PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Study protocol for a randomised controlled trial evaluating the effect of folic acid supplementation beyond the first trimester on maternal plasma unmetabolised folic acid in late gestation
AUTHORS	Sulistyoningrum, Dian; Green, Tim; Palmer, Debbie; Sullivan, Thomas; Wood, Simon; Makrides, Maria; Skubisz, Monika; Best, Karen

VERSION 1 – REVIEW

REVIEWER	Shi Wu Wen Ottawa Hospital Research Institute/University of Ottawa, Ottawa, Canada
REVIEW RETURNED	05-Jul-2020

GENERAL COMMENTS	BMJ Open-2020-040416 Study protocol for a randomised controlled trial evaluating the effect of folic acid supplementation beyond the first trimester on maternal plasma unmetabolised folic acid in late gestation 1. Review checklist: (Y/N) 1) Is the research question or study objective clearly defined? Yes 2) Is the abstract accurate, balanced and complete? Yes 3) Is the study design appropriate to answer the research question? Yes 4) Are the methods described sufficiently to allow the study to be repeated? Yes 5) Are research ethics (e.g. participant consent, ethics approval) addressed appropriately? Yes 6) Are the outcomes clearly defined? Yes 7) If statistics are used are they appropriate and described fully? Yes 8) Are the references up-to-date and appropriate? Yes

	9) Do the results address the research question or objective? N/A 10) Are they presented clearly? N/A 11) Are the discussion and conclusions justified by the results N/A 12) Are the study limitations discussed adequately? N/A 13) Is the supplementary reporting complete (e.g. trial registration; funding details; CONSORT, STROBE or PRISMA checklist)? Yes 14) To the best of your knowledge is the paper free from concerns over publication ethics (e.g. plagiarism, redundant publication, undeclared conflicts of interest)? Yes 15) Is the standard of written English acceptable for publication? Yes 2. General comments This protocol is for a study aiming to assess the effect of continued folic acid supplementation beyond the first trimester on maternal unmetabolized folic acid levels, thereby to provide biochemical evidence for clinical practices. The protocol is well structured, with clear descriptions for study population and eligibilities, and details pertaining to intervention assignment and study outcomes including safety outcomes. However, here are some concerns. 1. The authors stated that no sponsor for the trial, which is not the normal practice for a clinical trial. Usually the institute/hospital/university where the PI is affiliated should assume the role of sponsor. Please clarify. 2. The authors listed trial management committee and trial steering committee, but did not mention an independent safety and data monitoring committee, which is not the normal practice for a clinical trial. Please clarify. 3. Plasma homocysteine, and other relevant vitamin Bs are also a well-known factors associated with the metabolism of folic acid during the pregnancy, and it may be of value to include in the study protocol of these biomarkers to fully understand the underlying pathway regarding to the effect of late folic acid supplementation; 4. In the third paragraph of Introduction, the phrase ‘Without proven benefit and the suggestion of harm’ seems to be misleading. The authors may want to say ‘Without proven benefit and with the suggestion of harm’. 5. In the ‘Monitoring adherence to study treatment’ section, the authors mentioned ‘At each contact, women will be asked if they have missed any supplements in the last week’, which has a minor risk of introducing recalling bias but is generally acceptable and practical. However, Figure 1 shows that from timepoint t1a on until t6 women will only be contacted monthly. They might easily forget ‘if they have missed any supplements in a given week or how many

	have been missed' if they were not given a diary to record this. Also, can the authors clarify how will they utilize the measure of compliance (the proportion of supplements returned)? Will there be a cut-off point of the measure of compliance that excludes women of poorer compliance from PPS analysis? 6. In Figure 1, at the enrollment column and row (and related text), it should be '12 to 16 w' or '≥ 12 to ≤16'.

REVIEWER	Kristina Pentieva Ulster University Northern Ireland UK
REVIEW RETURNED	13-Jul-2020

GENERAL COMMENTS	The protocol describes a study aiming to address in a very systematic and robust way whether the discontinuation of of folic acid supplement usage after the first trimester of pregnancy (a period officially recommended for prevention of neural tube defects) would impact the appearance and concentration of unmetabolised folic acid (UMFA) in the circulation of pregnant women at 36th week of gestation. This is an important question considering that the presence of UMFA has been associated with some potential adverse health effects. The protocol is very well presented and provides all the necessary details required for the execution of a randomised controlled trial. I have only a few minor comments/suggestions. 1. Line 49-56: It should be acknowledged that the findings so far from different investigations are inconsistent and there are studies which did not find association between folic acid supplement use and these adverse health effects. 2. The activity of dihydrofolate reductase (DHFR) is essential for the metabolism of folic acid to natural folate forms. However, there is a great interindividual variability of DHFR activity which is probably partly a consequence of some common DHFR polymorphisms. According to the protocol from participation in the study will be excluded women with MTHFR 677TT genotype and I completely support this. However, you may wish to consider also to exclude or to control at the analysis stage for DHFR 19bp deletion polymorphism. 3. Different colour-coded study packs will be used for the intervention and control arm of the study. Please clarify how this will not affect the blinding of the study. 4. The primary outcome of the study is plasma UMFA concentration. Please clarify whether you intend to measure FA in red blood cells which would provide information for the situation in the tissues. 5. I was not able to see a date for commencement of the study. It could be that I have missed this information, but it should be included in the protocol.

VERSION 1 – AUTHOR RESPONSE

Reviewer(s)' Comments to Author:
Reviewer: 1
1. The authors stated that no sponsor for the trial, which is not the normal practice for a clinical trial. Usually the institute/hospital/university where the PI is affiliated should assume the role of sponsor. Please clarify.
Response:
Under Funding Statement section, we have inserted the following “This study is sponsored by the South Australian Health and Medical Research Institute (Adelaide, Australia).”

2. The authors listed trial management committee and trial steering committee but did not mention an independent safety and data monitoring committee, which is not the normal practice for a clinical trial. Please clarify.
Response:

The nutrient supplements used in this trial do not differ substantially from commercially available supplements used unmonitored by many Australian pregnant women. The study was deemed very low risk and in the views of the investigators and the ethics committee an independent safety and data monitoring committee was not necessary.

The following text as been added to the manuscript:

Data and Safety Monitoring
We do not anticipate any serious adverse events related to participation in this trial. Regardless, an independent (blinded) clinician will review all serious adverse events and determine whether there is any likelihood that involvement in the trial could have contributed to the event. Determinations of causality will be made from medical records retrieved for this purpose. All serious adverse events will be captured and reported to the Human Research Ethics Committee.

3. Plasma homocysteine, and other relevant vitamin Bs are also a well-known factors associated with the metabolism of folic acid during the pregnancy, and it may be of value to include in the study protocol of these biomarkers to fully understand the underlying pathway regarding to the effect of late folic acid supplementation;
Response:
This is a good point. We have approval from the ethics committee to measure other B-vitamins and related metabolites such as homocysteine involved in one carbon metabolism. We will store extra blood for future analyses if more funds become available. Homocysteine is not a particularly useful biomarker in pregnancy and has not been consistently associated with any adverse pregnancy outcome.

4. In the third paragraph of Introduction, the phrase ‘Without proven benefit and the suggestion of harm’ seems to be misleading. The authors may want to say ‘Without proven benefit and with the suggestion of harm’.
Response:
Revised.

5. In the ‘Monitoring adherence to study treatment’ section, the authors mentioned ‘At each contact, women will be asked if they have missed any supplements in the last week’, which has a minor risk of introducing recalling bias but is generally acceptable and practical. However, Figure 1 shows that from timepoint t1a on until t6 women will only be contacted monthly. They might easily forget ‘if they have missed any supplements in a given week or how many have been missed’ if they were not given a diary to record this. Also, can the authors clarify how will they utilize the measure of compliance (the proportion of supplements returned)? Will there be a cut-off point of the measure of compliance that

excludes women of poorer compliance from PPS analysis?
Response:
The reviewer is correct we will be contacting women monthly and ask them to recall the previous week. The purpose of this is twofold, to alert study staff if a participant has ceased their study supplements and enable a follow up phone call and to encourage compliance.

The primary indicator of adherence will be based on the number of supplements returned. The analysis will be intention to treat and no woman will be excluded due to poor compliance. Women will be classified as compliant if they take ≥ 80% of supplements. No per protocol analysis will be conducted and the percent of women compliant by treatment group will be described in the results as a measure of trial quality.
This has been clarified in the manuscript under Monitoring adherence to study treatment.

6. In Figure 1, at the enrollment column and row (and related text), it should be '12 to 16 w' or '≥ 12 to ≤16'.
Response
Revised to '≥ 12 to <16'

Reviewer: 2
1. Line 49-56: It should be acknowledged that the findings so far from different investigations are inconsistent and there are studies which did not find association between folic acid supplement use and these adverse health effects.
Response:
This paragraph has been revised as per Reviewer's suggestion for clarity. Supporting references have been added.

2. The activity of dihydrofolate reductase (DHFR) is essential for the metabolism of folic acid to natural folate forms. However, there is a great interindividual variability of DHFR activity which is probably partly a consequence of some common DHFR polymorphisms. According to the protocol from participation in the study will be excluded women with MTHFR 677TT genotype and I completely support this. However, you may wish to consider also to exclude or to control at the analysis stage for DHFR 19bp deletion polymorphism.
Response:
Thank-you for this useful comment. It seems reasonable that DHFR 19bp deletion would affect the levels of UMFA. However, other than Kalmbach et al (J Nutr 2008; 138:2323–7) which showed higher levels of UMFA in those homozygous for the condition, but only with high folic acid intakes we know of no other papers that have shown this. Indeed, Plumptre et al (Am J Clin Nutr 2015;102:848–57) showed no effect of this deletion in the fetus on cord blood UMFA.
Nevertheless, given the frequency of this deletion, close to 70% including heterozygotes and double deletions it would be interesting to measure this variant in the participants. However, due to limited funding availability and given the study has already started we would prefer not to include this in the protocol.

3. Different colour-coded study packs will be used for the intervention and control arm of the study. Please clarify how this will not affect the blinding of the study.
Response:
This information is added under the Blinding section for clarity. "The independent unblinded statistician (not involved in any other way in the trial) allocated two colours to the intervention group and two colours to the control group. Supplements were subsequently packaged and labelled by colour only by two unblinded staff members who have no other involvement in the trial. Research personnel, participants and their family, care providers, outcome assessors, and data analysts remain blinded to colour allocation and therefore randomisation group."

4. The primary outcome of the study is plasma UMFA concentration. Please clarify whether you intend to measure FA in red blood cells which would provide information for the situation in the tissues.
Response:
Red blood cell and plasma total folate levels will be measured at 36 weeks' gestation as stated under Secondary outcomes section using the folate microbiological assay harmonized by the Centers for Disease Control and Prevention. We are not familiar with the measurement of UMFA in red blood cells. We would presume that UMFA would be very low in red blood, cells, as for cells to retain folate it must be reduced to THF and polyglutamylated.

5. I was not able to see a date for commencement of the study. It could be that I have missed this information, but it should be included in the protocol.
Response:
This information is added under Study Population section of the protocol paper. '. Enrolment commenced on 18th December 2019 and recruitment is on-going. Data collection will continue through to May 2021.'

VERSION 2 – REVIEW

REVIEWER	Shi Wu Wen Ottawa Hospital Research Institute, Canada
REVIEW RETURNED	13-Sep-2020

GENERAL COMMENTS	The authors have addressed most of the issuses I raised before and I have no further comments.

REVIEWER	Kristina Pentieva Ulster University
REVIEW RETURNED	10-Sep-2020

GENERAL COMMENTS	The revised version of the manuscript has addressed my previous comments