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An ignored risk factor in toxicology: The total imprecision of exposure assessment*

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Abstract: Quality assurance of exposure biomarkers usually focuses on laboratory performance only. Using data from a prospective birth cohort study in the Faroe Islands, we have assessed the total imprecision of exposure biomarkers. As biomarkers of prenatal methylmercury exposure, mercury concentrations were determined in cord blood, cord tissue, and maternal hair. We determined their mutual correlations and their associations with the child's neurobehavioral effect variables at age 7 years. The exposure biomarkers correlated well with one another, but the cord-blood mercury concentration showed the best associations with neurobehavioral deficits. Because at least three exposure parameters were available, factor analysis and structural equation modeling could be applied to determine the total imprecision of each biomarker. For the cord-blood parameter, the total imprecision was 25–30 %, and almost twice as much for maternal hair. The total imprecision of these biomarkers much exceeded the normal laboratory variability of less than 5 %. Such imprecision can cause underestimation of dose-related toxicity, and data analysis should therefore include sensitivity analyses that take this factor into account. Ignoring preanalytical imprecision may cause serious bias.

Keywords: environmental exposure; imprecision; quality assurance; risk assessment; uncertainty.

INTRODUCTION

Exposure assessment is a key aspect of observational studies in human toxicology and epidemiology, where individual dosages are not part of the study design. In commonly used statistical analysis, the exposure variable is treated as an independent variable without error. Unfortunately, the frequently occurring non-differential errors in the exposure parameter tend to bias the dose–response relationship toward null [1]. Residence, occupation, and dietary questionnaire responses may be used as proxy variables that reflect an exposure. A better measure of individual exposure can be obtained from exposure biomarkers, i.e., contaminant concentrations in samples of human tissue or body fluids [2].

The validity of exposure biomarkers is usually expressed in terms of the laboratory uncertainty, where the imprecision is expressed as the coefficient of variation (CV) for repeated analyses of the sample [3]. However, this parameter reflects only the analytical variation, and the total imprecision also in-

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cludes biological or preanalytical sources of variation. The latter encompasses the variability associated with specimen sampling, toxicokinetic variability, storage, transportation, and related factors. The two sets of variability are independent, and improvement of laboratory performance does not automatically lead to a reduction of the total error [4,5]. Thus, the total imprecision may be underestimated when considered from laboratory variability only.

In laboratory quality assurance, validation of a laboratory analysis usually involves the analysis of a reference material and comparing the result obtained with the certified value for the material. This approach is insufficient when estimating the validity of a biomarker that may be affected by preanalytical variability. Correlations between related biomarkers have sometimes been used to estimate their validity, but interpretation of such data is difficult in the absence of a gold standard or certified value with which to compare the results. A supplementary approach is to assess the predictive validity of the biomarkers from their associations with known outcome variables [6].

Recent insight has suggested that some advanced statistical methods may be applied to estimate the total imprecision of biomarkers. In principle, the result for each exposure biomarker can be expressed as an intercept, an error function, and a loading factor multiplied by the unknown "true" exposure, i.e., similar to a regression equation. At least three sets of exposure indicators from a group of subjects are required to allow for a factor analysis to provide estimates of the unknown parameters [7]. The error function obtained will correspond to the total imprecision of the biomarker. A more sophisticated approach is to apply a structural equation model, where the influence of confounders and effect variables may be included, thereby utilizing all available information from a study to assess the parameter imprecision [8].

The impact of imprecision of exposure parameters has recently attracted attention in regard to methylmercury toxicology and is of general relevance to trace metal and other environmental exposures, where biomarkers are frequently used. In a prospective epidemiological study of a Faroese birth cohort [9], we found that two commonly used exposure biomarkers showed a linear relationship with scatter, thus suggesting that substantial degree of imprecision was occurring, despite excellent laboratory quality data [10]. Therefore, data from this study are applied to demonstrate a statistical approach to assessing exposure biomarker imprecision and providing proper adjustment for its consequences [11].

MATERIALS AND METHODS

Cohort formation and biomarker analyses

A birth cohort of 1022 subjects was formed from consecutive live births in 1986–1987 at the three Faroese hospitals [12]. In this fishing community, the traditional habit of eating pilot whale meat is the main source of methylmercury exposure. Information on the frequency of whale dinners during pregnancy was obtained from the mothers by questionnaire administered by the midwife [12]. In connection with each birth, we collected umbilical cord tissue, cord blood, and maternal hair. As an indication of the methylmercury exposure, cord blood and maternal hair were analyzed for total mercury [12]. The hair length corresponding to the complete pregnancy duration was analyzed, as was the proximal 2-cm segment that reflected the exposure during the third trimester [13]. For some cohort members, one or more specimens were not available, and some hair samples were sufficient only for the full-length analysis. For cord tissue, the dry weight of the sample was determined after freeze-drying [14]. The quality assurance data for the mercury analyses suggested a highly acceptable imprecision with a CV of approximately 5 % [10,15].

Clinical assessment of adverse effects

Follow-up of this cohort included an extensive neurobehavioral examination at age 7 years, where five main outcome tests were selected to represent different domains of brain functions [9]. Finger-tapping with the preferred hand (motor speed) was the main motor function test. Verbally mediated function encompassed Continuous Performance Test reaction time (attention); Bender Visual Motor Gestalt Test (visuospatial); Boston Naming Test (language); and California Verbal Learning Test–Children's short-term reproduction (verbal memory).

The study was carried out in accordance with the Helsinki convention and with the approval of the ethical review committee for the Faroe Islands and the institutional review board in the United States.

Statistical analysis

Logarithmic transformations were used for mercury concentrations with skewed distributions to reduce the impact of some very high mercury concentrations. In addition, this transformation is needed to obtain approximately linear relationships with homogenous scatter between the exposure biomarkers, as required by the subsequent analysis. Geometric means were used, and interrelationships between the transformed exposure biomarkers were determined by correlation coefficients.

Using the main outcomes at age 7 years, we carried out multiple regression analyses that included the same set of confounders that was originally selected [9,16]. As methylmercury exposure biomarkers, we used the mercury concentrations in cord blood, maternal hair, and cord tissue [6,8,14]. The mercury effect is expressed in terms of the change in the response variable relative to the standard deviation of the response that was associated with a doubling in the mercury concentration [6].

To assess the degree of uncertainty in exposure biomarkers, a confirmatory factor analysis was first carried out [7]. In this approach, each marker of mercury exposure (M-Hg) can be assumed to be a manifestation of the true (unobserved) exposure (Hg):

$$\log(M\text{-}Hg) = \alpha_m + \lambda_m \log(Hg) + \varepsilon_m \quad (1)$$

Thus, the log-transformed marker will depend linearly on the true (log-transformed) mercury exposure (Hg) and a measurement random error (ε_m). To comply with the requirement of at least three markers with independent error terms (ε) [7,17], we included the mercury concentrations in cord blood and maternal hair (full length) as well as the questionnaire response on the frequency of pilot whale consumption during pregnancy as the main source of methylmercury exposure. The regression coefficient λ_m—also known as the factor loading—was fixed at 1 for the cord-blood concentration so that the true exposure is expressed on the scale of this biomarker. Thus, a one-unit increase in log-Hg will on average lead to a one-unit increase in log cord-blood Hg. Because a natural log transformation is used, error standard deviations are mathematical approximations to the error CVs of the untransformed concentrations. Using these results, the biomarkers can be compared both in terms of their imprecision and from their estimated correlations with the true exposure [7]. Likelihood-based 95 % confidence limits for the error standard deviation were determined to quantify estimation uncertainty as described elsewhere [11].

Information from additional mercury biomarkers as well as outcome variables and covariates were then included in a structural equation model analysis [8]. In parallel to the factor analysis model, the observed variables are considered to be manifestations of one or more latent variables, which are not available for direct observation, but can be estimated from the observed variables. The structural equation models therefore allow estimation of causal relationships between the latent variables after possible adjustment for the effects of covariates. Thus, the structural equation model combines the (confirmatory) factor analysis and the path analysis [17].

In regard to the exposure model, this analysis included the above exposure indicators (mercury in cord blood and maternal hair, and frequency of whale dinners) and was, in an extended model, supplemented by the mercury concentrations in dry-weight cord tissue and proximal segment of the maternal hair. Similar to eq. 1, all exposure biomarkers are considered as manifestations of an underlying latent variable (Hg). In this type of analysis, measurement errors (ε_m) in different markers are usually assumed to be independent. However, we anticipated dependence between error terms in the two hair measurements and between errors in the two cord-based measurements. Adjustment for such local dependence is possible in structural equation models, and we therefore allowed ε_m to be correlated for the two sets of maternal hair concentrations and for the two cord concentrations (tissue and blood), respectively.

Based on *a priori* neurobehavioral considerations, outcome variables were separated into verbal and motor outcomes so that test results belonging to the same group could be assumed to represent the same latent functional variable, i.e., in the same way as the exposure markers. Exposure and outcome variables were then related by assuming a linear effect of the true exposure on both latent response functions. Potential confounders in the dose–response relationship were included as covariates, which were allowed to be associated both to the exposure and the outcome functions. Children with incomplete information on the study variables were included in a missing data analysis based on the maximum likelihood principle [18]. Compared to standard complete case analysis, this approach is more powerful and less likely to yield biased results.

The structural equation model induces a specific structure on the (expected) covariance matrix of the observed variables. Thus, apart from scale differences, the covariances between, say, a given motor score and a given verbal score will be the same for all pair of variables. The model parameters are estimated by examining all possible covariance matrices satisfying the model assumptions to identify the one closest to the covariance matrix of the observed data. An overall assessment of the model fit it then obtained by comparing the distance between the observed and the expected covariance matrix to a chi-square distribution. If this distance is statistically significant, then it is a sign that some model assumptions are violated.

RESULTS

In this population with substantial differences in methylmercury exposures, all exposure biomarkers showed the anticipated wide ranges (Table 1). The correlations between the biomarkers showed that mercury concentrations in cord tissue and cord blood were closely associated. The two hair parameters correlated well with one another, but somewhat less so with the cord-blood concentration.

Table 1 Geometric means, 25^{th}–75^{th} percentiles, and total ranges of mercury concentrations in specimens used as prenatal methylmercury exposure biomarkers in a Faroese birth cohort.

Exposure biomarker	N	Geometric mean	Interquartile range	Total range	Correlation with cord blood
Umbilical cord					
Blood (μg/l)	996	22.4	13.1–40.4	0.90–351	(1)
Tissue (μg/g dry weight)	447	0.210	0.132–0.36	0.000–1.28	0.940
Maternal hair at parturition					
Proximal segment (μg/g)	683	4.46	2.76–14.6	0.34–40.5	0.837
Full length (μg/g)	1019	4.17	2.52–7.7	0.17–39.1	0.784

The regression coefficients (Table 2) showed similar results for cord-tissue and cord-blood concentrations as predictors of neurobehavioral deficits. Because the mercury concentrations were logarithmically transformed, regression coefficients were calculated to allow comparison between the effects of a doubling of the concentration level. However, cord tissue was available only for less than one-half of the subjects, and some calculations were therefore based on small numbers. Except in regard to motor speed, the cord-based biomarkers appeared to be better than maternal hair in predicting toxicity risks.

Table 2 Numerical change (β, expressed as percent of the standard deviation) in five different response variables associated with a doubling of three different ε_m biomarkers after adjustment for confounders [6,14]. The direction of all effects is toward increasing deficit at higher exposures (p-values are two-sided).

Response	β (p)		
	Cord tissue	Maternal hair	Cord blood
Motor speed	3.00 (0.47)	5.99 (0.04)	5.37 (0.05)
Attention	29.6 (0.01)	8.99 (0.04)	15.9 (<0.0001)
Visuospatial	1.70 (0.66)	3.60 (0.21)	3.83 (0.15)
Language	11.3 (0.006)	7.47 (0.009)	10.5 (<0.0001)
Verbal memory	7.45 (0.08)	5.93 (0.05)	6.64 (0.019)

A factor analysis was carried out for the mercury concentrations in cord blood and maternal hair as well as the questionnaire information on the frequency of maternal pilot whale dinners during pregnancy. The results indicate that the cord-blood concentration had a smaller ε than the maternal hair concentration (Table 3). This difference in total imprecision was statistically significant with a p-value of 0.004 [7]. This finding is in agreement with—but independent of—the observation that the cord-blood concentration also showed stronger relations to the neurobehavioral outcome variables. Nonetheless, both biomarkers are associated with a total imprecision, which is substantially in excess of documented laboratory imprecision levels of about 5 %. Thus, even for the cord-blood marker which appeared to be the least imprecise exposure biomarker, the estimated imprecision CV had a 95 % confidence interval from 21 to 38 %.

Table 3 Factor loading (λ), standard deviation of ε and estimated correlation to the true exposure calculated for two major biomarkers of prenatal methylmercury exposure in a factor analysis model.*

Biomarker sample	Factor loading	Error standard deviation	Correlation to estimated truth
Cord blood	(1)	0.30	0.93
Maternal hair (full-length)	0.84	0.44	0.85

*The frequency of maternal pilot whale dinners during pregnancy was used as the third independent exposure indicator. Because of the logarithmic transformation of exposure variables, the error standard deviation is considered the same as the CV.

The advanced structural equation model showed a good fit to the data ($p = 0.067$ in overall test of lack of fit) and thus confirmed the results of the simpler factor analysis (Table 4). Again, the cord-blood measurement was less imprecise than the other exposure biomarkers ($p < 0.05$), and the full-length hair concentration had the strongest error component. As anticipated, the two cord measures differed little, as did the two hair measurements. The advanced analysis was in close agreement with the factor analysis results for cord blood and maternal hair (Table 3). Inclusion of additional exposure bio-

markers, covariates, and neurobehavioral outcomes led to small changes in estimated imprecision. In addition, the structural equation analysis confirmed the association between prenatal methylmercury effects and deficits in motor and verbal functions. Thus, a two-fold increase in the true mercury concentration (Hg) decreased the verbal function level by 10.5 % ($p = 0.001$) of the standard deviation while a similar exposure increase decreased the motor level by 10.8 % ($p = 0.02$) of the standard deviation in this outcome function. These findings are adjusted for the imprecision of the exposure parameters and also take into account several outcome variables, thereby avoiding any need for adjustment for multiple comparisons.

Table 4 Factor loading (λ), standard deviation of ε and estimated correlation to the true (latent) exposure calculated for biomarkers of prenatal methylmercury exposure in a structural equation model.*

Biomarker sample	Factor loading	Error standard deviation	Correlation to estimated truth
Cord blood	(1)	0.30	0.94
Cord tissue (dry weight)	0.89	0.33	0.91
Maternal hair (proximal)	0.89	0.36	0.89
Maternal hair (full-length)	0.85	0.45	0.84

*The model included confounders and outcome variables. Because of the logarithmic transformation of exposure variables, the error standard deviation is considered the same as the CV.

DISCUSSION

The overall result of this study was that the laboratory imprecision of no more than 5 % (CV) was misleading in regard to the total imprecision, which ranged up to 10-fold higher. This finding suggests that preanalytical sources of variation are of much greater significance that laboratory error under modern-day circumstances. This underestimation of the total imprecision may have substantial consequences. It should therefore be considered a serious risk to research in this field.

An imprecise exposure assessment will tend to underestimate the true effect of the exposure and may also complicate confounder adjustment [7,19]. In observational studies, where the exposure is not a matter of design, assessment of total exposure imprecision is a key to obtaining valid estimates. Even superb laboratory repeatability results are insufficient to ensure the validity of an exposure biomarker, but the additional imprecision due to preanalytical variability is usually unknown. The present study suggests that it should no longer be ignored.

In assessing the degree of imprecision, the simple correlation coefficients between exposure biomarkers and outcomes provide only limited guidance. In the absence of a gold standard, any disagreement between two correlated exposure parameters must rely on the result of a third, independent variable. When at least three variables are available, factor analysis can be carried out. Our factor analysis results show that the mercury concentration in cord blood provided lesser imprecision than that of maternal hair. The more detailed calculations using structural equations, with inclusion of information on covariates and outcomes, showed virtually unchanged results. While each model is based on certain assumptions, a test of the model fit showed that the structural equation model gave an adequate description of the data. Further, the results agreed with independent regression analyses, where cord blood tended to be the best predictor of neurobehavioral deficits at age 7 years. The recently completed 14-year examinations also confirmed these findings [20].

In regard to exposure assessment for methylmercury, our findings are plausible. The most frequently used sample for methylmercury exposure assessment today is scalp hair [15]. Sampling of hair is noninvasive and painless, and it is a feasible and efficient procedure under most field study condi-

tions. Depending on the rate of hair growth, the mercury concentrations along the hair shaft can represent a calendar of past exposures. Yet, environmental mercury vapor may bind to the hair [21], and hair permanent treatments can remove some endogenous mercury from the hair [22,23]. Also, hair color or structure may affect the incorporation of mercury into the hair [15]. These factors might well account for the greater overall imprecision of this biomarker.

The blood concentration of a contaminant is often considered the appropriate indicator of the absorbed dose and the amount systemically available, but this biomarker may also be subject to possible variation. Methylmercury binds to hemoglobin, and the high affinity to fetal hemoglobin results in a higher mercury concentration in cord blood than in maternal blood [24]. Further, whole-blood mercury concentrations are affected by the hematocrit, and some researchers therefore prefer to measure the mercury concentration in erythrocytes [24]. Routine analyses for total mercury concentrations reflect the sum of both methylmercury and inorganic mercury, but the cord-blood mercury concentration likely reflects the methylated form, for which the placenta does not constitute a barrier [25].

The umbilical cord is formed mainly during the second and third trimesters, and it reaches two-thirds of its full length already by the end of the second trimester [26]. Assuming a biological half-life of about 45 days for methylmercury [27], the cord-tissue mercury concentration is likely to represent a measure of the average mercury burden during the third trimester. The cord-tissue mercury concentration will likely be less sensitive to short-term changes than will the cord-blood mercury concentration. When expressed in terms of dry weight, variations in the content of blood and Wharton's jelly will probably have only a minor impact on the precision [14].

Other authors have shown a scattered association between maternal hair-mercury concentrations and subsequent mercury concentrations in the child's brain obtained at autopsy [28]. These data are in accordance with the size of measurement error for the hair-mercury parameter found in the present study. Our overall findings are therefore in agreement with the observation of cord blood as the best available indicator of prenatal methylmercury exposure.

Our results also suggest that even the best exposure biomarker may be much more imprecise than suggested by laboratory-quality data. Thus, attention to laboratory quality must be coupled with vigilance in choosing specimens for analysis, as guided by physiological information and documentation on exposure variability. Because the total imprecision may vary from study to study, and because the impact on study findings will depend on the total range of exposures covered, each study should ideally include at least three exposure indicators, so that the imprecision can be determined by factor analysis. If this is not feasible, an assumed imprecision level of at least 25 %, as indicated by Tables 3 and 4, should be used in sensitivity analyses.

Exposure imprecision and thus misclassification will generally be nondirectional, thereby leading to an underestimation of dose–effect relationships [29]. This problem may be exaggerated by potential confounders that are correlated with the exposure. In a regression analysis, inclusion of such variables may then further add to the bias toward the null hypothesis [7], even in cases where the potential confounder has no independent effect on the outcome.

When confounding variables are measured with imprecision, the consequences of ignoring imprecision are more complex. Because the effect of the confounder will be underestimated, the effect estimate for the exposure will be biased. However, the direction of the bias will depend both on the association between the exposure and the confounder and the direction of the confounder effect. Recent calculations to separate the beneficial effect of fish intake from the adverse effects of methylmercury showed that the calculated adverse effects of mercury increased, particularly when both the fish intake parameter and its assumed imprecision were taken into regard [30].

The issue of biomarker imprecision is crucial in regard to dose–response relationships and calculation of exposure limits. Neither of the two major risk assessments for methylmercury [31,32] considered this factor. Both reports applied benchmark dose calculations in deriving a safe exposure limit for methylmercury. Because benchmark dose results are biased, when the exposure imprecision is ignored,

the calculated exposure limits are too high. We have calculated elsewhere that an imprecision-adjusted exposure limit would be reduced by 50 % [11].

Blind reliance on exposure indicators, without adjustment for imprecision, will bias the study findings and any conclusions derived from them. Imprecisions of 25–50 % should be considered realistic and be incorporated in sensitivity analyses. Total biomarker imprecision may be assessed if at least three independent exposure indicators are included. Adjustment can then take place using factor analysis or structural equation models.

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