MATERIAL AND METHODS
4.1 Inclusion Criteria

- Subjects with clinically diagnosed, having angiographycally proven CAD with/without Type-II DM were included in the study.

- Prevalence of diabetes was assessed either by fasting blood glucose levels ≥126.0 mg/dl or treatment with antibiotic drugs or HbA1c levels was more than 6% or abnormal glucose tolerance test were included for study.

- BMI values were derived from Quetlet’s formula (weight in kg/height in m²), obesity considered with BMI >25.0 kg/m².

- Smoking was defined as regular smoking of cigarettes or beedies.

- Systemic HTN was considered to be present if the patient was taking anti-hypertensive treatment at the time of hospital admission or if BP was recorded ≥140 mmHg as SBP or ≥90 mmHg as DBP, at least thrice on examination during admission.

- A positive family history of premature CAD was defined as first degree relative that had documented CAD below the age of 55 years in males or 65 years in females.

- Dyslipidemia considered with patient who had serum levels of TC ≥240 mg/dl, or LDL-c ≥160 mg/dl, or HDL-c ≤30 mg/dl are considered as hyperlipedemics were included for the study.

- For lipid analysis, samples were obtained after an overnight fast at hospital admission.
# Patients had a positive ECG (electrocardiogram) were included.

2 **Exclusion Criteria**

# Patients with acute coronary syndrome during preceding 4 weeks.

# Patients with history of prior coronary artery bypass grafting.

# Patients with kidney disease and acute liver disease.

# Patients with history of jaundice, tremors, burning and pregnancy were excluded from the study.

4.2 **Study design**

The study was case-controlled in design. We have selected the patients as they were presented. Patients included in the present study were all admitted to the intensive coronary care unit (ICCU) or attending the outpatient department of M. Y. Hospital attached to M.G.M. Medical College, Indore, Madhya Pradesh, Chirayu Hospital and Heart speciality centre Bhopal, M.P. India and NSCB Medical College, Jabalpur, M.P. India, recruited from April 2007 to June 2009

**a) Clinical Study populations**

Two hundred seventy individuals were included in the study and divided into four groups. After careful clinical examination and confirmed diagnosis, 70 patients presenting CAD (Group 2), 70 patients presenting Type-II DM (Group 3) and 70 patients presenting Type-II DM with CAD (Group 4) were included in the study.

All patients selected, underwent medical examination by a physician. A careful medical history was taken to obtain information about other
diseases (particularly hypertension, coronary heart disease, myocardial infarction, stroke, peripheral vascular disease, and endocrine disorders). Body weight and height were measured with subjects in light clothing without shoes. Three blood pressure recordings were obtained from the right arm while in the supine position after 30 min of rest at 5-min intervals, and their mean value was calculated. Blood samples were drawn into Vacutainer tubes. Fully informed consent was obtained from patients and controls. The study was approved by the ethical committee of MGM Medical College and M Y Hospital Indore.

Patients matched 90 subjects as control (Group 1) and Group 3 was randomly selected from patients attending to Medicine OPD of institute. Of them Group 1 subjects had negative history of CAD, Type-II DM or had normal resting ECG.

Type –II DM in the present study was defined as a fasting blood glucose level of ≥126.0 mg/dl or post-prandial glucose level ≥200.0 mg/dl or self reported physician diagnosis of diabetes, or pharmacological treatment for diabetes on the basis of mentioned criteria Group 3 subjects were selected for the study.

Subjects in Group 2 and Group 4 were recruited from in patients and out patients departments of hospital. The diagnosis of CAD was made on the basis of clinical history (typical angina, history of myocardial infarction) and 12-lead standard ECG before subjecting them to coronary angiography. The presence of any stenosis > 30% according to coronary angiography by visual assessment of coronary artery was included in the study. In most of the cases patients had sustained the primary clinical event elsewhere and were referred for further management.

As described previously, a coronary angiography examination (standard Judkin technique) was performed, and images were interpreted by a
panel of experienced cardiologists who were blinded to novel markers particularly ADMA, NO. A stenosis estimated at greater than 30% in at least one major epicardial artery was considered positive for CAD. Whole population was further categorized in two groups on the basis of CAD findings. Diseased Group (included Group-2 & 4) and Non Diseased Group (included Group-1 & 3).

**b) Collection of samples**
Venous blood was collected from all subjects between 9.00 am and 11.00 am soon after fasting from 10.00 pm in the previous day. Plain and EDTA vacutainer were used for blood collection. Serum was isolated by low-speed centrifugation. The samples were stored at -20°C for ≤ 45 days prior to analysis. This work was carried out in the M.Y.Hospital, Indore, M.P. India and NSCB Medical College, Jabalpur, M.P. India.

4.3 **Biochemical analysis**

**Total Cholesterol**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecnno, Rome-Italy.

**Method:** Enzymatic, CHOD/PAP method with cholesterol esterase, cholesterol oxidase and 4-aminoantipyrine\textsuperscript{378}. Roche Diagnostics Ltd. (Boehringer Mannheim, Germany) manufactured reagents were used for analysis.

**Principle:** Cholesterol esterase (CE) hydrolyses cholesterol to form free cholesterol and fatty acids. Cholesterol oxidase (CHOD) catalyse the oxidation of cholesterol to form cholest-4-ene-3-one and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). In presence of peroxidase (POD), the hydrogen peroxide
formed effects the oxidative coupling of phenol and 4-aminoantipyrine (4-AAp) to form a red colure quinineimine dye.

**Reaction:**

Cholesterol esters + H₂O --- CE---→ Cholesterol + Fatty acids

Cholesterol + O₂ --- CHOD---→ Cholest-4-ene-3-one + H₂O₂

2H₂O₂ + 4-AAp + Phenol --- POD---→ Quinoneimene dye + 4 H₂O

The colour intensity of the red quinoneimine dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance at 520 nm.

**Expected values:** Recommendations of the NCEP Adult Treatment Panel for the following risk cut-off thresholds for the US American population

- Normal <200 mg/dl
- Borderline high 200-239 mg/dl
- High >240 mg/dl

**HDL-Cholesterol Direct**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minitecno, Rome-Italy.

**Method:** Enzymatic, CHOD/PAP method without sample pre-treatment. Roche Diagnostics Ltd (Boehringer Mannheim, Germany) manufactured reagents are used for analysis.
**Principle:** HDL-Cholesterol Direct is based on the adsorption of synthetic polyanions to the surface of lipoproteins. LDL-c, VLDL-c and chylomicrons are thereby transformed into a detergent-resistant form, whereas HDL-c is not. Combined action of polyanions and detergent solubilizes cholesterol from HDL-c, but not from LDL-c, VLDL-c and chylomicrons. Solubilize cholesterol is oxidized by the sequential enzymatic action of CE and CHOD. The hydrogen peroxide formed reacts with N, N-bis (4-sulfobutyl)-m-toludine (DSBmT) and 4-AAP in the presence of POD and forms a red colur quinineimnine dye.

**Reaction:**

LDL, VLDL, Chylomicrons + Polyanions $\rightarrow$ Lipoprotein-polyanion complex

HDL + Detergent $\rightarrow$ Micelle complexes

Micelle complexes$\rightarrow$CE/CHOD$\rightarrow$ Oxidized cholesterol + H$_2$O

H$_2$O$_2$ + 4-aminoantipyrine + DSBmT $\rightarrow$ POD$\rightarrow$ quinoneimine dye

The colour intensity of the red quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 552 nm.

**Expected values:**

Recommendation of laboratory standardization panel of the national cholesterol education program (NCEP).

<table>
<thead>
<tr>
<th></th>
<th>Good prognosis</th>
<th>Standard risk</th>
<th>Risk indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>&gt;65 mg/dl</td>
<td>45-65 mg/dl</td>
<td>&lt;45 mg/dl</td>
</tr>
<tr>
<td>Males</td>
<td>&gt; 55 mg/dl</td>
<td>35-55 mg/dl</td>
<td>&lt;35 mg/dl</td>
</tr>
</tbody>
</table>
**LDL-Cholesterol Direct**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minitecno, Rome-Italy.

**Method:** Enzymatic, CHOD/PAP method without sample pretreatment\(^3\). Roche Diagnostics Ltd (Boehringer Mannhiem, Germany) manufacture reagents are used for analysis.

**Principle:** HDL, VLDL and chylomicrons are specifically hydrolyzed by a detergent. The released cholesterol contained in these lipoproteins reacts immediately in the enzymatic action of CE and CHOD generating hydrogen peroxide. The latter is consumed by a POD in the presence of 4-aminoantipyrine to generate a colourless product. During this first step, LDL-c particles remain intact. The reaction of LDL-c is initiated by the addition of another detergent 3 together with a coupler, N, N-bis (4-sulfobutyl)-m-toludine (DSBmT). The second detergent releases cholesterol in the LDL-c particles which are subjected to the enzymatic reaction in the presence of coupler to produce a coloured product.

**Reaction:**

First Step

\[
\text{HDL, VLDL, Chylomicrons + Detergent 1} \rightarrow \text{released cholesterol}
\]

\[
\text{Cholesterol} \rightarrow \text{CE/CHOD} \rightarrow \text{Oxidized cholesterol + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} \rightarrow \text{POD} \rightarrow \text{Colourless product}
\]

Second Step

\[
\text{LDL + Detergent 2} \rightarrow \text{Released cholesterol}
\]

\[
\text{Cholesterol} \rightarrow \text{CE/CHOD} \rightarrow \text{oxidized cholesterol + H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine + DSBmT} \rightarrow \text{POD} \rightarrow \text{Quinoneimine dye}
\]
The colour intensity of the red quinoneimine dye formed is directly proportional to the LDL-c concentration. It is determined by measuring the increase in absorbance at 552 nm.

**Expected values:**
The following guideline apply to LDL-c (NCEP AT II categories)\(^{382}\)

<table>
<thead>
<tr>
<th>Category</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;130.0 mg/dl</td>
</tr>
<tr>
<td>Borderline high risk</td>
<td>130-160 mg/dl</td>
</tr>
<tr>
<td>High risk</td>
<td>&gt;160.0 mg/dl</td>
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</tbody>
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**Serum Triglycerides**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecno, Rome-Italy.

**Method:** Enzymatic, GPO/PAP method with glycerol phosphate oxidase and 4-aminophenazone\(^{383}\). Roch Diagnostcs Ltd (Boehringer Mannheim, Germany) manufactured reagents are used for analysis.

**Principle:** Triglycerides are hydrolyzed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP I a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and H2O2. In the presence of POD, hydrogen peroxide affects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form a red coloured quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample.
**Reaction:**

Triglycerides $\xrightarrow{\text{LPL}}$ Glycerol + Fatty acids  
Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol-3-phosphate + ADP  
Glycerol-3-phosphate + $O_2$ $\xrightarrow{\text{GPO}}$ Dihydroxyacetone phosphate + $H_2O_2$  
$2H_2O_2 + 4\text{-aminophenazone} + 4\text{-chlorophenol} \xrightarrow{\text{POD}}$ Quinoneimine dye + 4$H_2O$

**Expected values:**

According to NCEP: $<200.0 \text{ mg/dl}$

*Asymmetric Dimethylarginine (ADMA)*

**Analyzer:** Single strip reading semi automated strip ELISA Reader AUTOREADER-3011, Awareness Technology, USA was used.

**Method:** Enzyme Linked Immunoassay for the Quantitative Determination of Endogenous Asymmetric Dimethylarginine in serum only for research purpose. Manufacture by DLD DIAGNOSTICS GMBH was used for analysis.

**Principle:** The competitive ADMA-ELISA uses the microtiter plate format. ADMA is bound to the solid phase of the microtiter plate. ADMA in the samples is acylated and competes with solid phase bound ADMA for a fixed number of rabbit anti-ADMA antiserum binding sites. When the system is in equilibrium, free antigen and free antigen antiserum complexes are removed by washing. The antibody bound to the solid phase ADMA is detected by the anti-rabbit/peroxidase. The substrate TMB/peroxidase reaction is monitored at 450 nm. The amount of
antibody bound to the solid phase ADMA is inversely proportional to the ADMA concentration of the sample.

**Procedure:** As per the instructions provided with the kit.

**Preparation of Reagents and Samples**

**Acylation Reagent**
Dissolve the content of one bottle in 1.5 ml dimethylformamide (DMF) and shake for 5 minutes on a orbital shaker. After use the reagent has to be discarded. The Acylation Reagent has always to be prepared immediately before use. The second bottle allows a second run of the test. If the whole kit is to be used in one run it is recommended to pool the dissolved contents of the two vials of Acylation Reagent. Please note that DMF reacts with many plastic materials including plastic trays which are used as reservoir for multichannel pipettes. DMF does not react with normal pipette tips and with glass devices.

All other reagents are ready for use.

**Preparation of Samples (Acylation)**
1. Pipette each 20 µl standard A - F, each 20 µl control 1 & 2 and each 20 µl patient sample into the respective wells of the Reaction Plate.
2. Pipette 25 µl Acylation Buffer into all wells.
3. Pipette 25 µl Equalizing Reagent into all wells.
4. Mix the reaction plate for 10 seconds.
5. Prepare Acylation Reagent freshly and pipette 25 µl prepared Acylation Reagent each into all wells, mix **immediately**.
6. Incubate for 30 minutes at room temperature (approx. 20 °C) on an orbital shaker.
7. Dilute 1.5 ml prepared Equalizing Reagent in 9 ml dist. water, mix and pipette 100 µl each into all wells. (If you decide to run only half of the test kit dilute 0.75 ml Equalizing Reagent in 4.5 ml dist. water)

8. Incubate for 45 minutes at room temperature (approx. 20 °C) on an orbital shaker.

*Take each 50 µl for the ADMA-ELISA.*

**Test procedure ELISA**

Bring all reagents to room temperature and mix them carefully, avoid development of foam. A fibrinogen like clot will develop within the Reaction Plate, which should be moved to and fixed on the side of the wall of the Reaction Plate wells with the help of the pipette tips just before pipetting. Small particle will not interfere with the ELISA procedure but could block the pipette tip!!

1. **Sample Incubation**

Pipette each 50 µl prepared Standards A to F, 50 µl prepared controls and 50 µl prepared samples into the respective wells of the coated microtiter strips (duplicates are recommended). Pipette each 50 µl Antiserum into all wells and shake shortly on an orbital shaker. Cover the plate with adhesive foil and incubate Microtiter Strips for 15 –20 hours (overnight) at 2-8°C.

2. **Washing**

Discard or aspirate the contents of the wells and wash thoroughly with each 250 µl Wash Buffer (Shake shortly on an orbital shaker). Repeat the washing procedure 4 times. Remove residual liquid by tapping the inverted plate on clean absorbent paper.

3. **Conjugate Incubation**

Pipette each 100 µl enzyme conjugate into all wells. Incubate for 60 minutes at room temperature on an orbital shaker.
4. Washing
Repeat step 2.

5. Substrate Incubation
Pipette each 100 µl Substrate into all wells and incubate for 20 to 30 minutes at room temperature on an orbital shaker.

6. Stopping
Pipette each 100 µl Stop Solution into all wells.

7. Reading
Read the optical density at 450 nm (reference wavelength between 570 and 650 nm) in a microplate photometer.

8. Calculation of the Results
On a semilogarithmic graph paper the concentration of the standards (x-axis, logarithmic) are plotted against their corresponding optical density (y-axis, linear). The concentration of the controls and samples can be read directly from this standard curve by using their average optical density.

**Expected values:** 0.4 -0.75 µmol/l

*Myeloperoxidase (MPO)*

**Analyzer:** Single strip reading semi automated strip ELISA Reader AUTOREADER-3011, Awareness Technology, USA was used.

**Method:** Enzyme Linked Immunoassay for the Quantitative Determination of MPO in serum only for research purpose. Manufacture by ALPCO DIAGNOSTICS, SALEM was used for analysis.
**Principle:** The assay utilizes the two-site “sandwich” technique. Assay standards, controls and prediluted samples containing human MPO are added to wells of microplate that was coated with a high affinity polyclonal anti-human MPO antibody. After the first incubation period, antibody immobilized on the wall of micro wells captures human MPO in the sample. Then a biotinylated detection antibody, a monoclonal anti-human MPO antibody, is added to each microwell and a “sandwich” of capture antibody-human MPO-biotinylated detection antibody is formed. In the next step, a peroxidase labelled streptavidin is added. Tetramethylbenzidine (TMB) is used as a substrate for peroxidase. Finally, an acidic stop solution is added to terminate the reaction. The colour changes from blue to yellow and measured at 450 nm. The intensity of the yellow colour is directly proportional to the concentration of MPO in the sample.

**Procedure:** As per the instructions provided with the kit.

**Preparation of Samples**

Prior to analysis the serum should be diluted 1:40 with SAMPLEBUF (sample dilution buffer). 25µl sample + 975µl sample buffer.

**Test procedure**

1. Prior to use in the assay allow all reagents and samples to come to room temperature (18-26°C) and mix well.
2. Wash each well 5 times by dispensing 250 µl of diluted WASHBUF into each well. After the 5th step inverted microplate should be firmly tapped on absorbent paper.
3. Add 100 µl of STD/SAMPLE/CTRL in duplicate into respective wells. Use sample dilution buffer as STD 0 ng/ml
4. Cover the plate tightly and incubate for 1 hour at room temperature on a horizontal mixer.
5. Aspirate the contents of each well. Wash 5 times by dispensing 250 µl of diluted WASHBUF into each well. After the final washing step the inverted microplate should be firmly tapped on absorbent paper.

6. Add 10 µl of AB (detection antibody solution) into each well.

7. Cover the plate tightly and incubate for 1 hour at room temperature on a horizontal mixer.

8. Repeat step 5.

9. Add 100 µl CONJ (conjugate) into each well.

10. Repeat step 7.

11. Repeat step 5

12. Add 100 µl SUB (substrate) into each well.

13. Incubate for 20-30 minutes at room temperature in the dark.*

14. Add 50 µl of STOP (stop solution) into each well, mix thoroughly.

15. Determine absorption immediately with ELISA reader at 450 nm.

Expected values:

Normal < 300 µmol/l

Risk > 300 µmol/l

NO (Nitric Oxide)

Analyzer: Semi auto clinical chemistry analyzer, Model-Minictecno, Rome-Italy.

Method: Serum nitric oxide (as nitrite) was estimated by method of Cortas NK[383] by using Quantichrome™ Nitric Oxide Assay kit based on colorimetric determination of NO at 540 nm.

Principle: Nitric oxide is a reactive radical, since it is oxidized to nitrite and nitrate and it is easy to quantitate total NO₂/NO₃ as a measure for NO level, using kinetic cadmium reduction method or improved Griess
method (Griess reagent is consists of 1 part of 0.1% napthylenediamine dihydrochloride in distilled water plus 1 part 1% sulphanilamide (or sulfanilic acid) in 5% concentrated H₃PO₄)

**Procedure:** As per the instructions provided with the kit.

Deproteination of serum sample:
Mix 100 μl serum with 80 μl 75 mM ZnSO₄ in 1.5 ml tubes. If precipitation occurs, centrifuge 5 min at 14,000 rpm. Transfer supernatant to a clean tube containing 120 μl 55 mM NaOH. Protein precipitates again. Transfer 210 μl supernatant and mix with 70 μl Glycine Buffer in a 1.5 ml centrifuge tube.

If solution remains clear in these steps, deprotination is no required. Directly transfer 210 μl sample and mix with 70 μl Glycine buffer in a 1.5 ml centrifuge tube.

Activation of Cadmium:
Transfer Cd granules in a 50ml centrifuge tube. Wash Cd three times with water. Remove residual water with a pipette. Add 200 μl diluted 1× Activation Buffer per granule and incubate 5 min at room temperature. Swirl tube intermittently. Wash three times with water. Activated Cd should be used within 20 min.

Nitrate reduced Cd:
Dry the activated Cd granules on a filter paper. Add 3 Cd granules per sample and shake tubes intermittently. Incubate 15 min at room temperature. Transfer 2 × 100 μl samples into wells of the 96-well plate.

**ASSAY:**
Add 50 μl Reagent A to all wells and tap plate lightly to mix. Add 50 μl Reagent B and mix. Incubate 5 min at room temperature. Read OD at 500-550 (peak 540) nm. Signal is stable for >60 minutes.
**Expected values:**

Normal     > 30 μmol/L
Risk       < 30 μmol/L

**Lp(a) or Lipoprotein(a)**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecnno, Rome-Italy.

**Method:** Latex enhanced immunoturbidimetric method\(^{387}\), Using Diazyme's Lipoprotein(a) assay kit.

**Principle:** This test is based on a latex enhanced immunoturbidimetric method. Lp(a) in the sample binds to the specific anti-Lp(a) antibody, which is coated on latex particles, and causes agglutination. The degree of the turbidity caused by agglutination can be measured optically at 700 nm and is proportional to the amount of Lp(a) in the sample.

**Procedure:** As per the instructions provided with the kit.

**ASSAY**

1. Add R\(_1\) (Reagent 1) 180 μL to serum sample (4 μL).
2. Incubate for 5 minutes at 37°C temperature.
3. Add R\(_2\) (Reagent 2) 60 μL, mix well.
4. Take absorbance at 700 nm \(A_1\) and after 5 minutes \(A_2\).

**Expected value:**

Normal     15-30 mg/dl
Risk       > 30 mg/dl
**Homocysteine (Hcy)**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecno, Rome-Italy.

**Method:** Enzymatic homocysteine assay\(^3\) manufactured by Bio-Quant and supplied by Krisgen, Mumbai.

**Principle:** Enzymatic tHcy assay is based on a novel assay principle that assesses the co-substrate conversion product (a molecule that is not a substrate of the Hcy conversion enzyme, and does not contain any element from sample Hcy) instead of assessing co-substrate or Hcy conversion products of Hcy as described in the literature. In this assay, oxidized Hcy is first reduced to free Hcy which then reacts with a co-substrate, S-adenosylmethionine (SAM) catalyzed by a Hcy S-methyltransferase to form methionine (the Hcy conversion product of Hcy) and S-adenosylhomocysteine (SAH, the co-substrate conversion product). SAH is assessed by coupled enzyme reactions including SAH hydrolase, adenosinedeaminase and glutamate dehydrogenase, where SAH is hydrolyzed into adenosine (Ado) and Hcy by SAH hydrolase. The formed Hcy that is originated from the co-substrate SAM is cycled into the Hcy conversion reaction by Hcy S-methyltransferase. This forms a co-substrate conversion product based enzyme cycling reaction system with significant amplification of detection signals. The formed Ado is immediately hydrolyzed into inosine and ammonia which reacts with glutamate dehydrogenase with concomitant conversions of NADH to NAD+. The concentration of Hcy in the sample is indirectly proportional to the amount of NADH converted to NAD+ (\(\Delta A_{340nm}\)).

**Procedure:** As per the instructions provided with the kit.
**Expected values:**

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<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;15 micro mol/L</td>
</tr>
<tr>
<td>Risk</td>
<td>&gt;15 micro mol/L</td>
</tr>
</tbody>
</table>

**Hs-CRP (High sensitivity C-reactive protein)**

**Analyzer:** Single strip reading semi automated strip ELISA Reader AUTOREADER-3011, Awareness Technology, USA was used.

**Method:** High sensitivity Enzyme Immunoassay for the Quantitative Determination of CRP concentration in serum only for research purpose\(^{389}\). Manufacture by BioCheck, Inc. was used for analysis.

**Principle:** The Hs-CRP ELISA is based on the principle of a solid phase enzyme-linked immunosorbant assay\(^{389}\). The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the CRP molecule. This mouse monoclonal anti-CRP antibody is used for solid phase immobilization (on the microtitre wells). A goat anti-CRP antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the CRP molecules being sandwiched between the solid phase and enzyme –linked antibodies. After 45-minute incubation at room temperature, the wells are washed with water to remove unbound labelled antibodies. A tetramethylbenzidine (TMB) reagent is added and incubated for 20 minutes, resulting in the development of blue colour. The colour development is stopped with the addition of 1N HCl changing the colour to yellow. The concentration of CRP is directly proportional the colour intensity of the test sample. Absorbance is measured at 450 nm.
**Procedure:** As per the instructions provided with the kit.

**ASSAY**

1. Patient serum and control serum should be diluted 100 fold (5 µl of serum with 495 µl of sample diluent) prior to use.
2. Secure the desired number of coated wells in the holder.
3. Dispenses 10 µl of CRP standards, DILUTED specimens, and DILUTED controls into appropriate wells.
4. Dispense 100 µl of CRP Enzyme Conjugate Reagent into each well.
5. Mix for 30 seconds. Incubate at room temperature for 45 minutes.
6. Remove the incubation mixture by flicking plate contents into a waste container. Rinse and flick the microtiter wells 5 times with distilled water. Strike the wells sharply onto absorbent paper.
7. Dispense 100 µl TMB solutions into each well. Gently mix for 5 seconds.
8. Incubate at room temp. for 20 minutes.
9. Stop the reaction by adding 100 µl of Stop Solution to each well. Gently mix for 30 seconds. IT IS IMPORTANT TO MAKE SURE THAT ALL THE BLUE COLOUR CHANGES TO YELLOW COMPLETELY.
10. Read OD at 450nm with microtiter well reader within 15 minutes.

**Expected values:**

- Normal: <1mg/l
- Risk: >1mg/l

**Fibrinogen (FIB)**

**Analyzer:** Single strip reading semi automated strip ELISA Reader AUTOREADER-3011, Awareness Technology, USA was used.
Method: Highly sensitive two-site enzyme linked immunosorbent assay (ELISA) for measuring fibrinogen in human serum and plasma, only for research purpose. Manufacture by ALPCO DIAGNOSTICS, SALEM was used for analysis.

Principle: In this assay the fibrinogen present in the samples reacts with the anti-fibrinogen antibodies which have been adsorbed to the surface of the polystyrene microplate wells. After the removal of unbound proteins by washing, anti-fibrinogen antibodies conjugated with horseradish peroxidase (HRP) are added. This enzyme labelled antibodies form complexes with the previously bound fibrinogen. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3’5,5’-tetramethylbenzidine (TMB). The quantity of bound enzyme correlates directly with the concentration of fibrinogen in the sample tested; the absorbance at 450 nm is a measure of the concentration of fibrinogen in the sample. The quantity of fibrinogen in the sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

Procedure: As per the instructions provided with the kit.

ASSAY PROTOCOL

Dilution of samples
The assay for quantification of fibrinogen requires that samples be diluted before use. A 1/200 dilution is appropriate for most serum samples. A lesser or greater dilution might be required for absolute quantification of samples yielding results outside the range of the standard curve. If unsure of the sample level, it is highly recommended to do a serial dilution with one or two representative samples before running the entire plate.
1. To prepare a 1/200 dilution of sample, transfer 5 μl of sample to 995 μl of 1X Diluent. This yields a 1/200 dilution. Mix thoroughly.

2. To prepare a 1/10,000 dilution, transfer 5 μl of sample to 495 μl of 1X Diluent. This yields a 1/100 dilution. Next, add 5 μl of the 1/100 dilution to 495 μl of 1X Diluent. This yields a 1/10,000 dilution. Mix thoroughly at each stage.

PROCEDURE

1. **Bring all reagents to room temperature before use.**

2. Pipette 100 μl of:
   - Standard 0 (0 ng/ml) in duplicate
   - Standard 1 (12.5 ng/ml) in duplicate
   - Standard 2 (25 ng/ml) in duplicate
   - Standard 3 (50 ng/ml) in duplicate
   - Standard 4 (100 ng/ml) in duplicate
   - Standard 5 (200 ng/ml) in duplicate
   - Standard 6 (400 ng/ml) in duplicate

3. Pipette 100 μl of the prediluted samples (in duplicate) into the predesignated wells.

4. Incubate the microplate at room temperature for sixty (60+/−2) minutes. Keep plate covered and level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash solution and aspirate. Repeat three times, for a total of four washes. If washing manually - completely fill wells with 1X Wash solution, invert the plate, and then pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual solution. Repeat three times for a total of four washes.
7. Pipette 100 µl of appropriately diluted Enzyme Antibody Conjugate to each well. Incubate at room temperature for thirty (30+/−2) minutes. Keep plate covered, level, and in the dark during the incubation.

8. Wash and blot the wells as described in Steps 5 and 6.

9. Pipette 100 µl of Chromogen Substrate solution into each well.

10. Incubate in the dark at room temperature for precisely ten (10) minutes.

11. After ten minutes, add 100 µl of Stop solution to each well.

12. Determine the absorbance (450 nm) of the contents of each well.

Calibrate the plate reader to air.

**Expected values:**

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<tbody>
<tr>
<td>Normal</td>
<td>150-300 mg/dl</td>
</tr>
<tr>
<td>Risk</td>
<td>&gt; 300 mg/dl</td>
</tr>
</tbody>
</table>

*Albumin excretion rate or AER*

**Analyzer:** Esbach’s albuminometer

**Principle:** It is based on protein precipitation method in which picric acid is used to precipitate the protein and the amount of protein precipitate is measured by volume.

**Procedure:** As per the manual.

**Expected value:**

100 to 150 mg/24 hrs.

*Creatinine clearance rate or Ccr*

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecno, Rome-Italy.
Method: Jaffe’ reaction

Principle: Creatinine reacts with picric acid in alkaline medium-to form a reddish yellow complex, intensity of which is directly proportional to the concentration of creatinine in the sample and can be measured at 520 nm or using Green filter.

Using Formula
Creatinine clearance = UV/S × 1.73/A
U = mg/ml of urine creatinine
S = mg/ml of serum creatinine
V = ml of urine excreted per minute
1.73 = Standard average surface area of the normal individual
A = Surface area of the patient

Expected value
For males: 105 ± 20 ml/min
For Females: 95 ± 20 ml/min

Glycated haemoglobin A1c or HbA1c
Analyzer: DCA 2000+ANALYZER


Principle: For the measurement of total hemoglobin, potassium ferricyanide is used to oxidize hemoglobin in the sample to methemoglobin. The methemoglobin formed then complexes with
thiocyanate to form thiocyan-methemoglobin (a colored complex). The absorbance of thiocyan-methemoglobin is then measured. The extent of color developed at 531 nm is proportional to the concentration of total hemoglobin in the sample.

For the measurement of specific HemoglobinA1c, an inhibition of latex agglutination assay is used. An agglutinator (synthetic polymer containing multiple copies of the immunoreactive portion of HemoglobinA1c) causes agglutination of latex coated with HemoglobinA1c specific mouse monoclonal antibody. This agglutination reaction causes increased scattering of light that is measured as an increase in absorbance at 531 nm. HemoglobinA1c in whole blood specimens competes for the limited number of antibody-latex binding sites causing an inhibition of agglutination and a decreased scattering of light. The decreased scattering is measured as a decrease in absorbance at 531 nm. The HemoglobinA1c concentration is then quantified using a calibration curve of absorbance versus HaemoglobinA1c concentration.

Both the concentration of haemoglobinA1c specifically and the concentration of total haemoglobin are measured, and the ratio reported as percent haemoglobinA1c.

The percent HemoglobinA1c in the sample is then calculated as follows:

\[
\text{% HemoglobinA1c} = \frac{[\text{HemoglobinA1c}]}{[\text{Total Hemoglobin}]} \times 100
\]

The DCA 2000+ Analyzer performed all measurements and calculations automatically, and the screen displays percent HemoglobinA1c at the end of the assay. All of the reagents for performing both reactions are contained in the DCA 2000® Haemoglobin A1c (HemoglobinA1c) Reagent Cartridge (Figure).
**Procedure:** As per the instructions provided with the kit.

**Expected value:**
- Non Diabetics: 3-6%
- Controlled Diabetics: 6-8%
- Poorly controlled Diabetics: >8%

**Fructosamine**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecnno, Rome-Italy.

**Method:** NBT reduction method.

**Principle:** The fructosamine assay is a colorimetric test that determines the degree of glycation of serum proteins by measuring the degree of reducing activity of serum in alkaline medium\(^{304}\).

The test relies on the ability of Amodori compounds (fructose amine) to reduce nitoblu tetrazolium (NBT) to the tetrazinolyl radical $\text{NBT}^+$ which disproportionate to yield a highly colored formazan dye ($\text{MF}^+$) (monoformazzen), which can be measured at 530 nm.

**Expected value:** 1.8 to 2.8 micomol/L

**Globin bound Fructosamine**

**Analyzer:** Semi auto clinical chemistry analyzer, Model-Minictecnno, Rome-Italy.

Method: NBT reduction method.
**Principle:** The Globin bound fructosamine assay is a colorimetric test that determines the degree of glycation of serum proteins by measuring the degree of reducing activity of serum in alkaline medium\textsuperscript{394}. The test relies on the ability of Amodori compounds (fructose amine) to reduce nitroblue tetrazolium (NBT) to the tetrazinolyl radical NBT\textsuperscript+ which disproportionate to yield a highly colored formazan dye (MF\textsuperscript+) (monoformazen). This can be measured at 530 nm.

B L Somani et al modified fructosamine assay for the estimation of glycated hemoglobin/protein. By additionally modifying the NBT reagent by including p – Chloro mercuribenzoic acid (p – CMB) in the reaction mixture to bind free – SH groups so that same reagent could be used to quantify the serum glycated proteins as well as glycated hemoglobin. They modified the NBT reagent, using carbonate buffer (0.2 mol/L, pH 10.35) containing 500 \( \mu \)mol of NBT and 2 mmol of p-Chloro mercuribenzoic acid (p – CMB) per liter.

**Extraction of globin from hemolysate:**
Hemolysate containing 10 mg of hemoglobin was added to 10 ml of ice cold acidified acetone (prepare by adding 3 ml of 2N-hydrochloric acid to one litre of acetone). The tubes were vortexed and allowed to stand for 10 min at room temperature, then centrifuged at 1000× g rpm for 10 min. The supernatant was decanted, rim wiped with tissue paper. The precipitate (extracted globin) thus obtained was dissolved immediately in 1ml of normal saline within 1min. Then add NBT reagent and absorbance was taken at 530 nm.

**Expected value:**
1.8 to 2.8 micomol/l
**NT-proBNP**

**Analyzer:** Elecsys 2010 analyzer.

**Method:** Electrochemiluminescence immunoassay “ECLIA” using Roch Elecsys 2010 Analyzer.

**Principle:** It is based on Sandwich principle, Antigen in the sample, a biotinylated polyclonal NT-pro-BNP-specific antibody, and a polyclonal NT-pro-BNP-specific antibody labelled with a ruthenium complex from a sandwich complex during 1st incubation. After addition of strptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with Procell. Application of voltage to the electrode then induces chemiluminiscent emission which is measured by a photomultiplier$^{395}$.  

**Procedure:** As per the instructions provided with the kit. Bring the cooled reagent to approx $20^\circ C$ and place on the reagent disk of the analyzer. Avoid the formation of foam. The system automatically regulates the temperature of the reagents and the opening/closing of the bottles.

**Expected value:**

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;125 pg/ml</td>
</tr>
<tr>
<td>Increased Risk</td>
<td>&gt;125 pg/ml</td>
</tr>
</tbody>
</table>
4.4 Statistical analysis

All the data collected were statistically analyzed using the following tests where applicable: Analysis of variance test (ANOVA) and chi-square test. To test the significance's between the study and the control group, "χ²" test and student t – test were used. The univariate analysis performed is identifying risk factors of CAD, Type-II DM, and severity of disease. The multivariate logistic regression analysis (stepwise) was performed taking significant risk factors at univariate analysis independent variables to identify the final set of risk factors. For both univariate and multivariate analysis, cut-off values were used for differentiating the presence or absence of the disease. For categorical data

Statistical analysis was carried out using SPSS 14.3 version. All data are reported as the mean ± SD or as a percentage. Demographic and clinical variables were compared by unpaired t test. Prevalence was compared by the χ² test. Correlation analysis was performed by means of spearman test. Statistical significance was defined as P < 0.05.