Author's response to reviews

Title: Maternal iron metabolism gene variants modify umbilical cord blood lead levels by gene-environment interaction: a birth cohort study

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Author's response to reviews: see over
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Professor Neil Pearce  
The Environmental Health Editorial Team  
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Dear Dr. Pearce,

Thank you for reviewing our manuscript, ‘Maternal iron metabolism gene variants modify umbilical cord blood lead levels by gene-environment interaction: a birth cohort study’ (MS: 6827582541240797). We would like to extend our appreciation to the reviewers for their insightful comments and helpful suggestions, all of which have improved the quality of our work. Following this letter is a point-by-point description of the changes made, which address the concerns outlined by our reviewers. The revised manuscript has been resubmitted as directed.

The manuscript remains an original work, has not been previously published whole or in part, and is not under consideration for publication elsewhere. No animals were used in this research. Participation of human subjects did not occur until after informed consent was obtained. None of the authors have actual or potential competing interests to disclose. All authors have read the revised manuscript, agree the work is ready for resubmission to Environmental Health, and accept responsibility for the manuscript’s contents.

We thank you in advance for reviewing the revised manuscript and look forward to hearing from you.

Sincerely,

Matt Karwowski
Reviewer comments are numbered and listed in the order that they appear in the reviewer feedback files. All reviewer comments are addressed point-by-point.

Response to Comments from Reviewer 1:

1. The description of the study population and methodologies are fine, as is the approach to the statistical analyses. I found the first two paragraphs under “Potential Confounders” (page 8) to be somewhat unnecessary as it seems to me quite obvious that SES parameters would not influence the biological processes concerning maternal-fetal Pb transport. Indeed they did not when probed in sensitivity analyses in this paper. They could be deleted.

We have deleted the first two paragraphs under “Potential Confounders” (page 8) as suggested by the reviewer. We deliberately left our sensitivity analysis intact in order to assuage any concerns that factors extrinsic to the maternal-fetal system might confound the relationship between umbilical cord and maternal blood lead.

2. With regard to the results, the observation that maternal blood Pb (MBL) and umbilical cord blood Pb (UCBL) are strongly associated is not novel, and has been observed in many other study populations, a point which should be mentioned in the discussion.

We amended the beginning of the fifth paragraph of the Discussion section to reflect this point. We state that the association between maternal and umbilical cord blood lead has been observed in other studies and we provide several references as examples.

3. However, the finding that infants born to women with the hemachromatosis HFE C282Y gene variant have lower UCBLs is completely novel. The first paragraph of the discussion (page 13) ends by saying that this observation is “...both statistically and clinically significant.” I find this conclusion to be a bit strong (particularly in this population) and suggest that it be modified to indicate that it is “...potentially clinically significant in exposed populations.” To their credit, the authors do mention (in the limitations section) that the low range of BPBs in this population may limit the generalizability of the observations.

Given that the health effects of blood lead levels in the single-digits are subclinical, and that the range of blood lead levels seen in this population is low, we changed this portion of the discussion as recommended by the reviewer.

4. Page 5, line 76, should read: “…iron transport [16], while…

The sentences that precede and follow this phrase are meant to convey two separate thoughts:
The TF gene is located on 3q22.1 and codes for the glycoprotein transferrin, which is responsible for intercellular iron transport [16]. While the TF gene is highly polymorphic, a common variant is the P570S missense single nucleotide polymorphism (SNP) [17].

Thus, we feel that uniting them with a comma would confuse the reader.

5. Page 7, line 141: Define “...TE buffer...”

We added text after ‘TE’ to specify that it refers to Tris-EDTA buffer. The term ‘TE Buffer’ is also defined in the list of abbreviations that follows the conclusion section.

6. Page 9, line 172, should read: “...were available: serum ferritin...

We have made the appropriate change by specifying that we are referring to ‘serum’ ferritin.

7. Page 15, lines 309-310, should read: “...proteins and thereby lesson gastrointestinal lead absorption.”

We have amended the original descriptor, ‘environmental’, to read ‘gastrointestinal’, as suggested in the comment.

Response to Comments from Reviewer 2:

1. My concern relates to the reliance and possible over-interpretation of a single p value crossing the p<0.05 cut-off value. The “story” of the paper rests on finding effect modification of placental lead transfer by one of the three polymorphisms in iron metabolism genes examined, which gave a statistical interaction p value of 0.04 for mothers’ genotype. There was little consideration of the effect, or apparent lack of effect, for the other two polymorphisms in mothers. There was a mention of “While the other two maternal genotype interaction terms were not statistically significant, all three consistently trended in the same direction”. But that was it. There was little mention of the results as a whole. For example, were they surprised not to detect an interaction effect for the other two polymorphism, having seen it for one?

We thank the reviewer for raising this point. While we did not find statistically significant results for two or three polymorphisms, care must be taken not to misinterpret this ‘absence of evidence’ as ‘evidence of absence’.¹ Since the ability to demonstrate a statistically significant result depends in part on sample size, the reviewer is right to wonder about the utility of a power calculation. However, confidence intervals better inform readers about the possibility of an inadequate sample size than do post-hoc

power calculations. Indeed, parameter estimates for the two non-significant interaction terms in this study have wide confidence intervals, suggesting that a larger sample size would provide more stable estimates of the true interaction effect.

If we shift our focus from the p-value to quantification of the association, we see that the two non-significant interaction terms in this study have considerable effect sizes. Thus, we wrote, “While the other two maternal genotype interaction terms were not statistically significant, all three consistently trended in the same direction.” To provide context for this observation, we outline the existing literature on genetic modifiers of lead toxicokinetics and toxicodynamics (Discussion section, paragraph 3). Here we explain that:

“Genetic variability may influence bioavailability and susceptibility in a complex fashion, interrelating with other individual characteristics such as age and micronutrient status to produce differential outcomes.”

We have edited the text in this paragraph to clarify how these factors might explain why interaction effects were seen for one gene polymorphism, but not others. Space limitations restrict a more in depth exploration of this topic.

2. In particular, there was no mention of either type I error, whereby the finding for maternal HFE C282Y could have been a chance finding (multiple testing conducted), or a type II error, whereby the sample size was too small to detect small effect even if it was present. No sample size calculations were presented.

We have added text to the limitations section to reflect the possibilities of both Type I and Type II errors.

We carefully considered our approach to data analysis when designing this study. Though very few variables are likely to confound the relationship between maternal and umbilical cord blood lead levels, we thought that gene variant status was an important consideration.

We had two main concerns: 1) gene variant status was likely to be correlated between infants and their mothers, and 2) within any individual, the likelihood of carrying a single iron metabolism gene variant was hypothetically related to carrier status for related variants (i.e. the prevalence of HFE H63D mirrors that of HFE C282Y). Thus, our final multiple regression model accounts for all infant and maternal gene variants and their interactions with maternal blood lead (Table 4). In doing so, we created a multiple regression model that included thirteen independent variables and that raised concerns for both multicollinearity and Type I error related to multiple testing.

To address multicollinearity, we examined variance inflation factors and ran sensitivity analyses to assure ourselves that parameter estimates did not differ substantially between models. Assessing multiple testing proved more challenging. We ran two additional models, one including information limited to infant genotype and the other limited to maternal genotype – these models, each of which had seven independent variables (Additional Files 1 & 2 from original manuscript), confirmed our findings from the larger analysis. Furthermore, regressing UCBL on MBL, maternal HFE C282Y variant status, and the MBL-HFE C82Y interaction again yielded consistent findings ($P_{interaction} = -0.28; 95\% CI -0.5, -0.05; p = 0.016$). These data were not presented in the manuscript due to space limitations.

From a philosophical standpoint, adjusting for multiple comparisons can prove costly in terms of reductions in statistical power. Though the family-wise Type I error rate increases as predictors are added to a model, the probability of Type I error for each independent variable should remain unchanged so long as the sample size remains adequate.

3. My suggestion to the authors is that they be a little more cautious in their interpretation of the findings. Ideally, power calculations for the interaction effects should be included.

We have amended the language used to describe our conclusion as reflected in our response to Comment 3 from Reviewer 1. As described in prior responses to Reviewer 2, we feel that confidence intervals better inform readers about the possibility of an inadequate sample size than do post-hoc power calculations.

Minor essential revisions

4. In the Background section, mention of iron metabolism genes being involved in lead metabolism in line 73 seems to come “out of the blue”. I think the logic would be easier to follow if the sentence on lines 89-92 appeared before this.

We have added new text starting on line 73 that aims to provide a less abrupt shift to the introduction of iron metabolism genes.

5. In the methods section, there appeared to be a bit too much text on why the usual confounders were not likely to be important. Then you went ahead and adjusted anyway. I suggest you reduce a lot of the text between lines 156 and 168.

Please refer to our response for Comment 1, Reviewer 1.

6. Suggest you replace the term “dyads” in line 210 with “pairs”.

We have made the change.

7. In lines 264 to 269 you very nicely present a model to predict the beneficial influence of HYE C282Y variant. I suggest to use the 95% CI to predict the minimum and the maximum values.

We have calculated the 95% confidence interval and added the information to this section.

8. It would be useful if the title of table 1 included “mother-infant pairs”, perhaps instead of “study population”, or in brackets after.

We have substituted ‘mother-infant pairs’ for ‘study population’.

9. The number of mother-infant pairs should be added to Table 4.

We have specified the number of subjects included in the regression analysis by adding descriptive text to the title.
10. In the Discussion, I suggest you include possible methodological issues of repeat testing and statistical power.

Please refer to our response for Comment 2, Reviewer 2.

Response to Comments from Reviewer 3:

There remain concerns on the interpretation and evaluation of the results. The Discussion section is inconclusive. In the Introduction section more basic information should be provided.

Several aspects are inadequately addressed concerning what has been found, i.e., maternal HFE genotype has stronger effects on cord blood lead concentrations than infant HFE genotype:

1. A brief introduction into the HFE-282 mutation and how it affects iron and lead metabolism is missing, although it is stated on page 16 that HFE C282Y is a well characterized functional variant.

Please see paragraph 4 of the Background section, which now provides a brief introduction to both HFE H63D and C282Y at a level of detail that we feel is appropriate for this manuscript.

2. The „unique physiological state of pregnancy” (page 5, third par.) is not described further.

The phrase ‘unique physiological state of pregnancy’ is meant to refer to the normal adaptations seen in a number of molecular, cellular, and organ-system processes and functions during pregnancy. We feel that a thorough explanation is beyond the scope of this paper, but have added a brief explanation to the text to clarify our meaning.

3. Also the complex situation at the maternal-fetal interface has not been thought through regarding
- genetics: the placenta is fetal tissue/genotype
- the „metal transport machinery” (pages 9, 14). The only information provided on that „machinery” is that „HFE protein influences the expression of ...DMT1“ (page 5, sec. par), which in turn „mediates lead absorption in the intestine” (page 14, sec. par). It remains unexplained in which way HFE modulates DMT1 levels and at which level (RNA, protein?). Where is the link to the HFE mutations?
- localization of the involved transporters/receptors in the placenta layers together with the direction of transport [citations 36, 37 or e.g., Parkkila et al. (1997). Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. Proceedings of the National Academy of Sciences, 94(24), 13198-13202].

We greatly appreciate the reviewer’s detailed comments and suggestions. We have performed additional literature review related to the concerns outlined above and incorporated several new ideas
into the relevant sections. Please see paragraphs 5-7 of the Discussion, which largely consist of new text.

4. *Metallothioneins are not transporters (page 14, sec.par.) but ligands for divalent metal cations and thus involved in metal storage and detoxification.*

We appreciate the reviewer’s concern. We did not mean to imply that metallothioneins are transporters, but instead that they likely influence placental lead uptake. We have edited this sentence to clarify our meaning.

5. *An explanation for the circumstance that placentas were not collected and analysed is missing.*

We agree that placental collection would have been ideal, but they were not part of the original study aims. The aim of this study was to determine whether iron metabolism gene variants modified the placental transfer of lead from mother to fetus. We did not aim to explore the biologic basis for our findings. Since maternal and infant gene variant status could be defined using blood samples, we did not find it necessary to collect or analyze placental tissue for the purpose of this analysis.

6. *To know serum iron levels of mother-child dyades would also have increased the significance of the study.*

Although we did not have information on serum iron, we did assess the role of body iron burden in the analysis using serum ferritin, hemoglobin, and hematocrit in mother-infant pairs. Please refer to ‘Methods, Potential Confounders, paragraph 2’, ‘Results, paragraphs 4 and 5’, and ‘Discussion, paragraph 10’ for comprehensive discussion of maternal and fetal iron status in relation to the study results.