Author's response to reviews

**Title:** Calcium, phosphate, magnesium, and alkaline phosphatase gestational age-specific reference intervals for preterm infants

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**Author's response to reviews:** see over
Dear Ms Neilan,

Thank you for your correspondence regarding this paper. We have added the authors’ contributions and ensured that the sections of the paper are in the correct order.

We wish to extend our thank you to the reviewers for their time and helpful comments. We have addressed the reviewers comments in a revised manuscript and provided a point-by-point response to the reviewers comments.

Reviewer #1
1) In abstract, before conclusions, reference ranges had written wrongly. Such as calcium 2.1- 3.1 mgr/dl must be mmol/L  
Response: Yes, you are correct. Thank you for noticing our transposition error.

2) Is there these problems in exclusion criteria such as infants of diabetic mother (hypomagnesemia), antiacide (hypophosphatemia ) ?
Response: For the generation of the reference intervals, we intentionally excluded infants with potentially pathogenic conditions, when infants with those conditions were found to have different values compared to those without the conditions. We believe excluding those with conditions that altered the analytes levels provides more confidence that these reference ranges identify the desirable reference ranges for clinical care.

3) Information about vitamin D levels or preparation vitamin D must be stressed.
Response: Unfortunately we did not measure vitamin D levels in these cord blood samples. We used birth season as a surrogate measure of vitamin D status. In the analysis, birth season (summer versus winter) was not a significant predictor nor confounder for any of the analytes. We have added more explanation regarding this into the paper.

Reviewer #2
1) How did you arrive at the intervals that you selected? Were they somewhat arbitrary??
Response: Reference intervals were calculated using the mean ± 1.96 standard deviations by gestational age groups (23-27, 28-31, 32-34, 35-36 and >36 weeks). The gestational age categories were selected to reflect similarities in morbidity and mortality rates, and we acknowledge inconsistency exists regarding gestational age categories in the published literature.

2) Do the term babies simply represent a small control group? Why their inclusion?
Response: We included the term babies since they are the next logical age group.

3) You don’t report the SAP in the abstract results.
Response: We have now included the alkaline phosphatase results into the abstract.

Reviewer #3
Major Compulsory Revisions
1. - Throughout text The authors should clearly state throughout the title and text that the reference ranges are for cord blood of infants, i.e. not infant blood or serum. These can be significantly different.
Response: Thank you for pointing this out. We have clarified this throughout the paper.

2. The authors refer to Ca, P, Mg, Alb and ALP as bone forming minerals: this is incorrect and should be removed.
Response: Thank you for pointing this out; you are correct. We have corrected this.

3. The authors have measured total ALP, which reflects ALP as produced by the liver and bone. This should be stated as such.
Response: Yes, the ALP reflects both liver and bone produced ALP, which we have noted in the paper.

4 - Materials and methods: addition to para1: Procedure of cord blood collection: How long after delivery was cord blood collected; how was serum obtained (storage and duration of full blood; spinning conditions)
Response: Venous cord blood was collected in the deliver suite during delivery and transported to the adjacent laboratory for centrifugation and analysis. This 24hr stat laboratory carries out centrifugation (10min x 5000g) on receipt of specimens (there was no storage or delay prior to centrifugation).

5 - How was ensured that only blood from the infant partition of the cord was collection (i.e not from maternal side)
Response: The umbilical cord was routinely clamped with hemostats and the infant blood within the cord was sampled without risk of contamination by maternal blood.

Laboratory methods: para 4: pls add external QA scheme if adhered to.
Response: The laboratory is accredited by the College of Physicians and Surgeons of Alberta which uses the laboratory proficiency testing program of the College of American Pathologists.

6 - Selection of subjects:
Methods para 1 mentions that term cord bloods were collected from singleton pregnancies only, whereas cord bloods from pre-term infants were collected from singleton to triplet pregnancies. This should be mentioned clearly. The author should address how this will impact on the statistical analyses.
Response: The rates of multiple birth among the preterm infants ranged from 24 to 42% in the preterm categories (Table 1), compared to the expected rate of 2.2% of all births [1]. In a random sample of 54 term infants the chances of getting even one multiple birth are small, and the chances of a triplet would have been miniscule.

In terms of affecting the analysis, omitting multiple births shouldn't really affect the analysis for several reasons.

a) For the regression modelling one would not expect the deletion of one point to be very influential in the estimation of the regression coefficients - if it was we would call that high leverage and would consider downgrading the importance of that point in the estimation of the coefficients.
b) It also should not affect the estimation of the reference interval since this by definition the reference interval covers 95% of the observations so even if the multiple birth point(s) were extreme it would have been excluded from the reference interval.

7 - Statistical methods: Pls consult an expert statistician for the models used, particularly the impact of forward multiple regression analyses.
Response: A Statistical review has been completed by the fourth reviewer.

Minor Essential Revisions
8. The authors should tighten up some issues related to the use of short-hand in their language:
e.g 3rd para results; end of para
Response: We found a mistake in our methods section where we referred to the pathological variables being confounding so we have changed this.

9. Triplet: is not a condition
Response: Most perinatal research studies stratify deliveries into singleton and one or more groups of multiples. This captures the clinical variation, increased morbidity and mortality associated with the multiples in a manner that does not obscure analysis or prediction. We agree that triplet is not a condition, but we do feel it is an important category for analysis.

10. PIH is a maternal condition, not an infant condition
Response: Clarified.

11 – Results - Magnesium para, last sentence: Regression analyses is never used to provide proof for causative links; the last part of the sentence is unclear: rephrase.
Response: Rephrased

Reviewer # 4
This is a nice study with substantial potential practical impact. Certainly, it is sound to construct normal ranges (or intervals) for the cord blood serum values of bone minerals conditionally on not only gestational age, but also on relevant maternal and neonatal variables, as suggested in the paper. The choice of the variables seems
to be reasonable. The paper uses rather standard statistical methodology in a way that seems to be appropriate for both study goals and nature of the analyzed data.

1. The authors should clarify more explicitly their statement “... accounting for the intragroup correlation induced by twins and triplets ...” which appears on page 4. It seems that something like generalized estimating equation (GEE) methodology or a mixed model has been invoked here, but from the text, one cannot be sure how the correction was done.

Response: We have explained the adjustment for the intra subject correlation and included a reference for this.

2. The only slightly problematic aspect is related to the fact that the analysis upon which the paper’s results are based is performed only on babies coming from a single center. That is already an improvement compared to the previous situation when no such data were available, but the normal values should be verified more broadly. That will be necessary in order to prevent possible local influences upon the normal intervals (for various reasons, the local normal might not be entirely representative for population/national or even international normal). It is entirely OK to publish the pilot and pioneering results of this study, but the need for future validation on at least national level should be openly and explicitly mentioned somewhere in the text of the paper.

Response: This is a good point. We have added the statement: These results come from a single center, which could be influenced by local events or lab methodology. Therefore these results should be verified and validated by larger multicentre studies.

3. The word “multivariate”, is not used appropriately in the paper (e.g. in “...so both variables could not be included in the multivariate model.”, but also at other places, including a table caption). Most likely, a multiple regression model is meant (with a univariate response but several explanatory variables, instead of a multivariate model that implies a vector response taken simultaneously!).

Response: Clarified

4. The paper should be published after adopting the mild changes suggested.

Response: Thank you.

Sincerely,

Tanis Fenton RD PhD

Reference List