Chapter 3

Methods and Materials

Menopause, a natural phase in the life cycle of women, can increase the risk of non-communicable diseases among women. If the risk factors for NCDs are present at the onset of menopause, then it can worsen the condition. The increasing prevalence of mortality and morbidity due to NCDs among women is of grave concern and needs to be addressed exclusively**.** Early lifestyle management can prove an important step in reducing the risk of NCDs among women. Hence the research was planned and executed primarily to assess the nutritional discrepancies and prevalence of various risk factors of NCDs in pre and post-menopausal women. Subsequently the management of NCD risk factors was focussed using flaxseed as a functional food. The research plan to furnish these aims was divided into three phases as described below:

PHASES IN THE STUDY

PHASE I: Nutritional status of the pre and post-menopausal women (30-60y) of urban Vadodara

PHASE II: Identification of flaxseed variety for supplementation and estimation of its nutritive profile

PHASE III: Metabolic and inflammatory response to supplementation of whole roasted flaxseeds in pre-menopausal overweight/obese female subjects

PHASE I

3.1 NUTRITIONAL STATUS OF THE PRE AND POST-MENOPAUSAL WOMEN (30-60Y) OF URBAN VADODARA

A formative research to assess the nutritional status of the adult female population (30-60y) of urban Vadodara was performed with a focus on their menopausal status. The details of the experimental plan are depicted in Figure 3.1.

3.1.1 Study design

The study design was a cross sectional cum factorial design. To obtain a representative sample zonal distribution provided by Vadodara Urban Development Authority was used. According to this distribution urban Vadodara is divided into five zones i.e. east, west, central, north and south zone comprising 12 wards. Each zone is further distributed into two or three ward average. Two societies from each zone (one society from each ward except for west and east zone which have three wards each) was purposively selected for the study. Female subjects of 30-60 years of age were enrolled from the societies through snowball effect. Total 408 females were enrolled from which the data regarding general information, anthropometry and menopausal status was collected (Appendix I). As the primary objective of the study was to assess the nutritional status of the subjects in relation to their menopause status and obesity, the cases of pregnancy, peri-menopause, hysterectomy and underweight were excluded. Menopause was defined to the screened subjects as cessation on menstrual bleeding for more than one year (Harlow and Paramsothy, 2011).

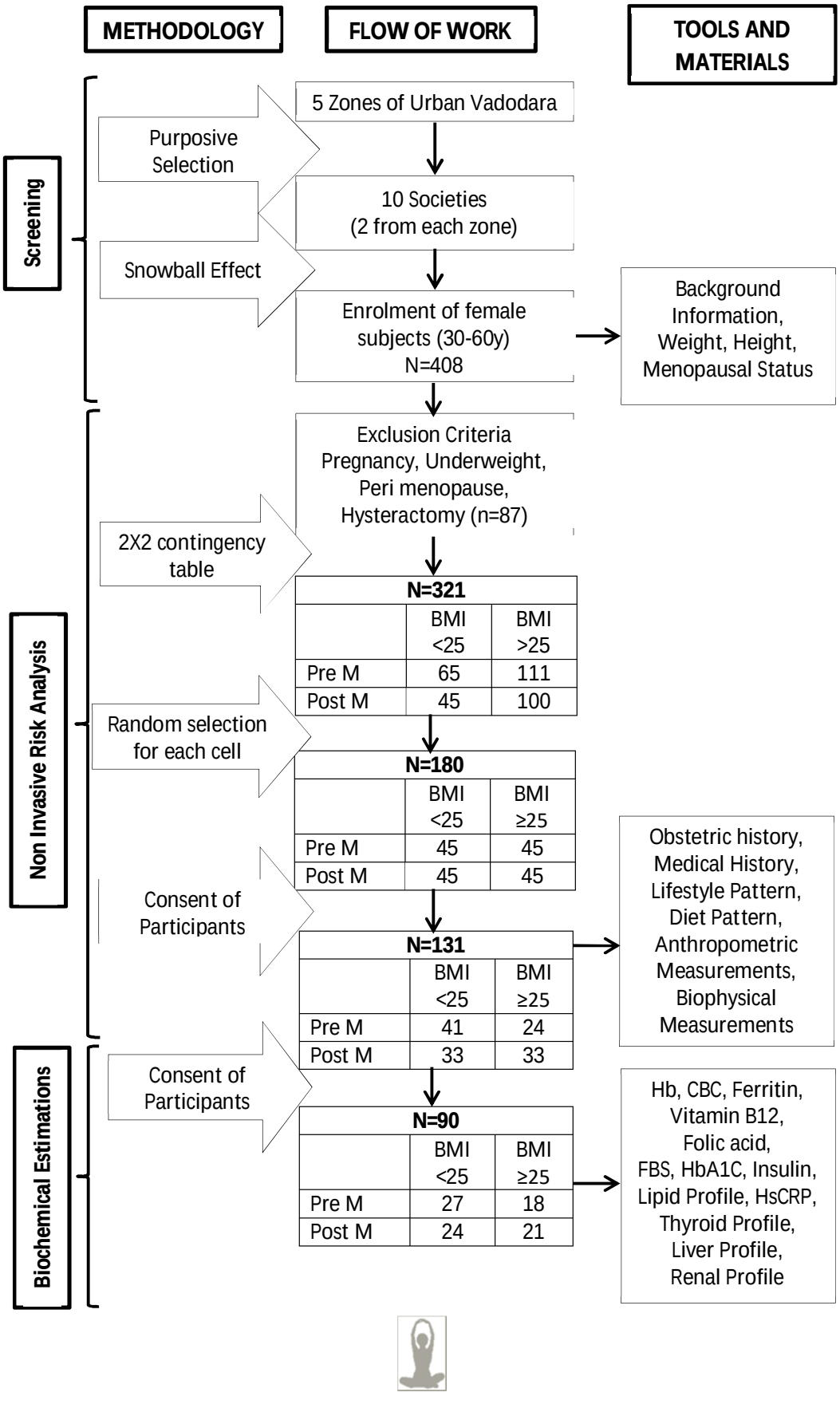
A factorial design was applied and the subjects were categorized into 2X2 contingency table (for menopause and BMI) to obtain a substantial number of subjects in each cell for statistical analysis later on. Further 45 subjects were randomly selected from each cell for detailed risk analysis of cardio metabolic diseases. The subjects were contacted to obtain consent for participation in the study and 131 subjects out of 180 gave the consent to participate in non-invasive risk analysis and 90 consented for biochemical estimations in addition to the non-invasive risk analysis. The details of the distribution of consented subjects for menopause and obesity have been provided in Figure 3.1.

3.1.2 Ethical clearance and considerations

The study was approved by the Institutional Ethics Committee for Human Research of Faculty of Family and Community Sciences, M.S. University, Baroda (No.: IECHR/2012/19). Prior written consent (Appendix IV) was taken from each participant through consent form in English or local language.

FIGURE 3.1: EXPERIMENTAL DESIGN

PHASE I



3.1.3 Data acquisition process

For the non-invasive risk analysis a prior appointment from the subjects was taken according to their convenience and the data regarding obstetric history, family and medical history, life style pattern, dietary habits, health seeking practices, physical activity, food frequency and 24 hour dietary recall was collected (Appendix II). The anthropometric measurements, blood pressure and body fat were also measured during this appointment. A next appointment was scheduled with the subjects who gave consent for biochemical estimations. They were asked to have 10-12 hour fasting before the withdrawal of blood sample. A trained lab technician was accompanied along with the researcher on the day of appointment and fasting blood was withdrawn. A disposable syringe was used for blood withdrawal at each time. The blood sample was appropriately labelled, stored at low temperature and transported to the laboratory for analysis. The biochemical parameters analysed during this phase of study were: Hemoglobin, Complete Blood Count, Ferritin, Vitamin B12, Folic Acid, Lipid Profile, Fasting Blood Glucose, Glycated haemoglobin, Insulin, Hs-CRP, Thyroid Profile, Liver function test and Kidney function test.

PHASE II

3.2 IDENTIFICATION OF FLAXSEED VARIETY FOR SUPPLEMENTATION AND ESTIMATION OF ITS NUTRITIVE PROFILE

3.2.1 Identification of flaxseed variety

PKV-NL 260 variety of the flaxseed was selected to use for the supplementation which was developed by BAIF Development Research Foundation, Pune in partnership with Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Bharati Vidyapeeth University, Pune and Ensigns Diet Care Private Limited under the project "A Value Chain on Linseed: Processing and Value Addition for Profitability - Production to Consumption System (PCS)" as a part of National Agricultural Innovation Project- Component 2 (Research on Production to Consumption Systems) funded by Indian Council of Agriculture Research. The details of the PKV-NL 260 flaxseed variety are provided in table 3.1.

TABLE 3.1: TAXONOMY OF PKV-NL 260 VARIETY OF FLAXSEED

Year of Identification	2009
Parent	R-552 x RLC-6
Breeding Method	Pedigree
Recommended ecology	Suitable for Rabi season : Rainfed – Last week of October, Irrigated – First week of November
Plant Height	45-63 cm
Varietal Character	Flower Blue colour
Duration	Average 105 days (97-111days)
Maturity Group	Early
Reaction of Major Pest/Diseases	Moderately resistant to alternaria, powdery mildew and budfly.
Seed Colour	Light Brown
Spacing	30 cm row to row distance
Plant Population	7-8 lakh per hectare
Fertilizer Dose	25:25:00 NPK kg/ha
Seed Rate	25 kg/ha
1000 Seed Wt.	7.6 gm
Oil Percentage	38 %
Average Yield	Average 963 kg/ha (900-1800 kg/ha)

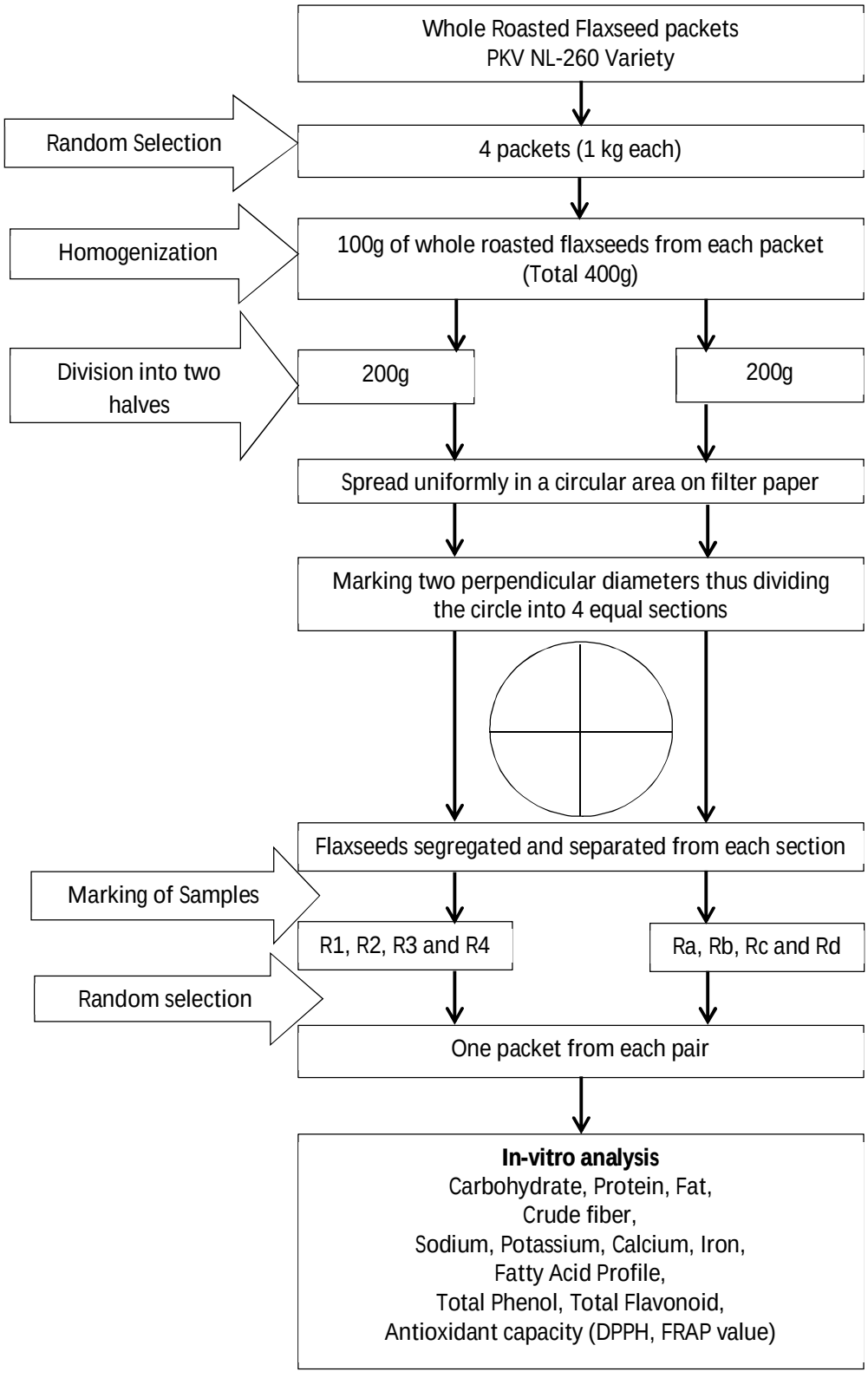
Source: National Agricultural Innovative Project, 2013

3.2.2 Estimation of Nutritive profile of flaxseeds

Out of total 32 packets (1kg each) of whole roasted flaxseeds 4 packets were randomly selected. 100g of flaxseeds from each packet were drawn and homogenized by mixing and making the sample amount to 400g. Further the flaxseeds were divided into two halves (200g each). Each part was spread uniformly in a circular area on filter paper. Two perpendicular diameters were marked on the filter paper thus dividing the circle into 4 equal sections. Flaxseeds were further segregated and separated from each section and marked. Samples of first half were marked as R1, R2, R3, R4 and samples of second half were marked as Ra, Rb, Rc, Rd. the details of sampling procedure of flaxseeds is provided in figure 3.2. The parameters for nutritive value evaluation included carbohydrates, fats, proteins, crude fiber, sodium, potassium, calcium, iron, fatty acid profile, total phenols, flavonoids and antioxidant capacity using DPPH and FRAP methods.

FIGURE 3.2: EXPERIMENTAL DESIGN

PHASE II



PHASE III

3.3 METABOLIC AND INFLAMMATORY RESPONSE TO SUPPLEMENTATION OF WHOLE ROASTED FLAXSEEDS IN PRE-MENOPAUSAL OVERWEIGHT/OBESE FEMALE SUBJECTS

3.3.1 Study Design

An open labelled parallel randomised controlled trial was performed to assess the impact of whole roasted flaxseeds on the lipid profile, insulin resistance and inflammation of premenopausal overweight/obese females. In the study the control arm was not provided with any kind of supplementation whereas group I was supplemented with 5g and group II was supplemented with 10g of roasted flaxseeds during the study period. Biochemical and anthropometric data was collected before and after the intervention.

3.3.2 Sample Size Estimation

For the sample size calculation total cholesterol was taken as the primary outcome. About 15% change (28mg/dl) in TC levels was anticipated. The standard deviation for total cholesterol of pre-menopausal women (34mg/dl) of Vadodara was taken from the data of Phase I of the study. Power was set as 80% with level of significance as <0.05 (two tailed). The sample size of 25 in each arm was computed using above details. Further attrition rate was taken as 20% of each arm which finally derived a sample size of 90 (30 in each arm) (Schoenfeld, 2013).

3.3.3 Ethical clearance and considerations:

The study was approved by the Institutional Ethics Committee for Human Research of Faculty of Family and Community Sciences, M.S. University, Baroda (No.: IECHR/2013/7). Prior written consent was taken from each participant through consent form in the local language (Appendix V).

The phase was divided into two parts as follows:

PHASE III (a): Screening and collection of baseline data

3.3.4 Data acquisition process

To identify the subjects for supplementation a screening of adult females of 30-50y was performed. For this purpose one society form urban Vadodara was purposively selected and nearby societies in a concentric manner were purposively selected. Health camps were organized at common plots or central area of the societies after seeking permission from the head. Camps were held during the evening and the data regarding medical history, menopausal history, life style pattern, frequency of consumption of n-3/ALA rich foods, physical activity pattern, anthropometry, blood pressure was collected during the camp (Appendix III). The enrolled subjects were asked to be in fasting state after night's dinner and come in the morning for fasting blood sugar testing. Digital blood sugar monitoring device was used to assess the fasting blood glucose. Alcohol swabs were used to clean the tip of finger and disposable lancets were used to draw blood.

The enrolled subjects (N=400) were further screened for identification of eligible subjects using inclusion and exclusion criteria for the study (Table 3.2).

TABLE 3.2: INCLUSION AND EXCLUSION CRITERIA FOR SUPPLEMENTATION STUDY

Inclusion Criteria	Exclusion Criteria
• Females (20-50 Years) • BMI: 23-29.9 • Pre menopause	• History Of Chronic Illnesses • Allergy to flaxseeds • Diabetes • Smoking or tobacco chewing • Pregnancy • Vigorous physical activity • Peri and Post menopause • Hysterectomy • Currently (For last 4 weeks) taking n-3 rich foods or supplements • Rapid or dramatic weight gain or loss in last year

A second appointment was scheduled with the subjects who were found eligible (n=151) for the supplementation trial. A detailed description was provided to them regarding the supplementation. They were informed about the possible benefits and

risks of consuming flaxseeds. The study protocol was briefed to them that if they give consent they will be randomly placed into any of the three groups and will be asked to consume specified doses of flaxseeds or will have to maintain their normal diet pattern without consuming flaxseeds for 8 weeks depending upon the group in which they have been selected. Information regarding biochemical estimations and anthropometric, biophysical measurements to be performed twice i.e. at the beginning and end of the supplementation was shared. Details were provided regarding the financial standings, biochemical tests to be included in the study, safety standards to be followed while withdrawing the blood, provision of availability of results, confidentiality and right to withdraw from the study at any point of time.

A third appointment for baseline biochemical estimation and anthropometric assessment was scheduled for the females who gave consent for participation in the study. Subjects were asked to have 10-12 hour fasting before the withdrawal of blood sample. A trained lab technician was accompanied along with the researcher on the day of appointment and fasting blood was withdrawn. A disposable syringe was used for blood withdrawal at each time. The blood sample was appropriately labelled, stored at low temperature and transported to the laboratory for analysis. Anthropometric and biophysical measurements were collected by the researcher. Data regarding 24 hour diet pattern was also collected from the subjects (Figure 3.3).

PHASE III (b): Randomised control trial to study the efficacy of whole roasted flaxseeds on lipid profile, insulin resistance and inflammation in pre-menopausal overweight/obese females

The subjects whose baseline data was collected were randomly divided into three group i.e. experimental group I, experimental group II and control group (Figure 3.4).

3.3.5 Doses of the flaxseeds

Experimental group I was asked to consume 5g of roasted flaxseeds and group II was asked to consume 10g of roasted flaxseeds for a period of 8 weeks. The control group was advised not to consume flaxseeds during the study period. The details of doses and the Alpha Lenolenic Acid (ALA) present in each dose are given in table 3.3.

TABLE 3.3: ALA CONTENT OF TWO DOSES OF FLAXSEED SUPPLEMENTATION

Control Group	Experimental Group I 5 g Flaxseeds	Experimental Group II 10 g Flaxseeds
--	1.17g ALA	2.34g ALA
No ALA supplementation	ALA provided by flaxseeds equivalent to RDA by IOM 1.1g/day (IOM, 2002)	ALA provided by flaxseeds equivalent to recommended average intake of ALA for Cardio-protective effect i.e. 1.5-3g/day established by AHA (Kris-Etherton et al, 2002)

3.3.6 Protocol for consumption of flaxseeds

The group I was asked to consume flaxseeds in one dose at any time of period during a day. As the consumption of 10g of flaxseeds was not feasible, group II was instructed to consume them into two divided doses in a day. Direction regarding proper chewing of flaxseeds was imparted to the subjects.

3.3.7 Protocol for distribution of flaxseeds

Sachet of 5g and 10g were made for distribution and every week 7 air tight sachet in a pouch were given to the subjects. For this purpose standardization of two test tubes was done to measure 5g and 10g of flaxseeds and marked properly. These test tubes were used every time to make the sachets.

3.3.8 Monitoring, compliance and drop outs

Weekly monitoring was done for the consumption of flaxseeds by the subjects. They were asked to return the empty sachet and any kind of side effects faced. A compliance sheet was also provided to the subjects to mark it daily after consuming flaxseeds (Appendix VI). The compliance of flaxseeds consumption was 100% among the subjects who completed the study protocol. There were total 4 drop outs during the study period out of which 2 were from experimental group I, 1 from experimental group II and 1 from control group. All the drop outs were due to non-compliance of study protocol.

After 8 week of supplementation data regarding biochemical estimations and anthropometric, biophysical measurements was collected following same protocol as for baseline data collection.

FIGURE 3.3: EXPERIMENTAL DESIGN

PHASE III (a)

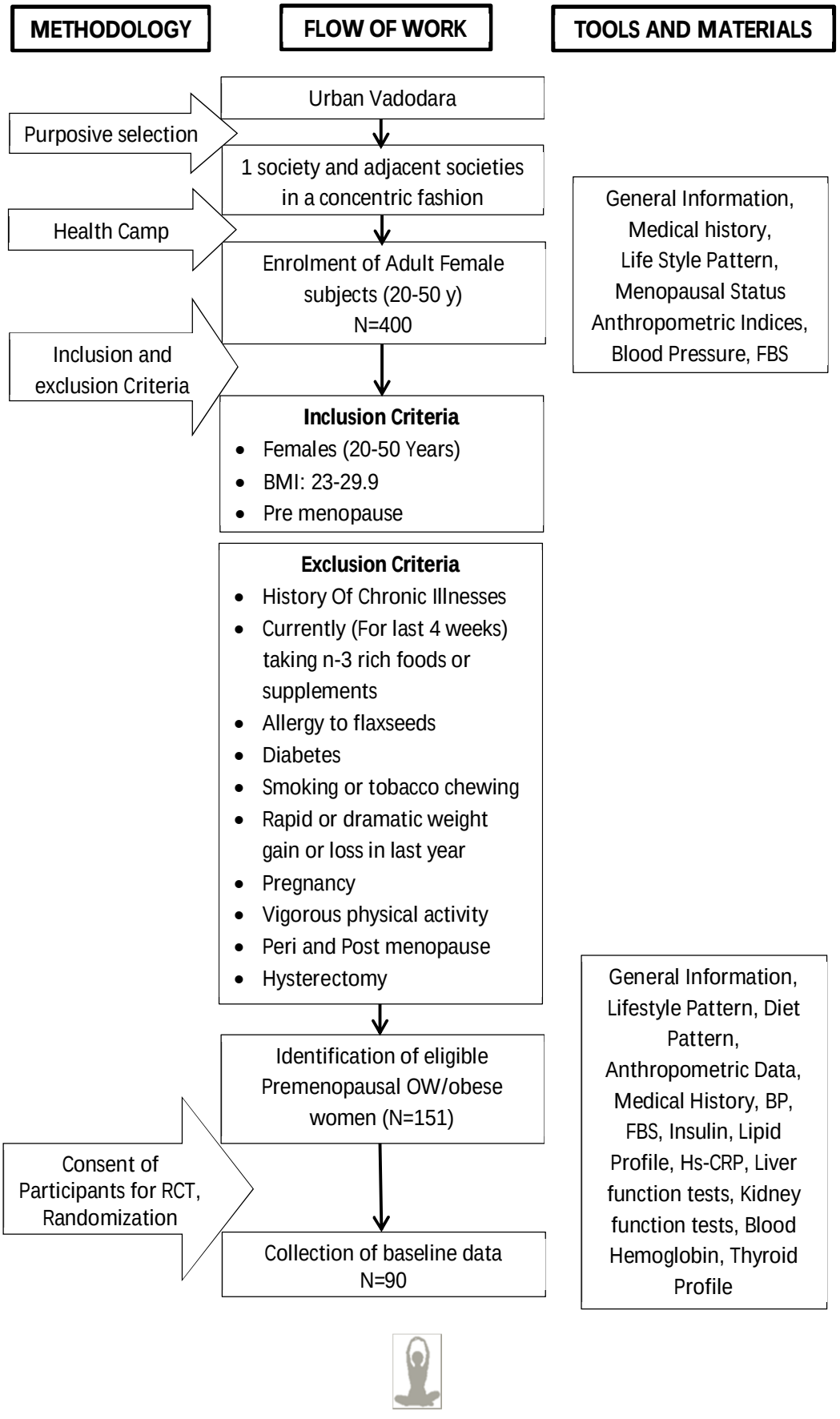


FIGURE 3.4: EXPERIMENTAL DESIGN

PHASE III (b)

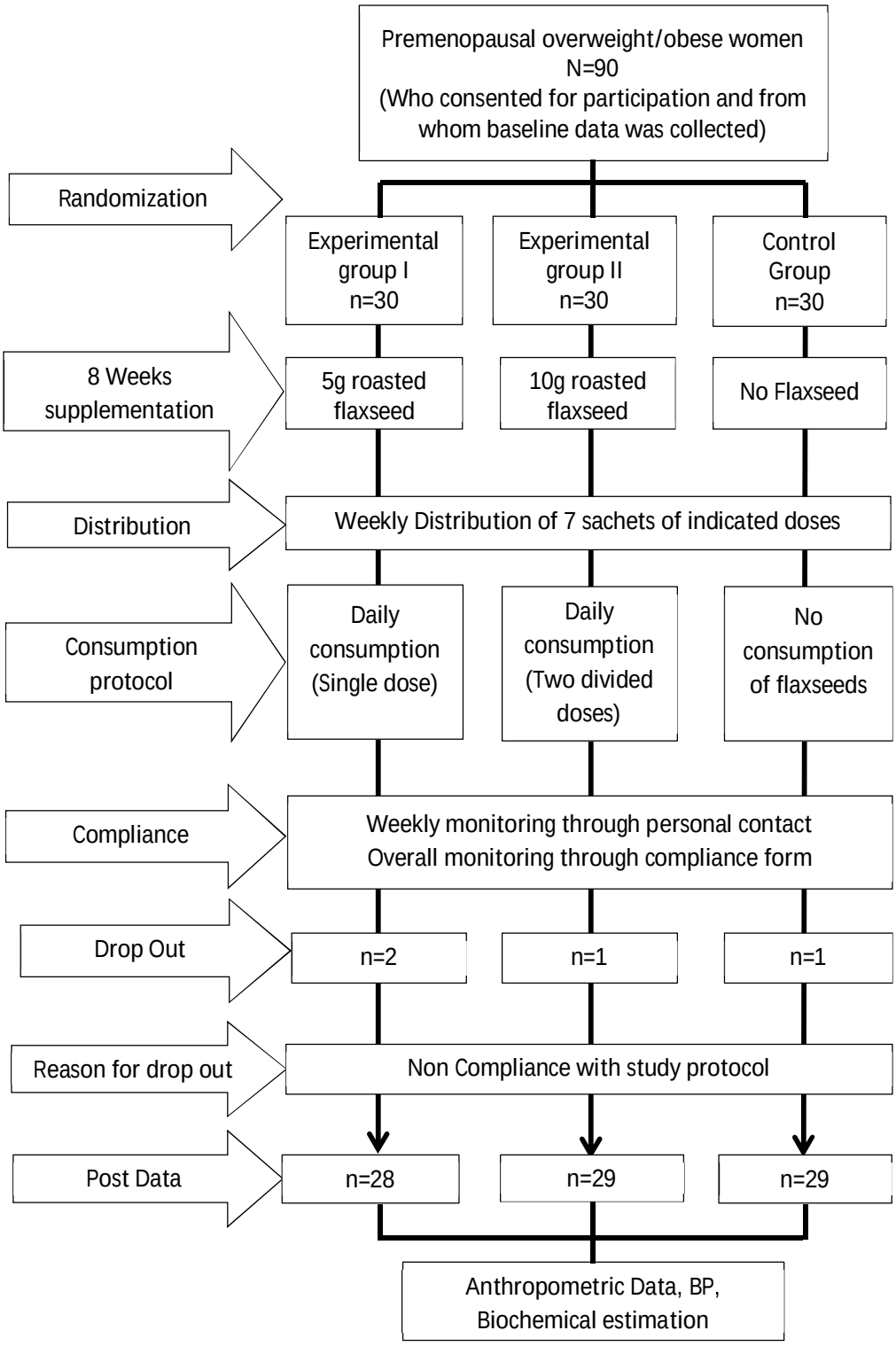


TABLE 3.4: METHODS AND TOOLS USED FOR DATA COLLECTION

Parameters	Techniques
Medical and Life Style History	
General Information, Medical history, Family History, Lifestyle variables, health seeking practices, obstetric history	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
WHR, WSR	Derived from WC, HC and height
Biophysical Measurements	
Blood Pressure	Mercury sphygmomanometer
Body Fat Analysis	Omron Body Fat Analyzer (HBF 306)
Nutritional Anemia	
Haemoglobin	Cyanmethemoglobin method
CBC	Differential Analyzer
Iron, Total iron binding capacity	Spectrophotometry
% Transferrin saturation	Calculated from iron and TIBC
Ferritin, Vitamin B12 and Folic acid	Chemiluminescence immune assay
Glucose Metabolism	
Fasting Blood Sugar	Enzymatic kit method
HbA1C (Glycated hemoglobin)	High Pressure Liquid Chromatography
Insulin	Solid phase radio immune assay
HOMA-IR, %β, %S	Derived from fasting glucose, insulin
Average blood glucose	Derived from HbA1c
Lipid Metabolism	
TC, HDL-C, TG	Enzymatic kit method
LDL-C	Derived from TC and TG
VLDL-C	Derived from TG
TC/HDL-C, LDL-C/HDL-C, TG/HDL-C, AIP	Derived from TC, TG, HDL-C and LDL-C
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, Serum calcium	Photometry

Parameters	Techniques
Liver Function Test	
Total Bilirubin, Direct Bilirubin, Indirect Bilirubin, SGOT, SGPT, GGT, serum Albumin, Total Protein	Photometry
Flaxseed Analysis	
Fatty Acid Profile	GC-MS
CHO, Protein, Fats	AOAC 2005 method
Crude fiber	Oxidative hydrolytic degradation
Antioxidant capacity	DPPH, FRAP
Total phenols	Folin–Ciocalteau colorimetric method
Flavonoids	Aluminium chloride colorimetric method
Minerals (Ca, Fe, Na, K)	ICP-OES

3.4 METHODS, TOOLS AND TECHNIQUES

3.4.1 BACKGROUND INFORMATION

3.4.1.1 Medical, life style and dietary history

General information regarding age, address, contact number, religion, type of family, educational level, per capita income, marital status, occupation, reproductive history, family and medical history, gastrointestinal ailments, nutritional deficiency symptoms, life style pattern, health seeking practices and dietary habits was collected using a pre tested semi-structured questionnaire by the researcher (Appendix II). Data was obtained using English, Hindi or Gujarati language.

Reproductive history included questions related to menarche, menopause, pregnancy, number of children, abortions and still births. Family and medical history was based on major non-communicable diseases and their metabolic risk factors. Data regarding common gastric ailments like gastritis, acidity, constipation and nutritional symptoms like fatigue, headache, loss of appetite, breathlessness, paleness, cramps/muscle weakness, numbness in feet, sudden weight gain or loss was collected. Life style pattern included habits regarding smoking, tobacco chewing, alcohol intake and sleep pattern. Questions regarding frequency of health check-up, awareness and practices regarding breast examination, breast cancer screening, use of hormonal replacement therapy, reverse osmosis water and nutritional supplements were asked to assess their health care practices. Dietary habits included questions related to vegetarianism, type of milk consumed, refrying of oil, use of single or multiple oils, total consumption of oil, sugar and salt in the household.

3.4.1.2 Physical activity

'International Physical activity Questionnaire (Short), 2002 was used to assess the physical activity pattern of the subjects. IPAQ short form is an effective tool for population surveillance of physical activity among adults (15-69 years). Three major components included in the IPAQ short form questionnaire are: walking, moderate-intensity and vigorous-intensity activity. Duration and frequency for these three

components were asked from the subjects and physical activity levels were calculated based on summation of all three.

MET values used for the analysis of IPAQ data were:

- Walking = 3.3 METs
- Moderate PA = 4.0 METs
- Vigorous PA = 8.0 METs

Total MET values for a week were calculated using the below mentioned formulas:

- Walking MET-minutes/week = 3.3 * walking minutes * walking days
- Moderate MET-minutes/week = 4.0 * moderate-intensity activity minutes * moderate days
- Vigorous MET-minutes/week = 8.0 * vigorous-intensity activity minutes * vigorous-intensity days

Total physical activity MET-minutes/week = sum of Walking + Moderate + Vigorous MET minutes/week scores.

Based on total MET values of a week, the activity level of the person was computed 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.

3.4.1.3 Food frequency

A semi-structured food frequency questionnaire was prepared for foods rich in trans fats, vitamin B12 and folic acid. The frequency of consumption was obtained as per daily, 3-4 times a week, weekly, fortnightly, monthly or occasionally basis (Appendix II) and the amount consumed per serving was noted. Further the data was

segregated into high frequency, moderate frequency and low frequency of consumption for the analysis purpose as below:

- Daily consumption or 3-4 times a week- High Frequency
- Weekly or fortnightly- Moderate frequency
- Monthly or occasionally- Low frequency

3.4.1.4. 24 hour dietary recall

The 24-h dietary recall is a quantitative method for measurement of food intake over a period of last 24 hours or one day (Appendix II). Subjects were asked to recall the type and amount of food consumed during major meals and snacks on previous day. Recall of any holiday/Sunday/festival was avoided. Standard measuring cups and spoons were used to note exact quantities of the food items consumed. In the end subjects were probed to recollect any beverage, fruit, snack sweets, pickle, papad etc. consumed in between the whole day which would have left out unintentionally. Later on the cooked volumes were converted into raw food items using standardized regional recipes. These values were entered into Diet Soft Software (Dietitian Gurdeep Kaur, AIIMS, 2007) to calculate the amount of various nutrients consumed by the person in a day.

3.4.2 ANTHROPOMETRIC MEASUREMENTS AND INDICES

Anthropometric measurements weight, height, waist circumference and hip circumference were directly measured from the subjects whereas indices like body mass index (BMI), waist hip ratio (WHR) and waist stature ratio were calculated using standard formulas.

3.4.2.1. Weight

Salter scale, which is portable digital weighing scale of research grade, was used to measure weight of the subjects. It was standardized using a standard 5kg weight and calibrated regularly. During measurement of body weight, the weighting scale was placed on an even flat surface. Subjects were asked to remove their footwear or

woollens (during winters) and stand straight, looking straight ahead and without touching or holding anything. The readings were recorded to the nearest 0.1kg.

3.4.2.2. Height

A non-stretchable fiberglass tape was vertically fixed on the wall for measuring the height. The subjects were asked to stand barefoot on the floor against the fiberglass tape with the feet parallel and heels placed together with the medial (inner) border of the feet at an angle of 60 degrees. It was ensured that the scapula and the buttock were in contact with the measuring wall. The subjects were asked to look straight ahead with the head held comfortably erect and the arms hanging loosely by the sides. A straight head piece was used which slightly crushed the hair to make contact with the top of the head. In this position, a mark was made on the wall and height was recorded with a measuring tape. The reading was recorded to the nearest 0.1cm.

3.4.2.3 Waist circumference

Waist circumference is an easy, inexpensive tool to measure abdominal obesity. The A non-stretchable fiberglass tape was used and standard protocol developed by National Health lung and blood Institute (2000) was followed for measuring waist circumference. The participants were asked to stand with their feet together and arms placed on the sides. The midpoint between the inferior margin of the last rib and the crest of the ilium in the mid auxiliary plane was identified. Before reading the tape measure it was ensured that the tape is snug, but does not compress the skin, and is parallel to the floor. The measurement was made at the end of a normal expiration to the nearest 0.1cm. Classification for waist circumference of adult females is given in Table 3.6.

3.4.2.4 Hip circumference

To measure the hip circumference the subjects were asked to stand straight and hip circumference was measured at the widest circumference over the buttocks and below the iliac crest. The measurement was recorded to the nearest 0.1cm.

3.4.2.5 Body Mass Index (BMI)

Body Mass Index is the most widely used anthropometric method to assess nutritional status of adults. It is a convenient and valid measure of adiposity. BMI was calculated using the standard formula:

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

Asia Pacific Classification (2004) of BMI was used to define overweight and obesity (Table 3.5).

TABLE 3.5: WHO ASIA PACIFIC CLASSIFICATION (2004) 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

3.4.2.6 Waist-hip Ratio (WHR)

Waist-Hip Ratio (WHR) is an important indicator of the risk of CVD (WHO, 2011). Classification for WHR of adult females is given in Table 3.6. WHR was calculated using the following formula:

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

3.4.2.7 Waist-stature ratio (WSR)

WSR is an important indicator of obesity which is significantly associated with all risk factors for obesity and metabolic syndrome and often shows better correlation than BMI (Ashwell and Hsieh, 2005). Classification for WSR of adult females is given in Table 3.6. WSR was calculated using the following formula:

WSR= Waist circumference (cm) / Height (cm)

TABLE 3.6: CUT OFFS OF ABDOMINAL OBESITY FOR WOMEN

Indices	Cut off value
Waist Circumference (WHO, 2000)	>80cm
WHR (WHO, 2000)	≥0.85
WSR (Ashwell and Hsieh, 2005)	>0.50

3.4.3 BIO-PHYSICAL MEASUREMENTS

3.4.3.1 Blood Pressure

Blood pressure measurements were taken after the subject rested quietly for at least 5 minutes. A cuff of suitable size was evenly applied to the exposed upper arm, with the bladder of the cuff positioned over the brachial artery. BP was measured in sitting position with support at back and arm rested on table/chair to bring it to heart level and bared without constrictive clothing. Subjects were asked not to talk or cross their legs while taking measurement. The cuff was inflated to 30 mmHg above the pressure at which the radial pulse disappears. Systolic BP was noted on appearance of the sound whereas for diastolic reading the disappearance of sound was used. The blood pressure was measured in right hand thrice with an interval of two minutes between every measurement using mercury sphygmomanometer. The column was read to nearest 2 mmHg. If initial readings were high, several further readings were taken after 5 minutes of rest (Pickering et al, 2005). JNC VIII classification (James et al, 2014) as described in table 3.7 was used to classify hypertensives.

TABLE 3.7: JNC VIII CLASSIFICATION 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

3.4.3.2 Body Fat Percentage

Body Fat Percentage is the total mass of fat tissue in the body denoted as a percentage of total body weight. It is an important indicator to assess the risk of NCDs like CVDs, hypertension and diabetes. Percentage body fat was calculated using the Omron body fat monitor (Model HBF-306). The instrument functions on the principal of bioelectrical impedance which is basically opposition to the flow of an electric current through body tissues. Personal data such as height, weight, age and gender were entered in the instrument for each analysis. Subject was asked to stand straight and hold the electrodes with hands upright at 90° angle to the body. The instruments provided the results within 7-10 seconds after pressing the start button which was noted down by the researcher. Classification for body fat percentage of adult females was used as provided in instrument manual (Table 3.8) as no standard values have yet been established.

TABLE 3.8: CUT-OFFS FOR PERCENT BODY FAT FOR WOMEN

Category	Percent body fat
Normal	15-25%
High risk	Over 30%

3.4.4 BIOCHEMICAL ASSAY METHODS AND INDICES

All the biochemical assays included in study were performed at an accredited laboratory of Vadodara "Thyrocare".

3.4.4.1 Total Cholesterol

Method: CHOD POD enzymatic colorimetric method

Principle: Cholesterol is measured enzymatically in serum or plasma in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reaction byproducts, H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a color. Absorbance is measured at 500 nm. The color intensity is proportional to cholesterol concentration (NHANES 2003-2004).

3.4.4.2 Triglycerides

Method: GPO enzymatic colorimetric method

Principle: Triglycerides are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase, and H_2O_2, one of the reaction products, is measured as described above for cholesterol. Absorbance is measured at 500 nm (NHANES 2003-2004).

3.4.4.3 High density lipoprotein (HDL) cholesterol

Method: Enzyme selective protection method

Principle: HDL is measured directly in serum. In this method, apoB containing lipoproteins in the specimen are reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL- is detected under the assay conditions.

The method uses sulfated alpha-cyclodextrin in the presence of Mg^{+2}, which forms complexes with apoB containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase for the HDL-cholesterol measurement (NHANES 2003-2004). Absorbance is measured at 600 nm.

3.4.4.4 Low density lipoprotein (LDL) cholesterol

Method: Homogenous enzymatic colorimetric method

Principle: Enzymatic colorimetric method is used for the direct estimation of LDL. HDL, VLDL and chylomicrons are specifically hydrolyzed by a detergent. The released cholesterol content in these lipoproteins reacts immediately in the enzymatic action of cholesterol esterase (CE) and cholesterol oxidase (CHOD) generating hydrogen peroxide. The latter is consumed by a peroxidase (POD) in the presence of 4-aminoantipyrine to generate a colorless product. During this first step, LDL particles remain intact. The reaction of LDL cholesterol is initiated by the addition of another detergent together with a coupler, N,N-bis(4-sulfonyl)-m-toluidine (DSBmT). The second detergent releases cholesterol in the LDL particles which are subjected to the

enzymatic reaction in the presence of coupler to produce a coloured product. The colour intensity of the red quinoneimine dye formed is directly proportional to the LDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 520nm (Sugiuchi J et al 1998). The classification of dyslipidemia provided by ATP III guidelines (2001) was used (Table 3.9) in the study.

3.4.4.5 Very Low Density Lipoprotein (VLDL) Cholesterol

Serum VLDL-C was derived from serum triglyceride values (Crook, 2006).

VLDL-C = Triglycerides/2.2

TABLE 3.9: NCEP ATP III (2001) CRITERIA FOR DIAGNOSING DYSLIPIDEMIA

Total Cholesterol -TC (mg/dl)	
<200	Desirable
200-239	Borderline high
>240	High
LDL Cholesterol (mg/dl)	
<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
>190	Very high
HDL Cholesterol (mg/dl)	
<50 (for women)	Low
Triacylglyceride- TG (mg/dl)	
<150	Normal
150-199	Borderline high
200-499	High
500	Very high

3.4.4.6 Lipid fractions and atherogenic index of plasma

- 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.

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

  Reference range for these lipid fractions are given in table 3.10.

TABLE 3.10: CUTOFF VALUES FOR LIPID RATIOS

Parameter	Value	Reference
TC/HDL-C	> 5	Laboratory reference value
LDL-C/HDL-C	> 3	Laboratory reference value
TG/HDL-C	> 3.5	Laboratory reference value

- Atherogenic Index of Plasma (AIP) was calculated by taking a log10 value of the TG/HDL-C ratio. The categorization of risk associated with AIP levels is given in table 3.11 (Dobiasova and Frohlich, 2001).

AIP= log10 (TG/HDL-C)

TABLE 3.11: CUTOFF VALUES FOR ATHEROGENIC INDEX OF PLASMA

Low risk	<0.11
Intermediate risk	0.11-0.21
High risk	>0.21

3.4.4.7 Fasting Blood Sugar Level

Method: Enzyme Hexokinase

Principle: The enzyme hexokinase (HK) catalyzes the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP). In the presence of nicotinamide adenine dinucleotide (NAD), G-6-P is oxidized by the enzyme glucose-6-phosphate dehydrogenase (G-6-PD) to 6-phosphogluconate and reduced nicotinamide adenine dinucleotide (NADH). The increase in NADH concentration is directly proportional to the glucose concentration and can be measured spectrophotometrically at 340 nm (NHANES 2003-2004). ADA (2011) classification for fasting blood glucose was used which is described in table 3.12.

TABLE 3.12: ADA 2011 GUIDELINES FOR DIAGNOSIS OF IMPAIRED GLUCOSE TOLERANCE AND DIABETES

Fasting Blood Sugar levels (mg/dl)		Blood Sugar levels After 2 hr of meal or 75g oral glucose load (mg/dl)	
Impaired Fasting Glucose (IFG)	100-125	and	<140
Impaired Glucose Tolerance (IGT)	<100	and	140-199
Pre-diabetes	100-125	And/or	140-199
Diabetes	>126	or	>200 (or random blood sugar)

3.4.4.8 Glycated haemoglobin (HbA1c)

Method: High Pressure Liquid Chromatography

Principle: Glycated proteins differ from non-glycated proteins by the attachment of a sugar moiety(s) at various binding sites by means of a ketoamine bond. Glycohemoglobin (GHb) thus contains 1,2-cis-diol groups not found in non-glycated proteins. These diol groups provide the basis for separation of glycated and non-glycated components by boronate affinity chromatography. In this analytical technique, a boronate such as phenylboronic acid is bonded to the surface of the column support. When a solution of proteins (e.g. hemolysate) is passed through the column, the glycated component is retained by the complexing of its diol groups with the boronate. After the unretained non-glycated component elutes from the column, the glycated component is eluted from the column with a reagent that displaces it from the boronate (NHANES 2001-2002).

Reference range: Levels <6 = Normal (Shimodaira et al, 2015)

3.4.4.9 Insulin

Method: Radioimmunoassay

Principle: Insulin radioimmunoassay is a double-antibody batch method. Insulin in the specimen competes with a fixed amount of ^{125}I-labelled insulin for the binding sites of the specific insulin antibodies. Bound and free insulin are separated by adding a second antibody, centrifuging, and decanting. The radioactivity in the pellet

is then measured. The radioactivity is inversely proportional to the quantity of insulin in the specimen (NHANES 2001-2002).

Laboratory reference range: 2.6-24.9µU/ml

3.4.4.10 Homeostasis model assessment of insulin resistance (HOMA-IR), %β cell activity (%β) and % insulin sensitivity (%S)

HOMA IR was calculated using the software HOMA2 Calculator version 2.2, released in 2004 by the Oxford Centre for Diabetes, Endocrinology and Metabolism, The University of Oxford. As no standard cut-point is available for HOMA IR, subjects were categorized as having insulin resistance with values above 75th percentile of the total study subjects.

3.4.4.11 Average blood glucose

Average blood glucose was calculated by the following formula:

Average blood glucose= 28.7 X HbA1C – 46.7

3.4.4.12 Metabolic Syndrome

Metabolic Syndrome is a cluster of various cardio-metabolic disease risk factors. International Diabetic Federation (Alberti et al, 2005) classification was used to categorize subjects into metabolic syndrome. The detail of classification is given in Table 3.13.

TABLE 3.13: METABOLIC SYNDROME IDF (2005) CLASSIFICATIONS FOR WOMEN

Metabolic risk factor	Levels
Fasting Glucose	>100mg/dL or previously diagnosed T2DM
BP	SBP ≥130 or DBP ≥85 mmHg or treatment of previously diagnosed hypertension
Triglycerides	≥150mg/dL or specific treatment for this lipid abnormality
HDL	<50mg/dL or specific treatment for this lipid abnormality
Central obesity	Waist circumference >80 cm (South Asians) or BMI>30kg/m^2
MS-definition	Abdominal obesity plus two or more risk factors

3.4.4.13 High Sensitive C-Reactive Protein (HsCRP)

Method: Nephelometry

Principle: In this method a soluble analyte and corresponding antibodies that are bound to polysterene 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. This method quantifies C-reactive protein (CRP) by latex-enhanced nephelometry. Particle-enhanced assays are based on the reaction between a soluble analyte and the corresponding antigen or antibody bound to polystyrene particles. For the quantification of CRP, particles consisting of a polystyrene core and a hydrophilic shell are used in order to link anti-CRP antibodies covalently. A dilute solution of test sample is mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP present in the test sample will form an antigen-antibody complex with the latex particles.

Light scattering, measured by a nephelometric procedure after 6 min, is proportional to the concentration of the analyte present in the sample. An automatic blank subtraction is performed. CRP concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve (NHANES 2007–2008). Reference range for HsCRP provided by AHA/CDC (Pearson et al, 2003) was used for categorization of risk associated with HsCRP levels (Table 3.14).

TABLE 3.14: AHA/CDC REFERENCE RANGE FOR HsCRP LEVELS

Category	HsCRP levels (mg/dl)
Low risk	<0.1
Average risk	0.1-0.3
High Risk	>0.3

3.4.4.14 Haemoglobin

Method: Cyanmethemoglobin method

Principle: Whole blood is diluted in cyanmethemoglobin reagent. This reagent hemolyzes the erythrocytes which releases haemoglobin in solution. The ferrous ions (Fe2+) of the haemoglobin molecules are oxidized by potassium ferricyanide to ferric ions (Fe3+). 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 cyanmethoglobin. 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 (NCCLS, 1994). The hemoglobin cut-offs used for diagnosing anemia were as per the WHO (2011) criteria (Table 3.15).

TABLE 3.15: WHO (2011) CRITERIA FOR IRON DEFICIENCY ANEMIA IN WOMEN

Categories	Hb (gm/dL)
Normal	> 12.0
Mild	11.0-11.9
Moderate	8.0-10.9
Severe	<8

3.4.4.15 Complete Blood Count (CBC)

Method/instrument: 5-Part Differential Analyzer

Principle: A suspension of blood cells passes through a small orifice simultaneously with an electric current. A small opening (aperture) between electrodes is the sensing zone through which suspended particles pass. In the sensing zone, each particle displaces its volume of electrolyte. 5-Part Differential Analyzer measures the displaced volume as a voltage pulse, the height of each pulse being proportional to the volume of the particle. The quantity of suspension drawn through the aperture is for an exact reproducible volume. 5-Part Differential Analyzer counts and sizes individual particles at a rate of several thousand per second. This method is

independent of particle shape, color, and density (NHANES 2003–2004). Table 3.16 provides the measurement details and reference range for various parameters and indices under CBC.

TABLE 3.16: MEASUREMENT DETAILS AND REFERENCE RANGE FOR PARAMETERS/ INDICES UNDER COMPLETE BLOODD COUNT

Parameters/ Indices	Measurement/ derivation or computation	Laboratory Reference Range
TLC ($\times 10^3$ /µl)	WBC bath	3.5-11 (<3.5 Leucopenia)
Total RBC ($\times 10^6$ /µl)	RBC bath	3.5-5
Platelet Count ($\times 10^3$ /µl)	RBC bath	150-400 (<150 Thrombocytopen-ia)
Neutrophils (%)	# cells inside NE area/# cells inside total cell area × 100	40-80
Lymphocyte (%)	# cells inside LY area/# cells inside total cell area × 100	20-40
Monocyte (%)	# cells inside MO area/# cells inside total cell area × 100	0-10
Eosinophils (%)	# cells inside EO area/# cells inside total cell area × 100	0-6
Basophils (%)	# cells inside BA area/# cells inside total cell area × 100	0-1
Hematocrit (Hct %)	RBC x MCV/10	>36
Mean cell volume (MCV fL)	# × size of RBC/total RBC	80-100 (<80 Microcytosis, >100 Macrocytosis)
Mean cell hemoglobin (MCH pg)	Hb/RBC × 10	34.9-46.9
Mean cell hemoglobin concentration (MCHC g/dl)	Hb/Hct × 100	33.4-37
Red cell distribution width (RDW %)	CV expressed in % of the RBC size distribution	11.5-14.5
Mean platelet volume (fL)	Mean volume of Plt population under the fitted curve × constant	6.5-12
PDW (%)	CV expressed in % of the platelet size distribution	>10
ESR (mm/hr)	Sedimentation rate (in mm) of RBC in one hour	0-20

3.4.4.16 Iron

Method: Spectrophotometry

Principle: 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 and the intensity is read at 562nm (NHANES 2001–2002).

Laboratory Reference range: >50µg/dl = Normal

3.4.4.17 Total iron binding capacity (TIBC)

Method: Spectrophotometry

Principle: Iron is quantitated by measuring the intensity of the violet complex formed in the reaction between ferrozine and Fe^{2+} in acetate buffer at 562 nm. Thiourea is added to complex Cu^{2+}, which can also bind with ferrozine and yield falsely elevated iron values. In TIBC tests, serum is mixed with 400 µg/dL iron solution to saturate the iron-binding sites of the serum transferrin molecules. Magnesium carbonate is used to remove excess iron. Centrifugation is used to precipitate the magnesium carbonate, and the supernatant is measured for iron content (NHANES 2001–2002)

Laboratory reference range: <450= Normal

3.4.4.18 % Transferrin saturation

It was calculated from the following equation:

% Transferrin saturation = (Fe/TIBC) × 100

Laboratory reference range: >15% = Normal

3.4.4.19 Ferritin

Method: Chemiluminescence immune assay

Principle: The method for measurement of Ferritin is a sandwich principle with a total duration time of 18 minutes. The 1st incubation uses 10 uL of sample, a ferritin-specific antibody and a labeled ferritin-specific antibody to form a sandwich

complex. The 2nd incubation occurs after the addition of microparticles that cause the complex to bind to the solid phase. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve (NHANES 2009-2010). Ferritin cut offs for diagnosing iron deficiency anemia was used as per WHO (2001) criteria.

Ferritin cut off: >15µg/L = Normal

3.4.4.20 Vitamin B12

Method: Chemiluminescence immune assay

Principle: The Vitamin B12 electrochemiluminescence immunoassay employs a competitive test principle using intrinsic factor specific for vitamin B12. The total duration of the assay is 27 minutes. The 1st step is to incubate 15 uL of sample with the vitamin B12 pretreatment 1 and pretreatment 2 to release bound vitamin B12. The 2nd incubation adds the ruthenium labeled intrinsic factor to the pretreated sample causing a vitamin B12-binding protein complex to form; the amount of which is dependent upon the analyte concentration in the sample. During the 3rd incubation, streptavidin-coated microparticles and vitamin B12 labeled with biotin are added and the still-vacant sites of the ruthenium labeled intrinsic factor become occupied. This forms a ruthenium labeled intrinsic factor-vitamin B12 biotin complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve which is instrument specifically generated by a 2 point calibration and a master curve provided via the reagent barcode (CDC laboratory procedure manual). Cutoffs for vitamin B12 deficiency are given in table 3.17 (Allen, 2012).

TABLE 3.17: CUT OFF FOR VITAMIN B12 DEFICIENCY

Category	B12 levels
Normal	>221 pmol/L (>300 pg/mL)
Marginal depletion	150–221 pmol/L (200–300 pg/mL)
Deficiency	<150 pmol/L (<200 pg/mL)

3.4.4.21 Folic acid

Method: Chemiluminescence immune assay

Principle: The Folate assay is a two-step assay for the quantitative determination of folate in human serum, plasma, and red blood cells (RBC) using Chemiluminescent Microparticle Immunoassay (CMIA) technology with flexible assay protocols, referred to as Chemiflex. Two pre-treatment steps mediate the release of folate from endogenous folate binding protein. In Pre-Treatment Step 1, sample and Pre-Treatment Reagent 2 (Dithiothreitol or DTT) are aspirated and dispensed into a reaction vessel (RV). In Pre-Treatment Step 2, an aliquot of sample/ Pre-Treatment Reagent 2 mixture is aspirated and dispensed into a second RV. Pre-Treatment Reagent 1 (potassium hydroxide or KOH) is then added. An aliquot of the pre-treated sample is transferred into a third RV, followed by the addition of Folate Binding Protein (FBP) coated paramagnetic microparticles and assay specific diluent. Folate present in the sample binds to the FBP coated microparticles. After washing, pteroic acid-acridinium labeled conjugate is added and binds to unoccupied sites on the FBP-coated microparticles. Pre-Trigger and Trigger Solutions are then added to the reaction mixture; the resulting chemiluminescent reaction is measured as relative light units (RLUs). An inverse relationship exists between the amount of folate in the sample and the RLUs detected. The cut off values for folic acid deficiency is provided in table 3.18 (Wadia et al, 2000).

TABLE 3.18: CUT OFF FOR FOLIC ACID DEFICIENCY

Category	Folic acid levels (ng/ml)
Normal	>4
Marginal depletion	3-4
Deficiency	<3

3.4.4.22 Thyroid Stimulating Hormone (TSH)

Method: sandwich chemiluminescent immuno assay

Principle: 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 (NHANES 2011-2012).

Reference Range: >4 μIU/ml =Subclinical hypothyroidism (Fatourechi, 2009)

3.4.4.23 Total Triiodothyronine (T3)

Method: sandwich chemiluminescent immuno assay

Principle: The Total T3 Assay is a competitive binding immunoenzymatic assay. Sample is added to reaction vessels with a stripping agent to dissociate T3 from the binding proteins. T3 in the sample competes with the T3 analogue coupled to biotin for anti-T3 alkaline phosphatase conjugate. Of the resulting antigen: antibody complexes, the T3 analogue: antibody complexes are bound to the streptavidin coated solid phase. Separation in a magnetic field and washing removes the sample T3; antibody complexes and other materials not bound to the solid phase. A chemiluminescent substrate, is added to the reaction vessel and light generated by the reaction is measured with a luminometer. The light production is proportional to the amount of enzyme conjugate bound to the solid support. The amount of analyte in the sample is determined by means of a stored, multi-point calibration curve (NHANES 2011-2012).

Laboratory Reference range 60- 200 ng/dl

3.4.4.24 Total Tetraiodothyronine (T4)

Method: sandwich chemiluminescent immuno assay

Principle: The method is a paramagnetic particle, chemiluminescent, competitive binding enzyme immunoassay (competitive binding immunoenzymatic assay) for the

quantitative determination of total thyroxine (T4) in human serum. A sample is added to a reaction vessel with anti-thyroxine antibody, thyroxine-alkaline phosphatase conjugate, and paramagnetic particles coated with goat anti-mouse capture antibody and a stripping agent to dissociate all T4 from serum-binding proteins. Thyroxine in the sample competes with the thyroxine-alkaline phosphatase conjugate for binding sites on a limited amount of specific anti-thyroxine antibody. Resulting antigen: antibody complexes bind to the capture antibody on the solid phase. Separation in a magnetic field and washing removes materials not bound to the solid phase. A chemiluminescent substrate, is added to the reaction vessel and light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of T4 in the sample. The amount of analyte in the sample is determined by means of a stored calibration curve (NHANES 2011-2012).

Laboratory Reference range: 4.5 - 12.0 µg/dl

3.4.4.25 Alkaline Phosphatase

Method: Photometry

Principle: 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 (NHANES 1999–2000).

Laboratory Reference range: 42-98 U/l

3.4.4.26 Total/ Direct Bilirubin

Method: Photometry

Principle: Total bilirubin is coupled with diazonium salt 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 (NHANES 1999–2000).

Laboratory Reference range:

Total Bilirubin- 0-1.20 mg/dL , Direct Bilirubin- 0-0.3 mg/dL

3.4.4.27 Indirect Bilirubin

Indirect Bilirubin is derived from serum total and direct Bilirubin values

Laboratory Reference range: 0-0.9 mg/dL

3.4.4.28 Aspartate Aminotransferase (AST) / Serum Glutamate Oxaloacetate Transaminase (SGOT)

Method: Photometry

Principle: α-Ketoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction uses the oxaloacetate for a kinetic determination of NADH consumption (NHANES 1999–2000).

Laboratory Reference range: 0-31 U/l

3.4.4.29 Alanine Transaminase (ALT) / Serum Glutamate Pyruvate Transaminase (SGPT)

Method: Photometry

Principle: α-Ketoglutarate reacts with L-alanine in the presence of ALT to form L-glutamate plus pyruvate. The pyruvate is used in the indicator reaction for a kinetic determination of the reduced form of nicotinamide adenine dinucleotide (NADH) consumption (NHANES 1999–2000).

Laboratory Reference range: 0-31 U/l

3.4.4.30 Gamma Glutamly Transferase (GGT)

Method: Photometry

Principle: In this rate method, 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 (NHANES 1999–2000).

Laboratory Reference range: Female: 0-30 U/l

3.4.4.31 Total Protein

Method: Photometry

Principle: In alkaline solution, a colored chelate forms between cupric ions and compounds containing at least two -$CONH_2$, -$CSNH_2$, -CH_2NH_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. Peptides of low molecular weight are present in serum, but their concentration is too low to cause interference (NHANES 1999–2000).

Laboratory Reference range: 6-8.3 gm/dL

3.4.4.32 Serum Albumin

Method: Photometry

Principle: At the reaction pH, the bromcresol purple in the albumin system reagent binds selectively with albumin. This reaction is based on a modification of a method described by Doumas. Although bromcresol purple is structurally similar to the conventional bromcresol green, its pH color change interval is higher (5.2–6.8) than the color change interval for bromcresol green (3.8–5.4), thus reducing the number of weak electrostatic dye/protein interactions. The bromcresol purple system eliminates many of the nonspecific reactions with other serum proteins as a result of the increased pH. In addition, the use of a sample blank eliminates background spectral interferences not completely removed by bichromatic analyses (NHANES 1999–2000).

Laboratory Reference range: 3.2-5 gm/dL

3.4.4.33 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 (NHANES 1999–2000).

Laboratory Reference range: 0.9-2.0

3.4.4.34 Serum Calcium

Method: Photometry

Principle: Calcium reacts with o-cresolphthalein complexone in the presence of 8-hydroxyquinoline-5-sulfonic acid to form a purple complex. The intensity of the final reaction color is proportional to the amount of calcium in the specimen (NHANES 1999–2000).

Laboratory Reference range: 8.8 - 10.6 mg/dl

3.4.4.35 Blood Urea Nitrogen (BUN)

Method: Photometry

Principle: Urea is hydrolyzed by urease to form CO2 and ammonia. The ammonia formed then reacts with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD+. The decrease in absorbance due to consumption of NADH is measured kinetically (NHANES 1999–2000).

Laboratory Reference range: 9-23 mg/dL

3.4.4.36 Serum Creatinine

Method: Photometry

Principle: This method, principally uses the Jaffe reaction. In an alkaline medium, creatinine forms a yellow-orange-colored complex with picric acid. The rate of color formation is proportional to the concentration of creatinine present and may be measured photometrically (NHANES 1999–2000).

Laboratory Reference range: 0.5-0.8 mg%

3.4.4.37 Uric Acid

Method: Photometry

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 and 4-aminophenazone in the presence of peroxidase to form quinone-imine dye and hydrogen bromide. The intensity of the red color is proportional to the uric acid concentration (NHANES 1999–2000).

Laboratory Reference range: 2.3-6.1 mg/dL

3.4.4.38 BUN/ Sr Creatinine ratio

It was derived from serum BUN and creatinine values.

Laboratory Reference range: 9:1- 23:1

3.4.5 NUTRIENT COMPOSITION ANALYSIS OF FLAXSEEDS

Nutrient analysis of flaxseeds (carbohydrate, fats, proteins, fiber, fatty acid, iron, calcium, sodium, potassium) was performed at Food Testing Laboratory [NABL Accredited Laboratory as per ISO/IEC 17025:2005], Indian Institute of Crop Processing Technology, Ministry of Food Processing Industries, Govt. of India,Thanjavur. Analyses of antioxidant capacity were performed at Faculty of Home Science, Sardar Patel University, Vidhyanagar.

3.4.5.1 Fats

Method: Soxhlet extraction method (AOAC, 2005)

Procedure: The ground sample was weighed accurately in thimble and defatted with petroleum ether in Soxhlet apparatus for 6 hours at 70^0C. The resultant ether extract was evaporated to remove traces of ether and lipid content was calculated.

$$\text{Fat content (g/100g)} = \frac{\text{Weight of ether extract}}{\text{Weight of the sample}} \text{ X } 100$$

3.4.5.2 Protein

Method: Micro-kjeldahl method (AOAC, 2005)

Procedure: Weighed sample was transferred to a digestion flask followed by the addition of 3 g of catalyst mixture (K_2SO_4 :$CuSO_4$:SeO_2 in 100:20:2.5) and 20 ml of concentrated sulphuric acid.

The content was then digested till transparent liquid was obtained. The volume of digested material was made up to 100 ml with distilled water. A blank digestion without the samplewas carried out and digest was made to 100 ml. Measured aliquot of digested material was distilled with excess of 40% NaOH solution and the liberated ammonia was collected in 20 ml of 2% boric acid solution containing 2-3 drops of mixed indicator (10 ml of 0.1 percent bromo cresol green + 2 ml of 0.1 percent methyl red indicator in 95 percent alcohol). The entrapped ammonia was titrated against 0.01 N hydrochloric acid. A reagent blank was similarly digested and distilled. Nitrogen content in the sample was calculated as follows and a factor of 6.25 was used to convert nitrogen to protein.

$$\text{Nitrogen \%} = \frac{\text{(Sample titre – Blank titre) X N of HCL X Volume made up of the digest X 100}}{\text{Aliquot of the digest taken X weight of the sample taken X 1000}}$$

Protein % = nitrogen % X 6.25

3.4.5.3 Carbohydrate

The total carbohydrate was determined by the AOAC (2005) difference method. The total carbohydrate such as sucrose, dextrose, dextrin, maltose and lactose percent by mass was calculated by difference method.

Total carbohydrate (Percentage by mass) = 100 - (A+B+C+D)

Where,

A = Percent by mass of moisture

B = Percent by mass of total protein

C = Percent by mass of fat

D = Percent by mass of total ash

3.4.5.2 Crude fiber

Method: Oxidative hydrolytic degradation (Sadasivam et al, 2005)

Procedure: During the acid and subsequent alkali treatment, oxidative hydrolytic degradation of the native cellulose and considerable degradation of lignin occur. The residue obtained after final filtration is weighed, incinerated, cooled down and weighed again. The loss in weight gives the crude fiber content. 2g of ground flaxseed was extracted with petroleum ether to remove fat (initial boiling temperature 35-38˚C and final temperature 52˚C) and further boiled the dried material with 200ml sulphuric acid for 30minutes with bumping chips. It was filtered through muslin cloth and washed with boiling water to remove acid. After that it was boiled with 200ml of sodium hydroxide solution for 30 minutes. The aliquot was filtered again and washed with 25ml of boiling 1.25% sulphuric acid, three 50ml portions of water and 25ml alcohol. The residue was dried for 2 hour at 130±2˚C, cooled and weighted. Then it was ignited for 30 minutes at 600±15˚C, cooled again and weighed.

$$\% \text{ crude fiber in ground sample:} = \frac{\text{Loss of weight on ignition (W2-W1)-(W3-W1)}}{\text{Weight of the sample}} \times 100$$

3.4.5.3 Fatty acid profile

Method: GC-MS

Procedure: The flaxseed sample was extracted with oil and the methylated extract was analyzed through GC-MS for the identification of fatty acid compounds.

GC Programme

- Column: Elite-5MS (5% Diphenyl / 95% Dimethyl poly siloxane), 30 x 0.25mm x 0.25m df
- Equipment: GC Clarus 500 Perkin Elmer
- Carrier gas: 1ml per min, Split: 10:1
- Detector: Mass detector Turbo mass gold-Perkin Elmer
- Software: Turbomass 5.2
- Sample injected: 2µl

Oven temperature Programme

- 110° C -2 min hold
- Up to 200° C at the rate of 10 ° C/min-No hold
- Up to 280 ° C at the rate of 5° C / min-9 min hold
- Injector temperature 250° C
- Total GC running time 36 min

MS Programme

- Library used NIST Version-Year 2005
- Inlet line temperature 200° C
- Source temperature 200 ° C
- Electron energy: 70 eV Mass scan (m/z): 45-450
- Solvent Delay: 0-2 min
- Total MS running time: 36 min

3.4.5.4 Minerals (Iron, calcium, sodium, potassium)

Method: ICP-OES (Barnes and Debrah, 1997)

Procedure: Microwave digestion of the flaxseed sample was performed using Teflon PFA digestion vessels HNO_3 and HCL.

Instrument Conditions for ICP-OES

- RF Power: 1100 W
- Nebulizer Flow: 0.950 L/min
- Auxiliary Flow:1.0 L/min
- Plasma Flow: 15 L/min
- Sample Flow: 1.0 mL/min
- Source equilibration time: 15 s
- Viewing Height: 15 mm
- Background correction: Manual selection of points
- Measurement processing mode: Area
- Auto integration: 1 s min–50 s max

- Read delay: 45 s
- Rinse delay: 45 s
- Number of replicates: 3

3.4.5.5 Total phenols

Method: Folin–Ciocalteau colorimetric method (Singleton et al, 1999)

Procedure: Phenols present in plant extract react with specific redox reagent (Folin–Ciocalteau reagent) to form blue chromophore constituted by a phosphotungstic phosphomolybdenum complex which is measured at 750 nm. Maximum absorbance of the chromophore is depends on the alkaline solution and concentration of the phenolic compounds. Different aliquots of sample were taken and volume was made up to 1.0 ml with distilled water. 0.5 ml of Folin–Ciocalteu reagent (50% diluted) was added and the contents were mixed using a vortex. After 3 min of reaction, 10 ml of 7.5% Sodium carbonate was added and the mixture was incubated at 37°C temperature for 1 hour. The blue colour complex was read at 750 nm. Different aliquots of known concentration of gallic acid were taken as standard. The results are expressed in mg Gallic acid equivalents/ 100 g (mg GAE/ 100 g).

3.4.5.6 Flavonoids

Method: Aluminium chloride colorimetric method (Zhishen et al, 1999)

Procedure: The natural flavonoid compounds present in the sample extracts reacts with sodium nitrite; the pink coloured flavonoid-aluminium complex developed using aluminium chloride in alkaline condition which is measured at 510 nm. Different aliquots of concentrated sample were taken and volume was made up to 5.0 ml with distilled water. 0.3 ml of 5% sodium nitrite was added and incubated at 37°C for 5 minutes. 0.6 ml of 10 % aluminium chloride was added and incubated at 37°C for 6 minutes. 2.0 ml of 1.0 N sodium hydroxide and 2.1 ml of distilled water was added. The resultant pink colour complex was read at 510 nm. Different aliquots of rutin standard was treated same as sample. The flavonoid content is expressed in terms of mg Rutin equivalents/ 100 g (mg RE/ 100 g).

3.4.5.7 DPPH Radical Scavenging Activity

Method: 2,2-diphenyl-1-picrylhydrazyl **(**Brand-Williams et al, 1995)

Procedure: 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a comparatively stable free radical and widely used to determine free radical scavenging activity of compounds and thus to evaluate the antioxidant activity. The antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore terminate the chain reaction of lipid peroxidation. The hydrogen atom or electron-donation ability of the corresponding antioxidant present in sample extract was measured from the bleaching of a purple-coloured methanolic solution of DPPH (Gulluce et al, 2007).

The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable DPPH free radical, was determined by the method described by Brand-Williams et al. (1995). 3.0 ml of 0.2 mM DPPH solution (Optical Density 517 nm=1.0) in methanol was added to different aliquots of sample after making up the volume to 1.0 ml with methanol. DPPH solution with methanol was used as control. After incubation at 37°C for 20 min, absorbance at 517 nm was measured against methanol as blank. The percent inhibition of activity was calculated as follows:

% Inhibition= [(Ac – Ae)/Ac] x 100

Where,

Ac = absorbance of control; Ae = absorbance of extract

Total antioxidant capacity of sample measured by DPPH RSA is expressed in terms of mg Trolox equivalents/ 100 g (mg TE/ 100 g).

3.4.5.8 FRAP (Ferric-Reducing Antioxidant Power)

Method: FRAP (Benzie and Strain, 1996)

Procedure: The FRAP assay is based on the reduction of ferric TPTZ (2, 4, 6-tris (2-pyridyl)-s-triazine) complex to the ferrous form at acidic pH. Electron-donating substances for which the half-reaction has a lower redox potential than Fe+3/Fe+2-TPTZ, drive the reaction and form the blue complex forward, which can be monitored by measuring the absorption change at 593 nm. To perform the assay, different aliquots of sample as well as trolox standard were taken and volume was made up 300 µl with distilled water. 1.8 ml of FRAP reagent was added and

incubated at 37°C temperature for 10 minutes. The coloured complex was measured at 593 nm using 300 µl of distilled water treated as blank. The total antioxidant capacity of sample by FRAP is expressed in terms of mg Trolox equivalents/ 100 g (mg TE/ 100 g).

3.4.6 STATISTICAL ANALYSIS

- The data was entered into Microsoft excel 2007 and verified. Statistical analysis was performed using Epi Info 7 and SPSS 16. All statistical analyses were considered significant at $p<0.05$ level.
- Data was described using descriptive statistics (mean and standard deviations) for continuous variables. Data was also depicted using proportions i.e. percentages in case of categorical variables and prevalence rates for continuous variables using well defined cut off points.
- The independent student's 't' test and Chi-square test were performed to compare continuous and categorical variables respectively. Univariate odds ratio analysis was performed to assess the risk burden of a particular risk factor and has been depicted with 95% confidence intervals.
- In multivariate analysis, analysis of covariance (ANCOVA) was performed to remove the effect of certain variables while comparing continuous data of two or more groups. Forward Logistic regression was applied to find out the variables that significantly affected the dependent variable independently irrespectively of other effect of other variables on this association.
- One way analysis of variance (ANOVA) was computed to find out the difference between baseline characteristics within groups of intervention trial. Paired 't' test was used to compare the difference between the pre and post intervention values of the outcome variables in the intervention phase.

3.4.7 DATA MONITORING AND MANAGEMENT

To ensure the quality of the research, data collection, monitoring and handling was done using checkpoints as described in table 3.19.

TABLE 3.19: 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	Collected empty pouches by the researcher on weekly basis.
Data handling	Safe and confidential storage at central level.
Data entry	Microsoft Excel
Data Analysis	Using Microsoft Excel 2007, SPSS (version 16) and Epi Info (version 7) by applying appropriate statistical techniques.