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WITHIN-PERSON REPRODUCIBILITY OF RED BLOOD CELL MERCURY OVER A 10–15 YEAR PERIOD AMONG WOMEN IN THE NURSES' HEALTH STUDY II

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Abstract

Background—Most epidemiologic studies of methylmercury (MeHg) health effects rely on a single measurement of a MeHg biomarker to assess long-term exposures. Long-term reproducibility data are, therefore, needed to assess the reliability of a single measure to reflect long-term exposures. In this study we assessed within-person reproducibility of red blood cell (RBC) mercury (Hg), a marker of methyl-mercury, over 10–15-years in a sample of 57 women.

Methods—Fifty-seven women from the Nurses' Health Study II provided two blood samples 10–15-years apart (median:12 years), which were analyzed for mercury levels in the red blood cells (B-Hg*). To characterize within-person reproducibility we estimated correlation and intraclass correlation coefficients (r and ICC) across the two samples. Further, we compared different prediction models, including variables on fish and seafood consumption, for B-Hg* at the first sample using leave-one-out cross-validation to assess predictive ability.

Results—Overall, we observed strong correlations over 10–15 years (r=0.69), as well as a high ICC (0.67; 95%CI: 0.49, 0.79). Fish and seafood consumption reported concurrently with the first B-Hg* sample accounted for 26.8% of the variability in that B-Hg*, giving a correlation of r=0.52.

Conclusions—Despite decreasing B-Hg* levels over time, we observed strong correlations and high ICC estimates across B-Hg* measured 10–15 years apart, suggesting good relative within-person stability over time. Our results indicate that a single measurement of B-Hg* likely is adequate to represent long-term exposures.

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Conflicts of Interest: None

Keywords

reproducibility; NHSII; methylmercury

Introduction

Methylmercury (MeHg) is a neurotoxicant able to cross the blood-brain barrier[1] as well as the placental barrier and has therefore been linked to adverse effects on the central nervous system and neurodevelopment, especially in fetuses and newborns.[2] Moreover, exposures to MeHg have also been linked to cancer,[3,4] and myocardial infarction,[5,6] although higher consumption of polyunsaturated fatty acids from fish –the principal route of exposure to MeHg–[7,8,9] has also been shown to decrease the risk of certain cardiovascular diseases.[6] MeHg has a reported half-life of approximately 50 days,[10] and MeHg exposure reflects increased fish and seafood consumption.[7,8,9]

Given the known toxicity of MeHg, the availability of a good biomarker for MeHg, rather than the less toxic total mercury (Hg), exposure is crucial, especially in large epidemiologic settings when often only a single biosample is available. Both inorganic Hg and MeHg can be measured in blood serum, whereas in red blood cells (RBC) 90% of Hg is in the form of MeHg.[11,12] Thus, RBC would be useful medium for analyzing total mercury concentrations as a MeHg exposure biomarker. Due to differences in the hematocrit of whole blood, moreover, the mercury concentration of the red blood cells is likely to be a more precise exposure biomarker.[12]

Many epidemiologic studies investigating the association between MeHg exposure and adverse health outcomes lack the resources to collect multiple blood samples for each subject. In addition, it is likely that study participants might have joined after a biologically meaningful exposure window, for instance mothers joining a study several years after having given birth. Most studies, thus, have to rely on single measurement of Hg or a measure in a surrogate time window. The degree to which a single measurement reflects long-term Hg levels, however, depends on the within-person variability of the biomarker over time. Larger within-person variability over time is likely to introduce measurement error, when using a single Hg measurement as an indicator of long-term exposure, and, hence, attenuate subsequent health effect estimates.[13,14]

In the present study, we assessed the reproducibility of RBC Hg levels in the Nurses' Health Study II (NHSII) over a 10- to15-year period. Furthermore, because retrospective dietary assessment of fish consumption could be another approach to assessing past MeHg exposure, we also assessed the correlation between fish and seafood consumption and RBC Hg. For this, we used concurrent dietary assessment, as this would represent a best case scenario, since reporting on past diet history would be expected to introduce more error.

Methods

Data Collection

Study Population—The NHSII began in 1989, when 116,430 female registered nurses 25–42 years old responded to a questionnaire about their health and lifestyle.

Between 1996 and 1999, 29,611 NHSII participants provided a blood sample. Each willing participant was sent a collection kit to have blood samples drawn by either a local lab or a colleague. The samples were returned to the laboratory via overnight courier, including an ice-pack or a frozen water bottle to keep the samples cool. The blood was then separated into plasma, RBC and white blood cell (WBC) components. and aliquoted into labeled cryotubes. From 2008–2011, 16,510 of the women from the initial blood cohort of NHSII returned a second blood sample using a similar protocol to the first collection.

To assess the blood Hg reproducibility and the ability to predict Hg levels 10–15 years apart we analyzed blood samples from 57 women who provided 2 samples (median time between blood samples = 12 years). Return of questionnaires with the blood samples constituted implied consent for the first sample. For the second sample, women had to provide written informed consent, reflecting the standards at the time of each collection. The Institutional Review Board of BWH approved this study.

Laboratory Methods—Aliquots of RBC were prepared for each blood sample and sent frozen on dry ice to the University of Southern Denmark. Total Hg concentration was determined on a Direct Mercury Analyzer: DMA-80 (Milestone Inc, Sorisole, Italy). The RBC samples were first thawed and the cell suspension was homogenized by mixing on a Vortex mixer and diluted with Milli-Q treated water (dilution ratio = 2:3 to approach a normal hematocrit and to facilitate analysis). The detection limit for the dissolved sample was estimated to be 0.05 μg/L, i.e., three times the standard deviation of the blanks. The total analytical imprecision was estimated to be 2.5, 2.5 and 1.9% at Hg concentrations of 1.97, 15.20 and 31.40 μg/L respectively. The samples were also analyzed for hemoglobin by spectrometry, and the Hg:hemoglobin ratio was calculated to adjust for differences in RBC preparation and specimen dilution. We then standardized the Hg concentration to the expected average hemoglobin concentration of 13.6 g/dL, as this number would approach the likely in vivo whole-blood concentration; we refer to this quantity as B-Hg*. Hg concentrations in μg/L can be converted to nmol/L by multiplying by 5.0.

Dietary Items—MeHg exposure mostly occurs through consumption of seafood and fish[7,8,9] and such dietary items have been used in the past to predict MeHg exposures.[15,16] We, therefore, also examined the association between seafood and fish consumption and B-Hg*.

We used nutrient-validated, semi-quantitative food frequency questionnaires (FFQ) that are administered to all NHSII participants every 4 years.[17] We included information on 4 seafood items: 1) dark fish meat, such as mackerel, salmon, sardines, bluefish or swordfish, 2) canned tuna, 3) other fish and 4) shrimp, lobster or scallops (as a main dish). Analyses used consumption of these food items as continuous variables, measured in times per month.

Participants were assigned values from either the 1995 or 1999 FFQ, whichever was closest to the date of their first blood sample.

Data Analysis

We log-transformed all B-Hg* values, to account for deviations from normality. Differences between paired samples were expressed as median (95% CI), and the average coefficient of variation (df = 1) was also computed. We used the Pearson and Spearman rank correlation coefficients and the intraclass correlation coefficient (ICC) to assess within-person reproducibility of B-Hg* levels across time, with ICC defined as the ratio of the between-person over the sum of the within- and between-person variability. High ICC values indicate higher between-person contributions to the total variability, and, thus, lower within-person variability and better within-person reproducibility. ICC values ≥ 0.75 indicate excellent, 0.4 ≤ ICC < 0.75 fair to good and <0.4 poor reproducibility.[18] We reported 95% confidence intervals (CIs) for the ICC using the exact method. We calculated weighted kappa statistics and 95% CIs to quantify the agreement between quintiles of B-Hg* levels across the two time points, i.e. quantify the likelihood that the B-Hg* levels of the two blood draws of each subject would fall into the same quintile.

We examined the association between fish and seafood consumption and B-Hg*, to assess whether information on such dietary habits concurrent with the first B-Hg* measurement or a later B-Hg* measurement would be a better predictor for an earlier B-Hg* levels, for studies in which these data are not available. To this end, we ran two different linear regression models, both with the log(B-Hg*) at the first measurement as the dependent variable. In the first model we included the following variables as predictors: age, BMI, smoking status and caloric intake at the time of the first blood draw, as well as seafood and fish consumption from the FFQ collected closest to the first blood draw. In the second model we included only the log(B-Hg*) at the second blood draw as the predictor.

To assess whether the second B-Hg* measurement or seafood/fish consumption at the time of the first blood collection better predicted the first B-Hg* measurement, we employed a leave-one-out cross-validation process.[19] Models included data from the FFQ collected closest to the first blood draw (either in 1995 or 1999) or the second B-Hg* in a linear regression model with the first measurement of the log-transformed B-Hg* as the outcome. Omitting one observation at a time, we re-fit each model and then predicted the Hg value for the omitted observation. The predictive ability of the models was quantified by the calculated R^2 between the predicted and observed first B-Hg* levels. We also assessed whether the slope between predicted and observed values was significantly different than 1, to assess potential under- or over-estimation.

All statistical analyses were conducted using the R Statistical Software, version 2.14.1 (Foundation for Statistical Computing, Vienna, Austria).

Results

The study participants were on average 42 years old (sd = 4.8 years) at the first blood collection. The characteristics of the study participants are presented in Table 1. Table 2

presents summary statistics for the Hg and B-Hg* at each blood collection. Overall, we observed slightly higher B-Hg* concentrations during the first blood collection, with a median difference in B-Hg* levels of −0.14 μg/L (95% CI: −1.78, 2.86) between the first and second collections. Even though the median B-Hg* concentrations decreased over time in our study sample, the variability in B-Hg* concentrations was higher in the second collection (Table 2). The average estimated within-person CV over time was 37.4% (sd = 24.2%).

We observed a strong correlation between the two B-Hg* samples (correlation coefficient r=0.69, Spearman r_s=0.70) and low within-person variability, relative to total variability, as shown by a high ICC = 0.67 (95%CI: 0.49, 0.79). In addition, we observed good agreement between quintile categories defined by the distribution of B-Hg* levels at each time point, with a weighted kappa statistic of 0.66 (95%CI: 0.53, 0.79). These results did not change after removing a potential outlier.

Summary statistics on fish/seafood consumption are presented in Table 3. We assessed whether fish and seafood consumption at the time of the first blood collection or the B-Hg* of the second blood collection better predicted the B-Hg* levels at the first blood collection (Table 4). We included age, BMI, caloric intake and smoking status at the time of the FFQ in the model of fish consumption as these improved the model fit. All variables included in the model accounted for 40.7% of the variability in the log(B-Hg*) levels and fish and seafood consumption alone accounted for 26.8%, corresponding to an r=0.52 between B-Hg* and fish and seafood consumption. The predictive ability of this model, however, was not strong: the cross validation R^2 between predicted and observed values was 0.29, and the predicted values were marginally under-predicted, as shown by the slope between predicted and observed values (β=0.75; 95%CI: 0.45, 1.05).

In comparison, B-Hg* from the second blood collection explained 46.8% of the variability in the first B-Hg* sample. The cross validation R^2 was 0.43. No significant over- or under-prediction was observed (β=0.96; 95%CI: 0.68, 1.24). Finally, a model including both fish and seafood consumption and the second B-Hg* measurement did not perform better than the model only including the second B-Hg* measurement.

Discussion

Overall, we observed a very good within-person stability of B-Hg* over 10–15 years, as shown by high values of the correlation coefficients, the ICC and the weighted kappa statistic between the two samples. Conversely, the observed average CV between repeat samples was higher than the laboratory CV. Potential changes in residence and dietary habits could contribute to the high value of the CV. Given the observed decrease in B-Hg* levels in our sample, a high CV value is not surprising; even if all participants' B-Hg* concentrations decreased by the same amount, which would preserve the exposure rankings and result in the correlation coefficients being approximately equal to 1, the CV would still be high and would increase as the average B-Hg* decreased.

For our analyses we used B-Hg* as a biomarker of MeHg exposure, a blood fraction usually discarded after analyses for other blood biomarkers in epidemiological studies. B-Hg* is ~90% MeHg, with demethylated MeHg comprising a large part of the remaining 10%, while total blood Hg has higher inorganic Hg contributions,[11,12,20] compared to B-Hg*. Hair Hg has also been considered a good biomarker for MeHg exposure.[11,21] In populations, however, that have been exposed to vapor Hg, use of hair Hg as a biomarker for MeHg exposure might be problematic, as vapor Hg may bind to the hair directly,[22] and hair treatments may also affect Hg concentrations in the hair.[23,24]

The B-Hg* levels of our participants were comparable to those previously reported. The geometric mean of total blood Hg levels among women 16–49 years old from the 1999–2000 National Health and Nutrition Examination Survey (NHANES) has been reported to be 1.02 μg/L.[9,25] For years 1999–2006 these levels were 0.93 μg/L,[26] and in 2009–2010 they were 0.86 μg/L.[27] Given these, the blood Hg levels seem to be decreasing over time.[27] The geometric mean of the first sample of our participants, which was collected between 1996–1999, was slightly higher than the reported 1999–2000 mean (1.06 vs. 1.02 μg/L), in agreement with a decreasing trend over time. This difference, however, was not statistically significant, as apparent by the widely overlapping CIs of the two means. The second measurement, collected in 2010, although lower than the first (geometric mean = 0.98 μg/L), was higher than the reported 2009–2010 levels by the U.S. Environmental Protection Agency (EPA);[27] EPA, however, only included women of childbearing age in their report (16–49 years), while our participants were older at the time of their second sample (mean age = 53 years) and, therefore, likely did not adjust their fish consumption habits to EPA and U.S. Food and Drug Administration (FDA) recommendations for pregnant or nursing women, published in 2001[28] and updated in 2004.[29]

Our results are in agreement with previously published work assessing the reproducibility of biomarkers of MeHg exposure over time. Hinners et al.,[30] for example, found a Pearson correlation coefficient of 0.75 between toenail samples provided 14 months apart in a sample of 43 Japanese women, as part of the Arsenic Mercury Intake Biometric Study. Similarly, Garland et al.,[31] also using toenail Hg as a MeHg biomarker, found a Spearman correlation coefficient of 0.56 between samples provided by 127 NHS participants over a 6-year period. To our knowledge, ours is the first study to assess reproducibility of MeHg exposure levels, using B-Hg* over a period of more than 10 years. Together, these results indicate that MeHg exposure levels are relatively stable across time and one sample is adequate to represent long-term exposures.

Though we observed high ICC values, they were still lower than 1, suggesting that relying on a single measurement of B-Hg* could still introduce measurement error in the subsequent health effects model and, hence, attenuate health effect estimates.[13] If a single B-Hg* measurement is used to reflect exposures longer than 10 years, the true effect (log(OR)) could be as high as 1.5 times the biased effect estimate (= 1/0.67), assuming no other misclassified variable or that B-Hg* is not strongly correlated with the other covariates in the model.[13] For example, if the true OR = 2.00, under the above assumptions, use of a single blood sample would yield OR* = 1.59 (= exp [log(OR) × ICC]), with narrower confidence intervals. When the above assumptions are not met, information on all variables

correlated with B-Hg* and/or on those measured with error should be included in the estimation of the ICC, to obtain corrected ORs and inferences.[13,32] Furthermore, Grandjean and Budtz-Jørgensen showed that failure to account for the total imprecision associated with use of a single MeHg biomarker in health analyses could also lead to biased health effect estimates.[33]

We assessed whether contemporaneous fish and seafood consumption, a widely recognized MeHg exposure pathway, [8,9,16] better predicts B-Hg* levels, as compared to B-Hg* samples obtained 12 years later. Given our small sample size, we were not able to build a better prediction model when using dietary items as predictors, as compared to other studies.[8,9,16] Still, we were able to explain a higher proportion of the variability in the B-Hg* levels by fish and seafood consumption than the one reported in a recent study by Golding et al. (26.8% in our study vs. 8.8%).[34] In their study, they assessed total blood Hg exposures, which is a less precise biomarker for MeHg exposure than B-Hg*, as it can also be influenced by inorganic Hg.[11] Further, they sampled women during pregnancy; pregnant women, however, have been found to have significantly lower Hg levels,[26] which might have reduced the ability to detect stronger associations.

In our analyses, a model including only the second B-Hg* measurement as a predictor performed much better than the contemporaneously collected fish consumption. Fish consumption data collected retrospectively would be expected to perform even worse. Thus, in a study setting where the aim is to assess past Hg exposure, measuring current B-Hg* should be better than retrospectively assessing earlier fish consumption. It should be noted, however, that for the dietary assessment in the NHS, fish were grouped according to their nutrient content (e.g. fish oil) and not mercury content. The dark fish group, therefore, includes both salmon (low MeHg content) and swordfish (high MeHg content). Imprecise estimates of specific fish consumption might have also affected our ability to build a strong and stable prediction model. In addition, these results might not be generalizable to other populations with potentially different dietary variability over time. Overall, we observed the highest predictive ability with the second measurement of B-Hg*, indicating that although the two blood samples were collected 10–15 years apart, their correlation is stronger than the one between B-Hg* levels and fish consumption.

Our results should be interpreted in light of the limitations of our study. First, any variability due to laboratory analysis will also be reflected in the ICC. Given the well-established analytic techniques employed and the low reported CV (4.4%), however, we would expect any such influence to be small. Furthermore, our sample consisted of 57 participants. The small sample size of our study is reflected in the wide confidence intervals of the ICC estimate and the weighted kappa statistic and likely affected our ability to build a better prediction model and use information on fish and seafood consumption to predict B-Hg*. In addition, our measurements were collected 10–15 years apart; our results, therefore, represent the reproducibility of Hg levels over a very long period of time. If shorter exposure durations are of interest, it is likely that the B-Hg* levels would be more stable. Finally, our subjects were predominantly white US women with an average age of 42 years. Our results, thus, might not be generalizable to other populations, especially younger

pregnant women, which is usually the population of interest in studies of post-natal developmental disorders.

In conclusion, we observed good reproducibility of B-Hg* levels over 10–15-years, suggesting stable long-term exposures to MeHg. To our knowledge, the present study is the first to assess reproducibility of B-Hg* levels, one of the more precise biomarkers for MeHg exposure.[11,12] The usefulness of B-Hg* as a proxy for MeHg is further supported by our higher correlations between samples 10–15-years apart than correlations previously reported, using toenail Hg as a MeHg biomarker, between samples over 6 years from a very similar population.[31] We would, therefore, recommend the use of B-Hg* to assess long-term MeHg exposures.

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Table 1

General characteristics of population at 1st and 2nd blood samples (n = 57)

	Mean	S.D.
Age (1st draw)	41.84	4.81
BMI (kg/m^2)		
1st draw	24.90	5.55
2nd draw	25.87	5.12
Smoking Status [n (%)]		
1st draw		
Past	13	(22.4)
Current	5	(8.6)
2nd draw		
Past	17	(29.3)
Current	2	(7.1)

Table 2

Hg (μg/L), hemoglobin (g/dL) and standardized B-Hg* (μg/L) levels.

	Median	2.5th – 97.5th percentile	Geometric Mean	2.5th – 97.5th percentile
		1st draw		
Hg	1.19	(0.30, 4.59)	1.26	(0.31, 5.18)
Hemoglobin†	16.21	(14.06, 18.00)	16.16	(14.04, 18.59)
B-Hg*‡	0.99	(0.25, 3.94)	1.06	(0.26, 4.40)
		2nd draw		
Hg	1.11	(0.24, 7.44)	1.19	(0.19, 7.51)
Hemoglobin†	16.61	(14.33, 17.75)	16.47	(14.64, 18.53)
B-Hg*‡	0.91	(0.20, 6.06)	0.98	(0.16, 6.14)

† In RBC fraction

‡ Standardized to a standard hemoglobin level of 13.6 g/dL

Table 3

Summary statistics on fish and seafood consumption (times/mo) at the first blood sample.

Fish/Seafood	Median	2.5th – 97.5th percentile
Canned Tuna	2.00	0.50 – 4.35
Dark Fish	0.50	0.50 – 2.00
Other Fish	0.50	0.50 – 2.00
Shrimp, etc	0.50	0.50 – 2.00

Table 4

Results of multivariate regression to predict B-Hg* levels at the first blood collection using fish and seafood intake as predictors.

Variable	Coefficient	95% CI	Partial R^2 (%)*
Age	0.034	(−0.006, 0.075)	5.6
BMI	−0.035	(−0.0065, −0.005)	12.6
Calories (per 10 kcal/d)	−0.002	(−0.005, 0.000)	5.7
Smoking, never	*Ref.*	–	
Smoking, past	−0.233	(−0.636, 0.170)	8.9†
Smoking, current	−0.544	(−1.101, 0.013)	
Canned tuna	−0.053	(0.012, 0.093)	12.2
Dark fish	0.126	(0.034, 0.218)	13.3
Other fish	0.070	(0.006, 0.135)	8.9

*Total R^2 = 40.7%

†Partial R^2 for the variable smoking (all levels)