

METHODS AND MATERIAL

Vitamin-D, also known as the sunshine vitamin, has received a lot of attention in recent times as a result of a meteoric rise in the prevalence of its deficiency. Vitamin-D deficiency (VDD) is now recognized as a global pandemic with the major cause being the lack of appreciation that sun exposure has been and continues to be the major source of vitamin-D for people of all ages. Publications have reported that vitamin-D plays a crucial role in a plethora of physiological functions and association of VDD with many acute and chronic illnesses including disorders of calcium metabolism, autoimmune diseases, some cancers, type 2 diabetes mellitus, cardiovascular disease and infectious diseases. Though the exact pathways are not clear yet, with the rise in incidences of these conditions it is important to identify the determinants of VDD among the high risk population. Also, there still remains some controversy regarding what blood level of 25-hydroxyvitamin D should be attained for both bone health and reducing risk for VDD associated acute and chronic diseases and how much vitamin-D should be supplemented (Wacker & Holick, 2013). Along with this it is also very important to sustain the serum vitamin-D levels in the desirable range. Whether this can be achieved through simple lifestyle changes needs to be investigated and knowledge regarding it should be propagated. Keeping these questions in mind it was thought appropriate to conduct the present research which aimed at mapping the prevalence of VDD among adult population, identify its determinants and see its association with various clinical conditions. Also an attempt was made to examine the impact of vitamin-D supplementation on various blood parameters among subjects with type-II diabetes mellitus. A NHE material was developed which can be used as an education tool for imparting knowledge regarding vitamin-D and its health benefits.

Thus the present research work was carried out in four phases as described below:

Phase I: Prevalence of vitamin-D deficiency and determinants of vitamin-D status among free-living adult population of Vadodara city

Phase II: Impact of Vitamin-D supplementation on cardio-metabolic profile of subjects with type-2 diabetes mellitus

Phase III: Comparison between diabetic and non-diabetic populations

Phase IV: Development of Nutrition Health Education (NHE) Material

The different phases comprised of the following objectives:

Phase I Cross-sectional study

1. To map the prevalence of vitamin D deficiency (VDD) among free living adult population of age 30-60 years in Vadodara city.
2. To identify the determinants of vitamin D status and study its association on other clinical conditions like overweight/obesity, hypertension & CVDs.
3. To study the metabolic aberrations in relation to vitamin D deficiency in the study population in terms of-
 - a) Anthropometry (BMI, WSR and % Body fat)
 - b) Blood Pressure (Systolic & Diastolic)
 - c) Carbohydrate metabolism (FBS & HbA1c)
 - d) Lipid metabolism (TC, TAG, LDL-C, HDL-C)
 - e) Inflammation (Hs-CRP)
 - f) Nutritional anemia (Hb)
 - g) Thyroid functions (TSH, T3, T4)
 - h) Liver and kidney functions

Phase II (a) Clinical study

1. To map the prevalence of vitamin-D deficiency in type II diabetic patients attending a diabetic clinic.
2. To identify the determinants of vitamin-D status among the subjects.
3. To study the association of the determinants with T2DM.

Phase II (b) Intervention study

1. To study the effect of vitamin-D supplementation on serum vitamin-D status of the subjects with T2DM.
2. To examine the impact on biophysical measurements, physical activity pattern and nutrient intake of the subjects.
3. To study the efficacy of the vitamin-D supplementation dose on the changes in the cardio-metabolic profile of subjects with T2DM.

Phase II (c) Washout effect

1. To study the washout effect of the vitamin-D supplementation on anthropometry, HbA1c levels and lipid profile of the subjects after eight weeks of post supplementation.

Phase III Comparison between diabetic and non-diabetic populations

1. To compare the vitamin-D status of the diabetic and non-diabetic populations.
2. To see the difference in biophysical measurements, physical activity pattern and nutrient intake of the subjects belonging to both the populations.
3. To compare the levels of various biochemical parameters among the subjects.

Phase IV Development of NHE

A nutrition health education material titled “Vitamin D and Type 2 Diabetes Mellitus: A lifestyle Management Approval” was developed.

PHASE I

**Prevalence of vitamin-D deficiency and determinants of vitamin-D status
among free-living adult population of Vadodara city**

This was a cross-sectional study to assess the vitamin-D status of adult population residing in Vadodara city. The city was divided into five zones- north, south, central, west & east. One society was selected purposively from each zone for the enrolment of the subjects in the age group 30-60 years. Then through snow ball effect the subjects who gave written consent for participation were included till the required sample size was attained. The inclusion criterion was subjects should be in the age group 30-60 years. They should be residents of Vadodara city and give a positive consent to participate in the study. Pregnant women and lactating mothers were excluded from enrollment. While the limitation was that urban slums were not considered for the enrollment of the adult subjects.

A total of 141 subjects (50 males and 91 females) were enrolled between the months of February to September 2012. Out of the enrolled subjects twelve subjects had T2DM, who were then not included in this phase of study for the data analysis. Hence the final study sample obtained was 129 subjects (47 males and 82 females). Background information was collected using a pre-tested semi-structured questionnaire. Other data was collected in terms of anthropometric measurements, blood pressure, 24-hour dietary recall and physical activity profile. After an over-night fast, blood samples were collected for biochemical estimations by a trained technician. Biochemical parameters included serum 25(OH)D, lipid profile, fasting blood glucose and HbA1c levels, thyroid profile, liver enzymes and kidney profile. Thus an attempt was made to identify the determinants of vitamin-D deficiency among the apparently healthy population. The study was approved by the Institutional Ethics Committee for Human

Research of Faculty of Family and Community Sciences, The M.S. University of Baroda (No.: IECHR/2012/21). The details of the experimental plan is given in Figure 3.1.

PHASE II

Impact of Vitamin-D supplementation on cardio-metabolic profile of subjects with type-II diabetes mellitus

A randomised control trial was performed to assess the impact of vitamin-D3 (cholecalciferol granules) supplementation on serum 25(OH)D status, HbA1c levels, lipid profile and other biochemical parameters of adult subjects (30-65 years) with type-II diabetes mellitus. The study was approved by the Institutional Ethics Committee for Human Research of Faculty of Family and Community Sciences, The M.S. University of Baroda (No.: IECHR/2013/04).

Phase II (a): Screening and collection of baseline data

To identify the subjects for supplementation study screening was conducted. A diabetic clinic in Vadodara city was purposively selected. Adult subjects with an inclusion criteria of age 30-65 years and having confirmed T2DM attending the clinic were enrolled between the months of September 2012 to March 2013. Subjects with Gestational diabetes i.e. pregnant women, Type-I diabetes (juvenile diabetes) were excluded. Biochemical estimations were not done on subjects taking vitamin D supplements.

A total of 209 subjects (101 males & 108 females) were enrolled. Background information and medical history was collected using a pre-tested semi-structured questionnaire and anthropometric measurements by standard procedures. Written informed consent was acquired from all the subjects before participating in the study for the biochemical estimations. Out of the enrolled subjects, 114 subjects gave positive consent to carry out the biochemical estimations. These subjects had not consumed vitamin-D supplements in any form from six months prior to the day of enrolment. After an over-night fast, blood samples were collected for biochemical estimations by a trained technician. Biochemical parameters included serum 25(OH)D, lipid profile, fasting blood glucose and HbA1c levels, thyroid profile, liver enzymes and kidney profile which were estimated in an accredited laboratory. Dietary information and physical activity profile was also obtained from the subjects. The Figure 3.2 illustrates the details of screening and baseline data collection.

**Figure 3.1: EXPERIMENTAL DESIGN
PHASE I**

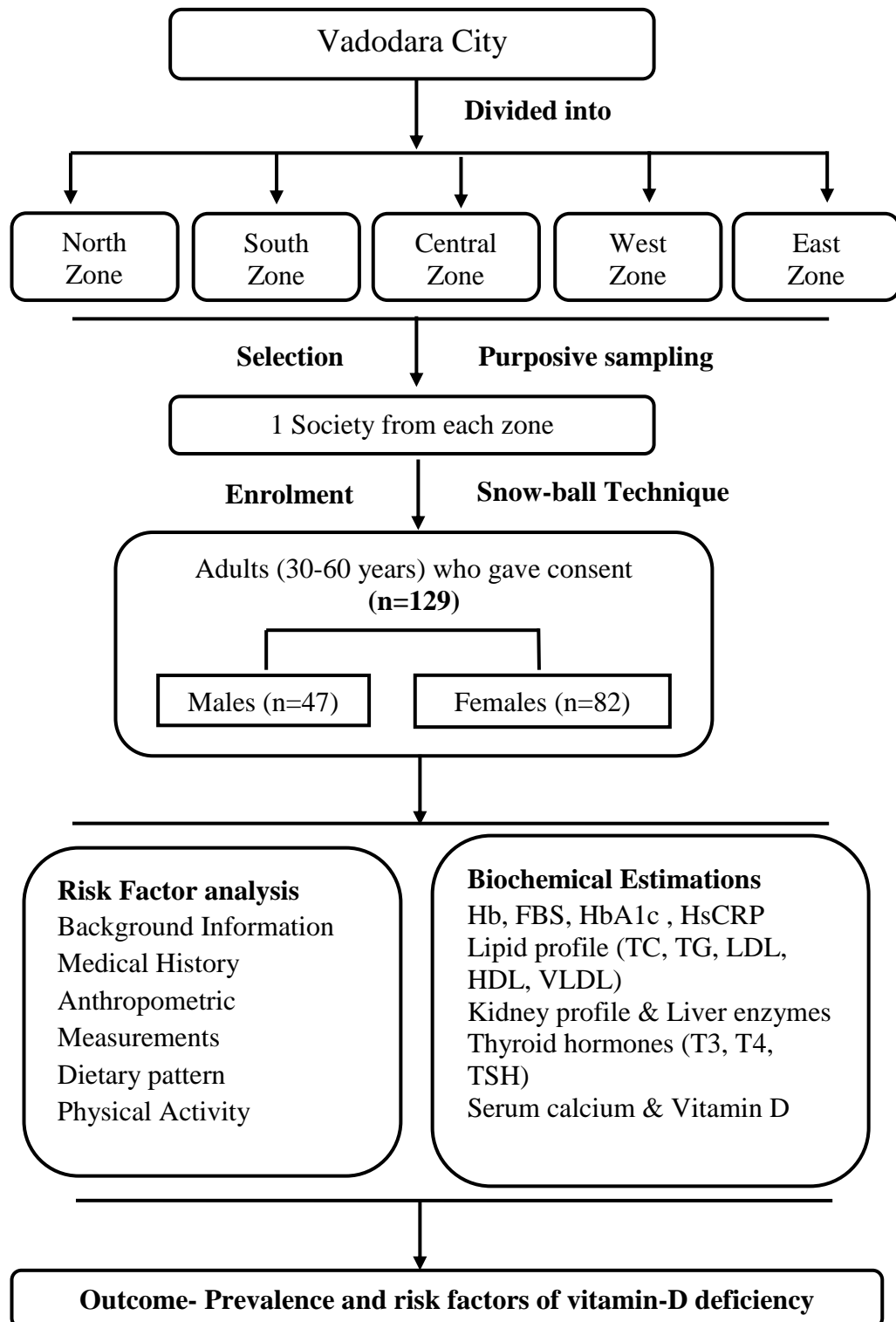
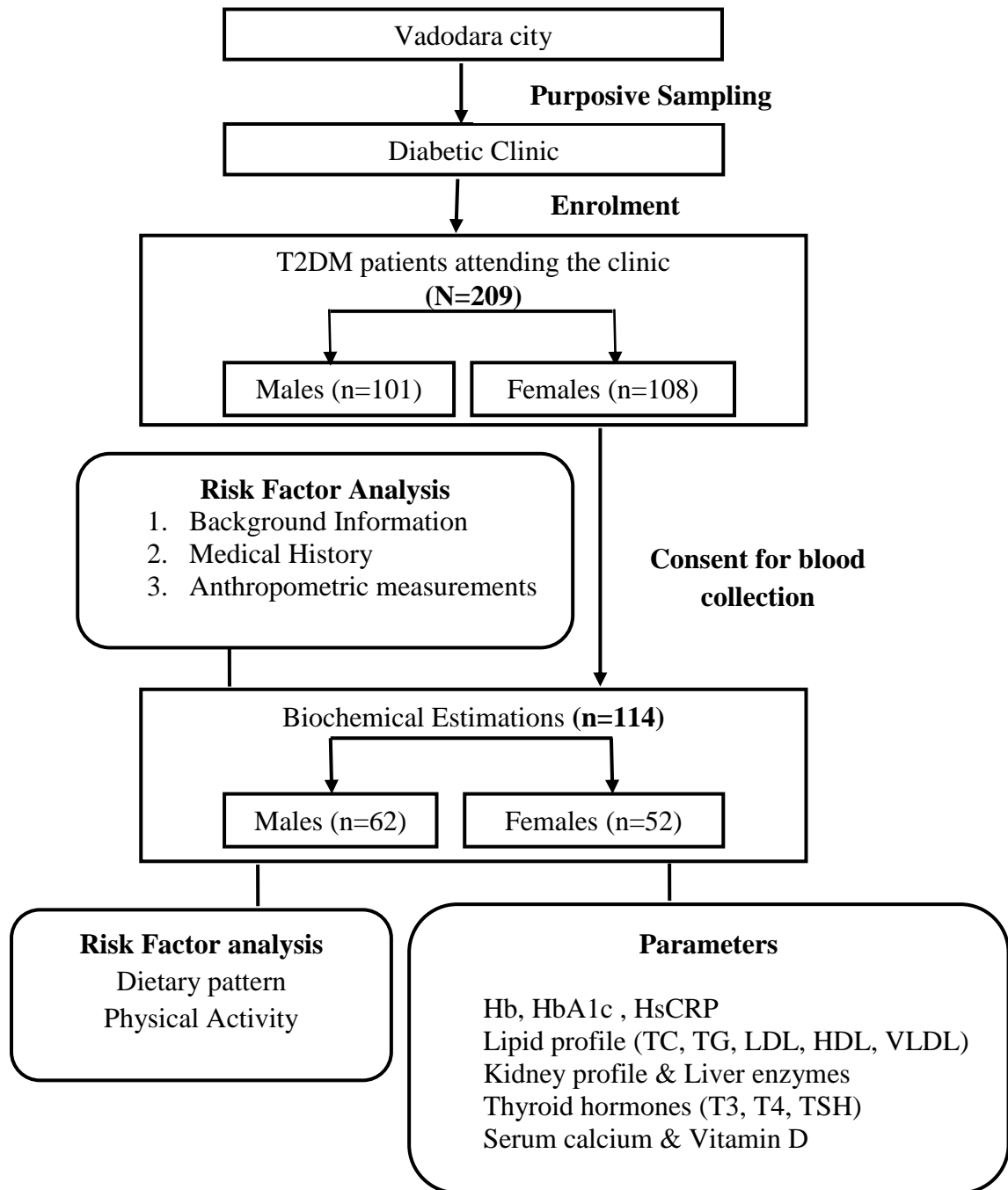


Figure 3.2: EXPERIMENTAL DESIGN**PHASE II (a)**

Phase II (b): Randomised control trial to study the impact of vitamin-D3 granules on serum 25(OH)D status and cardio-metabolic profile of subjects with T2DM

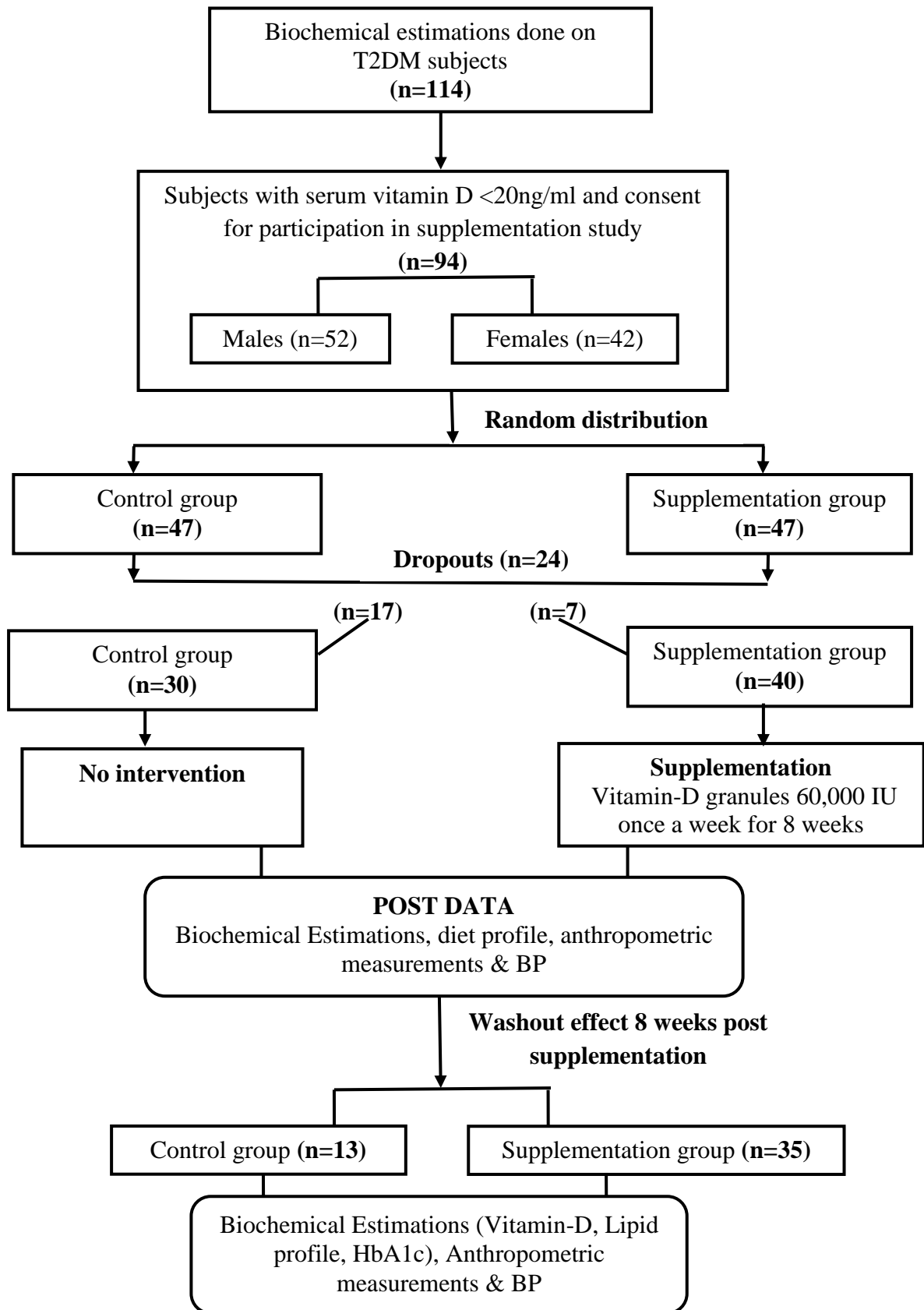
For this phase of the study all the subjects falling in the deficiency range of serum 25(OH)D (<20ng/ml) were included. Out of 114 subjects screened, 101 subjects had serum vitamin-D levels less than 20 ng/ml, however only ninety-four subjects gave consent for further participation, which were then enrolled for the supplementation study. They were randomly divided into supplementation group (SC) and control group (CG). A further drop out of twenty-four subjects (SG=7, CG=17) was reported due to non-cooperation (later backing out of the study) or initiation of vitamin-D supplements as prescribed by their doctor. Hence the remaining seventy subjects (40 in supplementation group & 30 in control group) completed the study. The supplementation group received 60,000 IU weekly vitamin-D3 (cholecalciferol) granules for eight weeks (May 2013 to July 2013) while the control arm didn't receive any intervention. The cholecalciferol sachets were procured from US Vitamins Ltd, Mumbai and were from one batch of manufacture. The subjects were asked to consume the whole sachet at one time with either milk or water. All the subjects in the study group reported of consuming it with water. Written consent was taken from the subjects for the phase. Post data in the form of anthropometric measurements, 24-hour dietary recall, physical activity profile and biochemical estimations was collected at the end of eight weeks in the months of August-September 2013.

Phase II (c) Washout effect of vitamin-D supplementation on serum 25(OH)D status of T2DM subjects

In continuation of the supplementation study, the sustainability of vitamin-D levels was assessed after sixteen weeks of supplementation (October-November 2013). The washout effect of supplementation was studied on the serum 25(OH)D status, lipid profile, HbA1c values and anthropometric measurements. For this phase data could be collected for forty-eight subjects only due to either non-availability of subjects or start of vitamin-D supplements in course of time and few didn't want to continue further in the study.

The detailed protocol of the supplementation trial both phase II (b) & II (c) is described in Figure 3.3.

**Figure 3.3: EXPERIMENTAL DESIGN
PHASE II (b & c)**



Phase III

Comparison between diabetic and non-diabetic populations

As observed from the experimental design of the previous phases; the study comprised of two distinct populations in respect to their disease profile. In phase-I the population was apparently healthy especially the subjects did not suffer from diabetes mellitus, while in phase-II the population was suffering from type-II diabetes mellitus. Hence it was thought to be appropriate to have a comparison of various parameters among the subjects belonging to both these populations. The primary parameter compared was the serum vitamin-D levels among them and hence mapping the prevalence of vitamin-D deficiency among the subjects. Other parameters included the biophysical measurements, lifestyle factors such as activity pattern and dietary intake and the various biochemical estimations such as their lipid profile, HbA1c, haemoglobin and thyroid hormone levels, liver function tests and kidney profile. For this phase of the study in all a total of 243 subjects were compared; 129 belonged to the non-diabetic group while 114 were in the diabetic group. The representative flowchart of the experimental design is given in Figure 3.4.

PHASE IV

Development of Nutrition Health Education (NHE) Material

In order to frame guidelines to restore vitamin-D status in sufficiency range, NHE material in a booklet form is developed which can be used in future for counselling the general population as well as population with T2DM in particular. The material was developed on the outline as discussed in Table 3.1. The NHE booklet is attached as Appendice 1.

The methods and tools used for the data collection as well as estimation of biochemical parameters are portrayed in Table 3.2 and explained henceforth.

**FIGURE 3.4: EXPERIMENTAL DESIGN
PHASE III**

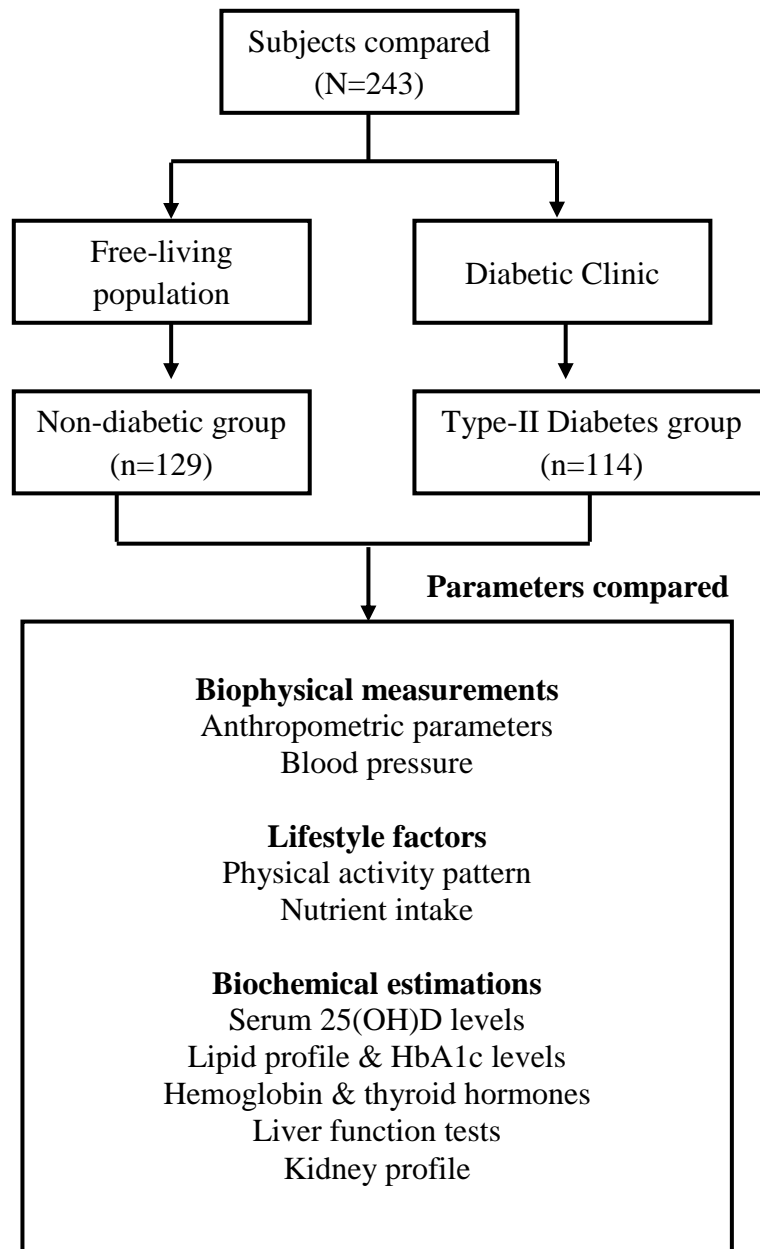


TABLE 3.1 COMPONENTS FOR DEVELOPMENT OF NHE
PHASE IV

Key Components	Details
T2DM	Signs and symptoms, causes, complications
	Modifiable risk factors
	Normal values for BMI, BP, blood sugar, HbA1c
	Dietary guidelines
	Lifestyle changes
Vitamin D	Signs and symptoms, causes, complications
	Vitamin D and T2DM
	Cutoffs for serum vitamin D levels
	Advocacy of foods rich in vitamin D
	Lifestyle changes (increased physical activity & sun exposure)

Table 3.2: METHODS AND TOOLS USED FOR DATA COLLECTION

Parameters	Techniques
Medical and Life Style History	
General Information, Medical history, Family History, Lifestyle variables	Pre tested Structured questionnaire
24 hours dietary recall	Semi structured questionnaire
Food Frequency	Semi Quantitative Structured questionnaire
Physical Activity	International Physical activity Questionnaire (Short), 2005
Anthropometric measurements	
Weight	Salter Electronic Weighing Scale
Height, Waist and Hip circumference	Non stretchable Fiber Glass Tape
Biophysical Measurements	
Blood Pressure	Digital BP meter (Omron HEM-7203)
Body Fat Analysis	Omron Body Fat Analyzer (HBF 306)
Vitamin-D status	
Serum 25-Hydroxy vitamin D	Chemiluminescence immune assay
Nutritional Anemia	
Haemoglobin	Cyanmethemoglobin method
Iron, Total Iron Binding capacity	Spectrophotometry
Glucose Metabolism	
Fasting Blood Sugar	Enzymatic kit method
HbA1C (Glycated hemoglobin)	High Pressure Liquid Chromatography
Lipid Metabolism	
Triglycerides, Total Cholesterol, High Density Lipoprotein-Cholesterol	Enzymatic kit method
Low Density Lipoprotein-Cholesterol	Derived from TC and TG
Very Low Density Lipoprotein-Cholesterol	Derived from TG
Inflammation	
High Sensitivity C-Reactive Protein	Nephelometry
Thyroid Profile	
TSH, Total T3, Total T4	Competitive chemiluminescence immune assay
Kidney Function test	
Creatinine, BUN, Uric Acid, Alkaline Phosphate	Photometry
Serum calcium	Ion Selective Electrode & Photometry
Liver Function Test	
Total Bilirubin, Direct Bilirubin, Indirect Bilirubin, SGOT, SGPT, GGT, Serum Albumin, Total Protein	Photometry

Methods for data collection

Consent from the subjects

In line to the ethics of research, written informed consent was taken from all the subjects for both- phase I and phase II of the study. The consent forms used are given in Annexure 1 and Annexure 2 for phases I and II respectively.

Background Information

Demographic information like religion, education level, employment status, family size and type of family etc., was collected from all the enrolled subjects using a pre-tested questionnaire (Annexure 3 & 4).

Medical History

Information on medical history of NCDs such as coronary heart disease, hypertension, diabetes, dyslipidemia, asthma, thyroid and cancer was collected using a pre-tested questionnaire. Medical history of any other condition was also noted down which was based on the present condition of the subjects (Annexure 3). For the supplementation study among the diabetics additional questions related to duration of diabetes, precipitating factors, symptoms and treatment regimen were also included (Annexure 4)

Lifestyle Practices

Prevalent lifestyle practices among the subjects from free living population and those enrolled from the diabetic clinic were assessed through a carefully planned pre tested questionnaire. This included personal habits like smoking, tobacco chewing, alcohol drinking, etc.

The physical activity pattern of the subjects was assessed using the ‘International Physical activity Questionnaire (Short), 2005. IPAQ short form is an instrument designed primarily for population surveillance of physical activity among adults. It has been developed and tested for use in adults (age range of 15-69 years). The items in the short IPAQ form were structured to provide separate scores on walking, moderate-intensity and vigorous-intensity activity. Computation of the total score for the short form requires summation of the duration (in minutes) and frequency (days) of walking, moderate-intensity and vigorous-intensity activities. The following values were used for the analysis of IPAQ data: Walking = 3.3 METs, Moderate PA = 4.0 METs and Vigorous PA = 8.0 METs. Using these values, four continuous scores are defined:

1. Walking MET-minutes/week = 3.3 * walking minutes * walking days
2. Moderate MET-minutes/week = 4.0 * moderate-intensity activity minutes * moderate days
3. Vigorous MET-minutes/week = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days
4. Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous METminutes/week scores.

The three levels of physical activity proposed to classify population were defined as follows

Low activity: Total physical activity score < 600 MET-minutes/week

Moderate activity: Any combination of walking, moderate-intensity or vigorous intensity activities achieving a minimum Total physical activity score of at least 600 MET-minutes/week.

High activity: Any combination of walking, moderate-intensity or vigorous-intensity activities achieving a minimum Total physical activity score of at least 3000 MET-minutes/week.

As one the objective was to identify the determinants of vitamin-D, information related to non-invasive factors such as history of fractures, sun exposure, use of sunscreen, type of clothing preferred while going out in sun, skin complexion, etc was also collected from the subjects (Annexure 3 & 4).

Dietary Habits

To assess the dietary habits of the subjects information regarding the type of oil used, periodic blending/ rotation of oil, use of left-over deep fried oil, the type of milk consumed, type of beverage consumed, etc was collected using the questionnaire.

The subjects were also asked to recall their intake of vitamin-D food sources and state the frequency with which they consumed them the information was filled in a pre tested food frequency questionnaire. For the detailed nutrient intake the subjects' one day dietary recall was taken. Holidays and days of fast were avoided and in such cases recall for another working day were recorded (Appendix 3 & 4). The 24-hour diet recall information was converted into raw ingredients using standardized recipes and then analysed using the *DietCal* software developed by Ms. Gurdeep Kaur, Chief Dietician at AIIMS, New Delhi.

Compliance of vitamin-D supplementation

The compliance of vitamin-D supplementation given in phase-II of the study was recorded using a compliance sheet (Annexure 5). The subjects were given vitamin-D sachets in two

visits during the period of eight weeks i.e. four sachets at a time. They had to record the date and day on which the sachet was consumed for the period of eight weeks in the sheet provided to them. Telephonic calls were made to them each week as a reminder to consume the sachet. At the end of four and eight weeks the empty sachets were collected from them and preserved as a compliance proof by the researcher.

Anthropometric Measurements

To assess the nutritional status of the subjects, direct parameter of assessment i.e. anthropometric measurements were recorded. The measurements included height, weight, percent body fat, hip and waist circumference. Based on these the body mass index (BMI), waist-to-hip ratio (WHR) and waist-stature ratio (WSR) were calculated. The detailed procedures are discussed below and the tools used are given in Table 3.2.

- **Height**

Height is a linear measurement of the body. It is associated with chronic insufficiency food intake, frequent infections and continued under nutrition.

A fiberglass tape was fixed perpendicular to the floor on the wall and the subjects were asked to stand on the floor against the fixed scale with the feet parallel and heels, shoulders and the back of head comfortably erect and not with the knees bent. The subjects were asked to look straight ahead with the head held comfortably erect and the arms hanging loosely by the sides. The head piece of the scale was gently lowered crushing the hair and making the contact with the top of the head. The head piece was at right angles to the scale. The height was measured to the nearest 0.1cm.

- **Weight**

Weight is a key anthropometric measurement of body mass. When expressed with respect to height, it serves as an index of current nutritional status of an individual.

The Salter electronic weighing scale was standardized with the help of standard weight of 5kg and the error was recorded. The subjects were asked to stand on the platform of the scale without touching anything, without foot wear and with minimum clothing. The subjects were asked to look straight ahead without bending down. The equipment was calibrated daily and the minimum weight that the instrument could measure was 0.1 kg.

- **Body Mass Index (BMI)**

Body Mass Index indicates current nutritional status. It is considered as an indicator of weight that is relatively independent on height. To calculate BMI, weight and height of the subjects were measured by the recommended methods. BMI was calculated using the standard formula:

$$\text{BMI} = \text{Weight (kg)} / \text{Height (m}^2\text{)}$$

Cut off levels for BMI based on Asia Pacific Classification were used to define overweight and obesity is given in Table 3.3.

Table 3.3 Asia Pacific Cut-Offs for Body Mass Index (BMI)

Classification	BMI (Kg/m²)
Underweight	<18.5
Normal Weight	18.5 – 22.9
Overweight	23 – 24.9
Obese- Grade 1	25 – 26.9
Obese- Grade 2	27 and Above

- **Waist Circumference**

Waist circumference is the measure of abdominal obesity. It is well correlated with visceral adipose tissue. It is also an easy, inexpensive and useful tool for identifying obesity and overweight (NCEP, 2001). The midpoint between the inferior margin of the last rib and the crest of the ileum in the mid-auxiliary plane was determined. The subjects were asked to stand with their feet together and arms placed on the side with palms of hands facing inwards. The subjects were also asked to breathe out and a constant tension tape was pressed through the point determined earlier and the measurement was taken to the nearest 0.1cm. The WHO cut offs used for abdominal obesity are given in the Table 3.4

- **Hip Circumference**

The subjects were asked to stand with their feet together, arms placed on sides with palms facing inwards and subjects breathing out. Hip circumference was defined as the widest circumference over the buttocks and below the iliac crest. A constant tension tape was pressed through the hips and the measurement was recorded to the nearest 0.1 cm.

- **Waist Hip Ratio: (WHR)**

Waist Hip Ratio is one of the widely used measures for abdominal obesity. Increased WHR may reflect both a relative abundance of abdominal fat (increased WC) relative risk of guttel muscle (decreased hip girth). WHR is a good indicator for obesity as it separates gynoid (pear shaped) obesity from anroid (apple shaped) obesity (Baldwin 2010). The cut offs for WHR is given in Table 3.4.

WHR= Waist circumference (cm) / Hip circumference (cm)

- **Waist Stature Ratio**

WSR, also known as Waist Height Ratio (WHR), was calculated by dividing the waist circumference by height. It is more accurate anthropometric index than BMI, for individuals who have ‘peer’ rather than ‘apple’ shaped obesity. WSR is the best simple anthropometric tool in predicting a wide range of CVD risk factors and related health conditions. The cut offs for classifying individuals as high risk risk group is given in Table 3.4.

Table 3.4 Cut-Offs for Abdominal Obesity

Criteria	Men	Women
Waist to hip ratio (WHO)	≥ 0.90	≥ 0.85
Waist Circumference (IDF)	≥ 90 cm	≥ 80 cm
Waist Stature Ratio	≥ 0.55	≥ 0.53

- **Body Fat Percentage**

Body Fat Percentage is the amount of fat tissues in the body as a percentage of total body weight. Due to differences in body composition the BMI is not necessarily an accurate indicator of body fat, for example in individuals with greater than average muscle mass will have a higher BMI. Body Fat Percentage is an accurate indicator to assess the health risk of CVD, hypertension and diabetes. Guidelines for body fat percentage are given in Table 3.5.

Percentage body fat was calculated using the Omron body fat monitor (Model HBF-306). The instrument measures body fat percentage based on the electric resistance and personal data such as height, weight, age and gender. After setting the data, body fat mass can be measured by simply holding the grip electrodes and pushing the Start button. The

measured results are displayed approximately 7 seconds after the Start screen is displayed.

Table 3.5 ADA Cut-Offs for Percent Body Fat

Category	Men	Women
Normal	10-20%	15-25%
High risk	Over 20%	Over 30%

Blood Pressure

The blood pressure of all the subjects enrolled in the study was checked by automatic blood pressure monitoring machine. The instrument used was ‘Omron HEM-7203 Automatic Blood Pressure Monitor’. The instrument uses the oscillometric method of blood pressure measurement. Three readings at an interval of five minutes each were recorded and a mean value was calculated, which was then used as the final reading. The cut-offs given by JNC VIII classification were used for assessing the prevalence of hypertension among the subjects. The classification for blood pressure by JNC VIII is given in Table 3.6.

Table 3.6 JNC VIII Cut-Offs for Blood pressure

Classification	Systolic BP (mmHg)	Diastolic BP (mmHg)
Normal	<120	<80
Pre HTN	120-139	80-90
Stage 1	140-159	90-99
Stage 2	≥160	≥100

Metabolic Syndrome

Metabolic Syndrome is a constellation of cardio-metabolic disease risk factors. The prevalence of this condition was also assessed among the subjects. Metabolic Syndrome was defined based on two criteria- the International Diabetes federation (IDF) classification, 2005 and the guidelines given by Adult Treatment Panel-III in 2001. The detail of these classifications is given in Table 3.7.

Table 3.7 Definition of Metabolic Syndrome

Parameters	ATP III (2001)	IDF (2005)
Fasting Glucose	>100mg/dL or previously diagnosed T2DM	>100mg/dL or previously diagnosed T2DM
BP	>130/85 mmHg	>130/85 mmHg
Triglycerides	150mg/dL or specific treatment for this	150mg/dL or specific treatment for this
HDL	Men: <40mg/dL Women: <50mg/dL	Men: <40mg/dL Women: <50mg/dL
Obesity BMI>30kg/m ² or Abdominal WC	South Asian men: >90cm South Asian women: >80cm	Waist circumference ≥90 cm (m), ≥80 cm (f) – South Asians
MS-definition	At least three risk factors	Abdominal obesity plus two or more risk factors

ANALYTICAL PROCEDURES FOR BIOCHEMICAL ESTIMATIONS

The biochemical parameters were performed in an accredited laboratory, Thyrocare Lab which is certified by ISO 9001:2008, NABL (India) and CAP (College of American Pathologist). All the methods and tools used for performing biochemical estimations with their detailed procedures were as mentioned below-

1. Vitamin D (25-hydroxy vitamin D)

The serum concentration of 25-Hydroxyvitamin-D [25(OH)D] is widely regarded as a ‘‘gold standard’’ indicator of vitamin D status as it is a good reflection of cumulative exposure to sunlight and dietary intake of vitamin D (Springbeet et al., 2010). VDD was defined as serum 25(OH)D concentration of <20ng/ml and categorized as insufficiency (20–≤30 ng/ml) and sufficiency (>30 ng/ml) (Lips, 2001).

Serum 25(OH)D was estimated by CLIA (Chemiluminescence Immunoassay). The ADVIA Centaur Vitamin-D Total (VitD) assay is for *in vitro* diagnostic use in the quantitative determination of total 25 (OH) vitamin D in human serum and plasma (EDTA, lithium-heparin, sodium-heparin) using the ADVIA Centaur and ADVIA Centaur XP systems. The ADVIA Centaur VitD assay is intended as an aid in the determination of vitamin D

sufficiency. The kit used for the estimation was of Simens – ADVIA Centaur. The specimen type is Human serum and plasma (EDTA, lithium-heparin, sodium-heparin)

Principle: The ADVIA Centaur VitD assay is a one-pass, 18-minute antibody competitive immunoassay that uses an anti-fluorescein monoclonal mouse antibody covalently bound to paramagnetic particles (PMP), an anti-25(OH) vitamin D monoclonal mouse antibody labeled with acridinium ester (AE), and a vitamin D analog labeled with fluorescein. An inverse relationship exists between the amount of vitamin D present in the patient sample and the amount of relative light units (RLUs) detected by the system.

Assay Procedure

Before placing samples on the system, ensure that samples have the following characteristics:

- Samples are free of fibrin or other particulate matter. Remove particulates by centrifugation at 1000 x g for 10 to 15 minutes.
- Samples are free of bubbles.

This assay requires 20 µL of sample for a single determination. This volume does not include the unusable volume in the sample container or the additional volume required when performing duplicates or other tests on the same sample.

The ADVIA Centaur and ADVIA Centaur XP systems automatically perform the following steps:

1. Dispenses 20 µL of sample into a cuvette, and incubates for 15 seconds.
2. Dispenses 200 µL of Ancillary Pack Reagent, and incubates for 4.5 minutes at 37°C.
3. Dispenses 50 µL of Lite Reagent, and incubates for 5.5 minutes at 37°C.
4. Dispenses 100 µL of Solid Phase reagent, and 50 µL of ancillary well reagent, and incubates for 2.75 minutes at 37°C.
5. Separates the Solid Phase from the mixture, and aspirates the unbound reagent.
6. Washes the cuvette with Wash 1.
7. Dispenses 300 µL each of Acid Reagent and Base Reagent to initiate the chemiluminescent reaction.

The cut offs given by Lips (2001) were used to classify the vitamin D status among the subjects (Table 3.8). Serum vitamin D is expressed in two units; nanogram/millilitre (ng/mL) and nanomole/litre (nmol/L). To convert ng/mL into nmol/L multiple the former with a factor of 2.5.

Table 3.8 Classification for vitamin D status

Classification	Serum 25(OH)D levels
Toxicity	> 100 ng/mL
Sufficiency	30-100 ng/mL
Insufficiency	20 - ≤ 30 ng/mL
Mild Deficiency	10 - <20 ng/mL
Moderate Deficiency	5- <10 ng/mL
Severe Deficiency	<5 ng/mL

2. Haemoglobin:

Blood haemoglobin was estimated using cyanmethemoglobin method in autoanalyser.

Principle: Whole blood was diluted in cyanmethemoglobin reagent. This reagent hemolyzes the erythrocytes which releases haemoglobin in solution. The ferrous ions (Fe^{2+}) of the haemoglobin molecules are oxidized by potassium ferricyanide to ferric ions (Fe^{3+}). This oxidation results in the formation of methemoglobin. Methemoglobin combines with cyanide ions (CN^-) to form cyanmethemoglobin, a stable compound. All haemoglobin derivatives except sulfhemoglobin are converted to cyanmethemoglobin. When measured spectrophotometrically at 540nm, the absorbance of cyanmethemoglobin follows Lambert-Beer's law and is directly proportional to the concentration of haemoglobin in blood.

The hemoglobin cut-offs used for diagnosing various severities of anemia was as per the WHO (2001) criteria, outlined in Table 3.9.

Table 3.9 WHO Criteria For Diagnosing Severity Of Anemia

Categories	Women (gm/dL)	Men (gm/dL)
Normal	> 12.0	>13.0
Mild	11.0-11.9	11.0-12.9
Moderate	8.0-10.9	8.0-10.9
Severe	<8	<8

3. Iron

Iron was estimated by Ferrozine method without deproteinization.

Iron (Fe^{3+}) is separated from transferrin by means of guanidinium chloride in the weakly acidic pH range and reduced to Fe^{2+} with ascorbic acid. Fe^{2+} then forms a colored complex with ferrozine. Ingested iron is absorbed primarily from the intestinal tract and is temporarily stored in the mucosal cells as Fe^{3+} -ferritin, a complex of ferric hydroxide-ferric phosphate attached to the protein apoferritin. On demand, iron is released from the mucosal cells into

the blood as Fe^{2+} -transferrin in equilibrium with a very small amount of free Fe^{3+} . Transferrin is the plasma iron transport protein that binds iron strongly at physiological pH levels.

Reference range Male: 70-180 and Female: 60-180

4. Total Iron Binding Capacity (TIBC)

Total Iron Binding Capacity (TIBC) is done indirectly by the Unsaturated Iron Binding Capacity (UIBC) method. A known ferrous iron standard 105 $\mu\text{mol/L}$ (586 $\mu\text{g/dL}$) incubated with serum at a pH of 7.9 saturates the available binding sites on serum transferrin. The unbound excess iron is then complexed with ferene® to form ferrous ferene, a blue complex, which is measured by the LX system. The UIBC is equal to the total iron added less the excess iron. TIBC is elevated in iron deficiency. The sensitivity of TIBC for iron deficiency is less than that of serum ferritin.

Reference range Male: 225-535 and Female: 215-535

5. Percent Transferrin Saturation

Percent Transferrin Saturation was derived from iron and TIBC values

The terms transferrin saturation and iron-binding capacity, saturation, are interchangeable. Total iron binding capacity (TIBC) is a blood test to examine the iron content in the blood. Iron moves through the blood attached to a protein called transferrin. This test helps to know how well transferrin can carry iron in the blood.

Reference range 13-45 %

6. Fasting Blood Sugar Level

It was estimated using GOD/POD method using an enzymatic kit from Ecoline.

Principle: Glucose is oxidised by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide (H_2O_2). H_2O_2 in the presence of peroxidase (POD), oxidises the chromogen, 4-aminoantipyrine/phenolic compound to a red coloured compound. The intensity of the red coloured compound is proportional to the glucose concentration and is measured at 500nm (490-530nm)

Reference range 70-110 mg/dl

7. HbA1c

Principle: It works on the principle of high-performance liquid chromatography (HPLC), which is considered the “Gold Standard” technology in the follow-up of the plasma glucose

concentration of diabetic patients over time, via the measurement of HbA1c (glycated hemoglobin fraction).

It is a chromatographic technique that can separate a mixture of compounds and is used to identify, quantify and purify the individual components of the mixture. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed and the solvent(s) used. Cation exchange columns employ the differences in ionic interactions between haemoglobin components to separate them in a span of 1.6 min. A step gradient elution is used to separate HbF, s- A1c, Total A1, and Hb variant with three types of G8 variant elution buffer His (G8 variant elution buffer His no. 1, 2, and 3 (S)) of different salt concentrations.

Reference range

1. Below 6 % Normal
2. 6%-7% Good Control
3. 7%-8% Fair Control
4. 8%-10% Unsatisfactory Control
5. >10 % Poor Control

8. Lipid Profile

Instrumentation used for the analysis was Olympus AU2700 and Advia 1800 and the detection technology was by Photometry.

Kits- Cholesterol, Triglyceride and HDL: Aggape

LDL: Rapid Diagnostics

The cut-points used for detecting hyperlipidemia were used from the NCEP ATP III guidelines (2001) given in Table 3.10.

Principle: It works on the principle of Beer's law and Lambert's law. Under suitable conditions, the amount of light absorbed by a coloured solution, when illuminated with a light of suitable wavelength, is directly proportional to the concentration of the coloured solution and the length of the light path through the solution. Therefore, the amount of light decreases exponentially with the increase in the concentration of the solution and with increase in thickness of the layer of solution through which light passes. Olympus AU2700 and Advia 1800 measures the components in a sample (serum). First the system mixes and stirs a sample

and a reagent, and then executes photometry of the absorbance of the mixture in which a reaction has occurred. The optical density is calculated in accordance with the absorbance.

Serum VLDL-C was derived from serum triglyceride values.

TC/HDL-C was derived from total cholesterol and HDL-C values.

LDL-C/HDL-C was calculated by dividing the former by the later value.

TAG/HDL-C was obtained by dividing TAG values by HDL-C values.

Atherogenic Index of Plasma (AIP) is calculated by taking a log₁₀ value of the TAG/HDL-C ratio.

Table 3.10 NCEP ATP III (2001) Criteria for diagnosing Dyslipidemia

Total Cholesterol (TC) mg/dL	≥ 200
LDL Cholesterol (LDL-C) mg/dL	≥ 100
HDL Cholesterol (mg/dL)	<50 (for women)
	<40 (for men)
Triacylglyceride (TAG) mg/dL	≥150
TC/HDL-C	≥ 5
LDL-C/HDL-C	≥ 3
TAG/HDL-C	≥ 3.5
AIP (log ₁₀ TAG/HDL-C)	≥ 0.11

9. High Sensitive C-Reactive Protein (HsCRP)

The high sensitivity assay of C - reactive protein (hs-CRP) in the serum was done using nephelometry on a BN II automated nephelometer (Dade Behring/Siemens).

Principle: Here a soluble analyte and corresponding antibodies that are bound to polystyrene particles are made to react. The test specimen is mixed with latex particles coated with monoclonal antibodies (anti-CRP antibodies), so the CRP present in the specimen will bind with the latex bound antibodies.

Reference range Adults ≥ 0.1 mg/dl (Pearson et al., 2003)

10. Thyroid Stimulating Hormone (TSH)

Detection Technology used for the estimation of TSH is ultra sensitive sandwich chemiluminescent immuno assay by Advia centaur CP and XP systems

Principle: Advia Centaur (BAYER, USA) works on the principle of chemiluminescence (CLIA). Antigen (Ag) present in the serum sample binds to the antibodies (Ab) present in the reagent. The reagent also has the paramagnetic microparticles to which the Ag-Ab complex

binds and remains attached to the walls of reaction vessel during the washing. The luminescence given out due to the reaction is enhanced by the Acid and Base reagent. The concentration of analyte is analyzed based on the intensity of the luminescence.

TSH assay is a two step immunoassay using direct chemiluminescent technology which uses constant amounts of two antibodies. The first antibody in the lite reagent is a monoclonal mouse anti-TSH antibody labeled with acridinium ester. The second antibody, in the solid phase is a polyclonal sheep anti-TSH antibody which is covalently coupled to paramagnetic particles. A direct relationship exists between the amount of TSH present in the patient sample and the amount of relative light units (RLUs) detected by the system.

The Advia Centaur is a fully automated, random access, instrument that features on-board storage of reagent packs in a refrigerated compartment; sample syringe arrangement probe tip for level sense detection, sample and reagent delivery, mixing, and probe cleaning to minimize carryover; barcode identification of specimens and reagent packs; temperature controlled reaction reactions; and measurement and analysis of the light signal generated by the chemiluminescent reaction (RLU).

Reference Range 0.30-5.5 μ IU/ml

11. T_3

Principle: The Advia Centaur (BAYER, USA) Total T_3 assay is a competitive binding immunoenzymatic assay. Sample is added to a reaction vessel with a stripping agent to dissociate T_3 from the binding proteins. T_3 in the sample competes with the T_3 analogue coupled to biotin for anti- T_3 alkaline phosphatase conjugate. Of the resulting antigen: antibody complexes, the T_3 analogue: antibody complexes are bound to the streptavidin coated solid phase. Separation in a magnetic field and washing removes the sample T_3 : antibody complexes and other materials not bound to the solid phase. Then, the chemiluminescent substrate Lumi-PhosTM 530 is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of total T_3 in the sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve.

Reference range 60- 200 ng/dl

12. T_4

Principle: Advia Centaur (BAYER, USA) works on the principle of chemiluminescence (CLIA). Antigen (Ag) present in the serum sample binds to the antibodies (Ab) present in the

reagent. The reagent also has the paramagnetic microparticles to which the Ag-Ab complex binds and remains attached to the walls of reaction vessel during the washing. The luminescence given out due to the reaction is enhanced by the Acid and Base reagent. The concentration of analyte is analyzed based on the intensity of the luminescence.

T₄ assay is a competitive immunoassay using direct chemiluminescent technology. T₄ in the patient sample competes with T₄, which is covalently coupled to paramagnetic particles in the Solid phase, for a limited amount of acridinium ester labeled monoclonal mouse anti-T₄ antibody in the Lite reagent. An inverse relationship exists between the amount of T₄ present in the patient sample and the amount of relative light units detected by the system.

Reference range 4.5 - 12.0 µg/dl

13. Alkaline Phosphatase

In the presence of magnesium ions, *p*-nitrophenylphosphate is hydrolyzed by phosphatases to phosphate and *p*-nitrophenol. The rate of *p*-nitrophenol liberation is proportional to the ALP activity and can be measured photometrically. Increased ALP activity is associated with two groups of diseases: those affecting liver function and those involving osteoblastic activity in the bones.

Reference range Male: 53-128 U/l and Female: 42-98 U/l

14. Total/ Direct Bilirubin

Technology used for estimation is Diazo method of Pearlman and Lee, endpoint. Total bilirubin is coupled with diazonium salt DPD (2,5-dichlorophenyldiazonium tetrafluoroborate) in a strongly acidic medium (pH 1-2). The intensity of the color of the azobilirubin produced is proportional to the total bilirubin concentration and can be measured photometrically. Bilirubin is an organic compound formed by the reticuloendothelial system during the normal and abnormal destruction of red blood cells.

Reference range

Total Bilirubin- 0.3-1.20 mg/dL

Direct Bilirubin- 0-0.2 mg/dL

15. Indirect Bilirubin

Indirect Bilirubin is derived from serum total and direct Bilirubin values

Reference range 0-0.9 mg/dL

16. Aspartate Aminotransferase (AST) / Serum Glutamate Oxaloacetate Transaminase (SGOT)

SGOT is estimated using Audit kit by Photometry method through the instruments- Olympus AU2700 & Advia 1800

Principle: SGOT is an aminotransferase, a group of enzymes which catalyse the reversible transformation of alpha-keto acids into amino acids by transfer of amino groups. SGOT transfers the amino group from aspartate to 2-oxoglutarate to form pyruvate and glutamate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of SGOT. The pyruvate enters a lactate dehydrogenase (LDH) catalysed reaction with NADH to produce lactate and NAD⁺. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the SGOT activity in the sample.

Reference range Male: 0-37 U/l and Female: 0-31 U/l

17. Alanine Transaminase (ALT) / Serum Glutamate Pyruvate Transaminase (SGPT)

SGPT is estimated using Audit kit by Photometry method through the instruments- Olympus AU2700 & Advia 1800. As a group, the transaminases catalyze the interconversion of amino acids and α -keto acids by transferring the amino groups.

Principle: SGPT is an aminotransferase, a group of enzymes which catalyse the reversible transformation of alpha-keto acids into amino acids by transfer of amino groups. SGPT transfers the amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of SGPT. The pyruvate enters a lactate dehydrogenase (LDH) catalysed reaction with NADH to produce lactate and NAD⁺. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the SGPT activity in the sample.

Reference range Male: 13-40 U/l and Female: 10-28 U/l

18. Gamma Glutamyl Transferase (GGT)

It is a rate method, in which L- γ -glutamyl-3-carboxy-4-nitroanilide is used as a substrate and glycylglycine as a acceptor. The rate at which 5-amino-2-nitrobenzoate is liberated is proportional to γ -GT activity and is measured by an increase in absorbance. γ -GT measurement is principally used to diagnose and monitor hepatobiliary disease. It is currently

the most sensitive enzymatic indicator of liver disease, with normal values rarely found in the presence of hepatic disease. It is also used as a sensitive screening test for occult alcoholism.

Reference range Male: 0-55 U/l and Female: 0-38 U/l

19. Total Protein

In alkaline solution, a colored chelate forms between cupric ions and compounds containing at least two $-\text{CONH}_2$, $-\text{CSNH}_2$, $-\text{CH}_2\text{NH}_2$ or similar groups, joined directly or through a carbon or nitrogen atom. In proteins, the chelate is formed between one cupric ion and about six nearby peptide bonds. The intensity of the color is proportional to the total number of peptide bonds undergoing reaction and thus to the total amount of protein present. This is similar to the biuret reaction. Although compounds undergoing the biuret reaction give colors ranging from pink to purple, the violet colors given by serum albumins and globulins are essentially the same. Total protein measurements are used in the diagnosis and treatment of a variety of diseases involving the liver, kidney, or bone marrow, as well as other metabolic or nutritional disorders

Reference range 6.6-8.3 gm/dL

20. Serum Albumin

Albumin constitutes about 60% of the total serum protein in normal, healthy individuals. At the reaction pH, the bromocresol purple (BCP) in the Roche Diagnostics (RD) albumin system reagent binds selectively with albumin. This reaction is based on a modification of a method described by Doumas (4). Although BCP is structurally similar to the conventional bromocresol green (BCG), its pH color change interval is higher (5.2–6.8) than the color change interval for BCG (3.8–5.4), thus reducing the number of weak electrostatic dye/protein interactions. The BCP system eliminates many of the nonspecific reactions with other serum proteins as a result of the increased pH. Albumin measurements are used in the diagnosis and treatment of numerous diseases primarily involving the liver or kidneys.

Reference range 3.5-5.2 gm/dL

21. Serum Albumin/ Globulin Ratio

The values are derived from serum albumin and protein values. The difference between total protein and albumin gives the content of globulins. To calculate albumin/globulin ratio divide the amount of albumin that by globulin.

Reference range 0.9-2.0

22. Serum Calcium

Detection technology for the estimation of serum calcium used is Photometry and Ion Selective Electrode. Total serum calcium is composed of three fractions: free or ionised calcium, 50%; protein bound calcium most of which is bound to albumin with only a small portion bound to globulins, 45%; and complex-bound calcium, mainly to phosphate, citrate, and bicarbonate, 5%. The ionized calcium is physiologically most significant, but has proven difficult to assay directly. It may be estimated from total calcium given knowledge of the protein content and pH of the blood, which strongly affect the level of ionised calcium. Calcium ions are important in the transmission of nerve impulses, as a cofactor in several enzyme reactions, in the maintenance of normal muscle contractility, and in the process of coagulation. A significant reduction in calcium ion concentration results in muscle tetany. A higher than normal concentration of calcium ions produces lowered neuromuscular excitability and muscle weakness along with other more complex symptoms.

Principle: It works on the principle of Beer's law and Lambert's law. Under suitable conditions, the amount of light absorbed by a coloured solution, when illuminated with a light of suitable wavelength, is directly proportional to the concentration of the coloured solution and the length of the light path through the solution. Therefore, the amount of light decreases exponentially with the increase in the concentration of the solution and with increase in thickness of the layer of solution through which light passes. Calcium ions form a coloured complex with Arsenazo III, which is measured at 658/694 nm. The amount of calcium present in the sample is directly proportional to the intensity of the coloured complex formed.



Reference range Adults : 8.4 - 10.6 mg/dl

23. Blood Urea Nitrogen (BUN)

Urea is synthesized in the liver from ammonia produced as a result of deamination of amino acids. This biosynthetic pathway is the human body's chief means of excreting surplus nitrogen. BUN measurements are used in the diagnosis of certain renal and metabolic diseases.

Reference range 7.2-20 mg/dL

24. Serum Creatinine

Principle: The creatinine (CREA) method is based on the reaction of picric acid with creatinine in an alkaline medium using Jaffe method. The creatinine reacts with the alkaline

picric acid and forms a colored complex. The rate of complex formation is measured at 505/571 nm and is proportional to the creatinine concentration.



Reference range Male: 0.6-1.1 mg% and Female: 0.5-0.8 mg%

25. Uric Acid

Uric acid is oxidized by the specific enzyme uricase to form allantoin and H_2O_2 . The H_2O_2 reacts with 2,4,6-tribromo-3-hydroxybenzoic acid (TBHB) and 4-aminophenazone in the presence of peroxidase to form quinone-imine dye and hydrogen bromide (HBr). The intensity of the red color is proportional to the uric acid concentration. Uric acid measurements are used in the diagnosis and treatment of numerous renal and metabolic disorders, including renal failure, gout, leukemia, psoriasis, starvation

Reference range Male: 3.5-7.2 mg/dL and Female: 2.6-6.0 mg/dL

26. BUN/ Sr Creatinine ratio

This is derived from serum BUN and creatinine values

Reference range 9:1- 23:1

STATISTICAL ANALYSIS

- The data was entered into Microsoft excel 2007 and verified.
- Statistical analysis was performed using Epi Info 7 and SPSS 20.
- Data has been described using descriptive statistics (mean and standard deviations) for continuous variables.
- Data has also been depicted using proportions i.e. percentages in case of categorical variables and prevalence rates for continuous variables using well defined cut off points.
- Wherever relevant, the 95% confidence intervals in which the means and proportions lie were calculated.
- The independent student's 't' test and Chi-square test were performed to compare continuous and categorical variables respectively.
- Univariate odds ratio analysis has been performed to assess the risk burden of a particular risk factor and has been depicted with 95% confidence intervals.
- In multivariate analysis, stepwise forward linear regression was applied to find out the variables that contributed to significant amount of variations on different dependent variables.
- One way analysis of variance (ANOVA) has been computed to find out the difference between baseline characteristics within groups of intervention trial.
- Paired 't' test has been used to compare the difference between the pre and post intervention values of the outcome variables in the intervention phase.
- Stratified analysis would be done to rule out residual confounding due to covariate imbalance.
- All statistical analyses were considered significant at $p < 0.05$ level.

DATA MONITORING AND MANAGEMENT

For any research it is very important to collect the data, monitor it periodically and manage it properly once collected. The storage of the data is equally crucial for its easy accessibility to all the investigators involved in the study and for ready reference. Also doing the analysis with appropriate software, applying the correct technique keeping the objectives in mind is required. Hence for the present research the following checkpoints as given in Table 3.11 were jotted down-

Table 3.11 Checkpoints for Data Monitoring and Management

Data Collection	Through pretested questionnaires and appropriate tools by the Investigator
Blood Collection	By Trained Technician of accredited laboratory
Supplementation	Delivered by the researcher at the start of every week
Compliance	<ul style="list-style-type: none"> • Telephonic calls made in-between and at the end every week. • The empty sachets were collected back at the end of the supplementation period and preserved by the researcher.
Data handling	Safe and confidential storage at central level.
Data entry	Microsoft Excel
Data Analysis	Using Microsoft Excel 2007, SPSS (version 20) and Epi Info (version 7) by applying appropriate statistical techniques.