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Prevalence of specific micronutrient deficiencies in urban school going children of India aged between 6-16 years: study protocol for a multicentric cross-sectional study

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ABSTRACT

Introduction

Childhood and adolescence are the period of rapid physical and cognitive growth and development, requiring adequate nutrition. Malnutrition in the form of undernutrition or micronutrient deficiency or overweight/obesity effects the health, cognition and educational achievement of this age group.The objective this study is to assess the prevalence of Calcium, Iron, Ferritin, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies in the serum and Hemoglobin and Lead levels and its association with reported dietary intake and cognitive abilities, in urban school going children aged 6 to 16 years in ten cities of India.

Methods and analysis

A multicentric cross sectional study will be conducted to recruit 2400 participants (240 per site). Participants will be selected using two stage random sampling technique and will be categorized into age groups of 6 to 11 years and 12 to 16 years, with equal distribution. Data on socio economic status, anthropometric measures and three-day dietary intake and cognitive performance will be collected. Blood samples will be collected for biochemical analysis of micronutrients. Findings will estimate the prevalence of micronutrient deficiencies and their association with dietary habits and cognitive functioning.

Ethics and dissemination

Study protocol has been reviewed and approvedby institutional ethics committee of all ten participating sites. Results will be shared and published in a peer-reviewed journal so that the findings will be helpful for the stakeholders in planning nutritional interventions for targeted groups.

Trial registration number: CTRI/2019/02/017783.

Key words

Malnutrition, micronutrients, school age children, adolescents, cognitive, diet

Strengths and limitations of this study

- Multicentric study with sites being representative of different geo-cultural regions of India.
- This protocol will clearly synthesize evidence on micronutrient deficiencies in school going children and adolescents.
- It will establish association between micronutrient status and cognitive performance of children.
- Chances of recall bias during self-reported dietary survey.

INTRODUCTION

Childhood and adolescence are the period of rapid physical and cognitive growth and development, requiring adequate nutrition. Any change in nutritional status during this age influences health, learning and physical fitness. Nutrients essential for normal growth and functioning of human body are macronutrients like carbohydrate, fat and proteins, required in large quantities and micronutrients like vitamins and minerals required in small quantities. Vitamins, categorized as fat soluble (A, D, E and K) and water soluble (B group and C), are synthesized in human body in quantities lesser than required. Minerals are required for growth, repair and regulation of vital functions of human body. They are major minerals like calcium, phosphorus, sodium, potassium, magnesium and trace elements like iron, iodine, ferritin, fluoride, zinc, copper, cobalt, chromium, manganese, molybdenum, selenium and nickel.

The term malnutrition addresses three broad groups of conditions:

- Undernutrition: Indicating wasting (low weight-for-height), stunting (low height-for-age) and underweight (low weight-for-age)
- Micronutrient-related malnutrition: micronutrient deficiencies or excess
- Overweight and obesity

Micronutrient deficiencies affect an estimated two billion people, or almost one-third of the world's population (1). Various studies had reported the sub optimal nutritional status of Indian children. Prevalence of anaemia in school children and adolescents is between 19-88% across five different cities in India (2) and may be attributed to inadequate food intake, poor stores and deficiencies of nutrients (3). Nutrition anaemia is caused by deficiency of iron, folic acid and vitamin B 12 (4). The association between nutritional status and health, cognition and

educational achievement is established among the school going children (5). Deficiency of micronutrients and nutrients, delays cognitive and motor development and is associated with low Intelligence Quotient (IQ) (6,7,8). Stunting is linked to compromised intellectual attainment and school performance. Long term malnutrition may also result into thinness, decreased muscular strength and work capacity and reduced bone density later in life (9).

In Indian scenario, there is definitely a need for well-planned, large-scale study using standardized methodologies to estimate the prevalence of micronutrient deficiencies with giving due importance to accurate evaluation of socio-economic status and representation of the different regions of the country (10).

Hence the present study will be conducted with an aim to assess the prevalence of deficiency of various vitamins (Folic acid, Vitamin A, 25 Hydroxy Vitamin D, Vitamin B12) and minerals (Calcium, Iron, Zinc, Selenium) and its association with reported dietary intake and cognitive abilities, in urban school children aged 6 to 16 years in ten cities of India.

METHODOLOGY

Ethical approval

Ethical approval from institutional ethics committees of all the ten respective sites has been taken. The study is registered prospectively with Clinical Trial Registry of India (registration number CTRI/2019/02/017783).

Study design

This is a multi-centric cross-sectional study. Itwill be conducted in ten major cities across India. Each site will recruit 240 participants, having equal proportion of gender and age group (Figure 1).

Study setting

Study sites at Bangalore, Bhubaneswar, Chandigarh, Dibrugarh, Jodhpur, Lucknow, Patna, Srinagar, Thiruvananthapuram and Udupi districts are selected as being representative of different geo-cultural regions of India (Figure 2). King George's Medical University (KGMU), Lucknow will be the central coordinating unit (CCU) for the study.

These ten cities have a total population of 24.1 million, which is 2% of country's total population. Study cohort population in these cities was 7.1 million (11). Demographic characteristics and key anthropometric indicators in urban areas of study sites are shown in table 1 (11,12).

Table 1: Demographic characteristics and key anthropometric indicators in urban areas of study sites

Study Site	Urban population of district (in millions)		Literacy rate (state)	Prevalence rate of (state) (age in years)					
				Severe thinness			Obesity		
	All ages	6 to 16 years		5-9	10-14	15-19	5-9	10-14	15-19
India	377.11	76.73	84.1	4.7	7.0	4.9	2.8	2.5	1.8
M S Ramaiah Medical College & Hospital Bangalore	8.75	1.42	85.8	5.2	3.4	4.0	2.4	1.5	8.0
Kalinga Institute of Medical Sciences, Bhubaneswar	1.08	0.20	85.7	1.5	2.5	0.4	8.9	9.1	4.5
Post Graduate Institute of Medical Sciences, Chandigarh	1.03	0.20	86.2	-	-	-	-	-	-
Assam Medical College, Dibrugarh	0.24	0.043	88.5	4.3	11.9	5.7	4.3	2.2	0.0
All India Institute of Medical Sciences, Jodhpur	1.27	0.29	79.7	4.3	7.4	5.4	1.3	1.5	1.7
King George's Medical University, Lucknow	3.04	0.64	75.1	5.9	6.6	3.3	2.0	1.0	0.0
All India Institute of Medical Sciences, Patna	2.52	0.62	76.9	5.5	7.6	3.2	0.8	0.2	0.2
Sher-i-Kashmir Institute of Medical Sciences, Srinagar	1.22	0.24	77.1	2.9	2.4	5.0	4.3	3.0	2.2
Government Medical College,	1.77	0.28	95.1	2.9	4.1	6.4	5.4	1.8	0.8

Thiruvananthapuram									
Kasturba Medical College, Manipal, Udupi	0.33	0.05	85.8	5.2	3.4	4.0	2.4	1.5	8.0

Objective

The primary objective of study is to assess the prevalence of Calcium, Iron, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels in urban school going children aged 6 to 16 years in ten cities of India.

Secondary objectives are:

(a) To assess the association of Calcium, Iron, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with anthropometric indicators in urban school going children aged 6 to 16 years in ten cities of India.

(b) To assess the association of Calcium, Iron, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with socioeconomic status in urban school going children aged 6 to 16 years in ten cities of India.

(c) To assess the association of Calcium, Iron, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with cognitive assessments in urban school going children aged 6 to 16 years in ten cities of India, by

- General Intelligence (Colored Progressive Matrices (CPM), Standard Progressive Matrices (SPM)
- Attention, concentration and Visuo-motor coordination (Coding Test)
- Working Memory (Digit span Test, Arithmetic test)

(d) To assess the association of Calcium, Iron, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with three-day dietary intake assessed by 24 hours recall method in urban school going children aged 6 to 16 years in ten cities of India.

Sample size computation

Assuming the prevalence of folate deficiency in India as 30.7% (13), precision of 2% and level of confidence 0.05, the calculated sample size is 2044 participants. After taking, attrition rate of 10% sample size will inflate to 2400 participants. This sample size will be equally divided in ten sites respectively.

Sampling technique

Participants will be selected by using two-stage sampling technique. In the first stage, schools will be selected, and in the second stage participants will be recruited from the selected schools.

Selection of participants

Each study site will provide a list of schools imparting co-education to children between 6 to 16 years of age and located within the urban limits of city. From this list six schools will be randomly selected, having at least one to a maximum of three private schools. Principals of recruited schools will be met to obtain written voluntarily informed consent. They will be asked to allocate a coordinating teacher from the school. With the help of coordinating teacher, a gender-wise list of students between 6 to 11 and 12 to 16 years of age will be prepared. From each of these lists, fifteen students who are apparently healthy and residing within five kilometers of radius from school will be randomly selected. They will be invited to participate into the study. Out of these, first ten participants whose parents will provide written informed consent will be included into the study. Rest will be kept as back-up in case of exclusions. Participants having body mass index (BMI) below 12.5% will be excluded from the study. Written assent will be obtained from all participants who will be 8 years or above of age.

Training of study team

Site team will consist of a social worker, a data entry operator, a Nutritionist and a Psychologist. Training of Nutritionist and Psychologist will be conducted at CCU Lucknow by project investigators and co-investigators. These will be oriented to train the site staff on study protocol, procedures, data collection instruments, data entry tools and standard operating procedures.

Data collection

The study opened for data collection in April 2019 and is still ongoing due to COVID-19 pandemic and is expected to complete by mid of 2021.

Demographic and socioeconomic data

Demographic and socioeconomic details of participant will be recorded by interviewing participants and their primary care giver. Revised Kuppuswamy's socioeconomic scale (14) will be used to assess socioeconomic status.

Anthropometric measurements

The height will be measured to the nearest 0.1 centimeter using Seca 213 Mobile Stadiometer (Seca, Hamburg, Deutschland). Participant will be asked to stand barefoot after removing hair barrettes & rubber bands, with the heels, back, and head touching the measuring rod. A head board placed above the head perpendicular to the ruler on measuring rod and parallel to the ground will be used to record height measure. Two measurements of height will be taken for each participant. If the difference between the two height measurements is greater than 5 mm, then a second set of two height measurements will be taken to obtain more precise values.

Weight will be measured to the nearest 0.1 kilogram using portable Seca 803 weighing scale (Seca, Hamburg, Deutschland). The unit will be standardized by calibrating it to zero before each measurement. The participants will be weighed barefoot with empty pockets and without any heavy items like woolen blazers or belts.

Body Mass Index (BMI) will be calculated using the standard equation:

$$\text{BMI}\left(\frac{\text{kg}}{\text{m2}}\right) = \text{Weight (in kg)/ [Height (in m)]}^2$$

For assessing anthropometric indicators, recommendations of the WHO expert committee will be used as shown in table 2.

Table 2: Recommendations of the WHO expert committee for assessing anthropometric indicators

Stunting	:	Height for age < –2 SD of the WHO Child Growth Standards median
Severe thinness	:	BMI for age < –3 SD of the WHO Child Growth Standards median
Thinness	:	BMI for age < –2 SD of the WHO Child Growth Standards median
Overweight	:	BMI for age > +1 SD of the WHO Child Growth Standards median
Obesity	:	BMI for age >+2 SD of the WHO Child Growth Standards median
Severe obesity	:	BMI for age >+3 SD of the WHO Child Growth Standards median

Cognitive assessment

Cognitive assessment will be administered at a mutually convenient time in a separate room to keep relaxed and pleasant environment. The assessment will be conducted in a single individual session. During the assessment session, the psychologist will first make the child comfortable by establishing good rapport. Each participant will be assessed for attention, concentration and

visuo-motor coordination throughcoding test (15), general intelligence through colored progressive matrices (CPM)(16)/ standard progressive matrices (SPM)(17) and working memory using arithmetic and digit span test (15).

Coding test: Sheet 'A' will be used for participants below 8 years of age, rest will use sheet'B' sheet.Participants will be given 120 seconds to complete the test. One point will be awarded for each correct response, excluding samples.

CPM/SPM: CPM will be administered to participants 6 to 11 years of age. Participants 12 years or above of age will be given SPM. Each participant will be given about 15 to 20 minutes to complete the assessment.

Arithmetic and digit span test: These will be undertaken by all the participants. 3 to 5 minutes will be given to complete digit span test and 10 minutes will be given for arithmetic test.

For scoring and interpretation of test results, standard tables from respective test's manual (15,16,17) will be referred.

Dietary assessment

General dietary assessment will include dietary habits, meal frequency and consumption of water, beverages, green leafy vegetables, fruits, and animal products. Frequencies of common food items, popular among children will also be captured.

Data on dietary intake of children will be collected using 24h recall method for two non-consecutive days and one Sunday, which are not fasting or feasting days. The intake will be recorded by interviewing participant along with his/her mother or primary caregiver. Previously standardized cups, glass and spoons willbe used as an aid to help in recalling the quantity of different foods consumed by the participants in a 24 hour period prior to the investigation.

For calculating the nutritive value of raw ingredients, Nutritive value of Indian foods will be used (18). Daily nutrient intake for all the nutrients will be calculated using DIETSOFT Software and will be compared with the Recommended Dietary Allowances (19).

Dietary intake will be assessed in terms of Nutrient Adequacy Ratio (NAR)(20). The NAR for a given nutrient is the ratio of a participant's intake to the current recommended allowance for each sex and age category. To estimate the nutrient adequacy of the diet, NAR will be calculated for all the nutrients using the equation:

$$\text{NAR} = \text{Participant's nutrient intake of a day} / \text{RDA of the respective nutrient}$$

Participants will be then categorized as having (a)Adequate NAR (NAR≥1.00), (b) Fairly adequate NAR (0.66> NAR <1.00) and Inadequate NAR (NAR<0.66), for various nutrients (20).

Dietary diversity (DD), defined as the number of different foods or food groups consumed in a day, will be measured using Dietary Diversity Score (DDS). DDS will be measured by categorizing the food items, consumed in a day, into fourteen groups. Simple counting of different types of food groups consumed in a particular day will give individual DDS, which would range from 1 to 14.

Thereafter, participantswill be categorized in three classes according to their Dietary Diversity Score (21)-

1. Low: ≤8
2. Moderate: 9
3. High: ≥10

The fourteen food groups that we would use areanimal meat, cereals and millets, fats and edible oils, meat and poultry, fishes and other sea foods, fruits, pulses and legumes, green leafy vegetables, milk and milk products, nuts and oil seeds, other vegetables, roots and tubers, sugars and miscellaneous foods.

Blood sample collection, processing and storage

Blood sampling will be done at school in presence of parent/s where available, by trained phlebotomists. Venous blood sample of 6 ml (4 ml in clot activator and 2 ml in EDTA) will be collected using vacuum-tube systems, preferably from cubital vein. Measures to prevent and counter any adverse event like syncope, hematoma or swelling will be adequately employed and recorded.

Blood sample transportation from school to study sites will be done maintaining temperature of 2°C to 8°C. One ml of EDTA sample will be send to a centralized laboratory (National Accreditation Board for Testing and Calibration Laboratories (NABL) and The College of American Pathologists (CAP) accredited) for complete blood count (CBC) assessment. The CBC assessment will include estimation of hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RDW-CV, platelet count, total leucocyte count, differential leucocyte count and absolute leucocyte count. Report of CBC assessment will be given to parents with relevant advice by site investigators. Rest of the samples will be processed at site to separate plasma, serum and packed cells. Plasma and serum will be stored below -20°C and packed cells between 2°C to 8°C, at the study sites, with restricted access. Samples from study sites to CCU will be transported in two batches of 120 each, maintaining required temperatures. Sample

transportation will be managed by professional agencies having expertise in handling and shipment of bio-medical samples.

Biochemical assessment

Biochemical analysis of blood samples will be carried out at Department of Biochemistry, KGMU. Levels of serum calcium, iron, ferritin, folic acid, Vitamin B-12, Vitamin D, Vitamin A, zinc, plasma selenium and lead in whole blood will be assessed.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) will be used to assess zinc, selenium and lead. Stock solutions for respective elements will be prepared at a concentration of 1000 mg/L. Working standard will be prepared by diluting stock solution in 2% nitric acid in desired range. Adding 0.5 ml of sample, 1.5 ml nitric acid, 0.5 ml of perchloric acid, 1.0 ml of hydrogen peroxide and 1.0 ml of Mili-Q water will do microwave digestion. A Multiwave Reaction System equipped with the Rotor 16HF100 (100 ml PFA vessels, 40 bar) and Pressure, Temperature (p/T) sensor, will be used for digestion. Clear solution obtained after microwave digestion will be analyzed by ICP-OES (Optima 8000, Perkin Elmer) maintaining operational conditions as shown in table 3.

Table 3: Operational conditions for Inductively coupled plasma-optical emission spectrometry

Plasma Gas Flow	:	8 L/min
Auxiliary Gas Flow	:	0.2 L/min
Carrier Gas Flow	:	0.55 L/min
RF Power	:	1300 W
View Distance	:	15 nm
Plasma view	:	Axial
Sample flow rate	:	1.0 ml/min

The value of metal (zinc, selenium, lead) will be calculated using the formula:

$$\text{Metal}\left(\mu\frac{g}{dl}\right) = (\text{Sample reading} - \text{Blank reading})\text{x dilution made x 100/ Sample taken (ml)}$$

Folic acid, Vitamin B 12, Vitamin D and ferritin levels will be determined using Chemiluminescence method using a fully automatic analyzer.Folic acid estimation will be done by using ARCHITECT Folate Reagent Kit (Abbot Diagnostics, Iceland), 1P74-27. The Immulite folic acid is a competitive analog immunoassay with incubation cycles of 2 × 30 minutes. For Vitamin B 12 assessment, ARCHITECT B12 Reagent Kit (Abbot Diagnostics, Iceland), 7K61-27 will be used. Immulite 1000 vitamin B12 is a solid-phase, two-site chemiluminescent

immunometric assay with incubation cycles of 1 × 60 minutes. These estimations depend on chemiluminescence reactions in which part of the chemical energy generated produces excited intermediates that decay to a ground state with the emission of photons. The emitted radiation is measured using a photomultiplier tube and the signal is converted into analyte concentration.

The ARTICHECT 25-OH vitamin D Assay (5P02, G5-6832/R03, Abbot Diagnostics, Iceland) uses chemiluminescent immunoassay technology. Specific antibody to vitamin D is used for coating magnetic particles (solid phase) and vitamin D is linked to an isoluminol derivative. During the incubation, 25-hydroxyvitamin D is dissociated from its binding protein and competes with labelled vitamin D for binding sites on the antibody. After the incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as relative light units and is inversely proportional to the concentration of 25-hydroxyvitamin D present in samples. Quantitative determination of ferritin in serum will be done using ARTICHECT Ferritin 7k59 kit, B7K590 (Abbot Diagnostics, Iceland).

Vitamin A in serum willanalyzed by immune-enzymatic assay (ELISA), using commercially available kit (CED051Ge, USCN Wuhan USCN Business Co., Ltd.) This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to retinol has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled retinol and unlabeled retinol (Standards or samples) with the pre-coated antibody specific to retinol. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of retinol in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of retinol in thesample.

Serum calcium will be measured by Fully Automatic Biochemistry Analyzer by Selectra PRO M, using Calcium Arsenazo III Colorimetric 30160, Labkit. Calcium with Arsenazolll (1, 8-Dihydroxy-3, 6-disulpho-2, 7-naphthalene-bis (azo)-dibenzenearsonic acid), at neutral pH, yields a blue colored complex. The intensity of the color formed is proportional to the calcium concentration in the sample.

Serum iron levels will be assessed using ELITech Clinical Systems Selectra Pro Series Analyzers (Iron ferene, FEFE-0203 *ELITech Group* empowering IVD, USA). Iron released from transferrin in acidic pH as ferric ion (Fe^{3+}), is reduced by the ascorbic acid into ferrous ion (Fe^{2+})

which eventually forms a colored complex with ferene. The 578 nm absorbance of the iron-ferene complex is proportional to their concentration in the sample.

Patient and public involvement

Patients and/or the public are not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Quality assurance

Robust mechanisms will be employed to maintain the quality of data collection. Data collection at sites will be done under direct supervision of Site Investigator / Co-Investigator. CCU will also monitor data quality by onsite monitoring to observe data collection process and ascertain work in accordance to laid SOPs. Scoring sheets for cognitive assessments will also be assessed for scoring and interpretation of results at CCU. Dietary recordswill be assessed for appropriateness of proportion of ingredients and entry in DIETSOFT software. Retraining will be imparted where lacunae will be identified. Controls will run daily, before biochemical analysis of blood samples.

Data management and statistical analysis

Data will be entered in MS excel (Double data entry), matched electronically and discrepancies will be rectified by referring the source documents. Point estimates and confidence intervals of proportions of different micronutrient deficiencies shall be evaluated. These estimates shall be found for overall proportion as well as city-wise proportion. Different measures of association (Kendall, Somer, Goodman and Kruskal etc.) shall be found for categorical data (micronutrient deficiency with cognitive function, socio economic status, dietary intake). Pearson's correlation coefficient shall be computed for measuring association for continuous variables etc.).

DISCUSSION

Micronutrient deficiencies are a major problem in developing countries and India is not an exception. It adversely affects the population health resulting in decreased national performance and productivity, adding financial burden to the country. Despite the steps taken by government through various food supplementation and food fortification programs, problem is still deep rooted. Large population of children and adolescents is still bearing the curse of micronutrient deficiencies (12,22-27). Several researchers have investigated the magnitude of problem in India at various time points. These studies were mostly isolated and were confined to anaemia, ferritin, folate, vitamin B12 and vitamin D. In the recent times, much interest has been generated in multiple micronutrient deficiencies, little is known about the magnitude and significance of this

problem. The evidence on interactions between micronutrients, however, clearly indicates a need for more work in this area (28). The present study, perhaps first of its kind, will provide the estimates of Calcium, Iron, Zinc, Selenium, Folic acid, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels its association with anthropometric indicators, socioeconomic status, cognitive abilitiesand dietary habits along with three-day dietary intake, at country level.

The dietary intake assessment of population provides important information on the frequency and distribution of diets and nutritional status. This information can be used in designing interventions targeting improvement in dietary habits at community level. A wide variety of methods are available for dietary assessment, each one having its own advantages and disadvantages. The 24-hour dietary recall method is the most widely used method. This is a subjective and retrospective method that requires face to face or telephonic interview and consists of precisely recalling, describing and quantifying the intake of foods and beverages consumed

in the 24-hour period prior to, or during the day before the interview. Although the most thorough, comprehensive and complete instrument for dietary assessment, 24-hour dietary recall method has extensive dependence on interviewer's skills and participant's memory (29). In current study, since we will be assessing the actual level of micronutrients through biochemical analysis, at the same time we can correlate the results to that of 24-hour dietary recall.

Cognitive development is a continuous and sequential process from birth through adulthood. Cognition compasses memory, association, concept formation, pattern recognition, language, attention, perception, action, problem solving and mental imagery as a process (30,31). These processes are mandatory and interrelated for any task acquisition. Intelligence varies from person to person because of differences in their environmental and biological components. Biological factors like genes, maternal age and environmental factors like socioeconomic status, malnutrition, etc. influence intelligence adversely. Deficiency of micronutrients is associated with impaired neuropsychological development and classroom performance (32,33). Malnutrition during the early part of life (1–5 years) delays physical growth, motor and cognitive development (34-36). Studies have shown that skipping breakfast interferes with cognitive performance of students.

While there are several identified micronutrients, the role of only Iron, Zinc, Iodine, and vitamins is studied well in India. The researches clearly indicate that the school age period is nutritionally significant because this is the prime time to build up body stores of nutrients in preparation for rapid growth of adolescence.

Hence, the findings of present study may add to the existing body of knowledge not only to the prevalence but also the association of micronutrient deficiencies with cognitive abilities, dietary patterns anthropometric indicators, socioeconomic status. These finding may be further scaled up to interventions to provide adequate nutrition to targeted groups, so as to promote health and cognitive abilities thus resulting in increased productivity.

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Competing interests

The authors declare that they have no competing interests.

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Author Contributions

SA conceived the study and is principal investigator, responsible for finalizing study protocol and manuscript. DK contributed to design of the final study protocol, drafted the initial manuscript and coordinated ethical approvals. SS, SD and AAM contributed to the technical design of final study protocol and revised the initial manuscript draft. GA provided biostatistical support. IMC are site investigators and contributed in the design of the final study protocol.

Patient consent for publication

Not required.

Ethics and dissemination

Ethics approval for this study was obtained from the respective institutional ethics committee of all participating sites. Written informed consent will be obtained from parents of all study participants. Findings will be disseminated with stakeholders and will be presented in national and international conferences. Results will be published in a peer-reviewed journal.

Data statement

Results from this protocol will be published in a peer reviewed journal. Alternatively, data can be provided on request to corresponding author.

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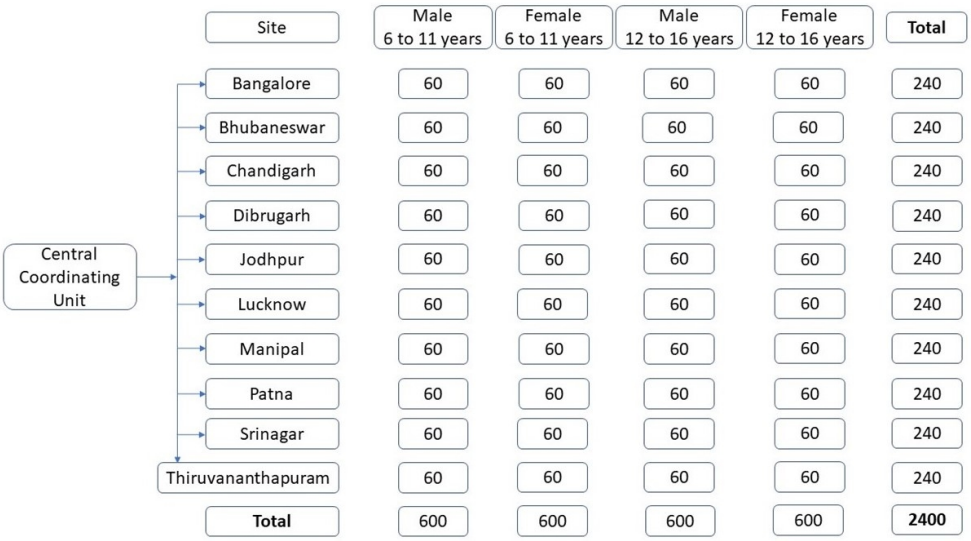

Site	Male 6 to 11 years	Female 6 to 11 years	Male 12 to 16 years	Female 12 to 16 years	Total
Bangalore	60	60	60	60	240
Bhubaneswar	60	60	60	60	240
Chandigarh	60	60	60	60	240
Dibrugarh	60	60	60	60	240
Jodhpur	60	60	60	60	240
Lucknow	60	60	60	60	240
Manipal	60	60	60	60	240
Patna	60	60	60	60	240
Srinagar	60	60	60	60	240
Thiruvananthapuram	60	60	60	60	240
Total	600	600	600	600	**2400**

Distribution of participants based on gender and age group across ten study sites.

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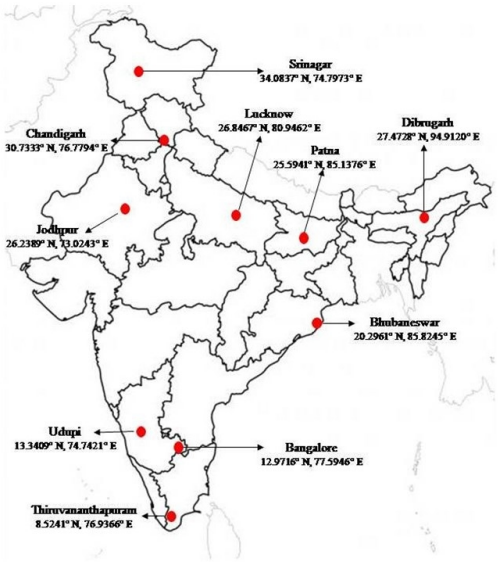

Study sites and their geo-coordinates

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Prevalence of specific micronutrient deficiencies in urban school going children of India aged between 6-16 years: study protocol for a multicentric cross-sectional study

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Prevalence of specific micronutrient deficiencies in urban school going children of India aged between 6-16 years: study protocol for a multicentric cross-sectional study

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ABSTRACT

Introduction

Childhood and adolescence are the period of rapid physical and cognitive growth and development, requiring adequate nutrition. Malnutrition in the form of undernutrition or micronutrient deficiency or overweight/obesity effects the health, cognition and educational achievement of this age group. The objective this study is to assess the prevalence of Calcium, Iron, Zinc, Selenium, folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies in the serum and Hemoglobin, Ferritin and Lead levels and its association with reported dietary intake and cognitive abilities, in urban school going children aged 6 to 16 years in ten cities of India.

Methods and analysis

A multi-centric cross-sectional study will be conducted to recruit 2400 participants (240 per site) across India. Participants will be selected using random sampling and will be categorized into age groups of 6 to 11 years and 12 to 16 years, with equal distribution. Data on socio economic status, anthropometric measures and three-day dietary intake and cognitive performance will be collected. Blood samples will be collected for biochemical analysis of micronutrients. Findings will estimate the prevalence of micronutrient deficiencies and their association with dietary habits and cognitive functioning.

Ethics and dissemination

Study protocol has been reviewed and approved by institutional ethics committee of all ten participating sites. Results will be shared and published in a peer-reviewed journal so that the findings will be helpful for the stakeholders in planning nutritional interventions for targeted groups.

Trial registration number CTRI/2019/02/017783.

Key words

Malnutrition, micronutrients, school age children, adolescents, cognitive, diet

Strengths and limitations of this study

- Multicentric study with sites being representative of different geo-cultural regions of India.
- As a concern of power of study, will enroll 2400 urban school going children.
- Use of standardized cognitive assessment tools adapted for Indian children.
- Chances of recall bias during self-reported dietary survey.

INTRODUCTION

Childhood and adolescence are the period of rapid physical and cognitive growth and development, requiring adequate nutrition. Any change in nutritional status during this age influences health, learning and physical fitness. Nutrients essential for normal growth and functioning of human body are macronutrients like carbohydrate, fat and proteins, required in large quantities and micronutrients like vitamins and minerals required in small quantities. Vitamins, categorized as fat soluble (A, D, E and K) and water soluble (B group and C), are synthesized in human body in quantities lesser than required. Minerals are required for growth, repair and regulation of vital functions of human body. They are major minerals like calcium, phosphorus, sodium, potassium, magnesium and trace elements like iron, iodine, ferritin, fluoride, zinc, copper, cobalt, chromium, manganese, molybdenum, selenium and nickel.

The term malnutrition addresses three broad groups of conditions:

- Undernutrition: Indicating wasting (low weight-for-height), stunting (low height-for-age) and underweight (low weight-for-age)
- Micronutrient-related malnutrition: micronutrient deficiencies or excess
- Overweight and obesity

Micronutrient deficiencies affect an estimated two billion people, or almost one-third of the world's population (1). Various studies had reported the sub optimal nutritional status of Indian children. Prevalence of anemia in school children and adolescents is between 19-88% across five different cities in India (2) and may be attributed to inadequate food intake, poor stores and deficiencies of nutrients (3). Nutrition anemia is caused by deficiency of iron, folate and vitamin B 12 (4). The association between nutritional status and health, cognition and educational achievement is established among the school going children (5). Deficiency of micronutrients and nutrients, delays cognitive and motor development and is associated with low Intelligence Quotient (IQ) (6,7,8). There is a consensus on the fact that iron deficiency has a negative impact on

cognition, behavior, and motor skills. It has been found that, hemoglobin though correlates to cognitive performance, iron supplementation improves cognitive functions regardless of the hemoglobin levels (9). Vitamin B12 deficiency is associated with poor cognitive development e.g. episodic memory and language ability (10) and growth in children (11). Zinc, Iron, folate, iodine, B12 and protein deficiency can also result in low IQ (12) and deficits in attention, learning, memory, and neuropsychological behavior (13,14). Lead, a well-known toxic heavy metal, though widely discontinued in many countries of world, is still a public health problem in developing countries like India. Worldwide every year 0.6 million cases of childhood intellectual disabilities are attributed to lead exposure. Iron deficiency, which is common in children can enhance lead absorption (15).

Various studies across the globe had tested the association between nutritional status biomarkers and dietary intake (16-20). Some significant association or role in progression of various malnutrition issues are already established. Plasma concentrations of vitamin B12 and folate are found to be associated with dietary intake provided that gender, age and energy intake are taken into account and this association is independent of physical activity of individual (21). A similar significant positive correlation is found between selenium intake and its blood level (22). Although some authors consider the nutritional assessment as a practical, noninvasive, and cost-effective tool for rapid nutritional evaluation (23), but others recommend concurrent collection of biological specimens to estimate levels of dietary biomarkers, so as to overcome possible sources of error, indigenously associated with every method of dietary intake assessment (24).

In Indian scenario, there is definitely a need for well-planned, large-scale study using standardized methodologies to estimate the prevalence of micronutrient deficiencies with giving due importance to accurate evaluation of socio-economic status and representation of the different regions of the country (25).

Hence the present study will be conducted with an aim to assess the prevalence of deficiency of various vitamins (folate, Vitamin A, 25 Hydroxy Vitamin D, Vitamin B12) and minerals (Calcium, Iron, Zinc, Selenium) and its association with reported dietary intake and cognitive abilities, in urban school children aged 6 to 16 years in ten cities of India.

METHODOLOGY

Study design

This is a multi-centric cross-sectional study. It will be conducted in ten major cities across India. Each site will recruit 240 participants, having equal proportion of gender and age group (Figure 1).

Study setting

Study sites at Bangalore, Bhubaneswar, Chandigarh, Dibrugarh, Jodhpur, Lucknow, Patna, Srinagar, Thiruvananthapuram and Udupi districts are selected as being representative of different geo-cultural regions of India (Figure 2). King George's Medical University (KGMU), Lucknow will be the central coordinating unit (CCU) for the study.

These ten cities have a total population of 24.1 million, which is 2% of country's total population. Study cohort population in these cities was 7.1 million (26). Demographic characteristics and key anthropometric indicators in urban areas of study sites are shown in table 1 (26,27).

Table 1: Demographic characteristics and key anthropometric indicators in urban areas of study sites

Study Site	Urban population of district (in millions) (26)		Literacy rate (state) (11)	Prevalence rate of (state) (age in years) (27)					
				Severe thinness*			Obesity**		
	All ages	6 to 16 years		5-9	10-14	15-19	5-9	10-14	15-19
India	377.11	76.73	84.1	4.7	7.0	4.9	2.8	2.5	1.8
M S Ramaiah Medical College & Hospital Bangalore	8.75	1.42	85.8	5.2	3.4	4.0	2.4	1.5	8.0
Kalinga Institute of Medical Sciences, Bhubaneswar	1.08	0.20	85.7	1.5	2.5	0.4	8.9	9.1	4.5
Post Graduate Institute of Medical Sciences, Chandigarh	1.03	0.20	86.2	-	-	-	-	-	-
Assam Medical College, Dibrugarh	0.24	0.043	88.5	4.3	11.9	5.7	4.3	2.2	0.0
All India Institute of Medical Sciences, Jodhpur	1.27	0.29	79.7	4.3	7.4	5.4	1.3	1.5	1.7

King George's Medical University, Lucknow	3.04	0.64	75.1	5.9	6.6	3.3	2.0	1.0	0.0
All India Institute of Medical Sciences, Patna	2.52	0.62	76.9	5.5	7.6	3.2	0.8	0.2	0.2
Sher-i-Kashmir Institute of Medical Sciences, Srinagar	1.22	0.24	77.1	2.9	2.4	5.0	4.3	3.0	2.2
Government Medical College, Thiruvananthapuram	1.77	0.28	95.1	2.9	4.1	6.4	5.4	1.8	0.8
Kasturba Medical College, Manipal, Udupi	0.33	0.05	85.8	5.2	3.4	4.0	2.4	1.5	8.0
* BMI for age < –3 SD of the WHO Child Growth Standards median ** BMI for age >+2 SD of the WHO Child Growth Standards median									

Objective

The primary objective of study is to assess the prevalence of Calcium, Iron, Zinc, Selenium, folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels in urban school going children aged 6 to 16 years in ten cities of India.

Secondary objectives are:

(a) To assess the association of Calcium, Iron, Zinc, Selenium, folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with anthropometric indicators in urban school going children aged 6 to 16 years in ten cities of India.

(b) To assess the association of Calcium, Iron, Zinc, Selenium, folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with socioeconomic status in urban school going children aged 6 to 16 years in ten cities of India.

(c) To assess the association of Calcium, Iron, Zinc, Selenium, folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels

with cognitive assessments in urban school going children aged 6 to 16 years in ten cities of India, by

- General Intelligence (Colored Progressive Matrices (CPM), Standard Progressive Matrices (SPM)
- Attention, concentration and Visuo-motor coordination (Coding Test)
- Working Memory (Digit span Test, Arithmetic test)

(d) To assess the association of Calcium, Iron, Zinc, Selenium, folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels with three-day dietary intake assessed by 24 hours recall method in urban school going children aged 6 to 16 years in ten cities of India.

Sample size computation

Assuming the prevalence of folate deficiency in India as 30.7% (28), precision of 2% and level of confidence 0.05, the calculated sample size is 2044 participants. After taking, attrition rate of 10% sample size will inflate to 2400 participants. This sample size will be equally divided in ten sites respectively.

Sampling technique

Participants will be selected by using two-stage sampling technique. In the first stage, schools will be selected, and in the second stage participants will be recruited from the selected schools.

Selection of participants

Each study site will provide a list of schools imparting co-education to children between 6 to 16 years of age and located within the urban limits of city. From this list, schools will be randomized repeatedly till we get a pool of six schools having at least one to three private schools and rest of the government schools. Principals of recruited schools will be met to obtain written voluntarily informed consent. They will be asked to allocate a coordinating teacher from the school. With the help of coordinating teacher, a gender-wise list of students between 6 to 11 and 12 to 16 years of age will be prepared. From each of these lists, fifteen students who are apparently healthy and residing within five kilometers of radius from school will be randomly selected. They will be invited to participate into the study. Out of these, first ten participants whose parents will provide written informed consent will be included into the study. Rest will be kept as back-up in case of exclusions. Participants having body mass index (BMI) below 12.5 will be excluded from the study and their parents will be advised to have a medical evaluation. Written assent will be obtained from all participants who will be 8 years or above of age. Any study specific assessment will be done after obtaining written informed consent.

Training of study team

Site team will consist of a social worker, a data entry operator, a Nutritionist and a Psychologist. Training of Nutritionist and Psychologist will be conducted at CCU Lucknow by project investigators and co-investigators. These will be oriented to train the site staff on study protocol, procedures, data collection instruments, data entry tools and standard operating procedures.

Data collection

The study opened for data collection in April 2019 and is still ongoing due to COVID-19 pandemic and is expected to complete by mid of 2021. Since, the school calendar of different states of India varies from another owing to their specific geography, culture and climate, time frame of data collection across the sites also varies accordingly.

Demographic and socioeconomic data

Demographic and socioeconomic details of participant will be recorded by interviewing participants and their primary care giver. Revised Kuppuswamy's socioeconomic scale (29) will be used to assess socioeconomic status.

Anthropometric measurements

Anthropometry will be done by qualified and trained nutritionists. The height will be measured to the nearest 0.1 centimeter using Seca 213 Mobile Stadiometer (Seca, Hamburg, Deutschland). Participant will be asked to stand barefoot after removing hair barrettes & rubber bands, with the heels, back, and head touching the measuring rod. A head board placed above the head perpendicular to the ruler on measuring rod and parallel to the ground will be used to record height measure. Two measurements of height will be taken for each participant. If the difference between the two height measurements is greater than 5 mm, then a second set of two height measurements will be taken to obtain more precise values.

Weight will be measured to the nearest 0.1 kilogram using portable Seca 803 weighing scale (Seca, Hamburg, Deutschland). The unit will be standardized by calibrating it to zero before each measurement. The participants will be weighed barefoot with empty pockets and without any heavy items like woolen blazers or belts.

Body Mass Index (BMI) will be calculated using the standard equation:

$$\text{BMI}\left(\frac{\text{kg}}{\text{m2}}\right) = \text{Weight (in kg)}/\ [\text{Height (in m)}]^2$$

For assessing anthropometric indicators, recommendations of the WHO expert committee will be used as shown in table 2.

Table 2: Recommendations of the WHO expert committee for assessing anthropometric indicators		
Stunting	:	Height for age < –2 SD of the WHO Child Growth Standards median
Severe thinness	:	BMI for age < –3 SD of the WHO Child Growth Standards median
Thinness	:	BMI for age < –2 SD of the WHO Child Growth Standards median
Overweight	:	BMI for age > +1 SD of the WHO Child Growth Standards median
Obesity	:	BMI for age >+2 SD of the WHO Child Growth Standards median
Severe obesity	:	BMI for age >+3 SD of the WHO Child Growth Standards median

Cognitive assessment

Cognitive assessment will be administered at a mutually convenient time in a separate room to keep relaxed and pleasant environment. The assessment will be conducted in a single individual session. During the assessment session, the psychologist will first make the child comfortable by establishing good rapport. Each participant will be assessed for attention, concentration and visuo-motor coordination through coding test (30), general intelligence through colored progressive matrices (CPM) (31)/ standard progressive matrices (SPM) (32) and working memory using arithmetic and digit span test (30).

Coding test: Sheet 'A' will be used for participants below 8 years of age, rest will use sheet 'B' sheet. Participants will be given 120 seconds to complete the test. One point will be awarded for each correct response, excluding samples.

CPM/SPM: CPM will be administered to participants 6 to 11 years of age. Participants 12 years or above of age will be given SPM. Each participant will be given about 15 to 20 minutes to complete the assessment.

Arithmetic and digit span test: These will be undertaken by all the participants. 3 to 5 minutes will be given to complete digit span test and 10 minutes will be given for arithmetic test.

For scoring and interpretation of test results, standard tables from respective test's manual (30,31,32) will be referred.

Dietary assessment

General dietary assessment will include dietary habits, meal frequency and consumption of water, beverages, green leafy vegetables, fruits, animal products. Frequencies of common food items, popular among children will also be captured.

Data on dietary intake of children will be collected using 24hour recall method for two non-consecutive days and one Sunday, which are not fasting or feasting days. The intake will be recorded by interviewing participant along with his/her mother or primary caregiver, preferably at their home. Previously standardized cups, glass and spoons will be used as an aid to help in

recalling the quantity of different foods consumed by the participants in a 24 hour period prior to the investigation.

For calculating the nutritive value of raw ingredients, Nutritive value of Indian foods will be used (33). Daily nutrient intake for all the nutrients will be calculated using DIETSOFT Software and will be compared with the Recommended Dietary Allowances (34).

Dietary intake will be assessed in terms of Nutrient Adequacy Ratio (NAR)(35). The NAR for a given nutrient is the ratio of a participant's intake to the current recommended allowance for each sex and age category. To estimate the nutrient adequacy of the diet, NAR will be calculated for all the nutrients using the equation:

NAR = Participant's nutrient intake of a day/ RDA of the respective nutrient

Participants will be then categorized as having (a) Adequate NAR (NAR≥1.00), (b) Fairly adequate NAR (0.66> NAR <1.00) and Inadequate NAR (NAR<0.66), for various nutrients (35).

Dietary diversity (DD), defined as the number of different foods or food groups consumed in a day, will be measured using Dietary Diversity Score (DDS). DDS will be measured by categorizing the food items, consumed in a day, into fourteen groups. Simple counting of different types of food groups consumed in a particular day will give individual DDS, which would range from 1 to 14.

Thereafter, participants will be categorized in three classes according to their Dietary Diversity Score (36)-

1. Low: ≤8
2. Moderate: 9
3. High: ≥10

The fourteen food groups that we would use are animal meat, cereals and millets, fats and edible oils, meat and poultry, fishes and other sea foods, fruits, pulses and legumes, green leafy vegetables, milk and milk products, nuts and oil seeds, other vegetables, roots and tubers, sugars and miscellaneous foods.

Blood sample collection, processing and storage

Blood sampling will be done at school in presence of parent/s where available, during early school hours, by trained phlebotomists. Venous blood sample of 6 ml (4 ml in clot activator and 2 ml in EDTA) will be collected using vacuum-tube systems using a certified stainless steel hypodermic

needle, preferably from cubital vein. Measures to prevent and counter any adverse event like syncope, hematoma or swelling will be adequately employed and recorded.

Blood sample transportation from school to study sites will be done maintaining temperature of 2°C to 8°C. One ml of EDTA sample will be send to a centralized laboratory (National Accreditation Board for Testing and Calibration Laboratories (NABL) and The College of American Pathologists (CAP) accredited) for complete blood count (CBC) assessment, through a professional agency having experience in handling and air-transportation of blood samples. The CBC assessment will include estimation of hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RDW-CV, platelet count, total leucocyte count, differential leucocyte count and absolute leucocyte count. Report of CBC assessment will be given to parents with relevant advice by site investigators. Rest of the samples will be processed at site to separate plasma, serum and packed cells, in a trace element free area. Plasma and serum will be stored in trace element-free cyro tubes, below -20°C and packed cells between 2°C to 8°C, at the study sites, with restricted access. Samples from study sites to CCU will be transported in two batches of 120 each, maintaining required temperatures. Sample transportation will be managed by professional agencies having expertise in handling and shipment of bio-medical samples. Samples will be prevented from exposure to light during this whole process.

Biochemical assessment

Biochemical analysis of blood samples will be carried out at Department of Biochemistry, KGMU. Levels of serum calcium, iron, ferritin, folate, Vitamin B-12, Vitamin D, Vitamin A, zinc, plasma selenium and lead in whole blood will be assessed.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) (Optima 8000, Perkin Elmer) will be used to assess zinc, selenium and lead. Stock solutions for respective elements will be prepared at a concentration of 1000 mg/L. Working standard will be prepared by diluting stock solution in 2% nitric acid in desired range. Adding 0.5 ml of sample, 1.5 ml nitric acid, 0.5 ml of perchloric acid, 1.0 ml of hydrogen peroxide and 1.0 ml of Mili-Q water will do microwave digestion. A clear microwave digested sample will use to measure analytes using ICP-OES, maintaining operational conditions as shown in table 3.

Table 3: Operational conditions for Inductively coupled plasma-optical emission spectrometry

Plasma Gas Flow	:	8 L/min
Auxiliary Gas Flow	:	0.2 L/min
Carrier Gas Flow	:	0.55 L/min

RF Power	:	1300 W
View Distance	:	15 nm
Plasma view	:	Axial
Sample flow rate	:	1.0 ml/min

The value of metal (zinc, selenium, lead) will be calculated using the formula:

$$\text{Metal}\left(\mu\frac{g}{dl}\right) = (\text{Sample reading} - \text{Blank reading})\text{x dilution made x } 100/\text{ Sample taken (ml)}$$

Folate, Vitamin B 12, Vitamin D and ferritin levels will be determined using Chemiluminescence method using a fully automatic analyzer. Folate estimation will be done by using ARCHITECT Folate Reagent Kit (Abbot Diagnostics, Iceland), 1P74-27. The Immulite Folate is a competitive analog immunoassay with incubation cycles of 2 × 30 minutes. For Vitamin B 12 assessment, ARCHITECT B12 Reagent Kit (Abbot Diagnostics, Iceland), 7K61-27 will be used. Immulite 1000 vitamin B12 is a solid-phase, two-site chemiluminescent immunometric assay with incubation cycles of 1 × 60 minutes. These estimations depend on chemiluminescence reactions in which part of the chemical energy generated produces excited intermediates that decay to a ground state with the emission of photons. The emitted radiation is measured using a photomultiplier tube and the signal is converted into analyte concentration.

The ARTICHECT 25-OH vitamin D Assay (5P02, G5-6832/R03, Abbot Diagnostics, Iceland) uses chemiluminescent immunoassay technology. Specific antibody to vitamin D is used for coating magnetic particles (solid phase) and vitamin D is linked to an isoluminol derivative. During the incubation, 25-hydroxyvitamin D is dissociated from its binding protein and competes with labelled vitamin D for binding sites on the antibody. After the incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as relative light units and is inversely proportional to the concentration of 25-hydroxyvitamin D present in samples. Quantitative determination of ferritin in serum will be done using ARTICHECT Ferritin 7k59 kit, B7K590 (Abbot Diagnostics, Iceland).

Vitamin A in serum will analyzed by immune-enzymatic assay (ELISA), using commercially available kit (CED051Ge, USCN Wuhan USCN Business Co., Ltd.) which is principally based on competitive ELISA. This assay employs the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific to retinol has been pre-coated onto a microplate. A competitive inhibition reaction is launched between biotin labeled retinol and unlabeled retinol

(Standards or samples) with the pre-coated antibody specific to retinol. After incubation the unbound conjugate is washed off. Next, avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of retinol in the sample. After addition of the substrate solution, the intensity of color developed is reverse proportional to the concentration of retinol in the sample.

Serum calcium will be measured by Fully Automatic Biochemistry Analyzer by Selectra PRO M, using Calcium Arsenazo III Colorimetric 30160, Lab kit. Calcium with Arsenazo lll (1, 8-Dihydroxy-3, 6-disulpho-2, 7-naphthalene-bis (azo)-dibenzenearsonic acid), at neutral pH, yields a blue colored complex. The intensity of the color formed is proportional to the calcium concentration in the sample.

Serum iron levels will be assessed using ELITech Clinical Systems Selectra Pro Series Analyzers (Iron ferene, FEFE-0203 *ELITechGroup* empowering IVD, USA.) Iron released from transferrin in acidic pH as ferric ion (Fe^{3+}), is reduced by the ascorbic acid into ferrous ion (Fe^{2+}) which eventually forms a colored complex with ferene. The 578 nm absorbance of the iron-ferene complex is proportional to the iron concentration of the sample.

Patient and public involvement

Patients and/or the public are not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Quality assurance

Robust mechanisms will be employed to maintain the quality of data collection. Data collection at sites will be done under direct supervision of Site Investigator / Co-Investigator. CCU will also monitor data quality by onsite monitoring to observe data collection process and ascertain work in accordance to laid SOPs. Quality of anthropometric measurements will be assured by routinely calibrating the equipment along with the resampling of participants. Scoring sheets for cognitive assessments will also be assessed for scoring and interpretation of results at CCU. Dietary records will be assessed for appropriateness of proportion of ingredients and entry in DIETSOFT software. Retraining will be imparted where lacunae will be identified. Internal quality assurance of bio chemical analysis will be done by using calibrated instruments and analyzing test specific standards. For interlaboratory comparison of the test results, 10% of the total samples will be sent to peer laboratory.

Statistical analysis

Data will be entered in MS excel (Double data entry), matched electronically and discrepancies will be rectified by referring the source documents. Point estimates and confidence intervals of proportions of different micronutrient deficiencies shall be evaluated. These estimates shall be found for overall proportion as well as city-wise proportion. To assess the association of micronutrient deficiencies (continuous variables) with anthropometric measures (height and weight), Pearson's correlation coefficient shall be used along with their confidence intervals. To assess the association of micronutrient deficiencies (continuous variables) with cognitive assessments (categorical variables), analysis of variance shall be employed. The dietary intake, for each participant, is recorded for three days, which will be converted into nutritional value using DIETSOFT software. Using the three observations as repeated measurement for each participant, the appropriate "descriptive" as well as "inferential" analysis shall be done. The hierarchical (nested) linear model shall be used for analyzing repeated measures, or longitudinal data.

Ethics and dissemination

The study is approved by the Institutional Ethics Committee for MS Ramaiah Medical College and Hospital Bangalore (approval reference number (ARN): MSRMC/EC/AP-02/02-2019), Institutional Ethics Committee for Kalinga Institute of Medical Sciences Bhubaneswar (ARN: KIMS/KIIT/IEC/112/2016), Institutional Ethics Committee for PGIMER Chandigarh (ARN: PGI/IEC/2019/000152), Institutional Ethics Committee (H) Assam Medical College (ARN: AMC/EC/1430), Institutional Ethics Committee for All India Institute of Medical Sciences Jodhpur (ARN: AIIMS/IEC/2017/765), Institutional Ethics Committee for King Georges Medical University (ARN: 9334/Ethics/R.Cell-16), Institutional Ethics Committee for Kasturba Medical College (ARN: IEC:388/2019), Institutional Ethics Committee for All India Institute of Medical Sciences Patna (ARN: IEC/AIIMS/PAT/153/2017), Institutional Ethics Committee for Sher-i-Kashmir Institute of Medical Sciences (ARN: IEC/SKIMS Protocol # RP 175/2018) and Human Ethics Committee Medical College Thiruvananthapuram (ARN: HEC.No.04/34/2019/MCT). The study is registered prospectively with Clinical Trial Registry of India (registration number CTRI/2019/02/017783). Written informed consent will be obtained from parents of all study participants. Findings will be disseminated with stakeholders and will be presented in national and international conferences. Results will be published in a peer-reviewed journal.

DISCUSSION

Micronutrient deficiencies are a major problem in developing countries and India is not an exception. It adversely affects the population health resulting in decreased national performance and productivity, adding financial burden to the country. Despite the steps taken by government

through various food supplementation and food fortification programs, problem is still deep rooted. Large population of children and adolescents is still bearing the curse of micronutrient deficiencies (27,37-42). Several researchers have investigated the magnitude of problem in India at various time points. These studies were mostly isolated and were confined to anemia, ferritin, folate, vitamin B12 and vitamin D. In the recent times, much interest has been generated in multiple micronutrient deficiencies, little is known about the magnitude and significance of this problem. The evidence on interactions between micronutrients, however, clearly indicates a need for more work in this area (43). The present study, perhaps first of its kind, will provide the estimates of Calcium, Iron, Zinc, Selenium, Folate, Vitamin A, 25 Hydroxy Vitamin D and Vitamin B12 deficiencies and blood Hemoglobin, Ferritin and Lead levels its association with anthropometric indicators, socioeconomic status, cognitive abilities and dietary habits along with three-day dietary intake, at country level.

The dietary intake assessment of population provides important information on the frequency and distribution of diets and nutritional status. This information can be used in designing interventions targeting improvement in dietary habits at community level. A wide variety of methods are available for dietary assessment, each one having its own advantages and disadvantages. The 24-hour dietary recall method is the most widely used method. This is a subjective and retrospective method that requires face to face or telephonic interview and consists of precisely recalling, describing and quantifying the intake of foods and beverages consumed in the 24-hour period prior to, or during the day before the interview. Although the most thorough, comprehensive and complete instrument for dietary assessment, 24-hour dietary recall method has extensive dependence on interviewer's skills and participant's memory (44). In current study, since we will be assessing the actual level of micronutrients through biochemical analysis, at the same time we can correlate the results to that of 24-hour dietary recall.

Cognitive development is a continuous and sequential process from birth through adulthood. Cognition compasses memory, association, concept formation, pattern recognition, language, attention, perception, action, problem solving and mental imagery as a process (45,46). These processes are mandatory and interrelated for any task acquisition. Intelligence varies from person to person because of differences in their environmental and biological components. Biological factors like genes, maternal age and environmental factors like socioeconomic status, malnutrition, etc. influence intelligence adversely. Deficiency of micronutrients is associated with impaired neuropsychological development and classroom performance (47,48). Malnutrition during the early part of life (1–5 years) delays physical growth, motor and cognitive development (49-51). Studies have shown that skipping breakfast interferes with cognitive performance of students.

While there are several identified micronutrients, the role of only Iron, Zinc, Iodine, and vitamins is studied well in India. The researches clearly indicate that the school age period is nutritionally significant because this is the prime time to build up body stores of nutrients in preparation for rapid growth of adolescence.

Hence, the findings of present study may add to the existing body of knowledge not only to the prevalence but also the association of micronutrient deficiencies with cognitive abilities, dietary patterns anthropometric indicators, socioeconomic status. These finding may be further scaled up to interventions to provide adequate nutrition to targeted groups, so as to promote health and cognitive abilities thus resulting in increased productivity.

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Competing interests

The authors declare that they have no competing interests.

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Author Contributions

SA conceived the study and is principal investigator, responsible for finalizing study protocol and manuscript. DK contributed to design of the final study protocol, drafted the initial manuscript and

coordinated ethical approvals. SS, SD and AAM contributed to the technical design of final study protocol and revised the initial manuscript draft. GA provided biostatistical support. IMC are site investigators and contributed in the design of the final study protocol.

Patient consent for publication

Not required.

Data statement

Results from this protocol will be published in a peer reviewed journal. Alternatively, data can be provided on request to corresponding author.

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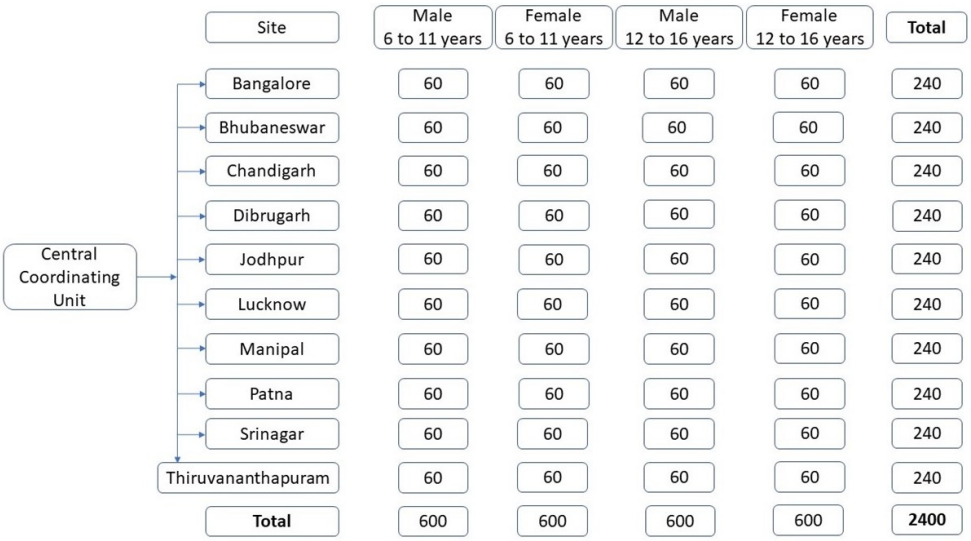
Figure 1: Distribution of participants based on gender and age group across ten study sites.

Figure 2: Study sites and their geo-coordinates



Site	Male 6 to 11 years	Female 6 to 11 years	Male 12 to 16 years	Female 12 to 16 years	Total
Bangalore	60	60	60	60	240
Bhubaneswar	60	60	60	60	240
Chandigarh	60	60	60	60	240
Dibrugarh	60	60	60	60	240
Jodhpur	60	60	60	60	240
Lucknow	60	60	60	60	240
Manipal	60	60	60	60	240
Patna	60	60	60	60	240
Srinagar	60	60	60	60	240
Thiruvananthapuram	60	60	60	60	240
Total	600	600	600	600	**2400**

Distribution of participants based on gender and age group across ten study sites.

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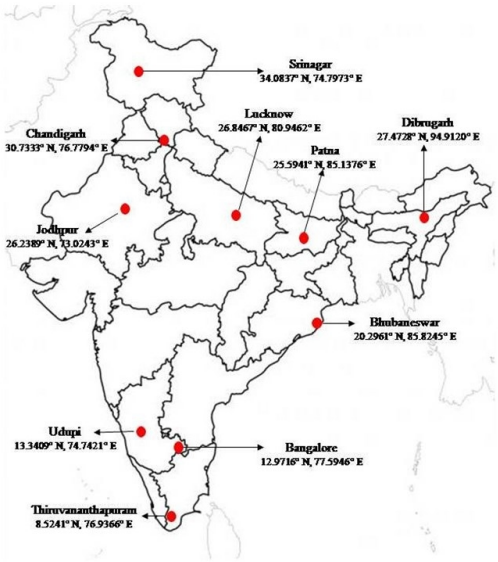

Study sites and their geo-coordinates

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