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Original Article

Docosahexaenoic acid status at 9 months is inversely associated with communicative skills in 3-year-old girls

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Abstract

The objective of the present observational study was to investigate if the docosahexaenoic acid (DHA) status assessed in infant erythrocytes (RBC) at 9 months was associated with the age when the infants reach developmental milestones and their psychomotor function at 3 years of age. Three hundred eleven healthy Danish children were followed from 9 months to 3 years of age (the SKOT cohort). RBC fatty acid composition was analysed by gas chromatography in 272 of the children. Milestone age was collected by questionnaires at 9 and 18 months and psychomotor development at 3 years of age was assessed by the parents using third edition of the Ages and Stages Questionnaire (ASQ-3). RBC DHA levels ranged from 2.2% to 12.6% of the RBC fatty acids. The age of reaching milestones correlated with psychomotor development, particularly with gross motor function at 3 years. An association between milestones and later personal and social skills was also observed, but only for girls. In girls, RBC–DHA was found to be inversely correlated with communication at 3 years of age (odds ratio = 0.69, 95% confidence interval: 0.56–0.86, $P = 0.001$), but no other associations with psychomotor development or milestones were found. The results from study indicate that DHA status at 9 months may not have a pronounced beneficial effect on psychomotor development in early childhood and that communicative skills at 3 years of age may even be inversely associated with early RBC–DHA levels in girls.

Keywords: n-3 fatty acids, brain development, communication, infant nutrition, cohort study.

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Introduction

The unique high content of n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA), primarily docosahexaenoic acid (DHA), in the central nervous system (CNS) has been shown to accumulate during the brain-growth spurt (Martinez 1992; Lauritzen *et al*. 2001). Studies have shown that breastfed children have higher levels of DHA in the brain (Farquharson *et al*. 1992; Makrides *et al*. 1994) and achieve higher scores in various developmental tests when compared with formula-fed children (Anderson *et al*. 1999; Sacker *et al*. 2006; Horta *et al*. 2007). However, randomized controlled trials with DHA-enriched infant formula have shown conflicting results (McCann & Ames 2005; Eilander *et al*. 2007). Possible reasons for the inconsistent results could be: timing of the supplementation, duration of the trial or the n-3 LCPUFA-status at baseline in the supplemented individuals (Calder *et al*. 2010). Furthermore, Caspi *et al*. (2007) and Steer *et al*. (2010) found that the cognitive advantages of the breastfeeding are modified by genetic differences in the LCPUFA metabolism (Caspi *et al*. 2007). The effect of DHA supplementation may also

be limited in groups with particular LCPUFA-metabolism gene variants (Tanaka *et al*. 2009).

Cohort studies have shown that early child development is positively associated with maternal seafood consumption during pregnancy (Hibbeln *et al*. 2007; Oken *et al*. 2008). Some studies have found a beneficial association between maternal fish oil supplementation during pregnancy and lactation and child development (Helland *et al*. 2003; Jensen *et al*. 2005; Judge *et al*. 2007), whereas others did not (Lauritzen *et al*. 2005). Some studies even points to a potential unbeneficial influence of high DHA intakes on language development in children (Scott *et al*. 1998; Lauritzen *et al*. 2005; Makrides *et al*. 2010; Cheatham *et al*. 2011). Most of the studies have focused on intake during pregnancy and the first months of life and few have included DHA status in erythrocytes (RBC) later in infancy. Two maternal fish oil-supplementation trials have found significant associations between high RBC–DHA levels and child development (Gibson *et al*. 1997; Dunstan *et al*. 2006), but again limited to the first months after birth.

A transition from breast milk as the main source of n-3 LCPUFA to fish products takes place during the complementary feeding period, when the child is adapting to the family diet. Observational studies suggest that plasma LCPUFA concentrations decrease as breast milk is replaced by solid food (Schwartz *et al*. 2009). The rate of CNS development and brain growth is still high during the complementary feeding period. We hypothesize that complementary feeding is a critical period with respect to n-3 LCPUFA, which affect structural and functional changes in the CNS with potential long-term programming consequences on development.

The aim of this study was to examine associations between the level of DHA in RBC at 9 months and the age by which the infant reach the World Health Organization-specified developmental milestones (Wijnhoven *et al*. 2004) and psychomotor developmental scores at 3 years of age, assessed by a questionnaire that determines communication, problem solving, fine and gross motor development, and personal and social abilities.

Methods

The study used data from the SKOT-cohort carried out in May 2007–October 2010 at the Department of Human Nutrition, University of Copenhagen (Madsen *et al*. 2010). The study was approved by the Scientific-Ethical Committees for Copenhagen and Frederiksberg (H-KF-2007–0003) and written informed consent was obtained from the parents of all participating children after they were informed about the study both orally and in writing.

Participants

The participants were recruited by age and address through the Danish Social Security Register and excluded if the child was a twin, born earlier than the thirty-seventh gestational week, or suffering from serious chronic diseases that could affect growth and dietary intake, or if the parents did not speak Danish. Invitations were send to 2211 families and we got 380 eligible positive responses, but in 46 cases, it was impossible to arrange meetings in accordance with the age limits for the child, four chose not to participate after all, and one was excluded because of late manifestation of a severe chronic disorder. Thus, 329 children were included and 18 of these dropped out before first examination visit, and one did not complete the background questionnaire (5%). The dietary

Key messages

- Docosahexaenoic acid (DHA) accumulates in the central nervous system (CNS) during early life. Diet can modulate CNS DHA content, which has developmental effect, especially on infant visual acuity. The importance for psychomotor development of intake of DHA in the complementary feeding period is unknown.
- DHA levels at 9 months are not associated with a pronounced beneficial effect on psychomotor development in early childhood. Communicative skills at 3 years of age may even be inversely associated with early DHA levels in girls.

Table 1. Birth and socio-demographic characteristics of the subjects

Variables	Mean ± SD	Range	*n*
Birth weight (g)	3541 ± 441		310
Boys	3639 ± 426	2500–4850	148
Girls	3451 ± 436	2404–4600	162
Birth length (cm)	52.1 ± 2.0		310
Boys	51.7 ± 2.0	48–58	148
Girls	52.6 ± 1.8	47–57	162
Age of the mother at delivery (years)	31.8 ± 4.6	19–46	310
Head circumference at 9 month (cm)	45.5 ± 1.4		310
Boys	46.2 ± 1.3	43.37–49.53	149
Girls	44.9 ± 1.2	41.73–49.67	161
Older siblings in the household	0.56 ± 0.81	0–6	310
Percent with none	58.7		180
1	31.0		96
≥2	10.3		32
Education – mother (years)	15.4 ± 1.8	7–17	305
Percent with <10 (primary to secondary school)	2.6		8
>10–13 (high school)	7.9		24
>13–15.5 (medium to long higher education)	49.5		151
>15.5 (long higher education)	40.0		122
Education – father (years)	15.3 ± 1.9	9–17	295
Percent with <10 (primary to secondary school)	1.7		5
>10–13 (high school)	10.8		32
>13–15.5 (medium to long higher education)	44.4		131
>15.5 (long higher education)	43.1		127
Household income (in 100 000 DK kr*)	%:		304
<2.0–3.49	13.8		42
3.5–6.49	28.0		85
6.5–7.49	23.0		70
7.5–>8.0	35.2		107

DKK, Danish Kroner; SD, standard deviation. *100 000 DKK equals approximately 13 500 Euro.

habits of the children at 9 months has been reported previously (Gondolf *et al*. 2012). The socio-demographic and birth characteristics of the infants are shown in Table 1.

Examinations were completed by 94%, 88% and 80% of the children at 9, 18 and 36 months of age, respectively. Anthropometric assessments, dietary recordings and questionnaires about growth, health and development of the child were performed at each visit. The 9-month examination also included a blood sample for which local anaesthetics (eutectic mixture of local anaesthetics patches with 1 g cream containing 25 mg g^{-1} lidocaine and 25 mg g^{-1} prilocaine, AstraZeneca AB, Södertälje, Sweden) was offered for the skin. Blood samples were successfully collected in 272 children at a mean age of 8.6 ± 0.3 months (range 7.5–9.4).

RBC fatty acid analysis

Within half an hour after blood sampling, RBC was isolated from a 2-mL lithium-heparin sample by centrifugation (10–15 min, 2300 × G, 4°C). Plasma and buffy coat were removed and the packed RBC was washed three times in 2–3 mL 150 mM NaOH, 1 mM ethylenediaminetetraacetic acid (EDTA) (5 min, 2300 × G, 4°C). The entire procedure was performed on ice and the final packed RBC were reconstituted 1:1 in 150 mM NaOH, 1 mM EDTA and two drops per millilitre RBC solution of 0.1% butylated hydroxytoluene in ethanol was added (final concentration of ~0.01%). The samples were blown with N_2 and frozen at –80°C. On the day of the analysis, lipid was extracted, fatty acid trans-methylated and the composition was determined by capillary gas chroma-

tography, as previously described (Andersen et al. 2011). The relative amounts of identified fatty acids are given as a percentage of the overall chromatogram area [percent of fatty acids (FA%)]. Blood sampling and RBC fatty acid analysis was successfully performed in 272 children.

Assessment of psychomotor development

Before attending the 9-month examinations, the parents filled out a questionnaire about the age at which their child reached milestones of gross motor development, as defined by the World Health Organization: Sitting without support, standing with assistance, hands-and-knee crawling, walking with assistance, standing alone and walking alone (Wijnhoven *et al*. 2004). The same questionnaire was answered before the 18-month examination to provide information about milestones achievements from 9 to 18 months of age. Of the children with RBC data, 262 children also had milestone data.

At 3 years of age, psychomotor development were assessed by the parent in the third edition of Ages & Stages Questionnaires® (ASQ-3™; Squires & Bricker 2009), which is designed as a screening tool for developmental delays within abilities, such as communication, gross motor skills, fine motor skills, problem solving and personal and social skills. The questionnaire was translated into Danish in corporation with a professor in psychology. The questionnaire consisted of 30 questions (six per ability), which could be answered with either 'yes' (=10), 'sometimes' (=5) or 'not yet' (=0) – resulting in a score of 0–60 for each ability. If more than two of the six questions were not answered, then that ability was not scored for the child. The average age for answering the ASQ-3 was 37.2 ± 1.3 months with a range of 34.5–40.6 months. However, 14 children were excluded because the ASQ-3 was filled out >38.9 months, leaving 241 for analysis of ASQ-3 skills vs. RBC–DHA.

Statistics

Data are presented as mean ± standard deviation for normally distributed variables, as percent for categorical variables or as median and interquartile range for not normally distributed variables. All statistical analysis was performed with STATA™ 10.0 (Stata Corporation, College Station, TX, USA) and level of significance was set to 5% (*two-sided tests*).

Several of the psychomotor development variables were dependent on gender and all analysis was thus, carried out separately for each gender. Student's *t*-test was used to compare means of continuous outcomes between boys and girls, whereas Mann–Whitney *U*-test was applied for not normally distributed continuous and ordinal variables. The ASQ-3 data were not normally distributed, and scores for each ASQ-3 skill was grouped into four approximately equal-sized groups (near-quartiles); 0–49.9, 50–54.9, 55–59.9 and 60. Correlation analysis between age of reaching milestones and 3-year ASQ-3 abilities scores was performed by Spearman's test.

Binary correlation analyses were also performed with RBC–DHA (FA%) and the outcome variables: the six milestone ages (as continuous variables in weeks) and the five ASQ-3 abilities (in score-interval groups). For the five ASQ-3 abilities, we furthermore performed ordinal logistic regression analysis, providing an estimate of the likelihood of obtaining a given score or more relative to a lower score with an increase in exposure.

Multiple regression analysis was performed for each of the six continuous milestone variables both controlling for sex and for boys and girls separately. Potential confounding factors and covariates to be included in the statistical analysis were picked if they had previously been shown to be, or they are theoretically inferred to be, associated with the outcome. We included all covariates of *a priori* interest, namely; infant sex, birth weight, maternal age at delivery, number of older siblings, head circumference at 9 months of age, maternal education, household income, age at introduction to fish and age at completion of the ASQ-3. Smoking in the home and during pregnancy has been shown to be potential confounders of psychomotor development (Auestad *et al*. 2003; Bakker *et al*. 2003; Oken *et al*. 2008). However, only four mothers had smoked 11–30 cigarettes per day during pregnancy and 19 < 10 cigarettes per day, six children came from a smoking home, and five children from an only almost non-smoking home (<1 cigarette

per day). Therefore, we did not include any of these smoking parameters in the analyses, and subsequent analysis showed that they did not appreciably change results (data not shown).

We performed multiple logistic regression analysis and analysis of covariance for each of the ASQ-3 abilities and age of reaching milestones, respectively. In both types of analysis, all the mentioned covariates were included in the first model, followed by a backward exclusion of non-significant variables ($P > 0.05$) one at a time by the variables with the highest P-value. Model plots of predicted values against standardized residuals were examined and a 'trumpet-shaped' fan opening pattern was seen for standing with support, so we used $\log_{10}$ of this variable to achieve a more random pattern.

Results

The majority of the children (64%) had fish introduced in their diet at around 8 months of age (♂ 8.1 ± 1.5 and ♀ 8.2 ± 1.6), and relatively few were given fish oil supplements (0.6% and 7.5% at 9 and 18 months of age, respectively). Table 2 show the overall RBC fatty acid composition at 9 months of age. DHA was found to range from 2.2 to 12.6 FA% (Fig. 1), and did not differ between boys and girls (data not shown).

We found that boys had faster milestone development – with respect to crawling and walking (Table 3). Girls were found to have higher scores at 3 years of age in the ASQ-3 abilities 'fine motor' and 'personal and social skills' (Table 3). We found no significant difference between boys and girls with respect to parental education and household income (data not shown).

No beneficial association was observed between RBC–DHA and age of reaching milestones (Supporting Information Table S1) and most of the ASQ-3 abilities at 3 years of age (Table 4). However, we did find an inverse association between RBC–DHA and the ASQ-3 ability 'communication', especially for girls (Table 4, Fig. 2), with the odds ratio for attaining a higher ASQ-3 score at 0.69 per percent increment in RBC–DHA.

Table 2. Erythrocyte fatty acid composition in the infants at 9 months of age

Fatty acid	Mean ± SD (FA %)
SFA	34.1 ± 3.0
MUFA	17.5 ± 2.0
PUFA	45.9 ± 4.4
n-6 PUFA	34.2 ± 3.5
18:2 n-6	12.93 ± 2.01
20:3 n-6	1.54 ± 0.40
20:4 n-6 (AA)	16.62 ± 2.90
22:4 n-6	3.16 ± 0.69
22:5 n-6	1.07 ± 1.01
n-3 PUFA	10.0 ± 2.4
18:3 n-3	0.23 ± 0.13
n-3 LCPUFA	9.80 ± 2.42
20:5 n-3 (EPA)	0.73 ± 0.37
22:5 n-3	2.26 ± 0.46
22:6 n-3 (DHA)	6.59 ± 1.97
Ratio n-6/n-3 PUFA	3.62 ± 1.01

SD, standard deviation; FA, fatty acid; n-3 LCPUFA, n-3 long-chain polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Values are given as mean ± SD ($n = 272$, 135 boys and 137 girls).

The ASQ-3 scores at 3 years of age correlated with age of reaching milestones (Table 5). For four out of six associations between the ASQ-3 ability 'gross motor skills' and milestones were significantly negative – i.e. those who reached the milestones fastest also had higher scores at 3 years of age. Separately, in the gender analysis, three of the milestone–ASQ-3 'gross motor' associations came out significant for the boys, whereas no correlations were seen for the girls. However, for the girls, we saw a significant negative correlation between age of reaching milestones and the ASQ-3 abilities 'fine motor' and 'personal and social skills' at 3 years of age.

Discussion

In this study of 272 healthy Danish children, we observed an inverse association between RBC–DHA level and communication for girls at 3 years of age, but no association was found for any other measures of psychomotor development. For boys, reaching milestones at young age correlated with higher gross motor development score at 3 years of age, whereas

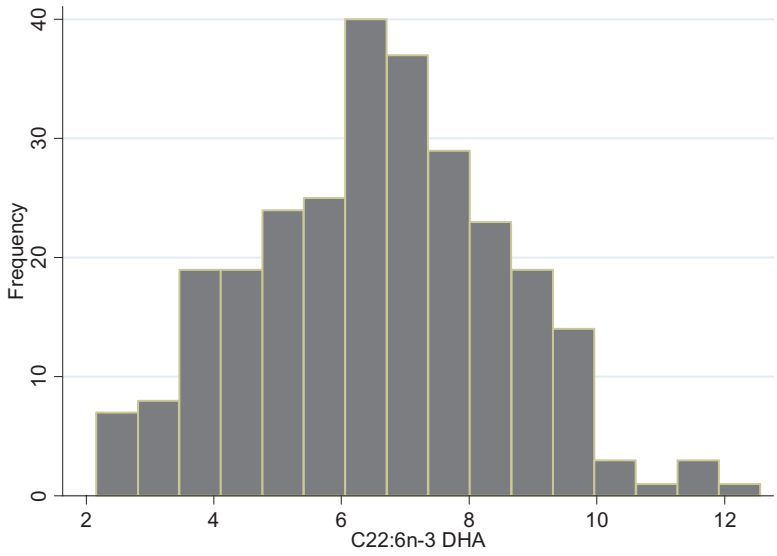

Fig. 1. Distribution histogram of erythrocyte docosahexaenoic acid levels in the children.

Table 3. Difference between the boys and the girls in psychomotor development assessed by age to reach World Health Organization milestones and ASQ-3 developmental scores at 3 years

	Girls	*n*	Boys	*n*	*P*-value
	Mean ± SD (weeks)		Mean ± SD (weeks)		
Age at reach of milestones					
Sitting without support	28.45 ± 4.55	160	28.56 ± 4.03	142	0.847
Standing with assistance	39.12 ± 8.04	140	38.28 ± 8.75	120	0.386
Hands- and knee-crawling	40.48 ± 9.76	136	38.36 ± 7.09	126	0.047
Walking with assistance	46.06 ± 8.94	129	43.56 ± 7.87	120	0.021
Standing alone	50.71 ± 6.91	119	49.66 ± 7.87	112	0.284
Walking alone	56.53 ± 6.95	133	54.67 ± 7.55	130	0.039
ASQ-3 sub-scales score	Median (IQR) ($n = 128$)		Median (IQR) ($n = 113$)		
Communication	50 (50–55)		50 (50–55)		0.142
Gross motor	60 (55–60)		60 (55–60)		0.802
Fine motor	55 (45–60)		50 (40–55)		0.002
Problem solving	55 (50–60)		55 (50–60)		0.940
Personal & social skills	60 (55–60)		55 (50–55)		<0.001

ASQ-3, Ages and Stages Questionnaire, third edition; SD, standard deviation; IQR, interquartile range. For age when reaching milestones for girls and boys the values in the groups are given as mean ± SD and *n* along with *P*-values from a Student's *t*-test comparison. Data for the ASQ-3 score for boys and girls at 3 years of age for each of the five ASQ-3 themes are not normally distributed, so values in the groups are given as median and IQR with 75–25 percentile along with *P*-values from Mann–Whitney *U*-test/two-sample Wilcoxon rank-sum comparison.

girls, who reached milestones early, had higher scores in fine motor development and personal and social skills at 3 years of age. Curiously, for each of the genders, milestones were associated with exactly the later psychomotor abilities where that gender seems to have their strong sides.

Our results point towards DHA as having a possible unbeneficial effect on language development, which is in conflict with studies that found a beneficial influence in relation to child development. However, results from earlier studies have looked at a number of different aspects of child development and the results in children born at term are inconsistent. Our results of an inverse association between DHA and communicative development in children are in line with previous studies (Scott *et al.* 1998; Lauritzen *et al.* 2005;

Table 4. The association between erythrocyte DHA and ASQ-3 score controlling for sex and for boys and girls separately

ASQ-3 score	Odds ratio	95% CI	*P*-value	Pseudo R^2
Communication	0.86†	(0.754–0.983)	0.026	0.0106
Boys‡	0.99	(0.839–1.188)	0.989	0.0000
Girls‡	0.69	(0.562–0.858)	0.001	0.0450
Gross motor	0.99†	(0.865–1.139)	0.916	0.0004
Boys‡	1.04	(0.859–1.249)	0.708	0.0007
Girls‡	0.94	(0.764–1.159)	0.564	0.0015
Fine motor	0.98†	(0.862–1.106)	0.709	0.0146
Boys‡	1.04	(0.872–1.226)	0.680	0.0007
Girls‡	0.91	(0.761–1.093)	0.318	0.0034
Problem solving	0.99†	(0.871–1.118)	0.829	0.0002
Boys‡	1.06	(0.897–1.248)	0.504	0.0016
Girls‡	0.90	(0.742–1.093)	0.290	0.0041
Personal & social skills	0.97†	(0.861–1.100)	0.664	0.0490
Boys‡	0.96	(0.824–1.131)	0.659	0.0007
Girls‡	0.98	(0.804–1.197)	0.847	0.0002

DHA, docosahexaenoic acid; ASQ-3, Ages and Stages Questionnaire, third edition; CI, confidence interval; FA%, percent of fatty acids. Values are given with odds ratio per FA% increment in erythrocyte DHA, CI along with *P*-value and pseudo R^2 †from ordinal logistic regression analysis controlling for sex and ‡from ordinal logistic regression analysis with boys and girls separately ($n = 212$, 109 girls and 103 boys). Pseudo R^2 is the part of the variance, which can be explained.

Makrides *et al*. 2010). In their most recent study, Makrides *et al*. found that girls exposed to a high dose of DHA *in utero*, via DHA supplementation of their pregnant mothers, had lower language scores assessed by the Bayley Scales of Infant Development (third edition) at 18 months of age and were more likely to have a delayed language development than girls from the control group (Makrides *et al*. 2010). Furthermore, in a previous study, we observed an inverse association between RBC–DHA at 4 months and MacArthur verbal comprehension at 12 months of age in children, whose mothers were supplemented with fish oil in the first 4 months of lactation (Lauritzen *et al*. 2005), but in this case, most pronounced in the boys.

At present, there are conflicting results on whether a negative effect of early intake of DHA on language development indicates a long-term overall slow development, or if the effect might just be transient. Scott *et al*. (1998) found an adverse effect on language development with the MacArthur Communicative Inventories at 14 months of age in children who were fed DHA-enriched formula (Scott *et al*. 1998), but this was only transient, as no difference was seen at 39 months of age in receptive and language expression assessed by the Revised Peabody Picture Vocabulary test (Scott *et al*. 1998; Auestad *et al*. 2003). On the other hand, in our previous study (Lauritzen *et al*. 2005), the decrement in language comprehension at 12 months was found to correlate with lower prosocial scores at 7 years of age – again only in boys (Cheatham *et al*. 2011).

Language acquisition in early childhood shows great variation, e.g. a Danish 3-year-old child has a mean vocabulary of 600 words with a variation of 500 words (Bleses *et al*. 2008b). Parental education has been shown to affect many aspects of psychomotor development (Kaplan *et al*. 2001), including language, which is found to correlate inversely with maternal education in both small children and toddlers (Fenson *et al*. 2000; Lauritzen *et al*. 2005). It is widely acknowledged that language acquisition occurs in interplay with innate abilities and experience with linguistic and social input. Therefore, it is possible that children of more educated parents are presented to a more complex language, prolonging their development process, but that they may speed up their language acquisition later. Of the five ASQ-3 abilities, only personal and social skills were correlated to years of maternal education in the present study (data not shown).

The assessment of psychomotor development in children is complex. Furthermore, comparison of

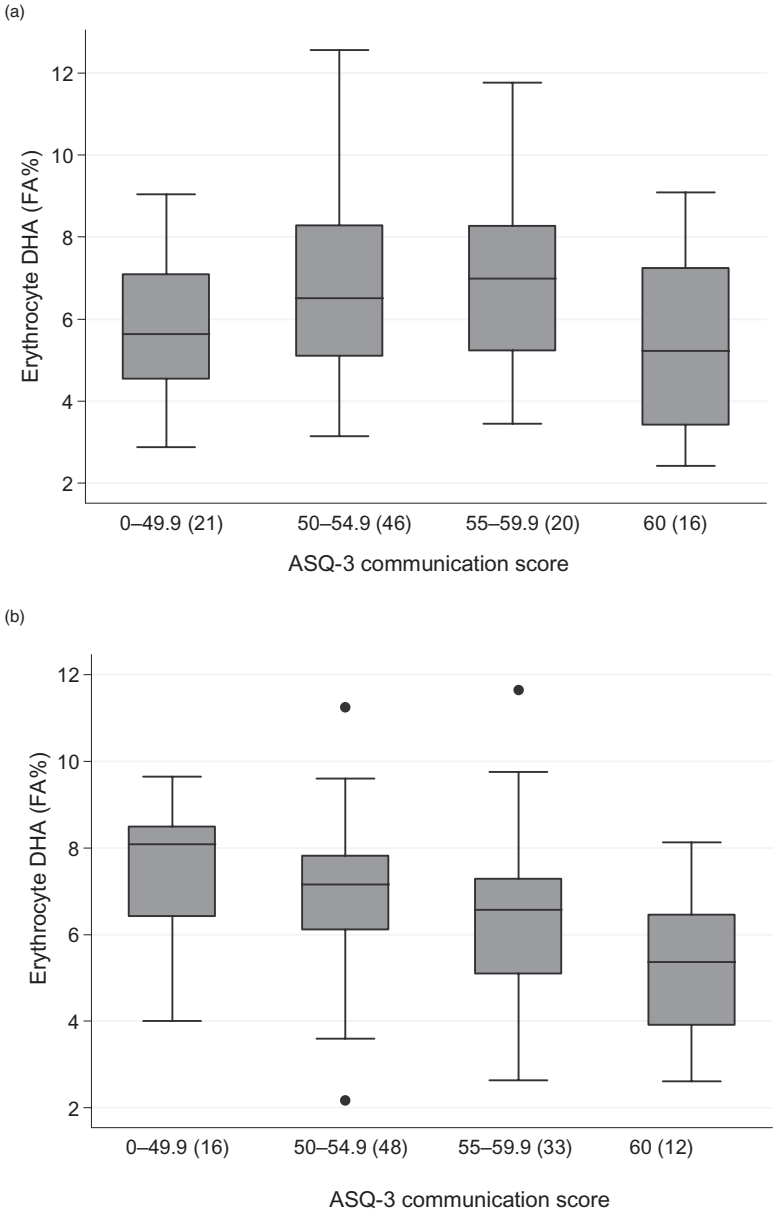

Fig. 2. Erythrocyte level of docosahexaenoic acid (DHA) in the different score groups for communicative ability assessed by Ages and Stages Questionnaire, third edition (ASQ-3) for boys (a) and girls (b). The boxes represent the individual values between twenty-fifth and seventy-fifth percentile, the horizontal being the median, the error bars gives the range of the values and dots represents outliers. DHA levels are given as percentage of all fatty acids in the erythrocytes (FA%).

results of different studies of the influence of DHA is often compromised by differences in age of supplementation, length of the follow-up period and types of tests. In the present study, child age at completion of the ASQ-3 questionnaire was explanatory for all skills except 'communication'. Thus, the developmental pattern for language and communication differed from that of other skills such as problem solving, personal and social skills, and gross motor development. Cultural differences could affect the appropriateness of an American questionnaire in Danish children. Compared with American children, Danish children spend more time in institutions from an early age, and this may contribute to a slow language development (Bleses *et al*. 2008a). Furthermore, the questions originate from other norms – e.g. one of the questions is whether the child when asked 'what is your name?' answers with their full name, which Danish children

Table 5. Correlations between 3-year ASQ-3 developmental score and age of reaching milestones for girls and boys separately and together

ASQ-3 score/milestones	Communication			Gross motor			Fine motor			Problem solving			Personal and social skills		
	Girls	Boys	All	Girls	Boys	All	Girls	Boys	All	Girls	Boys	All	Girls	Boys	All
Sitting without support	–0.164	0.101	–0.037	–0.163	–0.294 (0.002)	–0.227 (0.001)	–0.218 (0.015)	–0.082	–0.140 (0.032)	–0.065	–0.133	–0.093	–0.098	–0.100	–0.091
Standing with assistance	–0.139	–0.109	–0.119	–0.131	–0.225 (0.028)	–0.181 (0.009)	–0.278 (0.002)	–0.042	–0.139 (0.045)	–0.106	–0.129	–0.118	–0.187 (0.046)	–0.186	–0.144 (0.038)
Hands- and knee-crawling	–0.106	0.093	0.004	–0.018	–0.001	–0.012	–0.102	0.038	–0.011	–0.087	0.050	–0.021	–0.164	–0.081	–0.075
Walking with assistance	–0.139	–0.001	–0.049	–0.159	–0.136	–0.165 (0.017)	–0.133	0.039	–0.040	–0.144	–0.074	–0.121	–0.214 (0.024)	–0.157	–0.115
Standing alone	–0.079	–0.007	–0.030	–0.028	–0.207 (0.049)	–0.118	–0.011	–0.050	–0.025	–0.027	–0.114	–0.063	–0.229 (0.018)	–0.114	–0.136
Walking alone	–0.178	–0.041	–0.089	–0.069	–0.183	–0.132 (0.049)	–0.063	–0.017	–0.024	–0.022	–0.073	–0.042	–0.215 (0.019)	–0.046	–0.085

ASQ-3, Ages and Stages Questionnaire, third edition. The given values are correlations coefficients and *P*-values in brackets, if significant. Non-parametric correlations analysis: Spearman's rank correlations coefficients.

rarely do at any age. Moreover, as the questions are phrased, they assess the ability of the child to follow instructions in addition to actually understanding them. Thus, differences in the responses of girls and boys may be due to differences in temperament and upbringing.

With RBC fatty acid data from 272 children at 9 months of age, this study is unique in size compared with earlier studies. Furthermore, few studies have investigated associations during the complementary feeding period. The rather large variation in RBC–DHA in this cohort is a great advantage in an association study like the present study, as this increase power and the chance that it includes most of the relevant functional range. The variation in RBC–DHA in the present population is due in part to variation both in length of breastfeeding and intake of fish (L. Harsløf, 'unpublished observations'), which is relatively high in Danes compared with most other countries (Welch *et al.* 2002). This may compromise comparison with other studies exploring the relationship between early n-3 LCPUFA intake and cognitive function. It is also an advantage that the children are followed in a relatively long period with assessment of the development at various time points and both as age of reaching milestones and developmental scores at 3 years of age. The fact that we saw correlation between the two types of outcome measures shows a consistency in development and that the two questionnaires identify a continuum of development within the same domain. However, the children in this population approached the upper limits of the score range for many of the ASQ-3 abilities, gross motor development specifically, and this may have lowered the sensitivity of the ASQ-3 in relation to our research question. The ASQ-3 questionnaire was translated from English for the present study by professional linguists, and we therefore believe that previous validations on the questionnaire in English and other languages (Squires *et al.* 1997; Richter & Janson 2007; Gollenberg *et al.* 2010; and Guiberson *et al.* 2011) will also apply to this version, but we did not undertake a separate validation. Also, a limited sensitivity must be expected when using a screening questionnaire like the ASQ-3. The study has some other potential weaknesses, most notably the lack of a

measure of parental intelligence and assessment of the home environment; inclusion of these might have reduced noise. It is always reasonable to discuss whether a result could be due to chance, especially when only one of a number of outcomes turns out as significant. However, as the significant association was seen between RBC–DHA and communication, which is in line with earlier studies, the results should not just be disclaimed as due to chance.

Conclusion

Presently, we assume that the need for DHA the first 6 months of life is high, but we know little about the importance during complementary feeding. In this study, we wished to clarify the importance of RBC–DHA status during complementary feeding, but the results were not as hypothesized. Generally, no beneficial influence was seen for high DHA levels at 9 months on psychomotor development up to 3 years of age. However, communication deviated from the other skills as RBC–DHA level at 9 months of age was inversely associated with the ASQ-3 communication score at 3 years of age for girls. The study is the first study with RBC data from the weaning period and the results underline the need to look more into, whether the influence of DHA on communication is transient or linked to cognitive development in later childhood. Furthermore, the study raises a need to examine in more detail how and why boys and girls respond differently to high DHA levels.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Contributions

KFM and LL are responsible for the initial idea of the study, SE analysed and interpreted the data and wrote the initial draft of the manuscript with some assistance from KMHT and LL, who also provided statistical guidance in data analyses. LIH was responsible for the FA analysis, and he and KFM assisted in the interpretation of results. All co-authors participated in manuscript preparation and critically reviewed all sections of the text for important intellectual content.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Estimated linear relationships between docosahexaenoic acid status in erythrocytes at 9 month of age (FA%) and age of reaching developmental milestones in Danish boys and girls.