The present study was conducted in the Department of Biochemistry and Cardiology, Ganesh Shankar Vidhyarthi Memorial Medical College & L.L.R. Hospital Kanpur in collaboration with Department of Biochemistry, Subharti Medical College Meerut on 215 subjects (54 control and 161 patients) from Sep 2010 to Aug 2014.

**Study Design:** This was an Observational case-control study

**Selection of Control**

Selection criteria of control subjects will be-

- N=54 subject in the age group of 25 to 60 years.
- Healthy individuals of either sex are selected and they will be advised to take their usual meal.
- None of the control subject had clinical or laboratory evidence of any disease that might have affected the parameters to be measured.

**Selection of Patients**

The established CVD patients will be selected from a series of consecutive outdoor patients attending coronary clinic and indoor patients of Laajpat Singhania Institute of Cardiology of G.S.V.M Medical College, Kanpur (U.P).

**Criteria of selection of CVD patients would be:**

- N=161 subject in the age group of 25 to 60 years.
- Patients with abnormal lipid profile which includes dyslipidemia and hyperlipidemia were selected.
- Patients selected are divided into three sub-groups:
  1) Patients suffering from stable angina pectoris (SAP), N = 52.
  2) Patients suffering from unstable angina (UAP), N = 53 and
  3) Patients with acute myocardial infarction (AMI), N = 56.

Patients with Angina Pectoris (AP) who were scheduled for percutaneous coronary intervention were recruited from the outpatient clinic. Only patients with more than 50% stenosis of one or more of the main coronary branches (as proven by coronary angiography) were included. Evaluation of the coronary stenosis was performed by
MATERIALS & METHODS

Patients presented themselves with prolonged new-onset chest pain (<30 days), an accelerating pattern of chest pains or with chest pains occurring at lesser degrees of exertion or at rest. Angina Pectoris is also characterized by ischemic ECG changes (such as ST segment elevation, reciprocal ST segment depression, T wave inversion, or development of Q waves) with or without elevation of cardiac enzymes.

Patients with AMI presented themselves with an acute onset of chest pain, ischemic ECG changes, or elevation of cardiac enzymes. Diagnoses of AP or AMI were made by cardiologists blinded for the study aims.

Exclusion criteria: Patients with diabetes mellitus, renal diseases, respiratory diseases, thyroid disorders, acute infection or any other systemic illness and on lipid lowering drugs for the past 3 months were excluded. The same exclusion criteria were also applied for the selection of controls.

The study protocol was approved by Institutional Ethical Committee and Prior informed consent from the patients and controls were taken (Annexure-II). Family history of coronary artery disease, diabetes, hypertension, other major illness in the past, personal history of smoking, alcohol, dietetic history, drug history is recorded for each subject on a prefixed proforma (Annexure-III). Intake of milk and milk products are included in vegetarian group.

Sample collection and storage
Whole blood- 6 ml of the 10-12 hrs overnight fasting venous blood samples was drawn aseptically from each of the study subjects and collected in vials containing anticoagulant (EDTA).

Plasma- 3 ml blood was centrifuged at 15000 rpm for ten minutes at 4°C and plasma and plasma free packed cell volume were collected and kept stored at –20°C in aliquots till in use.

Preparation of Hemolysate- Plasma removed PCV (Packed cell volume) washed 3 times with normal saline and then cells were lysed by adding 1.0 ml chilled distilled water for 10 min., and was shaken vigorously for 2 min, and preserved by adding 0.5 ml of chloroform. Mixture was centrifuged at 3000 rpm for 20 min. Mixture clearly
separated into 3 layers, lower most layer was of chloroform, middle of cell stroma (mucus) and upper most layer was of clear hemolysate. Estimation/determination of each parameter under study was completed same day and hemolysate was preserved at –20°C in aliquots until use.

1) **Estimation of Total-cholesterol and HDL-cholesterol (T-C & HDL-C)**

Total cholesterol and HDL-C was estimated by the CHOD/POD kit Method similar to that proposed by Allain et al.\textsuperscript{140}

\[
\text{Cholesterol ester} \xrightarrow{\text{CHE}} \text{Cholesterol + Free Fatty acid}
\]

\[
\text{Cholesterol} \xrightarrow{\text{CHOD}} \text{Cholest-4-ene + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{AAP} \xrightarrow{\text{POD}} \text{Red Quinoneimine + H}_2\text{O}_2
\]

On addition of the precipitating reagent to the serum, followed by centrifugation, HDL fraction remains in the supernatant while the other lipoprotein precipitate out.

**REAGENTS**

1) Enzyme reagent (CHE, CHOD & POD)
2) Buffer solution
3) Precipitating reagents (Phosphotungusic acid and magnesium ions).
4) Cholesterol standard 200 mg/dl
5) Working reagent: 10 ml reagent 2 added in reagent 1 (each vial), mix gradually to constitute working reagent.
**PROCEDURE**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>0.2 ml plasma + 0.3 ml precipitating reagent in centrifuge tube mixed well by swirling and allowed to stand minimum for 5 min.</td>
</tr>
<tr>
<td>2)</td>
<td>Above centrifuged to get clear supernatant</td>
</tr>
</tbody>
</table>
| 3)    | Four tubes prepared as follows:  
  Reagents | Blank (B) | T-C | HDL-C | Standard (S) |
  Working reagent | 1.0 ml | 1.0 ml | 1.0 ml | 0.1 ml |
  Cholesterol Std. | - | - | - | - |
  Plasma | - | .01 ml | - | - |
  Supernatant | - | - | 0.1 ml | - |
  Water | 0.1 ml | 0.1 ml | - | - |
| 4)    | Mix well each tube and incubate for 5 min. at 37˚C and read OD at 505 nm against blank. |

**Calculation:**

\[
\text{T-C (mg/dl)} = \frac{\text{Absorbance of T-C}}{\text{Absorbance of S}} \times 200
\]

\[
\text{HDL-C (mg/dl)} = \frac{\text{Absorbance of HDL-C}}{\text{Absorbance of S}} \times 5
\]

2) **Estimation of LDL-C**

LDL-C was estimated in the sample by using Friedwald’s and Fredrichsen’s (1972) formula.  \(^{141}\)

\[
\text{LDL-C (mg/dl)} = \text{T-C} - (\text{HDL-C} + \text{VLDL-C})
\]

3) **Estimation of VLDL-C**

VLDL-C was estimated in the sample by using Friedwald’s and Fredrichsen’s (1972) formula.  \(^{141}\)

\[
\text{VLDL-C (mg/dl)} = \frac{\text{TG}}{5}
\]
4) **Estimation of Triglycerides (TG)**

By the method of GPO/POD End point Method similar to that proposed by Bucolo et al.\textsuperscript{142}.

\[
\text{Triglyceride} + \text{H}_2\text{O} \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acid}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-P} + \text{ADP}
\]

\[
\text{Glycerol-3-P} + \text{O}_2 \xrightarrow{\text{GPO}} \text{Dihydroxy acetone-P} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red quinoneimine dye} + \text{H}_2\text{O}
\]

**REAGENTS:** All the reagents were supplied with kit.

Reagent 1: Enzyme Reagent (Lipoprotein Lipase, Glycerol kinase, peroxidase, 4-Amino antipyrine and Glycerol phosphate oxidase).

Reagent 2: TG Standard (200 mg/dl).

**Procedure**

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>1000 μl</td>
<td>1000 μl</td>
<td>1000 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 2</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 & incubate for 20 min. at 20-25 °C or 10 min. at 37 °C. Measure the absorbance of standard and sample against reagent blank at 505 nm.

**Calculation:**

\[
\text{TG (mg/dl)} = \frac{\text{Absorbance of T} \times 200}{\text{Absorbance of S}}
\]

5) **Estimation of non-HDL cholesterol:** calculated by the formulae described by Frost PH et al.\textsuperscript{69}

\[
\text{Non-HDL-C} = \text{Total Cholesterol} - \text{HDL-C}
\]
6) **Estimation of Plasma Malonaldehyde (MDA) level:** by the method as described by Satho K, (1978).

**REAGENTS:**
- a) TCA (Trichloro acetic acid) 15% w/v
- b) TBA (Thiobarbituric acid) 0.375% w/v
- c) 0.25 N HCl
- d) 1N NaOH

The TCA-TBA-HCl solution will be freshly prepared by mixing equal volume of 15% TCA, 0.375% TBA and 0.25 N HCl.

**PROCEDURE**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>0.8 ml of Plasma + 1.2 ml of TCA-TBA-HCl reagent.</td>
</tr>
<tr>
<td>2)</td>
<td>Mixed immediately + kept in a boiling water bath for 10 minutes.</td>
</tr>
<tr>
<td>3)</td>
<td>Cooled + 2 ml of NaOH (freshly prepared) to eliminate centrifugation.</td>
</tr>
<tr>
<td>4)</td>
<td>OD at 535 nm against blank which contained normal saline in place of plasma.</td>
</tr>
</tbody>
</table>

**CALCULATION:**
The content of MDA was calculated using molar extinction co-efficient (1.56 X 10^5 cm\(^{-1}\)) and expressed as µmole of MDA per 100 ml of plasma.

7) **Estimation of CATALASE:** Colorimetric assay of catalase by Ashok K. Sinha (1971).

**Reagents Preparation:**
- **Dichromate acetic acid reagent:** 2.5 gm potassium dichromate in 50 ml distilled water + 150 ml glacial acetic acid (98-100%).
- **Hydrogen peroxide (0.2 M).**
- **Phosphate buffer (0.01 M) pH=7:** contains two chemicals, solution A and B mixed in required proportions.
  - a) 0.01 M KH2PO4 = 1.378 gm / lit.
  - b) 0.01 M Na2 HPO4 = 1.439 gm / lit.
MATERIALS & METHODS

PROCEDURE

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td><strong>Reaction mixture:</strong> 4 ml of H$_2$O$_2$ in small beaker + 5 ml of phosphate buffer + 1 ml of diluted sample (haemolysate 1:10) added rapidly + mixed gently by swirling motion.</td>
</tr>
<tr>
<td>2)</td>
<td>Taken 2 ml 0f dichromate acetic acid reagent in other test tubes labeled 1,2,3,4. + 1 ml of <strong>reaction mixture</strong> added to each test tube at an interval of 60 sec. Blue precipitate of perchromic acid obtained.</td>
</tr>
<tr>
<td>3)</td>
<td>Boil the test tubes for 10 min in water bath. Color of the solution changes to green due to the formation of chromic acetate. Cool the test tubes at room temperature and make up the volume upto 3 ml by adding distilled water.</td>
</tr>
<tr>
<td>4)</td>
<td>Read the OD at 570 nm.</td>
</tr>
</tbody>
</table>

**Procedure of Blank:**
2ml of dichromate acetic acid + 1 ml of distilled water.

**Calculation:**
The activity of Catalase was expressed as µmoles of H$_2$O$_2$ consumed/min/gm Hb or units/gm Hb and calculated by calibration curve.

8) **Estimation of Oxidized LDL**

**PRINCIPLE**

Mercodia Oxidized LDL Competitive ELISA is based on the monoclonal antibody 4E6. Oxidized LDL in the sample competes with a fixed amount of oxidized LDL bound to the microtiter well for the binding of the biotin-labeled specific antibodies. After a washing step that removes unreactive sample components, the biotin-labeled antibody bound to the well is detected by HRP-conjugated streptavidin. After a second incubation and an additional washing step, the bound conjugate is detected by reaction with 3,3′,5,5′-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm.
SAMPLE DILUTION

Samples must be diluted the same day as the assay performance. Separate tube was prepared for each patient sample. Each sample is diluted in one step to a final dilution of 41 times as follows:

Patient sample  25 µl  
Sample buffer  1000 µl

Samples are properly mixed before further use.

Sample diluted 41 times in sample buffer is stable for 1 day at 4°C.

TEST PROCEDURE

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add Calibrator 0 for B0</td>
<td>50 µl</td>
</tr>
<tr>
<td>Add Calibrator 0 for Blank</td>
<td>100 µl</td>
</tr>
<tr>
<td>Add Calibrators, Controls and Samples</td>
<td>50 µl</td>
</tr>
<tr>
<td>Add Antibody to all wells except the Blank</td>
<td>50 µl</td>
</tr>
<tr>
<td>Incubation</td>
<td>2 hours at 18–25°C on a plate shaker (700-900 rpm)</td>
</tr>
<tr>
<td>Wash plate with Washing Buffer</td>
<td>6 times</td>
</tr>
<tr>
<td>Add enzyme conjugate to all wells</td>
<td>100 µl</td>
</tr>
<tr>
<td>Incubation</td>
<td>1 hour at 18–25°C on a plate shaker</td>
</tr>
<tr>
<td>Wash plate with Washing Buffer</td>
<td>6 times</td>
</tr>
<tr>
<td>Add Substrate TMB</td>
<td>200 µl</td>
</tr>
<tr>
<td>Incubation</td>
<td>15 minutes (without shaking)</td>
</tr>
</tbody>
</table>
| Add Stop Solution                         | 50 µl  
Shake for 5 seconds to ensure mixing                                      |
| Measure absorbance at 450 nm.             | Evaluate Results                                                             |
CALCULATION
1. The absorbance (B) for Calibrators, Controls and Unknowns expressed as a percentage inhibition of the mean absorbance of the maximum binding at zero inhibition ($B_0$).

$$\%B_0 = \frac{B \text{ (of Calibrators, Controls or Unknowns)}}{B_0 \text{ (mean abs at zero inhibition)}} \times 100$$

2. Plot the calculated percentage values ($\%B_0$) obtained for the Calibrators against the log concentration and construct a calibrator curve.
3. Read the concentration of the Controls and Unknown samples from the calibrator curve.
4. Multiply the concentration of the Controls and the Unknown samples with the dilution factor (i.e. $\times 41$).

9) Estimation of Plasma Myeloperoxidase (MPO)

Principle
The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human MPO. Samples are pipetted into these wells. Non-bound MPO and other components of the sample should be removed by washing, then biotin-conjugated monoclonal antibody specific to MPO added. In order to quantitatively determine the amount of MPO present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) should be added to each microplate well. The final step, a TMB-substrate solution added to each well. Finally, a sulphuric acid solution is added and the resulting yellow colour product is measured at 450nm. Since the increases in absorbency is directly proportional to the amount of captured MPO.

Assay Procedure
1) Determine the number of 16-well strips needed for assay.
2) Add 300 µl of Incubation buffer to all wells and incubate the plate for 5 minutes at room temperature.
3) Thoroughly aspirate or decant the solution from the wells. Wash wells 2 times.
4) For the standard curve, add 100 µl of the standard to the appropriate microtiter wells. Add 100 µl of the Standard/Sample Dilution Buffer to zero wells.
5) Serum and plasma require at least 20 fold dilution in the Standard/Sample Dilution Buffer. And add 100 µl of samples to each well.

6) Cover the plate with the plate cover and incubate for 2 hours at room temperature.

7) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times.

8) Pipette 100 µl of “Working Secondary Antibody Solution” into each well.

9) Cover the plate with the plate cover and incubate for 1 hour at room temperature.

10) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times.

11) Add 100 µl “Working AV-HRP Solution” to each well.

12) Cover the plate with the plate cover and incubate for 30 minutes at room temperature.

13) Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times.

14) Add 100 µl of Substrate to each well. The liquid in the wells should begin to turn blue.

15) Incubate the plate at room temperature.

16) Add 100 µl of Stop Solution to each well. The solution in the wells should change from blue to yellow.

17) Read the absorbance of each well at 450nm. Read the plate within 20 minutes of adding the Stop Solution.

18) Plot on graph paper the absorbance of the standard against the standard concentration and draw the standard curve.

19) Read the human MPO concentrations for the unknown samples and controls from the standard curve plotted in step 18. Multiply value(s) obtained for the unknown sample by the dilution factor.

10) **DNA Isolation:** whole blood DNA was isolated by using phenol/chloroform method.\(^{145}\)

1) Take 600 µl of blood in a centrifuge tube.

2) Add 600 µl of Lysis buffer.

3) Mix gently and carefully.

4) Incubate at 37 °C for 30 min or 56 °C for 10 min.

5) Now centrifuge at 14000 rpm for 10 min.

6) Discard the supernatant.

7) Add 600 µl of Lysis buffer and centrifuge at 14000 rpm for 10 min.
8) Again discard the supernatant.
9) Add 500 µl of Distilled water.
10) Centrifuge at 12000 rpm for 5 min. and discard supernatant.
11) To the pellet added 200 µl of Proteinase K buffer and 10 µl of 10 % SDS.
12) Mix gently and frothing by repeated pipetting.
13) Added 100 µl of 5 M NaCl and tap.
14) Added 200 µl of distilled water and shake well for 2-3 times.
15) Added 400 µl of Tris saturated phenol + 100 µl of chloroform (4:1).
16) Mix well and centrifuge at 12000 rpm for 10 min.
17) Three layers will appear, collect the uppermost aqueous layer.
18) Added 1 ml of Absolute alcohol and centrifuge at 12000 rpm for 4 min.
19) Precipitated DNA (pellet) washed in 70 % ethanol twice for removal of traces of salt.
20) Finally centrifuged at 12000 rpm for 5 min. at room temperature.
21) DNA pellet obtained, keep at 37 °C to evaporate alcohol.
22) Added 100 µl of 1X TAE and keep at 37°C for overnight.
23) Quantitated and stored at -20 °C

11) **Amplification of Gene by Polymerase chain reaction**

Polymerase chain reaction (PCR) is an ingenious and highly sensitive tool in molecular biology. It is based on the in-vitro enzymatic synthesis/amplification of new DNA strands on the template using oligonucleotide sequences as primers. Polymorphisms were detected by using RFLP-PCR analysis as described earlier. A 350-bp DNA fragment was amplified using forward primer **MPOF (5’-CGG TAT AGG CAC ACA ATG GTG AG-3’)** and reverse primer **MPOR (5’-GCA ATG GTT CAA GCGATT CTT C-3’).**

**Reagents**
- Template cDNA
- Forward Primer
- Reverse primer
- dNTPs
- 10x PCR buffer
- Taq DNA polymerase
- Milli Q H2O

**Protocol**
Arranged PCR tubes in the rack and labeled them.

Thawed the vials of template DNA, primers, dNTPs, Taq, & 10X PCR buffer. Gave a short spin and placed on ice.

10µl reaction mix was prepared for n+1 samples (where n = no of samples) as follows:

- DNA sample: 2.0µl
- dNTPs: 1.0µl
- Taq / PCR Buffer: 1.0 µl
- Taq (3u/µl): 0.3 µl
- Primer F: 0.5 µl
- Primer R: 0.5 µl
- SDW (to make up the volume to 10.0µl): 4.7 µl

Closed the caps and gave a momentary spin.

Placed the tubes in the thermal cycler and set it to carry out the following temperature profile:

1. Initial denaturation 95°C for 2 min;
2. Denaturation at 94°C for 30 s
3. Annealing Temperature (62°C) for 30 sec.
4. Primer extension at 72°C for 30 s;
5. Goto step 2 and repeat for 35 cycles.
6. Final extension at 72°C for 3 min.

Separated PCR products on 2 % Agarose gel.

Visualized in UV transilluminator and took a careful record of the profile generated.

11) Restriction Digestion

Endonucleases are enzymes that produce internal cut, called cleavage, in DNA molecule. Some endonucleases cleave only one of the two strands of a DNA duplex; such cuts are usually known as nicks. In contrast many endonucleases cleave both the strand of the DNA molecule; many of such endonucleases cleave DNA molecule at random site. But a class of endonucleases cleaves DNA only within or near those
sites, which have specific base sequences; such endonucleases are known as restriction endonucleases and the site known as recognition site. Digestion of DNA with restriction endonucleases generates different restriction maps. An interested difference in the pattern of restriction is known as Restriction Fragment Length Polymorphism or RFLPs. The RFLP method is used in this study to identify different isoforms of a specific polymorphism.

**Reagents**

- PCR reaction mixture
- AciI (10u/µl) (Fermentas)
- 10X buffer O
- Nuclease free water

**Protocol**

- **Add:**
  
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR reaction mixture</td>
<td>10 µl</td>
</tr>
<tr>
<td>nuclease-free water</td>
<td>18 µl</td>
</tr>
<tr>
<td>10X Buffer O</td>
<td>2 µl</td>
</tr>
<tr>
<td>AciI 1</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

- Mix gently and spin down for a few seconds.
- Overnight incubation at 37°C.
- Prepared 2% Agarose gel for electrophoresis.
- Added appropriate amount of gel loading buffer in digested sample and loaded in Agarose gel.
- Also load 50 bp DNA ladder in gel for comparison.
- Electrophoresed the samples at 50V for 1 hr.
- Visualized the DNA bands by UV transilluminator and took a careful record of the profile.
Statistical Analysis:

All statistical analysis was conducted by software package SPSS version 17.0. Continuous variables of demographical and baseline characteristics were compared by the use of Students t-test for two groups or analysis of variance by using one way ANOVA for multiple comparisons. Univariate comparisons of categorical variables were performed with chi-square test for analyzing allele frequencies and genotypic distribution followed by multivariate analysis and the calculation of odd ratios with corresponding 95% confidence interval.