MATERIAL AND METHODS

SOURCE OF DATA:

- This study will be conducted at the department of anatomy, Rama Medical College, Hospital, Rama University, Kanpur.
- The subjects for study taken from the Department of Medicine at Rama Hospital.
- Before the study permission will be taken from the institution ethical committee and professor and head, Department of Medicine, Rama Medical College, Hospital.
- Anthropometric measurements will be collected from OPD and IPD in the Department of Medicine, Rama Medical College, Hospital.
- Venous blood will be collected from the subject to evaluate the biochemical and haematological parameters.

- **Socio-demography:-**

  - Age-35-60 yrs
  - Sex-Male/Female
  - **Type of occupation:** active/sedentary
  - **Educational level:** Illiterate/primary/secondary/graduate or above
  - **Area:** Rural/Urban
  - **Religion:** All religions.
  - **Smoking status:** once in a while/regular
  - **Diet habits:** Vegetarian/ Non-vegetarian
  - **Residence:** above poverty line/below poverty line
  - **Alcohol:** once in a while/regular
  - **Parity:** Uni/multi parity
  - **Past medical history:** - diabetes, CVD

- **Anthropometric parameter-**

  - **Height:** Height will measure using a portable stadiometer with a sliding head plate, a base plate and connecting rods marked with a measuring scale in millimeters.

  - **Weight:** To measure the subject's weighted; standard portable weighing machine will use.
Body Mass Index-

The formula for calculating BMI is:

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

<table>
<thead>
<tr>
<th>BMI (kg/m²)</th>
<th>Weight Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below 18.5</td>
<td>Underweight</td>
</tr>
<tr>
<td>18.5 to 24.9</td>
<td>Healthy weight</td>
</tr>
<tr>
<td>25.0 and above</td>
<td>Overweight</td>
</tr>
<tr>
<td>30.0 and above</td>
<td>Obese</td>
</tr>
</tbody>
</table>

➢ Circumference measurement:-

**Waist circumference:** The waist will defined as the midpoint between the lower rib and the upper margin of the iliac crest. It will measure using a tape with an insertion buckle at one end. The measurement will take twice, using the same tape (waist and hip measurements will alternate), and will record to the nearest millimeter. Where the two waist measurements will differed by more than 3cm, a third measurement will take.

**Hip circumference:** Tape should be placed around the point with the maximum circumference over the buttocks. Participant is asked to remove clothing, except for light underwear. Tight clothing, including belts, should be loosened and the pockets emptied. Participant should stand with feet fairly close together (about 12-15 cm apart) with weight equally distributed on each leg. Participant is asked to breathe normally; the reading of the measurement should be taken at the end of gentle exhaling.

**Waist hip ratio(WHR):-**

- Male: >0.9-risk
  - <0.9-normal
- Female: >0.8-risk
  - <0.8-normal

[16]
Body fat Testing With Skin fold Calipers

Prediction of body density or fatness from published equations: VALIDATION STUDY

Five empirically derived equations for the prediction of density or BF% from skin folds were tested. (1) Equations of Durnin and Rahaman et al, (1967) predicted density (kg/l): Boys= 1 1533-0 0643X (log sum of 4 skin folds) Girls= 1-1369-0-0598x (log sum of 4 skin folds) These equations were derived from empirical relationships between skin fold thickness and body density in adolescents (48 boys age range 12-7-15-7 years; 38 girls age range 13-2-16-4 years). Their validity in younger children was unknown, and as the methodology used was identical to that in the present study, a test of cross validation was justified[78]. (2) Equations of Slaughter et al, (1988) BF% for children with triceps and subscapular skin folds 3 5 mm: Boys=0-783 (sum of 2 skin folds)- 1-7 Girls=0 546 (sum of 2 skin folds)+9 7 These equations are based on an empirically derived multi component method utilizing measurement of body density, total body water, and bone mineral content of radius and ulna. The sample used to derive these particular equations consisted of 50 boys (mean age 9-8 years) and 16 girls (mean age 10 0 years) from the USA. The cross validity of the Slaughter et al equations has been reported to be high,’3 and these are effectively the 'standard' equations used in North America[79]. (3) Equations of Johnston et al, (1988) Predicted density (kg/l): Boys= 1-1660-0-0070X(log sum of 4 skin folds) Girls= 1-144-0 060X(log sum of 4 skin folds) These equations are based on empirically derived relations between skin folds and density of Canadian children aged 8-14 years (140 boys, 168 girls). The cross validity of these equations has not been reported[80]. (4) Equations of Brook et al, (1971) Predicted density (kg/l): Boys= 1-1690-00788x (log sum of 4 skin folds) Girls= 1-2063-0-0999X(log sum of 4 skin folds) These equations were derived from empirical relations between total body water and body density (predicted from equations for adolescents'6), in a sample of 13 obese children and 10 with short stature, age range 1-11 years. The equations are widely used in the UK[81]. (5) Equations of Deurenberg et al, (1990) Predicted density (kg/l) (prepubertal): Boys= 11133-0-0561 X(log sum of 4 skin folds)+ 17 (age X 10-3) Girls= 1-1187+0 063x (log sum of 4 skin folds)+ 1 9 (age X 10- 7) These equations are based on empirically derived relationships between skin folds and density in Dutch children (114 boys mean age 1 1.0 years; 98 girls mean age 10. 5 years). The equations are now widely used, but their cross validity has not been reported[82].

Men's Chart:

Chart #1 - Men
% Fat For Sum Of Measurements At All 4 Locations

<table>
<thead>
<tr>
<th>Sum in mm</th>
<th>Age 16-29</th>
<th>Age 30-49</th>
<th>Age 50+</th>
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<tbody>
<tr>
<td>20</td>
<td>8.1</td>
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<td>9.2</td>
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<td>28</td>
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<td>30</td>
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<td>Sum in mm</td>
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<td>Age 30-49</td>
<td>Age 50+</td>
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<td>14</td>
<td>9.4</td>
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Women's Chart:
Chart #2 - Women
% Fat For Sum Of Measurements At All 4 Locations

<table>
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<tr>
<th>Sum in mm</th>
<th>Age 16-29</th>
<th>Age 30-49</th>
<th>Age 50+</th>
</tr>
</thead>
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<tr>
<td>40</td>
<td>16.3</td>
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<td>22.8</td>
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<tr>
<td>200</td>
<td>46.6</td>
<td>47.6</td>
<td>52.3</td>
</tr>
</tbody>
</table>

**Back of the arm (triceps):**

The back of the arm, (Triceps). This is located midway between the shoulder joint and elbow joints. The fold is taken in a vertically.

**Front of the arm (Biceps):**

The front of the arm, (Biceps). This is taken exactly the same as the Triceps, except it is taken on the centre of the front of the arm.
Shoulder blade:

Back, below the shoulder blade. This is located just below the shoulder blade. Note that the skin fold is taken at 45 degrees angle as shown on the diagram.

Waist:

Waist (Suprailiac). This is located just above the iliac crest of hip bone, a little towards the front from the side of the waist. The fold is taken approximately horizontally as shown on the diagram.

➢ Physiological parameter

Blood pressure:

BP will measured by a physician using standard blood pressure measurement process after the patient had rested at least for 10 minutes. Two measurements will taken by a mercury sphygmomanometer. Hypertension will be defined as SBP >140 mmHg and DBP <90 mmHg without any medication.

Heart rate

Measuring by the radial pulse.

➢ Biochemical Analysis

Lipid profile- A volume of 5 mL of venous blood will be collected from subjects in the morning after an overnight fast, and then serum will used for biochemical tests. All biochemical tests were carried out at the laboratory in Rama Medical College, Hospital & Research Centre for Lipid parameters (TC, TG, LDL, and HDL) will estimated by enzymatic colorimetric methods.
Dyslipidemia will be defined according to the assess and Treatment of High Blood Cholesterol in Adults LDL cholesterol optimal (about 100 mg/dl), near optimal/above optimal (100–129 mg/dl), borderline high (130–159 mg/dl), high (160–189 mg/dl), very high (greater than 190 mg/dl); total cholesterol desirable (about 200 mg/dl), borderline high (200–239mg/dl), very high (greater than 240 mg/dl); HDL cholesterol low (about 40 mg/dl), high(greater than 60 mg/dl); triglyceride normal(about 150 mg/dl) (goal), borderline high (150–199 mg/dl), high (200–499 mg/dl), very high (greater than 500 mg/dl)[83].

RBS

Materials Required:

Collection of blood sample: About 2ml of patient’s blood will be collected by vein puncture into a tube, and the tube containing a mixture (anticoagulant mixture) of ethylenediaminetetraacetic acid (EDTA) and sodium fluoride in the ratio of 1:2 (W/W). 5 mg of the mixture is enough for 2ml of blood sample. The tube should be thoroughly shaken for complete mixing.

➢ Hematological parameter:-

Hemoglobin.

Normal value

• Adult males: 14 to 18 gm/dL & Adult women: 12 to 16 gm/dL.
• Men after middle age: 12.4 to 14.9 gm/dL & Women after middle age: 11.7 to 13.8 gm/dL.

Blood group

There are four blood groups : A, B, AB and O. The Rh blood group system is the second most significant system for blood grouping. Rh factor refer to Rh D antigen only. Determination of Rh factor along with ABO is essential for defining the Rh +ve or Rh -ve status of the individual. Around 85% of the human population is Rh +ve while 15% is Rh -ve. The ABO & Rh systems are the most significant blood group systems from the clinical point of view.

➢ GENETICAL EXAMINATION
MOLECULAR ANALYSIS:

Sampling: 5ml of venous blood was drawn from peripheral vein using Di sodium EDTA vaccutainers. All the samples are liquated and stored at -70°C until tested. The laboratory work will be carried out in the central research laboratory, Rama Medical College, Hospital & Research Centre. All the groups have their FTO gene analyzed for the sequence of intron 1 of chromosome16q12.2 SNP rs9939609.

Methodology:

1. Isolation of DNA:

DNA will be extracted from whole blood containing EDTA by Ponez et al., standard salting out procedure. The reagents required for DNA isolation are:

Chemicals used for DNA isolation

- 1 M tris buffer(ph-7.5)
- 1 M Mgcl2
- 1 MM Kcl
- 0.5 M EDTA
- 10% SDS
- 5 M Nacl
- Triton X 100
- Ethanol

Reagents

1) RBC LYSIS BUFFER:

- 10 mM of tris HCl
- 10 mM of KCL
- 10 mM of Mgcl2
- 2 mM of EDTA

2) NUCLEUS LYSIS BUFFER:

- 10 mM of tris HCL
- 10mM of KCL
Protocol for DNA isolation (salting out Method)

1. Thawed blood samples (5ml) from EDTA vaccutainers will be transferred into clean and sterile centrifuge tubes.
2. 10ml of RBC lysis buffer will be added and the volume will make upto 15ml.
3. 0.1% of triton X will be added
4. The centrifuge tubes will be incubated at 37\(^\circ\)C for 5 mins
5. The samples will be spun at 2000rpm for 15 mins in a cold centrifuge at 4\(^\circ\)C
6. After centrifugation the supernatant will be discarded off taking care of the pellet.
7. 10ml of RBC lysis buffer will be again added and vortex mixed
8. The samples will be spun at 2000 rpm for 15mins
9. The supernatant will be discarded off and a white pellet will be obtained
10. 1 ml of nucleus lysis buffer will be added then vortex mixed
11. About 20\(\mu\)l of 10\% SDS will be added and mixed properly
12. The mixture will be incubated in a water bath at 55\(^\circ\)C for 1 hour
13. After incubation, the contents will be transferred into 2ml eppendorf tubes.
14. 400\(\mu\)l of 5M Nacl will be added
15. The contents will be spun at 10,000 rpm for 15mins
16. After spinning the supernatant will be transferred into a new centrifuge tube leaving behind the pellet in the eppendorf tubes
17. About double the volume if cold absolute ethanol will be added
18. Large clumps of DNA will be obtained by gentle swirling of the tubes
19. The DNA will be scooped with the help of a sterile spatula or a sterile loop from the centrifuge tubes and transferred into eppendorf tubes
20. 200\(\mu\)l of 70\% ethanol will be added
21. The contents were centrifuged will be decanted and the eppendorf tubes with the DNA will keep for air drying
22. After drying, about150\(\mu\)l of TE buffer will be added and the DNA stored at -20\(^\circ\)C for later use.

Concentration and quality assurance of DNA:

- The quality of the DNA was analyzed by 0.8\% agarose gel electrophoresis and the quality assessed by standard spectrophotometer at 260 nm and 280 nm.
2. **Molecular characterization of polymorphism of FTO gene by Polymerase chain reaction:**

To determine the FTO genotype of cases and the control groups, the genomic DNA fragments will be amplified by PCR (T100 Biorad). The conditions required for amplification are mentioned below:

The flanking primer sequences as reported by Adeela Shahid et al. 2014 were;

**Forward primer:** 5' - AACTGGCTTGTGAATGAAATAGGATTCAGA -3';

**Reverse Primer:** 5' - AGAGTAACAGAGACTATCCAGTGAGTAC -3' [84].

Primers will be obtained from IDT, Hyderabad and will be reconstituted with sterile double distilled water based on the manufacturer’s instruction.

**PCR reaction mix (working concentration)**

The working conditions for PCR reaction mixture are consumed from IDT, Hyderabad, India:

1. 10x PCR Buffer: 1X
2. dNTPs: 200µM
3. Forward primer: 30pM
4. Reverse primer: 30pM
5. Taq polymerase: 1.5U
6. Template DNA: 50µg
7. Distilled water: to make up the volume

**PCR conditions:**

1) Initial denaturation: 94°C-5mins
2) Denaturation: 94°C-30secs
3) Annealing: 58°C-45secs
4) Extension: 72°C-45secs
5) Cycling condition: 30 cycles
6) Final extension: 72°C-7mins
7) Hold at 4°C
3. Agarose Gel Electrophoresis

- Once the amplification will be obtained, they will be subjected to 2% agarose gel electrophoresis with Ethidium bromide and the bands will visualize under UV light in gel documentation system (Biorad). The requirements for electrophoresis are:

Requirements for electrophoresis

1. Purified Agarose
2. 50x TAE buffer
3. Ethidium bromide
4. Bromophenol Blue

Composition of 50 X TAE Buffer (1000Ml)

1. Tris Base :242 g
2. Acetic acid :57.5ml
3. EDTA:18.6g
4. pH:7.2

Procedure:

1. 2% agarose gel Himedia will be prepared ,by mixing 2 g of agarose in 100ml of 1% buffer
2. The contents were boiled completely till a clear solution was obtained
3. About 100 µg of Ethidium bromide will be added and mixed properly
4. Agarose will be casted into specific trays and allowed to solidify
5. Specific combs will be used to make sample wells on the gel
6. The PCR products will be mixed with Bromophenol blue
7. 2µl of DNA ladder will be loaded into the first well
8. Then, samples will be loaded into the gels and run at 100v
9. After electrophoresis, The PCR products will be visualized under UV light in gel documentation system (Biorad). In the case of Deletion (D allele) and Insertion(I allele),a 190bp and a 490 bp fragment were obtained respectively and comparison was done with a DNA ladder on the first well.
METHOD OF COLLECTION OF DATA:

SAMPLE SIZE CALCULATION AND STATISTICAL ANALYSIS:

Sample size has been calculated in order to control type I & type II error. Assuming a minimum power 80% and 95% significance level the sample size has been calculated using this formula:

\[ n = \frac{2(p)(1-p)(Z\beta + Z\alpha/2)}{(p_1 - p_2)^2} \]

- \( p \) - Incidence of the disease (obesity)
- \( q = 1-p \)
- \((P1-p2)^2\) or \(d^2\) – Is the difference which we want to detect at a specified power & level of confidence.
- \( Z_\beta \) - power of statistical test we want to be minimum 80% for which is \( Z_\beta \) is 0.84.
- \( Z_{\alpha/2} \) –is the level of confidence we have chosen 95% confidence in this \( Z_{\alpha/2} \)=1.96.

When \( P \) indicates the incidence of the clinical condition e.g.: obesity.

Following the literature the incidence of obesity has been assumed between 11%.

The calculated minimum sample size for our study is 160.

The calculated minimum sample size for control group is 160.

In order to control loss of follow up and manual errors, which we rounded the sample size of 320 for each group.

Data will be collected and entered in MS excel worksheets and results will be analysed with appropriate statistical tools like, tests of significance, logistic regression analysis etc using SPSS version software.

Inclusion Criteria

1. Subjects of either sex having age in between 35 to 60 years.
2. Subjects having BMI more than 24.9kg/m²
3. Hereditary indisposition.

Exclusion Criteria

1. Subjects below 35 years and more than 60 years.
2. Subjects having severe cardiac anomalies.
3. Pregnant and lactating women.
4. Obesity produced due to certain secondary cause.
5. Subjects suffering from any grave disease and hospitalization in last 3 months.
6. Endocrinal origin obesity.
7. Subjects who are not wanting to participate in the study.