METHODS AND MATERIALS

Escalating chronic disease burden in women has links to complex underlying metabolic and endocrine disturbances. On one hand, there is plausible biological evidence about ovarian steroids having a protective effect on not only the cardiac health but a host of other body systems in a woman. Also menopause (mainly loss of the ovarian steroids due to decline in ovarian function) has been observed to disrupt the harmony in the cardio-metabolic and related systems. On the other hand, the disturbingly high statistics of cardio-metabolic events occurring in women worldwide raises a concern to address it promptly with utmost gravity. Hence this research was undertaken for studying the clinico-biochemical derangements in middle-aged menopausal women; describing the major risk factors, identifying most pressing risk factor and furnishing a natural remedy for its management. The initial formative research paved the way for identification of the most prevalent problem (hyperlipidemia) and the subsequent intervention research was directed at the management of this problem.

The outline of the study and the detailed experimental design are given below.

PHASES IN THE STUDY

I. **Formative Research:** Clinico-Biochemical Changes across Pre, Peri and Post Menopausal Women in

   *Part A* - Women From Free-Living Population in Vadodara

   *Part B* - Women Attending a Health Check-Up Facility in Ahmedabad.

II. **Follow-up Study:** The Immediate and Longitudinal Outcomes of Health Check-Up on Women’s Health Care Practices.
III. **Translational Research:** Analysis of Nutritional Quality of Wheatgrass Powder and its Incorporation in Different Recipes as a Functional Food and its Acceptability.

IV. **Experimental Research:** Impact of Wheatgrass Powder Supplementation on Lipoprotein Status in Primary Hyperlipidemic Women – An Open Label Randomized Controlled Trial.

**DETAILED EXPERIMENTAL DESIGN**

I. **FORMATIVE RESEARCH: CLINICO-BIOCHEMICAL CHANGES IN PRE-PERI AND POST MENOPAUSAL WOMEN IN A] FREE-LIVING POPULATION IN VADODARA AND B] THOSE ATTENDING A HEALTH CHECK-UP FACILITY IN AHMEDABAD.**

**Design:** By employing a cross-sectional & analytical study design, the formative research was conducted to study the distribution of clinico biochemical changes across pre, peri and post-menopausal women, with regard to the presence of cardio-metabolic risk factors. The experimental plan for Part A of Phase I is shown in Figure 3.1 and part B is shown in Figure 3.2. The study design was approved by the Institutional Ethics Review Committee of the Department of Foods and Nutrition, The Maharaja Sayajirao University of Baroda (No. Fc Sc/FND/ME 59 dated 30/9/2010).

**Sample-Size Estimation:** Since the objective of the present study was to study a number of major risk conditions, the sample size was estimated using the prevalence of metabolic syndrome, which represents clustering of major risk factors. Recent cross-sectional studies in literature conducted on Indian population suggest that the prevalence of metabolic syndrome is 25%. The sample size was determined employing the following formula (Daniel 1999):
Figure 3.1 EXPERIMENTAL DESIGN FOR PHASE I- PART A

Vadodara City

North Zone

West Zone

East Zone

South Zone

Women aged 30-40 years (N=44)

Women aged 40-50 years (N=66)

Women aged 50-70 years (N=76)

A total of 186 middle aged women were studied for dietary and lifestyle risk behaviors, anthropometric profile and biochemical risk factors

Anthropometry

- Height
- Weight,
- Waist Circumference,
- Hip circumference
- Waist Hip Ratio

Bio-chemical Parameters

- Blood Glucose,
- Blood Hemoglobin
- Serum Lipid Profile
- Serum TSH
- Serum Free T4
- Plasma Insulin
- hs-CRP

- Socio-Economic background
- Brief Medical and Obstetric history
- Dietary habits
- Blood pressure
Figure 3.2 EXPERIMENTAL DESIGN FOR PHASE I - PART B

From Health check-up section of a multi-specialty hospital in Ahmedabad

213 women aged 30-70 years enrolled from the period of December 2010 to March 2011

A total of 213 middle aged women were studied for dietary and lifestyle risk behaviors, anthropometric profile and biochemical risk factors

Anthropometry
- Height
- Weight,
- Waist Circumference,
- Hip circumference
- Waist Hip Ratio

Bio-chemical Parameters
- Blood Glucose,
- Blood Hemoglobin
- Serum Lipid Profile
- Serum TSH
- Serum Free T4
- Plasma Insulin
- hs-CRP

- Socio-Economic background
- Brief Medical and Obstetric history
- Dietary habits
\[ n = \frac{Z^2 \times p \times (1-p)}{d^2} \]

Where, \( n \) = estimated sample size,

\( Z \) = confidence interval, which was taken as the Z-score corresponding to 95% on the Gaussian distribution curve, therefore, \( Z = 1.39 \),

\( p \) = reported prevalence of the condition under study (obesity-50%, MS-25%),

\( d \) = error of estimation/precision, which was taken as 0.05.

Fitting the above values into the equation, gave the sample size to be 288.

**PART A - WOMEN FROM FREE-LIVING POPULATION IN VADODARA**

**Subjects and Sample Selection**

Thus, to study both the problems of concern described above and numerous other cardio-metabolic risk factors, a sample of 399 women aged between 30 and 65 years of age were enrolled for this phase, which was conducted in two parts: Part A and Part B.

In Part A, 186 women (44 women in the age group of 30-40 years, 66 women in the age group of 40-50 years and 76 women in the age group of 50-70 years) were enrolled from free-living population during the time span of October 2009 to April 2010 from each four zones of Vadodara city namely, north zone, south zone, east zone and west zone (zonal map outlined in Appendix I).

The information about the enrollment in the study was passed on employing the snowballing technique, wherein one or couple of individuals in each zone of the city was informed about the enrollment of subjects for the study. The potential subjects were notified that the incentives for participation in the study included a free health check-up at their households and provision of
the health report following the health check up. It was made clear to the subjects that they would not incur any cost for participating in the study. All the consenting individuals were asked to read and sign the consent form (Appendix II), those who were not able to read, were explained clearly, the objectives of the study, the information required to be provided by the subject upon enrollment, the fact that a fasting blood sample will be required and that it will be done using safe disposable syringes by a trained lab technician. When the required number of subjects from a particular zone was achieved (roughly 50), the enrollment was stopped.

**Data Acquisition Process**

Once the subjects were enrolled after obtaining the written consent, an appointment was scheduled for one or two hours as per their convenient time and during this allotted time period, the collection of information pertaining to socio-economic status, medical obstetric history, dietary habits & intake, lifestyle habits, physical activity, anthropometric measurements and blood pressure measurements were conducted at the subjects’ place of residence. Following this, another appointment was scheduled for drawing of blood for biochemical estimations within the next few days, which was also done at the subjects’ place of residence. The subjects were adequately informed about the level of fasting required (12 hours) before the blood sample was to be drawn and information on what all fluids were allowed to be ingested during the fasting hours was also provided. On the day of the scheduled appointment, a trained lab technician was taken along with the researcher during the morning to obtain a fasting blood sample from the subject, while care was taken to see that the blood sample was maintained at low temperature during the transportation till it reached the lab. The disposable syringe used for the collecting the blood sample was disposed off immediately after use.
PARAMETERS STUDIED

The parameters that were studied included biochemical ones, biophysical, physical and reported data. These were as follows

1. Reported Data
   - Information on socio-economic status
   - Medical obstetric history
   - Dietary habits & intake
   - Lifestyle habits
   - Physical activity

2. Physical Parameters
   - Height
   - Weight
   - Waist circumference
   - Hip circumference

3. Bio-physical Parameters
   - Blood pressure
   - Bone Mineral Density

4. Biochemical Parameters
   - Blood Hemoglobin
   - Plasma Glucose
   - Serum Lipid profile
   - Serum Thyroid Stimulating Hormone (TSH)
   - Serum Free Thyroxine (FT4)
   - Plasma Insulin
The methodological details of data collection techniques and the parameters studied are explained in the detailed Methods, Tools and Techniques section on page number 121.

**PART B - WOMEN ATTENDING A HEALTH CHECK-UP FACILITY IN AHMEDABAD**

In part B, the formative research was conducted in a clinical setting as opposed to part A where the subjects were from a free living population. The clinical site here was the health check-up facility of the multi-speciality hospital, Jivraj Mehta Smarak Foundation in Ahmedabad, from where the women who came for a health check up were studied during the period of December 2010 to March 2011. After being explained the objectives and nature of the research and the information that they will be required to divulge for the purpose of the research, the potential participants were asked for their consent in writing (Appendix III) for enrolling in the study.

**DATA ACQUISITION PROCESS**

The consenting people were interviewed in the hospital premises itself for obtaining reported data and the measurements for the physical and biophysical parameters under study were done on the same day. The subjects then provided a fasting blood sample in the laboratory associated with the health check up section for estimation of the biochemical parameters. On an average, 5 females had OPD appointments daily and the average response rate was 83.3%. In total, we interviewed 213 subjects aged 20-65 years.

**PARAMETERS STUDIED**

The parameters that were studied included biochemical ones, biophysical, physical and reported data. These were as follows
1. Reported Data
   - Information on socio-economic status
   - Medical obstetric history
   - Dietary habits & intake
   - Lifestyle habits
   - Physical activity

2. Physical Parameters
   - Height
   - Weight
   - Waist circumference
   - Hip circumference

3. Bio-physical Parameters
   - Blood pressure

4. Biochemical Parameters
   - Blood Hemoglobin
   - Fasting Plasma Glucose
   - Post Prandial Plasma Glucose
   - Serum Lipid profile
   - Serum Thyroid Stimulating Hormone (TSH)
   - Plasma Insulin

The methodological details of data collection techniques and the parameters studied are explained in the detailed Methods, Tools and Techniques section on page number 121.
II. FOLLOW-UP STUDY: THE IMMEDIATE AND LONGITUDINAL OUTCOMES OF THE HEALTH CHECK-UP ON WOMEN'S HEALTH-SEEKING PRACTICES

Of the 186 subjects enrolled from the free-living population in the formative research phase, 107 were followed up after a period of 2 years to observe what action pertaining to health was taken immediately after they obtained the results from the health check-up, and track the anthropometric changes undergone by them over a period of 2 years. The follow-up also tracked if the women had taken any health-seeking action after the health check-up till two years. In follow-up, the subjects whose contact details were valid after 2 years were called up for an appointment at a time convenient to them and at the scheduled appointment the reported data and the physical and biophysical measurements were collected.

**Parameters Studied**

The parameters that were studied included biochemical ones, biophysical, physical and reported data. These were as follows

1. Reported Data
   - Information on the action taken after the subjects got the results of the health check up

2. Physical Parameters
   - Height
   - Weight
   - Waist circumference
   - Hip circumference

3. Bio-physical Parameters
   - Blood pressure
The methodological details of data collection techniques and the parameters studied are explained in the detailed Methods, Tools and Techniques section on page number 121.

III. TRANSLATIONAL RESEARCH: EVALUATION OF NUTRITIVE VALUE OF WHEATGRASS POWDER, PRODUCT DEVELOPMENT BY INCORPORATION OF WHEATGRASS POWDER IN SELECTED INDIAN RECIPES AS A FUNCTIONAL FOOD AND EVALUATION OF ACCEPTABILITY OF THESE PRODUCTS

Freeze-dried nitrogen packed wheatgrass powder was procured from an exporting firm based in Vadodara. The nutrient component analysis (conducted by Analytical & Environmental Sciences, Vadodara) included quantitative testing of energy, protein content, total fat, fibre, iron, moisture, ash, carbohydrate & sugar content, ascorbic acid, and β carotene, the methodological details of which have been described in the Methods, Tools and Techniques section on page number 121.

Product development was carried out by incorporating freeze-dried wheatgrass powder in selected Indian recipes. The various recipes tested included Khakhra, Thepla, Muthiya, Dal and Buttermilk. All the recipes were standardized first (Appendix V) and then wheatgrass powder was incorporated at the levels 1g, 1.5g and 2g per unit in case of Khakhra and Thepla; and per serving in case of Muthiya, Dal and Buttermilk. Except Khakhra, Thepla & Muthiya, rest of the recipes did not involve heat application to wheatgrass powder in order to serve the objective preserving the heat-sensitive antioxidant compounds in the freeze-dried wheatgrass powder. Method of preparation of Khakhra involved roasting, with minimal amount of water content, Thepla was also prepared by roasting, but had higher water content than Khakhra; whereas Muthiya was steam cooked.
Following the product development, all the developed products were evaluated for their acceptability depending on the sensory attributes, by conducting a sensory evaluation using composite rating test (Appendix VI). The evaluation panel included 12 semi-trained members comprising of post-graduate and doctoral students of the department. The procedure required the panel members to be present for the testing at least an hour after the previous meal. The evaluation criteria for all the products included aroma, appearance, flavor, color, aftertaste, texture and overall acceptability. To rate the products, the panelists were asked to score each product on a 10 point scale based on degree of liking/dislike for the attributes of the product.

IV. INTERVENTION RESEARCH: IMPACT OF WHEATGRASS POWDER SUPPLEMENTATION ON LIPOPROTEIN STATUS IN PRIMARY HYPERLIPIDEMIC WOMEN – AN OPEN LABEL STUDY

The intervention was targeted at alleviating hyperlipidemia which was found to be the most prevalent problem among the women studied in the formative research phase.

**Design:** The study was conducted using an open label randomized design. The study had a control group, which was not given any intervention and an experimental group which was given the intervention: wheatgrass capsules; and the data was collected prior to and after the intervention (pre and post data). The study design was approved by the Institutional Ethics Review Committee (No. Fc Sc/FND/ME 158 Dated 30/9/2010)

**Preparation of the Treatments**

*Source of Wheatgrass:* Freeze-dried wheatgrass powder (subjected to dehydration at 0-5°C and ground using cold water jacketing) was procured from Aum Agri Freeze Foods, a local exporting firm based in Vadodara. The
powdered wheatgrass was nitrogen-packed so as to ensure minimal moisture accumulation and contamination.

**Encapsulation:** The powdered wheatgrass was encapsulated into 350mg gelatin capsules of size 0, courtesy Centurion Laboratories, Vadodara. Prepared capsules were hermetically packed in sterile plastic jars, each having a capacity to contain 100 capsules.

**Sample-Size Estimation:** Since the intervention was targeted at management of hyperlipidemia, the major outcome that was used to estimate the required sample size was the mean triacylglycerol levels. The targeted percent change in TAG levels was taken as 15%, attrition rate was considered 20% and the standard deviation in the TAG levels in the population was 45mg/dl. With the above mentioned specifications and at 80% power, the sample size was estimated using an online statistical model (Length 2006-2009) adapted for an open label controlled trial and it came out to be 28 in each arm.

**Subjects and Sample Selection:** For the supplementation study the major inclusion criteria was the subject to be a primary hyperlipidemic, therefore the subjects were to be selected from a population of primary hyperlipidemic women. For this reason, the subjects who were found to be hyperlipidemic in the formative research phase were approached for this trial, in addition to other women who had previously been found to have either high TC or high TAG levels subjects, were approached for this trial. All in all, there were 78 women who were identified as being mildly hyperlipidemic in the formative research phase. The potential participants were explained the nature and purpose of the research in a language comprehensible to them, and also the risks and benefits involved in the research, following which a written consent (Appendix IV) was obtained from those willing to participate. They were also explained clearly the fact that
they would not incur any costs for participating in the trial. There were 69 women who gave consent for participation in the study, rest 9 did not. The consenting women were subject to scrutiny by following a set of inclusion and exclusion criteria (Figure 3.4). Those meeting the inclusion criteria & not falling in the exclusion criteria were enrolled in the study, which included 64 women. After enrollment of the subjects, the baseline blood tests were conducted to ascertain hyperlipidemia, following which, 61 subjects were still hyperlipidemic. After this the subjects were randomized into the two study groups: control and experimental group. Randomization was carried out by following the chit method, the specifics of which have been delineated by Giesbrecht and Gumpertz (2004). Following the baseline data collection, 61 women were confirmed to be still mildly hyperlipidemic and after randomization, 31 women were allotted to the experimental group and 30 into the control group.

**DATA ACQUISITION PROCESS**

Data for all the parameters studied was collected in a fashion similar to the formative research phase. After the enrollment, an appointment was scheduled for one or two hours as per a time convenient to the subjects’ time and during this allotted time period, the collection of information pertaining to socio-economic status, medical obstetric history, dietary habits & intake, lifestyle habits, physical activity, anthropometric measurements and blood pressure measurements were conducted at the subjects’ place of residence. The collection of blood sample for biochemical estimations was done at another visit which was scheduled within the next 2-3 days of the first visit, & was done at the subjects’ place of residence. As with the drawing of the previous blood samples, this time too, the subjects were adequately informed about the level of fasting required (12 hours) before the blood sample was to be drawn and information on what all fluids were allowed to be ingested during the fasting hours was also provided. A trained
lab technician collected the venous blood samples from the subjects in the presence of the researcher on the morning of the scheduled appointment and care was taken to see that the blood sample was maintained at low temperature during the transportation till it reached the lab. The disposable syringes used for drawing the blood samples were disposed off immediately after use. The detailed experimental plan, sample selection process and the inclusion criteria for the supplementation study are shown in Figures 3.3, 3.4 and 3.5.

**Parameters Studied**

The parameters that were studied included biochemical ones, biophysical, physical and reported data. These were as follows:

1. **Reported Data**
   - Medical obstetric history
   - Dietary habits & intake
   - Lifestyle habits
   - Physical activity

2. **Physical Parameters**
   - Height
   - Weight
   - Waist circumference
   - Hip circumference

3. **Bio-physical Parameters**
   - Blood pressure
FIGURE 3.3 SELECTION OF SUBJECTS FOR THE WHEATGRASS SUPPLEMENTATION STUDY

78 mildly hyperlipidemic women identified from formative research

69 consented

9 did not give consent for participation

64 met inclusion criteria

5 did not meet inclusion criteria (3 started on statins, 2 diabetics)

Exclusion Criteria
women on statins, having diabetes, pituitary disorders, hyperthyroidism, taking HRT, taking medications for hypothyroidism and Diabetes excluded

Baseline

61 mildly hyperlipidemic

Randomization

Experimental Group n= 31

Control Group n= 30

2 dropouts, final n=29

3 reverted to normocholesterolemia, and no longer conformed to the inclusion criteria, so not enrolled

10 weeks intervention period

Post intervention Data
**Figure 3.4 Inclusion and Exclusion Criteria for the Subject Selection for the Wheatgrass Supplementation Trial**

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Females</td>
<td>• Diabetics</td>
</tr>
<tr>
<td>• Age between 30-60 years</td>
<td>• Taking Hormone Replacement Therapy</td>
</tr>
<tr>
<td>• Hyper-cholesterolemic defined either by serum TAG &gt;200mg/dl and/or serum AG ≥150mg/dl and/or serum LDL ≥100mg/dl</td>
<td>• Statins initiated &lt;3 months before trial</td>
</tr>
<tr>
<td>• Willingness to participate in the trial</td>
<td>• Pituitary disorders</td>
</tr>
<tr>
<td></td>
<td>• Hypothyroidism</td>
</tr>
<tr>
<td></td>
<td>• Genetic traits of Hyperlipidemia</td>
</tr>
<tr>
<td></td>
<td>• Polycystic Ovarian Disease (PCOD)</td>
</tr>
<tr>
<td></td>
<td>• Not willing to participate in the trial</td>
</tr>
</tbody>
</table>
**Figure 3.5 Experimental Design for the Open Label Randomized Controlled Supplementation Study with Wheatgrass Capsules in Primary Hyperlipidemic Women**

**Exclusion Criteria**
- women on statins, having diabetes, pituitary disorders, hyperthyroidism, taking HRT, taking medications for hypothyroidism and Diabetes excluded

61 women selected

Randomization

Group A - **Control** (N=31)

Group B - **Experimental** (N=30)

**Pre-Data**
- Medical History, Dietary and lifestyle habits
- Anthropometry (WC, WHR and BMI)
- Biochemical Parameters (Hb, Lipid profile, Apo a, Apo b, hs-CRP and FBS)
- Bio-physical parameters (SBP and DBP)

No Intervention

10 weeks

4 Wheatgrass capsules containing 350mg wheatgrass powder each daily

**Post-Data**
4. Biochemical Parameters

- Blood Hemoglobin
- Plasma Glucose
- Serum Lipid profile
- Serum Apo A
- Serum Apo B
- Serum hs-CRP

The methodological details of data collection techniques and the parameters studied are explained in the detailed Methods, Tools and Techniques section on page number 121.
### Table 3.1 Methods and Parameters for Data Collection

<table>
<thead>
<tr>
<th>Conditions &amp; Parameters Studied</th>
<th>Data Collected</th>
<th>Tools and Techniques used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bio-physical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Overweight/Obesity, Abdominal Obesity</td>
<td>Height, weight, Waist Circumference (WC)</td>
<td>Non-elastic fiberglass measuring tape, Salter research grade weighing scale</td>
</tr>
<tr>
<td>2 Hypertension</td>
<td>SBP, DBP</td>
<td>Sphygmomanometer</td>
</tr>
<tr>
<td>3 Osteoporosis</td>
<td>Bone Mineral Density at Calcaneus bone (quantitative Ultrasound-qUS)</td>
<td>Achilles EXP III (Lunar, General Electric, China) Ultrasonometer</td>
</tr>
<tr>
<td><strong>Bio-chemical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 2DM and Insulin Resistance</td>
<td>Fasting plasma Glucose and Plasma Insulin</td>
<td>Glucose Oxidase, Chemiluminescent assay</td>
</tr>
<tr>
<td>5 Dyslipidemia</td>
<td>Serum Fasting Lipids, Apc a, Apo b</td>
<td>Cholesterol oxidase, HDL direct precipitation, Friedewald equation, Nephelometry</td>
</tr>
<tr>
<td>6 Anemia</td>
<td>Blood hemoglobin</td>
<td>Cyanmethemoglobin</td>
</tr>
<tr>
<td>7 Sub-clinical Hypothyroidism</td>
<td>Serum Fasting TSH &amp; FT4</td>
<td>Sandwich Chemiluminescent assays</td>
</tr>
<tr>
<td>8 Arterial Inflammation</td>
<td>Serum hs-CRP</td>
<td>Nephelometry</td>
</tr>
<tr>
<td><strong>Dietary and Lifestyle Factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 Dietary &amp; Lifestyle Risk Behaviors</td>
<td>Semi structured protected questionnaire</td>
<td>One to one Interview</td>
</tr>
<tr>
<td>10 Food Intake</td>
<td>24 hour dietary recall method</td>
<td></td>
</tr>
<tr>
<td>11 Nutrient Intake</td>
<td>Calculated from food intake</td>
<td>Nutritive Value of Indian Foods’ Food Composition Tables</td>
</tr>
</tbody>
</table>
METHODS, TOOLS AND TECHNIQUES

A. BACKGROUND INFORMATION, MEDICAL HISTORY AND DIETARY & LIFESTYLE HABITS

Information pertaining to demographic details, medical history, use of medication, family history of chronic diseases, dietary practices, physical activity patterns and smoking status was elicited from each participant in an interview method using a pretested semi-structured questionnaire (Appendix VII). Physical activity level was assessed using an open ended questionnaire which was modified from the Behavioral Risk Factor Survey following standardization and pre-testing. The questions collected information on the number of days per week that the subjects devoted to physical activity and also the duration of activity per day were obtained.

All the questions were asked either in English, Hindi or Gujarati. Collection of all the data including standardized cardiometabolic risk factor screenings such as height, weight, BMI, WC, blood pressure, and estimations of blood hemoglobin, HDL cholesterol, triglycerides, fasting plasma glucose, serum thyroid stimulating hormone, plasma insulin and serum creatinine were carried out by trained health care professionals, including the researcher, who collected all the non-invasive data. The biochemical examinations were carried out at the national accredited diagnostic lab facility, Thyrocare.

B. BODY COMPOSITION PARAMETERS

1) Height and Weight

Height was measured by a non-elastic fiber-glass tape. Body weight was taken by research-grade, portable Salter scales standardized using 5 kg standard weights and readings were recorded to the nearest 0.1kg. BMI
was calculated directly by the standard formula: weight (kg)/height (m²). Then the patient’s Body Mass Index (BMI) was computed with the standard formula: BMI = Weight (kg)/ Height (m²)

For defining overweight and obesity the Asia-Pacific high cut-off points delineated by the WHO expert consultation (2004) were used in this study (Table 3.2).

2) **Waist and Hip Girth**

The waist and hip circumferences (WC and HC) were measured with a non-elastic fiberglass tape by trained examiners using a standard protocol (National Health Institute 1998). For WC, the participants were asked to stand with their feet together and arms placed on the side. The tape was placed through the midpoint between the inferior margin of the last rib and the crest of the ileum in the m.d - auxiliary plane and the measurement was taken to the nearest 0.1 cm. For HC, the subjects were asked to stand straight and the fibre glass tape was placed through the hips and the measurement at the widest point of the hip was recorded to the nearest 0.1 cm. The cut points used for increased WC was defined >80cm for women, as per the WHO 2000 recommendations (Table 3.3).

**Indices of Abdominal Obesity**

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

One of the widely used measures for abdominal obesity, WHR separates gynoid (pear shaped) obesity from android (apple shaped) obesity (Baldwin, 2010). As the name suggests, it’s the ratio of the waist and the hip circumferences

WHR = Waist girth (cm) / Hip girth (cm)
**Table 3.2 Classification of adults according to BMI (WHO, 2004)**

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI (Kg/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt; 18.5</td>
</tr>
<tr>
<td>Normal</td>
<td>18.5 – 22.99</td>
</tr>
<tr>
<td>Overweight</td>
<td>23.0 – 24.99</td>
</tr>
<tr>
<td>Pre-obese</td>
<td>25.0 – 26.99</td>
</tr>
<tr>
<td>Obese</td>
<td>≥27.0</td>
</tr>
</tbody>
</table>

**Table 3.3 Cut offs for Central Obesity**

<table>
<thead>
<tr>
<th>Cut-offs (WHO 2000)</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist to hip ratio</td>
<td>more than 0.95</td>
<td>more than 0.80</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>more than 90 cm</td>
<td>more than 80 cm</td>
</tr>
<tr>
<td>Waist Stature Ratio</td>
<td>more than 0.5</td>
<td>more than 0.5</td>
</tr>
</tbody>
</table>
• **Waist-stature Ratio (WSR)**

Another anthropometric index that has been found to correlate well with cardio-vascular mortality is the waist stature ratio. We used the WHO 2000 cut offs for waist hip ratio waist-stature ratio and waist circumference, the same are given in Table 3.3.

**C. BIO-PHYSICAL PARAMETERS**

• **Blood Pressure**

Systolic and diastolic blood pressure was assessed by a sphygmomanometer using standard protocol by trained personnel. The patient was asked to be seated for the measurement, relax the arm muscles and rest for 5 minutes prior to the measurement. Other things that were ensured before the measurement were that the arm did not have any clothing, was supported at heart level and the palm was facing up; also care was taken that the subjects’ legs were uncrossed. All readings were taken in triplicate. In this study, high blood pressure taken to be a systolic blood pressure value of 140 mm Hg or more, or a diastolic blood pressure 90 mm Hg based on JNC VII guidelines (Chobanian et al 2003), which are outlined in Table 3.4.

**Table 3.4 JNC VII Criteria for Diagnosing Hypertension**

<table>
<thead>
<tr>
<th></th>
<th>Optimal</th>
<th>Pre-hypertension</th>
<th>Stage I Hypertension</th>
<th>Stage II Hypertension</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SBP (mmHg)</strong></td>
<td>&lt;120</td>
<td>120-139</td>
<td>140-159</td>
<td>≥ 160</td>
</tr>
<tr>
<td><strong>DBP (mmHg)</strong></td>
<td>&lt;80</td>
<td>80-89</td>
<td>90-99</td>
<td>≥ 100</td>
</tr>
</tbody>
</table>
• **Bone mineral density**

The bone mineral mass of the subjects was measured through Quantitative Ultrasound (qUS) technique using the Achilles EXP III (Lunar, General Electric, Shanghai, China) ultrasonometer. The site of measurement was the heel bone *Calcaneus*. Despite the gold standard being Dual Energy X-ray Absorptiometry (DEXA) technique, qUS is widely used for the measurement of bone density because it does not involve the use of ionizing radiation, is relatively inexpensive (especially compared DEXA), correlates well with DEXA, is relatively simple to implement and process and the most importantly, it is portable and perfect for large-scale field surveys.

qUS can provide information about the density and elasticity of bone by measuring the velocity of sound through bone, and about the structure of bone by measuring the attenuation of the signal. Bone tissue can be characterized in terms of speed of sound and broadband ultrasound attenuation (BUA). Speed of sound and attenuation of a sound wave are affected by the density, compressibility, viscosity, elasticity, and structure of the material it is travelling through. The primary assumption made while using this technology is that bones with varying biomechanical properties have different attenuation and velocity values as determined by ultrasound. The propagation of the US wave through bone is affected by bone mass, bone architecture, and the directionality of loading among other factors.

It proves to be somewhat difficult to use ultrasound to measure common fracture sites (hip & vertebrae) because the depth of soft tissue surrounding these bones attenuates too much of the ultrasound signal so a reading cannot be obtained. However, such issues are not encountered for calcaneus. There is very little soft tissue which makes it easy to measure bone. It has a relatively flat surface which ensures good
contact between the heel and the transducers. It is similar in composition to the main fracture sites (approx 90% trabecular bone). It is easily accessible and requires very little patient preparation (Evans 2006). This makes calcaneus the most popular ultrasound measurement site.

**Principle:** Ultrasound imaging devices offer a parametric image of what is called as the Broadband Ultrasound Attenuation (BUA) at the calcaneus. For a given material the attenuation of the ultrasound wave will be constant, known as its BUA index. This is a measure of the increase in attenuation of the ultrasound wave as a function of increasing frequency. An ultrasound wave covering a range of frequencies is passed through a known thickness of sample. The amplitude spectrum of the received signal is then compared to the spectrum of a reference material. The difference between the two spectra is plotted against frequency and the slope is the BUA index (dB/MHz). If it is then divided by the thickness of the measured sample, it gives a volumetric parameter in (dB/MHz)/cm. Along with the BUA, the Achilles EXP III also gives the T-score, which is used to classify the individual as having adequate bone mineral density or not, using the WHO (2007) classification suggested by the WHO scientific consultation group on assessment of osteoporosis, as outlined in table 3.5.

**Table 3.5 WHO Criteria for Diagnosing Osteoporosis and Osteopenia**

<table>
<thead>
<tr>
<th>Category</th>
<th>T-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&gt; -1</td>
</tr>
<tr>
<td>Osteopenia</td>
<td>-1 to -2.5</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>&lt; -2.5</td>
</tr>
</tbody>
</table>
The precision of this technique was found to be 1.4 to 3.3 percent and at the calcaneus qUS and DEXA measurements have been found to have a correlation of approx. 0.8 to 0.85 (Gluer 1997). However there are also studies which found that the precision of qUS is not as good and changes in qUS of the heel may not reflect changes in BMD at the spine or hip. But, more importantly, studies also suggest that qUS is useful in determining fracture risk of hip and spine independently of BMD measurements, with qUS devices demonstrating significant age-adjusted odds ratios regarding the association with fractures and the diagnosis of osteoporosis (Hans, Hartl and Krieg 2003).

D. BIOCHEMICAL PARAMETERS

- **Blood Hemoglobin**

  Hemoglobin was estimated in whole blood using the cyanmethemoglobin method (Gowenlock 1988). The hemoglobin cut-offs used for diagnosing various severities of anemia was as per the WHO criteria, outlined in Table 3.6.

**Table 3.6 WHO Criteria for Diagnosing Severity of Anemia**

<table>
<thead>
<tr>
<th>Severity</th>
<th>Range (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>11.0-11.9</td>
</tr>
<tr>
<td>Moderate</td>
<td>8.0-10.9</td>
</tr>
<tr>
<td>Severe</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>
**Principle:** The popular cyanmethemoglobin method makes use of the fact that when the ferrous ions in the hemoglobin molecule are oxidized to ferric form, it results in formation of a compound called methemoglobin, which when made to react with cyanide ions, forms cyanmethemoglobin which is a colored compound proportional to the content of hemoglobin in the test sample and can be measured spectrophotometrically at the absorbance of 540nm. To remove the cell membrane barrier of the erythrocytes, the whole blood is combined with the Drabkin’s reagent which results in hemolysis of the erythrocytes and the hemoglobin is released into the solution containing potassium ferricyanide and the oxidation reaction of the ferric ions begins resulting in formation of cyanmethemoglobin.

- **Plasma Glucose**

Glucose was estimated in blood spectrophotometrically on an EM200 (Erbannhein, Germany) fully automated autoanalyzer using the glucose oxidase method (Trinder 1969), for which the diagnostics kits were obtained from Aggape Diagnostics, India. Diagnosis of diabetes and impaired glucose metabolism was made according the ADA 2011 guidelines, given in table 3.7.

**Principle:** In the glucose oxidase method, the aldehyde group of glucose molecule is acted upon by the enzyme glucose oxidase to form gluconic acid and hydrogen peroxide. Peroxide further undergoes a condensation reaction, catalyzed by horseradish peroxidase, wherein along with the colorless chromogen compound 4-amino phenazone, forms a red colored compound which can be photometrically read at 505nm.
## Table 3.7 ADA 2011 Guidelines for Diagnosis of Impaired Glucose Tolerance and Diabetes

<table>
<thead>
<tr>
<th></th>
<th>Fasting Blood Sugar levels (mg/dl)</th>
<th>Blood Sugar levels After 2 hr of meal or 75g oral glucose load (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impaired Fasting Glucose (IFG)</td>
<td>100-125 and 100-125</td>
<td>&lt;140 and 140-199</td>
</tr>
<tr>
<td>Impaired Glucose Tolerance (IGT)</td>
<td>&lt;100 and &lt;100</td>
<td>140-199</td>
</tr>
<tr>
<td>Pre-diabetes</td>
<td>100-125 And/or 100-125</td>
<td>140-199</td>
</tr>
<tr>
<td>Diabetes</td>
<td>&gt;126 or &gt;126</td>
<td>&gt;200 (or random blood sugar)</td>
</tr>
</tbody>
</table>
• **Plasma Insulin**

Plasma Insulin after a 12 hour fast was assessed using the Insulin Chemiluminescent immune assay (CLIA). Briefly, Chemiluminescence is another technique employed to follow antigen–antibody combination.

\[
\beta\text{-D-glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{Glucose oxidase}} \text{D-gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-amino phenazone} \xrightarrow{\text{Peroxidase}} \text{red colored product} + 4 \text{H}_2\text{O}
\]

Principle of Chemiluminescence makes use of emission of light caused by a chemical reaction, typically an oxidation, which gives rise to an excited molecule that emits light while decaying back to its ground state. A large number of molecules are capable of chemiluminescence, but the most common substances used are luminol, acridinium esters, ruthenium derivatives, and nitrophenylloxalates. When these substances are oxidized, typically using hydrogen peroxide and an enzyme as a catalyst, intermediates are produced that are of a higher energy state. These intermediates spontaneously return to their original state, giving off energy in the form of light. Light emissions range from a rapid flash of light to a more continuous glow that can last for hours. Acridinium esters, for example, emit a quick burst of flash of light, while the light remains for a longer time with luminol and dioxetane (Stovons 2010).

This type of labeling is used for heterogeneous and homogeneous assays, because labels can be attached to either antigen or antibody. In heterogeneous assays, competitive and sandwich formats are the ones most often used. Smaller analytes such as therapeutic drugs and steroid hormones are measured using competitive assays, while the sandwich format is used for larger analytes such as protein hormones.
Chemiluminescent assays have an excellent sensitivity, comparable to ELISA and RIA, and the reagents are stable and relatively nontoxic. The sensitivity of some assays has been reported to be in the range of attamoles (10-18mol) to zeptomoles (10-21mol). Because very little reagent is used, they are also quite inexpensive to perform. The relatively high speed of detection also means a faster turnaround time.

**Principle:** The insulin CLIA is a solid phase enzyme linked immunosorbent assay. The assay system utilizes one anti-Insulin antibody for solid phase (microtiter wells) immobilization and another anti-Insulin antibody in the antibody-enzyme conjugate solution, which has horseradish peroxidase. The standards and serum are added to the Insulin antibody coated microtiter wells. Then anti-Insulin antibody labeled with horseradish peroxidase conjugate solution is added. The Insulin present in the serum combines with the antibody on the well and the enzyme conjugate resulting in the Insulin molecules being sandwiched between the solid phase and enzyme-linked antibodies. After an hour of incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of chemiluminescent substrate is then added and read relative light units (RLU) in Luminometers. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of Insulin in the sample. By reference to a series of Insulin standards assayed in the same way, the concentration of Insulin in the unknown sample is quantified.

The cut-point used for identifying hyperinsulinemia in this study was an insulin concentration of >12mU/L (McAuley et al 2001).
• **Serum Total Cholesterol (TC)**

The total serum cholesterol was estimated photometrically on Olympus AU2700 (Beckman Coulter) chemistry analyzer using Aggape cholesterol oxidase kits. The cut-points used for detecting hypercholesterolemia were used from the NCEP ATP III guidelines (2001) given in Table 3.8.

**Principle**: Estimation was done by the cholesterol oxidase method, (Allain et al. 1974) which makes use of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidase (POD). In the first step, CE separates cholesterol esters in to cholesterol and fatty acids. In the second step, CO oxidizes the cholesterol into a ketone and hydrogen peroxide, which in the presence of peroxidase condenses the mixture of phenol and 4-aminocantipyrine (4-AA) are to form a red colored quinoneimine in what is called the Trinder’s reaction. This quinoneimine dye is proportional to the concentration of cholesterol in the sample and is read colorimetrically at 505 nm (green filter) to estimate the cholesterol in the sample.

\[
\text{Cholesterol esters} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{FA}
\]

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Choles-4-ene-3-one} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinonimine}
\]

• **Serum Triacylglycerols (TAG)**

Triacylglycerols in serum were estimated using the glycerol kinase (Buccolo and David 1973) diagnostic kits (Aggape) employing photometry on an Advia1800 (Siemens) chemistry analyzer.

**Principle**: Serum TAG were estimated using in a four step method, where first the TAG are split using lipoprotein lipase and the resulting
**Table 3.8 NCEP ATP III (2001) Criteria for Diagnosing Dyslipidemia**

<table>
<thead>
<tr>
<th>Total Cholesterol - TC (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;200</td>
<td>Desirable</td>
</tr>
<tr>
<td>200-239</td>
<td>Borderline high</td>
</tr>
<tr>
<td>&gt;240</td>
<td>High</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LDL Cholesterol (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100</td>
<td>Optimal</td>
</tr>
<tr>
<td>100-129</td>
<td>Near optimal/above optimal</td>
</tr>
<tr>
<td>130-159</td>
<td>Borderline high</td>
</tr>
<tr>
<td>160-180</td>
<td>High</td>
</tr>
<tr>
<td>&gt;190</td>
<td>Very high</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HDL Cholesterol (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50 (for women)</td>
<td>Low</td>
</tr>
<tr>
<td>&gt;60</td>
<td>High</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triacylglycerols- TAG (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;150</td>
<td>Normal</td>
</tr>
<tr>
<td>150-199</td>
<td>Borderline high</td>
</tr>
<tr>
<td>200-499</td>
<td>High</td>
</tr>
<tr>
<td>500</td>
<td>Very high</td>
</tr>
</tbody>
</table>
glycerols which are further phosphorylated using glycerol kinase. The glycerol phosphates are oxidized in to form Dihydroxy Acetone Phosphate (DHAP) and hydrogen peroxide, which in turn, following the Trinder’s reaction generates the red colored quinoneimine dye, which is colorimetrically read at 505 nm using the green filter.

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{lipase}} \text{Glycerol} + \text{FFA} \\
\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP} \\
\text{Glycerol-3-phosphate} + \text{O} \xrightarrow{\text{Glycerol-Phosphate Oxidase}} \text{DHAP} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{- Aminoantipyrine} + \text{Chlorophenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine}
\]

- **SERUM LOW DENSITY LIPOPROTEIN (LDL) CHOLESTEROL**

The LDL levels were estimated directly on an Olympus AU2700 (Beckman Coulter) chemistry analyzer using the Rapid Diagnostics’ LDL direct measurement diagnostic kit.

**Principle**: The principle for estimation employs two steps: first where the total lipoproteins are eliminated by cholesterol oxidase and in the second step, employing the cholesterol oxidase-peroxidase method, LDL is estimated as in the estimation of total cholesterol.

**Step 1- Elimination of lipoproteins other than LDL**

\[
\text{Cholesterol esters} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{FA} \\
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol-3-one} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\ \text{H}_2\text{O} + \text{O}_2
\]
Step 2 Measurement of LDL

\[
\begin{align*}
\text{Cholesterol esters} + \text{H}_2\text{O} & \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{FA} \\
\text{Cholesterol} + \text{O}_2 & \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol-3-one} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{-AAP} + \text{Phenol} & \xrightarrow{\text{Peroxidase}} \text{Quinonimine} + 4\text{H}_2\text{O}_2
\end{align*}
\]

- **Serum High Density Lipoprotein (HDL) Cholesterol**

  The HDL cholesterol assay was performed using the HDL cholesterol plus second generation assay for direct measurement using diagnostic kits (Aggapo) on an Olympus AU2700 (Bockman Coulter) chemistry analyzer.

  **Principle**: Briefly. The apo B containing lipoproteins in the specimen namely, very low density lipoprotein (VLDL) and the low density lipoproteins (LDL), are made to react with a blocking reagent that makes them non-reactive with the cholesterol enzyme reagents. Hereafter, running the cholesterol esterase detects only the HDL in the specimen.

- **Serum VLDL Cholesterol**

  The very low density lipoprotein cholesterol was arrived at using the Friedewald (1972) formula using the triglycerides, total cholesterol and LDL concentrations. The standard assumption made while using this formula is that there are no chylomicrons present in the serum while estimating other lipoprotein fractions to be used in the formula.

  \[
  \text{VLDL} = \text{TC} - \text{LDL} - \text{HDL}
  \]
• **Serum Apolipoprotein A (apo A) and Apolipoprotein B (apo B)**

The present apolipoprotein assays were done using a BN II automated nephelometer (Dade Behring/Siemens), which uses the N Antiserum to Human Apolipoprotein A-I and Apolipoprotein B as the reagent.

**Principle:** Serum concentrations of apolipoproteins a and b were estimated using Nephelometry. Nephelometry measures the light that is scattered at a particular angle from an incident beam as it passes through a suspension. The amount of light scattered is an index of the solution's concentration. Beginning with a constant amount of antibody, if the amount of antigen is increased, it results in an increase in antigen–antibody complexes. Thus, the relationship between antigen concentrations approaches linearity, as indicated by antigen–antibody complex formation and light scattering. Light scatter may be recorded in arbitrary units of “relative light scatter,” or it may be directly extrapolated by a computer to give actual concentrations in milligrams per deciliter (mg/dL) or international units per milliliter (IU/mL), based on established values of standards. Nephelometers measure light scatter at angles ranging from 10 degrees to about 90 degrees (Stevens 2010).

• **Serum Thyroid Stimulating Hormone (TSH) and**

The TSH estimation in serum was carried using a chemiluminescent two-site or ‘sandwich’ type assay. The cut-off used to detect low TSH levels was > 4μU/ml (Baskin et al 2002).

**Principle:** This CLIA uses two specific antibodies. The microtiter wells are coated with a monoclonal antibody specific for TSH and another monoclonal antibody specific for a different region of TSH is conjugated to horseradish peroxidase (HRP). In this one-step capture assay, the TSH from the test specimen and standards are allowed to bind simultaneously to the antibody coated wells and to the HRP conjugate.
The HRP conjugate is removed by washing after incubation and decantation. Following this, a luminescent substrate is added to the system, which leads to the phenomenon of light emission and the luminosity in terms of relative luminescence units (RLUs) are measured in a microtiter plate luminometer. The RLUs formed by the enzymatic reaction are directly proportional to the enzyme conjugate bound to the solid phase in the samples. A set of standards is used to plot a standard calibration curve which is used to determine the concentration of TSH in samples.

- **Serum Free Thyroxine (FT4)**

The unbound or free Thyroxine (FT4) was estimated in serum using the competitive CI IA. The cut-off level used to determine low FT4 levels was <0.9ng/dl (Baskin et al 2002).

**Principle:** The solid phase is the T4 antibody coated on the microtiter wells. To this, a conjugate of T4 and horseradish peroxidase along with the test specimen, in this case the subject’s serum, are added. Hereafter, the FT4 in the test specimen and the FT4 in the conjugate compete for the limited T4 antibody on the microtiter wells. After an incubation period of 60min at 37 degrees Celsius, the unbound T4-enzyme conjugate is washed off and the RLU is measured, as is it is inversely proportional to the FT4 in the test specimen.

- **Serum C- Reactive Protein high sensitivity Assay (hs-CRP)**

The high sensitivity assay of C - reactive protein (hs-CRP) in the serum was done using nephelometry on a BN II automated nephelometer (Dade Behring/Siemens).
Principle: Here a soluble analyte and corresponding antibodies that are bound to polystyrene particles are made to react. The test specimen is mixed with latex particles coated with monoclonal antibodies (anti-CRP antibodies), so the CRP present in the specimen will bind with the latex bound antibodies.

**BIOCHEMICAL INDICES**

- **HOMA 2**

  The index used for assessing insulin resistance was the modified version of Homeostasis Model Assessment or HOMA put forth by David Jonathan Levy et al (1998) called as HOMA 2. This new version is different from the first version (published by David Matthews et al in 1985), because it took into consideration the hepatic and peripheral glucose resistance variations and the increases in the insulin secretion curve for glucose levels higher than 180mg/dl, and also the contribution of circulating pro-insulin. It was calculated using the software HOMA 2 Calculator version 2.2, released in 2004 by the Oxford Centre for Diabetes, Endocrinology and Metabolism. The University of Oxford. The cut-point used for diagnosing insulin resistance using in this study, a HOMA 2 value greater than 1.4 was used (Geloneze, Vasques & Stabe 2009).

**E. NUTRIENT CONTENT ANALYSIS OF WHEATGRASS POWDER**

A sample of the freeze-dried nitrogen packed wheatgrass powder that was procured from an exporting firm in Vadodara was sent for evaluation of nutritional value. The nutrient component analysis (conducted by Analytical & Environmental Sciences, Vadodara) included quantitative testing of energy, protein content, total fat, fibre, iron, moisture, ash, carbohydrate & sugar content, ascorbic acid, and β carotene. For this, the
sample of wheatgrass powder was homogenized and a representative sample was taken after confirming that it was free from insect infestation and fungal growth. also that it conformed to the general PFA rules (1995). The estimation of nutritive value was conducted by employing methods conforming to the BIS standards. These methods have been described in Table 3.9.

STATISTICAL ANALYSES

- Statistical analysis has been carried out using the software packages Microsoft Excel 2007, Epi Info 3.4.1 and SPSS 17.
- Data has been described using descriptive statistics (means and standard deviations) for continuous variables. Data has also been described using proportions: percentages in case of categorical variables and prevalence rates for continuous variables by using cut-off points.
- Wherever relevant, the 95% confidence intervals in which the means and proportions lied have been indicated.
- The two sample Students’ t test and Chi-square test have been performed to compare continuous and categorical variables, respectively.
- Bivariate analyses including Odds ratios and Correlation analyses (Spearman for non parametric variables) have been performed to estimate associations between two variables.
- Partial Correlation and binomial logistic regression (for odds ratios) were computed to estimate true associations between variables/conditions, while adjusting for covariates to rule out residual confounding.
- Data has been segregated based on quintiles of anthropometric indices to observe the distribution of prevalence of risk factors across quintiles of BMI, WC and WSR.
- In multivariate analysis, Stepwise backward Multiple Linear regression has been applied to find out the set of variables that contributed significantly to
**Table 3.9 Methods used for Nutritional Analysis of Wheatgrass Powder**

<table>
<thead>
<tr>
<th>Test</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>Dehydration using hot oven</td>
</tr>
<tr>
<td>Ash</td>
<td>Dry Ashing using Muffle furnace</td>
</tr>
<tr>
<td>Fat</td>
<td>Soxhlet method</td>
</tr>
<tr>
<td>Protein</td>
<td>Kjeldahl method</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Difference method</td>
</tr>
<tr>
<td>Energy</td>
<td>Calculation</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Titremetry</td>
</tr>
<tr>
<td>Fibre</td>
<td>Colorimetry</td>
</tr>
<tr>
<td>Iron</td>
<td>Colorimetry</td>
</tr>
<tr>
<td>β-carotene</td>
<td>Spectrophotometry</td>
</tr>
</tbody>
</table>
to the variation in blood pressure and blood sugar levels.

- Paired ‘t’ test has been used to compare the difference between the pre and post intervention values of the outcome variables in the intervention study.
- One way analysis of variance (ANOVA) was computed to find the difference in the sensory evaluation scores across the categories of varying wheatgrass concentration of the wheatgrass incorporated recipes.
- All statistical analyses were considered significant at a 2 tailed significance level of P<0.05.