Author’s response to reviews

Title: Iron status and risk factors of iron deficiency among pregnant women in Singapore: A cross-sectional study

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Author’s response to reviews:

Reviewer 1:
Major comments:

1. What is the proportion of anaemia in non-pregnant women of obstetric age in Singapore? Is there a difference in the infant iron stores between women who are anaemic before pregnancy started and those who are not? Please add this information to the introduction.

Response: According to the WHO data repository in 2016, the prevalence of anaemia among non-pregnant women of reproductive age in Singapore was 22.2%. In humans, we are not able to identify any epidemiological study on investigating preconception anaemia/iron status and infant iron stores. However, a study on rhesus macaques suggest that the iron status of the infant after birth reflects that of the mother before pregnancy. This animal study has been cited in other review articles to highlight the importance of preconception iron status on neonatal iron stores. We have included this information in the ‘Introduction’.

Introduction section, Page 6, Line 150-153: However, a large proportion of pre-pregnant women or those of reproductive age have low iron stores [9,10], predisposing them to an increased risk of iron deficiency when becoming pregnant [8]. In Singapore, a developed country, one in five non-pregnant women of reproductive age were found to be anaemic [11].

Introduction section, Page 7, Line 159-162: Studies on rhesus macaques suggest that infants born to iron deficient mothers before pregnancy had low iron stores after birth [14]. In humans too, newborns from iron deficient mothers at delivery were found to have low iron stores, indicating there is a limited capacity for the fetus to accumulate iron from mothers with low stores [15].

2. In the introduction, please include information on the extent to which iron transport to the fetus is protected from lower maternal iron stores?

Response: We have included the relevant information in the ‘Introduction’.

Introduction section, Page 7, Line 156-159: It was previously thought that neonate was protected from iron deficiency as the developing fetus could acquire sufficient iron from the mother even when she was iron deficient [13]. However, it is now documented that neonatal iron stores can be compromised when the mother is iron deficient or anaemic [13].

3. From table 1, it can be derived that a majority of the women already had low Hb levels in early pregnancy. What were their ferritin levels in early pregnancy? What were their Hb levels in early third trimester?

Response: Thank you for raising this issue. Unfortunately, women in this study were not examined for ferritin levels in early pregnancy and Hb levels in early third trimester. We recognised this as a study limitation, and therefore, had included these points with their justifications under the ‘limitations’ section in the ‘Discussion’.

Discussion section, Page 15, Line 369-373: As we did not measure plasma ferritin in early pregnancy, the present findings should therefore be interpreted cautiously. However, the use of a plasma ferritin cut-off of <30 μg/L to define iron deficiency in the late second trimester is supported by van den Broek and colleagues [18], using bone marrow iron as a reference among pregnant women at mid and late pregnancy.
Discussion section, Page 15, Line 378-381: Measurement of Hb level at 26-28 weeks’ gestation is not part of routine clinical assessment in this setting, so data on maternal anaemia status was not available at the same time point as the plasma ferritin measurement. However, the use of a plasma ferritin cut-off of <15 μg/L has been documented as an indicator for iron deficiency anaemia [17].

4. Given that the great majority of women would be iron-depleted e.g. 74% but only 7% are severly iron depleted, are the cut-offs used in this study misleading? Are the cut-offs specific for the population used?

Response: The cut-off used for iron deficiency (<30 μg/L; 74%) in this study was determined based on the guidelines from The Committee for Standards in Haematology (United Kingdom), underpinned by a study using stained bone marrow aspirates to evaluate iron status among pregnant women at mid and late pregnancy (similar to the period of examination (26-28 weeks’ gestation) among pregnant women in this study). In Singapore, the same threshold is also applied in obstetric clinical practice to guide therapy for iron deficiency. We had added this point under the ‘Introduction’.

The cut-off for severe iron depletion (<15 μg/L; 7%) was determined based on the WHO guidelines, a threshold indicating iron depletion at all stages of pregnancy which has deemed to be appropriately use for all pregnant population around the world. Based on the American College of Obstetricians and Gynecologists practice, levels of ferritin less than 10-15 μg/L confirm the presence of iron deficiency anaemia. In addition, the cut-off of <15 μg/L has also commonly been applied in other studies. We had added this information under the ‘Introduction’.

Introduction section, Page 7, Line 170-173: The Committee for Standards in Haematology from United Kingdom recommends iron supplementation in pregnancy for plasma ferritin less than 30 μg/L [3], a threshold also widely used in clinical practice, including in Singapore, to guide therapy for iron deficiency in pregnancy [16].

Introduction section, Page 7, Line 168-170: According to the World Health Organization [5], plasma ferritin levels of lower than 15 μg/L indicate iron depletion (loss of iron stores) at all stages of pregnancy [3]. This threshold also confirms the presence of iron deficiency anaemia [17].

5. Dietary intake of iron through consumption of meat could be another factor that could determine iron status: what information is available on dietary iron intake? Could this potentially explain the higher proportion of women with modest iron deficiency that were of Indian/Malay ethnicity?

Response: We thank the reviewer for this helpful point. In dietary data from the Singapore National Nutrition Survey, Chinese participants were found to have highest intakes of meat, poultry and egg; Malay participants had lowest intakes of fruits and vegetables and had the lowest vitamin C intake; while Indian participants tended to follow a vegetarian diet. This dietary information could in significant part explain why Malays and Indians had lower iron status. We have added this information into the ‘Discussion’.

Discussion section, Page 14, Line 350-355: Among the ethnic groups, Chinese women have been reported to consume more meat, poultry and eggs, contributing to a rich source of haem-iron with a higher bioavailability; Malay women consumed fewer fruits and vegetables and had a lower vitamin C intake, which could reduce iron absorption; while Indian women tended to be vegetarian, where the absorption of non-haem might be inhibited by phytates present in vegetables and cereals [37,38].
6. Have Hb levels in early pregnancy been correlated with ferritin levels at 26-28 weeks gestation to see if there is a linear relationship between these two factors? Even if the majority of women with known anaemia in early pregnancy is "only" 20%, the levels indicate that there are quite a few women who are hovering just over the threshold and it would thus be interesting to see if there is a continuous relationship between the two parameters.

Response: We performed Spearman correlation to examine the continuous association between Hb in early pregnancy and plasma ferritin at 26-28 weeks’ gestation. This has been added under ‘Statistical analysis’ and ‘Results’.

Methods section (statistical analysis), Page 10, Line 242-243: Spearman correlation was used to analyse the continuous association between maternal Hb in early pregnancy and plasma ferritin at 26-28 weeks’ gestation.

Results section, Page 12, Line 294-295: A significant correlation was observed between Hb in early pregnancy and plasma ferritin at 26-28 weeks’ gestation (r=0.22, p<0.001).

7. If only the women were included with severe iron deficiency, were the same factors still significantly associated with iron depletion?

Response: Yes, for women with severe iron deficiency, the same factors were still significantly associated with iron depletion. This is because we performed the analysis using ordinal logistic regression which can help to increase statistical power by making full use of the structure of an ordinal scale, produce a more stable estimate with a broad interpretation, applicable across multiple dichotomizations of outcome, including the cut-off used for severe iron deficiency. Although the number of women with severe iron depletion was small (n=67), the proportional odds assumption was met and the model fitted the data well. The proportional odds ratio as presented in this study could be viewed as independent from the degree of severity used to classify the iron status and was thus, valid over all cut-points simultaneously. We have added this information in the ‘Statistical analysis’ section.

Methods section (statistical analysis), Page 10, Line 245-249: Compared to a series of binary logistic regression or using multinomial logistic regression, the use of an ordinal logistic regression model helps to increase the power by making full use of the structure of an ordinal scale, producing a more stable estimate and summary with a broad interpretation, applicable across multiple dichotomizations of outcome [22].

Methods section (statistical analysis), Page 11, Line 262-265: The fit of model and proportional odds assumption were checked and met. The proportional odds ratio as presented in this study could be viewed as independent from the degree of severity used to classify the iron status and was thus, valid over all cut-points simultaneously.

8. The inclusion of smoking status in the logistic regression, which is based on 24 women and n=2 in the severe iron depletion and n= 8 in the iron sufficient women, needs to be discussed in terms of how reliable that data is based on the small sample size of that analysis. It is unclear why this factor, which was not significant in the univariate analysis, was included in the multivariate analysis.

Response: The small number of smokers observed in this study restricted our reliability to draw conclusions. We have included this point in the ‘Discussion’. The reasons for including this variable in the multivariate analysis are justified below in point #9.
Discussion section, Page 15, Line 381-383: The proportions of women who reported smoking and a history of anaemia were small (≤5%) and insufficient to provide reliable conclusions regarding these groups.

9. Similarly, it is not clear why other factors that were not significant in the univariate analysis including BMI and history of anaemia were included in the multivariate analysis. It is common to only include significant factors in the multivariate model.

Response: Thank you for raising this query and enabling us to provide more clarifications. We included variables in the multivariable analysis using clinical judgement, prior literature review and by using a directed acyclic graph, but not depending on pre-determined p-value in the univariate analysis. Using univariate analysis (regardless of any p-value cut-off) to select variables to be included in the multivariable analysis has been shown to be inappropriate as univariate analysis is unable to correct for confounding or consider intercorrelations between independent variables. When risk factors (independent variables) are not truly independent of each other, a non-significant risk factor in univariate analysis is not necessarily non-significant in multivariable analysis. This has been proven by Sun et al. (1996) using both hypothetical and actual data. Without considering these issues, estimation of the effects of a risk factor on outcome can be biased and distorted. In this study, age and education were found not to be significant in the univariate analysis, but became significant in the multivariate analysis, though BMI, smoking and history of anaemia remained non-significant. We have included these justifications in the ‘Statistical analysis’ section.

Reference:

Methods section (statistical analysis), Page 10, Line 249-257: In determining variables to be included or excluded from the multivariable model, it has been shown that methods using pre-determined p-value criteria in the univariate analysis are inappropriate, and likewise for automated variable selection procedures (e.g. forward, stepwise) [23]. This is because confounding effects and inter-correlations between independent variables are not being considered, which can lead to biased and distorted outcomes [23]. A better way to determine which variables should be included in the multivariable model is by using clinical judgement [23], as done in other studies identifying risk factors of an outcome [24,25]. In this analysis, we selected the potential risk factors and built the model based on a literature review [26-28], clinical knowledge and by using a directed acyclic graph.

10. Lastly, why was the multivariate model not further refined: if stepwise backward or forward analysis would have been performed, it would be clear which factor is the most important determinant of circulating ferritin levels. This needs to be done to finalise the analysis presented in this manuscript.

Response: Using automated variable selection procedures (e.g. forward, stepwise) to include or exclude variables in the final model are not optimal as these methods are highly sensitive to specification of their stopping rules in the presence of confounding, potentially leading to result bias (evidence in the report of Sun et al., 1996, as reference above). Full-model fit using clinical knowledge has been suggested as a better approach in clinical research, which can correct for confounding and proper documentation of all variables considered. We have included this explanation in the ‘Statistical analysis’ section (page 10, Line 249-257), the same as stated in point #9.
Separately, we also performed analysis using a backward elimination procedure to refine the model to compare our findings. BMI, smoking status and history of anaemia were excluded from the model. However, when checking for model fitness based on the Pearson Chi-square and Pseudo-R2 statistics, this regression model did not appear to fit well with the data, though the final results (significant factors of iron deficiency) were similar compared with full-model fit. We present here the results for multivariable model using backward elimination for reference:

**Age:**
- <25y (OR 2.31; 95% CI 1.14, 4.70; p=0.021)
- 25-34y (OR 1.26; 95% CI 0.88, 1.81; p=0.198)
- ≥35 years (reference)

**Ethnicity:**
- Chinese (reference)
- Malay (OR 1.98; 95% CI 1.26, 3.10; p=0.003)
- Indian (OR 1.92; 95% CI 1.12, 3.30; p=0.019)

**Education:**
- None/ Primary/ Secondary (reference)
- University (OR 1.68; 95% CI 1.16, 2.44; p=0.007)

**Parity:**
- Nulliparous (reference)
- Multiparous (OR 1.69; 95% CI 1.22, 2.36; p=0.002)

**Iron-containing supplementation:**
- Yes (reference)
- No (OR 3.23; 95% CI 1.25, 8.31; p=0.015)

**Minor Comments:**
1. Abstract line 130, three-quarter should read three-quarters.

Response: The term has been revised as ‘three-quarters’.

Abstract, Page 6, Line 130: Nearly three-quarters of Singaporean women were iron deficient in the early third trimester of pregnancy.

2. Please define ID as iron deficiency when used for the first time in line 158.

Response: We have removed the ‘ID’ abbreviation and spelled out the full name as ‘iron deficiency’ throughout the manuscript.

3. In table 1, the actual median/mean BMI (and the spread around the median/mean) of the women in the different categories should be included.

Response: We have included the mean and SD of BMI for each category in Table 1.
Background:
1. You need to be consistent about using 'Iron deficiency' or the abbreviation ID. You also need to include (ID) in brackets after 'Iron deficiency' the first time of use.

Response: We removed the ‘ID’ abbreviation and spelled out the full name as ‘iron deficiency’ throughout the manuscript.

2. Some countries, e.g. UK do not recommend additional iron requirement during pregnancy (due to amenorrhoea and increased absorption), see COMA 1991. A discussion of this would be helpful in the introduction.

Response: Thank you for this suggestion. We have added this point in the ‘Introduction’.

Introduction section, Page 6, Line 146-149: For pregnancy, the European Food Safety Authority [6] and the UK Committee on Medical Aspects of Food Policy [7] recommend no increase in iron intake over that for non-pregnant women. The extra iron requirements during pregnancy are considered to be met through cessation of menstrual losses, increased intestinal absorption and mobilisation of maternal iron stores [8].

3. An explanation of anaemic & non-anaemic iron deficiency is needed.

Response: We have added the explanation for anaemic and non-anaemic iron deficiency in the ‘Introduction’.

Introduction section, Page 6, Line 139-141: Iron deficiency represents a spectrum ranging from iron depletion without anaemia (reduced iron stores with a normal haemoglobin (Hb) concentration) to eventual overt anaemia, where the iron supply is insufficient to maintain a normal Hb concentration [3].

Methods:
1. Is KK an abbreviation? If so, write in full first.

Response: KK is not an abbreviation, it is the name of the hospital. The full name of the hospital is ‘KK Women’s and Children’s Hospital’.

2. Is natural conception an inclusion criteria? (mentioned in results). If so, it needs to be indicated on the methods section.

Response: Natural conception was one of the inclusion criteria in this study, which has been added in the ‘Methods’.

Methods section, Page 8, Line 195: Those who conceived naturally were included in this study.

Results:
1. see comment about natural conception above.

Response: We had indicated ‘natural conception’ as one of the inclusion criteria of this study in the ‘Methods’ section, as stated above.
Discussion:
1. 299-300: Most anaemic women already had iron deficiency anaemia in early pregnancy - it is also likely that many women were anaemic prior to conception. Iron deficiency in women of child-bearing age is well documented and some discussion of this issue would be helpful here.

Response: We have added this information in the ‘Introduction’ and ‘Discussion’.

Introduction section, Page 6, Line 150-153: However, a large proportion of pre-pregnant women or those of reproductive age have low iron stores [9,10], predisposing them to an increased risk of iron deficiency when becoming pregnant [8]. In Singapore, a developed country, one in five non-pregnant women of reproductive age were found to be anaemic [11].

Discussion section, Page 13, Line 330-331: We found that one in five women reported having been anaemic in the first trimester, similar to the prevalence of anaemia (22.2%) in women of reproductive age in Singapore [11].

Discussion section, Page 13, Line 333-335: Since nearly 80% of anaemia in the Singapore pregnant population is due to iron deficiency [26], it is likely that most anaemic women in our study already had iron deficiency anaemia in early pregnancy, and even prior to conception.

2. 313-314: Can you expand on the differences in dietary practices in these ethnic groups?

Response: We have included details of dietary practices which would affect iron status across ethnicity in the ‘Discussion’.

Discussion section, Page 14, Line 350-355: Among the ethnic groups, Chinese women have been reported to consume more meat, poultry and eggs, contributing to a rich source of haem-iron with a higher bioavailability; Malay women consumed fewer fruits and vegetables and had a lower vitamin C intake, which could reduce iron absorption; while Indian women tended to be vegetarian, where the absorption of non-haem might be inhibited by phytates present in vegetables and cereals [37,38].

3. 320-323: Are iron supplements well absorbed? Could this be an explanation for poor iron status, despite supplementation?

Response: Data on iron-containing supplements included those taken as part of a multivitamin and mineral supplement or prenatal supplement. Previous studies have shown that the absorption of iron from these tablets is probably low due to the absorptive interaction of iron with other divalent metal ions contained in the tablets. We have included this explanation in the ‘Discussion’.

Discussion section, Page 15, Line 363-365: Otherwise, the absorption of iron from these iron-containing tablets is probably low due to the absorptive interaction of iron with other divalent metal ions in the tablets (e.g. zinc, manganese, calcium) [40].

4. Further discussion about how these findings should influence current practice would be helpful here, particularly from a public health perspective. What needs to be done to improve iron status of pregnant women/women of child bearing age at a population level?

Response: We have added relevant suggestions to improve iron status of pregnant women/women of
reproductive age in the ‘Conclusions’.

Conclusions section, Page 16, Line 397-400: Concerted efforts, including routine dietary advice (e.g. consuming plenty of iron-rich foods with a higher iron bioavailability, along with items containing vitamin C) and individual iron supplementation prophylaxis before and during pregnancy should be considered in this population for optimal maternal and offspring health.