Author's response to reviews

Title: Blood Manganese Concentrations in Jamaican Children with and without Autism Spectrum Disorders

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Author's response to reviews:

Response to the Reviewers’ Comments on Blood Manganese Concentrations in Jamaican Children with and without Autism Spectrum Disorders

Reviewer 1: Birgit Claus Henn

Reviewer's report:
“This manuscript describes a case-control study conducted to evaluate associations between blood manganese levels and risk of autism spectrum disorder (ASD) among Jamaican children. It appears to be one of the first reports to characterize Mn levels in a Jamaican pediatric population and to describe potentially unique dietary Mn exposure sources, and as such, would be a novel contribution to the literature. However, I have several concerns and suggestions for improvement detailed below.”

Major Compulsory Revisions

Abstract

Comment #1 of Reviewer #1

“As it is written, the introduction of the abstract only mentions the essentiality of Mn with no mention of its potential to be neurotoxic. I suggest providing a brief rationale for investigating Mn as a risk factor for ASD. Is there a proposed biological mechanism, for example, or mechanistic link that led authors to this hypothesis?”

Response to Comment #1 of Reviewer #1
We appreciate the reviewer’s suggestion. In the revised manuscript, we have added the following sentence to the Background subsection of the abstract:
Previous studies have shown neurotoxic effects in children exposed to higher levels of manganese.

Background

Comment #2 of Reviewer #1
“The background lacks text on what is known about environmental risk factors for ASD, and about Mn specifically as a potential risk factor for ASD. In contrast, there is extensive text covering general aspects of Mn, such as general sources of exposure, breakdown in the environment, and environmental fate and transport, which would be better suited in a different publication. The background should be streamlined and improved substantially, e.g., the first paragraph and the last 3 paragraphs of the background contain the crux of the introductory material.”

Response to Comment #2 of Reviewer #1
We thank the reviewer for highlighting this deficiency in the introduction of our manuscript. In the revised manuscript, we have streamlined the introduction as suggested by cutting much of the information on breakdown and transport of manganese. We have also shortened and combined the information on potential manganese sources in Jamaica to improve the relevance of the information presented in the introduction to the main objectives of this manuscript.

Comment #3 of Reviewer #1
“Authors describe Jamaican soils as having Mn levels that are >10% higher than “recommended amounts.” While it would be appropriate to describe Jamaican soil Mn levels relative to soil Mn in other areas of the world, I have a hard time understanding “recommendations” for soil Mn concentrations. Please clarify or rewrite.”

Response to Comment #3 of Reviewer #1
We regret this was not clear. To improve understanding of the comparison between soil manganese levels in Jamaica and the rest of the world, we have omitted the 10% reference and have edited the sentence to read as follows (Page 5):
Previous studies have reported that levels of manganese found in Jamaican soil are approximately twice the average levels reported for soils in other countries based on a world-wide comparison performed by the International Atomic Energy Agency [29].

Comment #4 of Reviewer #1
“There is a lack of reference values for blood Mn levels in children. I am not aware of a published blood manganese guidance value, or level that is defined to be “elevated,” as stated by authors. Any guidance values that have been
recommended by ATSDR are interim values, based on dietary balance studies in adults. Their relevance for children, especially in the age range of children in this study, is questionable. Therefore, I would urge the authors to omit citing the percentage of children with “elevated” blood Mn levels in their cohort, throughout the paper.”

Response to Comment #4 of Reviewer #1

We appreciate the reviewer’s insightful suggestion. We have removed all references to the percentage of children with elevated blood manganese levels as recommended.

Methods

Comment #5 of Reviewer #1

“This paper would be of much broader interest and importance if authors reported findings on the other metals they examined (Pb, Hg, As, Cd), in addition to Mn. Especially given the null findings for Mn, I strongly suggest adding results for the other metals that authors have not yet published (e.g., Pb, Cd). Because Cd has been reported to be a concern in Jamaican produce (Howe et al. 2005), I would think this question would be of interest and fit well with the current manuscript. I also urge authors to review Roberts et al. (2013), who created an index for overall measure of metals, and strongly suggest that they consider a similar type of overall measure.”

Response to Comment #5 of Reviewer #1

We agree with the reviewer that results on other metals investigated in this study, including lead and cadmium, would enrich this manuscript. A separate manuscript from our research in Jamaica focusing on cadmium has already been published in the journal of Research in Autism Spectrum Disorders. Additionally, another manuscript focusing on blood lead concentrations and three glutathione-S-transferase (GST) family genes (i.e., GSTT1, GSTP1, and GSTM1) is currently under review for possible publication in another journal. We plan to investigate the additive and interactive effects of joint heavy metal exposures using various proposed methods in a separate manuscript after using a larger dataset and additional data that will be obtained from the R01 phase of our study.

Comment #6 of Reviewer #1

“In the methods, it is unclear which variables were considered as potential confounders. As written, it seems that authors only considered paternal age, place of child’s birth, and dietary/water intake though these were only the variables included in final models). Please clarify in text. Were child’s age, gender, and maternal age considered? Blood Mn levels decline with age, and some studies have reported differences in internal Mn levels by sex. Also, authors have previously published on maternal and paternal age being jointly associated with ASD, so could the authors’ published recommendation of jointly modeling maternal and paternal ages be implemented in this study?”

Response to Comment #6 of Reviewer #1
We regret a lack of clarity regarding the variables that were considered as potential confounders in our analysis. Potential founders were determined by associations with both ASD case status and blood manganese concentrations, as evident by a P<0.25, and if these changed the regression coefficient by >10% when included in the model. We searched for potential confounders among several variables that included consumption of root vegetables, leafy vegetables, legumes, fruits, seafood, and grain products as well as drinking and cooking water source, maternal and paternal ages, and parental education levels. Additionally, because a disproportionate number of our controls were drawn from the Kingston area and parental education level is a known factor associated with ASD, place of child’s birth (Kingston parish vs. other parishes) and parental education level were considered as a priori potential confounders in our multivariable model. The potential cofounders included in our final model included paternal age, parental education levels, place of child’s birth, consumption of root vegetables, cabbage, saltwater fish, and cakes/buns. Also, we would like to clarify that because as part of our study design ASD cases and TD controls were matched based on age and sex, we did not control for these as potential confounders in our multivariable models. To clarify all of these important issues, we added additional information to the methods section. Additionally, although maternal and paternal ages are associated with ASD status, maternal age was not associated with the blood Mn concentrations in children. Therefore, this variable did not meet our aforementioned criteria for as a potential confounder and was not included in our final model.

We have attempted to address the comments of the reviewer by providing more explanation for our choice of confounders used in the final model and listing the group of variables that was considered on page 10. We also added results for an examination of the relationship between manganese levels by sex and found no associations. Results for this additional analysis are shown in Table 3. For this paper, since the main objective is to examine the possible associations between blood manganese concentrations and ASD, the role of maternal and paternal ages could be limited as potential confounders. However, we believe that joint modeling is not an appropriate method for the scope of this objective in our study.

Comment #7 of Reviewer #1

“How did authors take into account the possible beneficial effects of Mn in their models? Using a linear model, we may fail to observe a true association if the association is not linear. Authors should, for example, categorize Mn or model Mn as a smoothed term to allow for possible non-linearity.”

Response to Comment #7 of Reviewer #1

We would like to clarify that because of our case-control study design, the outcome variable is blood manganese concentrations in ASD cases and TD controls. Although blood manganese concentration is modeled as a continuous variable in a linear regression model, the main independent variable in our model is categorical (i.e., ASD case or TD control). In our situation the linear regression model results in a comparison between mean manganese concentrations in ASD
cases and the TD control groups. In addition, the potential confounders in the linear regression model are all categorical variables. Therefore, there is no assumption of linearity in any of the variables in our models.

Comment #8 of Reviewer #1

“Authors claim that blood Mn is a “reliable measure” of Mn exposure given the population’s continuous exposure to Mn in the diet. Only a small fraction of oral Mn, however, is absorbed, and this is held under tight homeostatic control. It is not known what blood Mn levels reflect, either environmental exposure or an underlying physiologic need/role or both. I suggest omitting this statement and including text on the limitations of blood Mn as a biomarker in the discussion section. Authors might consider referring to ATSDR, which states that, “elevated blood manganese alone does not constitute an adequate indicator of manganese overexposure”, however, “at high exposure levels, a higher level of blood manganese may be maintained.”

Response to Comment #8 of Reviewer #1

We thank the reviewer for these excellent comments and the suggestions. In the revised manuscript, we have removed the statement declaring blood manganese a “reliable measure” on page 19. Additionally, we have added text in the limitation section on the use of blood as a biomarker for exposure as follows:

Although we used blood manganese concentrations as our biomarker of exposure, we acknowledge that tissue markers of exposure, such as nervous tissue, may be better indicators of long term exposures. Though prior studies have indicated that hair manganese levels increase over years of exposure, these measurements may be influenced by external exposures as well [79]. Considering that the best biomarker of manganese exposures is still not established, we used blood manganese concentration, which is considered as an adequate biomarker for body burden [79,80].

Comment #9 of Reviewer #1

“The statement that blood and urine are the most frequently used biomarkers of Mn exposure may be true for occupational settings, but is probably not true for studies of environmental Mn exposure, where hair (and water) are also often used to measure exposure. Mn is excreted primarily via the bile and thus urine is only a minor excretion route for Mn. The statement should be qualified, or rephrased to something like, “blood and urine have frequently been used as biomarkers in occupational settings…”

Response to Comment #9 of Reviewer #1

Thank you for the suggestion. We have edited this sentence on page 15 to read as follows:

Though there are various potential biomarkers for measurement of manganese exposure, blood and urine have been frequently used to assess exposure in occupational settings [36].

Comment #10 of Reviewer #1
“Page 11: authors write, “we used GLMs to evaluate associations between Mn concentrations and exposures of interest.” By “exposures of interest,” do you mean possible sources of Mn exposure? Please clarify, here and throughout the manuscript.”

Response to Comment #10 of Reviewer #1
We regret for the lack of clarity. Yes, we did mean potential exposures to manganese. We have edited that section for clarification as follows (Page 10):
We also used GLMs to evaluate the association between manganese concentrations and potential exposures to manganese, including frequency of consumption of various food items by children.

Results

Comment #11 of Reviewer #1
“There seem to be several significant differences in characteristics of cases vs controls, including the place of child’s birth. For me, this raises the question of whether controls were appropriately selected to represent the source population that gave rise to the cases. Could there be selection bias? The majority of controls were selected from Kingston parish, while majority of cases selected from other parishes. I’m not familiar with these areas of Jamaica, but is it possible that controls were inadvertently selected in a way that was not independent of exposure, thereby making the control group look more similar to cases, and result in a null finding? Can the authors address this concern and provide more information about how controls were selected?”

Response to Comment #11 of Reviewer #1
We appreciate the reviewer’s concerns. We acknowledge that recruiting controls for this study was difficult. While parents of potential ASD cases were willing to travel far distances for re-assessment of their ASD status by our study team in Jamaica led by the PI of the subcontract at the University of the West Indies (UWI), parents of many TD controls were unwilling to travel far distances for participation in the study. Also, the selection of matched TD controls was not based on their area of residence. The issue of potential selection bias has been addressed in the limitations section of the revised manuscript. However, we do not believe this limitation will significantly impact the overall findings reported in this study. Specifically, we have shown in Table 3 of the revised manuscript that there were no statistically significant differences in the mean blood Mn concentrations of children from different parishes in both ASD cases and TD controls groups. Furthermore, in order to adjust for any potential differences by area of residence we have included parish of birth (Kingston vs. all others) as an a priori potential confounder in the multivariable model. We hope we have adequately addressed the reviewer’s concern. (Page 19)

Discussion

Comment #12 of Reviewer #1
“There is unnecessary repetition between text in the intro and discussion, which should be eliminated. (For example, paragraph on p. 16 on biomarkers is similar to section on assessing Mn exposure on p. 10)”

Response to Comment #12 of Reviewer #1
We appreciate the reviewer for highlighting this deficiency. In the revised manuscript we have streamlined both the introduction and the methods section to mitigate the repetition with the discussion section of the paper.

Comment #13 of Reviewer #1
“This section could be strengthened with additional consideration of possible reasons for failing to detect an association. For example, is it possible that prenatal and/or early life Mn exposure is more predictive of ASD, and that this exposure period was not adequately captured here?”

Response Comment #13 of Reviewer #1
We have added this into our limitations portion of the discussion section. (Page 19)

Minor Essential Revisions

Comment #14 of Reviewer #1
“By GLM, do you mean general linear model (i.e., standard linear model assuming normality of residuals) or generalized linear model (i.e., errors do not need to be normally distributed)? Perhaps describing the model rather than, or in addition to, using this terminology would help clarify.”

Response to Comment #14 of Reviewer #1
We previously defined GLM as General Linear Model in the abstract and methods section of the manuscript. For clarification, we have added a brief definition of GLMs to the methods sections as follows (Page 9):

To account for the age- and sex-matched study design when comparing BMC of ASD cases and TD controls, we used General Linear Models (GLM), which are equivalent to standard linear regression models with normality assumption of the residuals but allow assessment of random effects.

Comment #15 of Reviewer #1
“In Statistical Analysis, delete ‘not geometric means.’”

Response to Comment #15 of Reviewer #1
We have removed this portion of the sentence as suggested by the reviewer.

Comment #16 of Reviewer #1
“Reporting odds ratios, an informative estimate of the association between blood Mn and ASD, could be useful for comparison with other case-control studies reporting ORs.”
Response to Comment #16 of Reviewer #1

We agree with the reviewer that providing matched odds ratios will allow comparison with reported odds ratios (ORs) from other case-control studies that investigated the association between Mn and ASD. In the revised manuscript we added relevant information for using an alternative method to obtain matched odds ratios by modeling ASD status as the outcome variable in a conditional logistic model and blood manganese concentrations as an independent variable. In this alternative method of analysis, described in the methods section, we have categorized the BMC into four quartiles. Matched odds ratios from univariable and multivariable models along with their 95% CIs are reported in Table 5 (Page 10).

Comment #17 of Reviewer #1

“We “fit” GLMs, not “fitted”.”

Response to Comment #17 of Reviewer #1

We have corrected this per the suggestion of the reviewer. (Page 10)

Comment #18 of Reviewer #1

“Blood Mn levels decline with age. Was there an association between child’s age and blood Mn levels? If so, authors could consider reporting age-specific Mn levels, or reporting any observed trend. “

Response to Comment #18 of Reviewer #1

We investigated the role of child’s age in relation to blood manganese concentrations and added our findings in Table 3 of the revised manuscript. Although in our combined samples of ASD and TD controls we observed that blood manganese concentrations were actually lower for children over 4 years of age, there was no statistically significant association suggesting such a trend in the population. However, after stratification by ASD case status, we found that the lower manganese levels seen in children over 4 years of age was statistically significant only in the TD group (P=0.01).

Reviewer 2: Pam Factor-Litvak

Reviewer's report:

This paper assesses the associations between blood manganese and ASD using a case control design. The investigators are building on an emerging literature on the adverse associations between both deficiencies in manganese and excessive manganese exposure and adverse neurodevelopmental exposures. Although there are several studies, which examine this research question in the literature, results are conflicting, perhaps due to lack of variability in the exposure distribution of cases and controls. The investigators therefore exploit the unique Mn exposure pathways in Jamaica using an ongoing case control study of the relationships between heavy metal exposure and ASDs. My major concern regarding this manuscript is the lack of focus. The investigators intensively
investigate the sources of exposure to Mn, as they describe in detail the identification of confounders.

Major required revisions

Comment #1 of Reviewer #2

"Please either refocus the purposes of the paper to both identify the sources of Mn exposure and the associations between Mn exposure and ASDs, or focus on the latter with minimal discussion of the former."

Response to Comment #1 of Reviewer #2

We appreciate the reviewer’s suggestions. In the revised manuscript we have clarified that there are two objectives. In our first objective, we investigated whether there is an association between blood manganese concentrations and ASD in children living in or near Kingston, Jamaica. In our second objective, we estimated blood manganese concentrations in TD Jamaican children and identified factors associated with blood manganese concentrations in these children, with a particular focus on the food consumed by these children. Because this is the first study, to our knowledge, to report blood manganese levels in Jamaican children, we have also dedicated a portion of our discussion section to evaluating differences between the observed blood manganese levels in our samples as compared to that observed in other countries.

Comment #2 of Reviewer #2

"Unfortunately ASDs were not further defined according to type. Please discuss."

Response to Comment #2 of Reviewer #2

We agree with the reviewer’s comment that ASD subtypes are an important factor to investigate; however, the data we have does not permit us to do this with any accuracy or precision. Any subtypes we might define empirically would be arbitrary. Given current research, the DSM 5 no longer recognizes subtypes clinically. For these reasons we have not conducted any analysis of blood manganese concentrations by ASD subtypes.

Comment #3 of Reviewer #2

"Please provide details as to control selection. Were they chosen from the same geographic areas (it doesn’t seem so)? Please discuss any potential biases that the control selection may result in."

Response to Comment #3 of Reviewer #2

We appreciate the reviewer’s concerns. We acknowledge that recruiting controls for this study was difficult. While parents of potential ASD cases were willing to travel far distances for re-assessment of their ASD status by our study team in Jamaica led by the PI of the subcontract at the University of the West Indies (UWI), parents of many TD controls were unwilling to travel far distances for participation in the study. Therefore, the selection of TD control was not based on their area of residence. The issue of potential section bias has been addressed in
the limitation section of the revised manuscript. However, we do not believe this limitation will significantly impact the overall findings reported in this study. Specifically, we have shown in Table 3 of the revised manuscript that there were no statistically significant differences in the mean blood Mn concentrations of children from different parishes in both ASD cases and TD controls groups. Furthermore, in order to adjust for any potential differences by area of residence, we have included “parish of birth (Kingston vs. all others) as an a priori potential confounder in the multivariable model. We hope we have adequately addressed the reviewer’s concern.

Comment #4 of Reviewer #2
“The statistical analysis section is confusing. Please be specific about which model was used for which analysis.”

Response to Comment #4 of Reviewer #2
In the revised manuscript, we have added additional information to the statistical analysis section in order to clarify that we used General Linear Models (GLMs) when comparing blood manganese concentration of ASD cases and TD controls. We also used GLMs to evaluate the association between blood manganese concentrations and potential exposures to manganese while also comparing blood manganese concentrations within case and control groups using t-tests. We used Conditional Logistic Regression to examine the association between ASD case status and exposure variables. Lastly, we fit a multivariable GLM to investigate the relationship between ASD and blood manganese concentrations while adjusting for confounders including parish of birth (Kingston vs. all others), paternal age, parental education, and consumption of root vegetables (“yam, sweet potato, or dasheen”), “cabbage”, salt water fish, and cakes/buns.

Comment #5 of Reviewer #2
“The statistical power analysis for predicting blood Mn concentration is not necessary.”

Response to Comment #5 of Reviewer #2
We have removed this section as suggested by the reviewer.

Comment #6 of Reviewer #2
“It is unclear whether the associations between sources of Mn exposure and ASD are adjusted for variables such as social circumstances, which would be important. Please clarify and fix.”

Response Comment #6 of Reviewer #2
We examined parental education as a potential confounder and indicator of social circumstances; however, this variable did not meet our criteria for inclusion in the multivariable model, as this variable was found not to be associated with blood manganese concentrations (P = 0.82). We have however added this variable as a confounder to our multivariable models and revised the results in Tables 4 and 5.
Comment #7 of Reviewer #2
“The conclusions in the abstract suggest that the investigators found associations between BMC and neurodevelopment in Jamaican children. They did not, and this is misleading. Please fix.”

Response to Comment #7 of Reviewer #2
We have edited the abstract conclusion in accordance with the focus of this paper as follows:
While these results cannot be used to assess early exposure at potentially more susceptible time periods, our findings suggest that there is no significant association between manganese exposures and ASD cases status in Jamaica.

Minor required revisions

Comment #1 of minor required revisions of Reviewer #2
“On the bottom of page 1 of the background the authors cite soil Mn concentrations based on International Atomic Energy Agency levels. No reference is given. Please cite the actual reference.”

Response to Comment #1 of minor required revision of Reviewer #2
We have removed the statement on recommended soil Mn concentrations in response to a comment made by the other reviewer.

Minor discretionary revisions

Comment #1 of minor discretionary revisions of Reviewer #2
“Please edit the paper. It is repetitious, especially when reviewing the sources of Mn exposure and the biomarkers of Mn exposure. The discussion of biomarkers can be dramatically shortened and placed in the discussion.”

Response to Comment #1 of minor discretionary revisions of Reviewer #2
Per the reviewers’ requests, we have streamlined all sections of the paper removing all of the repetitions along with much of the discussion on biomarkers of exposure, breakdown, and transport of manganese.