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The influence of supplemental docosahexaenoic and arachidonic acids during pregnancy and lactation on neurodevelopment at eighteen months

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ABSTRACT

Docosahexaenoic acid (DHA) and arachidonic acid (AA) are important for neurodevelopment. The effects of DHA (220 mg/day, $n=41$), DHA+AA (220 mg/day, $n=39$) or placebo ($n=34$) during pregnancy and lactation on neurodevelopment at 18 months, and the relations between umbilical cord DHA, AA and Mead acid and neurodevelopment were studied. An age-specific, standardized neurological assessment for the evaluation of minor neurological dysfunction (MND), and the Bayley Scales of Infant Development (BSID) were used. The intervention did not influence any of the outcomes. Umbilical venous (UV) Mead acid was negatively and n-6 fatty acids were weakly positively associated to the BSID mental developmental index. Children with simple MND had lower UV DHA compared to normally classified children. We conclude that relatively short-term maternal DHA or DHA+AA supplementation does not influence neurodevelopment at toddler age, although some parameters of brain development are related to perinatal DHA and AA status.

1. Introduction

The long chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are considered important for brain development. DHA, ARA and eicosapentaenoic acid (EPA, 20:5n-3) are structural components of membrane phospholipids, modulators of gene expression and precursors of eicosanoids (ARA, EPA), resolvins (ARA, EPA, DHA) and (neuro)protectins (DHA) [1,2]. EPA and DHA are mainly derived from fish, while meat, poultry and eggs are the principal sources of ARA. Brain DHA and ARA contents increase rapidly from the last trimester of pregnancy up to 2 years postpartum [3]. There are many studies on the influence of DHA or fish oil supplementation on early brain development, but their outcomes are inconclusive in a meta analysis [4]. Since the beneficial effects of DHA in maternal supplementation studies do not seem dose-dependent, and effects are mainly found in older infants and toddlers and not in early infancy [5,6], it is conceivable that potentially positive effects of DHA on neurodevelopmental outcome first become expressed after early infancy.

In a previous study, postnatal supplemental LCPUFA did not affect neurological outcome at 18 months [7]. In the same study we showed that lower DHA and ARA in umbilical vessels were associated with a less favorable neurological condition as assessed with the Hempel technique, although no relations were demonstrable with the Bayley Scales of Infant Development [10]. The Hempel assessment [8,9] is an age-specific and standardized neurological assessment designed for the evaluation of minor neurological dysfunction (MND). Next to classic signs of neurological function, such as muscle tone and reflexes, ample attention is paid to the quality of motor behavior. The outcome is either a clinical classification in terms of MND or major neurological dysfunction, or a neurological optimality score (NOS). The NOS at 18 months proved sensitive for detecting subtle differences in prenatal environmental or nutritional changes, such as exposure to polychlorinated biphenyls (PCBs) [9], LCPUFA [10] and feeding with formula or breast milk [9,11].

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The lower umbilical vessel DHA and ARA of children with a less optimal neurological condition at ages 3 [12] and 18 [10] months support the idea that adequate prenatal LCPUFA status is important for neurological development. We therefore explored the effects of supplemental DHA and DHA+ARA during pregnancy and lactation on the children's neurological development at the age of 3 and 18 months. At the age of 3 months, we assessed neurological development by recording general movement (GM) quality. We found that 60% of the children exhibited mildly abnormal GMs in the DHA supplementation group. The neurological condition of infants in the DHA+ARA groups was similar (DHA+ARA 34%; placebo 31% mildly abnormal GMs) [13]. Taken together, these results suggested a typical but less optimal neurological condition in early infancy [14] following maternal supplementation with DHA-only. In the current study we report the neurological outcome of this trial at 18 months, assessed using the Hempel technique and Bayley Scales of Infant Development. In addition, we studied whether prenatal LCPUFA status was related to brain development.

2. Subjects and methods

2.1. Subjects

This study was part of a double-blind placebo-controlled randomized trial in which we explored the influence of DHA with or without ARA during pregnancy and lactation on infant neurological condition [13,15], maternal mood [15] and cognition as well as milk fatty acid composition [16]. The study design has been reported in detail elsewhere [16]. In short, apparently healthy women were enrolled between the fourteenth and twentieth weeks of pregnancy, with the majority (80%) being enrolled between 15.6 and 17.4 weeks postmenstrual age (mean 16.5 weeks). A vegan diet was an exclusion criterion and (gestational) diabetes mellitus and preterm birth, i.e. birth before 37 weeks of pregnancy, were termination criteria. At enrollment, women were randomized into 3 groups using block randomization. The initial research protocol and the follow-up were approved by the Central Committee on Research Involving Human Subjects (CCMO, Den Haag, The Netherlands; protocol number P03.1071C). All women gave written informed consent. The trial is registered under ISRCTN58176213.

Power analysis for this trial was based on the NOS [17] at the age of 2 weeks [13], which revealed that 64 children per group were needed to obtain sufficient power to detect a 2 point (=0.5SD) difference between the groups. Next to the NOS at 2 weeks, we analyzed GM quality. Due to the higher rate of mildly abnormal GMs at the age of 3 months in the DHA group, inclusion was discontinued early. From the 183 women included in the study, 58 dropped out during pregnancy due to lack of motivation to fill in questionnaires on a regular basis and take supplements daily (placebo, $n=23$; DHA, $n=20$; DHA+ARA, $n=15$). Six mother–infant pairs dropped out due to obstetric complications (placebo, $n=3$; DHA, $n=1$; DHA+ARA, $n=2$). Attrition was evenly distributed among the groups ($p=0.33$). At the age of 18 months, 5 additional children were lost to follow-up. One of the infants moved and was not examined due to logistical reasons (DHA group), and 4 of the infants were not examined due to parental lack of interest (placebo, $n=2$; DHA+ARA, $n=2$). For this current report, 114 children were evaluated. Due to the early discontinuation of inclusion, the trial might lack sufficient power to detect between-group differences in any of the parameters. However, at the time the study was designed, no data were available on the effects of maternal DHA and ARA supplementation on the used neurological outcomes at the age of 18 months and power analysis was based on the NOS at 2 weeks.

2.2. Dietary intervention

All women received a supplement of vitamins and minerals according to the Dutch recommended dietary allowances. The mixture of vitamins and minerals derived from FrieslandCampina and was produced under the same conditions as those used for infant formula. The women were instructed to take 2 capsules once daily from enrollment until 3 months after delivery. The DHA+ARA group received 220 mg DHA (Marinol D40, Lipid Nutrition B.V., Wormerveer, The Netherlands, a DHA-enriched purified fish oil) and 220 mg ARA (Wuhan Alking Bioengeneering Co. Ltd., Wuhan, China). The DHA group received 220 mg DHA and 1 capsule containing soy bean oil (Wuhan Alking Bioengeneering Co. Ltd., Wuhan, China). The placebo group received 2 capsules containing soy bean oil. The daily fatty acid intakes from the capsules for each of the 3 treatment groups are shown in Table 1. The daily dosages of DHA and ARA are within the range of typical Western intakes, i.e. adding the supplements to the diet doubled the intake of DHA and ARA. The dosages of linoleic acid and alpha-linolenic acid from the capsules are only a fraction of the daily Western intakes [18].

2.3. Developmental evaluation

Eighteen months after birth, neurodevelopmental status was assessed using 2 instruments, i.e. the neurological examination according to Hempel [8,9] and the Dutch version of the Bayley Scales of Infant Development, second edition (BSID-II-NL) [19]. The Hempel examination includes the observation of spontaneous motor behavior (grasping, sitting, crawling, standing and walking) and classical neurological tests, such as the assessment of muscle tone and reflexes. In this way, fine-motor function, gross-motor function, posture and muscle tone, reflexes and visuomotor function are evaluated. Multiple signs in one of these functions result in a dysfunctional domain (previously labeled 'cluster'; [20]), of which the isolated domain of dysfunctional reflexes has no neurological implications. The neurological examination resulted in 3 types of outcome. First, the neurological classification; children are classified as normal, as having simple MND, as having complex MND or as definitely abnormal. Definitely abnormal implies the presence of a neurological syndrome like cerebral palsy. Simple MND indicates the presence of 1 dysfunctional domain and is regarded as a typical but non-optimal form of brain function. Complex MND indicates the presence of at least 2 domains of dysfunction and is considered the clinically more relevant form of MND, as it is associated with pre- and perinatal adversities and learning and behavioral problems [20]. We also used the optimality concept to summarize neurological condition. Of 58 items of the neurological examination the optimal range

Table 1
Daily intakes of fatty acids (in mg) from the supplements.

	Placebo group	DHA group	DHA+ARA group
LA	535	274	46
ALA	60	32	7
ARA	0	15	220
EPA	0	34	36
DHA	0	220	220

LA, linoleic acid, 18:2n-6; ALA, alpha-linolenic acid, 18:3n-3; ARA, arachidonic acid, 20:4n-6; EPA, eicosapentaenoic acid, 20:5n-3; DHA, docosahexaenoic acid, 22:6n-3.

was defined. The number of items meeting the criteria for optimality was added to form the NOS. It is important to stress that optimality is not identical to normality and that non-optimal in general is not abnormal, as the range for optimal is narrower than the range for normality. The fluency score, describing the fluency of motor behavior in 13 items was extracted from the total NOS.

The BSID-II-NL was used to assess the mental (MDI) and psychomotor (PDI) developmental indices, which are based on the number of successfully completed items from the age-adjusted test.

2.4. Umbilical cord fatty acid analysis

At birth, 7–10 cm samples of the umbilical cord were taken and stored in saline at 4 °C for a maximum of 24 h until further processing. A 1 cm sample from the umbilical venous and the umbilical arterial wall was isolated from the umbilical cord for fatty acid profiling. The tissue sample was transferred to a Sovirel tube containing 2 mL methanol/HCl (5:1, v/v) and 5 mg butylated hydroxytoluene. Following transmethylation, the fatty acid compositions were determined using the capillary gas chromatographic method with flame-ionization detection [21]. Fatty acid compositions were calculated assuming that equal peak areas give rise to equal weight amounts [22]. The fatty acids are presented in relative amounts, i.e. g/100 g fatty acids (wt%) and ratios in wt/wt.

2.5. Statistics

Statistical analyses were performed with the SPSS software package, version 16. Unless otherwise indicated, a $p < 0.05$ was considered to be significant. The NOS and fluency score proved skewed and were transformed using $-\ln(59.5\text{-NOS})$ resp. $-\ln(14.5\text{-fluency score})$. Univariate between-group differences for $-\ln(59.5\text{-NOS})$, $-\ln(14.5\text{-fluency score})$, MDI and PDI were evaluated using a one-way ANOVA with Tukey's correction at $p < 0.05$.

Differences between intervention groups for maternal age, birth weight, gestational age at birth, testing age, and height, weight and head circumference at 18 months were calculated using a one-way ANOVA with Tukey's correction. For categorical data, i.e. maternal education (lower education vs. higher education), maternal parity at birth (0 or 1), and gender, a chi-square test was used.

Linear or logistic multivariate regression was applied to correct the effect of the intervention for potential confounders like gender, maternal education, maternal age, maternal parity at birth, duration of breastfeeding, birth weight and gestational age at birth, as well as for testing age for the Hempel data (not for the outcomes of the Bayley scales, which are normalized for age). Both the DHA and DHA+ARA groups were compared to placebo in the 'default' model, but to compare the differences between the DHA group and the DHA+ARA group, models were created in which the placebo group was not included.

Between-group differences for umbilical arterial (UA) and venous (UV) wall fatty acids were tested using the Mann–Whitney *U*-test at $p < 0.017$ to correct for multiple comparisons. The relations between UA and UV fatty acids and the NOS, the fluency score, the MDI and the PDI at 18 months were tested using the Spearman correlation. Significant correlation coefficients ≥ 0.3 were considered relevant, significant correlations with coefficients in-between 0.2 and 0.3 were considered weak. In addition, differences in umbilical cord fatty acids between children with different neurological classifications were established using the Mann–Whitney *U*-test ($p < 0.05$ was considered significant for comparison between 2 groups, $p < 0.017$ for comparison between 3 groups). We primarily focused on umbilical UA and UV DHA, ARA, Mead acid and the DHA/ARA ratio.

3. Results

Prenatal, perinatal and social characteristics as well as anthropometrics were similar in the 3 treatment groups (Table 2).

The developmental outcomes in the 3 groups are shown in Table 3. The NOS, the fluency score, the prevalence of simple and complex MND as well as the Bayley MDI and PDI scores did not differ between the groups. None of the toddlers suffered from an evident neurological syndrome such as cerebral palsy. Multivariate analyses which took into account the role of confounders, i.e. gender, gestational age at birth, duration of breastfeeding, birth weight, maternal education and maternal parity at birth, confirmed that the neurological condition in the 3 groups was similar (Table 4).

Umbilical cord fatty acid profiles were available from 94 of the 114 children (data shown in Table 5). Absence of umbilical cord samples was mainly caused by logistical reasons, i.e. parents and/or midwives forgot to collect and store a piece of umbilical cord. UA ARA was highest in the placebo group (median 13.18 wt%, range

Table 2
Prenatal, perinatal and social characteristics and anthropometrics.

	Placebo group ($n=34$)		DHA group ($n=41$)		DHA+ARA group ($n=39$)		p
	Mean	SD	Mean	SD	Mean	SD	
Testing age (months)	18.4	0.9	18.5	1.1	18.0	2.0	0.35
Maternal age at inclusion (years)	32.7	5.1	32.5	4.4	32.9	4.8	0.95
Gestational age at birth (weeks)	40.2	1.1	40.1	1.1	40.2	1.2	0.95
Birth weight (g)	3576	551	3592	465	3652	377	0.76
Duration of breastfeeding (months)	5.1	4.5	6.0	4.5	4.6	4.2	0.37
Weight at 18 months (kg)	11.5	1.1	11.3	1.4	11.5	1.3	0.67
Height at 18 months (cm)	84.0	3.8	82.8	4.7	83.6	2.9	0.39
Head circumference at 18 months (cm)	47.8	1.5	47.6	1.1	47.5	1.4	0.79
	n	%	*n*	%	*n*	%	*p*
Maternal higher education[a]	29	85.3	30	73.2	26	66.7	0.18
First born	21	61.8	25	61.0	18	46.2	0.30
Male gender	21	61.8	16	39.0	18	46.2	0.14

DHA, docosahexaenoic acid; ARA, arachidonic acid.

[a] At least High school completed.

Table 3
Developmental outcomes at 18 months of age.

	Placebo group ($n=34$)		DHA group ($n=41$)		DHA+ARA group ($n=39$)		p
	Median	Range	Median	Range	Median	Range	
Neurological optimality score	47.5	29–55	46	30–56	48	25–57	0.55[a]
Fluency score	10	6–12	9	5–12	10	4–12	0.44[a]
	n	%	*n*	%	*N*	%	*p*
Prevalence of normal neurological condition	20	58.8	24	58.5	28	71.8	0.39[b]
Prevalence of simple MND	9	26.5	14	34.1	6	15.4	0.19[c]
Prevalence of complex MND	5	14.7	3	7.3	5	12.8	[d]
	Mean	SD	Mean	SD	Mean	SD	*p*
Mental developmental index	115.2	11.6	113.7	13.0	112.8	12.6	0.72
Psychomotor developmental index	91.7	8.3	95.8	11.4	92.4[e]	8.8	0.14

DHA, docosahexaenoic acid; ARA, arachidonic acid; MND, minor neurological dysfunction.

[a] ANOVA testing was performed on the transformed data.
[b] Difference in prevalence of normal neurological condition vs those with simple and complex MND in the 3 groups.
[c] Difference in prevalence of children with simple MND vs children with a normal neurological condition in the 3 groups.
[d] The groups are too small for statistical analyses.
[e] PDI was not assessed from one of the infants.

Table 4
Effects of intervention after correction for potential confounders.

	Coefficient *B* (95% CI)	*p*	Explained variance r^2[a]
−ln (59.5-NOS)[b]			19.2%
Placebo vs. DHA	−0.092 (−0.322–0.137)	0.43	
Placebo vs. DHA+ARA	0.038 (−0.196–0.271)	0.75	
DHA vs. DHA+ARA	0.125 (−0.100–0.351)	0.27	
−ln (14.5-fluency score)[b]			12.3%
Placebo vs. DHA	−0.084 (−0.242–0.074)	0.30	
Placebo vs. DHA+ARA	0.023 (−0.138–0.184)	0.78	
DHA vs. DHA+ARA	0.107 (−0.048–0.262)	0.17	
Mental developmental index			16.2%
Placebo vs. DHA	−2.759 (−8.348–2.830)	0.33	
Placebo vs. DHA+ARA	−2.298 (−7.963–3.368)	0.42	
DHA vs. DHA+ARA	0.439 (−5.208–6.086)	0.88	
Psychomotor developmental index			8.0%
Placebo vs. DHA	4.086 (−0.534– 8.706)	0.08	
Placebo vs. DHA+ARA	0.529 (−4.200–5.258)	0.83	
DHA vs. DHA+ARA	−3.622 (−8.430–1.186)	0.14	
	Exp *B* (95% CI)	*p*	Nagelkerke r^2 (%); χ^2; p[a]
Presence of MND (simple or complex)[b]			17.3; 15.4; 0.119
Placebo vs. DHA	1.003 (0.369–2.722)	1.00	
Placebo vs. DHA+ARA	0.477 (0.165–1.378)	0.17	
DHA vs. DHA+ARA	0.513 (0.177–1.485)	0.22	
Presence of complex MND[b]			24.3; 15.1; 0.130
Placebo vs. DHA	0.472 (0.092–2.424)	0.37	
Placebo vs. DHA+ARA	0.698 (0.148–3.287)	0.65	
DHA vs. DHA+ARA	1.546 (0.284–8.424)	0.61	

DHA, docosahexaenoic acid; ARA, arachidonic acid; NOS, neurological optimality score according to Hempel; MND, minor neurological dysfunction.
To compare the DHA group to the DHA+ARA group, a new model was created in which the placebo group was excluded.
The first mentioned group functions as the reference group (i.e.=0). Effects of the intervention were corrected for gender, gestational age at birth, duration of breastfeeding, birth weight, maternal education and maternal parity at birth.

[a] In the models in which both the DHA and DHA+ARA groups were compared to placebo, the explained variance was assessed.
[b] Adjusted for testing age in addition to the below mentioned variables.

10.84–16.92) and lowest in the DHA group [12.18 (9.32–16.11); $p=0.004$]. UA ARA in the DHA+ARA group [12.45 (9.34–16.29)] did not differ from either the placebo group ($p=0.031$) or the DHA group ($p=0.50$). The UA DHA/ARA ratio was lowest in the placebo group [0.38 (0.24–0.44)] compared to the DHA group [0.41 (0.24–0.53); $p=0.002$]. The DHA+ARA group showed an intermediate arterial DHA/ARA ratio [0.39 (0.27–0.56)], which did not differ significantly from the placebo group ($p=0.16$) or the DHA group ($p=0.085$). There were no differences in UV fatty acid compositions.

Umbilical cord fatty acid compositions did not correlate with the NOS and fluency score. We observed a negative correlation between UV Mead acid and the MDI (correlation coefficient, cc= −0.32, $p=0.002$). In addition, significant though weak

Table 5
Umbilical cord fatty acid composition (wt% or wt/wt) in the 3 supplementation groups.

Umbilical artery	Placebo $n=25$		DHA $n=35$		DHA+ARA $n=33$	
	Median	Range	Median	Range	Median	Range
14:0	1.10	0.87–1.28	1.10	0.84–1.38	1.04	0.69–1.37
16:0	22.04	20.36–25.07	21.87	19.69–26.83	21.97	20.29–29.30
18:0	19.83	18.54–21.17	19.64	17.87–22.52	19.87	17.41–21.51
20:0	0.53	0.41–0.63	0.55	0.46–0.71	0.54	0.43–0.65
22:0	1.45	1.21–2.33	1.49	1.17–2.40	1.48	1.14–1.87
24:0	2.68	1.99–3.46	2.78	1.75–4.41	2.69	2.04–3.39
SAFA	47.86	45.48–51.64	47.42	45.60–54.27	47.46	44.52–54.28
22:5n-3	0.25	0.12–0.54	0.27	0.13–0.49	0.23	0.11–0.68
22:6n-3	5.02	3.44–5.95	5.09	2.83–6.19	4.79	3.54–8.03
Sum LCPn-3	5.23	3.63–6.26	5.24	3.10–6.49	5.02	3.73–8.72
Sum n-3	5.33	3.72–6.36	5.34	3.19–6.62	5.11	3.74–8.81
18:2n-6	1.83[a]	1.35–5.35	1.91[a, b]	1.30–3.33	1.67[b]	1.16–3.65
20:2n-6	0.22	0.11–0.53	0.23	0.11–0.41	0.20	0.11–0.36
20:3n-6	1.76[a]	1.30–2.36	1.77[a]	1.28–2.27	1.54[b]	1.21–2.43
20:4n-6	13.18[a]	10.84–16.92	12.18[b]	9.32–16.11	12.45	9.34–16.29
22:4n-6	3.29	2.14–5.79	3.14	2.14–4.80	3.08	2.11–4.62
22:5n-6	3.44	2.34–5.01	3.13	1.85–4.89	3.18	1.93–4.22
Sum LCPn-6	21.97[a]	18.01–28.84	20.78[b]	15.86–23.78	20.67[b]	15.85–24.74
Sum n-6	23.78[a]	19.83–32.19	22.91[a, b]	17.38–26.44	22.11[b]	17.02–26.55
18:1n-7	2.94	2.32–3.50	3.17	2.54–3.92	3.15	2.37–4.32
18:1n-9	12.78	9.10–16.19	13.55	11.65–17.33	13.70	10.63–17.81
20:1n-9	0.55	0.32–0.94	0.62	0.34–1.15	0.64	0.31–1.46
24:1n-9	4.09	2.91–5.09	4.04	3.35–5.58	4.16	3.30–5.68
20:3n-9	2.71	0.44–4.15	2.73	1.66–5.31	3.04	0.39–5.46
22:3n-9	1.35	0.30–2.28	1.47	0.80–2.36	1.70	0.47–2.73
Sum n-9	21.88	13.76–26.67	23.13	18.43–29.21	23.87	15.85–30.64
MUFA	20.31[a]	15.85–24.54	21.81[b]	18.48–26.37	21.83[a, b]	17.81–27.92
PUFA	32.75	29.71–39.34	32.83	26.56–35.95	32.25	28.68–35.71
DHA/AA	0.38[a]	0.24–0.44	0.41[b]	0.24–0.53	0.39[a, b]	0.27–0.56
Umbilical vein	$n=27$		$n=34$		$n=33$	
14:0	0.96	0.48–1.20	0.94	0.78–1.26	0.93	0.73–1.31
16:0	24.52	22.34–28.66	24.32	21.43–28.19	24.27	20.81–32.31
18:0	19.67	16.93–22.71	19.41	16.53–22.34	19.55	17.49–23.02
20:0	0.48	0.37–0.62	0.48	0.31–0.62	0.48	0.32–1.21
22:0	1.14	0.95–2.03	1.14	0.84–1.82	1.20	0.83–2.31
24:0	2.03	1.61–3.02	2.25	1.20–3.23	2.15	1.70–3.01
SAFA	48.56	44.67–55.17	48.39	44.95–55.88	48.13	45.60–60.17
22:5n-3	0.28	0.14–0.63	0.35	0.17–0.73	0.32	0.09–0.67
22:6n-3	4.66	2.60–6.16	4.97	3.72–6.11	4.83	2.01–7.59
Sum LCPn-3	4.90	2.85–6.54	5.33	3.98–6.69	5.12	2.19–8.26
Sum n-3	4.97	2.97–6.62	5.42	4.09–6.83	5.18	2.22–8.33
18:2n-6	2.71	1.89–4.89	2.84	2.11–4.33	2.62	1.69–3.67
20:2n-6	0.42	0.21–0.57	0.40	0.28–0.80	0.37	0.19–1.27
20:3n-6	2.43	1.83–3.19	2.54	1.67–3.45	2.28	1.50–3.45
20:4n-6	16.68	12.40–19.73	16.51	13.35–19.83	16.87	11.73–19.68
22:4n-6	5.14	2.27–7.13	5.10	3.70–7.32	5.33	3.08–7.18
22:5n-6	2.65	1.85–3.97	2.45	1.48–4.74	2.68	1.45–3.73
Sum LCPn-6	28.16	19.26–31.14	27.56	21.46–30.35	28.09	19.48–30.20
Sum n-6	31.04	21.16–33.53	30.10	24.28–33.57	30.73	22.08–32.98
18:1n-7	2.76	1.90–3.48	2.74	2.22–3.96	2.76	2.19–3.51
18:1n-9	10.17	8.80–13.80	10.33	8.47–16.35	10.20	8.51–13.18
20:1n-9	0.42	0.24–0.87	0.44	0.23–1.16	0.40	0.21–0.73
24:1n-9	3.86	3.08–5.19	4.13	2.79–4.82	3.81	3.12–4.63
20:3n-9	0.51	0.26–2.50	0.53	0.23–1.43	0.58	0.06–2.05
22:3n-9	0.52	0.25–1.07	0.51	0.20–1.48	0.52	0.17–1.31
Sum n-9	15.76	13.02–21.02	15.75	12.95–23.95	15.60	12.55–20.35
MUFA	17.13	14.89–21.61	17.72	14.62–25.17	17.39	14.31–21.56
PUFA	37.25	28.52–41.07	37.04	30.25–39.95	36.97	25.90–39.68
DHA/AA	0.29	0.19–0.35	0.30	0.23–0.43	0.29	0.17–0.45

DHA, docosahexaenoic acid; ARA, arachidonic acid; SAFA, saturated fatty acids; LCP, long chain polyunsaturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; different superscript letters indicate differences between the groups at $p<0.017$.

positive correlations (cc in-between 0.2 and 0.3) were found for n-6 fatty acids and the MDI (UA ARA, sum LCPUFAn-6, sum n-6 and sum polyunsaturated fatty acids as well as UV sum n-6) whereas n-9 fatty acids in general showed a weak but significant negative correlation (in-between −0.2 and −0.3; UA 18:1n-9, sum n-9, sum mono-unsaturated fatty acids and UV 20:1n-9, 22:3n-9 and sum n-9). The PDI correlated weakly to 20:2n-6 (cc=0.243, $p=0.020$).

UA fatty acid contents did not differ between children classified as neurologically normal, having simple MND or having

complex MND. UV DHA was lower in the simple MND group as compared to the normal group [simple MND: median 4.65 (range 2.83–6.15) versus normal: 5.00 (3.44–6.47); $p=0.012$]. UV ARA was significantly higher in the complex MND group compared to the children classified as normal ($p=0.017$). The infants classified as normal and as having simple MND showed similar UV ARA values.

4. Discussion

We found that supplementation of DHA or DHA+ARA during pregnancy and lactation did not influence neurological condition at 18 months as assessed using the Hempel technique and the Bayley MDI and PDI. UV Mead acid was negatively related to the MDI. DHA in umbilical veins of children with simple MND was lower as compared with children with a normal neurological condition. UV ARA was higher in children with complex MND, as compared to children with simple MND and those with a normal neurological condition.

At 18 months, we found no effect of supplemental DHA and DHA+ARA during pregnancy and lactation on neurodevelopmental parameters. This was in contrast to our findings at 3 months of age. At that time, infants in the DHA group showed more frequently mildly abnormal GMs compared to the placebo group and additional ARA counteracted this effect of DHA [13]. The presence of mildly abnormal GMs reflects a typical but non-optimal condition of the young brain [23]. The absence of either beneficial or negative effects of supplementation at the age of 18 months might be due to the small sample size. However, post-hoc power analyses revealed that even in large groups of 150–860 children per group, depending on the developmental parameter, no significant differences were to be expected using our techniques and the same doses of DHA and ARA.

We have no information regarding the children's diet after 3 months of age, except for the global duration of breastfeeding. However, since the participants were properly randomized, the diet of either mother or child is not expected to differ between the groups. Indeed, we found no between-group differences in maternal fish-intake per week during the intervention period, as estimated with food-frequency questionnaires (average fish consumption 0.94 times/week, 0.5 times fatty fish/week [15]). In addition, Bouwstra et al. [10] showed that prenatal fatty acid status is related to neurodevelopment at 18 months, in contrast to short-term postnatal LCPUFA supplementation to formula [7], which may suggest that intrauterine LCPUFA status has an important effect on postnatal development.

Maternal DHA supplementation studies during pregnancy or during pregnancy and subsequent lactation with doses ranging from 200 to 3300 mg DHA/day resulted in inconsistent outcomes that did not seem related to supplementation dose, duration of supplementation or testing age [5,6,24]. The discrepancy may relate to a complex interplay between a ceiling effect in the dose–outcome relationship, benefits to those with suboptimal baseline status only, and inter-individual differences in developmental potential [25]. A high variety in developmental tests, differing in functional background, is used in the various studies. Various brain regions differ in fatty acid composition, which may imply region-specific regulation of brain LCP contents. It might therefore be that DHA supplementation influences some brain regions, but not all, and thereby fails to influence the outcomes of each of the various tests. In addition, the relation between DHA status and neurodevelopment may be non-linear.

Our results may also indicate a transient effect of supplementation that is expressed at early age (3 months), but disappears at preschool age, possibly due to plasticity of the brain. The higher incidence of mildly abnormal GMs in the DHA supplementation group may also reflect a different developmental trajectory, which may or may not have consequences for functioning in later life [26]. The effect of LCPUFA supplementation on neurodevelopmental outcome has been studied best in full-term infants in studies dealing with supplementation of infant formula after birth. These studies indicated a positive effect of LCPUFA on outcome in early infancy, but positive effects on later outcome have not been demonstrated [27]. However, no data are available on long-term developmental effects. It is therefore also possible that effects of LCPUFA supplementation may not be found at the age of 1–2 years, but re-emerge at school age, similarly to the effect of breastfeeding on developmental outcome [7,28,29].

Children classified as normal had higher UV DHA as compared to those with simple MND. Simple MND is considered to reflect a normal (i.e. non-pathological) but non-optimal form of brain function in which for example the dopaminergic system may operate in a non-optimal manner [20]. Our findings are therefore in line with animal studies that indicated a relation between perinatal brain DHA and e.g. dopaminergic neurotransmission [30]. Nearly all children (93%) displaying simple MND showed a gross motor dysfunction, which is in accordance to previously suggested relations between notably DHA and motor behavior in rats [31–33] and humans [26,34]. Complex MND is a more severe condition and is suggested to be at least partly due to an interruption of connecting fiber systems [20]. The effects of LCPUFA are known to be subtle at best, and we are not aware of any etiological mechanisms that may explain the relation between ARA and complex MND.

The current study showed a negative association between UV Mead acid and the MDI score. The negative association between Mead acid and neurodevelopment is consistently found in our studies, since also the rate of MA GMs increased at increasing infant erythrocyte Mead acid contents at 3 months of age [26]. In addition, Dirix et al. [35] showed a negative association between UA Mead acid contents and parameters of fetal learning and development and Helland et al. [36] found a negative relation between umbilical plasma Mead acid and intelligence scores at 4 years of age. Mead acid is used as a parameter of essential fatty acid deficiency. During pregnancy, Mead acid is probably a consequence of the high *de novo* synthesis of oleic acid from the large amount of glucose that passes the placenta due to reduced maternal insulin sensitivity. The high neonatal and infant Mead acid contents likely reflect relative rather than absolute EFA and LCPUFA deficiency and may point at the importance of adequate insulin sensitivity and glucose homeostasis [37] in addition to sufficient, balanced, n-3 and n-6 fatty acids for optimal brain development .

Our study did not show between-group differences in the RCT, but we were able to demonstrate associations between the umbilical cord fatty acid compositions and developmental outcome at 18 months. Bouwstra et al. [7,10] also reported significant associations between umbilical cord fatty acid composition and neurodevelopmental status at 18 months. The associations may be explained by the fact that umbilical cord fatty acid compositions reflect long-term LCPUFA status, and are therefore more likely to show a relationship with neurodevelopment. The currently employed relatively short term, and relatively low maternal supplemental dose (i.e. 220 mg DHA/day) may not be sufficient to provide a higher, steady state, UV DHA content, despite higher maternal erythrocyte DHA contents [13] after DHA supplementation. Placental DHA supply to the fetus derives from both maternal intakes and stores [38]. Due to the low dose and short term of supplementation, infants in the placebo group may have higher DHA supply compared to infants in the DHA group when also the highly variable, but insufficient, background LCPUFA n-3 intake by

Dutch women (on average 84 mg LCPUFAn-3/day in 2003) [39] is taken into account.

It should be noted that especially short-term postnatal RCTs [40] but also prenatal maternal intervention studies [36,41] show merely subtle effects on neurodevelopment at toddler age, whereas association studies show relations between (parameters of) prenatal LCPUFA status and development [10,23,42–44]. DHA status might be no more than a proxy for socio-economic background, healthy life-style factors, or both [45], that may synergistically improve brain development. It is well known that cognitive development is related to socio-economic status. In addition, randomized controlled trials with single nutrients like LCPUFA, ignore the many possible interactions with other nutrients, and may therefore not be able to provide us with the proper information regarding intakes beneficial for e.g. brain development.

We conclude that relatively short-term maternal supplementation of a relatively low dose of DHA with or without ARA during pregnancy and lactation did not influence our neurological outcomes at toddler age. Long-term maternal LCPUFA intakes, as well as maternal insulin sensitivity during pregnancy, are likely to influence some parameters of brain development.

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