Author's response to reviews

Title: Maternal cortisol and stress are associated with birth outcomes, but are not affected by lipid-based nutrient supplements during pregnancy: an analysis of data from a randomized controlled trial in rural Malawi

Authors:

Christine P Stewart (cpstewart@ucdavis.edu)
Brietta M Oaks (boaks@ucdavis.edu)
Kevin D Laugero (Kevin.Laugero@ARS.USDA.GOV)
Ulla Ashon (ulla.ashorn@uta.fi)
Ulla Harjunmaa (Harjunmaa.Ulla.G@student.uta.fi)
Chiza Kumwenda (chizakumwenda@yahoo.co.uk)
David Chaima (davidchaima@gmail.com)
Kenneth Maleta (ken.maleta@gmail.com)
Per Ashorn (per.ashorn@uta.fi)
Kathryn G Dewey (kgdewey@ucdavis.edu)

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Author's response to reviews: see over
Maternal cortisol and stress are associated with birth outcomes, but are not affected by lipid-based nutrient supplements during pregnancy: an analysis of data from a randomized controlled trial in rural Malawi

Response to Reviewers

We would like to thank the reviewers for their careful review of our manuscript. Our responses are detailed below under each of their comments. We make reference to all of the changes to the line numbers in the marked version of the manuscript. We also have attached a clean copy of the manuscript for easier reading, though the line numbers do not match up to the changes described below.

Reviewer 1’s report:

No major revisions required.
Weakness recognized by authors is scattered timing of salivary sample collections. Unless, as seems clear, the saliva cortisols are secondary outcomes of a primary LNS study, the specifics of the intervention groups seem puzzling. Two of the groups have multiple micronutrients and are mainly distinguished by the lipids and modest calories in the LNS. It would be helpful to those unfamiliar with LNS and its present research status [or / and to this reviewer if his assumption is wrong!] if the reason for selection of interventions was clarified at some point in the manuscript.

Yes, you are correct. These analyses were a secondary outcome to the main trial. We have added a sentence to the first line of the methods to clarify (Line 145).
Reviewer 2's report:

This study assesses the effect of prenatal nutrition supplementation on maternal cortisol levels and, as secondary analysis, the association between maternal cortisol and birth size and gestational length. I read the paper with great interest as it aids in entangling the complex hormonal mechanism of nutrition, regulation and birth outcomes in a LMIC setting. There are some problems with the analysis that might lead (or not lead) to erroneous conclusions. Also, I feel that part of the general mechanism could be moved from discussion to introduction to leave more space to discuss what these finding add to the evidence on HPA and birth outcome.

Major
1. The introduction does not really hypothesize a mechanism for the 2 main research questions to underline why maternal cortisol was measured in relation to birth outcome. The part that explains this better is now situated in the discussion and parts of it could be moved to the introduction. This is especially interesting for readers without endocrinologic expertise.

We have made significant revisions to the introduction and discussion to reorganize the flow of text (Lines 64-132 and 416-450).

2. I believed that the essence of the HPA mechanism is situated at the placental level. Maternal cortisol gets converted to inactive cortisone by 11#-HSD2. Poor performance of that 11#-HSD2 system (because of eg. malnutrition) can lead to increased exposure of the fetus to cortisol which in turn could affect growth and/or gestational duration. The negative association between maternal cortisol and birth size is clearly stronger at 36 weeks compared to 28 weeks, which happens to be the timing when also 11#-HSD2 levels are getting lower. So might this imply that ‘damaging’ levels of maternal cortisol only then exert an influence on fetal size trough increasing fetal cortisol levels? However, papers from our group (cfr bottom) from West-Africa did not really demonstrate strong associations with birth size (at least not when adjusting for IGF-1/2 levels). One wonders if another mechanism can explain the association between late gestation maternal cortisol and birth size. It would be interesting to add this aspect to the discussions so it expands the chain of observations.

We agree that the placental 11B-HSD2 activity is a potentially very important pathway. Unfortunately, we do not have data in this cohort to test this hypothesis specifically. In addition to the pathway that you’ve mentioned, there are a few other potential pathways. Cortisol had a very strong association with duration of gestation, birth weight, risk of preterm delivery and risk of low birth weight, but no association with SGA. Therefore, the smaller size at birth is likely to be partially due to the earlier timing of delivery. Additionally, cortisol is elevated during periods of immune activation. Thus, it is also possible that the elevated cortisol that we observed was reflective of an inflammatory state that was contributing to the earlier timing of parturition and smaller size at birth. We found that adjustment for CRP and AGP attenuated many of the associations that we’d
observed, which would support this hypothesis. We describe this on lines 400-404.

3. Throughout the discussion authors need to clarify if it concerns maternal or fetal cortisol levels. Despite some publications reporting on positive correlations, it would conceptually be better to treat them separated given the role of the placental interface/barrier function.

We agree and have added clarifications throughout the discussion.

4. Table 3/4: There is a clear imbalance in cortisol levels at baseline. Why wasn’t an overall analysis conducted including all data? It would be interesting to see the impact of adjusting for baseline cortisol levels on the overall trend.

We regret that the footnote on Table 3 was accidentally deleted. We have added it back into the manuscript. We evaluated the models both with and without adjustment for baseline cortisol. The crude p-values are reported in the table; however the p-values for the differences between groups remained high after adjustment for baseline cortisol (28 wk: p=0.14, 36 wk: p=0.24). The adjusted means only differed from the crude values at the point of the third significant digit, so were not appreciably different than what was presented in the table.

5. Table 3/4: It also seems that at every time point a different subsample of the data is analyzed so comparing means between time points is problematic as we can’t really tell if differences are related to time points or different subjects. Either consider an overall analysis, or make sure we are looking at the results of the same sample.

We compared characteristics between groups of women who had complete baseline, 28 wk, and 36 wk cortisol data from those who had missing cortisol values at any of those time points. Differences between the women with missing 28 wk cortisol data compared to those with complete data at all visit timepoints have been reported in the text (Lines 282-287). We found that only the prevalence of anemia at baseline differed between groups. We also conducted the same type of analysis for those with missing 36 wk data and found no significant differences compared to those with complete data. We added text to describe this on lines 287-289.

Minor
1. L121: to what extent are nurses able to conduct complex ultrasound measurements? Was there a data quality control conducted by an obstetrician (X% of ultrasound pictures verified by an expert). Is there any data available on intra and inter-rater coefficient of variation of these measurements of these nurses?
The nurses were trained by two study physicians who periodically rechecked readings for some study participants. However, there was no formal quality control for this, i.e. no % verified and no intra or inter-rater CV available. We have added a sentence to lines 172-173.

2. L161: this needs to be specified better. What was the proportion measured within 24hrs, 48hrs, 72 hrs and was it different between intervention groups? Measuring birth weight within 24 hr is a quality criterion. Between 24-48hr birth weight can decrease with 50-75 g already. If there is a substantial spread in the timing when BW was recorded, it is recommended to adjust for this variable as it can enhance your precision.

In the main study, 89% of birth weights were recorded within 48 hours of delivery. The remaining weights measured within 14 days were back-translated to calculate birth weights. A sentence has been added to the methods to describe this (Line 212-213).

3. L174: were there any values below the limit of detection of the analytical tool, if yes, what strategy was followed to deal with these values?

There were no values below the limit of detection for the assay.

4. Add sample size calculation to situate what significant differences one is able to detect with these samples?

The overall sample size was determined for the main study outcomes of the trial. The initial sample size calculation was based on being able to detect differences between the three groups equivalent to an effect size of 0.30 (difference between groups, divided by the pooled SD) for each continuous outcome, assuming 80% power and alpha=0.05. This would have required 216 participants per group, for a total of 648 subjects. Allowing for up to 25% loss to follow-up, we estimated needing to recruit 864 subjects. We increased the sample size to allow for tests of interaction, but were limited due to budgetary constraints. The revised final sample size of 370 per group provided the study with 80% power to detect main effects of > 0.23 SD with 2-sided type I error rate of 5%. This corresponds to a detectable difference of 0.83 nmol/L in cortisol concentration and 1.2 point difference in PSS. We have added a brief description to the methods (Lines 225-228).

5. L198: why parity and not primiparity, the latter constituting of a particular sub-population. Both Huybregts et al and Roberfroid et reported higher cortisol concentrations in primiparous or primigravidae.
We used a dichotomous variable categorizing women as primiparous or multiparous in all of our models. We have added clarification to lines 251 and 286, Table 4 footnote b, and Table 5 footnote a.

6. L205: add “with robust estimation of SE”. Poisson distribution has the assumption that E(X)=Var(X) which is theoretically impossible for a binomial distribution.

We have added this clause to the methods (Line 260).

7. L206: add the analysis of SGA to be consistent with literature on prenatal interventions. Strangely SGA is mentioned in the discussion
8. L206: add that SGA was also analyzed
Thank you for catching this omission from the methods (now added, line 267). The results are included in Table 5.

9. L208: repetition of line 198

Deleted

10. L215: add which imputation method was used
We used the method described in Schafer JL. Multiple imputation: a primer. Stat Methods Med Res; 1999. We have added this reference to the methods (line 270).

11. L246: crude and adjusted analyses were conducted, it is unclear what results are shown in table 3.

Please see our response to point number 4 under the major revisions heading above.

12. There is a clear imbalance in cortisol levels at baseline. Could an overall analysis be conducted? I was thinking of a mixed effects model with mother as random intercept (and if needed random slope) that adjusts for baseline imbalances.

Please see our response to point number 4 under the major revisions heading above.

13. Table3: tables should be stand alone: add below table what analysis (model, covariates etc) was used

Added
14. Table 4: For coefficients (=point estimates), 95% confidence intervals are required (=interval estimates).

Revised

15. L262 and L273: a p-value cannot be statistically significant, rather a difference

Revised (Lines 320 and 331)

16. L290: Please report coefficients that were 10% different from the limited data analysis. If missingness is substantial so will be the uncertainty of the estimations at the expense of the gain in statistical power.

When the data were imputed and analyses repeated, no coefficients were $\geq$10% different from the limited data analysis. We have added this statement to the results (Lines 348-349).

17. L316 Compared to other two trials. Authors first mention that this study did not find a difference in maternal cortisol, so L336 is a little contradictory?

Our study found no differences in the main group comparisons, nor in any tests of interactions. This differed from the subgroup analyses in the Ghana trial. We have added a clause to clarify (Line 382).

18. Discussion part L345-379 gives an overall explanation of which part could be moved to the introduction as it is not directly linked to the observations. I recommend you go through this part and filter out a hypothesized mechanism model that made you decide to undertake this study on prenatal nutrition, maternal cortisol and birth outcome.

Thank you for this suggestion. We have substantially re-organized the flow of the introduction and discussion sections.

Refs
Note that Robefroid et al. reported increased cord cortisol concentration as a consequence of prenatal MMN in primiparous.

Thank you for drawing our attention to these papers. We have added some text to the discussion.
Reviewer 3’s report:
- Major Compulsory Revisions: None
- Minor Essential Revisions
Line 44: 'Lower newborn' may be replaced by 'shorter newborn'

**Done (Line 44)**

Line 136: this should be ‘a tool that has...’ instead of ‘at tool that has... ’

**Corrected (Line 186)**

Line 140: 'home during the 28 week visit' may be replaced by ‘during the 28 week home visit’

**Corrected (Line 190)**

Line 266: 'Lower newborn' may be replaced by 'shorter newborn'

**Done (Line 324)**

- Discretionary Revisions
In this paper, Christine Stewart and colleagues have examined the association between LNS supplements during pregnancy maternal salivary cortisol and whether maternal cortisol and stress are associated with birth outcomes. The question posed by the authors is well defined, the methods are appropriate, but some additional explorations could be done. Limitations of the work are clearly stated and the discussion and conclusions are well balanced and adequately supported by the data.

**Thank you.**