MATERIAL AND METHODS:

SOURCE OF DATA:

- This study will be conducted in the department of Anatomy in association with department of Ophthalmology and Central research laboratory at Rama medical college Hospital and Research Centre, Rama University Kanpur.
- Before the study, approval of project will be taken from the institutional ethical committee.
- Samples will be collected from the department of Ophthalmology and appropriate examination will be done to assess the type.

Detailed history of patient regarding the socio- demographic profile and present and past medical history will be recorded on pre designed pre tested proforma.

- Age
- Gender
- Weight
- Height
- Addiction : Alcohol: Yes/No
- Smoking yes/no
- Food style: vegetarian / non vegetarian
- Lifestyle: sedentary/working

The following biochemical investigation will be done by the ERBA Auto analyzer.

- Flouride vacutainers for Blood glucose by Glucose Oxidase-Peroxidase Method.
- Plain vacutainers for lipid profile;
  - Serum total cholesterol by CHOD-PAP Method.
  - Triglycerides by Enzymatic Method.
- HDL by Precipitation Method,
- LDL by LDL-C = total cholesterol - (HDL-C + VLDL-C) 
  (Friedewald formula)
- VLDL by triglycerides/5

Following genetic investigation will be done with subject’s venous blood.

MOLECULAR ANALYSIS

Sampling: 5ml of venous blood will be drawn from peripheral vein using Disodium EDTA vacutainers. All the samples will be aliquoted 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.

All the groups will be analyzed for APE1, XRCC1, OGG1 genes (OGG1rs1052133, XRCC1rs25487, and APE1rs1760944) (Homo sapiens).

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) 1 M tris buffer(ph-7.5)
2) 1 M MgCl2
3) 1 MM KCl
4) 0.5 M EDTA
5) 10% SDS
6) 5 M NaCl
7) Triton X 100
8) 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
- 10 mM of KCl
- 10 mM of MgCl2
- 2 mM 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°C.

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°C for 5 mins
5. The samples will be spun at 2000 rpm for 15 mins in a cold centrifuge at 4°C

6. After centrifugation the supernatant will be discarded off taking care of the pellet.

7. 10 ml 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 μl of 10% SDS will be added and mixed properly

12. The mixture will be incubated in a water bath at 55°C for 1 hour

13. After incubation, the contents will be transferred into 2 ml eppendorf tubes.

14. 400 μl 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.

**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 APE1, XRCC1, OGG1 gene by Polymerase chain reaction:**

To determine the APE1, XRCC1, OGG1 genotype of cases and the control groups, the genomic DNA fragments on the will be amplified by PCR. The condition required for amplification mentioned below:

**Primers for OGG1 polymorphism used in this study:**

<table>
<thead>
<tr>
<th>Gene OGG1</th>
<th>Forward: 5-TTGATGGGTCACAGAAGGG-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bp552</td>
<td>Reverse: 5-TGAGGTTAGTCACAGGGAGGC-3</td>
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</tbody>
</table>

**Primers for XRCC1 polymorphism used in this study:**

<table>
<thead>
<tr>
<th>Gene XRCC1</th>
<th>Forward: 5-TCCCTGCGCCGCTGCAGTTTCT-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bp 447</td>
<td>Reverse: 5-TGGCGTGTGAGGCCCTTACCTCC-3</td>
</tr>
</tbody>
</table>

**Primers for APE1 polymorphism used in this study:**

<table>
<thead>
<tr>
<th>Gene APE1</th>
<th>Forward: 5-GAGGAATTGG AGCGTTAACTGT-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bp168</td>
<td>Reverse: 5-GCTTATTACCACGAAIAGCC-3</td>
</tr>
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</table>

Primers will be obtained from Qiagen, Germany 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 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) Molecular characterization by Restriction fragment length polymorphism:

Following amplification, the PCR products will be digested with the restriction endonuclease (OGG1rs1052133,XRCC1rs25487,APE1rs1760944) New England Biolabs, Inc. Beverly, MA. Genotype will be determined by fragment by running 3% agarose gel to check the size of the digested product by RFLP

4) 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 be visualized under UV light. The requirements for electrophoresis will be;

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 was prepared, by mixing 2 g of agarose in 100 ml of buffer
2. The contents were boiled completely till a clear solution was obtained

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3. About 100 µg of Ethidium bromide was added and mixed properly
4. Agarose was casted into specific rays and allowed to solidify
5. Specific combs were used to make sample wells on the gel
6. The PCR products were mixed with bromophenol blue
7. 12µl of DNA ladder was loaded into the first well
8. The samples were then loaded into the gels and run at 100v
9. After electrophoresis, The PCR products were visualized under UV light. 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

**STATISTICAL ANALYSIS:**
Sample size has been calculated in order to control type I & type II error. Sample size has been calculated using this formula:

\[
n \geq \left[ Z_1 - \frac{\alpha}{2} \sqrt{2pq} + Z_1 - \beta \sqrt{p_1q_1 + p_2q_2} \right]^2 / (p_2-p_1)^2
\]

- p- Prevalence of the disease (Cataract)
- q- (1-p)
- p1= Control Group    p2= Study Group

The calculated minimum sample size for our study is 245.
The calculated minimum sample size for control group is 245.
In order to control loss of follow up and manual errors, we finalised the sample size 250 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.

**METHOD OF SAMPLING:**

2 groups are made

Group-I: Control group-This group will consist of age and sex matched non-diabetic healthy subjects and not affected with Cataract. They are free from any major ailment which could affect the parameters under study.

Group-II: Diagnosed case of Cataract and type II Diabetes Mellitus patients with cataract.

**INCLUSION CRITERIA:**

1. Patients affected with cataract.
2. Patients diagnosed as diabetic along with cataract from last 5 years or less.

**EXCLUSION CRITERIA:**

1. Patients with type 1 diabetes mellitus, Pregnancy and lactating females.
2. Patients with any thyroid disorder, tuberculosis and cancer.

**TYPE OF STUDY:**

Case Control study.