Author's response to reviews

Title: Diagnostic utility of zinc protoporphyrin to detect iron deficiency in Kenyan preschool children: a community-based survey

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Author's response to reviews:

BHEM-D-16-00039 Diagnostic utility of zinc protoporphyrin to detect iron deficiency in Kenyan preschool children - EM Teshome; AM Prentice; AY Demir; PEA Andango; H Verhoef

We wish to thank the Editor and Reviewers for taking time to read our manuscripts and for their valuable comments that have enabled us improve on this manuscript. Line numbers given below refer to the revised manuscript without 'track changes'.

1. EDITOR COMMENTS:

Please provide sample size calculation and include study type in the title

We included the study type in the title as requested. With regards to sample size calculation, we now included the following statement (LN. 169-175): 'We included all children enrolled in the trial in the present study. Our sample size calculations were based on our primary aim to show non-inferiority of the haemoglobin concentration response to home fortification with 3mg iron as NaFeEDTA compared with 12.5 mg iron as ferrous fumarate intention. Because this aim is irrelevant to the present study, these calculations are not reported here, although they are available elsewhere [Teshome EM, Andango PEA, Osoti V, Terwel SR, Otieno W, Demir AY, Prentice AM, Verhoef H. Daily home fortification with iron as ferrous fumarate versus NaFeEDTA: a randomised, placebo-controlled, non-inferiority trial in Kenyan children. BMC Med 2017 [in press]. We also refrained from reporting retrospectively calculated statistical power, which is generally acknowledged as being misleading (e.g. Goodman SN, Berlin JA. The

use of predicted confidence intervals when planning experiments and the misuse of power when interpreting results. Ann Intern Med 1994;121:200–06.).

REVIEWER REPORTS:

CRYSTAL D KARAKOCHUK, PHD, RD (REVIEWER 1):

This is a well-written manuscript describing a study in Kisumu-west district of Kenya. Authors aimed to identify factors associated with ZPP concentrations in young children (1-3 years) and to assess the diagnostic ability of ZPP (alone or with Hb) to identify iron deficiency.

No comments required.

I have few comments and suggestions for the authors.

1. The proportion of children with whole blood ZPP >70 umol/mol haem was very high (98%) similarly, erythrocyte ZPP >40 (97%). The authors describe in lines 349-356 how plasmodium infection causes elevated ZPP and further in lines 357-360 the suspected mechanism of a haemolysis-induced increase in erythropoietin activity in the presence of malaria that drives the demand for iron. Given these mechanisms, I'm curious to ask about the known prevalence of sickle cell, thalassemia, or glucose-6-phoshpate dehydrogenase deficiency in this study population - could these factors be contributing to the surprisingly high levels of ZPP observed in your study?

We now added a section in the Discussion that answers these questions (LN:396-410).

2. How did you assess/approach QC in the ZPP method? Has your method for ZPP been previously validated? (if so, can you provide details or reference).

We now added the following sentences in the Methods section (LN.126-130): ‘For quality control of the haematofluorometer, we used erythrocyte controls for low, medium and high ZPP values from the manufacturer (Aviv) and as per manufacturer’s instructions. Measurements were within the acceptable range throughout the study.’ Calibration could be done using calibrator material that is available from the manufacturer (Aviv), but this was neither done nor necessary because measurements of the controls remained within the acceptable range.

3. The authors clearly state why children with inflammation were excluded from some analyses (lines 366-70) and why other methods of correction were not applied (lines 372-386).

Inflammation was assessed based on AGP and CRP biomarkers (which is pretty standard) - how accurate do you think these biomarkers capture inflammation (especially among populations where infection is persistent and recurrent). Although I realize this is global standard practice to use these two biomarkers, I wonder, could this be a limitation in your conclusions?

The reviewer is correct that this is a limitation. We now added the following sentences in the Discussion section (LN.429-434): ‘Another limitation is the possibility that plasma ferritin concentration is possibly elevated at levels of inflammatory markers within the normal range (serum C-reactive protein concentration <5 mg/L or plasma α1-acid glycoprotein concentration <1.0 g/L).’

4. The exclusion criteria in lines 111-4 is not completely clear. 'Not at risk of malaria (e.g. children who received chemoprophylaxis against malaria because of HIV infection or sickle cell disease) - previously it was mentioned in inclusion criteria: absence of reported or suspected major systemic disorder. So perhaps the latter statement is not necessary. And second, "did not complete the second and third doses of dihydroartemisinin-piperaquine'. Please clarify.

This has now been clarified (LN. 161-167).

Minor points:

line 95: , should be .

Sentence rephrased (LN. 108-110)

Line 94: Is this region at sea level (<1000m)?

The region is 1350m above sea level, this information is now included (LN.110).

Line 103: Suggest to use 1-3 y for consistency (rather than 12-36 mo)

The text has been modified for consistency.

line 116: Was blood fasting?

No, there was no blood fast

Line 119: Was Hemocue also done in triplicate (or just ZPP). Or was hemocue Hb measured just once.

This has now been clarified (LN. 123-124).

Line 124: What type of rapid kit was used.

We used CareStart G0151 for detection of P. falciparum-specific lactate hydrogenase (pLDH), and CareStart G0171 for detection of P. falciparum-specific histidine-rich protein-2 (HRP2) and P. ovale/P. malariae/P. vivax-pLDH (LN. 137-141)

Line 126: 'further details are reported elsewhere.' - please can you include a citation here.

Reference included (Teshome EM, Andango PEA, Osoti V, Terwel SR, Otieno W, Demir AY, Prentice AM, Verhoef H. Daily home fortification with iron as ferrous fumarate versus NaFeEDTA: a randomised, placebo-controlled, non-inferiority trial in Kenyan children. BMC Med 2017 [in press].

Line 151: CRP and AGP are acronyms here but spelled out throughout the most of the manuscript.

This has now been corrected throughout the manuscript: we spelled out these acronyms in the text but retained them in the Tables, which contain explanatory footnotes.

Lines 469-70. Suggest to remove 'if asked, we can elaborate on our reasons with editor'

Correction made (LN. 538-539)

line 465: Period needed at end of sentence.

Correction made (LN.548)

MADELEINE VERHOVSEK (REVIEWER 2):

This study presents analysis of clinical and laboratory data collected as part of an rct. The authors are trying to determine the diagnostic utility of ZPP alone or in combination with other laboratory values (e.g. hemoglobin concentration) in identifying cases of iron deficiency. The manuscript is generally well written, however there is a focus on long descriptions of statistical analyses and presentation of results without sufficient placement in the clinical context.

We better explained the rationale and context for the study in our Introduction. No further comments required.

Major comments:

- overall the authors have not made it clear why this is an important clinical question, and how they feel zpp could add to the currently available tests. In particular, the authors note that both ZPP and ferritin are elevated in inflammatory states. Therefore ZPP seems to have the same test limitations as ferritin, which is a widely used and available test.

We better explained the rationale for our study. One notable difference between ZPP and ferritin is that ZPP test provides instant results, at low assay costs (see our Introduction). These issues are of critical importance in primary care, even more so in developing countries.

- the definition of iron deficiency was based on ferritin <12 ug/l, which is a very strict definition. This cut-off is likely quite specific but not sensitive, therefore missing out on cases of iron deficiency with higher ferritin levels.

This is most clear in the results section where it appears that very few of the children with inflammation or plasmodium infection were classified as having iron deficiency (32.1% in the no inflammation/infection group vs. 17.1% in the group as a whole). This is not clinically plausible and suggests that very many cases were missed by using exclusively the strict ferritin cut-off for case identification.

We agree with the reviewer that plasma ferritin concentration < 12 µg/L has low sensitivity for iron deficiency in the overall population. As we explained, however (LN 189-190): 'We defined iron deficiency as the absence or near-absence of storage iron, indicated by plasma ferritin concentration <12 µg/L [ref]. Because this definition is recommended by WHO to measure population iron status except where inflammation is prevalent [ref], we considered it to be valid only in children without inflammation, Plasmodium infection, or HIV infection.' We were also careful to point out that 'The analysis [of the diagnostic performance of ZPP to detect iron deficiency] was restricted to children without inflammation (i.e. plasma concentrations of C-

reactive protein < 5 mg/L and/or α1-acid glycoprotein < 1 g/L) and without Plasmodium infection.' (LN. 242-245).

- a major study limitation is the lack of ability to definitively identify and confirm all cases of iron deficiency. Understandably, the gold standard of hemosiderin stain on bone marrow sample would not b practical in this study setting.

We agree that collection of bone marrow samples is not practical in these apparently healthy children. We also agree and discussed in our paper that the absence of a reference standard for iron deficiency in the presence of inflammation is a limitation of our study (LN. 429-434).

Abstract

- no context or background is provided as to why this is an important study question.

This is now better explained .

- it is likely unnecessary to include all inclusion and exclusion criteria in the abstract - this listing takes up a large portion of the word allowance.

We believe that a listing of eligibility criteria is essential to define the study population.

Background

- page 4, last paragraph - the authors indicate that ZPP has "little diagnostic utility as a screening marker to manage iron deficiency in pregnant women". Please explain why you would then decide to study this test again in the current population

Findings from pregnant women do not necessarily apply to preschool children. Apart from physiological differences due to age and pregnancy (e.g. haemodilution), pregnant women may have less inflammation because of lower exposure to infections, or because they have acquired higher levels of protective immunity against such infections, resulting in a lower inflammatory response to infections.

Methods

- exclusion criteria - why were children with twin siblings and those with Hb <70g/l excluded?

We excluded twins for practical reasons (in many settings in developing countries, parents find it unacceptable that their children are allocated to different interventions; in addition, this would also likely result in cross-overs, i.e. mixing of interventions). We now explained that we excluded children with haemoglobin concentration <70 g/L for ethical reasons, because the trial had a placebo arm (LN. 160-161).

- earlier in the manuscript it is noted that this was an analysis of data collected in an rct. It would be helpful to the reader if you briefly describe the nature and purpose of the RCT

To provide context, we have now given more information about the trial (LN. 105-108).

- were the children tested for thalassemia?

No.

Results

- As noted above, the lower rates of iron deficiency identified in children with inflammation/infection vs. children who were well make it clear that ferritin alone was not an appropriate test in this population for case identification

See our reply to a similar question raised by reviewer 1 above.

- The authors have not explained the reasons for testing both whole blood ZPP and erythrocyte ZPP. Please explain the differences between these tests, and what the anticipated differences would be in test results.

This was explained in the Methods section (LN.132-135).