into four groups. Each bottle contained 50-100 g tissue. The representative samples were finally prepared by homogenization (Iyengar and Rapp 2001). The entire cord was stored in a clean glass tube without any preparation.

The mothers were finally asked to provide a sample of breast milk (more than 50 ml) one month after the delivery. A clean glass bottle was used for the shipping of breast milk.

Questionnaire. Several types of questionnaire were administered after the delivery. To assess the fish-intake and the general nutrition status of the mothers a food-intake frequent questionnaire (FFQ) for 122 individual foods and recipes (Date et al. 1996) and some additional items regarding seafood was administered. This is a standardized FFQ that enables the assessment of the intake of not only major nutrients but also several essential nutrients including retinol and folic acid in the Japanese population.

Other questionnaires were administered with the following items: educational background, occupation, income, smoking habit including passive smoking, alcohol consumption during pregnancy, hair treatments including bleaching, permanent wave and coloring, and dental amalgam treatment.

Neurodevelopment assessment. All testers who performed neurodevelopment assessments were not informed of exposure information including alcohol consumption/smoking habit, FFQ data, and feeding method.

The Brazelton Neonatal Behavioral Assessment Scale (NBAS) was administered when the infants were 3 days old. The testers had been trained in the training center at Nagasaki University School of Medicine, Japan.

Cognitive functions of the infants at 7 months old were evaluated using the Bayley Scale of Infant Development (BSID), second edition, the Kyoto Scale of Psychological Development (KSPD), and the Fagan Test of Infant Intelligence (FTII). BSID, an established psychodevelopmental test tool, consists of three major scales: the Mental Scale, the Psychomotor Scale, and the Behavior Rating Scale; only the first two scales are used. The mental scale assesses the infant's level of cognitive function (memory, learning, and problem solving), language development (expressive/receptive language, and vocalization), and personal/social development. The motor scale assesses fine and gross motor functions. Since there is no Japanese version of the standardized protocol of BSID, we translated the original manual into Japanese. To examine its reliability, the evaluation of testers were examined on the basis of the Gold Standard developed at the University of Rochester School of Medicine (Davidson et al. 1995). In addition, raw scores were used in the analysis because of the lack of Japanese age norms. KSPD is a Japanese standard developmental test (Maehara et al. 2002); therefore, the developmental performance of the infants is expressed as the developmental age (DA) for each behavior area and for all areas. The developmental quotient (DQ) is obtained by dividing the estimated DA by the chronological age and then multiplying the quotient by 100. FTII is a noninvasive test of information processing that may be applied to infants up to one year of age (Fagan and Detterman 1992).

BSID and KSPD were also used for the assessment of neurobehavioral development when the children were 18 months old. The Japanese version of Kaufman Assessment Battery for Children (K-ABC) was employed to assess the development and intelligence of children when they are 42 months old. The growth and development of the children will be followed up until they are 6-7 years old, but the battery of neurobehavioral tests is as yet undetermined.

Chemical determinations. Total mercury analysis was carried out by cold vapor atomic absorption spectrometry (Akagi and Nishimura 1991) with minor modifications. Briefly, without washing the hair samples, each sample, weighing approximately 20 mg, was acid digested with 0.5

ml of HNO_3, 0.5 ml of $HClO_4$ and 2 ml of H_2SO_4 at 200°C for 30 minutes. The resultant ionic mercury was then reduced to mercury vapor by adding 0.5 ml of 10% tin chloride to a flameless atomic absorption monitor (HG-201, Sanso Co., Ltd., Tokyo). Analytical accuracy was ensured by analyzing the Human Hair Reference Material NIES CRM No. 13 from the National Institute of Environmental Studies (Lot #650, Tsukuba). In fish-eating populations, total mercury in hair consists mostly of MeHg. Indeed, a few samples were analyzed to know the exact MeHg concentration by the method of Akagi and Nishimura (1991). MeHg in hair first extracted with hydrochloric acid and then with benzene. The organic layer was subjected to electron-capture detection gas chromatography (ECD-GC) at the National Institute for Minamata Diseases. The concentration of MeHg was confirmed to be more than 95% of the total mercury content. Total mercury analysis was also applied to other samples similarly.

Assessment of PCB exposure was performed by determining PCB levels in cord blood, placenta, breast milk, and maternal blood. All 209 PCB congeners were analyzed by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) using the isotope dilution method. The analytical method was as follows: after biological samples were spiked with the ^{13}C-labeled standard mixture of PCBs, lipids in a sample were extracted and weighed. The extract redissolved by an organic solvent was purified in a multi-layer silica gel column. The purified solution was concentrated and analyzed for PCB after the addition of ^{13}C-labeled syringe spike. Four nonplanar PCB congeners (International Union for Pure and Applied Chemistry (IUPAC) nos. 118, 138, 153, and 180) are the predominant congeners found in human tissues and typically account for approximately 50-60% of total PCB (data not shown). Some earlier epidemiological studies attempted to assess PCB exposure using the sum of the above four major PCB congeners. For comparison with those earlier studies, the sum is also calculated in the present study.

A reporter gene assay of the toxic potency of dioxins and related chemicals was used for the assessment of dioxins. The Chemically Activated LUciferase gene eXpression (CALUX) assay was developed by Xenobiotic Detection Systems (XDS, Durham NC, USA) using a patented recombinant mouse cell line that contains the luciferase reporter gene under the control of dioxin-responsive elements (Denison et al. 1998). This analytical process consisted of the first extraction process as in PCB analysis and then column purification using sulfuric acid-impregnated silica gel and activated carbon column. The last purified extracts were given to the cells to produce luciferase, and the amount of light generated by the luciferase was directly related to dioxin toxic equivalent (TEQ) value. This assay has several advantages including its high sensitivity, easy pretreatment, and rapid determination, in comparison with HRGC/HRMS. This assay also requires only a smaller sample volume, which is another important advantage for epidemiological studies.

Cadmium and lead were determined by graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry, respectively, after samples were digested in a microwave oven with ultrapure nitric acid. The standard reference material for analysis was NIST 1577b (bovine liver). Other major biochemical analyses of maternal and cord blood samples included those of plasma selenium and thyroid hormones including TSH, and total/free T4 and T3. Selenium was determined fluorometrically (Watkinson 1966). The assay of thyroid hormones were performed using a radioimmunoassay technique.

Potential confounders/covariates. The quality of the home environment was assessed using a questionnaire, the Evaluation of Environmental Stimulation (EES) (Anme et al. 1998), which has been established in Japan modified after the Home Observation for Measurement of the Environment (HOME) score (Caldwell and Bradley 2001).

HOME is a validated instrument for the assessment of the home environment, but there is no Japanese version that matches the Japanese cultural context. The EES is a questionnaire that directly evaluates the interaction between the child and the caregiver. It was shown that the results of EES highly correlated with those of HOME (Anme et al. 1998).

The parental socioeconomic status (SES) was rated using the Hollingshead Four Factor Index of Social Status (Hollingshead 1975) with several modifications to make the category and prestige of occupation match the Japanese economical context.

Maternal intelligence quotient was measured using the Raven standard progressive matrices. Only the Raven colored progressive matrices have already been introduced in Japan only for people older than 40 years old. We therefore used the original Raven standard version and analyzed results using the raw data.

Other major potential confounders included were as follows: age at examination (days), gestational age (weeks), and alcohol consumption/smoking habits during pregnancy for the mothers, and the Apgar score, neonatal illness/jaundice, spontaneous delivery, parity, chronic diseases, and duration of breastfeeding (months) for the infants.

TABLE 2. *Variables measured at the Tohoku study*

Measurement	Description
Exposure assessment	
PCBs	Cord blood, placenta, breast milk, and maternal blood
Dioxins	Cord blood, placenta, and breast milk, expressed by CALUX-TEQ
Pesticides	Breast milk and placenta, but the exact assay method has not been decided.
MeHg	Maternal hair at delivery, maternal blood, cord blood, and placenta
Heavy metals	Other heavy metals including Pb and Cd, in cord blood, maternal blood, and placenta
Other biochemical measurements	
Selenium	Cord blood and maternal blood
TSH, T4/T3	Cord blood and maternal blood
Neurodevelopment assessments	
NBAS	Infants at 3 days old
BSID	Infants at 7 and 18 months old
KSPD	Infants at 7 and 18 months old
FTII	Infants at 7 months old
K-ABC	Children at 42 months old
Confounders/covariates	
EES	A questionnaire regarding the home environment
SES	Hollingshead four factor index with modifications for application in Japan
Maternal IQ	Raven standard progressive matrices
FFQ	An interview method, with 122 single foods and recipes, and some additional seafood items
Questionnaires	Alcohol consumption/smoking during pregnancy, educational background, hair cosmetic treatments, dental amalgam, and duration of breastfeeding (months),
Other factors	**Mother**: age at delivery, spontaneous delivery/cesarean section, and chronic diseases **Infant**: Apgar score, body weight, body height, head circumference at birth, gestational age (weeks), neonatal illness/jaundice, parity, and age at examination (days)

Results and Discussion

The present report describes the study design and protocol for the prospective cohort study on the effects of perinatal exposures to MeHg and other environmentally POPs on neurobehavioral development in Japanese children. All variables measured are summarized in Table 2. To our knowledge, this is the first cohort study that examines these hazardous risks to children in Japan.

Recruitment. We recruited 687 healthy pregnant women between January 2001 and September 2003 at the obstetrical wards of two hospitals in Sendai, but the final number of babies registered in this study is not yet determined because the delivery of pregnant women registered in this study is ongoing. The percentage of babies fulfilling the criteria for inclusion with the mothers' consent to participate in the assessment using NBAS was 85%. The percentage of babies participating in the next assessment at 7 months old was 86% of those participating in the assessment using NBAS. This reduction was mainly due to family relocation from Sendai to other places. Sample size is essential for the statistical power, and this is especially important to test whether exposures to low levels of chemicals have the hazardous effects. In addition to the theoretical approach to decide the appropriate sample size, recent epidemiological studies that assessed neurobehavioral consequences of perinatal exposure to PCBs are useful in considering this issue. The Dutch cohort study was started with 418 healthy infants and 395 children were examined at 42 months of age (94% of the original cohort) (Patandin et al. 1999). The German cohort study consisted of 171 mother-infant pairs; 126 mothers provided milk samples and 91 mothers remained in the final examination of children at 42 months of age (approximately 70% of the mothers participating in the postnatal follow-up cohort) (Winneke et al. 1998). In the Faroe cohort study, PCBs could be analyzed in cord tissues from 435 of 1022 children who underwent neurodevelopment examination at 7 years old (Grandjean et al. 2001). These cohort studies showed a negative correlation between prenatal/postnatal PCB exposure and neurobehavioral development in children. Considering that the exposure level of Japanese women was similar to that of European women, and that the potential risk is almost identical, our sample size is probably sufficient.

Neurodevelopment assessment. There are six sets of cohort studies on health hazardous effects of perinatal PCB exposure in children, and all these studies approached this issue by the method of neurodevelopment assessment. Four sets of studies employed BSID to measure the development of infants, and three of them found a significant correlation between the outcomes of BSID and PCB exposure (Schantz et al. 2003). Based on these findings, BSID is expected to be a useful tool for evaluating the risks and the results can be easily compared among the studies. This was the reason why we employed BSID as one of the major components in our tests. On the other hand, BSID is a developmental test based on the developmental milestone concept, and there are no standardized data in Japan. Thus, BSID does not provide us information on MPI and PDI, the two standard indexes of the relative status of development in a population. We therefore used KSPD, the most commonly used neurodevelopmental test in Japan, to calculate DQ. Both BSID and KSPD were originally developed based on the work of Gessell (Ikuzawa et al. 1985; Black and Matula 1999). We also applied FTII and K-ABC to assess children at the ages of 7 and 42 months, respectively. The present study was the first trial to use FTII in Japan. FTII is a novelty preference task designed to predict the later development and intelligence of children (Fagan and Detterman 1992). These two intelligence tests were shown to be sensitive in detecting the adverse effects of low levels of perinatal PCB exposure (Jacobson et al. 1985; Patandin et al. 1999; Darvill et al. 2000; Walkowiak et al. 2001).

Chemical determinations. In a review (Schantz et al. 2003) of epidemiological studies on the possible adverse effects of perinatal expo-

sure to PCBs, it was concluded that a more complete information regarding the neurotoxicity of individual congeners or congener groups may be helpful for risk assessment. There are 209 PCB congeners, and a large number of these congeners were indeed found to be present in human tissues. Since their relative potency to produce nerve system effects is entirely unknown, a congener-specific analytical technique is essential for risk assessment. Despite the fact that several recent studies have used sophisticated congener-specific analytical techniques, there have been no attempts to analyze individual PCB congeners probably present in cord blood, mainly due to the lack of assay sensitivity. The delay of cognitive development may be more related to prenatal PCB exposure, as measured by the sum of concentrations of three or four major PCB congeners in either cord or maternal blood, but not with the postnatal PCB exposure, as measured by the sum of concentrations of PCBs in breast milk samples (Schantz et al. 2003). These findings suggest the importance of PCB congener-specific analysis in cord blood. In the present study, the detailed assessment of individual PCB congeners in cord blood and other samples was designed using a very sensitive HRGC/HRMS.

Only the Dutch cohort study (Patandin et al. 1999) examined the adverse effects of dioxin exposure on neurobehavioral development in children, in which the perinatal exposure, as measured by GC/MS in breast milk samples collected at 2 weeks postpartum, showed no noticeable correlation with cognitive functions measured later. However, the interpretation of these findings is complicated by the results that total PCB in breast milk samples showed no correlation with cognition functions, even though the same study showed negative correlation when total PCB in cord blood was used for analysis. These findings suggest that the characterization of prenatal exposure is more important to clarify the adverse effects of dioxins; the effects of prenatal dioxins exposure should be examined by analyzing levels of dioxins in cord blood. Because dioxins could not be measured by HRGC/HRMS in small volume of cord blood and maternal blood samples, the CALUX assay, a reporter gene assay to determine the all dioxin-like substances, is useful for this purpose. Previously, we already confirmed that data obtained by CALUX assay showed an extremely good correlation with TEQs obtained by HRGC/HRMS in environmental materials (Nakamura et al. 2002).

However, in practice, several problems in exposure assessment remain. First, the metabolites of PCBs are likely included in the adverse effects of PCB exposure. The main hypotheses are that PCB effects on neurodevelopment include the disruption of thyroid hormone homeostasis (Porterfield and Source 2000), and that candidate PCB congeners that may disturb the homeostasis may include several minor congeners and their OH-metabolites (Cheek et al. 1999; Chauhan et al. 2000). The measurement of all possible metabolites of PCBs is not realistic. Second, although there are limited available data describing the neurotoxicity of pesticides in humans, these chemicals may indeed affect the neurodevelopment of children (Schettler 2001). In the present study, however, the assay methods for pesticides including organochlorine and organophosphorus chemicals are not yet determined because the number of chemicals is too large. Third, the measurement of all chemicals including PCBs, dioxins, heavy metals and pesticides from cord blood is difficult because of the shortage of sample volume and the insufficient detection limit. Other biological samples such as placenta are promising for identifying the surrogate marker for exposure assessment. A recent report suggested a good correlation of total PCB in placenta with that in cord blood, maternal blood, and breast milk samples (Wang et al. 2004). Further studies are necessary in order to examine the importance and usefulness of placenta and cord tissues in the assessment of prenatal exposure effects.

Confounders. Despite the major source of MeHg and POPs is via fish intake, fish consumption itself is though to have several beneficial

aspects. Selenium is considered to play an essential role in protection against MeHg toxicity (Watanabe 2002). Fish is usually rich in selenium, and almost 70% of the daily total selenium is through the fish intake in Japan (Miyazaki et al. 2002). However, the bioavailability of fish-derived selenium is still controversial. Fish is also rich in PUFA which may be essential for the normal development of an infant brain (Horwood and Fergusson 1998). However, the beneficial effects of increased amount of PUFA in cord blood on the later developmental period are also still controversial (Bakker et al. 2003). In the present study, these confounding factors including selenium and PUFA were considered from nutritional perspectives in the risk assessment of eating fish.

The results of this cohort study will allow us to evaluate associations between the neurobehavioral development of children and perinatal exposures to MeHg and environmentally POPs in Japan. A recent report from the cohort at Faroe Islands (Murata et al. 2004) indicated that the adverse effects of prenatal exposure to MeHg were still observed in the children at age 14 years by neurophysiological tests, suggesting that some neurotoxic effects from prenatal exposures are irreversible. To clarify this issue, the subjects should be followed until their adolescent ages. The present report describes the study design for children aged 0 to 42 months. When any significant associations between child development and chemical exposures are observed in this study, the further follow-up is essential to know the persistency of adverse effects.

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DELAYED BRAINSTEM AUDITORY EVOKED POTENTIAL LATENCIES IN 14-YEAR-OLD CHILDREN EXPOSED TO METHYLMERCURY

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Objective To determine possible exposure-associated delays in auditory brainstem evoked potential latencies as an objective measure of neurobehavioral toxicity in 14-year-old children with developmental exposure to methylmercury (MeHg) from seafood.

Study design Prospective study of a birth cohort in the Faroe Islands, where 878 of eligible children (87%) were examined at age 14 years. Latencies of brainstem evoked potential peaks I, III, and V at 20 and 40 Hz constituted the outcome variables. Mercury concentrations were determined in cord blood and maternal hair, and in the child's hair at ages 7 and 14.

Results Latencies of peaks III and V increased by about 0.012 ms when the cord blood mercury concentration doubled. As seen at age 7 years, this effect appeared mainly within the I–III interpeak interval. Despite lower postnatal exposures, the child's hair mercury level at age 14 years was associated with prolonged III–V interpeak latencies. All benchmark dose results were similar to those obtained for dose-response relationships at age 7 years.

Conclusions The persistence of prolonged I–III interpeak intervals indicates that some neurotoxic effects from intrauterine MeHg exposure are irreversible. A change in vulnerability to MeHg toxicity is suggested by the apparent sensitivity of the peak III–V component to recent MeHg exposure. *(J Pediatr 2004;144:177-83)*

See related article, p 169.

Methylmercury (MeHg) is a worldwide contaminant of seafood and freshwater fish.[1] MeHg toxicity can produce widespread adverse effects within the nervous system, especially when exposures occur during brain development.[2,3] Early adverse effects have been characterized by administering neurobehavioral tests to children exposed in utero from maternal seafood diets.[4-6] Thus, a National Research Council (NRC) committee[7] concluded that intrauterine MeHg exposure was the most critical and emphasized the findings from a prospective birth cohort study carried out in the Faroe Islands.[5] The damage to the developing nervous system is thought to be potentially irreversible.[7] The possibility also exists that exposure during postnatal development may induce brain lesions; clinical[2,8] and experimental[9] information suggests that such effects would tend to be more focal and would particularly involve the sensory cortex and the granular layer of the cerebellum.

Current advisories on fish consumption issued by national and state authorities differ and mainly aim at pregnant women or women of reproductive age groups.[1] Because the risk to children from dietary MeHg exposure is unclear, some fish advisories in the United States also address "young children,"[10] or children as old as, for example, 8 years[11] or 15 years.[12]

As an indicator of MeHg neurotoxicity, delayed evoked potential latencies have been recorded in poisoning victims[13,14] and in laboratory animals.[15] In contrast with

BAEP	Brainstem auditory evoked potential	MeHg	Methylmercury
BMD	Benchmark dose	NRC	National Research Council
BMDL	Benchmark dose level	PCB	Polychlorinated biphenyl
BMR	Benchmark response		

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neuropsychologic test outcomes, this measure is thought to be independent of socioeconomic covariates.[16] As illustrated by environmental exposure to lead, evoked potential abnormalities constituted important objective evidence on neurotoxic effects in children.[17]

In an extended follow-up of the Faroese birth cohort, we have assessed brainstem auditory evoked potentials (BAEPs) at age 14 years. We previously showed that increased intrauterine MeHg exposures were associated with delayed peak III latencies at age 7 years.[5,18] We hypothesized that these delays would remain at age 14 years and that BAEP latencies would also be sensitive to MeHg from adolescent seafood diets.

METHODS

Study Population and Follow-up

A cohort of 1022 births was assembled in the Faroe Islands during a 21-month period in 1986 to 1987.[19,20] The primary indicator of intrauterine exposure to MeHg was the mercury concentration in cord blood, and concentrations in maternal hair at parturition were also determined.[19] MeHg exposures varied considerably: 15% of the mothers had hair mercury concentrations >10 μg/g, whereas 4% were below 1 μg/g, a level that corresponds with the exposure limit recommended by the NRC committee.[7] Concomitant exposure to polychlorinated biphenyls (PCBs) was determined from the concentration in umbilical cords from 438 cohort members.[5] The first follow-up examination was performed 7 years later and included hair mercury assessment, evoked potentials, and pediatric examination.[5,21]

At age 14 years, a total of 878 of 1010 live cohort members (86.9%) were examined. Most examinations took place at the National Hospital in Tórshavn, the capital of the Faroe Islands, from April to June of 2000 and 2001. For families who had moved, examinations were also offered in Odense, Denmark, in November 2000. Each day, four children were examined during the morning and four during the afternoon. The examinations were conducted by a team of health professionals who had no access to information on individual exposure levels. The 438 boys and 440 girls examined had an average age of 13.83 (SD 0.32) years.

Hair samples were again obtained, and the proximal 2-cm segment was analyzed by flow-injection cold-vapor atomic absorption spectrometry after digestion of the hair sample in a microwave oven.[5] The total analytical imprecision for this analysis was estimated to be 4.3% and 5.5% at mercury concentrations of 4.7 μg/g and 11.1 μg/g, respectively. Accuracy was ensured by participation in the Canadian Hair Mercury Quality Control Program; all our results were within 1 SD of the adjusted mean. The high analytical quality is comparable with previous performance.[5,19] Results in micrograms may be converted to nanomol by multiplying by 5.0.

The study protocol was approved by the ethical review committee for the Faroe Islands and the institutional review board at the US institution, and parental informed consent was obtained.

Neurologic Examination

A thorough pediatric examination included otoscopy and assessment of neurologic optimality. None of the children had current middle ear infection. A total of 18 children examined had neurologic disorders thought to be independent of MeHg exposure and were therefore excluded from the data analysis: congenital hypothyroidism, one; Tourette syndrome, one; dystonia, three; epilepsy, two; polyneuropathy sequelae, one; mental retardation, one; psychomotor retardation, four; meningitis sequelae, one; concussion, three; and deafness, one. None of the subjects examined had diabetes. The MeHg exposure of these subjects did not differ from that of other cohort members.

Brainstem auditory evoked potentials were determined in all participating subjects except one refusal (N = 859). We used a four-channel electromyograph (Medelec Sapphire-4ME, Surrey, United Kingdom) also used previously.[5,21] Click signals at an intensity of 65 dB hearing level (0.1-ms impulses of alternating polarity) were presented to the right ear through shielded ear phones at 20 Hz and 40 Hz (sampling time, 0.01 ms); the other ear was masked with white noise at an intensity of 45 dB HL. A frequency of 50 Hz was also attempted, but peak I was poorly defined at this click rate. Evoked potentials were recorded by using three standard electroencephalogram electrodes placed on the vertex, the right mastoid ipsilateral to stimulation, and the left mastoid (ground). Although 1024 responses were used 7 years before,[5,21] the number was increased to 2048 to improve the definition of peak I. Amplification and filtration were unchanged, and one replication of each condition was again performed for calculation of average peak latencies. The coefficients of variation for duplicate assessments remained higher for peak I (mean, 8.4%) than for peaks III and V (means, 4.3% and 3.7%, respectively). As an additional step for quality assurance, latencies from the first 250 children examined in Tórshavn in 2000 were scored twice by the same examiner (K. M.). The results of this blinded scoring–rescoring showed average coefficients of variation of 8.9%, 4.4%, and 3.7% for peaks I, III, and V, ie, similar to the duplicate assessments. Thus, although highly appropriate for latency measurement, the study circumstances did not allow accurate assessment of peak amplitudes. Peaks I, III, and V are thought to reflect the volume-conducted electric activity from the acoustic nerve, pons (superior olivary nucleus), and midbrain (inferior colliculi), respectively.[16]

Audiometry was performed by a trained nurse using Interacoustics Diagnostic Audiometer AD229 with a Peltor H7A headphone (Assens, Denmark) in a sound-insulated room. The patient-controlled Hughson-Westlake procedure was used in accordance with International Organization for Standardization 8253-1. A threshold was defined as two of three correct responses in a procedure with 5-dB increases and

Table I. Results of developmental methylmercury exposure biomarkers for 859 birth cohort members without neurological disease examined at age 14 years*

Biomarker	n	Geometric average	Interquartile range	Association with cord blood†
Cord blood (μg mercury/L)	835	22.6	13.2–40.8	(1)
Hair (μg mercury/g)				
Maternal, parturition	855	4.22	2.55–7.68	0.77
Child, 7 years	800	0.60	0.34–1.24	0.33
Child, 14 years	839	0.96	0.45–2.29	0.35

*Concentrations in μg may be converted to nmol by multiplying by 5.0.
†Correlation coefficient after logarithmic transformation.

10-dB decreases. Pure-tone air-conduction hearing thresholds were measured at 125, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, 6000, and 8000 Hz. Two children did not complete their audiometry examination.

Data Analysis

Pearson correlation coefficients were used to assess bivariate relationships between exposure variables. Regression analysis was used to determine the association of MeHg exposure with the outcome variables. Age and sex may be important predictors of BAEP latencies[16,21] and were therefore included as independent variables along with the exposure variables. In addition, confounders previously included in the analysis of neuropsychologic test results[5] were screened for possible associations with the outcomes in the current study, but no pattern was found. Further models included as an independent variable the latency result obtained 7 years previously along with the age at that examination. Additional analyses incorporated PCB and postnatal MeHg exposure parameters as explanatory variables. Because of skewed distributions, logarithmic transformation of the contaminant concentrations was used, and the mercury regression coefficients therefore correspond to the change in the dependent variable associated with a 10-fold increase in MeHg exposure. Significant exposure effects were further explored in generalized additive models, which do not require linearity assumptions while providing a smooth, nonparametric dose-response curve.[22]

Calculation of the benchmark dose (BMD) is increasingly used for comparison of dose-response curves at low dose levels and for determining exposure limits.[7,18] The BMD is the dose of a substance that increases the risk of an abnormal response by a benchmark response (BMR), ie, from P_0 (usually 5%) for an unexposed child to P_0 + BMR for a child exposed at the BMD.[23] The NRC committee used a BMR of 5% so that an exposure at the corresponding BMD will double the risk of an abnormal response.[7] To take the statistical uncertainty into account, a lower 95% confidence limit (BMDL) for the BMD is also determined. Using linear dose-response models, BMDLs expressed as the maternal hair mercury concentration were approximately 10 μg/g for the most sensitive neuropsychologic and BAEP outcomes in the Faroese children at age 7 years.[7,18,24] For comparison with these dose-response associations, we used the same default settings when calculating BMDL results for BAEP outcomes at age 14 years.

RESULTS

Prolonged Peak III and Peak V Latencies at Higher Prenatal Methylmercury Exposures Were Caused by Increased I–III Intervals That Were Prolonged Already 7 Years Before

Hair mercury concentrations at age 14 years (Table I) indicated that the children's current MeHg exposure had increased since the previous examination ($P < .001$). Approximately half of the children now exceeded the hair mercury limit of 1 μg/g, but the average corresponded to only one fourth of the concentrations in maternal hair at child birth. Nonetheless, the different sets of exposure biomarkers correlated well.

The BAEP latencies were similar to the results obtained at age 7,[5,18,21] and again differed as expected[16] between boys and girls. Age had no effect within the limited range studied.

Intrauterine MeHg exposure biomarkers showed several statistically significant associations with the BAEP latencies, especially peaks III and V at both frequencies (Table II). The same tendency was seen for the interpeak I–III latency, despite being affected by the greater imprecision of peak I determinations. Because peak I and interpeak III–V latencies were clearly not associated with the intrauterine exposure level, MeHg appeared to affect mainly the I–III interval. Neither sex nor age was associated with MeHg exposure levels, and confounder adjustment therefore did not affect the mercury regression coefficients.

Given the more robust findings for the full peak III latency (Fig 1), its better precision, and the parallel results for this outcome obtained at age 7 years,[5,18] this outcome parameter was selected for more detailed calculations. Inclusion of the postnatal exposure biomarkers as additional predictors did not affect the regression coefficients for the prenatal exposures. However, they were almost completely abolished when peak III latencies at age 7 were incorporated as predictors.

Table II. Mean results and regression coefficients for logarithmic transformations of mercury exposure biomarkers as predictors of latencies of brainstem auditory evoked potentials (ms) in 859 Faroese children at 14 years

		Regression coefficient* (*P* value)			
	Mean (SD)	Cord blood (n = 835)	Maternal hair (n = 855)	Hair at 7 y (n = 800)	Hair at 14 y (n = 839)
20 Hz					
I	1.770 (.129)	0.015 (.213)	0.001 (.942)	−0.005 (.622)	0.006 (.553)
III	3.952 (.161)	0.045 (.002)	0.037 (.014)	0.012 (.335)	0.001 (.907)
V	5.788 (.204)	0.049 (.006)	0.032 (.085)	−0.002 (.901)	0.018 (.159)
I–III	2.183 (.152)	0.027 (.051)	0.036 (.013)	0.017 (.150)	−0.004 (.631)
III–V	1.835 (.132)	0.004 (.722)	−0.005 (.686)	−0.014 (.181)	0.017 (.056)
40 Hz					
I	1.806 (.169)	0.027 (.089)	0.014 (.410)	0.007 (.602)	0.012 (.293)
III	4.054 (.178)	0.032 (.048)	0.023 (.169)	0.008 (.536)	0.002 (.847)
V	5.954 (.214)	0.048 (.009)	0.036 (.066)	0.006 (.686)	0.024 (.070)
I–III	2.248 (.190)	0.004 (.805)	0.009 (.614)	0.001 (.925)	−0.010 (.430)
III–V	1.900 (.148)	0.015 (.226)	0.013 (.383)	−0.002 (.852)	0.022 (.028)

*Adjusted for sex and age; because of the logarithmic transformation of the mercury concentrations, the regression coefficient indicates the change in the dependent variable associated with an increase in MeHg exposure by a factor of 10.

Current Methylmercury Exposures Were Associated With Prolonged III–V Interpeak Latencies

The regression coefficients also suggested an effect of recent MeHg exposure, but only on the III–V interpeak interval (Table II, Fig 2). This association was not affected by inclusion of prenatal exposure biomarkers, and neither did the lower mercury concentrations at age 7 years seem to affect this outcome parameter. At the same time, this interpeak variable was significantly associated with all other peak latencies, except for the peak I latency.

Inclusion of PCB exposure within the subset of the cohort for which this parameter was available did not affect any of the MeHg regression coefficients. In addition, the PCB parameter did not reach statistical significance in any of the analyses.

Audiometry results generally showed normal hearing, and hearing thresholds above 30 dB(A) were recorded for only approximately 2% of the children. Hearing thresholds were not associated with MeHg exposure, except for 4 kHz in the right ear (Table III). The association with the peak III latency (Table III) was caused by a prolonged latency for peak I at increased hearing thresholds, whereas the interpeak I–III interval was unaffected. PCB exposure and postnatal MeHg exposure were not associated with the audiometry results. Inclusion of the hearing threshold at 4 kHz as a predictor of BAEP latencies changed the mercury regression coefficients only marginally.

Benchmark Dose Results Were Similar to Those Seen at Age 7 Years

The relative magnitude of the regression coefficients (Table II) can be judged by comparison with the variability of the outcome variables. Thus, for peak III latencies, an average regression coefficient of approximately 0.04 corresponds to almost 25% of the SD. Because a logarithmic transformation of the mercury concentrations was used, the effect of a doubling of the exposure can be determined by multiplying the regression coefficient by 0.301. Accordingly, a doubling of the prenatal exposure results in a latency prolongation by about 7% of the SD. Similarly, a doubling of the current exposure level is associated with a prolongation of the interpeak III–V interval by about 5% of the SD.

Additional comparisons may be based on BMD calculations. Prenatal BMDL results for peak III at the two frequency conditions corresponded to an average of approximately 10 μg/g hair based on either cord blood or maternal hair. For the III–V interval, postnatal BMDLs averaged approximately 5 μg/g for the child's hair mercury concentration at age 14 years.

DISCUSSION

The developing brain is thought to be the organ most vulnerable to MeHg exposure.[1,7] Emphasis in risk assessment has therefore been placed on neurologic function of children with intrauterine exposure to this neurotoxicant, and previous studies have applied neuropsychologic function as a key measure of adverse effects.[4-6] In parallel, neurophysiologic tests, such as BAEP assessment, have been used in population studies as highly standardized, rapid, painless, and inexpensive procedures.[16,25] Prolonged BAEP latencies have been reported as an effect of exposure to MeHg[13-15] and other neurotoxicants, such as lead.[17,25]

We report that BAEP latency assessments were highly reproducible and that several latencies at age 14 years showed a positive association with MeHg exposure. Intrauterine exposure was mainly associated with delays in peaks III and V,

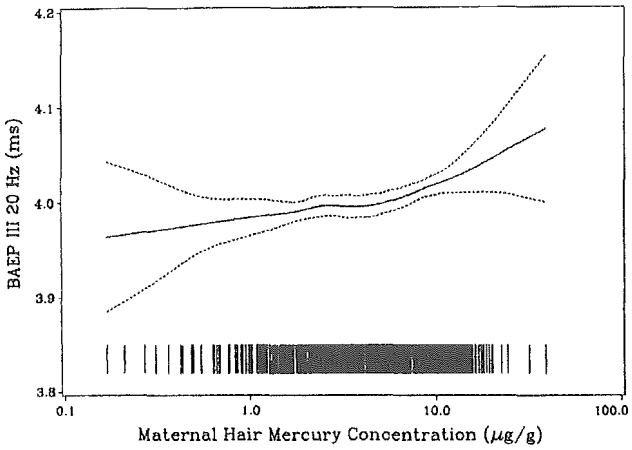

Fig 1. Prenatal dose-effect relationship between maternal hair mercury at birth and the peak III latency of the brainstem auditory evoked potentials in 859 Faroese children at 14 years, adjusted for sex and age. The association is estimated in a generalized additive model analysis in which a smooth nonparametric curve (equivalent degrees of freedom, 3) is fitted to the data while adjusting for confounders. The *broken lines* indicate the point-wise 95% confidence interval for the dose-response relationship. Each *vertical line above the horizontal axis* represents one observation at the exposure level indicated. To convert to nmol/g, multiply mercury concentration in μg/g by 5.0.

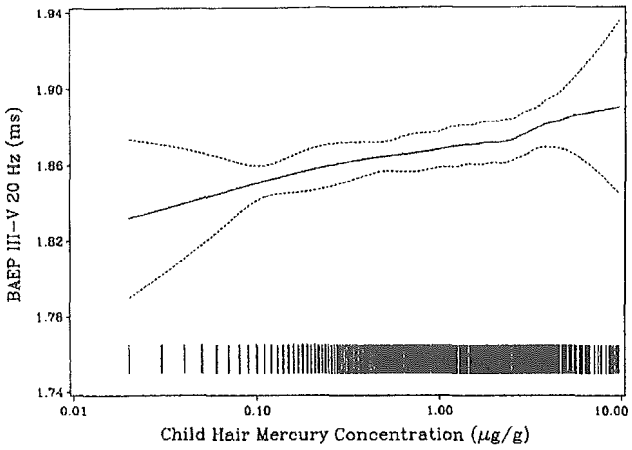

Fig 2. Postnatal dose-effect relationship between the child's current hair mercury and the interpeak III-V latency of the brainstem auditory evoked potentials in 859 Faroese children at 14 years, adjusted for sex and age. The association is estimated in a generalized additive model analysis as in Figure 1, but the horizontal scales differ. The *broken lines* indicate the point-wise 95% confidence interval for the dose-response relationship. Each *vertical line above the horizontal axis* represents one observation at the exposure level indicated. To convert to nmol/g, multiply mercury concentration in μg/g by 5.0.

and the I–III interpeak interval appeared to be most sensitive. This result is in accord with previously reported exposure-associated delays in the same cohort examined at age 7 years and in a cross-sectional study of 7-year-old children from another North Atlantic fishing population.[18,21] However, the regression coefficients at age 7[21] were approximately twice the magnitude observed 7 years later. Furthermore, adjustment of the most recent peak latency for the result obtained 7 years previously virtually abolished the mercury effect. These observations suggest a lasting neurotoxic effect of the intrauterine exposure, although the reduced regression coefficient may perhaps indicate some degree of compensation. The peak III results also suggest that this outcome is not affected by postnatal exposures at the levels occurring in this population.

More recent MeHg exposure, as reflected by the current hair mercury concentrations of the children, was associated with a prolonged interpeak III–V interval. This observation is noteworthy, because the children's current exposures averaged less than one fourth of the maternal levels during pregnancy, and a single hair analysis probably is a very inaccurate marker of the causative postnatal exposure levels. Although paired mother–child exposure data correlated well and thereby suggested relatively stable dietary habits within each household, only the recent exposure level was associated with this outcome. Hair mercury concentrations at age 7 years were the lowest and did not contribute to this association.

Despite the delayed BAEP latencies, the audiometry data suggested only limited, if any, effect of MeHg exposure on hearing thresholds. These results parallel those obtained at age 7 years.[26] All BAEP latencies were recorded with a sound pressure adjusted for audiometry results; no association between hearing thresholds and BAEP latencies was detected, except for peak I at a single sound frequency on one side only. Although deafness has been reported in severe congenital MeHg poisoning cases,[2] hearing loss is not a uniform finding in less serious childhood poisonings or in adult cases.[2,27]

The MeHg-associated prolongation of BAEP latencies in the current study was subtle and comparable with effects associated with lead exposure.[17,25] These changes are much less extensive than clinical findings in patient groups, such as the abnormal BAEP waves with poorly defined or absent peaks III and V in multiple sclerosis, and the markedly prolonged interpeak latencies in patients with acoustic neurinoma or diabetes mellitus.[28] However, the relative change parallels the extent of neuropsychologic deficits determined in the cohort children at age 7 years.[5] Thus, in several functional domains, a doubling of the intrauterine MeHg exposure showed a decrease in performance by 5% to 10% of the SD.[5,29] Subtle neurotoxic effects, sometimes expressed in terms of IQ points, have important societal implications in regard to educational achievement and earning potential.[30]

The BMDL represents a statistically defined point on the dose-response curve that allows comparison between low-range toxicity studies. However, the BMDL should not be interpreted as a threshold indicator. Indeed, significant exposure-related deficits on neuropsychologic tests at age 7 years were documented at maternal hair mercury concentrations below the BMDL.[5] Previous calculations[7,12,24,31] based on the most sensitive neurologic, neuropsychologic, and neurophysiologic endpoints all indicate a BMDL of about 10 μg/g maternal hair, ie, the same level as found for peak III delays at age 14 years. We found that the postnatal BMDL for the prolonged III–V interpeak interval was approximately

Table III. Cord blood mercury concentration (geometric mean) and peak III latency of the brainstem auditory evoked potentials measured at age 14 years (arithmetic means) in 857 Faroese cohort children in relation to the hearing threshold at 4 kHz on the right ear

Hearing threshold (dB[A])	n	Mercury concentration*	Peak III latency (ms) 20 Hz†	40 Hz†
<0	158	20.0	3.94	4.03
0	171	20.8	3.92	4.02
5	218	22.8	3.94	4.03
10	161	24.9	3.99	4.09
>10	136	25.4	3.98	4.10

P value for association (Spearman correlation coefficient) with hearing threshold: * < .01, † < .001.

one half of that. However, because of statistical uncertainty, this difference may not necessarily reflect the relative toxic potentials of prenatal and postnatal exposures.

The participation rate at age 14 years was very high, thereby reducing the concern that the results may have been affected by differential follow-up rates. An important strength of this study is that the examinations relied on the same methodology as 7 years before, and were performed by the same examiner, who was blinded to exposure data and previous peak latency results. In addition, the outcome measures were confirmed to be independent of socioeconomic confounders. The known[16] BAEP peak latency difference between boys and girls was replicated, but sex was not associated with MeHg exposure and therefore did not cause confounding. At age 7 years,[21] prolongations of the peak I latency occurred as a result of middle ear infection, but at age 14 years, this trait was absent.

An important limitation is that few postnatal exposure estimates were obtained and that the prenatal and postnatal exposure indicators were highly associated. Although dietary patterns may have been rather stable, the postnatal exposure biomarkers do not necessarily represent the magnitude of the toxic exposure at susceptible time windows. Any exposure misclassification would be mostly random and would tend to dilute the associations with the outcome variables, although this dilution would be limited by the wide exposure interval covered within this cohort. Despite this bias, the size of the cohort allowed separation of latency prolongations associated with intrauterine and recent MeHg exposures. The fact that peaks III and V at both frequencies showed clear associations with two independent indicators of prenatal MeHg exposure, and not with indicators of postnatal exposure, suggests that the findings are robust and credible for human health risk assessment. Likewise, although unanticipated, the association of the prolonged interpeak III–V interval with recent MeHg exposure only was also seen at both frequencies.

As previously reported for the results at age 7 years,[26] concomitant prenatal exposure to PCBs, which occur in whale blubber sometimes eaten in the Faroes, did not influence the BAEP outcomes. Developmental exposure to PCBs is now thought to affect primarily cochlear function and effect on BAEP amplitudes rather than latencies.[32] In addition, lead exposure was comparatively low and not associated with exposure to mercury.[19] The generalizability of this study would therefore not seem to be limited by concomitant exposures to other neurotoxicants.

Although a chance finding in multiple comparisons cannot be ruled out, the possibility that prenatal and postnatal MeHg exposure may affect different targets in the brain is supported by both experimental and clinical evidence. Prenatal exposure of rats to toxic amounts of MeHg results in severe lesions that include the brainstem, whereas effects of postnatal treatment are less diffuse and particularly involve the sensory cortex.[9] Similarly, neuropathologic and imaging evidence reveals a greater degree of focal cortical damage with postnatal MeHg exposure compared with congenital cases.[2,3,8] The results of this study would therefore seem to be plausible, although the specific vulnerability of the interpeak III–V interval to postnatal MeHg exposure was not predicted. Although the significance of postnatal MeHg exposure needs to be documented further in independent studies with more frequent exposure assessments, our results suggest that developmental vulnerability to MeHg neurotoxicity is likely to extend into the teenage period.

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EFFECTS OF PERINATAL EXPOSURE TO ENVIRONMENTALLY PERSISTENT ORGANIC POLLUTANTS AND HEAVY METALS ON NEUROBEHAVIORAL DEVELOPMENT IN JAPANESE CHILDREN: IV. THYROID HORMONES AND NEONATAL NEUROBEHAVIORAL STATUS

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Introduction

From several epidemiological studies, it has been reported that there are some associations between perinatal exposures to PCBs, dioxins and heavy metals, and neurobehavioral defects such as postnatal growth delay and poorer cognitive function[1]. We have started a prospective cohort study to examine the effects of perinatal exposures to environmentally persistent organic pollutants on neurobehavioral development in Japanese children[2].

Thyroid hormones (THs) are essential for normal brain development. A lack of THs in pregnancy can result in congenital hypothyroidism, which causes moderate to severe intellectual defects. It has been reported that perinatal exposure to PCBs adversely affects on children's intellectual functions. The chemical structures of some PCBs resembles thyroxine (T4), and therefore, it is suspected that the action mechanism of PCBs is disruption of TH function. Some PCBs and their metabolites are thought to bind with transthyretine (TTR)[3], which is necessary for the transfer of T4 into the brain, and this may cause a shortage of T4 in the developing brain. To examine the effects of perinatal exposure to PCBs on children's development, it is essential to evaluate the functions of THs at a fundamental level.

In this report, we examined the correlations of THs in maternal peripheral blood and cord blood, and the association between THs and neonatal neurobehavioral status.

Methods and Materials

The subjects of this study were 545 mother-infant pairs. Mean maternal age at the time of delivery was 31.40 (SD4.29). Mothers were recruited with their informed consent at obstetrical wards of two hospitals in Sendai, Japan. The infants consisted of 284 boys and 261 girls, and they were all singleton and born after full-term (36 to 42 weeks) gestation without congenital anomalies or diseases. Birth weight was 2500g or more. Information was obtained about pregnancy, delivery conditions and infant characteristics from their medical records. These protocols were described previously[2].

Maternal peripheral blood samples were collected at 28 weeks of pregnancy; umbilical cord blood samples were collected shortly after delivery. THs, including thyroid-stimulating hormone (TSH), total thyroxine (T4), triiodothyronine (T3), free T4 (FT4) and free T3 (FT3), were measured from plasma by SRL, Inc. (Tokyo, Japan), with the use of radioimmunoassay.

The Neonatal Behavioral Assessment scale (NBAS) was administrated three days after birth. Examiners of the NBAS were trained and certified to administer it at the Training Center for NBAS in the Nagasaki University School of Medicine in Japan. Reliability checks were conducted throughout data collection to maintain a 90% level of agreement.

In statistical analysis, we examined the correlations of THs between maternal and cord blood. Single regression analyses were performed to examine the associations between THs and the seven NBAS cluster scores. When significant associations were observed, multiple regression analyses were performed for controlling the effects of covariates, which included gender, birth weight, gestational age, Apager score 1 minute after delivery, maternal age at the time of delivery, delivery type, parity, alcohol drinking during pregnancy, smoking habit and NBAS examiners.

Results and Discussion

Figure 1 shows the distribution of THs in maternal and cord blood. T3 and T4 in both maternal and cord blood had almost normal distributions. FT3 and FT4 also showed similar distributions (data not shown). The concentration of TSH in the cord blood was about ten times higher than that of maternal blood (11.36 μU/ml in cord blood, 1.54 μU/ml in maternal blood).

There were some significant ($p<0.05$) correlations of THs between maternal and cord blood. Typical correlations are shown in Fig 2. There was a positive correlation between maternal T4 and cord T4, and a negative correlation between maternal TSH and cord FT4. There were no significant correlations of maternal TSH between cord blood TSH and T4.

Although TSH in maternal and cord blood had no significant association with any of the seven NBAS clusters, cord blood T3 and FT3 had significant positive correlations with the Orientation cluster (Fig 3). These remained significant after controlling for covariates. There were no significant associations of T4 and FT4 in maternal and cord blood with any of the NBAS clusters.

One possible hypothesis of the action mechanisms of persistent organic pollutants (POPs) is the disruption of thyroid function. The chemicals have been shown to alter the metabolism of THs in animal experiments[4]. Human data also suggested that background-level exposure to PCBs might have similar effects in newborns in the Netherlands[5], whereas no association between PCB exposure and the status of THs in cord blood was observed among North Carolina children[6]. Several studies have suggested an association of exposure to dioxin-like compounds with increased TSH in young children[7]. These reports suggest that exposure to POPs may disturb the hypothalamic-pituitary-thyroid regulatory system, and then affect the neurobehavioral status of children. In the present study, we examined the relationship between levels of THs and neonatal neurobehavioral status. Single regression analyses showed that there were several significant associations between THs and NBAS cluster scores. Although cord blood T3 and FT3 had significant positive correlations with the Orientation cluster, TSH, T4, and FT4 in the maternal and cord blood had no significant correlation with any of the NBAS cluster scores. These findings suggest that thyroid function is a modulator of the neurobehavioral status in newborns; however, the relation between chemical exposure and neonatal neurobehavioral awaits further investigation.

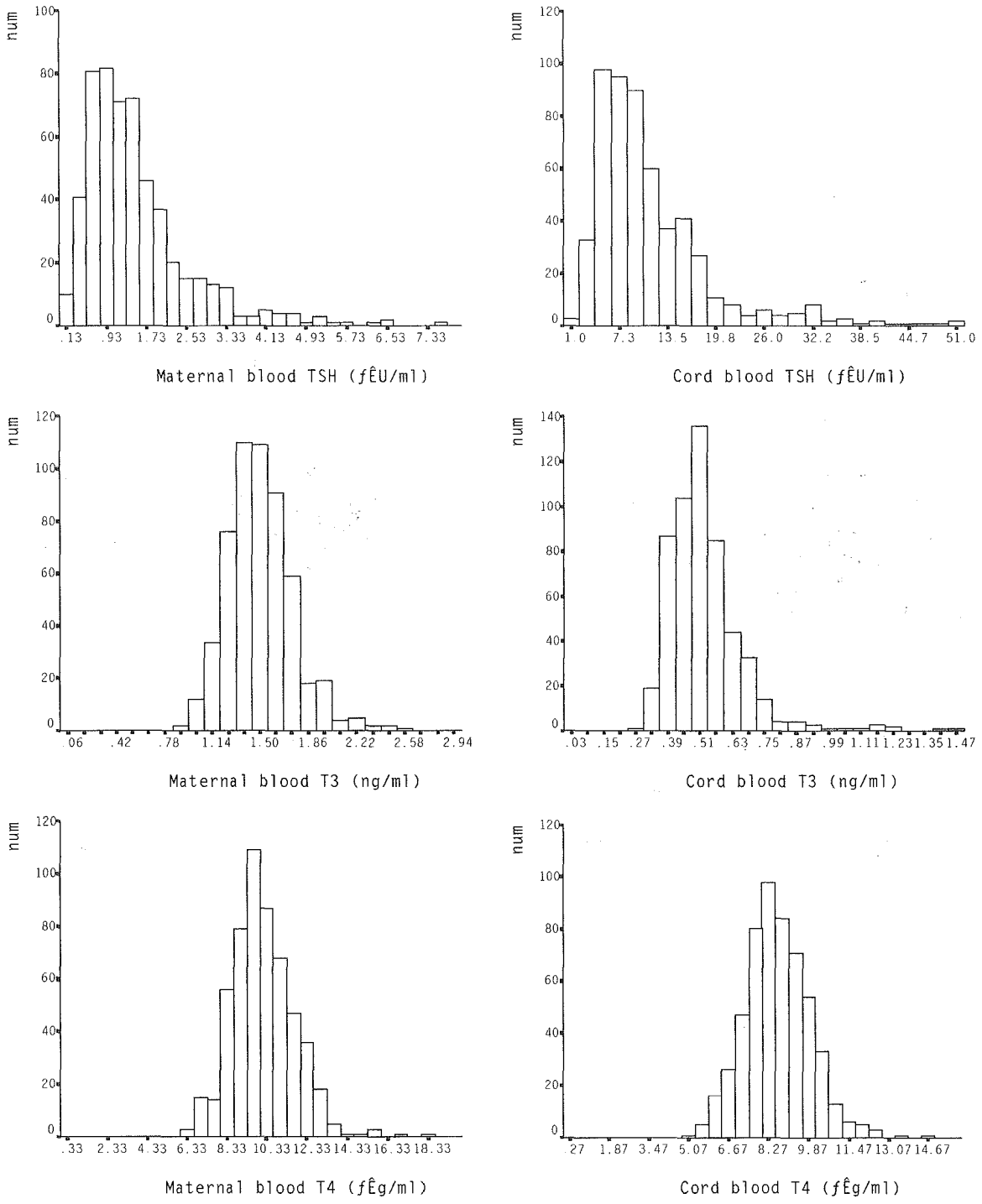

Fig 1. Population distributions of maternal blood and cord blood thyroid hormones

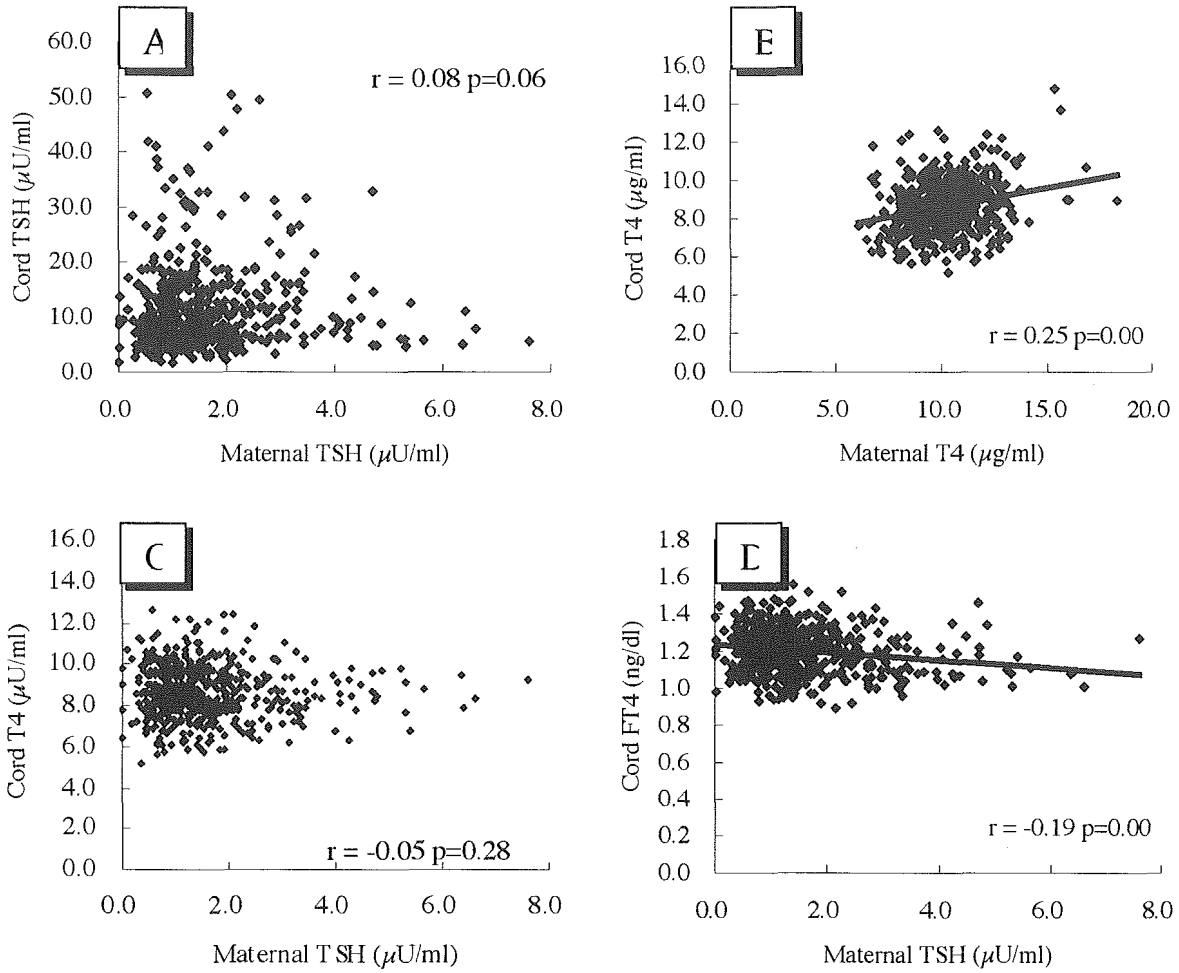

Fig 2. Correlations of thyroid hormones of maternal and cord blood