

Methodology

The methodology followed in the present study entitled “**Evaluation of a Functional Food Supplement on Body composition of Obese Young Adults and Influence of a selected PPAR Gamma Gene Polymorphism on its Outcome**” is outlined in the following phases.

A. PHASE I: SELECTION OF THE AREA AND GROUPING OF THE SUBJECTS

1. Selection of the area and grouping of subjects
2. Screening of the subjects for obesity and overweight
3. Grouping of the subjects as obese, overweight and normal categories

B. PHASE II: DEVELOPMENT, STANDARDIZATION AND EVALUATION OF FUNCTIONAL FOOD SUPPLEMENT.

1. Formulation and Standardization of the functional food mix
2. Organoleptic evaluation
3. Nutrient analysis
4. Identification and quantification of phytochemical components
5. Analysis of enzymatic and non-enzymatic antioxidant activities
6. Analysis of *in vitro* radical scavenging activity
7. Determination of pancreatic lipase inhibitory effect
8. Quality analysis of food supplement

C. PHASE III: ASSESSMENT OF SOCIO ECONOMIC, NUTRITIONAL STATUS AND BODY COMPOSITION MEASURES

1. Formulation of the questionnaire
2. Assessment of socio-economic status
3. Anthropometric assessment

4. Body composition assessment by Bioelectrical Impedance Analysis (BIA)
5. Assessment of food and nutrient intake

D. PHASE IV: COMPUTATION OF TOTAL ENERGY EXPENDITURE

1. Computation of Total Energy Expenditure (TEE)
2. Computation of energy balance

E. PHASE V: ANALYSIS OF PPAR GAMMA GENE POLYMORPHISM AND CONDUCT OF INTERVENTION STUDY

1. Analysis of Pro 12 Ala polymorphism of PPAR Gamma gene
2. Assessment of biochemical parameters
3. Assessment of metabolic syndrome as per ATP III Criteria
4. Components of the intervention study
5. Conduct of the intervention study
6. Evaluate the impact of intervention

F. VALIDATION AND INTERPRETATION OF DATA

The study was presented before the Institutional Human Ethical Committee of the Avinashilingam University and ethical approval was obtained (**AUW/IHEC13/FHP-02**). The trial was also registered in Clinical Trial Registry of India (CTRI) of ICMR and trial registration number was obtained (**CTRI/2014/11/005222**)

Information sheet clearly explaining the objective, nature and duration of the study was prepared in English and the local language Tamil and was distributed to all the participants. Oral as well as written consent was obtained from all the participants. The subjects were instructed to follow the procedures as per the protocol of the project. It explained that no incentives or monetary benefits will be provided for participating in the study. The subjects were given full freedom to withdraw at any point of time. The respondents were given confidentiality assurance with regard to the personal data collected.

A. PHASE I

SELECTION OF THE AREA AND GROUPING OF SUBJECTS

The map (figure 1) shows the location of study namely Tamil Nadu in India

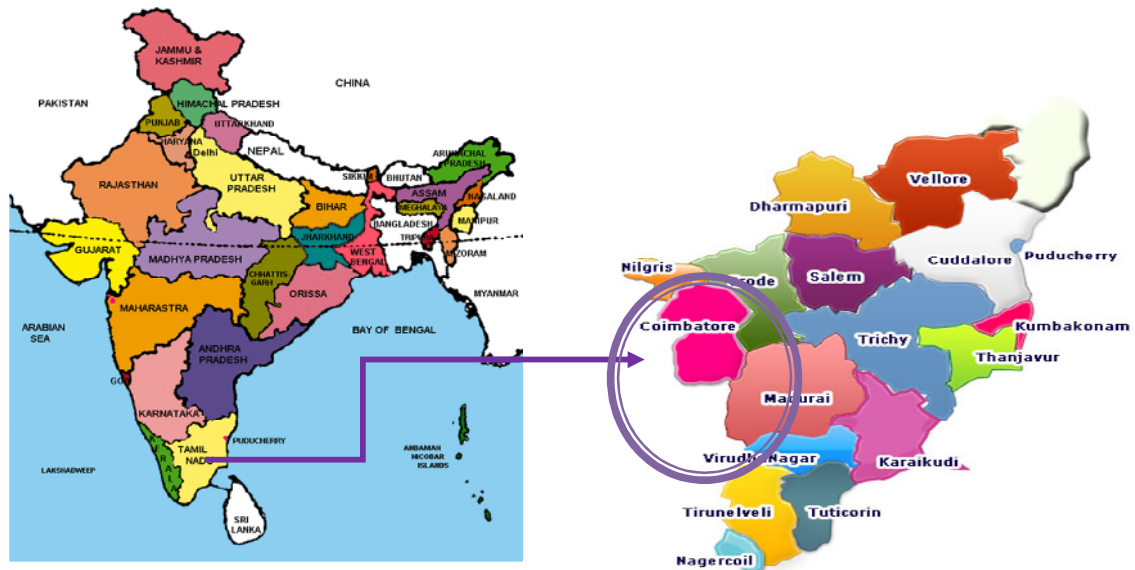


Figure 7
Map of India and Tamil Nadu

1. SELECTION OF THE AREA AND GROUPING OF SUBJECTS

The urban area of Coimbatore district was selected. Young adults in the age group of 19 – 24 years were recruited. Young adults were selected in order to inculcate lifestyle and food modification at the earliest time possible in their life. Creating awareness on obesity and overweight might also pave way for prevention strategies. Both male and female subjects were included in the study. The authorities of various colleges in Coimbatore were approached for their consent to recruit subjects and conduct the study. The duration, nature and aim of the study were explained to them for obtaining consent. A total of 1873

participants from different colleges were randomly selected for screening based on their willingness to participate in the study.



Plate 1

Participants signing consent form

2. SCREENING OF THE SUBJECTS FOR OBESITY AND OVERWEIGHT

The subjects were screened for obesity, overweight and normal category using the BMI latest Asian cut-off (WHO, 2000). Out of 1873 participants, a total of 150 subjects were chosen for further study. Based on the BMI, the subjects were divided into three groups viz obese, overweight and normal. Out of 150 subjects, 75 subjects were male and 75 subjects were females. Purposive sampling technique was used for this selection. Before recruitment of subjects for trial, the investigator explained the nature and duration of the study in regional language. Prior to the conduct of the study, the subjects were well informed about the kind of supplement, physical activities expected to perform, biochemical analysis, nutrition education sessions and all the procedures to be followed throughout the study. Oral as well as written consent were obtained from all the participants for the same. The inclusion and exclusion criteria were strictly followed for the recruitment of subjects.

The inclusion criteria were:

- Subjects willing to participate and sign consent.
- Subjects between 18- 24 yrs of age
- Subjects with BMI above 23

- Subjects with sound mental health

The exclusion criteria were:

- Subjects not willing to give a written consent.
- Subjects below 18 and above 23 yrs of age.
- Subjects enrolled in weight loss programmes.
- Subjects enrolled in other clinical trials
- Subjects with complication of DM or CVD

The subjects in each category were carefully scrutinized for participation in the study with the above inclusion and exclusion criteria.

a. Anthropometric measurements

Anthropometric indicators emerge as simple, easily accessible, and non-invasive measures that can diagnose central adiposity (Farb and Gocke, 2015)

Body Mass Index (BMI) is a measure of overall adiposity, whereas, Waist Circumference (WC), Waist-Hip Ratio (WHR), and Conicity Index (CI) are reliable proxy measures of abdominal fat (Kopelma, 2000). Studies indicate that BMI, WC and WHR could be used independently to identify overweight and obesity (Gill *et al.*, 2003). These measures of adiposity have also been widely recommended for epidemiological surveys because of their independent association with major cardiovascular and metabolic risk factors. Findings indicate that WHtR, WC, Conicity index, and BMI can be used as high-sensitivity screening methods for assessing abdominal adiposity (Sousa *et al.*, 2016). Body fat distribution can be assessed by different methods, especially by anthropometric measurements since they have the advantage of being relatively simple, inexpensive, and non-invasive

b. Measurement of height and weight

Height and weight were measured initially and BMI was calculated. The height of the subjects were measured in standing position with bare foot as measured to the nearest of 0.1 cm. Body weight was measured using digital weighing balance to the nearest of 0.1 kg. The subjects were asked to empty bladder before weight measurement and they were asked to wear light clothing

during measurement. The waist circumference was measured using a flexible inch tape to the nearest of 0.1 cm at midway between the lower costal margin and the iliac crest while the hip circumference was measured over the buttocks using the same. All the measurements were taken twice to ensure accuracy.

PLATE 1



Measurement of Height



Measurement of Weight

Plate 2

c. Calculation of Body Mass Index (BMI)

Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m²). BMI was calculated using the formula.

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

d. Measurement of Waist Hip Circumference

Waist circumference (WC) is an accurate and simple measure of abdominal obesity as compared to waist–hip ratio (WHR) Ahmad *et al.*, 2016).

Waist circumference was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest, using a stretch resistant tape. Hip circumference was measured around the widest portion of the buttocks, with the tape parallel to the floor.

For both measurements, the subject was instructed to stand with feet close together, arms at the side and body weight evenly distributed. The measurements were taken at the end of a normal expiration. Each measurement was repeated twice when the difference in the measurements were within 1 cm of one, the average was taken. When the difference between the two measurements exceeded 1 cm, the two measurements were repeated.

e. Calculation of Waist Hip Ratio

The waist to hip ratio (WHR) is a simple measure of central obesity. The score from the WHR predicts the risk of developing several conditions associated with excess abdominal fat

Waist hip circumference was calculated using the formula.

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

f. Calculation of Waist to Height Ratio

World Health Organization (WHO, 2008) guidelines state that alternative measures that reflect abdominal obesity such as WC, WHR, and waist-to-height ratio (WHtR) have been found to be superior to BMI. The waist-to-weight ratio (WHtR) is increasingly used as an indicator that considers the increase in waist relative to height (Faria *et al.*, 2014). Waist-to-height ratio (WHtR) was calculated by using the formula:

WHtR = Waist Circumference (cm)/Height (cm)

g. Calculation of Conicity Index(CI)

The Conicity Index (CI), which assesses the relationship between body weight, height, and waist circumference, is a key indicator of central obesity. Individuals with a lower accumulation of fat in the central area have a body shape like a cylinder and those with greater accumulation of fat have a body shape like a double cone (i.e., two cones with common base). Conicity index (C index) was determined by using weight, height, and waist circumference through the following mathematical equation:

$$\text{C index} = \text{WC (cm)} / 0.109 \sqrt{\text{body weight (kg)} / \text{height (m)}}$$

h. Calculation of Body Adiposity Index

BMI has been criticized as frequently being an inaccurate measure of body fatness. Recently, Bergman *et al.*, 2011 proposed a new index called the Body Adiposity Index (BAI) that reflects body composition. The Hip circumference and height were used to calculate the BAI using the following equation as suggested by Bergman *et al.* (2011).

$$\text{BAI} = \text{Hip circumference in cm} / \text{Height in m}^{1.5} - 18$$

3. GROUPING OF THE SUBJECTS AS OBESE, OVERWEIGHT AND NORMAL CATEGORIES

The subjects were divided into Experimental Group-I (obese), Experimental Group II (overweight) and Control (Normal) based on BMI cut off for Asians recommended by World Health Organization (WHO), International Association for the Study of Obesity (IASO) and International Obesity Task force (IOTF), 2000

TABLE 1
BODY MASS INDEX CUT-OFF FOR ASIANS

Criteria	Body Mass Index Cut off for Asians
Obese	< 25
Overweight	23 – 24.9
Normal	18.6 -22.9
Under weight	<18.5

Source:WHO, IASO, IOTF (2000)

a. Categorization of Groups Based on BMI

i. Experimental Group I (Obese)

Accordingly to the modified BMI cut-off (WHO, 2000), subjects who had BMI greater than 24.9 were grouped under this category. The group comprised a total of 50 subjects, comprising of anequal number of males (25) and females (25).

ii. Experimental Group II (Overweight)

According to the latest BMI of the subjects those who had BMI between 23 and 24.9 were categorised under experimental group II. It consisted of a total of 50 subjects, with equal number of males (25) and females (25).

iii. Control Group (Normal):

Normal healthy subjects served as control. Subjects who had a BMI of 18.5 to 22.9 were categorised under this group. The group consisted of a total of 50 subjects, with 25 males and 25 females.

B. PHASE II

DEVELOPMENT, STANDARDIZATION AND EVALUATION OF FUNCTIONAL FOOD SUPPLEMENT

The development of the functional food was carried out meticulously through the following steps

1. FORMULATION AND STANDARDIZATION OF THE FUNCTIONAL FOOD MIX

Oats, defatted soy flour, wheat bran, flaxseed, foxtail millet, horse gram dhal and jaggery were chosen to develop the functional food supplement. The ingredients were purchased on weekly basis from the market and cleaned thoroughly thrice. They were sundried for one day between 10 am to 2 pm to remove excess moisture. All the ingredients were roasted separately under slow flame in a shallow pan until raw smell disappeared and golden brown colour was developed. Care was taken not to overheat the ingredients. The roasted ingredients were ground into a powder separately in a mechanical pulverized mill. It was then spread evenly over a clean sheet to enable quick emission of heat, and brought to room temperature. The ground ingredients were mixed in various proportions for standardization of the functional food. Ten per cent of jaggery in the form of syrup was added to the functional food supplement. It was used for binding as well as improving the palatability of the food. The ground ingredients were kept in ziplock covers and placed in air tight containers to retain shelf life and to prevent quality deterioration of the product. The details of various ingredients included for preparation of the supplement are given below

a. Flax seeds (*Linum usitatissimum*)

Flax (*Linum usitatissimum*) belonging to family Lineaceae, is a blue flowering annual herb that produces small flat seeds varying from golden yellow to reddish brown color. Flaxseed possess crispy texture and nutty taste (Rubilar *et al.*, 2010). Flaxseeds are excellent source of natural antioxidants and ranked top among plant phenolic compounds (Kasote,

2013). India ranks first among the leading flaxseed producing countries in terms of acreage accounting 23.8 per cent of the total and stands third in production contributing to 10.2 per cent of the world's production. Flaxseed is considered as functional food owing to the presence of three main bioactive components namely alpha-linolenic acid, lignans and dietary fiber. (Kajla. *et al.*, 2015). Viscous dietary fibers have been shown to induce an increased sensation of satiety and lowered energy intake at the following meal (Lee *et al.*, 2006). Consumption of 50 g flaxseed reported a reduction of 8 per cent plasma cholesterol and fivefold increase in total urinary lignan excretion along with 30 per cent increase in bowel movements per week. Flaxseed under the condition of storage and processing technology is considered as harmless food product. Consumption of 50g/ day of flax seed showed no adverse effect in humans. (Martinchik *et al.*, 2012). Lucas *et al.* (2004) found the reduction of cholesterol in the plasma and arteriosclerotic lesions after incorporation of flax mucilage and alpha linolenic acid into diet. These characteristics with functional and bioactive properties make flax seed as an attractive source of functional ingredient for the preparation of food supplement (Rubilar *et al.*, 2010).

b. Defatted soy flour:

Soy and its products play a vital role as functional foods. According to FDA claim, 25g of soy protein per day, as part of a diet may reduce the risk of heart disease. Evidence suggests that diet rich in phytoestrogen (isoflavone and lignans) namely soy protein may have beneficial effect on many aspects of obesity and diabetes. (Bhathena and velasquez, 2002). Soy may improve obesity and diabetes by reducing insulin resistance and reduce adiposity by inhibiting insulin secretion from the pancreatic beta cells or by inhibiting lipogenesis and enhancing lipolysis in liver and adipocytes. Isoflavones and lignans may also exert beneficial effect on tissue lipids through their antioxidative actions. Various studies in animals and humans indicate that protein from soy exhibit antiobesity effects. Soy beans are well known sources of isoflavones.

Botanical compounds such as isoflavone may be agonists or activators of the “promiscuous” PPAR nuclear receptors regulating lipid metabolism in the cells and glucose tolerance. (Mezei *et al.*, 2003). Isoflavone intake alters lipid metabolism in a manner consistent with activation of PPAR gamma and also via PPAR gamma independent mechanism. (Mezei *et al.*, 2006). Possibly mediate by PPAR gamma activation. In particular genistein has been identified as a ligand of PPAR gamma receptor.

Epidemiological studies have proven that diet rich in soy isoflavones are associated with lower rates of CVD, osteoporosis, cancer and obesity related complication. FDA approves foodlabelling health claim for products containing 25g of soy. Specifically it helps in cholesterol reduction. Intake of 15 to 20g of soy / day containing about 50-90mg of isoflavone is recommended (Messina, 2004). Numerous nutritional intervention in animals as well as humans indicate that consumption of soy protein reduces body weight and fat mass in addition to lower

plasma cholesterol and triglycerides (Velasquez and Bhathena 2007). Deibert *et al.*, 2004) demonstrated a diet with high soy protein and low fat diet lowered LDL cholesterol as well as improve body composition by increasing the ratio of lean body mass to fat.

i. Preparation of Toasted Defatted Soy Flour

Toasted defatted soy flour is the enzyme inactive soy flour manufactured by using high quality soy bean seeds as raw material. Soy beans are cleaned twice to remove all foreign materials. After cleaning, the beans are conditioned, cracked, dehulled and rolled into flakes. The flakes are treated in a solvent bath that extracts the oils. After highly efficient extraction, it is desolventised and toasted to the required PDI of 20-35 per cent and ground to ultrafine quality through the ACM and sieved through vibro measures of particles size 80 – 200. The toasted defatted soy flour thus prepared was purchased and used for the development of functional food mix.

c. Wheat Bran



Wheat bran is a byproduct obtained during wheat processing. The bran fraction is a by-product of milling and has food (Curti *et al.*, 2013) and was commonly used for nonfood applications (Apprich *et al.*, 2013). The use of wheat bran for human consumption has increased gradually over the years. Globally, the number of wheat bran incorporated food products increased from 52 in 2001 to approximately 800 in 2011 (Pruckler *et al.*, 2014). Wheat bran is rich in minerals, fibre, B vitamins and bioactive compounds which are known to possess health-promoting properties (Reisinger *et al.*, 2013). Evidence from epidemiological studies suggests an inverse relationship between intake of dietary fibre, weight gain and obesity. Since fibre consumption is associated with increased satiety and decreased energy intake. (Freeland *et al.*, 2009). Studies using wheat bran also report that a reduction in food intake

following a test meal with wheat bran. Hence due to the above advantages, wheat bran was included for preparation of the functional food supplement

d. Oats



Oats (*Avenasativa* L.) have been identified as a whole grain and is defined as a cereal grain that is intact, ground, cracked, or flaked kernel with the endosperm, germ, and bran present in the same relative proportions as the intact grain. Compared with other cereal fibers, oats are rich in dietary fiber, which includes cellulose, arabinoxylans, and soluble fibers, especially β -glucan; oats also have relatively high levels of protein and unsaturated fats. β -Glucans are thought to be primarily responsible for the cholesterol-lowering property of oats as well as improving appetite control and increasing satiety. Those studies were done in adults and the effect on children has not been studied. Antioxidative components found in oats include vitamin E (tocopherols and tocotrienols), phenolic compounds, phytic acids, sterols, and flavonoids. Epidemiological and clinical data suggest that fiber can aid in weight maintenance and/or the prevention of weight gain and subsequent obesity over ideal body (Mozaet *al.*,2015). Chang *et al.*,(2013) demonstrated that daily supplementation of oat act as an adjuvant therapy for metabolic disorders. An increased intake of viscous soluble fiber (10-25 g/d) and plant stanols/sterols (2g/d) is recommended for the reduction of LDL cholesterol levels. (The Expert Panel, NCEP, 2001). Slavin (2005) opined that fiber intake is inversely associated with body weight as well as body fat and further fiber intake is inversely associated with body mass index at all levels of fat intake.

e. Foxtail Millet



Millet is considered as a miracle food for good health. It is rich in fibre and contains many essential nutrients. It is often considered as a power house of nutrients. Studies prove that millets are highly beneficial for obese and diabetic people. Small quantities of millets give satiety and thus can prevent increase in weight. This is a big plus as most lifestyle diseases are a result of being overweight and for metabolic disorder. Phosphorus creates energy and helps in fat metabolism and body tissue repair. Foxtail millet is a good source of protein (12.3 g/100g) and dietary fiber (14 g/100g). The carbohydrate content is low (60.9 g/100g). Besides, it is rich in minerals (3 g/100g) and phytochemicals and it is also a rich source of beta carotene. Based on the above studies on bioactive components and its effect on obesity, foxtail millet is included as an ingredient in functional food mix.

f. Horse gram dhal



Horse gram (*Dolichos biflorus*) is a common twining plant all over India. In traditional medicine, the plant is sighted as astringent, diuretic and tonic. It is an excellent natural fat burner, lowers cholesterol and regulates blood pressure and blood glucose. It is attributed to reduce weight in obese individuals and has the ability to reduce post-prandial hyperglycemia by slowing down carbohydrate digestion and reduce insulin resistance by inhibiting protein-tyrosine phosphatase 1 beta enzyme.

Positive effects in managing obesity by natural components, and selected foods have drawn attention due to the potential side effects of obesity drugs. The food industry has developed low-density foods to reduce energy intake. Now focus has been geared towards the development of foods that

possess more than one mechanism to alter the progression of obesity (Sunkara and Verghese, 2014). Functional foods are defined in various ways by several agencies and authorities. According to The International Food Technologists Expert Panel Report, Functional Foods Opportunities and Challenges, “functional foods” are defined as foods and food components that provide a health benefit beyond basic nutrition (for the intended population) Anonymous (2005). These substances are known to provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects. Hence, functional food supplement were developed by incorporating different proportion of the above ingredients.

Standardization

The developed food supplement was standardised. The ingredients were mixed in varied proportion to achieve three variations apart from the standard preparation. The proportion of flax seed added was different in all the variations from over that of the standard. The variation I, II and III had 15, 25, 30 per cent flax seed respectively while the standard formulation had 5 per cent flax seed. The addition of flax seed contributed to the change in taste hence based upon the palatability of the product, the proportion of flax seed added were adjusted. The various ingredients used and its proportion are given below.

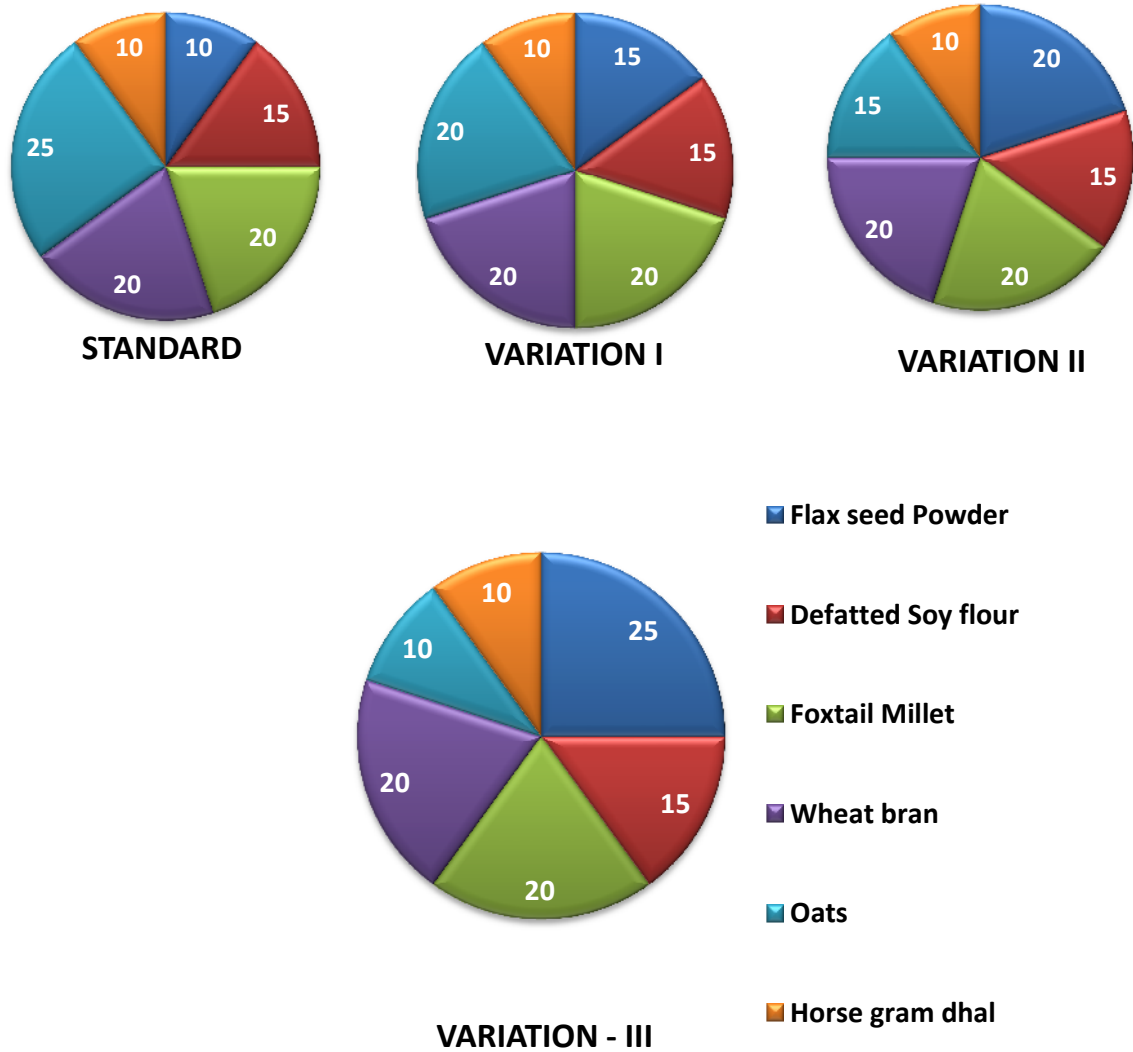


Figure 8

COMPOSITION OF FUNCTIONAL FOOD MIX VARIATIONS

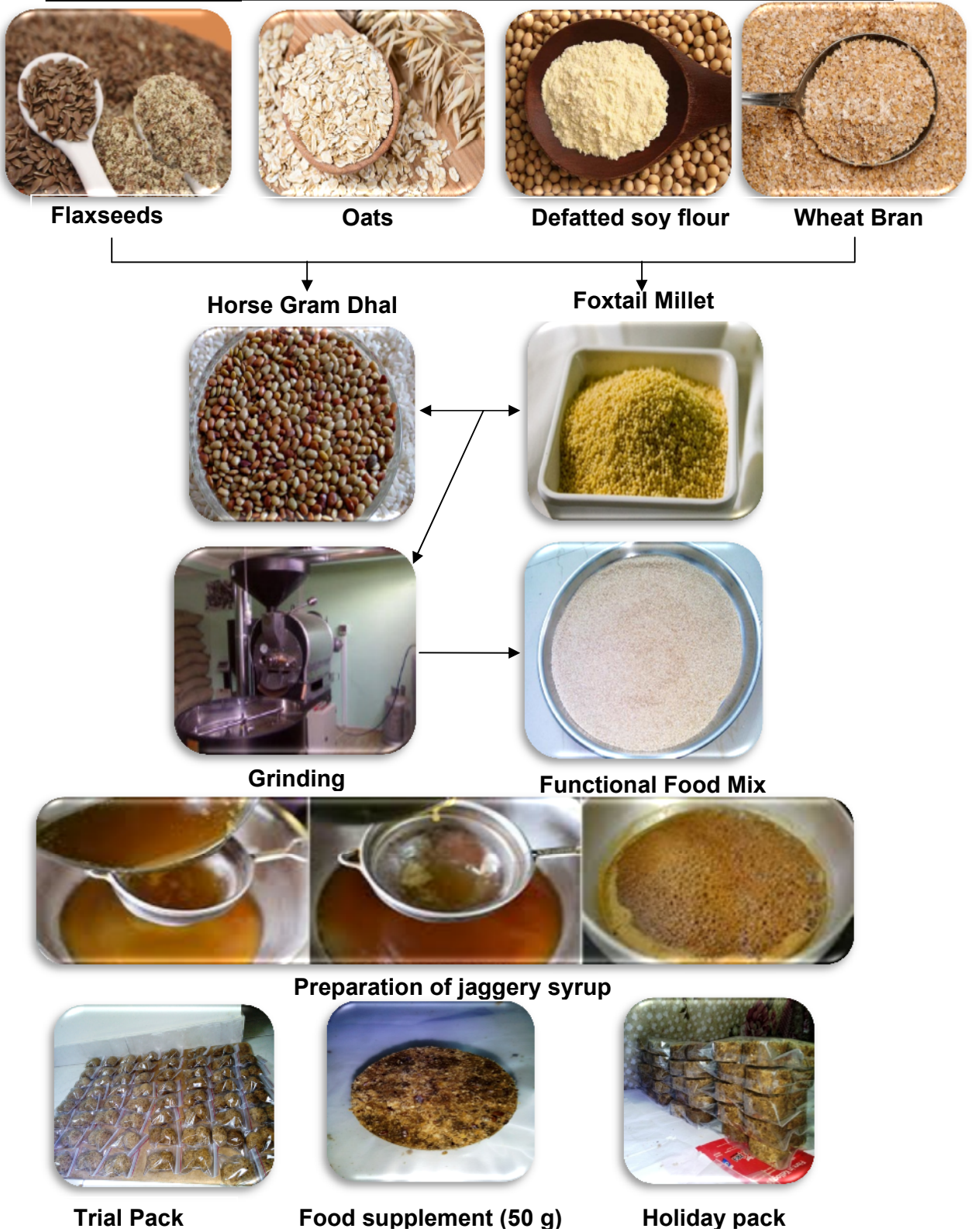


Figure 9
Preparation of Functional food Supplement

The ingredients were mixed in the above proportion viz standard, variation I, II and III respectively. 10 per cent of jaggery syrup was prepared (single thread stage) in batches and used for the preparation of the supplement. 50g of the supplement was weighed and placed in a mould and pressed firmly to get a definite shape. Each portion of supplement was checked for weight with a deviation restricting to 5g so that it contributed one serving of the supplement. The developed supplement was further subjected to organoleptic evaluation.

2. ORGANOLEPTIC EVALUATION:

Organoleptic evaluation was conducted to assess the organoleptic property of the prepared functional mix. The total of 50 semi trained panel members was included in the study for sensory evaluation. 9 point Hedonic rating scale was used for the same. The supplement was freshly prepared and displayed to thesemi skilledpanelists. A glass of water was also kept for the panelist to avoid bias in taste. They were requested not to take any other strongly flavoured food at least 1 hour before the test to minimize taste interaction that would influence the decision in evaluation. The variations were labelled as A, B, C and standard. The ingredients used, the proportion and nature of variation were not revealed to the panelists to minimize any kind of anticipation that would arise with regard to taste. Separate sheets with instructions were given to all panelists to evaluate the product. The variation with highest overall score was considered as the best accepted variation and it was chosen for further analysis and supplementation.

3. NUTRIENT ANALYSIS:

The best accepted variation based on sensory evaluation was further subjected to nutrient analysis. Proximate analysis such as energy, protein, fat, fibre were analysed by standard AOAC procedures. Iron, folic acid, calcium, magnesium, sodium was also assessed. Natural antioxidant vitamins such as vitamin A, E and C were analysed using standard procedures.

4. IDENTIFICATION AND QUANTIFICATION OF PHYTOCHEMICAL COMPONENTS

a. Phytochemical screening

The methanolic as well as aqueous extracts were used for phytochemical screening. Phytochemicals like alkaloids, glycosides, carbohydrates, proteins and amino acids, flavonoid, sterols, saponins, tannins, terpenoids, phenols were analysed by qualitative chemical method. Alkaloids – Dragendorff's test, Phenols – Ferric chloride test, Tannins – Ferric chloride test, Protein – Biuret test, Saponin – Foam test, Glycosides – Baljet's test, Flavonoids – Sodium hydroxide test, Carbohydrate – Molisch test, Thiols – sodium nitroprusside test, Sterols – Salkowski test, Steroids – LibermannBurchard method



Plate 3

Phytochemical screening – Qualitative analysis

b. Extraction

Soxhlet: 170 ml of the solvent namely ethanol was taken in a round bottom flask, 25 g of the sample were covered by muslin cloth and kept in soxhlet extractor. After completion of seven cycles, the respective samples were concentrated and transferred to the pre weighed beaker. Also the weight of the extract in the beaker was recorded. Further the extract yield per cent was calculated. Cold extraction was carried out. The sample was soaked for 24 hr in distilled water (Aqueous) and stored at 4°C. Finally the ethanolic and aqueous extract was used for the following experiments.



Soxhlet (ethanolic extract)



Cold extraction (Aqueous extract)



Ethanolic extract



Aqueous extract

Plate 4

Ethanolic and aqueous extract of functional food mix

c. Quantitative Analysis of Phytochemicals

Weighed 0.5 g of rice samples, ground in a mortar and pestle and prepared ethanolic and aqueous extracts of rice samples separately. The supernatant was collected in a volumetric flask after the extracts were centrifuged at 2000 rpm for ten minutes. The evaporated residue was then dissolved in water for the estimation of total phenolic and flavanoid content.

i. Estimation of Total Flavonoid content (TFC)

Flavonoids reacts with aluminium chloride in ethanolic solution forms a yellow colour which was read colorimetrically at 420nm (Ordonez et al., 2006). To 0.5 ml of sample solution, 0.5 ml of Two per cent $AlCl_3$ in ethanol solution was added and incubated at room temperature for one hour. UV visible spectrophotometer was used to measure the absorbance at 420 nm. A standard graph was prepared using the gallic acid and the total flavonoid content was expressed as gallicacid equivalent (mg/g).

ii. Estimation of Total Phenol content (TPC)

Singleton and Rossi (1965) method was used to estimate the total phenolic content. 0.1 ml of the sample extract was taken along with Three ml of distilled water and 0.5 ml of Folin- Ciocalteu reagent was added. Two ml of 20 per cent sodium carbonate was added exactly after three minutes and thoroughly mixed. Boiling water bath was prepared and the tubes were kept in it exactly for one minute. The tubes were cooled and measured at 650nm in spectrophotometer. The standard graphs were plotted with various concentrations of gallic acid.

iii. Estimation of Tannin content

Tannins are wide spread in nature and possibly in all plant materials. Tannin content was carried out by method given by Schenderl, (1970). Tannin reduces phosphomolybdic acid in alkaline condition to lower oxides of molybdenum when Na_2CO_3 and folin-Denis reagent are added.

iv. Estimation of saponin content

Sample of 5g was added to 20% ethanol and heated for 4hr at 55°C. Then the sample was filtered. The filtrate was reduced at 90°C (waterbath). Further the sample was separated by diethyl ether and the aqueous layer was retained. To the aqueous layer, n-butanol and 5% sodium chloride was added to collect the organic layer. Finally the organic layer was measured gravimetrically (Olayiwola. O, A, (2013)

v. Estimation of alkaloid content

5g of sample was weighed and dissolved in 10% acetic acid and kept it for (RT).The sample was filtered and the filtrate was kept in water bath for 100°C.Then few drops of Ammonium hydroxide solution was added to precipitate. This precipitate was filtered (pre weighed filter paper). Further the filter paper was washed with 1% Ammonium hydroxide solution. Finally the filter paper was kept in hot air oven (60 °C) for 30min and the filter paper was weighed (HarborneJB ,1973)

5. ANALYSIS OF ENZYMATIC AND NON- ENZYMATIC ANTIOXIDANTS

Preparation of enzyme extract

Functional food mix was homogenized (1g) in an electrical mixer with M/150 phosphate buffer at a pH 7.0 and centrifuged at 4°C. Cold phosphate buffer was added to the sediment and disturbed at regular intervals. This extraction is repeated once or twice. It should be ensured that the extraction time did not exceed 24 hours. The supernatant was extracted and used for analysis.

Enzymatic antioxidants like superoxide dismutase (SOD) (Das et al., 2000), Glutathione peroxidase (GPx) activity (Rotruck et al., 1973), catalase (Sinha, 1972), ascorbate oxidase (Vines and Oberbacher., 1965) were analysed for the functional food mix. Boyne and Ellman, (1972) method was used to estimate total reduced glutathione.

6. ANALYSIS OF *IN VITRO* RADICAL SCAVENGING ACTIVITY

The antioxidant property of the food mix was determined by the total antioxidant activity that is highly influenced by individual antioxidant properties of each single ingredient added. However processing would affect the same. Therefore, the developed food mix was subjected to Ferrous Reducing Antioxidant Assay (FRAP) developed by Benzie and Strain (1998), DPPH radical scavenging assay developed by Braca (2002)

a. DPPH radical scavenging assay

A simple method that has been developed and extensively applied to determine the antioxidant activity of foods utilizes the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The antioxidant activity of the extracts and the standard was assessed on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by modified method (Braca2002). The diluted working solutions of the test extracts were prepared in methanol. The samples were mixed with DPPH and solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm. Methanol with DPPH solution was used as blank and ascorbic acid was taken as reference standard. The percentage inhibition versus concentration was plotted.

$$\% \text{ Radical Scavenging activity} = [1(\text{Abs Control} - \text{Abs sample} / \text{Abs control OD}) \times 100]$$

b. Ferric Reducing Antioxidant Power Assay (FRAP)

FRAP assay was performed according to the methods of Benzie and Strain (1999) with a slight modification to suit the study. A known volume of the sample was made up to 3 ml with phosphate buffer and 1per centpotassium ferric cyanide. It was cooled and add 10 per cent TCA, distilled water and ferric chloride. It was kept for 10 minutes at room temperature and the absorbance at 700 nm against standard ascorbic acid equivalent

$$\% \text{ Radical Scavenging activity} = [1(\text{Abs Control} - \text{Abs sample} / \text{Abs control OD}) \times 100]$$

c. Reducing Power Activity (Oyaizu, 1986)

The reaction mixture contained 2.5 ml of various concentrations of functional food extract, 2.5 ml of 1 % potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture was incubated at 50°C for 20 min. and were terminated by the addition of 2.5 ml of 10 % (w/v) of trichloroacetic acid , followed by centrifugation at 3000 rpm for 10 min. 5.0 ml of the supernatant upper layer was mixed with 5.0 ml of deionized water and 1.0 ml of 0.1 % ferric chloride. The absorbance was measured at 700nm against blank that contained distilled water and phosphate buffer. Increased absorbance indicates increased reducing power of the sample. BHT was used as standard.

d. Nitric Oxide Scavenging Activity (Green et al., 1982)

Sodium nitroprusside in aqueous solution, at physiological pH, spontaneously generate nitric oxide, which interacts with oxygen to produce nitrite ions that is estimated spectrophotometrically at 546 nm. Various concentration of the extract was mixed with 2.5 ml of sodium nitroprusside and made upto 3.0 ml with PBS. Then the mixture was incubated for 15 minutes at 25°C. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent was added. Then the absorbance was measured at 546 nm. Vitamin C was used as standard. The percentage inhibition was calculated by comparing the results of the test with those of controls not treated with the extract, as per the following formula:

$$\% \text{ scavenging of Nitric oxide} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

7. DETERMINATION OF PANCREATIC LIPASE INHIBITORY ACTIVITY

One of the most promising strategies in the effort to reduce energy intake through gastrointestinal mechanisms without altering the central mechanism is the development of nutrient digestion and absorption inhibitors (Birari and Bhutani 2007).

Dietary fat is not directly absorbed by the intestine unless the fat has been subjected to the action of pancreatic lipase. Therefore pancreatic lipase is one of the most widely studied mechanisms for determining natural products potential efficacy as antiobesity agents (Birari and Bhutani, 2007). Pancreatic lipase is a key enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerols to monoacylglycerols and fatty acids. A variety of plants possess pancreatic lipase inhibitory effects. These pancreatic lipase inhibitory phytochemicals include mainly saponins, polyphenols, flavonoids and caffeine (Kim and kang 2005; Han *et al.*, 2006, Moreno *et al.*, 2006; Shimoda *et al.*, 2006).

Fatty acids are present in human diet and are found to be one of the important factors in the process of adipogenesis. However, all fats are not considered to cause ill effects. Certain PUFA are beneficial due to its pathways in the primary metabolism. Pancreatic lipase is the key enzyme that is known to play a vital role in the adipogenesis by increasing the metabolism of fat. In other words, it helps in the formation of fats. Therefore, the activity of pancreatic lipase has to be inhibited to prevent the formation of fat thereby preventing adipogenesis. Hence, the possible mechanism of pancreatic lipase activity should be suppressed directly or indirectly in order to prevent adipogenesis. The ability of a food to inhibit the pancreatic lipase activity determines the efficacy of the food in controlling adipogenesis. Therefore, it was of interest to determine the pancreatic lipase inhibitory activity of the developed food mix.

8. QUALITY ANALYSIS OF THE FUNCTIONAL FOOD SUPPLEMENT

The quality of the food should be good in order to be fit for consumption. The developed functional food mix was subjected to quality analysis. The quality analyses such as total bacterial count, total fungal count was carried out by pour plate method (TBC – IS 5402:2012 , TFC – IS 5403:1999) and peroxide value were carried out to ensure the quality of the developed food mix as per standard procedures.

C. PHASE – III**ASSESSMENT OF SOCIO-ECONOMIC, NUTRITIONAL STATUS AND BODY COMPOSITION MEASURES****1. FORMULATION OF THE QUESTIONNAIRE**

Background information of subjects would facilitate better insight into the contributing and influencing factors. A total of 130 subjects were selected for further study. Hence a pretested questionnaire was used to collect background information such as age, sex, community, dietary habits and personal habits. A separate questionnaire was administered for Males (N=75) and females (N=75) each focusing on different aspects based on gender. The basic information collected was same for both the genders. The questionnaire for males focused on the personal habits such as smoking and consumption of alcohol while the questionnaire for girls focused on menstrual health in order to rule out hormonal problems.

2. ASSESSMENT OF SOCIO ECONOMIC STATUS

Socio economic status affects health of the individuals in a variety of ways. Thus to assess the socio economic status, a pretested questionnaire was formulated. The details such as name, sex, date of birth, education, literacy level of the head of the family, monthly income, expenditure details were collected using the formulated questionnaire. The subjects were asked to assemble in a class room. The aim of the study was explained to all the participants both in Tamil and English. The questions were read out and explained to them. Their role and responsibility in filling up appropriate information and its importance was explained clearly. They were encouraged to clarify their doubts regarding the same. They were also insisted not to discuss with others. They were told that confidentiality at all levels will be maintained. After the questionnaire was filled up by the subjects, the investigator checked each questionnaire for completeness. If found incomplete, corresponding subjects were asked to answer the same. Care was taken to collect appropriate information from the subjects.

3. ANTHROPOMETRIC ASSESSMENT

Anthropometric measurements were measured using standard procedures

a. Height:

Height was measured using an anthropometer to the nearest of 0.01cm. The subjects were asked to stand erect with bare foot.

b. Weight

Weight was measured with a digital weighing balance corrected to the nearest of 0.1kg. The subjects mounted the balance with bare foot and light clothing

. Body Mass Index was calculated by using the formula

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

Waist and hip measurements were measured with a flexible measuring tape.

c. Waist circumference:

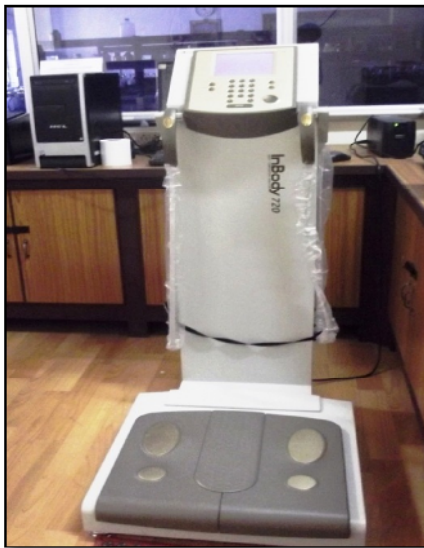
Waist circumference was measured at the smallest horizontal girth between the costal margins and the iliac crest during minimal respiration. The waist measurement was made at the midpoint between the top of the iliac crest (upper edge of the main pelvic bone) and the lower margin of the last palpable rib in the mid axillary line (lowest point of the ribcage that can be located by touch along the side of the body).

d. Hip circumference

Hip circumference was also measured in a similar manner, with the tape being passed around the hip at the widest circumference of the buttocks. In both cases the tape measurement was kept parallel to the floor. Waist hip ratio was calculated using the formula

$$\text{Waist Hip Ratio} = \text{Waist Circumference (cm)}/\text{Height (cm)}$$

4. BODY COMPOSITION ASSESSMENT BY BIOELECTRICAL IMPEDENCE ANALYSIS



Body composition was measured using INBODY 720 body composition analyzer. InBody720 analysis of body composition is based on the 4-Compartment Model. This 4-Compartment Model assumes that body is composed of four different elements: total body water, protein, minerals, and body fat. Total body water is separated into intracellular and extracellular water by cellular membranes. InBody720 assigns a quantitative value to

the various body compositional elements. These values demonstrate the weight of each body compositional element that makes up the examinee's total body weight. The estimated values were then compared with the standard values.

Bioelectrical impedance analysis (BIA) is a widely used method for estimating body composition. The technology is relatively simple, quick, and noninvasive. BIA measures the opposition of body tissues to the flow of a small (less than 1 mA) alternating current. Impedance is a function of two components (vectors): the resistance of the tissues themselves, and the additional opposition (reactance) due to the capacitance of membranes, tissue interfaces, and nonionic tissues. The measured resistance is approximately equivalent to that of muscle tissue.

a. Intracellular water(ICW), Extracellular water(ECW), Total Body Water(TBW)

Total Body Water(TBW) is measured by using a multi-frequency technique that separates TBW into ICW and ECW. Intracellular water (ICW) indicates the quantity of water within cellular membrane. Extracellular water (ECW) indicates

the total quantity of water in the interstitial fluid and blood. In the case of a healthy body, the proportion of ICW and ECW should be maintained at about 3:2.

b. Protein

Protein is a solid organic compound that consists of nitrogen and can be found in body cells. Protein is also the main component, along with body water, of Soft Lean Mass. Protein is directly related to intracellular water. Therefore, a lack of protein indicates a lack of intracellular water, which in turn implies poor cell nutrition.

c. Mineral

InBody720 analyzes two large groups of minerals: osseous minerals and non-osseous minerals. Osseous minerals are the minerals found in the bones while nonosseous minerals are those which are found in all other parts of the body. Osseous minerals account for about 80% of the body's total minerals. Mineral mass is closely related to soft lean mass. According to BIA principle, the mineral mass cannot be calculated in a direct way. It can be obtained from DEXA, a bone density scanner. Therefore, the mineral mass presented by the InBody720 is an estimated value. However, a comparative experiment with DEXA shows a very high correlation so that it can be utilized as a primary screening data.

d. Body Fat Mass

Body Fat Mass refers to the total quantity of lipids that can be extracted from fat and other cells. Body Fat Mass cannot be directly estimated using the BIA method, but rather is calculated by excluding Fat Free Mass (FFM) from body weight. $\text{Body Fat Mass} = \text{Body Weight} - \text{Fat Free Mass (FFM)}$ Body Fat Mass is stored under the skin, as well as between the abdomen and muscles. When an examinee's body fat mass is outside of the standard range, he/she is diagnosed as being obese.

e. Soft Lean Mass

Soft Lean Mass can be calculated by excluding the mineral found in the bones from Fat Free Mass.

f. Fat Free Mass

Fat Free Mass consists of the weight of the remaining components once Body Fat Mass has been excluded from body weight.

g. Weight

Weight consists of body water, protein, mineral and Body Fat Mass. Thus, body weight is the sum total of these four body components. $\text{Weight} = \text{Total Body Water} + \text{Protein Mass} + \text{Mineral Mass} + \text{Body Fat Mass}$

h. Muscle Fat Analysis

The MuscleFat Analysis consists of an estimation of the value of three elements, weight, skeletal muscle mass, and body fat mass. This analysis is also capable of carrying out relative comparisons of the above-mentioned body components using numbers and bar graphs. The numbers shown in the bar graphs indicate the measured values for each element while the length of the graph demonstrates the percentage of the standard value for each item. Thus, a score of 100 percent would indicate a standard value, with the standard weight calculated using the examinee's height. Therefore, the examinee's body composition balance can be ascertained simply by looking at the graphs and seeing if they are longer or shorter than the standard value of 100 per cent. The normal range is shown on the right side of the bar graph; it can be compared with the estimated value. If the lengths of bar graphs are alike, it means that your body composition is in balance

i. Skeletal Muscle Mass(kg)

100 per cent standard Skeletal Muscle Mass refers to the ideal quantity of Skeletal Muscle Mass for an examinee's standard weight. There are three types of muscle - cardiac muscle, visceral muscle and skeletal muscle. However, it is

the quantity of skeletal muscle that undergoes changes through exercise. As such, InBody720 displays Skeletal Muscle Mass separately from Soft Lean Mass. By comparing the percentage of Body Fat Mass and Skeletal Muscle Mass found in each body component, the level of obesity can be estimated in a more proactive and exact manner.

j. Body Fat Mass(kg)

100 per cent standard Body Fat Mass refers to the Body Fat Mass that an examinee should maintain for his/her standard weight. In general, the ideal Body Fat Mass is 15 per cent for males and 23 per cent for females.

k. Percent Body Fat (%)

Percent Body Fat indicates the percentage of body fat to body weight. Percent Body Fat (%) = $\text{Body Fat Mass(kg)} / \text{Body Weight(kg)} \times 100$ The standard Percent Body Fat is 15% for males and 23% for females while the standard range of Percent Body Fat for males is 10-20%, and 18-28% for females.

l. Waist-Hip Ratio

It is a useful indicator for comprehending the distribution of body fat. However, it causes the inconvenience of measuring the body and inaccuracy marked by the discrepancy in measurements taken by different measurers. Therefore, in reality, it is hard to measure it with measuring tapes for obesity treatment.

INBody720 uses its impedance index to provide a scientific estimation of the examinee's WHR. Given its high degree of reproduction and accuracy, InBody720's estimation of the ratio of abdominal fat can be used as an effective tool with which to treat obesity. Males and Females found to have Waist hip Ratio of 0.95 and 0.90 respectively are considered to suffer from abdominal obesity. An adult found to suffer from abdominal obesity is one who exhibits the excessive visceral fat mass due to increasing free fatty acid levels in the blood than in subcutaneous fat which causes hypertension, heart disease, diabetes and various other clinical diseases

m. Lean Balance

With the InBody720, one can measure the soft lean mass of body. It is achieved through the use of one of the INBody720's measuring principles, bio-electrical impedance measurements of body parts

n. Visceral Fat Area

Visceral Fat Area is defined here as the cross-sectional area of visceral fat found in the abdomen. When the area of visceral fat spans more than 100cm² and they are prone to develop abdominal obesity. Fat, depending on its location, can be divided into visceral, subcutaneous and inter-muscle fat. The area of the visceral fat is calculated in INBODY 720 analyzer.

o. Obesity Degree

Obesity Degree is the ratio of the current weight to the standard weight, and also serves as an index with which to evaluate the examinee's obesity level in accordance with their height and weight. The standard weight is calculated using the BMI method. Obesity Degree (%) = (Current Weight / Ideal Weight) × 100. Obesity Degree is an index used to evaluate an examinee's obesity based solely on their overall weight, and as such does not take into consideration the individual's body composition. Therefore, it is not of much help in evaluating the real state of an examinee's obesity, and only allows one to know if he/she is overweight. 90 ~ 110% is considered to be the standard, while 110 ~ 120% is considered to be overweight and 120% or more obese.

p. BMR (Basal Metabolic Rate)

Basal Metabolic Rate (BMR) indicates the minimum energy required for sustain vital functions while at rest. InBody720 makes it possible to estimate BMR using a known regression equation based on FFM. FFM is known to be closely related to BMR. BMR is usually calculated using indirect Calorimetry, which in turn, employs oxygen demand. However, InBody720 calculates BMR based on Fat Free Mass as follows:

$$REE = 21.6 \text{ FFM(kg)} + 370 \text{ (FFM=Fat Free Mass, kg)}$$

q. Precautions

- The test was conducted before a meal. In cases the examinee has already eaten, the test was taken after two hours of the last meal. This is because the mass of the food is counted as weight, and thus, may result in measurement errors.
- The volunteers were instructed to empty bladder before measurement to avoid biological errors.
- Volunteers were instructed to avoid strenuous exercise since it can cause temporary changes in body composition.
- Measurements of the female volunteers were not recorded during menstruation since the body water tend to increase during menstruation
- The test was conducted at an optimum temperature of 20 ~25 While the human body is stable at normal temperatures, body composition is susceptible to change in hot or cold weather

5. ASSESSMENT OF FOOD AND NUTRIENT INTAKE

a. Food Intake by Recall Method

Food intake of the individual greatly influences the degree of obesity. The types of fat, the amount of food taken all contribute to the quality of food. Food intake was recorded by 3 day recall method. The recall method was used to assess the actual energy intake. A set of cups and spoons were first standardized by the investigator following the procedure given by Thimmayamma and Rao (2003).

The subjects were asked to carefully note down the type and amount of food taken during last three days and report the same to the investigator. The subjects were explained about the importance of assessing their food as well as nutrient intake. The respondents were asked about the types of food preparations made throughout the day including breakfast, lunch, teatime and dinner. The amount of raw ingredients used for each menu was also noted. Information on the total cooked amount of each preparation and the quantity consumed by the respondent was then assessed using the standardized cups. The cups were

used to aid the respondent recall the quantities prepared and eaten. Subjects were asked to indicate the various measuring cups that mimic their actual intake. Later the raw food equivalents of the food consumed by the respondent were found out. The special occasions like fasting and feasting were avoided during assessment of food intake to avoid bias.

b. Mean Nutrient Intake

Nutrient intake was calculated from the data obtained through the 3 day recall method, equating the nutritive value present in the individual ingredients. The different food items consumed were converted into their raw equivalents; categorized into their respective food groups and the mean daily intake of energy, protein, fat, calcium, iron, beta- carotene and vitamin C were calculated from the values per 100 g of edible portion using the Nutritive Value of Indian Foods (2010). The calculated nutrient intake was compared with the recommended dietary allowances (RDA) for the respective age group.

D. PHASE IV

COMPUTATION OF THE TOTAL ENERGY EXPENDITURE

1. COMPUTATION OF TOTAL DAILY ENERGY EXPENDITURE (TEE)

The total energy expenditure (amount of calories needed per day) is composed of three primary factors:

1. Resting or basal metabolic rate
2. Thermic effect of food
3. Activities of Daily Living (ADL) - physical activity.

In other words, the total daily energy expenditure (TEE) is the product of the basal metabolic rate (BMR) and the physical activity level (PAL). The determination of the TEE has brought a measure of objectivity to the determination of the energy needs of groups of individuals, particularly since energy expenditure was adopted as the method of quantifying energy requirements, rather than energy intake (FAO/WHO/UNU, 1985, 2004).

a. Basal Metabolism

This comprises a series of functions that are essential for life, such as cell function and replacement; the synthesis, secretion and metabolism of enzymes and hormones to transport proteins and other substances and molecules; the maintenance of body temperature; uninterrupted work of cardiac and respiratory muscles; and brain function. The amount of energy used for basal metabolism in a period of time is called the basal metabolic rate (BMR), and is measured under standard conditions that include being awake in the supine position after ten to 12 hours of fasting and eight hours of physical rest, and being in a state of mental relaxation in an ambient environmental temperature that does not elicit heat-generating or heat-dissipating processes. Depending on age and lifestyle, BMR represents 45 to 70 percent of daily total energy expenditure, and it is determined mainly by the individual's age, gender, body size and body composition.

b. Metabolic Response to Food

Eating requires energy for the ingestion and digestion of food, and for the absorption, transport, interconversion, oxidation and deposition of nutrients. These metabolic processes increase heat production and oxygen consumption, and are known by terms such as dietary-induced thermogenesis, specific dynamic action of food and thermic effect of feeding. The metabolic response to food increases total energy expenditure by about 10 percent of the BMR over a 24-hour period in individuals eating a mixed diet.

c. Physical activity

This is the most variable and, after BMR, the second largest component of daily energy expenditure. Humans perform obligatory and discretionary physical activities. Obligatory activities can seldom be avoided within a given setting, and they are imposed on the individual by economic, cultural or societal demands. The term "obligatory" is more comprehensive than the term "occupational" that was used in the 1985 report (WHO, 1985) because, in addition to occupational work, obligatory activities include daily activities such as going to school, tending to the home and family and other demands made on children and adults by their

economic, social and cultural environment. Discretionary activities, although not socially or economically essential, are important for health, well-being and a good quality of life in general. They include the regular practice of physical activity for fitness and health; the performance of optional household tasks that may contribute to family comfort and well-being; and the engagement in individually and socially desirable activities for personal enjoyment, social interaction and community development.

d. Calculation of Basal Metabolic Rate

Basal metabolic rate (BMR) is the amount of energy required to maintain the body's normal metabolic activity, such as respiration, maintenance of body temperature (thermogenesis), and digestion. Specifically, it is the amount of energy required at rest with no additional activity. The energy consumed is sufficient only for the functioning of the vital organs such as the heart, lungs, nervous system, kidneys, liver, intestine, sex organs, muscles, and skin.

One way to estimate total energy expenditure calculated using prediction equation that uses information about the gender, age, height, weight, and activity level. Various prediction equations were used to estimate total energy expenditure.

1. The Harris-Benedict equation
2. World Health Organization Equations -1
3. World Health Organization Equations -2
4. Mifflin-St. Jeor Equation
5. Schofield's equations
6. Liu's Equation
7. Owen's Equation

i. The Harris-Benedict Equation

The Harris-Benedict equation is a method used to estimate an individual's basal metabolic rate (BMR) and daily calorie requirements (total energy expenditure). The estimated BMR value is multiplied by a number that corresponds to the individual's activity level. The resulting number is the recommended daily calorie intake to maintain current body weight. The equation

assumes a normal body composition, with an average ratio of muscle mass to fat mass, so it may be inaccurate for individuals who are very muscular (the formula underestimates true requirements) or for individuals with obesity (the equation overestimates true requirements).

Men

$$\text{BMR} = 88.362 + (13.397 \times \text{weight in kg}) + (4.799 \times \text{height in cm}) - (5.677 \times \text{age in years})$$

Women

$$\text{BMR} = 447.593 + (9.247 \times \text{weight in kg}) + (3.098 \times \text{height in cm}) - (4.330 \times \text{age in years})$$

The BMR is then multiplied by a Physical Activity Factor to estimate total energy needs

ii. World Health Organization Equation -1

The World Health Organization (WHO) developed an equation for estimating energy needs in the 1980s. The equation is based on a person's sex, age range and weight. WHO did not feel that height was necessary to include in its equation. The equations are as follows:

Females : Age 10 to 17 years = $12.2 \times (\text{Weight in kg}) + 746$
Age 18 to 29 years = $14.7 \times (\text{Weight in kg}) + 496$
Age 30 to 60 years = $8.7 \times (\text{Weight in kg}) + 829$
Age over 60 years = $10.5 \times (\text{Weight in kg}) + 596$

Males : Age 10 to 17 years = $17.5 \times (\text{Weight in kg}) + 651$
Age 18 to 29 years = $15.3 \times (\text{Weight in kg}) + 679$
Age 30 to 60 years = $11.6 \times (\text{Weight in kg}) + 879$
Age over 60 years = $13.5 \times (\text{Weight in kg}) + 487$

Again, these equations are multiplied by the same Physical Activity Factor to estimate daily caloric needs.

iii. World Health Organization Equations 2

Men

$$18-30 \text{ RMR} = 15.4 \times \text{weight} - 27 \times \text{height} + 717$$

$$31-60 \text{ RMR} = 11.3 \times \text{weight} + 16 \times \text{height} + 901$$

$$>60 \text{ RMR} = 8.8 \times \text{weight} + 1128 \times \text{height} - 1071$$

Women

$$18-30 \text{ RMR} = 13.3 \times \text{weight} + 334 \times \text{height} + 35$$

$$31-60 \text{ RMR} = 8.7 \times \text{weight} - 25 \times \text{height} + 865$$

$$>60 \text{ RMR} = 9.2 \times \text{weight} + 637 \times \text{height} - 302$$

iv. Mifflin-St. Jeor Equation

The Mifflin-St. Jeor equation was developed in 1990 and has been validated by more than 10 studies. The Mifflin-St. Jeor equation is gaining popularity among the nutrition professionals for accurately estimating the caloric needs. Frankenfield et al have advised the use of the Mifflin equation for overweight and obese subjects. The equation is as follows:

$$\text{For females} : 10 \times (\text{Weight in kg}) + 6.25 \times (\text{Height in cm}) - 5 \times \text{age} - 161$$

$$\text{For males} : 10 \times (\text{Weight in kg}) + 6.25 \times (\text{Height in cm}) - 5 \times \text{age} + 5$$

v. Schofield's equations

It is reported by the World Health Organization

Age	Men	Women
18–30 years	$15.057 \times \text{weight} + 692.2$	$14.818 \times \text{weight} + 486.6$
30–60 years	$11.472 \times \text{weight} + 873.1$	$8.126 \times \text{weight} + 845.6$
>60 years	$11.711 \times \text{weight} + 587.7$	$9.082 \times \text{weight} + 658.5$

These equations are also multiplied by the same Physical Activity Factor to estimate daily caloric needs

vi. Owen equation

Owen's equation for men and women are given separately.

$$\text{Men: kcal/day} = 879 + 10.2 (\text{wt})$$

$$\text{Women: kcal/day} = 795 + 7.2 (\text{wt})$$

vii. Liu's Equation

$$\text{Men : } 13.88 \times \text{BW} + 4.16 \times \text{Height} - 3.43 \times \text{Age}$$

$$\text{Women: } 13.88 \times \text{BW} + 4.16 \times \text{Height} - 3.43 \times \text{Age} - 112.40$$

e. Physical Activity Factors

Exercise Level	Details	Calorie Calculation (Daily Needs)
Little to no exercise		BMR x 1.2
Light exercise	1-3 days per week	BMR x 1.375
Moderate exercise	3-5 days per week	BMR x 1.55
Heavy exercise	6-7 days per week	BMR x 1.725
Very heavy exercise	twice per day, extra heavy workouts	BMR x 1.9

2. COMPUTATION OF ENERGY BALANCE

Based on energy intake and expenditure, energy balance is calculated

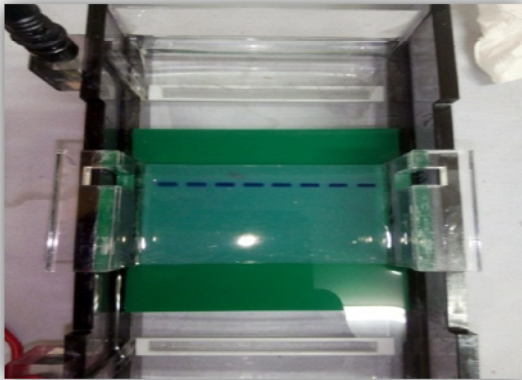
E. PHASE V**ANALYSIS OF PPAR GAMMA GENE POLYMORPHISM AND CONDUCT OF INTERVENTION STUDY****1. ANALYSIS OF PRO 12 ALA POLYMORPHISM OF PPAR GAMMA GENE**

Peroxisome Proliferator Activator Receptor (PPAR) Gamma polymorphism were analysed using Restricted Fragment Length Polymorphism (RFLP) technique.

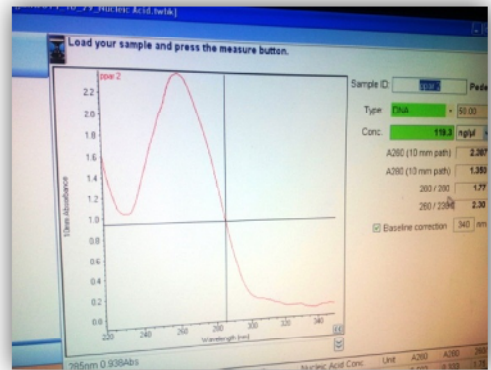
Genotyping of the patients was performed to identify the Pro12Ala (rs1801282) polymorphism in the PPAR gamma gene. The genomic DNA was first extracted from the blood samples of the study participants. The extracted DNA was rinsed with 70% alcohol and re-suspended in 40 µL of TE buffer (pH 8.0). Pro12Ala polymorphism was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The DNA was amplified by PCR using a sense primer (5'-GCCAATTCAAGCCCAGTC-3') and an antisense primer (5'-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGC TTTCCG-3') that flanked the region containing the 12-amino acid site of PPAR γ 2. The PCR product was 270 bp in length. The PCR conditions were an initial denaturation step at 94°C for 8 min, followed by 35 cycles of denaturation at 94°C for 50 sec, annealing at 59°C for 50 sec, and extension at 72°C for 1 min, with a final extension of 5 min at 72°C. After PCR amplification, the PCR product was subjected to restriction digestion by BstUI 1236 I enzyme at 37°C overnight, electrophoresed on a 12% polyacrylamide gel and stained with ethidium bromide. The digested RFLP products after gel electrophoreses was used to identify the study subjects with the Pro12Ala polymorphism. A single 270 bp fragment indicated the presence of CC (Pro12Pro) genotype while three fragments of 270, 227 and 43 bp product size confirmed the presence of GC (Pro12Ala) genotype, GG (Ala12Ala) genotype would be represented by two fragments of 227 and 43 bp. In order to ensure validity, the samples were run in polyacrylamide gel using an internal positive and negative control and replicating 10% of the samples randomly.



DNA extraction from blood



Gel Electrophoresis



Purity check – Nano count

Plate 5

Restricted Fragment Length Polymorphism (RFLP) technique

2. ASSESSMENT OF BIOCHEMICAL PARAMETERS

Parameters such as haemoglobin, fasting blood glucose, lipid profile, LDL, HDL, VLDL, triglycerides total cholesterol were assessed before the conduct of intervention study. Haemoglobin was assessed by cyanmethaemoglobin method, blood glucose by method, lipid profile by spectrophotometry. For collection of blood samples the subjects were instructed to assemble at the college at 6. 30 am. They were asked to come in fasting for collection of blood. Blood was collected by a trained lab technician separately for each analysis in respective tubes. After the blood collection, food was arranged for all of the subjects.



Plate 6

Measurement of Blood Pressure and Collection of Blood

3. ASSESSMENT OF METABOLIC SYNDROME AS PER ATP III CRITERIA

Metabolic syndrome is the presence of a group of symptoms. It is common among obese subjects. Therefore is of interest to find out the prevalence of metabolic syndrome. Adult Treatment Plan –ATP III criteria recommended by NCEP were used to assess the same. ATP III Criteria consist of 5 factors, presence of any 3 out of the 5 criteria was used to categorize as metabolic syndrome. ATP III considered the “obesity epidemic” as mainly responsible for the rising prevalence of metabolic syndrome. Obesity contributes to hypertension, high serum cholesterol, low HDL cholesterol, and hyperglycemia, and it otherwise

associates with higher CVD risk. Abdominal obesity especially correlates with metabolic risk factors. Excess adipose tissue releases several products that apparently exacerbate these risk factors. They include non-esterified fatty acids (NEFA), cytokines, PAI-1, and adiponectin. A high plasma NEFA level overloads muscle and liver with lipid, which enhances insulin resistance. High CRP levels accompanying obesity may signify cytokine excess and a pro-inflammatory state. An elevated PAI-1 contributes to a prothrombotic state, whereas low adiponectin levels that accompany obesity correlate with worsening of metabolic risk factors. The strong connection between obesity (especially abdominal obesity) and risk factors led ATP III to define the metabolic syndrome essentially as a clustering of metabolic complications of obesity (NCEP, 2001)

Table 2

ATP III CRITERIA (MODIFIED)

Risk factor	Defining level
Abdominal obesity (Waist Circumference)	>102 cm in men >88 cm in women
TGL	>150mg / dl
HDL cholesterol	< 40 mg/dl men < 50mg/dl women
Blood Pressure	>130/>85mm Hg
Fasting blood glucose	>110mg/dl

Apart from lipid profile, blood pressure was recorded for all the subjects using standard procedure. Blood pressure was recorded by a physician using an automatic electronic sphygmomanometer. Two consecutive reading was taken and average of the same was considered as final reading.

4. COMPONENTS OF INTERVENTION STUDY

The intervention study has three vital components to reduce excess weight. The three components are,

- a. Dietary intervention
- b. Physical activity intervention
- c. Nutrition education intervention

Though diet plays an important role in reducing weight, physical activity is equally advantageous than the diet *alone*. Along with the above, nutrition education was also included to create awareness on foods and nutrients as an essential component for reducing weight and maintain the same.

This group received all the three interventions viz. food supplement, physical activity and nutrition education.

a. Dietary Intervention - Food Supplement:

As part of the dietary intervention, 50 g of the functional food mix was supplemented to the Experimental groups I and II for a period of 4 months (120 days). The developed food mix was supplemented for the overweight and obese group. The supplements were freshly prepared and supplements were packed in a ziplock cover and distributed on daily basis. Initially, the subjects were asked to consume two 25gm supplements.

For familiarization before initiation of supplement the subjects were also assessed to evaluate organoleptic properties of the food supplement. Gradually after a week, 50gm supplement was given daily for a period of four months. The subjects were asked not to share their supplements with others and they were instructed to eat the whole supplement on the same day possibly on the same time. The subjects were also advised to drink more water during the supplementation. The subjects were also instructed not to eat outside they were also asked to exclude fried foods and any other snacks. The subjects were also insisted to take the supplements regularly and if there was deviation from the same. They were asked to report it immediately for compliance. A separate attendance sheet was also maintained for the same.

b. Physical Activity Intervention:

Physical activity intervention was the second component in the intervention study. Physical activity intervention was given for a period of four months to the experimental groups I and II. Twelve sets of exercises were instructed to the subjects for 45 minutes each day. The physical activity intervention was given for 3 days a week on alternate days. Physical activity levels were initially assessed then a physical activity intervention schedule was framed with the help of the physical instructor.

The physical activity training was given to subjects on hourly basis - 45 minutes intervention on alternate days for a period of 4 months. 45 minutes sessions was further divided into 3 sessions 15 minutes of each. First 15 minutes were used for warm up, while next 15 minutes for extensive training followed by 15 minutes of stretching exercise. The training was imparted with the help of a physical training instructor for all the subjects. A separate attendance was maintained for the same. The subjects were instructed to attend the physical activity intervention regularly.

c. Nutrition Education

The nutrition education was carried out in two phase.

Phase I : Group education

Phase II : Personalized education

i. Phase I : Group Education

In group education all the subjects were asked to assemble in a particular place at a stated time. General details of obesity status in India, their complications were described through PowerPoint. They were also briefed about the kind of fat available in food and nutrient content of commonly consumed food.



Plate 7

Nutrition Education in progress

All the three groups received Nutrition education on the other 3 days for a period of 4 months. Each session lasted for a period of 20-30 minutes depending upon the topic. Healthy snack recipes were demonstrated. Foods were displayed to explain their nutrient content. The subjects were imparted nutrition education on food groups, food pyramid, energy requirement, choosing right fat, avoiding unhealthy eating habits, Recommended Dietary Allowances, importance of being physically active, concept of Basal Metabolic Rate and factors affecting the same. The doubts of each individual were clarified. Nutrition Education was disseminated through visual aids such as posters, pamphlets; handouts and Power Point presentations.



Figure 10 a

Materials used for imparting nutrition education

Charts, posters, pamphlets were also distinguished to the participants. They were also encouraged not eat low fat diet. They were also advised to consume unhealthy snacks particularly deep fat fried items.

ii. Phase II: Personalised Nutrition Education

In the personalized nutrition education sessions each of the subjects were asked to clarify their doubts individually. Their food as well as nutrient intake was assessed and modified accordingly. They were asked to replace the high fat foods that they were consuming. Modification of diet related aspects were also

discussed in detail. They were asked to contact the investigator each week for personalised nutrition education. The nutrition education sessions were given twice per week for 30 minutes. During each session 20 minutes were utilised by the investigator for nutrition education and last 10 minutes were utilized for clearing doubts with regard to food, diet, and lifestyle.

5. CONDUCT OF THE INTERVENTION STUDY

Obesity is a result of long term high caloric intake and reduced energy expenditure overtime. Their causes can be complex varying from genetic alterations, environmental factors and gene interactions. Sedentary lifestyle and excess caloric intake due to obesogenic environmental changes coupled with genetic susceptibility likely contributed to the recent escalation of obesity rates. (Nalin.Setal 2013) .Hence, it was of interest to develop a food mix and supplement the same as a dietary intervention along with physical activity intervention and nutrition education intervention. The intervention study was conducted by supplementing the developed food mix to selected subjects of various groups such as obese, overweight and normal subjects .

6. EVALUATE THE IMPACT OF INTERVENTION

Impact of intervention was evaluated based on the changes in anthropometric, biochemical, body composition, metabolic syndrome parameters and physical activity pattern after supplementation.

The phenotype (obese, overweight and normal) Vs genotype variation based on single nucleotide polymorphism was taken as a primary criterion and other criteria such as diet, biochemical parameters physical activity parameters were considered as associated criteria. Thus correlation as well as association studies were carried out with respective statistical tools.

F. VALIDATION AND INTREPRETATION OF DATA

The experimental results are expressed as the Mean \pm SD. The data were subjected to Anova and t statistics to analyze the difference in various parameters before and after supplementation and Co-efficient of correlation was

also carried out using SPSS software 16.0. A value of $p < 0.05$ was considered to indicate a significant difference between groups.

The biochemical analysis and gene analysis in blood samples were standardized. The investigator herself carried out the entire analytical procedures and anthropometric measurements. She underwent prior training for taking measurements in body composition analyser. Further, she also underwent training in phytochemical analysis and PCR techniques. The investigator underwent training at the Centre for Molecular Medicine and Therapeutics (CMMT lab), PSG IMS&R, Coimbatore. Hence, there is no probability of bias or error in obtaining the analytical data in the present study.

Limitation of Methodology

The polymorphism was found to be in lower percentage in the selected sample. The study was conducted only among 19-24 years old subjects.

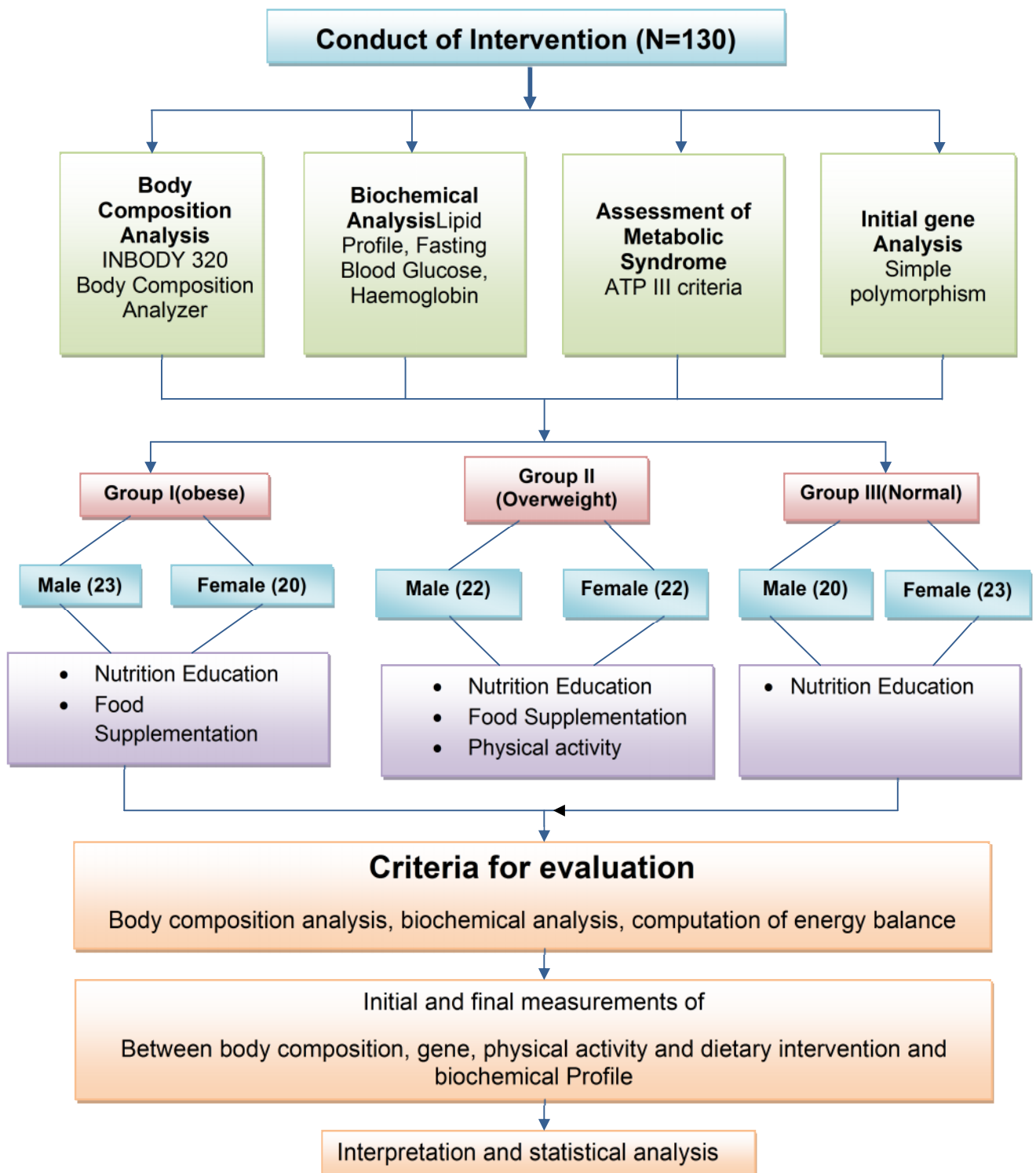


Figure 11
Methodology