The Journal of Nutrition
Nutrient Requirements and Optimal Nutrition

Prenatal DHA Status and Neurological Outcome in Children at Age 5.5 Years Are Positively Associated[1–4]

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Abstract

Beneficial effects of perinatal DHA supply on later neurological development have been reported. We assessed the effects of maternal DHA supplementation on the neurological development of their children. Healthy pregnant women from Spain, Germany, and Hungary were randomly assigned to a dietary supplement consisting of either fish oil (FO) (500 mg/d DHA + 150 mg/d EPA), 400 μg/d 5-methyltetrahydrofolate, both, or placebo from wk 20 of gestation until delivery. Fatty acids in plasma and erythrocyte phospholipids (PL) were determined in maternal blood at gestational wk 20 and 30 and in cord and maternal blood at delivery. Neurological development was assessed with the Hempel examination at the age of 4 y and the Touwen examination at 5.5 y. Minor neurological dysfunction, neurological optimality score (NOS), and fluency score did not differ between groups at either age, but the odds of children with the maximal NOS score increased with every unit increment in cord blood DHA level at delivery in plasma PL (95% CI: 1.094–2.262), erythrocyte phosphatidylethanolamine (95% CI: 1.091–2.417), and erythrocyte phosphatidylcholine (95% CI: 1.003–2.643). We conclude that higher DHA levels in cord blood may be related to a better neurological outcome at 5.5 y of age. J. Nutr. 141: 1216–1223, 2011.

Introduction

Long-chain PUFA (LC-PUFA)[12] such as DHA are essential constituents of the central nervous system and are incorporated into 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 on tests designed to measure cognitive and behavioral ability in animal studies (2,4,5). Benefits of LC-PUFA supply for visual and neurologic development in both term and preterm infants have been reported but not confirmed in all studies (6–8). A major part of the human main brain growth spurt and the related DHA incorporation into brain tissue occurs during the last trimester of pregnancy (1). Observational studies suggest that high prenatal DHA status also might have subtle positive effects on neurodevelopmental outcome beyond early infancy (9–13). In the last few years, research has centered on increasing the LC-PUFA supply to the fetus by supplementing maternal diets with (n-3) LC-PUFA. Randomized controlled trials have reported higher DHA levels in cord blood at birth of children born to supplemented women compared with 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. Whereas some studies report better performance on different neurological examinations by children whose mothers received supplements during pregnancy (17–19), others did not show such effects (20–23). Information on the long-term effects of supplementation is

[1] Supported by the Commission of the European Communities, RTD Programme "Quality of Life and Management of Living Resources," within the 5th Framework Programme (QLK1-CT-1999-00888 Nutraceuticals for a Healthier Life) and within the 6th Framework programme (priority 5.4.3.1. Food quality and safety. 007036-EARNEST Project. Early nutrition programming-long term follow up of efficacy and safety trials 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.
[2] Author disclosures: M. V. Escolano-Margarit, R. Ramos, J. Beyer, G. Csábi, M. Parrilla-Roure, F. Cruz, M. Pérez-García, M. Hadders-Algra, A. Gil, T. Decsi, B. V. Koletzko, and C. Campoy, no conflicts of interest.
[3] This trial was registered at clinicaltrials.gov as NCT01180933.
[4] Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
[12] Abbreviations used: AA, arachidonic acid; FO, fish oil; K-ABC, Kaufman Assessment Battery for Children; LC-PUFA, long-chain PUFA; MND, minor neurological dysfunction; 5-MTHF, 5-methyltetrahydrofolate; NOS, neurological optimality score; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; wt %, percentage by weight.
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Manuscript received July 23, 2010. Initial review completed September 16, 2010. Revision accepted March 17, 2011.
First published online April 27, 2011; doi:10.3945/jn.110.129635.

scarce. One trial examined neurologic development of children exposed to cod liver oil supplementation in early life after the age of 4 y (21).

Our 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 mo of postnatal life on the later neurologic development of children.

Participants and Methods

Study design. This study is part of a double-blind, randomized, controlled trial investigating the effects of prenatal and postnatal supplementation of (n-3) LC-PUFA and/or 5-methyltetrahydrofolate (5-MTHF) in healthy term infants. Details of the study design, recruitment of women, 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 wk 20 at 3 European centers (University of Munich Medical Centre, Germany, the University of Granada, Sapain, and the University of Pécs, Hungary). Women were randomly assigned to 4 different groups and received from wk 20 of pregnancy until delivery 1 sachet per day with 15 g of a milk-based supplement (Blemil Plus Matter; Ordesa Laboratories) containing modified fish oil (FO) providing 500 mg DHA and 150 mg EPA (Pronova Biocare), 400 μg 5-MTHF (BASF, Ludwigshafen, Germany), both, or placebo, 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 any unused sachets to the study center at wk 30 of gestation and at delivery. Detailed and standardized information on socio-demographic characteristics was collected at study entry. Dietary information was collected by FFQ both at wk 20 and 30 of gestation. Information on the course of pregnancy as well as information at delivery was obtained in standardized reports. Maternal venous blood samples (2 mL) were obtained at wk 20 and 30 of gestation, as well as maternal and umbilical cord blood samples at delivery, for fatty acid analyses in plasma phospholipids (PL) and in erythrocyte membrane phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

At birth, a trained physician performed a physical examination of the child and obtained information about the infants in standardized case report forms. Women were encouraged to breastfeed their infants. Infants who required supplements or substitution for breastfeeding were given infant formulas (Blemil Plus NF, Ordesa Laboratories) with a composition following European legislative standards until the infant was 6 mo of age. There were 2 formulas, both identical with the exception of the fatty acid composition. Children born to mothers in the FO and FO+5-MTHF groups received a formula containing 0.5% of total fatty acids as DHA and 0.4% as arachidonic acid (AA), whereas children in the placebo or 5-MTHF groups received a formula virtually free of DHA and AA. These 2 formulas were coded in the same way as the supplements for the mothers (1–4); thus, double-blinding was maintained.

At the ages of 4 and 5.5 y, the participating mothers and their 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 y the Hempel examination was used (25) and at 5.5 y, the Touwen assessment was applied (26). Potential confounders were assessed by using 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 5.5 y of age, as well as the fatty acid (DHA, AA, AA:DHA) levels in cord and maternal plasma and erythrocyte PL. Socio-demographic and clinical characteristics of the children and their parents, 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 follow-up of children at 4 y of age.

Fatty acid analyses. Blood was centrifuged at 3500 $\times$ *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 hemolyzed in distilled water. Plasma and erythrocytes were stored at −80°C until further analysis.

Lipids from erythrocyte membrane were extracted into chloroform/methanol and PL were isolated by TLC (27). The bands were stained with dichlorofluorescein, visualized under UV light, and scraped for transmethylation. Plasma lipids were extracted into chloroform/isopropanol according to the method of Kolarovic and Fournier (28) and the PL isolated by liquid chromatography on aminopropyl columns (Sep Pak Cartridges; Waters) (29).

The isolated PL were transesterified by reaction with methanolic hydrochloric acid (30). The quantification of FAME from erythrocyte was performed by high-resolution capillary GLC (model 9001 gas chromatography; Finnigan/Tremetrics) with split injection, automatic sampler (A200SE, CTC Analytic) and flame ionization detector with a DB-23 cyanopropyl column of 60 m length (J & W Scientific). Conditions during the analysis and standards used were recently described in detail elsewhere (31). For identification of sample peaks, we used 2 commercially available FAME calibration mixtures (Supelco 37 FAME mix and NU-CKECK GLC reference 463) containing the fatty acids measured in the present study. The analysis of FAME from plasma PL was performed by using GC (HP5890 Series II; Hewlett Packard) with a flame ionization detector with a 60-m long capillary column (0.32-mm diameter i.d. and 0.20-μm thickness) and impregnated with SP-2330 FS (Supelco; Bellefonte). Conditions during the analysis have been reported elsewhere (24). FAME 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 y of age, children were neurologically examined according to Hempel (25). The Hempel assessment is organized into 5 functional domains: fine motor function, gross motor function, posture and muscle tone, reflexes, and visuomotor behavior. The findings of the Hempel examination result in a clinical classification consisting of the following categories: *1*) 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; *2*) simple minor neurological dysfunction (MND) if only 1 domain is dysfunctional; *3*) complex MND if 2 or more domains show abnormal neuromotor signs; and *4*) 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 behavior.

Neurological assessment at 5.5 y of age was performed according to Touwen (26). The examination is organized into 8 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: *1*) neurologically normal, when none of the domains meet the criteria of deviancy or in case of the isolated presence of deviancy in the domain of reflexes; *2*) simple MND, when 1 or 2 domains are scored as dysfunctional; *3*) complex MND if 3 or more domains are deviant; or *4*) definitively abnormal neurological condition. Neurological condition can also be expressed in the form of NOS. The NOS of the Touwen assessment consists of 64 items with age-specific criteria for optimality (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 at the 4-y follow-up and 2.46 points of difference in the NOS at the 5.5-y 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.

A 3-factor repeated-measures ANOVA with the intervention group as between-subject factor and pregnancy time points (gestation wk 20, gestation wk 30, and delivery) as within-subject factors was performed to compare the effects of supplementation on DHA status. In case of significance, multiple comparisons with Bonferroni corrections were performed. Regarding baseline characteristics of participants, differences between intervention groups for numeric variables were assessed by ANOVA for the normally distributed variables and the Kruskal-Wallis test for the non-normally distributed variables. In case of significance, multiple comparisons with Bonferroni corrections were performed. For categorical variables chi-square tests were applied.

Univariate analyses of the differences in fatty acids levels between children with and without MND (simple and complex MND pooled) and children with an optimal neurological condition [the best NOS scores at the age of 4 y (56 points) and at 5.5 y of age (64 points)] and those with nonoptimal scores were performed by using the Student's *t* test 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, BMI, hematocrit, 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 breastfeeding, as well as BMI, health status of the children at 5.5 y of age, and site of investigation were also included. All control variables related to the outcome variable at $P < 0.2$ were entered in the model as covariables. The variable optimality (optimal vs. suboptimal) and 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.

Spearman correlations were also performed for the analyses of the association between fatty acid levels and the NOS.

$P \leq 0.05$ was considered significant. Statistical analyses were performed using SPSS 15.0 for Windows.

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 whom were excluded because they did not fulfill 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, with 89.5% of the women in the second trimester gestation and 87.4% in the 3rd trimester missing <5 d of supplementation. A total of 270 mother-infant pairs were invited for the neurological follow-up of children; 175 complied with the request at the age of 4 y and 157 complied at 5.5 y of age. Dropout rates were 35.18% at the age of 4 y and 41.9% at the age of 5.5 y, with no differences in the dropout rates between intervention groups. The 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 the age of 4 y and 5 of those examined at 5.5 y were born prematurely before wk 35 of pregnancy and were therefore excluded from the analyses. Except for 1 child who was born with a congenital left side anophthalmus, no other serious congenital disorder was observed. In the health screening questionnaire at 4 y of age, 1 child was reported to have left side deafness, another had developed craniosynostosis and had surgery at the age of 6 mo, and 1 child suffered from a developmental retardation of unknown etiology. These children were also excluded from the analyses, which left 167 and 148 children at the age of 4 and 5.5 y, respectively (**Supplemental Fig. 1**). No other severe illness or disability interfering with adequate functioning in normal life was observed. Children's baseline clinical and socio-demographic characteristics at 4 y of age are shown in **Table 1**. The social and obstetrical characteristics in the 4 intervention groups of the remaining study population at the 4- and 5.5-y follow-up were similar. Maternal basal dietary intakes of energy and nutrients at wk 20 and 30 of gestation also did not differ among the groups (**Supplemental Table 1**). There were no differences among groups in perinatal adverse events or illnesses in the first years of life. The children's ages at the Hempel and Touwen assessments were 50 ± 1.8 mo and 69.8 ± 2.0 mo, respectively.

Maternal and neonatal LC-PUFA levels in plasma and erythrocyte PL. Maternal baseline levels of AA and DHA (wt %) and the AA:DHA ratio in plasma or erythrocyte PL at wk 20 of gestation did not significantly differ among the 4 intervention groups. At 30 wk of gestation and at delivery, DHA levels in maternal plasma and erythrocyte PL and in cord blood were in general higher in the FO and FO+5-MTHF groups compared with the placebo and 5-MTHF groups. We did not find significant differences between 5-MTHF–supplemented groups and those that did not receive 5-MTHF in fatty acid percentages in maternal or cord plasma or erythrocyte PL (**Table 2**).

Clinical neurologic classification. None of the children had a definitely abnormal neurological condition. We did not find significant differences in clinical neurological condition among the 4 intervention groups at 4 or at 5.5 y. Given that the FO and FO+5-MTHF groups and the placebo and 5-MTHF groups had similar clinical and socio-demographic characteristics as well as similar AA, DHA, and AA:DHA levels in plasma and erythrocyte PL, we pooled the data of the FO and FO+5-MTHF groups and the data of the placebo and 5-MTHF groups. Likewise, neurological classification did not significantly differ between the groups. Data on the outcome of the neurological examination at the ages of 4 y and 5.5 y are shown in **Supplemental Table 2**.

There were no significant differences in cord blood DHA, AA levels, or the AA:DHA ratio between children classified as normal and those with MND at 4 y or 5.5 y of age. Furthermore, children with higher DHA or AA levels in cord blood (upper quartiles) had an incidence of MND similar to that of those with lower cord blood levels of these fatty acids (lower quartiles).

Relative DHA and AA concentrations and the AA:DHA ratio in plasma and erythrocyte PL did not differ between mothers of children with or without MND at the age of 4 y. Likewise, the percentage of DHA concentrations in plasma or erythrocyte PL did not differ between mothers of children classified as normal and those with MND at 5.5 y of age. However, mothers of children with MND at 5.5 y of age had lower AA concentrations in plasma PL at wk 20 (10.3 ± 1.8 vs. 9.0 ± 1.5%; $P = 0.028$) and in erythrocyte PC at wk 30 of gestation (6.9 ± 3.0 vs. 4.6 ± 2.5%; $P = 0.019$) compared with mothers of normal children. After adjustment for confounders in the logistic regression analyses, there was no association between AA and neurological classification.

NOS and fluency score. We did not find significant differences in the NOS or the fluency score at the age of 4 y or in the NOS at 5.5 y of age (Supplemental Table 2). We paid specific attention to children with an optimal neurological performance: $n = 22$ (13%) at the age of 4 y and $n = 14$ (9.9%) at 5.5 y of age. Plasma and erythrocyte DHA and AA concentrations and the AA:DHA ratio did not differ between children classified as optimal at the age of 4 y and those who were suboptimal. However, children classified as optimal at 5.5 y of age had significantly higher DHA levels in cord blood plasma PL than those classified as suboptimal

TABLE 1 Baseline characteristics of children at 4 y of age after randomization in the four intervention groups[1]

	FO	FO+5-MTHF	5-MTHF	Placebo
n	43	37	40	47
Center, *n (%)*				
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, *y*	29.6 ± 5.2	31.0 ± 4.9	31.0 ± 5.8	31.1 ± 4.0
wk 20 of gestation				
BMI	26.3 ± 3.7	25.4 ± 2.7	25.3 ± 2.4	25.0 ± 2.1
Hematocrit	0.35 ± 0.06	0.36 ± 0.04	0.36 ± 0.02	0.35 ± 0.02
Parity, *n (%)*				
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)
Smoked during pregnancy, *n (%)*	7 (16.3)	5 (13.5)	6 (15)	2 (4.3)
Gravidity risk (wk 20), *n (%)*				
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 (%)*				
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, *wk*	38.9 ± 1.5	38.5 ± 1.9	38.6 ± 1.6	39.0 ± 1.5
Sex, *n (%)*				
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 (%)*				
None	37 (86)	29 (78.4)	35 (87.5)	40 (85.1)
Preterm (>35 wk)	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 score (5 min)	10 (0.5)	10 (0.75)	10 (0.5)	10 (1)
Birth weight, *kg*	3.34 ± 0.40	3.12 ± 0.52	3.38 ± 0.39	3.39 ± 0.40
Birth length, *cm*	50.9 ± 2.0	50.7 ± 3.5	51.1 ± 1.4	51.0 ± 2.1
Birth head circumference, *cm*	34.9 ± 1.4	35.1 ± 1.9	34.9 ± 1.4	35.1 ± 1.3
Infant feeding, *n (%)*				
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 (%)*				
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[2], *n (%)*	20 (46.5)	13 (36.1)	23 (63.9)	22 (46.8)
Paternal education[2], *n (%)*	21 (48.8)	16 (44.4)	21 (52.5)	21 (44.7)
Age at Hempel evaluation, *mo*	50.2 ± 1.5	50.0 ± 1.5	50.0 ± 1.2	49.6 ± 2.1
Age at Touwen evaluation, *mo*	70.5 ± 2.0	70.7 ± 2.0	70.0 ± 2.3	70.1 ± 2.2
Childrens' BMI at 4 y of age	16.6 ± 2.1	15.7 ± 1.2	15.8 ± 1.1	15.9 ± 1.4

[1] Values are mean ± SD (continuous variables) and *n* (%) (categorical variables). Groups did not differ, $P > 0.05$.
[2] *n* (%) Parents with general qualification for university entrance or university degree.

(**Table 3**). DHA concentrations in maternal erythrocyte PL at delivery were higher and the AA:DHA ratios were lower in mothers of children classified as optimal at the age of 5.5 y compared with those considered suboptimal (Table 3). 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 5.5 y of age remained significant (**Table 4**). In addition, DHA levels in maternal plasma PL were significantly higher in the mothers of children classified as fluent by the Hempel examination at the age of 4 y (fluency score equal to 15) compared with mothers of those considered nonfluent (fluency score < 15) at wk 20 (4.7 ± 1.2 vs. 4.2 ± 0.9%; $P = 0.033$) and wk 30 (5.4 ± 1.4 vs. 4.8 ± 1.2%; $P = 0.031$) of pregnancy and at delivery (5.3 ± 1.5 vs. 4.4 ± 1.2%; $P = 0.005$). We observed no association between fatty acid levels in maternal plasma PL and optimally fluent movements in children at the age of 4 y after adjustment for confounders in the stepwise logistic regression analyses.

Correlation coefficients were positive between maternal DHA levels in plasma PL at delivery and the NOS ($r = 0.179$; $P = 0.026$) and fluency score ($r = 0.187$; $P = 0.02$) of children at 4 y 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 PL at delivery negatively correlated with the NOS of their children at 5.5 y of age ($r = -0.234$; $P = 0.006$). However, we did not

TABLE 2 Circulating fatty acid levels in newborns who were later evaluated at 4 y of age and in their mothers who were treated with FO, 5-MTHF, both, or neither during pregnancy[1]

	n	FO	FO+5-MTHF	5-MTHF	Placebo
wk 20 of gestation			*wt %*		
Plasma	159				
DHA		4.5 ± 1.1	4.8 ± 1.1	4.5 ± 1.0	4.9 ± 1.3
AA		10.6 ± 2.0	10.1 ± 1.6	9.9 ± 2.0	10.1 ± 1.5
AA:DHA		2.4 ± 0.7	2.2 ± 0.6	2.3 ± 0.7	2.2 ± 0.6
Erythrocyte PE	135				
DHA		5.1 ± 1.9	5.2 ± 2.1	5.0 ± 2.0	5.0 ± 2.0
AA		19.7 ± 5.8	18.6 ± 5.1	16.9 ± 5.0	18.6 ± 5.1
AA:DHA		4.2 ± 1.3	3.8 ± 1.0	3.7 ± 1.3	4.1 ± 1.2
Erythrocyte PC	117				
DHA		1.9 ± 1.0	2.1 ± 1.2	2.1 ± 1.2	2.3 ± 1.3
AA		6.8 ± 3.3	6.8 ± 2.4	6.3 ± 2.5	6.9 ± 2.7
AA:DHA		3.9 ± 1.7	3.9 ± 2.0	3.5 ± 1.5	3.7 ± 1.7
wk 30 of gestation					
Plasma	157				
DHA		6.1 ± 1.3^{a}	6.4 ± 1.1^{a}	4.3 ± 0.7^{b}	4.3 ± 1.0^{b}
AA		9.0 ± 1.4	8.9 ± 1.3	9.4 ± 1.8	9.2 ± 1.5
AA:DHA		1.6 ± 0.5^{b}	1.4 ± 0.3^{b}	2.2 ± 0.6^{a}	2.2 ± 0.7^{a}
Erythrocyte PE	142				
DHA		6.7 ± 2.4^{a}	7.0 ± 2.5^{a}	5.4 ± 1.8^{b}	5.8 ± 2.0^{ab}
AA		18.5 ± 4.3	17.0 ± 3.9	16.4 ± 4.1	18.8 ± 4.3
AA:DHA		2.9 ± 0.9^{bc}	2.6 ± 0.8^{c}	3.3 ± 1.1^{ab}	3.5 ± 0.9^{a}
Erythrocyte PC	130				
DHA		2.9 ± 1.7	2.9 ± 1.5	2.2 ± 1.3	2.5 ± 1.4
AA		6.5 ± 2.9	6.3 ± 2.9	6.6 ± 3.1	7.6 ± 3.1
AA/DHA		2.8 ± 1.2^{b}	2.6 ± 1.7^{b}	3.8 ± 2.0^{a}	3.8 ± 1.8^{a}
Delivery					
Plasma	155				
DHA		6.0 ± 1.2^{a}	6.2 ± 1.2^{a}	4.3 ± 1.3^{b}	4.3 ± 1.2^{b}
AA		8.9 ± 1.7	8.8 ± 1.2	9.1 ± 1.8	9.3 ± 1.5
AA:DHA		1.6 ± 0.5^{b}	1.5 ± 0.3^{b}	2.2 ± 0.7^{a}	2.3 ± 0.7^{a}
Erythrocyte PE	122				
DHA		8.9 ± 2.7^{a}	9.1 ± 3.0^{a}	6.5 ± 2.3^{b}	6.5 ± 1.9^{b}
AA		19.3 ± 4.4	17.8 ± 3.6	19.2 ± 3.4	19.5 ± 3.5
AA:DHA		2.3 ± 0.7^{b}	2.1 ± 0.6^{a}	3.2 ± 1.0^{a}	3.2 ± 0.9^{a}
Erythrocyte PC	113				
DHA		3.6 ± 1.7^{a}	3.9 ± 1.4^{a}	2.3 ± 1.2^{b}	2.4 ± 1.1^{b}
AA		7.1 ± 2.5	7.7 ± 2.4	7.3 ± 2.7	7.7 ± 2.7
AA:DHA		2.2 ± 0.7^{b}	2.1 ± 0.7^{b}	3.7 ± 1.4^{a}	3.8 ± 1.9^{a}
Newborn					
Plasma	146				
DHA		7.8 ± 1.7^{a}	7.0 ± 1.9^{ab}	6.2 ± 1.6^{b}	6.9 ± 1.6^{ab}
AA		16.8 ± 1.9	16.4 ± 2.0	17.3 ± 1.7	17.6 ± 1.7
AA:DHA		2.3 ± 0.5^{b}	2.5 ± 0.7^{ab}	3.0 ± 0.9^{a}	2.7 ± 0.8^{a}
Erythrocyte PE	101				
DHA		8.7 ± 2.6^{ab}	9.7 ± 2.9^{a}	7.4 ± 1.9^{b}	7.5 ± 1.9^{b}
AA		24.7 ± 6.6	24.9 ± 4.4	23.6 ± 4.0	25.1 ± 6.6
AA:DHA		3.0 ± 0.7^{ab}	2.8 ± 0.9^{b}	3.3 ± 0.8^{ab}	3.5 ± 0.8^{a}
Erythrocyte PC	98				
DHA		4.5 ± 1.5^{a}	3.9 ± 1.3^{ab}	2.6 ± 1.3^{c}	3.0 ± 1.3^{bc}
AA		12.9 ± 3.0	12.7 ± 2.8	11.4 ± 3.8	13.1 ± 4.1
AA:DHA		3.1 ± 0.9^{b}	3.5 ± 0.9^{b}	5.0 ± 1.6^{a}	4.8 ± 1.5^{a}

[1] Values are mean ± SD. Means in a row with superscripts without a common letter differ, $P < 0.05$.

find any significant correlation between maternal plasma or erythrocyte LC-PUFA levels (DHA, AA, AA:DHA) and the NOS or fluency scores at the ages of 4 y or 5.5 y 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 the 5.5-y follow-up (63% of fathers whose children were lost to follow-up in the FO group and 61% in the group not

TABLE 3 Fatty acid levels in children classified using the NOS as optimal or suboptimal at 5.5 y of age

Maternal RBC at delivery	Optimal (NOS = 64)	Suboptimal (NOS < 64)	*P*
Cord plasma PL DHA, *wt %*	8.1 ± 1.5	6.9 ± 1.7	0.015
PE DHA, *wt %*	11.0 ± 2.7	7.4 ± 2.5	0.002
PC DHA, *wt %*	5.0 ± 2.1	2.9 ± 1.4	<0.001
PE AA/DHA	1.8 ± 0.4	2.8 ± 0.9	0.003
PC AA/DHA	1.8 ± 0.5	3.0 ± 1.4	0.003

supplemented with FO had a general qualification for university entrance or a university degree, whereas only 41% of the followed children in the FO group and 43% in the group not supplemented with FO 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 lower in the group lost to follow-up at 4 y of age (weight: 3.15 ± 0.6 kg, length: 49.4 ± 3.2 cm, head circumference: 33.4 ± 2.5 cm) compared with the followed group (weight: 3.4 ± 4.2 kg, length: 51.1 ± 2.1 cm, head circumference: 34.8 ± 1.5 cm) ($P < 0.05$).

Regarding measured variables, DHA levels in plasma and erythrocyte PC at delivery were significantly higher in the group of mothers whose children continued in the study at the age of 4 y compared with 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%; $P = 0.031$). Mothers of children who dropped out at 5.5 y of age had higher AA levels in wk 30 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: 20.77 ± 3.15% vs 19.03 ± 3.60%, $P= 0.026$) in the non FO-supplemented group compared to the supplemented one. Cord blood DHA percentages in the plasma PL of the unsupplemented group were higher in the children continuing in the study at the age of 4 y (5.7 ± 1.5 vs. 6.6 ± 1.6%; $P = 0.011$) and 5.5 y (5.8 ± 1.6 vs. 6.6 ± 1.6%; $P = 0.018$).

The other baseline socio-demographic, obstetrical, and clinical characteristics of children who withdrew were similar to those of the children who 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 3rd trimester of gestation (1,3). Therefore, it seems that the effects of DHA supplementation to pregnant women during the 3rd trimester could be more beneficial for brain development than postnatal intake. Whether this supplementation actually improves the long-term neurological outcome of children remains a matter of discussion.

Bearing in mind its limitations, the current study showed no differences regarding the NOS, fluency score, or incidence of MND at the ages of 4 or 5.5 y between children whose mothers received FO supplements during pregnancy and those whose mothers were not supplemented.

The fact that we did not detect beneficial effects of prenatal supplementation cannot be attributed to the power of the study, because 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, often has been related to poorer neurological development of children. The group of fathers with a high educational level was underrepresented in both intervention groups, which should not induce bias. Children with lower weight and head circumferences were underrepresented in the nonsupplemented 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 that the neurologic assessment was performed by 3 different people in the 3 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 examination is reported to vary between 0.62 and 1.00 for the various items, with a mean value of 0.93 (35). The inter-assessor reliability of the Touwen examination varies between 0.75 and 1.00 for the various items, with a κ value of 0.76 for the assessment of MND (36). 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 a confounder in multivariate analyses.

The lack of association between supplementation and neurological outcome cannot be attributed to the sensitivity of the neurological examination, because 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

TABLE 4 Contribution of the DHA status of newborns and mothers at delivery to the neurological outcome of the children at 5.5 y of age assessed using the NOS[1]

	B[2]	*P*	OR (95% CI)[3]	Correct classification, %	Naegelkerker R Square, %
Cord DHA in plasma PL	0.453	0.014	1.09–2.26	89.8	14.5
Cord DHA in erythrocyte PE	0.845	0.017	1.09–2.42	94.2	19.0
Cord DHA in erythrocyte PC	0.488	0.049	1.00–2.64	91.7	10.8
Maternal DHA in erythrocyte PE at delivery	0.584	0.002	1.24–2.60	92.4	38.1
Maternal DHA in erythrocyte PC at delivery	0.945	0.001	1.45–4.66	92.8	37.1

[1] Logistic regression analysis 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).
[2] Logistic regression coefficient.
[3] Odds of children with the maximal NOS at 5.5 y of age for every unit increment in DHA level.

(35,37). Because the Hempel and Touwen examinations focus on the evaluation of neuromotor behavior (35), it could be hypothesized that LC-PUFA may have an effect in other specific developmental domains not assessed by these examinations. Interestingly, studies in animals have shown that the basal ganglia have the highest DHA accumulation in the brain of baboons (38–40) and may be specifically vulnerable to DHA deficiency. These areas are important to psychomotor behavior 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.

Also interesting in our results is the low MND prevalence at the age of 4 y compared with that at 5.5 y. At 4 y old, children are at the border between the Hempel assessment (upper age limit) and Touwen (lower age limit). At the age of 4 y, the Hempel assessment is more appropriate than the Touwen, but it suffers to some extent from ceiling effects. Therefore, the difference in prevalence of MND between the 2 ages may be attributed to age, because with increasing age more dysfunctions become expressed (41), and ceiling effects of the Hempel assessment at the age of 4 y.

Our finding that FO supplementation to pregnant women did 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 contrasts 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 assessed neurological outcome of children before school age. We are aware of only 1 randomized controlled trial assessing neurological outcome of children older than 4 y (21). The authors reported higher mental processing scores in the Kaufman Assessment Battery for Children (K-ABC) at 4 y of age in children whose mothers received (n-3) LC-PUFA supplements compared with those born to mothers receiving (n-6) LC-PUFA supplements, but no differences in the K-ABC scores at the age of 7 y. In our study, we gave a low DHA dose compared with other studies that have shown a benefit of supplementation (17,19). Although the supplementation with 500 mg/d 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 compared with data on fatty acid composition of venous cord blood PL in healthy, full-term infants from different populations (42).

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 (43). 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 the ages of 4 and 5.5 y 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 in children at 34 mo 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. (17) reported no association between neonatal DHA levels and the Mental Processing Composite of the K-ABC test at 4 y of age. However, they found a significant association between neonatal and maternal (n-3) LC-PUFA levels and the Score in the Sequential Processing Scale of the same test at 7 y of age (21). The logistic regression analyses in the present study showed a higher occurrence of an optimal neurologic condition at the age of 5.5 y with increasing DHA percentages in cord and maternal blood at delivery, which is in agreement with previous studies. It should be realized that the range for optimal behavior is narrower than that of normal behavior, because children classified as neurologically normal may show single signs of dysfunction in various neurological domains (35). Thus, although the FO supplementation in the second half of pregnancy did not 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 3 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 wk 20 of pregnancy and 89.2% at wk 30 achieved the recommended DHA intake of 200 mg/d (44), which has been associated with an optimal long-term developmental outcome in the ALSPAC study (Avon Longitudinal Study of Parents and Children) (45). 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 neurologic 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 5.5 y 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.

Acknowledgments

B.K., T.D., and C.C. coordinated research; M.V.E.M. conducted research, analyzed data, and wrote the paper; R.R., J.B., G.C., and M.P. conducted research; T.D., A.G., F.C., M.P., and M.H.A provided essential materials; and B.K., T.D., and C.C. had primary responsibility for final content. All authors read and approved the final manuscript.

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