Author’s response to reviews

Title: Association of urinary concentrations of early pregnancy phthalate metabolites and bisphenol A with length of gestation

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Author responses (in italics) to Reviewer Reports:

Reviewer #1: This is an analysis, using a cohort of 221 women for whom they collected repeat urine samples from the period of pre to post implantation. These data are novel and the analysis was well done overall. They measured phthalate levels, urinary levels of hCG, and duration of pregnancy. Previously, there have been reports of inverse associations of urinary phthalate levels and the duration of labor, and one report of higher phthalate levels being associated with longer gestation. This was an opportunity to assess this relationship based on exposure levels pre-conception, at time zero of implantation, and up to 5 weeks of pregnancy. A strength is use of Cox proportional hazards models to model duration of pregnancy as an outcome.

The early period in which they measured exposure is the time period of embryogenesis, formation of the placenta and the gestational sac. In these early stages, there is no direct communication between maternal blood and embryo/placenta. That which they measured as phthalate exposure using these urinary biomarkers might reflect long-term chronic exposure of the uterine and ovarian tissue, and the oocytes to phthalates, contemporaneous levels in the uterine glands, or general xenobiotic metabolism as an indicator of general uterine/pregnancy
health. Given the latter, it is not surprising that they had null effects of the monoester phthalates and only detected associations with MEHHP and MCPP, which are products of secondary and tertiary metabolism of the monoesters. One indication of this is the seeming opposite direction of association of MnBP and MCPP with outcomes. MCPP is considered a metabolite of DnBP/MnBP.

Author response: We did not consider our exposure measures to represent chronic exposure. While there is no direct communication between maternal blood and the embryo/placenta during the early pregnancy period, as noted by the reviewer, there is maternal blood flow to the uterus where the embryo is implanting and ovaries which produce hormones necessary to maintain early pregnancy. We think that maternal exposure to phthalates and BPA during this early pregnancy period could potentially affect the health of the overall pregnancy.

I would further suggest that authors create a molar sum of the DEHP metabolites (MEHHP, MEOHP, MECPP) and calculate the association with outcomes. If the association is the same as that of MEHP, then we can infer that these metabolites represent the same thing i.e. DEHP exposure. If the association differs, then we can infer that those metabolites represent something different than DEHP exposure, and may be confounded by metabolism differences between women.

Author response: We report the suggestion of longer gestation with higher concentrations of the pre-implantation DEHP metabolites, specifically pre-implantation MEHHP. We assessed the molar sum of the DEHP metabolites MEHHP, MEOHP, and MECPP in our model as suggested by the reviewer. The estimate for this composite measure was HR: 0.65, 95% CI: 0.42, 1.02. This association is similar to the association for the molar sum of the 4 measured DEHP metabolites, and we did not add this to the manuscript. Differences in the magnitude of association between MEHP and the molar sum of MEHHP, MEOHP, and MECPP may be due to differences in phthalate metabolism, but we are not able to confirm that in our data.

Comments:

1. The rationale behind the censoring by medical intervention is not clear to me. Was this considered as an intermediary variable which is why it is included in the analysis? Should it be a separate outcome assuming phthalates may have contributed to risk of intervention? What were the medical interventions?

Author response: Our main objective was to assess the association between phthalate metabolites and BPA with length of natural gestation. Some women in our cohort underwent a medical intervention that artificially shortened their length of gestation. To account for this artificial
shortening of pregnancy, we censored these women at the time of their medical intervention in our survival analysis because after the time of their medical intervention they were no longer at risk of spontaneous birth.

We did not consider medical intervention as a separate outcome because so few women reported a medical intervention that shortened their pregnancy in this cohort (n=24). We note that medical intervention included induced labor or Caesarean section without labor in the Methods section in the Study description and Outcome assessment subsections and the revised last paragraph of the Discussion section. To clarify we have revised the first sentence of the second paragraph of the Outcome assessment subsection to now read: “Length of gestation was quantified as the number of days from implantation to spontaneous birth with censoring for early delivery due to medical intervention (i.e., induced labor or Caesarean delivery without labor).”

2. I read the methods several times and don't understand how the imputation to fill in the missing values for medical intervention related to the overall analysis of phthalates and duration of gestation. Please make this clearer;

Author response: Yes, this was a bit unclear. We had incomplete information on medical interventions that shortened pregnancy for the participants in the study. For these women we do not know whether or not the date of the birth of their study baby was a spontaneous birth (our outcome of interest) or a birth occurring by medical intervention. We used the multiple imputation to fill in this information on medical intervention for the 27 women for whom it was missing.

We have added the following sentence to the Methods section, 3rd paragraph of the Statistical analysis subsection: “The multiple imputation allowed us to have data on medical interventions that artificially shortened pregnancy (labor induction or Caesarean section without labor) for all of the women in our study, so that we could assess length of natural pregnancy and censor pregnancies that did not end in spontaneous birth.”

3. It would be helpful to share the results with regards to correlations in phthalate levels over this time period. Authors mention a range, but could they show the correlations in a table or in a figure?

Author response: We have added a table with the correlations between the pre-implantation and post-implantation phthalate metabolites and BPA to the supplemental material. We reference Supplemental Table 1 in the sentence where we describe the range of correlation coefficients in the first paragraph of the Results section.
4. Did they have information on the sex of the baby at birth? HCG levels differ by sex of the baby as early as 3 weeks gestation so they may also see sex differences in these associations. Maybe they could impute fetal sex based on hCG?

Author response: We did have information on the sex of the baby, but only measured hCG in the mothers for 6 days past the day of implantation. In this cohort, sex of the offspring was not related to urinary hCG levels over the first week after implantation among the singleton live births (the study population analyzed in this paper) (Nepomnaschy, Weinberg et al. 2008). The sample size in this analysis was small and therefore we did not stratify by infant sex; however, offspring sex was not associated with length of gestation in this cohort (Jukic, Baird et al. 2013).

5. Underadjustment is better than overadjustment but it still might be important to adjust for maternal age if it reduces the standard error in the phthalate estimate. I agree that it would be likely confounder. The other ones would be maternal weight/BMI, race, smoking status, and infertility treatment.

Author response: We chose not to adjust for maternal age in our analysis because age was not associated with phthalate concentrations in our cohort. We also considered maternal BMI, race, parity, and smoking status as potential confounders, however, none of these factors were associated with gestational length (Jukic, Baird et al. 2013), so we did not adjust for these covariates. Additionally, the study population was relatively homogeneous with regard to these characteristics, 95% of the cohort was white, there were very few current smokers, and 80% had a normal BMI. None of the participants in the Early Pregnancy Study received fertility treatment because inclusion criteria for the study were that women had no history of fertility problems and were attempting a naturally conceived pregnancy.

We have added the following sentence to the Methods section, 2nd paragraph of the Statistical analysis subsection, stating our consideration of other maternal characteristics as potential confounders in addition to maternal age: “We considered maternal characteristics associated with the exposure and outcome in this cohort as potential confounders and adjust for maternal age in the analysis. Other maternal characteristics such as body mass index, smoking status, race, and parity were not associated with length of gestation in this cohort.”

6. In their previous paper, this group of authors report the association of phthalates with change in hCG levels over time. How does that finding relate this finding, either conceptually or statistically/analytically?

Author response: In a previous paper we found that higher concentrations of MBzP and the molar sum of the DEHP metabolites (MEHP, MEHHP, MEOHP, MECPP) were associated with
a slower initial rise in hCG over the first few days after implantation, but not with any differences by the end of the first week after implantation (Chin, Jukic et al. 2018). This was similar to the pattern seen in the DES daughters in our study, but those were excluded from the current analysis. It is possible that this slower initial rise affected long-term pregnancy health, however, a separate paper showed that neither the hCG concentrations on the day of implantation nor the hCG rise over the first 7 days after implantation was associated with natural length of gestation (Jukic, Baird et al. 2013).

7. Authors might consider some type of strategy to 1) adjust for confounding by phthalate metabolism/excretion, and 2) consider a summary measure of the phthalates.

Author response: 1) There is limited literature on incorporating a measure of phthalate metabolism in the analysis of phthalate exposure and human health outcomes. One example is to report estimates for the %MEHP, calculated as the proportion of MEHP using the 4 commonly measured DEHP metabolites (MEHP, MEHHP, MEOHP, and MECPP) (Meeker, Calafat et al. 2007, Hauser 2008). No method has been evaluated for validity. Therefore, as is done in most studies, we present results without adjusting for confounding by phthalate metabolism. 2) We only assessed the summary measure of DEHP metabolites in this paper. We did not assess a summary measure of all phthalate metabolites examined due to different routes of exposure, mechanisms of action, and potential differences in the direction and magnitude of their effect on gestational length.

8. The first paragraph of the Discussion section discusses the results of previously published reports. That seems odd. Usually, that paragraph starts with the primary findings and then offers an interpretation with regard to previous findings.

Author response: We have revised the first paragraph of the Discussion section to focus on results from the current analysis.

9. Did you learn something different by having pre vs. post implantation phthalate measures? Can you offer a summary or interpretation of that comparison?

Author response: Thank you for this suggestion. In reorganizing the first paragraph of the Discussion section as recommended by the reviewer, we clarify that the results for MEHHP and longer gestations was strongest for the pre-implantation measure and the association with MCPP and shorter gestations was seen for the post-implantation measure. However, the confidence intervals for the two estimates (pre-implantation and post-implantation) substantially overlap, which limits our ability to make conclusions about differences in the associations for each of the
time windows. We summarize these findings with the following sentence which has been added to the first paragraph of the Discussion: “These results suggest that there may be different effects of phthalate exposure during the pre-implantation window compared with the post-implantation window on length of pregnancy, however overlapping confidence intervals limit our ability to draw conclusions about differences in the associations for each of the time windows.”

10. Given the dynamic nature of these associations relative to time, could you include a figure or set of figures with time as the x-axis?

Author response: To address this comment, we have moved Table 2 to the supplementary material and included a figure (Figure 1) to graphically display the hazard ratios for the two time points where we have estimates (pre-implantation and post-implantation) side-by-side for each phthalate metabolite assessed and BPA.

11. How did the Cox models compare to a regression model with gestational age regressed on phthalates and interacted on time?

Author response: As the reviewer notes in the summary, the use of Cox proportional hazards models was a strength in this paper. We chose to use a Cox model because we were interested in modeling a time to event (birth) using gestational length as the time scale. We did not compare our results to a regression model because such an approach would require more parametric assumptions and would not have allowed us to account for the censoring that cut short 24 of the gestational lengths.

12. Were pre and post implantation phthalate levels correlated?

Author response: Yes. Please see response to Comment #3.

13. In previous papers, these authors studied DES exposure in utero in relation to these endpoints. How was that variable treated here? How do they conceptualize DES in this model?

Author response: In the Methods section we listed exclusions to the analytic dataset, which included women who were exposed in utero to DES (first paragraph of the Statistical analysis subsection). “Women exposed to DES in this cohort had irregular hormonal patterns and increased risk of early birth (Jukic, Baird et al. 2013).”
Reviewer #2: The authors have completed an interesting analysis of urinary concentrations of phthalates and BPA in a small sample of women, pre and post implantation, to evaluate the associations of these concentrations with a very precise measure of length of gestation. This analysis is important and contributes to the existing literature regarding the possible effects of exposure to these chemicals before and during pregnancy. The methods appear thorough and sound. Results are clearly stated, but could be improved a bit. The justification for this analysis could also be improved. It seems the authors have already done a very similar analysis (reference 31), and could perhaps explain early on (in the introduction) why this additional analysis is necessary. Perhaps they could point to their previous results and say that an analysis involving a more precise measure of gestational length could further help understanding of the effects of these chemicals on gestation/fetal development. It would also be helpful to see a diagram in the methods section. Finally, structure of the discussion could be improved. With these small improvements, I think the manuscript would make a nice publication. I will list some specific suggestions.

1. Page 3, lines 72-75 "In the North Carolina Early Pregnancy Study…length of pregnancy (10)" I had a hard time understanding this sentence, had to read it over several times. Could you rephrase it?

   Author response: We have split the referenced sentence into two sentences in order to be more clear. The sentences now read “In the North Carolina Early Pregnancy Study (EPS), a cohort of naturally conceiving women, early pregnancy events were predictive of length of gestation. These early events of pregnancy were hormonally defined and included the number of days between ovulation and embryo implantation and timing and pattern of post-implantation rise in progesterone (rescue of the corpus luteum).

2. Page 3, lines 75-76 "These findings suggest… length" Do you mean to say that early pregnancy hormonal events can have effects on gestational length? It is a bit unclear to just say "pregnancy events"

   Author response: Thank you for the opportunity to clarify our definition of early pregnancy events. We now note that the early pregnancy events were hormonally defined (see response to comment #1) in this study. We have also revised the referenced sentence to read “These findings suggest that characteristics of the hormonal changes necessary to maintain early pregnancy can be associated with gestational length.”
3. In general, the second paragraph of page 3 explains some research background about phthalates and gestational length, pregnancy hormones and gestational length, but it is missing any mention of previous research regarding BPA and gestational length. This is also where authors could improve justification of this analysis.

Author response: We have added information on the state of the literature regarding BPA exposure and length of gestation to the Background section of the paper: “Fewer studies have examined the association between BPA exposure and length of gestation. These studies focus on preterm birth as the outcome and also report inconsistent findings.” In addition, to provide a clearer justification for our current analysis, we revised the last sentence of the Background to read: “To further investigate early pregnancy exposures, we conducted an analysis to assess associations of urinary concentrations of phthalate biomarkers and BPA measured in early pregnancy with length of gestation in this cohort of women with no known fertility problems.”

4. Page 4, Exposure assessment: this type of exposure assessment is outside my specialty, so I have had a hard time understanding how many samples were collected and how many were used for this analysis. It would be very helpful to include a figure indicating samples collected pre-implantation, post-implantation, how many of those samples were used in this analysis, how the pools were made. I think I understand there are three Monday pre and post samples that were each pooled, per participant. But it was not easy to figure that out, the explanation could be clarified with a diagram.

Author response: We have added a figure that shows the three urine samples collected from each participant that was included in the pre-implantation and post-implantation pools (Supplemental Figures 1 and 2). The reviewer is correct that we pooled 3 samples from each woman during the pre-implantation period and 3 samples from each woman during the post-implantation period. The timings of these urine collections relative to menses and day of implantation are depicted in the new figures.

5. Page 5, first full paragraph: "Urine specimens were analyzed...". In this paragraph, it would be better to say here that you dichotomize the exposure variables. For me, it seems out of place in the statistical analysis section.

Author response: We chose to describe which metabolites were measured and how the urine specimens were analyzed by the laboratory in the exposure assessment section of the manuscript. We had continuous measure of the phthalate metabolites that we chose to dichotomize for the analysis. Because this was a decision made for the analysis phase, we would prefer to keep this information in the statistical analysis section of the paper.
6. Page 7, line 166 "However, 27 women were missing data on whether they had such …"
   It would be helpful if authors could indicate what % of the sample this was.

   Author response: 27 of the 125 women (22%) included in the analysis were missing data on whether they had a medical intervention. We have added the percentage to the above referenced sentence in the Methods section, Statistical analysis subsection.

7. Page 8, line 191 "…we present only unadjusted estimates." You have justified here why you did not adjust for age, but what about the other confounders such as maternal education, smoking, or parity? You could provide justification for why you did not adjust for these, or you could provide your results adjusted and unadjusted for comparison.

   Author response: See response to Reviewer #1, comment #5.

8. Table 1: The table is missing units. Also, I was curious to also see the concentrations of HCG pre and post implantation. Could that be added to the table?

   Author response: Thank you for bringing the missing units to our attention. The units for the creatinine adjusted biomarker concentrations are ng/mg of creatinine. We have added this information to Table 1.

   We did not report pre- and post-implantation hCG concentrations for the participants in our study. hCG concentrations become detectable at the time of implantation and was identified in this cohort using a highly sensitive assay with a limit of detection of 0.01 ng/ml (Wilcox, Baird et al. 1999). Because hCG is produced at the time of implantation, we did not have values for this hormone during the pre-implantation time window. The study’s hCG measurement protocol was for only the first week of hCG, capturing the first rapid rise.

9. Table 2: In the Biomarker column, you could indicate that this is a binary variable, and not continuous concentration. If I understand the analysis correctly, I think maybe this table should be more structured like an odds ratio table, indicating that the hazard ratio 1 is for the reference category (mom's below the median), then indicating the listing the HR for mom's with concentrations above the median. It is also not clear how this HR relates to days of gestation. You explain in the text that the MEHHP concentrations correspond to a 3 day longer pregnancy, but it is not easy to understand how this calculation was made, based on the HR. Perhaps you could provide the formula? It would also be helpful in the table to provide the N for mom's above and below the median for each biomarker.
Author response: We have replaced Table 2 with a figure (see response to Reviewer #1, comment #10) and included in the legend that hazard ratios represent the risk of birth for women with biomarker concentrations above the median compared with those below the median (reference). Footnote b of the table referenced, now Supplemental Table 2, also includes this information.

To calculate longer or shorter gestations based on higher concentrations of the exposure biomarker we used life table methods combining the imputed datasets used for the main analysis and compared the difference in median days gestation between the above the median and below the median groups.

We have added the following sentence to the end of the last paragraph of the Methods section: “The difference in the median days of gestation was used as an estimate of the difference in gestational length (number of days longer or shorter length) for women who had above median concentrations of the exposure biomarker compared to women with below median concentrations.” There were an equal number of women above and below the median of each biomarker examined and therefore we chose not to include the n for each group in this table.

10. Discussion: I would have structured this discussion a bit differently. In the first paragraph, you don't yet need to mention other literature, you can just summarize the most important findings from your analysis. Second paragraph: strengths, third paragraph: limitations, then after that you can get into how your findings compare to the literature. It would also be helpful to discuss suspected biological mechanism for these findings.

Author response: Please see response to Reviewer #1, comment #8 regarding restructuring the first paragraph of the discussion. We prefer to maintain the current organization of the rest of the Discussion section, first putting our findings in the context of the current literature and then discussing the strengths and limitations.

11. Page 11, line 281-283 "This cohort of naturally… birth." This whole paragraph is not really necessary, you already say it in the strengths paragraph.

Author response: Thank you. We have incorporated the information in the above referenced paragraph to other parts of the discussion section and deleted repetitive sentences.
References


