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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

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Keywords: Pregnancy, unmetabolised folic acid, folic acid, red blood cell folate, periconception, policy, prenatal supplementation

Abbreviations

UMFA	: unmetabolised folic acid
NTD	: neural tube defect
MTHFR	: methylene tetrahydrofolate reductase
RBC	: red blood cell
REDCap	: Research Electronic Data Capture
SAHMRI	: South Australian Health Medical Research Institute
HREC	: Human Research Ethics Committees
WCH	: Women's and Children's Hospital
FMC	: Flinders Medical Centre
GMP	: Good Manufacturing Product
DQES	: Dietary Questionnaire for Epidemiological Studies

ABSTRACT

Introduction: Taking folic acid containing supplements prior to and during early pregnancy reduces the risk of neural tube defects. Neural tube defects occur prior to 28 days post-conception, after which, there is no proven benefit of continuing to take folic acid. However, many women continue to take folic acid containing supplements throughout pregnancy. At higher intakes, folic acid is not converted to its active form and accumulates in circulation as unmetabolised folic acid (UMFA). Recently, concerns have been raised about possible links between late gestation folic acid supplementation and childhood allergy, metabolic disease and autism spectrum disorders. We aim to determine if removing folic acid from prenatal micronutrient supplements after 12 weeks gestation reduces circulating levels of maternal UMFA at 36 weeks gestation.

Methods and analysis: This is a parallel design, double-blinded randomised controlled trial. Women between 12 and 16 weeks' gestation with a singleton pregnancy and able to give informed consent are eligible to participate. Women (n=100; 50 per group) will be randomised to receive either a micronutrient supplement containing 0.8mg of folic acid or a micronutrient supplement without folic acid daily from enrolment until delivery. The primary outcome is plasma UMFA concentration at 36 weeks gestation. Secondary outcomes include red blood cell folate and total plasma folate concentration. We will assess whether there is a difference in mean UMFA levels at 36 weeks gestation between groups using linear regression with adjustment for baseline UMFA levels and gestational age at trial entry. The treatment effect will be described as a mean difference with 95% confidence interval.

Ethics and Dissemination: Ethical approval has been granted from the Women's and Children's Health Network Research Ethics Committee (HREC/19/WCHN/018). The results of this trial will be presented at scientific conferences and published in peer-reviewed journals.

Trial Registration Number: ACTRN12619001511123.

STRENGTHS AND LIMITATIONS OF THIS STUDY

- This is the first study to determine if continued supplementation of folic acid after 12 weeks of gestation results in excess maternal levels of unmetabolised folic acid; in a country with mandatory folic acid fortification.
- This study will provide contemporary data regarding maternal unmetabolised folic acid levels as a result of the common practice of self-supplementation throughout pregnancy and give some insight to the biochemical effects of this practice.
- This study will provide biochemical data critical to inform future research to investigate the effect of late gestation folic acid supplementation and unmetabolised folic levels on maternal and infant health outcomes.
- This study is not powered to detect the effect of continuing folic acid supplements after the first trimester on clinical outcomes.

INTRODUCTION

Evidence from randomised controlled trials (1,2) and a large public health intervention (3) showed that taking folic acid containing supplements prior to and during early pregnancy reduces the incidence of neural tube defects (NTD). Based on these findings, public health agencies around the world issued recommendations advising women to take folic acid supplements prior to conception and during early pregnancy.(4) For example, in Australia the government recommends that women trying to become pregnant take a folic acid supplement of 0.5 mg/day 12 weeks prior to conceiving and for the first 12 weeks of pregnancy.(5)

The neural tube closes in the first month of pregnancy, beyond this time there is no proven benefit of taking folic acid.(6) However, many women continue to take folic acid as part of a prenatal vitamin and mineral supplement throughout pregnancy.(7) In Australia, for example, a randomised controlled trial of pregnant women showed that more than 80% of women were taking a prenatal supplement containing folic acid at some time during their pregnancy,(8) with the market leading supplement containing 0.8 mg of folic acid. In addition, almost 80 countries, including Australia, have mandated the addition of folic acid to food staples, typically wheat flour, to reduce NTDs in unplanned pregnancies.(9) As such, the combination of food fortification along with prenatal supplement use may expose women and their fetus to excessive amounts of folic acid.

There is emerging evidence that higher intakes of folic acid in late pregnancy may have negative health effects on the offspring. Chief amongst these are concerns about an increased risk of childhood allergy,(10–12) but also increased rates of autism spectrum disorders (13–15) and insulin resistance.(16) Without proven benefit and the suggestion of harm, the amount of folic

acid in prenatal supplements may need to be reduced after the first trimester. Folic acid is the synthetic form of the vitamin folate that is used in supplements and fortified foods because of its high bioavailability and stability compared to naturally occurring folate in food.(17) Once consumed, folic acid must be converted into an active form, 5-methyltetrahydrofolate.(18) At higher intakes folic acid is not converted to its active form and accumulates in plasma as unmetabolised folic acid (UMFA).(19) Circulating UMFA has been proposed as a biomarker of excess folic acid intake.(20) We aim to determine if removing folic acid from prenatal multivitamin supplements after the first trimester (12 weeks gestation) reduces the accumulation of maternal UMFA measured at 36 weeks gestation.

Hypotheses

Removing folic acid from prenatal supplements after 12 weeks of gestation will limit the accumulation of UMFA in maternal plasma at 36 weeks of gestation.

METHODS AND ANALYSIS

Trial design

A multicentre two arm parallel design, double-blinded randomised controlled trial.

Participating Centres

The sponsoring institution and Trial Coordinating Centre is the South Australian Health and Medical Research Insititute (SAHMRI) based at the Women's and Children's Hospital (WCH). We will also seek approval to conduct the trial at Flinders Medical Centre (FMC), Adelaide, South Australia.

Study Population

Participants are pregnant women with a singleton pregnancy enrolled between 12 and 16 weeks gestation.

Eligibility Criteria

Inclusion Criteria

To be eligible for participation women must be:

1) Carrying a singleton pregnancy ≥12 and <16 weeks gestation and;

2) Currently taking a folic acid containing supplement and planning to continue this throughout pregnancy and;

3) Able to give informed consent.

Exclusion criteria

Women will be ineligible for trial participation if they meet any the following criteria:

1) Carrying a fetus with a confirmed or suspected fetal abnormality.

2) Unwilling to cease current folic acid containing supplement/s.

3) Past history of a NTD affected pregnancy.

4) Currently taking medication known to interfere with folate metabolism (e.g. methotrexate, sulphasalazine, anti-convulsants, antimalarials or barbiturates).

5) Known haemolytic anaemia or haemoglobinopathy.

6) Known to carry the TT variant of the methylene tetrahydrofolate reductase gene (MTHFR C77T) polymorphism.

7) Intolerance or allergy to prenatal vitamin and mineral supplements.

Study treatments

Participating women will be randomised to receive either a micronutrient supplement containing 0.8 mg folic acid (the dose in the most commonly used supplement in Australia) or an identical micronutrient supplement containing no folic acid. The composition of micronutrients within the intervention and control supplements are formulated to approximate leading brands of prenatal micronutrient supplements available in Australia, **Table 1**. Intervention and control supplements are identical in size, shape, colour and packaging and only differ in the removal of folic acid from the intervention supplement. Women will be asked to consume one supplement per day from enrolment (12-16 weeks of gestation) until delivery.

Manufacture of study supplements

Intevention and control supplements are manufactured in a licensed facility in accordance with the Code of Good Manufacturing Practice (GMP) for Medicinal Products and have been donated to the trial by Factors Group of Companies, Coquitlam, British Columbia, Canada. The capsules are packaged and labelled in accordance with GMP including an individual product identifier (ID), batch number, expiry date and the statement 'for clinical trial use only'. The pharmacist or the investigator's designee maintains accurate records of the dispensing of study product. Unused study supplements will be destroyed in compliance with applicable regulations.

Monitoring adherence to study treatment

Research personnel will maintain regular contact with participating women to monitor and encourage supplement adherence and study compliance and answer any questions as they arise. At each contact, women will be asked if they have missed any supplements in the last week and if so, how many have been missed. Women are asked to return unused capsules at the final study visit (36 weeks' gestation) and the proportion of supplements returned serves as an additional

measure of compliance. At this visit women will be issued with enough supplements to last until the delivery of their baby.

Outcome measures

The primary outcome is maternal plasma UMFA concentration at 36 weeks gestation.

Secondary outcomes

- Maternal plasma total and red blood cell (RBC) folate levels at 36 weeks' gestation.
- Gestational age at birth, birth weight, birth length, birth head circumference.

Safety outcomes

- Neonatal complications requiring admission to the neonatal unit.
- Pregnancy complications requiring hospital admission.
- Serious adverse events defined as: maternal or fetal (>20 weeks) deaths, fetal loss (< 20 weeks), maternal or neonatal admissions to intensive care and major congenital anomalies.

Participant timeline

Women will be randomised and asked to cease their current prenatal supplements immediately and for the duration of the study. At enrolment, following informed consent and prior to commencement of the study treatment, research personnel will collect baseline clinical and demographic data including: contact details, self-reported ethnicity, gravida, parity, age, supplement and prescription drug use, weight, height, highest level of education, occupation and smoking status. Maternal dietary intakes of folate and other one-carbon nutrients during early and late pregnancy will be collected with the use of an 80-item semi-quantitative food-frequency questionnaire – Dietary Questionnaire for Epidemiological Studies (DQES v3.2).(21) A 10ml venous blood sample will be collected by venepuncture to assess UMFA, folate status and full

blood count. The time the woman last ate and drank as well as the time her last supplement was taken will be recorded. Research personnel will contact the participant one week following the enrolment visit and then monthly to ensure adherence and record adverse events, Figure 1. At 36 weeks' gestation participating women will attend a clinic appointment for collection of venous blood sample for UMFA and folate analysis and full blood count. Participants will be asked to return unused supplements which will be counted as a measure of compliance. The food-frequency questionnaire will be repeated and women will be given enough supplements to last the remainder of their pregnancy. Following delivery, research personnel will extract details of pregnancy, labour and birth from the woman and her baby's medical records. Blood samples will be analysed for UMFA according to established methods.(22) Plasma folate (nmol/L) and erythrocyte folate (nmol/L) concentrations will be determined using the folate microbiological assay harmonized by the Centers for Disease Control and Prevention.(23)

Figure 1. Folic Acid Trial Schedule

	Screening	Enrolment	Allocation	+1 week		Post-allocation				
TIMEPOINT**		$-t_1$	0	t_1	t_{1a}*	t_2	t_3	t_4	t_5	t_6
ENROLMENT:	<12w	>12 to <16w		13 to17w	16w	20w	24w	28w	32w	36w
	Clinic	Clinic	Clinic	Phone	SMS	SMS	SMS	SMS	Phone	Clinic
Eligibility screen	X	X								
Consent to Contact	X									
Informed consent		X								
Randomisation			X							
Demographic data		X								
Allocation			X							
INTERVENTION:			←	—	—	—	—	—	→	
ASSESSMENTS:										
Maternal Folate status		X								X
Maternal UMFA status		X								X
Food Frequency Questionairre		X								X
Adverse events					X	X		X		X
Serious Adverse events										X
Compliance – maternal report					X	X	X	X	X	X
Compliance – Supplement count										X

Sample size

A sample size of 90 women (45 per group) will provide >90% power to detect a standardised difference in mean UMFA concentration at 36 weeks gestation between groups of 0.60 (two-tailed alpha=0.05, correlation between UMFA concentrations at baseline and 36 weeks of gestation=0.60).(24) Calculations were performed based on a standardised mean difference (mean difference divided by standard deviation of outcome at 36 weeks gestation) due to considerable variability in the literature in the reported standard deviation for UMFA concentration in pregnancy.(12,24) A standardised mean difference of 0.60 represents a medium effect size and

would demonstrate biologically excessive folic acid consumption. To allow for 10% loss to follow-up, we will randomise 50 women per group.

Recruitment

Pregnant women will be recruited through a combination of flyers,posters, a digital media campaign and through in-person recruitment at antenatal clinics. Women who meet elgibility crtiteria and agree to participate are invited to attend an enrolment appointment at our research clinics at the Women's and Children's Hospital or Flinders Medical Centre, Adelaide between 12 and 16 weeks gestation.

Randomisation procedures

Participants will be randomised using a secure web-based randomisation service. Allocation will follow a computer-generated randomisation schedule using balanced variable block sizes, prepared by an independent statistician who is not involved with trial participants or data analysis. A unique four-digit study identification number and a coloured coded study pack are assigned. The study identification number identifies the randomised woman and the coloured product identifies either intervention or control supplements, pre-packaged corresponding to the randomisation schedule. Stratification will be by gestational age at trial entry 12 to ≤14 weeks or >14 to 16 weeks gestation.

Blinding

Participants and their family, care providers, outcome assessors, research personnel and data analysts are blinded to randomisation group. The intervention and control supplements are identical in size, shape, colour, packaging and labelling and uniquely identified by the coloured product identification label (Yellow, Pink, Blue or Green). The randomisation code for an

individual participant may be unblinded by the independent statistician in the event of an emergency.

Data collection and trial management

Data are collected by trained research personnel and entered directly into an electronic case-report form with password protection and defined user-level access Research Electronic Data Capture (REDCap). A record of all women approached, screened for eligibility and consented will be recorded.(25) Once consented and randomised, REDCap automatically calculates study milestones for each participant. This information is readily available for clinical trial staff to enable scheduling of appointments and sample collection. Summary reports including screening data, enrolment, appointment attendance, sample collection, serious adverse events and study completion are generated from REDCap and reviewed at monthly trial steering committee meetings. Electronic data are stored on secure servers at South Australia Health and Medical Research Institute and released only to persons authorised to receive those data.

Statistical analysis

Statistical analyses will be performed on an intention-to-treat basis according to a pre-specified statistical analysis plan. For the primary outcome, we will assess whether there is a difference in mean UMFA levels at 36 weeks gestation between groups using linear regression, with adjustment for baseline UMFA and the stratification variable gestational age at trial entry (12 to ≤ 14 weeks or > 14 weeks). The treatment effect will be described as a mean difference with 95% confidence interval. Secondary outcomes will be analysed using linear and logistic regression models for continuous and binary outcomes, respectively, again with adjustment for gestational age at trial entry. Safety outcomes will be compared between groups using Fisher exact tests. In all analyses, a two-sided p-value <0.05 will be taken to indicate statistical significance.

ETHICS AND DISSEMINATION

Human Research Ethics Approval

This protocol, the informed consent and participant information document and all participant communication have been approved by the Women's and Children's Health Network Research Ethics Committee (HREC) (HREC/19/WCHN/018) and Governance (SSA/19/WCHN/080). Governance approval has also been obtained from FMC. Any subsequent modifications will be reviewed and approved by the HREC and governance of each study site. The study will be conducted in compliance with the current approved version of the protocol. Any change to the protocol document or informed consent form that affects the scientific intent, study design, patient safety, or may affect a participant's willingness to continue participation in the study will be considered a major amendment. All such amendments will be submitted to the HREC for approval. Any other changes to the protocol (such as administrative changes to dates and study personnel) will be considered minor amendments and will be notified to the HREC as appropriate.

Confidentiality

Participant confidentiality is strictly held in trust by the participating investigators, research staff and their agents. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Regulatory authorities may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records for the women and/or infants in this study subject to individuals having obtained approval/clearance through State/National Governments and HREC as required by local laws. Clinical information will not be released without written permission of the parent, except as necessary for monitoring by HREC or regulatory agencies.

Patient and public involvement

The study was supported by a consumer advisory group which provided input to the protocol. A Consumer representative from our SAHMRI Women and Kids Consumer Advisory Group partnered with us for the design of the study, informational material to support the intervention, and the burden of the intervention from the participant's perspective. We will meet with the consumer representative for this trial and the full Consumer Advisory group on a regular basis for the duration of the study. At the end of the study, the consumer advisory group will be given the opportunity to comment on the findings and contribute to the dissemination plan.

Dissemination Plan

Study findings will be submitted for peer-reviewed publication and for presentation at appropriate local and international conferences. In addition, study findings will be disseminated to participants through a one-page lay summary. Results will be made available to the wider community through social media avenues and the SAHMRI website.

Authors' contributions

KPB, TJG, MM, DP and MS conceived the trial and proposed the trial design; TS advised on sample size calculations, trial design, and analysis; SW and TJG designed the prenatal supplement and had it manufactured; DCS advised on analytical methodology; DCS, TJG and KPB drafted the protocol, all authors contributed to refinement of the study and approved the final manuscript.

Funding statement

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of Obstetricians and Gynaecologists supports MS. KPB is supported by a Women's and Children's Hospital Foundation, MS McLeod Postdoctoral Research Fellowship. DCS is supported by the Australian Government Research Training Program Scholarship from The University of Adelaide. The study product is donated by Factors Group of Companies, Coquitlam, British Columbia, Canada. The funder/s have no role in the study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication and have no authority over any of these activities.

Competing interest statement

Dr. Makrides reports that she has a financial relationship outside the submitted work with Trajan Nutrition as a member of the board. Simon Wood is a consultant for the Factors Group of Companies. DCS, TJG, DJP, TS MS, and KPB have nothing to disclose.

Data sharing statement

Once the primary trial is published, data will be available for data sharing to appropriately qualified investigators upon submission of a protocol and approval by the Trial Steering Committee. Please send requests to Dr Karen P Best (karen.best@sahmri.com).

Table 1. Ingredients of Supplements for Intervention and Control Groups

Ingredients	Intervention	Control	unit
folic acid	0	0.8	mg
calcium	250	250	mg
Iron	27	27	mg
thiamine	1.4	1.4	mg
riboflavin	1.4	1.4	mg
niacinamide	18	18	mg
vitamin B-6	1.9	1.9	mg
vitamin B-12	2.6	2.6	mcg
pantothenic acid	6	6	mg
biotin	30	30	mg
vitamin C	85	85	mg
vitamin E	13.5	13.5	IU
magnesium	50	50	mg
zinc	7.5	7.5	mg
manganese	2.0	2.0	mg
iodine	0.22	0.22	mg
copper	1	1	mg
chromium	30	1	mcg
selenium	30	30	mcg
Vitamin D3	10	10	mcg
b-carotene	2500	2500	IU

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	Yes
Protocol version	3	Date and version identifier	Supp.
Funding	4	Sources and types of financial, material, and other support	14-15
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	N/A
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	N/A
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	12, 13

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-5
	6b	Explanation for choice of comparators	4-5
Objectives	7	Specific objectives or hypotheses	5, 8
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	7
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	8, 9, 10 & Fig. 1

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	10, 11
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	11
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	11
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	11
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8, 11, 12
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11, 12
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	12
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8, 9, 12
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	12
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	12
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	12
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	13
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	13
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	13
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	13
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	13

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	8, 13
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	13
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	15
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	N/A
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Supp. PICF
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	14
	31b	Authorship eligibility guidelines and any intended use of professional writers	N/A
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Supp. PICF
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Supp. PICF

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

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

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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

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Abbreviations

UMFA	: unmetabolised folic acid
NTD	: neural tube defect
MTHFR	: methylene tetrahydrofolate reductase
RBC	: red blood cell
REDCap	: Research Electronic Data Capture
SAHMRI	: South Australian Health Medical Research Institute
HREC	: Human Research Ethics Committees
WCH	: Women's and Children's Hospital
FMC	: Flinders Medical Centre
GMP	: Good Manufacturing Product
DQES	: Dietary Questionnaire for Epidemiological Studies

Caption for Figure 1. Folic Acid Trial Schedule

ABSTRACT

Introduction: Taking folic acid containing supplements prior to and during early pregnancy reduces the risk of neural tube defects. Neural tube defects occur prior to 28 days post-conception, after which, there is no proven benefit of continuing to take folic acid. However, many women continue to take folic acid containing supplements throughout pregnancy. At higher intakes, folic acid is not converted to its active form and accumulates in circulation as unmetabolised folic acid (UMFA). Recently, concerns have been raised about possible links between late gestation folic acid supplementation and childhood allergy, metabolic disease and autism spectrum disorders. We aim to determine if removing folic acid from prenatal micronutrient supplements after 12 weeks gestation reduces circulating levels of maternal UMFA at 36 weeks gestation.

Methods and analysis: This is a parallel design, double-blinded randomised controlled trial. Women ≥12 and <16 weeks' gestation with a singleton pregnancy and able to give informed consent are eligible to participate. Women (n=100; 50 per group) will be randomised to receive either a micronutrient supplement containing 0.8mg of folic acid or a micronutrient supplement without folic acid daily from enrolment until delivery. The primary outcome is plasma UMFA concentration at 36 weeks gestation. Secondary outcomes include red blood cell folate and total plasma folate concentration. We will assess whether there is a difference in mean UMFA levels at 36 weeks gestation between groups using linear regression with adjustment for baseline UMFA levels and gestational age at trial entry. The treatment effect will be described as a mean difference with 95% confidence interval.

Caption for Figure 1. Folic Acid Trial Schedule

Ethics and Dissemination: Ethical approval has been granted from the Women's and Children's Health Network Research Ethics Committee (HREC/19/WCHN/018). The results of this trial will be presented at scientific conferences and published in peer-reviewed journals.

Trial Registration Number: ACTRN12619001511123.

STRENGTHS AND LIMITATIONS OF THIS STUDY

- We will determine if discontinuing folic acid supplementation after 12 weeks of gestation results in lower levels of unmetabolised folic acid.
- Unmetabolised folic acid is a biomarker of excess folic, and has been associated with a number of adverse pregnanacy outocomes.
- This study is not powered to deterimine the effect of continuing folic acid supplements after the first trimester on clinical outcomes.
- The study findings will be generalisable to countries which like Austrlia have mandatory folic acid fortificiation
- This research will inform the need for larger trials to determine if folic acid beyond the first trimester leads to adverse maternal and infant health outcomes.

Caption for Figure 1. Folic Acid Trial Schedule

INTRODUCTION

Evidence from randomised controlled trials (1,2) and a large public health intervention (3) showed that taking folic acid containing supplements prior to and during early pregnancy reduces the incidence of neural tube defects (NTD). Based on these findings, public health agencies around the world issued recommendations advising women to take folic acid supplements prior to conception and during early pregnancy.(4) For example, in Australia the government recommends that women trying to become pregnant take a folic acid supplement of 0.5 mg/day 12 weeks prior to conceiving and for the first 12 weeks of pregnancy.(5)

The neural tube closes in the first month of pregnancy, beyond this time there is no proven benefit of taking folic acid.(6) However, many women continue to take folic acid as part of a prenatal vitamin and mineral supplement throughout pregnancy.(7) In Australia, for example, a randomised controlled trial of pregnant women showed that more than 80% of women were taking a prenatal supplement containing folic acid at some time during their pregnancy,(8) with the market leading supplement containing 0.8 mg of folic acid. Furthermore, almost 80 countries, including Australia, have mandated the addition of folic acid to food staples, typically wheat flour, to reduce NTDs in unplanned pregnancies.(9) As such, the combination of food fortification along with prenatal supplement use may expose women and their fetus to excessive amounts of folic acid.

There is emerging evidence that higher intakes of folic acid in pregnancy may have negative health effects on the offspring including autism spectrum disorders (10–12) and insulin resistance. (13) An increased risk of childhood allergic disease is chief amongst these concerns with several studies reporting an inverse association with folic acid (14–16) (17–24) However, results are inconsistent and some studies report no relationship between folic acid intake and allergy outcomes in offspring (25–27) or a reduction in risk of allergic disease.(28) These

Caption for Figure 1. Folic Acid Trial Schedule

studies vary greatly in regard to the timing and measurement of exposure and only one study differentiated between maternal total plasma folate and maternal plasma unmetabolized folic acid (UMFA).(29)

Folic acid is the synthetic form of the vitamin folate that is used in supplements and fortified foods because of its high bioavailability and stability compared to naturally occurring folate in food.(30) Once consumed, folic acid must be converted into an active form, 5-methyltetrahydrofolate.(31) At higher intakes folic acid is not converted to its active form and accumulates in plasma as unmetabolised folic acid (UMFA).(32) Circulating UMFA has been proposed as a biomarker of excess folic acid intake.(33) Without proven benefit and with the suggestion of harm, the amount of folic acid in prenatal supplements may need to be reduced after the first trimester. We aim to determine if removing folic acid from prenatal multivitamin supplements after the first trimester (12 weeks gestation) reduces the accumulation of maternal UMFA measured at 36 weeks gestation.

Hypotheses

Removing folic acid from prenatal supplements after 12 weeks of gestation will limit the accumulation of UMFA in maternal plasma at 36 weeks of gestation.

METHODS AND ANALYSIS

Trial design

A multicentre two arm parallel design, double-blinded randomised controlled trial.

Participating Centres

Caption for Figure 1. Folic Acid Trial Schedule

The sponsoring institution and Trial Coordinating Centre is the South Australian Health and Medical Research Insititute (SAHMRI) based at the Women's and Children's Hospital (WCH). We will also seek approval to conduct the trial at Flinders Medical Centre (FMC), Adelaide, South Australia.

Study Population

Participants are pregnant women with a singleton pregnancy enrolled between ≥12 and <16 weeks gestation. Enrolment commenced on 18th December 2019 and recruitment is on-going. Data collection will continue through to May 2021.

Eligibility Criteria

Inclusion Criteria

To be eligible for participation women must be:

1) Carrying a singleton pregnancy ≥12 and <16 weeks gestation and;

2) Currently taking a folic acid containing supplement and planning to continue this throughout pregnancy and;

3) Able to give informed consent.

Exclusion criteria

Women will be ineligible for trial participation if they meet any the following criteria:

1) Carrying a fetus with a confirmed or suspected fetal abnormality.

2) Unwilling to cease current folic acid containing supplement/s.

3) Past history of a NTD affected pregnancy.

4) Currently taking medication known to interfere with folate metabolism (e.g. methotrexate, sulphasalazine, anti-convulsants, antimalarials or barbiturates).

5) Known haemolytic anaemia or haemoglobinopathy.

Caption for Figure 1. Folic Acid Trial Schedule

6) Known to carry the TT variant of the methylene tetrahydrofolate reductase gene (MTHFR C77T) polymorphism.

7) Intolerance or allergy to prenatal vitamin and mineral supplements.

Study treatments

Participating women will be randomised to receive either a micronutrient supplement in tablet form, containing 0.8 mg folic acid (the dose in the most commonly used supplement in Australia) or an identical micronutrient supplement containing no folic acid. The composition of micronutrients within the intervention and control supplements are formulated to approximate leading brands of prenatal micronutrient supplements available in Australia, **Table 1**. Intervention and control supplements are identical in size, shape, colour and packaging and only differ in the removal of folic acid from the intervention supplement. Women will be asked to consume one supplement per day from enrolment (≥12 and <16 weeks of gestation) until delivery.

Manufacture of study supplements

Intevention and control supplements are manufactured in a licensed facility in accordance with the Code of Good Manufacturing Practice (GMP) for Medicinal Products and have been donated to the trial by Factors Group of Companies, Coquitlam, British Columbia, Canada. The supplements are packaged and labelled in accordance with GMP including an individual product identifier (ID), batch number, expiry date and the statement 'for clinical trial use only'. The pharmacist or the investigator's designee maintains accurate records of the dispensing of study product. Unused study supplements will be destroyed in compliance with applicable regulations.

Caption for Figure 1. Folic Acid Trial Schedule

Monitoring adherence to study treatment

Research personnel will maintain regular contact with participating women to monitor and encourage supplement adherence and study compliance and answer any questions as they arise. At each contact, women will be asked if they have missed any supplements in the last week and if so, how many have been missed. Women will be asked to return unused supplements at the final study visit (36 weeks' gestation) and the proportion of supplements returned will serve as the primary measure of compliance. A woman will be classified as compliant if she takes greater than 80% of her study supplements. At this visit women will be issued with enough supplements to last until the delivery of their baby.

Outcome measures

The primary outcome is maternal plasma UMFA concentration at 36 weeks gestation.

Secondary outcomes

- Maternal plasma total and red blood cell (RBC) folate levels at 36 weeks' gestation.
- Gestational age at birth, birth weight, birth length, birth head circumference.

Safety outcomes

- Neonatal complications requiring admission to the neonatal unit.
- Pregnancy complications requiring hospital admission.
- Serious adverse events defined as: maternal or fetal (>20 weeks) deaths, fetal loss (< 20 weeks), maternal or neonatal admissions to intensive care and major congenital anomalies.

Participant timeline

Caption for Figure 1. Folic Acid Trial Schedule

Women will be randomised and asked to cease their current prenatal supplements immediately and for the duration of the study. At enrolment, following informed consent and prior to commencement of the study treatment, research personnel will collect baseline clinical and demographic data including: contact details, self-reported ethnicity, gravida, parity, age, supplement and prescription drug use, weight, height, highest level of education, occupation and smoking status. Maternal dietary intakes of folate and other one-carbon nutrients during early and late pregnancy will be collected with the use of an 80-item semi-quantitative food-frequency questionnaire – Dietary Questionnaire for Epidemiological Studies (DQES v3.2).(34) A 10ml venous blood sample will be collected by venepuncture to assess UMFA, folate status and full blood count. The time the woman last ate and drank as well as the time her last supplement was taken will be recorded. Research personnel will contact the participant one week following the enrolment visit and then monthly to ensure adherence and record adverse events, Figure 1. At 36 weeks' gestation participating women will attend a clinic appointment for collection of venous blood sample for UMFA and folate analysis and full blood count. Participants will be asked to return unused supplements which will be counted as a measure of compliance. The food-frequency questionnaire will be repeated and women will be given enough supplements to last the remainder of their pregnancy. Following delivery, research personnel will extract details of pregnancy, labour and birth from the woman and her baby's medical records. Blood samples will be analysed for UMFA according to established methods.(35) Plasma folate (nmol/L) and erythrocyte folate (nmol/L) concentrations will be determined using the folate microbiological assay harmonized by the Centers for Disease Control and Prevention.(36)

Caption for Figure 1. Folic Acid Trial Schedule

Sample size

A sample size of 90 women (45 per group) will provide >90% power to detect a standardised difference in mean UMFA concentration at 36 weeks gestation between groups of 0.60 (two-tailed alpha=0.05, correlation between UMFA concentrations at baseline and 36 weeks of gestation=0.60).(29) Calculations were performed based on a standardised mean difference (mean difference divided by standard deviation of outcome at 36 weeks gestation) due to considerable variability in the literature in the reported standard deviation for UMFA concentration in pregnancy.(16,29) A standardised mean difference of 0.60 represents a medium effect size and would demonstrate biologically excessive folic acid consumption. To allow for 10% loss to follow-up, we will randomise 50 women per group.

Recruitment

Pregnant women will be recruited through a combination of flyers,posters, a digital media campaign and through in-person recruitment at antenatal clinics. Women who meet eligibility crtiteria and agree to participate are invited to attend an enrolment appointment at our research clinics at the Women's and Children's Hospital or Flinders Medical Centre, Adelaide between 12 and 16 weeks gestation.

Randomisation procedures

Participants will be randomised using a secure web-based randomisation service. Allocation will follow a computer-generated randomisation schedule using balanced variable block sizes, prepared by an independent statistician who is not involved with trial participants or data analysis. A unique four-digit study identification number and a coloured coded study pack are

Caption for Figure 1. Folic Acid Trial Schedule

assigned to each participant. Stratification will be by gestational age at trial entry 12 to ≤14 weeks or >14 to 16 weeks gestation.

Blinding

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 with a colour 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.

The intervention and control supplements are identical in size, shape, colour, packaging and labelling and uniquely identified by the coloured product identification label (Yellow, Pink, Blue or Green) only. The randomisation code for an individual participant may be unblinded by the independent statistician in the event of an emergency.

Data collection and trial management

Data are collected by trained research personnel and entered directly into an electronic case-report form with password protection and defined user-level access Research Electronic Data Capture (REDCap). A record of all women approached, screened for eligibility and consented will be recorded.(37) Once consented and randomised, REDCap has been designed to automatically calculate study milestones for each participant. This information is readily available for clinical trial staff to enable scheduling of appointments and sample collection. Summary reports including screening data, enrolment, appointment attendance, sample collection, serious adverse events and study completion are generated from REDCap and reviewed at monthly trial steering committee meetings. Electronic data are stored on secure

Caption for Figure 1. Folic Acid Trial Schedule

servers at South Australia Health and Medical Research Institute and released only to persons authorised to receive those data.

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.

Statistical analysis

Statistical analyses will be performed on an intention-to-treat basis according to a pre-specified statistical analysis plan. For the primary outcome, we will assess whether there is a difference in mean UMFA levels at 36 weeks gestation between groups using linear regression, with adjustment for baseline UMFA and the stratification variable gestational age at trial entry (12 to ≤ 14 weeks or > 14 weeks). The treatment effect will be described as a mean difference with 95% confidence interval. Secondary outcomes will be analysed using linear and logistic regression models for continuous and binary outcomes, respectively, again with adjustment for gestational age at trial entry. Safety outcomes will be compared between groups using Fisher exact tests. In all analyses, a two-sided p-value <0.05 will be taken to indicate statistical significance.

ETHICS AND DISSEMINATION

Human Research Ethics Approval

This protocol, the informed consent and participant information document and all participant communication have been approved by the Women's and Children's Health Network Research

Caption for Figure 1. Folic Acid Trial Schedule

Ethics Committee (HREC) (HREC/19/WCHN/018) and Governance (SSA/19/WCHN/080). Governance approval has also been obtained from FMC. Any subsequent modifications will be reviewed and approved by the HREC and governance of each study site. The study will be conducted in compliance with the current approved version of the protocol. Any change to the protocol document or informed consent form that affects the scientific intent, study design, patient safety, or may affect a participant's willingness to continue participation in the study will be considered a major amendment. All such amendments will be submitted to the HREC for approval. Any other changes to the protocol (such as administrative changes to dates and study personnel) will be considered minor amendments and will be notified to the HREC as appropriate.

Confidentiality

Participant confidentiality is strictly held in trust by the participating investigators, research staff and their agents. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Regulatory authorities may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records for the women and/or infants in this study subject to individuals having obtained approval/clearance through State/National Governments and HREC as required by local laws. Clinical information will not be released without written permission of the parent, except as necessary for monitoring by HREC or regulatory agencies.

Patient and public involvement

The study was supported by a consumer advisory group which provided input to the protocol. A Consumer representative from our SAHMRI Women and Kids Consumer Advisory Group partnered with us for the design of the study, informational material to support the intervention,

Caption for Figure 1. Folic Acid Trial Schedule

and the burden of the intervention from the participant's perspective. We will meet with the consumer representative for this trial and the full Consumer Advisory group on a regular basis for the duration of the study. At the end of the study, the consumer advisory group will be given the opportunity to comment on the findings and contribute to the dissemination plan.

Dissemination Plan

Study findings will be submitted for peer-reviewed publication and for presentation at appropriate local and international conferences. In addition, study findings will be disseminated to participants through a one-page lay summary. Results will be made available to the wider community through social media avenues and the SAHMRI website.

Authors' contributions

KPB, TJG, MM, DP and MS conceived the trial and proposed the trial design; TS advised on sample size calculations, trial design, and analysis; SW and TJG designed the prenatal supplement and had it manufactured; DCS advised on analytical methodology; DCS, TJG and KPB drafted the protocol, all authors contributed to refinement of the study and approved the final manuscript.

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Caption for Figure 1. Folic Acid Trial Schedule

Program Scholarship from The University of Adelaide. The study product is donated by Factors Group of Companies, Coquitlam, British Columbia, Canada. The funder/s have no role in the study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication and have no authority over any of these activities.

Competing interest statement

Dr. Makrides reports that she has a financial relationship outside the submitted work with Trajan Nutrition as a member of the board. Simon Wood is a consultant for the Factors Group of Companies. DCS, TJG, DJP, TS MS, and KPB have nothing to disclose.

Data sharing statement

Once the primary trial is published, data will be available for data sharing to appropriately qualified investigators upon submission of a protocol and approval by the Trial Steering Committee. Please send requests to Dr Karen P Best (karen.best@sahmri.com).

Caption for Figure 1. Folic Acid Trial Schedule

Table 1. Ingredients of Supplements for Intervention and Control Groups

Ingredients	Intervention	Control	unit
folic acid	0	0.8	mg
calcium	250	250	mg
Iron	27	27	mg
thiamine	1.4	1.4	mg
riboflavin	1.4	1.4	mg
niacinamide	18	18	mg
vitamin B-6	1.9	1.9	mg
vitamin B-12	2.6	2.6	mcg
pantothenic acid	6	6	mg
biotin	30	30	mg
vitamin C	85	85	mg
vitamin E	13.5	13.5	IU
magnesium	50	50	mg
zinc	7.5	7.5	mg
manganese	2.0	2.0	mg
iodine	0.22	0.22	mg
copper	1	1	mg
chromium	30	1	mcg
selenium	30	30	mcg
Vitamin D3	10	10	mcg
b-carotene	2500	2500	IU

Caption for Figure 1. Folic Acid Trial Schedule

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Caption for Figure 1. Folic Acid Trial Schedule

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Caption for Figure 1. Folic Acid Trial Schedule

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Caption for Figure 1. Folic Acid Trial Schedule

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Caption for Figure 1. Folic Acid Trial Schedule

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Figure 1. Folic Acid Trial Schedule

	Screening	Enrolment	Allocation	+1 week		Post-allocation				
TIMEPOINT**		$-t_1$	0	t_1	t_{1a}*	t_2	t_3	t_4	t_5	t_6
ENROLMENT:	<12w	≥12 to <16w		13 to17w	16w	20w	24w	28w	32w	36w
	Clinic	Clinic	Clinic	Phone	SMS	SMS	SMS	SMS	Phone	Clinic
Eligibility screen	X	X								
Consent to Contact	X									
Informed consent		X								
Randomisation			X							
Demographic data		X								
Allocation			X							
INTERVENTION:			←	—	—	—	—	—	→	
ASSESSMENTS:										
Maternal Folate status		X								X
Maternal UMFA status		X								X
Food Frequency Questionairre		X								X
Adverse events					X	X		X		X
Serious Adverse events										X
Compliance – maternal report					X	X	X	X	X	X
Compliance – Supplement count										X

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	Yes
Protocol version	3	Date and version identifier	Supp.
Funding	4	Sources and types of financial, material, and other support	14-15
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	N/A
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	N/A
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	12, 13

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-5
	6b	Explanation for choice of comparators	4-5
Objectives	7	Specific objectives or hypotheses	5, 8
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	N/A
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	7
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	N/A
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	8
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	8, 9, 10 & Fig. 1

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	10, 11
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	11
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	11
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	11
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8, 11, 12
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11, 12
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	12
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8, 9, 12
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	7

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	12
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	12
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	12
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	13
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	13
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	13
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	13
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	13

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	8, 13
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	13
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	15
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	N/A
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Supp. PICF
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	14
	31b	Authorship eligibility guidelines and any intended use of professional writers	N/A
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Supp. PICF
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	Supp. PICF

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.