MATERIALS AND METHODS:

 SOURCES OF DATA COLLECTION –

This study will be conducted in the department of pharmacology, Rama Medical College, Hospital, Rama University, Kanpur.

The material for study consists of blood sample (fasting blood glucose level & glycolated hemoglobin) collected from Department of General Medicine at Rama Hospital.

Before the study permission will be taken from the institution ethical committee.

Blood sample will be collected and sent for the lab for estimation of fasting blood glucose level, glycolated hemoglobin, lipid Profile, liver profile and renal profile.

EDTA mixed venous blood is needed to evaluate the Transcription Factor 7-Like 2 (TCF7L2) gene polymorphism by simple PCR technique.

MATERIALS:

Type-2 Diabetes mellitus Patients

- Venous blood from Type-2 Diabetes mellitus Patients
- EDTA vaccuntainers
- Micro pipette
- Tips
- Syringes
- Spectrophotometer
- Electrophoresis unit
- Gel documentation system
- PCR machine
- Primers
- Calorimeter
- Kit for estimation of blood glucose.
• Kits for estimation HbA1c.
• Kits for estimation of Lipid profile
• Kits for estimation of Renal profile

**Study also required a detailed history of Diabetic patients regarding the socio demographic profile, present and past health history & it’s treatment will be recorded on pre-designed pre-tested proforma: a standard questionnaire will prepared and data will be collected on clinical variables like,**

- IP number
- Age
- Weight
- Height
- Blood glucose
- Duration of Diabetes
- Other systemic diseases

**Socio-economic status:**

- Area of patient: rural or urban
- illiterate or Literacy
- Economy: below or above poverty line
- Mode: Employee / non-employee
- Food style: Veg or Non-Veg

**Examination of blood (plasma) sample as under biochemical parameters:**

- Estimation of blood glucose.
- Estimation of HbA1c
- Estimation of Lipid profile (HDL, TG, TC, LDL,)
- Estimation of Renal profile (proteins, Urea, Creatinine)
STUDY GROUP
The subjects selected for study were grouped into as followed.

GROUP – 1 TCF7L2 gene polymorphism in type 2 diabetes mellitus patients

Group – 1A – Single Drug Treatment
- Metformin (500mg to 2000mg)
- Glimepiride (1mg to 4 mg)

Group – 1B – Double Drug Treatment
- Metformin (500mg to 2000mg) + Glimepiride (1mg to 4 mg)
- Metformin (500mg to 2000mg) + Teneligliptin (20 mg)
- Metformin (500mg to 2000mg) + Pioglitazone (15 mg)

Group – 1C - Triple Drug Treatment
- Metformin (500mg to 2000mg) + Glimepiride (500mg to 2000mg) + Voglibose (0.2 to 0.3mg)

GROUP – 2 NO TCF7L2 GENE POLYMORPHISM IN TYPE 2 DIABETES MELLITUS

Group – 2 A – Single Drug Treatment
- Metformin (500mg to 2000mg)
- Glimepiride (1mg to 4 mg)

Group – 2 B – Double Drug Treatment
- Metformin (500mg to 2000mg) + Glimepiride (1mg to 4 mg)
- Metformin (500mg to 2000mg) + Teneligliptin (20 mg)
- Metformin (500mg to 2000mg) + Pioglitazone (15 mg)
Group – 2C - Triple Drug Treatment

- Metformin (500mg to 2000mg) + Glimepiride (500mg to 2000mg) + Voglibose (0.2 to 0.3mg)

**INCLUSION CRITERIA.**

- Newly diagnosed type 2 diabetes mellitus patients.
- Glycosylated haemoglobin (HbA1c) >6.5%
  
  Or

- Fasting plasma glucose > 126 mg/dl (7.0 mmol/L)
  
  Or

- 2-hours plasma glucose > 200mg/dl (11.1 mmol/L)

**EXCLUSION CRITERIA**

- Type 1 diabetes mellitus
- Any other severe illness
- Patients already diagnosed with diabetes mellitus and on treatment
- Pregnancy
- Smokers and alcohol.
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:

\[
n = \frac{2(P)(1-P)(Z_{\beta} + Z_{\alpha}/2)^2}{(p1 - p2)^2}
\]

✓ \(n\)=sample size
✓ \(p\)=measure of variability
✓ \(Z_{\beta}\)- 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.
✓ \((P1-p2)^2\) or \(d^2\)-effect in size difference in proportions.
✓ When \(P\) indicates the incidence of the clinical conditions e.g.: diabetes mellitus.
✓ Following the literature the incidence of type-2 DM has been assumed as (8.7%).
✓ The calculated minimum sample size for our study is 131
✓ In order to control loss of follow up and manual errors, we will be taken the sample size of 150 in 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 software.
MOLECULAR ANALYSIS:

**Sampling:** 5ml of venous blood will be drawn from peripheral vein using Di sodium EDTA vaccuntainers. All the samples will be a liquated and stored at -80°C until tested.

The laboratory work will be carried out in the central research laboratory, Rama Medical College, Hospital & Research Centre.

**METHODOLOGY:**

**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
   - 10mM of MgCl2
   - 2mM of EDTA
   - 400 mM of NaCl

All the components will be mixed with sterile double distilled water and the prepared reagents will be stored at 25\(^\circ\)C.

**Protocol for DNA isolation (salting out Method)**

1) Thawed blood samples (5ml) from EDTA vactcuntainers will be transferred into clean and sterile centrifuge tubes.
2) 10ml of RBC lysis buffer will be added and the volume will make up to 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 2000 rpm 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 15 mins
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 15 mins
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) 200µl 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, about 150µl of TE buffer will be added and the DNA stored at -20°C for later use.

1. 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 TCF7L2 Polymerase chain reaction:

PCR reaction mix (working concentration)

The working conditions for PCR reaction mixture are consumed from Banglore Genei, India:

1) 10 x 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: 57.5 ml
3. EDTA: 18.6 g
4. pH: 7.2

Procedure:

- 2% agarose gel Himedia will be prepared by mixing 2 g of agarose in 100 ml of 1% buffer
- The contents will be boiled completely till a clear solution obtained
- About 100 μg of Ethidium bromide will be added and mixed properly
- Agarose will be casted into specific trays and allowed to solidify
- Specific combs will be used to make sample wells on the gel
- The PCR products will be mixed with Bromophenol blue
- 2μl of DNA ladder will be loaded into the first well
- Then, samples will be loaded into the gels and run at 100v
After electrophoresis, The PCR products will be visualized under UV light in gel documentation system (Biorad) and comparison will be done with a DNA ladder on the first well.

**BIOCHEMICAL ANALYSIS**

**Blood Glucose levels:**

- FBS
- Glycolated haemoglobin

**Liver profile**

- Serum albumin
- Serum Bilirubin
- SGOT.
- SGPT
- ALP

**Lipid profile**

- Cholesterol
- Triglycerides
- HDL
- LDL
- VLDL

**Renal profile:**

- Serum potassium
- Serum Creatinine
- Blood Urea
1. **ESTIMATION OF BLOOD GLUCOSE BY GOD POD METHOD (Bartham.D at al.,)**[42]

Glucose is the major carbohydrate present in blood. Its oxidation in cells is source of energy for body. Increased levels are found in hyperglycaemia.

Glucose + O2+ H2O → Glucose oxidase --Gluconic acid+ H2O2

H2O2+ Phenol+ aminoantipyrene----Peroxidase----Quinonimine

**Specimen;** Serum/Plasma

**Procedure;** pipette in to clean dry test tube labeled as blank (B), standard (S) and test (T)

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>S</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme reagent</td>
<td>1ml</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Standard</td>
<td>------</td>
<td>10 µl</td>
<td>------</td>
</tr>
<tr>
<td>Sample</td>
<td>------</td>
<td>-------</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37°C for 10min, measure the absorbance of standard (Abs.S) and test (Abs.T) against the reagent blank at 505nm.

Calculation; glucose mg/dl = Abs test/Abs of standard*100

2. **ESTIMATION OF GLYCOXYLATED HAEMOGLOBIN LEVELS IN BLOOD (GHB).**

(Trivelli, L.A. et al)[43]

**Method;** Ion exchange resin method

Specimen; venous blood is collected with EDTA/ heparin using aseptic condition. GHB in the blood is found to be stable for one week at 2-8c.

**Test procedure:**

**Step-1 hemolysate preparation;**

1. Dispence 250µl lysing reagent into tubes and to be labelled.
2. Add the 50 µl of the well mixed blood into the appropriately labelled tubes. Mix unit completely until is evident.
3. Allow To stand for 5 minutes.

**Step-2 GHB separation;**

1. Remove the cap from the resin tubes and labeled as test.
2. Add 100 µl of hemolysate from step-1 in to appropriately labeled to exchange resin tubes.
3. Insert a resin separator in to each tube so that the rubber sleeve to approximately 1 cm above the liquid level of the resin suspension.
4. Mix the tubes on a rocker, rotator or a vortex mixture continuously for 5 min.
5. Allow the resin to settle, and then push the resin separator into the tubes until the resin is firmly packed.
6. Pour or aspirate each supernatant directly into a cuvette and measure each absorbance against distilled water at 415 nm (405-420).

**Step-3** Total hemoglobin (THB) fraction

1. Dispence 5.0 ml of distilled water into tubes labeled as test.
2. Add to it 20 µl of hemolysate (from step-1) appropriately labeled tube. Mix well.
3. Read each absorbance against distilled water at 415 nm (405-420)

**Calculations;** \[ GHB\% = \frac{Abs\ of\ GHB}{Abs\ of\ THB} \times 4.61 \]

**3. ESTIMATION OF TOTAL CHOLESTEROL BY MODIFIED ROESCHLAU’S METHOD - (Allain C et al.,)[44]**

**Method:** Modified Roeschlua’s Method.

**Principle:** Estimation of cholesterol involves as following enzymatic catalytic reactions

1. Cholesterol ester \[ \text{CE} \text{cholesterol} + \text{fatty acids} \]
2. Cholesterol+o2 \[ \text{CHOD} \text{cholest}-4\text{-en-3-one}+\text{H2o2} \]
3. 2H2o2+4aap+phenol \[ \text{POD} 4\text{H2o}+\text{Quononeimine} \]
Absorbance of Quononeimine formed is directly proportional to cholesterol concentration in the specimen.

**Storage & stability:** Unopened reagents and standard are stable till the expiry date stated on the label when stored at 2-8°C.

**Specimen collection & handling:** Use serum, plasma (heparin, EDTA)

**Stability:** In serum/plasma; at 20-25°C –7 days.

at -20°C –3 months.

**ASSAY PROCEDURE**

<table>
<thead>
<tr>
<th>Pipette in to tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000µl</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20µl</td>
<td>……</td>
<td>……</td>
</tr>
<tr>
<td>Standard</td>
<td>……</td>
<td>20µl</td>
<td>……</td>
</tr>
<tr>
<td>Test</td>
<td>……</td>
<td>……</td>
<td>20µl</td>
</tr>
</tbody>
</table>

Mix well incubate at 37°C for 10 min. aspirate the blank followed by standard and test. Read the absorbance of standard and each test against blank at 505nm or 505/670 nm on bichromatic analyzer.

**Calculation**

Cholesterol= absorbance of test/Absorbance of standard × concentration of standard (mg/dl)

4. **ESTIMATION OF HDL CHOLESTEROL BY PHOSPHOTUNGSTIC ACID METHOD, END POINT - (Burstein m., et al)^{[45]}**

**Method:** phosphotungstic acid Method, end point
**Principle:** Chylomicrons LDL, VLDL are precipitated from serum by Phosphotungstate in the presence of divalent of cations such as magnesium. The HDL cholesterol remains unaffected in the supernatant and is estimated using ERBA cholesterol reagent.

\[
\text{Serum/ Plasma} \quad \textbf{Phosphotungstate} \quad \text{HDL} \quad + \quad \text{(LDL, VLDL, and Chylomicrons)} \\
. \quad \text{Mg}^{2+} \quad \text{(supernatant)} \quad \text{(precipitate)}
\]

**Storage & stability:** Unopened reagents vials and standards are stable till the expiry date stated on the label when stored at 2-8\(^\circ\)c.

**Specimen collection:** Unhaemolysed serum or EDTA plasma.(citrates and heparin should not be used as anticoagulants) ; samples are stable for 7 days at 2-8\(^\circ\)c or one month at -20\(^\circ\)c.

**ASSAY PROCEDURE**

<table>
<thead>
<tr>
<th>Pipette in to tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000(\mu_l)</td>
<td>1000(\mu_l)</td>
<td>1000(\mu_l)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50(\mu_l)</td>
<td>......</td>
<td>......</td>
</tr>
<tr>
<td>HDL Standard</td>
<td>......</td>
<td>50(\mu_l)</td>
<td>......</td>
</tr>
<tr>
<td>Supernatant</td>
<td>......</td>
<td>....</td>
<td>50(\mu_l)</td>
</tr>
</tbody>
</table>

Mix well incubate at 37\(^\circ\)c for 10 min. Read the absorbance of standard and each test against blank at 505nm or 505/670 nm on bichromatic analysers against reagent blank.

**Calculation:**

HDL Cholesterol (mg/dl) = absorbance of test/Absorbance of standard x concentration of standard (mg/dl) x dilution factor.

\[
= \frac{\text{abs of test}}{\text{abs of standard}} \times 25 \times 3
\]

\[
= \frac{\text{abs of test}}{\text{abs of standard}} \times 75
\]
5. ESTIMATION TRIGLYCERIDES BY TINDER METHOD, END POINT (McGowan et al.,) [46]

**Method:** This reagent is based on the method of wako and the modifications by mc gowan et al and fossati et al.

**Principle:**

Triglycerides + H₂O \(_{\text{LPL}}\) Glycerol + Free fatty acids

Glycerol + ATP \(_{\text{GK}}\) glycerol-3- Phosphate + ADP Mg\(^{2+}\)

Glycerol-3- Phosphate + O₂ \(_{\text{GPO}}\) DAP + H₂O₂

H₂O₂+ 4AAP+ 3, 5 DHBSPeroxidase Quononeimine dye + H₂O₂

**Storage & stability:** Unopened reagents and standards are stable till the expiry date stated on the bottle and kit label when stored at 2-8°C.

**Specimen collection:** Use Unhaemolysed serum or EDTA plasma. (EDTA and heparin) samples are stable for 7 days at 4-8°C or one year at -20°C.

**ASSAY PROCEDURE**

<table>
<thead>
<tr>
<th>Pipette in to tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000µl</td>
<td>1000µl</td>
<td>1000µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10µl</td>
<td>......</td>
<td>......</td>
</tr>
<tr>
<td>Standard</td>
<td>......</td>
<td>10µl</td>
<td>......</td>
</tr>
<tr>
<td>Test</td>
<td>......</td>
<td>....</td>
<td>10µl</td>
</tr>
</tbody>
</table>

Mix well & incubate at 37°C for 10 min. Read the absorbance of standard and each test at 505nm (500-540 nm) or 505/670 nm on bichromatic analysers against reagent blank.

**Calculations:**

Triglycerides (Mg/dl) = Abs. Test / absorbance of standard x concentration of standard (mg/dl)
6. ESTIMATION OF BLOOD UREA BY BERTHELOT METHOD - (Chaney et al.,) [47]

Urea is the end product of protein metabolism in the body. The importance of the urea concentration in the blood lies in its values as an indicator of kidney function.

**Principle:**

Urea is hydrolyzed by ureases into ammonia, carbon dioxide. The ammonia generated reacts with alkaline hypochlorite and sodium salicylate in the presence of sodium nitroprusside as a coupling agent to yield a green chromophore. The intensity of the colored formed is proportional to the concentration of urea in the sample.

**Specimen:**

Serum/ Heparinized or EDTA plasma.

**Test procedure:**

Pipette into clean dry test tubes labeled as Blank (B), Standard(S), Test(T).

<table>
<thead>
<tr>
<th></th>
<th>(B)</th>
<th>(S)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Working reagent</strong></td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td><strong>R3 standard</strong></td>
<td>..........</td>
<td>10 µl</td>
<td>..........</td>
</tr>
<tr>
<td><strong>Serum sample</strong></td>
<td>..........</td>
<td>..........</td>
<td>10 µl</td>
</tr>
<tr>
<td><strong>Mix and incubate for</strong></td>
<td>5 min at 37°C(10min)</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td><strong>R2 alkaline buffer</strong></td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td><strong>Mix and incubate for</strong></td>
<td>5 min at 37°C</td>
<td>Or (10min Room tem)</td>
<td></td>
</tr>
</tbody>
</table>

Mix and read the absorbance of standard (S) test (T) against Blank (B) at 578 nm (570-620 nm) or with yellow filter.

**Calculations:** urea in mg/dl \(\text{ABS of test/abs of stand} \times 40\)
Creatinine is synthesized in the body at a fairly constant rate from Creatinine, which is produced during the muscle contraction from Creatinine phosphate. Creatinine in the blood is then removed by filtration through the glomeruli of the kidney for excretion in the urine.

Elevated levels of Creatinine in serum are usually associated with renal disease.

**Specimen;** Serum/ Heparinised or EDTA plasma.

**Pipette in to test tubes labeled as Test (T), Standard(S)**

<table>
<thead>
<tr>
<th></th>
<th>(S)</th>
<th>(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Working reagent</strong></td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td><strong>R4 standard</strong></td>
<td>100 µl</td>
<td>..........</td>
</tr>
<tr>
<td><strong>Serum sample</strong></td>
<td>..........</td>
<td>100 µl</td>
</tr>
<tr>
<td><strong>R3 acid reagent</strong></td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Mix well and keep at 37oc for 5min. Read absorbance A1, for standard and test against distilled water at 520nm or with green filter (505-570nm)

Mix well and keep at RT for 5min. read the absorbance A1 for standard and test against distilled water at 520 nm or with green filter (505-570nm)

**Calculations,** serum Creatinine in mg/dl= ΔAT/ΔAS*2