Reviewer: 1

Comments to the Corresponding Author
The authors describe the folate deficiency in Queensland from information based on outpatient laboratory measurements before (2004-2008) and after 2010-2015) mandatory fortification was introduced.

The revised paper is improved with additional explanation on definitions of folate status, potential biases that could have been introduced by the method used to assess folate concentrations. However, the paper still needs to address some important issues that require clarification:

Dear Reviewer 1,

We would like to thank you for the time and effort you invested in our manuscript. We absolutely understand your concerns and would like to highlight that all of your suggestions would really improve the content of our manuscript. However, we are facing some limitations regarding the availability of the data regarding most of the analyses you were asking for: Since we're dealing with secondary health data i.e. routinely collected data, this type of data has the inherent disadvantage that not all information which would have probably been collected in a specific dedicated study is available. The advantage is that usually – and especially also in our case – a large amount of data is available for analysis. Thus very specific questions (estimation / adjustment for specific potential influencing factors etc.) cannot be answered by this type of data and thus not in our study. In the present paper we were able to show a very robust pre/post comparison overall, based on rather large numbers of tests. Especially with that large amount of data it could be assumed that various potentially influencing factors will smooth out and especially are not able to bias the overall robust estimate to any relevant extent. It remains unquestionable that further dedicated studies should confirm this assumption in prospective data collections. We hope that you might understand and accept our apologies that accordingly we were unfortunately not able to address all your comments in a satisfactory way.

Comment 1:
1. Methods section. As stated in the methods section: "folic acid values were investigated as red cell folate (RCF) or serum folate (SFOL) during the observation period……based on the cut-off values of the assays".
a. Provide cut-off points and state the biomarker used.
Answer comment 1:
We strongly agree that this is important information of our study and included the respective assays and cut-off in a supplemental file to our article).

Laboratory values

During the observation period, several changes in folic acid measurement assays occurred and different parameters (red cell folate values (RCF), as well as serum folate (SFOL)), were measured. Due to several assay changes during the study period only dichotomous test results were analysed (deficient vs. non-deficient). This status was determined separately for each individual and based on

the test method and parameter used (please refer to supplementary information, Tables s1 and s2 for further details on cut-off points and biomarkers).

2.

Comment 2:

Main points

1. Folate levels could be impacted by the intake of different drugs such as drugs for epilepsy, cancer, HIV,etc. Because the information used for this assessment is based on outpatient laboratory results in routine care, it is important to remove those patients that are receiving prescriptions that could affect folate concentrations.

Answer comment 2:

We absolutely agree with you that this would be an important sensitivity analysis of our data but unfortunately we do not have any information regarding prescriptions in this study. Since we excluded repeated measurements from our analysis and concerning the high number of analysed individuals, we assume that it is unlikely that our estimates in general are biased by drug intake. We included a corresponding comment in the limitations section of our article.

Limitations

The present study constitutes a secondary analysis of routinely collected health data with limitations related to data quality and subsequent exclusion of invalid data. Moreover, although this study is currently the largest post-mandatory fortification program investigation of folic acid values in remote populations in Australia, our sample was not population-based and therefore may not be considered representative of the Australian remote population. Remote populations may be affected by regional variations among demographic parameters such as ethnicity, age and gender. Furthermore, the main influencing variable, remoteness, was based on postcode information rather than prospectively gathered information on the real life circumstances of participants - As such, using postcodes as an indicator of remote or urban living must be viewed with some degree of caution. The available data were also limited regarding information on current pregnancy status, folic acid supplementation prescriptions, drug intake, and comorbidities. These parameters are likely to affect folic acid status but were not available in this routine dataset and these influences were not accounted for in our statistical analyses. It is also possible the decline in folic acid deficiency might be independent of the mandatory fortification program and influenced by factors not included in this study such as changes in nutrition behavior or food supply. We were however, still able to show a very robust pre-/post-comparison overall, based on large numbers of tests. With this large amount of data it could be assumed that various potentially influencing factors will smooth out and decrease the risk of bias to the overall

estimates. Further dedicated studies could confirm this assumption with prospective data collections.

In 2007, a 1.4-fold increase in folic acid testing was observed in the study population. Possible reasons for the increase and the extent of differences in the size of increases between population subgroups were investigated using sensitivity analyses. We found the increases were non-differential and thus underlying selection bias does not seem to have influenced the observed prevalence of folic acid deficiency. Finally, a recent review by Rogers et al. raised concerns about the comparability of different folic acid assays. Data were analysed in a binary fashion (deficient vs. non-deficient) to account for assay changes in the current investigation.(Rogers, et al., 2018)

Comment 3:

2. When assessing folate deficiency (serum or RBC) you are assessing a clinical status, it is required to define the cut-off point for serum or RBC folate deficiency. It is incorrect to define folate deficiency as based on folic acid values. Folic acid is the chemical used to fortify a specific staple. The impact of folic acid intake is on serum and RBC folate concentrations. In addition, the determination of folate deficiency needs to be done separately for each type of folate because they represent a different stage in the biochemical process. Serum folate concentrations reflect the circulating folate after food intake. In contrast, RBC folate concentration measure the storage of folate in the red blood cell. This process takes about 12 weeks. Serum folate concentrations are affected when blood is drawn in non-fasting conditions, but RBC folate concentrations are not. Therefore, prevalence status can be impacted by the type of biomarker indicator used. So, it is important to present this prevalence separately to understand what we are talking about. That means that table 1, figure 2 need to be revised to address these issues as well as its description in the results section.

Answer comment 3:

Thank you for this comment. We determined the status of "folate deficiency" separately for each individual based on the test method used and the respective cut-off value for this specific test. Since test methods as well as the preferred parameter of measurement changed during the observation period we decided to illustrate a combined prevalence for all parameters and assays based on dichotomized folic acid measurements (deficient vs. non-deficient). We clarified this point in the methods section of our manuscript:

Laboratory values

During the observation period, several changes in folic acid measurement assays occurred and different parameters (red cell folate values (RCF), as well as serum folate (SFOL)), were measured. Due to several assay changes during the study period only dichotomous test results were analysed (deficient vs. non-deficient). This status was determined separately for each individual and based on

the test method and parameter used (please refer to supplementary information, Tables s1 and s2 for further details on cut-off points and biomarkers).

Comment 4:
3. In addition, the median age of the population was 52 yrs of age. Stratified analysis should have included women 15-49 years of age and not limited to women 15-34 yrs. Women of reproductive age are one of the main targets of folic acid fortification
Answer comment 4:
Thank you for this excellent suggestion. We added a second age range in our analyses including all women 35-49 years of age. Please refer to table 1.

3. RBC folate insufficiency.
Comment 5:
a. Folate concentrations are impacted by pregnancy status. Analyses presented among women of reproductive should be stratified by pregnancy status. The authors do not distinguish between these 2 groups and when present prevalence information for serum or RBC folate deficiency and RBC folate insufficiency.
Answer comment 5:
Regarding your above request we hope you understand and might accept our apologies that data on pregnancy status and folic acid supplementation were not available in the respective routine data sets. We absolutely agree that the information you were asking for would be helpful for a better understanding and interpretation of the presented results. We, however, tried to address the issue in the limitations section. See comment 3.

Comment 6:
b. Was the population provided with folic acid supplementation? If yes, how did it affect folate concentrations? When did they received the supplements?
Answer comment 6:
Please refer to comment 5.

4. Discussion.

Comment 7:
a. The discussion should be revised based on comments 1, 2 and 3. In addition, the decrease in folate deficiency is important but the main point of fortification with folic acid is to decrease the risk of neural tube defects among women of reproductive age. This paper does not address these issues. Do the authors have information on vitamin B12 status of these population? And how can it impact folate status?
Answer comment 7:
Unfortunately also these data were not available in our study!

Comment 8:
b. Information was collected on whether or not the women were taking a folate containing supplement. Were any analyses done to compare the status of those women consuming a folate containing supplement vs. not?
Answer comment 8:
Please see comment 5.

Comment 9:

3. Do the authors have data to indicate how much of the “folate” anemia is due to folate/B12 deficiencies vs. iron deficiency (or other causes)?

Answer comment 9:

Also these data were not available in our study, please accept our apologies and see the new paragraph in the limitations section.

Reviewer: 2

Comments to the Corresponding Author

Thank you for the opportunity to review the manuscript. The mandatory fortification has a long history of use as a strategic program in public health policy for increasing folate intake and status, and reducing the occurrence of folate-preventable neural tube defects. The current manuscript intends to assess the impact of mandatory fortification policy in remote vs regional urban areas and Indigenous vs non-Indigenous populations in northern Queensland – Australia

Dear Reviewer 2,

Thank you for the time and effort you invested in the very detailed review of our manuscript. We really appreciated your suggestions, which contributed to improve the content and accuracy of our article. We hope that you find all your comments and concerns adequately addressed. We are, however, happy to include any further suggestions.

Comment 1:
Title: Please add the Country's name in the title. My suggestion is "…in Northern Queensland-Australia"
Answer comment 1:
Thank you for this suggestion, we revised the title accordingly:
Low Proportions of Folic Acid Deficiency after Introduction of Mandatory Folic Acid Fortification in Remote Areas of Northern Queensland - Australia: A Secondary Health Data Analysis

Comment 2:
Abstract
Lines 18-21
The method topic is succinct. Please, give more details about the methods of this study.
Answer comment 2:
Thank you for this comment, we extended the method section in the abstract of hour article. Due to space restrictions we were not able to include further details in the abstract.

Methods

Exposure definition

`Remote areas' were defined by postcodes 4895, 4890, 4876, 4875, 4874, 4871 and 4825; and compared with the two main regional urban centres, Cairns (postcode: 4870) and Townsville (postcode: 4810). Please refer to https://postcodes-australia.com/state-postcodes/qld for further information about postcode areas.

Comment 3:
Lines 25 – 26
Please, specify the prevalence.
Answer comment 3:
The overall prevalence was 3.2% (235/7,282) in urban centres compared with 7.2% (480/6,647) in remote areas (p<0.001), and 9.3% (393/4,240) in the Indigenous population compared with 3.2% (273/8,451) in the non-Indigenous population (p<0.001).

Comment 4:
Lines
I am not sure if RRR was adequately used. Please, read my comments below.
Answer comment 4:
Please refer to comment 16.

Comment 5:
Page 6-7
Lines 54-57 (page 6) and Lines 1-13 (page 7)
Please, the authors could show the prevalence of folic acid deficiency reported in the reference cited.
Answer comment 5:
Thank you for this suggestion. Prevalence is now included:

In 1995, federal legislative changes in Australia facilitated a *voluntary* program of folic acid fortification for a number of foods including bread and breakfast cereals. A 2001 evaluation showed relatively few of the recommended foods had been fortified nevertheless, a general Australian population study reported that the program had halved the prevalence of folic acid deficiency from 39% at baseline to 17%.(Abraham B, 2001, Hickling, et al., 2005)

Comment 6:
Page 7
Lines 42-45
What about Indigenous vs. non-Indigenous populations? Please, the authors need to mention it in the objective.
Answer comment 6:
Thank you for this accurate observation. We changed the objective accordingly:

The aim of this study is to evaluate the impact of mandatory folic acid fortification in remote and regional urban areas and Indigenous vs non-Indigenous populations in northern Queensland, Australia.

Comment 7:
Line 60
I think the date "1st January 2000" is wrong. The authors mentioned 2004 throughout this manuscript. Please, check the correct year!
Answer comment 7:

Thank you for this observation. We extracted all data from 2000-2015 but due to data limitations, only data from 2004-2015 were analyzed. Thus we corrected the dates in the methods section.

The study was set in the northern part of the state of Queensland, Australia and constitutes a retrospective analysis of routinely collected data on all folic acid measurements conducted by pathology Queensland (AUSLAB) from 1 January 2004 to 31 December 2015.

Comment 8:
Page 8
Lines 7-10
It is not clear "Pathology Queensland (AUSLAB). Is it a Public health center? Is it a private lab? Please, elucidate this issue considering the foreign readers.
Answer comment 8:
Thank you! We included this information in the methods section of our article:

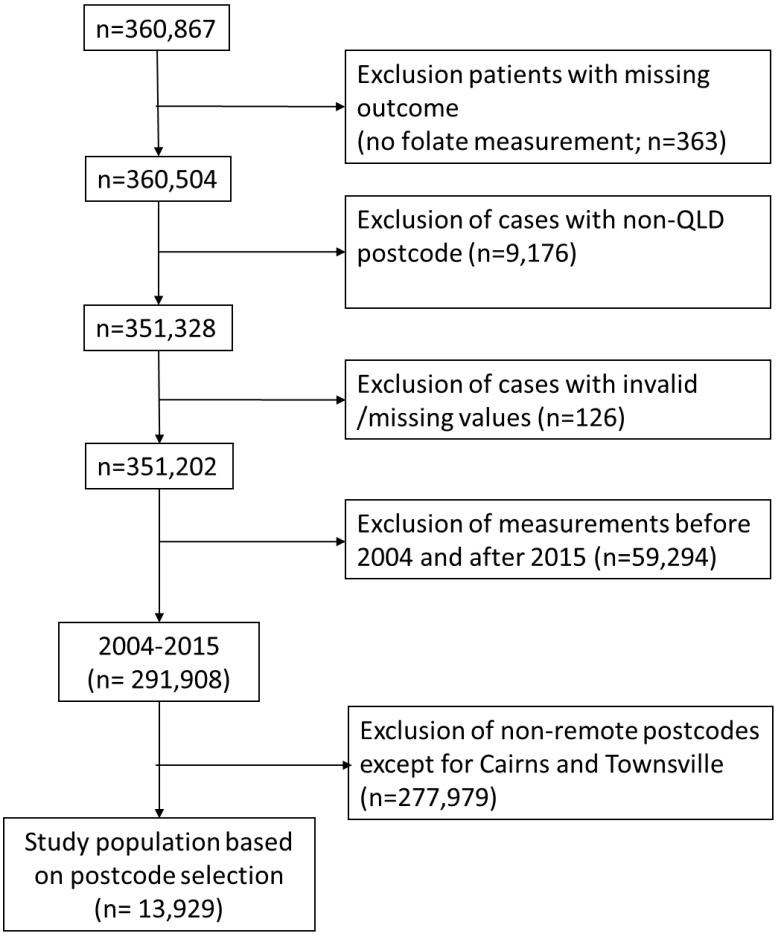
The study was set in the northern part of the state of Queensland, Australia and constitutes a retrospective analysis of routinely collected data on all folic acid measurements conducted by pathology Queensland (AUSLAB, public laboratory) from 1 January 2004 to 31 December 2015.

Comment 9:
Lines 38-43
Please, give additional information about the postcode. The authors could name the area or calculate the distance from postcode to the urban areas. For foreign readers, the postcode does not mean anything. Maybe the authors could show a map with some marks considering the postcodes.
Answer comment 9:
We included a link to further information about Queensland postcodes and area names. We considered to include area names but since really remote areas are included, there were many sub-areas included in these postcodes and therefore we would have had to name many areas.

Remote areas' were defined by postcodes 4895, 4890, 4876, 4875, 4874, 4871 and 4825; and compared with the two main regional urban centres, Cairns (postcode: 4870) and Townsville (postcode: 4810). Please refer to https://postcodes-australia.com/state-postcodes/qld for further information about postcode areas.

Comment 10:
Lines 48-57
Please, rewrite the paragraph and modify the figure 1. The authors could start with number of folic acid measurements in the study period keep only the first measurements exclude the remote postcode except Cains and Townsville exclude the invalid cases and show the final sample.
Answer comment 10:

We revised figure 1 'patient flow' and included additional information in the text:



Comment 11:
Page 9
Lines 7-8
Please, verify the period of this study. 2000 or 2004?
Answer comment 11:
Please see comment 7.

Comment 12:
Lines 24 – 32
The risk ratio or relative risk is the ratio of the probability of an outcome in an exposed group to the probability of an outcome in an unexposed group, in others words, the ratio of the incidence in an exposed group to incidence in an unexposed group. Please, the authors could mention some references that support the calculation of RR using prevalence.
Answer comment 12:
Thank you for this comment, we changed the term "risk ratios" to "prevalence ratios" and hope you might agree with this.

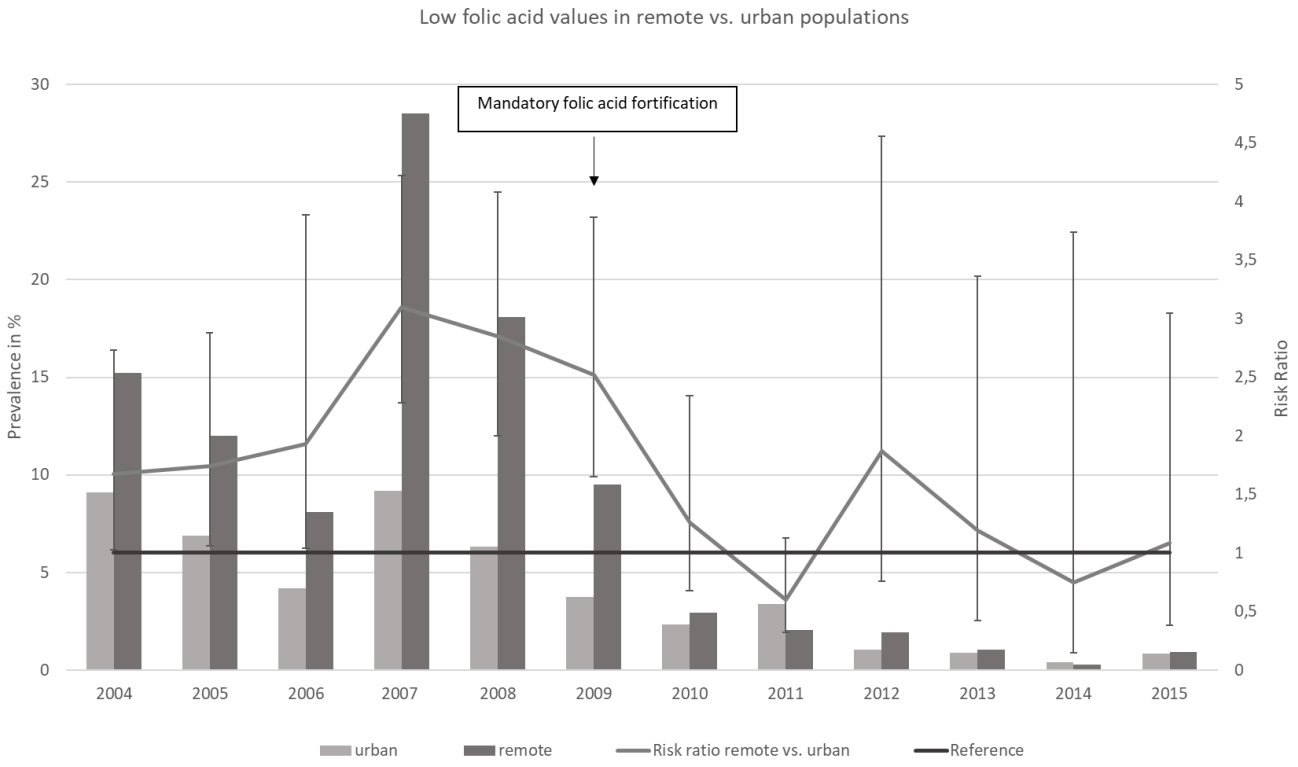
Exact binomial confidence intervals (95%-CIs) for proportions as well as prevalence ratios of proportions were calculated. The 95%-CIs for prevalence ratios were calculated based on Miettinen and Nurminen.[16] All analyses were conducted using SPSS Statistics Version 25.

Comment 13:
Lines 34-35
The authors can provide a new table with the characteristics of the sample (sex, age groups and ethnicity) for all
population and according the urban and remote area by year. It is important to check these frequencies before the assessment of folic acid deficiency. If it is possible, add the p-values for statistic differences between groups (urban vs. remote). See my suggestion below:
Answer comment 13:
We included a new table with demographic characteristics over time in the new supplement 2 to this article.

Comment 14:
Lines 44-45
I do not think the frequency is right. Please, check the values "5.1% identified as Indigenous (n=4,240)"
Answer comment 14:
Thank you very much for this observation, we apologize for this error.

Of all 13,929 measurements, 52.3% were conducted in one of the two main urban centres and 47.7% in remote areas. Females represented 54.2% of the study population (n=7,555), median age was 52 years (IQR: 35-69 years) and 30.4% identified as Indigenous (n=4,240).

Comment 15:
Page 10
Lines 5-17
Please, add the statistic difference of prevalence of folic acid deficiency between urban and remote area considering only the indigenous population.
About the table 1, I think the authors could improve the data visualization. I suggest the authors do 3 line graphs (visualization of data over time): Graph 1 (all population, urban and remote area), Graph 2 (urban indigenous, urban non-indigenous, remote indigenous and remote non-indigenous) and Graph 3 (all women 15-34 y, remote women 15-34y and urban women 15-34y). Format: horizontal axis is year, vertical axis is frequency and each category will be a line. In addition, you can add a dashed vertical line in 2009 indicating the mandatory fortification moment. However, the authors could add this complete table (prevalence of folic acid deficiency and 95% CIs) as supplemental material.
Answer comment 15:
We considered all these graphs during the drafting of the manuscript but in the end decided that a table would illustrate our findings better and more comprehensively than three graphs. The illustration of the different prevalences and confidence intervals is, however, quite confusing due to a substantial overlap between the groups. We now included the figure below in order to illustrate prevalences and risk ratios over time for the main study question and hope that you might agree with us.



Comment 16:
Lines 29-60
The use of RRR is not adequate in this study. The authors consider prevalence the same of incidence. Please, elucidate this analysis (RRR) for prevalence, and add references that support this use in the materials and methods.
Answer comment 16:
In our data, we cannot distinguish between new cases and prevalent cases. Thus we refer to the observed proportions as prevalence. (Epidemiology, Petra Büttner, Reinhold Müller, Oxford University Press).

Comment 17:
Page 11
Lines 59-60
Please, write the abbreviation "TSI"
Answer comment 17:
A study investigating 26 rural and remote Indigenous communities in northern Queensland prior to the 2009 mandatory program showed low folic acid levels occurred more often in 2,524 Indigenous persons (prevalence 25.6% in Aboriginal and 14.8% in Torres Strait Islander participants compared with the general Queensland population (2.5%) (Li, et al., 2012).

Comment 18:
Page 12
Lines 1-2
Please, mention the prevalence in general population according of reference.
Answer comment 18:
A study investigating 26 rural and remote Indigenous communities in northern Queensland prior to the 2009 mandatory program showed low folic acid levels occurred more often in 2,524 Indigenous

persons (prevalence 25.6% in Aboriginal and 14.8% in Torres Strait Islander (TSI) participants) compared with the general Queensland population (2.5%) (Li, et al., 2012).

Comment 19:
Page 14
Lines 12-24
This paragraph is very…very important!!! I am very glad to read it!
Answer comment 19:
Thank you for this compliment, we are very happy about it!

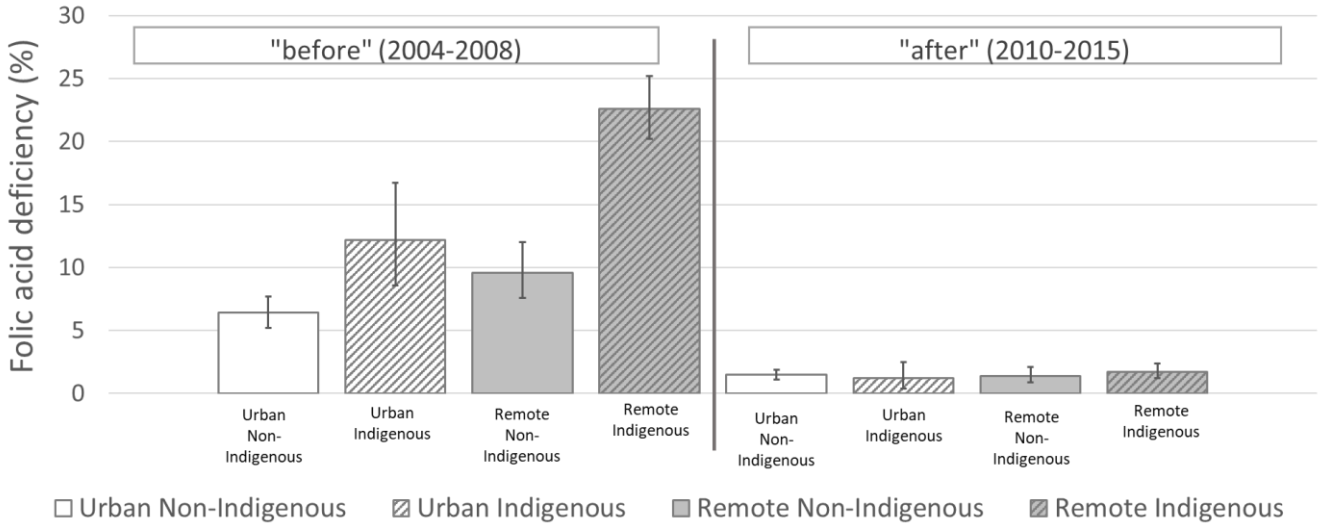
Comment 20:
Limitation
Do you have the information how long the people live in the same postcode? If not, add this issue as limitation of
this study. Sometimes, some participants could have lived for long time in remote area but, at the moment of the analysis, they were in urban area.
Answer comment 20:
Thank you for this excellent comment. We included this limitation accordingly:

Furthermore, the main influencing variable, remoteness, was based on postcode information rather than prospectively gathered information on the real life circumstances of participants - As such, using postcodes as an indicator of remote or urban living must be viewed with some degree of caution.

Comment 21:
References
The references are numerical throughout this text. However, at the end of manuscript, the references are in alphabetical order. Please, check the journal's instructions and revise them.
Answer comment 21:
We apologize for this and revised the references. Thank you for this comment.

Comment 22:
Figure 2
Please, add the label "before" (2004-2009) and "after" (2010-2015) the mandatory fortification in the graph, not
only in the figure legend.

Answer comment 22:
Thank you, please find below the revised figure:


Folic acid deficiency (%)
30
25
20
15
10
5
0
"before" (2004-2008)
"after" (2010-2015)
Urban Non-Indigenous
Urban Indigenous
Remote Non-Indigenous
Remote Indigenous
Urban Non-Indigenous
Urban Indigenous
Remote Non-Indigenous
Remote Indigenous
Urban Non-Indigenous
Urban Indigenous
Remote Non-Indigenous
Remote Indigenous