Author’s response to reviews

Title: Transcriptome-wide analysis of changes in the fetal placenta associated with prenatal arsenic exposure in the New Hampshire Birth Cohort Study

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Author’s response to reviews:

Reviewer reports:

Reviewer #1:

This manuscript concerns associations of fairly low-level arsenic exposure in pregnant women with differentially expressed genes in their placentas collected at delivery. The manuscript is well-written, particularly the sections about the gene expression analyses. However, more information is needed concerning other parts of the study, e.g. urine and placenta sampling and arsenic analysis and evaluation.
A major weakness of the study is the exposure assessment, which is based on the sum concentration of arsenic metabolites in spot urine samples (one from each woman) collected 3-4 months before delivery and the sampling of placenta biopsies. Even the most elegant and detailed measure of gene expression is of no use, is the exposure measure imprecise or irrelevant. The problem is that most of the inorganic arsenic metabolites have very short half-lives in the body (hours-days), why the sum concentration of arsenic metabolites in a spot urine sample is not a very exact measure of the exposure during pregnancy, and even less so, if there is a critical time period for the changes in placental gene expression. Furthermore, the metabolism of inorganic arsenic by methylation is markedly increased during pregnancy (see e.g. Gardner et al., Reprod Toxicol, 2011), which will affect the toxicity (induction of gene expression in this case), as well as the excretion rate. Therefore, urinary arsenic metabolites may be useful for exposure assessment on a group basis only, unless it can be ascertained that the exposure through drinking water and food is constant over time. In the present study, only 24+24 placentas have been analyzed, why the relevance of the exposure assessment must be thoroughly discussed. I would strongly recommend a separate section on arsenic exposure assessment in Materials and Methods, where the choice of exposure measure and its relevance for gene expression in the placenta at delivery is motivated.

Our selection criteria included the requirement that participants had not moved since their last menstrual period and were not planning to move before delivery, such that exposure from household water would be expected to remain relatively constant. Household water arsenic was shown to be strongly associated with maternal urinary arsenic in the NHBCS (Proc Natl Acad Sci U S A. 2011 Dec 20; 108(51), consistent with it being a major source of exposure. (Although, as mentioned in that report, food sources such as rice were also important.) We have added a new Methods section, “Arsenic exposure assessment”, as suggested, containing this information. We acknowledge that arsenic metabolism is different in pregnancy, and that this may affect the accuracy of the urine analysis due to increased excretion rates. However, since we are not comparing our results to a non-pregnant group, we feel this does not greatly alter our conclusions as regards effects on gene expression.

Also, it should be discussed (in Methods or in Discussion) to what extent the different exposure groups may represent different dietary intakes, and thus exposure to different forms of arsenic, e.g. DMA through rice, as well as other toxic exposures and nutritional factors. Potential confounding? Did the authors compare the different urinary arsenic metabolites between exposure groups?

We compared the proportions of different arsenic species in urine between high and low groups and found no difference, except in the case of AsV in males, which was slightly higher in the low arsenic group (P = 0.032). We have included these data as Additional file 1 (referenced in Methods section “Urine sample collection and arsenic measurement”, and mention this as a potential limitation in the Discussion (page 18 lines 4-6).
It would be a much stronger study, had the exposure assessment (maternal urinary arsenic in gestational weeks 26-28) also been based on the arsenic concentrations in the placentas, which apparently have been measured. Punshon et al. (2019) reported a range of 0.006-18.35 ng/g of arsenic in NHBCS placetas. A comparison might have generated additional results and conclusions. Also, such a design would enable an evaluation of other toxic exposures, e.g. cadmium, which is accumulated in the placenta much more than arsenic.

The disadvantage with using placental arsenic for exposure assessment is that arsenic levels in this tissue are low, and therefore we can detect only total arsenic, rather than individual arsenic species, as are measured in urine. Therefore, the recorded levels in the placenta will also include arsenobetaine, which is considered to be non-toxic (e.g., see Environ Res. 2011;111(1):110–118.) and therefore is excluded from our total maternal urinary arsenic measurement. According to a study of a representative US population (J Expo Sci Environ Epidemiol. 2009 Jan;19(1):59-68.), arsenobetaine can constitute a high proportion (from 16.2 to 62.7%) of total urinary arsenic, and thus likely also constitutes a considerable proportion of placental arsenic. Therefore, in the current study, we chose to focus on participants with the highest and lowest maternal arsenic levels. However, we strongly agree that an equivalent study using placental arsenic levels should also be performed, and hope to address this in future work. We did check the levels of placental arsenic in our samples, and did not find a strong association with urinary arsenic levels. This may be due to a confounding effect of arsenobetaine, or variations in exposure between mid-gestation, when urinary arsenic levels were recorded, and placental sampling at delivery, as the reviewer suggests. We have included these points in a new paragraph at the end of the Discussion, as recommended by the reviewer.

Important, it has to be made clear throughout the manuscript that the results represent associations only, not proven effects.

We appreciate the reviewer’s point and have revised the manuscript wording accordingly.

Title: Is it really the prenatal exposure that is studied? On page 6, line 7, it is mentioned that only maternal urinary arsenic is measured. At least, it has to be explained how this can be interpreted as the fetal exposure.

Please see our response to the reviewer’s first question. We have added a new Methods section, “Arsenic exposure assessment”, to address the reviewer’s concerns.
Abstract: The introduction is far too long and much information missing in methods and results, especially the exposure assessment and more results presented in the manuscript. In particular, how many women were studied? What did the exposure come from? How was it measured? How were placentas collected and treated? I would recommend reporting differentially expressed genes at FDR < 0.05, not p<0.05 in the abstract. The marked difference is not discussed anyhow. How many differentially expressed genes were common for female and male placentas? Were they generally up-regulated or down-regulated? Which genes showed strongest association? Were potential confounders considered?

We have revised the Abstract as suggested.

Background

Page 3, line 20: It is a misunderstanding that studies in areas with prevalent high levels of contamination, e.g. Mexico and Bangladesh, cover high-level arsenic exposure only. In fact, most studies have a wide range of exposure, from very low to very high.

We have revised the first paragraph of the Background section to correct this error.

Page 4, first sentence. It is not clear from the writing that 10 µg/L refers to the drinking water guideline.

We have corrected this (now page 4, line 10).

Page 4, line 18: How is "low birth weight" defined?

Low birth weight is usually defined as less than 2500 grams, which is rare in our cohort. More correct terms for what we focus on in this study would be “reduced” or “decreased” birth weight. We have altered the wording throughout our manuscript accordingly. We thank the reviewer for bringing our attention to this matter.

Materials and methods

Page 6, line 4: What was the reason for selecting 24+24 placentas? Was any power calculation performed?

We did perform a power analysis, which indicated adequate power to detect a 2.1-fold change, and have now moved that information to this paragraph (page 6, lines 13-16).

Page 6, line 5: Check "each consisting of", which now refers to participants?
This does refer to participants. We have attempted to clarify the wording of this sentence (page 6, lines 10-13).

Page 6, lines 9-10: As written, I think the exclusions can be questioned. At least, they are not well justified. For example, what is a "urinary arsenic sampling error"? Also, it should be clarified that it was "an outlier" in the gene expression, not in the arsenic exposure. As mentioned above, a section on exposure assessment would markedly facilitate the reading.

We apologize for the confusion regarding the “urinary arsenic sampling error” text. A participant that was originally selected for the study was later found not to have provided a urine sample. We have clarified this in the text. We also have clarified that the female sample was an outlier as regards gene expression. We have added the section, “Arsenic exposure assessment”.

Page 6, line 19: How does diethyldithiocarbamate stabilize arsenic species? How would they change at -80°C?

Diethyldithiocarbamate is a chelating agent that binds to the unstable trivalent methyl arsenic species, MMAIII and DMAIII, and improves their stability (see Chem Res Toxicol. 2004 Jan;17(1):95-103.). This was not strictly necessary in the current study as we did not measure these species. We have added this reference. Samples were stored at -80°C to replicate the conditions used in that study (which used -50°C), as closely as possible. Previous work indicates that arsenic species in urine are more stable at −20° or less (Cancer Epidemiol Biomarkers Prev. 2002 Nov;11(11):1427-33.).

Page 7, lines 2-4: How many samples had measurements below zero? What is meant by "flagged"?

We have added the information regarding numbers of samples below zero. We have deleted references to the flags since they were not used in the analysis. We apologize for causing confusion.

Page 7, line 4: I would recommend not to use "total urinary arsenic" for the sum of inorganic arsenic metabolites. Even if defined here, it might be misleading for those reading other parts of the manuscript. The abbreviations of the different metabolites are already explained on the previous page.

We have altered the wording here (page 8, line 5) and elsewhere to clarify that U-As refers to the sum of arsenic metabolites excluding arsenebottaine. We have removed the repetition of metabolite definitions.

Page 7, lines 10-12: The collection of placentas and placenta biopsies need to be described in much more detail. As the expression "fetal placenta" is frequently used throughout the manuscript, it is assumed that only the fetal part of the placentas is collected. Please, clarify.
Who collected the biopsies "at the time of delivery", and how was it done? Biopsy size? Time periods between placenta delivery, biopsy collection, immersion in RNAlater, and freezing?

We have expanded this section (page 8, lines 11-15) to include the above information.

Page 7, line 19: What "experiments"?

“Experiments” refers to the RNA-seq assays. We have clarified this (page 8, line 22).

Page 8, lines 4-6: Incomplete sentence. Median of what? Also the following sentence is hard to understand. What is "raw read counts ~ groups"? Was maternal age the only covariate considered (several are mentioned at the bottom of page 5)? How were other potential confounders evaluated? Also, a reference to Figure 1 already here would probably help the reader to follow the analytical approach.

The phrase “according to median” was included in error and has been deleted. The phrase "raw read counts ~ groups" means that for each gene, gene count was modeled as a function of the arsenic exposure groups, i.e. high and low exposure. For the analysis of arsenic exposure, the model was adjusted for maternal age at enrollment, while in the birth weight analysis, both maternal and enrollment age were included in the model. Both were also adjusted for RNA-seq assay batch. As mentioned in this section, these adjustments were based on a previous study we performed of NHBCS samples (Environ Health. 2013 Jul 16;12:58.) in which we used a series of linear regression models to evaluate potential confounders. We found these variables to be associated with U-As/birth weight and gene expression in these models. We did not include other confounders in the models, in order to optimize our statistical power. We have added the reference to Figure 1, and thank the reviewer for this suggestion.

Page 8, line 11-15: Again, how were the potential confounders in the analysis of associations between gene expression and birth weight evaluated and selected?

Please see the previous answer.

Page 8, line 18: What effect size? Gene expression or birth weight? What does a 2.1-fold change mean?

We have now moved this passage to the “Study cohort” section of the Methods, in response to your earlier question. The effect size referred to a 2.1-fold change in gene expression in the exposure analysis.
Results

Page 10, lines 11-12: The authors need to be more cautious about mentioning "effects" of arsenic. Only associations are evaluated. Also, why is FDR <0.1 used here, but <0.05 elsewhere? Does this mean that there were no gene sets that were significant at <0.05?

We have corrected the language as suggested here and elsewhere. FDR <0.1 was initially used here, as there were only a small number of gene sets (7) positively associated with arsenic exposure in males. However, based on your suggestion, we have opted to use FDR <0.05 for consistency, as we found this did not alter the main points of interest within the data. We have revised the manuscript and Additional files 6, 7, and 8 accordingly.

Page 12, lines 3-4. Again, how is "low birth weight" defined? Are there really "many" previous studies indicating "low birth weight" in relation to arsenic exposure? No references are given here. "Low birth weight" is also mentioned on lines 10-11, without definition.

As mentioned in response to your previous comment, we have removed the use of “low birth weight” throughout the manuscript. We have altered the wording here, and have added references.

Discussion

The whole first paragraph is repetition of previous statements and can be omitted.

We have removed this paragraph as suggested.

Page 14, line 12: The type of studies where "arsenic has been shown to activate the UPR" should be mentioned, especially as the authors conclude that it "has been shown".

This was shown in mouse and human cell culture studies. We have added this information (now page 15, line 5).

I don't have much to comment on the rest of the discussion on the associations with gene expression, which is rather detailed and somewhat uncritical. However, the relevance of the exposure measure needs a thorough discussion, including the stability of the exposure over time and the changes in arsenic metabolism during pregnancy. Also, the time point of induction of gene expression changes and their stability over time need comments. Other potential confounding needs to be considered in the section on limitations (see my comments above).

We thank the reviewer for pointing out these issues and have added these limitations to the Discussion section (penultimate and final paragraphs) as recommended.
Reviewer #2: This is an interesting study that seeks to better understand potential mechanisms of the adverse effects of arsenic on birth weight by using RNAseq from fetal placental samples to identify genes whose expression are affected by arsenic exposure. The major limitation of the study, which the authors rightfully acknowledge, is that RNA sequencing was performed on separately on males and female placentas so one cannot rule out that differential findings between males and females are related to experimental variation rather than true sex-differences.

Title

1) I suggest condensing the title so that it's easier to read quickly and reflects the overall scope of the project

We have revised the title as suggested.

Abstract

2) Please include the number of participants/fetal placentas included in this study.

This information has been added.

3) In the abstract, it is unclear why results are only presented as sex-stratified.

We have added a sentence (page 3, lines 10-12) to explain this.

Intro

4) Page 5, Lines 9-10. Other than arsenic exposure, how did the 46 infants compare to the other infants in the cohort?

We have now included a comparison of demographic data from these infants with the subcohort of 312 as Table 1 (referenced in the Methods section under “Study cohort”).

Methods

5) Page 6, line 4. What is meant by the use of "subcohort" in the sentence beginning: "Out of the NHBCS subcohort of 312,"
The NHBCS is an ongoing study involving over 2000 participants; however, our participants were selected from a group of 312 participants recruited between 2012 and 2013 (the “subcohort”). We have added this information to the first paragraph of this section, and to the Background section.

6) Page 6, line 6. Can you please provide more context for the range of arsenic exposure in this cohort and define the range for being in the "high" and "low" arsenic exposure groups.

We have reworked the first sentence of this paragraph (page 6, lines 10-13) to try to better describe how the participants were selected. Unfortunately, there was not any predefined range for inclusion in the “high” or “low” groups; we simply selected the 12 participants at the top or bottom of the range of U-As levels for each sex. This is clearly another weakness of the study and we have added this to the Discussion section on limitations.

7) When used as a continuous measure, urinary As is usually right skewed. For the highly exposed groups, since the standard deviation is much larger than the mean, it appears that the distribution is likely still highly right skewed. Please provide the median and interquartile ranges for each of the groups instead of mean and SD so that readers will get a better idea of the distributions of exposure?

We thank the reviewer for this suggestion and have made this change (page 6, lines 18-22 and Table 1).

8) Page 7, line 1-2. Please include detection limits for each arsenic species and also include how many samples were below the detection limit.

We have added this information (page 8, lines 1-3).

9) Page 7, line 3-4. For samples that were below the detection limit but above zero, what happened after samples were flagged?

As mentioned in the response to Reviewer 1, we have deleted references to the flags, since they were not used in the analysis. We apologize for the confusion.

Results

10) Page 10, line 4-7. Please explain why results were validated using qPCR only for female samples. Was this a post hoc analysis? Were the new tissues samples from an adjacent location near the original subsection of the placenta?

Yes, this was a post hoc analysis. Unfortunately, we did not have sufficient male placenta samples left for qPCR validation. For females, the validation was performed using tissue samples collected from the same region of the placenta, at the same time.
11) Table 1. It would be helpful to include how many DE genes were included in each gene set as a column.

In GSEA, both DE and non-DE genes can contribute to the enrichment of a particular gene set. Genes are ranked according to z score, which takes into account both the significance and size of the expression change. Significant gene sets are those that contain genes close to the top or bottom of the ranked list. (See Proc Natl Acad Sci U S A. 2005 Oct 25;102(43):15545-50 for more details.)

12) Table 1 and 2. Remove underscores so that the gene set names are easier to read.

We thank the reviewer for this suggestion, and have made this change.

13) Table 2. Several U-As and birthweight FDR p-values are listed as 0, but this is likely not the true p-value, please use exact p-values.

Since the p-value is calculated based on permutation (as opposed to parametric methods), the raw p-value can be exactly 0 (as can the FDR) when all permutations score higher than the observed one. This was the case here.