Author’s response to reviews

Title: Very Low-Level Prenatal Mercury Exposure and Behaviors in Children: The HOME Study

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Author’s response to reviews:
On behalf of my co-authors, I am pleased to submit the revised manuscript entitled “Very Low-Level Prenatal Mercury Exposure and Behaviors in Children: The HOME Study” for consideration of publication in Environmental Health. We thank the reviewers for their valuable time to provide much helpful and constructive comments. In the below document, we have responded point-by-point to the comments and made changes to the manuscript accordingly highlighted in yellow.

Reviewer - 1 Comments:

Comment 1: Justification should be provided for the choice of cut-points for maternal age and income. Were these, like the one used for HOME scores, based solely on the distributions of these variables or were these motivated by some other consideration?

Response: We have added the justification for the choice of cut-of points for the mentioned two variables. They were primarily categorized based on frequency distribution. For maternal age, maternal and fetal risks are higher when the maternal age is over 30 years compared to <30 years [1, 2].


Text added:

“Maternal age at delivery and annual household income were categorized based on the frequency distribution of the variables.” (page 7, lines 5 & 6)

Comment 2: The mixed models combined children's BASC scores at 2, 3, 4, 5, and 8 years. It is likely that BASC scores are not equally sensitive at all ages, especially for internalizing behaviors. Were analyses conducted that focused solely on scores at 8 years of age (all BASC
scores, not just the anxiety scale), as I would expect that scores at this age might provide the best opportunity to evaluate an association with prenatal mercury.

Response: We conducted analyses for all BASC composite scores and their subscales at 8 years of age. We did not find any statistically significant associations between prenatal mercury exposure and any other scores [except anxiety] at 8 years of age. As found in another study [1], associations between prenatal exposure to environmental toxicant and externalizing behaviors had a fairly good stability through 2-8 years of age, but the association was not stable for cognitive and psychomotor behaviors. Hence, it is possible that the association between prenatal environmental exposures and internalizing behaviors such as anxiety are not stable through 2-8 years. Hence, the associations at 8 years of age could be more meaningful for the anxiety and internalizing behaviors.


Text added:

"Not only anxiety, we also examined the association of prenatal mercury concentrations and other composite scores/subscales at 8-years of age.” (page 9, lines 9-16)

“We did not find any statistically significant association between prenatal mercury concentrations and other BASC composite scores/subscales at 8 years of age.” (page 12, lines 14-16)

Comment 3: Table 1 could be made more informative. Given that 320 women contribute data to both columns, the absence of important differences found it is not surprising. It would be more helpful to compare the 320 women/children for whom data were available to the 69 of the women who delivered singleton children. In the text (first paragraph of Results), who the 320 dyads are needs to be explained.

Response: We thank the reviewer for noting this. We compared the 320 dyads with at least one mercury and behavior assessment with 69 dyads not included in the analyses because of either missing mercury or behavioral data. The 320 dyads were more older (>30 years), white, educated
(completed Bachelor’s degree), less depressed (BDI≤13), had higher income at baseline (> $40,000) and higher HOME score (≥40) compared to 69 dyads without at least one mercury and behavior assessment. This would lead to selection bias in the study, hence, we have added that in the discussion. We also clarified who the 320 dyads are in the first paragraph of the Results.

Text added/modified:

Column added to the table 1 - Dyads not included due to missing either mercury or behavior assessment data (n=69). (Page 23)

“We compared the 320 dyads with at least one mercury and behavior assessment with 69 dyads not included in the analyses because of missing either mercury and behavior assessment (page 7, lines 21-22 and page 8, 1).”

“However, the 320 dyads were more older (>30 years), white, educated (completed Bachelor’s degree), less depressed (BDI≤13), had higher income at baseline (> $40,000) and higher HOME score (≥40) compared to 69 dyads not included in the analyses because of missing either mercury and behavior assessment.” (page 10, lines 9 to 12)

“Fourth, 320 dyads included in our study were statistically different in some maternal and child characteristics from the 69 dyads not included due to missing either mercury or behavior assessment data, which may result in a selection bias in our study.” (page 16, lines 20-22)

Comment 4: The difference in the directions of the findings for parent reports and child reports of anxiety is interesting. I agree that the findings of less anxiety among children with higher prenatal mercury is probably spurious. I think children under the age of 10 tend not to be very reliable informants of their own strengths and weaknesses. This cannot explain the direction of the association, of course, but it does reduce confidence that the association is real.

Response: We agree with the reviewer that the self-reported internalizing behaviors at 8 years of age tend to be less reliable, hence, the results could be spurious and should be used with caution. We have added the text to the manuscript to capture this important point.

Text Added:
“However, self-reported behaviors at 8 years of age could be less reliable. So, the results of the association between higher prenatal mercury exposure and lower self-reported anxiety levels should be interpreted with caution.” (page 16, lines 4-6)

Comment 5: When the critical exposure biomarker is measured in the mother and the critical endpoint is maternal ratings of child behavior, one always has to consider whether the toxicant might have affected maternal perceptions and interpretations of that behavior. The inclusion of (self-rated) maternal depression as a covariate appears to have been an effort to consider this, but there are other possibilities. Some mention of this in the Discussion could be considered. Also, use of a higher cut-off other than 13 (minimal depression) could be considered, as it is not obvious that that is the best choice.

Response: Again, we thank the reviewer for pointing out this important point. We agree that although we adjusted for maternal depression, there could be residual confounding from other variables such as maternal anxiety. That could have affected mother’s perception and interpretation of child’s behaviors such as anxiety. We have elaborated this limitation in the manuscript.

We have only 22.3% of the mothers with mild, moderate, or severe depression with very few who had severe depression. We did our preliminary analyses with higher cut off point, however, the cut-off point at 13 for maternal depression seemed appropriate based on the frequency distribution for a stable regression model.

Added Text highlighted in yellow:

“Second, although we could control for an extensive number of potential confounders in this study, residual confounding due to unknown, unmeasured, or misclassified confounders cannot be ruled out. For instance, we measured prenatal mercury concentrations in mothers and they were also a part of parents-reported children behaviors. It is possible that the mercury exposure might have affected mother’s perceptions and interpretation of child’s behaviors. We attempted to address this by adjusting for maternal depression, however, it could have been affected through other pathways such as maternal anxiety. This would have biased our result either towards or away from the null [43].” (page 16, lines 10-17)
Reviewer - 2 Comments:

Comment 1: The biological processes related to the different periods of the brain development during pregnancy are not well described. This would be needed to justify the use of the different MeHg sampling times in the analyses.

Response: We thank the reviewer for catching this. In general, fetuses are at more risk of developing adverse effects from exposure to environmental toxicants because they have rapidly developing organ system, different pharmacokinetics and higher exposure dose per body surface area compared to adults. Any disruption during pregnancy can significantly results in adverse outcomes in growth and development. The magnitude of adverse outcomes also depends on the timing of the exposure [early vs late pregnancy exposure] and these periods of heightened sensitivity are toxicant-specific. This makes it necessary for researchers to study and understand these periods of heightened vulnerability/window of vulnerability [1]. For mercury, these windows of vulnerability are not well studied/known. Hence, we used different sampling times in the analyses. We have added this to the manuscript.


Added text highlighted:

“We hypothesized that very low-level prenatal whole blood mercury concentrations (< 4 – 5 µg/L) would be associated with behavior problems among children from 2 to 8 years of age. In general, fetuses may be more susceptible to the adverse effects of exposure to environmental toxicants because they have rapidly developing organ system, different pharmacokinetics and higher exposure dose per body surface area compared to adults. Any disruption during pregnancy can significantly results in adverse outcomes in growth and development. The magnitude of adverse outcomes also depends on the timing of the exposure [early vs late pregnancy exposure] and these periods of heightened sensitivity are toxicant-specific. This makes it necessary for researchers to study and understand these periods of heightened vulnerability/window of vulnerability [23]. For mercury, these windows of vulnerability are not well studied/known. Hence, we also sought to identify windows of heightened vulnerability during fetal development using serial blood samples collected during pregnancy and at delivery.” (page 3, 4)

Reference added:
Comment 2: Since methylmercury was measured this term should be used throughout the manuscript.

Response: We measured total whole blood mercury concentrations in our cohort. Although methylmercury represents the significant amount of the total whole blood mercury concentrations in the United States, we used the term mercury concentrations throughout the manuscript since it was the whole blood mercury concentrations which also contains inorganic mercury.

We have corrected the error in the methods section [under Measurement of mercury exposure] to represent the whole blood mercury concentrations.

Text corrected to:

“Total whole blood mercury was quantified using inductively coupled plasma mass spectrometry (ICP-MS) [24].” (page 4, line 21)

Comment 3: When moving from the composite score to the scales, like in the case of anxiety, I would expect a clear a priori hypothesis. Here I don't understand why this particular sub-scale of the BASC-2 was suddenly used. Only Figure 2 suggests that this sub-score might be relevant. That's cherry-picking if no justification is given, that anxiety is a vulnerable dimension since previous studies showed that. Also mechanistic data could be used to support a more specific hypothesis on this association.

Response: We thank the reviewer for bringing this lack of clarity to our attention.
We did have a priori hypothesis that the prenatal mercury exposure would be associated with adverse behavior outcomes (including anxiety). As found in another study [1], associations between prenatal exposure to environmental toxicant and externalizing behaviors had a fairly good stability through 2-8 years of age, but the association was not stable for cognitive and psychomotor behaviors. Hence, it is possible that the association between prenatal environmental exposures and internalizing behaviors such as anxiety are not stable through 2-8 years. Hence, the associations at 8 years of age could be more meaningful for the anxiety and internalizing behaviors. Additionally, we examined anxiety at 8-years of age because there is some evidence suggesting an association between prenatal mercury exposure and anxiety [2, 3]. Although this was at higher prenatal mercury levels, it was important for us to examine if such association exists at very low – yet representative – levels of prenatal mercury exposure.

Additionally, as secondary analyses, we conducted age-specific analyses at 8-years of age for all composite BASC scores and subscales. We did not find any statistically significant associations between prenatal mercury exposure and any other composite scores/subscales [except anxiety] at 8 years of age. We have added some text in the manuscript to support our analyses methods.


Added text highlighted:

“Not only anxiety, we also examined the association of prenatal mercury concentrations and other composite scores/subscales at 8-years of age. We examined anxiety at 8-years of age in more depth because there is some evidence suggesting an association between prenatal mercury exposure and anxiety [16, 34]. Although this was at higher prenatal mercury levels, it was important for us to examine if such association exists at very low – yet representative – levels of
prenatal mercury exposure as we found some evidence of positive association between mean prenatal mercury concentrations and BASC-2 anxiety subscale” (page 9, lines 9-16)

“We did not find any statistically significant association between prenatal mercury concentrations and other BASC composite scores/subscales at 8 years of age.” (page 12, lines 14-16)

Reference added:


Comment 4: From a statistical perspective I wonder why the means of a variable that is obviously skewed and needs log-transformation are given. I wonder if a model using a cut-off or quartiles of the measurements would be more appropriated. This could be used as an initial model and later the more fine-graded exposure values might be used of a NOAEL derivation of the most sensitive endpoint, here BASC-2 anxiety score.

Response: We presented the means of a variable in the paper before the log-transformation for easier comparison and interpretations with other studies that only reported arithmetic means. For instance, we wanted to compare our research findings with other studies if they found similar association at the low-level of mercury concentrations. Also, we wanted to compare if mean mercury concentrations in our cohort are representative of the pregnant women in the United States.

That is an excellent suggestion to use a model using a cut-off or quartiles of the measurement model initially. This method is extremely common in contemporary epidemiology because the results can be interpreted in terms of low, medium, and high-risk group and relative risks can be calculated [1]. However, these benefits are outweighed by disadvantages and its use is criticized in most cases [1, 2, 3]. Some disadvantages could be higher, particularly when the distribution is skewed [1]. As suggested by the researchers, the natural alternative to this method is using linear regression [1]. If the association is non-linear, LOESS/spline regression methods are recommended.
To examine the shape of the relationship between our exposure and outcome(s), we created locally weighted scatterplot smoothing (LOESS) graphs. We have added this figure 3 in the manuscript which provides an evidence of approximately linear relationships. Additionally, using the SAS macro provided by Desquilbeta and Mariottib [4], we constructed restricted cubic splines linear regression models to examine the assumption of linearity. From this macro, all the statistical outputs for non-linearity came statistically insignificant meaning the associations were linear. We have added a spline regression [Supplementary figure – 1] output for anxiety for your reference which demonstrates linear as well as dose-response relationship. It is restricted, cubic splines using linear regression at three knots at 5, 50, and 95th percentile and adjusted for confounders. These justify our linearity assumption and the use of linear mixed models.

There is an animal study [5] and another human study [6] that found some association between prenatal mercury concentrations and anxiety. However, this was at very higher levels of mercury concentrations (13.4 times higher than our study cohort). In our best knowledge, this is the first study to understand the effects of prenatal mercury exposure with anxiety (and other behavior outcomes). As mentioned in the manuscript, we found mercury concentrations are associated with higher parents-reported and lower self-reported anxiety scores at 8 years of age. It can be argued that self-reported anxiety scores are not reliable at 8 years of age as children at this age might not be reliant about their own limitations and strengths of their behaviors, the inconsistent results and multiple comparisons could suggest that these results need to be supported by other studies at similar [low and representative] levels of prenatal mercury concentrations and we need to be cautious interpreting these results. Hence, we did not arrive at NOAEL.

To conclude, for the above-mentioned disadvantages of quantile regression and evidence of linearity though loess and splines, we strongly believe our selection of using linear regression is appropriate for this particular study.


Modifications to the manuscript:

Figure 3 added to represent the Locally weighted scatterplot smoothing (LOESS) plots for the relationship between mean prenatal [log2-transformed] mercury concentrations and BASC-2 scores through 2-8 years. (Page 30)

Text added:

“Locally weighted scatterplot smoothing (LOESS) analyses revealed approximately linear relationships between log2-transformed prenatal mercury concentrations and BASC-2 behavioral outcomes (Figure 3).” (Page 11, lines 3-6)

Comment 5: While most of the covariables were used as binary variables, the exposure was log-transformed and used as continuous variable.

Response: We have some continuous covariables in the model such as child blood lead concentrations, pre-natal serum cotinine concentrations, and post-natal child mercury concentration. Some variables [in data collection] were categorical in nature only such as ethnicity, fish intake, education, marital status, child sex. We categorized maternal age, HOME score, income, and maternal depression for easier interpretation. Predictors in multivariable linear regression models can be binary, categorical, or discrete numeric, as well as continuous numeric [1]. Also, the regression outputs of only exposure variable (prenatal mercury concentrations) are reported in this paper.

Modifications: None

Comment 6: Moreover, the associations derived from the model need to be presented graphically. The authors mentioned these scatter plots (see page 9) but they are not given! At least as a supplement I would expect the comparisons of the various models that were used (see page 9). The process of how the final regression model was derived needs to be more transparent.

Response: We agree with the reviewer that the associations when presented graphically can help better understand the association between an exposure and outcome. We have added a LOESS graph (Figure 3) in the manuscript. We have also included a figure 2 representing outcomes from different regression models we used. We selected our best model based on the lowest AIC value. As such seen in the image, multivariable model 1 vs 2 would not have much difference in the results or interpretation.

We thank the reviewer for identifying a lack of clarity in explaining the process of model selection. We have added some text to make the process more clear.

Modifications:

Figure 3 added to represent the Locally weighted scatterplot smoothing (LOESS) plots for the relationship between mean prenatal \([\log_2\text{-transformed}]\) mercury concentrations and BASC-2 scores through 2-8 years. (Page 30)

Figure 2 added to the manuscript to represent Comparison of different regression models on association between prenatal mercury \([\log_2\text{-transformed}]\) concentrations and BASC-2 scores through 2-8 years. (Page 29)
“We constructed linear mixed models for composite scores. First, we constructed unadjusted models. Then we added fish intake in the unadjusted models to test for confounding with whole blood mercury. Next, we added participants’ demographics and characteristics, child blood lead concentrations, maternal serum cotinine concentrations with and without child mercury concentrations to the unadjusted models and called this multivariable model 1. We added fish intake to the multivariable model 1 and call this model multivariable model 2. As mentioned before, the choice of the inclusion of the variables in the models was based on the criteria of confounders (associated with both mercury exposure and behavior changes in children but were not known to be on the causal pathway) and also guided by previous research [16, 18, 29]. The findings of the different regression models are presented in Figure 2. Multivariable model 2 had the lowest Akaike’s information criterion (AIC) values compared to multivariable model 1 and considered this our primary regression model. We then constructed primary regression model for all corresponding BASC-2 subscales. We used locally weighted scatterplot smoothing (LOESS) analysis to examine the shape of the relationship for blood mercury and behaviors in adjusted models. The relationships appeared to be linear (Figure 3).” (Page 8 – 9)

“Multivariable model 2 had the lowest AIC value and hence selected as the primary regression model (Figure 2). Locally weighted scatterplot smoothing (LOESS) analyses revealed approximately linear relationships between log2-transformed prenatal mercury concentrations and BASC-2 behavioral outcomes (Figure 3).” (page 11, lines 3-6)

Comment 7: In Figure 1 the labels of the x-axis are not correct (-0.5).

Response: We have fixed the labels of the x-axis in the Figure.

Modifications: The updated Figure 4 now reads the correct label on the x-axis (-0.5).

Comment 8: When using the self-reported anxiety of the children (Figure 3) I wonder why this analysis wasn't done with the prenatal MeHg measures. According to their hypothesis and results
presented so far, the early MeHg exposure, measured with the 16 weeks value, should predict this behavior. Moreover, the box plot of the postnatal MeHg blood values indicated that (a) this exposure period was extremely low, and (b) linear regression modeling might be not appropriated.

Response: We apologize this was not clear. The analyses in Figure 3 [now figure 5] were conducted with all prenatal mercury concentrations, including 16-weeks value.

The higher amount of prenatal mercury concentrations could be attributed to maternal fish consumption. That could explain lower postnatal mercury concentrations (no/little fish consumption) compared to prenatal mercury concentrations.

The primary purpose of our paper was to examine the impact of prenatal mercury concentrations. However, we examined the association of post-natal mercury concentrations in secondary analyses. But since we did not find any significant association, we mentioned in the text under the results section about this finding. The Figure 3 [now figure 5] represents the results of prenatal mercury concentrations only.

Modifications: None

Comments 9: The mean T-scores and also the SDs of the BASC-2 composite scores are absolutely normal! This needed to be mentioned and addressed in the discussion.

Response: We thank the reviewer for pointing this out. We agree with the reviewer that the mean T-scores and the SDs are normal. We have added this in the manuscript.

Added Text:

“The mean T-scores and the SDs of the BASC-2 composite scores are normal (Table 2).” (Page 10, lines 15-16)