The present study was a supervised double blind randomized clinical trial conducted on unmarried or married (who still reside with their parents, i.e. prior to ‘Gauna’) anaemic adolescent girls aged 11-18 years residing in Kirti Nagar Slum Cluster of West Delhi.

The trial involved screening of study volunteers in Kirti Nagar slums for identification of mild or moderately anaemic adolescent girls. Those anaemic girls, who met the inclusion criteria and expressed willingness to participate were then randomly allocated to either of the two study groups, viz. Group 1 and Group 2. Both the groups were given Iron Folic acid tablets, along with either Vitamin B12 or placebo once weekly for 26 weeks. The data collection was initiated in January, 2012 and completed in March, 2013. The tablets/ capsules (i.e. Iron Folic acid tablets; Cyanocobalamin 500 mcg; Cyanocobalamin 15 mcg and Placebo) required for the trial was supplied by M/s Cyano Pharma Pvt. Ltd, Indore, Madhya Pradesh. Deworming tablet ‘Zybend’ containing 400 mg Albendazole manufactured by M/s Zydus Cadila Healthcare Limited was used in the trial. All the biochemical parameters (at baseline and after the supplementation period) were analysed at NABL accredited laboratory at ICMR’s “Centre for Promotion of Nutrition Research and Training with special focus on North-East, Tribal and Inaccessible population”, New Delhi.

3.1 STUDY DESIGN

3.1.1 Study Locale: The study was carried out in Jhuggi Jhumpri (JJ) cluster at Kirti Nagar, West Delhi which is a notified JJ colony as per ‘Delhi Urban Shelter Improvement Board’ under Government of NCT of Delhi. The JJ cluster at Kirti Nagar consists of approximately 11,500 small units, covering an area of approx.
1,60,000 square metre (Source: http://delhishelterboard.in/main/). The slum is densely populated having a poor hygiene, sanitation, ventilation and drainage facilities. People use community toilets and collect drinking water mostly from community taps.

The slum area at Kirti Nagar is divided into 10 main blocks/areas namely Damkal Kendra, Chunna Bhatti, Jawahar Camp, Kamla Nehru Camp, Furniture Block, Reshma Camp, 5/35, 8/35, Sanjay Camp and Barar Square.

Inhabitants of the cluster are mostly either from northern or eastern part of the country. Men are daily wage laborers at nearby marble and furniture shops or employed in surrounding factories. Others are Rickshaw pullers or work as laborers at construction sites. Women mostly stay at homes and those who go out for work are mainly employed as housemaids in nearby colonies or daily wage workers in surrounding factories. The average family size was found to be 5.6 ± 1.65 members/family and the average monthly income to be approx. Rs. 5000/-.
Fig 3.1 MAP OF KIRTI NAGAR SLUM CLUSTER
Criteria for selection of locale

- **Heterogeneity among people** - the slum is densely populated with migratory population from Bihar, Uttar Pradesh, Madhya Pradesh, Rajasthan and thus comprise a heterogeneous sample.

- **Homogeneity among study volunteers** – the sample was homogenous with respect to their socio-economic status, living conditions, and basic amenities.

- **Easy accessibility** - the slum was within easy reach of the workplace and the centre where blood samples were transported for the analysis of various biochemical parameters.

- **Rapport with the community** - the “Centre for Promotion of Nutrition Research and Training, with special focus on north-east, tribal and inaccessible population” (ICMR) has been working in this slum area for the last nine years and has a good rapport with the community. Therefore, identification of adolescent girls, making them understand the purpose of the study and enrolling them in the study was feasible.

### 3.1.2 Sample size calculation:

The sample size was calculated with the assumption of reducing prevalence of anaemia by 30%. The level of confidence was 95% and the power of test was 90%. The following formula was used:

\[
N = \frac{(Z_\alpha + Z_\beta)^2}{K^2}
\]

*Zα and Zβ are constants*

*α=90% (value= 1.64)*

*β=95% (value= 1.96)*

*K= assumption of reducing anaemia by 30%

The estimated sample size was 144 adolescent girls in each group. It was assumed that there will be a loss to follow up for approx. 20% subjects, therefore, it was proposed that 180 mild or moderately anaemic girls would be enrolled in
each of the two groups. However, due to migratory nature of the slum population, frequent visits to their native village etc, the sample size was later increased after due approval from the ethics committee and 230 adolescent girls were enrolled in each of the two groups.
Fig 3.2 STUDY DESIGN

Selection of Kirti Nagar (Purposive sampling)

Development of rapport with community leaders and community & Linkages with existing health system

Door-to-door survey to identify adolescent girls

IEC approval, Approvals sought from DCGI for conduct of Clinical Trial, manufacture of drugs, registration at CTRI and NIH, constitution of DSMB**

Screening to identify mild or moderately anaemic girls

Those who met the inclusion criteria and willing to participate

Using pre-tested questionnaire

Household and subject Information, Demographic Profile

Using calibrated equipments

Anthropometric information (Wt, Ht, WC, HC, MUAC)

Using pre-tested questionnaire

Dietary Information (24-hour dietary recall & FFQ)

Using validated methods

Biochemical (Hb, Ferritin, Folic Acid and Vitamin B12)

Random allocation to 2 group; de-worming given to both before the supplementation

Experimental Group
Iron (100 mg), Folic Acid (500 µg) and Vitamin B12 (500 µg for 6 wks; 15 µg for 20 wks) once weekly for 26 weeks

Control Group
Iron (100 mg) and Folic Acid (500 mcg) once weekly for 26 weeks + placebo

Monthly once IEC sessions given to both groups

At end of 6 months repeat survey to assess the impact

Biochemical (Hb, Ferritin, Folic Acid and Vitamin B12)

Anthropometric information (Wt, Ht)

Dietary Information (24-hour dietary recall)

Data Analysis and Interpretation
3.1.3 Identification of study volunteers:

3.1.3.1 Screening of Adolescent girls- Door-to-door survey to identify adolescent girls was carried out in 6 out of the 10 major blocks of Kirti Nagar JJ cluster based on their situation, accessibility and safety concerns. These areas were Damkal Kendra, Chunna Bhatti, Jawahar Camp, Kamla Nehru Camp, Furniture Block and Reshma Camp. The girls and their parents/guardians were contacted and explained the purpose of the study and anticipated benefits and those who were willing to participate, were screened for hemoglobin levels to assess the prevalence of anaemia (hemoglobin <120 g/L). Mild (Hb: 100-119 g/L) and moderately anaemic (Hb: 70-99 g/L) girls were identified, while the severely anaemic girls (Hb:<70 g/L) were advised to consult physician.

3.1.3.2 The anaemic subjects who were identified were tested for following inclusion and exclusion criterias:

**Inclusion Criteria**

a) Willingness to participate  
b) Unmarried or married (who still reside with their parents, i.e. prior to ‘Gauna’) adolescent girls.

**Exclusion Criteria**

a) Adolescent girls with severe anaemia  
b) Pregnant Adolescent girls  
c) Adolescent girls suffering from any medical conditions like TB, Cancer etc

The mild or moderately anaemic subjects who met the inclusion criteria were randomly divided into two groups using Computer generated list by software ‘nQuery’.
1) **Group 1** (n=230): One tablet of iron folic acid containing 100 mg of elemental iron and 500 mcg of folic acid along with one placebo capsule was given once *every week* for 26 weeks. Monthly IEC sessions were also organized.

2) **Group 2** (n=230): One tablet of iron folic acid containing 100 mg of elemental iron and 500 mcg of folic acid along with one capsule containing cyanocobalamin (Vitamin B12) was given to adolescent girls once *every week* for 26 weeks. For the first 6 weeks of intervention, 500 mcg of cyanocobalamin was given (once in a week) followed by a maintenance dose of 15 mcg cyanocobalamin for remaining 20 weeks. Monthly IEC sessions were also given.

*Rationale for Vitamin B12 dose*- In a study by Yajnik et al, 2007 effect of vitamin B12 supplementation was studied on plasma homocysteine concentration. Oral Vitamin B12 (500 mcg) was given every alternate day for a period of 6- weeks to women aged 20- 50 yrs. It was found that plasma vitamin B12 concentration plateaued within two weeks of supplementation, and there was no further increase in the following four weeks. The authors suggested that this could be due to diminished intestinal absorption of vitamin B12 due to ‘saturation’ of the absorptive mechanisms, or due to equilibrium between absorption and tissue clearance. Therefore, in the present study high doses of Vitamin B12 was given to ensure an effect in a short period of time. Thereafter, small maintainence dose was given weekly.

In all study groups deworming tablets was given at the initiation of the intervention.
3.1.4 APPROVALS/ CLEARANCES

3.1.4.1 Clearance of Ethics Committee: The study protocol was approved by the Ethics Committee, Lady Irwin College, University of Delhi. The written informed consent was obtained from the subjects and countersigned by their parents/ Guardians in the presence of a witness. The subjects and their parents/ Guardians were explained the purpose and aim of the study and were provided information sheet in hindi bearing the address and contact number of person they may contact incase of any emergency. The subjects were communicated in written the results of all the biochemical tests performed, both at the baseline and after the intervention.

Copy of information sheet (Hindi) and consent form (Hindi) are placed at Annexure 7.1 & 7.2 respectively.

3.1.4.2 Permission to manufacture the tablets/ capsules and to conduct clinical trial: Since the study involved a clinical trial, permissions from State Licensing Authority and Drug Controller General of India (DCGI) for conducting the trial and manufacturing the drugs required for the trial were obtained before the initiation of the study.

The trial involves use of following drugs:
Tablet 1 : Iron Folic Acid (100 mg elemental Iron and 500 mcg Folic Acid)
Capsule 1: Cyanocobalamin 500 mcg
Capsule 2: Cyanocobalamin 15 mcg
Capsule 3: Placebo (Starch IP and Lactose IP)

Cyanocobalamin 15 mcg came under the category of new drug, as it is not currently available in the Indian market in the required dosage. Cyanocobalamin
500 mcg though available in the market was not being currently manufactured by the pharmaceutical company who was identified for supply of medicines for the trial. Therefore, permission was required from Drugs Controller General of India for conducting a clinical trial on human volunteers as well as for manufacturing cyanocobalamin capsules (1 & 2) mentioned above. Following actions were taken in this regard:

3.1.4.2.1 Pharmaceutical company for supply of tablets/ capsules- Cyano Pharma Pvt Ltd, Indore (M.P.) who is an authorized supplier of Government of India for Iron Folic Acid tablets under the National Programme for control of anaemia was approached for the supply of medicines required under the trial. The firm agreed to supply/ manufacture all the required tablets/ capsules for the said trial.

**Fig 3.3 Steps taken to obtain the required permissions for the trial**

1. **Application was submitted to DCGI for permission to conduct the clinical trial and for issuing ‘No Objection Certificate’ to manufacture cyanocobalamin capsules**

2. **No objection certificate was issued by DCGI to manufacturer (M/s Cyano Pharma Pvt. Ltd, Indore, MP) for the manufacture of the drug**

3. **The manufacturer applied and obtained license from State Licensing Authority, Bhopal to manufacture the trial batch medicines for stability exercises**

4. **The results of stability studies carried out by M/s Cyano Pharma to assess the various parameters like disintegration time, stability of salt etc for cyanocobalamin capsules was submitted to DCGI**

5. **DCGI scrutinized the application along with the stability data submitted by M/s Cyano Pharma Pvt Ltd and granted the permission for the conduct of the trial and for manufacture of the tablets**
3.1.4.2.2 Submission of application to DCGI for permission to conduct the trial and for manufacturing of drugs- Application was submitted to DCGI, New Delhi for following:

- Permission to conduct the trial
- Permission to manufacture cyanocobalamin capsules, 500 mcg and 15 mcg.

3.1.4.3 Registry of the trial:
Before the enrollment of the study volunteers for the trial, it was registered at following portals:

3.1.4.3.1 “Clinical Trial Registry of India” (ID: CTRI/2011/12/002217) under Indian Council of Medical Research, Ministry of Health and Family Welfare, Govt. of India.

3.1.4.3.2 Clinical Trial Registry at “National Institute of Health”, USA (ID: NCT01490944).

3.1.4.4 Since the study involved a clinical trial, a Data Safety Monitoring Board (DSMB) was constituted. The Board met twice during the course of the trial for evaluating the progress and for reviewing the results of the trial.

3.1.5 Double-Blinding
Since, the study involved a Double Blind clinical trial, it was ensured that the placebo as well as cyanocobalamin capsules were exactly of the same colour, shape, size and odour. A person nominated by DSMB and having no conflict of interest with the trial was requested to carry out the coding-decoding for the present study. For the double blinding, the procedure followed is given under:
The IDs of the subjects who were identified as mild or moderately anemic after the screening were randomly divided into one of the 2 groups, i.e. ‘A’ or ‘B’ using computer generated list using software ‘nQuery’ by the person responsible for double blinding.

Since IFA was given to all the subjects irrespective of the groups, therefore, no blinding was done.

For the distribution of Vitamin B12 or placebo capsules, sealed envelopes were received by the Investigator, bearing the subject ID and containing a card mentioning either ‘A’ or ‘B’.

Simultaneously, the cyanocobalamin or placebo capsules procured from Cyano Pharma Pvt. Ltd. were put into two glass bottles and labeled as ‘A’ or ‘B’ by the person who was assigned the responsibility of blinding. The codes were not told to the Investigator.

The Investigator by looking at the cards in the sealed envelopes distributed the capsules (A or B) to the subjects.

The codes for the study were kept with the person responsible for blinding and a copy of the same was kept with the Chairperson of Data Safety Monitoring Board.

Since the dose of cyanocobalamin capsules was changed after 6 weeks of supplementation, the whole process was repeated before the 7th dose was given to the subjects.

Decoding was done after the completion of the supplementation period and bio-chemical analysis.

3.1.6 Methodology followed for distribution of tablets:

1. After the hemoglobin analysis, biochemical reports were distributed door-to-door to all the girls (whether anaemic or non-anaemic). Anaemic girls were identified and those with severe anaemia were advised for therapeutic treatment and excluded from the study population.
2. Those having mild or moderate anaemia were identified and an area wise list of all such subjects was prepared bearing details like their name, age, father’s name, address and contact number. Parents/guardians of all such subjects were contacted, bio-chemical findings were discussed with them and on their consent, they were also informed about the tentative date of start of supplementation.

3. Before the initiation of Iron, Folic acid and Vitamin B12 supplementation, all the subjects were given de-worming tablet ‘Zybend’ (Albendazole 400 mg) procured from Zydus Cadila Healthcare Limited. The tablet was chewable and the girls were asked to chew the tablets in front of the investigator.

4. Since the study area was scattered over approx. 4 Km, it was divided into smaller areas for the ease of supplementation, and one tentative day was allotted to each area as the ‘Main Day’ of supplementation and the subjects were informed about the same. For eg., Saturday for Kamla Nehru; Sunday for Chunna Bhatti and Monday for Jawahar Camp.

5. ‘Spare days’ were kept for those who were not available on the ‘Main Day’ of the supplementation. Those who were not available even on the ‘spare day’, were contacted over telephone to find a suitable time to visit them or if only 1 or 2 subjects were left in an area, the medicine was left with the volunteers appointed from within the field with the instruction to go and make the subject to consume the medicine in her presence.

6. Iron, Folic acid and Vitamin B12 supplementation was initiated within one week of administration of de-worming tablet. For the supplementation, the subjects list prepared was followed and all subjects were contacted door-to-door. The subject was given the medicine and drinking water by the investigator and was asked to consume the tablets then and there to ensure that the medicine has been consumed.
7. It was ensured that the medicine is given to the subjects after the meal (mostly in the afternoon) to minimize the side-effects.

8. It was observed that most of the subjects were school-going and also attend tuition classes or other hobby classes mainly ‘Salai’, ‘Beautician’, ‘Computer course’ etc in their own locality or nearby areas in the evening. Therefore, a record of their tentative time-table was prepared and the subjects were also followed in their tuition, computer or other classes to ensure compliance.

9. Under no circumstance was the medicine given to parents/guardian or left with any other member of the family. However, since the population often visit their native places, those who went to their native village during the supplementation period, were given the medicine and told to consume it once every week. The subjects were then followed vigorously over telephone. The data from such subjects has been analyzed seperately.
Supervised administration of medicines
(Water and glass provided by the investigator)

Subject consuming the tablet

Supervised administration of medicines
Supervised administration of medicines

Subject consuming the tablet
3.1.7 IEC SESSIONS- Information, Education and Communication sessions were given to all subjects throughout the period of supplementation. The frequency of such sessions was once every month and the sessions were given in small groups of not more than 15 girls. Those who missed the sessions, were briefed about the same during the weekly supplementation. The existing IEC material like posters, booklets and pamphlets focussing on anaemia and general health prepared by UNICEF, State or Central Government were utilized for imparting the education. Besides, to make the sessions interesting, audio (Meena Radio Episode 47 ‘Rajkumari Ki Kahani’) and video films (‘Amma Ji Kya Kehti Hain’) in Hindi, developed by UNICEF addressing issues like anaemia, cleanliness, good hygiene practices, care during menstruation were shown to the subjects.

3.2 TOOLS AND TECHNIQUES:

Demographic, household and dietary intake information were collected by developing appropriate questionnaires. Anthropometric measurements which included weight, height, waist circumference, hip circumference and mid upper arm circumference were taken using calibrated equipments like electronic weighing balance, anthropometric rod and non stretchable fibre measuring tape. Biochemical parameters were assessed by estimating them in blood/serum samples of subject using appropriate well standardized methods. Internal and external quality assurance programme was an integral part of the centre’s NABL accredited laboratory.
Showing video film “Amma Ji Kya Kehti Hai” focusing on symptoms and prevention of anaemia

Explaining symptoms of anaemia to subjects using poster

Delivering general talk on anaemia and hygiene practices
3.2.1 QUESTIONNAIRE:

Suitable questionnaire was developed. The questionnaire was pretested on few subjects who were not a part of the actual study. Based on pretesting, the required modifications in the questionnaire were made (Annexure 7.3).

3.2.1.1 DEMOGRAPHIC INFORMATION: Demographic information was collected from the subjects which included age, sex, family profile, family income, occupation and educational qualification.

3.2.1.2 HOUSEHOLD INFORMATION: Information was gathered from the study volunteers regarding household details like type of house, source of water collection, treatment of water before drinking, hygiene practices etc.

3.2.1.3 ASSESSMENT OF DIETARY INTAKE: In the present study, information regarding the dietary pattern and dietary intake was obtained using 24-hour dietary recall and food frequency questionnaire methods.

3.2.1.3.1 24 hour dietary recall: This is one of the most frequently used techniques to characterize diet. This method is simple, relatively inexpensive, less time consuming and provides data which is easy to interpret.

It is well documented that to calculate nutrient intake from dietary data with increased accuracy, it is necessary to assess portion sizes of every individual, differentiate between small, medium and large portions and assess average portion weights (Joseph and Joseph, 1986). The subjects were asked to report all kinds of foods consumed by them on the previous day starting from getting up till last meal of the day. To assist the subjects in describing the serving size and for better precision in quantity, various types of standardized utensils were shown to them consisting of katoris, glasses, cups, teaspoon, tablespoon and serving spoon. Pictorial representation of the chapati/parantha size was used. The
method of preparation of the food items was noted recording each ingredient used. Thus, this method was used to assess the current usual daily food intake of the subjects.

**3.2.1.3.2 Food Frequency Questionnaire:** This was used to obtain qualitative descriptive information about usual food consumption patterns before the diagnosis of the diabetes in patients. This method estimated how frequently certain foods were eaten by the subjects during a specified period of time i.e. 6 months. Thus, information was collected regarding the frequency of consumption of cereals (refined/whole), pulses, milk and milk products, vegetables (leafy/roots/others), fruits, meat and poultry, nuts and oilseeds, sugars, beverages, snacks and fast foods prior to the development of diabetes.

**3.2.1.3.3 Other diet related information:** Data was also collected from the subjects regarding their dietary habits (Vegetarian/Ovo-vegetarian/Non-vegetarian), types of cooking oil consumed, amount of oil consumed, type of salt consumed, their awareness regarding consumption of Iodized salt and type of milk consumed etc.

**3.2.3 ANTHROPOMETRIC MEASUREMENTS:**

In the present study, anthropometric status was assessed by measuring height, weight, waist circumference, hip circumference and mid upper arm circumference of the subjects (Gibson, 1990). Body Mass Index (BMI) and Waist-to-Hip ratio (WHR) was calculated. The measurement techniques for the same are described below:

**3.2.3.1 BODY HEIGHT:** The height of the subjects was measured using the vertical measuring rod (anthropometer). The subject was asked to stand erect looking straight on a levelled surface without shoes, with heels together and toes apart. The anthropometric rod was placed behind the subject in the centre of the
heels perpendicular to the ground. The investigator stood on left side of the subject firmly holding the chin of the subject with her left hand and the occiput of the subject with her right little finger in the Frankfurt horizontal plane (an imaginary line joining the tragus of the ear and the eye). The moving head piece of the anthropometer was placed in the sagital plane over the head of the subject applying a slight pressure to reduce the thickness of the hair. The reading was taken when the anthropometer rod was still in position. The average of three measurements was taken as the final measurement (Rao and Vijayaraghavan, 1996).

3.2.3.2 BODY WEIGHT: The body weight of the subjects was measured using the electronic weighing balance (Galaxy, India). The subject was asked to stand erect on the weighing machine looking straight and breathing normally. The body weight (kg) was carefully noted. The following precautions were taken to measure the body weight:

- The subjects worn minimal clothing, and was without shoes.
- The subjects did not lean against or held anything while the weight was recorded.

3.2.3.3 BODY MASS INDEX: BMI is based on measurement of weight (in Kg) with minimal clothing and height (in meters) without shoes (Ahuja, 2002). It is calculated as follows:

\[ \text{BMI} = \frac{\text{Weight (in Kgs)}}{\text{Height (in meters)}^2} \]

3.2.3.4 WAIST CIRCUMFERENCE: Waist circumference was measured using a non stretchable fibre measuring tape (Galaxy, India). The subject was asked to stand comfortably with his or her weight evenly distributed on both feet, and the feet about 25-30 cm apart. The measurement was taken mid way between the inferior region of the last rib and the crest of the ilium, in a horizontal plane. Each landmark was palpated and marked and the mid point determined with a tape
and marked. The investigator sat by the side of the subject and fitted the tape snugly but not so tightly as to compress underlying soft tissues. The circumference was measured to the nearest 0.1 cm at the end of normal expiration (Rao and Vijayaraghavan, 1996; Ahuja et al, 2002).

3.2.3.5 HIP CIRCUMFERENCE: The subject was asked to stand erect with arms at the side and feet together. The investigator sat by the side of the subject so that the level of maximum extension of the buttocks could be seen and placed the tape measure around the buttocks in a horizontal plane. The tape was snug against the skin but not compressing the skin tissues. The measurement was recorded to the nearest 0.1 cm (Rao and Vijayaraghavan, 1996; Ahuja et al, 2002).

3.2.3.6 WAIST TO HIP CIRCUMFERENCE RATIO (WHR): It was calculated as follows:

\[ WHR = \frac{\text{Waist Circumference}}{\text{Hip Circumference}} \]

3.2.3.7 MID UPPER ARM CIRCUMFERENCE (MUAC): For measurement of MUAC, the subjects were asked to stand erect, with the arms hanging freely at the sides of the trunk and palms towards the thighs. MUAC was taken on the left hand. The circumference was measured at the mid point of the arm. To locate the mid point, the subject's elbow was flexed to 90° with the palm facing upward. The mid point between the tip of the acromion of scapula and the tip of the olecranon of the forearm bone, ulna was marked with a pen. The arm was left hanging freely and the fibre glass tape was gently but firmly placed embracing the arm without exerting too much pressure on the soft tissues. The reading was taken to the nearest millimetre, with the tape still in position (Rao and Vijayaraghavan, 1996; Ahuja et al, 2002).
3.2.4 BIOCHEMICAL ANALYSIS

Biochemical analysis was carried out twice, i.e. once before the initiation of the supplementation and then at the end of the intervention. The following biochemical parameters were assessed in subjects:

- Hemoglobin
- Serum Ferritin
- Serum Folic Acid
- Serum Vitamin B12

3.2.4.1 SAMPLE COLLECTION: Blood samples were collected in BD vacutainers for the biochemical investigations. 5 ml of venous blood was drawn from the subjects with a disposable syringe using 21 gauge needle. The blood collected was then dispensed into following types of vials:

- **Plain vial** - 4 ml blood (for the analysis of Ferritin, Folic acid and Vitamin B12)
- **EDTA Vial** - 1 ml blood (for the analysis of Hemoglobin)

The blood collected in vials was allowed to stand at room temperature for approximately 30 minutes. Under the field conditions, using portable centrifuge, the vials were centrifuged at 2000 rpm for 5 minutes. While centrifuging the samples, it was ensured that the machine was properly placed and not vibrating. Also, while putting the tubes in the rotor of the centrifuge machine, it was ensured that the tubes were balanced.

After centrifuging the blood samples, the tubes were carefully removed from the centrifuge rotor, and supernatent was separated in pre-labelled tubes from the clot with the help of a micropipette. For each sample separation, a new micropipette tip was used to avoid contamination. The samples after separation
were immediately stored in dry ice in the dark. The tubes were then transported to the ICMR lab where the samples were transferred to -80°C until analysis. WHO manual was followed for the collection of blood samples and disposing off the used syringes, gloves etc (WHO, 1994 and WHO, 2004).

All the biochemical analysis was done at NABL accredited laboratory at “Centre for Promotion of Nutrition Research and Training, with special focus on North-East, Tribal and Inaccessible Population” (Indian Council of Medical Research), New Delhi.

3.2.4.2 SAMPLE STORAGE: All the samples collected were stored in the deep freezer at -80°C until analysis.

3.2.4.3 ANALYTICAL METHODS

3.2.4.3.1 HEMOGLOBIN

Equipment Used: Spectrophotometer (Electronic Corporation India Ltd., Hyderabad)

Apparatus & material Required: Whatman No. 1 filter paper, Glass test tubes (15 x 125 mm), Test tube stand, Pipette and pipette tips (500-5000µl), Tissue paper.

Chemicals required:

- Drabkin’s Solution/ Hemoglobin Reagent (HEMOCOR-D) – Ready to use Hemoglobin diluting reagent.
- Stock Hemoglobin standard (HEMOCOR) having concentration of 60mg/dL
Analysis:

**Principle:** Potassium ferricyanide converts the hemoglobin in the sample to methemoglobin. The methemoglobin further reacts with potassium cyanide to form a stable cyanmethemoglobin complex. Intensity of the complex formed is directly proportional to the amount of hemoglobin present in the sample.

**Methodology:** 5 ml of Drabkin solution was pipetted into a test tube and 20 mcl of whole blood was pipetted into the Drabkin’s solution. The tubes were vortexed for exactly 60 seconds and then left for atleast 15 minutes. After 15 minutes, the reaction mixture from the test tube was carefully transferred into a cuvette. The absorption was read on spectrophotometer at 540nm wavelength against the reagent blank (Drabkin’s solution was used as a reagent blank). The OD of the pure stock hemoglobin standard was also recorded for each run.

**Calculations:** The hemoglobin is calculated in g/dL by using the following formula:

\[
Hb(\text{g/dL}) = \frac{\text{Abs at} \ 540\text{nm of test sample} \times \text{Conc. of std. mg/L} \times \text{Dilution factor}}{\text{Abs at} \ 540\text{nm of standard}} \times X \ 1000
\]

Dilution factor = 251 (total reaction Vol. (5.02mL) / Sample Vol. (0.02 mL))

1000 = Multiplication factor to convert mgs. to grams.

Concentration of std (mg/L) = 60.

### 3.2.4.3.2 FERRITIN, FOLIC ACID AND VITAMIN B-12

**Equipment used:** Immulite 1000

**Chemicals and Reagents**

**Adjustors:**

- **Ferritin:** Two vials (low and high) each of 2.5ml ferritin in a human protein-based matrix with preservative. These are ready to use.
• **Folic Acid:** Two vials (low and high) of lyophilized folic acid in a human protein based matrix, with preservative.

• **Vitamin B12:** Two vials (low and high) of lyophilized vitamin B-12 in a human protein based matrix, with preservative.

**Controls:**

• **Ferritin Control:** It is an assayed bi-level control. The pack contains a set of two vials (2 ml each), containing different concentrations of ferritin in a protein based matrix, with preservative.

• **CON6:** CON6 is a multivalent (25 constituents) tri level control module. It is human serum based. This control can be used for Folic Acid and Vitamin B12. It is intended for in vitro diagnostic use as an aid in monitoring the day-to-day accuracy and precision of the tests. There are 2 sets of 3 vials in a pack.

**Reagents:**

• **Ferritin:** 7.5 ml of alkaline phoshatase (bovine calf intestine) conjugated to polyclonal goat anti-ferritin in buffer, with preservative.

• **Folic Acid:** The following reagent are used for the analysis of folic acid in human serum samples:
  i) **LFOA:** One wedge (7.5 ml) containing folic acid binding protein, with preservative.
  ii) **LFOB:** One wedge (7.5 ml) containing alkaline phosphatase (bovine calf intestine) conjugated to anti-ligand in buffer, with preservative.

The following reagents are required for preparation of working solution:

iii) **Borate-KCN Buffer solution (LB CN):** 125ml of Borate –KCN Buffer solution, with preservative. Stable for 2-8°C for 30 days after opening.
iv) **Dithiothreitol (LDTT):** 3ml of a dithiothreitol solution. Stable at 2-8°C for 30 days after opening.

v) **Ligand Labeled Folate:** 5ml of ligand-labeled folate in buffered human protein based solution, with preservative. Stable at 2-8°C for 30 days after opening.

- **Vitamin B-12:** The following reagents are used for the analysis of Vitamin B-12 in human serum samples:

  i) **LVBA** - One wedge (7.5ml) containing B-12 binding protein (purified hog intrinsic factor), with preservative.

  ii) **LVBB** - One wedge (7.5ml) containing alkaline phosphatase (bovine calf intestine) conjugated to murine monoclonal anti-hog intrinsic factor antibody in buffer, with preservative.

  The following reagents are required for preparation of working solution:

  iii) **Borate-KCN Buffer solution (LB CN) 125ml of Borate** – KCN Buffer solution, with preservative. Stable for 2-8°C for 30 days after opening.

  iv) **Dithiothreitol (LDTT):** 3ml of a dithiothreitol solution. Stable at 2-8°C for 3 days after opening.

**ANALYSIS**

**Principle:** The analysis is a solid-phase, two-site chemiluminescent immunometric method.

Luminescence is the phenomenon where photons (light) are emitted. This occurs in nature in Firefly (which emits light in night) and is referred as Bioluminescence. When this phenomenon is achieved in laboratory using chemical reactions it is referred as Chemiluminescence. When Chemiluminescence is achieved by using
acid and alkali (acid-base reaction) it is referred as Direct Chemiluminescence, which occurs only for a fraction of second.

The short or flash chemiluminescence can be enhanced by using enzymes and is referred as Enhanced Chemiluminescence. This phenomenon, achieved by enzymatic reaction, stays for 5 minutes and thus offers better sensitivity and reproducibility.

First the alkaline phosphatase conjugate (reagent) is bound to the bead (within the test unit) during the immunological reaction. The amount of alkaline phosphatase captured is directly proportional (for sandwich assay), or is inversely proportional (for competitive assay) to the concentration of the analyte in the patient sample. Once the test unit is washed, a luminogenic substrate is added to the test unit and it is moved onto the Luminometer chain. 10 minutes later, the test unit arrives in front of the photomultiplier tube (PMT), where the light generated by luminogenic reaction is measured. Unlike chemiluminescent reactions involving acridinium esters (which produce flash of light) the enzyme-amplified reaction in the immulite system produces a prolonged glow.

Procedure:

**Ferritin:** No sample pretreatment is required for analysis of ferritin. Pipette 200 μl of serum directly into the sample cup for analysis.

**Folate and Vitamin B12:**

A) **Preparation of working solution:** The amount of each component depends on the number of tests to be performed. The volumes required, in micro liters per test, are tabulated below. Multiply these volumes by a number slightly greater than the number of tests to be run.
Preparation of working solution for Folic Acid

<table>
<thead>
<tr>
<th>Chemical</th>
<th>μl/test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borate-KCN Buffer Solution</td>
<td>1,000</td>
</tr>
<tr>
<td>Ligand Labelled Folate</td>
<td>20</td>
</tr>
<tr>
<td>Dithiothreitol Solution</td>
<td>20</td>
</tr>
</tbody>
</table>

Preparation of working solution for Vitamin B-12

<table>
<thead>
<tr>
<th>Chemical</th>
<th>μl/test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borate-KCN Buffer Solution</td>
<td>1,000</td>
</tr>
<tr>
<td>Dithiothreitol Solution</td>
<td>20</td>
</tr>
</tbody>
</table>

**Important:** The working solution should be prepared on daily basis. If not used immediately, it should be refrigerated at 2-8°C for a period of not more than 24 hours.

**B) Sample Pretreatment:**

- Pipette 200μl of serum sample into a test tube (same procedure to be followed for controls and adjusters too).

- Add 1000μl of the working solution to all tubes. Slightly shake the tubes.

- Cover the tubes with foil paper and place them in a covered water bath at (100°C) for exactly 20 minutes.

- Remove the tubes from water bath and cool them by keeping them at room temperature. This takes approximately 20-30 minutes.

- When cool, pipette at least 300μl of the treated sample into the sample cup.
3.2.4.4.2 External Quality Assurance: The laboratory had enrolled in External Quality Assurance Programmes to assess and improve the ability to accurately and precisely measure various biochemical parameters in serum/plasma samples.

- Enrolled in Bio- Rad Clinical Chemistry EQAS programme for Ferritin, Folic Acid and Vitamin B12.
- Enrolled in AIIMS EQAS programme for Hemoglobin estimation.

The performance in the above mentioned programmes for all the biochemical parameters was satisfactory.

3.2.4.5 STANDARDISATION OF METHODOLOGIES: The methodologies for the determination of above mentioned biochemical parameters were well standardized at ICMR’s “Centre for Promotion of Nutrition Research and Training with special focus on North east, Tribal and Inaccessible population”. The standardization of the analytical methodologies involved various steps and these had been critically evaluated. Each instrument’s performance with respect to its capability for measurement of analyte, sensitivity and linearity were all analyzed and had been found to be satisfactory so as to suggest that they could be reliably used for the estimation of various analytes. Precision and accuracy of all the methods was found to be good.

3.3 DATA ANALYSIS

Entire data was collected on a predesigned proforma and managed on Microsoft Access and Excel sheets. Rechecking of the data was done to assure data entry accuracy. The data was analyzed both quantitatively as well as qualitatively. Frequency and percentage was calculated for age, educational status, occupation, family income etc. Appropriate statistical methods like T-test (Independent and paired), chi-square test were used to determine the change in
the hemoglobin levels and other bio-chemical parameters as well as the prevalence of deficiencies before and after the intervention period both within and between the groups. Incase of serum ferritin and Vitamin B12, where Standard Deviation was found to be very high, log transformations were done to create normal distribution. The analysis were performed using SPSS package.

For analysis of the dietary data collected by the 24 hours recall method, cooked food items in household measures were converted to raw food weights using standard recipes available at Lady Irwin College (Kashyap and Narula, 2002) (Annexure 7.4). Thus, dietary data obtained by 24 hours dietary recall was converted into equivalent raw weights. The various foods were grouped together under appropriate food groups and compared for dietary accuracy with ICMR (2010) recommendations for balanced diets for adults. Mean intake of Energy, Protein, Fat, Iron, Calcium, Vitamin A, Vitamin C, Vitamin B1 and Vitamin B2 for each subject was then calculated using ‘Dietsoft’ software based on Nutritive Value of Indian Foods (Gopalan et al, 2004). The values thus obtained were assessed for adequacy by comparing with respective RDA’s (ICMR, 2010) and percentage adequacy was calculated.

3.3.1 CUT OFFS USED

3.3.1.1 ANTHROPOMETRIC PARAMETERS:

3.3.1.1.1 BMI for Age- WHO Child Growth Standards, 2007 were used for comparing the BMI for Age. The following WHO classification was used for distribution (%) of subjects according to age specific BMI centiles.

<table>
<thead>
<tr>
<th>BMI CENTILES</th>
<th>NUTRITIONAL GRADES</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; Median - 3SD</td>
<td>Severe Thinness</td>
</tr>
<tr>
<td>&lt; - 3SD to - 2SD</td>
<td>Moderate Thinness</td>
</tr>
<tr>
<td>- 2SD to +1SD</td>
<td>Normal</td>
</tr>
<tr>
<td>+1SD to +2SD</td>
<td>Overweight</td>
</tr>
<tr>
<td>≥ Median +2SD</td>
<td>Obese</td>
</tr>
</tbody>
</table>
The data was also compared with Indian Standard given by Marwaha et al, 2007.

**3.3.1.1.2 Height for Age**: height-for-age data was compared with both WHO Child Growth Standards, 2007 and reference values for Indians given by Khadilkar et al, 2009 to assess the prevalence of stunting

**3.3.1.2 BIOCHEMICAL PARAMETERS**: Biochemical investigations included hemoglobin, serum ferritin, folic acid and Vitamin B12 at the baseline and after the supplementation. Following cut offs were used:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cut-Off</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>≥120 g/L</td>
<td>WHO, 2001</td>
</tr>
<tr>
<td>Serum Ferritin</td>
<td>≥ 15 ng/ml</td>
<td>WHO, 2011</td>
</tr>
<tr>
<td>Serum Folic acid</td>
<td>≥4 ng/ml</td>
<td>WHO, 2008</td>
</tr>
<tr>
<td>Serum Vitamin B12</td>
<td>≥203 pg/ml</td>
<td>WHO, 2008</td>
</tr>
</tbody>
</table>