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Neurodevelopmental Effects of Maternal Nutritional Status and Exposure to Methylmercury from Eating Fish during Pregnancy

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Abstract

Fish contain nutrients that promote optimal brain growth and development but also contain methylmercury (MeHg) that can have toxic effects. The present study tested the hypothesis that the intake of selected nutrients in fish or measures of maternal nutritional status may represent important confounders when estimating the effects of prenatal methylmercury exposure on child development. The study took place in the Republic of Seychelles, an Indian Ocean archipelago where fish consumption is high. A longitudinal cohort study design was used. A total of 300 mothers were enrolled early in pregnancy. Nutrients considered to be important for brain development were measured during pregnancy along with prenatal MeHg exposure. The children were evaluated periodically to age 30 months. There were 229 children with complete outcome and covariate data for analysis. The primary endpoint was the Bayley Scales of Infant Development-II (BSID-II), administered at 9 and 30 months of age. Combinations of four secondary measures of infant cognition and memory were also given at 5, 9 and 25 months. Cohort mothers consumed an average of 537 gm of fish (9 meals containing fish) per week. The average prenatal MeHg exposure was 5.9 ppm in maternal hair. The primary analysis examined the associations between MeHg, maternal nutritional measures and children's scores on the BSID-II and showed an adverse association between MeHg and the mean Psychomotor Developmental Index (PDI) score at 30 months. Secondary analyses of the association between the PDI and only MeHg alone or nutritional factors alone showed only a borderline significant association between MeHg and the PDI at 30 months and no associations with nutritional factors. One experimental measure at 5 months of age was positively associated with iodine status, but not prenatal MeHg exposure. These findings suggest a possible confounding role of maternal nutrition in studies examining associations between prenatal MeHg exposures and developmental outcomes in children.

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Keywords

Prenatal Methyl mercury; Child Development; Fish Consumption; Maternal Nutritional Status; Seychelles Child Development Study (SCDS)

Introduction

Methylmercury (MeHg) is a well-documented prenatal neurotoxicant at sufficiently high dosages. The level of exposure resulting from maternal consumption during pregnancy of a diet high in fish that can result in exposures to MeHg that adversely affect child neurodevelopment is not presently known.

Epidemiological studies from New Zealand, where shark was the primary exposure source (Kjellstrom, et al., 1989), and the Faeroe Islands, where whale meat was the primary exposure source (Grandjean, et al., 1997; Debes, et al., 2006), have reported subtle impairments in some measures of children's neurological development. The mean prenatal MeHg exposure measured in maternal hair was 6 ppm in New Zealand and 4 ppm in the Faeroe Islands. Our studies in the Republic of Seychelles, where the primary exposure is from the consumption of a wide variety of fish, have not identified consistent neurodevelopmental or neuropathological impairments (Davidson, et al., 1998; Myers, et al., 2003; Lapham, et al., 1995). In Seychelles the prenatal exposure is about 6 ppm of MeHg measured in maternal hair. For some endpoints in the Seychelles, we found that enhanced child development was correlated with increasing maternal hair MeHg levels in the range being studied. We postulated that this apparently anomalous finding might be related to the beneficial effects of nutrient intake from fish. .

Fish contain nutrients that are important for brain growth and development (Daniels, et al., 2004; FAO, 2002; Strain, et al., 2004). These include pre-formed docosahexaenoic acid (DHA), an Ω-3 long chain polyunsaturated fatty acid (LCPUFA). DHA is associated with enhanced cognitive neurodevelopment (Dunstan, et al., 2006; Gibson, et al., 2001; Helland, et al., 2003;; Jacobson et al., 2008; O'Connor, et al., 2001; Willatts, 2002). Both DHA and arachidonic acid (AA), an Ω-6 LCPUFA, have important structural and functional roles within the human central nervous system (Marszalek, et al., 2005). Deficiencies of DHA have been reported during pregnancy even in Western societies like Canada (Innis & Friesen, 2008). Fish is also a rich source of choline, and trace elements such as iodine and iron (Fe), nutrients linked to motor or cognitive development (Cao, et al., 1994; Colombo, et al., 2004; Grantham-McGregor, 2001; Rioux, et al., 2006; Zeisel, et al., 2006).

Fish is an important source of nutrition worldwide. The Food and Agricultural Organization of the United Nations estimates that there are as many as one billion people around the world who depend on fish for nutrition and notes that many of these individuals have limited options for nutritional alternatives (FAO, 2000).

Recent risk-benefit analyses have suggested that the benefits of fish consumption during pregnancy may balance the possible adverse effects of MeHg on neurodevelopment (Cohen, et al., 2005; Mozaffarian, et al., 2006; Institute of Medicine, 2006). The limited numbers of studies that have tested this hypothesis have not directly measured maternal nutrient intake or nutritional status, but have used fish intake (in meals per unit time) as a proxy (Budtz-Jørgensen, et al., 2007; Oken, et al., 2005). A more direct test of this hypothesis requires measurement of maternal nutritional status during pregnancy because the nutrient content of fish varies (USDA, National Nutrient Database) and the maternal status of critical nutrients varies with the overall diet and is also influenced by pregnancy related factors (Picciano, 2003).

Although there is uncertainty about the association between maternal prenatal exposure to small amounts of MeHg from maternal fish consumption and postnatal neurodevelopment, some governmental advisory bodies have recommended limiting consumption of fish during pregnancy, especially those that may contain higher levels of MeHg, (US EPA, 2004). Clarifying the relationship between maternal nutritional status and MeHg exposure from fish consumption is thus of substantial public health interest. The broader context of this study is the consideration of possible confounding of a potentially adverse neurotoxicant exposure with a potential beneficial exposure to nutrients in food, a very understudied topic (Clarkson & Strain, 2006).

We report a study designed to test directly the hypothesis that maternal consumption of nutrients present in fish and elsewhere in the diet promotes fetal and infant neurodevelopment, thereby obscuring the possible association between prenatal MeHg exposure and adverse developmental outcomes. Our focus was on prenatal exposures only. This paper describes the primary results of the study and a companion paper (Strain, et al., in review) reports secondary analyses examining associations among maternal concentrations of Ω-3 and Ω-6 LCPUFAs during pregnancy and their association with child development, and prenatal MeHg exposure.

Methods

Setting

The study was conducted in the Republic of Seychelles, an Indian Ocean archipelago with about 85,000 inhabitants of mostly mixed African, European and East Asian origins. The Seychellois diet is varied and is characteristically high in fish and fruit (Bonham, et al., in review, a). Seychellois health care, education and social services are free, readily available and comparable to westernized societies. The population is not exposed to significant levels of other neurotoxic pollutants such as polychlorinated biphenyls (PCBs), pesticides and lead (Shamlaye, et al., 2004).

The MeHg concentrations in local fish are similar to those of fish commercially available in the United States (Robinson, et al., 2004) and sea mammals are not consumed. The five fish species most frequently consumed in the Seychelles are Karang (Carangoides *gymnostethus*), Shoemaker (Sigamus *sutor*), Tuna (Thunnus *albacares*), Mackerel (Rastrelliger *kanagurta*) and Barracuda (Sphyraena *jello*) (Robson, et al., 2004). In all ocean fish, both MeHg and nutrient concentrations vary considerably both within and across species (Bonham, et al., in review, a; Robinson, et al., 2004).

Overall Study Design

The study was designed to test the hypothesis that developmental outcomes in children exposed prenatally to MeHg from high levels of maternal fish consumption were affected by both MeHg and maternal nutritional status. Maternal nutritional status was assessed during pregnancy and at delivery by taking blood samples at enrollment, at 28 weeks gestation and at delivery. Maternal dietary intake was assessed by a food use questionnaire and 4 day diet diary completed at 28 weeks gestation. Cohort children were evaluated using the Bayley Scales of Infant Development-II (**BSID-II**) at 9 and 30 months of age and other measures of infant cognition and memory at 5, 9, and 25 months of age.

The study protocol was approved by the Seychelles Ethics Board, by the Cornell University Committee on Human Subjects, and by the University of Rochester Research Subjects Review Board. Informed consent was obtained in writing from parents of all participants. Investigators who collected developmental data and biological samples from subjects were blinded to MeHg concentrations, and laboratory personnel responsible for analyzing hair MeHg or nutritional

status were blinded to subjects' developmental data. Family members of cohort children were blinded to the mother's maternal hair MeHg concentration and to the child's developmental status.

Power

We estimated the required sample size to detect a 5-point difference on the BSID-II, approximately one-third of the test's standard deviation. We assumed that half of the cohort mothers would have a hair MeHg level of >5 ppm based on experience with previous Seychellois cohorts. The calculation indicated that a sample size of 250 subjects would afford 80% power to detect a 5-point difference between the low and high MeHg exposure groups using a two-sided test and a significance level of 0.05.

Participants

In 2001 all women making a first visit to their local Antenatal Clinic were invited to participate if they were Seychellois and at least 16 years of age. Enrollment was completed when 300 volunteers had consented. Maternal age at enrollment ranged from 16 to 43 years and gestational age ranged from 14 to 24 weeks. Among these subjects, 4 women were not pregnant, and 13 pregnancies terminated in fetal death. There were 283 live births. Two mothers delivered outside Seychelles thus precluding biological sample collection at delivery. Six infants were excluded, 4 with major congenital anomalies and 1 set of twins.

Mothers and children were recalled for evaluation at 5, 9, 25 and 30 months of age. A total of 265 children completed the first study visit at age 5 months, but the numbers of attendees at subsequent visits varied. A total of 229 children completed the 30 month evaluation, had complete covariate data (113 males and 116 females) and were available for the primary analysis. Subjects for whom full data were unavailable did not differ significantly on any variables from the remaining cohort.

Developmental Assessment

The ***main*** developmental endpoint was the **BSID-II** given at ages 9 and 30 months (Bayley, 1993), but a total of 16 neurodevelopmental endpoints were measured. The BSID-II is a well-standardized measure of infant development and yields two primary endpoints, a Mental Developmental Index (**MDI**) and a Psychomotor Developmental Index (**PDI**).

Additional assessments examined more specific aspects of infant cognition. The Fagan Infantest (**FTII**; Fagan & Singer, 1983) measures novelty preference and gives two endpoints, a Mean Fixation Duration and an Overall percentage Novelty Preference. The Visual Expectation Paradigm (**VEXP**; Canfield, et al., 1997; Jacobson, et al., 1992) measures visual recognition memory and gives two endpoints, an Overall Mean Reaction Time and an Overall Percentage Anticipatory Saccades. The FTII and the VEXP were given at 5 and 9 months. The **A-not-B** (Diamond, 1990; Piaget, 1954) and the Delayed Spatial Alternation (**DSA**) tests were administered at 25 months to measure aspects of planning, inhibition, attention and working memory. Each test yields two endpoints, an Overall Percentage Correct Reaches and a Percentage of Lose-Stay Errors. Three specially trained pediatric nurses collected the neurodevelopmental data.

Reliabilities—The first author (PWD) was present during BSID-II testing to complete reliabilities on 5% of the cohort. Mean agreements ranged from 92.3% to 98%. Inter-tester agreement was ascertained on an additional 10% of the BSID-II tests and mean agreements ranged from 85.9% to 93%.These reliabilities were conducted every week as a means to improve the likelihood that skill sets to administer the BSID remained continuously high. The experimental tests were all videotaped and scored in the US by one of the authors (RLC).

Maternal Nutrition

We focused on a limited number of nutrients that could be measured in maternal blood, are present in fish, and have been directly linked to children's development (Strain, et al., 2004). We measured LCPUFA as total lipids (including phospholipids) in maternal serum samples taken at 28 weeks and at delivery. This study focused on DHA and AA, the most important LCPUFA for normal brain growth and development (Marszalek, et al., 2005). Other LCPUFA were measured as described in a companion paper (Strain, et al., in review). We used the geometric mean of the 28 week gestation and delivery values for DHA and for AA for analysis because the transfer of LCPUFA from the maternal circulation to the fetus occurs mainly during the third trimester (Montgomery, et al., 2003). The geometric mean averages the logarithm of the two pertinent values, before exponentiating to put the final value on the original scale. Among the 229 subjects with otherwise complete data, maternal serum DHA and AA status were missing for 6% of the cohort at 28 weeks and for 20% at delivery. As described later, missing LCPUFA values at single time points were imputed before calculation of geometric means.

Non-fasting blood samples (30 ml) were taken from mothers at 28 weeks gestation, and again one day after delivery. Samples were collected by antecubital venipuncture into evacuated serum tubes, then placed on water ice and allowed to sit for 30 minutes prior to being centrifuged at 1000g for 15 minutes. Aliquots of samples were stored at −80°C until analysis. Biochemical analyses of nutrients were done at the University of Ulster and associated laboratories. Samples were processed immediately after thawing and analyzed using gas chromatography mass spectrometry (Folch, et al., 1957). Lipid extracts were methylated and quantified using a ThermoFinnegan TRACE MS with 30m FAMEWAX capillary column with an internal diameter of 0.25mm and a 0.25-μm film thickness.

Maternal iodine status was measured at 28 weeks gestation as thyroid stimulating hormone (TSH) and free thyroxine (T4) using radiometric immunoassay and competitive immunoassay respectively. Maternal Fe status was determined as total body Fe stores calculated from soluble transferrin receptor and ferritin (Cook, et al., 2003). We assessed Fe status at enrollment, prior to the start of maternal supplementation, a routine practice in the Seychelles.

We measured maternal fish consumption using a food use questionnaire (FUQ) and a 4 day diet diary. The FUQ was designed to provide information on frequency of consumption of fish and fish containing meals over a retrospective two week period. The questionnaire was developed in the Seychelles and piloted among nurses working in ante natal clinics in Seychelles; it was produced to augment the data obtained from the food diaries. At 28 weeks gestation, each mother completed a 4-day food diary (two consecutive weekdays and two weekend days) to ascertain intake of fish and fish products in grams per day (Robson, et al., 2004). Nurses, trained by nutritionists from the University of Ulster, explained to all subjects the procedures involved with completing food diaries. Subjects were asked to record in the food diaries, the amounts of foods and beverages consumed in terms of household measures (e.g. spoons, cups etc), fractions or multiples of commercially packaged foods (e.g. cans, bottles etc), and fractions or multiples of non-packaged commodities (e.g. eggs, fruits, vegetables etc). For the determination of quantities of meat and fish eaten in mixed dishes such as stews or curries, small wooden cubes in three different sizes were shown to the subjects to help determine amounts eaten. Nurses reviewed the diaries within one week of completion, and errors and omissions were clarified with subjects. Literacy level in the Seychelles is about 90%. Diaries were completed in Creole, the language spoken in most Seychellois homes, or English depending upon maternal preference. They were reviewed with the mothers by project team members upon completion. Dietary data analysis was undertaken at the University of Ulster by trained nutritionists. They used the program WISP version 2 augmented with composition data for foods consumed in Seychelles (Athar, et al., 2003) and local recipe and portion size

information. Estimated maternal dietary choline intake (mg/day) was calculated from the food diaries and used as an indirect measure of choline status.

Dosimetry

We used average maternal hair Hg during pregnancy as the bio-marker for prenatal MeHg exposure. Maternal hair Hg is known to correlate with infant brain Hg levels (Cernichiari, et al., 1995) and is believed to reflect the species of Hg that is transported across the blood-brain barrier (Clarkson & Magos, 2006).

We determined mercury in maternal hair samples obtained at delivery. We used the hair sample that covered as much of the gestation as possible, assuming a hair growth rate of 1.1 cm/month. Total and inorganic Hg levels were measured using the Magos reagents (Cernichiari, et al., 1995). The selected hair segment was weighed and digested in 2 mI of 45% (w/v) solution of NaOH and 1 ml of 1% (w/v) cysteine at 95°C until the sample was dissolved. Stannous chloride was used to selectively reduce inorganic Hg to vapor which was then measured by atomic absorption spectroscopy. Total Hg (organic plus inorganic) was reduced to vapor by a cadmium chloride, stannous chloride reagent. The volume as made up to 10 mI with 0.9% NaCI solution. MeHg analyses were performed using a Laboratory Data Control Mercury Monitor Mode11235 (Cernichiari, et al., 1995). The average Hg was then calculated based on the length of the hair sample.

The University of Rochester's Mercury Analytical Laboratory regularly implements internal and external quality control programs. Certified Reference Materials from the European Commission and from the International Atomic Energy Agency (IAEA) are used to check the performance of instruments and technical personnel.

Statistical Analysis

Analysis Plan—The association among maternal nutrients and dietary indicators, prenatal MeHg exposure and each of the 16 neurodevelopmental endpoints was estimated using multiple linear regression analysis. Only models with an overall F-test significant at the 0.05 level were examined further and are reported here. Within each model we used a level of $p \leq 0.05$ to determine the significance of independent variable effects[1]. All tests were two-tailed.

Covariates—All models were adjusted for the same list of covariates, factors known to be associated with child development. Socioeconomic status (**SES**) was estimated using the Hollingshead Four-Factor Socioeconomic Status modified for use in the Seychelles (Davidson, et al., 1998). Home environment was assessed using the *Pediatric Review of Children's Environmental Support and Stimulation* (**PROCESS**). Maternal intelligence was assessed using the Matrices subtest of the Kaufman Brief Intelligence Test (**K-BIT**). The tester for each child was included in all models involving all endpoints except the BSID-II (for which reliability was conducted). **Birth weight** (**BW**) and **maternal age** were included as covariates because of their independent influence on child development. **Sex** of the child has been associated with prenatal MeHg exposure in some previous studies. **Both parents living with the child** was included because the number of nuclear family members living with the child

[1]A decision rule of 0.05 was adopted for several reasons. First, it is consistent with all previous Seychelles Child Development Study analysis plans and has been used in most epidemiological studies. There was no compelling reason to adjust this practice *a priori* in the present analysis plan. Second, we wanted to afford some protection against making a Type I error in rejecting a true null due to multiple comparisons. Indeed, we ran several models on each of 16 endpoints and should have found one or two significant results by chance alone. Adopting a probability level of 0.10 for example, roughly doubles the risk of committing a Type I error. There was also the possibility that the study was slightly underpowered, increasing the likelihood of making a Type II error by adopting p=0.05 as the decision rule. But both power and decision rules were set *a priori* based on a power calculation that indicated an 80% (sufficient) power to detect a 5 point difference in Bayley endpoints between the low and high MeHg exposure groups at p=0.05. In our analyses, this was the only endpoint that was associated with Hg.

varied in the Seychellois culture and could have an independent influence on developmental outcome. By 30 months of age, about one third of parental partnerships change and care-giving arrangements no longer involved both parents. Our dichotomous index measured whether or not both parents resided with the enrolled child at age 9 months. All analyses for each endpoint followed the same procedures irrespective of results at any stage.

Primary and Secondary Analyses—The **primary analysis** tested the association between each endpoint and the mean prenatal MeHg exposure and also included six nutrition indictors: DHA, AA, TSH, and Fe stores, maternal fish consumption and choline intake. The primary and secondary models did not include any interactions. Two **secondary analyses**[2] were also conducted. The first replicated the analysis from our earlier studies (Davidson, et al., 1998;Myers, et al., 2003) testing the covariate-adjusted association between MeHg and each endpoint without adjusting for any nutrition indicators (MeHg model). The second tested the covariate-adjusted association between each endpoint and the six measures of maternal diet and nutrition without correcting for MeHg (nutrition model).

Among the 170 women with LCPUFA measured at both 28 weeks of gestation and delivery, the mean DHA level declined from 0.19 mg/ml (SD=0.06) to 0.16 mg/ml (SD=0.06) and the mean AA level declined from 0.63 mg/ml (SD=0.14) to 0.60 mg/ml (SD= 0.15). Such declines are known to occur during the third trimester (Montgomery, et al., 2003) making a single LCPUFA value at one time point not representative of the third trimester average. Therefore, we imputed a single value for each missing DHA and AA value based on the relationships between each LCPUFA at the two time points in the observed data. We assumed that the joint distribution of each LCPUFA at the two time points was bivariate normal on the logarithmic scale. This assumption appeared to be quite reasonable in this dataset. A mother's missing LCPUFA value at one time point was imputed to be the predicted value conditional upon the observed LCPUFA value for that mother at the other time point. The geometric mean of the observed and estimated values (e.g. the estimated LCPUFA value on the untransformed scale) was then used in the analysis.

Tertiary Analyses for the 30-month PDI—Several tertiary analyses were conducted to clarify results of the primary analysis of the 30-month BSID-II PDI. These analyses involved modifying the primary and secondary analyses by (1) replacing TSH with T4, an alternative biomarker for maternal iodine status (Model 1); (2) excluding nutrients measured from the mothers' diet diaries (choline and fish intake) in favor of using only biological measures of nutritional status during pregnancy (Model 2); (3) replacing TSH with T4, and excluding choline and fish intake (Model 3); (4) including interactions of MeHg with both DHA and AA, when DHA and AA were included as indicator variables to distinguish between tertiles of their sample distribution(Model 4); and (5) using multiple imputation to account for the uncertainty in estimating the mean DHA and AA when one value was missing (Model 5. Multiple imputation using 10 multiply imputed datasets was carried out (Little and Rubin, 2002; Schafer, 1997), assuming that DHA and AA at both time points were model covariates. For each imputation, we sampled from the posterior predictive distribution of the unobserved LCPUFA on the logarithmic scale at each time point, conditional on MeHg, the other nutritional and dietary status indicator variables, the other model covariates, and the BSID-II MDI and PDI at 9 and 30 months. For each imputed dataset, the geometric mean of each LCPUFA over the two time points was then used as the covariate in the regression model, putting the LCPUFA back to their original scale. Regression estimates and their standard errors from the 10 multiply

[2]The secondary analyses described here are different from those secondary analyses reported in the companion paper by Strain, et al., in this number of the Journal.

imputed datasets were then combined in the usual way (Little and Rubin, 2002; Schafer, 2001) for inference.

Check of Regression Assumptions—Every analysis included a check for regression assumptions. If the assumption of normally distributed errors with constant variance was violated, we used a log transformation of the outcome to stabilize the variance and produce more normally distributed errors. For each model statistical outliers (defined as observations with standardized residuals greater than 3 in absolute value) and influential points (defined as observations with a Cook's distance larger than 0.50) were identified. Affected models were then run with and without each. Variance inflation factors (VIF) were used as a check for collinearity among variables. Model results are reported using all observations unless otherwise noted. When results differed substantially with the inclusion or exclusion of outliers or influential points the differences are noted.

Results

The mean maternal hair mercury concentration was 5.7 ppm (SD=3.7, range=0.2–18.5) and the mean birth weight was 3.24 kg. Table 1 shows the means (SDs) and ranges for the maternal diet and nutrition status indicators and developmental endpoints. There were no clinical signs of nutritional deficiencies in the cohort. Prenatal MeHg was significantly but not highly correlated with mean DHA (r=0.32, $p<0.0001$) but not with mean AA (r=0.07, $p=0.27$). Correlations between MeHg and all nutrition variables are given in Table 2. Maternal fish intake averaged 537 g/week (an average of 9 fish containing meals per week) and was not significantly correlated with prenatal MeHg (r=0.07, $p= 0.32$) or with any dietary or nutritional status indicator except dietary choline intake (r=0.36, $p<0.0001$). The developmental endpoints exhibited the expected variability and the means were in the normal range. However, the mean scores for the main endpoints, the BSID-II MDI and PDI declined nearly one SD from 9 months to 30 months. The Pearson correlations between the BSID scores at 9 and 30 months were 0.12 for the MDI and 0.27 for the PDI, values below those expected (Bayley, 1993). Conversely, the correlations between PDI and MDI were 0.35 at 9 months and 0.57 at 30 months, consistent with test standardization data (Bayley, 1993).

We examined the data further to see if this drop in BSID-II scores, as well as the low correlation between the scores at the two time points could be due to a tester effect. We did this by subdividing the 9-month and 30-month data into categories based on the tester at each of the two time points. The correlation between PDI scores at the two time points for subjects examined by the same tester both times ranged from 0.22 to 0.39, whereas this correlation among subjects examined by two different testers ranged from 0.13 to 0.35. Furthermore, within these categories, the mean PDI at each of the two time points was remarkably similar to the overall PDI means as given in Table 1. Taken together, this suggests that the mean drop and the low correlation between the BSID-II scores at the two age points was not due to a tester effect.

Results from the Primary and Secondary Regression Analyses

Prenatal MeHg exposure was not significantly associated with any endpoint at any age in all models that did not include adjustment for dietary intake and nutritional status indicators using a p value of 0.05 as the cutoff for significance The association between MeHg and one endpoint, the PDI at 30 months, was borderline significant ($p=0.07$), In general, those models replicated the analyses reported in our earlier evaluations of the Seychelles Child Development Study Main Cohort (Davidson, et al., 1998; Myers, et al., 2003) where measures of maternal dietary intake or nutritional status were not obtained. Similarly, in the models with dietary intake and nutritional status indicators that did not adjust for MeHg, there were no significant associations

at any age with any endpoint. Statistical outliers occurred in some analyses and are reported below, but Influential points did not appear in the regression analysis for any endpoint. There was no evidence of multiple collinearity as variance inflation factors were all <2.0. The FT-II Fixation Duration, VEXP Reaction Time and Overall Percentage Anticipatory Saccades variables were log transformed at ages 5 and 9 months to meet the regression assumptions.

Five Months—The overall F test for the primary analysis model of the **VEXP** Overall Percentage Anticipatory Saccades was significant. The association between prenatal MeHg and Percentage Anticipatory Saccades was not significant. There was a significant association ($p = 0.04$) between maternal TSH and Percentage Anticipatory Saccades. Increasing maternal TSH was associated with a decrease in Percentage Anticipatory Saccades, indicating improved performance. A 1 mlU/l increase in maternal TSH was associated with a 0.22 point decrease on the log-transformed 5-month Percentage Anticipatory Saccades. The TSH association with Anticipatory Saccades was not significant in the secondary analysis when adjusted for MeHg. Likewise, the primary and secondary analyses for all other endpoints were either not significant, or indicated no significant effects for MeHg or any of the maternal dietary intake or nutritional status indicators.

Nine Months—At age 9 months none of the primary or secondary models for the BSID-II MDI were significant. All of the primary and secondary models for the BSID-II PDI were significant at $p \leq 0.03$. However, there were no statistically significant associations between the PDI and either prenatal MeHg or any maternal dietary intake or nutritional status measure. The primary model indicated that the PDI increased with increasing birth weight (p=0.0008) and was higher in girls than boys (p=0.02). The secondary models indicated no significant associations between the endpoints and either MeHg or any dietary intake or nutritional status measure. There were two outliers in the PDI analyses, but their removal did not affect the results.

Twenty-Five Months—No significant effects of either MeHg or any maternal nutrient or dietary intake or nutritional status indicator were found in any primary or secondary model for any endpoint at 25 months.

Thirty Months—At age 30 months, the overall models for the primary and secondary analyses of the BSID-II MDI were statistically significant but none showed an effect of either MeHg or any maternal dietary intake or nutritional status indicators. The 30 month MDI increased with increasing home environmental stimulation (p=0.0001) and with increasing birth weight (p=0.04). The results of the primary and secondary analyses of the 30-month BSID-II PDI were also significant and are summarized in Table 3. A 1-ppm increase in prenatal MeHg exposure was associated with a 0.55-point decrease in the PDI ($p = 0.035$, an adverse effect). As noted earlier, without adjustment for dietary intake and maternal nutrient status indicators, the relationship between MeHg and the PDI at 30 months was borderline significant (p=.07). However, unlike results from models fit to the SCDS Main cohort: a 1-ppm increase in MeHg was associated with a decrease of 0.44 point on the PDI. This was the only significant adverse effect of MeHg at any age on any endpoint in the Primary analysis, as summarized in Table 4.

The primary analysis did not show a significant association with DHA (p=0.34) or AA (p=0.49). However, the direction of these associations was as expected. A 0.1 mg/ml increase in DHA was associated with a 2.5 point increase in the PDI (p=0.34) and a 0.1 mg/ml increase in AA was associated with a 0.6 point decrease in the 30 month PDI (p=0.49).

Girls scored higher on the PDI than boys (p=0.0001), as they did at 9 months. The 30-month PDI also increased with increasing maternal age (p=0.02).

Tertiary Analyses for 30-month BSID-II—Model 1, in which T4 was used in place of TSH, was significant. However, T4 was not a significant predictor of 30-month PDI and coefficients for other model covariates showed little change from the primary analysis model. Models 2 and 3 that did not adjust for dietary measures (fish and choline) were significant and closely paralleled the primary analysis models whether T4 or TSH was used as the measure of maternal iodine status. In Model 4, the interactions between MeHg and DHA and between MeHg and AA were not significant. (p=0.53 and p=0.30 for the two-degree of freedom tests for MeHg*DHA and MeHg*AA respectively). Model 5, which used multiple imputations for missing DHA and AA values, was also significant. The estimated LCPUFA effects on 30-month PDI and their *p*-values from the multiple imputation models were similar to the primary model. The estimated DHA and AA coefficients were −24.84 (p=0.33) and −5.95 (p=0.51) per mg/ml respectively using multiple imputation. The slope for the MeHg effect in the multiple imputation model was −0.56 (p=0.03) per ppm in maternal hair which was also similar to the estimated effect from the primary model.

Discussion

The present study showed that prenatal MeHg exposure adversely affected one of 16 neurodevelopmental endpoints. At 30 months of age the PDI decreased as MeHg increased. This association appeared to be significant only when both MeHg and nutritional indicators were included in the same statistical model, although the association between MeHg and the 30-month PDI was borderline significant when not adjusted for nutrient and dietary status indicators. Moreover, in separate models that included MeHg by itself together with covariates, no significant association was present, paralleling results of previous research involving similar cohorts (Davidson, et al., 1998; Myers, et al., 2003). The 30 Month PDI was the only outcome significantly related to MeHg exposure. No other associations were present at any other age on any of the 3 other primary or 12 other secondary endpoints. We found some direct evidence that prenatal exposure to iodine may exert a positive influence on at least one measure of early postnatal cognitive development but that association was unrelated to MeHg.

These results need to be confirmed, but could be of importance for two reasons. First, they suggest that nutritional status and MeHg exposure may simultaneously influence developmental outcomes in opposite directions, and these effects can be separated only by careful measurement and statistical modeling. Second, these results would help to clarify the apparently anomalous association between increasing prenatal MeHg exposure and better neurodevelopmental outcomes seen at age 66 months in the Seychelles Main Cohort (Davidson, et al., 1998) and by the Faeroes study at age 1 year (Grandjean, et al., 1995). Our findings suggest that the beneficial influence of nutrients derived from fish and the overall diet need to be considered in order to fully interpret the risk of a neurodevelopmental effect from prenatal MeHg exposure from fish consumption.

These results do not directly clarify which nutrients or dietary indicators may be exerting an influence on performance or what the mechanism or mechanisms of influence may be. Hibbeln and colleagues (2007) in the ALSPAC study showed that fish consumption during pregnancy of >340 grams/ week was associated with beneficial effects on child development. In addition, Butz-Jørgensen and colleagues (2007) reported that fish consumption in the Faeroese cohort, mainly as North Atlantic cod, correlated with cord blood mercury levels and was associated with beneficial effects on child development. They noted that when correction was made for the confounding due to fish consumption, the adverse association between MeHg and child development was more significant. They also noted that patterns of fish consumption may vary among different populations and that it might be preferable to measure specific nutrients from fish. This variation in consumption appears to occur in the Seychelles population, since we found no correlation of maternal hair MeHg concentration and fish consumption, but we did

find correlations and confounding with specific nutritional indicators such as DHA. This is not surprising because fish species vary substantially in concentrations of both MeHg and nutrients. Consequently the mother's nutritional status may not show a direct relationship with fish or nutrient intake because there are many intrinsic and extrinsic factors that are involved such as bioavailability and physiological state. These factors are not taken into account when measuring only fish meals. The results of this study suggest that there may be biological and neurodevelopmental consequences of the interplay between DHA, other nutrients, and MeHg when fish is consumed.

Although the DHA and AA effects did not reach statistical significance, the confidence intervals indicated improving performance on the BSID-II PDI at 30 months associated with increasing DHA and declining performance associated with increasing AA. There might be several reasons to explain the lack of significant *p* values for DHA and AA effects. First, of course, our hypothesis that LCPUFA may counterbalance an effect of MeHg could be incorrect. But a lack of significance could also have resulted from underpowering. Indeed, the coefficients in the Nutrition Model associated with DHA and AA are in opposite directions and if the coefficients were the same in a larger study they would be significant. These associations are of potential importance and deserve further study. Several authors have suggested that Ω-3 LCPUFA may play a unique role in promoting central nervous system development (Gibson, et al., 2001; O'Connor, et al., 2001; Rioux, et al., 2006; Strain, et al., 2004). However, our finding that their influence on the developing brain may change in relation to prenatal MeHg exposure raises questions about the relationship between the two compounds. More data are needed before the underlying biological relationship between LCPUFA and MeHg can be clarified. Our statistical modeling also suggested that after accounting for DHA, AA may have an adverse effect on the BSID-II PDI. Recent studies in the non-human primate CNS have highlighted the importance of AA and DHA in the basal ganglia and brainstem and related them to changes in motor development (Diau, et al., 2005). These findings could help explain the complex interplay between AA and DHA and why increasing AA was associated with decreasing PDI scores in the present study.

Our results indicating improved performance on VEXP Overall Percentage Anticipatory Saccades at 5 months with better maternal iodine status are consistent with previous studies of iodine supplementation (O'Donnell, et al., 2002).

The suggestion of combined effects of MeHg and nutritional status indicators occurred primarily on an endpoint measuring psychomotor rather than cognitive development. The reasons for this result are unclear. It may be that the principal impact of beneficial effects of many of the nutritional status indicators we measured occurs on visually mediated behaviors, as has been reported (Dunstan, et al., 2006; Gibson, et al., 2001; Helland, et al., 2003; O'Connor, et al., 2001; Willatts, 2002).

The drop in BSID-II scores from 9 months to 30 months was not expected for normal infants and toddlers (Harris, et al., 2005; Niccols and Latchman, 2002) and may be important. This finding does not appear to be related to administration of the test as scoring reliabilities were uniformly high and the variability around the BSID-II means was similar to what would have been expected if US children had been examined. The drop in mean scores from 9 to 30 months might have been the result of cultural bias. At 30 months, the Mental Scale is comprised of more language based itmes than at earlier ages, perhaps creating a disparity between the Seychellois cohort and the normative population. Such a circumstance would not explain the comparable drop in the PDI, which measures fine and gross motor skills and perceptual motor responses. This finding deserves further study.

The premise tested in this study was that prenatal co-exposures to MeHg and nutrients in fish are confounded and if separated, might reveal different effects on developmental outcomes. The study was not designed to consider also the influence of postnatal exposures to either MeHg or nutrients because the focus of public health concern has been on prenatal MeHg exposure. Postnatal MeHg exposure was therefore not measured. Measuring postnatal MeHg exposure presents many challenges. There is no agreed upon metric aside from the convenience measure of recent exposure (usually amounting to hair or blood MeHg measured at the time of a particular postnatal developmental evaluation). In the Main Cohort Study (Davidson, et al., 1998; Myers, et al., 2003) we adjusted for postnatal MeHg first at 5.5 years. By that age, the children in the Seychelles appear top have established their own pattern of fish consumption. We also know from previous work that prenatal and postnatal MeHg levels are not correlated (r=0.08, Myers, et al., 2003). We expected that any effects of nutrients were likely to be mainly prenatal (Strain et al, 2004).

Our study has some important strengths and limitations. We were able to measure specific nutrients including LCPUFA, iodine, and other nutritional parameters during pregnancy and examine their associations with the children's developmental outcomes. Some but not all psychosocial covariates known to influence child development had the expected effect on our developmental endpoints. However, the sample size of the study was smaller than we had planned. About half of the subjects with complete data did have hair MeHg levels ≥5 ppm but the difference in mean BSID-II scores between the low and high MeHg groups was small and ranged from 0 for the 30-month MDI to 2.7 for the 30-month PDI. Although we selected a number of nutrients known to affect neurodevelopment, other nutrients may be influential in determining developmental outcomes in a fish consuming population. Direct assessment of choline in maternal blood might have been important, but logistic and technical problems precluded this. Finally, the study involved multiple examinations at several different ages using several different endpoints. We found only one statistical association with MeHg and the four primary endpoints. Follow-up examinations of our cohort at later ages may reveal different results. A larger scale confirmatory study may be needed to further explore some of these issues.

In conclusion, this study suggests that nutrients present in fish and maternal diets may act as confounders in detecting associations between prenatal MeHg exposure from fish consumption and child development. These results suggest that MeHg and nutrition have competing roles in relationship to child development. Nutrients may protect the developing brain from the toxic action of MeHg or alternatively MeHg exposure from fish consumption may diminish the efficacy of the beneficial action of nutrients. These competing explanations of our findings deserve further study and if confirmed could have important public health implications.

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Table 1

Summary Statistics for Nutrition and Developmental Measures

Variable	N**	Mean	SD	Range
MeHg (measued in mothers hair in ppm)	229	5.7	3.7	0.2–18.5
TSH @ 28 wks (mlU/l)	229	1.2	0.6	0.1–3.7
Choline in mg/day (from Diet Diary)	229	223.2	81.6	26.3–537.9
Fish intake in g/day (from Diet Diary)	229	76.7	47.0	0–346.3
DHA (mg/ml)				
28 wks gestation	216	0.19	0.06	0.07–0.4
Delivery	183	0.16	0.06	0.06–0.3
Geometric Mean of 28 wks and Delivery*	229	0.17	0.05	0.07–0.3
AA (mg/ml)				
28 wks Gestation	216	0.63	0.14	0.4–1.2
Delivery	184	0.60	0.15	0.3–1.2
Geometric Mean of 28 wks and Delivery*	229	0.61	0.13	0.4–1.1
Fe (Total Body Stores at Enrollment in mg/kg bodyweight)	229	15.3	3.1	5.5–23.9
Bayley MDI 9 mo	226	102.9	8.3	72–122
Bayley PDI 9 mo	225	105.7	10.4	68–141
Bayley MDI 30 mo	228	85.0	9.5	56–115
Bayley PDI 30 mo	225	89.8	13.8	50–123
Fagan Fixation Duration 5 mo (sec)	215	2.3	0.7	0.8–5.3
Fagan % Looking Time 5 mo	214	56.8	6.9	39.1–75.7
Fagan Fixation Duration 9 mo (sec)	220	1.6	0.4	0.8–2.8
Fagan % Looking Time 9 mo	220	56.9	6.4	39.5–77.6
VEXP Mean Reaction Time 5 mo (sec)	182	335.6	55.5	221.6–544.4
VEXP % Anticipatory Saccades 5 mo	179	12.1	9.8	1.0–57.0
VEXP Mean Reaction Time 9 mo (sec)	197	300.7	45.1	202.8–440.7
VEXP % Anticipatory Saccades 9 mo	197	14.5	8.4	1.1–45.2
A not B % Correct (All Trials)	218	68.5	9.2	41.0–97.1
A not B % Lose-Stay Errors	218	14.4	5.3	2.9–38.2
DSA % Correct (All Trials)	185	61.2	7.9	25.0–96.0
DSA % Lose-Stay Errors	185	14.9	9.0	0–50.0

* DHA and AA were measured in maternal blood at 28 weeks and delivery. The geometric mean of the two measures was used in the analysis. If a mother had a measurement at only one time point, the missing measurement was estimated as described in the text.

** Not all 229 subjects complete data.

Table 2

Correlations of Prenatal MeHg and Maternal Nutritional and Dietary Status

Nutritional and Dietary Status Measure	Pearson r
AA (Mean of 28 wks and Delivery in mg/ml*)	0.073
DHA (Mean of 28 wks and Delivery in mg/ml*)	0.32
TSH @ 28 wks (mlU/l)	−0.025
Choline in mg/day (from Diet Diary)	0.094
Fish intake in g/day (from Diet Diary)	0.066
Fe (Total Body Stores at Enrollment in mg/kg bodyweight)	−0.0061

Table 3

Regression Coefficients (*p* values)* for BSID PDI Main Analyses at 30 Months

Model Component	Primary Analysis Model		Secondary Analysis	
			MeHg Model	Nutrition Model
	All data N=225	Excluding Outlier, N=224	All data* N=225	All data** N=225
Prenatal MeHg (ppm in maternal hair)	**−0.55 (0.04)**	**−0.60 (0.02)**	−0.44 (0.07)	--
DHA (mg/ml)	24.97 (0.34)	29.70 (0.24)	--	5.41 (0.82)
TSH (mlU/l)	−1.39 (0.35)	−1.35 (0.35)	--	−1.25 (0.40)
AA (mg/ml)	−6.31 (0.49)	−9.70 (0.28)	--	−2.90 (0.75)
Choline (mg/day)	0.01 (0.70)	0.01 (0.60)	--	0.003 (0.83)
Fish Intake (g/day)	0.02 (0.29)	0.03 (0.20)	--	0.02 (0.32)
Iron (Σ body stores in mg/kg body weight)	0.08 (0.77)	0.14 (0.62)	--	0.06 (0.83)
Sex (girls)	**7.79 (<0.0001)**	**7.90 (<0.0001)**	**8.14 (<.0001)**	**7.68 (<0.0001)**
Family Status (< 2 parents)	1.56 (0.39)	1.97 (0.27)	1.35 (0.45)	1.76 (0.33)
Maternal Age (years)	**0.35 (0.03)**	**0.31 (0.05)**	**0.36 (0.02)**	**0.31 (0.05)**
Maternal Intelligence (K-BIT)	0.04 (0.58)	0.06 (0.37)	0.03 (0.66)	0.03 (0.69)
SES (Hollingshead)	0.04 (0.64)	0.04 (0.65)	0.03 (0.73)	0.04 (0.67)
Birth Weight (gms)	0.004 (0.06)	0.003 (0.09)	0.004 (0.06)	0.004 (0.07)
Home Environment (PROCESS)	0.03 (0.67)	0.02 (0.76)	0.03 (0.60)	0.02 (0.74)

* Significant coefficients are bolded.

** There were no outliers in secondary analysis models

Table 4

Effects of MeHg on Endpoints from the Primary Model

Outcome	Direction of improved score	n	Correlation with MeHg	MeHg coefficient in Primary Model	*P*[*]
5 months					
Mean fixation duration[**]	−	215	−0.001	0.01	0.42
novelty preference	+	214	0.13	0.02	0.91
Mean reaction time[**]	−	182	−0.01	−0.005	0.18
Percent anticipatory saccades[**]	+	179	0.01	0.02	0.19
9 Months					
MDI[***]	+	226	−0.03	−0.16	0.34
PDI[***]	+	225	0.08	−0.17	0.39
Mean fixation duration[**]	−	220	−0.13	−0.004	0.43
novelty preference	+	220	0.10	0.03	0.83
Mean reaction time[**]	−	197	−0.05	−0.0003	0.92
Percent anticipatory saccades[**]	+	197	0.05	0.002	0.91
25 Months					
A not B percentage correct reaches	+	218	−0.03	−0.05	0.80
A not B percentage lose stay errors	−	218	0.01	−0.07	0.49
DSA percentage correct reaches	+	185	−0.05	0.07	0.69
DSA percentage lose stay errors	−	185	0.04	−0.19	0.36
30 Months					
MDI[***]	+	228	0.03	−0.17	0.35
PDI[***]	+	225	0.03	**−0.55**	**0.04**

Significant coefficients are bolded

[**] Outcome variable transformed to log scale

[***] Primary endpoints