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Supplementation of vitamin B_{12} in pregnancy and postpartum on growth and neurodevelopment in early childhood: Study Protocol for a Randomized Placebo Controlled Trial

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Abstract

Introduction: Vitamin B12 is crucial for normal cell division and differentiation, and necessary for the development and myelination of the central nervous system. Pregnant mothers in resource poor settings often lack vitamin B12. Poor vitamin B12 status in infancy is linked to poor growth and neurodevelopment. The most important time for neurodevelopment starts in utero.

Methods and analysis: Individually randomized double-blind placebo controlled trial in pregnant Nepalese women at risk of poor vitamin B12 status. Participants will be randomized in a 1:1 ratio. 600 pregnant women will be enrolled as early as possible, but no later than in week 15 of pregnancy. A daily dose of 50 µg of vitamin B12 or placebo will be given to women from early pregnancy until 6 months after birth and monthly visits will be conducted in order to record compliance, growth and morbidity. Neurodevelopment will be measured by the Bayley Scales of Infant and Toddler Development 3rd ed. at 6 and 12 months of age, and growth by measuring gestational age and birth weight at delivery and monthly weight and height of the infants up to one year of age. We will compare the mean Bayley-III scores (total score and scores on the subscales: cognitive, language and motor, with the motor scale analyzed both separately for fine and gross motor development and as composite measure) between the vitamin B12 group and the placebo group. For growth, the main outcome will be measured when the child is 12 months of age.

Ethics and dissemination: National Health and Research Council, Nepal and Regional Committee for Medical and Health Research Ethics of Western Norway have approved the study. The results from this study may support new dietary guidelines for Nepalese and possibly South Asian pregnant women that can lead to improved pregnancy outcomes, neurodevelopment and cognitive functioning in children

Protocol date: 4 December, 2016

Keywords: vitamin B12, pregnant mother, clinical trial, neurodevelopment, Nepalese infants

Strengths and limitations of this study

- Deficiency of vitamin B12 during pregnancy is associated with adverse pregnancy outcomes, poor growth and neurodevelopment among infants as it is crucial for cell division and myelination of the central nervous system. This study will add to the knowledge of the potential beneficial effect of vitamin B12 to improve pregnancy outcomes and neurodevelopment in infants.
- Many children in low and middle income countries fail to reach their developmental potential due to many factors such as poverty, illiteracy, malnutrition and lack of stimulation. One of the main outcomes of this study is neurodevelopment in young children by using the best available cognitive tools.
- A potential caveat in this study is that not all women will have poor vitamin B12 status and if this proportion is high, it will reduce our statistical power.
- The primary outcomes will be measured when the children are 12 months old. A beneficial effect of improving vitamin B12 status of the mother might not be measurable until later in life.
- It is a challenge to measure neurodevelopment during infancy, particularly when the tools have been developed in different settings.

Background

Cobalamin (Vitamin B12) deficiency is common in many low- and middle-income countries [1, 2]. This is not surprising as the main source of vitamin B12 is animal source foods, which are expensive and for cultural and religious reasons often not eaten at all. We have in several studies in women and children demonstrated that poor vitamin B12 status is common in South Asia [3-5]. There is also compelling evidence that vitamin B12 deficiency occurs frequently during pregnancy [2, 6, 7], and case studies have demonstrated harmful effects of severe vitamin B12 deficiency on the developing infant brain [8, 9]. The consequences of mild or subclinical vitamin B12 deficiency are less clear but it has been shown to be associated with decreased cognitive performance in both the elderly and children [10-13]. Three randomized controlled trials (RCT) have measured the effect of vitamin B12 supplementation on neurodevelopment in children: In a Norwegian trial, an intramuscular injection of B12 substantially improved motor development in six weeks old infants after one month [14]. Another intervention study in low birth weight children in Norway recently confirmed these findings [15]. The infants in these studies had evidence of suboptimal vitamin B12 status, but none was severely deficient. We found a beneficial effect of vitamin B12 supplementation for six months on neurodevelopment in young North Indian children [16]. In this study, where the children were supplemented daily with two recommended daily allowances (RDA) for six months, the effect of vitamin B12 supplementation was more apparent in children who had evidence of vitamin B12 deficiency at the start of the study.

During pregnancy, vitamin B12 is concentrated in the fetus and stored in the liver [17, 18]. Infants born to vitamin B12-replete mothers have stores of vitamin B12 that are adequate to

sustain them for the first several months postpartum. Consequently, vitamin B12 deficiency rarely occurs before the infant is about four months old if the mother has adequate vitamin B12 status during pregnancy. However, infants of vitamin B12-deficient breastfeeding mothers are vulnerable to B12 deficiency from an early age [19].

Maternal vitamin B12 deficiency has been associated with increased risk of common pregnancy complications, including spontaneous abortion, low birth weight, intrauterine growth restriction, and neural tube defects [20-22]. Children born to vitamin B12-deficient women are at increased risk for adverse health outcomes, including developmental abnormalities and anemia [9, 23]. In a recent RCT in Bangalore, India, daily maternal vitamin B12 supplementation (50 μg/d) during pregnancy through six weeks postpartum, substantially improved maternal vitamin B12 status and increased breast milk and infant plasma vitamin B12 concentrations [24]. In this study the proportion of children being born small for gestational age was lower in the vitamin B12 group than in the placebo group (25% vs 34%), however no difference was found in cognitive development in infants at 9 months of age [25]. Similar improvements in vitamin B12 status was found among Bangladeshi mothers and infants when supplemented with 250 μg of vitamin B12 from 11-14 weeks of pregnancy through 3 months postpartum [26]. In this study women that were randomized to receive vitamin B12 also had an improved immune response to the pandemic influenza A (H1N1) vaccine.

Vitamin B12 works with folate and together they are required for cell division. Improper red blood cell production due to impaired cell growth is the main mechanism behind anemia in severe vitamin B12 deficiency. However, mild deficiency may have consequences through other metabolic pathways: Vitamin B12 is important for intracellular energy production and for the generation of methionine, which again is needed in the production of neurotransmitters and

myelin. Myelin is the principal component of white matter in the brain, and important for nerve conductivity. Disruptions in myelination alter the speed of conduction in multiple systems of the central nervous system. Myelination can also be impaired due to accumulation of metabolites when folic acid is supplemented to individuals with vitamin B12 deficiency [27].

The first 1000 days after conception are regarded as the most critical time for brain development. During this period brain growth is rapid, increasing the susceptibility to influences from the environment [28]. This is also the period when most of the myelination of the brain occurs [9]. In infants, moderate and severe vitamin B12 deficiency has been associated with demyelination and brain atrophy [29, 30]. Little is known about the consequences of maternal vitamin B12 deficiency for early brain myelination, neurodevelopment and cognitive function. In this randomized double-blind placebo controlled trial in pregnant Nepalese women, we hypothesize that daily supplementation of 50 µg of vitamin B12 given to mothers from early pregnancy until 6 months after birth improve offspring neurodevelopment and growth in first two years of life.

Methods

Study design and aims

We will undertake a double-blind randomized placebo-controlled parallel group superiority trial to test this hypothesis. The trial will also estimate the efficacy of cobalamin on other health related outcomes in the mothers and children (see below).

Specific objectives

Primary objectives:

1) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves scores of the Bayley scales of infant and toddler development 3rd. ed. (Bayley-III) in the infants at 6 and 12 months of age.

2) Measure whether daily maternal administration of vitamin B12 from early pregnancy until six months postpartum improves an infant's z-scores length for age, weight for age and weight for length at 12 months.

Secondary objectives

1) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves cognitive function of infants as measured by the Bayley-III at 24 months of age and the Ages and Stages questionnaire 3rd edition measured at 6 and 12 months of age.

2) Measure whether daily maternal administration of vitamin B12 from early pregnancy until six months postpartum improves an infant's z-scores length for age, weight for age and weight for length at birth, after 1 month and at 3, 6 and 9 months.

3) Measure whether daily maternal administration of 50 µg vitamin B12 from early pregnancy until six months postpartum improves hemoglobin concentration of the mother and infant.

4) In the subgroup of children whose mothers had a B12 concentration < 150 pmol/L: Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves scores of an infant's Bayley-III at 6 and 12 months of age.

5) In the subgroup of children whose mothers has a B12 concentration < 150 pmol/L: Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves the z-scores weight for age, weight for length and length for age.

6) Measure whether daily maternal administration of B12 from early pregnancy until 6 months postpartum improves sleep, physical activity, parent-child interaction and maternal depression.

7) Explore if sleep, heart rate variability, physical activity, parent-child interaction and maternal depression serve as mediators/moderators between vitamin B12 and the main outcomes of the study.

Population and study setting

We will include 18-40 years old pregnant women from early pregnancy, not later than 15 weeks pregnant, residing in Bhaktapur municipality and surrounding areas in Bhaktapur district. Those who give consent for participation will be screened and will be enrolled.

Enrollment plan

Prior and throughout the study period, we will identify pregnant mothers through a hospital and community based surveillance system (Figure 1). Records of newly married couples in the study area will be maintained and updated quarterly. Information regarding pregnancies will be obtained monthly by household surveys or phone calls.

Exclusion criteria

1. Taking dietary or multivitamin supplements containing vitamin B12,
2. Known cases of chronic disease like tuberculosis, diabetes, hypertension, hypo or hyperthyroidism, pernicious anemia, Crohn's disease and current use of anticonvulsant drugs
3. Severe anemia (hemoglobin concentration <7 g/dL),
4. Suffering from any condition that requires treatment with vitamin B12.

Randomization and blinding

We will randomize women in a 1:1 ratio in blocks of 8. The vitamin B12 supplements and the placebos will be produced specifically for this trial and be identical in taste and appearance. Each woman will be assigned a packet of supplementation according to her id number. This packet will only be labeled with general information about the study and a unique id number. The list that links the id number to the randomization code will be kept with the company that produces the intervention and the placebo, and with the scientist who will generate the randomization code. This scientist will otherwise not be involved in the study. None of the investigators will have access to this list until completion of data collection, analysis and interpretation. We will use the STATA (Stata inc. College Station, TX) and the stata-script "randomize" (net from http://folk.uib.no/mihtr/stata/) to generate the randomization list.

Confirmation of pregnancy and assessment of gestational age

Once the pregnancy is detected by standard HCG kits, ultrasonography (USG) will be performed to confirm pregnancy as well as for the estimation of gestational age. Follow up USG will be performed between 18 to 22 weeks for anomaly scan.

Intervention/co-intervention and rational for choosing 50 µg dose of vitamin B12

The enrolled women will receive daily oral supplement of 50 µg vitamin B12 or placebo from enrollment until 6 months postpartum. All pregnant women will also be given iron, folic acid and calcium supplements according to national guidelines. Based on results from previous studies in this area, we expect the intake of vitamin B12 among women in our study to be low, both due to a predominant plant based diet, but also because of poor gut function that again may disturb vitamin B12 absorption and increase dose requirements. Absorption of vitamin B12 is strictly regulated, and the body is not able to absorb more than 2-4 µg per day. The vitamin B12

supplementation study in pregnancy from South India [24], and the study from Bangladesh [26] used very high doses of 20-100 x RDA with no reported adverse effects. There are other, large RCTs where similarly high doses of vitamin B12 have been given together with other micronutrients [31, 32]. These studies found that the micronutrient mixture that included high doses of vitamin B12 reduced the risk for SGA with no adverse effects. In Europe and the US, vegans and vegetarians are aware that there is a risk of B12 deficiency that can result in poor neurodevelopment and other adverse outcomes [33]. Many therefore take additional supplements with high doses of B12 also during pregnancy.

Laboratory procedures

From the mother, blood samples will be obtained at enrollment (baseline) and at the end of supplementation (6 months post partum). In the infants we will collect blood samples at 6 months. Three mL of blood will be collected into vials containing EDTA as anticoagulant. The plasma will be centrifuged at approx. 700 g at room temperature for 10 minutes, separated and transferred into storage vials, and stored at -70 degrees before analysis.

The blood samples will be analyzed for

1) Hemoglobin concentration by using HemoCue B immediately after blood collection (HemoCue, Vedbæk, Denmark).

2) Plasma vitamin B12 and plasma folate concentrations will be estimated by microbiological assays using a chloramphenicol resistant strain of Lactobacillus casei and colistin sulphate resistant strain of Lactobacillus leichmannii, respectively.

3) The plasma will be stored and used for other biomarkers (nutrients, markers of inflammation, epigenetic markers, and markers related to neurodevelopment).

Follow up

We will keep track of the enrolled women in a GPS/mobile based surveillance system. Enrolled women will be visited every week in the period of supplementation and the field worker will ask questions about her physical condition, compliance and adverse events. The family/women will be asked to inform us when she goes into labor and we will ensure transportation to an appropriated hospital where she will give birth. This way we will also ensure that we get information about the pregnancy outcome. We will not try to influence the choice of place to deliver. However, we will encourage the women to deliver in a health facility rather than at home. In the Kathmandu valley, less than 5% of deliveries are at home.

After delivery we will follow the child at home every month until 2 years of age. We will collect information on breastfeeding and complementary feeding (frequency, whether other drinks or solids/semi solids have been given). We will also ensure that the child receives standard vaccines as per the EPI schedule. We will measure growth and dietary recalls every three months. Neurodevelopment will be measured at different time points during infancy.

Safety considerations, safety monitoring and adverse event reporting

All adverse events (AE) and serious adverse events (SAE) will be recorded by the study staff. AEs are graded from 1 to 5 according to their severity according to Common Terminology Criteria for Adverse Events (CTCAE) [34]. All immediate adverse events following each dose of intervention will be documented for all women.

All adverse events will be followed up till resolution or stabilization as judged by gynecologist or pediatrician (site investigator) and the principal investigator. All SAEs will be followed up until satisfactory resolution or until the treating physicians and the principal investigator deems the event to be chronic or the participant to be stable.

SAE, that includes critical or life threatening illness or death, will be documented from the time of enrolment, throughout the study period. The pregnant women will be visited every week. During each contact the study staff will collect data to ascertain SAEs and illness requiring hospitalization. All SAEs of death, development of signs of critical illness and severe illness requiring hospitalization and AEs as described above will be reported to the Ethics Committees, the Data and Safety Monitoring Board (DSMB) and to the sponsors of the study within 24 hours of awareness of the event followed by a final report within 10 days. SAE relatedness to the administration of vitamin B12 or placebo will be judged by the investigator/designee, the Ethics Committee and the DSMB who will have access to all relevant investigations, clinical assessments and management details**.**

Data and Safety Monitoring Boards (DSMBs)

The DSMB is comprised of a pediatrician, public health expert and a biostatistician. The DSMB will be independent from the sponsor and will have no competing interests. The DSMB members will prepare a charter and decide *a priori* on study stopping rules, and will review SAEs and AEs reported in the study periodically. They will examine all pregnancy complications, cases of severe illness, infant deaths and other SAEs to decide if the study should be continued, based on the pre-decided stopping rules. After one third of the study participants have been enrolled and completed follow-up, the DSMBs will review the data and make recommendations concerning continuation, modification or termination of the study due

to unexpectedly large beneficial effects or serious side effects and can suggest extension of the trial should the primary outcomes occur less often than anticipated.

Sample size calculations

Bayley-III scores at 12 months of age.

A standardized effect size of .25 SD corresponding to a difference of 3.75 composite Bayley scores (with an expected standard deviation of 15) is programmatically relevant. With these assumptions we need data from 506 mother children pairs (253 per group) with a power of 80%. Assuming a loss to follow up of 15% we need 600 mother children pair. Sample sizes adjusted for expected losses to follow up of the various reasons are shown below.

Expected losses to follow up		
	Late abortions	5%
	Drop - outs during pregnancy	4%
	Drop -outs during infancy, including infant mortality	6%

These figures are based on national figures (Nepal Demographic Survey 2011) but our experiences from the field site indicated that infant morbidity and mortality is expected to be lower because of the proximity to the capital Kathmandu and close regular follow up.

Z-scores weight for length, weight for age, length for age at 12 months of age.
A difference of 0.25 z-scores is programmatically relevant. We expect the SD of the z-scores to be 1. With these assumptions we also need valid data from 506 children with a power of 80%. Our experience with similar studies is that the SDs of our study populations is lower than what is expected, so we believe that our sample size estimates are conservative. The power to detect larger and even more programmatically relevant differences is higher.

Outcomes

Anthropometry

Weights will be measured with a portable electronic scale that measures to the nearest 0.01 kg (portable Seca weight). Height and length (portable Seca board) will be measured according to standard guidelines. Both length and weight will be measured in their homes immediately after birth and at 1, 3, 6, 9, 12, 18 and 24 months.

Neurodevelopment

Bayley Scale of Infant and Toddler development 3rd ed. (Bayley-III) is a comprehensive assessment tool of developmental functioning in infants and toddlers age 1-42 months [35]. It is administered directly with the child and provides information on functioning in cognition, language, fine and gross motor abilities and social-emotional functioning. The Bayley-III is considered to be the gold standard in developmental assessment of this age-group, and is widely used for research purposes. Summary scores of the Bayley-III at 6 and 12 months of age will be the primary outcome in the current study, while assessment at 24 months will serve as secondary outcome.

The Ages and Stages Questionnaire – 3rd ed. (ASQ-3) is an easily administered checklist of developmental status standardized for children 1-66 months [36]. The screening system includes age-appropriate questionnaires for every two/three-month intervals. Each questionnaire contains 30 items in five domains: communication, gross motor, fine motor, problem solving and personal social. The questionnaires are designed to be completed by caregivers, but can also be administered by a trained examiner which will be done in the current study. The ASQ-3 will be administered in the homes of the families and provide us the opportunity to assess developmental trajectories.

Actigraph (the Actiwatch 2 (Phillips)) is a wristwatch-like device that records motion data. Using a validated scoring algorithm, the actiware® software translates the activity data to sleep-wake-patterns and activity level. Sleep problems and sleep patterns will be further corroborated by questionnaire data (the Brief Infant Sleep Questionnaire (BISQ)). The BISQ has demonstrated good psychometric properties as a brief sleep screening tool for clinical and research purposes in infants and toddlers (0-30 months) [37].

Alarm Distress Baby Scale (ADBB) is a measure of social withdrawal in infants and young children 2-24 months of age, and provides information of the infant social abilities and the quality of the ongoing parent-child interactions through standardized observations of the child in interaction with a stranger.

Heart rate variability (Vagal tone) is a marker of parasympathetic activity. Vagal tone has been suggested to be a sensitive marker of self-regulation and cognitive control and associated with the status of vitamin B12 during pregnancy [38], and may be an important, subjective measure of early risk identifier of child development and we plan to measure in children at 3, 12 and 24 months of age.

Eye tracking involves the process of measuring points of gaze (where one is looking). For this purpose, a sensor bar with infrared diods will be placed below a video screen (e.g., a laptop or other monitor). All visual stimuli are harmless and without strong emotional content. The sensors measure gaze direction and fixation duration with high temporal resolution, and is considered a measure of early cognitive functioning.

Test for Infant Motor Performance (TIMP) is a test of functional motor behavior which can be used up to 4 months of age [39, 40]. It consists of 42 items that are scored as present or absent based on observation. It has shown good predictive validity for later neurodevelopmental outcomes [41].

Questionnaires/Inventories

The Home Observation for the Measurement of the Environment (HOME Inventory) is an internationally recognized tool for measuring both the quality and quantity of stimulation and support available to the child in the home environment, which has been used successfully in over 100 countries and validated in several low and middle income countries [42]. It consists of 6 subscales; Parental Responsivity; Acceptance of Child; Organization of the Environment; Learning Materials; Parental Involvement, and Variety in Experience. A disadvantaged environment is among the many factors identified as influencing child cognition and development, and studies have demonstrated that the HOME inventory is a predictor of later school achievement [42]. Administration of the HOME inventory requires both interviewing the mother as well as observing the household. It takes approximately 45 minutes to 1 hour to complete, and trained field workers will conduct this assessment. Information about the child's environment will be collected so that we can account for the effect on child development in our study.

The Caregiver Knowledge of Child Development Inventory (CKCDI) is a brief questionnaire to assess parental knowledge of child developmental milestones and knowledge of developmental stimulation [43]. Parental knowledge on child developmental milestones may be of importance to promote adequate development in children. In this study we will use the CKCDI in the 3rd

trimester and at 12 months postpartum to account for the effect of the caregivers knowledge on child development.

Self-Report Questionnaire (SRQ-20) is a screening of mental health developed by the World Health Organization (WHO) especially for low to middle income countries [44]. The scores on the SRQ-20 may serve different purposes in the current study. We can estimate the association between maternal depressive scores and child development, and in addition, we may investigate possible effects of maternal vitamin B12 supplementation on depressive symptoms.

Quality control of field activities

The fieldwork will be monitored by skilled supervisors, medical doctors, gynecologists, and qualified psychologists. For all outcomes the study staff will undergo training before initiation of the project and staff will be utilized based on their skills. For a specified proportion (typically 4-6%) of the primary outcomes, a study supervisor or scientist will monitor the workers` performance. Some of these assessments will also be done independently. For example, when a field worker has measured weight and length of a child, a supervisor or scientist will undertake the same measurement and compare their findings with that of the field worker. For all main outcomes we will compare the reliability and precision of each examiner with the reliability of a gold standard. For example, for measuring length we will have training sessions where the field workers and a gold standard will measure each child twice. In a typical training session, each worker measures 10 children twice. The intra-individual variability (mean squared difference) of a trainee will be compared to that of the gold standard. For length measurements we will accept no more than 50% higher SDs of the examiner compared to the gold standard. In this exercise we can also compare the mean deviations of absolute differences between the gold standard and the worker. The mean deviation will also be compared to the mean variability of the

measurements of the gold standard. The deviations will also be compared on a qualitative scale to assess whether there are systematic higher of lower readings.

Quality control for the neurodevelopmental tools

All methods that will be used to measure neurodevelopment have been translated, adjusted and/or piloted in studies at the current study site. Medical doctors and qualified psychologists will train and monitor fieldworkers to perform the neurodevelopmental assessments such as the ASQ-3, HOME-assessment and the various questionnaires. Some of the comprehensive neurodevelopmental tools such as Bayley III will be administered by the medical doctors and psychologists. Ahead of study start, we will arrange training sessions and measure the performance of the staff compared with a gold standard. Trainings will build on existing competence and experience from ongoing studies at the field site. The trainings will be supervised and monitored by experienced psychologists from the Regional Center for Child and Adolescent Mental Health and Welfare, Uni Research Health, Bergen, Norway. Video recording will be done for all Bayley assessment and 10% of the videos will be scored independently for quality control.

Data management

All information will be collected in structured forms designed specifically for this project. A relational database will be designed using Microsoft Access. All forms will be checked manually by the study supervisors before they are sent for computer entry. All data will be entered twice by two different data operators within one week of data collection.

Data will be stored in secure servers in the field office and in a data management centre (DMC) in Kathmandu. A substantial proportion of the forms will also be captured using ipads or similar

tablets using the iformbuilder system (iformbuilder.com). We have used this electronic data capture system with success in many previous studies. Only computer data entry staff and supervisors will have access to the data. In the data-system, the entered data will be processed in several steps and the system will be designed to identify inconsistencies between different variables and different forms. The system will also be able to detect entries that are out of range and notify when information is missing. In this system we will also include reminders about study activities, i.e. the study staff will be notified when a birth or a scheduled vaccine is due. We will collect GPS coordinates and phone numbers from several family members. By this we can more easily reach the families and will probably reduce the numbers of missed home visits.

Data tracking, cleaning, and quality checks

The DMC will be responsible for initial cleaning of the data. Interim tabulations and scatter plots for some variables will be made at regular intervals to identify data errors. Special checks will be made on observations that are more than two or three standard deviations from the mean. There will be a regular feedback of errors from the DMC to the clinical sites.

Record Retention and Archival

All the study documents including participant's source data and documents will be archived by the study sites after the completion of the study, till the time the sponsor informs in writing to the study sites that they no longer need to maintain the study documents.

Plan of analysis

All analyses will initially be done on an intent-to-treat- basis. All randomized participants will be included in the analyses if the relevant outcome variables have been collected. The main

outcomes are continuous and expected to be normally distributed. We will use the Bayley-III scores at 6 and 12 months in separate analyses where B12 supplementation is the main exposure. We will compare the mean Bayley-III scores (total score and scores on the subscales: cognitive, language and motor, with the motor scale analyzed both separately for fine and gross motor development and as composite measure) between the vitamin B12 group and the placebo group.

For growth, the main outcome will be measured when the child is 12 months of age. For the secondary outcomes we will use a variety of statistical approaches. Following the model of postulated mechanisms (figure 3) there is a range of possible analyses where the data may be used as predictors, mediators or moderators. Before such analyses commence, we will lay out a plan of analysis for each of the research questions that will be addressed.

We will analyze the effect of vitamin B12 separately in the following subgroups:

Vitamin B12 status

-based on cobalamin, (cut off: 150 pmol/L)

-based on low total Homocysteine (cut off: 10 µmol/l)

-based on low Methyl Malonic Acid (cut off: 0.26 µmol/L)

-based on low composite B12 status score (<-.5) [67]

Maternal BMI (cut off: 18.5)

Vegetarian (yes / no)

Birth weight (cut off: 2500g)

Per protocol analysis

In addition to a standard per protocol analysis, we will use Instrumental Variable Analysis (IVA) in an attempt to estimate the true effect of cobalamin had it been given to all women in the

scheduled doses and intervals. The random allocation will be the instrument in these analyses. For per protocol analysis, women who receive less than 50% of the projected doses during pregnancy will not be included in the analyses, well acknowledging that the ensuing effect estimates may not only be biased but will certainly represent an effect higher than what can be achieved even in our well-resourced study setting.

Trial Status

The study will recruit its first pregnant women on 1st March 2017.

Discussion

This proposed study measure the effect of vitamin B12 supplementation started during early pregnancy and post-partum on early neurodevelopment and growth among 600 Nepalese infants. Randomized clinical trials on vitamin B12 in pregnancy is called for as concluded in a recent systematic review and meta-analysis the recent systematic review and meta-analysis [22]. This trial will add to the knowledge on the potential beneficial effect of vitamin B12 to improve pregnancy and perinatal outcomes [21, 45]. The results will be used to inform both regional dietary guidelines for Nepalese and possibly South Asian pregnant women. Further, The World Health Organization recently published the report "WHO recommendations on antenatal care for a positive pregnancy experience" recommend only iron/folic acid to all pregnant women [46] and our results possibly impact for update of this guideline.

Anemia presumably due to iron deficiency is still a major problem particularly among pregnant women residing in developing countries [47]. Since 2002, Government of Nepal intensified free iron folic acid supplementation program through female community health workers for pregnant and lactating mothers. In the last demographic survey, 80% of pregnant mothers in Nepal reported consumption of iron folic acid supplementation during last pregnancy and among them

56% took 90+ days [48] contributing to substantial reduction on maternal anemia. Despite this reduction in maternal anemia, recent data support that there are also a high burden of anemia possibly due to deficiencies of other nutrients [49]. In this proposed study, we will able to address how much burden of maternal anemia resolved by additional supplementation of vitamin B12.

Universal folic acid supplementation is recommended in early pregnancy for prevention of neural tube defects in offspring. Additional folic acid many not utilized effectively when it is given to the population with vitamin B12 deficiency possibly due to folate trap mechanism [27]. The imbalance of high folate and low vitamin B12 status during pregnancy increased insulin resistance in children [50] and low birth weight and other negative pregnancy outcomes [20, 51, 52]. Folate intake and status among women in our study area is high [4] so in our proposed study we will able to analyze its effect particularly among women who are having sub-optimal vitamin B12 status.

Government of Nepal with partnership of different organization such as WHO, UNICEF initiated many programs on mother and child care. There are great concerns that many children in low and middle income countries fail to reach their developmental potential due to many factors such as poverty, illiteracy, malnourish and lack of stimulation [53, 54]. One of the main outcomes of our study is improvement in neurodevelopmental in young children by using the best available tools which also may help to further integrate government of Nepal program on first 1000 golden days.

Competing Interests

The authors declare that they have no competing interests

Authors contributions

TS, IK, MH, RKC, conceived the study, contributed to study design, sample size calculations and analytical plans. TS, RKC, IK, MH drafted the manuscript. MU, RKC, TS, MS, SB, LS will initiate the project, have assisted in developing the protocol, and help with implementation. All authors read and approved the final manuscript

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Registration:

Universal Trial Number: U1111-1183-4093

Trial registration: clinicaltrials.gov: NCT

Figure 1. Study flow chart- Recruitment, supplementation and follow up plan

Figure 2. Estimated required total sample sizes based on relevant effect sizes at 80 and 90 % power.

Figure 3. Postulated indirect pathways between vitamin B12 status and Neurodevelopment and growth

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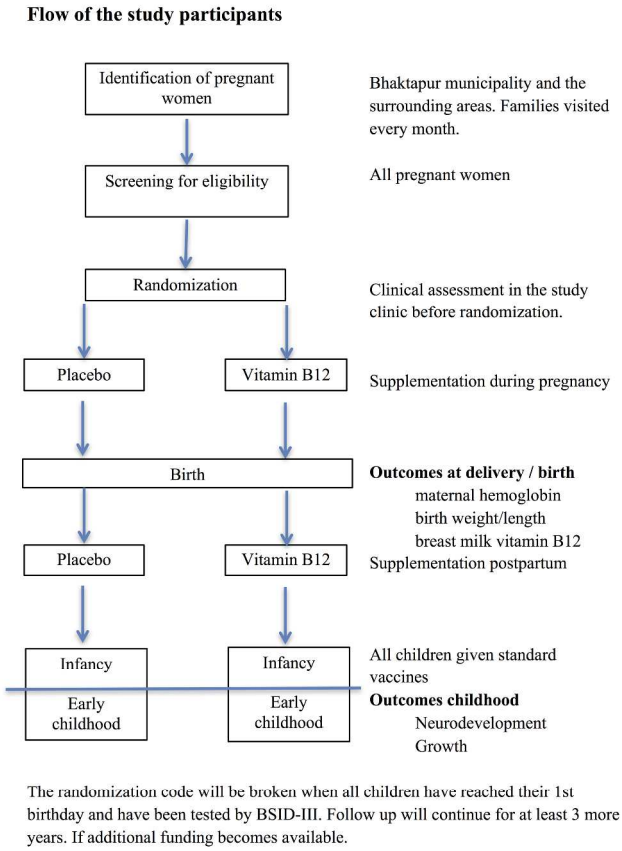

Figure 1. Study flow chart- Recruitment, supplementation and follow up plan‼ ┼

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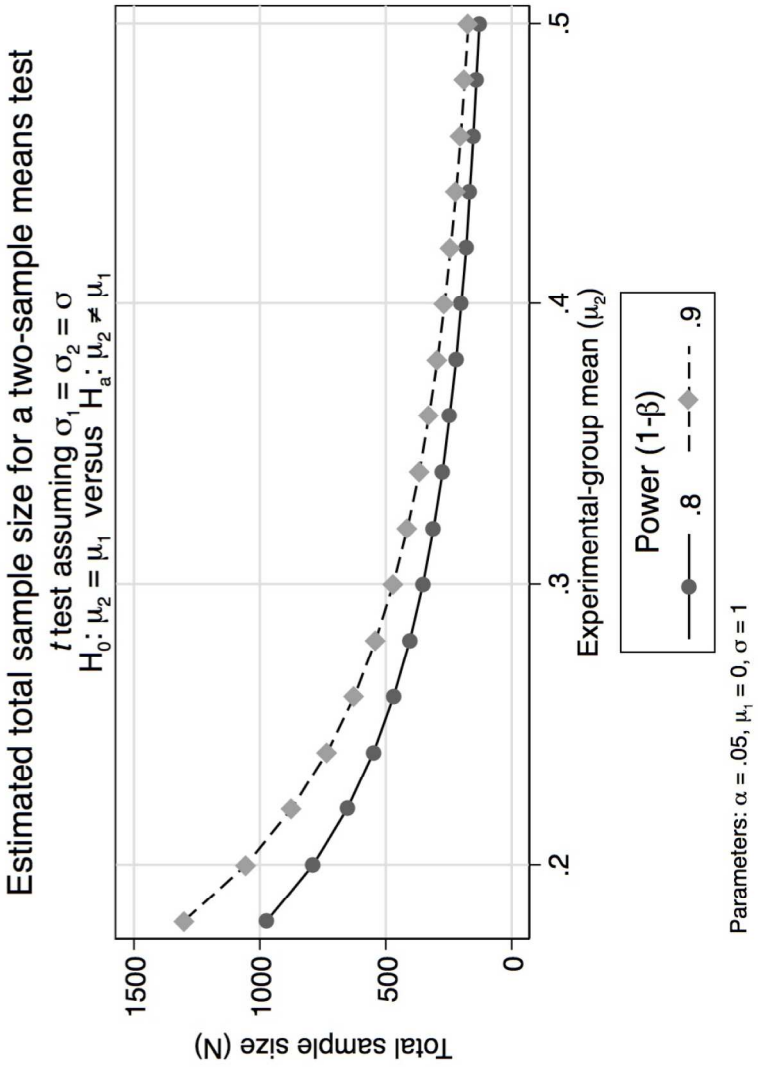

Figure 2. Estimated required total sample sizes based on relevant effect sizes at 80 and 90 % power.

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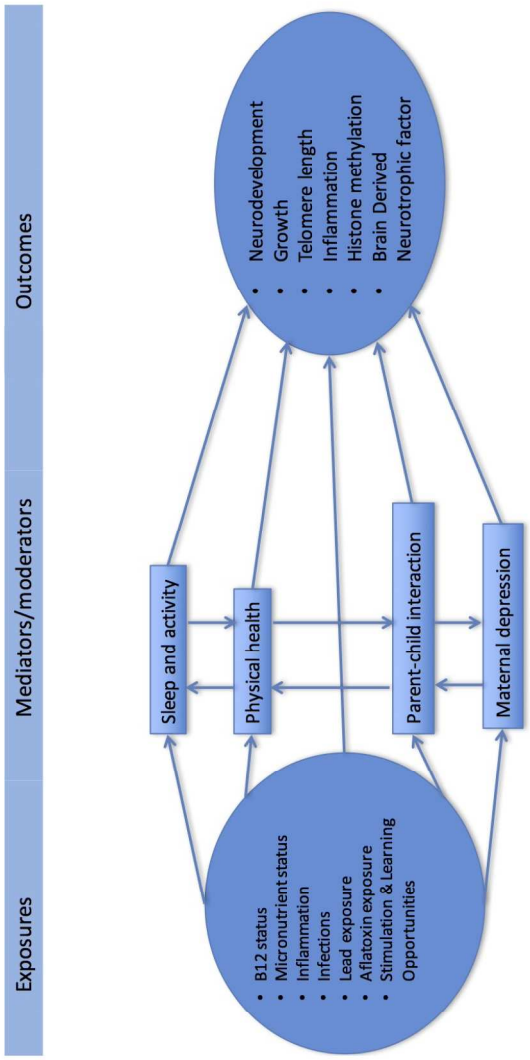

Figure 3. Postulated indirect pathways between vitamin B12 status and Neurodevelopment and growth

297x420mm (300 x 300 DPI)

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	1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	23
	2b	All items from the World Health Organization Trial Registration Data Set	23
Protocol version	3	Date and version identifier	3
Funding	4	Sources and types of financial, material, and other support	23
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,23
	5b	Name and contact information for the trial sponsor	23
	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	22
	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)	19

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
	6b	Explanation for choice of comparators	
Objectives	7	Specific objectives or hypotheses	10
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)	8
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	6
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)	7
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)	12
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	9 and 17
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	8
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	6-7
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)	Figure 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	13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	8
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	9
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	9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	9
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	9
	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	14-15
	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	11

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	13
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	20
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	21
	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)	21
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	19
	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	19
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	12
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	21
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)	

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
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	_____19_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____
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	_____19_____
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	_____
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	_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
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	_____10-11_____

*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

Supplementation of vitamin B12 in pregnancy and postpartum on growth and neurodevelopment in early childhood: Study Protocol for a Randomized Placebo Controlled Trial

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Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Global health, Paediatrics, Public health, Reproductive medicine, Neurology
Keywords:	Cobalamin, Nepal, Pregnancy, Supplmentation, Developmental neurology & neurodisability < PAEDIATRICS

Supplementation of vitamin B_{12} in pregnancy and postpartum on growth and neurodevelopment in early childhood: Study Protocol for a Randomized Placebo Controlled Trial

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Abstract

Introduction: Vitamin B12 is crucial for normal cell division and differentiation, and necessary for the development and myelination of the central nervous system. Pregnant mothers in resource poor settings are at risk for poor vitamin B12 status. Poor vitamin B12 status in infancy is linked to poor growth and neurodevelopment. Brain development starts from conception, and pregnancy is a period of rapid growth and development for the brain.

Methods and analysis: The study is an individually randomized double-blind placebo controlled trial in 800 pregnant Nepalese women randomized in a 1:1 ratio. A daily dose of 50 µg of vitamin B12 or placebo is given to women from early pregnancy, not later than week 15, until 6 months after birth. Weekly visits are conducted in order to record compliance, growth and morbidity. The primary outcomes are scores on the cognitive, language and motor subscales of the Bayley Scales of Infant and Toddler Development 3rd ed. measured at 6 and 12 months of age, and growth (length and weight) measured at 6 and 12 months of age.

Ethics and dissemination: National Health and Research Council, Nepal (NHRC 253/2016) and Regional Committee for Medical and Health Research Ethics of Western Norway (2016/1620/REK vest) have approved the study. Investigators who have contributed to the conceptualizing, conducting, as well as being involved in the data analyses and manuscript writing will be eligible for authorship and be responsible to share outcomes with different stakeholders through publications and workshops. The results from this study may support new dietary guidelines for Nepalese and possibly South Asian pregnant women that can lead to improved pregnancy outcomes, neurodevelopment and cognitive functioning in children.

Registration:

Universal Trial Number: U1111-1183-4093

Trial registration: clinicaltrials.gov: NCT03071666

Protocol date: version 1.2, 1st June, 2017

Keywords: vitamin B12, pregnant mothers, clinical trial, neurodevelopment, Nepalese infants

Strengths and limitations of this study

- This is a large-scale randomized controlled trial to investigate the effect of maternal vitamin B12 supplement during pregnancy through 6 months postpartum on early child development and growth.
- We are performing a comprehensive neurodevelopmental assessment by tools that have been previously used in the same population.
- A potential caveat in this study is that not all women will have poor vitamin B12 status, and if the proportion of women with adequate status is high, this will reduce our statistical power which may result in failure to detect important differences.
- The primary outcomes will be measured when the children are 6 and 12 months old, beneficial effects of improved vitamin B12 status of the mother might not be measurable in the children until later childhood.

Background

Cobalamin (Vitamin B12) deficiency is common in many low- and middle-income countries [1,2]. This is not surprising as the main source of vitamin B12 is animal source foods, which are expensive and for cultural and religious reasons often not eaten at all. We have in several studies in women and children demonstrated that poor vitamin B12 status is common in South Asia [3-5]. There is also compelling evidence that vitamin B12 deficiency occurs frequently during pregnancy [2,6,7] and case studies have demonstrated harmful effects of severe vitamin B12 deficiency on the developing infant brain [8,9]. The consequences of mild or subclinical vitamin B12 deficiency are less clear but it has been shown to be associated with decreased cognitive performance in both the elderly and children [10-13]. Three randomized controlled trials (RCT) have measured the effect of vitamin B12 supplementation on neurodevelopment in children: In a Norwegian trial, an intramuscular injection of B12 substantially improved motor development in six weeks old infants after one month [14], another intervention study in low birth weight children recently confirmed these findings [15]. The infants in these studies had evidence of suboptimal vitamin B12 status, but none was severely deficient. We found a beneficial effect of vitamin B12 supplementation for six months on neurodevelopment in young North Indian children [16]. In this study, where the children were supplemented daily with two recommended daily allowances (RDA) for six months, the effect of vitamin B12 supplementation was more apparent in children who had evidence of vitamin B12 deficiency at the start of the study.

During pregnancy, vitamin B12 is concentrated in the fetus and stored in the liver [17,18]. Infants born to vitamin B12-replete mothers have stores of vitamin B12 that are adequate to

sustain them for the first several months postpartum. Consequently, vitamin B12 deficiency rarely occurs before the infant is about four months old if the mother has adequate vitamin B12 status during pregnancy. However, infants of vitamin B12-deficient breastfeeding mothers are vulnerable to B12 deficiency from an early age [19].

Maternal vitamin B12 deficiency has been associated with increased risk of common pregnancy complications, including spontaneous abortion, low birth weight, intrauterine growth restriction, and neural tube defects [20-22]. Children born to vitamin B12-deficient women are at increased risk for adverse health outcomes, including developmental abnormalities and anemia [9,23]. In a recent RCT in Bangalore, India, daily maternal vitamin B12 supplementation (50 µg/d) during pregnancy through six weeks postpartum, substantially improved maternal vitamin B12 status and increased breast milk and infant plasma vitamin B12 concentrations [24]. In this study the proportion of children being born small for gestational age was lower in the vitamin B12 group than in the placebo group (25% vs. 34%), however no difference was found in neurodevelopment in infants at 9 months of age [25]. Similar improvements in vitamin B12 status was found among Bangladeshi mothers and infants when supplemented with 250 µg of vitamin B12 from 11-14 weeks of pregnancy through 3 months postpartum [26]. In this study women that were randomized to receive vitamin B12 also had an improved immune response to the pandemic influenza A (H1N1) vaccine.

The first 1000 days after conception are regarded as the most critical time for brain development. During this period brain growth is rapid, increasing the susceptibility to

influences from the environment [27]. This is also the period when most of the myelination of the brain occurs [9]. In infants, moderate and severe vitamin B12 deficiency has been associated with demyelination and brain atrophy [28,29]. Little is known about the consequences of maternal vitamin B12 deficiency for early brain myelination, neurodevelopment and cognitive function.

Hypothesis to be tested: In Nepalese women, daily supplementation of 50µg vitamin B12 from early pregnancy, not later than week 15, and until 6 months postpartum improves linear and ponderal growth as well as cognitive, language, and/or motor scores of the Bayley Scales of Infant and Toddler Development 3rd. ed. (Bayley-III) in their infants.

Specific objectives

Primary objectives:

1) Measure whether daily maternal administration of 50µg vitamin B12 from early pregnancy until 6 months postpartum improves cognitive, language and motor scores of the Bayley-III measured at 6 and 12 months of age.

2) Measure whether daily maternal administration of 50µg vitamin B12 from early pregnancy until six months postpartum improves the infant's z-scores length for age, weight for age and weight for length at 12 months.

Secondary objectives

1) Measure whether daily maternal administration of 50 µg vitamin B12 from early pregnancy until six months postpartum improves hemoglobin concentration of the mother and

infant, and also assess the relationship between maternal and infant hemoglobin concentration on cognitive outcomes at 6 and 12 months of age.

2) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves cognitive function of infants as measured by the Bayley-III at 24 months of age.

3) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves development as measured by the Ages and Stages questionnaire 3rd edition at 6 and 12 months of age.

4) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum improves motor function of infants as measured by the Infant Motor Performance test at 45 days.

5) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum reduces the risk of complicated deliveries.

6) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum reduces the risk of giving birth to a child small for gestational age.

7) Explore the efficacy of vitamin B12 on growth and development in various subgroups (defined before the randomization code that links the group treatment to a study participant is broken).

8) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum alters sleep and activity patterns of the infant.

9) Measure whether daily maternal administration of vitamin B12 from early pregnancy until 6 months postpartum affects heart rate variability.

Methods

Study setting

The study site is located in Bhaktapur, 15 km east of the capital city Kathmandu. Bhaktapur city is an ancient city famous for its traditional temples and buildings. It is listed under UNESCO world heritage and one of the main tourist attractions in Nepal. Bhaktapur is a homogenous community consisting mostly of practicing mixed Hindus and Buddhists. It is a peri-urban agricultural based community located 1400 m above sea level with a population predominated by the Newar ethnic group. In the Bhaktapur municipality, domestic migrant workers from diverse ethnic groups work in several carpet factories. The local climate is humid subtropical with a wet and hot season (monsoon) from May to August, and a dry and cool season from October to March. The annual average rainfall is 78.3 mm, and the temperature ranges from -2 to 35 ^{o}C. The majority of the residents in Bhaktapur consume foods grown in the community. Rice is the staple food. The eating pattern varies with season, workload in the field and availability of foods. A variety of local green leafy vegetables is widely consumed mainly in the winter and spring season.

Eligibility criteria

1. 20-40 years old pregnant women from early pregnancy, not later than 15 weeks pregnant,
2. Residing in Bhaktapur municipality and surrounding areas in Bhaktapur district, and
3. Availability of informed consent.

Exclusion criteria

1. Taking dietary or multivitamin supplements containing vitamin B12,

2. Known cases of chronic disease under treatment like tuberculosis, diabetes, hypertension, hypo- or hyperthyroidism, pernicious anemia, Crohn's disease and current use of anticonvulsant drugs,

3. Severe anemia (hemoglobin concentration <7 g/dL). Blood will be analyzed for hemoglobin concentration immediately after collection at enrollment by using HemoCue,

4. Suffering from any condition that requires treatment with vitamin B12 such as pernicious anemia and strict vegans.

Intervention

The supplements are produced by GC Rieber Compact inc. (Bergen, Norway). The vitamin and placebo tablets are, except for the cyanocobalamin content, identical in composition, taste, smell, and appearance. The compositions of the tablets follow the recipe of the commercial product "Seven OceanS® Emergency Ration". Each daily dose (placebo or 50µg vitamin B12) also contain 31 kcal from the 6.95 g vehicle which mainly consists of dextrose and palm oil. First dose of supplementation will be given by research staff at the hospital, and mothers will then be instructed to properly store the supplements, and consume these everyday preferably in the morning. During the weekly visits, our research staff will record consumption of supplement during the last 7 days including iron, folic acid and calcium, and also count the amount of remaining supplements. We will also record if there was vomiting after supplementation, and reasons why the supplements was not taken. During the antenatal visits or other visits at the hospital, the study gynaecologists will verify the compliance. Additionally, study supervisors will randomly select days (5% of total days) to make

independent visits to participants mother's home in order to record compliance with supplementation.

Co-intervention

All pregnant women will also be given folic acid (0.4 mg) for the first two months of pregnancy followed by iron (60 mg elemental iron) and calcium supplements (500 mg) until 45 days after delivery according to the WHO guidelines [30].

Recruitment procedure, confirmation of pregnancy and assessment of gestational age

Prior and throughout the study period, we will identify pregnant mothers through a hospital and community based surveillance system (Figure 1). We have records of newly married couples in the study area that are updated quarterly. Information regarding pregnancies will be obtained monthly by household surveys or phone calls. Women with history of interrupted menstruation cycle or pregnancy detected by standard HCG kits are screened in the hospital. In these women, pregnancy and gestational age is estimated by ultrasonography (USG). Follow-up USG are performed between 18 to 22 weeks for anomalies scanning.

After screening of eligibility, a detailed information regarding the study will be provided focusing on the duration, collection of biological samples, and the follow-up plan of the study. The consent forms will be filled by study gynaecologists or supervisors, preferably in the presence of husband. A copy of the consent form along with the information sheets will be provided to each participant. In the consent form, there is a separate provision for consent of biological samples where the participants may agree or refuse. In case of illiterate

participants, we will obtain thumbprints after getting signature from an impartial witness who is not part of research team.

Allocation, randomization and blinding

The randomized allocation list was generated in STATA (Stata inc. College Station, TX) using the script "randomize" (net from http://folk.uib.no/mihtr/stata/). We randomized the women in a 1:1 ratio in blocks of 8. Each woman is assigned a packet of supplementation according to her id number which is sequentially numbered. This packet is only labelled with general information about the study and the unique id number. The list that links the id number to the randomization code is kept with the company that produces the intervention and the placebo, and with the scientist who generated it who is otherwise not involved in the study. We will ensure allocation concealment throughout the study as none of the investigators will have access to this list until completion of data collection. This is only after the database with the main outcomes (growth and neurodevelopment scores) have been cleaned and locked, and after the plan of analysis for the main outcomes is finalized and approved by all involved scientists.

Rationale for choosing 50 µg dose of vitamin B12

Based on results from previous studies in this area, we expect the intake of vitamin B12 among women in our study to be low, both due to a predominant plant based diet, but also because of poor gut function that again may disturb vitamin B12 absorption and increase dose requirements. Absorption of vitamin B12 is strictly regulated, and the body is not able to absorb more than 2-4 µg per day. The vitamin B12 supplementation study in pregnancy from

South India [24], and the study from Bangladesh [26] used very high doses of 20-100 x RDA with no reported adverse effects. There are other, large RCTs were similarly high doses of vitamin B12 have been given together with other micronutrients [31,32]. These studies found that the micronutrient mixture that included high doses of vitamin B12 reduced the risk for SGA with no adverse effects. In Europe and the US, vegans and vegetarians are aware that there is a risk of B12 deficiency that can result in poor neurodevelopment and other adverse outcomes [33]. Many therefore take additional supplements with similar high doses of B12 during pregnancy.

Timeline

First participant in the study was enrolled in March 2017 and we plan to enroll for two years. End of follow up of infants (for collecting primary outcomes) will be completed approximately 18 months after the last enrollment.

Laboratory procedures

From the mother, blood samples will be obtained at enrollment (baseline) and at the end of supplementation (6 months postpartum). In the infants we will collect blood samples at 6 months by a trained lab technician with direct supervision of study pediatrician. Three mL of blood will be collected into vials containing EDTA as anticoagulant. The plasma will be centrifuged at approx. 700 g at room temperature for 10 minutes, separated and transferred into storage vials, and stored at -70 degrees before analysis.

The blood samples will be analyzed for

1) Hemoglobin concentration by using HemoCue B immediately after blood collection (HemoCue, Vedbæk, Denmark).

2) Plasma vitamin B12 and plasma folate concentrations will be estimated by microbiological assays using a chloramphenicol resistant strain of Lactobacillus casei and colistin sulphate resistant strain of Lactobacillus leichmannii, respectively.

3) The plasma will be stored and used for other biomarkers (nutrients, markers of inflammation, epigenetic markers, and markers related to neurodevelopment).

Follow up

We keep track of the enrolled women in a GPS/mobile based surveillance system. Enrolled women will be visited every week in the period of supplementation and the field worker will ask questions about her physical condition, compliance among both study groups and adverse events. The family/women are asked to inform us when she goes into labor, and we will ensure transportation to hospital where she will give birth. This will also ensure that we get information about the pregnancy outcome. We will not try to influence the choice of place to deliver, however, we will encourage the women to deliver in a health facility rather than at home. In the Kathmandu valley, less than 5% of deliveries are at home.

After delivery we will follow the child at home every month until 2 years of age. We will collect information on breastfeeding and complementary feeding (frequency, whether other drinks or solids/semi solids have been given). We will also ensure that the child receives standard vaccines as per the EPI schedule. Growth will be measured and dietary recalls

undertaken every three months. Neurodevelopment will be measured at different time points during infancy.

Outcomes

Anthropometry

Weights will be measured with a portable electronic scale that measures to the nearest 0.01 kg (portable Seca weight). Height and length (portable Seca board) will be measured according to standard guidelines. Both length and weight will be measured in their homes immediately after birth and at 1, 3, 6, 9, 12, 18 and 24 months.

Neurodevelopment

With the exception of the *Test for Infant Motor Performance (TIMP)*, all the planned assessment tools are currently in use in our ongoing studies in Nepal. Thus, the study benefits from the fact that the necessary translations, adaptations, piloting and psychometric evaluations have been completed for the study setting.

Bayley Scale of Infant and Toddler development 3rd ed. (Bayley-III) is a comprehensive assessment tool of developmental functioning in infants and toddlers age 1- 42 months [34]. It is administered directly with the child and provides information on functioning in cognition, language (receptive and expressive), motor (fine and gross) abilities and social-emotional functioning. The Bayley-III is considered to be the gold standard in developmental assessment of this age-group, and is widely used for research purposes worldwide. Summary scores of the Bayley-III cognitive, language and motor subscales at 6 and 12 months of age

will be the primary outcome in the current study, while the assessment at 24 months will serve as secondary outcome.

The Ages and Stages Questionnaire – 3rd ed. (ASQ-3) is an easily administered checklist of developmental status standardized for children 1-66 months [35]. The screening system includes age-appropriate questionnaires for every two/three-month intervals. Each questionnaire contains 30 items in five domains: communication, gross motor, fine motor, problem solving and personal social. The questionnaires are designed to be completed by caregivers, but can also be administered by a trained examiner which will be done in the current study. Since the ASQ-3 is a screening tool developed to identify children at risk for delay, there is a possibility for a ceiling effect. To avoid possible ceiling effects, children who get maximum scores will be administered the next level checklist. We have used this tool in the current and at a similar study setting [36] previously, and find the tool to be a feasible addition to the assessment of neurodevelopment in young children. The ASQ-3 will be administered in the homes of the families and provide us the opportunity to assess developmental trajectories.

Actigraph (the Actiwatch 2 (Philips)) is a wristwatch-like device that records motion data. Using a validated scoring algorithm, the actiware® software translates the activity data to sleep-wake-patterns and activity level. Sleep problems and sleep patterns will be further corroborated by questionnaire data (the Brief Infant Sleep Questionnaire (BISQ)). The BISQ has demonstrated good psychometric properties as a brief sleep screening tool for clinical and research purposes in infants and toddlers (0-30 months) [37].

Heart rate variability (Vagal tone) is a marker of parasympathetic activity. Vagal tone has been suggested to be a sensitive marker of self-regulation and cognitive control and associated with the status of vitamin B12 during pregnancy [38], and may be an important, subjective measure of early risk identifier of child development. We plan to measure the vagal tone at 3, 12 and 24 months of age.

Test for Infant Motor Performance (TIMP) is a test of functional motor behaviour which can be used up to 4 months of age [39,40]. It consists of 42 items that are scored as present or absent based on observation. The TIMP has shown good predictive validity for later neurodevelopmental outcomes [41]. We will pilot the test in the current study setting ahead of study start to ensure that it is appropriate for the study setting. Three psychologists and medical doctors will be trained through an online course and standardised to perform this test, which will be conducted when the children are 45 days to estimate early signs of effect of the maternal vitamin B12 supplementation.

Questionnaires/Inventories

The Home Observation for the Measurement of the Environment (HOME Inventory) is an internationally recognized tool for measuring both the quality and quantity of stimulation and support available to the child in the home environment. The tool has been used successfully in over 100 countries and validated in several low and middle income countries [42]. It consists of 6 subscales; Parental Responsivity; Acceptance of Child; Organization of the Environment; Learning Materials; Parental Involvement, and Variety in Experience. A

disadvantaged environment is among the many factors identified as influencing child cognition and development, and studies have demonstrated that the HOME inventory is a predictor of later school achievement [42]. Administration of the HOME inventory requires both interviewing the mother as well as observing the household. It takes approximately 45 minutes to 1 hour to complete, and trained field workers will conduct this assessment. Information about the child's environment will be collected so that we can account for the effect on child development in our study.

The Caregiver Knowledge of Child Development Inventory (CKCDI) is a brief questionnaire to assess parental knowledge of child developmental milestones and knowledge of developmental stimulation [43]. Parental knowledge on child developmental milestones may be of importance to promote adequate development in children. In this study we will use the CKCDI in the 3rd trimester and at 12 months postpartum to account for the effect of the caregivers knowledge on child development.

Self-Report Questionnaire (SRQ-20) is a screening of mental health developed by the World Health Organization (WHO) especially for low to middle income countries [44]. The scores on the SRQ-20 may serve different purposes in the current study. We can estimate the association between maternal depressive scores and child development, and in addition, we may investigate possible effects of maternal vitamin B12 supplementation on depressive symptoms.

Quality control of field activities

The fieldwork will be monitored by skilled supervisors, medical doctors, gynecologists, and qualified psychologists. For all outcomes the study staffs have received training before initiation of the project, and the staff will be utilized based on their skill level. For a specified proportion (5%) of the primary outcomes, a study supervisor or scientist monitor the worker's` performance. Some of these assessments are also done independently. For example, when a field worker has measured weight and length of a child, a supervisor or scientist will undertake the same measurement and compare their findings with that of the field worker. For all main outcomes we compare the reliability and precision of each examiner with the reliability of a gold standard. For example, for measuring length we have training sessions where the field workers and a gold standard measure each child twice. In a typical training session, each worker measures 10 children twice. The intra-individual variability (mean squared difference) of a trainee will be compared to that of the gold standard. For length measurements we will accept no more than 50% higher intra-individual variability of the examiner compared to the gold standard. In this exercise we can also compare the mean deviations of absolute differences between the gold standard and the worker. The mean deviation is also compared to the mean variability of the measurements of the gold standard. The deviation is also compared on a qualitative scale to assess whether there are systematic higher or lower readings. In case of poor agreement, extra training will be carried out until acceptable agreement were achieved.

Quality control for the neurodevelopmental tools

All methods that will be used to measure neurodevelopment have been translated, adjusted and/or piloted in studies at the current study site. The comprehensive neurodevelopmental

tools such as the Bayley-III and TIMP will be administered by the medical doctors and psychologists. Medical doctors and qualified psychologists will train and monitor fieldworkers to perform the neurodevelopmental assessments such as the ASQ-3, HOME-assessment and the various questionnaires. Ahead of study start, we will arrange training sessions. The trainings will be supervised and monitored by experienced psychologists from the Regional Center for Child and Adolescent Mental Health and Welfare, Uni Research Health, Bergen, Norway. Standardisation exercises will be conducted measuring the inter-rater reliability of the assessor compared with a gold standard until satisfactory level of agreement. Video recording will be done for all Bayley assessments and 10% of the videos will be scored independently for quality control. For all other instruments, 5% of the assessments will be double scored. Once a month inter-rater reliability will be analyzed for quality control.

Safety considerations, safety monitoring and adverse event reporting

All adverse events (AE) and serious adverse events (SAE) will be recorded by the study staff. AEs are graded from 1 to 5 according to their severity according to Common Terminology Criteria for Adverse Events (CTCAE) [45]. All immediate adverse events following each dose of intervention will be documented for all women.

All adverse events will be followed up till resolution or stabilization as judged by gynecologist or pediatrician (site investigator) and the principal investigator. All SAEs will be followed up until satisfactory resolution or until the treating physicians and the principal investigator deems the event to be chronic or the participant to be stable.

SAE, that includes critical or life threatening illness or death, will be documented from the time of enrolment, throughout the study period. The pregnant women will be visited every week. During each contact, the study staff will collect data to ascertain SAEs and illness requiring hospitalization. All SAEs of death, development of signs of critical illness and severe illness requiring hospitalization and AEs as described above will be reported to the Ethics Committees, the Data and Safety Monitoring Board (DSMB) and to the sponsors of the study within 24 hours of awareness of the event followed by a final report within 10 days. SAE relatedness to the administration of vitamin B12 or placebo will be judged by the investigator/designee, the Ethics Committee and the DSMB who will have access to all relevant investigations, clinical assessments and if require unblinding allocated interventions**.**

Data and Safety Monitoring Boards (DSMBs)

The DSMB is comprised of a pediatrician, public health expert and a biostatistician. The DSMB is independent from the sponsor and has no competing interests. The DSMB members have prepared a charter and decided the study stopping rules, and will review SAEs and AEs reported in the study periodically. They will examine all pregnancy complications, cases of severe illness, infant deaths and other SAEs to decide if the study should be continued, based on the pre-decided stopping rules. After one third of the study participants have been enrolled and completed follow-up, the DSMBs will review the data and make recommendations concerning continuation, modification or termination of the study due to unexpectedly large beneficial effects or serious side effects and can suggest extension of the trial should the primary outcomes occur less often than anticipated. Any important protocol modification such

as changes to eligibility criteria, outcome analyses will be thoroughly communicated with DSMB members and will obtain amendment clearance from the IRB.

Sample size calculations

We expect that as many as 15% of the enrolled women will be lost to follow up because of abortions, stillbirths, and migration (see box below).

Expected losses to follow up due to different conditions during the study period

Expected losses to follow up		
	Late abortions	**5%**
	Drop - outs during pregnancy	**4%**
	Drop - outs during infancy, including infant mortality	**6%**

We have calculated the sample sizes based on standard formulas [46] to compare two means which are used in the "power" function in STATA (Stata inc. College Station, TX). For an effect size of .25 SD we would need to analyze 676 infants to achieve a statistical power of 90% (p-values of 0.05) we will therefore target 800 women-infant dyads [676/(1-0.15)]. In these calculations we assumed equal standard deviations in the placebo and vitamin B12 groups. The power to detect differences between the study groups are larger than what is projected here as we will measure most of the outcomes more than once and also use these repeated measurements in the same analyses, adjusting for interdependence within a child

using mixed effects models, generalized estimating equations, or other appropriate methods. Various required total sample sizes according to meaningful differences and powers of 80 and 90% are depicted in Figure 2.

These figures are based on national figures and our experiences from the field site. Infant morbidity and mortality is expected to be lower than national figures because of the proximity to the capital Kathmandu and because of our close follow up.

Data management

All information will be collected in structured forms designed specifically for this project. A relational database has been designed. All forms are checked manually by the study supervisors before they are sent for computer entry. All data are entered twice by two different data operators within one week of data collection.

Data are stored in secure servers in the field office and in a data management centre (DMC) in Kathmandu. A substantial proportion of the forms is captured using ipads or similar tablets using the cloud-based data management system. We have used this electronic data capture system with success in many previous studies. Only computer data entry staff and supervisors will have access to the data. In the data-system, the entered data is processed in several steps and the system will be designed to identify inconsistencies between different variables and different forms. The system can also detect entries that are out of range and notify when information is missing this system also include reminders about study activities, i.e. the study staff will be notified when a birth or a scheduled vaccine is due. We are collecting GPS

coordinates and phone numbers from several family members. By this we can more easily reach the families and will probably reduce the numbers of missed home visits.

Data tracking, cleaning, and quality checks

The DMC is also responsible for initial cleaning of the data. Interim tabulations and scatter plots for some variables will be made at regular intervals to identify data errors. Special checks are made on observations that are more than two or three standard deviations from the mean. There is a regular feedback of errors from the DMC to the study sites.

Record Retention and Archival

All the study documents including participant's source data and documents will be archived by the study sites after the completion of the study, till the time the sponsor informs in writing to the study sites that they no longer need to maintain the study documents.

Plan of analysis

All analyses will initially be done on an intent-to-treat-basis. All randomized participants will be included in the analyses if the relevant outcome variables have been collected. The main outcomes are continuous and expected to be normally distributed. We will use the Bayley-III scores at 6 and 12 months in separate analyses where B12 supplementation is the main exposure. We will compare the mean Bayley-III scores (scores on the cognitive, language and motor subscale scores, with the language scale analyzed both separately for receptive and expressive language and as a composite measure, and the motor scale analyzed both separately for fine and gross motor development and as a composite measure) between the

vitamin B12 group and the placebo group. For growth, the main outcome will be measured when the child is 12 months, linear growth, expressed as cms and HAZ, and weight expressed as kgs, WAZ, and WHZ will be compared between the study groups. If baseline differences are observed, we will adjust these imbalances in multiple linear regression models, adjusted and unadjusted effect estimates will then be presented.

We will analyze the effect of vitamin B12 separately in the following subgroups:

Vitamin B12 status

-based on cobalamin, (cut off: 150 pmol/L)

-based on low total Homocysteine (cut off: 10 µmol/l)

-based on low Methyl Malonic Acid (cut off: 0.26 µmol/L)

Maternal BMI (cut off: 18.5)

Vegetarian (yes / no)

Birth weight (cut off: 2500g)

In these subgroup analyses the differences in the effect between the levels of the subgroups will be measured by including 2-way interaction terms in multiple regression models. Statistically significant effect modification will be when the p-value of the interaction term is <0.05.

Per protocol analysis

In addition to a standard per protocol analysis, we will use Instrumental Variable Analysis (IVA) in an attempt to estimate the true effect of cobalamin had it been given to all women in

the scheduled doses and intervals. The random allocation will be the instrument in these analyses. For per protocol analysis, women who receive less than 50% of the projected doses during pregnancy will not be included in the analyses, well acknowledging that the ensuing effect estimates may not only be biased but will certainly represent an effect higher than what can be achieved even in our well-resourced study setting.

Discussion

This proposed study measures the effect of vitamin B12 supplementation from early pregnancy through 6 months postpartum on early neurodevelopment and growth in 800 Nepalese infants at 6 and 12 months. Randomized clinical trials on vitamin B12 in pregnancy is called for as concluded in a recent systematic review and meta-analysis [22]. This trial will add to the knowledge on the potential beneficial effects of vitamin B12 on pregnancy and perinatal outcomes [21]. Our results can be used to inform both regional dietary guidelines for Nepalese and South Asian pregnant women. The recently published report "WHO recommendations on antenatal care for a positive pregnancy experience" only recommend iron and folic acid to pregnant women [30], the results from our study possibly contribute to necessary updates of these guidelines.

The primary concern raised by the Norwegian IRB was that this RCT was violating the principle of clinical equipoise. The IRB was accordingly concerned that we were withholding necessary vitamin B12 treatment for those who were randomized to the placebo. This concern was based on comments from an expert reviewer appointed by this IRB and we are dealing with this concern, in part, by measuring functional motor behaviour by the TIMP when the

children are 45 days in infancy in the first 100 children. If we find an unexpected beneficial effect of vitamin B12 on the results of this test, the DSMB will consider stopping the trial. The TIMP was added solely based on the concerns raised by the IRB, and this motor test will mainly be used as a tool for the DSMB when deciding whether the study should continue or not. It should also be noted that before approving our protocols, the IRB engaged a second external expert who did not share the concerns regarding clinical equipoise.

The Government of Nepal in partnership with different organizations such as WHO and UNICEF, have initiated many programs in mother and child care. There are great concerns that many children in low and middle income countries fail to reach their developmental potential due to poverty related factors such as malnutrition, micronutrient deficiencies, parental illiteracy, and lack of stimulation and learning opportunities [47]. Results from our study will provide a valuable contribution to the field of early risk and development in disadvantaged children and their families, and may also help to further integrate the Government of Nepal program on the first 1000 golden days.

Competing Interests

The authors and principal investigators declare that they have no financial and other competing interests for the overall trial.

Authors contributions

TS, IK, MH, RKC, conceived the study, contributed to study design, sample size calculations and analytical plans. TS, RKC, IK, MH drafted the manuscript. MU, RKC, TS, IK, MH, MS,

SB, SR, LS have assisted in developing the protocol, will initiate the project, and help with the implementation. All authors read and approved the final manuscript

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Figure 1. Study flow chart- Recruitment, supplementation and follow up plan

Figure 2. Estimated required total sample sizes based on relevant effect sizes at 80 and 90 % power.

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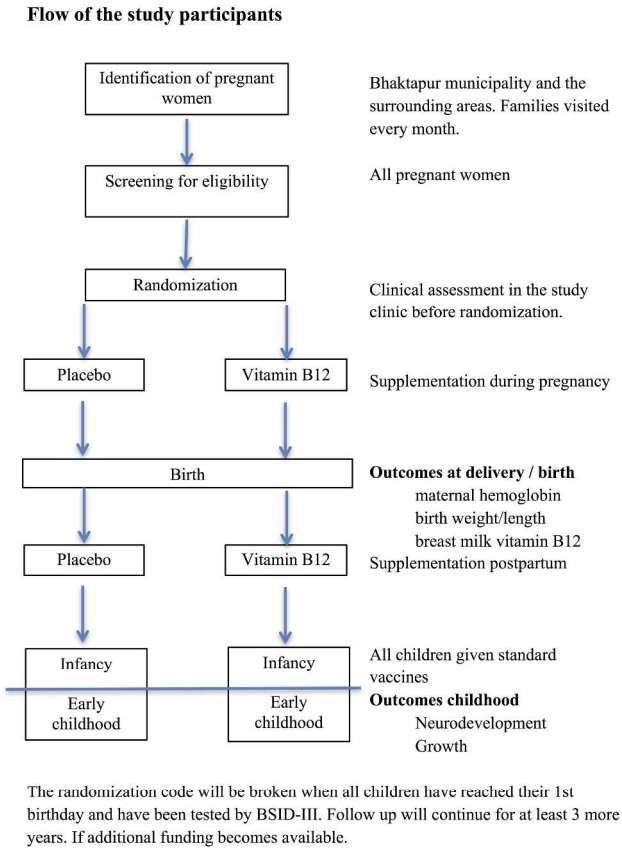

Figure 1. Study flow chart- Recruitment, supplementation and follow up plan‼ ┼

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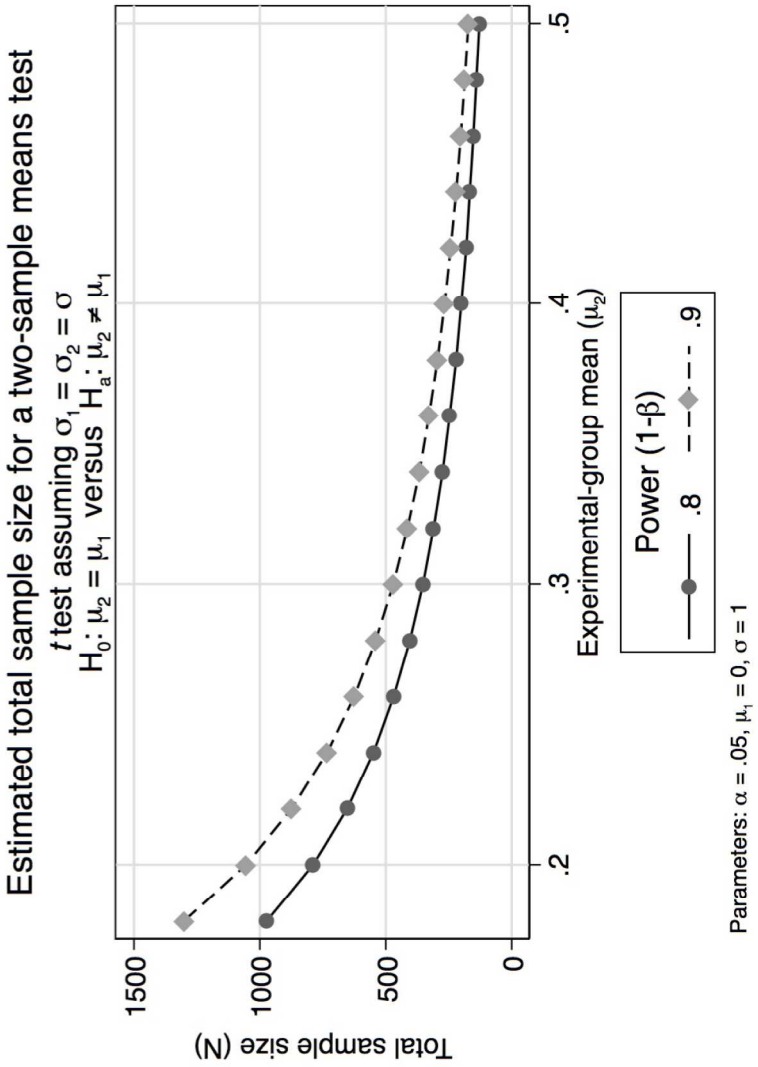

Figure 2. Estimated required total sample sizes based on relevant effect sizes at 80 and 90 % power.

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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	2-3
	2b	All items from the World Health Organization Trial Registration Data Set	3
Protocol version	3	Date and version identifier	3
Funding	4	Sources and types of financial, material, and other support	27
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,26-27
	5b	Name and contact information for the trial sponsor	27
	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	20
	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)	22

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
	6b	Explanation for choice of comparators	n/a
Objectives	7	Specific objectives or hypotheses	6
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)	2
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	8
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)	8-9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9-10
	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)	19
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	9-10
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	10
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	6-7
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)	12

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	21-22
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	10
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	11
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	11
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	21
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	10-11
	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	13

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	22
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	23-24
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	24-25
	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)	23-24
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	23
	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	23-24
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	20
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	20
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	2
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)	20-21

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	____11_____
	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	_____19_____
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	_____28____
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	_____19_____
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	____n/a_____
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	____2_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	_____29_____
	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	_____11_____
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	_____10-11_____

*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.