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Impact of vitamin B12 on neurodevelopment and cognitive function from early life into school age, extended follow-ups from randomized controlled trials

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ABSTRACT

Introduction

As many as 250 million children under the age of five may not being reaching their full developmental potential partly due to poor nutrition during pregnancy and the first 2 years of life. Micronutrients, including vitamin B12, are important for the development of brain structure and function; however, the timing, duration, and severity of deficiencies may alter the impact on functional development outcomes. Consequently, to fully explore the effect of vitamin B12 on cognitive function it is crucial to measure neurodevelopment at different ages, in different populations and with vitamin B12 supplementation at different times during the critical periods of neurodevelopment.

Methods and analysis

In this project we follow-up children from four recently completed randomized-placebo controlled trials of oral vitamin B12 supplementation, two in India and two in Tanzania, to explore the long-term effects on neurodevelopmental outcomes and growth. All the included trials provided at least two recommended dietary allowances (RDA) of oral vitamin B12 daily for at least six months. Vitamin B12 was supplemented either during pregnancy, early infancy or early childhood. Primary outcomes are neurodevelopmental status, cognitive function and growth later in childhood. We apply validated and culturally appropriate instruments to identify relevant developmental outcomes. All statistical analyses will be done according to intention to treat principles. The project provides an excellent opportunity to examine the effect of vitamin B12 supplementation in different periods during early life and measure the outcomes later in childhood.

Ethics and dissemination

The study has received ethical approvals from all relevant authorities in Norway, USA, Tanzania and India and complies fully with ethical principles for medical research. Results will be presented at national and international research and policy meetings, and published in peer-reviewed scientific journals, preferably open access.

Registration Original trials are registered. There are no new exposures in the follow-up study. Original trial registrations are NCT00641862 (Bangalore), NCT00717730, updated CTRI/2016/11/007494 (Delhi), and NCT00197548 and NCT00421668 (Dar es Salaam).

Key words

Vitamin B12; neurodevelopment; cognitive function; event related potential; executive function, growth

STRENGTHS AND LIMITATIONS OF THE STUDY

- The current project takes advantage of recently completed randomized-placebo controlled trials to study long-term effects of oral vitamin B12 supplementation on neurodevelopmental outcomes and growth in children in Tanzania and India.
- The project provides an excellent opportunity to examine the effect of vitamin B12 supplementation during different periods of the critical first 1000 days and measure the outcomes later in childhood.
- The studies were not designed to follow the children up beyond the time of supplementation, which may lead to a somewhat high rate of loss to follow-up.
- The effect measures may be difficult to standardize across the different studies.

INTRODUCTION

New estimates based on proxy measures of stunting and poverty indicates that as many as 250 million children under the age of five from developing countries are at risk of not fulfilling their developmental potential partly because of poor nutrition during pregnancy and the first 2 years of life.(1) Malnutrition and micronutrient deficiencies represent a major challenge to child health in many low- and middle-income countries (LMICs) and are associated with suboptimal cognitive function and poor growth.(2) Many children in South Asia and Africa suffer from deficiencies of several nutrients including vitamin B12.(3-7)

Vitamin B12 and neurodevelopment

There is ample evidence that vitamin B12 is important for cognitive development.(8) In observational studies among adults and elderly, low levels of vitamin B12 are associated with cognitive decline and dementia.(9) Although there are fewer observational studies in children, the association between vitamin B12 status and cognitive functioning has also been documented from early infancy to adolescence. Neonatal severe vitamin B12 deficiency causes failure to thrive and possible irreversible neurological manifestations.(10, 11) A study in the Netherlands showed that infants fed on a macrobiotic diet had delayed gross motor, speech and language development compared to infants on an omnivore diet.(12) In adolescence, these same children who were fed a macrobiotic diet for the six first years of life had poorer performance on cognitive tests, independent of their current

vitamin B12 status, compared to adolescents who were fed on an omnivorous diet.(13) In an Indian infant cohort, we also demonstrated that poor vitamin B12 status was associated with lower scores on the Mental Development Index in the Bayley Scales of Infant and Toddler Development 2nd edition.(4) In a study from Nepal, we were able to demonstrate associations between early vitamin B12 status (2-12 months) and cognitive functioning five years later.(14) These long-term associations remained strong, even after adjusting for several potential confounders. There is also evidence for the significance of vitamin B12 to the developing brain in a few clinical trials. In a RCT where Indian children aged 6-30 months were administered placebo, vitamin B12 and/or folic acid for six months, children provided with vitamin B12 and folic acid scored higher on a neurodevelopment assessment compared to the placebo group.(15) The effects were most prominent in stunted children or children less than 2 years of age. A favorable effect of vitamin B12 was also demonstrated on motor development in two RCT`s involving Norwegian infants with developmental regressions and signs of vitamin B12 deficiency one and six months following a 400 µg injection of vitamin B12.(16, 17)

Why vitamin B12 may be important for the developing brain

Vitamin B12 plays a key role in normal brain development and function and is required for the folate-dependent enzyme, methionine synthase, which is necessary for the synthesis of methionine from homocysteine (tHcy).(18) Methionine in its activated form, S-adenosylmethionine, is the major methyl group donor used in human methylation reactions, including methylation of DNA and RNA. Deficient methylation reactions in the central nervous system can impair the methylation of myelin basic protein in the central as well as peripheral nervous system.(19) The production of myelin is a key component of brain development from gestation, throughout childhood and well into middle age.(20) The myelination of the brain is of importance for multiple brain systems and is highly related to neurodevelopment and subsequent cognitive functioning.(8) Vitamin B12 also serves as a cofactor in numerous catalytic reactions in the human body, which are required for neurotransmitter synthesis and functioning. Vitamin B12 deficiency may cause pernicious anemia with similar effects on cognitive development and functioning as anemia caused by iron deficiency.(11) Vitamin B12 deficiency can also result in neuropathy through degeneration of nerve fibers and irreversible brain damage.(21) However, albeit subtle vitamin B12 deficiency is very common, the degree to which it has significant consequences for neurodevelopment and cognitive function is not established.(22, 23)

Knowledge gaps

There is a need to clarify to what extent improving vitamin B12 status impacts developmental outcomes in children in LMICs. It is also important to identify populations in which such interventions can be beneficial and at what age vitamin B12 administration is most effective.(8) Most studies in

this field have used only motor function tests or general neurodevelopment assessments (15, 16, 24), while less is known on the effect of B12 on specific cognitive functions such as executive functions, attention, and sensorimotor and visuospatial abilities.(14) The functions and areas under development are most sensitive to negative influences, and will provide the most specific outcomes in assessments.(25, 26) Consequently, to fully understand findings in developmental assessments, we must consider the developmental timing of an exposure, in this case vitamin B12 supplementation (or lack thereof), as well as the timing of the assessment.(25) Measuring neurodevelopment at different ages, in different populations and the effect of exposure at different times during the critical periods of neurodevelopment is needed to understand the role of B12 in cognition.

Research on the impact of B12 on the neurophysiological outcomes in children is scarce. Event Related Potential (ERP) are non-invasive, reliable measures of neurophysiological brain function(27), that provide direct measure of underlying neural activity of higher mental processes. Behavioral assessments may sometimes not be sensitive enough to detect these small changes in brain functions(28) and these neurophysiological processes may be important correlates of the cognitive abilities and give new insight into the nutritional impact on the developing brain.(29)

STUDY OBJECTIVES

The aim of the current study, the Vitabeginning project, is to determine the long-term effects of vitamin B12 supplementation given during the first 1000 days of life on neurodevelopmental outcomes and growth.

General objective

- To provide evidence for the role of vitamin B12 on neurodevelopment in vulnerable children in low and-middle income countries (LMICs).

Specific objectives and objectives of the separate studies

- In North Indian children 6 to 30 months at enrollment, measure to what extent six months' administration of vitamin B12 with or without folic acid improves neurodevelopment and cognitive function five years after the end of supplementation.
- In South Indian children, measure to what extent administration of vitamin B12 in pregnancy and early lactation improves neurodevelopment, cognition and neurophysiological function in childhood.
- In Tanzanian children, measure to what extent administration of vitamin B12 given with other

vitamins from early or mid-pregnancy until delivery improves neurodevelopment and cognitive function up to school age.

- In Tanzanian children, measure to what extent administration of vitamin B12 given with other vitamins from week 6 to 18 months improves neurodevelopment and cognitive function up to school age.
- To estimate the effect of vitamin B12 administration on certain domains of neurodevelopment using pooled data from all of the above studies.

METHODS AND ANALYSIS

Study design and interventions

This study is a follow-up of four recently completed double blind randomized-placebo controlled trials conducted in lower socioeconomic, semi-urban and urban populations in Delhi, India(15, 30), Bangalore, India(31) and in Dar es Salaam, Tanzania(32, 33) in 2001 to 2011. All trials provided at least two recommended dietary allowances (RDA) of vitamin B12 daily for at least six months, with no B12 supplementation in the placebo groups. The two studies that enrolled pregnant women provided the highest dose of vitamin B12 (50µg daily, approximately 20 times the RDA). An overview of the original trials and the follow-ups is presented in Table 1.

In Bangalore, South-India, pregnant women before or at 14 weeks gestational age were randomized to receive a daily dose of oral B12 (50 µg) or a placebo through 6 weeks post-partum. The primary objective was to determine the effect of vitamin B12 supplementation in improving maternal B12 status. Enrollment was completed in September 2010, and the last enrolled infant was born in August 2011. Oral B12 supplementation of women throughout pregnancy and early lactation, in combination with standard prenatal care with routine supplementation of iron and folate, significantly increased the B12 status of women and their offspring.(31) Neurodevelopment was measured at 9 and 30 months. In this follow-up study, we will measure the effect of maternal B12 supplementation on neurodevelopment and cognitive function several times 5-7 years after supplementation, and on neurophysiological outcomes using event-related potential (ERP).

Table. 1 Overview of study populations, study period and exposure in the original trials and in the Vitabeginning follow-up study

Characteristics	Bangalore	Delhi	Dares Salaam	
ORIGINAL TRIALS				
Trial registration number	NCT00641862	NCT00717730	NCT00197548	NCT00421668
Intervention period	09.2010-08.2011	01.2010-03.2011	08.2001- 02.2005	07.2007- 05. 2011
Original sample size	366	1000	8468	4400
Exposure	B12 [a]	B12 and/or folic acid [b]	Maternal MV [c]	MV and/or zinc [d]
Timing of exposure	Pregnancy	Infancy/early childhood	Pregnancy	Infancy
VITABEGINNING FOLLOW-UPS				
Expected sample size	230	800-900	366	446
Age range at follow-up	5-6.5 yrs	7-9 yrs	11-15 yrs	7-10 yrs
Start date of enrollment	01.2015	10.2016	07.2015	07.2015
Expected date for study completion	Estimated 07.2017	Estimated 11.2017	Estimated 11.2017	Estimated 11.2017

[a] *From < 14 wk gestational age to 6 wk post-partum B12 50 µg daily. In addition, all women were given iron and folic acid supplementation throughout pregnancy*
[b] *From 6-30 months for 6 months; four group: B12, B12 + folic acid, folic acid and placebo, doses >12 months: B12 1.8 µg and Folic acid 150 µg (half doses for <12 months).*
[c] *From 12-27 wk gestational age until delivery: Multivitamin (MV) B12 50 µg, B1 20mg, B2 20mg, B6 25 mg, Niacin 100mg, Vit-C 500 mg, Vit-E 30 mg, Folic acid 0.8 mg*
[d] *From 6 wk to 18 months, four groups; multivitamin (MV), MV + Zinc, Z and placebo.MV<6m: B12 1mg, B1 0.5mg, B2 0.6mg, B60.6mg, niacin 4mg, folic acid 130µg, Vit-C 60mg, Vit-E 8mg. Zinc5 mg. > 6 m MV and zinc doses were double*

In Delhi, North India, children 6-30 months of age were randomized to receive daily (i) B12, (ii) B12 and folate, (iii) folate, or (iv) placebo for six months. The primary objective was to measure the effect of these interventions on the incidence of diarrhea and pneumonia. Neurodevelopment was a predefined secondary outcome and was measured in a sub-sample of 422 children. In total 1000 children were enrolled between January 2010 and September 2011. B12 and folic acid supplemented children scored significantly higher on neurodevelopment scores compared to those who received placebo.(15) In this follow-up study we will measure to what extent early supplementation of folic acid and/or B12 improves neurodevelopment and cognitive function 5-6 years after supplementation.

In Dar es Salaam, Tanzania, infants 6-10 weeks of age born to HIV-negative mothers were randomized to receive daily (i) zinc, (ii) multivitamin, (iii) zinc +multivitamin, or (iv) placebo. Multivitamins contained on average two times the recommended daily allowance of B-vitamins, including B12 in addition to vitamin E and C. Children were followed for 18 months (i.e. until age 19.5 months). The primary objective was to measure the incidence of diarrhea and respiratory tract infections. Enrollment was completed in December 2009, and follow-up ended in May 2011. Neurodevelopmental outcomes were assessed in a subset of children at 15 months.(32) Daily zinc supplementation lowered the burden of diarrhea and respiratory tract infections. No added benefit was seen from the provision of multivitamins.(33)

In another trial in Dar es Salaam, Tanzania on micronutrients and adverse pregnancy outcomes, HIV-negative pregnant women at 12-27 weeks gestational age were randomized to receive daily oral supplementation of B-vitamins, including B12 in addition to vitamin E and C, or placebo. All women received prenatal iron and folate supplementation. The primary objective was to measure the effect of vitamin supplementation on fetal loss, low birthweight and severe preterm birth. Enrollment was completed in July 2004, and the last enrolled infant was born in February 2005. Multivitamin supplementation reduced the incidence of low birth weight and small for-gestational-age births, but had no significant effects on prematurity or fetal death.(34) The two studies from Tanzania are well suited for follow-up studies on potential impact of micronutrient supplementation on child health and neurodevelopmental outcomes in older children.

Outcomes

Neurodevelopment, cognitive function, and linear growth will be key outcomes in the different studies.

Neurodevelopment

Neurodevelopmental status in young children has been assessed by the Bayley Scales of Infant and Toddler Development 3rd ed. (Bayley- III)(35) and the Ages and Stages Questionnaire 3rd ed. (ASQ-3) in the original studies.(15, 36) In the follow-up, each site use their own unique collections of tests and questionnaires (Table 2). General cognitive functioning is assessed by a modified Kaufmann ABC II (Bangalore and Dar es Salaam), the Wechsler Preschool and Primary Scale of Intelligence –III (WPPSI III) (Bangalore) or the Wechsler Intelligence Scale for Children VI, Indian version (WISC-IV ^{India}) (Delhi). These are complemented by tests on specific cognitive functions by subtests from the developmental neuropsychological test battery NEPSY-II (Delhi), and social maturity such as the Vineland Social Maturity Scale (VSMS) (Bangalore), mental health and behavior such as the Strengths and Difficulties Questionnaire (SDQ) and executive functions by Behavior Rating Inventory of Executive Function (BRIEF (all sites). In addition, in one study (Bangalore) we study the effect of the maternal B12 supplementation on Event Related Potential (ERP) in children aged 6 years. See table 2 for details on the assessments at each site.

Anthropometry

Weight and height are measured using standard methods. In Delhi, height, weight and skin fold thickness were measured in children, using Seca scales (hight/weight) and Holtain Calipers. In Bangalore weights of mothers and children were recorded using a digital balance (Salter's 9016; Tonbridge) to the nearest 100g, and the heights were measured using a stadiometer to the nearest 0.1 cm.

Table 2. Overview over inventories and data-collection tools utilized in the different studies and the age of assessment

Outcomes to be measured	**Bangalore**	**Delhi**	**Dares Salaam**	
Vitamin B12 exposure	Pregnancy	Child	Pregnancy	Child
Outcomes to be measured	Age, yrs	Age, yrs	Age, yrs	Age, yrs
General abilities				
Modified KABC-II [a] EACABT®	5, 6		11-15	7-10
WISC-IV[INDIA]		7-9		
Verbal abilities				
Crichton Vocabulary Scale, Hindi edition		7-9		
Neurophysiological tests				
Event related potential [b]	6			
Neuropsychological tests				
NEPSY II		7-9		
Questionnaires				
Brief [c]	5.5	7-9	11-15	7-10
Strengths and Difficulties Questionnaire (SDQ)	6.5	7-9	11-15	7-10
Vineland Social Maturity Scale (VSMS)	5			
Maternal assessment				
Demography	5	7-9	11-15	7-10
Anthropometry	5, 5.5, 6, 6.5		11-15	7-10
Home environment	5.5		11-15	7-10
Nutrition				
24 hour diet recall form	5, 5.5, 6, 6.5			
Anthropometry	5, 5.5, 6, 6.5	7-9	11-15	7-10
Household food security questionnaire	5, 6			
Medical morbidity				
Morbidity questionnaire	5, 5.5, 6, 6.5		11-15	7-10
Biomarkers				
B12	5, 6	7-9		6-24m *
CBC, MMA, HcY, RBC folate, CRP	5, 6	7-9		
H	5, 6	7-9	11-15	7-10

[a] In Bangalore: Atlantis, Number recall, Word Order, Pattern Reasoning and Triangles from KABC and Koh's Block Design Test and Verbal Fluency in addition, in Dar es Salaam: The East African Cognitive Assessment battery (EACABT©) with permission from Savings Brain Multisite study (WHO/BMGF) including Atlantis, Hand Movements, Footsteps, Story completion, KILIFI Naming test, ROCF, NOGO, Shift, People Search, Literacy and Numeracy, HOME, Brief-P (modified from MAL-ED), SDQ, Behavior Questionnaire for Parents (BQP), in addition to Verbal Fluency and, Koh's Block

[b] ENBIO 32, Brain monitoring and stimulation technologies, mis-match negativity, P300

[c] Different versions utilized in different sites: in Bangalore: Brief Parent, In Delhi: Brief 2nd ed., in Dares Salaam: Saving Brains/Gates/WHO modified version from the malaria study,

** On stored serum samples from Child II cohort (subject to funding)*

Sample size calculation

The data for our most relevant tests are expected to be normally distributed. We have decided that a standardized effect size of 0.35 standard deviations (SD) is the minimally clinically relevant effect for the main psychometric tests. If the smallest group in any of our comparisons includes at least 130

children in each arm, and assuming a two-sided alpha error of 0.05 we will have 81%, 89%, and 98% power to detect differences for effect sizes of 0.35, 0.4, and 0.6, respectively. As a result, all of the described studies have sufficient power to detect important differences between vitamin B12 and placebo recipients, between individuals with poor and adequate vitamin B12 status or between other groups of other exposures as long as each of the groups contain at least 130 children. Statistical powers according to standardized mean effect sizes of .35, .4 and .6 and total sample sizes are depicted in figure 1. This graph was generated using the "power twomeans" command in STATA version 13, it assumes equal group sizes and equal variances, and a significance level of 0.05.

Data collection

Data-collection for the primary and secondary outcomes are synchronized and selected to capture the same domains of development using different tools. Assessment tools for cognitive functions such as general abilities, achievement, verbal-, visual spatial and sensorimotor skills, memory, executive functioning, general behavior and social perception and maturity are dependent on the child's age. We have carefully selected well-validated and developmentally sensitive instruments to ensure detection of the relevant predictors according to age and research questions. Instruments are adapted to ensure psychometric qualities, as well as cultural and linguistic appropriateness of the test at each site. Clinically useful tests will be prioritized, to improve sustainability of test material and knowledge of developmental assessment at the specific sites. A group of experienced scientists with expertise in developmental, neuropsychological and neurophysiological assessments are responsible for training and standardization in the different sites.

Analysis plan

Several domains will be measured and compared between the study groups within each of the studies. In these analyses, we will initially use the Students t-test for the crude analyses, but also multiple linear regression models to adjust for potential baseline differences and when measuring effect modification.

Each of the studies has several outcomes as we will compare both linear and ponderal growth, in addition to all the above-mentioned neurodevelopmental measures between the study groups. Thus, there will be several comparisons from each study and negative and positive effects will be reported in order to avoid focusing on spurious positive findings.

For each of the planned publications, we will make a detailed plan of analysis before commencing the analysis. In these plans, we will include sections on how to deal with multiple comparisons and

whether post hoc adjustments will be done. In addition to these standard per protocol analysis, we will consider Instrumental Variable Analysis (IVA) in an attempt to estimate the true effect of vitamin B12 had it been given to all participants in the scheduled doses and intervals. The random allocation will be the instrument in these analyses. For per protocol analysis, participants who received less than 50% of the projected doses during the period of intervention 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.

For our subgroup analyses, we will include interaction terms to measure whether or not the subgrouping variable significantly modifies the effect of the exposure of interest. All of our analyses will initially be done according to intent to treat.

We will not be able to retain the complete number of children from these studies. We will compare the features of the population that is included in this analysis with the population that we failed to reenroll into the study. We will also detect risk factors for poor neurodevelopment in multiple linear or binomial regression models. A significance level of 0.05 will be used.

ETHICS AND DISSEMINATION

Ethical and safety considerations

The exposures under investigation in this study were included in the original trials. We have obtained ethical approval of all new research activities in this follow-up study, from Norway and from the participating countries. Informed written parental consent will be taken from one or both parents of participating children prior to enrollment in the follow-up study and assent will be obtained from children older than 7 years in the Delhi site. Parents unable to read or write will be encouraged to bring along a literate relative or neighbor as an impartial witness.

Relevance and benefit to society

The current project takes advantage of four recently completed randomized-placebo controlled trials, to study the long-term effects of vitamin B12 supplementation on neurodevelopmental outcomes and growth in children in low- and middle-income countries. When measuring the effect of vitamin B12 on growth and development long term follow-up is important: Vitamin B12 can be stored for years in the body, and the effect of an increased intake for a relatively short period such as 6 months may accordingly last much longer. Furthermore, for many of the neurodevelopmental outcomes, it might not be possible to estimate the effect of early life exposures until later in

childhood because of limitations in the assessment tools. Thus, to follow children for a long time as in this project is important to fully understand the role of vitamin B12 for brain development and growth.

In most studies, follow-up for several years is not possible, so the results from our studies will be of great importance. Since none of the RCTs originally were designed for such long term follow-up, there is risk that loss to follow-up can bias our effect estimates. There is, however, no reason to believe that the loss to follow-up rate will be different according to randomized regimen in any of the described studies.

If positive effects of supplementation are observed, this may constitute important contributions to improve childhood nutrition in many low and middle-income countries. Our findings, however, must be interpreted in light of the results from other RCTs that are measuring the effect of vitamin B12 supplementation on growth and development. If anything, our findings will guide our next step in understanding the role of vitamin B12 nutrition on child growth and development: Should there be additional follow-ups in the ongoing studies, or will the results from our studies discourage further studies on vitamin B12 and child health?

It should also be emphasized the value of negative findings from this large project. If none, or very few, of our several outcomes respond to vitamin B12 supplementation, alone or when given in combination with several other micronutrients, then poor vitamin B12 status is likely not an important contributor to impaired neurodevelopment.

Millions of children across the world grow up malnourished lacking essential nutrients, with high burden of infectious diseases and where parents may not have the resources to provide an optimal environment for nurturing care. Acting in early childhood, these factors may result in poorer chances for later success in school and work. The results from the RCTs can lead to dietary recommendations that can improve learning and academic achievements, which again can lift individuals from the vicious cycle of poverty and malnutrition. Programs designed to prevent or treat micronutrient deficiencies can be targeted toward specific recommendations. Any further evidence for the long-term effect of dietary supplements has potentially high impact and may provide sustainable improvements in health and equity.

The suggested studies are geared towards rapid dissemination of results into national and international child health promotion programs. We will actively use the potential influence of the

international collaborators to ensure that our results reach the relevant health authorities.

Planned publications

Study results will be presented at national and international research and policy meetings, and published in peer-reviewed scientific journals, preferably open access. We will also discuss alternative strategies to inform the public. We will publish scientific papers as a consortium, specifically directed towards developmental and neuropsychological assessment methodology, and site specific publications. We expect each of the sites to generate approximately five publications in high ranked international peer reviewed journals.

AUTHOR´S CONTRIBUTIONS

TAS, IK, CPD, and WF took the initial initiative for the study. TAS, IK, CPD, WF, BAW, SK, KM, ST, MH, NB and ES, were involved in developing the design and the study protocol, ST, SK and KM are responsible for setting up the study conduct in each site, with support from AD, ST and SB. The statistical approach of the study was drafted by CS, MH, TAS, and WFMH, IK, ES, DCB, and SK were responsible for the cognitive assessments included in the protocol. All authors approved the final version of the protocol. BAW, IK and TAS drafted the current manuscript. All authors have reviewed and accepted the final version of the manuscript.

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COMPETING INTERESTS STATEMENT

None declared

FIGURE LEGENDS

Figure 1. Estimated required total sample sizes based on relevant effect sizes

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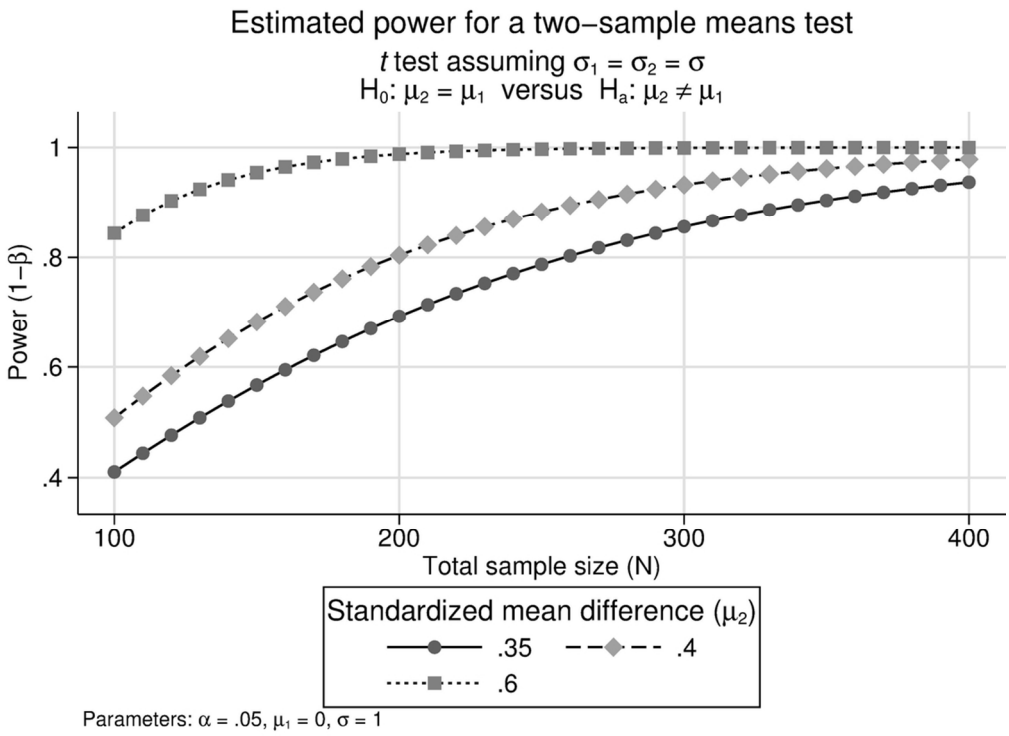

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Does early vitamin B12 supplementation improve neurodevelopment and cognitive function in childhood and into school age; a study protocol for extended follow-ups from randomized controlled trials in India and Tanzania

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ABSTRACT

Introduction

As many as 250 million children under the age of five may not being reaching their full developmental potential partly due to poor nutrition during pregnancy and the first 2 years of life. Micronutrients, including vitamin B12, are important for the development of brain structure and function; however, the timing, duration, and severity of deficiencies may alter the impact on functional development outcomes. Consequently, to fully explore the effect of vitamin B12 on cognitive function it is crucial to measure neurodevelopment at different ages, in different populations and with vitamin B12 supplementation at different times during the critical periods of neurodevelopment.

Methods and analysis

In this project we follow-up children from four recently completed randomized-placebo controlled trials of oral vitamin B12 supplementation, two in India and two in Tanzania, to explore the long-term effects on neurodevelopmental outcomes and growth. All the included trials provided at least two recommended dietary allowances (RDA) of oral vitamin B12 daily for at least six months. Vitamin B12 was supplemented either during pregnancy, early infancy or early childhood. Primary outcomes are neurodevelopmental status, cognitive function and growth later in childhood. We apply validated and culturally appropriate instruments to identify relevant developmental outcomes. All statistical analyses will be done according to intention to treat principles. The project provides an excellent opportunity to examine the effect of vitamin B12 supplementation in different periods during early life and measure the outcomes later in childhood.

Ethics and dissemination

The study has received ethical approvals from all relevant authorities in Norway, USA, Tanzania and India and complies fully with ethical principles for medical research. Results will be presented at national and international research and policy meetings, and published in peer-reviewed scientific journals, preferably open access.

Registration Original trials are registered. There are no new exposures in the follow-up study. Original trial registrations are NCT00641862 (Bangalore), NCT00717730, updated CTRI/2016/11/007494 (Delhi), and NCT00197548 and NCT00421668 (Dar es Salaam).

Key words

Vitamin B12; neurodevelopment; cognitive function; event related potential; executive function, growth

STRENGTHS AND LIMITATIONS OF THE STUDY

- The current project takes advantage of recently completed randomized-placebo controlled trials to study long-term effects of oral vitamin B12 supplementation on neurodevelopmental outcomes and growth in children in Tanzania and India.
- The project provides an excellent opportunity to examine the effect of vitamin B12 supplementation during different periods of the critical first 1000 days and measure the outcomes later in childhood.
- The studies were not designed to follow the children up beyond the time of supplementation, which may lead to a somewhat high rate of loss to follow-up.
- The effect measures may be difficult to standardize across the different studies.

INTRODUCTION

New estimates based on proxy measures of stunting and poverty indicates that as many as 250 million children under the age of five from developing countries are at risk of not fulfilling their developmental potential partly because of poor nutrition during pregnancy and the first 2 years of life.[1] Malnutrition and micronutrient deficiencies represent a major challenge to child health in many low- and middle-income countries (LMICs) and are associated with suboptimal cognitive function and poor growth.[2] Many children in South Asia and Africa suffer from deficiencies of several nutrients including vitamin B12.[3-7]

Why vitamin B12 may be important for the developing brain

Vitamin B12 plays a key role in normal brain development and function and is required for the folate-dependent enzyme, methionine synthase, which is necessary for the synthesis of methionine from homocysteine (tHcy).[8] Methionine in its activated form, S-adenosylmethionine, is the major methyl group donor used in human methylation reactions, including methylation of DNA and RNA. Deficient methylation reactions in the central nervous system can impair the methylation of myelin basic protein in the central as well as peripheral nervous system.[9] The production of myelin is a key component of brain development from gestation, throughout childhood and well into middle age.[10] The myelination of the brain is of importance for multiple brain systems and is highly related to neurodevelopment and subsequent cognitive functioning.[11] Vitamin B12 also serves as a cofactor in

numerous catalytic reactions in the human body, which are required for neurotransmitter synthesis and functioning. Vitamin B12 deficiency may cause pernicious anemia with similar effects on cognitive development and functioning as anemia caused by iron deficiency.[12] Vitamin B12 deficiency can also result in neuropathy through degeneration of nerve fibers and irreversible brain damage.[13] However, albeit subtle vitamin B12 deficiency is very common, the degree to which it has significant consequences for neurodevelopment and cognitive function is not established.[14 15]

Vitamin B12 and neurodevelopment

There is ample evidence that vitamin B12 is important for cognitive development.[11] In observational studies among adults and elderly, low levels of vitamin B12 are associated with cognitive decline and dementia.[16] Although there are fewer observational studies in children, the association between vitamin B12 status and cognitive functioning has also been documented from early infancy to adolescence. Neonatal severe vitamin B12 deficiency causes failure to thrive and possible irreversible neurological manifestations.[12 17] A study in the Netherlands showed that infants fed on a macrobiotic diet, which is low on vitamin B12, had delayed gross motor, speech and language development compared to infants on an omnivore diet.[18] In adolescence, these same children who were fed a macrobiotic diet for the six first years of life had poorer performance on cognitive tests, independent of their current vitamin B12 status, compared to adolescents who were fed on an omnivorous diet.[19] In an Indian infant cohort, we also demonstrated that poor vitamin B12 status was associated with lower scores on the Mental Development Index in the Bayley Scales of Infant and Toddler Development 2nd edition.[4] In a study from Nepal, we were able to demonstrate associations between early vitamin B12 status (2-12 months) and cognitive functioning five years later.[20] These long-term associations remained strong, even after adjusting for several potential confounders. There is also evidence for the significance of vitamin B12 to the developing brain in a few clinical trials. In a RCT Indian children aged 6-30 months were administered placebo or approximately twice the recommended daily allowances of vitamin B12 and/or folic acid for six months. Children provided with vitamin B12 and folic acid scored higher on a neurodevelopment assessment compared to the placebo group.[21] The effects were most prominent in stunted children or children less than 2 years of age. A favorable effect of vitamin B12 was also demonstrated on motor development in two RCT`s involving Norwegian infants with developmental regressions and signs of vitamin B12 deficiency one and six months following a 400 µg injection of vitamin B12.[22 23]

Knowledge gaps

There is a need to clarify to what extent improving vitamin B12 status impacts developmental

outcomes in children in LMICs. It is also important to identify populations in which such interventions can be beneficial and at what age vitamin B12 administration is most effective.[11] Most studies in this field have used only motor function tests or general neurodevelopment assessments[21 22 24], while less is known on the effect of B12 on specific cognitive functions such as executive functions, attention, and sensorimotor and visuospatial abilities.[20] The functions and areas under development are most sensitive to negative influences, and will provide the most specific outcomes in assessments.[25 26] Consequently, to fully understand findings in developmental assessments, we must consider the developmental timing of an exposure, in this case vitamin B12 supplementation (or lack thereof), as well as the timing of the assessment.[25] Measuring neurodevelopment at different ages, in different populations and the effect of exposure at different times during the critical periods of neurodevelopment is needed to understand the role of B12 in cognition.

Research on the impact of B12 on the neurophysiological outcomes in children is scarce. Event Related Potential (ERP) are non-invasive, reliable measures of neurophysiological brain function[27], that provide direct measure of underlying neural activity of higher mental processes. Behavioral assessments may sometimes not be sensitive enough to detect these small changes in brain functions[28] and these neurophysiological processes may be important correlates of the cognitive abilities and give new insight into the nutritional impact on the developing brain.[29]

STUDY OBJECTIVES

The aim of the current study, the Vitabeginning project, is to determine the long-term effects of vitamin B12 supplementation given during the first 1000 days of life on neurodevelopmental outcomes and growth.

General objective

- To provide evidence for the role of vitamin B12 supplementation in pregnancy or early childhood on neurodevelopment in vulnerable children in low and-middle income countries (LMICs).

Specific objectives and objectives of the separate studies

- In North Indian children 6 to 30 months at enrollment, measure to what extent six months' administration of vitamin B12 (1.8 µg) with or without folic acid (150 µg) improves neurodevelopment and cognitive function five years after the end of supplementation. Age range at follow-up is 7-9 years.
- In South Indian children, measure to what extent administration of daily vitamin B12 (50 µg) in

combination with iron and folic acid from 14 weeks gestational age through six weeks post-partum improves neurodevelopment, cognition and neurophysiological function in childhood. Age range at follow-up is 5 - 6.5 years.

- In Tanzanian children, measure to what extent administration of vitamin B12 (50 µg) given with other vitamins from 11-17 weeks gestational age until delivery improves neurodevelopment and cognitive function up to school age. Age range at follow-up is 11-15 years.
- In Tanzanian children, measure to what extent administration of vitamin B12 (1 mg) given with other vitamins from week 6 to 18 months of age improves neurodevelopment and cognitive function up to school age. Age range at follow-up is 7-10 years.
- To estimate the effect of vitamin B12 administration on certain domains of neurodevelopment using pooled data from all of the above studies.

METHODS AND ANALYSIS

Study design and interventions

This study is a follow-up of four recently completed double blind randomized-placebo controlled trials conducted in lower socioeconomic, semi-urban and urban populations in Delhi, India[21 30], Bangalore, India[31] and in Dar es Salaam, Tanzania[32 33] in 2001 to 2011. All trials provided at least two recommended dietary allowances (RDA) of vitamin B12 daily (2 x 2.*6* µg for adults and 9 µg for small children) for at least six months, with no B12 supplementation in the placebo groups. The two studies that enrolled pregnant women provided the highest dose of vitamin B12 (50 *µg* daily, which is approximately 20 times the RDA). An overview of the original trials and the follow-ups is presented in Table 1.

In Bangalore, South-India, HIV-negative pregnant women recruited before or at 14 weeks gestational age were randomized to receive a daily dose of oral B12 (50 *µg*) or a placebo through 6 weeks post-partum. The primary objective was to determine the effect of vitamin B12 supplementation in improving maternal B12 status. Enrollment was completed in September 2010, and the last enrolled infant was born in August 2011. Oral B12 supplementation of women throughout pregnancy and early lactation, in combination with standard prenatal care with routine supplementation of iron and folate, significantly increased the B12 status of women and their offspring.[31] Neurodevelopment was measured at 9 and 30 months. In this follow-up study, we will measure the effect of maternal B12 supplementation on neurodevelopment and cognitive function several times 5-6.5 years after supplementation, and on neurophysiological outcomes using event-related potential (ERP).

Table. 1 Overview of study populations, study period and exposure in the original trials and in the Vitabeginning follow-up study

Characteristics	Bangalore	Delhi	Dares Salaam	
ORIGINAL TRIALS				
Trial registration number	NCT00641862	NCT00717730	NCT00197548	NCT00421668
Intervention period	09.2010-08.2011	01.2010-03.2011	08.2001- 02.2005	07.2007- 05. 2011
Original sample size	366	1000	8468	4400
Exposure	B12 [a]	B12 and/or folic acid [b]	Maternal MV [c]	MV and/or zinc [d]
Timing of exposure	Pregnancy (daily supplement from <14 wk ga through 6 wk postpartum)	Infancy/early childhood (daily supplement for 6 m from 6-30 m)	Pregnancy (daily supplement from 11-17wk until delivery)	Infancy (daily supplement from 6 wk to 18 m)
VITABEGINNING FOLLOW-UPS				
Expected sample size	230	800-900	366	446
Age range at follow-up	5-6.5 yrs	7-9 yrs	11-15 yrs	7-10 yrs
Start date of enrollment	01.2015	10.2016	07.2015	07.2015
Expected date for study completion	Estimated 07.2017	Estimated 11.2017	Estimated 11.2017	Estimated 11.2017

[a] *From < 14 wk gestational age to 6 wk post-partum B12 50 µg daily. In addition, all women were given iron and folic acid supplementation throughout pregnancy*
[b] *From 6-30 months for 6 months; four group: B12, B12 + folic acid, folic acid and placebo, doses >12 months: B12 1.8 µg and Folic acid 150 µg (half doses for <12 months).*
[c] *From 12-27 wk gestational age until delivery: Multivitamin (MV) B12 50 µg, B1 20mg, B2 20mg, B6 25 mg, Niacin 100mg, Vit-C 500 mg, Vit-E 30 mg, Folic acid 0.8 mg*
[d] *From 6 wk to 18 months, four groups; multivitamin (MV), MV + Zinc, Z and placebo.MV<6m: B12 1mg, B1 0.5mg, B2 0.6mg, B60.6mg, niacin 4mg, folic acid 130µg, Vit-C 60mg, Vit-E 8mg. Zinc5 mg. > 6 m MV and zinc doses were double*

In Delhi, North India, children 6-30 months of age recruited from the were randomized to receive daily (i) B12, (ii) B12 and folate, (iii) folate, or (iv) placebo for six months. The supplementation included B12 (1.8 μg) and/or folate (150 μg) and with half doses for children < 12 months. The primary objective was to measure the effect of these interventions on the incidence of diarrhea and pneumonia. Neurodevelopment was a predefined secondary outcome and wasmeasured in a sub-sample of 422 children. In total 1000 children were enrolled between January 2010 and September 2011. B12 and folic acid supplemented children scored significantly higher on neurodevelopment scores at the age of 12-36 months, compared to those who received placebo.[21] In this follow-up study we will measure to what extent early supplementation of folic acid and/or B12 improves neurodevelopment and cognitive function 5-6 years after supplementation. The study is powered to measure the effect of, vitamin B12, folic acid and the two vitamins combined on neurodevelopmental outcomes. The follow-up study will also measure to what extent vitamin D status in early life is associated with neurodevelopmental scores in early school years.

In Dar es Salaam, Tanzania, infants 6-10 weeks of age born to HIV-negative mothers were randomized to receive daily (i) zinc, (ii) multivitamin, (iii) zinc +multivitamin, or (iv) placebo. Multivitamins included B12 1 mg, B1 0.5 mg, B2 0.6 mg, B6 0.6 mg, niacin 4 mg, folic acid 130 μg, Vit-C 60 mg and Vit-E 8 mg and was provided alongside zinc 5 mg. Doses were doubled after 6 months. Children were followed for 18 months (i.e. until age 19.5 months). The primary objective was to measure the incidence of diarrhea and respiratory tract infections. Enrollment was completed in December 2009, and follow-up ended in May 2011. Neurodevelopmental outcomes were assessed in a subset of children at 15 months.[32] Daily zinc supplementation lowered the burden of diarrhea and respiratory tract infections. No added benefit was seen from the provision of multivitamins.[33]

In another trial in Dar es Salaam, Tanzania on micronutrients and adverse pregnancy outcomes, HIV-negative pregnant women at 12-27 weeks gestational age were randomized to receive daily oral supplementation of multivitamins including B12 or placebo. Multivitamins included B12 50 μg, B1 20mg, B2 20 mg, B6 25 mg, Niacin 100 mg, vit-C 500 mg, Vit-E 30 mg and folic acid 0.8 mg. All women received prenatal iron and folate supplementation. The primary objective was to measure the effect of vitamin supplementation on fetal loss, low birthweight and severe preterm birth. Enrollment was completed in July 2004, and the last enrolled infant was born in February 2005. Multivitamin supplementation reduced the incidence of low birth weight and small for-gestational-age births, but had no significant effects on prematurity or fetal death.[34] The two studies from Tanzania are well suited for follow-up studies on potential impact of micronutrient supplementation on child health

and neurodevelopmental outcomes in older children.

Outcomes

Neurodevelopment, cognitive function, and linear growth will be key outcomes in the different studies.

Neurodevelopment

Neurodevelopmental status in young children has been assessed by the comprehensive assessment tool Bayley Scales of Infant and Toddler Development 3rd ed. (Bayley- III)[35] and the easily administered screening tool Ages and Stages Questionnaire 3rd ed. (ASQ-3) in the original studies.[21 36]

In the follow-up, each site use their own unique collections of tests and questionnaires (Table 2). General intellectual functioning is assessed by a modified Kaufmann ABC II (Bangalore and Dar es Salaam), the Wechsler Preschool and Primary Scale of Intelligence–III (WPPSI III) (Bangalore) or the Wechsler Intelligence Scale for Children VI, Indian version (WISC-IV [India]) (Delhi). These are complemented by tests on specific cognitive functions by subtests from the developmental neuropsychological test battery NEPSY-II including attention and executive functioning, language, social perception, sensorimotor and visuospatial processing (Delhi), and adaptive functioning by the Vineland Social Maturity Scale (VSMS) (Bangalore). Mental health and behavior problems are measured by the parent reported screening instrument Strengths and Difficulties Questionnaire (SDQ), and executive functions by the parent reported questionnaire Behavior Rating Inventory of Executive Function (BRIEF (all sites). Finally, in one study (Bangalore) we measure neurophysiological functions using event related potentials as this may yield additional information on the effects of nutritional deficiencies on brain function. In the present study we propose to use two well characterized ERPs, P-300 and Mis-match Negativity (MMN) that are known to reflect higher cognitive functions of attention and memory. See table 2 for details on the assessments at each site.

Anthropometry

Weight and height are measured using standard methods. In Delhi, height, weight and skin fold thickness were measured in children, using Seca scales (hight/weight) and Holtain Calipers. In Bangalore weights of mothers and children were recorded using a digital balance (Salter's 9016; Tonbridge) to the nearest 100g, and the heights were measured using a stadiometer to the nearest 0.1 cm.

Table 2. Overview over inventories and data-collection tools utilized in the different studies and the

age of assessment

Outcomes to be measured	Bangalore	Delhi	Dares Salaam	
Vitamin B12 exposure	Pregnancy	Child	Pregnancy	Child
Outcomes to be measured	Age, yrs	Age, yrs	Age, yrs	Age, yrs
General abilities				
Modified KABC-II [a] EACABT®	5, 6		11-15	7-10
WISC-IV[INDIA]		7-9		
Verbal abilities				
Crichton Vocabulary Scale, Hindi edition		7-9		
Neurophysiological tests				
Event related potential [b]	6			
Neuropsychological tests				
NEPSY II		7-9		
Questionnaires				
Brief [c]	5.5	7-9	11-15	7-10
Strengths and Difficulties Questionnaire (SDQ)	6.5	7-9	11-15	7-10
Vineland Social Maturity Scale (VSMS)	5			
Maternal assessment				
Demography	5	7-9	11-15	7-10
Anthropometry	5, 5.5, 6, 6.5		11-15	7-10
Home environment	5.5		11-15	7-10
Nutrition				
24 hour diet recall form	5, 5.5, 6, 6.5	7-9		
Anthropometry	5, 5.5, 6, 6.5	7-9	11-15	7-10
Household food security questionnaire	5, 6			
Medical morbidity				
Morbidity questionnaire	5, 5.5, 6, 6.5		11-15	7-10
Biomarkers				
Vitamin B12	5, 6	7-9		6-24m *
**CBC, MMA, HcY, RBC folate, CRP	5, 6	7-9		
***H	5, 6	7-9	11-15	7-10

[a] In Bangalore: Atlantis, Number recall, Word Order, Pattern Reasoning and Triangles from KABC and Koh's Block Design Test and Verbal Fluency in addition, in Dar es Salaam: The East African Cognitive Assessment battery (EACABT©) with permission from Savings Brain Multisite study (WHO/BMGF) including Atlantis, Hand Movements, Footsteps, Story completion, KILIFI Naming test, ROCF, NOGO, Shift, People Search, Literacy and Numeracy, HOME, Brief-P (modified from MAL-ED), SDQ, Behavior Questionnaire for Parents (BQP), in addition to Verbal Fluency and, Koh's Block

[b] ENBIO 32, Brain monitoring and stimulation technologies, mis-match negativity, P300

[c] Different versions utilized in different sites: in Bangalore: Brief Parent, In Delhi: Brief 2nd ed., in Dares Salaam: Saving Brains/Gates/WHO modified version from the malaria study,

** On stored serum samples from Child II cohort (subject to funding)*

*** CBC, complete blood count; MMA, methylmalonic acid; HcY, homocysteine; RBC folate, red blood cell folate analysis; CRP, c reactive protein*

**** H, hemoglobin*

Sample size calculation

The data for our most relevant tests are expected to be normally distributed. We have decided that a

standardized effect size of 0.35 standard deviations (SD) is the minimally clinically relevant effect for the main psychometric tests. If the smallest group in any of our comparisons includes at least 130 children in each arm, and assuming a two-sided alpha error of 0.05 we will have 81%, 89%, and 98% power to detect differences for effect sizes of 0.35, 0.4, and 0.6, respectively. As a result, all of the described studies have sufficient power to detect important differences between vitamin B12 and placebo recipients, between individuals with poor and adequate vitamin B12 status or between other groups of other exposures as long as each of the groups contain at least 130 children. Statistical powers according to standardized mean effect sizes of .35, .4 and .6 and total sample sizes are depicted in figure 1. This graph was generated using the "power twomeans" command in STATA version 13, it assumes equal group sizes and equal variances, and a significance level of 0.05.

< Insert figure 1 about here >

Data collection

Data-collection for the primary and secondary outcomes are synchronized and selected to capture the same domains of development using different tools. Assessment tools for cognitive functions such as general abilities, achievement, verbal-, visual spatial and sensorimotor skills, memory, executive functioning, general behavior and social perception and maturity are dependent on the child's age. We have carefully selected well-validated and developmentally sensitive instruments to ensure detection of the relevant predictors according to age and research questions. Instruments are adapted to ensure psychometric qualities, as well as cultural and linguistic appropriateness of the test at each site. Clinically useful tests will be prioritized, to improve sustainability of test material and knowledge of developmental assessment at the specific sites. Assessments are administered by trained psychologists in the Indian sites and by trained health care workers in the Tanzania site. A group of experienced scientists with expertise in developmental, neuropsychological and neurophysiological assessments are responsible for the training and standardization in the different sites.

Analysis plan

Several domains will be measured and compared between the study groups within each of the studies. In these analyses, we will initially use the Students t-test for the crude analyses, but also multiple linear regression models to adjust for potential baseline differences and when measuring effect modification.

Each of the studies has several outcomes as we will compare both linear and ponderal growth, in addition to all the above-mentioned neurodevelopmental measures between the study groups. Thus, there will be several comparisons from each study and negative and positive effects will be reported in order to avoid focusing on spurious positive findings.

For each of the planned publications, we will make a detailed plan of analysis before commencing the analysis. In these plans, we will include sections on how to deal with multiple comparisons and whether post hoc adjustments will be done. In addition to these standard per protocol analysis, we will consider Instrumental Variable Analysis (IVA) in an attempt to estimate the true effect of vitamin B12 had it been given to all participants in the scheduled doses and intervals. The random allocation will be the instrument in these analyses. For per protocol analysis, participants who received less than 50% of the projected doses during the period of intervention 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.

For our subgroup analyses, we will include interaction terms to measure whether or not the subgrouping variable significantly modifies the effect of the exposure of interest. All of our analyses will initially be done according to intent to treat.

We will not be able to retain the complete number of children from these studies. We will compare the features of the population that is included in this analysis with the population that we failed to reenroll into the study. We will also detect risk factors for poor neurodevelopment in multiple linear or binomial regression models. We will include socioeconomic and seasonal factors and dietary intake as exposures in the analyses. A significance level of 0.05 will be used.

ETHICS AND DISSEMINATION

Ethical and safety considerations

The exposures under investigation in this study were included in the original trials. We have obtained ethical approval of all new research activities in this follow-up study, from Norway and from the participating countries. Informed written parental consent will be taken from one or both parents of participating children prior to enrollment in the follow-up study and assent will be obtained from children older than 7 years in the Delhi site. Parents unable to read or write will be encouraged to bring along a literate relative or neighbor as an impartial witness.

Relevance and benefit to society

The current project takes advantage of four recently completed randomized-placebo controlled trials, to study the long-term effects of vitamin B12 supplementation on neurodevelopmental outcomes and growth in children in low- and middle-income countries. When measuring the effect of vitamin B12 on growth and development long term follow-up is important: Vitamin B12 can be stored for years in the body, and the effect of an increased intake for a relatively short period such as 6 months may accordingly last much longer. Furthermore, for many of the neurodevelopmental outcomes, it might not be possible to estimate the effect of early life exposures until later in childhood because of limitations in the assessment tools. Thus, to follow children for a long time, as in this project, is important to fully understand the role of vitamin B12 for brain development and growth.

In most studies, follow-up for several years is not possible, so the results from our studies will be of great importance. Since none of the RCTs originally were designed for such long term follow-up, there is risk that loss to follow-up can bias our effect estimates. There is, however, no reason to believe that the loss to follow-up rate will be different according to randomized regimen in any of the described studies. The risk of confounding of the main exposures (Vitamin B12 supplementation) is small because of the RCT design and the large number of randomization units. Substantial imbalances of potential confounding factors across study groups are unlikely.

If positive effects of supplementation are observed, this may constitute important contributions to improve childhood nutrition in many low and middle-income countries. However, several factors may have profound impact on long-term neurodevelopment outcomes and growth and potentially dilute the effect of optimizing B12 status in early life. Our findings, however, must be interpreted in light of the results from other RCTs that are measuring the effect of vitamin B12 supplementation on growth and development. If anything, our findings will guide our next step in understanding the role of vitamin B12 nutrition on child growth and development: Should there be additional follow-ups in the ongoing studies, or will the results from our studies discourage further studies on vitamin B12 and child health?

It should also be emphasized the value of negative findings from this large project. If none, or very few, of our several outcomes respond to vitamin B12 supplementation, alone or when given in combination with several other micronutrients, then poor vitamin B12 status is likely not an important contributor to impaired neurodevelopment.

Millions of children across the world grow up malnourished lacking essential nutrients, with high burden of infectious diseases and where parents may not have the resources to provide an optimal environment for nurturing care. Acting in early childhood, these factors may result in poorer chances for later success in school and work. The results from the RCTs can lead to dietary recommendations that can improve learning and academic achievements, which again can lift individuals from the vicious cycle of poverty and malnutrition. Programs designed to prevent or treat micronutrient deficiencies can be targeted toward specific recommendations. Any further evidence for the long-term effect of dietary supplements has potentially high impact and may provide sustainable improvements in health and equity.

The suggested studies are geared towards rapid dissemination of results into national and international child health promotion programs. We will actively use the potential influence of the international collaborators to ensure that our results reach the relevant health authorities.

Planned publications

Study results will be presented at national and international research and policy meetings, and published in peer-reviewed scientific journals, preferably open access. We will also discuss alternative strategies to inform the public. We will publish scientific papers as a consortium, specifically directed towards developmental and neuropsychological assessment methodology, and site specific publications. We expect each of the sites to generate approximately five publications in high ranked international peer reviewed journals.

AUTHOR´S CONTRIBUTIONS

TAS, IK, CPD, and WF took the initial initiative for the study. TAS, IK, CPD, WF, BAW, SK, KM, ST, MH, NB and ES, were involved in developing the design and the study protocol, ST, SK and KM are responsible for setting up the study conduct in each site, with support from AMD, ST, TK, AK and SB. The statistical approach of the study was drafted by CRS, MH, TAS, and WFMH, IK, ES, DCB, and SK were responsible for the cognitive assessments included in the protocol. All authors approved the final version of the protocol. BAW, IK and TAS drafted the current manuscript. All authors have reviewed and accepted the final version of the manuscript.

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Funding of the original trials

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COMPETING INTERESTS STATEMENT

None declared

FIGURE LEGENDS

Figure 1. Estimated required total sample sizes based on relevant effect sizes

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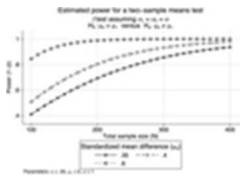

Figure 1. Estimated required total sample sizes based on relevant effect sizes

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