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in Two Cohorts of Children

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Short title: Benchmark dose for mercury

Delays in evoked potential latencies were observed at increased exposures to methylmercury from seafood in two cohorts of children. Because this outcome parameter appeared to be virtually independent of confounders, including cultural differences, a joint analysis of benchmark doses was carried out. Comparable cohort members included 382 Faroese and 113 Madeiran children without middle ear infection or neurological disease at age 7 years. Maternal hair-mercury concentrations at parturition in the Faroese children ranged from 0.6 to 39.1 :g/g (geometric average, 4.49 :g/g). In Madeira, mothers who had not changed their diet since pregnancy had current hair mercury concentrations ranging from 1.1 to 54.4 :g/g (geometric average 10.14 :g/g). The mercury-associated delay in peak III latencies at two frequencies (20 and 40 Hz) showed similar regression equations in the two groups of children, and benchmark dose calculations were therefore carried out for the two groups separately and jointly. For a doubling of a 5% prevalence of abnormal results of the peak III latencies at 40 Hz in a linear dose-response model, the benchmark dose for the maternal hair mercury concentration was 8.79 :g/g for the Faroese children; 8.04 :g/g for the Madeiran children; and 9.46 :g/g for both groups. Results were similar for the 20 Hz condition. Benchmark dose results were substantially lower using a logarithmic or square root curve function, although the difference in fit between the curves was far from statistically significant. The benchmark results using evoked potential latencies are in close agreement with results based on neuropsychological test performance.

KEY WORDS: Methylmercury; benchmark dose; brainstem auditory evoked potentials; neurotoxicity; human health risk assessment

1. INTRODUCTION

Methylmercury is a well documented neurotoxicant, which can cause serious adverse effects on brain development, especially when exposures occur prenatally.[1,2] While current risk assessment efforts have emphasized neuropsychological test results as important outcome variables,[2,3] delays in evoked potential latencies recorded from the brain have provided useful supplementary evidence of pathological changes in methylmercury poisoning.[4-8] The neurophysiological response parameters require only minimal cooperation by the subject and are unlikely to be affected by socioeconomic or cultural differences.[9] Recent studies have reported latency delays at increased exposures to methylmercury from seafood and freshwater fish.[10-12] This evidence suggests that the latency of peak III on the auditory brainstem response may be particularly sensitive to mercury.

We have been involved in epidemiological studies of 7-year-old children in two geographically separate fishing communities exposed to methylmercury from contaminated seafood.[11,12] In both island cohorts, increased latencies of evoked potentials were associated with higher maternal exposures but were not affected by the child's current exposure.[11,12] In the Faroe Islands, increased maternal exposures originate mainly from pilot whale meat,[13] while the exposure in Madeira is from certain species of marine fish, such as black scabbard.[14] Both communities are rather stable and homogeneous, and the participation rate in the studies was high. Because the children were examined by similar methods and by the same clinical team, a joint analysis of the data seemed indicated.

Dose-response modeling for risk assessment purposes often uses benchmark dose calculations,[15] and substantial information is now available for mercury-associated effects on neurological development and performance on psychological tests.[2,16-18] The current study was therefore carried out to determine benchmark dose levels using similar default conditions for the neurophysiological results and to apply the benchmark approach for joint analysis of results from two separate studies. Because the shape of the dose-response functions for methylmercury neurotoxicity is unknown,[2] different curve functions were applied.

2. MATERIALS AND METHODS

2.1. Study Populations and Exposure Assessment

The two studies have been described in detail elsewhere.[11,19] In the Faroes, the prospective study included 382 children (187 boys and 195 girls) from a birth cohort generated in 1986-1987 and examined at age 7 years in 1993; six children did not complete the examination (one child at 20 Hz only), and children with neurological disease (N = 6) or current middle ear infection (N = 45) were excluded.[12] The mercury exposure

biomarkers available for these children included the mercury concentration in maternal hair obtained at parturition (geometric mean, 4.52 :g/g; range, 0.6-39.1 :g/g). This parameter is thought to reflect the maternal exposure during pregnancy and is therefore also a risk indicator for fetal neurotoxicity. While the children were also exposed to PCB, this neurotoxicant did not show any clear association with peak latencies.[20]

The cross-sectional study in Madeira involved children in first grade at the two schools at the fishing harbor of Câmara de Lobos, Madeira, Portugal.[11] The children examined in 1994 were born in 1987. Two children were excluded because of neurological disease; middle ear infection was not present. As indicator of prenatal methylmercury exposure, we used the current mercury concentration in maternal scalp hair. Children whose mothers had changed their diet since pregnancy were therefore excluded (N = 29). For the remaining 113 children (53 boys and 60 girls), the maternal hair mercury concentration varied from 1.1 to 54.4 :g/g (geometric mean, 10.14 :g/g).

The mercury analyses of the Faroese samples involved the length of hair strands thought to represent the full pregnancy period, i.e., in most cases 8-9 cm. The analysis was carried out by an experienced laboratory which has also been responsible for similar analyses in other population studies.[21] For the Madeira children, a 3-cm proximal hair segment was analyzed by a similar method.[11] Both laboratories have documented high analytical quality, and the methods are thought to be entirely comparable. The total analytical imprecision has been estimated to be about 3-4% (as coefficient of variation) at the average hair-mercury concentrations seen in the two populations.[11]

2.2. Evoked Potential Assessment

For determination of evoked potential latencies, we used a 4-channel electromyograph (Medelec Saphire-4ME). BAEP was measured in the children in comfortable reclined position. Click signals with an intensity of 65 dB HL (0.1 ms impulses of alternating polarity) were presented to the right ear through earphones at a rate of first 20 Hz, then 40 Hz.[11,22] The other ear was masked with white noise at 45 dB HL. The BAEP was recorded using three standard EEG electrodes placed on the vertex, the right mastoid ipsilateral to stimulation and the left mastoid (ground). The responses were averaged 1,024 times after amplification and filtration, with one replication at each condition. Peaks I, III and V reflect the volume-conducted electric activity from the acoustic nerve, pons and midbrain, respectively.[23] Delays in peak I and subsequent peaks may therefore occur in the case of middle ear infection.

The imprecision of latency assessments was determined from the coefficient of variation of duplicate measures in the Faroese children. The latency of peak I showed imprecisions of 14.6% and 16.0% at 20 and 40Hz, respectively, while the corresponding imprecisions of Peak III latencies were 8.4% and 7.6%. Daily examinations over a 16-day period had previously shown coefficients of variation of the peak I latency that were more than twice the level of peak III.[24] Although the interpeak latency I-III would be preferable to avoid interference from inner ear disease,[22] we therefore used the total peak III latency as outcome parameter in the present study.

2.3. Regression Analyses

Regression analyses were performed using the maternal hair mercury concentration as the dose parameter. The distribution of the mercury results was skewed, and an improved fit of the dose-effect association was obtained using logarithmically transformed mercury data.[11,12,19] Sex and age were included as potential confounders, although they had only a small effect on the response variables. Other covariates, such as maternal smoking and alcohol intake during pregnancy, were far from significant as predictors of the latencies and were therefore not included in the multiple regression analyses.[11,12]

2.4. Benchmark Calculations

As proposed by Crump,[15] the benchmark dose (BMD) is the dose of a substance that results in an increased probability of abnormal test performance by a benchmark response (BMR), i.e., from P_0 for an unexposed child to $P_0 + BMR$ for a child at the BMD. Previous applications of this method[2,16-18,25,26] have used a P_0 of 5%, i.e., an abnormal test performance defined by a probability of 5% in an unexposed population. This definition of P_0 does not take into account any medical considerations. A lower confidence limit (BMDL) for BMD is then calculated as the statistical 95% lower bound of the BMD. Thus, the BMDL depends on the statistical uncertainty of the BMD determination and will therefore tend to increase when the size of a study increases. Recent calculations have used a BMR of 5% as an appropriate choice, as it corresponds to a doubling of the prevalence of abnormal test performance.[2,26] As the magnitude of the BMR substantially affects the calculations, we have also used BMRs of 2% and 10%.

The benchmark concept was originally developed in the context of highly standardized carcinogenesis trials with a dichotomous outcome.[15] In applying the method for graded responses in epidemiological studies, the influence by confounders must be taken into account in addition to the exposure of interest. The preferable

adaptation of the benchmark concept to this situation is to use the same unexposed risk (P_0) for each child and a linear multivariate model for the dependence on the confounders. Accordingly, for each child the level of abnormal test performance depends on the individual values of the confounders for that child. This approach avoids dependence of the BMD and BMDL on the confounders.

In the two studies considered, the dose is expressed as the mercury concentration in maternal hair. A proper regression model would then be:

$$Y_i = \forall + \exists_1 * sex_i + \exists_2 * age_i + :(d_i) + ,_i,$$

where Y_i denotes the response of child i, and $\exists$ is the regression coefficient for a confounder, while the error term $,_i$ is normally distributed with a mean of zero. The function $:(d_i)$ gives the expected change in latency at the child's dose level d_i compared to an unexposed child with the same confounder values. Values of BMD and BMDL may be calculated using a statistical dose-response model where : is a power function[15] for the dependence of a child's expected latencies of evoked potentials on the mercury dose parameter (d):

$$:(d) = \exists \cong d^K$$

The power parameter K is restricted to values equal to or above 1, thus allowing the dose-response curve to be nonlinear. This approach has the advantage of avoiding unreasonably low BMDLs, which may otherwise occur if the dose-response curve becomes infinitely steep at low doses. However, the parameters do not enter the model in a linear fashion, thus complicating the calculation of the BMDL. We therefore used the parametric bootstrap method[27] for this purpose. Two-thousand new data sets were simulated from the distribution of the original data. Each of these data sets has covariate values equal to the original data, whereas the response is given by the function of the covariates plus the random error. Each simulation allows a determination of BMD, and the BMDL is then derived as their 5th percentile.

Using this approach, a power of 1 generally provided the best fit to the Faroese neuropsychological test data, i.e., a linear association between dose and response.[2,26] In accordance with the recent applications,[2,26] we therefore also included the linear dose-response curve. Because of the difference in calculations, the BMDL may be slightly different from the one estimated for the K power function with K = 1.

A logarithmic dose parameter provided a slightly better fit for neuropsychological outcome parameters[24] and had been used in previous regression analyses of the evoked potential data from both populations.[11,12] In examining the robustness of the benchmark calculations, we therefore also used a logarithmic and a square root dose parameter. To avoid problems with an infinite slope of these curves at zero dose, the dose

was transformed after addition of 1 to the mercury concentration. The following three additional dose models were therefore used:

Linear dose-response model: $:(d) = \exists \cong d$

Square root dose-response model: $:(d) = \exists \cong \{ \sqrt{(d+1)} - 1 \}$

Logarithmic dose-response model: $:(d) = \exists \cong \log(d+1)$ The fits of these three models was determined pairwise, i.e., each model was tested against an expanded model that also included another model. A high p-value in this test indicates that the model at hand does not fit the data significantly worse than the expanded model. Because the expanded model in all cases fitted the data only slightly better than the best of the individual models, the three models can be compared simply by calculating their -2log(L) difference and then judging this value from a chi-square table. The fit of the K-power model could not be compared to the rest of the models by formal asymptotic likelihood testing. However, as the K-power model gives approximately the same fit as the linear model but includes one more parameter, at best this model can be considered only as good as the linear model.

3. RESULTS

Three hundred and eighty-two Faroese children and 113 Madeiran children without middle ear infection or nervous system disease were included in the benchmark calculations. The mercury concentrations in maternal hair at child-birth were significantly related to the III peak latencies at both 20 and 40 Hz after adjustment for age and sex (Table I) as previously reported.[11,12] The average latencies were slightly greater at the Faroes than at Madeira.

When comparing the two regression equations with confounder adjustments, the residual variance was found to be similar. More importantly, the regression coefficients (betas) for mercury did not differ, as suggested by the standard deviations. The lowest p-value for a difference between the two groups of children was 0.08 for the 40 Hz results using the logarithmic dose scale. Likewise, the small effects of age and sex were similar in all equations compared.

The two sets of data were therefore collapsed, while allowing for different intercepts for the two groups. Table I shows the joint regression coefficients for the logarithmic transformation of the mercury concentration. The effects of mercury measured on the linear or the square root dose scales also showed p-values of 0.001 or below. Figs 1 and 2 depict the association of peak III latencies at the two test conditions with the maternal

hair-mercury concentration after adjustment of the response variable for age and sex. A logarithmic dose scale has been used because of the skewed distribution of the data.

Tables II-III show the results of the benchmark calculations for peak III latencies of the BAEPs at 20 and 40 Hz using the four curve models. As expected from Table I, the 20 Hz results for the Faroes and Madeira are quite similar, but the BMDs and BMDLs are lower in Madeira than in the Faroes for the 40 Hz test condition. The collapsed population results show BMDs close to the average for the two populations, but the BMDLs are closer to or slightly greater than the largest of the two individual results.

The K power model showed the best fit at K = 1 or slightly above 1. The BMDs and BMDLs were therefore quite similar to those obtained with the linear dose function. The fit of the logarithmic models linear, square root, and logarithmic models were compared from likelihood function results (data not shown). Despite the fact that the benchmark results in Tables II and III are clearly different, the difference in model fits was far from statistically significant.

4. DISCUSSION

The developing brain is thought to constitute the most vulnerable organ in regard to methylmercury exposure.[1-3] Emphasis in risk assessment has therefore been placed on the neurological function of children with developmental exposure to this neurotoxicant. However, results of psychological tests may not be comparable between cultures and languages, and age standardization may not be meaningful if tables from a different country are employed. In this regard neurophysiological tests may be preferable to the extent that they can be applied in population studies. Assessment of evoked potential latencies is one such method, which has been shown to be sensitive to methylmercury[4-8] and other neurotoxicants.[9,28] In the two different populations of 7-year-old children examined, we found that indicators of prenatal methylmercury exposure were significantly associated with the peak III latency of the BAEPs. The peak III latency reflects axonal conduction in auditory tracts rather than synaptic activation within brainstem auditory nuclei, and delays may imply segmental demyelination and axonal and neuronal loss.[23] Accordingly, evoked potential latencies are used for clinical purposes in diagnosing early stages of diseases such as multiple sclerosis.[23] The magnitude of mercury-associated delays is similar to effects previously attributed to exposure to other neurotoxicants.[9,28-30] For example, delayed BAEP latencies were documented in asymptomatic children exposed to lead, thus suggesting subclinical pathology of the auditory pathway rostral to the cochlear nucleus.[29,30] Although the latency results obtained have not revealed any frank

abnormality in the children examined, the evidence on methylmercury neurotoxicity and previous findings on BAEP latencies indicate methylmercury may cause adverse effects on the auditory tracts.

The maternal hair-mercury concentration is probably a rather imprecise measure of the amount of methylmercury that has reached the child's brain during the critical time period of maximum vulnerability.[31] Although uncertainty about the effective dose in general will affect the validity of the benchmark results, current error models[32] are not easily applicable for estimation of the possible magnitude of any bias. Some general observations should be noted in this regard. While the Madeira study was cross-sectional in design, information on developmental exposure levels was obtained at birth for the Faroese cohort. The range of mercury exposure in both groups spanned approximately two orders of magnitude, thus possibly limiting the impact of exposure misclassification. Despite the different sampling times for the exposure biomarker, the two populations showed similar and statistically significant mercury-associated delays in peak III latencies. It is noteworthy that mercury exposure levels differed between mother and child, but only the maternal exposure level was a significant risk factor for delayed evoked potential latencies in the child.[11,12] In other communities where individual differences in exposure levels were thought to be relatively stable, cross-sectional studies of children have also shown clear associations between current mercury exposure levels and neurobehavioral deficits.[33,34]

The cross-cultural similarity of the mercury-association in the two populations is in accordance with the assumption that evoked potential latencies are independent of language, education, and socioeconomic factors.[21] However, the total peak III latency was about 0.1 ms shorter in Madeira than in the Faroes, i.e., a difference corresponding to the size of the regression coefficients shown in Table I. Because mercury exposures in Madeira were higher than in the Faroes, this difference cannot be attributed to mercury. Other factors, including genetic differences or temperature,[23] must be suspected. This finding emphasizes the importance of including subjects with low-level exposures from each population as a built-in control group.

Two different conditions of the BAEP were used. The benchmark results based on the Faroese data are very similar for the two conditions. At 20 Hz, the results obtained for the linear model are slightly higher when based on the Madeira results, but the reverse was seen at the 40 Hz condition. However, these differences are well within the uncertainties expected from the standard deviations of the regression coefficients (Table I). When merging the data, the resulting BMDs are quite similar to the averages for the two groups if account is taken that the Faroese population was three times larger than the Madeiran. However, the joint BMDL results are closer to the larger of the two, in some cases greater, as the combined number of observations causes a decrease in the

statistical uncertainty.

In the original approach to benchmark calculations,[15] a power function was proposed for the dose-response curve, with the power being greater or equal to 1. When using neuropsychological test results[25] as the outcome variable, the best fit was obtained at a power of 1, e.g., a linear association. The present study showed similar results, although two of six K values are slightly above 1. Accordingly, the benchmark results obtained for the power function are almost the same as those seen with the linear function and does not provide a better fit.

Because the shape of the dose-response curve will depend on the outcome variable and the range of exposures covered, concave curves may also be envisaged. As in previous calculations,[25] we therefore included for comparison a logarithmic and a square root function. Due to the skewed distribution of mercury exposures and the improved fit obtained, the log transformation had been used in the original analysis of the neurobehavioral data from the two studies.[11,12,19,20] The BMDL results are much lower for the logarithmic curve shape than for the linear one, with the square root results being intermediate between the two. Although no statistically significant difference in curve fits was observed, this finding emphasizes the substantial dependence of the BMDL results on the default conditions chosen. The same observation was made when considering the neuropsychological outcomes as dependent variables,[25] and these results therefore emphasize the need to obtain better *a priori* information on the dose-effect functions.

We also determined visual evoked potentials (VEPs) in both populations.[11,12,19] However, VEP latencies are known to be affected by nutritional factors, including the supply of essential fatty acids during early development.[35] Nutritional factors were not assessed in our studies, where delayed VEP peaks were significantly associated with mercury exposure in Madeira,[11] but not in the Faroes.[12] In Madeira, the most sensitive VEP parameter seemed to be the N145 peak at the 15' condition.[11] Although these results are therefore not included in the present paper, we carried out supplementary benchmark calculations for this parameter in Madeira children who were not wearing glasses. At a BMR of 5%, the BMDL for the linear model is 12.3 :g/g (data not shown). This result is very similar to the results obtained for the BAEP latencies.

A BMDL of approximately 10 :g/g maternal hair is similar to recently calculated BMDLs for other neurological outcome variables in the Faroese children[2,25] and in a New Zealand population.[2,17] Higher BMDLs were reported from a study in the Seychelles, where clear effects on psychological tests have not been detected so far.[2,18] However, in the absence of a detected mercury effect, the Seychelles results may not be directly comparable with those from studies with clear evidence of mercury toxicity. Using several curve functions, an average BMDL

of about 10 :g/g was also calculated for crude neurological abnormalities in children exposed in connection with the poisoning incident in Iraq.[3,16] The most sensitive neurological, neuropsychological, and neurophysiological endpoints therefore seem to agree in regard to the BMDL.

When applying the BMDL results for standard setting, an uncertainty factor must be used.[2,3,26] The BMDL should not be considered an approximate threshold. Thus, with a BMR of 5% as the default condition, the BMD corresponds to the dose that causes a doubling of the prevalence of abnormal results. While no medical information has been included in determining the definition of an abnormal result, current evidence on neurotoxicant effects on BAEP[9,28-30] would suggest that early adverse effects may be detectable by this outcome parameter. When defining a safe exposure level, an uncertainty factor will therefore be necessary, and the magnitude of this factor must take into account the steepness of the dose-response curve below the BMD. If one chooses the linear dose-response function, the uncertainty factor must also take into account that much lower BMDLs are obtained using other curve functions that may be equally appropriate. Taking into regard as well differences in individual susceptibility and the possibility that other organs could potentially be more vulnerable than the developing brain, authorities have chosen an uncertainty factor of 10.[2,3,26] Although several studies now offer substantial support for a methylmercury BMDL corresponding to approximately 10 :g/g hair, much less evidence is available to guide the choice of the uncertainty factor.

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Figure legends:

Fig. 1. Peak III latency on the brainstem auditory evoked potentials at 20 Hz for 382 Faroese and 113 Madeiran children aged 7 years in relation to the maternal hair-mercury concentration. The slopes of the two regression lines are not statistically different.

Fig. 2. Same as Fig. 1 but at the 40 Hz condition.

Table I. Average (and Standard Deviation, SD) Brainstem Auditory Evoked Potential Peak III Latencies (in ms) in 382 Faroese and 113 Madeiran Children Without Middle Ear Infection or Neurological Disease after Adjustment for Age and Sex, and the Regression Coefficients (Betas with SD) for the Logarithmic Transformation of the Mercury Concentration in Maternal Hair

Frequency	Population	Mean (SD)	Beta (SD)	P Value
20 Hz	Faroes	4.18 (0.27)	0.113 (0.042)	0.008
	Madeira	4.09 (0.29)	0.165 (0.079)	0.038
	Both	4.16 (0.28)	0.122 (0.037)	0.001
40 Hz	Faroes	4.32 (0.31)	0.111 (0.048)	0.022
	Madeira	4.23 (0.35)	0.294 (0.094)	0.002
	Both	4.30 (0.32)	0.149 (0.043)	<0.001

Table II. Benchmark Dose (BMD in :g Mercury/g of Hair) and its Lower 95% Confidence Limit (BMDL) at Different Benchmark Response (BMR) Levels and According to Four Different Dose-response Models for BAEP Peak III Latency at 20 Hz in 382 Faroese, 113 Madeiran Children, and Both Groups

Model		Power[1]		Linear		Square root		Logarithmic	
		BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
BMR=0.02	Faroes	10.70	4.62	5.55	3.47	2.84	1.54	1.11	0.58
	Madeira	9.13	5.25	9.13	4.89	3.40	1.54	0.83	0.39
	Both	7.26	5.04	7.26	4.80	3.14	1.84	1.05	0.61
BMR=0.05	Faroes	16.31	9.59	11.94	7.45	8.37	4.17	3.98	1.68
	Madeira	19.62	11.18	19.62	10.52	10.28	4.19	2.67	1.03
	Both	15.60	10.72	15.60	10.32	9.38	5.13	3.69	1.78
BMR=0.10	Faroes	21.68	14.83	20.00	12.48	18.83	8.82	13.72	4.20
	Madeira	32.85	18.53	32.85	17.61	23.50	8.85	7.81	2.27
	Both	26.12	17.86	26.12	17.29	21.28	11.05	12.30	4.53

[1]K = 1.81 for Faroes, and 1 for Madeira and both

Table III. Benchmark Dose (BMD in :g Mercury/g of Hair) and its Lower 95% Confidence Limit (BMDL) at Different Benchmark Response (BMR) Levels and According to Three Different Dose-response Models for BAEP Peak III Latency at 40 Hz in 381 Faroese, 113 Madeiran Children, and Both Groups

Model		Power[1]		Linear		Square root		Logarithmic	
		BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
BMR=0.02	Faroes	7.33	4.43	7.33	4.09	3.91	1.83	1.43	0.66
	Madeira	5.87	4.01	5.87	3.74	1.91	1.11	0.49	0.29
	Both	6.79	4.66	6.38	4.40	2.76	1.70	0.96	0.57
BMR=0.05	Faroes	15.75	9.41	15.75	8.79	12.04	5.08	5.76	1.98
	Madeira	12.61	8.60	12.61	8.04	5.34	2.90	1.34	0.73
	Both	14.20	9.97	13.71	9.46	8.11	4.68	3.23	1.65
BMR=0.10	Faroes	26.37	15.42	26.37	14.72	27.85	10.93	23.55	5.22
	Madeira	21.11	14.37	21.11	13.47	11.55	5.94	3.16	1.50
	Both	23.33	16.59	22.96	15.83	18.19	9.99	10.21	4.12

[1]K = 1 for Faroes and Madeira, and 1.04 for both