Our comments are found in line with the reviewer comments and are highlighted.

Reviewer 2

Comments and Suggestions for Authors

This is a report analyzing mercury exposure and immunological reaction, and it is valuable because further consideration including nutritional status has been approached. This reviewer believes that such analysis including combined effects will bring important knowledge.

Firstly, the authors are requested to check whether the vaccine formulation does not contain mercury compounds such as Thimerosal. Thimerosal and its metabolite ethylmercury may affect the assessment of mercury exposure using hair samples.

It is important to point out that mercury levels related to thimerosal exposure from vaccines has a half-life of 4-6 days and is non detectable 30 days after vaccination according to research in infants and children [1, 2]. Results are similar for influenza vaccines [3]. Thus, the comment by the reviewer is somewhat overstated as hair mercury assessment reflects a 1-2 month exposure window and we exclude children who received vaccine in the past 20 days. Regardless, in Peru, thimerosal has been phased out from all vaccines except for the flu vaccine, similar to the US. As this exposure was not mentioned in the manuscript we have added these details to the first paragraph of the introduction (lines 70-72).

Line 99-102: The authors reported that "Data are from an observational cohort study in Madre de Dios (MDD), Peru, a gold-mining region where the majority of the population have hair mercury content exceeding a level associated with impaired child development (1.2 μg/g) [31-33]." Then, they set this hair mercury level of 1.2 ppm as a cut-off point for the following analysis. However, this reviewer cannot identify the ground in the references 31-33. Furthermore, they also used 2 ppm as another cut-off point. Please show the theoretical basis of these numbers. Similar questions are also raised in other places (Line 233-234). How do the authors convert the EPA RfD value from cord blood mercury content or maternal hair content to that of child hair?

The 1.2 μg/g hair mercury content level was derived using a blood level associated with adverse neurological impacts in children (5.8 μg/L) and the assumption of a hair:blood ratio of 200:1. The blood level was initially calculated by the National Research Council and is recognized by the US Environmental Protection Agency as a blood level associated with adverse child cognitive impacts. The relationship between blood and hair mercury has been documented by the World Health Organization to vary from 200-300:1 (hair:blood), with most studies reporting ratios around 200:1.

The 2.0 μg/g hair mercury content level is a level recognized by Peru’s government and the WHO previously. This level has been calculated using a higher benchmark dose and the assumption of a hair:blood ratio of 250:1. The higher benchmark dose refers to the upper range of the BMDL (range: 4.6-7.9 μg/L). Additionally, WHO uses 2.2 ug/g based on the 2006 Joint WHO/FAO Expert Committee on Food Additives (JECFA). They adopted the Provisional Tolerable Weekly Intake of 1.6 μg/kg bodyweight per day for methylmercury to protect the most sensitive population of pregnant women and infants, which, accounting for adjustments for inter-individual variability, is associated with 2.2 μg total mercury / g hair.

To better inform readers as to our choice of the 1.2 and 2.0 μg/g hair mercury content levels we have added calculation details to the supplemental materials.

Since the RfD had been determined based on the adverse effects on neurobehavioral changes, do the authors consider analyzing mercury values simply such as dividing it into quartiles?

We considered analyzing the data using quartiles as such a practice is commonly used in epidemiology and is useful in communicating relative risk in lower and higher exposure groups. However, the usage of quartiles can increase the risk for multiple testing and makes the assumption that risk does not vary within a category ([4]). Varying risk within a category was a concern of our as the distribution of hair Hg was asymmetric, positively skewed. We ultimately decided to assess the impact of mercury two ways: 1) using a continuous variable, to allow for inferences on how incremental changes in hair Hg impact specific immune antibodies, and 2)
using a categorical variable to make inferences directly tied to reference levels associated with adverse impacts.

Lines 75-77: In the sentence “Anemic conditions related to mercury exposure may occur from impaired hemoglobin function from mercury competing with iron for binding sites [14] and have been observed in the same region”, what have been observed in the “same region”? When we say “same region” we actually mean data from the same study using children under 12. This was just poor wording and thank the reviewer for identifying this to be clarified. We have adjusted this sentence and the sentence prior be clearer. It now reads “Mercury exposure is also hypothesized to increase the risk of nutrient deficiencies, such as anemia due to impaired hemoglobin function from mercury competing with iron for binding sites. In our study region, elevated mercury content in hair was associated with decreased hemoglobin levels in children under 12 years old”.

2.3. Hair mercury analysis: Since inhalation exposure to mercury vapor is possible (Line 120-121), did the authors wash the hair samples prior to Hg determination to exclude the possibility of external contamination of hair materials with mercury vapor? We discussed washing methods to remove potential dust and exogenous Hg on hair samples, but ultimately decided to not wash the hair because the efficacy of washing steps has not been well established. Also, certain washing agents could strip endogenous mercury from the hair specimen (cite reference). As such, total Hg content in unwashed hair has been an accepted protocol for the evaluation of Hg exposure ([5]). This is the same in the mercury determination using toenail samples. Do the authors use the Hg data from toenail samples? Toenail samples were washed to remove potential contamination from metals in the soil. We agree with this reviewer that the results from the toenail data were not highlighted in this manuscript. We have decided to remove reference to these samples as they ultimately weren’t discussed because the sample size using hair Hg data was much greater (68 vs 37 younger children and 92 vs 59 older children).

Lines 249-250 & Figure 3: Since antibody concentrations are log-transformed, the figures indicating the antibody concentrations should be shown logarithmically. We agree with the reviewer that it would be more appropriate to show the log-transformed antibody concentrations and have updated Figure 3.

4(A), The results are complicated and they seem to be inconsistent sometimes. For example, mercury exposure exceeding 1.2 ppm increased the antibody concentration of Pertussis, and that exceeding 2.0 ppm decreased the concentration. Although the authors showed these phenomenon in Discussion, what mechanism do the authors think about? Figure 4 was created to show the average beta direction for a variable. For example the antibody direction indicated for a child exceeding 1.2ug/g hair Hg is the average of all the betas for the models where the nutrition variable varies (Hb, anemia, stunting, etc). As such, to fully compare the direction of specific models we refer readers to the Supplemental tables, because as the reviewer pointed out there is a lot of data to try to present concisely. In the case of the mercury variables for the older children with respect to Pertussis similar models show the same directionality. Hg when considered with hemoglobin levels has a negative association, while when considered with the other nutritional status variables has a positive relationship.

If the authors integrate the factors of malnutrition into one index, it is easy to understand. HAZ, WAZ and WHZ are established markers of nutrition used in both clinical medicine and research to classify various forms of under (or over) nutrition. We disagree with the use of an overall index as it would obfuscate the differential effects on one measure vs. another.

Line 380: The meaning of the sentence “Associations with parental hair mercury were also observed.” is unclear.
We have expanded on this sentence to read “Replacing child mercury level with parental Hg level resulted in similar relationships identified for pertussis and diphtheria, reflecting the strong child-parent Hg correlation.” And included a reference to the Supplemental table that includes these results.

Figure 5: The explanation of the vertical axis of the graph is incorrect; “non-reponder” will be non-responder.

We thank the reviewer for catching this typo. The axis of Figure 5 has been updated accordingly.


