CHAPTER 3

MATERIALS AND METHODS

There is unprecedented upsurge in the utilisation of vitamin B12 and omega-3 fatty acids rich food in the efficacious management of cognitive impairment. In the current times, the methylcobalamin form of Vitamin B12 and flaxseeds as the omega-3 rich source truly hold great promise in enhancing the cognition status. Hence, these two components have specifically emerged on the research front owing to their health benefits as memory boosters, anti-glycemic, anti-lipemic and probable risk modulators for neuropsychiatric disorders notably Alzheimer's disease, dementia, etc. The present study in focus is entitled as, "Vitamin B12 and Omega-3 fatty acid Interventions for Cognition in Elderly- a V.O.I.C.E. Trial". This chapter deals with the framework of experimental design and elaborates on materials and methods used for fulfilling the objectives under the study in the succeeding three phases.

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Intervention and impact evaluation of the MCI elderly group with Omega -3 fatty acids and Vitamin B_{12} supplementation.

Section3.3:	**Dual therapeutic interventional approaches with food formulation and methylcobalamin dosage**
Section 3.3.1:	Statistical Analysis and Impact Outcome

PHASE I

Baseline assessment of diet, nutrition and health status of cognitively impaired elderly with MCI.

The formative phase of study included preliminary screening for identification of the elderly with MCI (mild cognitive impairment). A number of 402 subjects (males and females) aged 60 -85 years (termed elderly [United Nations {2015} & WHO {2015}] were selected from the out-patient department (O.P.D.) of private & University Health Centre of The Maharaja Sayajirao University of Baroda (govt.) hospitals by purposive sampling. The targeted subjects were studied for the prevalence of MCI by employing the semi-structured questionnaire, based on cognitive impairment tests namely Addenbrooke's Cognitive Examination-Revised (ACE-R) falling $\leq$ 86 score represented MCI having the sensitivity 93% and specificity of 71% (Mioshi E, 2006), Mini Mental State Examination (MMSE) falling in the range of 21-26 score points indicating MCI with the sensitivity 71.1% and specificity of 81.3% (Folstein, 1975), Yamaguchi Fox Pigeon Imitation Test (Y.F.P.I.T.{Yamaguchi, 2010}) and Mini Nutritional Assessment (MNA) tool (Nestle Nutrition Institute, 2009).These combination tools are standardised universal predictors for M.C.I progression, individually used by diverse neurological societies. Thereafter, the entire information was elicited through one-to-one interviews and patient medical records, individualised anthropometric measurements and biophysical determinations.The patients fulfilling inclusion criteria and not receiving treatment for vitamin B12 were chosen. Succeeding these assessments, the risk factor analysis employing bio-chemical estimations comprised of the Fasting Blood Sugar (FBS), Complete Blood Count (CBC), glycated haemoglobin (HbA_{1c}), serum vitamin B12 and serum lipid profile for

assisting the assessment of cognitive functioning levels. From the total of 197 confirmed MCI patients (as adjudged by ACE), the group of 120 consenting Vitamin B12 deficient and borderline to moderately cholesterolemic patients were enrolled for the trial. Experimental design of the study is represented in the Figure 3.1.1.

3.1.1: Ethical clearances

The study has been approved by the Institutional Medical Ethics Committee of the Department of Foods and Nutrition, The Maharaja Sayajirao University of Baroda and granted with the Institutional Medical Ethics Committee No. **IEHCR/2012/22.** This clinical study trial has also been retrospectively registered in the Clinical Trial Registry of India (CTRI) No**. CTRI/2015/03/005602.** Informed written consent was taken from all the willing respondents on the consent form as per the WHO informed consent guidelines for clinical studies prior to enrolment in the trial. The MCI patients and their families were informed about the requisite of their blood sample for the biochemical analysis (Appendix I [i-iii]).

3.1.2: Estimation of the sample size for the study

The present study had the primary objective of prevalence detection of cognitive impairment and the interplay of neurological and metabolic aberrations in the study region. The cross-sectional researches recently conducted in the Indian population report a MCI rate of 29.8%. So, the sample size was estimated by the formula (Mahajan 2010):

$$n = \frac{4pq}{L^2}$$

Where, n= required sample size,

p= approximate reported prevalence rate of the disease under study (MCI =29.8%),

q= (1-p)

L = permissible error in the estimate/precision of p, which was taken as 0.05

On plugging all of these above values into the equation, the sample size arrived at 335 and an extra buffer of 20% was set aside to counter balance the dropout or attrition rate. Thus, the final sample size came out to be 402.

3.1.3: Selection of the study participants

To execute the prevalence check along with a neurological cross-examination tests' battery, the sample comprised of a number of 402 female and male subjects falling in the age range between 60 to 85 years. The patients attending the University Health Centre, The Maharaja Sayajirao University of Baroda (government clinic) and private clinics of Dr. Bhavin Upadhyay (Neurophysician), Dr. Kritgna Sinh Waghela (Psychiatrist), Dr. Vipul Bhavsar (Physician) and Dr. Dharmesh Dhamat (Dental Surgeon) at Shree Vallabhacharyajee Hospital, Vadodara were enrolled for this phase during the time span of June 2013 to February 2014.

Prior written permissions were sought for visiting the Out Patient Departments O.P.D's of these clinics as per their scheduled timings and working days (Appendix II [i-ii]). During the visits, the subjects were firstly informed about the genesis and intention of the study. They were informed that on their inclusion in the study, a neurologically structured medical check-up and the accompanying blood test reports would both be provided free as the study incentives. The clarification was made that under no circumstances the patient be held liable for bearing any charge towards the blood test profiles and vitamin B12 (methylcobalamin) injectable or flaxseed supplementations on participating in the study.

The willing respondents were handed and briefed over the consent form prepared in accordance to the ICMR guidelines for their consent approval (Appendix I [i-iii]). After satisfying all related queries of the subjects, each of the consenting respondents were asked to thoroughly read (this was done in presence of their relatives/family members) and then give their written informed consent before enrolment into the study. Those respondents unable to read were explained of the study objectives and guided about fasting blood sample collection requirements to be operated before and after the interventional phases of the study, usage of disposable needles, being conducted by an accreditated laboratory's experienced phlebotomist (technician trained to draw blood from patients for clinical or research purposes) in case of their agreement towards the consent form. The subjects fulfilling the inclusion criteria were asked for their participation in this interventional trial.

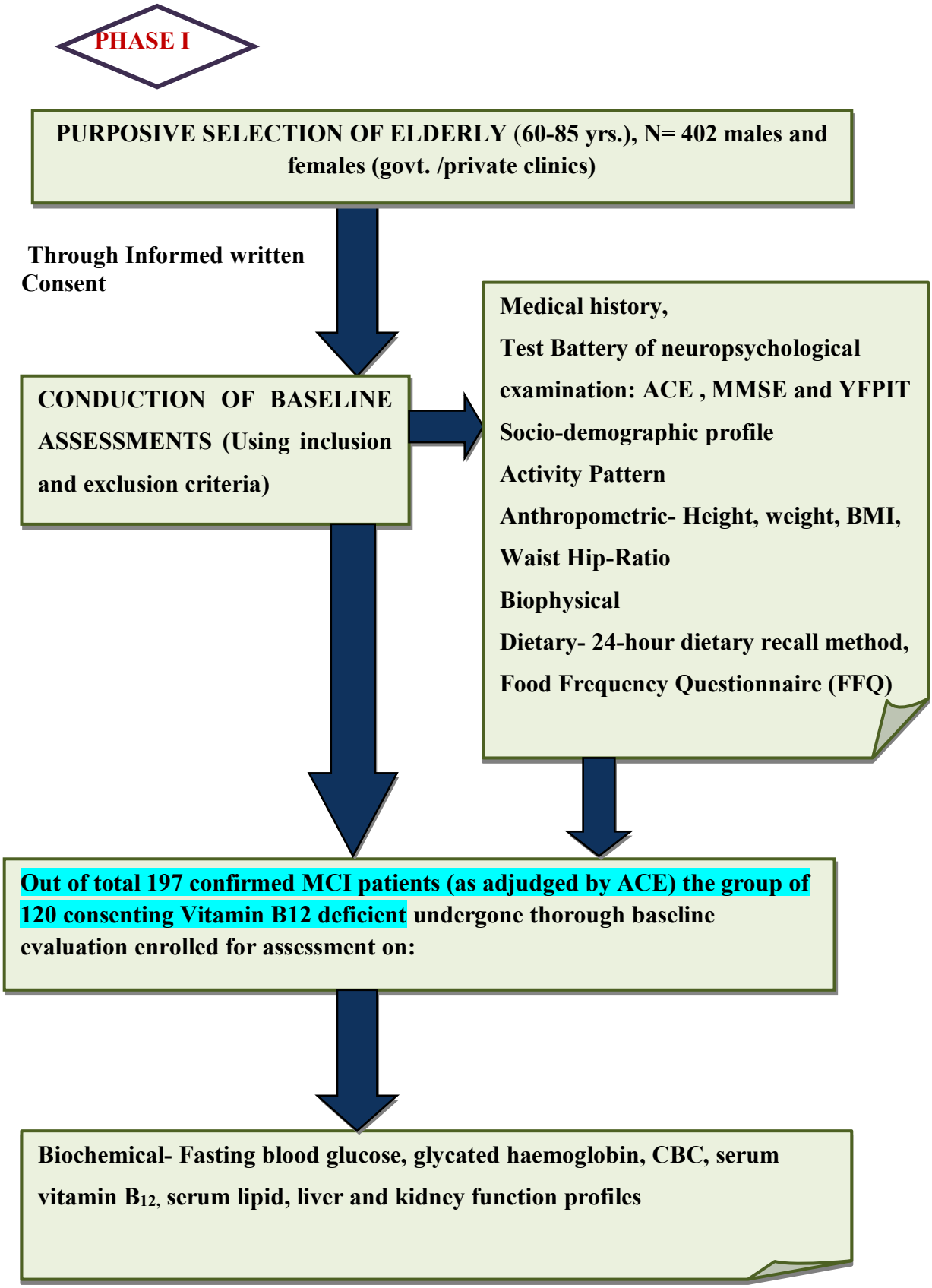


Figure 3.1.1: Experimental Design of Phase I

3.1.4: Inclusion and exclusion criteria for the study subjects

Inclusion criteria

- Patients of both sex in the age range of 60-85 years
- Patients belonging to any income group (HIG, MIG and LIG)
- Patients who attend O.P.D. of govt. or private clinics
- Patients having Cognitive Impairment as assessed by:-
  - Clinical assessment
  - Addenbrooke's Cognitive Examination(ACE) total scoring out of 100 based on age wise sub-scores
  - MMSE score ranging < 26 points
- Patients having serum Vitamin B_{12} levels < 200pmol/l
- Patients having Total Cholesterol levels falling between 200- 239 mg/dL
- Patients willing to undergo vitamin B12 and flaxseed supplementation

Exclusion criteria

- Patients suffering from any of the following ailments as:
  - Severe dementia, depression, stroke, psychiatric illnesses or medications affecting cognition
  - Major medical conditions *viz.* cancer, oral difficulties, genitourinary problems, endocrinal disorders, angina, unstable diabetes mellitus, Parkinson's disease, hypercholesterolemia, angioplasty, lumbar spine deformities (kyphosis)
- Patients allergic to flaxseeds

3.1.5: Study Protocol

Under the present study, 402 female and male subjects falling in the age range between 60 to 85 years were selected. These included the patients attending the University Health Centre, The Maharaja Sayajirao University of Baroda (government clinic), Shri Vallabhacharyajee and private clinics of Vadodara region. The informed written consent was obtained from all the subjects preceding the conduction of the formative study phase. The prevalence of MCI was established by employing a pre-

tested semi-structured questionnaire (based on the cognitive impairment tests namely Mini Mental State Examination (MMSE), Addenbrooke's Cognitive Examination (ACE) and Yamaguchi Fox Pigeon Imitation Test (YFPIT). Thereafter the diagnosis was performed for the disease severity incorporating the severity of the disease condition, socio-demographic, activity pattern (WHO-global recommendations on physical activity on health, 2011) and the checklist of major diseases. These subjects were then studied for anthropometric, biophysical, dietary and biochemical measurements. Under the preliminary anthropometric screening, the subjects were assessed for their BMI, waist and hip circumference. For the biophysical assessment, three systolic and diastolic blood pressure readings were successively measured in a relaxed atmosphere for each patient through the sphygmomanometer at five minute intervals by the investigator at both the pre and post intervention phases during the hospital visits (Appendix III). Thereafter the mean of these readings, was taken into consideration. The dietary analysis was performed using the three day consecutive 24-hour dietary recall and food frequency questionnaire (FFQ). Following these assessments, bio-chemical estimations at fasting levels for risk factor analysis were performed at both the pre- interventional and post-interventional study stages respectively with subjects' consented approval. The panel of tests consisted of fasting blood glucose, HbA1c, complete blood count (CBC), serum B_{12}, serum lipid profile, liver and kidney function tests (encompassed the array of tests namely blood urea, blood urea nitrogen (BUN), serum creatinine, serum uric acid, BUN/serum creatinine ratio, alkaline phosphatase, bilirubin direct, bilirubin total, bilirubin indirect, gamma glutamyl transferase (GGT), aspartate amino transferase (SGOT), alanine transaminase (SGPT), protein total, serum albumin, serum globulin, serum albumin/globulin ratio, estimated glomerular filtration rate (eGFR) exclusively for those desirous for flaxseed supplementation) for assisting the assessment of cognitive functioning levels. Subsequent to obtaining the preliminary results and willingness of subjects towards participation in the study, an experimental sample of 120 MCI patients was hence enrolled for the present trial. The proximate analysis was included next with the development of food product from the flaxseeds. 120 elderly willingly consenting for the trial were conveniently enrolled under two supplementation arms of 60 subjects each viz. first arm of vitamin B12 (n=60) and second arm having vitamin

B12 in combination with flaxseeds (n=60) based on their serum vitamin B12 and serum lipid values for a period of 6 months on a dose dependent basis. The subjects were instructed to similarly pursue their respective dietary intakes, physical activity schedules and suggested to immediately report the occurrence of any discomfort or side effects being experienced. Patients with kyphosis were unwilling to be a part of the regular compliance check and thus, were excluded. The family members were asked to fill a three- day diet record of the patient's routinely dietary intakes before and after each of the interventional arms except for holidays, fasts or outings (Appendix V). Every week a follow-up was slated for compliance check and observance of any side effect (if present), was accounted.

Primary variable: Cognition scoring levels

Secondary variables: Age, BMI, WHR, biophysical, serum Vitamin B12, serum lipid, glycated haemoglobin, FBS and dietary intake status.

3.1.6: Assessment by Battery of Neuropsychological Tests and Administration of Semi-structured Questionnaire

The respondents matching to the inclusion criteria were explained of the objective and benefits of the study and encouraged to participate in the study through their approval in the form of written informed consent (Appendix I [i-iii]). A battery of neuropsychological tests was exercised to detect the episode of Mild Cognitive Impairment (MCI) in the elderly.

i. **Addenbrooke's Cognitive Examination (ACE-R):** The ACE-R as described by Mioshi et al (2006) is a brief cognitive test that assesses five cognitive domains, namely attention/orientation, memory, verbal fluency, language and visuo-spatial abilities. Total score is 100, higher scores indicates better cognitive functioning. Administration of the ACE-R takes, on average, 15 minutes (Appendix IV [i]).

Table 3.1.6.1: Lower limit of normal (cut-off scores) for total ACE-R and sub-scores according to age (50-59, 60-69, 70-75, 80-85), showing control mean minus two standard deviations.

Age range	Education (years)	Total ACE-R score	Attention/ Orientation	Memory	Fluency	Language	Visuo-spatial
50-59	12.7	86	17	18	9	24	15
60-69	12.9	85	17	19	8	21	14
70-79	12.1	84	16	17	9	22	14

ii. Mini-Mental State Examination (MMSE): It was developed by Folstein et al (1975) was one of the tests used for cognitive assessment of the MCI condition (Appendix IV [i], V). It records responses in the category of constructs i.e. orientation, registration, attention and calculation, recall, language and visuo spatial abilities. The maximum score obtained is 30. Categorization of respondents was subjected to the following cut-offs:

Table 3.1.6.2: Cut-offs representing Mini-Mental State Examination (MMSE) scores

Criteria	MMSE score
Normal cognitive function	27-30
Mild cognitive impairment	21-26
Moderate cognitive impairment	11-20
Severe cognitive impairment	0-10

iii. **Yamaguchi Fox Pigeon Imitation Test (YFPIT):** A hand-gesture imitation test, YFPIT as proposed by Yamaguchi et al (2010) consisted of a simple one-handed sign of a 'fox' and a complex two-handed sign of a 'pigeon', as a rapid, game-like test for detecting MCI within one minute. The test measures the visuomotor function, which deteriorates in the early stages of AD (Appendix V).

The YFPIT consists of a hand-gesture imitation of a 'fox' (Figure 3.1.2 a) contiguous with a 'pigeon' (Figure 3.1.2 b). The protocol is as follows:

(1) The examiner sits face-to-face with a subject.

(2) The examiner gives a simple instruction: ‘Watch my hand gesture carefully and imitate it.’ The instruction can be repeated if necessary.

(3) Then, the examiner makes the ‘fox’ sign using his/her left hand: fingers III and IV touching the thumb on flexion of the metacarpophalangeal joints with fingers II and V held up (Figure 3.1.2 a).

(4) The examiner maintains the gesture for 10 seconds. The subject imitates the gesture concurrently with the examiner. Say nothing during the 10 seconds of the test. Be careful not to say the words ‘fox’, or the instruction.

(5) The examiner judges whether or not the subject produces the same sign within 10 seconds of demonstration; the subject may use either hand.

(6) For ‘pigeon’, the examiner gives the same instruction, and then makes a ‘pigeon’ sign using both hands: crossing the hands, palms facing the body, with fingers II–IV extended upward and the two thumbs crossing each other (Figure 3.1.2 b).

(7) The examiner maintains the gesture without saying anything, especially the word ‘pigeon’ nor instructions, during the 10 seconds of the test.

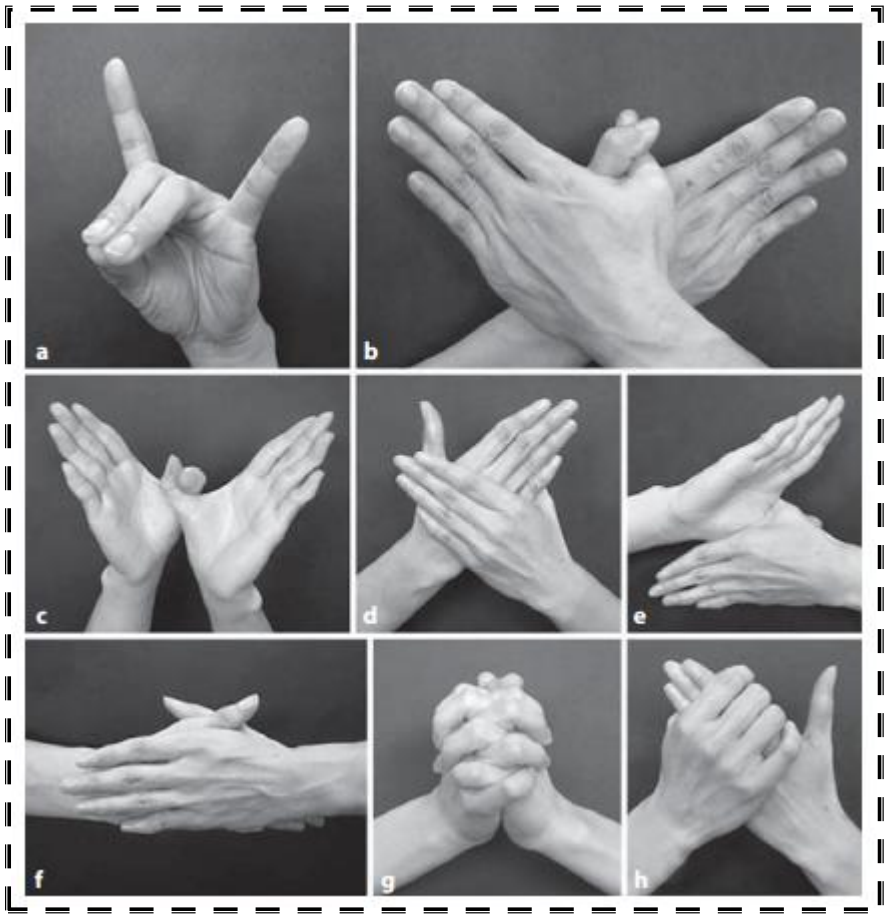
(8) The examiner judges whether or not the subject concurrently makes the same sign within 10 seconds of demonstration. Points for judgment are as follows:

(a) The direction of the arm and fingers II–V should be upward: hand positions in horizontal or downward directions are judged as failures (Figure 3.1.2 f).

(b) Both cases are acceptable: right hand in an outward position or vice versa.

(c) Both palms should be facing the body.

(d) Thumbs should be crossing each other.



Source: Yamaguchi et al 2010

Figure 3.1.6: Step-wise instructions for Yamaguchi Fox Pigeon Imitation Test

Subsequent to the neuropsychological examination, a pre-tested semi-structured questionnaire was administered to elicit the baseline information from the subjects (Appendix II).

a) Background information

Basic information with reference to age, sex, marital status, educational level, occupation, religion, type of family, per capita income was obtained from the subjects.

b) Lifestyle pattern

The information under this category was gathered under the following sub-heads:

i) Activity pattern

To delve into the better understanding of neurodegenerative diseases like MCI, dementia and Alzheimer's, it becomes essential to identify the physical activity pattern. The physical activity profile of the subjects was assessed using the Global Recommendations on Physical Activity for Health (GRPAH) given by the WHO 2011. The participation in physical activity was assessed according to the following cut-offs.

Table 3.1.6.3: Cut-offs for Physical Activity Levels

Age range	Physical activity per week Criteria	Physical activity minutes per week
18-64 years	Moderate-intensity	≥ 150 minutes
65 years- above	Vigorous-intensity	≥ 225 minutes

WHO 2011

ii) Addiction pattern

The information on the addiction of the subjects was collected for the consumption of cigarette or bidi, alcohol and tobacco or gutkha. The age of initiation of these addictions was also asked.

3.1.7: Anthropometric Measurements

Anthropometry is the measurement of the physical dimensions characterising skeletal and tissue development and effect of relationship between nutrient and level of well-being of the body is assessed. The anthropometric measurements were conducted on the basis of the standard reference guidelines by Lohman et al 1988.

a) Weight: It is the most widely used and simplest reproducible anthropometric measurement. Weight indicates the body mass and is a composite of all body constituents like water, minerals, fat, protein, bone, etc. (Robinson et al 1998).

Technique Used – A platform weighing scale to the nearest 100 g was used to measure the weight. The subject was weighed in standard indoor clothing, bare feet and without

leaning against or holding anything. Scale was 'zeroed' before taking any weight, and was calibrated using standard weights after every third subject.

a) Height: It is a linear measurement made up of the sum of four components, i.e. Legs, Pelvis, Spine and Skull (Jellife 1966).

Technique Used – A spring-loaded non-stretchable tape was used to measure the height of the subjects. A convenient flat wall was identified at the clinic site for the measurement of the height. The subject was made to stand barefoot with the arms hanging freely by the side. Heels of the feet were placed together with the medial (inner) border of the feet at an angle of 60 degrees. The scapula and the buttock were ensured to be in contact with the measuring wall. The head was held in the Frakfort plane (with the tragus of the ear and the lateral angle of the eye in a horizontal line). Height was recorded to the nearest 0.1 cm after the subject inhaled fully and maintained the erect position without altering the load on the heels. In this position, a mark was made on the wall and height was recorded with a measuring tape. Two consecutive readings were taken.

b) Waist circumference: Circumference of the waist is an important indicator of the risk of cardiovascular disorders (CVDs) when calculated with Hip circumference to give Waist-Hip Ratio (WHR) (Walter et al 1996).

Technique Used – The subject was made to stand erect with the abdomen relaxed and arms at the sides. The circumference was recorded using the constant tension, spring loaded tape at the narrowest part of the abdomen between the ribs and iliac crest. This was done with measurer facing the subject and identifying the natural waist (i.e. the point of narrowing). The measurement was taken to the nearest 0.1 cm at the end of a normal expiration, without the tape compressing the skin.

c) Hip circumference:

Technique Used – Hip circumference (HC) was measured at the point yielding the maximum circumference over the buttocks. The constant-tension spring loaded

measuring tape was placed around the buttocks in a horizontal plane at this level without compressing the skin. The measurement was noted to the nearest 0.1 cm.

Computation of Anthropometric Indices

d) Body Mass Index (BMI): The BMI is a convenient and valid measure of adiposity. It is calculated as-

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

Table 3.1.6.4: Cut-offs for Body Mass Index (BMI)

Category	BMI
Underweight	<18.5
Normal	18.5-22.9
Overweight	23-24.9
Obese grade I	25-29.9
Obese grade II	30-34.9
Obese grade III	≥35

Asia Pacific Classification 2004

e) Waist-Hip Ratio (WHR): This ratio gives an idea of central adiposity. The calculation is done by-

$$\text{WHR} = \frac{\text{Waist Circumference (cm)}}{\text{Hip Circumference (cm)}}$$

Table 3.1.6.5: Cut-offs for Waist Hip Ratio (WHR)

Category	Males	Females
Waist (cm)	>90	>80
WHR > 0.9 (Males)	Obese	-NA-
WHR > 0.8 (Females)	-NA-	Obese

WHO Asia Pacific criteria for abdominal obesity (WHO Expert Consultation 2004)

3.1.8 Bio-physical Investigations

Blood Pressure: Blood pressure is the lateral pressure exerted by blood on the vessels walls while flowing in it. The blood pressure of the subjects was measured in relaxed sitting position on the left arm using the gold standard mercury gravity sphygmomanometer.

Technique Used- The blood pressure was measured after the subject had quietly seated for 5 minutes with legs uncrossed, back and arm supported using a stethoscope with *Diamond* mercurial sphygmomanometer in accordance to the American Heart Association Council on High Blood Pressure Research Recommendations. The standard cuff was suitable for the length and width of the subjects' arm and ensured that it should encircle 80 percent or more of the patient's arm circumference. The middle of the cuff on the upper arm was at level with the right atrium, at the midpoint of the sternum. Mercury column was deflated at 2 to 3 mm per second. The first and last Korotkoff's sounds were recorded as systolic and diastolic pressure respectively. Measurements were given to the nearest 2mm/Hg. It was observed that neither the patient nor the investigator was talking during the entire procedure (Pickering et al 2005). A range of three readings were taken after a 5 minute interval between each of the successive measure and thereafter an average of these was taken into consideration. The recommendations of the Joint National Committee VIII (JNC8) were adopted for screening of the blood pressure.

Table 3.1.8: Classification of Blood Pressure for Adults

Blood Pressure Classification	SBP (mmHg)	DBP (mmHg)
Normal	<120	<80
Prehypertension	120-139	80-89
Stage 1 Hypertension	140-159	90-99
Stage 2 Hypertension	≥160	≥100

Joint National Committee VIII (JNC8), 2014

3.1.9 Biochemical Estimations and Assay Methods

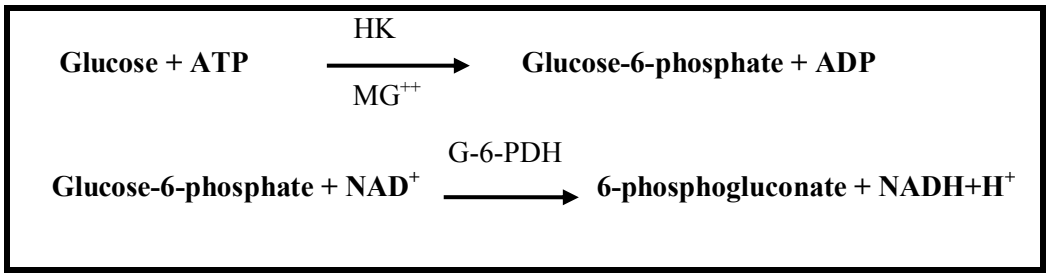
The subjects were instructed for an overnight fasting before the collection of their venous blood sample was collected in vacutainers by the use of disposable syringes having the respective EDTA, clot activator or gels for serum separation. These tubes may contain additional substances that preserve the blood before its processing. The serum was separated in these vacutainers and stored at a temperature of -80°C until analysed. The blood was then analysed for complete blood count, fasting blood glucose, glycated haemoglobin, vitamin B12, lipid profile, liver and kidney function tests using standardised kits.

a. Complete blood count (CBC)

The methods used to derive CBC parameters were based on the Beckman Coulter (1956) method of counting and sizing, in combination with an automatic diluting and mixing device for sample processing, and a single beam photometer for hemoglobinometry. The lytic reagent used for the complete blood count (CBC) parameters prepared the blood so the system can count leukocytes and sense the amount of haemoglobin. The lytic reagent rapidly and simultaneously destroys the erythrocytes and converts a substantial proportion of the haemoglobin to a stable pigment while it leaves leukocyte nuclei intact. The absorbance of the pigment is directly proportional to the haemoglobin concentration of the sample (ICSH, 1978).

b. Fasting blood sugar (FBS)

FBS was estimated using the accepted enzymatic reference method with hexokinase (Kunst et al 1984). Hexokinase (HK) catalyses the phosphorylation of glucose in the presence of adenosine-5'-triphosphate (ATP) and magnesium to form glucose-6-phosphate (G-6-P) and adenosinediphosphate (ADP). G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of nicotinamide adenine dinucleotide (NAD) to produce 6-phosphogluconate and NADH. One mole of NAD is reduced to one mole of NADH for each mole of glucose present. The absorbance due to NADH (and thus the glucose concentration) is determined using a bichromatic (340 and 383 nm) endpoint technique.



c. Glycated haemoglobin (HbA_{1c})

HbA_{1c} was quantified and assayed using IFFC and FDA approved automated dedicated high performance liquid chromatography (HPLC) technique (Geistanger 2008). The principle involved ion exchange of HPLC. The samples are automatically diluted on the d-10, injected into the analytical flow path, and applied to the analytical cartridge. The d-10 delivered a programmed buffer gradient of increasing ionic strength to the cartridge, where the haemoglobin is separated based on their ionic interactions, then pass through the flow cell of the filter photometer, where the change in the absorbance at 415 nm are measured. The d-10 software performs reduction of raw data collected from each analysis; two level calibrations are used for quantization of HbA_{1c} values. A sample report and a chromatograph are generated for each sample.

The A_{1c} area is calculated using an Exponentially Modified Gaussian (EMG) algorithm that excludes the labile A_{1c} and carbamylated peak areas from the A_{1c} peak area. HbA_{1c} covers all fractions; this includes labile HbA_{1a}, HbA_{1b} and HbA_{1c}. The

former two fractions are liable and hence do not represent the stable or long term change. HbA_{1c} represents the true long term glycemic control.

Table 3.1.9.1: Cut offs for HbA_{1c}

Classification	HbA_{1c}
Good	≤ 6
Borderline	7-8
Poor	> 8

American Diabetes Association 2007 standards

d. Vitamin B12

Serum Vitamin B12 was measured using electron chemiluminescence immunoassay (ELISA). The ADVIA Centaur CP VB12 assay is a competitive immunoassay using direct chemiluminescent technology in which vitamin B12 from the patient sample competes with vitamin B12 labeled with acridinium ester in the Lite Reagent, for a limited amount of purified intrinsic factor, which is covalently coupled to paramagnetic particles in the Solid Phase. Subsequently, adding of the starter reagents a flash chemiluminescent reaction is initiated. A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurs. This effects the separation of the antibody bound fraction after decantation or aspiration. Here, streptavidin $_{C.W.}$ refers to the streptavidin immobilized on well and immobilized complex means the sandwich complex bound to the solid surface. The light signal is measured by a photomultiplier as RLU within 3 seconds and is proportional to the concentration of VB12 present in samples (Tietz 1999). The assay uses Releasing Agent (sodium hydroxide) and DTT to release the vitamin B12 from the endogenous binding proteins in the sample & cobinamide to prevent rebinding after the Solid Phase is added to the sample. (Chen et al 1987).

$$AgAb_{Btn.} + {}^{Enz}AgAb_{Btn} + \underline{Streptavidin}_{C.W.} \Rightarrow \underline{immobilized\ complex}$$

Table 3.1.9.2: Cut offs for Serum Vitamin B12

Classification	Serum vitamin B12
Good	> 350 pg/ml
Borderline	≤ 350 pg/ml
Poor	≤ 200 pg/ml

WHO 1968, Lindenbaum 1988 standards

e. Total Cholesterol (TC)

Total cholesterol was estimated using end point enzymatic colorimetric technique. Cholesterol esterase (CE) catalyses the hydrolysis of cholesterol esters to produce free cholesterol which, along with preexisting free cholesterol, is oxidized in a reaction catalysed by cholesterol oxidase (CO) to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidise (HPO), the hydrogen peroxide thus formed is used to oxidize N, N diethylaniline-HCl/4-aminoantipyrine (DEA-HCl/AAP) to produce a chromophore that absorbs at 540 nm. The absorbance due to oxidized DEA-HCl/AAP is directly proportional to the total cholesterol concentration and is measured using a polychromatic (452,540, 700nm) endpoint technique.

Cholesterol esters $\xrightarrow{CE}$ **Cholesterol + Fatty Acids**

Cholesterol + O_2 $\xrightarrow{CO}$ **Cholest-4-ene-3-one + H_2O_2**

2 H_2O_2 + DEA•HCl/AAP $\xrightarrow{HPO}$ **4 H_2O + Oxidised DEA•HCl/AAP**

Table 3.1.9.3: Cut offs for Serum Cholesterol

Classification	Cholesterol Value (mg/dL)
Desirable	<200
Borderline High	200-239
High	≥ 240

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2002

f. Triglycerides (TG)

Enzymatic colorimetric method (GPO/PAP) with glycerol phosphate oxidase and 4-aminophenazone was used to assess triglycerides. Triglycerides are hydrolysed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol- 3- phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). In the presence of peroxidise (POD), hydrogen peroxide affects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form red coloured quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample (Fossati and Prencipe, 1982).

Triglycerides $\xrightarrow{\text{LPL}}$ **glycerol + fatty acids**

Glycerol + ATP $\xrightarrow{\text{GK}}$ **glycerol-3-phosphate + ADP**

Glycerol-3-phosphate + O_2 $\xrightarrow{\text{GPO}}$ **dihydroxyacetone phosphate + H_2O_2**

2 H_2O_2 + 4- aminophenazone + 4-chlorophenol $\xrightarrow{\text{POD}}$ **quinoneimine + 4 H_2O**

Table 3.1.9.4: Cut offs for Triglycerides

Classification	Triglycerides Value (mg/dL)
Desirable	<150
Borderline High	150-199
High	200-499
Very High	≥500

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2002

g. HDL Cholesterol (HDL-c)

HDL fraction of cholesterol was determined using enzymatic, colorimetric method (CHOD/PAP) without sample pre-treatment. The principle of HDL Cholesterol direct is based on the absorption of synthetic polyanions to the surface of lipoproteins. LDL, VLDL, chylomicrons are thereby transformed into a detergent resistant form, whereas HDL is not. Combined action of polyanions and detergent solubilises cholesterol from HDL, but not from LDL, VLDL, chylomicrons. Solubilized cholesterol is oxidized by the sequential enzymatic action of cholesterol esterase (CE) and cholesterol oxidase (CHOD). The hydrogen peroxide formed reacts with N, N-bis (4-sulfonyl)-m-toluidine (DSBmT) and 4-aminoantipyrine (AAP) in the presence of peroxidise (POD) and forms a red quinoneimine dye. The colour intensity of the red quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 552nm (Sugiuchi et al 1995).

LDL, VLDL, chylomicrons +polyanions ⟶ lipoprotein-polyanion

HDL + detergent ⟶ micelle complexes

Micelle complexes $\xrightarrow{\text{CE/CHOD}}$ **oxidised cholesterol + H_2O_2**

H_2O_2 + 4-aminoantipyrine + DSBmT $\xrightarrow{\text{POD}}$ **quinoneimine dye**

Table 3.1.9.5: Cut offs for HDL Cholesterol

Classification	HDL Cholesterol Value (mg/dL) Males	HDL Cholesterol Value (mg/dL) Females
Low	<45	<35
Optimal	45-55	35-45
High	>55	>45

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2002

h. LDL Cholesterol (LDL-c)

Enzymatic colorimetric method (CHOD/POD) was used for the direct estimation of LDL, HDL, VLDL and chylomicrons are specifically hydrolysed 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 colourless 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 a 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 et al 1995).

Step 1: **LDL, VLDL, chylomicrons +detergents ⟶ release cholesterol**

Cholesterol $\xrightarrow{\text{CE/CHOD}}$ **oxidised cholesterol + H_2O_2**

H_2O_2 + 4-aminoantipyrine + DSBmT $\xrightarrow{\text{POD}}$ **colourless product**

Step 2: **LDL + detergent ⟶ released cholesterol**

Cholesterol $\xrightarrow{\text{CE/CHOD}}$ **oxidised cholesterol + H_2O_2**

H_2O_2 + 4-aminoantipyrine + DSBmT $\xrightarrow{\text{POD}}$ **quinoneimine dye**

Table 3.1.9.6: Cut offs for LDL Cholesterol

Classification	LDL Cholesterol Value (mg/dL)
Optimal	<100
Near optimal/ Above optimal	100-129
Borderline High	130-159
High	160-189

NCEP- Adult Treatment Panel (NCEP-ATP III) Guidelines, 2002

Kidney and liver function tests

The blood samples were aspirated from the vein. Serum from each sample was separated and the biochemical parameters of liver and kidney functions tests included the following methods:

Kidney function tests

i. Blood urea

Blood urea in the test serum was detected by standard enzymatic method (UV KINETIC/GLDH). The ready-to-use urea agent is added in test tubes kept in a series forming ammonium and carbonate. α-Ketoglutarate and ammonium together with Nicotinamide adenine dinucleotide (NADH) gave glutamate and coenzyme NAD by catalysing with glutamate dehydrogenase (GLDH). The results were expressed in mg/dL (Fawcett and Scott 1960).

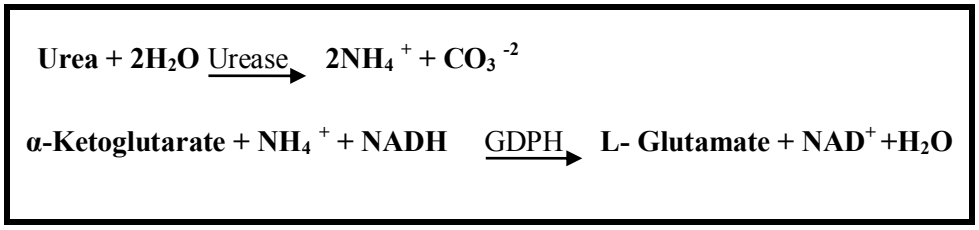


Table 3.1.9.7: Cut offs for Blood Urea (60-90years)

Classification	Blood Urea Value (mg/dl)
Normal	15-40
High	>40

Rose 1933

ii. Serum creatinine

The creatinine in test serum was estimated by standard enzymatic (Picric acid) method. The creatinine in alkaline solution reacts with picrate to form a red-orange compound. Under the specific conditions of the assay, the rate of development of the color is proportional to the concentration of creatinine in the sample when measured at 500nm (Henry et al 1974).

Creatinine + Picrate $\xrightarrow{\text{Alkaline medium}}$ **Orange color**

Table 3.1.9.8: Cut offs for Serum Creatinine (60-90years)

Classification	Serum Creatinine Value (mg/dl)
Normal	0.8-1.2
High	>40

Liver function tests

i. Aspartate amino transferase (SGOT)

The Aspartate amino transferase in the test serum was tested by standard enzymatic method (Siemens kit). The L-aspartate was added to α-ketoglutarate resulting in oxaloacetate and glutamate in presence of Aspartate Aminotransferase (AST). The reaction of oxaloacetate, NADH and hydrogen ion with catalyst Malate Dehydrogenase (MDH) yielded malate and coenzyme NAD. The result values were expressed in IU/L (IFFC, 1986a).

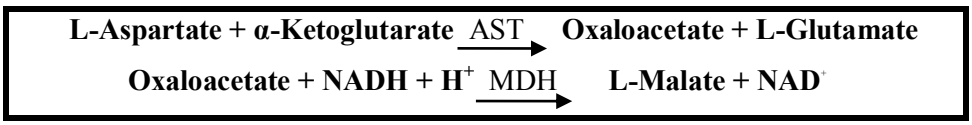


Table 3.1.9.9: Cut offs for SGOT (AST) (60-90years)

Classification	SGOT Value for Males (U/L)	SGOT Value for Females (U/L)
Normal	19 -48	9-36
High	>48	>36

ii. Serum Glutamic-Pyruvic Transaminase (SGPT) or Alanine transaminase (ALT)

The SGPT estimation was determined by standard enzymatic method (Siemens kit). The L-aspartate was added to α-ketoglutarate resulting in oxaloacetate and glutamate in presence of Alanine Aminotransferase (ALT). The reaction of oxaloacetate, Nicotinamide adenine dinucleotide (NADH) and hydrogen ion with catalyst Lactate Dehydrogenase (LDH) yielded malate and coenzyme NAD. Test values were expressed in IU/L (IFCC, 1986 b).

Table 3.1.9.10: Cut offs for SGPT (ALT) (60-90years)

Classification	SGPT Value (U/L)
Normal	30-65
High	>465

iii. Bilirubin (direct, total and indirect)

Conjugated (Direct) Bilirubin present in serum reacts with diazotized Sulphanilic Acid to yield Azobilirubin which absorbs at 546 nm. Total Bilirubin present in serum reacts

with diazotized sulphanilic acid in presence of activator to yield Azobilirubin, which absorbs at 546 nm (Jendrassik and Groff, 1938). The difference of the two values showed the indirect bilirubin value (Ochei & Kolhatkar, 2000).

Bilirubin + Diazotized Sulfanilic acid ⟶ Azobilirubin compound

Table 3.1.9.10: Cut offs for Bilirubin (direct, total and indirect)

Classification	Bilirubin (Total Value (mg/dl)	Bilirubin Direct Value (mg/dl)	Bilirubin Indirect Value (mg/dl)
Normal	0.3-1.2	0.-0.4	0-0.8
High	>1.2	>0.4	>0.8

3.1.9: Dietary Assessment

a. Food Frequency Questionnaire (FFQ):

The comprehensive listing of foods was bifurcated according to the food groups viz. vitamin B12 rich and omega-3 rich foods and the number of 8 vitamin B12 rich and 10 omega-3 rich foods were enlisted. The frequency of their consumption was acquired as per daily, two/three times a week, four/five times a week, once a week, occasionally and never basis (Appendix V). The details for each respective food item was thus elicited using this food frequency questionnaire

b. 24 hr Dietary Recall:

The 24 hr dietary recall procured from the group of individuals can be used to describe the 'customary' dietary pattern (foods that respondents consume on a typical day basis) of the population from where they are sampled, since the intra-individual dietary variation is insignificant as far as examination of the group level dietary pattern is concerned. A consecutive three day 24-hr dietary recall was taken at two junctures i.e. before and after the intervention (Appendix V). The questionnaire was administered by the interviewer owing to the better results obtained by this approach

as the interviewer's probing makes for forgotten or missed items to being possibly mentioned.

The 24 hr dietary recall is used to estimate an individual's food intake over a 24 hour period, referring to the previous day/night. All the subjects were asked for their 24 hour dietary recall to elicit information on the intake of nutrients as calories, proteins, fats, carbohydrates, vitamin B12, vitamin C, iron and omega- 3 fatty acids. The family members or relatives in presence of the subjects had to report of all the meals consumed throughout the previous day and the consumption of sweets, beverages, pickles, snacks, etc. was also recorded with addition sugar or salt, if any. The probing was done to cross check whether any food item is not missed out from the 24 hour dietary recall and it was ensured that no expression of opinion, feeling or suggestion was made as it could have affected the respondent's answer. The standardised set of utensils were shown before the respondents who were then encouraged to respond to the quantity, number and size of the food item consumed on its basis. The account of raw ingredients used in each of the reported preparations was obtained and weighed on Braun Scale with 10g sensitivity.The nutrient content of the diet was calculated by the ICMR food composition tables as described in the 'Nutritive Value of Indian Foods' (Gopalan et al 2004) and by using NSI Diet Calculator (2013).

PHASE II

Food product development using omega-3 fatty acid rich flaxseeds and sensory trials.

Section 3.2.1: Selection of locale for procurement of flaxseeds

Raw flaxseeds from the Agricultural Produce Marketing Cooperative (APMC) market and other auxiliary ingredients for developing the food products were procured from the local grocery market of urban Vadodara. The seeds were then cleaned, graded and stored in pet jars till further use.

Section 3.2.2.: Identification of food products for preparation

An exercise was conducted for recognition of roasted flaxseeds incorporated Indian food items being consumed at household levels and suitable for elderly population.

They were then subjected to the organoleptic assessment based on the substitution method. A total number of four food items were subsequently finalised on the basis of their suitability for integration. These food products included for roasted flaxseeds incorporation were *Khichdi, Porridge, Globs* having varied addition of roasted flaxseeds and *Mukhwaas*, which had an amount of 20g roasted flaxseeds. These all developed food products were assessed for their organoleptic attributes.

Section 3.2.2: Procurement details of auxiliary raw ingredients for food product development

Apart from the raw flaxseeds, else all of the other ingredients for developing the food product were acquired from the local grocery market of urban Vadodara. The inclusive list of raw ingredients with their respective procurement sources depending on the type of recipe is enlisted in Table 3.2.2.1.

Table 3.2.2.1 Inclusive list of raw ingredients with their procurement sources used for product development

Product	Ingredients	Brand name/ Procurement source
*Khichdi**	Rice	Laxmi
	Green gram dal	Laxmi
	Oil	Ankur
*Porridge**	Milk	Amul Taaza
	Gruel wheat	Uttam Fada
	Sugar	Madhur
*Globs**	Flaxseeds	APMC market, Baroda
	Jaggery	Krutika
*Mukhwaas**	Flaxseeds	APMC market, Baroda

* The detailed recipes of developed food products are affixed in Appendix VI

Section 3.2.2: Proximate and fatty acid analysis through gas chromatography (GC) of raw versus roasted flaxseeds

Under this study phase, the proximate and fatty acid analysis was performed to establish the nutrient and fatty acid profile of the raw versus roasted flaxseeds before being incorporated in food items to be developed for the MCI elderly. These are sub-divided into the following sections:

Section 3.2.2.1: Calculation of nutrient values of raw and roasted flaxseeds

The nutrient analysis of flaxseeds was carried out owing to paucity of literature citing the composition of Indian flaxseeds and considered worthwhile, for formulating the food product attuned to geriatrics with morbidities. Standard procedures were applied for the estimation of energy (FAO 2003) and carbohydrate (FAO 2008) content by the researcher in an ISO 9001:2008 accreditated lab. The protein analysis was performed using Kjeldahl method (Bureau of Indian Standards 1985). Fat was determined by the Soxhlet extraction method in which fat dissolved in solvent (petroleum ether) is estimated by vapourizing the solvent (Bureau of Indian Standards 2003). Crude fibre was estimated using acid and alkaline digestion method (Bureau of Indian Standards Reaffirmed 2005). The calcium content was being analyzed by volumetric method (Bureau of Indian Standards Reaffirmed 2003).

Section 3.2.2.2: Comparative estimations of α-linolenic acid (ALA) content in raw as well as roasted flaxseeds by GC technique

This section of the study was concerned with the comparative determination of α-linolenic acid (ALA) content in raw as well as roasted flaxseeds by GC technique.

a) Analytical procedure for samples:

The samples were analysed by CIC, 2011 which is based on procedure described by AOCS (2005) method.

Principle: GC technique used for the analysis of ALA content of raw and roasted flaxseeds worked for the quantification of saturated, unsaturated, polyunsaturated fatty acids, etc. by using the capillary column of high polarity and Flame Ionisation Detector. The first step was oil extraction from the flaxseeds i.e. roasted and raw for the purpose of loading them separately into the gas chromatogram (GC). On passing through the GC tube, the liquid sample vaporised into gas and subsequently came across with inert gas, acting as a carrier. Many constituents of the sample gas travelled through the long coiled tube at different rates and reached the detector at the different points of time. The detector was able to take all the signals and showed them on the

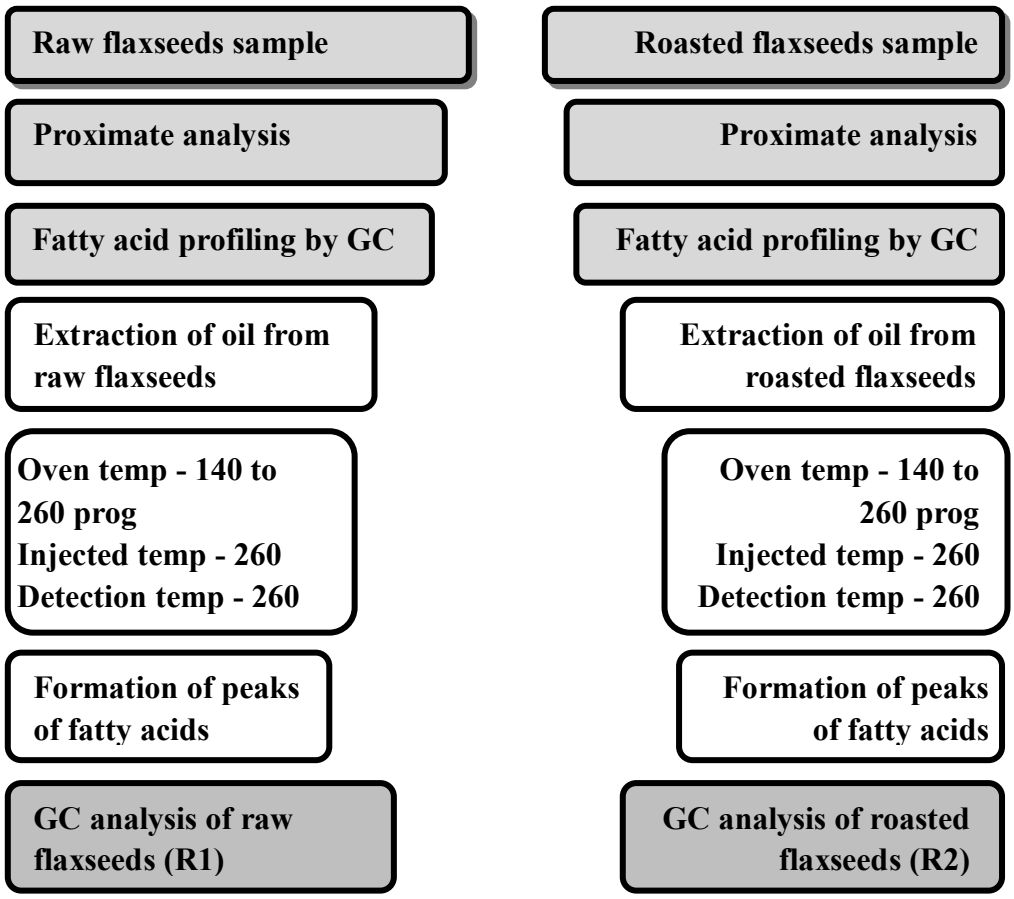
chromatograph depicting the amount in which a particular constituent was received and the times taken by each to finally reach there.

Determination of the ALA content in the samples:

a) Calculation and interpretation of the results: The ALA content in the samples was calculated by plugging the values in the area percentage peaks on the basis of the formula:

$$\text{ALA Area \% in Raw/ Roasted Flaxseeds} = \frac{\text{Area of Peak (alpha-linolenic acid) x 100}}{\text{Area of Peak (alph linolenic+linoleic+arachidonic+oleic+stearic+palmitic)}}$$

b) Estimation of final percentage of flaxseeds substitution



American Oil Chemists Society (AOCS), 2005

Figure 3.2.2.1: Analytical procedure for raw and roasted flaxseeds using GC technique

Section 3.2.2.3 Standardisation and percent substitution of flaxseeds into food products:

a) *Khichdi:* Khichdi was made adding 200 grams of rice and 100 grams of green gram dal. Then water was put in the 1:6 ratio to get the required consistency (Appendix IV). The base materials were substituted with roasted flaxseeds at the 10g, 15g and 20g levels.

b) *Porridge:* Porridge was prepared using 100 g of wheat gruel, 200 ml of milk, 10 g of sugar cooked over medium flame (Appendix IV). The roasted flaxseeds stood-in at the 10g, 15g and 20g levels with the base materials.

c) *Globs:* The flaxseeds were subjected to roasting on medium flame for up to 7 min and ground coarsely. The jaggery was melted in a pan with 5 ml of water and ground flaxseeds were added. The mixture was stirred uniformly for about 2 min. The globs were then rolled out while they were warm for instant binding. The globs were cooled at room temperature (30°C) (Appendix IV). At the 10g, 15g and 20g levels the roasted flaxseeds were appended with the base materials.

d) *Mukhwaas:* The flaxseeds were roasted on medium flame for up to 7 min (Appendix IV). Thereby choosing of the 20 g level of mukhwaas.

Section 3.2.2.4: Organoleptic evaluation of the roasted flaxseeds formulated recipes

The recipes developed were assessed for the organoleptic evaluation of the roasted flaxseeds formulated recipes (Figure 3.2.2.6.1 [a-d]). The tests used are explained as follows:

3.2.2.5: Screening of assessors based on training of organoleptic evaluation

a) **Selection of panel members:** Under this section, the set of semi-trained and untrained panellists were chosen. Semi-trained panellists constituted of Professors and doctoral students from the Department of Foods and Nutrition. They were

research personnel possessing strong background in the sensory food evaluation. The jury consisted of 30 semi-trained and 100 untrained panellists each (Figure 3.2.2.6.2). The semi trained panellists were in the range of 30–62 years whereas the untrained panellists were in the age group of 60–85 years. They measured the consumer acceptability of the developed food products and critically evaluated the dimensions of individual quality characteristics discriminating the samples with reference to the hedonic scale and composite scoring test. Afterwards, the results were tallied for preference using both of these scales from the untrained panellists also who conducted sensory evaluation of the flaxseed formulated products.

3.2.2.6 Categorisation of organoleptic tools for undertaking sensory evaluation

a) **Hedonic rating scale (Swaminathan 1995):** Hedonic rating scale refers to a range of the nine point hedonic rating scale. The panel members were subjected to measure the palatability effect of flaxseed globs on ‘‘like extremely’’ to ‘‘dislike extremely’’. The former response comprised a score of 9 whereas the latter carried a score of 1 (Appendix VI [i]).
b) **Composite rating score (Joshi 2006):** Composite rating score is termed as the 7-point composite rating score applied to ascertain the most suitable variant characterised from the specific organoleptic attributes namely; taste, appearance, odour, texture, absence of defects, suitability of serving size and overall acceptability. Absence of defects is referred to the suitable roasting, appropriateness in consistency, inexistence of any bad odour and flavour, lack of non-edible stuff (as husk/chaff, grit, worms, beevils, plastic films, dirt, and hair), etc. It’s not describing appearance here as appearance has been put under a separate attribute category for score determination. The selected panellists rated the variants separately on their specific attributes. The individual scoring was assigned. Out of a total score of 100, maximum score kept for taste was 20, 10 for appearance, 10 for odour, texture scored 15, 10 for absence of defects, suitability of serving size was 15 and overall acceptability at 20 (Appendix VI [ii]).
c) **Score card preparation for organoleptic evaluation of formulated products:** Score cards were developed for organoleptic evaluation (Appendix) for both of the sensory rating scales used in the study (Appendix VI [i]), VI [ii]).

3.2.2.8 Study product and mode of intervention

It was ensured prior that the developed food product was hypolipidemic, nutritious, easy to cook, palatable, acceptable and low in cost. The pilot study was carried on 50 subjects for determination of acceptability of roasted flaxseeds. Thereafter the patients were advised to consume 10 g of roasted flaxseeds each post lunch and post dinner.

Section 3.2.2.9: Statistical analysis

The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS 20.0v) software. Results obtained were expressed as mean ± standard deviation values for all four roasted flaxseeds food formulations. The analysis of variance (ANOVA) and Spearman's correlation coefficient were used to analyse the significant difference between the four levels of constitutions. Percent increase and decrease were also observed to evaluate the strength of likeability amongst all four variants.

a) Khichdi

b) Porridge

c) Globs

d) Mukhwaas

Figure 3.2.2.6.1(a-d): Roasted flaxseeds incorporated *khichdi*, *porridge*, *globs* and m*ukhwaas*

Figure 3.2.2.6.2: Semi-trained panelists performing organoleptic evaluation of formulated flaxseed recipes

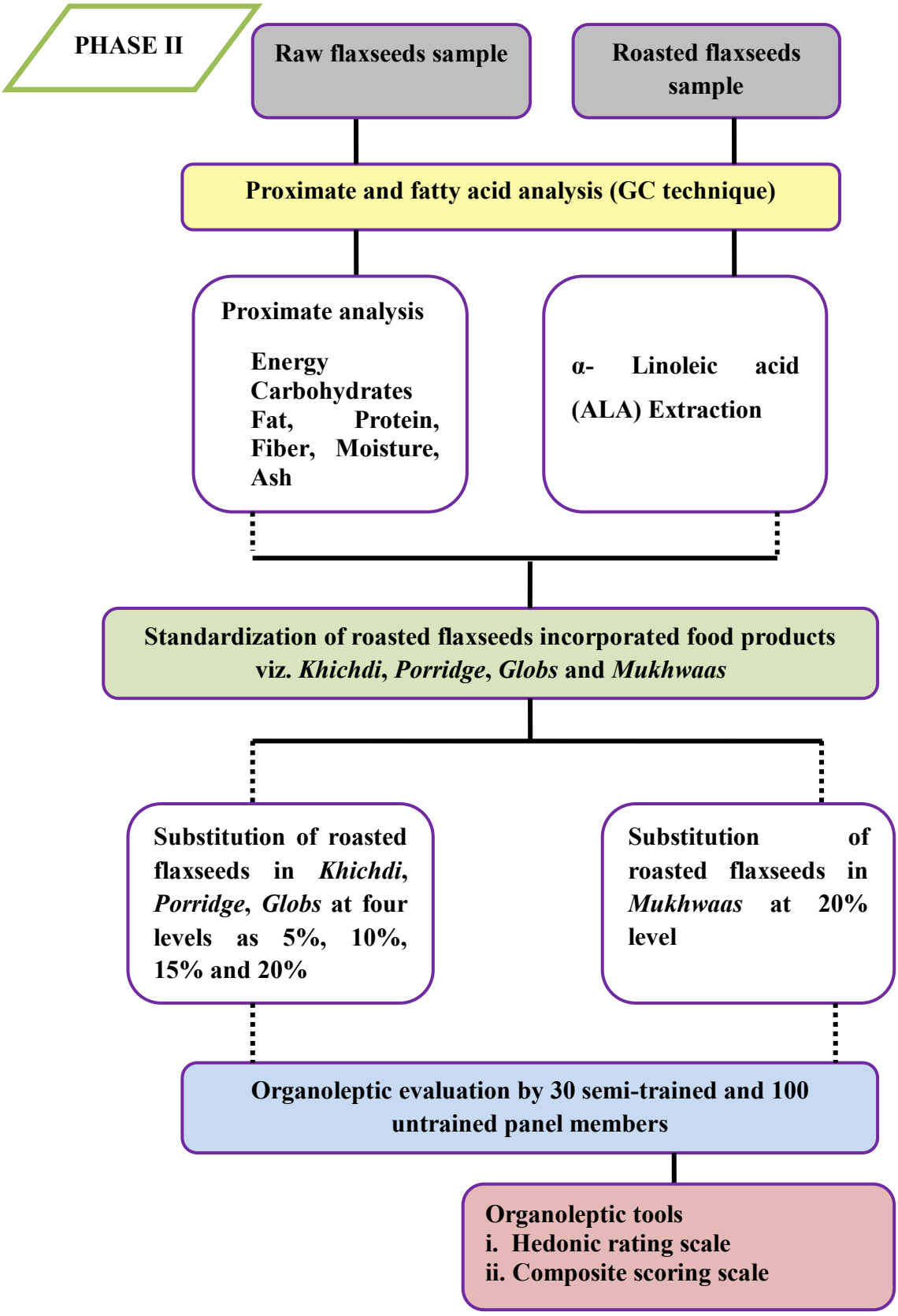


Figure 3.2.1: Experimental design of Phase II of the study

PHASE III

Intervention and impact evaluation of the MCI elderly group with Omega -3 fatty acids and Vitamin B_{12} supplementation.

Section 3.3: Dual therapeutic interventional approaches with food formulation and methylcobalamin dosage

The interventional trial was conducted in double arms of vitamin B12 (methylcobalamin) injectable doses in the first arm and second arm consisted of vitamin B12 (methylcobalamin) injectable doses with roasted flaxseeds (20 g) on a dose-dependent basis. The dose for first group taking B_{12} intramuscular injections was under the prescribed regime by the API (Association of Physicians of India, 2012) as 1000 µg everyday for one week, followed by 1000 µg every week for 4 weeks and then 1000 µg monthly dose for a period of four months; second group was supplemented with omega-3 fatty acid (20 gm flaxseed per day providing approx. 2.6 mg of ALA). In the second group, to rule out any toxic effects posed by 20 g of flaxseed per day, a pilot study was conducted on the patients having supplemented with 20 g flaxseeds and no deleterious effects of flaxseeds were assessed on their kidney and liver function profiles. Some willing patients for the trial were restricted from intervention owing to deaths, migration abroad and morbidities. Age classification for intervened elderly was based on criteria by Pantelic et. al. 2012.

After the period of six months, the outcome was observed on the parameters namely, cognitive function tests, nutritional status, blood pressure, complete haematological profile, fasting blood glucose, glycated haemoglobin, serum lipids, serum B_{12}, kidney and liver function tests particularly for those supplemented with flaxseeds for a thorough check of any detrimental health effect if caused, post flaxseed supplementation.

Section 3.3.1: Statistical Analysis and Impact Outcome

The data entry was done in MS Excel spreadsheet. The data was cleaned, sorted, verified and thereafter subjected to appropriate statistical analysis. Data was analysed using Statistical Package for Social Sciences (SPSS 20.0v) software. Results were expressed as mean ± standard deviation values. Paired *t*-test was applied to analyse the

supplementation effects on both the groups on anthropometric, biophysical, dietary, glycemic, lipemic, atherogenic, serum vitamin B12 and CBC, neuropsychological test battery. The level of significance was estimated at 5% two-tailed testing. Student *t*-test was performed between the Group1 of vitamin B12 and Group 2 of vitamin B12 plus flaxseeds supplementation for comparative analysis of numerous anthropometric, biochemical and neuropsychological testing parameters. Pearson's correlation was determined amidst glycemic factors with serum vitamin B12 and ALA intake. Also for change in anthropometric and biophysical factors, neurological and nutritional assessment scores with change in serum vitamin B12 and ALA levels.

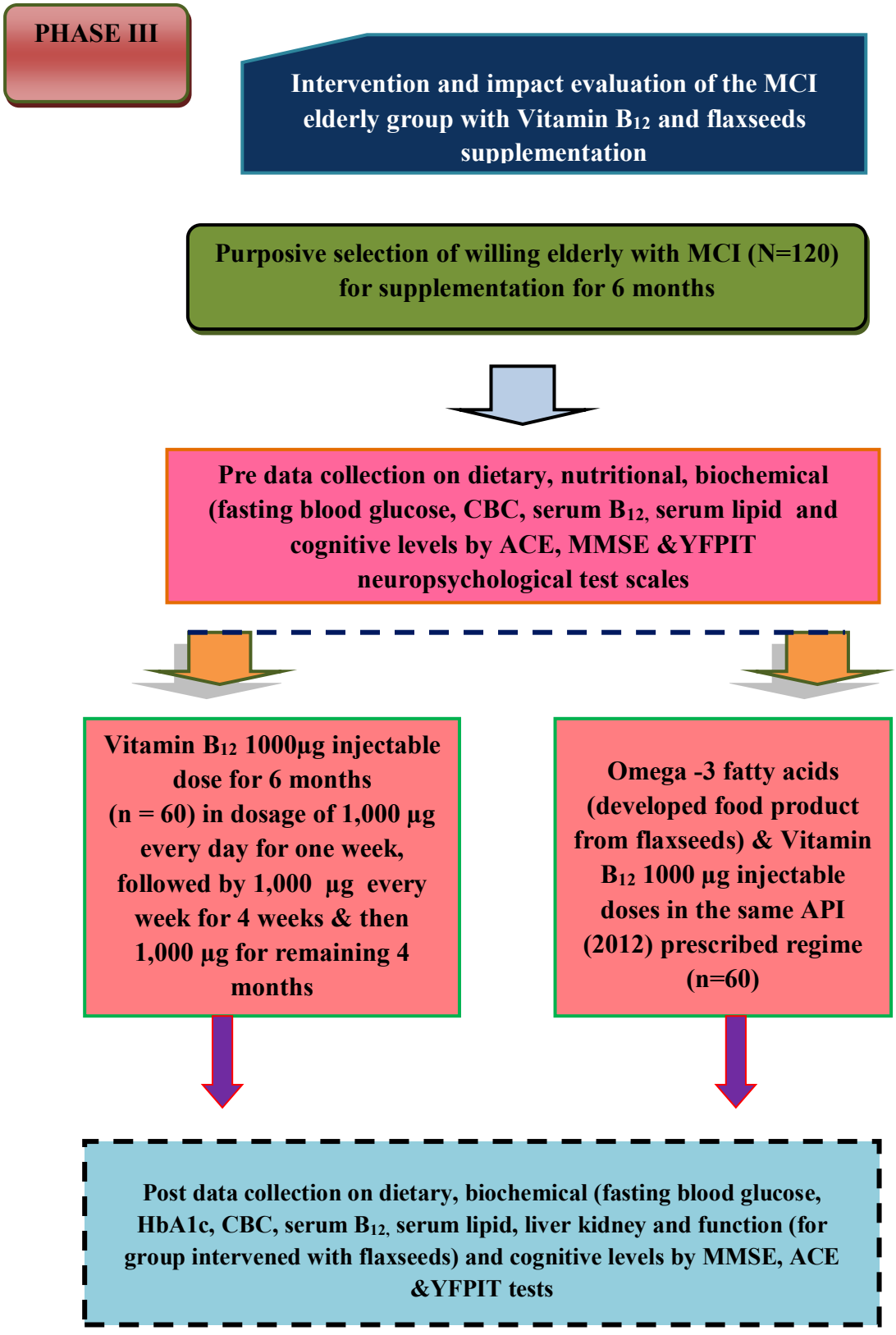


Figure 3.3.1: Experimental design of Phase III of the study