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A study of associations between early DHA status and fatty acid desaturase (*FADS*) SNP and developmental outcomes in children of obese mothers

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Abstract

DHA from diet or endogenous synthesis has been proposed to affect infant development, however, results are inconclusive. In this study, we aim to verify previously observed fatty acid desaturase gene cluster (*FADS*) SNP-specific associations with erythrocyte DHA status in 9-month-old children and sex-specific association with developmental outcomes. The study was performed in 166 children (55 % boys) of obese mothers. Erythrocyte fatty acid composition was analysed in blood-samples obtained at 9 months of age, and developmental outcomes assessed by the Ages and Stages Questionnaire at 3 years. Erythrocyte DHA level ranged from 4·4 to 9·9 % of fatty acids, but did not show any association with *FADS* SNP or other potential determinants. Regression analysis showed associations between erythrocyte DHA and scores for personal–social skills (β 1·8 (95 % CI 0·3, 3·3), $P=0·019$) and problem solving (β 3·4 (95 % CI 1·2, 5·6), $P=0·003$). A tendency was observed for an association in opposite direction between minor alleles (G-variant) of rs1535 and rs174575 and personal–social skills ($P=0·062$ and 0·068, respectively), which became significant when the SNP were combined based on their previously observed effect on erythrocyte DHA at 9 months of age (β 2·6 (95 % CI 0·01, 5·1), $P=0·011$). Sex–SNP interaction was indicated for rs174575 genotype on fine motor scores ($P=0·016$), due to higher scores among minor allele carrying girls ($P=0·043$), whereas no effect was seen among boys. In conclusion, DHA-increasing *FADS* SNP and erythrocyte DHA status were consistently associated with improved personal–social skills in this small cohort of children of obese mothers irrespective of sex, but the sample was too small to verify potential sex-specific effects.

Key words: Fish oil: *n*-3 PUFA: Cognitive function: Child development: Programming

Early intake of DHA (C22 : 6*n*-3) has been shown to affect the rate of DHA accumulation in the brain during the early brain growth spurt and has thus been hypothesised to affect cognitive development in children[1,2]. Many studies have investigated this hypothesis; but the findings are inconsistent. Some randomised controlled trials with maternal fish oil supplementation during pregnancy and/or lactation and trials with addition of DHA to infant formulas have shown beneficial effects on cognitive development, whereas others have failed to show a relationship[1].

The main source of dietary DHA during infancy is breastmilk, whereas fatty fish takes over as the dominating source later in life. Furthermore, like other long-chain PUFA (LCPUFA), DHA can also be formed endogenously from dietary α-linolenic acid. The endogenous DHA synthesis is generally low, but is up-regulated in pregnant women and young infants[2]. SNP in the gene cluster that encodes for the fatty acid desaturases (*FADS*) responsible for the endogenous formation of LCPUFA have been shown to modify LCPUFA concentrations in the blood of pregnant women and infants as well as in breastmilk. Studies generally show that minor allele carriers of *FADS* SNP exhibit a decreased synthesis of LCPUFA, as reflected mainly by an increase in dihomo-γ-linolenic acid (20 : 3*n*-6) relative to arachidonic acid (20 : 4*n*-6) in breastmilk and erythrocytes, whereas DHA levels are less affected[1]. However, we have demonstrated differential effects of three *FADS* tag-SNP on erythrocyte DHA status in 9-month-old infants, as minor allele carriers (the G-variant) of rs1535 had an increased status, whereas minor alleles of rs174448 (G-variant) and rs174575 (G-variant) had lower DHA, but none of the SNP were associated with erythrocyte DHA status at 3 years of age[3].

Abbreviations: FA%, percentage of the overall chromatogram area, equivalent to percentage of fatty acids by weight; *FADS*, fatty acid desaturase gene cluster; LCPUFA, long-chain PUFA (≥20 carbon atoms and ≥3 double bonds); SKOT, Småbørns Kost Og Trivsel.

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Observational studies have rather consistently shown that breast-feeding is associated with improved intelligence quotient (IQ) in childhood[4]. The effect of breast-feeding on IQ has been shown to be modified by SNP in the *FADS* gene cluster, although the results of these studies may differ[1]. Some of the randomised trials on the effect of maternal pre- or postnatal LCPUFA supplementation on child cognitive development have shown different outcomes in boys and girls[5–7]. We have in a Danish cohort, the Småbørns Kost Og Trivsel (SKOT)-1 Cohort, also found sex-dependent associations between erythrocyte DHA at 9 months and cognitive outcomes at 3 years of age[8]. Furthermore, these associations were found to be even more pronounced when examined according to the *FADS* genotype (rs1535, rs174448 and rs174575) and the effects differed between SNP in agreement with their effects on erythrocyte DHA during infancy[9]. DHA-increasing *FADS* alleles were found to result in improved communication and problem-solving abilities in boys, but reduced scores in girls.

A systematic review has shown that obese mothers are less likely to initiate breast-feeding and also breast-feed for a shorter duration compared with normal-weight mothers[10] and studies have observed a decline in cognitive scores of children with increasing maternal BMI[11]. The sub-optimal developmental scores could to some extent be linked to a reduced intake of DHA, but the differences could also merely reflect differences in social class and educational level[11]. The aim of the present study was to see if we could verify previously observed associations between erythrocyte DHA status at 9 months, *FADS* SNP and developmental outcomes assessed by the 36-month Ages and Stages Questionnaire (ASQ) among children of obese mothers in the SKOT-2 Cohort[12]. Our hypothesis was that minor alleles (G) of rs1535 would have effects in the opposite direction of rs174448 and rs174575, and that DHA-increasing alleles would increase developmental scores in boys, whereas it would tend to have the opposite effect in girls.

Methods

The present study used data from the SKOT-2 Cohort carried out from January 2011 to March 2015 at the Department of Nutrition, Exercise and Sports, University of Copenhagen[12]. The study was approved by the Research Ethical Committee for the Capital Region of Denmark (journal no. H-3-2010-122) and has been registered on ClinicalTrials.gov (NCT02377973). Written informed consent was obtained from the parents after they were informed about the study.

Participants

Children recruited to the SKOT-2 Cohort were born from mothers who had participated in the Treatment of Obese Pregnant women (TOP) Study at Hvidovre hospital (journal no. H-D-2008-119). Participants included in the TOP Study were pregnant women with a pre-pregnancy BMI $\geq 30\,kg/m^2$ and the aim of this randomised trial was to investigate if physical activity and/or dietary intervention could diminish gestational weight gain[13]. If gestational diabetes was diagnosed, care continued in the diabetic clinic, but the patient was not excluded from the study. The children were excluded from SKOT-2, if they were born premature that is before the 37th week of gestation; or as a twin; or were suffering from severe chronic diseases expected to affect growth and dietary intake; or if their parents were non-Danish speakers. Recruitment for the SKOT-2 study was initiated 1 year and 7 months after commencement of the TOP study.

Invitations were sent to 208 families in the TOP study (of a total of 425), who were eligible for the SKOT-2 study, and we received a positive response from 184 families. In all, eleven families dropped out before the first examination, and twelve families dropped out during the study period. A total of 166 children participated in the first examination when the children were 9 months of age (90 %), and a total of 129 families (70 %) completed the third examination at 3 years of age.

All assessments of the children were conducted similarly to the SKOT-1 Cohort study[8]. In short, at each examination, parents completed questionnaires regarding background variables, such as education and physical activity, in addition to questions regarding breast-feeding and introduction of solid foods since the last visit. Maternal weight and blood pressure were measured at all examinations. Anthropometrics, blood pressure and dietary intake of the children were obtained at all examinations. In addition, blood samples were collected at the 9-month examination. Before blood sampling, EMLA patches (Astra Zeneca AB) with local anaesthetic were applied at home by the parents, in order to minimise discomfort for the child. Blood samples were successfully obtained from 130 children (78 %) at the 9-month examination.

Erythrocyte DHA fatty acid analysis

Blood samples for fatty acid analysis were collected in lithium–heparin-coated tubes in order to prevent coagulation. Erythrocytes were isolated from the blood samples, which before the purification process (maximum 3 h) and during the procedure were stored on ice. The lithium—heparin blood (2–4 ml) was centrifuged for 10 min at 2300 ***g***, 4°C followed by removal of plasma. Thereafter, the packed erythrocytes were washed three times. At each wash, 2–4 ml of 150 mM-NaCl mixed with 1 mM-EDTA were added to the erythrocytes, and then, centrifuged for 5 min at 2300 ***g***, 4°C. After centrifugation, the supernatant and any residual 'buffy coat' that is thrombocytes and leucocytes were removed using a pipette. When the third wash was completed, 150 mM-NaCl mixed with 1 mM-EDTA was added in the same amount as erythrocytes plus two drops 0·1 % butylated hydroxytoluene in ethanol per ml of blood. Finally, the tube was purged with N_2 before the lids were closed, and stored at −80°C until further analysis at the Technical University of Denmark.

On the day of the analysis, the erythrocytes were haemolysed in redistilled water, and the lipids were extracted by the Folch procedure[14]. The extracted fatty acids were trans-methylated with BF_3 in methanolic sodium hydroxide followed by an extraction of the fatty acid methyl esters with heptane. The composition of the fatty acids was determined by GLC on an HP-6800 (Hewlett-Packard) equipped with an SP2380 column. The injector and detector temperatures were 270°C and the

initial oven temperature was 70°C for 30 s. He was used as the carrier gas with a constant flow of 1·2 ml/min. To determine the fatty acid methyl esters on the resulting chromatograms the peak areas were identified from retention times of commercial standards (Nu-Chek-Prep Inc.). Approximately 97 % of the chromatogram areas were identified. Calculation of the relative content of specific fatty acids in the erythrocytes was based on their contribution to the total identified chromatogram area and expressed as the percentage of the overall chromatogram area that is percentage of fatty acids (FA%)[9,15].

Selection of SNP and genotyping

In the present study, we aimed to verify our previous findings and therefore we analysed the same three SNP (rs1535, rs174575 and rs174448) as in our earlier study. We have reported the rationale for choosing the three selected *FADS* SNP in our previously published study[3]. The three selected SNP are tag-SNP and together they tag more than twenty-eight SNP in the 100-kb genomic region of the *FADS1*, *FADS2* and *FADS3* gene cluster according to the international HapMap data for European ancestry (release no. 28, NCBI build 36).

Buffy coat was isolated from EDTA-treated blood samples and kept at −80°C. DNA was extracted by LGC DNA extraction services and diluted to a concentration of 100 ng/μl (LGC). Participants (n 131) were genotyped by the Illumina Infinium HumanCoreExome Beadchip platform (Illumina). Genotypes were called using the Genotyping module (version 1.9.4) of GenomeStudio software (version 2011.1; Illumina). Data quality control involved ethnicity checks using principal component analysis and checks on heterogenicity. We removed individuals identified as ethnic outliers or with extreme positive or negative inbreeding coefficient, sample duplicates or samples with missing or wrong sex code, leaving 115 individuals who passed all quality control criteria. We applied a 95 % genotype call-rate criteria for inclusion of SNP. Two variants, rs1535 and rs174448 were retrieved from the directly genotyped data set. Additional genotypes were successfully imputed into 1000 genomes phase 1[16] using impute 2[17] for 112 individuals. The imputation quality was high (>0·95) for the imputed variant rs174575 that is included in the current analysis with its dosage information.

Assessment of behaviour

Following the third examination, the children's cognitive development was assessed using the ASQ-3, '36-month' questionnaire. The parents were instructed by an investigator about how to answer the questionnaire at home. The parents were encouraged to fill it out when the child was fed and rested in order to make the testing a playful and fun activity for both parent and child.

The ASQ-3 questionnaire is a screening tool designed to identify potential areas in the child's development that cause concern or demand extra attention[18]. The original American ASQ-3 questionnaire has been translated into Danish. The '36-month' questionnaire consists of thirty questions divided into five sub-categories: gross motor, fine motor, communication, problem solving and personal–social skills. Within each sub-category, the level of difficulty increases from question 1 to 6. The questions should be answered with a mark at 'yes', 'sometimes' or 'not yet', which were awarded 10, 5 or 0 points, respectively, making 60 the maximum obtainable score in each of the five sub-categories. The questionnaire also included some overall questions where parents were able to express possible concerns regarding the child's development for example hearing, vision or medical concerns.

Of the 129 children who participated in the 36-month examination, 113 subjects returned successfully answered ASQ-3 questionnaires. However, there was a slight variation in sample size (n) between the five sub-categories, as some of the questionnaires were insufficiently answered. If more than two of six questions within one ASQ sub-category were unanswered, the entire sub-category was excluded for that specific child. Thus, two children were excluded from the sub-categories 'communication', one from 'fine motor' and three from 'problem solving'.

Statistics

Data are presented as means and standard deviations or medians and interquartile range as appropriate. Sample size varied from a total of 166 participating infants to 111 in analyses of *FADS* SNP *v.* erythrocyte DHA as outcome and from seventy-five to eighty-three *v.* ASQ outcomes, and this was further reduced by 23 % and 7–11 % in analysis with covariates (mainly due to lack of information on birth weight and parity). Unpaired t tests were used to compare means of normally distributed continuous variables in boys and girls, whereas the non-parametric Mann–Whitney U test/two-sample Wilcoxon rank-sum comparison was used to compare medians between non-normally distributed continuous or ordinal variables. All statistical analyses were carried out using R Studio (version 0.98.1091 – 2009-2014R; Studio Inc.), and the level of significance (P) was set to 0·05.

Associations between erythrocyte DHA level and cognitive development were examined by multiple linear regression analyses. The analyses were performed separately for the five ASQ sub-categories. The ASQ scores were non-normally distributed as the distribution were skewed to the left, therefore, the scale was reversed and log transformed (as log (61–score)) to achieve an acceptable normal distribution of the data. The following potential confounding factors and covariates were included in the initial multiple linear regression models: infant sex, birth weight, head circumference at 9 months of age, maternal weight at the first examination, maternal age at delivery, number of older siblings, household income, highest household education, duration of exclusive breast-feeding and age at introduction to fish. Smoking was not included, as only four mothers smoked during pregnancy and smoking only occurred in the home of six children. Backward elimination of non-significant variables ($P>0·05$) was performed. Model control was performed by examining plots of residuals for the assumption of linearity, homoscedasticity and normal distribution. The final model used to examine the association between DHA status and cognitive development included five covariates besides erythrocyte DHA, that is duration of exclusive

breast-feeding, age at introduction to fish, number of older siblings, sex and head circumference at 9 months of age. Potential main effects and interactions between DHA status and infant sex were examined in separate analyses with *P*-value cut-off points set to <0·05 and <0·1, respectively.

The degree of linkage between the three *FADS* SNP in our study population was assessed by Pearson's correlation coefficient. Hardy–Weinberg equilibrium was calculated using χ^2 tests. Multiple linear regression analyses were performed to examine the importance of *FADS* SNP as determinants of erythrocyte DHA status at 9 months of age and their associations with ASQ outcomes. In the first of these analysis, we used the variables, which were significantly associated with erythrocyte DHA status in our previous study when available, that is all three *FADS* SNP, sex, birth weight, parity, still breast-fed at 9 months and duration of breast-feeding. Age at introduction to fish was used, as fish intake was not available in all subjects. Due to the low number of minor allele homozygotes, we used a dominant model, comparing minor allele carriers (homozygotes plus heterozygotes) with major allele homozygotes in the three-SNP models for the ASQ outcomes. Number of older siblings in the household and highest household education was included in the ASQ analyses together with the three *FADS* SNP, sex, birth weight and duration of exclusive breast-feeding. Furthermore, interaction between *FADS* SNP and sex were included in separate ASQ models.

Results

Despite being born of obese mothers, who maintained a mean body weight of about 100 kg at 9 months after delivery, the mean birth characteristics of the children were generally within normal range, although with some variation (Table 1). Breast-feeding was initiated in most of the children (97 %), but only 30 % were still breast-fed at 9 months of age. However, 42 % of those who were still breast-fed at 9 months were also given infant formula. The median duration of exclusive breast-feeding was 4·0 months for girls and 3·2 months for boys, but the difference was not significant ($P = 0·188$). The children were introduced to fish at about 7 months of age, and no difference was found between boys and girls ($P = 0·612$).

The erythrocyte fatty acid composition at 9 months of age was successfully analysed in all blood samples (online Supplementary Table S1). The erythrocyte DHA level in the children ranged from 4·4 to 9·9 FA% with a mean of 6·5 FA% (Fig. 1). Girls had higher erythrocytes total *n*-3 PUFA and DHA status compared with boys (*n*-3 PUFA: 9·5 (SD 1·2) *v.* 9·1 (SD 1·2) FA%, $P = 0·076$ and DHA: 6·7 (SD 1·0) *v.* 6·4 (SD 0·9) FA%, $P = 0·047$, *n* 130).

We had directly genotyped information on *FADS* SNP rs1535 and rs174448 in 115 of the subjects, and imputed dosage information for rs174575 in 112 of the children (online Supplementary Table S2). The three SNP were found to be moderately linked

Table 1. Characteristics of the participants
(Mean values and standard deviations or total (*n*) for males (M) and females (F) separately; medians, 25th and 75th percentiles; percentages)

	Mean	SD	Total (*n*)
Birth, breast-feeding and fish intake			
Sex, M:F (*n*)	91:75		
Birth weight (g)	3678	572	145
Birth length (cm)	52·3	2·4	144
Head circumference at 9 months (cm)	45·0	1·4	166
Breast-feeding initiation (%)	97·0		166
Duration of exclusive breast-feeding (months)			159
Median	4·0		
25th and 75th percentiles	1·0, 5·0		
Infants still breast-fed at 9 months (%)	30·0		160
Age for cessation of breast-feeding among the non-breast-fed at 9 months			112
Median	3·5		
25th and 75th percentiles	1·5, 6·5		
Age at introduction to fish (months)			160
Median	7·0		
25th and 75th percentiles	6·0, 8·0		
Fish oil supplementation at 9 months (%)	0·6		166
Maternal characteristics			
Age at delivery (years)	31·7	4·5	145
Parity, primiparous:multiparous (%)	60·4:39·6		144
Gestational weight gain (kg)	11·5	7·1	141
Maternal weight at first (9 months) examination (kg)	99·0	13·9	157
Smoking during pregnancy (%)	2·8		145
Highest household education (%)			166
Primary or secondary school, vocational education, specialised worker (≤11·5 years)	0·6		1
Secondary school, apprentice, vocational education (>11·5 years)	22·9		38
Short academic education (<3 to 12–15 years total)	10·2		17
Medium-long academic education (3–4 to 12·5–16 years total)	37·4		62
Long academic education (>4 to 17–18 years total)	25·3		42
Older siblings in the household (%)			166
0	62·1		103
1	33·1		55
≥2	4·8		8

(*r* 0·51–0·73, online Supplementary Table S3). A multivariate analysis of erythrocyte DHA at 9 months of age *v.* potential determinants; that is the three *FADS* SNP and breast-feeding status at 9 months of age, duration of exclusive breast-feeding, age at introduction of fish, sex, birth weight and parity; did not show any significant association for the *FADS* SNP or any of the other potential determinants (online Supplementary Table S4).

The developmental outcomes were successfully determined by ASQ-3 in 113 of the children at 3 years of age (Table 2). Girls generally performed superior to boys for all sub-categories except gross motor skill. This may not be surprising as most of the children (61% of the boys and 57% of the girls) had a maximum score in the gross motor category, thus, giving rise to a low power to discriminate and consequently, gross motor development was not examined for any correlations with erythrocyte DHA or *FADS* SNP.

Multiple linear regression analyses of associations between erythrocyte DHA and the ASQ sub-categories adjusted for duration of exclusive breast-feeding, age at introduction to fish, number of older siblings, sex and head circumference at 9 months of age showed significant associations for problem solving and personal–social skills (Fig. 2). No association was observed for communication and fine motor scores (β 1·83 (SE 1·60), $P=0·258$, adjusted R^2 0·126 and β 0·03 (SE 0·95), $P=0·977$, adjusted R^2 0·025, n 77). Furthermore, no interaction was observed between sex and erythrocyte DHA for any of the four ASQ-3 sub-scores ($P>0·1$), but the associations for problem solving and personal–social skills were mostly driven by the boys (data not shown).

The regression analysis for *FADS* SNP and the ASQ scores adjusted for sex, birth weight, duration of exclusive breast-feeding, number of older siblings and highest household education showed essentially zero explained variance for communication and problem solving (see online Supplementary Table S5) and thus, could not give rise to any meaningful associations. A tendency was observed for a borderline significant association in opposite direction between the personal and social skill scores and two of the *FADS* SNP, rs1535 and rs174575 with an effect size of about 5-score-point difference between minor allele carriers and major allele homozygotes (Table 3). A significant association was observed when the three *FADS* SNP were combined based on their previously observed effect on erythrocyte DHA at 9 months of age[3], showing increasing personal and social skill scores at increasing number of DHA-increasing *FADS* alleles (Fig. 3). No effect modifications by sex were indicated for personal–social skills neither in the three-SNP model nor in the combined DHA-increasing allele model ($P>0·1$). However, sex–SNP interactions were indicated for rs174575 on the fine motor score (Table 3). In the subsequent stratified analysis, this interaction was found to be driven mainly by significantly higher scores in minor rs174575 allele carrying girls (16·88 (95% CI 0·64, 33·11), $P=0·043$), whereas minor allele carrying boys did not differ from the major allele homozygotes (−5·12 (95% CI −22·11, 12·42), $P=0·556$). As the effect could potentially be mediated by differences in birth weight and breast-feeding, these analyses were also performed without these covariates with essentially similar results (online Supplementary Table S6).

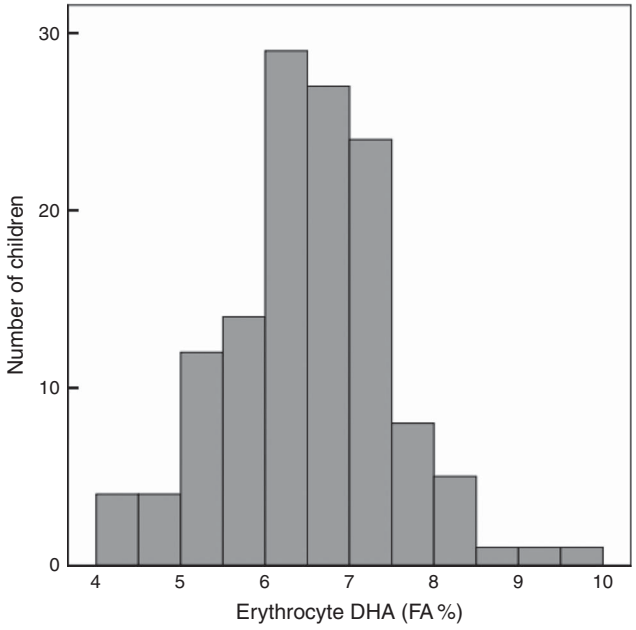

Fig. 1. Distribution histogram of erythrocyte DHA in percentage of all fatty acids (FA%) at 9 months of age (n 130). Mean = 6·5, SD = 1·0.

Discussion

There was a broad range in erythrocyte DHA levels among the children included in this present analysis, but no associations

Table 2. Scores from the Ages and Stages Questionnaire at 3 years of age* (Medians, 25th and 75th percentiles, ranges and percentages)

	Boys					Girls					
	Median	25th and 75th percentiles	Range‡	*n*	%	Median	25th and 75th percentiles	Range	*n*	%	*P*†
Communication	50	45, 53	30–60	63	11·1	50	50, 55	40–60	49	16·3	0·012
Gross motor	60	54, 60	20–60	64	61·0	60	50, 60	30–60	49	57·1	0·484
Fine motor	45	30, 50	10–60	63	8·0	50	45, 60	25–60	49	34·7	<0·001
Problem solving	50	40, 55	15–60	62	22·6	55	50, 60	25–60	48	33·3	0·028
Personal–social skills	50	45, 55	35–60	64	15·6	55	55, 60	40–60	49	40·8	<0·001

* Children with a total score of 60, which is the highest obtainable score.
† *P*-values from Mann–Whitney *U* test/two-sample Wilcoxon rank-sum comparison.
‡ Minimum and maximum scores obtained.

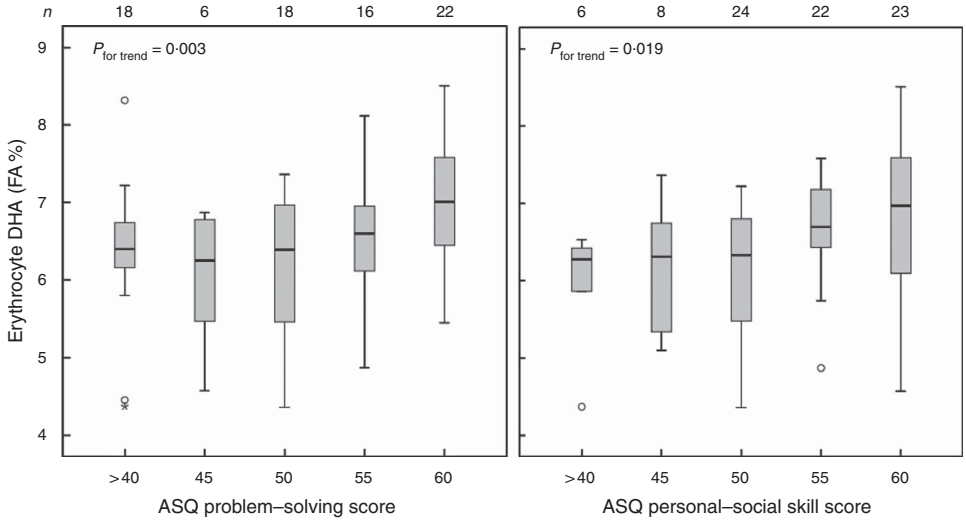

Fig. 2. Boxplot of erythrocyte DHA in percentage of all fatty acids (FA%) plotted against scores in the sub-categories 'problem solving' and 'personal–social skills' from the Ages and Stages Questionnaire (ASQ) at 3 years of age. The numbers on top of the plot represents the number of children (*n*) in each bar, total *n* in problem solving = 80 and personal–social skills = 83. The *P*-values derived from multiple linear regression analyses of associations between the two ASQ sub-categories and erythrocyte DHA adjusted for duration of exclusive breast-feeding, age at introduction to fish, number of older siblings, sex and head circumference at 9 months of age (back-transformed regression coefficient (β) 3·4 (95 % CI 1·2, 5·6) point increase in score per 1 FA% increase in DHA $P = 0{\cdot}003$, *n* 75 for problem solving and β 1·8 (95 % CI 0·3, 3·3), $P = 0{\cdot}019$, *n* 78 for personal–social skills).

Table 3. Association between scores in the Ages and Stages Questionnaire at 3 years of age and SNP in the fatty acid desaturase (*FADS*) gene cluster* (Back-transformed difference for minor allele carriers *v.* major allele carriers (reference), standard errors and 95 % confidence intervals; adjusted R^2; *n* 67)

	rs1535				rs174575				rs174448				
	Difference	SE	95 % CI	*P*	Difference	SE	95 % CI	*P*	Difference	SE	95 % CI	*P*	R^2
Fine motor													
Main effect	1·42	6·28	−11·17, 14·01	0·822	2·93	5·90	−8·90, 14·75	0·622	−2·38	4·70	−11·80, 7·04	0·614	0·050
Sex–SNP interaction	15·79	13·26	−10·81, 42·39	0·239	−31·56	12·67	−56·98, 6·14	0·016	2·36	9·93	−17·56, 22·28	0·813	0·114
Personal–social skills													
Main effect	5·35	2·81	−0·27, 10·97	0·062	−4·90	2·64	−10·18, 0·38	0·068	−3·05	2·10	−7·25, 1·16	0·153	0·251
Sex–SNP interaction	7·28	6·09	−4·94, 19·50	0·238	−3·92	5·82	−15·59, 7·76	0·504	−2·38	4·56	−11·53, 6·77	0·604	0·229

* The associations were examined by multiple linear regression analyses with inclusion of all three *FADS* SNP and adjusted for sex, duration of exclusive breast-feeding, number of older siblings, highest household education and birth weight. Results for main effects and sex × SNP interactions are from separate analyses.

were observed with *FADS* SNP or any of the other potential determinants. Regression analysis showed associations between erythrocyte DHA at 9 months and the 3-year ASQ scores for personal–social skills and problem solving. A tendency was observed for an association in opposite direction between *FADS* SNP rs1535 and rs174575 on personal and social skill scores, which became significant, when the SNP were combined based on observed effects on erythrocyte DHA at 9 months of age in the SKOT-1 Cohort[3]. A sex–SNP interaction was indicated for rs174575 on fine motor scores due to higher scores among minor allele carrying girls and lower scores in boys relative to major allele homozygotes of the same sex.

No associations were observed between *FADS* SNP and erythrocyte DHA in the present study, thus, not verifying the previously observed DHA-increasing effect of rs1535 minor alleles as opposed to the DHA-decreasing effects of minor alleles of rs174448 and rs174575 in 9-month-old infants, although the levels and range in erythrocyte DHA was similar to that in this previous study[3]. The present analysis does therefore not support the rationale behind our subsequent analyses of associations with ASQ outcomes. The lack of association could, however, be due to the lower number of children in the present study, which due to the linking of the SNP greatly reduce the number of children in the different haplotypes on which the SNP differentiation is based on. Furthermore, in the present study, we could not find associations between erythrocyte DHA status and breast-feeding or fish intake, which has been well-proven in a number of studies[3,19–21]. These effects should normally be detectable in eighty-six children, especially since our population had a broad range in erythrocyte DHA and breast-feeding. A possible explanation could be that the effect of breast-feeding may presumably be reduced now that most commercial infant formulas contain LCPUFA, and a major part of this shift took place between this and our previous study. In addition, almost half of the breast-fed children were also given infant formula. Some imprecisions were also introduced as we had to use age of introduction of fish instead of actual fish intake because dietary

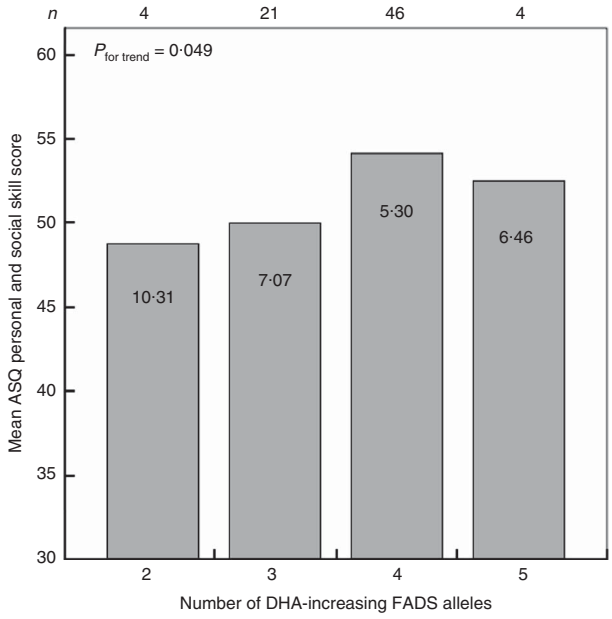

Fig. 3. Barplot of number of DHA-increasing alleles in the fatty acid desaturase gene cluster (*FADS*) plotted against the mean personal and social skill score from the Ages and Stages Questionnaire (ASQ) at 3 years of age. The numbers on top of the plot represents the number of children (*n*) in each bar, total *n* 75 and the numbers within each bar represents the standard deviation. The given *P*-value is from a multiple linear regression analysis of association between number of DHA-increasing *FADS* alleles and the personal and social skill score adjusted for infant sex, birth weight, duration of exclusive breast-feeding, number of older siblings and highest household education (back-transformed regression coefficients (*β*) was 2·55 (95 % CI 0·01, 5·09) score points per DHA-increasing allele, *n* 67).

information was missing in 15 % of the children[12]. The median intake of fish for the infants with data was 7 g/d (mean: 8·3 (SD 6·8) g/d) at 9 months of age, which is similar to what we previously have found in Danish infants[3]. As the present study could not verify the influence of these tested major determinants on erythrocyte DHA status, we cannot deduce anything specific from the lack of an effect of *FADS* SNP or differences between SNP on erythrocyte DHA.

To increase robustness we used a dominant model, comparing minor allele carriers (the few homozygotes plus the heterozygotes) with major allele homozygotes in the models with *FADS* SNP, although minor alleles of various *FADS* SNP have been shown to exert an additive effect on blood PUFA levels[3]. Furthermore, we performed multiple tests and did not employ any adjustments, as many of the tests were to some degree related (because of associations between the different cognitive outcomes and exposures), which means that the correction would be much smaller than a standard Bonferroni correction. The association between erythrocyte DHA status and *FADS* SNP with developmental outcomes was somewhat in agreement with expectations from the SKOT-1 Cohort[9]. Opposite effects of rs1535 and the two other SNP were seen only with rs174575 on personal–social skills (i.e. in 17 % of the tests *v.* 50 % in SKOT-1). This may be due to the lower number of children (sixty-six *v.* 179 in SKOT-1) and a generally low explained variance in the models, except for personal–social skills, where the explained variance was as high as that for communication and problem solving in the SKOT-1 Cohort (about 20 %).

The main limitation of our study is the low number of individuals and the resulting poor haplotype differentiation, which gives a low power, specifically in relation to the detection of potential interactions with sex. A significant interaction between sex and *FADS* SNP was only observed for one SNP (rs174575) on fine motor skills (in 11 % of the interaction tests as opposed to 47 % in SKOT-1). The results from the SNP analysis in Table 3 was supported by dose–response analysis for erythrocyte DHA and DHA-increasing alleles, thus, to some extent making up for the low power. The observed associations for erythrocyte DHA and *FADS* SNP on personal–social skills were consistent, as an increase – or expected increase based on our previous *FADS* *v.* erythrocyte DHA analysis – in erythrocyte DHA was associated with higher scores. The SNP–sex interaction on fine motor score (with an explained variance of 11 %) showed an increased score among girls who carried minor alleles (the G-variant) of rs174575, which in our previous study was also found to decrease erythrocyte DHA, and opposite effects in boys – thus in line with a sex equalising effect of DHA. The results are in the SKOT-1 Cohort, which showed that the communication and problem solving scores of boys were improved towards girl levels with increasing number of DHA-increasing *FADS* alleles, whereas scores in girls were slightly decreased at increasing DHA[9]. After DHA supplementation during pregnancy, Makrides *et al.*[7] found reduced scores for language and adaptive behaviour among girls, whereas the frequency of mildly impaired cognitive scores (<85) were reduced in boys – that is again decreasing the difference between boys and girls. In contrast, Makrides *et al.*[5] found that postnatal DHA supplementation in preterm infants improved Mental Development Index score in girls and Lauritzen *et al.*[6] found decreased vocabulary comprehension and sentence complexity only among boys after maternal fish oil supplementation during the first 4 months of lactation – that is effects that increased sex differences. It is possible that these differences in the results from the different studies indicate a dependence on whether the effect was exerted pre or postnatal.

Erythrocyte DHA gives a stable measure of status over the past months, whereas the *FADS* SNP profile will affect the endogenous PUFA desaturation from before birth to 3 years of age. It is therefore not possible to know if the continuous LCPUFA supply is important for the effect or whether the observed effects are due to some type of early programming from *FADS* SNP profile in early life when LCPUFA synthesis is at its highest[2]. *FADS* genotype and thereby desaturase activity will not only give rise to an increase in DHA, but will also affect other PUFA – including arachidonic acid. It is therefore not possible from *FADS* SNP analysis to exclude potential effects from these PUFA. However, the opposite effects of rs1535 compared with rs174448 and rs174575, the strengthened association when the SNP were combined based on their previously observed effects on erythrocyte DHA at 9 months, and the corroborate associations with *FADS* SNP and erythrocyte DHA in this and our previous study[3,8] indicate that DHA is the most likely mediator, as all the examined *FADS* SNP reduced erythrocyte arachidonic acid in the SKOT-1 Cohort[9].

The main strength of our paper is the use of *FADS* SNP, which in theory should eliminate potential confounding issues. The combined effect of the three used *FADS* SNP on erythrocyte DHA status at 9 months of age was in our previous study found to be of similar size as that of breast-feeding[3]. In the present study these three SNP was found to have a combined effect of 2·5-score point per DHA-increasing allele in personal and social skills, which is equivalent to 15-point difference between the 25th and the 75th percentile, which indeed would be of potential relevance. The affected developmental outcomes in this and the previous studies[6,7,9,22] were to some extent all related to language, social behaviour and problem solving/general cognition. It is unknown which differences between the studies that could account for the differences in affected outcomes, but it might be relevant how the specific functions were examined – for example by parent answered questionnaires or investigator assessment such as Bayley. It is also possible that parental socio-economic status or child raising principles could have an effect. Anyway, the validity of early tests, specifically the more global tests like Bayley and ASQ, with respect to later cognitive functions and IQ may not be reliable within normally developing children[23]. An effect between *FADS* genotype and PUFA intake and risk of attention-deficit hyperactivity disorder has also been proposed[1] and may be in line with the previously indicated sex difference as this diagnosis is most prevalent among boys[24]. The potential sex difference in the effect of DHA supply in early childhood could complicate the interpretation with respect to future implications for the children. Thus, there is a need for more and larger studies to investigate the indicated sex interaction and the association between both dietary DHA and *FADS* SNP and a range of specific developmental outcomes in order to increase our understanding of it and how an increased DHA supply effects brain development and function.

In conclusion, DHA-increasing *FADS* SNP and erythrocyte DHA status were associated with improved personal–social skills in this small cohort of children of obese mothers irrespective of sex, but the sample was too small to verify potential sex-specific effects on other developmental outcomes.

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Supplementary material

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