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Fetal learning and memory: Weak associations with the early essential polyunsaturated fatty acid status ☆

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ABSTRACT

To study the potential associations between fetal brain functions and the early essential polyunsaturated fatty acid (ePUFA) status, fetal learning and memory were assessed by repeated habituation rate measurements (HR) in fetuses of 30, 32, 34 or 36 weeks gestational age (GA). HR tests were repeated 10 min later. Both measurements were replicated in a second session at GA 38. Fetal short-term memory (STM) and long-term memory (LTM) were calculated from these habituation rates and related to concentrations of ePUFAs and their status markers, measured in umbilical artery wall phospholipids. The only relevant associations observed were positive trends ($0.010 < p < 0.050$) between STM measured before 38 weeks GA and concentrations of the ePUFA status markers Mead acid and Mead acid+dihomo-Mead acid, and between LTM and levels of Osbond acid, a marker of the n-3 LCPUFA status. Although these weak associations may imply some negative relationships between fetal brain functions and the early ePUFA status, we concluded that physiological differences in the availability of these fatty acids may probably not determine the differences in these primitive brain functions during the third trimester of fetal development.

1. Introduction

The essential long-chain polyunsaturated fatty acids (LCPUFAs), arachidonic acid (20:4n-6, AA) and docosahexaenoic acid (22:6n-3, DHA), are considered of great importance for brain development and function [1,2]. During pregnancy, the last trimester is noted for rapid development of the fetal brain and high accretion rates of AA and DHA [3]. To obtain these LCPUFAs, the fetus depends primarily on the placental transfer, and thus on the maternal supply, of these fatty acids [4]. However, since pregnancy is associated with a reversal decrease in the LCPUFA status of the mother, the fetal LCPUFA status may not be optimal [5], which may have consequences for the development of the fetal brain. We, therefore, assessed fetal brain functions and related these to the fetal exposure to essential fatty acids (EFAs) and their LCPUFAs, collectively called essential polyunsaturated fatty acids (ePUFAs) [5]. These fatty acids and their status markers were measured in the phospholipids (PLs) of umbilical cord artery walls. To assess fetal brain functions, we measured fetal habituation, which is a non-invasive method to test the integrity of the fetal central nervous system functions [6] and can also be used to assess fetal memory [7]. Fetal habituation is the decrease in, and ultimate cessation of, a fetal response to repeated stimulation. It is considered to represent a form of learning and requires an intact and functioning central nervous system [8].

2. Patients and methods

2.1. Study design and population

Learning capacity and memory performance of fetuses were derived from habituation data, available from a previous study [9]. By means of unadjusted and multivariable-adjusted regression analyses, these early brain functions were related to the ePUFA status of the fetuses as reflected by selected fatty acid concentrations measured in the arterial wall PLs of their umbilical cords collected directly after delivery. The study was approved by the Ethics Committee of the Maastricht University Medical Centre and all included mothers gave their written informed consent. Initially, 5 groups of 20 women were included. Pregnancy duration at the start of the habituation measurements was 30–38 weeks.

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Details of in- and exclusion for the habituation data have been published before [9]. In addition, we excluded volunteers if the umbilical cord could not be collected at birth.

2.2. Fetal habituation method

All habituation tests were performed by the same examiner (C.E.H. D.) as described before [9]. Briefly, the fetal trunk was visualized by an ultrasound scanner and every 30 s a vibroacoustic stimulus (VAS) of 1 s duration was applied to the maternal abdomen above the fetal legs. A general movement of the fetus within 1 s of application of the stimulus was considered a positive response. A lack of response to 4 consecutive stimuli was taken to indicate habituation. We allowed a maximum of 24 stimuli in each habituation test and when fetal habituation was identified, stimulation was stopped. The habituation rate (HR) was defined as the number of consecutive stimuli applied before a fetus stopped responding. Habituation tests, in which fetuses reacted inconsistently to the VAS so that habituation could not be established, were considered missing and as a consequence these data were ignored in calculating and analyzing the results. Mothers were excluded if their fetuses did not respond to VAS at the initial habituation test and replaced by other volunteers of similar pregnancy durations.

2.3. Habituation protocol and fetal learning and memory calculations

Fetal habituation rates were measured for the first time (HR-A) during a session at gestational ages (GA) of 30 (group 30), 32 (group 32), 34 (group 34) or 36 weeks (group 36) and tests were repeated 10 min later (HR-B). Gestational age was determined using the last menstrual period or by ultrasound when dates were uncertain. Both measurements were replicated under the same conditions during a second test session at GA 38 (HR-C and HR-D).

The first habituation test outcome of each fetus (HR-A) was taken to reflect its learning capacity. The difference in habituation rates of a given fetus between the two tests in each session was regarded a reflection of its short-term (10 min) memory and expressed as a percentage of the initial habituation rate. Thus, fetal short-term memory during the first test session (STM-1) was calculated as $100 \times [(\text{HR-A}-\text{HR-B})/\text{HR-A}]$. The short-term (10 min) memory during the second session (STM-2) was calculated as $100 \times [(\text{HR-C}-\text{HR-D})/\text{HR-C}]$. However, if fetuses did not respond to the initial VAS stimulus at the second session (HR-C = 0), percentage calculation would require division by zero, which is not possible. Therefore, 0.5 was added to all HR values measured to calculate STM-1 and STM-2.

As mentioned before, habituation of the fetuses of groups 30–36 was measured at 38 weeks GA again. This allowed us to asses the long-term memory (LTM) of these fetuses, since this is reflected by the difference between HR-A and HR-C. However, this difference not only results from 'memorizing' the earlier habituation measurements at GA 30–36, but also from the normal GA-associated brain development during the 2–8 weeks between both test sessions. To correct for this, the difference between HR-A and HR-C values (in % of HR-A) was decreased by the difference (also in % of HR-A) between each individual HR-A value and the median HR-A value (11.0) of a control group of 20 fetuses measured at 38 weeks GA. Consequently, individual long-term memory values were calculated according to the equation $\text{LTM} = \{100 \times [(\text{HR-A}-\text{HR-C})/(\text{HR-A})]\} - \{100 \times [(\text{HR-A}-11.0]/(\text{HR-A})]\}$.

2.4. Cord sampling and fatty acid measurements

From the fetuses of groups 30–36, a piece of the umbilical cord was collected immediately after birth, rinsed with saline and stored at −80 °C until fatty acid analysis. It was decided to analyze only PLs of the artery walls, because this tissue represents the lowest essential fatty acid concentration available to more 'upstream' tissues in the fetal body. These fatty acid compositions were determined by capillary gas–liquid chromatography as described elsewhere [4,10] and expressed as relative values (wt% of total identified PL-associated fatty acids). Fatty acid values $<0.05\%$ were considered too low for reliable detection and treated as missing. The selected fetal ePUFAs of interest were the major LCPUFAs for brain development, AA and DHA, their respective dietary precursors, the EFAs linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) and three ePUFA status markers [11] (Osbond acid, 22:5n-6, ObA; Mead acid, 20:3n-9, MA; dihomo-Mead acid, 22:3n-9, DHMA).

2.5. Covariables

Parity [12], maternal smoking [13] and drinking during pregnancy [14], socio-economic status (SES) [15] and infant sex [16] were included in the multivariable-adjusted regression analyses as potential confounding factors. These characteristics were obtained via study questionnaires and medical records, as detailed before [9]. In the regression analyses two dummy variables for parity were used, one for parity = 1 and one for parity $\geqslant 2$, with parity = 0 as reference category. Maternal smoking during pregnancy was categorized as 0 = non-smoking and 1 = 1–5 cigarettes per day. Maternal drinking during pregnancy was classified as 1 = 1 glass per week and 2 = 2–7 glasses per week, with 0 = no alcohol use as reference category, and infant sex as boy = 0 and girl = 1. Exact information on SES was not available. Therefore, parental SES was measured by proxy, using the variable 'highest educational level' [17]. For this variable, the education levels from both parents were compared and the highest education level (measured on an 8-point scale) was chosen as the value for the socio-economic status.

2.6. Statistical analyses

All data are presented as median (25th–75th percentile), unless otherwise mentioned.

Associations between various fetal learning and memory outcome measures and the neonatal fatty acid concentrations of interest were analyzed with unadjusted and multivariable-adjusted regression analyses. In these analyses, fetal learning (HR-A), STM-1, STM-2 and LTM were the dependent variables and the relative proportions of the selected neonatal fatty acids LA, ALA, AA, DHA, ObA, MA, DHMA and MA+DHMA measured in PLs of the umbilical artery wall were the independent variables. Parity, SES (parental education), maternal smoking and drinking habits during pregnancy and infant sex were included as potential confounders.

At first, the selected ePUFA status markers were validated, based on our own fatty acid data, using Spearman's rank correlation test to check if it was appropriate to select these fatty acids as deficiency markers for the ePUFA status. For this test the markers (MA, DHMA, MA+DHMA and ObA) were correlated with LA, AA, DHA and the sums of the n-3 and n-6 fatty acids.

Habituation rates of the various groups were correlated to GA using Spearman's rank correlation test to check if habituation was GA-dependent. Since this was the not the case ($p > 0.050$), fetuses of groups 30–36 were combined to one group. However, as an

extra check the variable 'group' was added to the list of potential confounders to correct for this factor, if appropriate.

Distributions of the dependent variables appeared skewed (Shapiro–Wilk test). Therefore, the distributions were optimized towards normal by means of transformations of the various datasets (natural log, square root, square or 1/square). Subsequently, obvious outliers ($\pm$4 standard deviations (SD) outside the mean) were removed, after which the normality of the distributions was checked again. Since they still appeared not normal, the 4-SD outliers were inserted again and outliers were then removed if their values were more than 3 interquartile ranges (IQR) below or above the median. Although these procedures improved the distributions, they did not normalize them. Nonetheless, linear regression analyses were performed, but since none of the residuals were normally distributed, results could not be accepted. Therefore, all dependent variables were dichotomized ($\geqslant$median vs. $<$median) and logistic regression analyses were performed.

Unadjusted logistic regression analyses were carried out with the same subjects as included in the corresponding multivariable-adjusted regression analyses. Because of occasionally missing observations, this limited the number of cases for analysis. Therefore, to increase the number of available cases, irrelevant covariables were removed by stepwise backward multivariable-adjusted regression analyses, performed for each fatty acid–brain function combination. This procedure has been described in detail before [18] and the successive steps were continued until all remaining covariables were either significant or were characterized as confounders. For each particular combination of fatty acid and fetal learning or memory criterion, these various steps were performed with the same dataset. However, since removal of the irrelevant covariables implied less missing values and, consequently, a larger number of cases available for analysis, the ultimate regression analyses were finally repeated with the maximum number of complete cases available for each combination of fatty acid and fetal brain function.

To check whether the relationships between dependent and independent variables were comparable for the added cases and the initial study population (a prerequisite for acceptance of this procedure), interaction analyses were performed as detailed before [18]. If the added cases were significantly different, the final model with the larger number of cases could not be accepted. Since these interaction analyses revealed no significant differences between initial and additional cases, all final backward models could be approved. Cook's distances were calculated to check for influential data points and data with Cook's distances >1 were removed.

Subjects with incomplete information were nevertheless included. Therefore, not all data analyses were based on the same number of subjects. For both correlation studies, a p-value <0.05 was considered significant. For all regression analyses, a p-value <0.010 was required for significance, to correct for multiple testing, whereas a p-value <0.050 was considered to indicate a (non-significant) trend. All statistical analyses were performed using the statistical package SPSS 11.5 for Windows (release 11.5, SPSS Inc., Chicago, Illinois).

3. Results

From the 80 participants included in groups 30–36, nine had to be excluded because of various pregnancy complications or because their fetuses did not react to the VAS at the initial habituation test of the first session (HR-A). Due to time restrictions, five of them could not be replaced, leaving 75 volunteers who completed the study. Three additional cases were excluded because cords could not be collected. Consequently, the data of 72 fetuses were left for analysis. In the control group (group 38), 2 fetuses were excluded because they did not react to the VAS at HR-A, remaining 18 fetuses instead of 20. All included neonates were in good health after birth, with a 5-min Apgar score $\geqslant$8 and a birth weight $>$10th percentile, and no congenital anomalies were detected.

The relevant maternal and neonatal characteristics of groups 30–36 and the control group are listed in Table 1. Results for fetal learning and memory are presented in Table 2. The relative contents (%wt/wt) of the selected umbilical artery wall PL fatty acids are reported in Table 3. In most cases ALA levels were below the level of reliable detection and therefore ALA was left out of the statistical analyses. The unadjusted and multivariable-adjusted analyses were performed with the same number of complete cases (all (co)variables available). In general, increasing the power by including all available cases in the unadjusted regression analyses hardly affected the outcome of these analyses (results not shown).

3.1. Validation of the ePUFA status markers

Using Spearman's rank correlation test, it was observed that the ePUFA status markers MA, DHMA and MA+DHMA were

Table 1
Maternal and infant characteristics[a].

Characteristics	Group 30–36		Control group (group 38)	
	n	Median (25th–75th percentile)	*n*	Median (25th–75th percentile)
Maternal characteristics				
Age (years)	72	31 (28–34)	18	29.5 (27.5–32.3)
Educational level	71	6 (5–8)	18	6.0 (4.0–8.0)
Smoking during pregnancy (no/1–5 cigarettes per day, *n*)	72	70/2	18	18/0
Alcohol use during pregnancy (no/1 glass per week/2–7 glasses per week, *n*)	72	63/7/2	18	17/1/0
Parity (0/1/2/3)	72	39/21/10/2	18	9/7/2/0
Infant characteristics				
Birth weight (g)	72	3540 (3058–3945)	18	3630 (3320–3825)
Gestational age at delivery (weeks)	72	40.1 (39.3–40.7)	18	40.4 (39.9–41.0)
Sex m/f	72	25/47	18	10/8
Apgar score after 5 min	71	10 (10–10)	18	10 (10–10)

[a] Data are given as median (25th–75th percentile), unless otherwise mentioned.

Table 2
Results for fetal learning and memory variables[a].

Variables	*n*	Median (25th–75th percentile)
Fetal learning	71	10.0 (5.0–16.0)
STM-1	65	76.2 (53.4–88.9)
STM-2	65	66.7 (22.9–90.9)
LTM	66	53.6 (0–102.5)

[a] Fetal learning = non-transformed results of first habituation test outcome (HR-A); STM-1 and STM-2 = fetal short-term (10 min) memory, calculated as the difference in habituation rates between two successive habituation rates measured with a 10-min interval in respectively the first (HR-A and HR-B) and second session (HR-C and HR-D), and expressed in % of HR-A and HR-C, respectively; LTM = long-term memory, defined as the HR difference (%) between HR-A and HR-C, corrected for the normal GA-associated brain development.

Table 3
Relative contents (wt%) of selected fatty acids isolated from arterial cord phospholipids ($n = 72$).

Fatty acid[a]	Median (25th–75th percentile)
LA	1.15 (0.98–1.28)
AA	13.7 (12.4–15.2)
DHA	5.81 (5.13–6.52)
ObA	3.19 (2.72–3.46)
MA	2.96 (2.39–3.68)
DHMA	1.57 (1.26–1.91)
MA+DHMA	4.54 (3.81–5.49)

[a] LA = linoleic acid, 18:2n-6; AA = arachidonic acid, 20:4n-6; DHA = docosahexaenoic acid, 22:6n-3; ObA = Osbond acid, 22:5n-6; MA = Mead acid, 20:3n-9; DHMA = dihomo-Mead acid, 22:3n-9.

significantly negatively correlated with LA, AA, DHA and the sums of n-3 and n-6 fatty acids ($p < 0.001$; $.421 < r < .866$). For the status marker ObA significant negative correlations were observed with LA ($p = 0.029$; $r = .260$), DHA ($p = 0.047$; $r = .235$) and the sum of n-3 fatty acids ($p = 0.012$; $r = .294$). These results clearly demonstrate the suitability of MA, DHMA and MA+DHMA as general ePUFA status markers. ObA, on the other hand, appears a more specific status marker of n-3 LCPUFAs in general, although the correlations were relatively weak.

3.2. Relationship between fetal learning (HR-A) and selected fatty acids

Neither in unadjusted, nor in fully adjusted or backward logistic regression analyses was fetal learning significantly associated with any of the fatty acids investigated, nor did they indicate a trend.

3.3. Relationship between short-term (10 min) memory and selected fatty acids

Unadjusted logistic regression analyses revealed trends between STM-1 and the fatty acids LA ($n = 64$; $B = -2.067$; $p = 0.042$; odds ratio (OR) = 0.127; 95% confidence interval (CI) = (0.017; 0.926); $r^2 = 0.094$), MA ($n = 61$; $B = 0.714$; $p = 0.033$; OR = 2.041; 95% CI = (1.060; 3.932); $r^2 = 0.106$) and MA+DHMA ($n = 64$; $B = 0.430$; $p = 0.049$; OR = 1.538; 95% CI = (1.002; 2.360; $r^2 = 0.085$). However, after adjustment for all covariables all these trends disappeared. After removal of irrelevant covariables by the stepwise backward procedure two positive trends were observed again for MA ($n = 65$; $B = 0.716$; $p = 0.026$; OR = 2.046; 95% CI = (1.090; 3.842); $r^2 = 0.108$) and MA+DHMA ($n = 65$; $B = 0.452$; $p = 0.039$; OR = 1.571; 95% CI = (1.024;2.410); $r^2 = 0.093$). For these two backward analyses no covariables remained in the final model.

No other associations or trends between short-term (10 min) memory and the neonatal fatty acids of interest were found.

3.4. Relationship between long-term memory and selected fatty acids

In the unadjusted logistic regression analyses between LTM and ObA a positive trend was observed ($n = 61$; $B = 1.148$; $p = 0.032$; OR = 3.153; 95% CI = (1.105; 8.991); $r^2 = 0.112$). After full adjustment this positive association even became significant ($n = 61$; $B = 2.740$; $p = 0.005$; OR = 15.482; 95% CI = (2.330; 102.877); $r^2 = 0.183$). After the stepwise backward procedure, 'maternal drinking during pregnancy' and 'group' were left as confounders, and a positive trend remained ($n = 63$; $B = 1.808$; $p = 0.012$; OR = 6.097; 95% CI = (1.489; 24.967); $r^2 = 0.137$).

No other associations or trends between long-term memory and the neonatal fatty acids of interest were found.

4. Discussion and conclusions

The aim of this study was to investigate whether there are significant associations between fetal learning and memory, as assessed by fetal habituation measurements, and the fetal ePUFA status, reflected by the concentrations of AA, DHA, their dietary precursors and three ePUFA status markers, measured in cord artery wall PLs. We observed no distinct relations with AA or DHA, which are thought to be important LCPUFAs for brain development and function [1,2]. Also no significant associations or trends were observed between LA and fetal learning or memory. On the other hand, positive trends were observed between fetal STM-1 and levels of the ePUFA status markers MA and MA+DHMA and between fetal LTM and the n-3 LCPUFA status marker ObA. If causal, these relationships indicate that fetal short-term (10 min) memory measured before 38 weeks GA may be better, the lower the ePUFA status of the fetus, as reflected by higher MA and MA+DHMA levels. Likewise, fetal long-term memory would be better the lower the n-3 LCPUFA status, as indicated by higher ObA concentrations [19]. These interpretations are in striking contrast with current opinions, however.

Several human studies investigated the associations between maternal or neonatal LCPUFA concentrations measured during pregnancy or directly after delivery and children's brain development. In the majority of cases these studies addressed visual and cognitive development. Some of these studies observed positive associations, especially for DHA [20,21], whereas others found no significant relationships [22,23]. Also from a number of reviews it can be concluded that there is evidence for potential benefits of LCPUFAs on visual and cognitive development, but results are limited and often inconsistent [24–27]. It is difficult to compare these previous studies directly with our present one, since study designs and brain function measurements are so different. Furthermore, it must be kept in mind that the subjects of the present study were fetuses, whereas all other studies included infants in the age range from birth till a couple of years.

As far as we know, only a few studies used a brain function assessment procedure (the Fagan Test of Infant Intelligence [28]) more or less similar to the method we applied. In the habituation phase of this Fagan test, the investigator shows the infant two identical pictures of an infant's face, until habituation is reached. In the test phase, the original stimulus is then paired with a novel stimulus (picture of second face) and the investigator records the infant's looking direction and looking time at each stimulus. From

these data the 'novelty preference' is calculated (the percentage of the total test time in the test phase that the infant spent looking at the novel stimulus). This test reflects the infant's ability to encode a stimulus into memory, to recognize that stimulus and to look preferentially at a novel stimulus. Oken and coworkers used this technique to assess associations between maternal fish and seafood intake during the second trimester and infant cognition at 6 months of age. The results of this study showed that higher fish consumption of mothers during pregnancy was associated with better visual recognition memory of their infants at 6 months of age, especially after adjustment for maternal hair mercury levels [29]. Since a higher fish intake during pregnancy has been shown to be associated with a higher n-3 LCPUFA status of mothers and their neonates [30], this study suggests that the early availability of n-3 LCPUFAs may promote early brain development and function. Furthermore, also positive associations were found between cord plasma DHA concentrations and the Fagan test of novelty preference in a study of Jacobson et al. [31]. On the other hand, in several LCPUFA supplementation studies no significant effect of n-3 LCPUFA supplementation of babies or pregnant and lactating women on the Fagan test [32–34] were observed, although O'Conner found a positive influence for supplements containing both DHA and AA [35].

The Spearman rank correlation test outcome showed that the habituation rates were GA-independent and therefore fetuses of groups 30–36 were combined to one group. However, we added the variable 'group' as an extra check to the list of potential confounders to correct for this factor, if necessary. Indeed, in some multivariable analyses, 'group' appeared a confounder. Since habituation rates were GA-independent, this might indicate that other undefined factors besides gestational age are related with 'group' and influence the association between several forms of brain function and the selected fatty acids.

One of the selected ePUFA status markers, ObA, is thought to be synthesized when there is a functional shortage of DHA [36,37]. However, it needs to be realised that there is some evidence suggesting that ObA may not always be a useful biochemical measure of a low DHA status under all conditions [38]. On the other hand, we observed negative correlations ($p < 0.050$) for the relationships between the concentrations of ObA and those of DHA and the sum of the n-3 fatty acids. This demonstrates that it was appropriate to choose ObA as a status marker for the sum of n-3 fatty acids in particular, but it must be kept in mined that results were rather weak. For MA, DHMA and MA+DHMA levels strong negative correlations were observed with all ePUFAs. These latter results show that it was a correct decision to use these fatty acids as markers for the ePUFA status.

As mentioned before, in the present study only three trends were found for the associations between fetal brain functions and the early ePUFA availability. These trends imply negative associations between several fetal brain functions and the early ePUFA status, as reflected by higher concentrations of ObA, MA and MA+DHMA. Because of this small number of weak associations observed, our results might indicate that habituation-based brain functions are probably not related to the presence of the selected fatty acids, possibly because habituation is such a basic function, that it is optimal early in fetal development already. Indeed, these elementary forms of learning and memory are already present in such primitive animals as worms and snails [39,40], which have a relatively simple neural network.

In conclusion, since only a few trends were observed for the associations between habituation-related fetal brain functions and the early ePUFA status, we concluded that physiological differences in the availability of these fatty acids may probably not determine the differences in these primitive brain functions during the third trimester of fetal development.

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