Author’s response to reviews

Title: Exploring telomere length in mother-newborn pairs in relation to exposure to multiple toxic metals and potential modifying effects of nutritional factors

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Author’s response to reviews:

Replies to comments from the reviewers of our manuscript BMED-D-18-01412

We appreciate very much the very good comments of the reviewers (changed to italic to facilitate the reading) and have included detailed answers point by point below. Major changes in the manuscript have been cited. We feel that the manuscript has improved markedly.

We submit a clean version of the manuscript and one version where track changes are included. In the latter, we have kept some of the comments, in order to facilitate tracking of changes (especially deletions). In addition, some of the information asked for by the reviewers are included in Supplemental material.

Reviewer reports:

Reviewer #1: In a mother-child cohort in northern Argentina (n=169), the authors measured multiple toxic metals and micronutrients in maternal blood or urine (average across pregnancy), placenta and cord blood. Zinc status showed the strongest association with telomere length with positive association in the mothers (~1 SD increase/5 mg/L in whole-blood zinc), and inverse in the placenta (-1.2 SD/10 mg/kg) and in the newborns (-1.6 SD/2 mg/L in cord blood). Serum folate was inversely associated with telomere length.

The study is a mix of different exposures and matrixes. I am wondering whether the authors might consider multiple exposure models to integrate different exposures.
Reply: Indeed, in most of the models we included multiple toxic metals and nutrients. We then selected the most influencing factors, in order not to overload the models, given the rather limited number of mothers and infants. So, for example, in Table 3a (maternal exposure and maternal rTL) we additionally adjusted for zinc, folate cobalamin, lithium, boron, arsenic and antimony in models with lithium, boron, arsenic, and antimony as appropriate (i.e., all except the one investigated as dependent variable). As mentioned in the results section, a few of the toxic elements in the studied exposure media were correlated, as they occurred together in some of the water sources. However, there were different water sources in the 10 different villages, also different within some villages, which lowered the biomarker correlations. Also, we used different biomarkers, e.g. serum boron and whole blood lithium to decrease the problem with collinearity. We considered writing separate manuscript for nutrients and toxic metals, as done in previous studies (even single nutrients or toxic substances), but decided to use a more comprehensive approach to understand the complex interplay between the combined exposures and telomere length.

Crucial information on telomere length measurements is missing such as which primers have been used and coefficient of variation among repeats of the assay; but I am confident that the authors can complete this. Details should be provided here.

Reply: The primers used were as follows: telomere forward: 5’-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3’, reverse: 5’-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT - 3’; HBB forward: 5’-TGT GCT GGC CCA TCA CTT TG- 3’, and reverse: 5’-ACC AGC CAC CAC TTT CTG ATA GG-3’. The thermocycling profiles were as follows for telomere: 95 °C for 3 min, followed by 25 cycles of 95 °C for 15 s and 56 °C for 1 min; and for HBB: 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 20 s.

This information is now included in Supplementary Table 1. For proper table please see attached version.

Supplementary Table 1. Primers and thermocycling profiles of qPCR for telomeres and HBB.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Thermocycling profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telomere Forward</td>
<td>5’-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT - 3’</td>
</tr>
<tr>
<td>HBB Forward</td>
<td>5’-TGT GCT GGC CCA TCA CTT TG- 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-ACC AGC CAC CAC TTT CTG ATA GG-3’</td>
</tr>
</tbody>
</table>
Telomere 95 °C 3 min + 25 cycles (95 °C 15 s + 56 °C 1 min)

HBB 95 °C 3 min + 40 cycles (95 °C 3 s + 60 °C 20 s)

Coefficient of variation for the telomere measurement was 8.0% based on 11 repeats of the reference sample. This is now included in the Methods section.

Some of the classical confounders were not taken into account including age of the father and newborn sex. I am aware that in the statistical analysis it is noted that these variables (among others) did not change the estimates more than 10%. I am not sure that this is a fully justified to not include them? Were these variables statistically significant?

Reply: Newborn sex was not associated with rTL in cord blood or placenta. We considered to include it as standard basic adjustment. Because of the small cohort, we decided not to do it, as long as it didn’t change the effect estimates. However, for the modeling of lead and rTL in cord blood, there were apparent sex differences in the estimate. In the end of the Result section we wrote: “Stratifying this model by newborn sex (46 boys and 36 girls) showed about ten times stronger association in boys (B= -0.077, 95% CI -0.135; -0.018, p=0.012) than in girls (B= -0.006, 95% CI -0.062; 0.050, p=0.824). The difference was statistically significant (p=0.015, Wald test).” We also commented in the Discussion upon the association between fetal lead exposure and cord blood rTL, although we tried to be cautious.

The age of the fathers was not associated with cord blood rTL (Spearman rS=0.08), in contrast to previous observations, but it was weakly correlated with placenta rTL (rS=0.22; p=0.046), which represent mainly fetal tissue. To note, the fathers in this cohort were rather young, 90% below 37 years of age (99% < 44 years). We didn’t include it in the final models as we had the age of the fathers for only 84 of the newborns with cord blood rTL data. Also, father’s age was strongly correlated with mothers´ age (rS=0.77). However, we did include paternal age in sensitivity analyses. In the end of the methods section we added the following: “In sensitivity analyses, we tested adjusting the cord blood models also for maternal rTL, as that could mediate the associations with cord blood rTL. Also, we evaluated additional adjustment with age of the fathers, which previously has been found to be associated with longer telomeres in the offspring (14, 31).” The new reference 31 being: Eisenberg DTA, Kuzawa CW. The paternal age at conception effect on offspring telomere length: mechanistic, comparative and adaptive perspectives. Philos Trans R Soc Lond B Biol Sci. 2018;373(1741).

At the end of the result section for placental rTL, we included the following: “In sensitivity analysis, we tested the influence of paternal age (26.9±7.3 years) in the models with placental rTL as dependent variable. Paternal age had little influence on the associations with arsenic (e.g., similar estimate for placental arsenic model 2c,e: B= 0.053; 95%CI 0.014; 0.091, p=0.008), but paternal age was significant in the model (B= 0.010, 95%CI 0.002; 0.018, p=0.019). Including paternal age in the model for Vitamin D slightly increased the estimate for this vitamin (model 2d: B= 0.0048, 95%CI 0.0018; 0.0078, p= 0.002), and the estimate for paternal age was very similar (B=0.011). For cobalamin, on the other hand, including paternal age in the model
decreased the estimate slightly and it was no longer statistically significant (B= -0.0004, 95% CI -0.0008; 0.0001, p= 0.102).”

Including paternal age in the cord blood rTL model for zinc didn’t change the estimate. In the model for lead, including paternal age decreased the estimate from -0.044 to -0.038. However, this was largely a consequence of missing 10% of the samples; the estimate for the model was 0.041 when restricting the original analysis (without adjusting for paternal age) to the newborns for whom we had the age of the fathers. At the very end of the Result section for cord blood we inserted: “Also, we tested the impact of the age of the fathers; however, this did not markedly change the estimates for lead (model 2d) or zinc (model 2c) (-0.038 and -0.090, respectively; n=77/71). Fathers’ age was not statistically significant in the models.”

In the third paragraph of the discussion (starting “In line with telomere physiology and function …”), we added at the very end: “Paternal age, known to be associated with longer telomeres in the offspring (14, 31), was associated with longer rTL in placenta, which is largely of fetal origin. However, we found no clear association with rTL in cord blood, possibly due to variations in leukocyte types. Obviously, many factors may affect the leukocyte profile, including pregnancy, inflammation etc., but also exposure to arsenic, especially early in life (37).”

In order to follow-up on this, we moved here the paragraph on arsenic and telomere length, and shortened this paragraph somewhat. A couple of references, which were already mentioned in the introduction were deleted. So, we continued directly after ref 37 (as a new 4th paragraph of the discussion) “Besides being immunosuppressive, arsenic is a potent carcinogen, and early life exposure appears to markedly increase the cancer risk later in life (38). In the present study, arsenic concentrations in maternal blood and placenta were positively associated with placental rTL, and the associations did not change markedly when nutritional factors or paternal age were accounted for. We have previously found positive associations between arsenic exposure and telomere length in blood of adults in the same Andean area (39, 40). Similar findings were reported for adults in Bangladesh (41) and India (42) and among adolescents in Nepal (20). In our mother-child cohort in rural Bangladesh, the children’s urinary arsenic below a spline knot at 45 µg/l was positively associated with TL at 9 years of age (23). At higher concentrations than 45 µg/L, both the prenatal and childhood arsenic exposure was inversely associated with TL. In support, in vitro studies using human cord blood showed that low concentrations of arsenic (sub-nM) increased telomerase mRNA and protein expression, while 1µM arsenic decreased telomerase expression and TL (24). The mechanism behind an arsenic-related elongation of telomeres in placenta may be through stimulating the expression of TERT, the catalytic subunit of telomerase, which was found to be positively associated with arsenic exposure in adults in the same area as the present study (39). However, what longer telomeres mean for placental function or child development is not known; indeed, the knowledge about placental rTL is sparse (5).”

Thereafter, we continue with the paragraph on lead exposure and telomere length in cord blood.

It would be of interest to have an analysis on maternal telomere length as a potential determinant of newborn telomere length or as an important mediator to understand the observed changes in the newborns between exposure and newborn telomere length.
Reply: A very relevant comment. Indeed, maternal TL could act as a mediator between exposure and cord TL. In the first paragraph of the result section we report: “Cord blood rTL correlated moderately with rTL in placenta (rS=0.37, p=0.0007) and weakly with maternal blood (rS=0.20, p=0.08), while rTL in maternal blood and placenta were not correlated (rS=-0.10, p=0.38)”. We have now tested if including maternal rTL in the models with cord blood rTL as the dependent factor changed any of the significant covariates (lead and zinc). The estimate for cord blood lead changed from 0.044 to 0.046 and the estimate for cord blood zinc changed from 0.098 to 0.111. We added the following in the end of the results section: “In an additional sensitivity analysis, we found that adjusting for maternal leukocyte rTL slightly increased the estimates for lead (model 2e) and zinc (model 2c) (-0.046 and -0.111, respectively; n=76/72), indicating that the associations were not mediated through the maternal rTL.”

Reviewer #2: This is an interesting analysis in a small birth cohort in rural Argentina. The authors collected maternal blood/urine, plancentae, and cord blood samples in varying degrees and measured relative telomere length (rTL), a variety of nutritional factors and a variety of toxic metals. They then evaluated the associations between these ‘exposures’ and rTL in each media. I have several comments pertaining to the methods used, and the interpretation of the results.

1. The authors need to discuss the merits of rTL compared to actual TL measured in base pairs using Southern Blot analysis, as there is substantial controversy regarding the reliability of rTL measures. More detail is required on the laboratory QA/QC methods for the rTL measures, especially compared to other established laboratories.

Reply: We have now included coefficient of variation as a marker of repeatability in the Method section. A comparison between qPCR and Southern blot (TFR) methods was performed when the qPCR method for relative telomere length was first published (Cawthon 2002). In that paper, Cawthon showed that the two methods produced similar results with a fairly high correlation (correlation coefficient R2=0.677). Another comparison was performed between the methods by Aviv and co-workers (2011) where duplicate aliquots were measured blindly in two different laboratories on two occasions with both TRF and qPCR methods. The methods showed high repeatability (r>0.9); and the results were correlated (r=0.847); however, the qPCR method showed higher variation than the TRF method (inter-assay CV=6.45% for qPCR and 1.74% for TRF). The pros and cons of the methods for telomere measurement have been discussed (Montpetit et al. 2014, Lai et al. 2018). Here, we chose qPCR due to limited amount of DNA sample.

2. I was not sure of the point of the paper. Was it exploratory, to evaluate a multitude of exposures or to evaluate specific hypotheses or to evaluate perhaps mediation of the toxic metal to rTL association by nutritional factors? The authors need to lay out specific hypotheses or indicate that the analysis is exploratory prior to describing the results.

Reply: There are previous indications that toxic metals, in particular those with pro-oxidative properties, may affect telomere length, so we based our hypotheses on these. We have now tried to clarify the background to the present aims in the introduction leading to the very last sentences.
which now read: “Taken together, these findings indicate that early-life telomeres may be susceptible to toxic metals. Therefore, the aim of the present study was to assess the potential effect of environmental exposure to multiple toxic metals on TL in maternal and cord blood and placenta, as well as interactions with nutritional factors. Because of the sparse information on the importance of various nutritional factors on TL, we have also evaluated those separately in a more exploratory way.” Also, we write in the beginning of the second section of the results (Associations of biomarkers of toxic metals and nutritional factors with rTL in maternal blood) as follows: “We first evaluated the associations of various nutritional factors on the rTL in the different media. In the subsequent main analyses of associations with toxic metals, we additionally adjusted the regression models for the nutritional factors of importance.”

To further facilitate the reading and interpretation, we changed the discussion to focus first on the associations with the toxic metals and then the potential alleviation of nutritional factors and how the latter associated with rTL.

3. Expanding upon comment 2, the authors make quite a number of comparisons. If the point of the study is to explore associations then these need to be adjusted either with a False Discovery Rate or with a Bonferroni correction. If there are specific hypotheses then it is fine to evaluate at p < .05, but please indicate as such.

Reply: Please, see answer to the previous comment. In addition, we have been more cautious in the interpretation and revised the discussion. In particular, we have toned down the associations with borderline significance and those that were weakened by covariate adjustment. To that end, we have deleted discussions about antimony, leaving a statement when describing the results that the association diminished after covariate adjustment.

4. I was concerned that no differences were found between boys and girls for rTL (in cord blood). This is established in the literature (see, e.g. Factor-Litvak, et al). Can the authors give a possible reason why this was not found?

Reply: rTL was very similar for newborn boys (mean 1.26±0.12) and girls (1.28±0.12); however, we only had data for 56 boys and 41 girls. Thus, we did not have the power to detect subtle differences. I notice from the article by Factor-Litvak et al. (2016) that the difference was quite small, 9.44 for boys and 9.58 for girls; which probably was statistically different because there were 274 boys and 216 girls included in the study.

5. Maternal rTL is a strong predictor of child rTL in cord blood and perhaps should be evaluated as a possible covariate in the models.

Reply: We commented the following to the similar issue raised by reviewer 1: A very relevant comment. Indeed, maternal TL could act as a mediator between exposure and cord TL. In the result section we report: “Cord blood rTL correlated moderately with rTL in placenta (rS=0.37, p=0.0007) and weakly with maternal blood (rS=0.20, p=0.08), while rTL in maternal blood and
placenta were not correlated (rS=-0.10, p=0.38). We have now tested if including maternal rTL changed any of the significant factors (lead and zinc) associated with cord blood rTL. The estimate for cord blood lead changed from 0.044 to 0.046 and the estimate for cord blood zinc changed from 0.098 to 0.111. We added the following in the end of the results section: “In an additional sensitivity analysis, we found that adjusting for maternal leukocyte rTL slightly increased the estimates for lead (model 2e) and zinc (model 2c) (-0.046 and -0.111, respectively; n=76/72), indicating that the associations were not mediated through the maternal rTL.”

Possibly, there are ethnic differences in the relationship between parental and offspring telomere length, as indicated in the study by Factor-Litvak et al. (2016). Obviously, there are multiple differences in dietary habits, life-styles and environmental exposures between the present Andean mother-child cohort, living at high altitude, and a similar cohort in New York. Hopefully, future studies will further disentangle influencing factors for telomere biology.

6. I am also concerned about the very small sample size of the study, and the possibility of selection issues regarding exposure levels.

Reply: Indeed, this is a relevant point. As mentioned under point 3 above, we have been more cautious in the interpretation and removed in the discussion associations with borderline significance or those that were weakened by covariate adjustment. In particular, we have deleted all discussions about antimony, leaving a statement when describing the results that the association diminished after covariate adjustment.

We acknowledge that this is a small mother-child cohort, but it is well-characterized in terms of background characteristics and the women had limited exposure to possible confounders and factors that may affect fetal rTL (no tobacco smoking, no alcohol, minimal industrial or traffic pollution, etc.). It should be noted that performing field studies in this region is very complicated and limits large-scale studies. Also, the total population in the study was about 8000 individuals with a yearly birth rate of about 200. The study area included the main village San Antonio de los Cobres with highly varying and generally more elevated exposure, as well as nine much smaller villages, located at a distance of 40-216 k, with similar background characteristics but lower exposure levels.

Reviewer #3: This is a cross-sectional study in a mother-child cohort in Argentina aimed to investigate whether certain toxic metals and micronutrients in body tissues (blood, urine, placenta) relate to relative telomere length. The authors conclude that many tested exposures, either nutritional factors or toxic metals, influence relative telomere length in mother-newborn pairs in a tissue-specific manner. The underlying hypothesis of the paper and the analytical methodology is sound. However, there are some main pitfalls.

- Lack of pre-specified hypothesis. The authors need to specify what hypothesis are they testing, and construct statistical models accordingly. If thinking on a particular nutrient/toxic (hypothesis testing), please clearly state the rationale for doing so in the
"introduction" and show unadjusted and adjusted models, including (if needed) other exposures as covariates. In contrast, if planning an "exploratory analysis" (i.e. does any micronutrient / toxic relate to telomere length? If so, which ones?), use stepwise (parsimonious) models adjusting for risk factors, allowing the entrance of pre-selected nutrients / toxics. Working with patterns or clusters seems more interesting that focusing on a particular exposure, unless (as said before) the authors are working with a pre-specified hypothesis. With the current approach the results might also be prone to positive results multiple hypothesis testing.

Reply: We have now clarified the aim of the study (in the last paragraph of the introduction), against the previously indicated effects on TL by different toxic metals. As the previously findings concerned metals with pro-oxidative properties, we thought it would be important to test potential alleviating effects of nutrients involved in anti-oxidative defense, and the one carbon metabolism. Thus, the end of the introduction is now as follows: “Data on the impact of toxic chemicals on early life TL is even more limited. A few studies have concerned air pollution (16, 17) and persistent organic pollutants (18). Also, certain toxic metals, especially those causing oxidative stress, have been found to affect telomere length. Cadmium exposure was shown to be inversely associated with placental TL (19) and salivary TL in adolescents (20), and lead exposure was inversely associated with blood TL in children aged 6-10 years (21). Exposure to methylmercury in pregnancy was, however, not associated with TL in the mothers or their children (22). Low level childhood arsenic exposure, on the other hand, was found to be positively associated with blood TL in 9-year-old children, while the association turned inverse at urinary arsenic concentrations exceeding 45 µg/L (23). Maternal arsenic exposure in early pregnancy and earlier childhood exposure showed mainly inverse association. In support, in vitro studies using human cord blood showed that very low concentrations of arsenic (sub-nM) increased telomerase mRNA and protein expression, with female cord blood cells being more sensitive than male ones, while 1 µM arsenic decreased telomerase expression and TL (24). Taken together, these findings indicate that early-life telomeres may be susceptible to toxic metals. Therefore, the aim of the present study was to assess the potential effect of environmental exposure to multiple toxic metals on TL in maternal and cord blood and placenta, as well as interactions with nutritional factors.”

We did consider evaluating clusters, but there was no clear clustering, as the different metals have different sources. Nevertheless, the potential impact of the specific exposures is of interest. We have mentioned that in particular lithium and boron were correlated, but this is not anything typical for drinking water. The water in the main water source in San Antonio de los Cobres does contain arsenic, besides lithium and boron. However, a filter that reduces the arsenic concentration was installed some years ago. Unfortunately, this filter does not reduce the concentrations of lithium and boron. Lead exposure is likely from other water sources or from food, as is cadmium and antimony.

- In line with this, the paper would be much improved if the authors discussed the clinical relevance of one/two main findings rather than unduly expanding the amount of shown data. The Discussion, which is too long, would improve if built around a clear and concise message.
Reply: We have now tried to focus on key findings and to shorten the discussion. However, in this study we did not measure telomere length in relation to the development or the prognosis of a disease, and therefore it is difficult to discuss the clinical relevance of our findings. Nevertheless, telomere biology is involved in many processes of the cell and is a measure of the cellular fitness. Subtle changes, due to elevated toxic or reduced nutritional factors may therefore influence the fitness and subsequent disease later in life. This is now commented upon in the discussion, page 16, second paragraph.

When shortening the discussion, we also changed the order of some paragraphs in order to improve the line of the statements and discussions. We realize that the version of the manuscript with track changes is why we want to summarize it as follows. After the first summarizing paragraph, we included a paragraph on the clinical relevance, as mentioned above. Then is the related paragraph about telomere biology (starting “In line with telomere physiology and function …”), to which a couple of sentences have been added (as described above), to bridge over to the paragraph on arsenic exposure and rTL. Thereafter, we have the paragraph on lead exposure and rTL (slightly shortened), and then lithium and boron (much the same as before). We deleted the long discussion about antimony as the associations are indeed not very convincing. Similarly, we deleted the discussion about cobalamin and rTL. The focus of the paragraph on nutrients is now on zinc, folate, vitamin D and BMI.

Table 3a. Statistical analyses are oddly explained in the footnote. For instance, why the model with "cobalamin" as exposure is adjusted for "zinc, folate, homocysteine and COBALAMIN"? This reviewer presumes that the models were reciprocally adjusted (included all covariates except the dependent variable), but this should be better explained.

Reply: We have revised the footnotes as suggested.

- Did the authors calculate the needed sample size? If not, this limitation should be acknowledged.

Reply: It has now been acknowledged that useful data for initial power calculations were not available. In the limitation section we have included as follows: “Also, we had no useful data for initial power calculations.”

- The study is entirely descriptive / associative and provides neither a cause-effect relationship nor novel mechanistic insight. Hence, authors need to be sure that they do not imply a cause-effect relationship. Only associations can be identified in cross-sectional data. So, please avoid using words (in relation to obtained data) such as "impact" (e.g. lines 85, 338...), "effect" (e.g. lines 339, 346…) or "influence" (e.g. lines 53, 54, 341).

Reply: This is true indeed. We have gone through the discussion with this in mind and changed whenever effect or impact is mentioned. For example: Line 338: “This study is the first to evaluate association of multiple toxic elements with nutritional factors ....”. Line 339:
“Importantly, the associations appeared to be exposure and tissue-specific.” Line: 346: As we have written “indicated effects” we think it can remain as written as this is a discussion.

Minor points:

- Line 40. Please define "ICP-MS".

Reply: We have included “Inductively Coupled Plasma Mass Spectrometry (ICP-MS)”

- Table 2. Why superscript "a" appears for first time after "b"?

Reply: Thank you for the notification. We have changed so “a” now appears before “b”.

References


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Declarations

- Ethics approval and consent to participate
- Consent to publish
- Availability of data and materials
- Competing interests
- Funding
- Authors' Contributions
- Acknowledgements

Reply: All these sections have been considered and included in the manuscript