Responses to reviewers' comments

We greatly appreciate the insightful comments from the Reviewer on the manuscript. We have incorporated changes to reflect the suggestions provided and have highlighted the changes in yellow throughout the manuscript. In addition, we added information on the amount of missing data, which is also highlighted in yellow. Language imperfections were corrected throughout, mainly in discussion. Please see green highlights.

Reviewer #2:

The manuscript, Essential trace elements status in Portuguese pregnant women and their association with maternal and neonatal outcomes: a prospective study from the IoMum cohort." Shows very interesting data in an impressive cohort. A cohort of 635 mother:child pairs very selected and observed regarding selected essential trace element concentrations in urine and if those impact the birth outcome. The introduction is clear and especially the table is great as it contains a lot of info about the trace elements that are presented short and precise. In the material and method section the study is well explained, and the flowchart is helpful to understand the study.

1. *In line 136, the authors state that "random urine sample was collected". And here lies the biggest issue in the whole study. Firstly, urine is no good biomarker for trace element status, if it is used then it would be more reliable to use 24-hour urine. Secondly, there should be any normalization parameters, like creatinine concentration. Please discuss on this issue in the manuscript*.

We agree that urine may not be a good biomarker for trace element status, as pointed out by us in the first version of the manuscript as a limitation of the study (please see lines 570 – 571 of the first version of the manuscript, now lines 606-607 of the revised version). Adjustment for creatinine excretion is sometimes seen as a way to adjust for urine concentration, to reduce the bias due to possible daily variations in spot urine concentration. Despite this, creatinine adjustment has also limitations, as its excretion also depends on age [1], muscle mass [1], nutrition status [2] and BMI [3,4]. Given the Reviewer's comment we decided to add data on urinary ETE's adjusted for creatinine. Please see supplementary tables S3, S4, S6, S7, S8 and S10 which we now refer to throughout the results and discussion sections.

In fact, in our sample population creatinine excretion had a weak but statistically significant negative correlation with maternal age ($r = -0.147$; $p < 0.001$, Spearmen's correlation), and it was higher for higher BMI ($p < 0.001$, Kruskal-Wallis) and for weight gain above recommendations for the first trimester ($p=0.019$, Kruskal-Wallis). In addition, we could observe that creatinine excretion in our sample also varied with food intake such as cheese, offal and nuts ($p = 0.039$ (Kruskal-Wallis); $p = 0.033$ (Mann-Whitney); $p = 0.021$ (Kruskal-Wallis), respectively). In view of this, we consider that the results based on adjustment of urinary ETEs to creatinine may be biased by many relevant variables. However, in our study, adjustment of urinary ETEs concentration for creatinine excretion did not substantially change the main conclusions drawn. These thoughts were added to the discussion section. Please see 608-620.

2. The analytical method should be discussed more detailed, which ICP-MS, sample preparation, where LODs and LOQs measured daily or only once etc.

We acknowledge this comment and agree that analytical method could be more discussed. We have added more information about the biochemical analyses (section 2.3), based on the points made by the Reviewer. Please see lines 154-164, 167-196.

3. *There is no description in the material and method section about the intake data, those data suddenly appear in table 5 without any additional information.*

We totally agree with the Reviewer and so we added information on intake data collection to the methods section (section 2.2). Please see lines 133-136.

4. *Section 2.5 is clear, as here a big cohort was observed, the use of Shapiro wilk test could be more robust.*

To the best of our knowledge, and as recently described [5], Kolmogorov-Smirnov test is usually used for larger sample sizes (n≥50), as it is the case of our cohort, and the Shapiro-Wilk is more adequate for smaller sample sizes (n<50). In every case, we are aware of the possibility of false positives or negatives with these approaches. This is the reason why we complemented the analysis with visual inspection of the histograms for symmetry/asymmetry check. This was referred in section 2.5.

5. *In line 261, the authors write about positive association, but here only significances were compared. So please consider the use of the terms association and significances, as an association needs an correlation analysis.*

In agreement with the Reviewer's comment, we removed the terms "positive association" and "negative association" throughout the results section. In replacement of these terms, we described whether the relationship observed as direct or inverse (for example (line 325): "urinary Mo levels increased with increasing educational level"). Please see lines 325, 328, 330, 367, 370, 372.

6. *In figure 2 IF is introduced, but at this point it is not clear which single ingredient was in the formula. With the presentation of data in that way it implies that, the respective trace element has been applied. Please explain here more precisely.*

We apologize for this misinformation. Isolated formulations (IF) in figure 2 do not represent the use of the isolated trace element in each case (panels A to D). In fact, in all panels IF corresponds to the use of a supplement with isolated iodine, isolated folic acid or isolated vitamin D. Because of this, IF condition is really not very informative in the context of our results and so we decided to remove it from Figure 2. The important conclusion about the type of formulation use is that using multivitamin/multimicromineral formulations (MV/MMF) seems to decrease the levels of Cu and Mn, despite their Cu or Mn content. So, our suggestion regarding the putative competition between Zn (which is the major mineral in those MV/MMF) and Cu or Mn for intestinal absorption is still valid. For clarification, we added a supplementary table with the composition of the reported MV/MMF. Please see supplementary table S1.

7. *Why are only the elements Co, Cu, Mn, and Zn shown in figure 2, where is Mo? Which significance test was used here, and what do the stars represent.*

As referred in the beginning of section 3.3 (line 349-350), none of the pregnant women included in the study reported the use of Mo-containing supplements, so this element was excluded from the subsequent statistical analysis. We are very sorry for the omission of the test and the meaning of the stars from the figures. This information was added to the all the graphs. Please see figures 2 to 4.

8. *For figure 3 it is the same, where are the other trace elements? And define the stars here as well.*

We acknowledge also this comment. For simplicity we did not present as graphs data on pregnancy complications that did not reveal statistically significant differences. We agree that we must refer to the absence of these associations. As such we added this reference to the manuscript. Please see lines 393-394).

9. *Table 7 is highly interesting, but it is still no association it is a comparison of the significances again.*

We removed the terms association as explained above. Please see lines 438-443.

10. *In table 8 some values are displayed in bold. Why? Please make this clear.*

We agree that the bold is of no use, so we removed it. Please see table 8.

11. *The discussion too long and should be more precise and shortened.*

We totally agree with the Reviewer. As such, we have shortened the discussion.

From first version of the manuscript, text was removed in lines: 406 to 417, 423, 480 to 484, 506 to 509, 518 to 524, 528 to 529, 547 to 555, 462 to 464.

Also, some rearrangements of the discussion and conclusions were made, please see lines 588-593, 602 – 605, 608-6120, 633, 637-639.

12. *Citation 41 is not complete.*

We apologize for this lapse. All the citations have been reviewed again, one by one, to make sure there are no more mistakes. Quote 41 has been corrected.

To conclude, in my opinion this manuscript should be rated with minor revision.

References

1. Jędrusik, P.; Symonides, B.; Gaciong, Z. Performance of 24-hour urinary creatinine excretion-estimating equations in relation to measured 24-hour urinary creatinine excretion in hospitalized hypertensive patients. *Sci Rep* **2019**, *9*, 3593, doi:10.1038/s41598-019-40416-w.
2. World Health Organization; Food Agriculture Organization of the United Nations. Trace elements in human nutrition and health. **1996**.
3. Chang, A.R.; Zafar, W.; Grams, M.E. Kidney Function in Obesity-Challenges in Indexing and Estimation. *Adv Chronic Kidney Dis* **2018**, *25*, 31-40, doi:10.1053/j.ackd.2017.10.007.
4. Fotheringham, J.; Weatherley, N.; Kawar, B.; Fogarty, D.G.; Ellam, T. The body composition and excretory burden of lean, obese, and severely obese individuals has implications for the assessment of chronic kidney disease. *Kidney Int* **2014**, *86*, 1221-1228, doi:10.1038/ki.2014.112.
5. Mishra, P.; Pandey, C.M.; Singh, U.; Gupta, A.; Sahu, C.; Keshri, A. Descriptive statistics and normality tests for statistical data. *Ann Card Anaesth* **2019**, *22*, 67-72, doi:10.4103/aca.ACA_157_18.