Author’s response to reviews

Title: Bisphenol A-associated epigenomic changes in prepubescent girls: a cross-sectional study in Gharbiah, Egypt

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Version: 2 Date: 21 February 2013

Author’s response to reviews: see over
Dr. D. Ozonoff, Boston University
Dr. P. Grandjean, University of Southern Denmark

Environmental Health, Editors-in-Chief

Re: Submission of revised manuscript MS: 1411799745884946 titled “Bisphenol A-associated epigenomic changes in prepubescent girls: a cross-sectional study in Gharbiah, Egypt”

Dear Drs. Ozonoff and Grandjean:

Please find enclosed our revised manuscript entitled “Bisphenol A-associated epigenomic changes in prepubescent girls: a cross-sectional study in Gharbiah, Egypt” by Kim et al., resubmitted for publication in Environmental Health (MS: 1411799745884946). We thank the reviewers for their remarks and the Environmental Health editors for the opportunity to submit a revised version of our manuscript along with this detailed document addressing the reviewers’ concerns.

Thank you for your consideration of this revised manuscript, and please feel free to contact us if you have any further questions.

Sincerely,

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Reviewer's report (Comment 3611302929143262)

**Title:** Bisphenol A-associated epigenomic changes in prepubescent girls: a cross-sectional study in Gharbiah, Egypt  
**Version:** 1  
**Date:** 7 February 2013  
**Reviewer:** Laura Vandenberg

**Reviewer's report:**
Kim and colleagues have examined BPA concentrations in the urine of a small population of girls in Egypt, and compared global gene methylation patterns based on urinary concentrations. The authors find some evidence that higher urinary BPA concentrations are associated with decreased genome methylation. They specifically identified changes in methylation patterns on the X chromosome. The report examines a subject that has been explored largely in animal models, and there is evidence that developmental exposures to BPA can alter methylation of genes in mice and rats. Thus, the rationale for the study is sound, and the study is novel in its human approach. The writing is technical but clear. The figures could be improved by better labeling (see my notes below). I am not an expert in epigenetics, so I am not comfortable making conclusions about the methodologies related to the methylation patterns. Instead, my comments will focus more on data presentation and interpretation.

**Major Compulsory Revisions:**
1. Background: The authors have presented an incomplete (and thus incorrect) interpretation of reference 21. In this study, Stahlhut et al examined BPA concentrations in urine based on fasting time. They found that levels did not drop off / cease even when fasting was for 8+ hours. These results suggest that *either* BPA bioaccumulates OR that BPA exposures are non-dietary OR both. To date, there is no evidence that BPA bioaccumulates.

   - Thank you for your comment. We agree and have revised the manuscript accordingly (Lines 128-129).

2. Results/Discussion: the authors have not considered what effect methylation is expected to have on gene expression. Was gene expression for any of the candidates examined?

   - A limitation of our study is the lack of functional validation of epigenetic targets with gene expression. Saliva DNA samples were collected using Oragene kits, which stabilizes field-collected DNA for later use. We were unable to collect and store RNA in Egypt during our sampling season. We have added a sentence in the Discussion (Lines 506-508) detailing this limitation and also further discuss our use of the CTD database to overcome this limitation (see next bullet).

   - Utilizing the Comparative Toxicogenomics Database (CTD), we were able to extract expression information with BPA exposure from multiple published studies. We identified candidate genes with concurrent gene expression
changes in other studies and listed them in Supplementary Table 6. The text discussing this approach is located in Lines 509 - 526.

2b. For the methylation patterns affected on the X-chromosome, what role does X-inactivation play in the interpretation of these results?

- The methylation of CpG islands in chromosome X is known to be associated in X chromosome inactivation and maintenance in female mammals. One of the significantly hypomethylated cytobands in chromosome X was Xq13 (p-value < 0.02, Supplementary Table 5), where the X Inactivation Center (XIC) is located. The XIC is known to play a role in \textit{Xist} expression, a major effector of X inactivation. We have now included additional text on this topic in the Discussion (Lines 491-498).

\textbf{Minor Essential Revisions:}

1. Background: references 3-5 are books. Is there a journal article that can be cited to support the idea that mammary growth is highly proliferative and tightly regulated during the pre-pubertal period?

- Thank you for this comment. We have now added 2 journal article references:
  
  

2. Background: I suggest the authors add 1-2 sentences that explains why this cohort was selected. Are you interested in controlling for genetic background? Ethnicity?? Would you expect the results to be specific to this population for some reason, or is there more general applicability?

- This study originated from a pilot study addressing environmental exposures in vulnerable populations (developing children) in a developing nation (Colacino et al 2011 and Nahar \textit{et al} 2012, both published in \textit{Environmental Health}). Text clarifying the cohort origination has been added to the Methods (Lines 151-152).

- While this population is expected to exhibit more genetic homogeneity than corresponding US populations, we did not explicitly address genetic variation in our study and our relatively small sample size precludes gene-environment interaction analysis. Rather, we sought to identify epigenetic differences between individuals exposed to high levels of BPA compared to lower levels of BPA. Controlling for ethnicity/race, sex, and age may help identify exposure based methylation profiles. We have revised text present in the Discussion to reflect this approach (Lines 444-445).

3. Figure 2: It would be best to specify the axes better. For Y-axis: frequency, write what the frequency is of… Also, the X-axis should be labeled “p-value”
Thank you for the suggestion. Figure 2 is modified accordingly.

4. Figure 3: the label for “n=” is confusing. These are individuals that contributed the samples, correct, and not the number of genes? Consider a better label.

   Thank you for the suggestion. Figure 3 is modified accordingly.

5. Discussion: the authors should consider addressing whether a spot urine sample is representative of exposures. Discuss inter-person variability.

   Urinary BPA will change throughout the day and also throughout the week based on the exposures. All urine samples were collected between 12-4pm to reduce variability arising from various exposures throughout the day. However, there exists interpersonal variation dependent on a number of factors, including the amount of water consumed as well as output of urine. Text has been added to the Methods (Lines 157-159) and the Discussion (Lines 452-457) acknowledging this limitation.

Reviewer's report (Comment 7937559519137887)
Title: Bisphenol A-associated epigenomic changes in prepubescent girls: a cross-sectional study in Gharbiah, Egypt
Version: 1 Date: 7 February 2013
Reviewer: Lori A Hoepner
Reviewer's report:
Overall, a well written and thorough manuscript which addresses effects of pre-adolescent exposure to BPA, an area in need of further investigation.

Minor Essential Revisions
1) This article leads strongly with the DOHaD hypothesis but there are holes in the logic of the first paragraph taking the reader from DOHaD to adolescent exposures and outcomes. It reads as though pre-adolescent and adolescent exposures are part of the DOHaD hypothesis. The introductory paragraph could use a better transition from DOHaD to adolescence.

   DOHaD often focuses on early life exposures such as fetal and early post-natal development, but programming can occur at any time of major development including puberty. We have edited the introductory paragraph and added a reference indicating DOHaD hypothesis focuses on both fetal and child development (Lines 89-98):

2) Please describe and cite the equipment used for field-collected anthropomorphic measurements (lines 149, 153-154).

- The measurements, including height, weight, waist, and hip circumference, were taken by trained nurses, when the subjects arrived to the Gharbiah Cancer Society Hospital for interviewing and specimen collection. Each measurement was taken twice. Height and weight were taken without shoes. If the difference in the two height measurements was > 0.5 centimeters (cm), height was measured a third time. We discarded the most discrepant value and averaged the other two. If the difference in weight was > 0.2 kilograms, a third weight measurement was taken. If the difference in waist circumference was > 0.5 cm, then a third measurement was taken. If the difference in hip circumference was > 0.5 cm, a third measurement was taken. We discarded the most discrepant value and averaged the other two. The participants clothing at the time of measurement was recorded. Training was provided to standardize anthropometric measurement tools and techniques as part of this and other ongoing studies with the collaborating institution in Egypt [Soliman and Schairer, 2012 and Schairer et al. (In Press)] and are now cited and described (Line 159-164):


3) line 155 - All 60 girls were pre-pubertal? Please explain how this was ascertained. What is the average age of female puberty in Egypt?

- All 60 girls were pre-menstrual. This was ascertained by asking the each mother about any prior menstrual cycles of her daughter. The average age of menarche in Egypt is 12.44 ± 1.3 [Ghali et al., 2008]. Text and a citation have been added to Methods Lines 154-156.


4) While the hypothesis is clearly stated in the abstract, it is missing from the Background section.
Thank you for the suggestion. We have added the hypothesis to the background section (Lines 135-137).

**Discretionary Revisions**

5) lines 299-300 - "Cluster 1...contained more than half of BPA-high samples" - Clarify is the importance that the cluster had >50% high BPA? or that >50% of the high BPA samples were in this cluster?

- Thank you for the clarification. We meant to say that more than half of BPA samples were categorized into the cluster 1, while the least number of BPA-high samples were categorized into the cluster 2. We reflected this clarification in the results (Lines 314-315).

6) The use of spot urine and the rapid excretion of BPA in comparison to epigenetic changes should be noted as a limitation of the study.

- We thank you for the reviewer’s suggestion. As addressed in Reviewer 1’s final comment, we have now indicated the use of spot urine as one of the limitations of the study. Text has been added to the Methods (Lines 157-159) and the Discussion (Lines 452-457) acknowledging this limitation.

7) The relevance of the reference in line 424 to cohort studies conducted in "relatively healthy populations" is not clear given the current study’s subjects are described as "healthy" in line 146.

- There are 3 references in line 424 (now line 439), two studies were conducted in healthy subjects exposed to low-level benzene and pollutants, and one study was conducted in general practice patients without major health issues. Thus, we removed a word “relatively” from the manuscript.

**Level of interest:** An article of outstanding merit and interest in its field  
**Quality of written English:** Acceptable  
**Statistical review:** No, the manuscript does not need to be seen by a statistician.  
**Declaration of competing interests:**  
I declare that I have no competing interests.