CHAPTER - 4

MATERIALS
&
METHODS
STUDY POPULATION

A. Cases:

Fist degree relatives of type-2 diabetes mellitus patients above 15 years of age, who are not previously diagnosed to have either type-2 diabetes mellitus or, abnormal glucose tolerance.

I regret to tell that I have not conducted auto antibodies study to categorize type-2, Diabetes mellitus patients. However, according to a study conducted by Kirsten A. McAuley and associates (91), type-2 diabetes mellitus patients are those who are diagnosed as diabetes after 30 year of age and who are not requiring insulin during the first 6 months from diagnosis. Socioeconomic status, activity and level of education of participants have not been included as parameters of the study, so these were not studied.

First degree relatives, by definition, include individuals having 50% genome common to the index case i.e., parents, siblings and offsprings.

INCLUSION CRITERIA

- Individuals includes should be the first degree relatives of Type-2, Diabetes patients registered in diabetic clinic, admitted in Medicine Department, attended OPD of Medicine Department, at C.S.M. Medical University, Lucknow.
- Included individuals should be more than 15 years of age.
- Individuals should not belong to the class of secondary diabetes due to pancreatic diseases, hormonal abnormalities, drug induced, genetic syndromes etc.
EXCLUSION CRITERIA:

- First degree relatives of Type-2 diabetic patients of less than 15 years of age.
- Pregnancy and lactation.
- Individuals belonging to the class of secondary diabetes, as mentioned above.

B. Controls:

The controls were healthy individuals; not suffering from type-2 diabetes mellitus nor having any family history of type-2 diabetes mellitus; not suffering from any acute or chronic cardiovascular diseases; not taking any drugs believed to alter plasma glucose level.

RESEARCH DESIGN AND METHODS:

A Total number of 450 cases and 450 controls gave verbal consent to participate in the study after explaining the purpose of the study.

Identification of individuals to be screened was kept in terms of number, age, sex, habits, addresses, phone number (s), if any, for easy accessibility.

Age: as stated by the patient and no documentary proof had been entertained.

Anthropometric Measurements

After 8-hour overnight fast, each participant was assessed for morphological built up which included the following parameters:

- Body weight (in Kg)
- Height (in meters)
- Body mass index (Weight/Height$^2$) (in Kg/m$^2$)
- Waist circumference (in cm)
- Hip circumference (in cm)
- Waist- Hip ratio.

Cases and controls were weighed in the same weighing machine and were measured for their heights in the same height machine.

Body mass index (BMI) is calculated by dividing the participant’s weight in kilogram by his/her height in Meter Square (kg/m$^2$). The advantage of this index rather than using the weight alone for estimation of obesity is that it is height dependent.
Chapter - 4 : Material & Methods

The internationally accepted (WHO) range of BMI shown in the following information box was used for estimation of obesity.

<table>
<thead>
<tr>
<th>Class</th>
<th>BMI (Kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under Weight</td>
<td>&lt; 18.5</td>
</tr>
<tr>
<td>Normal</td>
<td>18.5 – 24.9</td>
</tr>
<tr>
<td>Over Weight</td>
<td>25.0 – 29.9</td>
</tr>
<tr>
<td>Obese Class-I(moderate)</td>
<td>30.0–34.9</td>
</tr>
<tr>
<td>Obese Class-II(severe)</td>
<td>35.0–39.9</td>
</tr>
<tr>
<td>Obese Class-III(very severe/morbid obese)</td>
<td>≥ 40</td>
</tr>
</tbody>
</table>

WAIST HIP RATIO

It was taken for measuring abdominal or central obesity as it more closely relates to disease risk than BMI alone.

Waist circumference was measured as the minimum circumference between the iliac crest and the rib cage with the subject standing by a soft tape.

Hip circumference was measured at the maximum width over the greater trochanters.

Then, waist hip ratio (WHR) was calculated from these measurements. WHR greater than 0.9 in males and greater than 0.85 in females was taken as abdominal or central obesity.

PLASMA GLUCOSE ESTIMATION

Plasma glucose estimation was done by autozyme stat glucose test based on enzymatic method using glucose oxidase and peroxidase as enzymes.

The kits for measurement were purchased from ACCUREX Biomedical Pvt. Ltd., 212, Udyog Mandir, Mumbai.

Principle: Glucose oxidase (GOD) converts glucose to gluconic acid. Hydrogen peroxide formed in this reaction in presence of peroxidase (POD) oxidatively couples with 4-aminocoumarin or, phenol to produce red quinoneimine dye.

This dye has absorbance maximum at 505 nm. The intensity of the color complex is directly proportional to the glucose in specimen.
\[ \beta-D\text{-Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \]
\[ H_2O_2 + 4\text{-amino antipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red dye} + H_2O \]

**Fasting Lipid Measurement**

I. **Triglyceride**

Serum triglyceride was measured by an infinite liquid triglyceride method based on enzymatic determination using lipoprotein lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase as enzymes. The kits for measurement were purchased from ACCUREX Biomedical Pvt. Ltd. 212, Udyog Mandir, Mumbai. Lipoprotein lipase hydrolyses triglycerides to yield glycerol and fatty acids. Glycerol kinase converts glycerol to glycerol-3-phosphate, which is oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-amino antipyrine and 4-chlorophenol to produce red quinolinimine dye. The intensity of red colour formed is directly proportional to the concentration of triglycerides in the specimen and is measured photometrically.

II. **HDL-Cholesterol**

Serum HDL cholesterol was measured by autozyme HDL-Cholesterol precipitating reagent method based on enzymatic determination using phosphotungstate as enzyme.

The kits for measurement were purchased from ACCUREX Biomedical Pvt. Ltd.; 212, Udyog Mandir, Mumbai. Cholestest N HDL is a liquid reagent that directly measures the HDL-cholesterol concentration in serum by a new method that is based on the selective solubilizing effect of proprietary detergent to the different lipoproteins. In the assay system, only HDL is solubilized by a special detergent; other lipoproteins are not disrupted. After HDL is selectively disrupted, HDL cholesterol is measured enzymatically.

III. **Total Cholesterol**

Serum total cholesterol was measured by autozyme Cholesterol precipitating reagent method based on enzymatic determination.
The kits for measurement were purchased from ACCUREX Biomedical Pvt. Ltd. 212, Udyog Mandir, Mumbai. The detailed literature is available on http://www.accurex.com. Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase (CE) to cholesterol and free fatty acids. Free cholesterol, including that originally present, is then oxidized by cholesterol oxidase (CO) to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4-aminoantipyrine in the presence of peroxidase (POD) to form a chromophore (quinoneimine dye) which may be quantitated at 500-505 nm. The intensity of red colour formed is directly proportional to the concentration of total cholesterol in the specimen and measured spectrophotometrically.

IV. LDL-Cholesterol

Cholestest LDL is a liquid reagent that directly measures the concentration of LDL-cholesterol by a new homogeneous method based on an innovative detergent technology.

The serum LDL cholesterol level was calculated using Friedewald equation as follows:

\[ \text{LDL Cholesterol} = \text{Total Cholesterol} - \left(\frac{\text{HDL Cholesterol} + \text{Triglyceride}}{5}\right) \]

V. VLDL-Cholesterol

Serum VLDL cholesterol is calculated by following formula

\[ \text{VLDL cholesterol} = \frac{\text{Triglyceride}}{5} \]

FASTING PLASMA INSULIN LEVEL

Fasting plasma insulin was measured by $^{125}$I-radio immunoassay (RIA) technique. The RIA technique is based upon the competition of unlabelled insulin in the standard or samples and radio-iodinated ($^{125}$I) insulin for the limited binding sites on a specific antibody.

Procedure

5 ml of blood was drawn from the participant and was allowed to clot at room temperature. The plasma was separated and stored at -20°C. The plasma insulin level was measured using radio-immunoassay kit (kit for insulin was purchased from Board
of Radiation and Isotope Technology, BARC, VASHI complex, Navy Mumbai-05)

At the end of incubation, the bound antibody and free insulin was separated by the second antibody polyethylene Glycol (PEG) aided separation method. Insulin concentration of samples was quantitated by measuring the radioactivity in gamma counter.

Normal value: 0-30 μU/ml

**MEASUREMENT OF INSULIN RESISTANCE**

Though fasting insulin level alone is accurate at predicting insulin resistance, we applied the following methods for higher sensitivity and specificity when predicting insulin sensitivity.

1. **HOMEOSTASIS MODEL ASSESSMENT OF INSULIN RESISTANCE (HOMA<sub>IR</sub>)**
   The following formula is used —
   \[ \text{HOMA}_{IR} = \frac{\text{Fasting insulin (μU/ml)} \times \text{fasting plasma glucose (mmol/l)}}{22.5} \]

   Insulin resistance is defined as the highest quartile of HOMA<sub>IR</sub> index.

2. **McFarland Index or Insulin sensitivity Index (ISI) corrected for Fat free mass (Mffm/I)**
   According to a study conducted by McAuley et al (91), the variables that best predicted insulin sensitivity were fasting insulin level and fasting triglyceride level. According to them,
   \[ \text{Mffm/I} = \exp [2.63 - 0.28 \ln (\text{insulin}) - 0.31 \ln (\text{TG})] \]
   Where, I.insulin is in μU/ml and T.G. is triglyceride in mmol/l

3. **Insulinogenic index**
   \[
   \frac{\text{Fasting Insulin level (μU/ml)}}{\text{Fasting Plasma glucose (mmol/L)}}
   \]

4. **Bennett index**
   \[
   \frac{1}{\text{Fasting Plasma glucose (mmol/L)}}
   \]
In (Fasting glucose level (mmol/l)) In (fasting level (μu/ml))

5. **Quantitative Insulin Sensitivity Check Index (QUICKI)**
   The QUICKI is based on the logarithmic transformation:
   
   \[
   \text{QUICKI} = \frac{1}{\log \text{insulin} + \log \text{glycemia in mg/dl}}
   \]

**MEASUREMENT OF LEPTIN LEVELS**

The fasting leptin concentration was measured using human leptin RIA kit purchased from Linco Research, Inc (St Charles, MO) USA.

**Principle:** A fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody bound free from tracer and counting one or the other, or both fractions. A calibration or standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated.

Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

**ASSAY PROCEDURE**

**Assay Set-up**

**Day One**
Chapter - 4 : Material & Methods

1. Pipette 300 μl of assay buffer to the non-specific binding (NSB) tubes (3-4), 200 μl to reference (Bo) tubes (5-6), and 100 μl to tubes 7 through the end of the assay.

2. Pipette 100 μl of standards and quality controls in duplicate (see flow chart).

3. Pipette 100 μl of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when Leptin concentrations are anticipated to be elevated or when sample size is limited. Additional assay buffer should be added to compensate for the difference so that the volume is equivalent to 100 μl, e.g., when using 50 μl of sample, add 50 μl of buffer).

4. Pipette 100 μl of 125I-Human Leptin to all tubes.

5. Pipette 100 μl of Human Leptin antibody to all tubes except total count tubes (1-2) and NSB tubes (3-4).

6. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

Day Two

7. Add 1.0 ml of cold (4°C) precipitating reagent to all tubes (except total count tubes).

8. Vortex and incubate 20 minutes at 4°C.

9. Centrifuge, 4°C, all tubes [except total count tubes (1-2)] for 20 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, the time of centrifugation must be increased to obtain a firm pellet (e.g., 40 minutes). Multiple centrifuge runs within an assay must be consistent.

Conversion of rpm to xg:

\[ xg = (1.12 \times 10^{-5}) \times (rpm)^2 \]

\[ r = \text{radial distance in cm (from axis of rotation to the bottom of the tube)} \]

\[ \text{rpm} = \text{rotational velocity of the rotor} \]

10. Immediately decant the supernatant of all tubes except total count tubes (1-2), drain tubes for at least 15-60 seconds (be consistent
between racks), and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.

11. Count all tubes in a gamma counter for 1 minute. Calculate the ng/ml of Human Leptin in unknown samples using automated data reduction procedures.
## ASSAY PROCEDURE FLOW CHART

| Tube Number | Day One | | | Day Two | | | | | |
|-------------|---------|---------|---------|---------|---------|---------|---------|---------|
|             | Step 1  | Step 2-3 | Step 4  | Step 5  | Step 6  | Step 7  | Step 8  | Step 9-11 |
|             | Add Assay Buffer | Add Standard/ QC Sample | Add $^{125}$I-Leptin Tracer | Add Leptin Antibody | Vortex and Incubate 20-24 hr at 4°C | Add Precipitating Reagent | Vortex, and Incubate 20 min. at 4°C | Centrifuge for 20 min, Decant & Count Pellets |
| 1,2         | -       | -       | 100 µl  | -       | -       | -       | 1.0 ml  |           |
| 3,4         | 300 µl  | -       | 100 µl  | -       | -       | 1.0 ml  |           |           |
| 5,6         | 200 µl  | -       | 100 µl  | 100 µl  | 100 µl  | 1.0 ml  | 1.0 ml  |           |
| 7,8         | 100 µl  | 100 µl of 0.5 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 9,10        | 100 µl  | 100 µl of 1 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 11,12       | 100 µl  | 100 µl of 2 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 13,14       | 100 µl  | 100 µl of 5 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 15,16       | 100 µl  | 100 µl of 10 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 17,18       | 100 µl  | 100 µl of 20 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 19,20       | 100 µl  | 100 µl of 50 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 21,22       | 100 µl  | 100 µl of 100 ng/ml | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 23,24       | 100 µl  | 100 µl of QC 1   | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 25,26       | 100 µl  | 100 µl of QC 2   | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 27,28       | 100 µl  | 100 µl of unknown | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
| 29-n        | 100 µl  | 100 µl of unknown | 100 µl  | 100 µl  | 1.0 ml  |           |           |           |
MEASUREMENT OF ADIPONECTIN LEVELS

Serum adiponectin was estimated using the radioimmunoassay kit (RIA-K-1) for adiponectin provided by Linco Research Inc, USA.

Principle: A fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A calibration or standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated.

Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

ASSAY PROCEDURE

A. Dilute the 10X Assay Buffer with 450 ml distilled or deionised water to prepare working concentration of 1X Assay Buffer.

B. Human Adiponectin Standard Preparation

Using an Eppendorff pipette, reconstitute the Human Adiponectin Standard with 1 ml distilled or deionized water into the glass vial to give a 200 ng/ml concentration of Standard. Mix well.

Label eight glass tubes 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 ng/ml. Add 0.5 ml Assay Buffer to each of the eight tubes. Prepare serial dilutions by adding 0.5 ml of the 200 ng/ml reconstituted standard to the 100 ng/ml tube, mix well and transfer 0.5 ml of the 100 ng/ml Standard to the 50 ng/ml tube, mix well and transfer 0.5 ml of the 50 ng/ml Standard to the 25 ng/ml tube, mix well and transfer 0.5 ml of
the 25 ng/ml Standard to the 12.5 ng/ml tube, mix well and transfer 0.5 ml of the 12.5 ng/ml Standard to the 6.25 tube, mix well and transfer 0.5 ml of the 6.25 ng/ml Standard to the 3.125 ng/ml tube, mix well and transfer 0.5 ml of the 3.125 ng/ml Standard to the 1.56 ng/ml tube, mix well and transfer 0.5 ml of the 1.56 ng/ml Standard to the 0.78 ng/ml tube and mix well.

<table>
<thead>
<tr>
<th>Standard Concentration ng/ml</th>
<th>Vol. of Deionised Water to Add</th>
<th>Vol. of Standard to Add</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>1 ml</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard Concentration ng/ml</th>
<th>Vol. of Assay Buffer to Add</th>
<th>Vol. of Standard to Add</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.5 ml</td>
<td>0.5 ml of 200 ng/ml</td>
</tr>
<tr>
<td>50</td>
<td>0.5 ml</td>
<td>0.5 ml of 100 ng/ml</td>
</tr>
<tr>
<td>25</td>
<td>0.5 ml</td>
<td>0.5 ml of 50 ng/ml</td>
</tr>
<tr>
<td>12.5</td>
<td>0.5 ml</td>
<td>0.5 ml of 25 ng/ml</td>
</tr>
<tr>
<td>6.25</td>
<td>0.5 ml</td>
<td>0.5 ml of 12.5 ng/ml</td>
</tr>
<tr>
<td>3.125</td>
<td>0.5 ml</td>
<td>0.5 ml of 6.25 ng/ml</td>
</tr>
<tr>
<td>1.56</td>
<td>0.5 ml</td>
<td>0.5 ml of 3.125 ng/ml</td>
</tr>
<tr>
<td>0.78</td>
<td>0.5 ml</td>
<td>0.5 ml of 1.56 ng/ml</td>
</tr>
</tbody>
</table>

C. **Human Adiponectin Quality Control 1 And 2 Preparation**

Using an Eppendorff pipette, reconstitute each of the Human Adiponectin Quality Control 1 and Quality Control 2 with 1 ml distilled or deionised water into the glass vials and mix well.
D. Assay Set-Up,

Day One

1. Pipet 300 µl of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 µl to the Reference (Bo) tubes (5-6), and 100 µl to tubes 7 through the end of the assay.
2. Pipet 100 µl of Standards and Quality Controls in duplicate
3. Pipette 100 µl of each diluted Sample in duplicate.
4. Pipette 100 µl of 125I-Adiponectin to all tubes.
5. Pipet 100 µl of Adiponectin antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
6. Vortex, cover, and incubate overnight (20-24 hours) at room temperature (20-25°C).

Day Two

7. Add 10 µl of Rabbit Carrier to all tubes except Total Count tubes (1-2).
8. Add 1.0 ml of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, 4°C, all tubes [except Total Count tubes (1-2)] for 20 minutes at 2,000-3,000 xg
    Conversion of rpm to xg:
    \[ xg = (1.12 \times 10^{-5}) \ (r \ (rpm))^2 \]

    \[ r = \text{radial distance in cm (from axis of rotation to the bottom of the tube)} \]

    \[ \text{rpm} = \text{rotational velocity of the rotor} \]

11. Immediately decant the supernatant of all tubes except Total Count tubes (1-2), drain tubes for at least 15-60 seconds (be consistent between racks), and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.
12. Count all tubes in a gamma counter for 1 minute. Calculate the ng/ml of Adiponectin in unknown samples using automated data reduction procedures.
## ASSAY FLOW CHART

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Day One</th>
<th></th>
<th>Day Two</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step 1</td>
<td>Step 2-3</td>
<td>Step 4</td>
<td>Step 5</td>
<td>Step 6</td>
<td>Step 7</td>
<td>Step 8</td>
<td>Step 9</td>
</tr>
<tr>
<td></td>
<td>Add Assay Buffer</td>
<td>Add Standard / Sample</td>
<td>Add $^{125}$I-Adiponectin Tracer</td>
<td>Add Adiponectin Antibody</td>
<td>Vortex, Cover, and Incubate 20-24 hrs at RT</td>
<td>Add Rabbit Carrier</td>
<td>Add Precipitating Reagent</td>
<td>Vortex, and Incubate 20 min. at 4°C</td>
</tr>
<tr>
<td>1, 2</td>
<td>-----</td>
<td>-----</td>
<td>100 µl</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>3, 4</td>
<td>300 µl</td>
<td>-----</td>
<td>100 µl</td>
<td>-----</td>
<td>-----</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>5, 6</td>
<td>200 µl</td>
<td>-----</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>7, 8</td>
<td>100 µl</td>
<td>100 µl of 0.78 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>9, 10</td>
<td>100 µl</td>
<td>100 µl of 1.56 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>11, 12</td>
<td>100 µl</td>
<td>100 µl of 3.125 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>13, 14</td>
<td>100 µl</td>
<td>100 µl of 6.25 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>15, 16</td>
<td>100 µl</td>
<td>100 µl of 12.5 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>17, 18</td>
<td>100 µl</td>
<td>100 µl of 25 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>19, 20</td>
<td>100 µl</td>
<td>100 µl of 50 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>21, 22</td>
<td>100 µl</td>
<td>100 µl of 100 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>23, 24</td>
<td>100 µl</td>
<td>100 µl of 200 ng/ml</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>25, 26</td>
<td>100 µl</td>
<td>100 µl of QC-1</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>27, 28</td>
<td>100 µl</td>
<td>100 µl of QC-2</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
<tr>
<td>29, n</td>
<td>100 µl</td>
<td>100 µl of unknown</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>10 µl</td>
<td>1.0 ml</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

82
STATISTICAL ANALYSIS

In the present study parametric data has been represented as mean±SD, proportions have been shown as frequencies and percentages. Following statistical tests have been employed:

1. **Pearson's Chi-square test for proportions**: This test is employed to check the significance of difference between two or more sample proportions. Chi-square checks the difference between observed and expected values. The formula used for chi-square test is:

   \[ \chi^2 = \frac{(f_1 - F_1)^2}{F_1} + \frac{(f_2 - F_2)^2}{F_2} \]

   where \( f_1 \) and \( f_2 \) are the sample counts of subjects who do and do not possess the attribute being investigated (observed), the corresponding hypothetical frequencies being \( F_1 \) and \( F_2 \) (expected).

   The two deviations, then, are

   \[ f_1 - F_1 \]
   \[ f_2 - F_2 \]

2. **Student 't' test**: To test between equality of two means:

   \[ t = \frac{\bar{X}_1 - \bar{X}_2}{S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \]

   where

   \[ S^2 = \frac{1}{n_1 + n_2 - 2} \times \left[ S_1^2 \left( n_1 - 1 \right) + S_2^2 \left( n_2 - 1 \right) \right] \]

   \( \bar{X}_1 = \text{Mean 1} \)

   \( \bar{X}_2 = \text{Mean 2} \)

   \( S_1 = \text{Standard deviation 1} \)

   \( S_2 = \text{Standard deviation 2} \)
\[
\begin{align*}
n_1 &= \text{Number of values in Group 1} \\
n_2 &= \text{Number of values in Group 2}
\end{align*}
\]

In present study, a number of parameteric variables such as leptin concentration, HOMA_{IR}, insulin and plasma glucose levels etc. have been compared in different groups (such as obese and non-obese) by using mean levels as central tendency of the group.

3. Pearson's Correlation: This was employed to find the bivariate correlation between two variables. The value of Pearson's correlation coefficient ("r") ranges between -1 and +1; it tries to check whether change in the magnitude of one variable brings about change in the other variable. A correlation coefficient value of -1 indicates that positive change of one unit in one of the two factors brings about one unit of negative change in the other factor that means the two variables are perfectly correlated negatively. Similarly, a coefficient of correlation value of +1 indicates that one unit change in one of the two variables brings about one unit change in the other variable and in the same direction. We have tried to correlate the levels of fasting and post prandial plasma glucose, triglyceride, HDL, LDL, VLDL, total cholesterol, insulin, leptin and adiponectin in order to find out a mathematical relationship amongst each other.

4. Multivariate Linear Regression: Multiple Regression analysis was done to see the correlation of HOMA_{IR} with fasting insulin level, QUICKI, ISI, Insulinogenic Index and Bennett Index. This was performed to find whether the outcome variable is significantly associated with a multitude of variables in question. It is seen that two or more factors may associate with the outcome in a univariate environment but when the two or more factors are commonly associated, they may in effect, regress each other's effect. Under such circumstances, one set of variables either associates more powerfully than the other set of variables or sometimes regresses effect of each other. Multivariate regression is in effect representation of actual real life circumstances where
more than one variables are working towards the outcome. The concept of multivariate association revolves around co variations and correlations amongst a number of variables and then tries to provide quantified values for association of each of the variables under study. In the present study, dependence of HOMA\textsubscript{IR} was evaluated in relation to the impact of fasting insulin levels, QUICKI, insulinogenic index, Bennett index and McFarland index levels.

5. **Receiver-Operator Curves (ROC):** Receiver-Operator Curves or commonly known as ROC, are plots between sensitivity and 1-specificity of a test variable for an outcome. Each value of the test variable is plotted to depict the sensitivity and specificity of the test variable at that cut-off point. The area under curve depicts the optimum sensitivity of the test variable. These curves were used to find out the cut-off for various insulin resistance markers under study.

6. **Odds Ratio:** Odds ratios are probability of happening of an event. It is the proportion between happening of an event and not happening of that event. Odds ratio of insulin resistance in the view of presence of various independent variables has been assessed.

All the above tests were performed on a desktop computer using Statistical Package for Social Sciences (SPSS) version 13.0. The confidence limit of the study was kept at 95%, hence a "p" value less than 0.05 depicted statistically significant results.