthesis of S-adenosylmethionine (the most important methyl-group donor). Decreased S-adenosylmethionine concentrations results in DNA hypomethylation, which influences gene transcription and genomic stability. Methylation is reproduced every time the gene is copied. Thus, changes in dietary availability of methyl-groups induce stable changes in gene methylation, altering gene expression and resulting phenotype (21).

5-MTHF and vitamin B12 are required cofactors in the reaction catalyzed by methionine shynthase for the synthesis of methionine from homocysteine, as a result, plasma homocysteine concentrations are reduced (21). Homocysteine levels are, therefore, strongly influenced by the nutritional status of these vitamins and to a lesser extent vitamin B6 and riboflavin (73). A large number of studies have found an inverse association between folate and homocysteine levels in plasma (74-76). Furthermore, studies have consistently shown a reduction in homocysteine levels after folic acid supplementation (75;76) The folic acid dose required to achieve the maximal reduction in plasma homocysteine has been reported to be 0.8 mg/day in a meta-analysis of randomized controlled trials. The 0.4 mg/day supplementation used in the current study has been associated with 90% of this maximal effect (76). In addition to the metabolic pathway previously described there is an alternative choline-dependent pathway for the methylation of homocysteine to methionine. Betaine, derived from dietary choline, is the methyl-group donor in this reaction catalyzed by the enzyme betaine homocysteine methyltransferase (the product of the

BHMT gene). These two pathways act in parallel, and intersect at the point that homocysteine is converted to methionine. Both, choline and folate metabolic pathways lower homocysteine concentrations and because of their relation, perturbing metabolism of one results in compensatory changes in metabolism of the other (Fig. 3) (21).

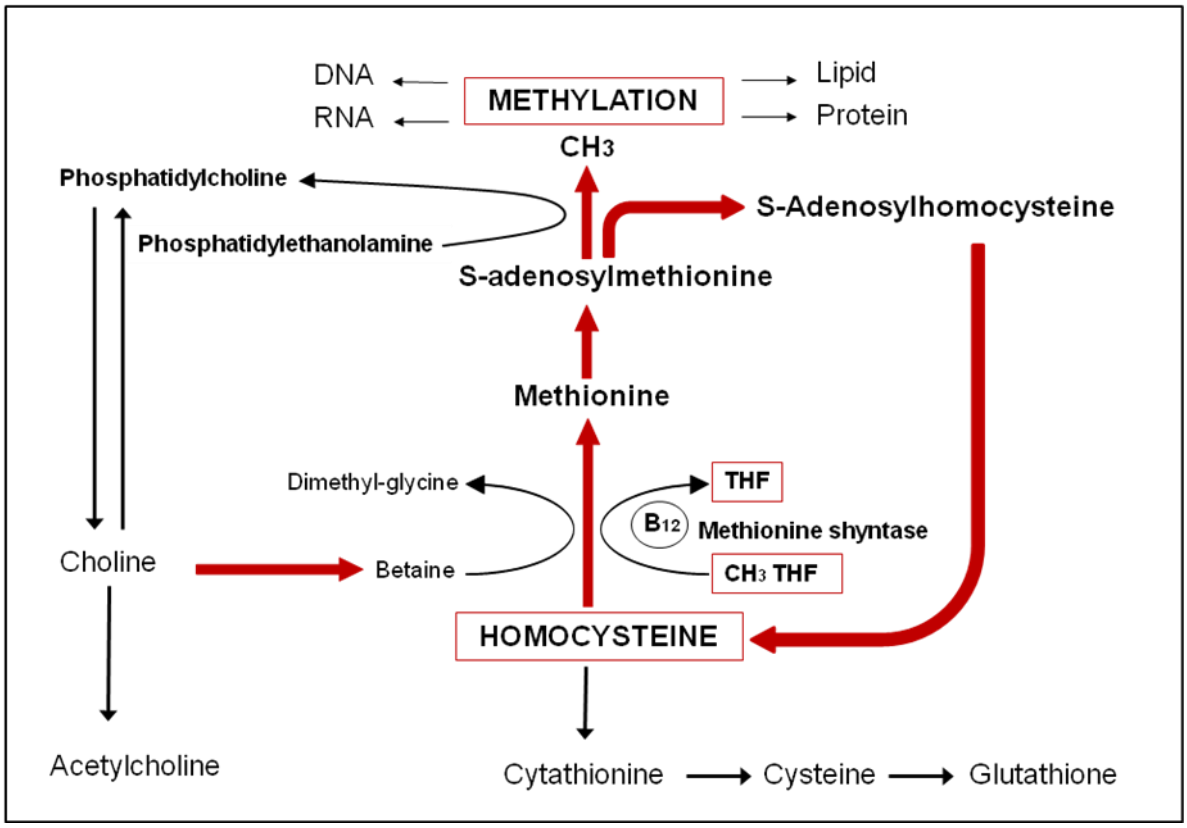

Fig 3. Metabolism of methionine. THF, tetrahydrofolate.

Thus, variation in dietary folate intake could influence fetal outcome by at least three distinct mechanisms: alteration of DNA biosynthesis, accumulation of toxic levels of homocysteine and perturbation of methylation reactions (21).

Genes of folate metabolism are polymorphic, thus, variants are relatively common, and some can increase dietary requirements for folate. Homocysteine concentration is therefore determined by both, nutritional and genetic factors. There are two common SNPs of the gene encoding the methyltetrahydrofolatereductase (MTHFR), the MTHFR677 C->T and MTHFR 1298 A->C, which result in a reduced

enzymatic activity. The former occurs in as many as 8-15% of the population and produces a thermally unstable enzyme with 50% reduction of enzymatic activity in homozygous individuals. The most significant genetic predictor of homocysteine has been reported to be the common 677C->T polymorphism of the MTHFR gene (21;76). Individuals having these MTHFR polymorphisms have greater risk of neural tube defects. In addition, a genetic variant of MTHFD1, 653 R->Q is also associated with increased maternal risk for neural tube defects (21).

Studies have demonstrated a significant decrease of folic acid and vitamin B12 along gestation with lower concentrations at delivery compared to the second trimester gestation. Furthermore homocysteine levels have been found to increase during the course of gestation. However, serum concentration of homocysteine has been reported to be significantly lower in cord blood than in the maternal blood at delivery, whereas significantly higher concentrations of folate have been observed in cord blood than in maternal blood at delivery. Folate have been reported to be the strongest predictor of homocysteine levels in maternal and cord blood (74). These results are consistent with an increased demand of folate during gestation which is extremely important for fetal development such that mothers may be clinical deficient on folate at the end of pregnancy. Nevertheless, fetal folate requirements seem to be paramount, to the point that cord blood folate is maintained even when maternal folate status is low.

In addition to plasma homocysteine, some authors have found that folate deficiency also modifies plasma and tissue fatty acid composition. A positive correlation between erythrocyte folate levels and plasma DHA levels (77) as well as a negative correlation between plasma DHA and homocysteine concentrations (78) have been reported in humans. Moreover, folic acid supplementation has been reported to restore normal fatty acid composition in patients on dialysis (79). However, results from a recent randomized controlled trial showed no effect of the

supplementation with folate, vitamin B12 and vitamin B6 on n-3 levels in plasma PC (80). The effect of 5-MTHF on fatty acids might be attributable to the increased methionine availability present after 5-MTHF supplementation, which stimulates EFA metabolism. Sugiyama et al. suggested that methionine, by stimulating phosphatidylethanolamine N-methylation to phosphatidylcholine, altered the liver microsomal profile of PLs and the phosphatidylcholine to phosphatidylethanolamine ratio which, in turn, increased the activity of Δ-6 desaturase. The detailed mechanism by which the Δ-6 desaturase activity is influenced by the microsomal PL profile remains unclear (81;82). The effects, although present in both, n-3 and n-6 fatty acid series, have been reported to be more marked in the n-3 metabolic pathway than in that of the n-6 series (79).

In addition, moderately elevated plasma homocysteine concentrations have been linked to vascular damage (73;83), thus reduction of elevated plasma concentrations of homocysteine during pregnancy might improve placental vascularisation and hence efficacy of materno-fetal substrate transfer. Indeed, significant correlations between maternal plasma total homocysteine concentrations and birth weight as well as placental weight have been observed. Furthermore, total homocysteine concentrations in maternal plasma have been reported to negatively correlate with DHA levels in erythrocyte PLs of their offspring which may reflect the placental vascular integrity (84).

As above described folate decreases plasma homocysteine levels and hence may improve the DHA status of the mother and the neonate. Therefore we intended to maximise the DHA status of pregnant women and their neonates by means of adding 400 μg 5-MTHF/day to the fish oil supplementation.

1.4 Dietary DHA and folate

The main natural source of LA and ALA are vegetable oils, particularly safflower, sunflower and corn oils. LA is primarily stored in plant seeds while ALA is associated with photosynthesis and is found in the green parts of the plants. AA and DHA are not found in plants, they are synthesized by animals, but only small mammals accumulate high proportions of them. Meat and eggs are rich sources of AA, by contrast, there is little DHA in muscle and organ lipids in large land mammals with the exception of brain and testes. Marine food is rich in n-3 PUFA as algae are the primary producers of DHA and EPA in the ecosystem. Fish consume algae and therefore are rich in EPA and DHA, fish content of these fatty acids is dependent on its position in the food chain, thus larger marine animals concentrate higher amounts of DHA (30).

Since agricultural revolution and especially since the industrial revolution, we have gradually changed our dietary habits. Two important changes are the decrease in the intake of micronutrients such us folic acid and the changes in the fatty acid composition of the diet consisting on increased intakes of n-6 fatty acids, saturated fatty acids and trans fatty acids and decreased n-3 fatty acids intakes. Thus, occidental diets are characterized by low n-3 fatty acids intakes and high amounts of n-6 fatty acids, as a result, EPA and DHA demands cannot be adequately supplied (25;30). The World Health organisation (WHO) recommends 6-10% of daily energy intake to be PUFAs with an optimal balance between n-6 PUFAs (5-8% of daily energy intake) and n-3 PUFAs (1-2% of daily energy intake) (85).

On the basis of evidence based consensus, the European Commission recently advised that pregnant and lactating women should achieve and average DHA intake of at least 200 mg/day. The Commission recommends the consumption of one or two portions of sea fish weekly (oily fish like herring, mackerel and salmon) in order to

reach the recommended DHA intake. Fish contain contaminants like mercury, dioxins and organochlorides which are in higher levels in large fish at the top of the food chain. Fishes with the highest methylmercury contents are predator fishes like marlin, pike, swordfish, tuna and shark and those with the highest levels of dioxins and organochlorides are herring and salmon. Women can decrease the levels of methylmercury in their bodies by reducing the intake of the most contaminated foods in the months prior to and during pregnancy. On the contrary, dioxin compounds as well as organochlorides are stored in the body and it takes a long period of time to decrease levels of these contaminants, thus food choice during pregnancy does not avoid fetal exposure to these compounds. However, the European Food Safety Authority concluded that pregnant women consuming two portions of fish weekly are unlikely to reach the provisional tolerable weekly intake of dioxin and dioxin-like compounds. Nevertheless, due to the high levels of contamination found in herring and salmon from the Baltic Sea, women of childbearing age should limit the consumption of these fishes to no more than one portion per week (86;87).

The average omnivore dietary intake of DHA has been estimated in several countries and it is in the same range as the calculated combined rate of maternal and fetal DHA utilisation of 200 mg/day (31). A study on the dietary intake of DHA in our cohort have reported mean dietary intakes of 259 mg/day in Germany, 403 mg/day in Spain and 317 mg/day in Hungary, all of them above recommendations (1). However, within populations there are much lower and higher intakes. Particularly important is the group of vegetarians, which has been estimated to be 11-12% of women in childbearing age in Europe and the majority of the population in countries like India. The exclusion of fish and meat from the diet can result in very low intakes of DHA (31).

Folate is found in vegetables, especially in green vegetables such us spinach, as well as in grains, legumes, soybeans and fruits. Bread and potatoes are not especially rich in folate, but consumed in high amounts can provide a significant portion of the total folate intake. The main source of folate for pregnant women in USA has been reported to be grains and cereals as they are generally fortified with folic acid in USA (1).

During pregnancy takes place a rapid maternal and fetal cellular growth and development which is associated with enhanced folate requirements. Current Dietary Reference Intakes (DRI) for folate are based primarily on metabolic studies in which erythrocyte folate concentration was considered the major indicator of adequacy. On the basis of these studies the Recommended Dietary Allowance (RDA) for folate have been reported to be 600 μg/day folate equivalents (88). Recommended daily intake of folate for pregnant women in Europe varies between 300 and 600 μg/day depending on countries and DRI recommended by the Food and Nutrition Board (2001) in US for pregnant women is 600 μg folate equivalents per day (89). To achieve the recommended amount pregnant woman should couple a varied diet that includes either folate-dense foods with sources of synthetic folic acid, such as fortified food products, or the available non-prescription supplements (88). Generally, reports on average folate intake in pregnant or childbearing women all over the world show low folate intakes compared to recommendations (90-95). Franke et al published average folate intake in the NUHEAL cohort and concluded that only 6% of the participants reached the recommended folate intake of 600 μg/day being the mean folate intake in Germany 254 μg/day, in Spain 304 μg/day and 396 μg/day in Hungary (1).

1.5 Maternal and neonatal LC-PUFA status during pregnancy

1.5.1 Maternal lipid metabolism during pregnancy

Glucose is quantitatively the most important nutrient transferred to the fetus during pregnancy followed by aminoacids. However, although quantitatively less important, lipids crossing the placenta play an important role in fetal development.

The mother must adapt her metabolism during pregnancy to support fetal demands of substrates. In the first two thirds of gestation there is an anabolic condition of maternal lipid metabolism that leads to the accumulation of fat depots and maternal net body weight gain. During this period an enhanced lipoprotein lipase activity is described, as a result triglycerides in plasma lipoproteins are hydrolysed and the hydrolysis products are taken up by the adipose tissue. This enhanced lipoprotein lipase activity, together with hyperphagia and increased lipogenesis occurring in the first trimesters of pregnancy lead to the storage of dietary LC-PUFAs in maternal adipose tissue. During the last trimester of gestation, maternal lipid metabolism switches to a catabolic condition characterized for the accelerated breakdown of fat depots, coinciding with the maximal fetal growth period. Lipolytic activity is enhanced during the last trimester of pregnancy which contributes to the maternal hyperlipidemia observed in normal pregnant women in late gestation. It mainly corresponds to increases in plasma triglyceride concentrations, with smaller rises in phospholipids and cholesterol. Free fatty acids in plasma increase in the same period of gestation, but if compared to the amount of LC-PUFAs in the different lipidic fractions they represent a negligible amount. The lipidic moieties that show the greater decline in LC-PUFAs content after delivery compared to the third trimester pregnancy are the triglycerides present in the VLDL, LDL and HDL lipoproteins. The changes found in phospholipids and esterified

cholesterol are milder and no significant changes for the LC-PUFAs concentration in the FFA fraction have been observed (27).

Lipolytic products, free fatty acids (FFA) and glycerol, are conducted to maternal liver where they can be used either in the synthesis of triglycerides or as energy substrates to support maternal and fetal metabolism. Glycerol may be used for glucose synthesis and FFA for β-oxidation to ketone bodies. These metabolic pathways are enhanced under food deprivation conditions in maternal liver which saves the use of aminoacids for gluconeogenesis and warrants their availability for fetal growth. An enhanced liver production of triglycerides develops in the last third of pregnancy which, together with the decreased removal form the circulation secondary to a reduced lipoprotein lipase activity in adipose tissue, contributes to the characteristic hypertriglyceridemia observed during late pregnancy. Synthesized triglycerides are the main source of EFA for the fetus and are released into maternal circulation from the liver as VLDLs. In addition, other lipoproteins like LDL and HDL which normally transport triglyceride in very small proportions increase their triglyceride content as a result of the enhanced activity of the cholesterol ester transfer protein and the decreased activity in the hepatic lipase.

All this changes are proposed to be conducted by two main mechanism; the increased circulation estrogens and the insulin levels and sensitivity changes occurring throughout pregnancy (27;96). During early pregnancy an increased insulinotropic effect of glucose is observed secondary to a heightened activity of pancreatic β-cells. Lipogenesis from glucose is an insulin sensitive metabolic pathway, thus maternal hyperinsulinemia in early pregnancy may contribute to fat deposition. During the last third of gestation an insulin resistant condition develops, this condition is responsible for the enhanced lipolytic activity and the decreased lipoprotein lipase activity in the adipose tissue, which despite maternal hyperinsulinemia leads to the breakdown of fat depots. The enhanced estrogens

concentrations during late pregnancy are the main activator of liver production of VLDL and they are also responsible for the decrease in the activity of hepatic lipase in the liver (96) (Fig. 4).

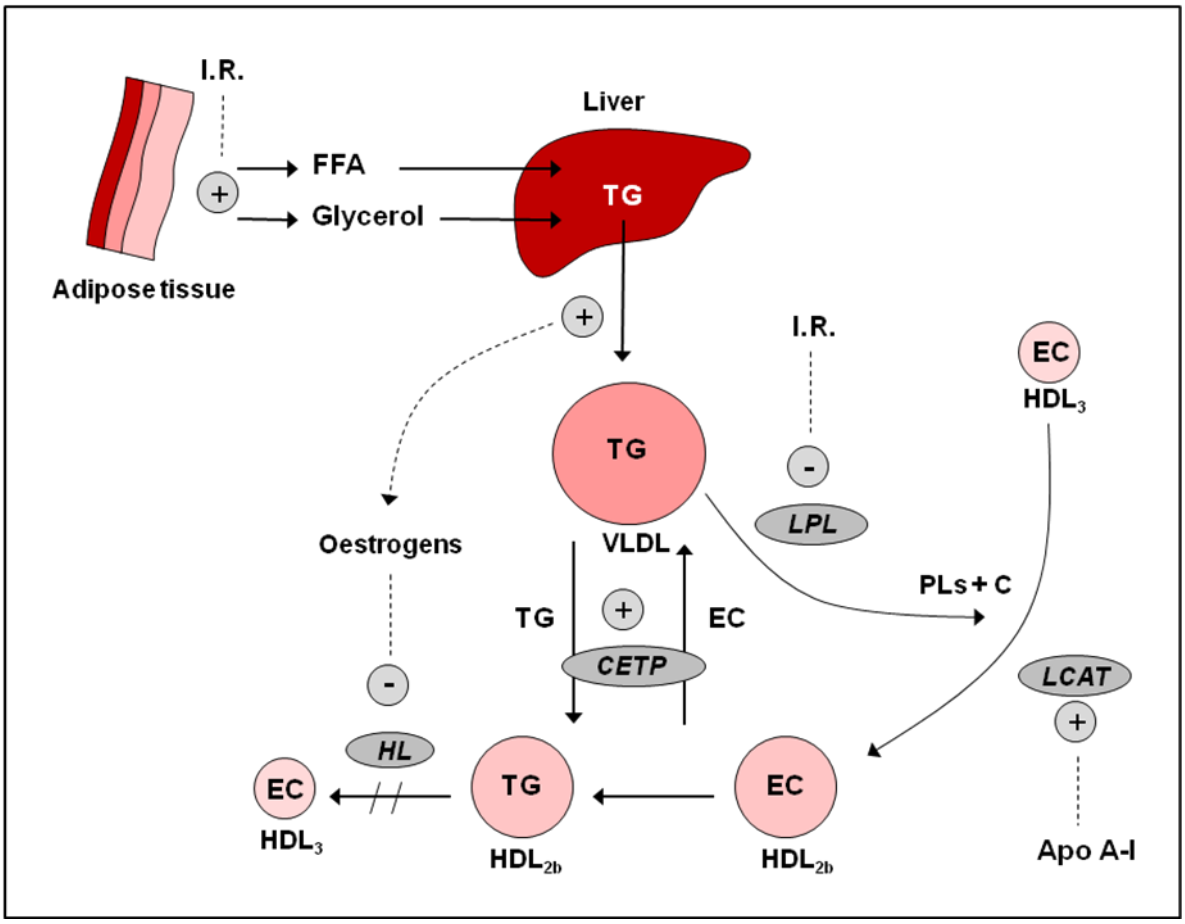

Fig. 4. Lipoprotein metabolism during pregnancy (Adaped from Herrera, Placenta 2002). I.R., insulin resistance, PLs, phospholipid; C, free cholesterol, EC, esterified cholesterol; TG, triglycerides; LPL, lipoproteinlipase; HL, hepatic lipase, CETP, cholesteryl ester transfer protein; LCAT, lecitin cholesterol acyltransferase.

1.5.2 Maternal LC-PUFA status during pregnancy

Several observational studies have described plasma and erythrocyte fatty acid behaviour during the course of gestation. In general, all studies agree that total amount of fatty acids (mg/L) in maternal plasma and erythrocyte increase during the course of pregnancy, which could be a direct consequence of the physiological hyperlipidemia occurring in pregnancy (62;63;97). Individual fatty acids separately considered show similar patterns and increase as gestation progresses, although

there are differences in the proportional increments. The increment for DHA has been reported to be the highest (98). This increment cannot be explained by modification of dietary habits, the most accepted theory to explain this fact is the increased mobilization of DHA from maternal adipose tissue stores but an increased activity of enzymes involved in its synthesis cannot be excluded. Despite the increments of the absolute fatty acid concentrations, declines in the EFA and DHA status indexes have been observed along pregnancy. These observations suggest that pregnancy is associated with reduced EFA and DHA status and may deplete maternal DHA stores (63;99;100).

When the analyses are performed on the basis of relative fatty acid values expressed as % weight of total fatty acids (wt%) some differences in the evolution pattern among fatty acids are observed. Studies show that monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) relative concentrations (wt%) in plasma PLs increase while PUFA decrease during the course of gestation (62;63;97;101). Relative concentrations of AA have been reported to diminish throughout pregnancy and, possibly as a consequence, a similar behaviour is reported for n-6 PUFA (62;63;97;101). Regarding relative DHA and n-3 PUFA, concentrations (wt%) in plasma PLs have been generally reported to increase or remain stable, specially in the first half of pregnancy, and to subsequently decrease in the second half of pregnancy (56;62;63;97;102). In general, the overall changes in fatty acid relative concentrations during pregnancy are comparable between plasma and erythrocyte PLs, with the exception of AA and n-6 PUFA which has been reported to increase (63) or remain stable (62;98) in erythrocyte PLs. Although the comparability has been reported to be less towards the end of gestation, the same behaviour reported for fatty acids and fatty acid combinations in plasma PLs has been observed in erythrocyte fatty acids (62;63). Relative levels of MUFA, SFA, DHA and n-3 PUFA increase while PUFA decrease in erythrocyte PLs during the course of pregnancy.

Most of the studies conducted have described the fatty acid evolution during the second and third trimester gestation but very few studies report the fatty aid profile in early pregnancy. Otto et al. studied the changes in the maternal EFA profile during the first 10 weeks of gestation and reported a significant increase in the total amount and in most of the individual fatty acids (mg/L) of plasma PLs as early as in the first weeks gestation. The percentage values (% of total fatty acids) of n-3 PUFA, n-6 PUFA, DHA and AA have also been described to increase in plasma PLs during the first trimester gestation while percentages of ΣMUFA, ΣSFA and ΣPUFA remained similar to those reported in prepregnancy. Because plasma PLs mainly originate from hepatic synthesis, these authors have suggested that the increase in DHA concentrations during pregnancy may reflect a pregnancy associated adjustment in the hepatic synthesis of DHA, with an enhanced or selective incorporation of this fatty acid into the PLs. No significant changes have been reported in the total fatty acid amount and most individual fatty acids levels expressed as mg/L in erythrocyte during the first trimester pregnancy but DHA and n-3 PUFA percentages of total fatty acids (wt%) in erythrocyte membranes were higher in the 10th week gestation compared to those observed in prepregnancy (56).

Studies conducted in different centres with different populations and as a result, different metabolic and dietary characteristics, show a similar pattern of change in fatty acid composition during pregnancy. Thus, it can be hypothesised that fatty acids have similar evolution patterns during pregnancy across different ethnic and diet groups. An international comparative study on fatty acid status in pregnancy showed differences in the fatty acid status among 5 countries but the pattern of fatty acid changes over the time were comparable among the 5 different populations (97).

The pattern of fatty acids during pregnancy varies widely among different lipid fractions in plasma. DHA has been reported to show a marked relative

enrichment in the phosphatidylcholine (PC) PL fraction during pregnancy, while no enrichment has been found in the triglyceride fraction. On the contrary, ALA concentrations increase in the triglyceride fraction but not in the PC PLs. Relative increments in the n-6 PUFA, although less marked than those reported for the n-3 PUFA, have been described in both triglycerides and PC PLs. Regarding the non esterified fatty acids (NEFA) fraction no differential distribution of fatty acids has been reported. Plasmatic NEFA are the result of the triglyceride hydrolysis occurring in the adipose tissue and are the precursors for the synthesis of triglyceride and PLs in the liver. No selective mobilization of individual fatty acids from adipose tissue has been described, however, the important differences observed in the fatty acids profiles of plasma triglycerides and PLs suggest a selective utilization of individual fatty acids in their formation in the liver (103). The fatty acid composition of cholesteryl esters (CE) is less influenced by pregnancy than the fatty acid composition of plasma PLs. Cholesterol esterification takes place in plasma by transfer of the fatty acids from the sn-2 position in PC PLs to the 3-β-OH-group of cholesterol. This reaction is catalyzed by the enzyme lection-cholesterol acyltransferase (LCAT) which generally utilizes the fatty acid in the sn-2 position normally occupied by linoleic acid. Thus, LCAT preferentially utilizes linoleic acid, which is the predominant fatty acid in CE. However, when AA or DHA are present in the sn-2 position, LCAT prefers the sn-1 group, which explains the small amount of DHA found in CE (101).

In addition, maternal LC-PUFA status has been demonstrated to significantly depend on maternal dietary intake of these fatty acids. Some observational studies examined the relation between dietary fatty acid intake reported by food frequency questionnaires and their concentration in plasma or erythrocyte. In general, these studies observed no correlations between plasma or erythrocyte fatty acid composition and dietary intake of SFA and MUFAs, probably because these two classes of fatty acids could be endogenously synthesized from carbohydrates. The

strongest correlation between dietary intake and plasma or erythrocyte fatty acid concentration has been always observed for the n-3 PUFA (37;40;41;44;49). However the ranges of correlation coefficients reported vary widely among studies, probably due to methodological differences, which makes it difficult to drawn conclusions. Associations between dietary n-6 PUFA intake and their levels in maternal blood, although present, have been less consistently reported (40;42;49;53). Although the PUFA composition of phospholipids is associated with their dietary intake, it is also affected by metabolic steps, such as absorption, lipoprotein metabolism in the intestine, liver and blood, and uptake in tissue cells. Dietary PUFA are metabolised as energy sources, distributed to circulation via VLDL formation and some are incorporated into structural membrane lipids. In addition, PUFA may be further desaturated and elongated in the liver which also contributes to the composition of PUFA in plasma PLs (23). In addition, changes in the human metabolisms, like those occurring during pregnancy, may also alter the association between fatty acid intake and fatty acid levels in maternal tissues. Only a few studies have been conducted in pregnant women. In general a good correlation is also reported between intake and plasma and erythrocyte n3-PUFA levels in pregnant women at delivery (42;45;53). By contrast, the association between dietary intake and maternal fatty acid levels in early pregnancy is not so clear. While some studies showed a positive association between frequency of self-reported fish consumption and EPA and DHA relative content in maternal erythrocyte in the first trimester gestation (55), other studies showed no association between dietary intake and maternal plasma and erythrocyte fatty acid content in early pregnancy (56). On the other side, it is well known from interventional studies that maternal PUFA status varies with n3-PUFA consumption during pregnancy, mothers consuming n3-PUFA supplements have higher n3-PUFA levels in both, plasma and erythrocytes, during pregnancy and at delivery (57-61;104;105).

It can be concluded that the availability of fatty acids to the fetus depends not only on the maternal dietary intake but also on the function of the placenta and the physiological and biochemical adaptations occurring during the course of pregnancy.

1.5.3 Placental transfer of LC-PUFA

As mentioned above, maternal lipoprotein triglycerides are the main source of essential fatty acids to the fetus. However, esterified fatty acids cannot directly be transferred across the placenta. In the diffusion of their fatty acids to the fetus, NEFA have to be released from lipoproteins by the hydrolase/lipoprotein lipase activity. Placental trophoblast cells contain specific binding sites for circulating lipoproteins which transport triglyceride and other esterified lipids. The lipoprotein receptors bind maternal circulating lipoproteins and mediate their metabolism before the resulting NEFA are taken up by the placenta. Although HDL receptor have been demonstrated in human placenta, the most abundant lipoprotein receptors are VLDL/Apo E receptors and LDL receptors (27;31;96;106;107). A phospholipase A2 activity has also been described in the placenta, thus PLs may also be hydrolysed as sources of NEFA for the fetus (107).

Once available in the microvillous membrane, NEFA resulting from the above mentioned hydrolysis processes, as well as those released into maternal blood following mobilisation from maternal adipose tissue must be transported across the placenta. Their placental transfer occurs either via a saturable protein mediated transport mechanism or via passive diffusion depending on the NEFA concentration gradient between the fetus and the mother (108). The NEFA concentration difference between the maternal and fetal circulation increases throughout gestation until term when the concentration of NEFA in the mother is approximately three times that in the fetal circulation. In addition the concentration of the primary NEFA carrier, albumin, is 10-20% higher in fetal than in maternal circulation which enhances the

gradient for the maternal to fetal transfer of fatty acids (31). Although NEFA can easily cross the placenta by simple diffusion, several fatty acid binding proteins (FABPs) have been identified in the membrane and cytoplasm of placental cells. These proteins are thought to facilitate the transfer of fatty acids across membranes. The main FABP are the plasma-membrane fatty acid binding protein (FABPpm), the fatty acid translocase (FAT/CD36) and the fatty acid transfer protein (FATP) (27;31;106;109). FAT/CD36 and FATP are related to the transport of fatty acids across the trophoblast membrane by means of a facilitated membrane translocation process. Six FATP genes have been to date identified in human genomes, from them, the FATP-1 and 4 are highly expressed in the placenta (106). The FABPpm is thought to act as an extracellular fatty acid acceptor. A placenta specific protein (p-FABPpm) has been identified in the maternal facing membranes, information about its structure remains to date unclear but it has been proposed to be involved in the preferential LC-PUFA uptake occurring in the placenta (109). Some other FABP have been recently observed in the syncytiotrophoblast, the family of the cytosolic FABP. They were first identified in other tissues than placenta, to date only the heart-FABP (H-FABP) and the liver-FABP (L-FABP) forms have been identified in placenta. Once in the cytosol of trophoblast NEFA are either reesterified and subsequently hydrolyzed by means of intracellular lipase activities or cross the tissue bound to the cytosolic proteins (109) (Fig. 5).

On the basis of placental perfusion studies some authors have suggested that there might also be a mechanism of exportation of the esterified fatty acids in PL, TG and CE to the fetus, which takes longer time than the transfer of non esterified fatty acids bound to cytosolic FABP. A in vivo study using 13C-labeled fatty acids showed a higher 13C enrichment of cord blood fatty acids than in placenta fatty acids (108). Further studies are required to better understand this mechanism of esterified fatty acids transfer to the fetus.

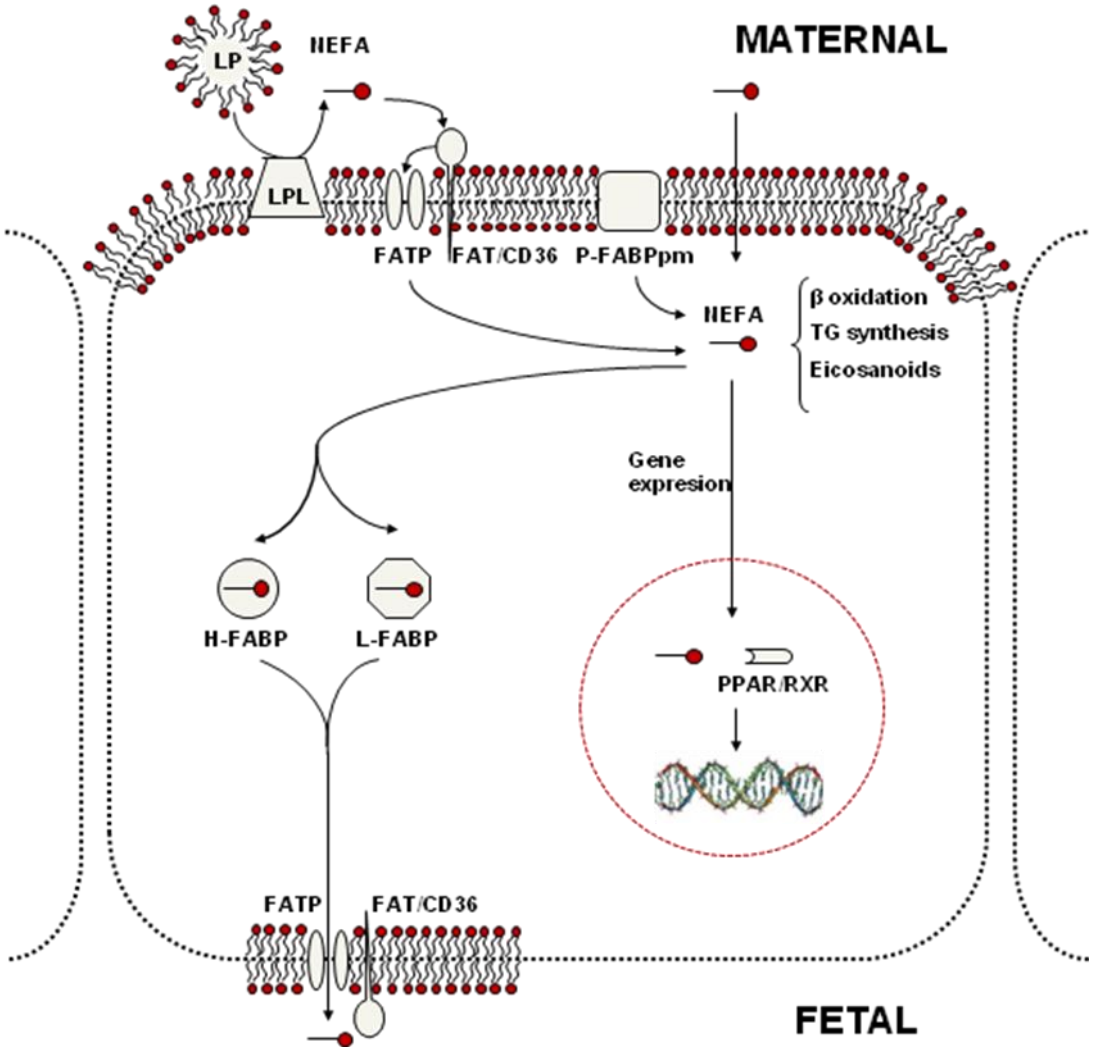

Fig.5. Model of placental fatty acid transport (Adapted from Hanebutt Clin Nutr 2008). LP, lipoprotein; LPL, Lipoproteinlipase; NEFA, non esterified fatty acids; TG, triglyceride; FATP, fatty acid transport protein; FAT, fatty acid translocase; P-FABPpm, placental plasma membrane fatty acid binding protein; L-FABP, liver-fatty acid binding protein; H-FABP, heart-fatty acid binding protein; PPAR, peroxisome proliferator activated receptor; RXR, retinois X receptor.

Some authors have also suggested that maternal erythrocyte may play a role as potential store of LC-PUFA and as a vehicle for the transport of these nutrients to the placenta, as erythrocytes have particular advantages as potential store and delivery vehicle. Primarily, they are relatively rich in AA and DHA and can enrich themselves through dynamic interaction with the plasma environment. Secondarily, erythrocyte will be able to transport PUFA to the placenta without the involvement of mediators (110).

Many studies have demonstrated that the accumulation of AA and DHA by the fetus elevates the fetal levels of these fatty acids above those in the mother. Thus, maternal plasma and erythrocyte lipids at birth contain higher relative levels of essential fatty acid precursors, ALA and LA, but lower percentage values for LC-PUFA than the cord lipids of their infants (57;96;106;111-113). There is evidence from in vivo and in vitro studies that placenta can selectively channel LC-PUFA to the fetal circulation. Human placenta perfusion studies have shown a preferential placental transport of fatty acids to the fetus which has been reported to be DHA>AA>ALA>LA (31). This selectivity has also been determined in vivo studies with the approximate order being DHA > ALA > LA > oleic acid > AA (27;31). Furthermore, recent in vivo studies using 13C-labeled fatty acids have demonstrated a preferential sequestration of DHA in the placenta relative to other fatty acids (108). The mechanisms for this selective materno-fetal transfer of LC-PUFAs is to date not fully understand. The above described protein system existing in the placenta for the fatty acid transfer might be responsible for this preferential uptake of fatty acids. FAT/CD36 and FATP do not appear to have any preference for LC-PUFA. However, Larque et al. (114) reported that the mRNA expression of the membrane proteins FATP-1 and FATP-4 in placental tissue was positively correlated with the uptake of maternal DHA into placental and cord blood phospholipids. It was therefore proposed than FATP-4 plays an important role in the LC-PUFA transport. The most important protein in the selective placental fatty acid transfer is the pFABPpm which has been described to have a preferential biding affinity for DHA ≥ AA > LA > ALA > oleic acid. Thus, the pFABPpm facilitates the selective transfer of fatty acid to the syncytiotrophoblast and drives to the DHA and AA enrichment at the microvillous border in the placenta (31;108;114). The fatty acid uptake in the placenta is also regulated through fatty acid activated nuclear transcription factors that modulate gene transcription and the mRNA expression of placental transport proteins. The so called peroxixome proliferator activate receptors (PPAR) are critical regulators of placental function and may control the placental fatty acid transport (106). Another

possible mechanism suggested for the placenta selective transfer of fatty acids is the existence of placental lipase selectivity for the release of LC-PUFA from triglyceride. In addition, the lipoprotein lipase is known to preferentially hydrolyse triglyceride fatty acids in the sn-2 position which are generally more unsaturated that the sn-1 and sn-3 position (31;115). Some authors suggest the existence of a different compartmentalization of individual fatty acids in various lipid fractions in the placenta as a mechanism for this selective fatty acids transport (108). Studies have shown that the pattern of enrichment of individual fatty acids varies widely among different lipid pools. As a result DHA is preferentially carried in plasma PLs, while ALA is enriched in the triglyceride fraction and AA and LA in both triglyceride and PLs, which may enable differential delivery to the fetus by means of a selective placental utilisation of maternal lipid fractions with a high LC-PUFA content (103).

Once in fetal circulation fatty acids are bound to the alpha-fetoprotein and taken to fetal liver where they are esterified and released in fetal circulation as triglycerides. There is evidence that esterified fatty acids in fetal circulation cannot be reuptaken in the placenta. A further mechanism for the selective concentration of certain LC-PUFA in fetal circulation may be a preferential incorporation of LC-PUFA into esterified lipids in the placenta before they are transferred to the fetus and/or the LC-PUFA esterification in fetal liver that would trap these LC-PUFA in fetal circulation (31).

In addition to its role in fatty acids transfer to the fetus, the placenta is also thought to be able to modulate its own fatty acid supply depending on fetal demands. The placenta regulates the fatty acid mobilization from maternal adipose tissue by means of releasing placental leptin into fetal and maternal circulation which stimulates lipolysis (31).

1.5.4 Relation between maternal and neonatal LC-PUFA status

Several observational studies have shown positive highly significant correlations between levels of most maternal fatty acids in plasma and erythrocyte PLs and their respective levels in cord blood at delivery (53;54;63;116). Some authors report stronger correlations for the association between erythrocyte fatty acid levels than for the association between fatty acid levels in plasma PLs (63). It is also well known from interventional studies that mothers consuming n-3 LC-PUFA supplements during pregnancy have higher n-3 LC-PUFA levels in both, plasma and erythrocyte, throughout pregnancy and at delivery (57;59-61;104;105;117). However, only those children whose mothers received high DHA doses (>1 g/day) have higher plasma and/or erythrocyte n-3-LC-PUFA levels in umbilical cord (117-120). Thus, PUFA concentrations in maternal plasma lipids have shown to be good predictors of PUFA concentrations in umbilical plasma lipids, which have been demonstrated to be a reasonable reflection of the intrauterine fatty acid supply (116).

Maternal LC-PUFA levels and therefore fetal LC-PUFA levels have been related to maternal fatty acid intake. However maternal adipose tissue has also been suggested to play an important role in fetal fatty acid supply, especially in the last trimester of gestation when fetal demands increase. A significant amount of mothers do not ingest sufficient DHA in the last trimester of pregnancy to meet the estimated maternal and fetal requirements. Fatty acid mobilisation from maternal adipose tissue may ensure a constant supply of fatty acids to the fetus and may protect the fetus against a low LC-PUFA intake during the critical periods of fetal LC-PUFA accretion (31). It is interesting that, while increases in cord blood n-3 LC-PUFA require high dose DHA supplements in the last trimester of pregnancy to be significant (59;117;118), observational studies have shown that modest differences in the n-3 LC-PUFA content of normal diets can have significant effects on cord blood fatty acid levels (97). On the basis of these observations, some authors have

suggested that improvements in LC-PUFA supply to the developing fetus may be more easily achieved by small changes in habitual maternal dietary intakes of DHA than by means of high dose LC-PUFA supplementation in late pregnancy (31).

Although it is true that the fetus depends on the mother for its fatty acid supply, a certain degree of autonomy of the fetus with respect to the establishment of its fatty acid status has been suggested. An international comparative study on maternal and neonatal EFA status showed that differences in the maternal EFA status between countries were only partially reflected in the neonates. A strong association between maternal and neonatal n-3 LC-PUFA levels was found whereas the relationships between maternal and cord blood n-6 LC-PUFA were significant only in some countries. This suggests some kind of autonomy of the fetus with respect to establishing its AA status which seems less pronounced for the DHA (97;100).

In many cases, erythrocyte and plasma PLs fatty acids levels in cord blood are lower than those in maternal blood. The absolute total amount of fatty acids in both plasma and erythrocyte PLs have been reported to be lower in cord blood than their respective levels in the mother at delivery in observational studies (63). When considering relative levels of fatty acids as percent of total fatty acid weight (wt%), some authors have also reported MUFA and PUFA showing higher levels in maternal than in umbilical blood (63) while some others report no differences between maternal and cord blood MUFA and PUFA levels (101). However, relative concentrations of SFA and LC-PUFA in maternal plasma and erythrocyte PLs have consistently shown higher levels than those in maternal blood (63;101). Paradoxically, the percentages, but not the absolute concentrations, of the LC-PUFA in cord blood are higher than their proportion in maternal blood, whereas the proportion of their precursors (LA and ALA) is lower in fetal than in maternal samples (101;112;116). The high proportion of LC-PUFA in cord plasma does not

seem to result from an active placental fetal LC-PUFA synthesis as the activity of both the Δ6- and Δ5-desaturases and LC-PUFA biosynthesis in the placenta and in the newborn, although active, appears to be limited. Thus, it appears more likely to result from a preferential maternal-fetal LC-PUFA transfer (112;116) which has been previously described.

LC-PUFA levels in the fetus have been reported to increase with gestational age. However, this gestational age-dependent increase has only been reported to happen in cord triglyceride but not in other fetal lipid classes like NEFA, cholesterol esters or PLs. The constant LC-PUFA levels in all lipid fractions other than triglycerides can be explained by the low activity of enzymes like lipase and LCAT in the fetus. Since lipolytic activity is considered to be low in the adipose tissue of newborns, fetal circulating NEFA are thought to be mainly derived from lipolysis of TG in the mother prior to their transport to the fetus. The low LCAT activity in the fetus may be the cause of the constant levels of LC-PUFA in cholesterol esters, as fetal plasma cholesterol esters are mainly synthesized by transfer of fatty acids from PC to cholesterol. Concerning PLs, they are constantly incorporated into fetal membranes during the course of pregnancy which may be the reason for the constant LC-PUFA levels in fetal plasma PLs. By contrast, fetal circulating triglycerides increase during the course of pregnancy as a result of an increased placental transfer in late gestation and the endogenous synthesis as VLDL in fetal liver. In addition, the maturation of the desaturase activity observed in the fetal liver during late gestation is responsible for the gestational age related gradual increase of n-3 LC-PUFA in fetal triglycerides. By contrast, most of the n-6 LC-PUFA in cord triglycerides show stable levels independent from gestational age, which is consistent with a higher substrate specificity of the Δ6-desaturase for the n-3 PUFA series (112).

Studies have shown that infants born with higher blood levels of DHA and AA maintain this advantage for several weeks after birth, which suggests that fatty

acids are accumulated during pregnancy in fetal tissues and do contribute to the circulating fatty acids pool after birth (113).

1.6 Effects of n-3 LC-PUFA supplementation

1.6.1 Effects of n-3 LC-PUFA supplementation on fatty acid status

Several intervention studies with different n-3 fatty acids have been conducted in order to increase the LC-PUFA status of individuals. A cross study metaregresion analysis of plasma PLs n-3 fatty acids content after ALA supplementation showed that ALA supplementation with up to 14 g/day resulted in a modest apparently linear dose dependent ALA increase in plasma PLs. The variability observed, especially at low ALA doses, is attributed to differences in the amount of LA concurrently administered in the diet, because LA reduces ALA accumulation. Small increases in EPA after ALA supplementation have also been reported in the study; however plasma PLs DHA concentrations do not detectably increase after ALA supplementation with ALA doses up to 14 g/day (25). In conclusion, even high doses of ALA result in only modest increases in ALA plasma concentrations, small changes in EPA and no effect on DHA under conditions of typical Occidental diets (25;121). Studies in vegans who consume high amounts of ALA but no EPA and DHA have shown low but stable DHA concentrations in plasma. These findings are consistent with the high oxidation and low conversion rates above described and suggest that humans can convert low quantities of ALA to EPA and DHA, particularly in the presence of a deficiency or low dietary n-6 fatty acids intakes. As described above diets containing low amounts of n-6 fatty acids can alter conversion rates (25).

Many others studied the effects of supplementation with DHA. Arterburn et al. comprised in their metaregression analysis 12 different studies with doses ranging from 0.2 to 6 g DHA/day for 1 to 6 months. This analysis showed a dose dependent

saturable increase of plasma PLs DHA concentrations in response to supplementation. DHA content in plasma PLs is highly sensitive to DHA intakes up to 2 g/day, above this amount, plasma DHA concentrations approaches saturation and increases very slowly. DHA also showed to increase EPA concentrations, presumably through retroconversion, with EPA concentrations increasing by 0.4 g/100 g fatty acid for each 1 g of DHA intake. DHA supplementation was also found to reduce plasma PLs AA concentrations in a dose dependent saturable manner, although AA response is more variable among studies (25). Studies that used EPA as supplement showed significant increases in EPA plasma PLs, but no increase in DHA concentrations, which is consistent with the poor enzymatic conversion of EPA to DHA.

Studies with fish oil supplements containing both DHA and EPA have also been conducted. They all agree that fish oil supplementation increases EPA and DHA levels in plasma and, although slower, in erythrocyte PLs (66;121). Dose-response analysis of fish oil supplementation suggests a near linear increase in plasma EPA concentrations and an apparent saturable increase in DHA concentrations after supplementation in normal individuals. AA concentrations also showed a dose dependent decrement in response to fish oil supplementation (25). Blonk et al. in their dose response study in males with DHA and EPA doses up to 6 g and 2:3 DHA:EPA ratios, report a DHA saturation dose of 1,2 g/day, which is lower than saturation dose when DHA is supplemented alone and suggest a possible displacement with EPA in plasma PLs (25;122).

A decrease in AA levels has generally been reported after fish oil or DHA supplementation (25;121). AA decreases in a gradual dose dependent manner and reaches equilibrium concentrations afterwards. Some studies have tried to avoid this side effect by adding n-6 PUFA to DHA supplements. Otto et al. supplemented in a controlled trial 24 pregnant women with both DHA and AA with a AA:DHA ratio

close to 1:2.2, they observed higher DHA levels in plasma and erythrocyte PLs in the supplemented group compared to control and no differences in AA levels between groups (61). Thus, concomitant AA supplementation has shown to be effective in avoiding the AA decrement observed after DHA and EPA supplementation. However an excessive AA intake should be cared as it might increase eicosanoid production. Geppert et al. conducted a trial that compared the effects of a fish oil and γ-linolenic acid blend with placebo and reported increases in plasma DHA levels without impairing AA status in a group of non pregnant women (123).

A cross-study comparison of effects of supplementation with similar doses of ALA, EPA and DHA on fatty acids in plasma PLs showed that ALA is relative inefficient for raising any n-3 fatty acid in plasma PLs while EPA and DHA increase their respective fatty acid in plasma to a similar degree. It was also found that both EPA and DHA supplementation result in a similar reduction in plasma PLs AA concentrations (25).

The effects of n-3 supplementation in tissues have also been reported in animal and human studies. DHA supplementation has demonstrated to produce a dose dependent increase in erythrocyte membrane DHA concentrations. In addition, strong correlations have been reported between plasma PLs and erythrocyte content of fatty acids, especially DHA and EPA (25;63). Some other studies in animals report correlation between plasma or erythrocyte DHA content and the levels of this fatty acid in other tissues such as cerebral cortex and cheek cells (25). Because of the limited accessibility of human tissues in experimental studies, there is limited experience on the direct effects of supplementation in tissues. However, the above mentioned correlations suggest that plasma or erythrocyte DHA content is a reasonable biomarker for tissue DHA concentrations. Furthermore, animal studies have shown increased content of DHA and a concomitant AA decrease in brain, retina, heart, liver, skeletal muscle, red blood cell and bone marrow of DHA

supplemented animals (25). Together, all this studies suggest that as well as plasma EPA and DHA, tissue concentrations of these fatty acids increase after supplementation.

Studies on the kinetics of incorporation and clearance of n-3 fatty acids in plasma and tissues after supplementation showed a rapid increase in plasma PLs concentrations of DHA in a dose dependent manner after daily supplementation that reaches equilibrium 1 month after the start of supplementation, this response to supplementation was slightly slower with low dose supplementation. Once equilibrium is attained, concentrations are maintained at a steady state concentration throughout the supplementation period. Erythrocyte kinetics follows a similar pattern; however it has been reported to take 4 to 6 months after the start of supplementation to reach steady state concentrations, probably due to the slower turnover of fatty acids in blood cells compared to plasma lipoproteins (25). Regarding other fatty acids EPA has also shown to incorporate more rapidly into plasma than into erythrocyte. Incorporation half-life of EPA into cholesteryl esters have been reported to be 4.8 days and plateau after 4-8 weeks after the start of supplementation. The rate of incorporation of EPA into cholesteryl esters could depend on the turnover rate of LDL which is the main lipoprotein carrying cholesteryl esters in serum. Although red blood cell lifetime is about 120 days, incorporation of EPA into erythrocyte have been shown to have a half-life of 28 days with a steady state reached after 180 days, nevertheless an increment in EPA concentration in erythrocyte have been shown already at 3 days after the start of supplementation. This is consistent with PLs or fatty acids exchange of red blood cells with their environment (66). However, DHA accumulates more slowly and erratically with larger individual variations in the percentage increases in erythrocyte PLs than does EPA. DHA turnover in erythrocyte membranes is slower probably because of the location of DHA in the inner leaflet of PLs bilayer membrane in cells which makes it difficult the exchange with plasma PLs. Moreover, it has been

observed that individuals with higher baseline DHA concentrations tend to take up DHA at slower rate than those with lower baseline concentrations, this finding suggests that erythrocyte membrane DHA concentration might be to some degree regulated (66;121). Differences in the accumulation of plasma DHA and EPA have been bound to the fact that DHA is mainly stored in PLs which is a more stable lipid molecule in plasma, while EPA is more equally distributed between triglycerides, cholesteryl esters and PLs (25).

Studies on the kinetics of DHA and EPA washout after supplementation also show a slower decrease for DHA levels compared to EPA and in erythrocytes compared to plasma. DHA and EPA decrease have been observed to have a steeper slope in the first week post supplementation, followed by a more gradual decline afterwards to the baseline concentration before supplementation (25;66;121).

From the above described it could be concluded that erythrocyte membrane fatty acids levels may be a better biomarker for long term intake of n-3 fatty acids, while plasma is more sensitive to short term changes in their intake.

However, fatty acid status of humans depends only partially on intake, nondietary factors should also be taken into account. Genetic and metabolic factors as well as lifestyle determinants may affect fatty acids metabolism and therefore concentrations in human tissues. Pregnancy may modify plasma and erythrocyte fatty acids concentrations as a result of the metabolic changes occurring during the course of gestation.

1.6.2 Effects of maternal LC-PUFA supplementation on maternal fatty acid status

Many observational studies have documented that maternal dietary n-3 LC-PUFA intake is significantly correlated with n-3 fatty acid levels in maternal plasma and/or erythrocyte PLs (42;45;53;55;124;125). In addition, interventional studies on FO or DHA supplementation during pregnancy consistently show an increase in plasma or erythrocyte DHA relative levels in supplemented groups compared to placebo. Furthermore, DHA relative levels in maternal blood at delivery are consistently higher in supplemented groups (57;59;61;104;105;117;118). By contrast, a decrease in AA relative levels has also been often reported in DHA supplemented groups compared to placebo with lower AA relative levels in both maternal plasma and erythrocyte at delivery. Thus, a competition between fatty acids of the n-3 and n-6 series is evident. However, the concomitant AA decrease in supplemented groups is mainly observed when high doses of DHA are administered (59;104;117;118). Some authors have tried to avoid this side effect by supplementing with n-6 PUFA as well as DHA. Otto et al. (61) conducted a trial in which pregnant women received a DHA/AA blend as supplement, the authors of this study report an increase in DHA levels without impairing the AA status. A summary of intervention studies on the effects of LC-PUFA supplementation during pregnancy on maternal and neonatal fatty acid profiles is shown in Table 1.

Table 1a. Summary of intervention studies on the effects of LC-PUFA supplementation during pregnancy on maternal and neonatal fatty acid profiles

Author/ Publication year	Groups	Intervention		Biochemical outcome parameter		Attrition	BI	R	C	Results		
		LC-PUFA dosage/day	Period	Maternal	Umbilical					DHA	AA	Neonatal LC-PUFA
Helland et al. 2006 Norway	S_1: 168 S_2: 160	S_1: FO. DHA 1.183 g + EPA 803 mg S_2: CO. LA 4.747 g + ALA 92 mg	18 to 35 w gestation	Plasma PLs (mg/L)	Plasma PLs (mg/L)	94%	+	+	+	↑ 35.7% in S_1 and 7% in S_2 $S_1 > S_2$ at delivery	↓ in S_1 = in S_2 $S_1 < S_2$ at delivery	DHA S1 > DHA S2. AA S1 < AA S2.
Montgomery et al 2003 Scotland	S: 50 C:50	FO: DHA 0.2 g + EPA 0.04 g	15 w gestation to Delivery	RBC (%) (mg/L) Plasma PLs (%) (mg/L)	RBC (%) (mg/L) Plasma PLs (%) (mg/L)	44-58%	?	+	+	↑ in S and ↓ in C S > C at delivery in RBC S = C at delivery in Plasma PLs.	↓ in S and C S = C at delivery in RBC and Plasma PLs.	S = C. RBC and Plasma PL DHA (%) > in umbilical than in maternal blood.
Sanjurjo et al. 2004 Spain	S: 8 C:8	FO: DHA 0.2 g + EPA 0.04 g	3rd trimestre to Delivery	Total plasma (%)	Total plasma (%)	80%	?	+	- *	↔ in S and C S > C at delivery	↓ in S and C S = C at delivery	S = C
Dunstan et al. 2004 Australia	S: 52 C:46	FO: DHA 2.2 g + EPA 1.1 g	20 w gestation to delivery	RBC PLs (%)	RBC PLs (%)	70-80%	?	+	+	↑ in S and ↔ in C S > C at delivery	↓ in S and ↔ C S < C at delivery	DHA S > DHA C. AA S < AA C. RBC DHA and AA (%) > in umbilical than in maternal blood. Positive materno-fetal correlation for DHA and AA %.
Bergmann et al. 2008 Germany	S_1: 48 S_2: 48 C: 48	S_1: FO. DHA 0.2 g + EPA low + FOS S_2: FOS	21 w gestation to 3 m lactation	RBC PLs (%)	_	80%	+	+	+	↑ in S_1 , S_2 and C; > in S_1 compared to S_2 and C. $S_1 > S_2$ y C at 37 w gestation.	Non significant ↓ in S_1 , S_2 and C.	_
Innis et al. 2008 Canada	S: 67 C: 68	S: DHA 0,4 g C: Soy-bean oil	16 w gestation to Delivery	RBC PLs (%)	_	?	+	+	+	↑ in S and C. S > C at 36 w gestation.	↓ in S and C. S < C at 36 w gestation.	_

Table 1b. Summary of intervention studies on the effects of LC-PUFA supplementation during pregnancy on maternal and neonatal fatty acid profiles

Author/ Publication year	Groups	Intervention		Biochemical outcome parameter		Attrition	BI	R	C	Results		
		LC-PUFA dosage/day	Period	Maternal	Umbilical					DHA	AA	Neonatal LC-PUFA
Van Houwelingen et al. 1995 Denmark	S: 23 C_1:16 C_2:10	S: 2.7 g n-3 (DHA+EPA) C_1: olive oil	30 w gestation to Delivery	Plasma PLs (%)	Plasma PLs (%)	?	+		+	↑ in S	↓ in S	DHA S > DHA C. AA S < AA C. Positive materno-fetal correlation for DHA and AA %.
Smuts et al. 2003 US	S: 142 C: 149	S: DHA enriched eggs (133 mg DHA/egg) C: Ordinary eggs	24-28 w gestation to Delivery	RBC (%)	RBC (%)	83%	+	+	+	S=C	-	DHA S > DHA C.
Velzing-Aarts et al. 2001 The Netherlands Antilles	S_1: 15 S_2: 24 S_3: 20 C: 57	S_1: 177 mg EPA + 123 mg DHA S_2: 293 mg EPA + 185 mg DHA S_3: 531 mg EPA + 369 mg DHA C: No supplement	18-23 week gestation to Delivery		Fatty acids in umbilical vein and artery	79%	-	?	+	-	-	S_1=C. DHA S_2 > DHA C in UV and UA. DHA S_3 > DHA C in UA. No AA differences between groups. 500-1000 mg n-3 LC-PUFA daily increases n-3 LC-PUFA status without affecting n-6 LC-PUFA status.
Otto et al. 2000 The Netherlands	S: 12 C:12	DHA 0.57 g + AA 0.26 g	In the 2nd trimester during 4 w	RBC (%) (mg/L) Plasma PLs (%) (mg/L)	_	?	+	+	+	No differences in the total amount (mg/L) of plasma and RBC PLs between groups. ↑ Plasma and RBC PLs (%) in S.	S = C	_

S, supplemented group; C, control group; LC-PUFA, long chain polyunsaturated fatty acids; FO, fish oil; CO, corn oil; DHA, docosahexahenoic acid; AA, arachidonic acid; EPA, eicosapentanoic acid; LA, linoleico acid; ALA, α-linolenic acid; PLs, phospholipids; RBC, red blood cells; UV, umbilical vein; UA, Umbilical artery; Bl, Blind intervention; R, randomization; C, adjusted for confounders; (%), total weight of fatty acids.
*Higher maternal age in supplemented group.

1.6.3 Effects of maternal LC-PUFA supplementation on fetal fatty acid status

There has been in the last years growing interest in increasing maternal DHA intake throughout gestation, in order to increase maternal DHA supply to the developing fetus.

Observational studies have shown that dietary intake of DHA and EPA positively significantly correlate with the same fatty acid in umbilical vessel walls as well as maternal and umbilical plasma PLs (117), furthermore, positive significant correlations between maternal and neonatal DHA levels in plasma and erythrocyte PLs have been reported (3;59;63;97). Data from these studies show that placental transfer of DHA is highly influenced by maternal DHA levels which have been associated with maternal DHA intake.

Interventional studies have demonstrated that DHA supplementation during pregnancy associated or not to EPA supplementation, increases neonatal DHA status. Most of the studies on DHA supplementation published to date report higher DHA and lower AA levels in cord blood from supplemented women (59;117;118). Thus, a competition between fatty acids of the n-3 and n-6 series seems to be evident. However those studies that used lower DHA supplementation dosages did not find any differences in cord blood fatty acid levels between supplemented and non supplemented women (57;60). This finding is consistent with a minimal supplementation dose required to increase fetal and neonatal DHA status. Velzing-Aarts et al. (119) supplemented pregnant women with three different FO doses and measured fatty acids in umbilical vein and artery, they concluded that 500-1000 g n-3 LC-PUFA supplement daily effectively increase fetal n-3 LC-PUFA status without affecting n-6 LC-PUFA. Supplementation with both DHA and AA has proven to increase the DHA levels without decreasing the levels of AA (61).

Apart from determining the optimal dosage and fatty acid mixture, the period of supplementation should also be considered. Some authors suggest that maternal nutritional status during early embryonic development has a greater effect on the fetus than maternal nutrition during the last 2 trimesters of pregnancy, as the increment on DHA levels described during the first period of pregnancy has been attributed to mobilization of maternal fatty acid depots in adipose tissue. Therefore, an early intervention in early pregnancy or even before conception, may be more effective in altering the neonatal EFA status than supplementation after complete maturation of the placenta (31;100).

1.6.4 The risks of n-3 LC-PUFA supplementation

Since dietary fatty acids during pregnancy play an important role in controlling the supply of LC-PUFA to the fetus, supplementation with LC-PUFA rich oils during the last trimester of gestation has been advised. However, an excessive LC-PUFA intake during pregnancy may alter balance of n-3 PUFA and n-6 PUFA, which may have negative effects on the offspring. An excessive n-3 LC-PUFA intake may decrease AA levels, which has been related to lower birth weight. However, the fetus is capable to some kind of regulation of its own AA levels, which may protect it from variations in maternal AA levels. In addition, and excessive LC-PUFA intake may reduce the antioxidant capacity in the mother and the fetus by means of increasing consumption of α-tocopherol, which would enhance susceptibility to oxidative damage. Studies in animals and cell cultures have shown a reduced antioxidant capacity and enhanced susceptibility to oxidative damage in the presence of excessive LC-PUFA. However, several studies in humans have shown that dietary supplementation with FO does not increase in vivo lipid peroxidation. Whether high n-3 LC-PUFA intake increases lipid peroxidation continues to be controversial (27;96;126-128).

Further concern is given to the fact that oils of marine origin contain organochlorides which may be harmful to the fetus if ingested in sufficient amount by the mother. Particular concern is given to the polychlorinated byphenils (PCBs), which are among the most ubiquitous organochloride contaminants in the environment. Organochlorides are stored in high amount in the adipose tissue where they are relatively harmless, but the high mobilisation of fat depots occurring in the mother during pregnancy and in the fetus in the first postnatal months may increase their concentration in blood to dangerous levels for the fetus and the newborn. Furthermore, cord blood levels of organochlorides have been positive correlated to the concentrations in maternal blood and adipose tissue (31). Maternal fish consumption has also been associated to an increased mercury exposure (129), results from studies have demonstrated that both, mercury and DHA are transferred to the fetus with a positive correlation between maternal and neonatal mercury concentrations. Furthermore, a positive correlation between methylmercury and DHA concentrations in fetal circulation has been reported (130). Exposure to high levels of mercury and PCBs in fetal life, as reported from past accidental poisoning in humans, has been associated to severe neurological damage, specially important are impairments of motor functions, particularly those involving the cerebellum such us balance and coordination. Lower level exposures to mercury and PCB observed in epidemiological studies have shown some indications of motor impairments, although these low level prenatal and/or perinatal exposures to mercury and PCBs have been mainly related to deficiencies in infant cognitive development, including decrements in attention, language verbal memory, motor speed and visuospatial function (129;131;132). Integrative analysis of the overall effect of prenatal mercury exposure on intelligence have estimated that each 1 μg/g increase in maternal hair mercury was associated with a decrement of child intelligence quotient between 0.18 and 0.7 points (129). Mercury is known to damage cerebellum in both, animals and humans but the effects of PCBs on cerebellum are not so widely studied. However, some authors suggest that cerebellum is particularly vulnerable to combined

mercury and PCBs exposure (132). However, studies examining the overall effects of fish consumption on neurological development have reported beneficial effects of fish consumption on neurological outcome up to the age of 14 years. Investigators conclude that women should continue to consume fish during pregnancy, but should avoid fish most highly contaminated with mercury (87;129).

1.7 LC-PUFA and the central nervous system

1.7.1 LC-PUFA and the brain

The development of the human brain starts with neurogenesis between the 14th and the 25th weeks of pregnancy, during this time takes place the proliferation of cells in the germinal layer and posterior migration to their final places of destination where they start to differentiate. Differentiation is a complex process that includes the formation of dendrites and axons, the production of neurotransmitters and synapses, and the elaboration of intracellular signalling machinery and the neural membranes and it is particularly active in the last trimester gestation and the first months of postnatal life. In addition glial cell production occurs in the second half of pregnancy and axonal myelination takes place especially between the second trimester of pregnancy and the end of the first postnatal year, although it is completed around the age of 30 years (133).

Approximately 50-60% of human brain dry weight is constituted by lipids, about 35% of the lipids are PUFA and most of them are LC-PUFA. In humans, the growth spurt of the brain takes place from the beginning of the third trimester of gestation to 18 months of postnatal age. During this period of time the size of the brain increases from about 100 mg at the beginning of the third trimester to 1,100g at 18 months after birth and the total amount of DHA increases in a parabolic manner from about 3000 nmol/g brain in the 25th week of pregnancy to 10,000 nmol/g at the

postnatal age of 2 years. Thus, the relative increment in the total amount of DHA in the brain is higher than the increment in its size. Given all this, the DHA enhancement in the brain is not only due to the increment in brain size but also to the enrichment in the relative content of DHA in the brain. Clandinin and co-workers estimated the brain DHA accretion to be 15-22 mg/week during the last trimester of pregnancy and 3.6 mg/week postnatally (29).

High proportions of DHA in brain ethanolamine PLs and phosphatidylserine, reaching as high as 35% of fatty acids in synaptic membranes, is a characteristic feature of the mammalian brain, even among herbivores, and regardless of low concentrations of DHA in plasma and hepatic lipids. Plasma lipid levels of DHA, on the other hand, are low in most terrestrial animals, including humans, suggesting the brain has particular mechanisms to concentrate DHA (134). Fatty acids in the brain can either be taken up from the blood as preformed DHA or synthesized from ALA inside the brain. Studies in animal and human tissues suggest that the brain is able to take up EFA and convert them to LC-PUFA. Studies in rats have demonstrated that the rat brain contains the elongase and desaturase enzymes necessary for the synthesis of LC-PUFA from. Furthermore, studies on adult human brain found the presence in brain of a Δ6-desaturase mRNA. In vitro neurons have only a small capacity of elongation and are not capable of DHA synthesis, by contrast, astrocytes are able to synthesize DHA and release it unesterified or in choline containing PLs and endothelial cells have demonstrated their capacity to metabolise ALA to EPA but their capacity to synthesize DHA is very limited. Some authors have found Δ6-desaturase activity in the choroid plexus. Some authors have suggested that conversion of EFA to LC-PUFA could be integrated as part of the brain EFA uptake mechanism but the way how EFA are taken up across the brain-blood barriers remains unknown (29;134). Most likely, DHA is taken up from plasma. Thus, the main source of PUFAs for the brain seem to be plasma albumin-associated NEFAs and not the complex lipids bound to lipoproteins as it is in other tissues. It has been

demonstrated that a substantial part of the albumin-associated DHA is esterified in the sn-2 position of PC PLs which is the second most prevalent PL in plasma. In vitro and in vivo studies in animals have shown that PUFA bound to PC PLs are preferentially transferred across the in vitro blood-brain barrier and incorporated in the brain rat relative to non esterified PUFAs. Before DHA reaches neurons, it has to be transported across the blood-brain barrier. The presence of an n-3 and n-6 fatty acid selective uptake mechanism has been proposed but it has not yet been found, although the transport has been thought to be aided by binding of LC-PUFAs to transport proteins. Neurons and glial cells appear to express an intramembranous FATP and cytoplasmic FABP, but the function of this transport system remains unknown (29).

Several hypotheses have been proposed to explain the role of DHA in the brain, which in general can be divided into properties conferred by lipid-bound DHA in the membrane bilayer and those related to unesterified DHA. Changes in the membrane DHA content can, on one hand, alter membrane physicochemical properties and, on the other hand, membrane DHA have a direct interaction with membrane proteins, thus impacting the signal transduction pathway, neurotransmission, and formation of lipid rafts. The high proportion of DHA in neural membranes also raises the possibility that n-3 LC-PUFA deficiency may impair membrane biogenesis, influencing neurogenesis, neuronal migration, and outgrowth. Unesterified DHA seems to have roles in regulating gene expression, ion channel activities, can be further metabolized to neuroprotective metabolites in the brain and it has been suggested to have an important role in neurogenesis. LC-PUFAs are natural ligands for the nuclear transcription factors PPARγ and RXR. PPARγ is highly expressed in embryonic mouse brain and neural stem cells, and seems to be important in regulating the early brain development, through effects that include regulation of stem cell proliferation. The RXR together with its retinoic acid receptor (RAR) plays important roles in many aspects of development, including

neurogenesis during embryogenesis, morphological differentiation of catecholaminergic neurons, and activity-dependent plasticity. Early changes in gene expression leading to altered molecular and morphological development could have long-term implications to brain function. Other recent studies have shown that DHA promotes differentiation of neural stem cells into neurons by promoting cell-cycle exit and suppressing cell death. Thus, n-3 fatty acid deprivation may affect brain development at multiple levels (membrane biogenesis, through gene expression, protection against oxidative stress, and altered neurotransmission), with the effects differing, and the potential for recovery dependent on when the deficiency is imposed (134;135).

Studies in animals have shown that maternal dietary n-3 fatty acid restriction during pregnancy alters neurogenesis in the fetal rat cerebral cortex (134). Moreover, several studies have shown that monoaminergic and cholinergic systems are affected in rodents chronically deprived of n-3 fatty acids during development (135). N-3 fatty acid deficiency has shown to affect dopaminergic neurotransmission in the frontal cortex of the rat as well as in hippocampus. This structures show lower endogenous dopamine concentrations with a reduced number of dopaminergic vesicles in the presinapse, higher extracellular metabolites and increased density of D2 receptors while nucleus accumbens is affected in the opposite direction as the dopaminergc activity in this region is under tonic inhibitory control of the mesocortical dopaminergic pathway on frontal cortex. Functions like learning and memory involve the frontal cortex and the hippocampus and the nucleus accumbens has an important role in cognitive, attentional, motivational and emotional processes which also influence learning abilities. As a result, n-3 deficiency might have negative effects in learning processes (29). Studies on baboons have shown that the basal ganglia have the highest DHA accumulation in the brain and may be specifically vulnerable to DHA deficiency. These areas are important to psychomotor behaviour and are particularly related to coordination and fine manipulative ability

(136;137). In addition, n-3 fatty acid deficient rats have also been reported to have changed pineal melatonin levels which might alter their circadian circle. N-3 LC-PUFAs have also been suggested to modulate nerve conductance by means of affecting the ion pumps and channels in neuron membranes. Furthermore, peroxisomal disorders are associated to both, low brain levels of DHA and myelination abnormalities, which is also very important for nerve conduction velocity (29).

1.7.2 LC-PUFA and the retina

The highest DHA levels in the body have been found in the rod outer segment membranes of the retina, where it comprises about 50% of total esterified fatty acids. DHA accretion in human retina starts at the beginning of the third trimester of pregnancy and seems to reach a plateau at 36-40 weeks gestation, although some observations suggest that it may continue after birth. Most of the DHA in the retina appears to derive from synthesis in the liver, but the retina has been demonstrated to have also some local synthetic capacity. The transfer of n-3 fatty acids from blood to the photoreceptors have been studied in frogs and it seems to occur through capillary endothelium and retinal pigment epithelium where they are converted to DHA. DHA is then selectively incorporated in photoreceptor PLs in the base of the rod and targeted to the rod outer segment membranes by cotransport with rhodopsin. Rhodopsin is the dominant membrane protein in rod outer segment. This molecule functions as a photon receptor coupled to a G-protein and suffers an important conformational change upon photoactivation when it is converted to metarhodopsin II. The light-induced conformational change in rhodopsin triggers a biochemical cascade, finally leading to membrane depolarization. One of the most important effects of membrane DHA is its role in the photoreceptor signal transduction process. The current hypothesis is that the high content of DHA in rod outer segment membrane PLs enhances elasticity of the membrane which accommodates the

necessary changes in membrane thickness and curvature occurring as a result of the high frequency conformational changes and volume expansion undergone by rhodopsin. The corresponding functional effect is the increase in retinal sensitivity to light associated to DHA supply (28;29). The rod outer segment membrane is a very dynamic structure, 10% of the membrane is phagocytized daily by the retinal pigment epithelium and then replaced by new membrane at the base of the rod. Nevertheless, once retinal development is complete the DHA content of the retina remains unaffected by n-3 fatty acid status. DHA is efficiently retained in the retina by some system involved in recycling DHA of the rod outer segment membranes from the retinal pigment epithelium back to the retina (29).

1.7.3 Brain development and assessment of neurological function

Brain develops mainly in the second half of pregnancy and the first year of life, but neural circuitries continue to change significantly afterwards. Neurological changes occurring during preschool age are growth of dendrites of cortical neurons, axonal elimination in long projection fibbers like corpus callosum and corticospinal tract, myelination and synaptic reorganization which correlates with functional neurodevelopment. Around 18 month of age the child has achieved the basic motor skills and is able to reach, grasp (including the pincer grasp), crawl, sit, stand, walk and uses a few words. During the preschool age, hardly any new motor function is added. Changes occurring at this period are qualitative rather than quantitative and the child learns to use these skills more efficiently and in increasingly complex situations. Apart from the acquisition of more complex motor functions such as run, jump, climb a stairs with alternating feet, throw a ball overhand and use scissors, social and emotional abilities show important changes at the age of 4. During the preschool period an evolution in the child's ability to deal with strangers is observed. At early preschool age the child has a preference for familiar caretakers and shows stranger and separation anxiety. Around 2 years of age the child is more likely to

approach a stranger and at 4 years of age forms relationships with others outside of the caretaking environment which allows the child to play with other children, to go alone to the toilet and to complete most parts of dressing and undressing. In addition, language develops exponentially during the preschool age, so that at 4 years of age the child is able to tell a story. The development of cognitive capacities is reflected in the acquisition of numerical abilities, at 4 years of age the child is able to understand ordinal relationships including processes as simple addition or subtraction (133).

From the above mentioned, it can be concluded that children have an age specific nervous system which makes it necessary an age specific neurological examination to evaluate their neurological condition. Moreover, the age specificity of children nervous system affects the way in which neural dysfunction is expressed. There are sigs of neurological dysfunction traditionally expressed in infancy and preschool age that often disappear with increasing age, as their expression is countered by advancing neurological development. Other signs cannot be expressed at preschool age, as the neural circuitries involved in their expression have not become functionally active yet and can first be found at school age. The progressive developmental changes occurring in the nervous system make it possible that dysfunctional signs at preschool age may disappear later on as the nervous system may be able to find solutions to overcome early dysfunctions. However, the opposite can also happen and children with no dysfunctional signs early in life may develop a functional deficit with increasing age (133).

Neurological assessment of young and older children basically consists on the examination of the child general appearance, assessment of cranial nerves, presence or absence of abnormal movements, assessment of muscle power and tone, reflexes and sensations and evaluation of posture and gross motor dysfunction. Prior to examination the child needs time to get accustomed to the unfamiliar situation, the

examination room and the examiner. It is important to achieve the cooperation of the child as behavioural state during the neurological assessment affects the expression of neurological functions. During infancy children are only partially aware of the strangeness of a neurological examination and let it pass as just another event. At school age the child is aware of the situation but has developed the social and cognitive abilities to cope with it. Preschool children are aware of the unusual of the situation of a neurological examination but lack the social and cognitive abilities and usually refuse being touched. Thus, preschool examination has been described as difficult because of the time needed to achieve child's cooperation and it is recommended to observe the child's spontaneous motor behaviour during play with carefully selected toys (133).

1.7.4 LC-PUFA supplementation during pregnancy and development of brain function

On the basis of the already presented data, it can be suggested that an inadequate supply of n-3 LC-PUFA to the fetus during the period when brain develops, can compromise brain function. Several animal studies have also shown that dietary DHA is readily incorporated into lipids of the developing brain, both before and after birth (134). Before birth, DHA is transported to the fetus from the mother across the placenta and after birth, the infant is provided with DHA in mother's milk. The effects of LC-PUFA on neurodevelopmental outcome have been widely studied and have also been reviewed in several articles.

A lot of studies on the effects of LC-PUFA supplementation after birth in term and preterm infants have been conducted. Simmer et al. (67) concluded in a Cochrane review that most of the well conducted randomized controlled trials did not show benefits of LC-PUFA supplementation in term infants on neurological outcome. They reviewed 14 randomized controlled trials comparing the effects of

LC-PUFA supplemented with non-supplemented formula milk on neurological outcome in term infants. 11 studies assessed neurological function at different ages. 8 of them used the BSID and only one showed a better performance of supplemented infants in the test compared to controls. Furthermore, a meta-analysis on the data did not show statistical significant differences on mental or psychomotor developmental indexes between supplemented and non supplemented infants. A study using the Fagan Infant Test at 9 months of age to assess neurodevelopment reported better novelty preference in those children who received LC-PUFA supplemented milk. Another study reported better problem solving at 10 months in the supplemented group compared to controls. However, no differences in the developmental quotient assessed with Brunet and Lezine developmental test was found between groups by other authors. Thus, the authors of the revision concluded that, on the basis of the current evidence, supplementation of milk formula to improve neurodevelopmental outcome in term infants cannot be recommended.

The Cochrane have also reviewed studies conducted in preterm infants (68). Most of the revised trials used the BSID to assess neurological outcome, 4 studies evaluating neurodevelopment between 9 and 24 months of age did not find differences between the group receiving supplemented formula milk and the group receiving the control formula. Two other studies reported higher psychomotor and mental developmental indexes (PDI and MDI) in the supplemented group compared to the control at 12 and 18 months of age. Interestingly one of the studies showed lower PDI and lower novelty preference in the Fagan test in those infants who received supplemented formula milk compared to controls, however these authors reported more looks and shorter duration of the looks in supplemented children, which they linked to a more rapid visual information processing. The meta-analysis conducted in the Cochrane review of 4 studies using BSID at 12 months of age post-term (n=364) and 3 studies using it at 18 months (n=494) showed no significant effect

of supplementation on neurodevelopment. Thus, no clear long term benefits have been demonstrated for preterm infants receiving LC-PUFA supplemented formula.

As the incorporation of DHA in the developing brain is particularly high in the prenatal period, n-3 LC-PUFA supplementation to expecting women, particularly DHA supplementation, may enhance n-3 LC-PUFA supply to the fetus and thereby improve its neurological function.

Studies on animals have shown lower DHA concentrations in the brain and poorer cognitive and behavioural capacities in severe n-3 LC-PUFA restricted animals compared to controls. Although studies comparing n-3 LC-PUFA supplementation in n-3 LC-PUFA restricted animals with n-3 restricted controls have reported a significantly better performance in cognitive and behavioural tests in the supplemented group, only a few studies have studied the effects of LC-PUFA supplementation of normal diets during pregnancy or lactation on brain development. While some studies reported an acceleration of visual development, others showed a poorer auditory and motor development after DHA or FO supplementation. However, learning capacity does not seem to be affected by supplementation. The negative effects have been proposed to be related to the decreased levels of AA secondary to the competition between fatty acids of the n-3 and n-6 series (133-135;138).

Epidemiological studies on the overall effects of fish consumption showed that, although higher fish consumption was indeed associated to higher mercury levels, higher fish consumption was also related to better performance of children in different neurological tests up to the age of 14. Oken et al. (139;140) associated higher maternal fish consumption with higher infant scores on the visual recognition memory test at the age of 6 months as well as with higher scores in the Peabody Picture Vocabulary test and the Wide Range Assessment of Visual Motor Abilities at the age of 3 years. The ALSPAC study (141) showed that mothers who ate less than

340 g of seafood per week had higher risk of having children in the lowest quartile for verbal intelligence quotient at 8 years. They also related low maternal seafood intake to suboptimal outcomes for prosocial behaviour, fine motor, communication and social developmental skills at 7. The study conducted by Budtz-Jorgensen et al. (142) in the Faroe Islands associated higher maternal seafood intake with higher scores of motor and spatial function in children at 14. These authors did not find any significant association between maternal fish intake and other skills such as attention, verbal performance and memory. These studies suggest that the benefits o increased maternal fish consumption during pregnancy would be greater if they chose seafood with low mercury content.

Observational studies have associated higher DHA and AA levels in umbilical blood or the wall of umbilical vessels at birth with more mature sleep pattern in the neonate (143), shorter latencies in the brainstem auditory evoked potentials at 1 month of age (144), a better visual acuity at 6 months of postnatal life (65), a better visual habituation at the ages of 4 and 6 months and better attention at 12 and 18 months of life (145) and a better neurological condition assessed by means of the Prechtl neurological examination (10-14 days) (146), the assessment of general movements (3 months) (147) and the Fagan (6 month) (65), Bayley (11 and 18 month) (65;148) and Hempel examinations (18 month) (148). Thus, many observational studies have reported positive effects of LC-PUFA during fetal development on neurological development assessed early in life, but very few of them have followed children development later in life. Higher grade stereopsis at 3 ½ years of age has been reported in children whose mothers ate oily fish during pregnancy compared to those children whose mothers did not eat oily fish irrespective of later infant feeding practice (149). Furthermore, higher levels of DHA at birth have been associated with lower levels of internalising problem behaviour and a better quality of movements in the Maastricht's Motor Test at 7 years of age (150). However, two observational studies evaluating cognitive function of full-term infants at 4 and 7 years of age could

not establish a relationship between neonatal LC-PUFA status at birth and the scores obtained in the Kaufman Assessment Battery for Children (K-ABC) (151;152). All these studies suggest that prenatal DHA status might have subtle positive effects on neurodevelopmental outcome, but whether these effects are maintained beyond early infancy remains a matter of discussion.

Several randomized controlled trials have been conducted to examine the effects of n-3 LC-PUFA supplementation during pregnancy on children neurologic outcome (Table 2). It is controversial whether supplementation actually improves neurologic development in children. Some studies have reported higher developmental outcomes in children whose mother received n-3 LC-PUFA supplements during pregnancy whereas others found no differences compared to control children. Three studies have assessed neurological outcome in the first year of life after n-3 LC-PUFA supplementation in pregnancy, none of them report a better performance in neurologic tests in the supplemented group. Two studies report no differences between the supplemented and control group performance in the Fagan Test of Infant Intelligence (FTII) at 6 and 9 months of age (58;153) and the third report no differences in the BSID at the 10th postnatal month (154). However, there are some data suggesting that n-3 LC-PUFA in the perinatal period may influence neurologic function. Helland et al. (58) report no differences in electroencephalogram (EEG) maturity at 2 days and 3 months after birth between groups, but higher EPA and DHA levels were associated with more mature EEG pattern. Judge et al. (153) report a positive effect of supplementation on the 2 step problem solving test in the 9th month after birth which has been related to intelligence quotient in early childhood. Moreover, Dunstan et al. report a better eye and hand coordination in the Griffiths Mental Development Scales (GMDS) in the supplemented group at 2 ½ years of age as well as a significant positive association with n-3 LC-PUFA in cord blood. Interestingly the authors also report an inverse correlation between eye and hand coordination and performance score at 2 ½ with

AA in cord blood (155). To our knowledge, only one trial has reported long term effects of supplementation to date. Helland et al. report a better performance in the K-ABC in the supplemented group compared to control at 4 years of age; however, they did not find any differences in IQ at 7 years of age using the same test (K-ABC) to asses it. The authors also report a significant positive correlation between IQ at 4 years of age and DHA levels in infant plasma at the 4th week of life as well as an association between maternal DHA levels in the 35 week gestation and IQ in the children at 7 years of age (156;157).

The difficulty of measuring mental development during infancy may partially explain the conflicting results. Furthermore, the studies widely differ methodologically which make it difficult to drawn conclusions. The fact that different tests measure different aspects of the neurological function and that the central nervous system of infants acquires the various cognitive skills with age progression, must be taken into account in the interpretation and comparison of results among studies.

Table 2a. Summary of controlled clinical trials evaluating the influence of n-3 LC-PUFA supplementation during pregnancy on neurological development

Author/ Publication year	Groups	Intervention		Biochemical outcome parameter	Neurologic Examination		Attrition	B	R	C	Results
		LC-PUFA dose/day	Period		Test	Age					
Dunstan et al 2008 Australia	S: 33 C: 39	S: FO (2.2 g DHA + 1.1 g EPA) C: olive oil	20 w gestation to delivery	Fatty acid RBC PLs (%)	GMDS PPVT CBCL	2 ½ y	26%	+	+	+	GMDS: S group higher eye hand coordination. No differences for mean general quotient and other subscales. Significant positve correlation between eye and hand coordination and n-3 LC-PUFA in cord blood. Inverse correlation between eye and hand coordination and performance score with AA in cord blood. PPVT: No differences. CBCL: No differences.
Tofail et al 2006 Bangladesh	S: 125 C: 124	S: FO (1.2 g DHA + 1.8 g EPA) C: soy oil (2.25 g LA + 0.27 g LNA)	25 w gestation to delivery		BSID HOME	10 m	38%	+	+	+	No differences
Judge 2007 US	S: 14 C: 15	S: 214 mg DHA C: corn oil	24 w gestation to delivery		2 step problem solving test FTII	9 m		+	+	+	Problem solving: S Group better performance. FTII: No differences (recognition memory abilities).
Helland 2001 Norway	S: 301 C: 298	S: FO (DHA 1.183 g + EPA 803 mg C: Corn oil (LA 4.747 g + ALA 92 mg)	17-19 w gestation to 3 m lactation	Fatty acids in plasma PLs (mg/L)	EEG FTII	2 d, 3m 6m, 9m	62% 71%	+	+	+	EEG: No differences in maturity. Children with higher umbilical EPA and DHA had a more mature EEG. FTII: No differences.

Table 2b. Summary of controlled clinical trials evaluating the influence of n-3 LC-PUFA supplementation during pregnancy on neurological development

Author/ Publication year	Groups	Intervention		Biochemical outcome parameter	Neurologic Examination		Attrition	B	R	C	Results
		LC-PUFA dose/day	Period		Test	Age					
Helland 2003 Norway	S. 48 C: 52	S: FO (DHA 1.183 g + EPA 803 mg C: Corn oil (LA 4.747 g + ALA 92 mg)	17-19 w gestation to 3 m lactation	Fatty acids in plasma PLs (mg/L)	K-ABC	4 y		+	+	+	S group higher score on the Mental Processing Composite. Significant positive correlation between maternal DHA and EPA intake and children´s mental processing scores. Significant positive correlation between intelligence score and DHA in plasma PLs at 4 w age.
Helland 2009 Norway	S: 82 C: 61	S: FO (DHA 1.183 g + EPA 803 mg C: Corn oil (LA 4.747 g + ALA 92 mg)	17-19 w gestation to 3 m lactation	Fatty acids in plasma PLs (mg/L)	K-ABC	7 y		+	+	+	No differences in the K-ABC scores. Tendency towards higher scores in sequential processing in S group (no significant). Positive significant correlation between maternal DHA levels in 35 w pregnancy and umbilical EPA levels at birth and scores on the sequential processing scale.

S, supplemented group; C, control group; LC-PUFA, long chain polyunsaturated fatty acids; FO, fish oil; CO, corn oil; DHA, docosahexahenoic acid; EPA, eicosapentanoic acid; LA, linoleico acid; ALA, α-linolenic acid; PLs, phospholipids; RBC, red blood cells; Bl, Blind intervention; R, randomization; C, adjusted for confounders; (%), total weight of fatty acids.

GMDS: Griffiths Mental Development Scales, PPVT: Peabody Picture Vocabulary Test, CBCL: Child Behaviour Check List, BSID: Bayley Scales of Infant Development, HOME: Home Observation for Measurement of Enviroment, FTII: Fagan Test of Infant Intelligence Test for recognition memory), K-ABC: Kaufman Assessment Batery for Children .

2 OBJECTIVES

We aim to study the biochemical and potential clinical and functional effects of supplying 500 mg of preformed n-3 LC-PUFA (DHA+EPA) and the biologically active form of folic acid (5- MTHF) to pregnant women during the second half of pregnancy.

Hypotheses to be tested:

- Supplementing pregnant women with DHA (500 mg/d), 5-MTHF (0.4 mg/d) or both during the second half of pregnancy has biochemical effects in mothers and infants.
- Supplementing pregnant women with DHA (500 mg/d), 5-MTHF (0.4 mg/d) or both has long term effects on the neurological development of infants.

To test these hypothesis DHA content in plasma and erythrocyte PLs from the mother at different time-points of pregnancy and from newborn infants at birth (cord blood) was measured. If supplemented DHA is efficiently absorbed and transferred across the placenta the percentage levels of DHA in cord blood is expected to increase in supplemented groups. Furthermore, an influence of 5-MTHF on the placental transfer should also introduce an effect on the DHA content. Changes of DHA relative levels in maternal plasma and erythrocyte PLs, as well as the response of other fatty acids to supplementation between study entry and the time of delivery was also studied. As habitual diets are assumed to be different between the centres participating in the study, the effect of supplementation can be evaluated above different dietary backgrounds.

The Hempel test for toddler, the Touwen Infant Neurological Examination (TINE) and the Kaufman Assessment Battery for Children (K-ABC) were performed as functional tests of the central nervous system to detect the influence of n-3 LC-PUFA supplementation on long term neurological development.

3 MATERIALS AND METHODS

3.1 Study design

The present study was conducted to asses the effects of n-3 LC-PUFA and/or folate supplementation during the second half of pregnancy on maternal and fetal fatty acid status, as well as on long term neurological development of their offspring. To address this question, a multicenter, randomized, double blind placebo controlled study was designed. Expecting women from three different European countries with different basal dietary intakes of fatty acids (Spain with high n-3 LC-PUFA intake, Germany with medium n-3 LC-PUFA intake and Hungary with low n-3 LC-PUFA intake) were supplemented from the 20th week gestation until delivery with either FO, 5-MTHF, both or placebo. Pregnant women were enrolled before the 20th week of pregnancy and randomly assigned to one of the four supplementation groups. Detailed and standardized information on socio-demographic conditions was collected at study entry, information on the course of pregnancy at the 20th and the 30th week, as well as information on delivery, was also obtained in standardized reports. Dietary information was collected by food frequency questionnaires (FFQ) both at the 20th and the 30th week of gestation. In addition, 10 ml of maternal venous blood were collected into EDTA at the 20th and the 30th week of gestation and 12 ml of maternal venous blood, as well as 12 ml venous cord blood samples, were also collected at delivery for the fatty acid analyses.

After birth, women were encouraged to breast feed their infants. The supplement is assumed to affect the composition of breast milk and hence the early postnatal nutrient supply to breast-fed infants. Infants who required supplements or substitution of breast feeding were provided with infant formulas until the 6th month

of postnatal age, either containing n-3 LC-PUFA or not, in an attempt to match as far as possible the supply with that of the breast fed babies.

All children were examined by a trained physician at birth and at the ages of 2 and 6 months. Data on the infants, postnatal diseases and visits to paediatrician were obtained in standardized reports; a physical examination and somatometric measures of the infants were as well performed in each visit.

At the age of 4 years the participating mothers and their respective infants were again approached and asked to participate in the neurological follow-up of the children (NUHEAL follow-up). Children neurological development was assessed at the age of 4 using the Hempel neurological examination, at 5½ with the Touwen Examination and at 6 ½ by means of the K-ABC. Assessment of potential confounders was made by means of a health screening questionnaires to obtain information on possible diseases of the children that could influence neurologic development. In addition, children were examined by a trained physician who also obtained anthropometric measures in each visit. Some other information not used in the present work was also collected in the different visits. At the age of 4 visual evoked potentials (VEP) and children FFQs were obtained, at 5 ½ years of age the Children Behaviour Checklist (CBCL) was filled in by parents and at 6 ½ FFQs were obtained (Fig. 6).

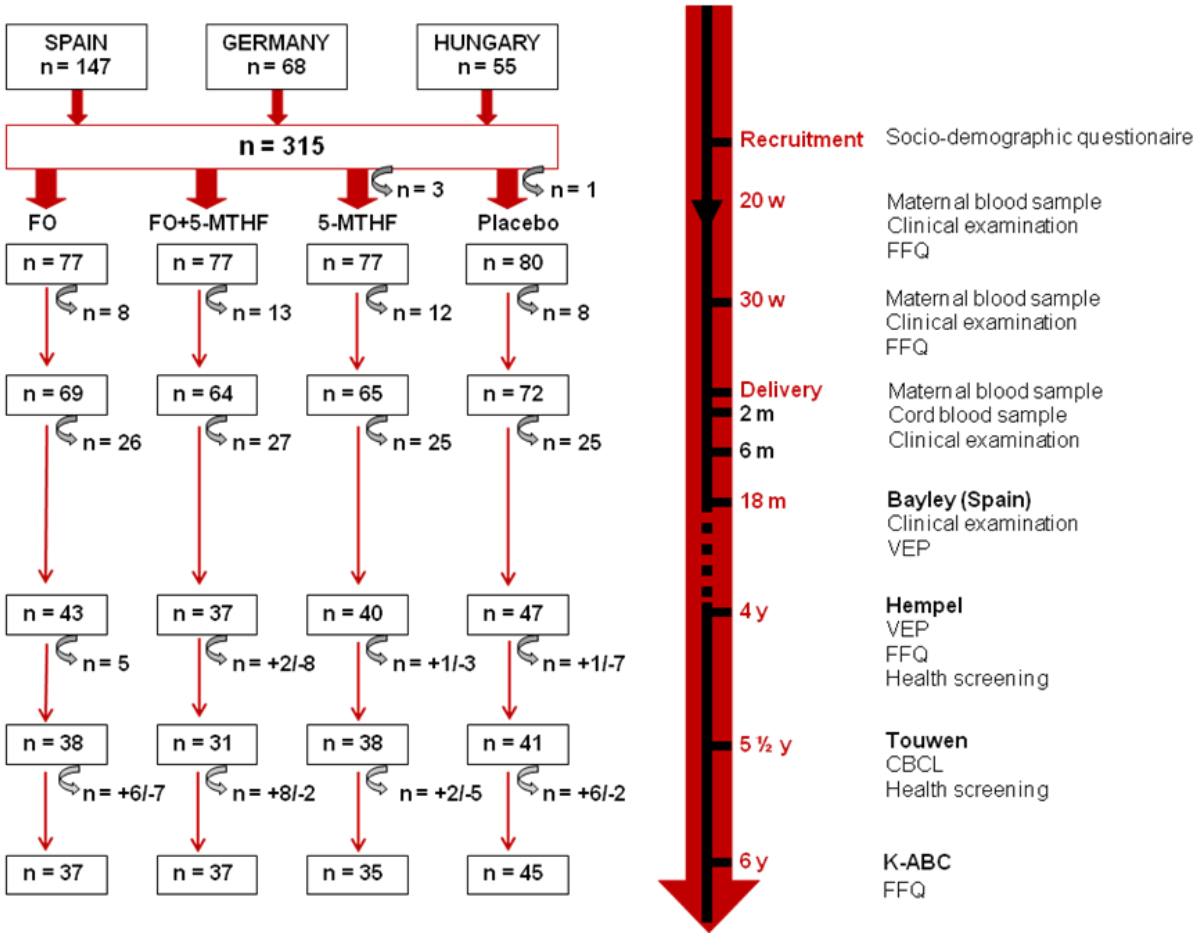

Fig 6. Study design and summary of participants flow. FFQ, food frequency questionnaire; VEP, visual evoked potentials; CBCL, Children Behaviour Checklist, K-ABC, Kaufman Assessment Battery for Children.

Main maternal outcome variables were fatty acid relative levels in plasma and erythrocyte membrane PLs at the 20th and 30th week of pregnancy and at delivery. The primary infant outcome variables were fatty acid relative content in cord plasma and erythrocyte PLs at delivery and neurological outcome obtained by age specific neurological examinations at 4, 5 ½ and 6 ½ years of age. Socio-demographic data, as well as indicators of the course of pregnancy and delivery, occurring complications and health screening of infants including information on children illnesses since birth were determined as potentially confounding factors and may be considered as safety parameters.

The study protocol was approved by the Medical Ethics Committees of all centres participating in the study. After careful explanation of the study details,

written informed consent was obtained from all participants at study entry and at the beginning of the children follow up at 4 years of age.

3.2 Subjects

The study participants were the cohort of women taking part in the NUHEAL Study and their respective children. The subjects taking part in the NUHEAL study consisted of a prospective cohort of pregnant women from three different European countries: University of Granada (Spain), Ludwig Maximilians University in Munich (Germany) and the University of Peçs (Hungary). Eligible for the study were pregnant women, who fulfilled the inclusion criteria and were not participating or were going to participate in any other clinical trial:

- Apparently healthy women with uncomplicated singleton pregnancies in < 20 week of gestation before enrollment, the expected delivery date and gestation week were determined by using Naegeles´s rule.
- Intention to give birth in one of the centres participating in the study.
- Age between 18 and 40 years at study entry.
- Body weight between 50 and 92 kg at study entry.
- No use of any n-3 fatty acid supplements since the beginning of pregnancy.
- No regular use of folate or vitamin B12 supplements after the 16th week of gestation.

Women were recruited between November 2001 and March 2003. Pregnant women attending antenatal care clinics between the 12 and the 20th week of gestation were approached by the stud personal and informed about the study. All subjects were carefully informed of the study by means of written information and oral explanation of the study details by the attending physician. All questions were clarified and it was made clear to the participants that they could withdraw consent

at any time without any negative consequences for their medical treatment before written consent was obtained.

Women who agreed to participate in the study were assigned a study code number and were randomly assigned to one of the 4 different intervention groups individually for each participating centre. Stratified by centre randomization was performed in blocks of 20 numbers by means of a closed box containing 20 cards with 1 of the 4 numbers according to the 4 intervention groups. By drawing envelopes, each subject identity number was assigned to the intervention group number of the card. The procedure was identical in the three participating centres.

As the study was double blind designed, the group number corresponding to each supplement was only know by the producer of the formulation, who provided each centre with a sealed envelope containing the information. The envelope should only be opened in case of emergency by the physician responsible for the study. The infant formulas provided to the infants not receiving breast milk were coded in the same way as the supplements for the mothers (1, 2, 3, 4), thus double blinding was maintained.

Children were followed postnatally until the age of 18 months. At the age of 4 participating mothers and their infants were again approached and asked to participate in the neurological follow up of their children. Children born before the 35^{th} week of pregnancy and those with a congenital disorder or severe illness interfering with adequate functioning in daily life were excluded from the analyses. The flow of participants during the course of the study is shown in figure 3.

3.3 Intervention

After randomization to one of the 4 different intervention groups participating women received from the 20th week of pregnancy until delivery 15 g of a milk based supplement (Blemil Plus Matter; Odesa Laboratorio, Barcelona, Spain) containing either fish oil providing 500 mg DHA and 150 mg EPA (Pronova Biocare, Lysaker, Norway), 400 μg 5-MTHF (BASF, Ludwigshafen, Germany), both or placebo together with vitamins and mineral in amounts meeting the recommended intakes during the second half of pregnancy for European mothers (158) (Table 3). The different supplements were not distinguishable with respect to the appearance of the sachets or to their content and had to be dissolved in any non acidic liquid drink before consumption. Detailed instructions were given in Spanish, German and Hungarian on the label of each sachet. Each woman was provided at 20th week gestation with 90 sachets containing the corresponding 15 g supplement of which she had to consume 1 per day until the following visit in the 30th week gestation when she was provided with another 90 sachets to be consumed until delivery. Subjects were instructed to return the leftover sachets to the study centre where they were counted for the assessment of compliance.

After birth, women were encouraged to breast feed their infants. Infants who required supplements or substitution of breast feeding were provided with infant formulas (Blemil Plus NF, Laboratorios Ordesa) with the recommended composition in Europe until the infant was 6 months of age. There were two available formulas, both identical with the exception of the fatty acid composition. Children born from mothers in the FO and FO+5-MTHF groups received a formula containing 0.5 % of total fatty acids as DHA (and 0.4 % of arachidonic acid) while children in the placebo or 5-MTHF groups received a formula virtually free of DHA and AA (Table 4).

Table 3. Composition of supplements

Nutrient content per 100 g	FO	FO+5-MTHF	5-MTHF	Placebo
Energy (Kcal)	472.5	472.5	465.5	465.5
Protein (g)	16.7	16.7	19.5	19.5
Fat (g)	20.9	20.9	19.5	19.5
Saturated (g)	7.4	7.4	10.5	10.5
Monousaturated (g)	5	5	5.6	5.6
Poliunsaturated (g)	6.4	6.4	0.5	0.5
Linoleic acid (g)	0.3	0.3	0.4	0.4
DHA (g)	3.3	3.3	0	0
Carbohydrates (g)	54.4	54.4	53	53
Minerals				
Natrium (mg)	250	250	250	250
Calcium (mg)	2,000	2,000	2,000	2,000
Phosphorus (mg)	1,600	1,600	1,600	1,600
Magnesium (mg)	620	620	620	620
Zinc (mg)	20	20	20	20
Iodine (μg)	400	400	400	400
Vitamins				
Vitamin A (μg)	2,200	2,200	2,200	2,200
Vitamin D (μg)	10	10	10	10
Vitamin E (mg)	20	20	20	20
Vitamin B_1 (Thiamin) (mg)	2.4	2.4	2.4	2.4
Vitamin B_2 Riboflavin (mg)	10	10	10	10
Vitamin B_3 (Niacin) (mg)	30	30	30	30
Vitamin B_6 (mg)	12.7	12.7	12.7	12.7
Vitamin B_{12} (μg)	23.3	23.3	23.3	23.3
Vitamin C (mg)	1,800	1,800	1,800	1,800
Folic acid	5.3	0	5.3	0

Table 4. Basic nutrient content of infant formulae

Nutrient content per 100 g	FO and FO + 5-MTHF	5-MTHF and Placebo
Energy (kcal)	514	511
Protein (g)	12.0	12.0
Carbohydrates (g)	58.0	58.6
Fat (g)	26.0	25.4
DHA	0.5% of total fatty acids	0.0%
AA	0.4% of total fatty acids	0.0%
Minerals (g)	2	2
Taurine (mg)	32	32
Nucleotides (mg)	19.1	19.1
Carnitine (mg)	17	17
Minerals		
Natrium (mg)	175	175
Potassium (mg)	535	535
Chlorine (mg)	290	290
Calcium (mg)	420	420
Phosphorus (mg)	230	230
Magnesium (mg)	42	42
Iron (mg)	6	6
Zinc (mg)	4.4	4.4
Copper (μg)	400	400
Manganese (μg)	50	50
Iodine (μg)	70	70
Selenium (μg)	10.7	10.7
Vitamins		
Vitamin A (μg)	640	640
Vitamin D (μg)	10.3	10.3
Vitamin E (mg)	25	25
Vitamin K (μg)	42	42
Vitamin B1 (μg)	520	520
Vitamin B2 (μg)	620	620
Vitamin B6 (μg)	825	825
Vitamin B12 (μg)	2.0	2.0
Vitamin C (mg)	60	60
Folic acid (μg)	42	42
Calciumpantothen. (mg)	3.2	3.2
Nicotinamide (mg)	6	6
Biotin (μg)	16	16

3.4 Data and biological samples collection

Data and samples from participating women were collected at study entry, prior to the beginning of supplementation in the 20th week of pregnancy, as well as in the 30th week of gestation and at delivery. Socio-demographic data were obtained at study entry with standardized questionnaires which included information about maternal age, parental ethnic, residence area, education level, type of work and maternal smoking habits as well as information on parental diseases. Dietary information was collected by FFQ at the 20th and the 30th week of gestation. Clinical information on the course of pregnancy was collected by a trained physician who performed standardized examinations and interviews on the 20th and 30th week of gestation. Information on parity and gravidity, maternal body weight and height, blood pressure, risk factors and pregnancy complications such us bleeding, polihydramnios and oligohydramnios, cervical incompetence, anaemia, rhesus incompatibility and the existence of severe illnesses, as well as data from routine blood and urine analysis were recorded. Indicators of the course of delivery including duration of pregnancy, proteinuria, blood pressure, cardiotocography, mode of delivery, blood loss, cord arterial pH and delivery complications such us carditocographic pathology, the existence of amnionitis, eclampsia or preeclampsia, premature rupture of membranes and cephalo-pelvic disproportion, was obtained in standardized reports. All examinations and interviews were performed according to a standardised scheme and documented in categorised items, as far as possible. Maternal indicators of the course of pregnancy and routine laboratory analyses as well as complications at parturition were recorded as safety parameters and potentially confounding factors.

Biological material was obtained from mothers at 20th and 30th week of pregnancy and at delivery. 10 ml of maternal venous blood samples at the 20th and the 30th week of gestation, as well as 12 ml at delivery were obtained into EDTA

containing tubes by venipuncture for the erythrocyte and plasma fatty acid analyses. In addition, 12 ml venous cord blood samples were also collected at delivery after clamping of the umbilical cord and before parturition of placenta by puncture and aspiration of a long cord segment for the erythrocyte and plasma fatty acid analyses.

Data on the infants of the participating women were collected at birth, at the age of 8 weeks, at the age of 6 months and at the age of 18 months (Spain). At birth a trained physician examined the children and obtained data on the infants in standardized reports. At the age of 2 and 6 months a physical examination and anthropometric measurement of the infants were performed and data on postnatal diseases and visits to paediatrician were also obtained.

Children were again evaluated at the age of 4, 5 ½ and 6 ½ years of age. Children neurological development was assessed at the age of 4 with the Hempel, at 5½ with the Touwen examination and at 6 ½ with the K-ABC. In addition, a trained physician explored and measured the children and obtained information on children health status in standardized health screening questionnaires in each visit.

3.5 Dietary evaluation of mothers

Dietary information was collected by a FFQ containing standard portion sizes, which was based on previous studies evaluating dietary intakes (MONICA study, nutrition protocol of Freiburg, GISELA study). The questionnaire included thirty-three food items primary focused on dietary sources of DHA and folate. Women were also asked to report the mode of food preparation and special dietary habits such as vegetarian diets. Multivitamin juice/pill, beer yeast, wheat bran, flax-seeds and evening primrose oil supplements were also recorded.

Single food items were combined in several food groups such as meat, seafood, vegetables, fats, fruits, soy products, cheese, eggs, bakery and potatoes. The frequency of intake was reported as never, 1 or 2-3 times a day, 1 or 2-3 or 4-6 times a week o 1-3 times a month. Intake of nutrients was calculated form the portion size and frequency of food consumption using the German nutrient database Bundeslebensmittelschlüssel version II.3. The same nutrient database was used in all participating centres.

3.6 Biochemical analyses

3.6.1 Sample collection procedure

10 ml maternal blood were obtained into EDTA containing tubes by venipuncture after an overnight fast in the 20th week (prior to supplementations) and in the 30th week gestation. At delivery 12 ml maternal blood was obtained as described for the samples at the 20th and 30th week of pregnancy. 12 ml placental venous cord blood samples were also collected in EDTA containing tubes at delivery after clamping of the umbilical cord and before parturition of placenta by puncture and aspiration of a long cord segment. Blood was centrifuged at 3500 rpm for 10 min at room temperature within 2 h. Obtained plasma samples were frozen in liquid N_2 at -196ºC in 100 and 200 µl aliquots and stored at -80 ºC until fatty acids analyses. Blood cells were washed three times in isotonic sodium chloride solution. The last sediment was haemolysed in 1 ml of distilled water fro 20 min at room temperature, and then 2 ml ice-cold isopropylic alcohol with 0.5% butylated hydroxytoluene was added as antioxidant. Thereafter the samples were stored at -80ºC until further analyses.

3.6.2 Fatty acid analyses in plasma and erythrocyte phospholipids

Initial total lipids extraction from plasma was performed according to the method of Kolarovic and Fournier (158). 0.5 ml plasma plus 0.5 ml water were vortexed for 30 s with 100 μl of internal standard (0.857 g/L diheptadecanoyl phosphatidylcholine dissolved in chloroform). Lipids were extracted then four times; initially with n-hexane/2-propanolol (3:2, by vol) containing 25 mg/L of butylated hydroxytoluene as antioxidant, then three times with pure hexane. The pooled extracts were dried under vacuum and dissolved in 200 μl hexane:methyl-tert-butyl-ether:acetic acid (100:3:0.3, by vol) and PLs were isolated by liquid chromatography with the use of aminopropyl columns (Sep Pak Cartridges; Waters, Milford, MA) as described by Agren et al. (159).

Lipids from erythrocyte were extracted by adding 3 ml chloroform and the two internal standards (phosphatidylethanolamine-heptadecanoate and phosphatidylcholine-pentadecanoate esters dissolved in methanol). The mixture was shaken on vortex and the lower layer was aspirated and evaporated under nitrogen stream. The dry lipid extract was resolved in chloroform and added to Silica gel plates (Merck 60, 10 x 20 cm). The runner solvent for the first run was hexane: diethylether: chloroform: acetic acid (21:6:3:1, volume/volume). The plate was dried under hood at ambient temperature and ran again with chloroform: methanol: water (65:25:4, volume/volume). (160). For positioning the PC and PE ester bands, the proper oleates were ran in parallel in every plate. The bands were stained with dichlorofluorescein, visualised under ultraviolet light and scrapped for transmethylation.

Fatty acid methyl esters from individual fractions were obtained by reaction with 3 N HCl-methanol at 84ºC for 40 min according to the method of Lepage and Roy (161).

The quantification of fatty acid methyl esters from plasma PLs was performed at the University of Granada by gas chromatography using a gas chromatograph (HP5890 Series II; Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector. Chromatography was performed using a 60 m long capillary column with 0.32 mm internal diameter and 0.20 μm thickness and impregnated with SP-2330 FS (Supelco; Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250 and 275ºC respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Fatty acid methyl esters were identified by comparison of retention times with those of known standards.

Analysis of fatty acid methyl esters from erythrocyte PE and PC was performed at the University of Peçs by high-resolution capillary gas-liquid chromatography (model 9001 gas chromatography; Finnigan/Tremetrics, Austin, TX) with split injection, automatic sampler (A200SE, CTC Analytic, Switzerland) and flame ionisation detector with a DB-23 cyanopropyl column of 60 m length (J & W Scientific, Folsom, CA). Conditions during the analysis were as follows: temperature of injector at 80°C for 0.1 min, temperature increase by 180°C per min up to 280°C, temperature of column area at 60°C for 0.2 min, temperature increase by 40°C min up to 180°C, a 5-min hold period, temperature increase by 1.5°C per min up to 200°C, an 8.5-min hold period, temperature increase by 40°C per min up to 240°C and a 13-min hold period. The constant linear velocity was 0.3 m/s referred to 100 °C. For identification of sample peaks we used two commercially available fatty acid methylester calibration mixtures (Supelco 37 FAME mix and NU-CKECK GLC reference 463) containing the fatty acids measured in the present study.

Results were expressed as percentages by weight (wt%) of total detected fatty acids with 14-24 C-atoms.

3.7 Neurological examination

Age specific neurological examinations were performed by three researchers, one in each study centre. They had been trained and were supervised by means of recording on video the examinations. All examiners were unaware of the supplementation that had been given to children's mothers. The room of examination is standardized with respect to size, furniture, light, floor (carpet on the floor) and a friendly atmosphere was created with pictures on the walls. Examiners avoided the typical appearance of a doctor's dress and examined the children in the presence of their parents. Bare feet were required at the beginning of the examination and the degree of further undressing was adapted to the need of the moment. The recording of the findings was standardized in precoded scales in order to facilitate comparison of findings between children. Addressed below are the descriptions of the specific neurological tests performed in each visit.

3.7.1 Hempel Neurological Examination

At 4 years of age children were neurologically examined according to Hempel (162). The method aims in particular at the detection of minor neurological dysfunction (MND), as children with MND are considered at risk for learning and behavioural problems. However, the test allows the detection and description of major neurological abnormalities as well. The test was especially designed for children between 1 ½ and 4 years of age and focuses on the observation of spontaneous motor behaviour of the child in a standardized free-field situation. The child moves freely in a standardized room and during his play various toys are presented in a standardized sequence in order to elicit particular motor performances. The examination takes an experienced examiner about 30 min and the procedure of examination can be learned in half a day. In the first part of the assessment games are introduced by presentation of standardized toys which enable

the examiner to call forth the motor pattern that he wants to observe. The manipulative part (resistance to passive movements, reflexes and responses) is postponed until the end of the session in order to achieve the cooperation of the child.

Examination consisted on the observations of the following neurological functions:

1. Prehension, including the assessment of the mode of grasping, posture of arm and shoulder, quality of arm and shoulder movements, posture of hands and fingers, adjustment of hand opening, presence of hindering associated movements, and the quality of hand motility.
2. Sitting behaviour, including the ability to sit up, posture of head, trunk, legs and feet, trunk rotation (spontaneous and elicited), fluency of trunk movements, and quality of accelerations and decelerations.
3. Crawling behaviour, including symmetry of movements, posture of the head, coordination of arm and leg movements, variability in speed, and fluency of trunk movements.
4. Standing behaviour, including the ability to stand up, variability in standing up, posture of head, trunk and limbs, distance between feet, balance while moving or not moving, trunk rotation (spontaneous or elicited), fluency of trunk movements, reaction to push.
5. Walking behaviour, including the ability to walk, fluency of trunk and leg movements, reciprocal arm swing, posture of head, trunk and legs, gait width, balance, abduction of shoulders, spontaneous walking on tiptoe, variability of speed, manoeuvrability, ability to avoid objects.
6. Head, including the assessment of cranial nerve function, including position and movements of the eyes, nystagmus, optokinetic nystagmus, pupilary reactions,

visual fields and acuity, hearing, facial expression and symmetry, drooling, and quality of speech.

7. Sensorimotor function by means of manipulation, including the assessment of muscle tone, muscle power, range of movements, intensity and threshold of deep tendon reflexes, and the foot sole response.

The findings of the Hempel examination lead to a clinical diagnosis in 4 different categories. In addition, an optimality score and fluency subscore can be obtained. Children can be classified into the clinical categories of major neurological dysfunction, complex MND, simple MND or normal. Major neurological dysfunction implies that a defined neurological syndrome associated with disability and/or social limitations was found. MND denotes the presence of mild abnormalities which do not result in a handicapping condition in daily life. Simple MND is present in about 15-20% of the children and reflects the expression of a normal but not optimally developed brain. Complex MND indicates a functional impairment resulting in some degree of disability and/or social limitation which is strongly related to developmental coordination, learning and behavioural disorders. For the classification in simple or complex MND, the signs of dysfunction are grouped into 5 functional clusters: fine motor function, gross motor function, posture and muscle tone regulation, reflexes and visuomotor function (Table 5). A child is classified as normal if none of the cluster is scored as deviant or in case of the isolated presence of dysfunction in the cluster of reflexes. Simple MND signifies the presence of one dysfunctional cluster and complex MND denotes the presence of more than one dysfunctional cluster. Children classified as normal might show single signs of dysfunction but not a whole dysfunctional cluster.

Table 5. Cluster profile of the Hempel neurological examination

Cluster	Based on	Criteria for deviant cluster
Fine motor function	- Uni- or bilaterally absent pincer grasp - Exclusive hand preference - Yoke movements present - Abnormal quality of arm/shoulder movements - Inadequate adjustment of hand opening - Abnormal quality of hand mobility	2 out of 6 criteria scored positive
Gross motor function	- Limited spontaneous trunk rotation during sitting - Uni- or bilaterally absent or slow response during lateral supporting reaction in sitting position - Broad or narrow distance between feet during standing and walking - Needs correction movements for balance during standing with movements - Limited spontaneous trunk rotation during standing - No or moderate balance at reaction to push against shoulders - Abnormal quality of leg movements during walking - Absent plantigrade gait - Limited balance during walking - Walks often or continuously on tip-toe - Limited variability in walking speed - Limited manoeuvrability during walking and limited ability to avoid objects	4 out of 12 criteria scored positive
Posture and muscle tone	- Consistently present mild abnormalities of posture of head, trunk, arms or legs - Mild hypotonia or mild hypertonia of arms and/or legs	Both criteria scored positive
Reflexes	- High or low intensity of reflexes in arms and/or legs - High or low thresholds of reflexes in arms and/or legs - Footsole response: uni- or bilateral dorsiflexion	2 out of 3 criteria scored positive
Visuomotor	- Deviant position of eyes (consistently or inconsistently present) - Deviancies in movements of the eyes - Presence of spontaneous nystagmus - Absent or asymmetrical optokinetic nystagmus - Absent or asymmetrical papillary reaction	2 out of 5 criteria scored positive

In addition to the clinical diagnosis, neurological findings can be evaluated by assessing performance on 56 representative items in terms of optimality. For each item criteria for optimality are defined (163). For each child a neurological optimality score (NOS) was obtained by counting the number of items considered optimal. It

has to be taken into account that optimality is not equal to normality, as the range for optimal behaviour is narrower than that of normal behaviour. Thus, a reduced optimality score does not means abnormality or dysfunction. NOS is therefore a good instrument to detect subtle deviations in neurodevelopment. The fluency subscore consists of 15 items of the NOS focused on the fluency of motor behaviour (163). The quality of movements was evaluated through this fluency subscore which is the part of the NOS most easily affected by subtle neurological dysfunction (Table 6).

Table 6a. Items of the Hempel neurological optimality score (NOS)

Items	Criteria of optimality
Prehension	
1. Mode of grasping Pincer grasp	Present in left and right hand
2. Handpreference	Preference for either left or right hand
3. Posture arm/ shoulder	Normal, variable posture
4. Yoke movements	Absent
5. Quality of arm/ shoulder movements *	Smooth
6. Posture hands/fingers	Normal, variable posture
7. Adjustment handopening	Good
8. Associated movements (hindering) *	Absent or, if present, they do not hinder
9. Quality of hand mobility *	Smooth
Sitting	
10. Sitting (up)	Can sit (up) without help
11. Posture head/ trunk / legs / feet / toes a	Normal, variable, and well-adapted posture
12. Trunk rotation, spontaneous *	Trunk rotation > 45º
13. Trunk rotation, elicited *	Trunk rotation > 45º
14. Fluency of trunk movements *	Smooth
15. Lateral supporting reactions	Quick, adequate reactions
16. Acceleration / deceleration *	Smooth
Standing	
17. Standing up/ free	Can stand up without help with object in hands /stands free
18. Variability in standing up	Various ways of standing up
19. Posture head/arms/ trunk / legs / feet / toes	Normal, variable, age-adequate, and well-adapted
20. Distance between feet	Medium
21. Balance without movements	No correction movements visible
22. Balance with movements	No correction movements visible
23. Trunk rotation, elicited *	Trunk rotation >45º
24. Fluency of trunk movements *	Smooth
25. Reaction to push against shoulders	Good balance
26. Dyskinesia *	No dyskinesia

Table 6b. Items of the Hempel neurological optimality score (NOS)

Items	Criteria of optimality
Walking	
27. Ability to walk and toddling *	Able to walk without help, and no toddling in the walking pattern at any speed
28. Fluency of trunk movements *	Smooth
29. Fluency of leg movements *	Smooth
30. Reciprocal arm swing *	Present
31. Posture head/arms/ trunk / legs / feet / toes	Normal, variable, age-adequate, and well-adapted posture
32. Gait width	Medium
33. Plantigrade walk	Present
34. Balance during walking	Good balance, no correction movements needed
35. Abduction shoulders	No abduction of the shoulders
36. Walking on tip-toe *	No or sometimes walking on tiptoe involuntarily
37. Variability of speed	Variable speed
38. Manoeuvrability	Changing direction in sharp turns
39. Ability to avoid objects	Avoids obstacles adequately without interrupting walking
Head	
40. Eyes, position	Symmetrical and centred position
41. Eyes, movements	Smooth, symmetrical movements
42. Nystagmus (spont. / direct.)	No nystagmoid movements
43. Optokinetic nystagmus	Symmetrical present both horizontally and vertically
44. Pupils size and shape/ reaction to light	Round, medium sized pupils /immediate reaction
45. Visual fields	Apparently intact
46. Vision	Apparently intact
47. Hearing	Quick and adequate reaction to sounds
48. Facial mobility	Normal, alert, and symmetrical
49. Drooling	Absent
50. Speech/language	Normal, age-adequate speech- and language development
Manipulative examination	
51. Resistance against passive movements	Moderate resistance
52. Active muscle power	Adequate for age
53. Range of movements	Medium range
54. Tendon reflexes	Normal intensity
55. Reflex thresholds	Medium threshold
56. Footsole response	No movements or plantar flexion of big toe

* *Items considered for the fluency score*

3.7.2 Touwen Neurological Examination

At 5 years of age neurological assessment was performed according to Touwen (164). The method can be used for neurological evaluation of school age children from 4 years onwards and is focused on the detection of MND.

Neurological examination includes the assessment of the following:

1. Sitting behaviour, including the ability to sit up, posture of head, trunk, legs and feet and posture during extension of arms in pronation and supination, presence of voluntary movements, mouth opening finger spreading phenomenon, kicking and response to being pushed. This part also includes the assessment of muscle tone and power, range of movements and reflexes.
2. Standing behaviour, including the posture of head, trunk, legs and arms, reaction to being pushed, Romberg test and test of involuntary movements. During this part also most test of coordination and fine manipulative ability are performed (disdiadochokinesis, finger-nose test, fingertip-touching test, finger opposition test, circle test, follow a finger test).
3. Walking behaviour, including the ability to walk, posture of head, trunk, arms, legs and feet, gait width and heel-toe gait, the ability to walk on a straight line, walking on tiptoe and on heels, the ability to stand on one leg and hope and the knee-heel test.
4. Sensory system, including graphesthesia, kinaesthesia, sense of position and knowledge of body scheme.
5. Head and cranial nerves, including facial motility, position and movements of the eyes, fixation, nystagmus, pupilary reactions, visual fields, hearing, tongue motility, quality of speech and pharyngeal arches.

The findings of the Touwen examination lead to a similar clinical classification to that described for the Hempel test. The Touwen examination is organized in 8 functional clusters. Children without major neurological dysfunction can be classified into the clinical categories of simple MND, complex MND and normal depending on the number of dysfunctional clusters (Table 7). A child is classified as neurologically normal when none of the clusters meet criteria of dysfunction or in case of the isolated presence of deviation in the cluster reflexes. Prior to puberty a child is classified as simple MND when 1 or 2 clusters are dysfunctional and complex MND when 3 or more clusters are scored deviant.

Table 7a. Cluster profile of the Touwen neurological examination

Cluster	Based on	Criteria for deviant cluster
Posture and muscle tone	- Consistent mild deviation in posture during sitting, standing and walking - Mild deviation of muscle tone in legs - Mild deviation of muscle tone in arms	Two or more
Reflexes	- Intensity tendon reflexes arms: high, low or asymmetrical - Threshold tendon reflexes arms: high, low or asymmetrical - Intensity tendon reflexes legs: high, low or asymmetrical - Threshold tendon reflexes legs: high, low or asymmetrical - Foot-sole response: uni- or bilateral Babinski sign - Plantar grasp: uni- or bilaterally present - Abdominal skin reflex: asymmetry	Presence of at least two signs
Involuntary Movements	- Marked, consistent choreiform movements of distal muscles - Marked, consistent choreiform movements of proximal muscles - Marked choreiform movements of face, eyes and/or tongue - Marked, consistent tremor - Consistent athetotiform movements in distal muscles While assessing: - Spontaneous motor behaviour - Test with extended arms - Movements of face, eyes, tongue	Presence of at least one

Table 7b. Cluster profile of the Touwen neurological examination

Cluster	Based on	Criteria for deviant cluster
Coordination and Balance	- Finger-nose test - Fingertip-touching test - Diadochokinesis - Kicking - Knee–heel test - Reaction to push, sitting - Reaction to push, standing - Romberg - Tandem gait - Standing on one leg - Hopping on one leg	Three or more tests inappropriate for age
Fine Manipulation	- Finger-opposition test: smoothness - Finger-opposition test: transition - Follow-a-finger test - Circle test	Two or more tests inappropriate for age
Associated movements	Presence of an excessive amount of associated movements for age during: - Diadochokinesis age in at least three tests - Finger-opposition test - Walking on toes - Walking on heels	Three or more test with excessive amount of associated movements for age
Sensory deficits	- Mouth-opening-finger-spreading-phenomenon - Graphesthesia - Kinaesthesia - Sense of position - Hearing - Visual fields	Two or more sensory functions dysfunctional
Cranial nerve dysfunction	- Motor behaviour of face, eyes pharynx and tongue	Mild cranial nerve palsy

In addition to the clinical classification, an optimality score can also be obtained with the Touwen examination (Table 8) (165).

Table 8. Items of the Touwen neurological optimality score (NOS)

Items	Age	Criteria of optimality
Posture, power and muscle tone		
1. Sitting, standing and walking	≥ 4	Ability to perform independently
2. Posture while sitting	≥ 4	Normal posture of head, trunk, arms and legs
3. Extending arms in pronation and supination while sitting	≥ 4	Ability to stabilize the arms in space
4. Voluntary relaxation	≥ 4	Easy
5. Muscle power in head, trunk, arms and legs	≥ 4	Typical power for age
6. Muscle tone of head and trunk	≥ 4	Typical muscle tone
7.Muscle tone of the arms and legs	≥ 4	Typical muscle tone
8. Range of head, trunk, arms and legs movements	≥ 4	Typical range
9. Posture while standing	≥ 4	Typical posture of head, trunk, arms and legs
10. Posture while walking	≥ 4	Typical posture of head, trunk, arms and legs
11. Walking on toes and walking on heels	≥ 4	Able to walk on toes and on heels.
Reflexes		
12. Reflex threshold of biceps reflex, triceps reflex, knee jerk and ankle jerk	≥ 4	All tresholds in typical range
13. Reflex intensity of biceps reflex, triceps reflex, knee jerk and ankle jerk	≥ 4	All intensities in typical range
14. Foot sole response	≥ 4	Simetrical plantar flexion or no response
15. Plantar grasp	≥ 4	Bilaterally absent
16. Abdominal skin reflex	≥ 4	Simetrically present
Involuntary movements		
17. Presence of choreiform movements during sitting with or without extended arms	≥ 4	Absent
18. Presence of athetotiform movements during sitting with or without extended arms	≥ 4	Absent
19.Presence of tremor during sitting with or without extended arms	≥ 4	Absent
20. Distal choreiform movements (standing)	≥ 4	Absent
21. Proximal choreiform movements (standing)	≥ 4	Absent
22. Athetotiform movements (standing)	≥ 4	Absent
23. Tremor (standing)	≥ 4	Absent
24. Choreiform movements of eyes during fixation and pursuit	≥ 4	Absent
25. Choreiform movements of face during fixation and pursuit	≥ 4	Absent
26. Choreiform movements of tongue while sticking out the tongue	≥ 4	Absent

Table 8b. Items of the Touwen neurological optimality score (NOS)

Items	Age	Criteria of optimality
Coordination		
27. Kicking while sitting	≥ 4	Typical performance
28. Reaction to push while sitting	≥ 4	Typical performance
29. Reaction to push while standing	≥ 4	Typical performance
30. Romberg	≥ 4	Typical performance
31. Diadochokinesis	≥ 4	Typical performance
32. Finger-nose test	≥ 4	Typical performance
33. Fingertip-touching test	≥ 4	Typical performance
34. Walking on a straight line	≥ 4	Typical performance
35. Standing on one-leg	4	Both legs ≥ 5 seconds
	5	Both legs ≥ 10 seconds
	6	Both legs ≥ 15 seconds
	7-9	Both legs ≥ 20 seconds, no swaying
	>10	Both legs ≥ 20 seconds, no swaying and no toe flexion
36. Hopping on one leg	4	Both legs ≥ 5 hops
	5	Both legs ≥ 10 hops
	6	Both legs ≥ 15 hops
	7-9	Both legs ≥ 20 hops
	>10	Both legs ≥ 20 hops, at the same spot and on toes
37. Knee-heel test	4	Also optimal when not able to perform the test
	≥ 5	Typical performance
Fine manipulative ability		
38. Finger opposition test, smoothness	4	Also optimal when not able to perform the test
	≥ 5	Typical performance
39. Finger opposition test, transition	4	Also optimal when not able to perform the test
	≥ 5	Typical performance
40. Follow-a-finger test	≥ 4	Typical performance
41. Circle test, opposite direction	≥ 4	Typical performance
42. Circle test, same direction	4	Also optimal when not able to perform the test
	≥ 5	Typical performance
43. Circle test, transition	4	Also optimal when not able to perform the test
	≥ 5	Typical performance
Associated movements		
44. Mouth- opening-finger spreading	≥ 4	Typical performance
45. Associated movements during diadochokinesis	≥ 4	Typical performance
46. Associated movements during finger- opposition test	4	Also optimal when not able to perform the test
	≥ 5	Typical performance
47. Associated movements during walking on toes	≥ 4	Typical performance
48. Associated movements during walking on heels	≥ 4	Typical performance

Table 8c. Items of the Touwen neurological optimality score (NOS)

Items	Age	Criteria of optimality
Sensory Systems		
49. Graphestesia	4	Also optimal when not able to perform the test
	≥ 5	Adequate performance
50. Kinaesthesia	4	Also optimal when not able to perform the test
	≥ 5	Adequate performance
51. Sense of position	4	Also optimal when not able to perform the test
	≥ 5	Adequate performance
52. Vision	≥ 4	Typical, no need of visual correction
53. Hearing	≥ 4	Typical performance
Cranial nerve function		
54. Facial motility	≥ 4	Typical
55. Position of the eyes	≥ 4	Typical
56. Fixation of the eyes	≥ 4	Typical
57. Pupillary reactions	≥ 4	Typical
58. Pursuit movements of eyes	≥ 4	Typical in all directions
59. Nystagmus	≥ 4	Typical
60. Visual fields	≥ 4	Typical
61. Tongue motility	≥ 4	Typical
62. Speech	≥ 4	Typical
63. Pharyngeal arches	≥ 4	Typical
64. Quality of walking	≥ 4	Typical gait width, typical quality of gait and adequate heel-toe gait

3.7.3 Kaufman Assessment Battery for Children (K-ABC)

The K-ABC is designed for children aged 2.5 to 12.5 years and measures intelligence and achievement. This is the method that we used in our study to evaluate cognitive function at 6 ½ years of age. The K-ABC is composed by 16 subtests but children should only perform those convenient for their age (Fig. 7). The time needed for the evaluation varies with the age of children; it takes about 60 minutes to perform the test at 6 ½ years of age.

	Age (years)										
K-ABC	2-6	3	4	5	6	7	8	9	10	11	12
Book 1 Mental Processing											
1. Magic window	■	■	■								
2. Face Recognition	■	■	■								
3. Hand Movements	■	■	■	■	■	■	■	■	■	■	■
4. Gestalt closure	■	■	■	■	■	■	■	■	■	■	■
5. Number Recall	■	■	■	■	■	■	■	■	■	■	■
6. Triangles			■	■	■	■	■	■	■	■	■
Book 2 Mental Processing											
7. Word Order			■	■	■	■	■	■	■	■	■
8. Analogous Matrix				■	■	■	■	■	■	■	■
9. Spatial Memory				■	■	■	■	■	■	■	■
10. Photo Series					■	■	■	■	■	■	■
Book 3 Achievement											
11. Expressive Vocabulary	■	■	■								
12. Faces and Places	■	■	■	■	■	■	■	■	■	■	■
13. Arithmetic		■	■	■	■	■	■	■	■	■	■
14. Riddles		■	■	■	■	■	■	■	■	■	■
15. Reading/Decoding				■	■	■	■	■	■	■	■
16. Reading/Comprehension						■	■	■	■	■	■

Fig. 7. Summary of subtests in the Kaufman-ABC depending on children's age in order of performance (Batería de Evaluación de Kaufman para Niños 1997).

The results of the K-ABC comprise 4 scales: Sequential Processing Scale, Simultaneous Processing Scale, Achievement Scale and the Nonverbal Scale. The Sequential Processing Scales measures children's ability to solve problem that require the arrangement of stimuli in sequential or serial order. The Simultaneous Processing Scale was designed to measure children's ability to solve spatial, analogical or organizational problems that require processing of many stimuli simultaneously. These two scales are hypothesized to reflect the children's stile of problem solving and information processing. The Sequential and Simultaneous Processing Scales are combined to form the Mental Processing Composite, which serves as a measure of intelligence. The Nonverbal Scale is composed of the subtests of the Sequential and the Simultaneous Processing Scales that do not require words. The Achievement Scales evaluate the knowledge of the facts, language and skills learned at home or in

the school. Raw scores are transformed into standard scores with means of 100 and SD of 15. These scores can also be transformed to percentile scores (166).

3.8 Statistics

3.8.1 Power calculation

The estimation of the sample size was focused on the outcome variable, DHA percentage in cord blood phospholipids. We considered a 0.8 % difference in DHA as a significant effect. Concerning the variation of DHA in cord blood we assume a standard deviation of 1.5 %, which is the average from several reports (118;167;168).

We aimed for an effect size of 0.8 % difference in DHA content. Based on the statistical model of a two factorial analysis of variance (4 different diets and 3 centres) we achieved a statistical power of 82 % to detect 0.8 % DHA difference between any of the supplements if 300 pregnancies were studied (for all estimations an error level of 0.05 is assumed).

Thus we aimed for 100 pregnant women to complete the clinical trial in each of the 3 study centres (corresponding to 75 subjects per dietary group, overall 25 cases per cell). If one assumes a drop out rate of 33 % for compliance to the supplement intake, giving child birth at the obstetrical unit of the study centre, and postnatal follow up of the infant, 150 women will need to be recruited at each study centre (450 total).

3.8.2 Statistical analyses

Normality of variables was checked with the Kolmogorov-Smirnov test for samples with more than 50 subjects and Shapiro-Wilk test for smaller samples. In case of deviations from normality non parametric test were applied in the analyses.

Results are given in percent of total cases for qualitative variables, crude means and standard deviation for continuous variables and medians and interquartile ranges for not continuous variables.

Baseline characteristics including basal dietary fatty acid intake were compared among the four intervention groups. Differences among intervention groups for numeric variables were assessed with the analyses of variance in the normally distributed variables and Kruskal-Wallis test in the not normally distributed variables. For categorical variables Chi square tests were applied.

To evaluate the effects of supplementation on maternal plasma and erythrocyte fatty acid levels the following statistical analyses were performed. The effects of supplementation with time were compared by using the general lineal model for repeated measures with the factor type of supplementation (FO, FO+5-MTHF, 5-MTHF and placebo) as between subject factors and time with the three pregnancy time-points (20^{th} and 30^{th} week of gestation and delivery) as within subjects factor. The equality of variances was tested with the Mauchy´s test of Sphericity and for the adjustment of the degrees of freedom Sphericity assumed, lower bound and Greenhouse-Geiser corrections were applied afterwards. If significant effects were observed over time, single time-points comparisons in gestation week 30 and delivery with baseline values in the 20^{th} week of pregnancy as well as pairwise intergroup comparisons (between the different supplementation

groups) with Bonferroni corrections for multiple comparisons at the different pregnancy time-points were tested.

The effects of supplementation on cord blood plasma and erythrocyte fatty acid content were evaluated separately with a one-way-analysis of variance or Kruskal-Wallis tests depending on the normality of variables to detect significant differences between groups. For the evaluation of pairwise intergroup differences Students-t-test or Mann-Whitney U test with corrections according to Bonferroni were applied.

For the identification of association between plasma and erythrocyte fatty acid levels in maternal and fetal blood, correlation coefficients according to Spearman were calculated. The same procedure was performed for the identification of a relationship between fatty acid relative content in maternal plasma and erythrocyte and their concentration in cord plasma an erythrocyte at delivery.

With respect to neurological outcome, differences among intervention groups were assessed with the Chi Square test for the clinical conclusion of the Hempel and Touwen tests. Kruskal-Wallis tests were used for the assessment of differences in the NOS and fluency scores between intervention groups. One way analysis of variance was used for the assessment of differences between groups in the outcome scores of the K-ABC. In case of significance multiple comparisons with Bonferroni corrections were performed.

To evaluate the association between maternal and cord fatty acid concentrations in plasma and erythrocyte PLs and neurological outcome the following statistical analysis were performed. Stepwise logistic regression analysis were performed to study the association between cord and maternal plasma and erythrocyte concentrations of n-3 LC-PUFA and neurological clinical classification of

the Hempel and Touwen tests. The analyses of the association between maternal and umbilical fatty acid percentage concentrations and the NOS and fluency scores were performed by means of raw and adjusted for confounders correlation coefficients which were calculated using Spearman test. Spearman correlation coefficients were also calculated between the scores of the K-ABC and the fatty acid percentages in cord and maternal plasma and erythrocyte PLs. Stepwise multiple lineal regression analyses were performed for the adjustment of confounders with the standardized scores of the K-ABC (MPC, Sequential Processing Scale and the Simultaneous Processing Scale) as dependent variables and each of the LC-PUFA separately as independent variables. All possible confounders were included in the model as covariables.

Maternal age, parity, body mass index, haematocrit at the 30th week of pregnancy and smoking habit during pregnancy, as well as length of gestation, gravidity risk factors, delivery complications and parental educational attainment and work status were taken into account in the statistical analyses as possible confounders. Infant weight, length and head circumference at birth, Apgar score and perinatal morbidity, sex and breast feeding, as well as BMI and health status of the children at 6 ½, were also included. Due to the high number of control variables only those related to the outcome variable at *P*-values <0.2 were entered as covariables in the analysis. For the identification of dependencies between clinical neurological outcome and confounding variables Chi Square test were used for categorical variables and the t-Student or Mann-Whitney tests for numerical variables. To identify dependencies between confounding variables and neurological scores (NOS, fluency score, MPC, Sequential Processing Scale, Simultaneous Processing Scale) Mann-Whitney test was used for categorical variables and coefficients according to Spearman were calculated for numerical variables.

In all cases the significance was assumed, if *P*-values were smaller or equal to 0.05.

All computations were performed with SPSS statistical software version 15.0 (Statistical Package for Social Sciences, SPSS Inc. Chicago IL, USA).

3.9 Ethical considerations

The dietary intervention could be considered without risk, even in pregnant women. We supplemented women with natural constituents of common European diets and the applied dosages did not have any known side effect. The dosage of DHA is 500 mg per day does involve any risk neither for pregnant women nor for infants. Studies on healthy adult volunteers have reported no side effects with DHA doses up to 6 g per day (169), furthermore studies in pregnant women with supplementation doses of more than 1 g DHA daily did not report any negative side effect in women or infants (59;117;154). With respect to folic acid, the recommendations for daily folic acid intake are below 1000 μg, because a higher intake might probably mask a Vitamin B 12-deficiency (170). For this reason we use MTHF which is not masking this deficiency. Additionally the dosage of 400 μg is less than the maximum recommended intake of folic acid.

The information collected was treated strictly confidential and was used only for the project.

The only invasive test in the study was maternal blood sampling, which involved a minimal risk of minor complications. In addition, no invasive test were performed in children, the only samples collected from children consisted of cord blood at delivery. Thus, there was no risk for the mothers or the babies.

During the enrolment pregnant women and their partners were informed about the study and a written informed consent was obtained prior to entry into the study and before the beginning of the neurological follow up of children. It was made clear to the subject that she could withdraw her consent at any time without any consequences for her medical treatment.

The study protocol was approved by the local ethical committees of each of the participating clinical study centres.

Although no risks were anticipated insurance coverage of any damage to the subjects was provided, the insurance contract was signed by Laboratorios Ordesa with Catalana Occidente SA SEG. REAS, 08190 Sant Cugat, Barcelona (Contract number 83115540).

4 RESULTS AND DISCUSSION

The following chapter is organized in 3 different publications comprising results of the present doctoral thesis.

The first publication deals with the biochemical effects of n-3 LC-PUFA supplementation on mothers and on the fetus. N-3 LC-PUFAs are essential for fetal growth and development, particularly important is DHA which is an essential constituent of the brain. Occidental diets are characterized by low n-3 fatty acids intakes and high amounts of n-6 fatty acids, as a result DHA intake may not adequately supply fetal demands. The first publication describes the fatty acid evolution profile in maternal plasma PLs and erythrocyte membrane PC and PE during the second half of pregnancy and analyzes the differences between supplemented and not supplemented women. The association between maternal and fetal fatty acids levels is also an issue in this publication. It analyzes the effects of n-3 LC-PUFA supplementation on fetal DHA status and the strength of the association between maternal and fetal fatty acid levels at delivery. This is important to better understand the fatty acid changes taking place during pregnancy and how we can influence the fatty acid supply to the fetus by the dietary supplementation of mothers. This publication also discuss the relationship between fatty acids in plasma PLs and fatty acids in erythrocyte membrane PLs and tries to elucidate the blood fraction that better reflects maternal and fetal DHA status.

The second and third publication discuss the effects of supplementation on neurological outcome of the children up to the age of 6 ½ years. The interest in children chronic conditions that interfere their daily life, such as developmental coordination disorders and attention deficit and hyperactivity disorder (ADHD) has

grown in the last years. The developmental coordination disorder refers to children with normal intelligence but poor motor coordination without evidence of frank neurological pathology. Such motor problems are so serious that they may affect daily activities at home and at school. The second publication deals with the association between maternal and fetal DHA status and the presence in children of MND which has been related to the presence of developmental coordination disorders and learning and behavioural disorders, such as ADHD. The third publication analyzes the effects of n-3 PUFA on cognitive performance of children in the K-ABC which evaluates intelligence. The effects of n-3 LC-PUFA on IQ may have limited clinical significance on individual basis but may have epidemiological importance.

4.1 Publication 1:

Effects of fish oil supplementation on the fatty acid profile in erythrocyte membrane and plasma phospholipids of pregnant women and their offspring: a randomised clinical trial

Submitted to: Clin Nutr

EFFECTS OF FISH OIL SUPPLEMENTATION ON THE FATTY ACID PROFILE IN ERYTHROCYTE MEMBRANE AND PLASMA PHOSPHOLIPIDS OF PREGNANT WOMEN AND THEIR OFFSPRING: A RANDOMISED CLINICAL TRIAL

Escolano-Margarit MV[1], Campoy C[1], Ramírez-Tortosa MC[2], Demmelmair H[3], Miranda MT[5], Gil A[2], Décsi T[4], Koletzko B[3]

[1]University of Granada, Department of Paediatrics, Granada, Spain

[2]University of Granada, Department of Biochemistry and Molecular Biology, Spain

[3]Dr. von Haunersches Kinderspital, University of Munich, Munich, Germany

[4]University of Pécs, Department of Paediatrics, Pécs, Hungary

[5]University of Granada, Department of Biostatistics, Granada, Spain

Corresponding author: Cristina Campoy, Prof., MD,
Department of Paediatrics. School of Medicine
University of Granada
Avda. de Madrid, 11
18012-Granada, Spain
Tel.: +34629308695
Fax.:+34958240740
e-mail: ccampoy@ugr.es /ccfolgoso@gmail.com

Abstract

Given the importance of the long chain polyunsaturated fatty acids (LC-PUFA) in fetal development, increasing interest has been given in the last years to maternal fatty acid status and its relation to fetal fatty acid levels. Our objective was to investigate the effects of n-3 LC-PUFA supplementation to pregnant women on maternal and fetal fatty acid profile in plasma and erythrocyte phospholipids (PLs).

Study design: A multicenter, randomized, double blind, controlled trial was conducted. Healthy pregnant women from three European centers were randomly assigned to receive from 20 weeks of gestation until delivery a daily dietary supplement with either fish oil (FO) [500 mg docosahexaenoic acid (DHA) + 150 mg eicosapentaenoic acid (EPA)], 400 μg 5-methyltetrahydrofolate (5-MTHF), both or placebo. Fatty acids in plasma and erythrocyte PLs were determined in maternal blood at the 20th and the 30th week of pregnancy and at delivery, as well as in cord blood at delivery.

Results: FO supplementation significantly increased DHA relative concentrations in maternal and cord plasma and erythrocyte PLs. We observed positive significant correlations between plasma and erythrocyte fatty acid levels for most fatty acids in both, maternal and cord blood. We found significant correlations between maternal and neonatal fatty acids relative levels at delivery/birth in both plasma and erythrocyte PLs. Correlation coefficients were higher in the erythrocyte PE compared to those observed in plasma.

Conclusions: Both, plasma and erythrocyte, appear to be suitable to evaluate the fatty acid status of mothers and neonates.

KEY WORDS: Docosahexaenoic acid (DHA), polyunsaturated fatty acids (PUFA), long chain polyunsaturated fatty acids (LC-PUFA), pregnancy, folate, erythrocyte membrane phospholipids, plasma phospholipids.

Introduction

Fetal metabolism and development depend on the nutrients crossing the placenta, and therefore, on maternal nutritional status and diet during pregnancy. The essential fatty acids (EFA) and their derivates, long chain polyunsaturated fatty acids (LC-PUFA), are essential constituents of membranes, especially in the brain and retina (1;2) and must be provided to the fetus.

Given the importance of LC-PUFA in fetal development, growing interest has been given in recent years to maternal fatty acid status. It is well known form interventional studies that LC-PUFA supplementation to pregnant women significantly increases LC-PUFA concentrations in both, plasma and erythrocyte phospholipids (PLs) in the mother as well as in the fetus (3-8). However, few studies have assessed the effects of supplementation on plasma and erythrocyte PLs in the same population (9;10). It is generally accepted that LC-PUFA in plasma PLs reflect tissue LC-PUFA status. Recent discussion has centered on whether erythrocyte membrane PLs might be preferred to assess fatty acid status (11-13).

This study was performed to investigate the effects of supplementation with fish oil (FO) and/or 5-metyltetrahydrofolate (5-MTHF) from the 20th week gestation until delivery on the fatty acid profile of plasma and erythrocyte PLs of pregnant women during the course of pregnancy and fetal LC-PUFA status.

Methods

Study design

Information regarding study design, recruitment, inclusion criteria, dietary intervention, and collection of data and biological material has been reported

elsewhere (14;15). Briefly, a multicenter, randomized, double blind controlled trial was conducted. 315 healthy pregnant women were recruited before gestation week 20 at three different European centers [Ludwig Maximilians University, Munich (Germany); the University of Granada (Spain) and the University of Pecs (Hungary)]. They were randomly assigned to 4 different groups and received daily a dietary supplement, from gestation week 20 until delivery, consisting of FO [500 mg docosahexaenoic acid (DHA)+150 mg eicosapentaenoic acid (EPA)] (Pronova Biocare, Lysaker, Norway), 400 μg 5-MTHF (BASF, Ludwigshafen, Germany), both or placebo together with vitamins and minerals in amounts meeting the recommended intakes during the second half of pregnancy for European women. The mothers were physically examined at the 20th and the 30th week of gestation and data on the course of pregnancy and dietary intake were collected by means of standardized questionnaires. At delivery, information about delivery and data on the newborn were collected. 10 ml of maternal venous blood were collected into EDTA at the 20th and 30th week of gestation and 12 ml of maternal venous blood as well as 12 ml venous cord blood were collected at delivery. Main outcome variables were fatty acid relative concentrations (wt %) in plasma and erythrocyte membrane PLs of cord and maternal blood.

The study protocol was approved by the Medical Ethics Committees of all centers participating in the study. Written informed consent was obtained from all participants.

Biochemical analyses

Sample Collection Procedure

10 ml maternal blood were obtained into EDTA containing tubes by venipuncture after an overnight fast in the 20th week (prior to supplementations) and in the 30th week gestation. At delivery 12 ml maternal blood was obtained as

described for the samples at the 20th and 30th week of pregnancy. 12 ml placental venous cord blood samples were also collected in EDTA containing tubes at delivery after clamping of the umbilical cord and before parturition of placenta by puncture and aspiration from a long cord segment. Blood was centrifuged at 3500 rpm for 10 min at room temperature within 2 h. Plasma samples were frozen in liquid N_2 at -196ºC in 100 and 200 µl aliquots and stored at -80 ºC until fatty acids analyses. Blood cells were washed three times in isotonic sodium chloride solution. The last sediment was haemolysed in 1 ml of distilled water for 20 min at room temperature, and then 2 ml ice-cold isopropylic alcohol with 0.5% butylated hydroxytoluene were added as antioxidant and samples were stored at -80ºC until further analyses.

Analyses of fatty acids in maternal and umbilical cord plasma and erythrocyte phospholipids

Total lipid extraction from plasma was performed according to the method of Kolarovic and Fournier (16). 0.5 ml plasma plus 0.5 ml water were vortexed for 30 s with 100 µl of internal standard (0.857 g/L diheptadecanoyl phosphatidylcholine dissolved in chloroform). Lipids were extracted then four times; initially with n-hexane/2-propanolol (3:2, by vol) containing 25 mg/L of butylated hydroxytoluene as antioxidant, then three times with pure hexane. The pooled extracts were dried under vacuum and dissolved in 200 µl hexane:methyl-tert-butyl-ether:acetic acid (100:3:0.3, by vol) and PLs were isolated by liquid chromatography with the use of aminopropyl columns (Sep Pak Cartridges; Waters, Milford, MA) as described by Agren et al (17).

Lipids from erythrocyte were extracted by adding 3 ml chloroform and the two internal standards (phosphatidylethanolamine-heptadecanoate and phosphatidylcholine-pentadecanoate esters dissolved in methanol). The mixture was shaken on vortex and the lower layer was aspirated and evaporated under nitrogen stream. The dry lipid extract was resolved in chloroform and added to silica gel

plates (Merck 60, 10 x 20 cm). The runner solvent for the first run was hexane:diethylether:chloroform:acetic acid (21:6:3:1, volume/volume). The plate was dried under hood at ambient temperature and ran again with chloroform:methanol:water (65:25:4, volume/volume). For positioning the PC and PE ester bands, the proper oleates were ran in parallel in every plate. The bands were stained with dichlorofluorescein, visualised under ultraviolet light and scrapped for transmethylation.

Fatty acid methyl esters from individual fractions were obtained by reaction with 3 N HCl-methanol at 84ºC for 40 min according to the method of Lepage and Roy (18).

The quantification of fatty acid methyl esters from plasma PLs was performed by gas chromatography using a gas chromatograph (HP5890 Series II; Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector. Chromatography was performed using a 60 m long capillary column with 0.32 mm internal diameter and 0.20 μm thickness and impregnated with SP-2330 FS (Supelco; Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250 and 275ºC respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Fatty acid methyl esters were identified by comparison of retention times with those of known standards.

Analysis of fatty acid methyl esters from erythrocyte PE and PC was performed by high-resolution capillary gas-liquid chromatography (model 9001 gas chromatography; Finnigan/Tremetrics, Austin, TX) with split injection, automatic sampler (A200SE, CTC Analytic, Switzerland) and flame ionisation detector with a DB-23 cyanopropyl column of 60 m length (J & W Scientific, Folsom, CA). Conditions during the analysis were as follows: temperature of injector at 80ºC for 0.1 min, temperature increase by 180ºC per min up to 280ºC, temperature of column area at

60°C for 0.2 min, temperature increase by 40°C min up to 180°C, a 5-min hold period, temperature increase by 1.5°C per min up to 200°C, an 8.5-min hold period, temperature increase by 40°C per min up to 240°C and a 13-min hold period. The constant linear velocity was 0.3 m/s referred to 100 °C. For identification of sample peaks we used two commercially available fatty acid methylester calibration mixtures (Supelco 37 FAME mix and NU-CKECK GLC reference 463) containing the fatty acids measured in the present study.

Results are expressed as percentages by weight (wt %) of total detected fatty acids with 14-24 C-atoms.

Statistical analyses

Kolmogorov-Smirnov or Shapiro-Wilk tests were used to test normality of variables. Baseline characteristics were compared among intervention groups by using the Kruskal-Wallis test for continuous data and chi-square test for ordinal data. A 3 factor repeated measures ANOVA with the intervention group as between subject factor and pregnancy time points (gestation week 20, gestation week 30 and delivery) as within subject factors was performed to compare the effects of supplementation with time. Differences in cord blood fatty acid levels were assessed separately by using the ANOVA analysis for normally distributed variables and Kruskal-Walis test for not normally distributed variables. If significant effects were observed multiple comparisons with Bonferroni correction were performed.

Rho Spearman correlations were performed to determine correlations between plasma and erythrocyte relative levels of fatty acids and between maternal fatty acids status at delivery and neonatal fatty acid status at birth. *P* values under 0.05 were considered significant.

Statistical analyses were performed using SPSS statistical software (version 15.0, Chicago, Ill).

Results

Sample description

Information regarding sample description, compliance, drop outs and baseline characteristics of study participants has been previously reported (14;15). Briefly 315 healthy women with single pregnancies were recruited before the 20th week of gestation. 4 of them did not fulfil inclusion criteria and were therefore excluded. 41 women did not complete the study; main reasons for dropping out were noncompliance, relocation and bad taste of the supplement (FO: 10.4%, FO+5-MTHF: 16.9%, 5-MTHF: 16.6% and Placebo: 10%; P=0.47). Compliance was good, 89.5% of the subjects in the second trimester of pregnancy and 87.4% in the third missed less than 5 days supplementation. 270 maternal blood samples could be drawn at the 30th week of pregnancy and 243 at delivery. Available cord blood samples for analyses were 220.

Baseline characteristics of study participants are shown in table 1. Maternal clinical or socio-demographic characteristics were similarly distributed in the four intervention groups (P>0.05). There were no significant differences with respect to the course of pregnancy or delivery complications between groups (P>0.05). Habitual dietary intake of energy and nutrients of participating mothers was similar in the four groups throughout the study (Table 2).

Fatty acid patterns in maternal plasma and erythrocyte phospholipids during pregnancy and in umbilical plasma and erythrocyte phospholipids at delivery

Mean values of fatty acids in maternal plasma and erythrocyte PLs during pregnancy and in umbilical plasma and erythrocyte PLs at delivery are shown in tables 3, 4 and 5.

DHA (wt %) in maternal plasma and PLs and erythrocyte PC significantly increased during the second half of pregnancy in the FO and FO+5-MTHF groups (P<0.001) compared to the placebo and 5-MTHF groups. DHA (wt %) in maternal erythrocyte PE significantly increased during the second half of pregnancy in all intervention groups. The increments observed in FO supplemented groups (FO: 3.54±2.63 and FO+5-MTHF: 3.87±2.46) were significantly higher than those observed in the not FO supplemented groups (5-MTHF: 1.44±1.75 and Placebo: 1.15±2.04) (P<0.001). DHA levels significantly increased between the 20th and the 30th week of pregnancy in plasma PLs and erythrocyte PC but remained stable afterwards. In contrast, DHA levels in erythrocyte PE continued to significantly rise between the 30th week of gestation and delivery.

The degree of these changes was different in the various lipid classes with higher percentage changes in erythrocyte PE (FO: +67.8%, FO+5-MTHF: +71.8%) and PC (FO: +77.6%, FO+5-MTHF: 67.1%) than in plasma PLs (FO: +28.4%, FO+5-MTHF: +28.6%) (P<0.001).

Correlations between fatty acids relative levels (% of total fatty acids) in plasma and erythrocyte PLs after randomization in 4 supplementation groups

Spearman rank correlations between plasma and erythrocyte fatty acids relative levels in the four intervention groups (FO, FO+5-MTHF, 5-MTHF and

placebo) are shown in table 6. In general, no relevant differences were observed during the course of gestation among the four intervention groups.

Correlations between maternal and neonatal fatty acids relative levels in plasma and erythrocyte phospholipids

Spearman rank correlation coefficients for the association between maternal and neonatal relative levels of DHA, AA and fatty acid combinations in plasma and erythrocyte PLs (PE and PC) at delivery are given in table 8.

Fatty acid percentage levels of most fatty acids in cord blood correlated significantly with fatty acid values in maternal blood at delivery in both, plasma and erythrocyte PLs. Global correlation coefficients were higher in the erythrocyte PE compared to those observed in plasma or erythrocyte PC for DHA and AA ($P<0.02$). Linoleic acid, ΣPUFA, ΣMUFA and ΣSFA showed higher global correlation coefficients in erythrocyte PE compared to plasma ($P<0.03$) but similar to those observed in PC.

No relevant differences in the correlation coefficients were observed among intervention groups.

Discussion

It is well known from interventional studies that mothers consuming n-3 LC-PUFA supplements have higher n-3 LC-PUFA levels in both, plasma and erythrocytes during pregnancy and at delivery (4;5;9;10;14;15;19). Higher levels of n-3 LC-PUFA are also reported in umbilical cord plasma and erythrocyte PLs in supplemented mothers (14;19;20). Furthermore, DHA levels in cord blood plasma and erythrocytes have been demonstrated to correlate with maternal blood DHA

levels (5;8;21;22). In agreement with previous interventional studies (23-25) FO supplementation in the second half of pregnancy significantly increased DHA levels in maternal and umbilical plasma and erythrocyte PLs. Supplementation did not significantly modified AA levels in umbilical erythrocyte membrane PLs but AA levels in umbilical plasma PLs were significantly lower in the FO supplemented groups. Supplementation with 500 mg/day of DHA in populations with a mean daily DHA intake above the recommended 200 mg/day (1;26), may lower AA in the fetus. The supplementation with a AA/DHA blend has proven to increase DHA levels without decreasing the levels of AA and may be a better way of avoiding the AA fall following the n-3 PUFA supplementation (27).

Human fatty acid status depends only partially on dietary intake. Genetic and metabolic factors, as well as lifestyle determinants, may affect fatty acid concentrations in human tissues. Furthermore, pregnancy may modify plasma and erythrocyte fatty acid concentrations as a result of the occurring metabolic changes. Several studies have been conducted in order to establish the most confident compartment for the assessment of the LC-PUFA status of individuals. A revised meta-analysis of interventional studies concluded that both, plasma and erythrocyte phospholipids, appear to be good markers for DHA status in humans, but special consideration should be given to particular population subgroups (28). Only a few studies evaluate the influence of n-3 PUFA supplements during the course of pregnancy in both erythrocyte and plasma in the same population (9;10).

Studies on the kinetics of fatty acid incorporation into plasma PLs and erythrocytes after DHA supplementation show a dose dependent rapid increase of DHA in plasma PLs with a subsequent steady state concentration 1 month after the start of supplementation. Red blood cells follow a similar pattern but it takes 4-6 months to reach equilibrium (29-31). In addition, most of the PE in erythrocyte membranes are located in the inner layer, whereas PC are in the outer layer and can

therefore interact more easily with circulating plasma lipids. Thus, PC reflects more directly the fatty acid composition of plasma PLs, whereas the FA composition of the PE appears to be more dependent on a selective incorporation of LC-PUFA into PE. A study on DHA supplementation in healthy adult vegetarians showed strong correlations between erythrocyte PC and plasma PL fatty acid changes, while erythrocyte PE and plasma PLs fatty acid changes were less closely related (32). After the first 10 weeks of supplementation in the present study, DHA levels in maternal plasma and erythrocyte PC may have risen to their steady state levels and consequently do not continue to increase in the last weeks of pregnancy. DHA in erythrocyte PE may need longer to reach the steady state concentration and therefore continues to increase until delivery. Given the slow turnover of fatty acids in cell membranes, erythrocyte PLs may be a more reliable measure of long term fatty acid intake, whereas plasma PLs appear more sensitive to short-term changes in the intake of LC-PUFAs and may be more suitable to monitor the fatty acid status of individuals in intervention studies. Both, plasma and erythrocyte fatty acids, appear adequate to asses the fatty acid status in pregnant women and their neonates but the type of study is a major consideration when determining which body compartment reflects a better measure of the fatty acid status.

It should be taken into account that studies on the kinetics of incorporation of fatty acids in plasma and erythrocyte PLs have not been conducted in pregnant women. Most studies conducted in pregnancy report a similar evolution pattern of fatty acids in both plasma and erythrocyte and report positive correlations between maternal plasma and erythrocyte fatty acid levels and fatty acid changes throughout gestation (21;22;33), which is consistent with our results. In general, changes observed in erythrocyte membrane PLs mirror those occurring in plasma PLs but we observed that the degrees of DHA percentage changes were different in the various lipid fractions. Geppert et al. in their study in healthy adult vegetarians reported that relative changes of DHA 8 weeks after supplementation were greater in plasma and

erythrocyte PC than in erythrocyte total lipids and erythrocyte PE (32), which contrast with our finding of greater changes in erythrocyte PE and PC compared to plasma PLs. Vlaardingerbroek et al. (22) observed in their study that the amount of most fatty acids continued to increase in erythrocyte after the 32 week of pregnancy while the amounts in plasma hardly increased or even decreased, which is consistent with our findings. These authors suggested that the increment observed in erythrocyte PLs could be explained by the replacement during pregnancy of erythrocyte PLs classes with one ester linked fatty acid moiety by PLs classes carrying two ester linked fatty acids, such as PC and PE (34). As a result, plasma lipids could be more readily adsorbed to the erythrocyte membrane which could explain the higher percentage increase of DHA in erythrocyte PC and PE. The increment of PE and PC in erythrocyte membrane may be an adaptation mechanism during pregnancy to increase the transfer of LC-PUFA to the fetus. However, further research is needed to elucidate the possible role of erythrocyte in providing LC-PUFA for the placenta to transfer to the fetus.

We observed positive significant correlations between plasma and erythrocyte fatty acid levels for most fatty acids in both, maternal and cord blood, which is consistent with previously reported data (21;22;33). Our correlation coefficients were considerably lower to those reported in non pregnant individuals (32). Fatty acids bind to triglycerides in high proportion during pregnancy (35) and the fact that we did not consider the LC-PUFA fraction bound to triglyceride in the current study could explain our lower correlation coefficients.

Correlation coefficients between maternal and cord fatty acid relative levels at delivery were significantly higher in erythrocyte PE compared to the correlation coefficients in plasma PLs for most fatty acids. Plasma levels of lipoproteins in cord serum are low, with HDL being the major lipoprotein and LDL and VLDL present in low and very low concentrations (36;37). Thus, it could be hypothesised that

erythrocyte membrane PLs are important carriers of LC-PUFA in fetal blood which could explain the higher correlation coefficients between maternal and fetal fatty acid percentage levels in erythrocyte membrane PLs. These results suggest that erythrocyte PLs seem to be a more reliable biomarker for the prediction of fetal/neonatal fatty acid status on the basis of maternal fatty acid levels.

In conclusion both, plasma and erythrocyte fatty acid levels, seem to be suitable to asses maternal fatty acid status. More information on the physiological changes taking place in the erythrocyte membrane during gestation, as well as further knowledge about fetal metabolism and the kinetics of fatty acid accretion in fetal tissues is needed. Nevertheless, fatty acid levels in erythrocyte PLs seem to be a more reliable biomarker compared to those in plasma PLs to predict fetal/neonatal fatty acid status on the basis of maternal blood fatty acid levels.

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Table 1. Study population base line characteristic after randomisation to intervention groups.

	FO (n=69)	FO + 5-MTHF (n=64)	5-MTHF (n=65)	Placebo (n=72)	*P*
Center n (%)					n.s.
Spain	38 (55.1%)	36 (56.2%)	35 (53.8%)	38 (52.8%)	
Germany	18 (26.1%)	16 (25%)	14 (21.5%)	20 (27.8%)	
Hungary	13 (18.8%)	12 (18.8%)	16 (24.6%)	14 (19.4%)	
Age (years)	31(7)	31 (8)	30 (10)	31 (6)	n.s.
BMI 20 w (kg/m2)	26.0±4.0	25.2±2.9	25.2±4.1	24.9±3.3	n.s.
BMI 30 w (kg/m2)	28.3±4.4	27.1±3.1	27.2±4.4	27.2±3.6	n.s.
Parity					n.s.
0	40 (58.0%)	30 (46.9%)	32 (49.2%)	37 (51.4%)	
≥1	29 (42.0%)	34 (53.1%)	33 (50.8%)	35 (48.6%)	
Smoking	9 (13.2%)	9 (14.5%)	10 (15.6%)	4 (5.6%)	
Gestational age (w)	39 (2)	39 (2)	38 (3)	39 (2)	n.s.
Ethnia (%)					n.s.
Caucasian	69 (100%)	63 (98.4%)	64 (98.5%)	70 (97.2%)	
Others	0 (0%)	1 (1.6%)	1 (1.5%)	2 (2.8%)	
Residence area (%)					n.s.
City area	32 (46.4%)	29 (45.3%)	32 (49.2%)	37 (51.4%)	
Farm area	37 (53.6%)	35 (54.7%)	33 (50.8%)	35 (48.6%)	
Gravidity Risk (20 w)					n.s.
no risk factor	19 (27.5%)	20 (31.2%)	18 (27.7%)	28 (38.9%)	
≥1 risk factor	50 (72.5%)	44 (68.8%)	47 (72.3%)	44 (61.1%)	
Delivery risk					n.s.
no risk factor	30 (43.5%)	35 (54,7%)	35 (53.8%)	45 (62.5%)	
≥1 risk factor	39 (56.5%)	29 (45.3%)	30 (46.2%)	27 (37.5%)	

Results expressed as n (%) for categorical variables, mean±SD for continuous variables and median (interquartile range) for non continuous variables.
n.s. (*P*>0.05). FO: Fish oil; 5-MTHF: 5-methyltetrahydrofolate, w: week.

Table 2. Basal dietary intake of energy and nutrients in participating women at the 20th and the 30th week of pregnancy.

	FO	**FO+5-MTHF**	**5-MTHF**	**Placebo**	***P***
20th week pregnancy	(n=69)	(n=60)	(n=64)	(n=70)	
Energy (Kcal)	2905±870.7	2960±990.6	3038±1237.5	2792±925.4	n.s.
Protein (g)	112±35.9	110±37.9	114±44.8	107±38.4	n.s.
Fat (g)	15±58.0	162±54.8	169±79.9	152±63.3	n.s.
Carbohydrate (g)	249±81.1	259±111.0	258±105.5	242±93.6	n.s.
ΣSFA (g)	46.7±15.8	48.9±15.9	51.8±25.2	44.1±16.9	n.s.
ΣMUFA (g)	68.4±26.1	68.1±26.3	72.2±36.4	64.9±29.4	n.s.
ΣPUFA (g)	34.4±19.9	35.0±18.5	34.6±19.6	33.8±20.0	n.s.
22:6n3 (g)	0.38±0.17	0.42±0.23	0.49±1.06	0.39±0.30	n.s.
20:4n6 (g)	0.82±0.32	0.82±0.40	0.81±0.48	0.77±0.40	n.s.
20:5n3 (g)	0.15±0.09	0.17±0.12	0.22±0.64	0.17±0.17	n.s.
Folate (µg)	370±182.7	357±156.1	365±182.6	345±137.6	n.s.
30th week pregnancy	(n=67)	(n=61)	(n=63)	(n=69)	
Energy (Kcal)	2905±1044.8	2831±977.2	2725±993.3	2816±975.5	n.s.
Protein (g)	106±38.5	109±43.6	103±30.2	104±34.0	n.s.
Fat (g)	160±61.2	155±59.9	150±63.2	153±60.9	n.s.
Carbohydrate (g)	253±103.7	241±89.6	234±94.7	249±95.2	n.s.
ΣSFA (g)	48.8±20.5	45.5±17.7	47.3±20.7	45.9±18.7	n.s.
ΣMUFA (g)	66.6±27.0	66.6±30.0	62.4±29.6	64.5±28.3	n.s.
ΣPUFA (g)	35.1±19.0	33.9±14.6	31.3±14.2	33.5±15.6	n.s.
22:6n3 (g)	0.38±0.22	0.40±0.19	0.36±0.17	0.43±0.43	n.s.
20:4n6 (g)	0.74±0.34	0.77±0.35	0.73±0.36	0.81±0.45	n.s.
20:5n3 (g)	0.15±0.12	0.18±0.10	0.14±0.10	0.18±0.25	n.s.
Folate (µg)	353±212.2	365±161.6	317±106.7	331±131.1	n.s.

FO: Fish oil; 5-MTHF: 5-methyltetrahydrofolate; ΣPUFA: Polyunsaturated fatty acids; ΣMUFA: Monounsaturated fatty acids; ΣSFA: Saturated fatty acids; n.s: $P>0.05$.

Table 3. Relative fatty acid composition of maternal and neonatal plasma phospholipids.

		20 week		30 week		Delivery		Newborn
18:2n-6	FO	21.22±2.79		20.50±2.95		19.98±3.01		8.02±1.91
	FO+5-MTHF	21.69±2.43	A	20.71±2.34	B	19.99±2.92	B	7.86±1.21
	5-MTHF	21.55±2.53		21.38±2.18		20.82±2.39		7.41±1.02
	Placebo	21.33±2.71		21.43±2.25		20.77±2.83		7.34±1.06
18:3n-3	FO	0.04±0.11		0.07±0.12		0.05±0.11		*
	FO+5-MTHF	0.04±0.11		0.04±0.11		0.06±0.13		*
	5-MTHF	0.02±0.09	B	0.08±0.13	A	0.11±0.14	A	*
	Placebo	0.04±0.12		0.09±0.13		0.05±0.11		*
20:4n-6	FO	10.41±1.82	A	8.88±1.47	B	8.93±1.90	B	16.47±2.33 [ab]
	FO+5-MTHF	10.40±1.59	A	8.89±1.27	B	8.94±1.86	B	16.39±2.04 [b]
	5-MTHF	10.24±2.12	A	9.20±1.73	B	9.08±1.77	B	17.38±1.80 [a]
	Placebo	10.18±1.47	A	9.28±1.56	B	9.46±1.63	B	17.58±1.88 [a]
22:6n-3	FO	4.43±1.05	B	5.87±1.61 [a]	A	5.69±1.42 [a]	A	7.53±1.69 [a]
	FO+5-MTHF	4.54±1.09	C	6.26±1.17 [a]	A	5.84±1.30 [a]	B	7.09±1.83 [ab]
	5-MTHF	4.46±0.86		4.43±0.81 [b]		4.31±1.20 [b]		6.06±1.62 [c]
	Placebo	4.66±1.14	A	4.19±0.99 [b]	B	4.20±1.24 [b]	B	6.53±1.62 [bc]
ΣSFA	FO	43.75±2.32		43.71±1.27		44.19±1.21		48.95± 1.71
	FO+5-MTHF	43.76±1.13	AB	43.44±1.22	B	44.14±1.53	A	48.75± 1.50
	5-MTHF	43.77±1.25		43.68±1.05		43.89±1.20		49.30± 1.55
	Placebo	43.68±1.49		43.94±0.90		44.09±1.35		49.32± 2.08
ΣMUFA	FO	13.37±2.49	B	14.46±1.69	AB	14.80±1.73 [ab]	A	12.58±2.21
	FO+5-MTHF	13.20±2.34		14.21±1.68		14.49±1.79 [b]		13.58±2.60
	5-MTHF	13.62±2.34	B	15.11±1.63	A	15.31±2.00 [a]	A	13.12±2.03
	Placebo	13.49±2.18		14.71±1.60		14.98±1.69 [ab]		12.55±2.96
ΣPUFA	FO	41.92±3.20	A	41.09±2.01 [ab]	A	40.26±1.82	B	37.61±2.35
	FO+5-MTHF	42.50±2.92	A	41.70±1.70 [a]	A	40.71±1.72	B	36.58±2.69
	5-MTHF	41.93±3.09	A	40.43±1.50 [b]	B	40.06±2.02	B	36.57±2.06
	Placebo	42.21±3.05	A	40.60±1.60 [b]	AB	40.31±1.91	B	36.87±2.44
AA/DHA	FO	2.47±0.69	A	1.63±0.57 [b]	B	1.67±0.57 [b]	B	2.30± 0.65 [b]
	FO+5-MTHF	2.41±0.66	A	1.48±0.39 [b]	B	1.61±0.53 [b]	C	2.46± 0.71 [b]
	5-MTHF	2.38±0.65	A	2.14±0.58 [a]	B	2.23±0.66 [a]	A	3.10± 0.99 [a]
	Placebo	2.32±0.65		2.35±0.71 [a]		2.40±0.69 [a]		2.85± 0.76 [a]
Σn-6/Σn-3	FO	6.85±1.66	A	4.65±1.55 [b]	B	4.90±1.46 [b]	B	4.13± 0.99 [c]
	FO+5-MTHF	6.78±1.80	A	4.30±1.12 [b]	C	4.73±1.34 [b]	B	4.35±1.26 [bc]
	5-MTHF	6.74±1.39		6.35±1.40 [a]		6.40±1.65 [a]		5.45±1.65 [a]
	Placebo	6.58±1.98		6.70±1.87 [a]		6.86±1.97 [a]		4.91±1.30 [ab]

a, b, c: Differences between intervention groups. Values with different letters are statistically different (P<0.05)
A, B, C: Differences between pregnancy time points. Values with different letters are statistically different (P<0.05)
*Concentration of 18:3n-3 in cord plasma PLs was too low to be detected
FO (n=57), FO+5-MTHF (n=51), 5-MTHF (n=56), placebo (n=53).
FO: Fish oil; 5-MTHF: 5-methyltetrahydrofoate; ΣPUFA: Polyunsaturated fatty acids; ΣMUFA: Monousaturated fatty acids; ΣSFA: Saturated fatty acids; AA/DHA: arachidonic acid/docosahexaenoic acid ratio; Σn-6/Σn-3: n-6 PUFA/n-3 PUFA ratio.

Table 4. Relative fatty acid composition of maternal and neonatal erythrocyte phosphatidylcholine.

		20 week		30 week		Delivery		Newborn
18:2n-6	FO	15.67±2.68		15.35±2.86		15.70±2.72		6.88± 2.32
	FO+5-MTHF	15.62±2.60		15.43±2.74		15.48±2.56		6.72±1.17
	5-MTHF	15.55±2.08		14.78±2.72		15.06±2.77		6.52±1.28
	Placebo	15.35±2.79		15.51±2.48		15.65±2.63		5.95±1.01
18:3n-3	FO	0.11±0.09		0.15±0.13		0.16±0.13		0.04±0.04
	FO+5-MTHF	0.13±0.10		0.12±0.06		0.14±0.09		0.08±0.09
	5-MTHF	0.18±0.14		0.17±0.16		0.17±0.12		0.11±0.24
	Placebo	0.14±0.13		0.14±0.11		0.13±0.10		0.04±0.04
20:4n-6	FO	6.64±3.13		7.07±2.57 ^{b}		7.02±2.56		12.11±3.43
	FO+5-MTHF	7.34±2.41		6.73±2.48 ^{b}		7.37±2.17		12.62±2.51
	5-MTHF	7.40±2.21		7.50±2.58 ^{b}		7.93±2.43		11.51±3.93
	Placebo	7.56±2.41	B	8.69±2.20 ^{a}	A	8.13±2.57	AB	12.88±3.97
22:6n-3	FO	1.92±1.00	B	2.95±1.57	A	3.41±1.70	A	3.97±1.62 ^{a}
	FO+5-MTHF	2.22±1.13	B	3.19±1.37	A	3.71±1.42	A	3.96±1.47 ^{a}
	5-MTHF	2.41±1.02		2.43±1.12		2.59±1.11		2.71±1.27 ^{b}
	Placebo	2.41±1.15		2.75±1.20		2.51±0.94		2.82±1.31 ^{b}
ΣSFA	FO	53.92±4.12	A	52.95±3.46	AB	52.62±3.54	B	54.98±4.45
	FO+5-MTHF	53.46±4.81		52.53±3.28		52.02±3.46		54.73±3.78
	5-MTHF	52.38±2.90		52.28±2.95		51.75±3.19		55.68±4.36
	Placebo	52.99±3.89		51.82±3.57		51.95±3.12		55.92±4.52
ΣMUFA	FO	16.18±2.26		15.70±2.15		15.40±2.17		13.95±2.22 ^{ab}
	FO+5-MTHF	15.41±2.32	AB	15.92±2.49	A	15.26±2.02	B	13.54±1.81 ^{b}
	5-MTHF	16.03±1.67		16.45±2.25		15.94±2.14		14.91±2.16 ^{a}
	Placebo	15.52±2.13		14.84±1.47		15.43±1.82		14.20±2.32 ^{ab}
ΣPUFA	FO	27.55±4.66	B	29.03±4.38	AB	29.83±4.46	A	27.88±5.30
	FO+5-MTHF	28.69±4.02		29.19±4.43		30.44±3.54		28.32±4.04
	5-MTHF	29.22±3.43		28.69±4.96		29.84±4.19		25.63±5.52
	Placebo	29.09±4.31	B	31.12±3.48	A	30.27±3.61	AB	26.19±5.64
AA/DHA	FO	4.01±1.76	A	2.84±1.14 ^{bc}	B	2.36±0.82 ^{b}	C	3.44±1.44 ^{b}
	FO+5-MTHF	3.94±1.85	A	2.47±1.61 ^{c}	B	2.17±0.76 ^{b}	C	3.47±0.96 ^{b}
	5-MTHF	3.48±1.30		3.53±1.30 ^{ab}		3.44±1.24 ^{a}		4.75±1.62 ^{a}
	Placebo	3.76±1.88		3.71±1.58 ^{a}		3.59±1.37 ^{a}		5.14±1.77 ^{a}
Σn-6/Σn-3	FO	12.16±7.88	A	7.93±4.47 ^{ab}	B	6.94±4.02 ^{bc}	B	5.43±2.28 ^{b}
	FO+5-MTHF	10.01±6.17	A	7.27±6.76 ^{b}	A	6.00±2.98 ^{c}	B	5.18±1.44 ^{b}
	5-MTHF	8.62±4.13	B	9.68±9.15 ^{a}	A	8.10±3.89 ^{ab}	B	7.09±2.60 ^{a}
	Placebo	8.70±4.57		8.77±6.67 ^{ab}		9.00±6.59 ^{a}		7.00±2.50 ^{a}

a, b, c: Differences between intervention groups. Values with different letters are statistically different (P<0.05)
A, B, C: Differences between pregnancy time points. Values with different letters are statistically different (P<0.05).
FO (n=36), FO+5-MTHF (n=31), 5-MTHF (n=31), placebo (n=30).
FO: Fish oil; 5-MTHF: 5-methyltetrahydrofoate; ΣPUFA: Polyunsaturated fatty acids; ΣMUFA: Monousaturated fatty acids; ΣSFA: Saturated fatty acids; AA/DHA: arachidonic acid/docosahexaenoic acid ratio; Σn-6/Σn-3: n-6 PUFA/n-3 PUFA ratio.

Table 5. Relative fatty acid composition of maternal and neonatal erythrocyte phosphatidylethanolamine.

		20 week		30 week		Delivery		Newborn
18:2n-6	FO	6.22±1.17	A	5.75±1.09 [b]	B	5.99±1.37	AB	2.74±0.90
	FO+5-MTHF	6.37±1.13	A	5.86±0.93 [ab]	B	5.90±0.94	B	2.78±0.67
	5-MTHF	6.04±0.98		6.12±0.93 [a]		6.21±1.05		2.72±0.60
	Placebo	5.97±0.95		6.15±0.90 [ab]		6.12±1.13		2.60±0.83
18:3n-3	FO	0.21±0.19		0.19±0.16		0.21±0.20		0.14±0.18
	FO+5-MTHF	0.28±0.26		0.26±0.20		0.24±0.23		0.21±0.30
	5-MTHF	0.23±0.24		0.25±0.24		0.22±0.16		0.20±0.24
	Placebo	0.20±0.17		0.22±0.21		0.23±0.21		0.12±0.12
20:4n-6	FO	20.36±5.34	A	19.20±4.14 [ab]	AB	19.19±4.23 [ab]	B	24.30±6.02
	FO+5-MTHF	19.49±4.86	A	18.04±4.04 [ab]	B	18.13±3.32 [b]	B	23.85±4.18
	5-MTHF	18.82±4.97	A	17.31±4.34 [b]	B	18.97±3.59 [ab]	AB	24.35±5.14
	Placebo	19.65±4.83		19.64±4.54 [a]		20.09±3.66 [a]		25.19±5.89
22:6n-3	FO	5.22±1.76	C	7.20±2.33 [ab]	B	8.76±2.65 [a]	A	8.64±2.41 [ab]
	FO+5-MTHF	5.47±1.80	C	7.88±2.73 [a]	B	9.40±2.65 [a]	A	9.39±2.53 [a]
	5-MTHF	5.43±2.15	B	5.60±2.12 [c]	B	6.89±2.21 [b]	A	7.76±1.90 [b]
	Placebo	5.42±1.99	B	5.94±1.91 [bc]	AB	6.54±1.81 [b]	A	7.57±1.91 [b]
ΣSFA	FO	36.43±5.27		36.83±5.15		35.15±4.11		39.04±6.06
	FO+5-MTHF	37.34±6.36	A	35.91±5.04	AB	34.56±3.95	B	38.35±4.55
	5-MTHF	37.02±5.36	AB	38.07±5.67	A	35.35±4.62	B	38.95±5.39
	Placebo	37.56±6.12		36.18±4.74		35.42±3.94		39.31±6.11
ΣMUFA	FO	24.15±2.99		23.36±2.53		23.20±2.19		17.62±2.35
	FO+5-MTHF	23.72±2.87		24.62±3.29		23.98±3.02		17.62±2.51
	5-MTHF	24.76±3.07		25.02±3.74		24.61±3.06		17.87±2.25
	Placebo	23.87±3.02		24.48±2.99		24.19±2.56		17.61±2.04
ΣPUFA	FO	36.92±7.09		37.51±6.27		39.55±5.56		41.12±7.44
	FO+5-MTHF	36.46±7.13	B	37.23±7.39	AB	39.31±5.69	A	41.69±5.68
	5-MTHF	35.54±7.37	AB	34.16±6.81	B	37.68±5.65	A	40.50±6.56
	Placebo	36.02±7.00		37.03±6.69		38.14±5.02		40.49±7.31
AA/DHA	FO	4.17±1.23	A	2.90±1.02 [b]	B	2.37±0.80 [b]	C	2.95±0.79 [ab]
	FO+5-MTHF	3.75±0.88	A	2.47±0.63 [b]	B	2.04±0.56 [b]	C	2.73±0.87 [b]
	5-MTHF	3.83±1.17	A	3.38±1.03 [a]	A	2.95±0.81 [a]	B	3.26±0.81 [a]
	Placebo	3.96±1.17	A	3.51±0.84 [a]	B	3.23±0.87 [a]	B	3.45±0.82 [a]
Σn-6/Σn-3	FO	3.65±0.85	A	2.70±0.75 [b]	B	2.34±0.67 [b]	C	2.95±0.75 [bc]
	FO+5-MTHF	3.46±0.83	A	2.45±0.61 [b]	B	2.06±0.52 [b]	C	2.72±0.65 [c]
	5-MTHF	3.49±1.04	A	3.17±0.88 [a]	A	2.79±0.71 [a]	B	3.28±0.73 [ab]
	Placebo	3.49±0.85	A	3.27±0.79 [a]	AB	3.04±0.77 [a]	B	3.40±0.67 [a]

a, b, c: Differences between intervention groups. Values with different letters are statistically different (P<0.05). A, B, C: Differences between pregnancy time points. Values with different letters are statistically different (P<0.05). FO (n=46), FO+5-MTHF (n=42), 5-MTHF (n=33), placebo (n=39).

FO: Fish oil; 5-MTHF: 5-methyltetrahydrofoate; ΣPUFA: Polyunsaturated fatty acids; ΣMUFA: Monousaturated fatty acids; ΣSFA: Saturated fatty acids; AA/DHA: arachidonic acid/docosahexaenoic acid ratio; Σn-6/Σn-3: n-6 PUFA/n-3 PUFA.

Table 6. Spearman correlations between erythrocyte and plasma phospholipid fatty acid concentrations (wt%)

		20 week		30 week		Delivery		Newborn	
Plasma		PC	PE	PC	PE	PC	PE	PC	PE
20:4n-6	FO	0.297[a]	0.293[a]	0.029	0.199	0.190	0.369[b]	0.432[b]	0.267
	FO+5-MTHF	0.290	0.234	0.218	-0.034	0.153	0.570[b]	0.455[a]	0.381[a]
	5-MTHF	0.466[b]	0.290[a]	0.092	0.194	-0.143	0.296	0.596[b]	0.523[b]
	Placebo	0.048	0.299[a]	0.051	0.155	0.166	0.296[a]	0.397[a]	0.275
	Total	0.276[b]	0.280[b]	0.134	0.147[a]	0.100	0.388[b]	0.439[b]	0.353[b]
22:6n-3	FO	0.194	0.315[a]	0.387[b]	0.403[b]	0.558[b]	0.571[b]	0.344[a]	0.293
	FO+5-MTHF	0.085	-0.013	0.112	0.038	0.336[a]	0.372[a]	0.270	0.428[a]
	5-MTHF	0.167	0.112	0.567[b]	0.427[b]	0.502[b]	0.278	0.269	0.291
	Placebo	0.382[a]	0.205	0.312[a]	0.242	0.471[b]	0.286	0.255	0.153
	Total	0.202[b]	0.158[a]	0.326[b]	0.379[b]	0.582[b]	0.535[b]	0.354[b]	0.348[b]
ΣSFA	FO	0.186	0.032	-0.044	0.041	0.164	0.166	-0.006	0.000
	FO+5-MTHF	0.090	0.100	0.029	-0.143	-0.088	-0.333[a]	0.348	0.231
	5-MTHF	0.183	0.034	0.224	0.169	0.166	-0.150	0.094	0.011
	Placebo	0.083	0.126	0.112	-0.137	0.139	0.065	-0.069	0.173
	Total	0.129	0.079	0.088	-0.015	0.115	-0.060	0.101	0.085
ΣMUFA	FO	0.019	0.017	0.351[b]	-0.080	0.278	0.092	0.063	0.002
	FO+5-MTHF	0.049	0.009	0.149	-0.070	0.651[b]	0.524[b]	0.144	0.399[a]
	5-MTHF	0.016	0.152	0.134	0.082	0.362[a]	0.472[b]	0.023	0.485[b]
	Placebo	0.107	-0.021	0.152	0.187	0.432[b]	0.079	0.471[b]	0.103
	Total	0.064	0.039	0.221[b]	0.025	0.432[b]	0.306[b]	0.208[a]	0.249[b]
ΣPUFA	FO	0.224	0.133	0.358[b]	0.073	0.300	0.202	0.202	-0.027
	FO+5-MTHF	0.308	0.156	0.330[a]	-0.004	0.163	0.246	0.208	0.381[a]
	5-MTHF	0.239	0.214	0.361[a]	0.317[a]	0.350[a]	0.173	0.231	0.130
	Placebo	0.034	0.094	0.242	0.259	0.440[b]	0.051	0.047	0.052
	Total	0.196[b]	0.156[a]	0.294[b]	0.134	0.328[b]	0.169[a]	0.207[a]	0.144
AA/DHA	FO	0.470[b]	0.568[b]	0.475[b]	0.513[b]	0.590[b]	0.553[b]	0.537[b]	0.320[a]
	FO+5-MTHF	0.401[a]	0.424[b]	0.239	0.149	0.457[b]	0.365[a]	0.576[b]	0.492[b]
	5-MTHF	0.478[b]	0.393[b]	0.446[b]	0.403[b]	0.553[b]	0.520[b]	0.654[b]	0.594[b]
	Placebo	0.545[b]	0.362[b]	0.648[b]	0.483[b]	0.672[b]	0.453[b]	0.371[a]	0.166
	Total	0.478[b]	0.428[b]	0.549[b]	0.529[b]	0.698[b]	0.604[b]	0.595[b]	0.478[b]

a $P<0.05$; b $P\leq0.001$

PE: phosphatidylethanolamine phospholipids; PC: phosphatidylcholine; FO: Fish oil; 5-MTHF: 5-methyltetrahydrofoate; ΣPUFA: Polyunsaturated fatty acids; ΣMUFA: Monousaturated fatty acids; ΣSFA: Saturated fatty acids; AA/DHA: arachidonic acid/docosahexaenoic acid ratio.

Table 7. Spearman correlations between cord and maternal erythrocyte or plasma fatty acid concentrations (wt%) at delivery.

		PC	PE	Plasma
		r	r	r
22:6n-3	FO	0.469 [b]	0.669 [b]	0.593 [b]
	FO+5-MTHF	0.408 [a]	0.631 [b]	0.138
	5-MTHF	0.543 [b]	0.818 [b]	0.468 [b]
	Placebo	0.329	0.408 [a]	0.601 [b]
	Total	0.488 [b]	0.686 [b]	0.523 [b]
20:4n-6	FO	0.509 [b]	0.697 [b]	0.546 [b]
	FO+5-MTHF	0.354	0.597 [b]	0.541 [b]
	5-MTHF	0.281	0.622 [b]	0.502 [b]
	Placebo	0.409 [a]	0.667 [b]	0.489 [b]
	Total	0.381 [b]	0.690 [b]	0.550 [b]
ΣSFA	FO	0.788 [b]	0.793 [b]	0.244
	FO+5-MTHF	0.723 [b]	0.654 [b]	0.306 [a]
	5-MTHF	0.471 [b]	0.702 [b]	0.331 [a]
	Placebo	0.547 [b]	0.787 [b]	0.446 [b]
	Total	0.632 [b]	0.728 [b]	0.329 [b]
ΣMUFA	FO	0.273	0.326	0.137
	FO+5-MTHF	0.379 [a]	0.613 [b]	0.369 [b]
	5-MTHF	0.212	0.571 [b]	0.356 [b]
	Placebo	0.384	0.172	0.199
	Total	0.340 [b]	0.452 [b]	0.244 [b]
ΣPUFA	FO	0.716 [b]	0.842 [b]	0.144
	FO+5-MTHF	0.534 [b]	0.582 [b]	0.039
	5-MTHF	0.479 [b]	0.738 [b]	0.541 [b]
	Placebo	0.329	0.658 [b]	0.279 [a]
	Total	0.542 [b]	0.724 [b]	0.253 [b]
AA/DHA	FO	0.448 [b]	0.627 [b]	0.693 [b]
	FO+5-MTHF	0.514 [b]	0.699 [b]	0.302 [a]
	5-MTHF	0.612 [b]	0.796 [b]	0.545 [b]
	Placebo	0.502 [b]	0.477 [b]	0.697 [b]
	Total	0.595 [b]	0.650 [b]	0.653 [b]

a $P<0.05$; b $P\leq0.001$

PE: phosphatidylethanolamine phospholipids; PC: phosphatidylcholine; FO: Fish oil; 5-MTHF: 5-methyltetrahydrofoate; ΣPUFA: Polyunsaturated fatty acids; ΣMUFA: Monousaturated fatty acids; ΣSFA: Saturated fatty acids; AA/DHA: arachidonic acid/docosahexaenoic acid ratio.

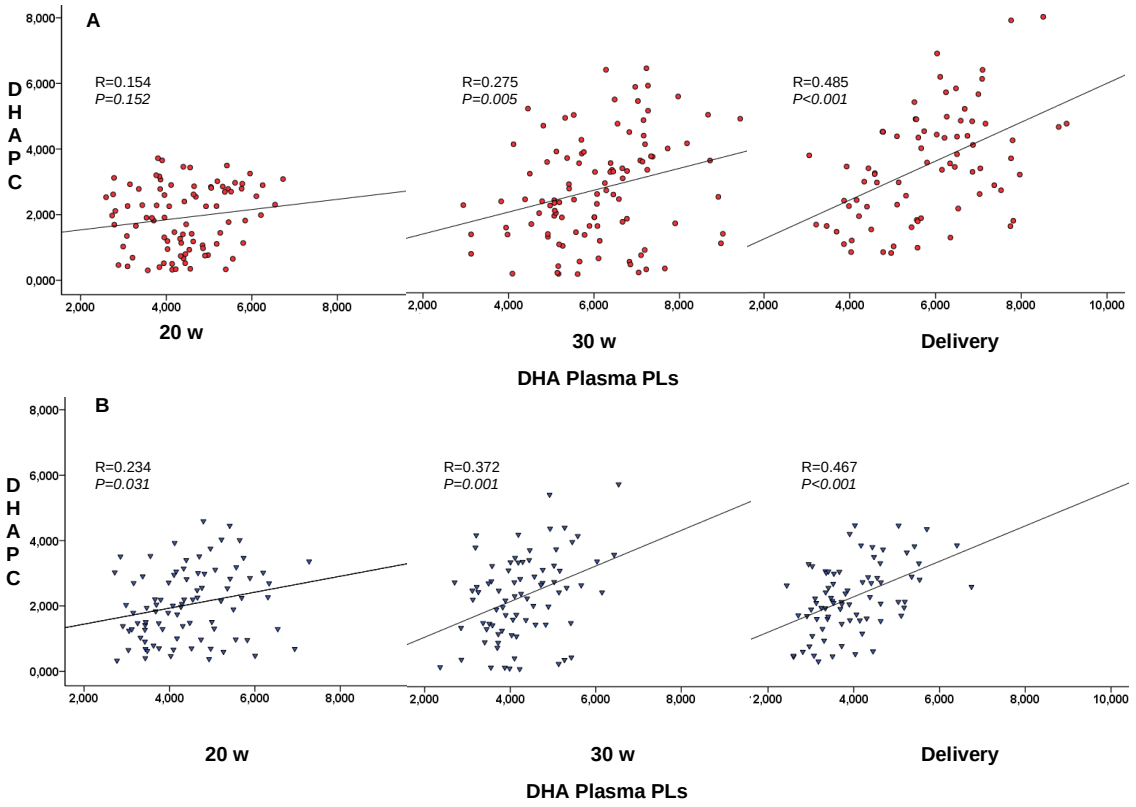

Fig 1. Scattered data graph of docosahexaenoic acid (DHA) percentage concentrations in plasma and erythrocyte membrane phosphatidylcholine (PC) during the course of pregnancy in the FO and not FO supplemented groups.

A: FO supplemented groups (FO and FO+5-MTHF); B: Not FO supplemented groups (Placebo and 5-MTHF); PLs: phospholipids; DHA PC: docosahexaenoic acid levels in erythrocyte membrane phosphatidylcholine.

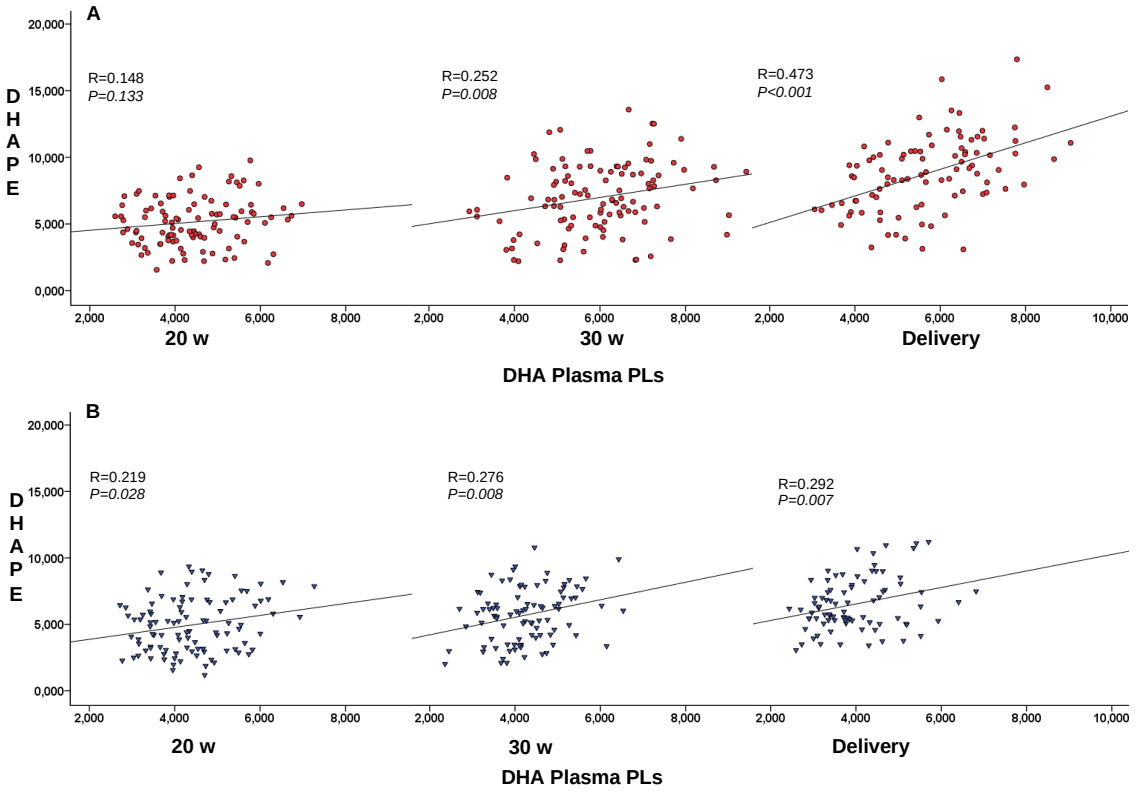

Fig 2. Scattered data graph of docosahexaenoic acid (DHA) relative concentrations in plasma and erythrocyte membrane phosphatidylethanolamine during the course of pregnancy in the FO and not FO supplemented groups

A: FO supplemented groups (FO and FO+5-MTHF); B: Not FO supplemented groups (Placebo and 5-MTHF); PLs: phospholipids; DHA PE: docosahexaenoic acid levels in erythrocyte membrane phosphatidylethanolamine.

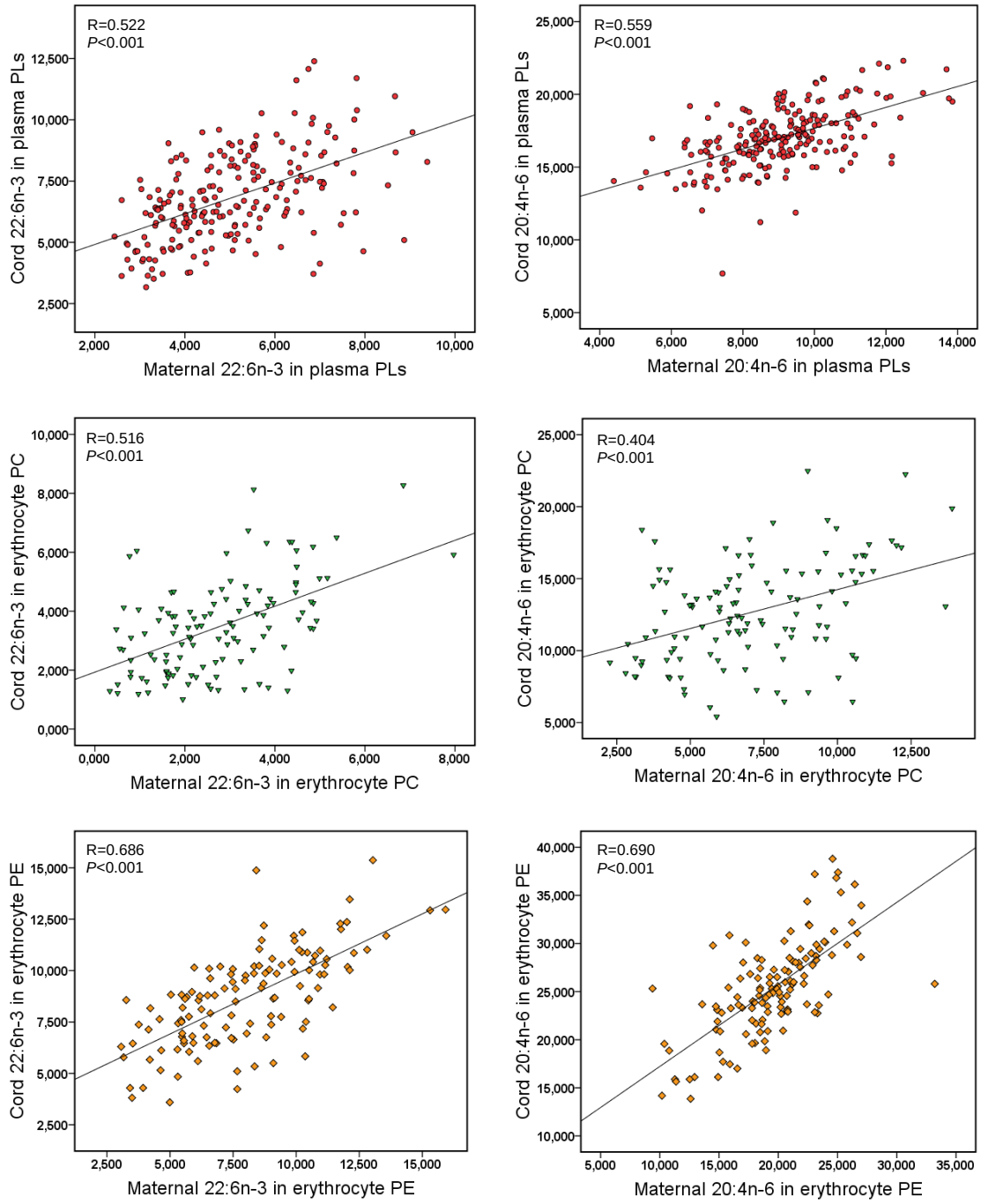

Fig 3. Lineal regression models for the relation between maternal arachidonic acid (AA) and docosahexaenoic acid (DHA) levels at delivery and their levels in cord plasma and erythrocyte membrane phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

4.2 Publication 2:

Fish oil supplementation to pregnant women and neurological outcome of their children at 4 and at 5 ½ years of age

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FISH OIL SUPPLEMENTATION TO PREGNANT WOMEN AND NEUROLOGICAL OUTCOME OF THEIR CHILDREN AT 4 AND AT 5 ½ YEARS OF AGE

Escolano-Margarit, MV[1], Ramos R[2], Beyer J[3], Csábi Gy[4], Parrilla-Roure M[1], Cruz F[5], Pérez-García M[5], Gil A[6], Decsi T[4], Koletzko B[3], Campoy C[1]

[1]University of Granada, Department of Paediatrics, Granada, Spain

[2]CIBER de Epidemiología y Salud Pública (CIBERESP), Spain, Laboratory of Medical Investigations, Clinical University Hospital San Cecilio, Granada, Spain.

[3]Dr. Von Haunersches Kinderspital, Ludwig Maximilians University of Munich, Munich, Germany

[4]University of Pécs, Department of Paediatrics, Pécs, Hungary

[5]University of Granada, Department of Clinical Psychology, Evaluation & Personality, School of Psychology, Granada, Spain

[6]University of Granada, Department of Biochemistry and molecular Biology, Granada Spain

Corresponding author: Cristina Campoy, Prof., MD,
Department of Paediatrics. School of Medicine
University of Granada
Avda. de Madrid, 11
18012-Granada, Spain
Tel.: +34629308695
Fax.:+34958240740
e-mail: ccampoy@ugr.es /ccfolgoso@gmail.com

Abstract

Some studies have reported beneficial effects of docosahexaenoic acid (DHA) on neurological outcome in early infancy. The present study aimed to asses the effects of DHA supplementation to expecting women on long term neurological development of their children. Healthy pregnant women from three European centers (Spain, Germany and Hungary) were randomly assigned to 4 different supplementation groups and received a dietary supplement consisting of fish oil [500 mg DHA + 150 mg eicosapentaenoic acid], 400 μg 5-methyltetrahydrofolate, both or placebo from the 20th week of gestation until delivery. Fatty acids in plasma and erythrocyte phospholipids (PLs) were determined in maternal blood at the 20th and the 30th week of pregnancy and in cord and maternal blood at delivery. Neurological development was assessed with the Hempel examination at 4 and the Touwen neurological examination 5 ½ years of age and expressed in terms of minor neurological dysfunction (MND), neurological optimality score (NOS) and fluency score. No significant differences were observed among intervention groups at both ages with respect to MND, NOS and fluency. Logistic regression analyses showed positive significant associations between an optimal neurological condition at 5 ½ and DHA content in cord blood PLs at delivery. The current study shows no beneficial effects of maternal FO supplementation on long term cognitive development of their offspring. Nevertheless, higher DHA levels in cord blood at delivery are related to a better neurological outcome in the children at 5 ½ years of age. We speculate that long chain polyunsaturated fatty acids status prior to the 20th week of gestation might be relevant for children neurological development.

Introduction

Long chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA) are essential constituents of the central nervous system and are incorporated in the brain mainly during the last trimester of pregnancy and the first year of postnatal life (1-3). An inadequate supply of n-3 LC-PUFA during fetal life has been associated with poorer performance in tests designed to measure cognitive and behavioural ability in animal studies (2;4;5). Recent reviews of studies in humans have showed no clear benefits of LC-PUFA supplemented formula on neurologic outcome of both, term and preterm infants (6-8). However, although brain growth and development continues after birth, the main brain growth spurt and DHA incorporation in the brain occurs in the last trimester of pregnancy (1). Observational studies suggests that high prenatal DHA status might have subtle positive effects on neurodevelopmental outcome also beyond early infancy (9-13). Thus, in the last few years, research has centered on increasing the LC-PUFA supply to the fetus by supplementing maternal diets with these fatty acids. Randomized controlled trials have reported higher DHA levels in cord blood at birth of children born from supplemented women compared to those whose mothers did not receive DHA supplements during pregnancy (14-16), but the potential beneficial effects of maternal DHA supply on neurologic outcome of their children remains controversial. While some studies report better performance of children whose mothers received supplements during pregnancy on different neurological examinations (17-19), others failed to show such effects (20-23). Information on long term effects of supplementation is scarce. We are aware of only one trial following neurologic development of children above 4 years of age (21).

The present study was conducted to asses the long term effects of DHA supplementation to pregnant women during the second half of pregnancy and

infants during the first 6 months of postnatal life on later neurologic development of children.

Subjects and methods

Study design

This study is part of a double-blind randomized controlled trial investigating the effects of prenatal and postnatal supplementation of LC-PUFA and 5-MTHF in healthy term infants. Details of the study design, recruitment of subjects, inclusion criteria, dietary intervention and collection of data and biological material has been reported elsewhere (24). Briefly, 315 healthy pregnant women were recruited before gestation week 20 at three different European centers (Ludwig Maximilians University of Munich, the University of Granada and the University of Pécs). Women were randomly assigned to 4 different groups and received from the 20th week of pregnancy until delivery one sachet per day with 15 g of a milk based supplement (Blemil Plus Matter; Ordesa Laboratories S.L., Barcelona, Spain) containing either modified fish oil (FO) providing 500 mg DHA and 150 mg EPA (Pronova Biocare, Lysaker, Norway), 400 μg 5-MTHF (BASF, Ludwigshafen, Germany), both or placebo, always together with vitamins and minerals in amounts meeting the European recommended intakes during the second half of pregnancy. Compliance was assessed by asking the mothers to return unused sachets to the study center at the 30th week of gestation and at delivery. Detailed and standardized information on socio-demographic characteristics was collected at study entry. Dietary information was collected by food frequency questionnaires both at the 20th and at the 30th week of gestation. Information on the course of pregnancy as well as information at delivery was obtained in standardized reports. 10 ml of maternal venous blood were collected into EDTA at the 20th and the 30th week of gestation for the fatty acid analyses. 2 ml of maternal venous blood samples at the 20th and the 30th week of

gestation, as well as maternal and umbilical cord blood samples at delivery were collected for the erythrocyte and plasma fatty acid analyses.

At birth a trained physician performed a physical examination of the child and obtained information of the infants in standardized case report forms. Women were encouraged to breast feed their infants. Infants who required supplements or substitution of breast feeding were provided with infant formulas (Blemil Plus NF, Ordesa Laboratories S.L., Barcelona, Spain) with a composition following European legislative standards, until the infant was 6 months of age. There were two formulas, both identical with the exception of the fatty acid composition. Children born from mothers in the FO and FO+5-MTHF groups received a formula containing 0.5% of total fatty acids as DHA and 0.4% of AA while children in the placebo or 5-MTHF groups received a formula virtually free of DHA and AA. These two formulas were coded in the same way as the supplements for the mothers (1-4), thus double blinding was maintained.

At the age of 4 and 5½ years the participating mothers and their respective infants were approached again and asked to participate in the neurological follow-up of the children. Children's neurological development was assessed with standardized and age-specific assessment techniques: at the age of 4 years the Hempel examination was used (25) and at 5½ Touwen assessment was applied (26;27). Assessment of potential confounders was made by means of questionnaires to obtain information on possible diseases and socio-demographic characteristics of children and parents.

Outcome variables in the current study are the results of the neurological examination at 4 and 5 ½ years of age, as well as the fatty acid (AA, DHA, AA/DHA) levels in cord and maternal plasma and erythrocyte phospholipids (PLs). Children

and parents socio-demographic and clinical characteristics, as well as obstetrical factors, were included in the analyses as potential confounders.

The study protocol was approved by the Medical Ethics Committees of all centers participating in the study. Written informed consent was obtained from all participants at study entry and at the beginning of the children follow up at 4 years of age.

Fatty acid analyses

Blood was centrifuged at 3500 g for 10 min at room temperature within 2 h. Plasma was removed and the remaining erythrocyte mass was washed in isotonic sodium chloride solution and haemolysed in distilled water. Plasma and erythrocyte were stored at – 80ºC until further analysis.

Lipids from erythrocyte were extracted by adding 3 ml chloroform and the two internal standards (phosphatidylethanolamine-heptadecanoate and phosphatidylcholine-pentadecanoate esters dissolved in methanol). The mixture was shaken on vortex and the lower layer was aspirated and evaporated under nitrogen stream. The dry lipid extract was resolved in chloroform and added to Silica gel plates (Merck 60, 10 x 20 cm). The runner solvent for the first run was hexane:diethylether:chloroform:acetic acid (21:6:3:1, volume/volume). The plate was dried under hood at ambient temperature and ran again with chloroform:methanol:water (65:25:4, volume/volume) (28). The bands were stained with dichlorofluorescein, visualised under ultraviolet light and scrapped for transmethylation. Plasma lipids were extracted into chloroform/isopropanol. 0.5 ml plasma plus 0.5 ml water were vortexed for 30 s with 100 μl of internal standard (0.857 g/L diheptadecanoyl phosphatidylcholine dissolved in chloroform). Lipids were extracted a first time with n-hexane/2-propanolol (3:2, by vol) containing 25

mg/L of butylated hydroxytoluene as antioxidant and then three times with pure hexane (29). The extracts were dried under vacuum and dissolved in 200 µl hexane:methyl-tert-butyl-ether:acetic acid (100:3:0.3, by vol). PLs were isolated by liquid chromatography on aminopropyl columns (Sep Pak Cartridges; Waters, Milford, MA) (30).

Fatty acid methyl esters from individual fractions were obtained by reaction with 3 N HCl-methanol (31). The quantification of fatty acid methyl esters from erythrocyte was performed by high-resolution capillary gas-liquid chromatography (model 9001 gas chromatography; Finnigan/Tremetrics, Austin, TX) with split injection, automatic sampler (A200SE, CTC Analytic, Switzerland) and flame ionisation detector with a DB-23 cyanopropyl column of 60 m length (J & W Scientific, Folsom, CA). Conditions during the analysis were as follows: temperature of injector at 80°C for 0.1 min, temperature increase by 180°C per min up to 280°C, temperature of column area at 60°C for 0.2 min, temperature increase by 40°C min up to 180°C, a 5-min hold period, temperature increase by 1.5°C per min up to 200°C, an 8.5-min hold period, temperature increase by 40°C per min up to 240°C and a 13-min hold period. The constant linear velocity was 0.3 m/s referred to 100 °C. For identification of sample peaks we used two commercially available fatty acid methylester calibration mixtures (Supelco 37 FAME mix and NU-CKECK GLC reference 463) containing the fatty acids measured in the present study. The analysis of fatty acid methyl esters from plasma PLs was performed by gas chromatography (HP5890 Series II; Hewlett Packard, Palo Alto, CA, USA) with flame ionization detector with a 60 m long capillary column (0.32 mm internal diameter and 0.20 µm thickness) and impregnated with SP-2330 FS (Supelco; Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250 and 275ºC respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Fatty acid methyl esters were identified by comparison of retention times with those of known standards. Results were expressed as percentages by weight (wt %) of total detected fatty acids.

Neurological assessment

At 4 years of age children were neurologically examined according to Hempel (25). It is based on the observation of spontaneous motor behaviour of the child in a standardized free-field situation. Essential in the diagnostics of MND is the presence of coherent clusters of signs. Single signs do not have clinical significance; signs only have significance when they co-occur with other signs within a functional domain. The Hempel assessment is organized into 5 functional domains: fine motor function, gross motor function, posture and muscle tone, reflexes and visuomotor behaviour. Each domain is assessed as deviant or not, according to specific criteria based on the presence of a number of dysfunctional signs. The findings of the Hempel examination result in a clinical classification consisting of the following categories: a) neurologically normal if none of the domains is scored as deviant or in case of the isolated presence of dysfunctions in the domain of reflexes; b) simple minor neurological dysfunction (MND) if only 1 domain is dysfunctional; c) complex MND if 2 or more domains show abnormal neuromotor signs; and d) major neurological dysfunction, which implies the presence of a defined neurological syndrome associated with disability and/or social limitations. Neurological findings can be also summarized with the neurological optimality score (NOS) by assessing performance on 56 representative items of the neurological examination (32). The NOS is defined as the sum of the total number of items with outcomes considered optimal according to a predefined optimal range. Besides the NOS, the fluency subscore was also calculated; it consists of 15 items of the NOS focused on the fluency of motor behaviour.

Neurological assessment at 5½ years of age was performed according to Touwen (26;27). The examination is organized into eight functional domains: posture and muscle tone, reflexes, the presence of involuntary movements, coordination and balance, fine manipulative ability, the presence of associated movements, sensory

deficits and cranial nerve function. The examination results in a clinical classification. Children are classified as: a) neurologically normal, when none of the domains meets the criteria of deviancy or in case of the isolated presence of deviancy in the domain of reflexes; b) simple MND, when 1 or 2 domains are scored as dysfunctional; c) complex MND if 3 or more domains are deviant; or d) definitively abnormal neurological condition. Neurological condition can also be expressed in the form of a NOS. The NOS of the Touwen assessment consists of 64 items with age specific criteria for optimality (27;33;34).

Statistics

The power calculation showed that the size of the remaining groups allowed for a detection of at least 2.25 points of difference in the NOS and 0.37 points in the fluency score (0.44 SD) at 4 years follow up and 2.46 points of difference in the NOS (0.47 SD) at 5½ years follow up with a *P*-value of 0.05 and a power of 80%.

Normality of variables was assessed by means of the Shapiro-Wilk tests.

Differences among intervention groups for numeric variables were assessed with the analyses of variance in the normally distributed variables and Kruskal-Wallis test in the not normally distributed variables. For categorical variables Chi square tests were applied. In case of significance multiple comparisons with Bonferroni correction were performed.

Univariate analyses of the differences in fatty acids levels between optimal/suboptimal children and children with and without MND (simple and complex MND pooled) were performed with the t-Student or Mann-Whitney test depending on the normality of variables. Multivariate analyses were carried out by means of stepwise logistic regression analyses which allowed correction for potential

confounders. Maternal age, parity, body mass index, maternal haematocrit and smoking habit during pregnancy, as well as length of gestation, gravidity risk factors, delivery complications and parental educational attainment and work status were taken into account in the statistical analyses. Infant weight, length and head circumference at birth, Apgar score and perinatal morbidity, sex and breast feeding, as well as BMI and health status of the children at 6 ½, were also included. All control variables related to the outcome variable at *P*-values <0.2 were entered in the model as covariables. The variables optimality (optimal vs suboptimal) or clinical conclusion (normal vs MND) were separately entered in the models as dependent variables and each LC-PUFA together with control variables as independent variables.

The analyses of the association between maternal or umbilical fatty acid levels and neurological scores were performed by means of raw and adjusted for confounders correlation coefficients which were calculated using Spearman tests.

A *P*-value of 0.05 or less was considered statistically significant. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL).

Results

Study participants

Baseline characteristics of the mothers participating in the study and their infants at birth have been reported elsewhere (24). Briefly, 315 women were recruited, 4 of them were excluded because they did not fulfil inclusion criteria and 41 did not complete the study (FO: n=8, 10.4%, FO+5-MTHF: n=13, 16.9%, 5-MTHF: n=12, 15.6% and Placebo: n=8, 10%; *P*=0.47). Compliance was good, 89.5% of the subjects in the second trimester gestation and 87.4% in the third missed less than 5

days of supplementation. Two hundred seventy mother-infant pairs were invited for the neurological follow up of children. One hundred seventy five complied with the request at 4 and one hundred fifty seven at 5½ years of age. Drop out rates were 35.18% at 4 and 41.9% at 5½ with no differences in the dropout rates between intervention groups (*P=0.827* and *P=0.539*). Main reasons for dropping out were relocation (n=3), loss of contact (n=65, n=76) and unwillingness to continue in the study (n=27, n=34). Four of the children examined at 4 and 5 of those examined at 5½ were born prematurely before the 35th week of pregnancy and were therefore excluded from the analyses. Except for one child who was born with a congenital left side anophthalmus, no other serious congenital disorder was observed. In the health screening questionnaire at 4 years of age, one child was reported to have left side deafness, another had developed craniosynostosis and was operated at the age of 6 months, and one child suffered from a developmental retardation of unknown aetiology. These children were also excluded from the analyses, which left 167 and 148 children at 4 and 5½ respectively (Fig. 1). No other severe illness or disability interfering with adequate functioning in normal life was observed. Children baseline clinical and socio-demographic characteristics at 4 years of age are shown in Table 1. The social and obstetrical characteristics in the 4 intervention groups of the remaining study population at 4 and 5½ years follow up were similar. Maternal basal dietary intake of energy and nutrients at the 20th and 30th week of gestation was also similar in the 4 intervention groups (Appendix 1). There were no differences among groups with respect to perinatal adverse events or to illnesses in the first years of life. Mean ages at Hempel and Touwen assessments were 50±1.79 and 69.83±1.97 months, respectively.

Maternal and neonatal LC-PUFA levels in plasma and erythrocyte phospholipids

As expected, at the 20th week of gestation maternal baseline relative levels (wt %) of AA, DHA and the AA/DHA ratio did not differ significantly between the 4

intervention groups. DHA relative levels in plasma and erythrocyte PLs were in general higher in the FO and FO+5-MTHF groups compared to the placebo and 5-MTHF groups at the 30th week of pregnancy and at delivery as well as in cord blood. We did not find significant differences between 5-MTHF supplemented groups and those that did not receive 5-MTHF with respect to fatty acid percentages in maternal or cord plasma or erythrocyte PLs (Table 2).

Clinical neurologic classification

None of the children had a definitely abnormal neurological condition. We did not find significant differences in the neurological clinical conclusion among the 4 intervention groups (P=0.169). Given that the groups FO and FO+5-MTHF and the groups placebo and 5-MTHF showed similar clinical and socio-demographic characteristics as well as similar AA, DHA and AA/DHA levels in plasma and erythrocyte PLs, we pooled the FO and FO+5-MTHF groups and the placebo and 5-MTHF groups to two different groups of children, who received the FO supplement or not. Likewise, we did not find significant differences in the neurological clinical conclusion between these two groups (P=0.382). Data on the outcome of the neurological examination at 4 and 5 ½ years of age are shown in Table 3.

There were no significant differences for cord blood DHA, AA or AA/DHA ratio between children classified as normal and those with MND at 4 or at 5½ years of age. Furthermore, children with higher DHA or AA levels in cord blood (upper quartiles) had a similar incidence of MND than those with lower cord blood levels of these fatty acids (lower quartiles).

Regarding fatty acid (AA, DHA, AA/DHA) relative concentrations in maternal plasma and erythrocyte PLs, we did not observe significant differences between children with MND at 4 and those classified as normal by the Hempel examination. Likewise, there were no differences in maternal plasma or erythrocyte PLs DHA

percentage concentrations between children classified as normal and those with MND at 5½ years of age. However, AA levels in maternal plasma at the 20th week of gestation and in maternal erythrocyte PC at the 30th week of pregnancy were higher in normal children compared to children with MND at 5½ years of age (plasma AA at the 20th week: 10.27±1.76 vs 9.00±1.55, *P*=0.028; erythrocyte PC AA at the 30th week: 6.95±2.99 vs 4.64±2.49, *P*=0.019). After adjustment for confounders in the logistic regression analyses no significant association between AA and neurological clinical conclusion was observed.

NOS and Fluency Score

We did not find significant differences in the NOS at 4 (*P*=0.661) or at 5 ½ years of age (*P*=0.874) or in the fluency score (*P*=0.187) at 4 between intervention groups (Table 3).

We considered it interesting to analyze the fatty acid status of those children with the best neurological performance in the tests. With this purpose we made two different groups of children, those with the highest NOS (NOS=56 at 4 and NOS=64 at 5 ½) were classified as optimal and children with lower scores as suboptimal. We found 22 (13%) optimal children at 4 years and 14 (9.9%) at 5 ½. We observed no differences in plasma or erythrocyte fatty acid percents (AA, DHA, AA/DHA) between children classified as optimal at 4 and those suboptimal. However, children classified as optimal at 5½ had significantly higher DHA percents in cord blood plasma and erythrocyte PLs than those classified as suboptimal (DHA plasma 8.09±1.47 vs 6.95±1.72, *P*=0.015; DHA PE 10.60±3.61 vs 8.08±2.12, *P*=0.009). DHA relative concentrations in maternal erythrocyte PLs at delivery were higher (PE 11.04±2.71 vs 7.40±2.53, *P*<0.001; PC 5.05±2.06 vs 2.92±1.41, *P*<0.001) and the AA/DHA ratios lower (PE 1.85±0.39 vs 2.79±0.92, *P*=0.003; PC 1.84±0.50 vs 3.03±1.43, *P*=0.003) in optimal children at 5½ compared to the suboptimal. After adjustment for

confounders in a stepwise logistic regression analysis the association between cord blood and maternal DHA levels at delivery and the occurrence of optimality at 5½ years of age remained significant (Table 4). In addition, maternal DHA levels in plasma PLs in the 20th and the 30th week of pregnancy and at delivery were significantly higher in children classified as fluent by the Hempel examination at 4 (fluency score=15) compared to those non-fluent (fluency score<15) (20th week: 4.75±1.17 vs 4.25±0.89, *P*=0.033; 30th week: 5.37±1.43 vs 4.76±1.22, *P*=0.031; Delivery: 5.30±1.51 vs 4.43±1.17, *P*=0.005). Furthermore, maternal AA/DHA ratios in plasma PLs in the 20th and 30th week of pregnancy and at delivery were significantly lower in the group of children classified as fluent (20th week: 2.24±0.64 vs 2.57±0.76, *P*=0.02; 30th week: 1.81±0.62 vs 2.17±0.74, *P*=0.019; Delivery: 1.83±0.64 vs 2.31±0.78, *P*=0.003). We observed no association between fatty acid levels in maternal plasma PLs and optimally fluent movements in children at 4 years after adjustment for confounders in the stepwise logistic regression analyses.

The bivariate analyses showed significant positive correlation coefficients between maternal DHA levels in plasma PLs at delivery and the NOS (r=0.179, *P*=0.026) and fluency score (r=0.187, *P*=0.02) at 4 years of age. The corresponding AA/DHA quotients were negatively correlated with the NOS (r= -0.185, *P*=0.021) and the fluency score (r= -0.249, *P*=0.002). In addition, maternal AA levels in plasma PLs at delivery negatively correlated with the NOS at 5½ years of age (r= -0.234, *P*=0.006). However, we did not find any significant correlation between maternal plasma or erythrocyte LC-PUFA levels (DHA, AA, AA/DHA) and the NOS and fluency scores at 4 or at 5½ years of age after adjustment for confounders.

Analysis of attrition

There was a higher attrition of children whose fathers had a high educational level in both intervention groups at 5 ½ years follow up (63% of fathers whose

children were lost to follow up in the FO group and 61% in the not FO supplemented one had a general qualification for university entrance or a university degree, while only 41% of the followed children in the FO group and 43% in the not FO supplemented group had a high educational level, $P<0.05$). In addition, weight, length and head circumference at birth in the group of children without FO supplementation were significantly lower in the group lost to follow-up at 4 years of age (weight: 3148.8±601.6, length: 49.44±3.2, head circumference: 33.40±2.5) compared with the followed group (weight: 3361.3±425.5, length: 51.11±2.1, head circumference: 34.78±1.5) ($P<0.05$).

Regarding measured variables, DHA levels in maternal plasma and erythrocyte PC at delivery were significantly higher in the group of children that continued in the study at 4 compared to those who were lost to follow-up in the FO supplemented group (plasma: 5.44±1.49 vs 6.05±1.19, $P=0.016$; PC: 2.90±1.82 vs 3.73±1.57, $P=0.031$). With respect to the group of children examined at 5½ years of age, AA levels were significantly higher in the group of lost to follow-up children in maternal erythrocyte PE in the 30th week of pregnancy (PE: 19.46±5.02 vs 17.51±4.04, $P=0.033$) and at delivery (Plasma: 9.72±1.82 vs 8.97±1.50, $P=0.013$; PE PLs: 20.77±3.15 vs 19.03±3.60, $P=0.026$) in the non supplemented children. Cord blood DHA percents in plasma PLs of the non supplemented group were significantly higher in the group of children continuing in the study at 4 (5.74±1.48 vs 6.56±1.64, $P=0.011$) and a 5 ½ (5.82±1.56 vs 6.58±1.62, $P=0.018$) years of age.

The rest of baseline socio-demographic, obstetrical and clinical characteristics of children who withdrew were similar to those of children that continued in the study in all intervention groups separately considered. Likewise, the intervention was not associated with attrition.

Discussion

The incorporation of DHA in the developing brain is thought to be particularly important in the third trimester of gestation (1;3). Therefore, it seems that the effects of DHA supplementation to pregnant women in the third trimester may be more beneficial for brain development than postnatal intake. Some observational studies have shown a positive association between neurological development of children and DHA levels in umbilical or maternal blood at birth (10-13;35-37). Several randomized controlled trials have been conducted in the last years in order to examine the effects of n-3 LC-PUFA supplementation during pregnancy on children's neurologic outcome. Most of the trials have increased DHA supply to the fetus by supplementing maternal diet during the second half of pregnancy (14-16). However, whether this supplementation actually improves long-term neurological outcome of children remains a matter of discussion to date.

Bearing in mind its limitations, the current study showed no differences regarding the NOS, the fluency score or the incidence of MND at 4 or at 5½ years of age between children whose mothers received FO supplements during pregnancy and those whose mothers were not supplemented.

The fact that we were unable to detect beneficial effects of prenatal supplementation cannot be attributed to the power of the study, as the power analyses showed that the size of the remaining groups allowed for the detection of at least 2.46 points of difference in the NOS with a *P*-value of 0.05 and a power of 80%. Apart from that, the high attrition could have induced bias resulting in an incorrect lack of association between supplementation and neurological outcome. The fact that the group of children with higher DHA relative concentrations was overrepresented in the group without supplementation could have induced a selection bias. A lower parental educational level, as well as lower birth weight and head circumference has

been often related to poorer neurological development of children. The group of fathers with high educational level is underrepresented in both intervention groups which should not induce bias. Children with lower weight and head circumferences were underrepresented in the non supplemented group. However, we did not find any association between supplementation and neurological outcome after adjustment for these confounders.

Another limitation of the present study is fact that neurologic assessment has been performed by 3 different persons in the three countries participating in the study which could induce an inter-observer error. The inter-assessor reliability of both tests has been reported to be satisfactory. The inter-rater agreement of the Hempel and is reported to vary between 0.62 and 1.00 for the various items with a mean value of 0.93 (38). The inter-assessor reliability of the Touwen varies between 0.75 and 1.00 for the various items with a Kappa value of 0.76 for the assessment of MND (39). All examiners were trained by the same expert assessor who also supervised some of the assessments by means of video-recordings. In addition, the center was taken into account as confounder in multivariate analyses.

The lack of association between supplementation and neurological outcome can neither be attributed to the sensitivity of the neurological examination, as both, the Hempel and Touwen examinations, focus on the detection of minor degrees of neurological dysfunction and have proven to be sensitive enough to detect subtle differences in the neurodevelopmental outcome (27;38). As the Hempel and Touwen examinations focus on the evaluation of neuromotor behaviour (38), it could be hypothesised that LC-PUFAs may have an effect in other specific developmental domains not assessed with these examinations. Interestingly, studies in animals have shown that the basal ganglia have the highest DHA accumulation in the brain of baboons (40-42) and may be specifically vulnerable to DHA deficiency. These areas are important to psychomotor behaviour and are particularly related to complex

movements such as those involved in fine manipulative ability. Thus, it seems conceivable that potential advantages of supplementation could have been detected with the tests.

It is also interesting in our results the low MND prevalence observed at 4 compared to that at 5 ½ years of age. At the age of 4 years children are at the border between the Hempel assessment (upper age limit) and Touwen (lower age limit). At the age of 4 the Hempel assessment is more appropriate than the Touwen, but it suffers to some extent of ceiling effects. Therefore, the difference in prevalence of MND between the two ages may be attributed to 1) age, as with increasing age more dysfunctions become expressed (43), and 2) ceiling effects of the Hempel assessment at the age of 4.

Our finding that FO supplementation to pregnant women do not influence neurodevelopment is consistent with the outcome of 3 other randomized trials (20-23) in which pregnant women received DHA supplementation in the second half of pregnancy, but it also contrast with the results of 3 other controlled trials (17-19). These studies widely differ methodologically which makes it difficult to make comparisons. Moreover, most of the trials have assessed neurological outcome of children before the school age. We are aware of only one randomized controlled trial assessing neurological outcome of children above the 4th year of age (21). The authors reported higher mental processing scores in the Kaufman Assessment Battery for children (K-ABC) at 4 years of age in children whose mothers received n-3 LC-PUFA supplements compared to those born from mothers receiving n-6 LC-PUFA supplements, but no differences in the K-ABC scores at 7 years. In our study we gave a low DHA dose compared to other studies that have shown a benefit of supplementation (17;19). Although the supplementation with 500 mg/day DHA significantly increased DHA levels in umbilical blood at birth (24), our LC-PUFA levels in cord blood are within the range of normal variability in all groups when

comparing them with data on fatty acid composition of venous cord blood PLs in healthy, full term infants from different populations (44).

Some authors have suggested that the improvement in the n-3 LC-PUFA supply to the developing fetus may be more easily achieved by small changes in habitual maternal dietary intakes of n-3 LC-PUFA than by means of high-dose supplementation of these fatty acids late in pregnancy (45). Therefore, comparing the fatty acid levels in blood between neonates could be a better way of looking at the relation between fatty acids and neurological outcome at 4 and 5½ years old, than just evaluating the intervention. Some observational and interventional studies have related high neonatal n-3 LC-PUFA status to a better performance in different neurological examinations (10-13;19;21). Dunstan et al. (19) showed a positive significant association between the eye and hand coordination score in the Griffiths Mental Development Scales (GMDS) in children at 34 months of age and DHA composition of cord blood erythrocytes. These authors also reported an inverse correlation between the mentioned score and AA levels in cord blood erythrocytes. Helland et al. reported no association between neonatal DHA levels and the Mental Processing Composite of the K-ABC test at 4 years of age (17). However, they found a significant association between neonatal and maternal DHA levels at birth and the Score in the Sequential Processing Scale of the same test at 7 years of age (21). The logistic regression analyses in the present study showed higher occurrence of an optimal neurologic condition at 5 ½ years with increasing DHA percentages in cord and maternal blood at delivery, which is in agreement with the previous studies. It should be realized that the range for optimal behaviour is narrower than that of normal behaviour, as children classified as neurologically normal may show single signs of dysfunction in various neurological domains (38). Thus, although the FO supplementation in the second half of pregnancy did not show to improve neurological function of children, the logistic regression analyses showed a positive

association between high maternal and fetal DHA status and optimal neurological condition.

The current study was conducted in a large heterogeneous cohort from three different European countries with corresponding differences in dietary intake of LC-PUFA and other nutrients in order to provide evidence for the applicability to the general population in Europe. However, 84.4% of the mothers at the 20th week of pregnancy and 89.2% at the 30th achieved the recommended DHA intake of 200 mg/day (46) which has been associated with an optimal long term developmental outcome in the ALSPAC study (47). In addition, parental level of education was relatively high. It is possible that beneficial effects of DHA supplementation during pregnancy might be less evident in well educated mothers who already have an optimal DHA supply.

The present randomized multicenter trial showed neither beneficial nor harmful effects of maternal FO supplementation during the second half of pregnancy on long term cognitive development of children. However, higher DHA levels in fetal and maternal blood during the course of pregnancy were related to a better performance on neurological examinations of the children at 5½ years of age. Although, further research is necessary to elucidate the long term effects of LC-PUFA, education programs related to nutrient intake in the population should, in our opinion, encourage the intake of DHA rich nutrients.

Acknowledgements and conflict of interest

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Table 1. Baseline characteristics of children at 4 years of age after randomization in the 4 intervention groups.

	FO (n=43)	FO+5-MTHF (n=37)	5-MTHF (n=40)	Placebo (n=47)	*P*
Center n (%)					n.s.
Spain	29 (67.4%)	24 (64.9%)	23 (57.5%)	30 (63.8%)	
Germany	9 (20.9%)	8 (21.6%)	9 (22.5%)	11 (23.4%)	
Hungary	5 (11.6%)	5 (13.5%)	8 (20%)	6 (12.8%)	
Maternal age	29 (7)	30 (7)	31.5 (7.75)	31 (5.5)	n.s.
BMI 20th WG	26.3±3.7	25.4±2.7	25.3±2.4	25.0±2.1	n.s.
Haematocrit 30th WG	34.0±3.7	33.4±2.5	32.5±6.3	33.3±2.9	n.s.
Parity					n.s.
0	23 (53.5%)	16 (43.2%)	19 (47.5%)	23 (48.9%)	
≥1	20 (46.5%)	21 (56.8%)	21 (52.5%)	24 (51.1%)	
Smoking in pregnancy	7 (16.3%)	5 (13.5%)	6 (15%)	2 (4.3%)	n.s.
Gravidity Risk (20 w)					n.s.
No risk factors	13 (30.2%)	9 (24.3%)	10 (25%)	18 (38.3%)	
≥1 risk factors	30 (69.8%)	28 (75.7%)	30 (75%)	29 (61.7%)	
Delivery risk					n.s.
No risk factors	17 (39.5%)	16 (43.2%)	24 (60%)	30 (63.8%)	
≥1 risk factors	26 (60.5%)	21 (56.8%)	16 (40%)	17 (36.2%)	
Gestational age (w)	39 (1.5)	39 (3)	39 (2)	39 (2.5)	n.s.
Sex					n.s.
Female	20 (46.5%)	22 (59.5%)	23 (57.5%)	18 (38.3%)	
Male	23 (53.5%)	15 (40.5%)	17 (42.5%)	29 (61.7%)	
Perinatal morbidity					n.s.
None	37 (86%)	29 (78.4%)	35 (87.5%)	40 (85.1%)	
Preterm (>35 w)	4 (9.3%)	6 (16.2%)	4 (10%)	3 (6.4%)	
Others	2 (4.6%)	2 (5.4%)	1 (2.5%)	4 (8.5%)	
Apgar 5 min	10 (0.5)	10 (0.75)	10 (0.5)	10 (1)	n.s.
Birth weight	3338.4±403.5	3107.4±519.7	3378.1±386.8	3388.6±405.1	n.s.
Birth length	50.9±2.0	50.7±3.5	51.1±1.4	51.0±2.1	n.s.
Birth head circumference	34.9±1.4	35.1±1.9	34.9±1.4	35.1±1.3	n.s.
Infant feeding					n.s.
Breastfed	23 (57.5%)	19 (57.6%)	18 (48.6%)	23 (53.5%)	
Mixed	13 (32.5%)	8 (24.2%)	12 (32.4%)	12 (27.9%)	
Formula	4 (10.0%)	6 (18.2%)	7 (18.9%)	8 (18.6%)	
Residence area (%)					n.s.
City area	21 (48.8%)	17 (45.9%)	19 (47.5%)	19 (40.4%)	
Farm area	22 (51.2%)	20 (54.1%)	21 (52.5%)	28 (59.6%)	
Maternal education [a]	20 (46.5%)	13 (36.1%)	23 (63.9%)	22 (46.8%)	n.s.
Paternal education [a]	21 (48.8%)	16 (44.4%)	21 (52.5%)	21 (44.7%)	n.s.
Age Hempel (months)	50.21±1.51	50.04±1.48	49.98±1.16	49.61±2.15	n.s.
Age Touwen (months)	70.5±2.0	70.7±2.0	70.0±2.3	70.1±2.2	n.s.
Children BMI 4y	16.6±2.1	15.7±1.2	15.8±1.1	15.9±1.4	n.s.

Results are expressed as mean±SD, median (interquartile range) or n (%) for continuous, non continuous and categorical variables respectively.

n.s. (*P*>0.05), FO (Fish oil), 5-MTHF (5-Methyltetrahydrofolate), WG (weeks of gestation), w (week)

a n (%) General qualification for university entrance or university degree.

Table 2. Fatty acids (wt%) in umbilical plasma and erythrocyte phospholipids at birth and fatty acids (wt%) in maternal plasma and erythrocyte phospholipids at the 20th week of pregnancy, 30th week of pregnancy and at delivery in the groups of children evaluated at 4 (mean±SD).

	FO	FO+5-MTHF	5-MTHF	Placebo	*P*
20th week gestation					
Plasma (n=159)					
22:6n-3	4.54±1.08	4.78±1.09	4.48±0.96	4.86±1.31	n.s.
20:4n-6	10.64±1.97	10.15±1.62	9.95±1.99	10.06±1.46	n.s.
AA/DHA	2.45±0.67	2.23±0.64	2.34±0.75	2.20±0.62	n.s.
Erythrocyte PE (n=135)					
22:6n-3	5.07±1.89	5.19±2.08	5.05±2.05	5.01±1.99	n.s.
20:4n-6	19.71±5.78	18.56±5.06	16.89±5.05	18.56±5.06	n.s.
AA/DHA	4.23±1.33	3.79±1.00	3.72±1.27	4.09±1.25	n.s.
Erythrocyte PC (n=117)					
22:6n-3	1.95±1.00	2.15±1.16	2.11±1.16	2.26±1.28	n.s.
20:4n-6	6.77±3.30	6.77±2.39	6.29±2.51	6.89±2.66	n.s.
AA/DHA	3.88±1.72	3.91±2.03	3.50±1.49	3.72±1.70	n.s.
30th week gestation					
Plasma (n=157)					
22:6n-3	6.07±1.28 ^{A}	6.42±1.06 ^{A}	4.33±0.75 ^{B}	4.34±0.99 ^{B}	<0.001
20:4n-6	9.01±1.43	8.89±1.26	9.40±1.78	9.17±1.49	n.s.
AA/DHA	1.56±.46 ^{B}	1.42±0.30 ^{B}	2.24±0.61 ^{A}	2.24±0.70 ^{A}	<0.001
Erythrocyte PE (n=142)					
22:6n-3	6.70±2.45 ^{A}	7.03±2.48 ^{A}	5.40±1.84 ^{B}	5.78±2.05 ^{AB}	0.003
20:4n-6	18.54±4.27	16.97±3.89	16.45±4.10	18.80±4.29	n.s.
AA/DHA	2.88±0.86 ^{BC}	2.61±0.79 ^{C}	3.33±1.09 ^{AB}	3.51±0.92 ^{A}	<0.001
Erythrocyte PC (n=130)					
22:6n-3	2.90±1.70	2.94±1.47	2.17±1.27	2.52±1.44	n.s.
20:4n-6	6.54±2.91	6.33±2.86	6.63±3.07	7.58±3.12	n.s.
AA/DHA	2.76±1.16 ^{B}	2.62±1.66 ^{B}	3.82±2.01 ^{A}	3.77±1.82 ^{A}	<0.001
Delivery					
Plasma (n=155)					
22:6n-3	5.96±1.21 ^{A}	6.16±1.18 ^{A}	4.31±1.28 ^{B}	4.35±1.22 ^{B}	<0.001
20:4n-6	8.93±1.67	8.81±1.18	9.08±1.76	9.28±1.49	n.s.
AA/DHA	1.57±0.52 ^{B}	1.48±0.34 ^{B}	2.25±0.71 ^{A}	2.28±0.69 ^{A}	<0.001
Erythrocyte PE (n=122)					
22:6n-3	8.94±2.69 ^{A}	9.09±3.04 ^{A}	6.48±2.29 ^{B}	6.53±1.91 ^{B}	<0.001
20:4n-6	19.32±4.45	17.84±3.64	19.23±3.36	19.52±3.50	n.s.
AA/DHA	2.32±0.70 ^{B}	2.11±0.62 ^{B}	3.24±0.97 ^{A}	3.19±0.95 ^{A}	<0.001
Erythrocyte PC (n=113)					
22:6n-3	3.59±1.69 ^{A}	3.89±1.44 ^{A}	2.29±1.18 ^{B}	2.42±1.15 ^{B}	<0.001
20:4n-6	7.15±2.49	7.73±2.38	7.31±2.69	7.66±2.69	n.s.
AA/DHA	2.24±0.75 ^{B}	2.15±0.75 ^{B}	3.74±1.41 ^{A}	3.83±1.93 ^{A}	<0.001
Newborn					
Plasma (n=146)					
22:6n-3	7.76±1.73 ^{A}	6.99±1.91 ^{AB}	6.20±1.60 ^{B}	6.89±1.63 ^{AB}	0.005
20:4n-6	16.79±1.94	16.40±2.02	17.30±1.67	17.56±1.74	0.042
AA/DHA	2.26±0.52 ^{B}	2.50±0.69 ^{AB}	3.00±0.91 ^{A}	2.70±0.76 ^{A}	0.001
Erythrocyte PE (n=101)					
22:6n-3	8.70±2.58 ^{AB}	9.72±2.89 ^{A}	7.45±1.89 ^{B}	7.47±1.86 ^{B}	0.002
20:4n-6	24.66±6.59	24.86±4.41	23.61±3.97	25.15±6.65	n.s.
AA/DHA	2.97±0.75 ^{AB}	2.79±0.93 ^{B}	3.33±0.85 ^{AB}	3.46±0.85 ^{A}	0.023
Erythrocyte PC (n=98)					
22:6n-3	4.46±1.50 ^{A}	3.87±1.34 ^{AB}	2.57±1.27 ^{C}	2.96±1.28 ^{BC}	<0.001
20:4n-6	12.87±3.02	12.72±2.76	11.42±3.78	13.09±4.13	n.s.
AA/DHA	3.13±0.93 ^{B}	3.52±0.88 ^{B}	4.98±1.65 ^{A}	4.83±1.46 ^{A}	<0.001

A, B, C: Differences between groups, values with different letters are significantly different ($P<0.05$), n.s. ($P>0.05$). FO (fish oil); 5-MTHF (5-methyltetrahydrofolate); PE (Phosphatidylethanolamin); PC (phosphatidylcholine). AA/DHA (arachidonic acid/docosahexaenoic acid).

Table 3. Neurological examinations at 4 and 5½ years of age in the four intervention groups.

	FO/FO+5-MTHF	**Placebo/5-MTHF**	***P***
HEMPEL	(n=80)	(n=87)	
Clinical conclusion			0.898
Normal	74 (92.58%)	81 (93.1%)	
Simple MND	5 (6.2%)	4 (4.6%)	
Complex MND	1 (1.2%)	2 (2.3%)	
NOS	51 (6)	51 (5)	0.661
Fluency score	15 (0)	15 (0)	0.187
TOUWEN	(n=69)	(n=79)	
Clinical conclusion			0.903
Normal	48 (69.6%)	55 (69.9%)	
Simple MND	20 (29%)	22 (27.8%)	
Complex MND	1 (1.4%)	2 (2.5%)	
NOS	59 (6)	59 (7)	0.874

Results are expressed as n (%) or mean (interquartile range).
FO (Fish oil). 5-MTHF (5-Methyltetrahydrofolate). MND (minor neurological dysfunction). NOS (Neurological optimality score).

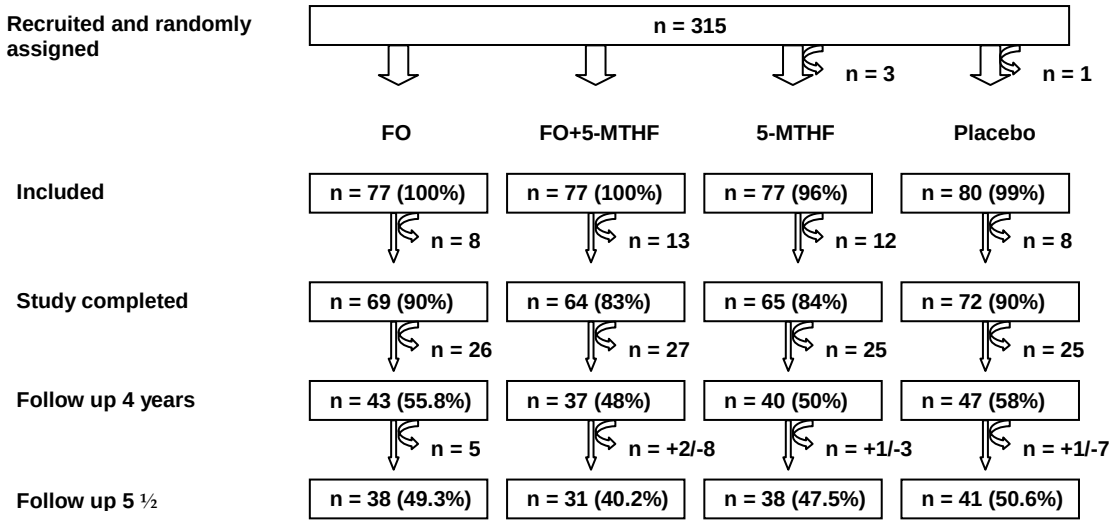
Table 4. Results of logistic regression analyses of the contribution of docosahexaenoic acid (DHA) status to the occurrence of optimality at 5 ½ years of age corrected for potential confounders (residence area, maternal age, risk factors during pregnancy, risk factors at delivery, perinatal morbidity, length of gestation, maternal status at work, parental educational level and center).

	Exp (B) (95% CI)*	***P***	**% correct classification**	**Naegelkerker R Square**
Cord DHA in plasma PLs	1.094-2.262	0.014	89.8%	14.5%
Maternal age				
Cord DHA in erythrocyte PE	1.091-2.417	0.017	94.2%	19%
Cord DHA in erythrocyte PC	1.003-2.643	0.049	91.7%	10.8%
Maternal DHA in erythrocyte PE at delivery	1.235-2.603	0.002	92.4%	38.1%
Maternal age				
Maternal DHA in erythrocyte PC at delivery	1.445-4.664	0.001	92.8%	37.1%
Maternal age				

*A standardized coefficient >1 means higher risk of the occurrence of optimality and <1 means lower risk of the occurrence of optimality.

CI: confidence interval; PLs: phospholipids; PE: phosphatidylethanolamine, PC: phosphatidylcholine.

Fig. 1. Flow of participants



FO (fish oil); 5-MTHE (5-methyltetrahydrofolate); (E) (Excluded); (W) (Withdrawn).

Appendix 1. Daily dietary intake of energy and nutrients in mothers of children evaluated at 4 years of age obtained by means of a Food Frequency Questionaire in the 20th and the 30th week of pregnancy (excluding supplements).

	FO	**FO+5-MTHF**	**5-MTHF**	**Placebo**	***P***
20th week pregnancy					
Energy (kcal)	2997±628.8	2739±940.5	3034±1335.1	2615±910.8	n.s.
Protein (g)	116±26.0	104±37.2	110±42.6	100±33.5	n.s.
Fat (g)	165±46.4	150±54.0	170±87.2	142.±59.8	n.s.
Carbohydrate (g)	256±64.8	236±102.5	259±111.5	228±86.8	n.s.
ΣSFA (g)	48.7±13.7	45.8±15.5	51.2±27.3	40.6±16.5	n.s.
ΣMUFA (g)	72.2±23.0	64.7±27.3	73.9±40.6	63.1±29.3	n.s.
ΣPUFA (g)	34.0±14.8	31.0±16.9	34.8±21.9	29.7±15.1	n.s.
22:6n3 (g)	0.40±0.14	0.39±0.23	0.37±0.18	0.37±0.32	n.s.
20:4n6 (g)	0.89±0.31	0.83±0.45	0.78±0.41	0.75±0.38	n.s.
Folate (μg)	174±68.1	158±74.4	182±109.4	157±60.2	n.s.
AA/DHA	2.61±1.45	2.31±1.27	2.28±1.10	2.44±1.35	n.s.
30th week pregnancy					
Energy (kcal)	2972±1077.8	2868±1101.0	2680±1075.7	2742±1001.9	n.s.
Protein (g)	107±36.4	108±51.1	101±34.0	101±33.7	n.s.
Fat (g)	167±63.2	163±65.6	151±73.0	150±62.1	n.s.
Carbohydrate (g)	255±106.4	237±100.6	226±89.4	241±95.8	n.s.
ΣSFA (g)	51.3±23.0	46.39±18.70	47.14±23.28	45.50±20.36	n.s.
ΣMUFA (g)	71.0±27.8	70.71±32.90	63.15±33.46	63.31±27.51	n.s.
ΣPUFA (g)	34.7±17.8	35.69±16.43	30.92±16.48	32.34±15.38	n.s.
22:6n3 (g)	0.37±0.13	0.42±0.22	0.37±0.20	0.46±0.50	n.s.
20:4n6 (g)	0.81±0.36	0.80±0.37	0.73±0.36	0.83±0.46	n.s.
Folate (μg)	162±86.1	173±95.4	147±64.8	148±62.0	n.s.
AA/DHA	2.20±0.84	2.02±0.69	2.46±2.48	2.19±1.32	n.s.

n.s. (P>0.05), FO (fish oil), 5-MTHF (5-Methyltetrahidrofolate), ΣSFA (saturated fatty acids), ΣMUFA (monounsaturated fatty acids), ΣPUFA (polyunsaturated fatty acids), 22:6n3 (docosahexaenoic acid), 20:4n6 (arachidonic acid), AA/DHA (arachidonic acid/docosahexaenoic acid).

4.3 Publication 3:

Effects of fish oil supplementation to pregnant women on cognitive development of their children at 6 ½ years of age

Submited to: Am J Clin Nutr

EFFECTS OF FISH OIL SUPPLEMENTATION TO PREGNANT WOMEN ON COGNITIVE DEVELOPMENT OF THEIR CHILDREN AT 6 ½ YEARS OF AGE

Cristina Campoy[1], María V. Escolano-Margarit[1], Rosa Ramos[2], Györgyi Csábi[3], Jeannette Beyer[4], María C. Ramirez-Tortosa[5], Tamas Decsi[3], Berthold V. Koletzko[4].

[1]University of Granada, Department of Paediatrics, Granada, Spain

[2]CIBER de Epidemiología y Salud Pública (CIBERESP), Spain, Laboratory of Medical Investigations, Clinical University Hospital San Cecilio, Granada, Spain.

[3]Dr. von Haunersches Kinderspital, Ludwig Maximilians-University of Munich, Munich, Germany.

[4]University of Pécs, Department of Pediatrics, Pécs, Hungary.

[5]University of Granada, Department of Biochemistry and molecular Biology, Granada Spain

Corresponding author: Cristina Campoy, Prof., MD,
Department of Pediatrics, School of Medicine
University of Granada
Avda. de Madrid, 11
18012-Granada, Spain
Tel.: +34629308695
Fax.:+34958240740
e-mail: ccampoy@ugr.es /ccfolgoso@gmail.com

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Running head:

Fish oil supplementation & child neurodevelopment

Abstract

Background: Long chain polyunsaturated fatty acids (LC-PUFA) have important structural and functional roles in the human brain. The main LC-PUFA accretion in the brain takes place in the last trimester of pregnancy and continues during the first year of life.

Objective: To assess the long term effects of n-3 LC-PUFA supplementation to pregnant women on cognitive development at 6 ½ years of age.

Design: Healthy pregnant women were recruited in three different European centers and randomly assigned to 4 different intervention groups. They received from the 20th week of pregnancy until delivery a daily supplement of either 500 mg docosahexaenoic acid (DHA) + 150 mg eicosapentaenoic acid (EPA), 400 μg 5-methyltetrahydrofolate, both or placebo. Fatty acids were determined in maternal plasma and erythrocyte membrane phospholipids (PLs) at the 20th and the 30th week of pregnancy and at delivery, as well as in cord blood. Cognitive function was assessed at 6 ½ years of age with the Kaufman Assessment Battery for Children (K-ABC).

Results: We observed no significant differences in the scores of the K-ABC between intervention groups. Higher DHA in maternal erythrocyte at delivery was associated with a Mental Processing Composite over the 50th percentile in their children.

Conclusions: Although we observed no significant effect of n-3 LC-PUFA supplementation during the second half of pregnancy on cognitive function, this study suggests that maternal DHA status is related to later cognitive function in children. We speculate that DHA availability prior to the 20th week of gestation may also be of importance.

Introduction

Over the past decades increasing concern has been given to the need of an adequate supply of long chain polyunsaturated fatty acids (LC-PUFA), especially docosahexaenoic acid (DHA), to the developing fetus and the infant. The developing fetus requires fatty acids as a source of energy as well as for the conformation of membranes in which they play an important role in maintaining their physical and chemical properties (1;2). Approximately 50-60% of human brain dry weight is constituted by lipids, about 35% of the lipids are PUFA and most of them are LC-PUFA (3). The growth spurt of the human brain and hence, rapid DHA accretion, takes place from the beginning of the third trimester of gestation to the 18th month of postnatal age (4). Before birth, DHA is mainly transported to the fetus from the mother across the placenta and after birth, the infant is provided with DHA in mother's milk (2).

Studies on postnatal LC-PUFA supplementation could not establish an unequivocal beneficial effect of LC-PUFA supplemented formulas on neurodevelopment in both, term and preterm infants (5;6). Observational studies have associated higher maternal fish consumption during pregnancy, as well as higher DHA concentrations in cord blood at birth, with a better neurological outcome (7-14), but very few of them have followed children development later in life. Interventional studies on prenatal supplementation to expecting women have conflicting results. N-3 LC-PUFA supplementation during pregnancy has been related to a better performance on a problem solving task at 9 months of age (15), a better eye and hand coordination in the Griffiths Mental Development Scales (GMDS) at 2 ½ years of age (16) and higher Mental Processing Composite score in the Kaufman Assessment Battery for Children (K-ABC) at 4 (17). Some other interventional studies did not show any association between child's neurodevelopment and supplementation (18-20). These inconclusive results could be

attributable to differences in the dosage and timing of supplementation, differences in the control formula and differences in the methodology to assess neurological condition in the children. To our knowledge, there is only one study assessing the long term effects of supplementation (17;20).

Umhau et al reported that folate deficiency modifies plasma and tissue fatty acid composition (21). The effect of 5-methyltetrahidrofolate (5-MTHF) on fatty acids might be attributable an increased methionine availability present after 5-MTHF supplementation, which modifies fatty acid metabolism (22). In addition, 5-MTHF has been demonstrated to lower homocysteine concentrations in plasma. Moderately elevated plasma homocysteine concentrations have been linked to vascular damage (23;24), thus reduction of plasma homocysteine concentrations during pregnancy might improve placental vascularisation and hence the efficacy of materno-fetal substrate transfers.

The present study approaches the effects of n-3 LC-PUFA and/or 5-MTHF supplementation to pregnant women during the second half of pregnancy and to the infants in the first 6 months of life on long term neurological condition of the children at 6 ½ years of age.

Subjects and Methods

Study design

The study design has been previously described (25). Briefly, healthy pregnant women attending antenatal care clinics were recruited before the 20th week of pregnancy in three different European centres (Ludwig Maximilians University of Munich, the University of Granada and the University of Pécs). Inclusion criteria were apparently healthy pregnant women aged 18 to 40 years with uncomplicated

singleton pregnancies and a body weight between 50 and 92 kg at study entry. Women should not have used fish oil supplements since the beginning of pregnancy or folate and/or vitamin B12 supplements after the 16th week of gestation. Blockwise randomization was performed by using stratification by centre for the allocation of participating women in 4 different supplementation groups. Women received from the 20th week of pregnancy until delivery one sachet per day with a milk-based supplement (Blemil Plus Matter; Ordesa Laboratories S.L., Barcelona, Spain) containing either modified fish oil (FO) providing 500 mg DHA+150 mg EPA (Pronova Biocare, Lysaker, Norway), 400 μg 5-MTHF (BASF, Ludwigshafen, Germany), both or placebo together with vitamins and minerals in amounts meeting the European recommendations for pregnant women in the second half of pregnancy. Neither participating women nor the study personal knew the content of the sachets. Detailed information on socio-demographic data were collected at study entry in standardized reports. Information on the course of pregnancy and delivery was also collected at the 20th and 30th week of pregnancy and at delivery. Food frequency questionnaires were performed at the 20th and 30th week of pregnancy. Maternal blood samples were obtained by venipuncture into EDTA containing tubes at the 20th and 30th week of pregnancy as well as at delivery. Cord blood samples were obtained at delivery after clamping of the umbilical cord and before parturition of placenta by puncture and aspiration from a long cord segment. After birth women were encouraged to breastfeed their infants. Infants requiring supplementation or substitution of breast feeding were provided with two different formulas. These two formulas followed the European legislative standards and were identical in all components with the exception of the fatty acid composition. Children born from FO supplemented women received a formula containing 0.5% of total fatty acids as DHA and 0.4% of arachidonic acid (AA) while children in the placebo or 5-MTHF groups received a formula virtually free of DHA and AA.

Cognitive assessment of the children was performed with the K-ABC at the age of 6 ½ years. Potential cofounders were assessed by means of standardized questionnaires containing information on children's health status.

The study protocol was approved by the Medical Ethics Committees of all centres participating in the study. Written informed consent was obtained from all participants at study entry and at follow-up.

Study population

The study described is part of the NUHEAL (Nutraceuticals for a Healthier Life) follow-up project that has been previously described (25). 270 women took part in the study until giving birth, baseline characteristics of the initial study population have been previously reported (25). Study participants where again approached and asked to participate in the neurological assessment of their children at 6 ½ years of age and 161 complied with the request. There were no differences in the dropout rates between intervention groups (P=0.554). Main reasons for dropping out were relocation (n=3), loss of contact (n=74) and unwillingness to continue (n=30). 4 of these children were born prematurely before the 35th week of pregnancy, one of them was born with a congenital left side anophthalmus, one child developed craniosynostosis and another was reported to have left side deafness. All these children were excluded from the analyses which left 154 children. No other illness or disability interfering normal neurological development was observed. There were no differences concerning baseline characteristics between followed children from the different intervention groups (**Table 1**). Mean dietary intake of DHA of the participating women was similar in all intervention groups at the 20th and the 30th week of pregnancy. The study population did not differ from the not tested population concerning maternal parity, BMI, smoking habits, obstetrical risk factors, complications at parturition or perinatal morbidity in any intervention group. There

was a higher attrition of children whose fathers had a high educational level in the placebo and FO+5-MTHF groups (76.9% of children with fathers with general qualification for university entrance in the placebo group and 70.8% in the FO+5MTHF withdrew at 6 ½ years follow up, while only 40% of the fathers of followed children in the placebo group and 30.6% in the FO+5-MTHF group had this qualification, *P*=0.003). Length of gestation in the 5-MTHF and placebo groups was significantly higher in the group of children continuing in the study than in the group of dropouts (5-MTHF: 38.71±1.6 vs 37.63±2.6 weeks, *P*=0.041; placebo: 39.13±1.5 vs 38.12±2.1, *P*=0.024). Birth length in the 5-MTHF group was also higher in the study population taking part in the follow-up (49.22±3.5 vs 51.10±1.8, *P*=0.011). Head circumference at birth in both, placebo and 5-MTHF groups, were significantly higher in the groups of children participating in the follow-up (placebo: 33.75±2.0 vs 34.85±1.3, *P*=0.021; 5-MTHF: 33.48±2.9 vs 34.74±1.4, *P*=0.041). There were no differences between tested and not tested children with respect to paternal educational level, length of gestation or length and head circumference at birth in the rest of intervention groups. Mean age at K-ABC assessment was 79.16±12.68 months.

Fatty acid analyses

Blood was centrifuged at 3,500 g for 10 min at room temperature within 2 h. Plasma was thereafter removed and stored at – 80ºC. The remaining erythrocyte mass was washed in isotonic sodium chloride solution, haemolysed in distilled water and kept at – 80ºC with isopropylic alcohol and butylated hydroxytoluene until further analysis. Lipids extraction from plasma was performed by vortexing 0.5 ml plasma plus 0.5 ml water and 100 µl of internal standard (0.857 g/L diheptadecanoyl phosphatidylcholine dissolved in chloroform). Lipids were extracted initially with n-hexane/2-propanolol (3:2, by vol) containing 25 mg/L of butylated hydroxytoluene as antioxidant, then three times with pure hexane. (26). The pooled extracts were dried under vacuum and dissolved in 200 µl hexane:methyl-tert-butyl-ether:acetic acid

(100:3:0.3, by vol). PLs were isolated by liquid chromatography with the use of aminopropyl columns (Sep Pak Cartridges; Waters, Milford, MA) (27). Lipids from erythrocyte were extracted by adding 3 ml chloroform and the two internal standards (phosphatidylethanolamine-heptadecanoate and phosphatidylcholine-pentadecanoate esters dissolved in methanol). The mixture was shaken on vortex and the lower layer was aspirated and evaporated under nitrogen stream. The dry lipid extract was resolved in chloroform and added to Silica gel plates (Merck 60, 10 x 20 cm). The runner solvent for the first run was hexane: diethylether: chloroform: acetic acid (21:6:3:1, volume/volume). The plate was dried under hood at ambient temperature and ran again with chloroform:methanol:water (65:25:4, volume/volume) (28). For positioning the PC and PE ester bands, the proper oleates were ran in parallel in every plate. The bands were stained with dichlorofluorescein, visualised under ultraviolet light and scrapped for transmethylation. The isolated PLs were transesterifed by treatment with methanolic hydrochloric acid (29).

Analysis of fatty acid methyl esters from plasma PLs was performed by gas chromatography using a gas chromatograph (HP5890 Series II; Hewlett Packard, Palo Alto, CA, USA) with a flame ionization detector. Chromatography was performed using a 60 m long capillary column with 0.32 mm internal diameter and 0.20 μm thickness and impregnated with SP-2330 FS (Supelco; Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250 and 275ºC respectively; nitrogen was used as carrier gas, and the split ratio was 29:1. Fatty acid methyl esters were identified by comparison of retention times with those of known standards. The quantification of fatty acid methyl esters from erythrocyte PE and PC was performed by high-resolution capillary gas-liquid chromatography (model 9001 gas chromatography; Finnigan/Tremetrics, Austin, TX) with split injection, automatic sampler (A200SE, CTC Analytic, Switzerland) and flame ionisation detector with a DB-23 cyanopropyl column of 60 m length (J & W Scientific, Folsom, CA). Conditions during the analysis were as follows: temperature of injector at 80ºC for 0.1 min,

temperature increase by 180°C per min up to 280°C, temperature of column area at 60°C for 0.2 min, temperature increase by 40°C min up to 180°C, a 5-min hold period, temperature increase by 1.5°C per min up to 200°C, an 8.5-min hold period, temperature increase by 40°C per min up to 240°C and a 13-min hold period. The constant linear velocity was 0.3 m/s referred to 100 °C. For identification of sample peaks we used two commercially available fatty acid methylester calibration mixtures (Supelco 37 FAME mix and NU-CKECK GLC reference 463) containing the fatty acids measured in the present study. Results were expressed as percentages by weight (wt %) of total detected fatty acids.

Assessment of cognitive function (K-ABC)

The K-ABC test is designed for children between 2.5 and 12.5 years of age to evaluate intelligence and achievement. The test comprises 16 subtests: 10 mental processing subtests and 6 achievement subtests, although not all of them are used for every age group. Neurological findings are summarized with three scores: Sequential Processing Scale, Simultaneous Processing Scale and Achievement Scale (not used in the present study). The Sequential Processing Scale primarily measures short-term memory and consists of subtests evaluating children's ability to solve problems that require the arrangement of stimuli in sequential order. The Simultaneous Processing Scale examines problem-solving skills that require processing of many stimuli at once. The Sequential and Simultaneous Processing Scales are combined to form the Mental Processing Composite (MPC), which is a global measure of the child's cognitive ability and is considered equivalent to the intelligence quotient. Raw scores are transformed into standard scores with a mean of 100 and a standard deviation of 15 and percentile scores (30).

Statistics

The power calculation showed that the size of the remaining group allowed for a detection of 6.85 (0.7 SD) points of difference in the standard MPC score obtained with the K-ABC at 6 ½ years follow up with a *P* value of 0.05 and a statistical power of 80%.

Outcome variables were assessed for normality. Differences among intervention groups for numeric variables were assessed with the analyses of variance in the normally distributed variables and Kruskal-Wallis test in the not normally distributed variables. For categorical variables Chi square tests were applied. In case of significance, multiple comparisons with Bonferroni corrections were performed.

Maternal age, parity, body mass index, haematocrit at the 30th week of pregnancy and smoking habit during pregnancy, as well as length of gestation, gravidity risk factors, delivery complications and parental educational attainment and work status were taken into account in the statistical analyses. Infant weight, length and head circumference at birth, Apgar score and perinatal morbidity, sex and breast feeding, as well as BMI and health status of the children at 6 ½, were also included. Due to the high number of control variables only those related to the outcome variable at *P*-values <0.2 were entered as covariables in the analysis.

Spearman correlation coefficients were calculated between the scores of the K-ABC and the fatty acid percentages in cord and maternal plasma and erythrocyte PLs. Stepwise multiple lineal regression analyses were performed for the adjustment of confounders with the standardized scores of the K-ABC (MPC, Sequential Processing Scale and the Simultaneous Processing Scale) as dependent variables and

each of the LC-PUFA separately as independent variables. All possible confounders were included in the model as covariables.

The percentile scores could not be transformed to normality and were dichotomized. Stepwise logistic regressions were performed on the dichotomized scores (over and under the 50th percentile) as dependent variable and LC-PUFA percentage concentrations together with confounders as independent variables.

A *P*-value of 0.05 or less was considered statistically significant. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL).

Results

LC-PUFA percentage concentrations (wt %) in cord and maternal blood

Mean LC-PUFA relative concentrations (wt %) in maternal and cord blood are shown in **Table 2**. Mothers receiving FO supplements during the second half of pregnancy had significantly higher DHA percentages in plasma and erythrocyte PLs at delivery than those receiving 5-MTHF supplements or placebo ($P<0.01$). Likewise, children born from supplemented mothers had significantly higher DHA percentages in cord plasma and erythrocyte PLs compared to those born from not FO supplemented women ($P<0.01$). AA levels in plasma and erythrocyte PLs were similar between intervention groups. There were no significant differences regarding DHA content in maternal and cord plasma and erythrocyte PLs between the FO and FO+5-MTHF groups as well as between the placebo and 5-MTHF groups. Thus, 5-MTHF supplementation does not seem to influence DHA percentage concentrations in maternal and fetal plasma or erythrocyte PLs.

AA/DHA ratio in maternal plasma PLs in the placebo group at the 20th and 30th week of pregnancy, as well as at delivery and in cord blood was significantly lower in the children taking part in the study than in those who withdrew (20th week: 2.71±0.71 vs 2.12±0.59, *P*=0.001; 30th week: 2.69±0.71 vs 2.12±0.56, *P*=0.001; delivery: 2.75±0.67 vs 2.20±0.61, *P*=0.002; cord blood: 3.34±0.78 vs 2.64±0.64, *P*=0.001).

Cognitive function

There were no statistical differences in the K-ABC scores at 6 ½ years of age between the 4 intervention groups (**Table 3**). The groups FO and FO+5-MTHF and the placebo and 5-MTHF groups were similar with respect to their basal clinical and socio-demographic characteristics and LC-PUFA (wt %) in cord blood. We pooled together the groups with and without FO supply to increase the power of the study for detecting differences, but we found no statistically significant differences.

We did not find significant bivariate correlations between intelligence scores and fatty acid percentages in umbilical plasma or erythrocyte PLs. There were significant bivariate correlations between the MPC score at 6 ½ years of age and DHA percentage concentrations in maternal erythrocyte PE at the 20th and 30th week of pregnancy (20th week: r=0.207, *P*=0.021; 30th week: r=0.182, *P*=0.039). The AA/DHA ratio in maternal erythrocyte PE at the 20th week of pregnancy was also significantly correlated with the MPC (r= -0.262, *P*=0.003). However, stepwise multiple regression analyses with the MPC as dependent variable and maternal fatty acid relative concentrations in all pregnancy time points as independent variables together with confounding factors as covariables did not show any association between maternal LC-PUFA in blood and intelligence scores at 6 ½ years of age.

We observed that children with MPC scores over 50th percentile had mothers with significantly higher DHA percentage concentrations in erythrocyte PE (20th

week: 4.09±1.85 vs 5.16±1.98; *P*=0.035, 30th week: 5.06±1.98 vs 6.42±2.25; *P*=0.014, Delivery: 5.95±1.68 vs 7.85±2.58; *P*<0.001) and PC (30th week: 1.52±1.09 vs 2.85±1.52; *P*=0.001, Delivery: 2.09±0.92 vs 3.23±1.58; *P*<0.001) during the course of pregnancy. There were no statistical differences concerning cord blood fatty acid relative concentrations.

Adjustment for confounders in the stepwise logistic regression (**Table 4**) showed that children whose mothers had higher DHA percentages in erythrocyte PE at delivery were more likely to have a MPC score over the 50th percentile. Likewise higher AA/DHA ratio in maternal erythrocyte PE at delivery was associated with a Mental Processing Composite scores under the 50th percentile.

Discussion

Almost all studies conducted to date investigating the effects of prenatal n-3 LC-PUFA supplementation on neurological condition have assessed neurological development early in life. The evidence for benefits of LC-PUFA supplementation in healthy children at older ages is too limited to allow unequivocal conclusions. To our knowledge, only one of these studies has followed children over the age of 4 (17;20). The current study approaches this relationship at 6 ½ years of age.

Helland et al. reported higher scores on the MPC of the K-ABC at 4 years of age in children born from mothers receiving cod oil supplements during the second half of pregnancy and three months after delivery compared to those whose mothers received corn oil supplements (17). The fact that we did not find differences could be explained by the differences between studies regarding n-3 LC-PUFA supplementation dosage and timing. We supplemented our women with 500 mg DHA daily from the 20th week of pregnancy until delivery while Helland et al. provided their study population with a far higher dose of 1,183 mg DHA per day

from the 18th week of pregnancy until the 3rd month after delivery. DHA concentrations in cord plasma PLs in the FO supplemented group reported in the Norwegian study (63.7±26.8 μg/ml) were higher than ours (54.9±1.8 μg/ml mean DHA in the FO and FO+5-MTHF). Furthermore, while Helland and co-workers report a mean difference of 21.3 μg/ml between the cod liver oil and the corn oil supplemented groups, we found a difference of only 6.5±2.5 μg/ml between the FO supplemented groups and those who received 5-MTHF or placebo. It appears possible that higher n-3 LC- PUFA doses are needed to have a measurable impact on cognitive development. Although the dose of n-3 LC-PUFA administered in the current study was high enough to induce a significant increment of DHA percentage levels in maternal and cord plasma and erythrocyte PLs, the DHA percentage concentrations in cord blood were within the range of normal variability for healthy term infants. Nevertheless, in spite of the higher DHA concentrations in supplemented groups reported by Helland et al., they observed no differences in the scores of the K-ABC between supplementation groups at the age of 7 (20).

Most of the studies detecting significant effects of supplementation on neurological outcome have been conducted earlier in life. The lack of differences observed in our study could also be due to differences in the tests used to assess neurological development. Due to the difficulties in the assessment of the cognitive function during infancy most of the tests used in the first stages of life focus on the assessment of the psychomotor function. The K-ABC is a measure of intelligence and hence of cognitive function. Bakker and Ghys (31;32) did not establish a significant association between LC-PUFA in umbilical plasma PLs at delivery and the cognitive performance in the K-ABC at 4 and 7 years of age in their observational study. However, they found a positive relation between umbilical plasma DHA and the quality of movements of the Maastricht Motor Test at 7 years in the same study population (33). LC-PUFA might exert effects on motor function, and thereby the K-ABC may not be suitable to detect such effects. Another possible explanation for our

results is that that the effects of supplementation are transient and can be detected only in younger children. Neurological development is influenced by many factors, such us genetics, social stimulation, diseases or nutrition in the first years of life. Thus, the subtle beneficial effects of LC-PUFA supplementation may be overshadowed by factors intervening between infancy and school age that may influence neurological outcome.

Given the high attrition in our study we were able to detect a difference of 4.5 points in the MPC with the remaining sample size at 6 ½ years follow up after pooling up together FO and the not FO supplemented groups. This is slightly over the 4.1 points of difference that Helland and co-worker could detect in their study. We consider the power of the study high enough to detect clinically relevant differences between groups. Furthermore, we did not see a trend in our data. Thereby we do not think that a larger sample would have leaded us to find significant differences. The high attrition could also have induced bias that may have made it difficult to detect differences. Children born from fathers with high educational level were underrepresented in the placebo and FO+5-MTHF groups and those with higher length of gestations, head circumference and length at birth were overrepresented in the not FO supplemented groups (placebo and 5-MTHF groups). Both, parental educational level and birth anthropometry have been previously related to cognitive development (31). However, logistic regression analysis did not show any association between these covariables and intelligence sores in the present study.

The logistic regression analysis showed that high DHA (wt %) in maternal erythrocyte PE are related to MPC scores over the 50th percentile. Our results are in agreement with some other studies reporting a positive association between DHA and neurological development (7-13). The few of them providing information beyond infancy have controversial results. High maternal consumption of seafood

has been reported to be beneficial for child's neurodevelopment (14;34;35). Moreover, higher DHA in cord blood at birth have been associated with better eye-hand coordination in the Griffiths Mental Development Scales (GMDS) at 2 ½ years of age (16) and higher quality of movements in the Maastricht Motor Test (33) as well as lower internalising problem behaviour at 7 (36). Helland et al. also reported a significant correlation between intelligence scores of the K-ABC at 4 and DHA concentrations in child's plasma PLs 4 weeks after delivery, as well as between the intelligence scores at 7 and DHA concentration in maternal plasma PLs in the 35th week of pregnancy (17;20). In contrast, the previously mentioned observational studies did not establish any association between cord blood DHA and intelligence scores of the K-ABC neither at 4 not at 7 (31;32). The detected association between cognitive function with DHA in erythrocyte PLs but not with plasma PLs in our study could reflect a more beneficial effect of long term LC-PUFA intake rather than just receiving supplementation during pregnancy. Therefore, we speculate that maternal DHA status prior to the 20th week of gestation may be of importance for the child outcome.

Our results do not provide evidence for a beneficial effect of n-3 LC-PUFA supplementation during the second half of pregnancy and the first months of postnatal life on cognitive performance at 6 ½ years of age. Prenatal DHA status seems to have subtle positive effects on neurodevelopment, but further research is needed to assess whether these effects are maintained beyond early childhood. The optimal doses for efficacy at different developmental stages also require consideration.

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All authors certify that there is no actual or potential conflict of interest in relation to this article.

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Table 1. Baseline characteristics of children at 4 years of age after randomization in the 4 supplementation groups.

	FO (n=37)	FO+5-MTHF (n=37)	5-MTHF (n=35)	Placebo (n=45)	*P*
Center n (%)					n.s.
Spain	29 (78.4%)	27 (73%)	22 (62.9%)	31 (68.9%)	
Germany	7 (18.9%)	8 (21.6%)	9 (25.7%)	11 (24.4%)	
Hungary	1 (2.7%)	2 (5.4%)	4 (11.4%)	3 (6.7%)	
Maternal age	30 (6.25)	30 (6.5)	32 (7)	32 (4)	n.s.
BMI 20th WG	26.0±3.6	24.9±2.4	25.0±3.4	24.9±2.5	n.s.
BMI 30th WG	28.5±4.4	26.8±2.4	27.2±3.7	27.0±2.7	n.s.
Haematocrit 30th WG	34.4±3.6	34.3±2.6	33.8±5.1	33.7±2.4	n.s.
Parity					n.s.
0	32 (94.1%)	29 (87.9%)	29 (82.9%)	40 (90.9%)	
≥1	2 (5.9%)	4 (12.1%)	6 (17.1%)	4 (9.1%)	
Smoking in pregnancy	7 (18.9%)	7 (18.9%)	6 (17.1%)	4 (8.9%)	n.s.
Gravidity Risk (20 w)					n.s.
No risk factors	10 (29.4%)	9 (27.3%)	10 (28.6%)	17 (38.6%)	
≥1 risk factors	24 (70.6%)	24 (72.7%)	25 (71.4%)	27 (61.4%)	
Delivery risk					n.s.
No risk factors	13 (38.2%)	16 (48.5%)	20 (57.1%)	29 (65.9%)	
≥1 risk factors	21 (61.8%)	17 (51.5%)	15 (34.1%)	15 (42.9%)	
Weeks of gestation	39 (2)	39 (2.5)	39 (2)	39 (2)	n.s.
Sex					n.s.
Female	19 (51.4%)	20 (54.1%)	19 (54.3%)	16 (35.6%)	
Male	18 (48.6%)	17 (45.9%)	16 (45.7%)	29 (64.4%)	
Perinatal morbidity					n.s.
None	32 (86.5%)	31 (83.8%)	31 (88.6%)	38 (84.4%)	
Preterm (>35 w)	3 (8.1%)	5 (13.5%)	4 (11.4%)	3 (6.7%)	
Others	2 (5.4%)	1 (2.7%)	0 (0%)	4 (8.9%)	
Apgar 1 min	9 (1)	9 (0)	9 (0)	9 (0)	n.s.
Apgar 5 min	10 (0)	10 (0)	10 (0)	10 (0)	n.s.
Birth weight	3372.6±453.7	3284.8±473.2	3420.0±347.3	3369.7±379.2	n.s.
Birth length	51.4±2.4	50.9±2.9	51.3±1.7	51.2±2.2	n.s.
Birth head circumference	34.7±1.4	34.6±1.7	35.0±1.4	34.8±1.3	n.s.
Infant feeding					n.s.
Breastfed	19 (57.6%)	16 (53.3%)	15 (46.9%)	23 (57.5%)	
Mixed	4 (12.1%)	6 (20.0%)	6 (18.8%)	8 (20.0%)	
Formula	10 (30.3%)	8 (26.7%)	11 (34.4%)	9 (22.5%)	
Residence area (%)					n.s.
City area	14 (37.8%)	17 (45.9%)	16 (45.7%)	16 (45.7%)	
Farm area	23 (62.2%)	20 (54.1%)	19 (54.3%)	29 (64.4%)	
Maternal education [1]	14 (41.2%)	17 (53.1%)	17 (48.6%)	21 (47.7%)	n.s.
Paternal education [1]	15 (44.1%)	11 (34.4%)	16 (45.7%)	18 (40.9%)	n.s.
BMI 6 ½ years	17.2±2.9	16.7±2.3	16.4±1.9	16.8±2.3	n.s.
Age K-ABC (months)	81.7±3.8	81.6±3.3	80.6±2.5	80.3±3.5	n.s.

Results are expressed as mean±SD, median (interquartile range) or n (%) for continuous, non continuous and categorical variables respectively. n.s. (*P*>0.05), FO (Fish oil), 5-MTHF (5-Methyltetrahydrofolate), WG (weeks of gestation), BMI (Body Mass Index), w (week).

[1] n (%) general qualification for university entrance or university degree.

Table 2. Fatty acids (wt%) in umbilical plasma and erythrocyte phospholipids at birth and fatty acids (wt%) in maternal plasma and erythrocyte phospholipids at the 20th week of pregnancy, 30th week of pregnancy and at delivery in the groups of children evaluated at 4 (mean±SD).

	FO	FO+5-MTHF	5-MTHF	Placebo	*P*
20th week gestation					
Plasma (n=146)					
22:6n-3	4.71±1.05	4.82±1.03	4.51±0.96	4.97±1.28	n.s.
20:4n-6	10.45±1.73	10.29±1.49	9.73±1.97	9.96±1.47	n.s.
AA/DHA	2.31±0.59	2.22±0.53	2.26±0.67	2.12±0.59	n.s.
Erythrocyte PE (n=123)					
22:6n-3	5.00±2.11	5.07±1.99	4.80±1.96	4.99±2.00	n.s.
20:4n-6	19.19±5.72	17.85±4.85	16.65±5.06	18.50±4.95	n.s.
AA/DHA	4.28±1.44	3.78±0.98	3.87±1.31	4.09±1.25	n.s.
Erythrocyte PC (n=103)					
22:6n-3	1.87±0.95	2.42±1.29	2.10±1.20	2.23±1.26	n.s.
20:4n-6	6.57±3.18	7.27±2.60	6.13±2.67	6.91±2.65	n.s.
AA/DHA	4.02±1.86	3.61±1.53	3.52±1.52	3.72±1.67	n.s.
30th week gestation					
Plasma (n=147)					
22:6n-3	5.99±1.31 ^{A}	6.45±1.04 ^{A}	4.40±0.73 ^{B}	4.44±0.94 ^{B}	<0.001
20:4n-6	8.90±1.27	8.80±1.09	9.22±1.75	9.02±1.42	n.s.
AA/DHA	1.56±0.44 ^{B}	1.40±0.29 ^{B}	2.15±0.55 ^{A}	2.12±0.56 ^{A}	<0.001
Erythrocyte PE (n=128)					
22:6n-3	7.00±2.73 ^{A}	6.63±2.22^{AB}	5.27±1.72 ^{B}	5.85±1.89^{AB}	0.012
20:4n-6	18.64±4.30^{AB}	16.09±3.30 ^{B}	16.32±4.21^{B}	19.02±3.94 ^{A}	0.003
AA/DHA	3.00±1.19 ^{AB}	2.61±0.71 ^{B}	3.35±1.05 ^{A}	3.46±0.88 ^{A}	0.002
Erythrocyte PC (n=114)					
22:6n-3	2.82±1.57	3.00±1.71	2.16±1.32	2.55±1.42	n.s.
20:4n-6	6.61±2.60	6.46±2.91	6.59±3.36	7.66±2.98	n.s.
AA/DHA	2.75±0.96 ^{AB}	2.69±1.68 ^{B}	3.87±2.12^{A}	3.72±1.82^{A}	0.001
Delivery					
Plasma (n=145)					
22:6n-3	5.83±1.34 ^{A}	6.05±1.29 ^{A}	4.42±1.28 ^{B}	4.41±1.20 ^{B}	<0.001
20:4n-6	8.74±1.52	8.70±1.15	8.90±1.78	9.16±1.32	n.s.
AA/DHA	1.58±0.48 ^{B}	1.51±0.34 ^{B}	2.13±0.62 ^{A}	2.20±0.61 ^{A}	<0.001
Erythrocyte PE (n=114)					
22:6n-3	8.95±2.74 ^{A}	8.27±2.59 ^{A}	6.47±2.22 ^{B}	6.54±1.78 ^{B}	<0.001
20:4n-6	19.61±4.52	17.34±3.18	19.20±3.60	19.58±3.64	n.s.
AA/DHA	2.37±0.79 ^{B}	2.27±0.73 ^{B}	3.20±0.90 ^{A}	3.13±0.76 ^{A}	<0.001
Erythrocyte PC (n=103)					
22:6n-3	3.42±1.76^{AB}	3.77±1.71^{A}	2.56±1.08 ^{B}	2.41±1.07 ^{B}	0.002
20:4n-6	7.22±2.50	7.59±2.47	7.84±2.55	7.51±2.44	n.s.
AA/DHA	2.43±0.84 ^{B}	2.29±0.84 ^{B}	3.46±1.38 ^{A}	3.59±1.46 ^{A}	<0.001
Newborn					
Plasma (n=187)					
22:6n-3	7.86±1.81^{A}	7.44±1.59^{A}	6.33±1.57^{B}	6.99±1.57^{AB}	0.004
20:4n-6	16.79±1.67	16.55±1.71	17.21±1.77	17.47±1.61	n.s.
AA/DHA	2.23±0.51 ^{C}	2.35±0.65 ^{BC}	2.89±0.78 ^{A}	2.62±0.64^{AB}	<0.001
Erythrocyte PE (n=93)					
22:6n-3	8.56±2.67 ^{AB}	9.54±2.90 ^{A}	7.34±1.94 ^{B}	7.65±1.84 ^{B}	0.009
20:4n-6	25.34±6.55	24.43±3.09	23.11±3.80	25.66±6.70	n.s.
AA/DHA	3.14±0.90 ^{AB}	2.78±0.85 ^{B}	3.31±0.85^{AB}	3.44±0.84 ^{A}	0.025
Erythrocyte PC (n=87)					
22:6n-3	4.25±1.62^{A}	4.12±1.43^{AC}	2.51±1.28 ^{B}	3.02±1.13^{BC}	<0.001
20:4n-6	13.00±2.85	12.63±2.57	11.58±4.06	13.44±4.34	n.s.
AA/DHA	3.46±1.53 ^{A}	3.33±0.94 ^{A}	5.10±1.54 ^{B}	4.72±1.28 ^{B}	<0.001

A, B, C: Differences between groups, values with different letters are significantly different ($P<0.05$), n.s. ($P>0.05$). FO (fish oil); 5-MTHF (5-methyltetrahydrofolate); PE (Phosphatidylethanolamin); PC (phosphatidylcholine). AA/DHA (arachidonic acid/docosahexaenoic acid).

Table 3. Kaufman Assessment Battery for Children standardized scores at 6 ½ years of age in the 4 intervention groups.

	FO (n=37)	FO+5-MTHF (n=37)	5-MTHF (n=35)	Placebo (n=45)	*P*
Mental Processing Composite	110 (11)	108 (10.5)	108 (12)	110 (14.5)	0.821
Sequential Processing Scale	108 (12)	104 (17)	104 (14)	106 (19)	0.569
Simultaneous Processing Scale	112 (10.5)	110 (10.5)	109 (14)	112 (11.5)	0.884

FO (fish oil), 5-MTHF (5-methyltetrahydrofolate)

Table 4. Logistic regression analyses of the contribution of maternal DHA status to the occurrence of Mental Processing Composite Score over the 50th percentile at 6 ½ years of age corrected for potential confounders (parental cultural level, maternal status at work, length of gestation, perinatal morbidity, sex).

	Exp (B) (95% CI)*	*P*	% correct classification	Naegelkerker R Square
Erythrocyte PE DHA levels at delivery	1.094-2.449	0.017	90	0.429
Erythrocyte PE AA/DHA at delivery	0.130-0.821	0.017	92.2	0.390

*A standardized coefficient >1 means higher risk of being over the 50th percentile.
PE (phosphatidylethanolamine), DHA (docosahexaenoic acid), AA (arachidonic acid)

5 CONCLUSIONS

CONCLUSIONS

1. The daily fish oil (500 mg docosahexaenoic acid +150 mg icosapentaenoic acid) supplementation during the second half of pregnancy effectively increases docosahexaenoic acid levels in maternal plasma and umbilical plasma and erythrocyte phospholipids. It should be taken into account that supplementation with docosahexaenoic acid doses of 500 mg/day in European populations with mean docosahexaenoic acid intakes over the recommended 200 mg/day may lower AA levels in the fetus with possible consequences in the offspring.

2. Supplementation with 400 μg/day of 5-methyltetrahydrofolate has no effects on fetal docosahexaenoic acid supply.

3. Both, plasma and erythrocyte fatty acids, appear adequate to asses the fatty acid status in pregnant women and their neonates but the type of study is a major consideration when determining which body compartment reflects a better measure of the fatty acid status. Erythrocyte phospholipids seem to be a more reliable measure of long term fatty acid intake, whereas plasma phospholipids appear more sensitive to short-term changes in long chain polyunsaturated fatty acids intake and may be more suitable to monitor the fatty acid status of individuals in intervention studies

4. Fatty acid levels in erythrocyte phospholipids seem to be a more reliable biomarker compared to those in plasma phospholipids to predict fetal/neonatal fatty acid status on the basis of maternal blood fatty acid levels.

5. The present randomized multicentre trial showed no evidence for beneficial or harmful effects of fish oil (500 mg docosahexaenoic acid + 150 mg eicosapentaenoic acid) and/or 5-methyltetrahydrofolate supplementation during the second half of pregnancy and the first months of postnatal life on long term motor and cognitive development of children at 4, 5 ½ and 6 ½ years of age. The optimal doses for efficacy at different developmental stages require consideration.

6. Docosahexaenoic acid dietary intake in Europe is in general above the 200 mg of the daily recommended intake. It appears to be no additional benefit of higher DHA supply from supplements.

7. Higher maternal and fetal docosahexaenoic acid status during pregnancy appears to be related to better performance on neuromotor tests at 5 ½ and on cognitive examination at 6 ½ years of age. Thus, long chain polyunsaturated faty acids status prior to the 20th week of gestation might be relevant for children neurological development. Although further research is needed to asses whether these effects are maintained beyond early childhood, education programs related to nutrient intake in the population should, in our opinion, encourage the intake of docosahexaenoic acid rich nutrients during pregnancy.

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